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Bioconjugate Chemistry

EDITORIAL: What's New

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It is a pleasure to acknowledge the contributions of the reviewers of manuscripts submitted in 2000 for publication in *Bioconjugate Chemistry*. Their unselfish service has been essential in the shaping of the eleventh volume of the journal. The scientific community is fortunate that its members are willing to share their time and specialized knowledge in this way. The reviewers are listed on the following page.

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Hemin (Fe^{3+})– and Heme (Fe^{2+})–Smectite Conjugates as a Model of Hemoprotein Based on Spectrophotometry

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Hemin (Fe^{3+}) was adsorbed onto synthetic smectite (clay mineral) in acetone to form a hemin–smectite conjugate. The hemin–smectite conjugate became soluble in water to form a transparent colloidal solution with a dark brown color. Its absorption spectrum in water showed a sharp Soret band at 398 nm with the molar extinction coefficient as $\epsilon_{398\text{nm}} = 11.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, which is in good agreement with $\epsilon_{398\text{nm}} = (12.2 \pm 3) \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ of monomeric hematin (1). Hemin (Fe^{3+})–smectite conjugate had a peroxidase-like activity in the presence of hydrogen peroxide (a hydrogen acceptor) and guaiacol (a hydrogen donor) in aqueous solution and its activity was higher than that of hematin. Hemin (Fe^{3+})–smectite conjugate in water was reduced by adding sodium dithionite to form a heme (Fe^{2+})–smectite conjugate which is also a transparent colloidal solution in water. Its absorption spectrum in aqueous solution was surprisingly in close agreement with that of oxyhemoglobin. Its peak positions of α , β , and Soret bands were located in only a 9–3 nm shift to shorter wavelengths in comparison with those of oxyhemoglobin. Therefore, heme (Fe^{2+})–smectite conjugate was bound to O_2 to form O_2 –heme (Fe^{2+})–smectite conjugate. The addition of carbon monoxide, CO, to O_2 –heme (Fe^{2+})–smectite conjugate caused the formation of CO–heme (Fe^{2+})–smectite conjugate with a similar absorption spectrum of carboxyhemoglobin (HbCO) accompanied by shifting 8–10 nm to shorter wavelength. Therefore, the transformation of O_2 –heme (Fe^{2+})–smectite conjugate to CO–heme (Fe^{2+})–smectite conjugate was accompanied by shifting of 7, 4, and 3 nm to shorter wavelengths in the α , β , and Soret bands respectively, which are similar to the spectral change from oxyhemoglobin to carboxyhemoglobin. Also the ratio (1:1.6) of the molar extinction coefficient of Soret band of O_2 –heme (Fe^{2+})–smectite conjugate and CO–heme (Fe^{2+})–smectite conjugate was surprisingly agreement with ratio (1:1.5) of oxyhemoglobin and carboxyhemoglobin. The phenomenon shown above was unexpectedly found during the course of study of bioconjugate of a bioactive substance, hemin (Fe^{3+}) or heme (Fe^{2+}), and a clay mineral, smectite, in place of the protein of globin in hemoglobin.

Human hemoglobin with the molecular weight of 64 500 in red blood cells consists of 6% heme and 94% globin and plays, in vivo, an important role in transport of oxygen. In its oxygenation form it is called oxyhemoglobin, HbO_2 ,¹ and when the oxygen is displaced by carbon monoxide, it is called carboxyhemoglobin, HbCO (3). This splendid function depends on the cooperative action of heme and globin protein ($\alpha_2\beta_2$) having a specific conformation composed of primary (α chain, 141 amino acids; β chain, 146 amino acids), secondary (helix content, 70%), tertiary (the steric conformation), and quaternary structures (four subunits) (4, 5). The hemoglobin molecule has a compact spheroidal structure of 6.4 by 5.5 by 5.0 nm in dimension (6). When one of amino acids in hemoglobin molecule is deleted or substituted by another amino acid by mutation, the normal function of hemoglobin can be harmed to form what is called abnormal hemoglobin (7). On the other hand, heme (Fe^{2+}) is a prosthetic group of hemoglobin and is obtained by reducing hematin (Fe^{3+}) in alkali solution by sodium dithionite (Figure 1a) (2). The absorption spectrum of monomeric

hematin in aqueous solution could be measured at pH 6.8 and found to have a sharp Soret band with a molar extinction coefficient as $\epsilon_{398\text{nm}} = (12.2 \pm 3) \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, as reported by Inada and Shibata (1).

The smectite (hectorite), which was hydrothermally synthesized, had unique physical and chemical properties such as high transparency in water, cation exchange, and the ability to form organic and inorganic interlayer complexes (8, 9). Expandable phyllosilicates have a structure composed of alternating 2:1 layer, which consists of two sheets of linked SiO_4 tetrahedra sandwiching octahedral cations, Mg, between them as shown in Figure 1b. The 2:1 layers which have a net negative charge (layer charge), are loosely tied together by interlayer cations. Water also is present between the layers. The properties of smectite prepared by Torii and Iwasaki (8–10) are as follows: elemental composition Si 8.00, Mg 5.65, Li 0.70, and Na 1.05; transmittance 95% in 1% aqueous solution at 500 nm; and methylene blue absorption 101 milliequiv/100 g which is obtained from Co-op Chemical Co., Ltd (Tokyo, Japan).

Chlorohemin is soluble in dilute potassium hydroxide solution to form hematin. The monomeric hematin molecule is unstable and has a marked tendency to take a polymerized form. The hemin–smectite conjugate was prepared by adding 300 mg of smectite powder to 33 mL

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¹ Abbreviations: HbO_2 , oxyhemoglobin; HbCO, carboxyhemoglobin; SME, smectite.

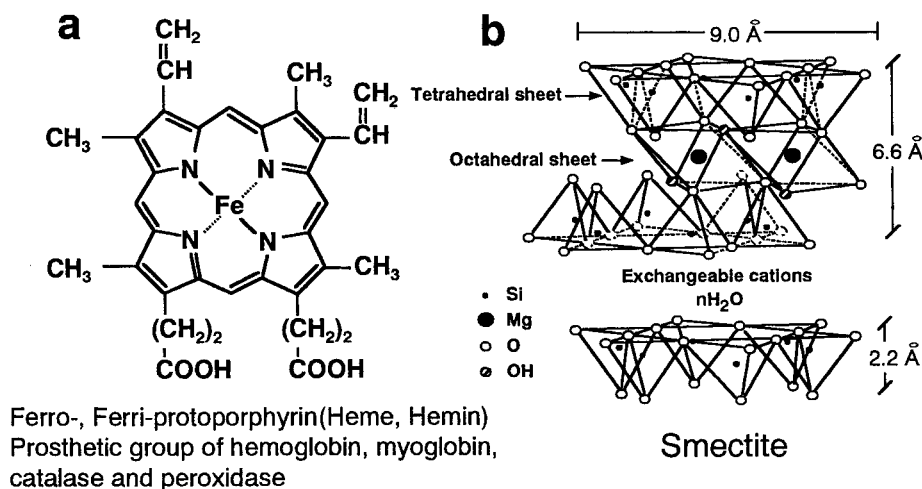


Figure 1. (a) Heme or hemin consists of protoporphyrin being made up of four pyrrole groups and an iron ion. The iron atom in heme or hemin binds to the four nitrogens in the center of the protoporphyrin ring. The iron can form two additional bounds. Their bonding sites are termed the fifth and sixth coordination positions. The iron atoms in the heme can be in the ferrous (Fe^{2+}) or the ferric (Fe^{3+}) oxidation state. (b) The chemical structure of smectite in a crystal state consists of two sheets of linked SiO_4 tetrahedra sandwiching octahedral cation (Mg) between them, and the interlayer. Elemental composition Si 8.00, Mg 5.65, Li 0.70, Na 1.05; transmittance 95% in 1% aqueous solution at 500 nm; and methylene blue absorption 101 milliequiv/100 g.

of acetone solution containing chorohemin (0.85 mg). The suspension was then shaken for 1 h at 25 °C to establish the adsorption equilibrium between hemin and smectite. The hemin (Fe^{3+})-smectite conjugate thus formed was collected by centrifugation and was dried under reduced pressure. The amount of hemin adsorbed onto smectite was spectrophotometrically determined by measuring the absorbance of the supernatant liquid obtained by centrifugation of the sample suspension using the molar extinction coefficient of hematin. The hemin-smectite conjugate, in which amount of hemin (0.2 mg) was adsorbed onto 100 mg of smectite, was dissolved in water to form transparent solution with dark brown color. Absorption spectrum of hemin (Fe^{3+})-smectite conjugate (1.14% in water) shows a sharp Soret band at 398 nm and two peaks at 490 and 610 nm as shown in Figure 2a. And its molar extinction coefficient at 398 nm as $\epsilon_{398\text{nm}} = 11.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ was in good agreement with that of monomeric hematin (1). The absorption spectrum of hematin, dashed line in Figure 2a, shows a polymerized form of hematin with a broad Soret band at approximate 360 nm. Figure 2b shows the peroxidase activities of hemin (Fe^{3+})-smectite conjugate (closed circle) and hematin (open circle) against incubation time using hydrogen peroxide (a hydrogen acceptor) and guaiacol (a hydrogen donor) in aqueous solution. The activity of the hemin (Fe^{3+})-smectite conjugate was increased linearly with time, while the activity of hematin was lowered with time. The peroxidase-like activity of the conjugate was well retained at high concentration of H_2O_2 and in high temperature at more than 60 °C in the reaction system in comparison with that of natural peroxidase composed of protein and hemin.

Hemin (Fe^{3+})-smectite conjugate in the thunberg tube was deaerated by successive freeze-pump-thaw cycles and was reduced by the addition of sodium dithionite to form heme (Fe^{2+})-smectite conjugate. Absorption spectrum of heme (Fe^{2+})-smectite conjugate in water is shown by solid line in Figure 3a, which is quite different from that of hemin (Fe^{3+})-smectite conjugate shown by dashed line in Figure 3a. The peak positions of α , β , and Soret bands in heme (Fe^{2+})-smectite conjugate were located at 568, 534, and 411 nm, respectively, which represent an only approximately 9–3 nm shift to shorter wavelengths compared with those of oxyhemoglobin

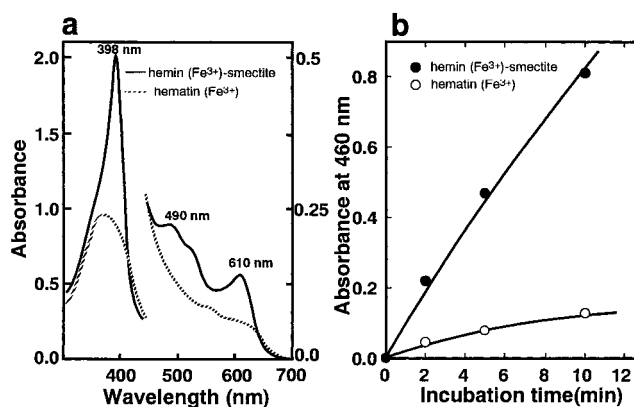


Figure 2. Absorption spectra of hemin (Fe^{3+})-smectite conjugate and hematin (Fe^{3+}) in water (a) and peroxidase activity of hemin-smectite conjugate in the presence of hydrogen peroxide and guaiacol in aqueous solution (b). (a) The spectrum of hemin (Fe^{3+})-smectite conjugate (17 μM) is shown by solid line with a sharp Soret band at 398 nm. The absorption spectrum of hematin at the same concentration as the hemin (Fe^{3+})-smectite conjugate is shown by dashed line with a broad band at about 360 nm as a Soret band which is a polymerized form of hematin. (b) The peroxidase activity was spectrophotometrically determined by measuring the absorbance at 460 nm caused by the oxidation of guaiacol (0.3 mM) with hydrogen peroxide (3.2 mM) in 3.1 mL of 0.1 M phosphate buffer (pH 7.0) in the presence of hemin-smectite conjugate (8.6 μM) or hematin (8.2 μM). The absorbance at 460 nm was plotted against incubation time (min) at 25 °C.

(Table 1). To confirm the binding of O_2 to heme (Fe^{2+})-smectite conjugate to form O_2 -heme (Fe^{2+})-smectite conjugate, states of heme (Fe^{2+}) and hemin (Fe^{3+}) conjugated with smectite were studied by measuring the ESR spectrum with a Japan Electron Optics Laboratory spectrometer model JES-FA 200 operating at 100 kHz. The ESR spectrum of hemin (Fe^{3+})-smectite conjugate in water showed the concomitant formation of the two high-spin states of ($g = 6.160$) and ($g = 5.865$). On the other hand, heme (Fe^{2+})-smectite conjugate obtained by adding sodium dithionite to hemin (Fe^{3+})-smectite conjugate showed a reduced ESR signal in high-spin state ($g = 6.160$). A similar phenomenon had been observed in transformation of methemoglobin (Fe^{3+}) to oxyhemoglobin (Fe^{2+}) in which ESR spectrum with high-spin state

Table 1. Spectrophotometric Characteristics of Hemin (Fe^{3+})– and Heme(Fe^{2+})–smectite Conjugates in Comparison with Those of Hemi(Fe^{3+}) and Hemo(Fe^{2+}) Proteins^a

compound	oxidation state of Fe	ligands		λ_{max}			ϵ (mM)
		fifth	sixth	α	β	Soret	
O_2 -heme-smectite	Fe^{2+}	(SME	O_2)	568	534	411	93
CO-heme-smectite	Fe^{2+}	(SME	CO)	561	530	408	148
oxyhemoglobin ⁽¹⁾	Fe^{2+}	His	O_2	577	541	414	125
carboxyhemoglobin ⁽³⁾	Fe^{2+}	His	CO	570	538	418	191
hemin-smectite	Fe^{3+}	(SME	Cl^-)	610	490	398	116
monomeric hematin ⁽¹⁾	Fe^{3+}	OH-	H_2O	—	—	398	122
hemoglobin ⁽³⁾	Fe^{3+}	His	H_2O	630	500	406	

^a The absorption spectra of hemin– and heme–smectite conjugates in water, a transparent colloidal solution, was measured with a Shimadzu spectrophotometer MPS-2000 (Kyoto, Japan). Soret (ϵ , mM): the peak position (λ_{max}) of Soret band with molar extinction coefficient (ϵ , mM).

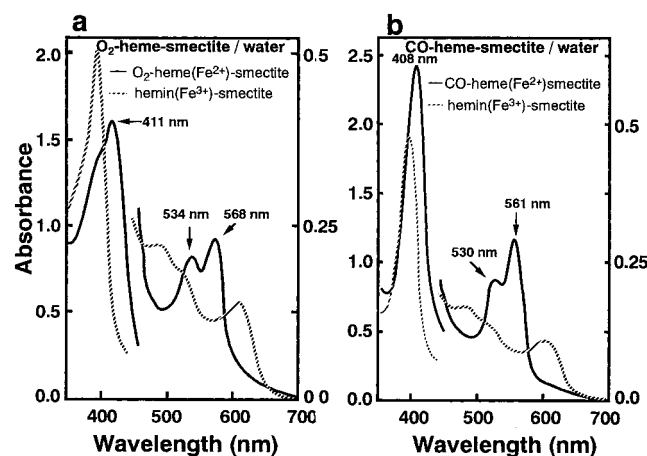


Figure 3. Absorption spectra of O_2 -heme (Fe^{2+})–smectite conjugate (a) and CO-heme (Fe^{2+})–smectite conjugate (b) in water. (a) Spectrum of O_2 -heme (Fe^{2+})–smectite conjugate in water (17 μM) obtained by adding dithionite to hemin (Fe^{3+})–smectite conjugate. The peak positions are located at 568, 534, and 411 nm. (b) Spectrum of CO-heme (Fe^{2+})–smectite conjugate (17 μM) obtained by adding carbon monoxide, CO, to O_2 -heme (Fe^{2+})–smectite conjugate. The absorption bands are at 561, 530, and 408 nm which are located at shorter wavelengths compared with the spectrum of O_2 -heme (Fe^{2+})–smectite conjugate. Dashed lines shown in panels a and b indicate the spectrum of hemin (Fe^{3+})–smectite conjugate having the peak positions of 610 and 490 nm and the Soret band at 398 nm.

($g \approx 6$) of methemoglobin (Fe^{3+}) is hardly detected in oxyhemoglobin (HbO_2) due to diamagnetism (11). It suggests the possibility of heme (Fe^{2+})–smectite conjugate being bound to oxygen to form O_2 -heme (Fe^{2+})–smectite conjugate. Furthermore, the addition of carbon monoxide, CO, to O_2 -heme (Fe^{2+})–smectite conjugate caused the formation of CO-heme (Fe^{2+})–smectite conjugate with the absorption maxima at 561, 530, and 408 nm, which are also similar to carboxyhemoglobin (HbCO) with peak positions at 570, 538, and 418 nm (Figure 3b and Table 1).

It is well-known that oxyhemoglobin changes easily to carboxyhemoglobin (HbCO) in the presence of carbon monoxide, CO, with the spectral change as shown in Table 1. A similar phenomenon was observed in heme (Fe^{2+})–smectite conjugate, in a form of a spectral change of O_2 -heme (Fe^{2+})–smectite to CO-heme (Fe^{2+})–smectite from 568, 534, and 411 nm to 561, 530, and 408 nm, respectively, with a 7–3 nm shift to shorter wavelength as shown in Table 1. The ratio of the molar extinction coefficient of O_2 -heme (Fe^{2+})–smectite conjugate and CO-heme (Fe^{2+})–smectite conjugate was 1:1.6 which is closely agreement with that of oxyhemoglobin and carboxyhemoglobin, 1:1.5 (Table 1). From the results obtained above, it may be concluded that the heme (Fe^{2+})–

smectite conjugate prepared by the above method is bound to an oxygen molecule to form O_2 -heme (Fe^{2+})–smectite conjugate.

It was previously known in a similar line of study conducted extensively by us that conjugate of tetrapyrrole ring including magnesium or chlorophylls adsorbed onto clay minerals such as bentonite and smectite is closely associated with the development of the photochemical function of chlorophylls (10, 12–14). It is well-known that chlorophylls *a* and *b* extracted with organic solvents from fresh green leaves are quite unstable and are rapidly bleached by light-illumination, although chlorophylls bound to protein in chloroplast are quite stable in vivo. In expectation of making chlorophylls much more stable against light, chlorophylls were adsorbed onto clay minerals such as bentonite or smectite in place of protein. Chlorophyll *a*–smectite conjugate was quite photostable, and the absorption maximum of the conjugate approached 678 nm, which is the absorption maximum of intact spinach leaves. And the chlorophyll–smectite conjugate becomes soluble in aqueous solution although chlorophylls are never soluble in water. The conjugate of chlorophyll *a*, poly(vinylpyrrolidone), and smectite had an absorption maximum at 677 nm thus becoming more photostable than chlorophyll–smectite conjugate and catalyzing hydrogen gas evolution by visible light in aqueous solution. The similar line of study was conducted by Wang (15): heme embedded in a hydrophobic matrix of polystyrene and 1-(2-phenylethyl)-imidazole has the ability of O_2 and CO binding.

During the course of the study mentioned above, we happened to prepare hemin (Fe^{3+})– and heme (Fe^{2+})–smectite conjugates in which smectite, soluble in water, was used in place of the protein of globin in hemoglobin. It was unexpected that the heme (Fe^{2+})–smectite conjugate is bound to O_2 and CO in a manner similar to hemoprotein. Soon, bioconjugate compounds of smectite with chlorophylls are expected to make artificial photosynthesis possible and that of smectite with heme serves as an oxygen carrier in place of hemoglobin.

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REVIEWS

Bifunctional Chelators for Therapeutic Lanthanide Radiopharmaceuticals

Shuang Liu* and D. Scott Edwards

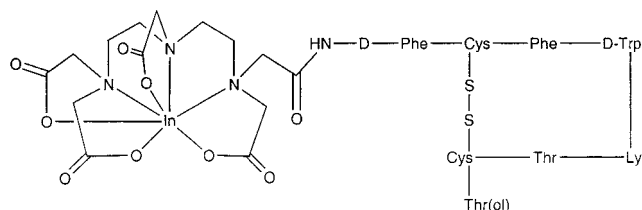
Medical Imaging Division, DuPont Pharmaceuticals Company, 331 Treble Cove Road, North Billerica, Massachusetts 01862. Received June 21, 2000

INTRODUCTION

Therapeutic radiopharmaceuticals are radiolabeled molecules designed to deliver therapeutic doses of ionizing radiation to specific disease sites (1, 2). Therapeutic doses of radiation can be delivered to sites of disease such as cancer in three ways: external beam irradiation, implantable “seeds” or systemic administration. Brachytherapy involves the use of seeds, which are physically placed at the tumor site and will remain there unless they are surgically removed. Brachytherapy plays a vital role in the care of prostate cancer patients. It is only useful for the treatment of accessible tumor mass. The systemic administration of radiopharmaceuticals that are designed for specific localization at tumor sites provides the opportunity for the treatment of disseminated disease (2–7). Most systemically administered therapeutic radiopharmaceuticals are small organic or inorganic compounds with definite composition (4, 5). They can also be macromolecules, such as monoclonal antibodies or antibody fragments labeled with a radionuclide. Ideally, the radiopharmaceutical should localize at the tumor sites in sufficient concentration to deliver a cytotoxic radiation dose to the tumor cells and clear rapidly from the blood and other normal organs to minimize radiation damage to normal tissues.

Radiotherapy has been around for over four decades starting with the use of radioiodine for the treatment of thyroid disorders. The main obstacle to radiotherapy assuming a wider role in clinical practice are the availability of therapeutic isotopes and techniques for their specific localization in diseased tissues. The introduction of [^{111}In]DTPA-octreotide (OctreoScan in Figure 1) for the diagnosis of somatostatin receptor positive tumors has spurred the search for new receptor-based target specific therapeutic radiopharmaceuticals. A number of radiolabeled small peptides (8–33) have been studied for their potential use in tumor therapy. These studies have provided an impetus to research in the production and chemistry of new therapeutic radionuclides (34–37), as well as new bifunctional chelators (BFCs).

Metalloradiopharmaceuticals are pharmaceuticals composed of a metallic radionuclide (e.g., ^{67}Cu , ^{90}Y , $^{99\text{m}}\text{Tc}$, ^{111}In , ^{177}Lu , ^{186}Re , ^{188}Re , and ^{212}Bi). In general, a target-specific metalloradiopharmaceutical (Figure 2) can be divided into four parts: a targeting biomolecule, a linker, a BFC, and a metallic radionuclide. The targeting biomolecules can be macromolecules such as antibodies or antibody



^{111}In -DTPA-Octreotide (OctreoScan®)

Figure 1. Structure of [^{111}In]DTPA-octreotide (OctreoScan).

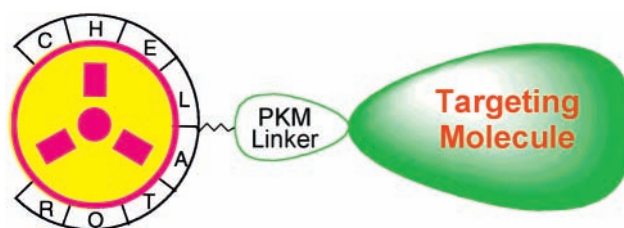


Figure 2. Schematic presentation of a target-specific metalloradiopharmaceutical. The linker is often used as a pharmacokinetic modifier (PKM).

fragments, or small peptides, peptidomimetics, or non-peptide receptor ligands. Between the targeting biomolecule and the radionuclide is the BFC, one end of which is covalently attached to the targeting molecule either directly or through a linker while the other strongly coordinates to the metallic radionuclide. Selection of a BFC is largely determined by the nature and oxidation state of the metal ion. The linker is often used for modifying the pharmacokinetic properties of the radiopharmaceutical.

The choice of a linker depends on the pharmacokinetic requirements for the radiopharmaceutical. Figure 3 shows several types of linkers (cationic, anionic, neutral or metabolically cleavable). The linker can be a simple hydrocarbon chain to increase lipophilicity, a peptide sequence (such as polyglycine, polyserine, or polyaspartic acid) to improve the hydrophilicity and renal clearance, or a poly(ethyleneglycol) linker to slow extraction by the hepatocytes. It has been reported that linker groups have significant effect on biodistribution of ^{111}In - and $^{99\text{m}}\text{Tc}$ -labeled antibodies (38–40). Metabolizable linkers have been used for ^{111}In -labeled somatostatin analogues (41). A tetrapeptide linker Gly-Gly-Gly-L-(p-NO₂)-Phe-CONH₂ that is cleaved between Gly and Phe residues has been used to modify pharmacokinetics of ^{90}Y -labeled antibodies (42–45). Depending upon the radionuclide and the BFC, linker groups capable of rapid metabolism can increase

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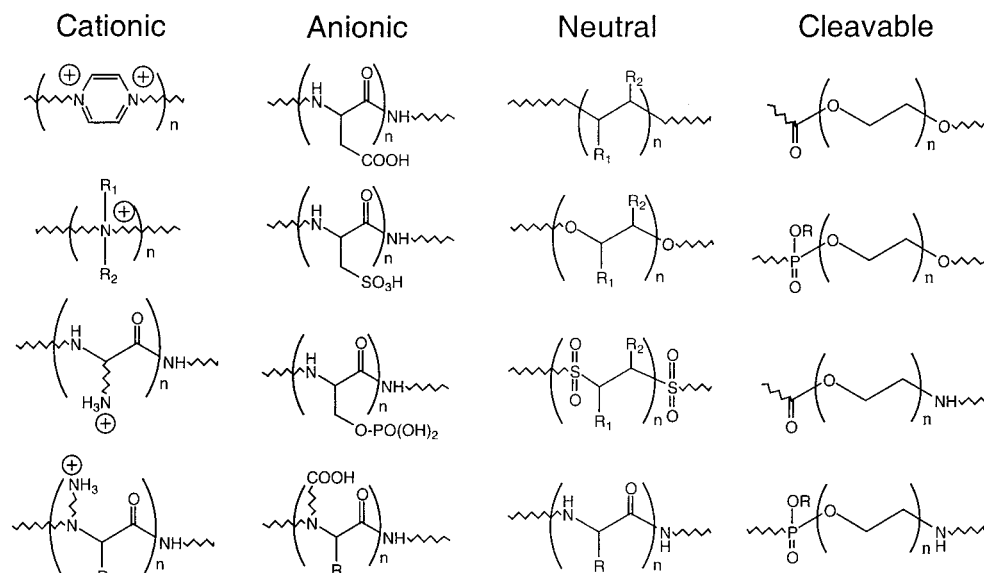


Figure 3. Linkers for modification of pharmacokinetics of radiopharmaceuticals.

Table 1. Radionuclides Useful for Radiotherapy

nuclide	half-life (days)	energy (MeV)	maximum range (mm)	gamma (keV)	source	specific activity ^a
⁶⁷ Cu	2.58	0.575	1.8	185 (40%)	accelerator	low
⁹⁰ Y	2.66	2.27	12.0		reactor/generator	high
¹⁰⁹ Pd	0.56	1.03	5.0	88 (5%)	reactor	low
¹¹¹ Ag	7.47	1.05	4.8		reactor	low
¹⁴⁹ Pm	2.21	1.07	5.0	286 (3%)	reactor	low/medium
¹⁵³ Sm	1.95	0.80	3.0	103 (28%)	reactor	low/medium
¹⁶⁶ Ho	1.1	1.6	8.0	81 (6.3%)	reactor/generator	high
¹⁷⁷ Lu	6.7	0.497	1.5	208 (28%)	reactor	medium/high
¹⁸⁶ Re	3.7	1.02	5.0	137 (9%)	reactor/accelerator	low/medium
¹⁸⁸ Re	0.71	2.12	11.0	155 (15%)	reactor/generator	high

^a The specific activity of a radionuclide depends on the source and method of production, as well as the technique of separation. In general, generator-produced radionuclides, such as ⁹⁰Y and ¹⁸⁸Re, have a higher specific activity than those accelerator- or reactor-produced radioisotopes. High specific activity can also be achieved by chemical purification of the desired radionuclide from the parent element after direct (n,γ)-activation of the target.

the clearance of the radiopharmaceutical from the blood via the renal system.

The use of metallic radionuclides offers many opportunities for designing new radiopharmaceuticals by modifying the coordination environment around the metal with a variety of chelators. The coordination chemistry of the metallic radionuclide will determine the geometry and solution stability of the metal chelate. Different metallic radionuclides have their different coordination chemistries, and require BFCs with different donor atoms and ligand frameworks. The metal chelate can significantly impact the tumor uptake and biodistribution of radiopharmaceuticals based on small biomolecules, due to the fact that in many cases the metal chelate contributes greatly to the overall size and molecular weight of the radiopharmaceutical. Therefore, the design and selection of the BFC is very important for the development of a clinically useful therapeutic agent.

Several reviews have appeared recently covering a broad range of topics related to diagnostic and therapeutic radiopharmaceuticals (46–49). This review will focus on the critical aspects to the development of clinically useful agents, the fundamentals of lanthanide chemistry, the design and synthesis of new BFCs for lanthanide radionuclides, and the factors influencing the ⁹⁰Y-labeling of DTPA- and DOTA-conjugated biomolecules (DTPA-BM and DOTA-BM, respectively).

General Considerations for Therapeutic Radiopharmaceuticals. In the development of a new thera-

peutic radiopharmaceutical, several factors need to be considered to satisfy the clinical need and to comply with FDA (Food and Drug Administration) regulations. While tumor imaging relies heavily on a high target-to-background ratio for the best contrast, tumor therapy depends largely on a high concentration of radioactivity in the tumor for a long duration. Thus, the new radiopharmaceutical must have high and fast tumor uptake, high tumor-to-background ratio, long tumor residence time, and fast renal clearance. High tumor uptake and fast renal clearance are particularly important to improve the tumor-to-background ratio and to reduce the radiation burden to other normal organs such as the kidney, liver and bone marrow. The new radiopharmaceutical must have high radiochemical purity (RCP > 90%) and high solution stability. Unlike diagnostic radiopharmaceuticals, which are typically made by radiopharmacists just before injection, therapeutic radiopharmaceuticals have to be manufactured and released under GMP conditions, and then delivered to the clinic. Therefore, the therapeutic radiopharmaceutical must retain its chemical and biological integrity during storage and transportation. This requires that the BFC bind strongly to the radionuclide and form a metal chelate with both thermodynamic stability and kinetic inertness.

Choice of Radionuclides. Table 1 shows some radionuclides potentially useful for radiotherapy. Among them, lanthanide radioisotopes are of particular interest. There are several lanthanide isotopes to choose, including

low-energy β -emitter ^{177}Lu , medium-energy β -emitters, ^{149}Pm and ^{153}Sm , and high-energy β -emitters, ^{166}Ho and ^{90}Y . Yttrium and lanthanide metals share similar coordination chemistry. The chelator technology and their coordination chemistry are well developed and well understood.

Identifying the most appropriate isotope for radiotherapy is often a difficult task and requires weighing a variety of factors (50–52). These include tumor uptake and retention, blood clearance, rate of radiation delivery, half-life and specific activity of the radionuclide, and the feasibility of large-scale production of the radionuclide in an economical fashion. The key point for a therapeutic radiopharmaceutical is to deliver a tumoricidal dose of radiation to the tumor cells while not causing unmanageable side-effects.

The physical half-life of the therapeutic radionuclide should match the biological half-life of the radiopharmaceutical at the tumor site. If the half-life of the radionuclide is too short, most of the dose will have been delivered before the radiopharmaceutical has reached the maximum target/background ratio. In this situation, attaining a sufficient integrated tumor dose for sterilization (>50 Gy) will prove problematic (50–52). On the other hand, too long a half-life would cause unnecessary radiation dose to normal tissues. Ideally, the radionuclide should have a half-life long enough so that a minimal dose rate (>0.4 Gy/h) is achieved and all cells are irradiated during the most radiation sensitive phases of the cell cycle. In addition, the half-life of a radionuclide has to be long enough to allow adequate time for manufacturing, release, and transportation.

Other practical considerations in selecting a radionuclide for a given targeting biomolecule for tumor therapy are availability and quality. The purity has to be sufficient and reproducible, as trace amounts of impurities can affect the radiolabeling and radiochemical purity of the radiopharmaceutical. The target receptor sites in tumors are typically limited in number. This requires that the chosen radionuclide have high specific activity. The specific activity depends primarily on the production method. Trace metal contaminants must be minimized as they often compete with the radionuclide for the BFC and their metal complexes compete for receptor binding with the radiolabeled BFC–BM conjugate.

For tumor therapy, both α - and β -emitters need to be considered. α -Particles are particularly good cytotoxic agents because they dissipate a large amount of energy within one or two cell diameters. Most α -emitters are heavy elements that decay to hazardous daughter products and their penetration range is limited to only 50 μm in tissue (52). The short-range α -emitters are more attractive if the radiopharmaceutical is internalized into tumor cells. Auger electron emitters are shown to be very potent but only if they can cross the cell membrane and come into close proximity with the nucleus, which creates extra challenges for the design of new metalloradiopharmaceuticals. β -Particle emitters have relatively long penetration range (2–12 mm in the tissue) depending the energy level. The long-range penetration is particularly important for solid tumors with high heterogeneity. The β -particle emitters yield a more homogeneous dose distribution even when they are heterogeneously distributed within the target tissue. Depending on the tumor size and location, the choice of the β -emitter may be different. For example, medium- or low-energy β -emitters such as ^{153}Sm and ^{177}Lu are better for smaller metastases while high-energy β -emitters such as ^{90}Y are used for larger tumors.

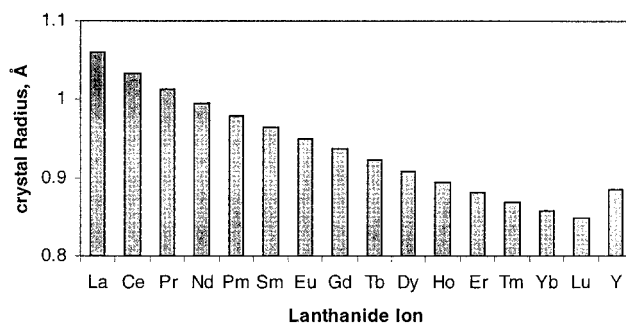


Figure 4. Ionic radius (Å) for trivalent yttrium and lanthanide metal ions.

Some β -emitters contain a small percentage of γ -emission. There is an ongoing controversy over the use of the γ -emission for dosimetry determination and staging of radiotherapy. Some favor the use of γ -emission for these purposes, and some are strongly against it mainly because of the additional radiation burden to the patient. In general, γ -emission should be avoided in selecting a suitable radionuclide since it is only useful for treatment planning not for treatment monitoring or staging of radiotherapy. There are better tracers or imaging modalities to monitor cancer therapy. For dosimetry determination, one can find a closely related diagnostic surrogate, which shares the similar coordination chemistry to that of the therapeutic radiopharmaceutical. For example, the $^{99\text{m}}\text{Tc}$ complexes are often used for the dosimetry determination of $^{186/188}\text{Re}$ radiopharmaceuticals.

For systemic cancer radiotherapy, ^{90}Y is of particular interest because it is a high-energy pure β -particle emitter. The half-life of 2.7 days is short enough to achieve a critical dose rate and long enough to allow the radiopharmaceutical to be manufactured and delivered for clinic use. For quantitative imaging, the corresponding ^{111}In BFC–BM complex is often utilized as a surrogate to determine the biodistribution and the dosimetry of ^{90}Y BFC–BM (10, 14, 18, 20, 22).

Fundamentals of Yttrium and Lanthanide Chemistry. Yttrium and lanthanide (La to Lu) metals all favor the +3 oxidation state. Due to its similar charge, ionic radii (Figure 4) and coordination chemistry, yttrium is often treated as a “pseudo-lanthanide” metal. In contrast to the d -block transition metals, the $4f$ electrons are inner electrons, shielded from external influences by the overlying $5s^2$, $5p^6$, and $6s^2$ -electron shells. The ligand field effect is relatively weak, and the magnetic properties of the metal ion are not significantly affected by the coordination environment. Since $4f$ electrons are not involved in bonding, the interactions between donor atoms and lanthanide metal ions are predominately ionic.

Metal Ions in Solution. Yttrium and lanthanide metal ions in solution are usually indicated as M^{3+} , as though they were “naked” cations suspended in structureless medium. In fact, they exist in aqueous solution in their complexed forms with a number of bonded water molecules. The number of water molecules bound directly to the metal ion, its coordination number, is very similar to the number of donor atoms found in its metal chelate in the solid state and depends largely on the size of the metal ion. Metal chelate formation involves replacement of the coordinated water molecules by a chelating agent. Due to their large size, the coordination numbers of yttrium and lanthanide ions are typically between 7 and 10. Very few six coordinate species are known while coordination numbers of 8 and 9 are common.

Table 2. Selected Solubility Product Constants (K_{sp})^a for Lanthanide Compounds

compd	log K_{sp}	compd	log K_{sp}
Y(OH) ₃	-22.1	Gd ₂ (CO ₃) ₃	-32.2
Gd(OH) ₃	-25.6	YPO ₄	-22.0
Yb(OH) ₃	-23.6	LaPO ₄	-22.43
Y ₂ (CO ₃) ₃	-30.6	GdPO ₄	-22.26
La ₂ (CO ₃) ₃	-33.4		

^a Martell, A. E., and Smith, R. M. (1974) *Critical Stability Constants*. Vol. 4, Plenum, New York.

Hydrolysis and Precipitation. One of the characteristics of yttrium and lanthanide metal ions in aqueous solution is their easy precipitation with commonly occurring anions such as hydroxide, phosphate or carbonate. Table 2 lists the solubility constants of lanthanide complex precipitates. Both phosphate and carbonate are able to compete for the metal ions with a BFC-BM conjugate, while the effect of metal hydroxide formation may not play a significant role in the release of the radionuclide from the metalloradiopharmaceutical. This is mainly due to the presence of more phosphate and carbonate anions in the blood circulation. The high affinity of lanthanide metal ions for the phosphate anion may also explain their affinity for localizing in bone (53).

Thermodynamic Stability. For a therapeutic radiopharmaceutical, the concentration in the blood circulation may become so low that dissociation of the radiometal from the metal chelate will eventually become favored, particular in the presence of many native chelators (54, 55). The loss of radiometal from the chelate may result in the accumulation of radioactivity in nontarget organs. It has been reported that ⁹⁰Y and lanthanide isotopes are readily deposited on the bone (53). Therefore, the BFC must form a metal chelate with high thermodynamic stability to retain its integrity in competition with natural chelators, such as transferrin. However, high thermodynamic stability is not the sole requirement because it only reflects the direction, not the rate, of the reaction. As a matter of fact, the solution stability of a metalloradiopharmaceutical in the blood stream is predominantly determined by the kinetic inertness, not the thermodynamic stability, of the metal chelate.

Conditional Stability Constants. The solution behavior of lanthanide radiopharmaceuticals should be considered in the context of pharmacokinetics, protein binding, excretion, and safety. The coordination chemistry of the radionuclide and BFC in the circulation is extremely complicated. There are many factors influencing the dissociation of the radionuclide. These factors include the competition of proton and metal ions such as Ca²⁺, Cu²⁺, Zn²⁺, and Fe³⁺, which are abundant in the blood stream, for the coordinated BFC, hydroxide, phosphate, and native chelators such as amino acids and transferrin for the radionuclide (54, 55). The concentration of Ca²⁺ is as high as 5 mM in the blood stream. Therefore, the conditional stability constant of a metal chelate is more predictive of the solution behavior of the radiopharmaceutical.

The thermodynamic stability constant K_{ML} of a metal complex is defined by Equation 1. [ML] is the concentration of the metal complex, [M] is the concentration of the metal ion, and [L] is the concentration of the polydentate chelator. Considering the factors influencing the stability of the metal complex, the conditional constant K_{con} can be calculated using eq 2. Due to the competition of proton and metal ions in the blood stream, the concentration of the chelator [L_{tot}] can be calculated by eq 3, where K_1 , K_2 , ..., K_n are the stepwise protonation constants of the

Table 3. Stability Constants, LD₅₀, and ¹⁵³Gd Bone Uptake Data for Selected Gd(III) Complexes (data from ref 60)

ligand	LD ₅₀	% ID/gram	log K_{GdL}	log K'_{GdL} (pH 7.4) ^d
EDTA	0.3 ^a	0.8	17.7	14.7
DTPA	5.6 ^a	0.005	22.46	17.7
DTPA-BMA	14.8 ^a	0.03	16.85	14.9
DOTA	11 ^b	NDR ^c	25.3	18.33
DO3A	7–9 ^c	0.008	21.0	14.97

^a Reference 57. ^b Reference 56. ^c Reference 58. ^d Only pH effect was considered in the calculation of conditional stability constants. ^e Nondetectable radioactivity.

ligand while K_{CaL} , K_{CuL} , K_{ZnL} , and K_{FeL} are stability constants of the corresponding Ca²⁺, Cu²⁺, Zn²⁺, and Fe³⁺ complexes, respectively. Due to hydrolysis (hydroxide), formation of precipitate (phosphate), and coordination of the metal ion by native chelators such as amino acids and transferrin (54, 55), the concentration of the metal ion [M_{tot}] has to be calculated using eq 4, where β_1 , β_2 , and β_3 are formation constant of the metal hydroxide, K_{sp} is the solubility constant of the phosphate precipitate, and K_{ML} is the stability constant of the metal complex of any given native chelator.

$$M + L = ML, \text{ where } K_{ML} = [ML]/([M][L]) \quad (\text{formal stability constant}) \quad (1)$$

$$M + L = ML, \text{ where } K_{con} = [ML]/([M]_{tot}[L]_{tot}) \quad (\text{conditional stability constant}) \quad (2)$$

$$[L_{tot}] = [L](1 + K_1[H^+] + K_1K_2[H^+]^2 + \dots K_1K_2\dots K_n[H^+]^n + K_{CaL}[Ca^{2+}] + K_{CuL}[Cu^{2+}] + K_{ZnL}[Zn^{2+}] + K_{FeL}[Fe^{3+}] \dots) = [L]\alpha_L \quad (3)$$

$$[M_{tot}] = [M](1 + \beta_1[OH^-] + \beta_1\beta_2[OH^-]^2 + \beta_1\beta_2\beta_3[OH^-]^3 + K_{sp}^{-1}[PO_4^{3-}] + \dots K_{ML}[L] \dots) = [M]\alpha_M \quad (4)$$

$$K_{con} = [ML]/([M]_{tot}[L]_{tot}) = \frac{[ML][M][L]\alpha_M\alpha_L}{[ML]\alpha_M\alpha_L} = K_{ML}\alpha_M\alpha_L \quad (5)$$

Table 3 shows the conditional stability constants of Gd complexes of various polydentate chelators and the amount of ¹⁵³Gd found in the rodent skeleton at 7 days postinjection for various complexes (56–58). If proton competition is the only factor taken into consideration, the conditional constants of these complexes are calculated according to eq 5. While thermodynamic (K_{ML}) and conditional constant (K_{con}) values for [Gd(EDTA)(H₂O)_n][−] ($K_{con} = 14.9$) and [Gd(DTPA-BMA)(H₂O)][−] ($K_{con} = 14.7$) are comparable (59, 60), the amount of Gd-153 deposited in bone is significantly different (~0.8% ID/g for [¹⁵³Gd(EDTA)(H₂O)_n][−] and 0.03% ID/g for [¹⁵³Gd(DTPA-BMA)(H₂O)][−]). For [Gd(DO3A)(H₂O)], the conditional stability constant is very low due to low denticity; the bone uptake of [¹⁵³Gd(DO3A)(H₂O)] is extremely small (0.03% ID/g) (58). Obviously, thermodynamics alone is not sufficient to explain and predict the biological properties of these complexes.

Kinetic Inertness. The term kinetic inertness refers to the rate of dissociation of the radionuclide from the metalloradiopharmaceutical. As noted in the previous sections, dissociation kinetics plays a significant role for the in vivo stability of a metal chelate. While fast dissociation kinetics are characteristic of metal complexes

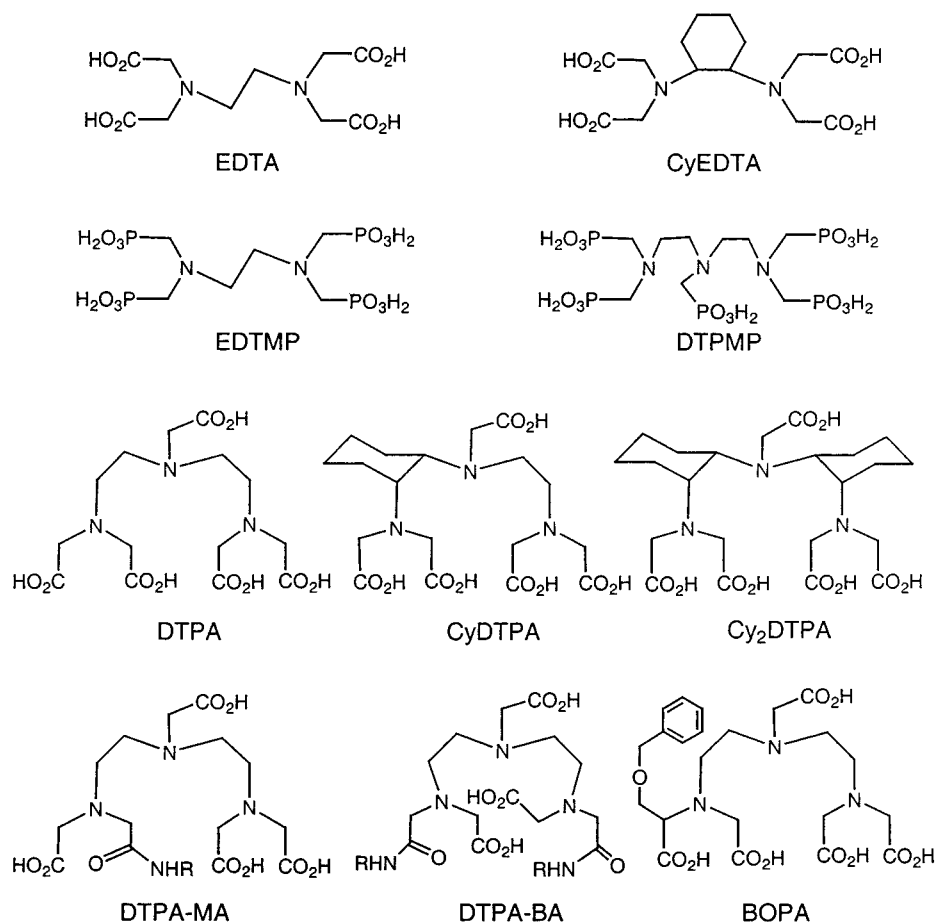


Figure 5. Structures of selected acyclic chelators.

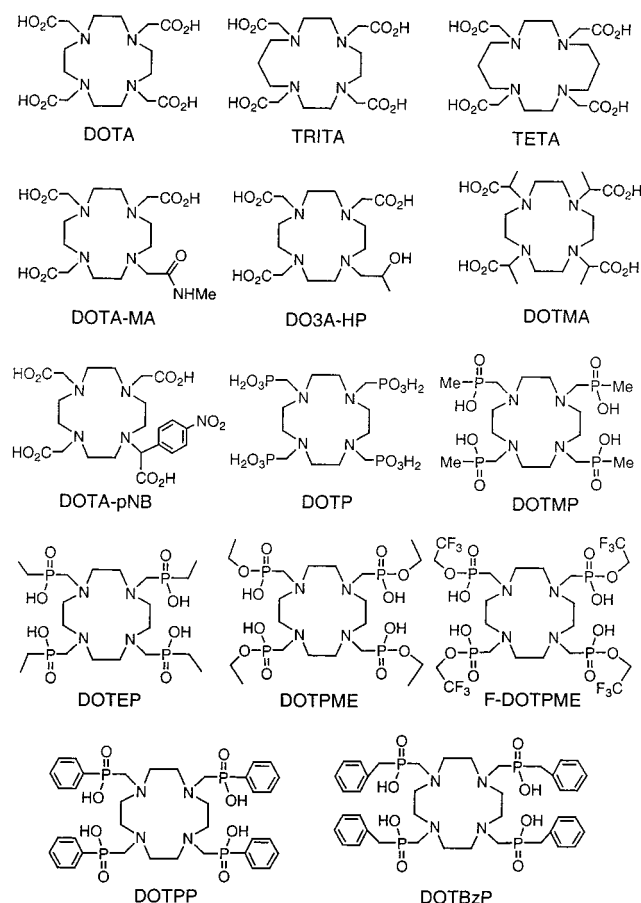
of acyclic chelators (Figure 5), an accumulated body of literature has shown that metal complexes containing macrocyclic chelators (Figure 6) are much more kinetically inert (59–66). This has been clearly demonstrated by the extremely high solution stability of ^{90}Y -labeled macrocycles such as DOTA and DOTMP even though the thermodynamic stability constants of $\text{Y}(\text{DOTA})^-$ and $\text{Y}(\text{DOTMP})^-$ are comparable to that of $\text{Y}(\text{DTPA})^{2-}$.

It should be noted that for metalloradiopharmaceuticals containing a macrocyclic chelator such as DOTA as BFC, acid-catalyzed dissociation of radionuclide from the metal chelate should be minimal in the blood circulation, and transmetalation is not anticipated to be an important mechanism for bone marrow toxicity. Recently, McMurry and co-workers studied ^{89}Y -labeled antibodies with a series of backbone-functionalized DTPA BFCs, and found that the acid-catalyzed dissociation is not the dominant pathway for the in vivo release of ^{89}Y (67). There are many receptors in the bone marrow or on the bone surface for the binding of radiolabeled biomolecules. Some small metal chelates of polyaminocarboxylates, such as HEDTA (*N*-hydroxyethylethylenediamine-*N,N',N'*-triacetic acid), often show very high bone uptake (68–71). The accumulation of radioactivity in the bone may not be caused by the loss of radionuclide from its metal chelate. The overwhelming importance on acid-catalyzed dissociation of radionuclide found in the literature is probably a little oversimplified. Biological studies in different animal models still remain the most appropriate method for evaluating the in vivo stability of the radiopharmaceutical.

The choice of kinetic characteristics of the metal chelate of a BFC is also dependent on the pharmacoki-

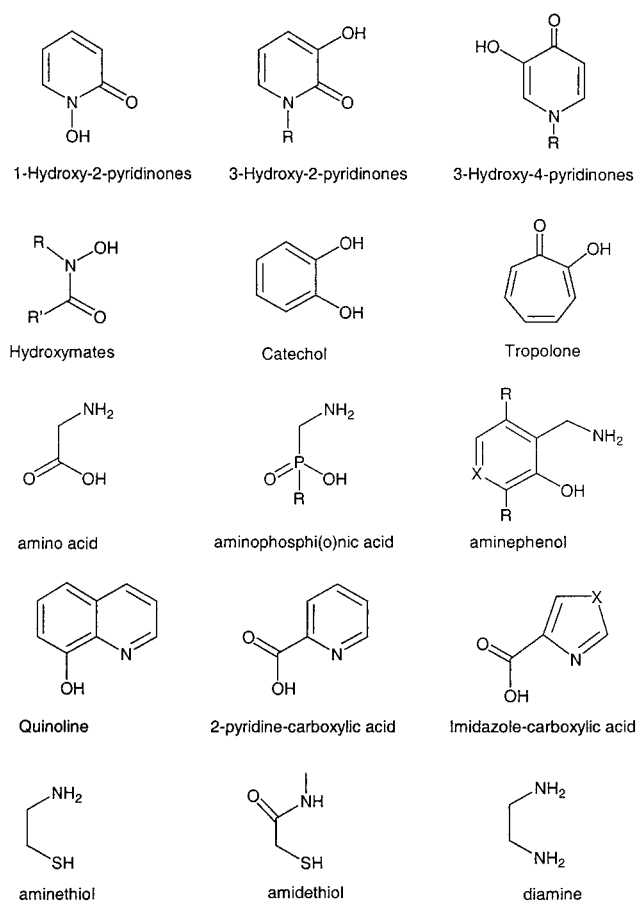
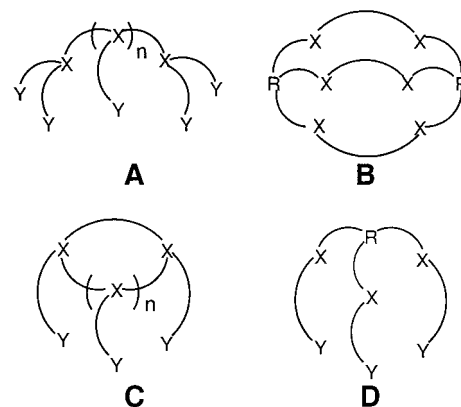
netics of the radiopharmaceutical. For radiolabeled antibodies, that often have long biological half-lives in the blood stream and at the tumor site, and are often metabolized in the liver, the metal chelate must have extremely high thermodynamic stability and kinetic inertness to withstand the competition from metal ions and native chelators in circulation and to tolerate the hepatobiliary metabolism. For radiolabeled small molecules, the biological half-life in the circulation is normally much shorter than that of radiolabeled antibodies. The requirement of kinetic inertness of the BFC metal chelate may not be as demanding. The main goal in choosing a successful BFC is to minimize the in vivo dissociation of radionuclide from the metal chelate of the radiopharmaceutical.

Basic Requirements for BFC. A BFC usually contains three parts: binding unit, ligand framework, and a conjugation group. There are several requirements for an ideal BFC. The BFC has to be able to withstand radiolysis because a large dose of β -radiation can produce very reactive free radicals and result in a significant amount of decomposition of the metal chelate during the manufacturing process and transportation. The BFC must form a metal chelate with high thermodynamic stability and kinetic inertness at neutral pH in order to keep the metal chelate intact under physiological conditions. Decomposition of the metal chelate produces free lanthanide metal ion, which may deposit on the bone and cause bone marrow toxicity. The BFC must form a metal chelate with a minimum number of isomers. The tumor uptake of a radiopharmaceutical depends not only on the receptor binding affinity of the biomolecule but also on pharmacokinetics, which is determined by the physical

**Figure 6.** Structures of selected macrocyclic chelators.

and chemical properties of both the biomolecule and the metal chelate. Formation of isomers may have a significant impact on the biological properties of the radiopharmaceutical. The BFC must have high hydrophilicity, which helps to improve the blood clearance and renal excretion of both the labeled and unlabeled BFC–BM conjugate. Fast renal clearance of the unlabeled BFC–BM will minimize its competition with the radiolabeled BFC–BM for the receptor. Finally, the conjugation group should be easily attached to the biomolecule.

Denticity Requirement. The most common way to increase the thermodynamic stability and kinetic inertness of a metal complex is to use a polydentate chelator. The denticity requirement of a BFC is largely dependent on the size and coordination geometry preference of the metal ion. Yttrium and lanthanide metal ions are large and need eight to nine donor atoms to complete the coordination sphere and form stable complexes with macrocyclic chelators, such as DOTA and its derivatives. It should be emphasized that the denticity requirement of a BFC for therapeutic lanthanide radiopharmaceuticals is not the same as that for MRI contrast agents. For chelators used in lanthanide MRI contrast agents, they are most likely hepta- or octadentate, leaving at least one site open for water ligand exchange to enhance the water proton relaxation rates. For BFCs in radiopharmaceuticals, higher denticity (9, 10) may provide enhanced thermodynamic stability and the improved kinetic inertness, particularly when extra donors are incorporated into a chelating arm attached to the macrocyclic cyclen framework. By adding extra donor atoms, all coordination sites of the lanthanide metal ion are occupied. Kimura et al. (72) studied the thermodynamic and kinetic properties of lanthanide complexes of a series of polydentate

**Figure 7.** Bonding units for building polydentate chelators.**Figure 8.** Ligand frameworks for polydentate chelators. X and Y represent donating heteroatoms incorporated in the ligand framework.

chelators, including DOTA, PEPA, HEHA, and DTPA. It was found that both PEPA and HEHA form Y^{3+} complexes with less kinetic inertness mainly due to the increased flexibility of the macrocyclic chelator framework. Thus, the design of new BFCs should be focused on those using cyclen as the macrocyclic chelator framework.

Bonding Unit and Donor Atoms. A polydentate chelator is built by the appropriate arrangement of the bonding units on a chelator framework. Figure 8 shows several bidentate bonding units, which are often used for building polydentate chelators. In the last two decades, a large number of chelators have been synthesized and studied for their complexation with various metal ions, including catechols, 8-hydroxyquinoline, aminephenols, hydroxamates, aminocarboxylates, aminophosphonates,

and hydroxypyridinones. In general, chelators with two ligating oxygen atoms (catechols, hydroxypyridinones, and hydroxamates) or aminocarboxylates and aminophosphonates have been more widely studied than the other classes.

Catechol binds strongly to trivalent metals such as Fe^{3+} , In^{3+} , and Ln^{3+} (73–78). However, the pK_a values for the two phenol groups are considered too high (9.2 and 13.0). The competition between the metal ion and protons leads to formation of a range of species at neutral or lower pH values. 8-Hydroxyquinoline possesses a high affinity for trivalent cations, but is not selective, as it also binds to Cu^{2+} tightly (78). Hydroxyquinoline is toxic probably due to its extreme lipophilicity. In addition, hydroxylated aromatics are very susceptible to radiolytic decomposition. Therefore, catechol and 8-hydroxyquinoline bonding units are not suitable as BFCs for therapeutic metalloradiopharmaceuticals.

Hydroxypyridinones have been used as substitutes for catechol in drug design (78–80). Like hydroxamates, the hydroxypyridinones are monobasic and form stable metal complexes with hard trivalent metal ions, such as Fe^{3+} , In^{3+} , and Ln^{3+} . Hydroxy-pyridinones have relatively low pK_a values (5–9). For example, the pK_a for 1-hydroxypyridin-2-one is only 5.8. It forms a highly stable ML_3 complex with Fe^{3+} at pH 3–9. They also have high selectivity for trivalent metal ions. As a result, chelators based on hydroxypyridinones have been used for metal detoxification, and their metal complexes have been studied as diagnostic imaging agents. It is surprising that very little information is available on the use of hydroxypyridinones as BFCs. The utility of hydroxypyridinones as binding units for the development of BFCs may be limited by the lipophilicity imparted by the aromatic ring. The same limitations may also apply to pyridinecarboxylates and imidazolecarboxylates. However, the combination use of hydroxypyridinones, pyridinecarboxylates and imidazolecarboxylates with other more hydrophilic bonding units may prove to be useful in designing new BFCs.

The hydroxamate moiety has been widely found in bacteria and fungi siderophores (78–80). The stability constants for 1:1 ML complexes are much lower than the corresponding values for catechols due to the fact that only one oxygen is protonated ($\text{pK}_a = 9.35$) under physiological conditions. As discussed above, the complex formation with metal ions can be considered as a competition reaction with proton, and consequently the charged 1:1 and 1:2 species dominate under acidic conditions even if an excess of hydroxamate is added. At pH 7.0, the 1:3 complex dominates and the stability is further enhanced in the presence of excess ligand. Although polydentate hydroxamate ligands were reported to form highly stable lanthanide complexes (81), there are very few examples of hydroxamate ligands used as BFCs.

Aminocarboxylate groups have been widely used to build polydentate chelators such as EDTA and DTPA (Figure 5). In general, aminocarboxylate groups possess a high affinity toward large cations, particularly In^{3+} . For lanthanide metal ions, EDTA and DTPA are not large enough to completely envelop the metal ion, leaving the remaining coordination sites occupied by water molecules. It should be noted that EDTA has a high affinity for divalent metal ions such as Zn^{2+} , Ca^{2+} , and Cu^{2+} . As a matter of fact, EDTA has higher affinity for Cu^{2+} than it does for La^{3+} and Gd^{3+} . Thus, they are not specific for lanthanide metals (78, 79). High selectivity of polyaminocarboxylate chelators for lanthanide metal ions can be

achieved by using macrocyclic frameworks with eight to nine donor atoms.

Aminophosphonate groups have been reported to have high affinity for hard cations such as Ca^{2+} . Metal complexes of polyaminophosphonates often localize in bone. Due to their high bone uptake, lanthanide complexes of polyaminophosphonates have been studied as therapeutic radiopharmaceuticals for bone-pain palliation and for the treatment of bone cancer metastasis. The complex [^{153}Sm]EDTMP (Quadramet) has recently been approved by FDA for bone pain palliation (82, 83). The complex [^{166}Ho]DOTMP is under clinical investigation for both bone pain palliation and the treatment of bone metastases (84, 85). In general, aminophosphonates form less stable metal complexes than their aminocarboxylate analogues. For example, EDTMP has lower affinity for La^{3+} than EDTA. The localization of metal complexes of polyaminophosphonates at bone and the resulting bone marrow toxicity makes this class of chelators not suitable as BFCs for the radiolabeling of biomolecules with ^{90}Y and lanthanide radionuclides.

Aminethiols and amidethiols form stable complexes with $[\text{Tc}=\text{O}]^{3+}$ and $[\text{Re}=\text{O}]^{3+}$ cores (86, 87). Depending upon nature of the chelator, the technetium and rhenium complexes of diaminedithiols or diamidedithiols can be cationic, neutral, or anionic. Both diaminedithiols and diamidedithiols have been used in BFCs for the radiolabeling of biomolecules with $^{99\text{m}}\text{Tc}$. Polyaminethiols have also been used for coordination of trivalent “hard” metal ions such as Ga^{3+} , Fe^{3+} , In^{3+} , and Ln^{3+} (88–92). Even though thiolate-S is considered as a “soft” donor, polyaminethiols were found to form very stable Ga^{3+} and In^{3+} complexes. It should be noted that the bonding between the trivalent metal ion and the donor atoms is predominantly ionic. Once the thiolate-S is deprotonated, it becomes anionic. Therefore, it is not surprising that polyaminethiols form very stable complexes with trivalent “hard” metal ions such as Ga^{3+} and In^{3+} (88–92).

Ligand Framework. The ligand framework is the spatial arrangement of the bonding units; several examples are shown in Figure 8. Polydentate ligands (panels B–D in Figure 8) with three-dimensional cavities are of particular interest because of their capability to adopt a preorganized conformation in the uncomplexed form. The higher the degree of preorganization of an uncoordinated ligand, the more stable the metal complex. Preorganization of the ligand framework also improves the kinetic inertness, which, as discussed in previous section, is the determining factor for the release of radionuclide from the metalloradiopharmaceutical.

Preorganization minimizes the freedom of motion of the donor atoms and the ligand framework during the complexation process in such a way that the free ligand has a conformation more similar to that in the complex (93–95). Because of the restricted freedom of motion, the loss of entropy in forming the complex is much less, which leads to the increased thermodynamic stability of the metal chelate. Although preorganization is a concept usually applied to macrocyclic and cryptate metal complexes, it is also of some importance for open-chain chelators. For example, metal complexes of CDTA (trans-cyclohexanediaminetetraacetic acid) are often 2–3 orders of magnitude more stable than those of EDTA (ethylenediaminetetraacetic acid) because of the restricted motion of the iminodiacetic chelating arms in CDTA (95).

It should be noted that preorganization of a polydentate chelator results in not only high thermodynamic stability but also increased kinetic inertness of its metal complex. This has been exemplified by the fact that the

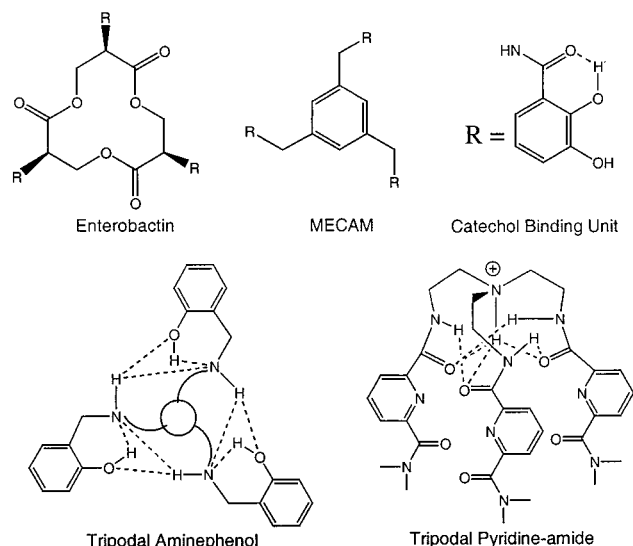


Figure 9. Selected examples of preorganized ligand frameworks.

half-life for $\text{Gd}(\text{DOTA})^-$ in 0.1 M HCl is 60.2 h while the complex $\text{Gd}(\text{DTPA})^{2-}$ having comparable thermodynamic stability decomposes rapidly under acidic conditions ($K_{\text{obs}} = 1.2 \times 10^{-3} \text{ s}^{-1}$; $t_{1/2} \approx 1 \text{ min}$) (61). The highly preorganized macrocyclic framework of DOTA forces the four acetate chelating arms to adopt a conformation that “wraps” the metal ion in an N_4O_4 donor set (96). At the same, it is more difficult for the coordinated acetate to be dissociated from the metal center. Therefore, preorganization should be an important factor in the design of new BFCs for the radiolabeling of biomolecules.

There are several ways to achieve a high degree of preorganization for a polydentate chelator. These include the use of a macrocyclic ligand framework, the use of hydrogen bond(s) to enforce a three-dimensional cavity for metal coordination, and the choice of appropriate chelating arms. Polyaminocarboxylate ligands based on cyclen are known to be well preorganized and form highly stable lanthanide complexes due to the endocyclic orientation of the nitrogen donors. The siderophore enterobactin (Figure 9) forms much more stable Fe^{3+} complex than MECAM because of the cyclic triester ligand framework and hydrogen bonding (97, 98). Tripodal peptides with chiral conformations were found to be stabilized by interstrand hydrogen bonds (99–104). Orvig et al. (105–109) reported a series of tripodal aminephenol ligands and found that hydrogen bonding plays a significant role in the conformation of the uncoordinated ligands. The use of a carbon atom as the bridgehead instead of a tertiary nitrogen atom also limits the motion of the three aminephenol chelating arms (Figure 9). Renaud et al. (110) reported C_3 symmetrical lanthanide podates organized by intramolecular trifurcated hydrogen bonds.

Polyazamacrocycles have been widely used as chelators for a variety of transition metal ions. Macrocyclic BFCs, such as DOTA, are of particular interest for several reasons. The macrocyclic ligand framework is well organized so that they form metal complexes with high thermodynamic stability and kinetic inertness. The pK_a values for the carboxylic groups are in the range 2–5. Lower pK_a values result in less competition from proton, high stability of the metal complex, and minimum acid-assisted demetalation, even under acidic (pH 2) conditions. A functional group for conjugation of a BM can be attached to either one of the four nitrogen donor atoms

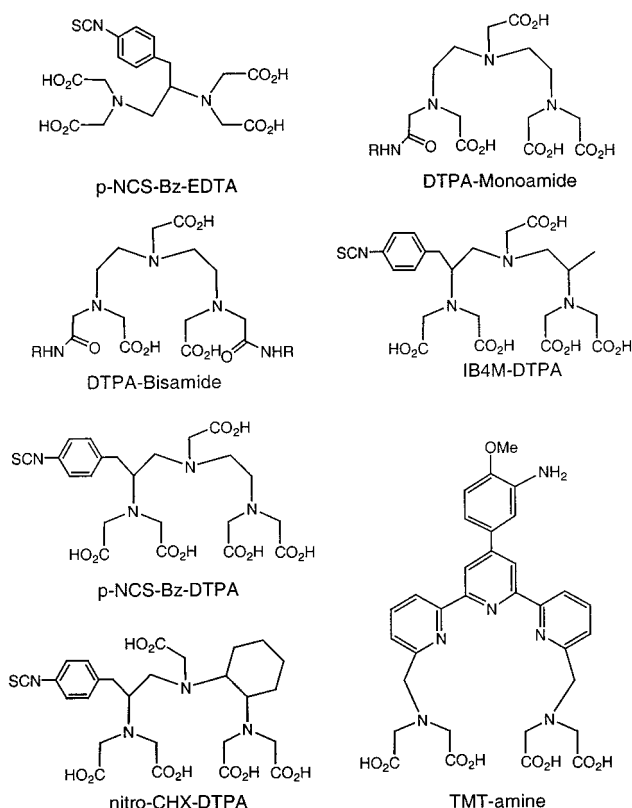
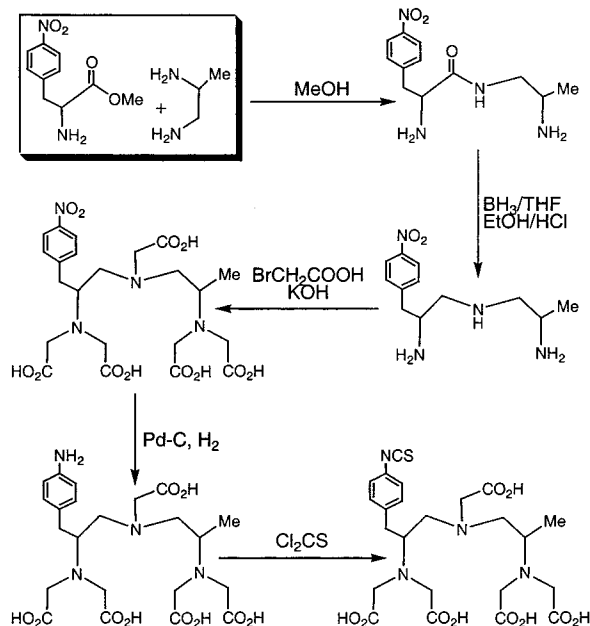


Figure 10. Structures of selected acyclic BFCs.

or one of C-atoms of the macrocyclic backbone. The acetate groups attached to the nitrogen donor atoms have low molecular weight and are very hydrophilic. Therefore, the contribution of the BFC to the overall molecular weight of the BFC–BM conjugate is minimized. The high hydrophilicity of the BFC is will favor faster blood clearance for small molecule radiopharmaceuticals, resulting in reduced competition between the labeled and unlabeled BFC–BM in binding to the targeted receptor. If the biomolecule is an antibody or antibody fragment, attachment of the BFC may not have such a dramatic impact on physical and biological properties of the BFC–protein conjugate as it does for small biomolecules.

Synthetic Strategies for DTPA Analogues. EDTA-based BFCs (Figure 10) were first developed by Sunberg and co-workers in the 1970s (111). Krejcarek and Tucker developed an activated DTPA analogue via a mixed anhydride, which can be linked to proteins (112). The cyclic anhydride of DTPA has also been used by many nuclear medicine researchers since (113). These linear BFCs (Figure 10) bond to a variety of metal ions, such as ^{111}In or ^{90}Y , forming thermodynamically stable metal complexes. A major advantage using DTPA analogues as BFCs is fast labeling kinetics, which is extremely important for antibodies and antibody fragments due to their sensitivity toward heating. However, the metal chelates of linear BFCs are often kinetically labile, which is believed to contribute to the loss of radionuclide for the metal chelate and often leads to significant bone marrow toxicity (114–116).

Considerable efforts have been made to produce polyaminocarboxylate BFCs that form complexes with ^{90}Y with improved thermodynamic stability and kinetic inertness (117–121). As a result, several functionalized DTPA analogues (Figure 10) have been developed as BFCs for the radiolabeling of biomolecules. For example, Gansow and co-workers reported the synthesis of 2-(p-

Chart 1. Synthesis of p-SCN-Bz-DTPA

isothiocyano-benzyl)DTPA (1B-DTPA), in which all eight donor atoms are available for metal ion bonding, and the conjugation group is located on the diethylenetriamine backbone (118, 120).

In general, synthesis (Chart 1) of open-chain polyaminocarbonylate BFCs is straightforward. The most important step is the preparation of the functionalized triamine. The alkylation of the triamine can be achieved in one step by reaction with excess bromoacetic acid in the presence of NaOH or in two steps using *tert*-butyl bromoacetate in DMF in the presence of sodium or potassium carbonate. Following the alkylation, the crude product is purified by cation-exchange chromatography. The major advantage of using *tert*-butyl bromoacetate as the alkylating agent is that the polyester intermediate can be easily purified using reversed-phase chromatography. In both cases, the alkylation step and subsequent steps have to be performed in acid-washed glassware or metal-free plasticware to avoid adventitious sequestering of divalent metals, especially calcium.

Synthetic Strategies for Macrocyclic BFCs. Macrocyclic compounds, $(XCH_2CH_2)_n$ ($X = O, NH$, and S ; n

$= 4, 5$, and 6), have been known for many years. It was not until 1967, however, that crown ether chemistry was really born when Pederson (122) first reported the synthesis and unique cation complexing properties of a number of macrocyclic compounds. Since that time, a number of macrocyclic chelators with a variety of donor atoms have been prepared and investigated for the coordination chemistry with metal ions. Among various macrocyclic chelators, tetraazamacrocycles and their N- or C-substituted derivatives (Figure 11) are of particular interest as BFCs because of the high thermodynamic stability and kinetic inertness of their metal complexes (123–127). Due to large body of literature in this area, it is very difficult to cover all the procedures for synthesis of these macrocycles. In the following section, we will focus on syntheses of some selected tetraazamacrocycles and their N- and C-substituted derivatives.

Richman–Atkins Method. In 1974, Richman and Atkins (128) first reported the synthesis of polyazamacrocycles (Chart 2) by reacting tosylated polyamines with respective dihalogeno or ditosyl fragments in the presence of a base, such as NaH, in DMF at elevated temperatures. This method has been used to produce a variety of tri-, tetra-, penta-, and hexaazamacrocycles. It remains one of the most prevalent synthetic methods for C-functionalized tetraazamacrocycles. When sodium was replaced by tetramethylammonium cation, the yield decreased. It has been suggested that the large tosyl groups tend to restrict free rotation in the starting materials. As a consequence, there is a small internal entropy loss on cyclization, allowing for macrocyclization to occur in good yield (50–80%) even when no apparent preorganization is involved by a template ion. This approach does not require high dilution conditions. Rather concentrated solutions of reactants appear to give the best yields. The yields are solvent-dependent; DMF has always been the solvent of choice and should be dried carefully prior to use (128, 129).

There is usually more than one synthetic route (Chart 2) to produce the same macrocycle. It has been suggested that the use of precursors of similar length is needed for high yields in the cyclization step (122, 128). It seems that the relative stability, solubility, and water-sensitivity of different tosylates and sulfonamides salts are the most significant factors affecting the yield. The choice of preparative method usually depends on the availability

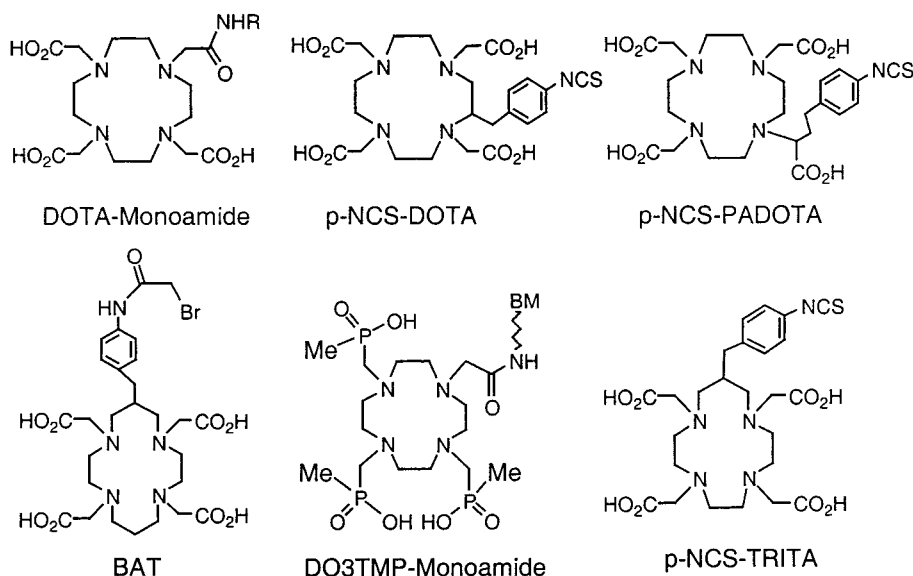
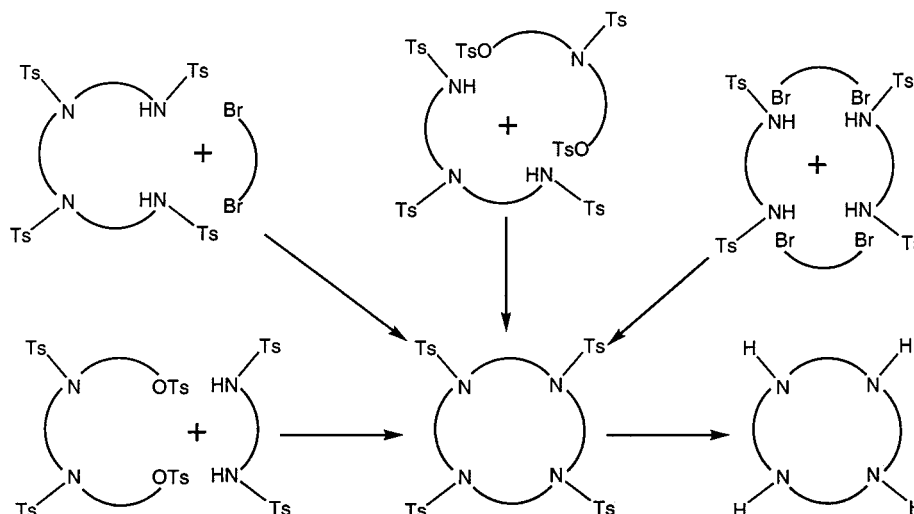
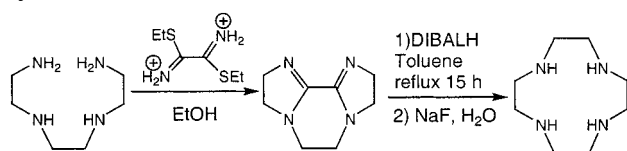
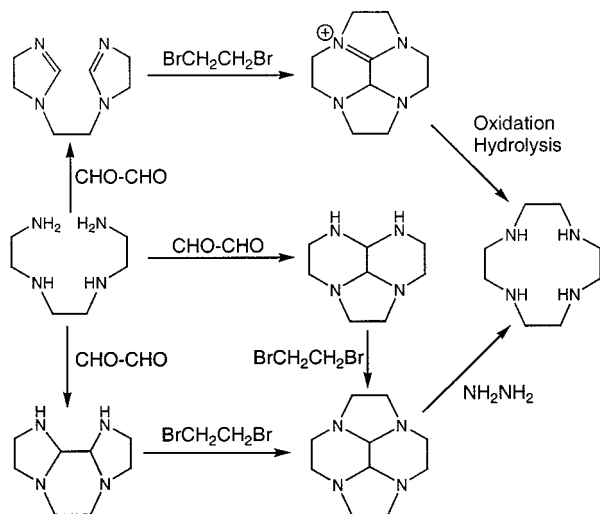
**Figure 11.** Structures of selected macrocyclic BFCs.

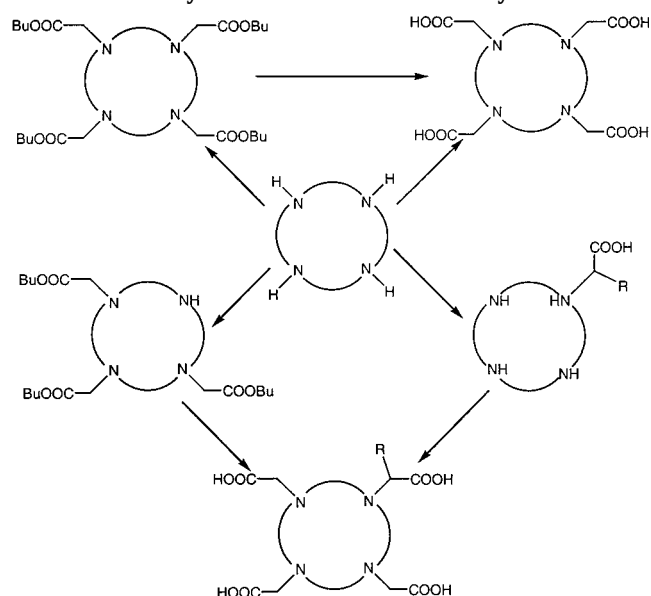
Chart 2. Richman-Atkins Method for the Synthesis of Tetraazamacrocycles**Chart 3. Weisman's Method for the Synthesis of Cyclen****Chart 4. Schiff Base Method for the Synthesis of Cyclen**

and ease of preparation of the precursor diols, amine diols, and polyamines.

Weisman's Methods for the Synthesis of Cyclen.

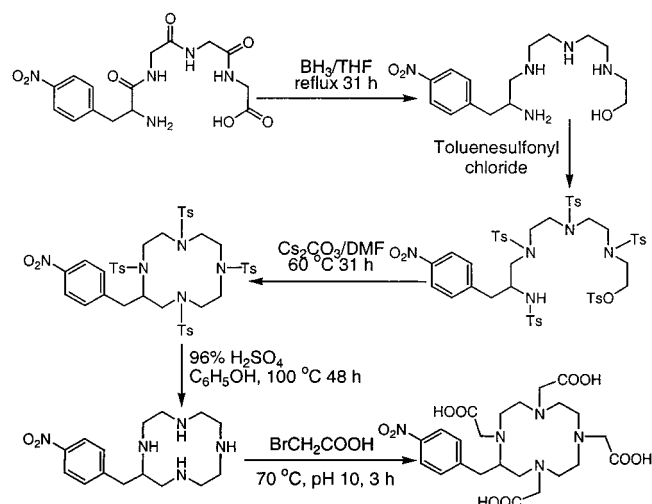
Weisman and Reed (130) recently reported a two-step synthesis of cyclen from triethylenetetraamine and dithiooxamide (Chart 3). This synthetic route is much easier to carry out, and also avoids the use of transition metal templates and the use of a large volume of dry DMF solvent. The starting materials are cheaper and readily available from commercial sources. The overall yield of cyclen is 57% at 100 mg to 5 g scale. Similar approaches (Chart 4) have also been disclosed recently using triethylenetetraamine and glyoxal. In all the cases, cyclen can be prepared in high yield and high quality (131, 132).

N-Functionalized Tetraazamacrocycles. Functionalization of tetraazamacrocycles is much more complicated than that of open-chain triamines. The most widely

Chart 5. N-Alkylation of Tetraazamacrocycles

used method is the alkylation of secondary amine-nitrogen atoms (133–140). However, the resulting product is often a mixture of macrocycles with variable number of substituents depending upon reaction conditions (such as solvent, pH, reaction time, and heating temperature) and the relative ratio of alkylating agent and the tetraazamacrocycle. Chart 5 shows synthesis of some examples of N-functionalized tetraazamacrocycles.

The alkylation (Chart 5) of a tetraazamacrocycle can be achieved by reacting with excess alkyl bromide in an aprotic solvent in the presence of base (e.g., Et_3N). For example, DOTA can be prepared in two steps by reacting with excess *tert*-butyl bromoacetate in DMF in the presence of potassium carbonate or triethylamine. The tetrabutyl ester of DOTA is then hydrolyzed with anhydrous TFA or a mixture of TFA and dichloromethane to give the crude product, which is then purified by cation-exchange chromatography. The advantage of using *tert*-butyl bromoacetate as the alkylating agent is that the tetrabutyl ester can be easily purified using reverse phase chromatography. An important intermediate in the reaction mixture is DO3A-(OEt)₃, which can be selectively prepared by adjusting the relative ratio of cyclen and ethyl bromoacetate (140).

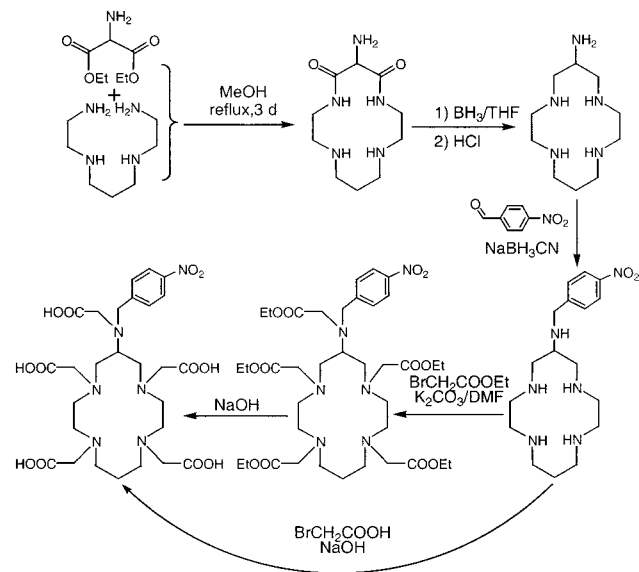
Chart 9. Meares Peptide Method for the Synthesis of *p*-NO₂-BzDOTA

can be easily reduced with excess borane (BH₃) to afford the macrocyclic tetraamine: 6-(4-nitrobenzyl)-1,4,8,11-tetraazacyclotetradecane. Reaction of the macrocyclic tetraamine with bromoacetic acid in the presence of NaOH gives the product: 6-(4-nitrobenzyl)-1,4,8,11-tetraazacyclotetradecane-*N,N',N'',N'''*-tetraacetic acid. The nitro group is then converted to an amino group for conjugation of biomolecules (152, 153).

Meares and co-workers (154) also reported a synthetic route involving formation of a linear tetrapeptide, reduction of the peptide bonds and intramolecular tosylamide ring closure (Chart 9). In this approach, the amino acid starting materials are readily available. The use of amino acids also allows one to vary the side chains. Racemization is unlikely under the conditions involved in the synthesis. Because of Meares' pioneering efforts in this area, many macrocyclic BFCs have been synthesized and studied for their use in the radiolabeling of biomolecules.

Parker and co-workers used the similar approach to prepare a series of C-substituted macrocyclic tetraamines for the radiolabeling of antibodies (155–157). Kimura and co-workers (66) also used the peptide approach to prepare C-functionalized BFCs (Chart 10). First, a dilute solution of diethyl aminomalonate hydrochloride and linear tetraamine in methanol was refluxed for 3 days to give the dioxoaza cyclic amine in ~30% yield. Reduction of the dioxoaza amine with BH₃–THF afforded the corresponding macrocyclic amine, which was then allowed to react with 4-nitro-benzaldehyde in the presence of NaBH₃CN to afford 4-nitrobenzylamino-substituted TRITA or TETA. Moreau and co-workers (158) also used this approach to synthesize 6-(4-aminobenzyl)-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (H₂NBn-TETA).

McMurphy and co-workers (159) used the peptide approach (Chart 11) to prepare the 12-membered tetraaza macrocycle, 2-(4-nitrobenzyl)-1,4,7,10-tetraazacyclododecane, and its N-substituted analogue, 2-(4-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid. The reaction of 4-nitrobenzyl-ethylenediamine with carbamate-protected amino disuccinimido ester under high-dilution conditions afforded the diamide in ~40% yield. The ready availability of carbamate-protected amino diesters from the corresponding aminoacetic acids, as well as the substituted diamine, makes this an efficient route for the construction of C-functionalized tetraaza macrocycles. Deprotection of the Boc groups was achieved by stirring the macrocycle in anhydrous HCl(g)/dioxane.

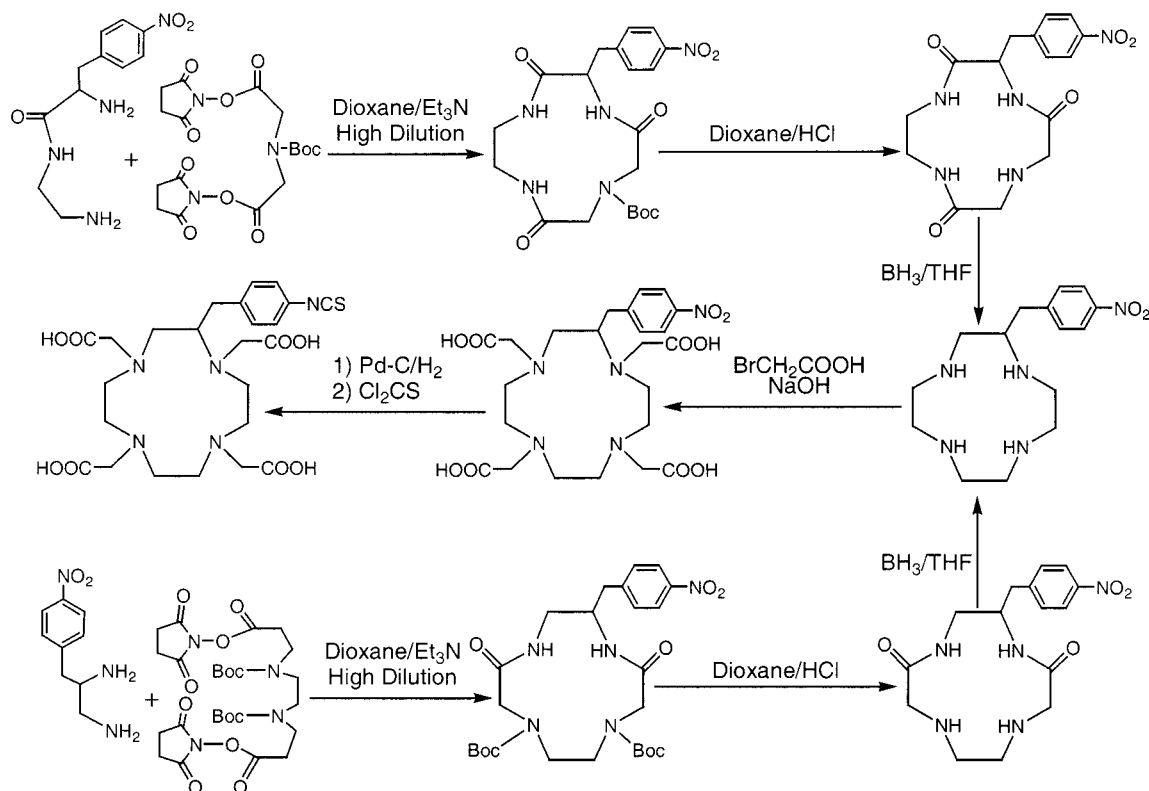
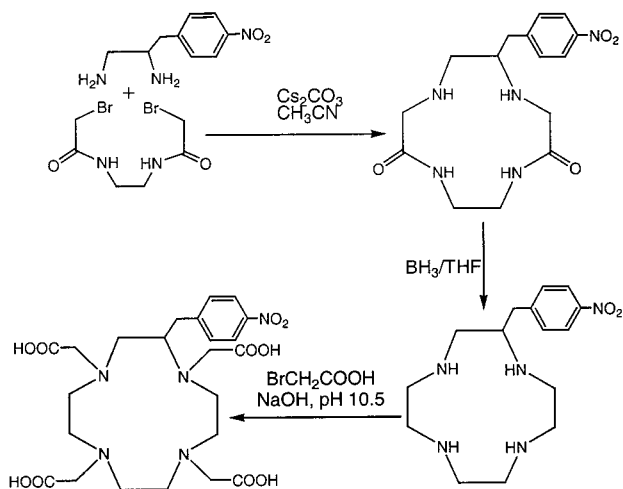
Chart 10. Kimura Peptide Method for the Synthesis of Tetraazamacrocycles

Reduction of the macrocyclic diamide produces the corresponding macrocyclic tetraamine. The 12-membered macrocyclic tetraamine was also prepared by an independent route employing the same cyclization chemistry but with a different diamine starting material (Chart 11). The cyclic diaminediamide was reduced with excess borane (BH₃) to produce the macrocyclic tetraamine, 2-(4-nitrobenzyl)-1,4,7,10-tetraazacyclotetradecane. Alkylation of the macrocyclic tetraamine to form the corresponding aminocarboxylate was accomplished in one step by using bromoacetic acid (pH 8.5) or in two steps by using *tert*-butyl bromoacetate (Na₂CO₃/DMF) followed by deprotection with TFA.

Mishra and co-workers (160) recently reported a new approach for the synthesis of 2-(4-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (Chart 12). This approach is a combination of the Richman-Atkins' method and peptide method. Reaction of 4-nitrobenzylethylenediamine with *N,N'*-bis(bromoacetyl)-ethylenediamine in the presence of cesium carbonate afforded the diamide in ~40% yield. High dilution conditions were not required for the reaction. The cyclic diaminediamide was easily reduced with excess borane (BH₃) to afford the corresponding macrocyclic tetraamine, 2-(4-nitrobenzyl)-1,4,7,10-tetraazacyclotetradecane. Reaction of the macrocyclic tetraamine with bromoacetic acid in the presence of a base afforded 6-(4-nitrobenzyl)-1,4,8,11-tetraazacyclotetradecane-*N,N',N'',N'''*-tetraacetic acid. The crude product was purified using a reverse phase HPLC method.

Isomerism of Lanthanide Chelates. Stereochemical nonrigidity is characteristic of metal complexes with coordination number of 7 or greater; there is very little energy barrier between different coordination geometries. However, the use of a polydentate chelator makes it difficult for different isomers to interconvert. As a result, it is not uncommon that metal complexes with coordination number of 7 or greater show isomerism both in the solid state and in solution.

In the past decade, many acyclic and macrocyclic BFCs have been used for the radiolabeling of antibodies and peptides. Very little information is available on isomerism of the metal chelate. In many cases, ITLC is the only analytical tool for the final product analysis. Radio-HPLC chromatograms are not presented nor are the HPLC

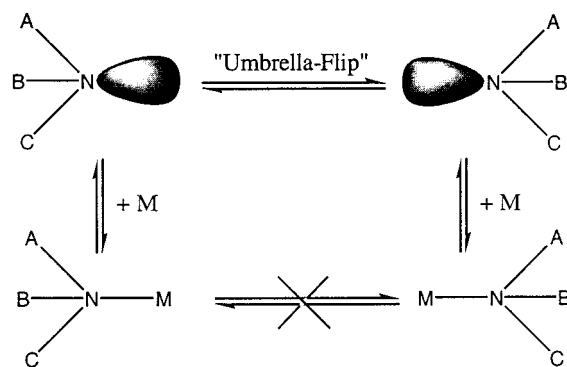
Chart 11. McMurtry Method for the Synthesis of *p*-SCN-Bz-DOTA**Chart 12. Mishra Peptide Method for the Synthesis of *p*-NH₂-Bz-DOTA**

methods used for the characterization of the radiolabeled biomolecule. Therefore, it is not surprising that there has been no detailed discussion about isomerism and the impact of isomerism on the biological properties of the radiolabeled biomolecule.

For radiolabeled antibodies, the attachment of the BFC and the radionuclide does not disturb the tertiary structure of the polypeptide; thereby, the biological and receptor binding properties are not significantly affected. For small biomolecules, however, the metal chelate (radionuclide and BFC) contributes greatly to the overall size and molecular weight of the radiopharmaceutical. Isomerism may have a significant impact on the biological performance (receptor binding and biodistribution) since the distribution of the radiopharmaceutical is dependent on the physical and chemical properties of both the biomolecule and the metal chelate.

Solid state structures provide a wealth of information about the coordination chemistry of a specific chelating system. However, the solution structure may be different from the solid state structure because of possible dissociation of certain donor atom or due to the coordination of water molecules. The isomerism observed in the solid state structure may not be seen in solution due to interconversion of isomers or fluxionality of the ligand framework. On other occasions, only one isomeric form is found in the solid state due to the crystal packing effect, and more isomers are observed in solution. Thus, it is imperative to confirm the solution structure in order to understand the biological properties of a metalloradiopharmaceutical. Recently, Caravan et al. (60) has reviewed extensively both the solid state and solution structures of lanthanide complexes of various acyclic and macrocyclic chelators. In this review, we only discuss some specific topics associated with the isomerism of lanthanide complexes of bifunctional polyaminocarboxylate chelators.

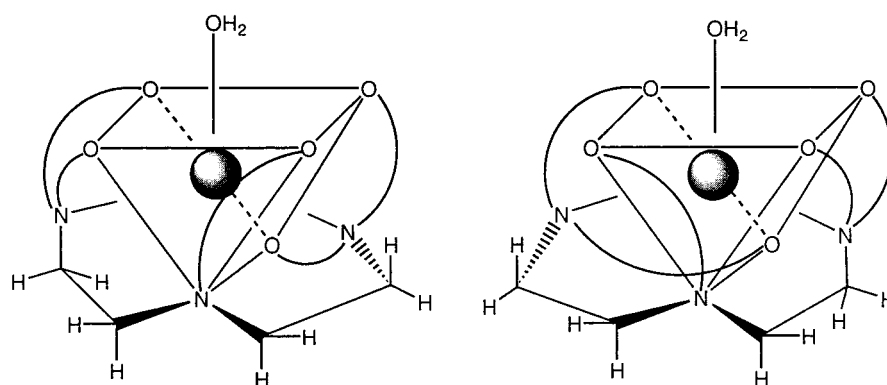
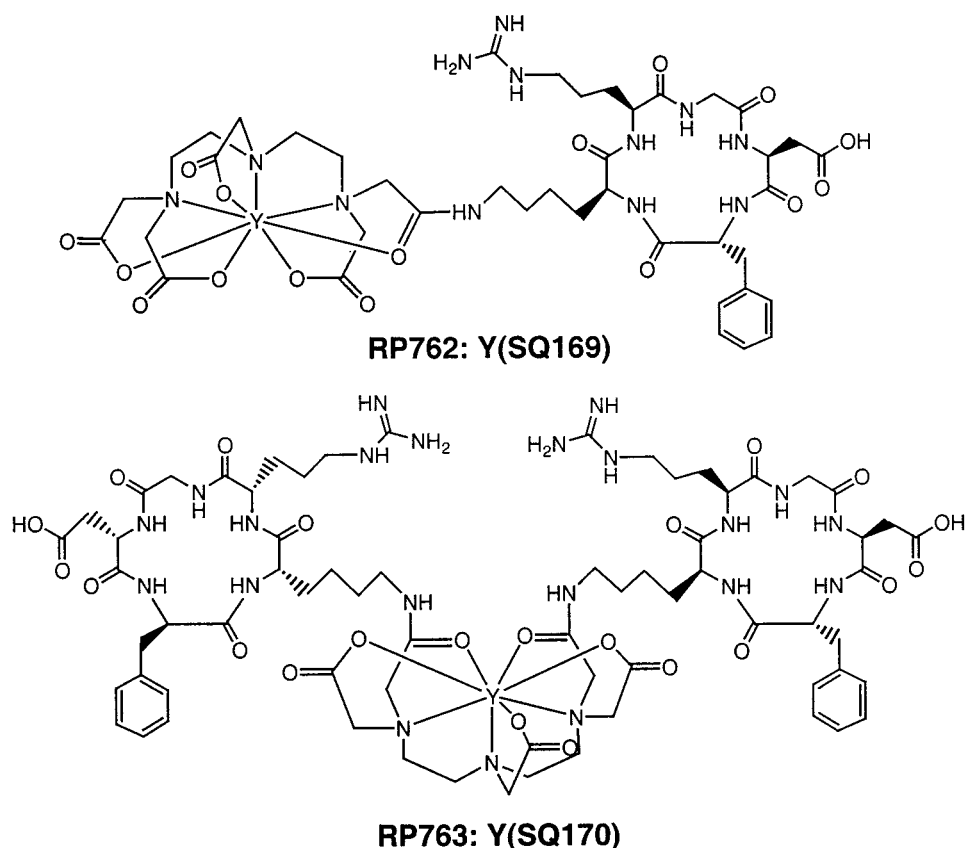
Inversion at the Coordinated Nitrogen. The pyramidal environment at coordinated amine-nitrogen atoms has been conclusively established by X-ray methods (161). Rapid inversion at monodentate amine nitrogen atoms is very common (Chart 13). Once the nitrogen atom in a polydentate chelator is bonded to the metal ion, the inversion becomes more difficult and involves dissociation and reformation of the coordination bond. Therefore, coordination of amine-nitrogen often results in formation of isomers in yttrium and lanthanide complexes of polyaminocarboxylate chelators. For example, DTPA, DTPA-MA, and DTPA-BMA contain three amine-nitrogen donors. In principle, this will result in formation of eight possible isomers. In solid state, only some of them were observed by X-ray crystallography (162–169). In solution, all eight isomers have been observed by NMR at low temperature (162–172). Some of them interconvert via a partial dissociation at elevated

Chart 13. Inversion at the Coordinated and Uncoordinated Amine-Nitrogen

temperatures. For example, the proton NMR spectra of the Pr^{3+} and Eu^{3+} complexes of DTPA show that at room temperature there are only two main isomers (Figure 12), which undergo rapid exchange in solution at tempera-

tures higher than 95°C (162, 163). For the Lu^{3+} chelate of DTPA-BMA, however, the temperature for rapid exchange of two isomers is lower (85°C), reflecting the weak bonding of carbonyl-oxygen as compared to carboxylate-oxygen donors (171, 172). The major feature of the exchange process involves the shuffling of coordinated acetate, accompanied by a flip of the backbone ethylene bridges between staggered conformations.

If the biomolecule in DTPA-monoamide is stereochemically pure, the yttrium complex will have the same number of isomers as the Y-DTPA complex. Recently, we used a reversed-phase HPLC method to characterize two DTPA-peptide conjugates (SQ169 and SQ170) (173). It was found that the ^{90}Y complex, RP762 (Figure 13), shows two radiometric peaks (Figure 14), suggesting that there are two detectable isomeric forms (A and B) in solution at room temperature. The formation of RP762 at room temperature is almost instantaneous, and the initial peak ratio is 50:50. The peak ratio changes over time until the equilibrium is reached with the peak

**Figure 12.** Two major isomers for lanthanide complexes of DTPA.**Figure 13.** Structures of two ^{90}Y -labeled vitronectin receptor antagonists: RP762 (top) and RP763 (bottom).

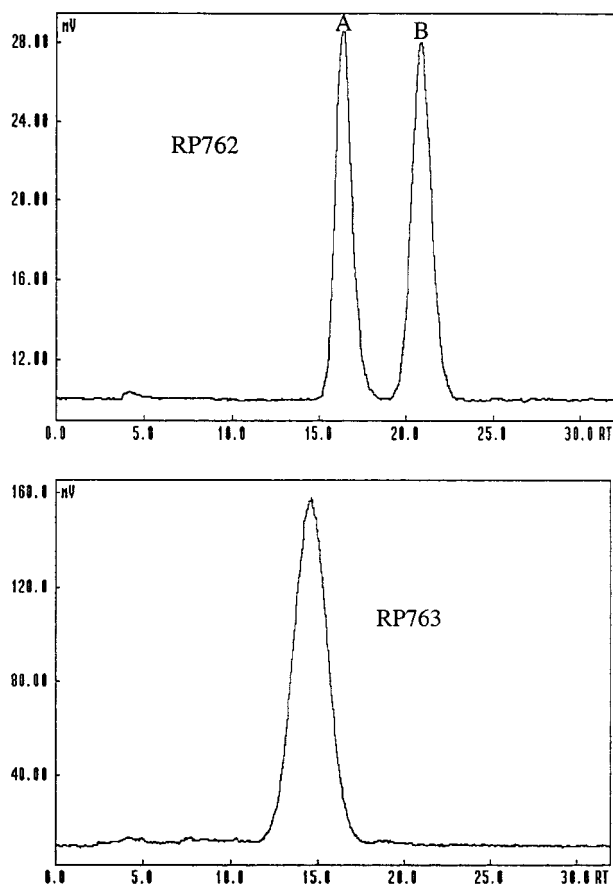


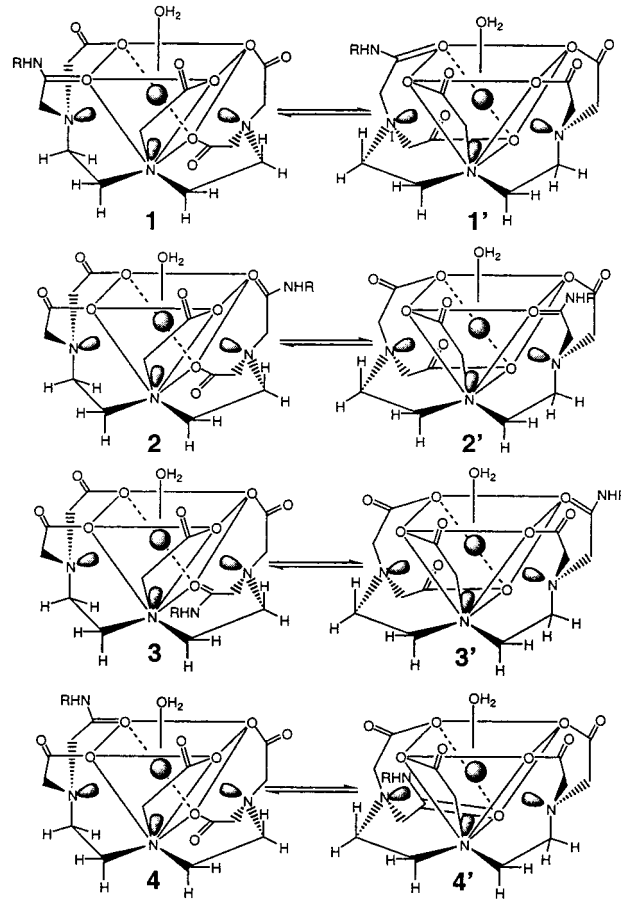
Figure 14. Typical radio-HPLC chromatograms of RP762 (top) and RP763 (bottom). The y-axis represents the signal intensity while the x-axis indicates retention time of a radiolabeled compound (data from ref 173).

A/peak B ratio of 48:52. The rate of interconversion is slow at room temperature. At a higher temperature (54 °C), the rate of interconversion is accelerated, and it took about 6.5 h for peak A to reach the 48:52 (peak A/peak B) ratio. However, it took a much longer time (>7.5 h) for peak B to reach the equilibrium, suggesting that isomer B is thermodynamically more stable. Both peak A and peak B remained stable in the HPLC mobile phase (containing ~10% acetonitrile) at 54 °C for more than 6 h, suggesting that interconversion of these two isomers is intramolecular.

A slightly different mobile phase was used to analyze RP763 (Figure 13) due to its higher lipophilicity (173). RP763 shows only one broad peak with the half-width of ~3 min (Figure 14). There are two possibilities for the large half-width of the peak. The first possibility is that the peak is a combination of several unresolved peaks. If that were true, it would have been possible to separate them using a long gradient method or isocratic chromatographic conditions. We tried several different gradients, and found that RP763 always show one broad peak with different half-widths depending upon the HPLC chromatographic conditions. The second possibility is that there is a rapid interconversion between different isomers at room temperature.

In general, the lanthanide complexes of DTPA analogues assume distorted tricapped trigonal prism (TTP) geometries. In the TTP arrangement, the neutral donor atoms with longer bond length will prefer to occupy face capping position rather than a prismatic corner. However, since it is not possible for all three amine-nitrogen donors to occupy capping positions, the central nitrogen

Chart 14. Eight Possible Isomers of the Complex Y(DTPA-Monoamide)

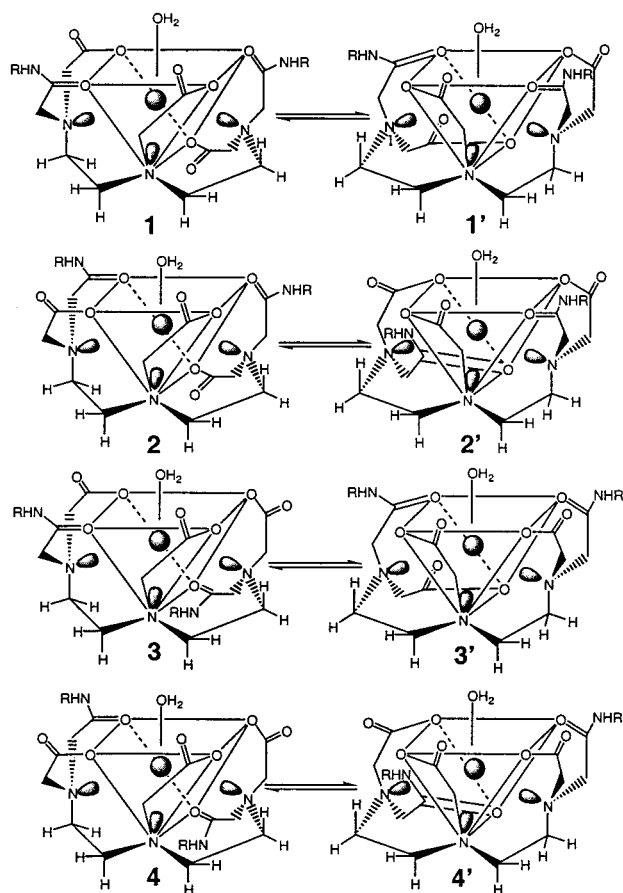


atom tends to occupy a prismatic corner (Figure 12) while the two terminal amine-nitrogen atoms occupy capping positions. Because of distortion, the actual coordination geometry is, in most cases, between TTP and monocapped square antiprism (CSAP).

^1H and ^{13}C NMR studies of lanthanide complexes of DTPA and DTPA-bisamide derivatives have revealed a rapid interconversion of two wrapping isomers (Figure 12). It has been proposed that the rapid exchange process involves "wagging" of the diethylenetriamine backbone and shuffling of coordinated acetate groups. Inversion at the coordinated nitrogen atoms has been excluded due to the fact that the ^1H signals of methylene hydrogens of the acetate and acetamide groups remains as AB quartets even at 85 °C (165, 166). This strongly suggests that the energy barrier for the inversion at coordinated amine-nitrogen donors is much higher than that for interconversion of two "wrapping isomers".

It should be noted that the concentration of the lanthanide complexes for NMR studies ranged from 0.05 to 0.2 M. Dissociation of acetate-oxygen and amine-nitrogen donors is not significant. At the tracer level, however, the concentration of RP762 in the collected HPLC mobile phase is only $\sim 10^{-10}$ M. At this concentration, it is quite possible for acetate-oxygen, carbonyl-oxygen, and amine-nitrogen donors to become dissociated. Partial dissociation of these donor atoms makes the interconversion between "wrapping isomers" much easier. That may account for the different solution characteristics of lanthanide complexes of DTPA-MA and DTPA-BA at different concentration levels.

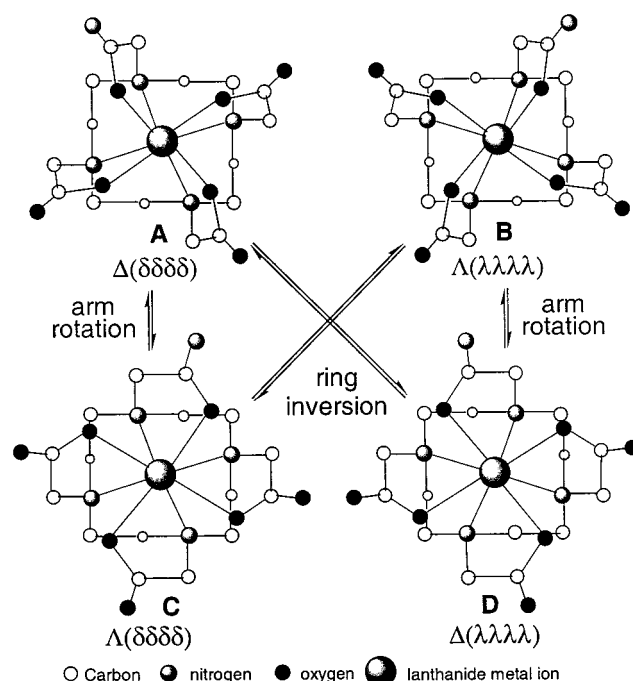
Among eight possible isomers of Y(DTPA-monoamide) (Chart 14), four of them are enantiomers. If one assumes

Chart 15. Eight Possible Isomers of the Complex Y(DTPA-Bisamide)

that the rapid interconversion occurs between “wrapping isomers” at room temperature, isomer **1** is readily converted to **1'** via “shuffling” of the two NO₂ donor sets and “wagging” of the diethylenetriamine backbone. The same is true for **2** to **2'**. Since **1'/2** and **2'/1** are the two enantiomer pairs, these four isomers are not separable by radio-HPLC, and are expected to appear as single peak while the remaining four isomers (**3**, **3'**, **4**, and **4'**) as another single peak in the HPLC chromatogram. This is completely consistent with the presence of two radiometric peaks in the HPLC chromatogram of RP762 (Figure 14).

It should be noted that the relative orientation of the acetamide group to the acetate arm at the central nitrogen atom is different (cis and trans, respectively) in isomers **1** and **4** (Chart 14). The same is true for isomers **1'** and **4'**, **2** and **3**, **2'** and **3'**. For cis and trans isomers to interconvert, inversion at the terminal amine-nitrogen atoms has to occur. This suggests that the NO₂ donor set have to be dissociated and accompanied by a rapid “umbrella-type” inversion at the nitrogen atom. That explains the fact that both radiometric peaks of RP762 interconvert and the rate of interconversion is temperature dependent. Since RP762 remains stable for at least 6 h at 54 °C without a significant decomposition, it is reasonable to assume that the remaining part of the DTPA-monoamide conjugate remains firmly bonded to Y(III) when one of the two NO₂ donor sets undergoes a rapid “umbrella-type” inversion at the terminal nitrogen atom.

SQ170 is a DTPA-bisamide and bonds to yttrium(III) as an octadentate chelator using two carbonyl-oxygen, three amine-nitrogen, and three acetate-oxygen donor

**Figure 15.** Four possible isomers of lanthanide complexes of DOTA.

atoms. Due to the chirality of the three coordinated amine-nitrogen atoms, DTPA-bisamide forms Y(III) complexes with eight isomers (four pairs of enantiomers in Chart 15). Compared to acetate-oxygen, the carbonyl-oxygen is a weak donor for yttrium(III). Thus, it is easier for the two NO₂ donor sets to be dissociated in solution, which results in faster interconversion between “wrapping isomers” and inversion at coordinated amine-nitrogen atoms. As a result, RP763 shows only one broad radiometric peak in its HPLC chromatogram (Figure 14).

Chiral Centers at Carbon Atoms. Substitution at carbon atoms of the chelator backbone or acetate chelating arms results in formation of more isomers due to the presence of stereogenic carbon at the point of substitution. For example, BOPTA has a benzyloxymethyl (BOM) group at one acetate group, which in combination with three prochiral amine-nitrogen atoms are expected to form 16 possible stereoisomers for lanthanide complexes (174). The introduction of chiral centers of equal configuration (*RRRR* or *SSSS*) to one or all four acetate arms of DOTA results in four pairs of diastereomers upon chelation to lanthanide metals. Two isomers were also observed for the *RRRR*-DOTMA by the proton NMR and high-resolution luminescence of the Eu³⁺ complex. A rapid exchange process between the two isomers was established by ¹H EXSY (175).

DOTA and Its Derivatives. In the solid state, lanthanide complexes of DOTA are often nine-coordinate with four amine-nitrogen and four carboxylate-oxygen atoms arranged in a square antiprismatic geometry and the monodentate water-O capping the face of the four carboxylate-oxygen donors (176–182). In principle, there are four possible isomers (panels A–D in Figure 15) for lanthanide metal complexes of DOTA. Isomers A and B are enantiomers and indistinguishable by NMR. Isomers C and D represent the other pair of enantiomers. Isomers A and B are close to square prismatic with the acetate arms in a different layout and the cyclododecane ring conformation being identical to those of C and D. Interconversion within each enantiomeric pair (A ↔ B or C ↔ D) occurs through inversion of the ethylenic groups and

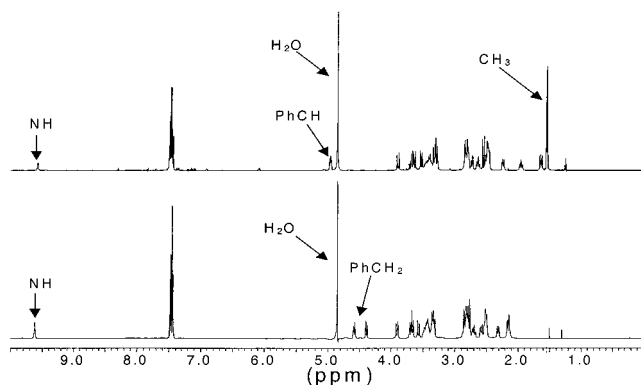


Figure 16. Room temperature ^1H NMR spectra of complexes Y(DOTA-MBA) (top) and Y(DOTA-BA) in 90% D_2O .

concerted or stepwise rotation of the acetate arms (180). This process is easily observed in the VT ^{13}C NMR spectra since it results in the equilibration at high temperatures of the two distinct resonances for the ethylenic carbons. On the other hand, the exchange between diastereomers may take place either by rotation of the acetate arms while maintaining the same conformation of the macrocyclic ring or by inversion of the ethylenic groups without changing the orientation of the acetate arms.

Recently, we prepared two model compounds, Y(DOTA-MBA) (MBA = methyl-benzylamine) and Y(DOTA-BA) (BA = benzylamine), for the radiolabeled DOTA-BM conjugates (183). Theoretically, DOTA-MBA should form lanthanide complexes with four possible isomers. Unlike those of lanthanide DOTA complexes, these four possible isomers are no longer enantiomers but diastereomers, which are expected to show distinctive NMR spectra. The lack of axial symmetry removes the signal degeneracy in the NMR spectra, and a large number of resonance signals are expected for each isomer. However, the ^1H (Figure 16) and ^{13}C NMR data show clearly that the complex Y(DOTA-MBA) exists predominantly as one isomer in solution. There are some small satellite peaks on either the right or left side of each main peak, suggesting that there may be a minor isomer for Y(DOTA-MBA). The percentage of the minor isomer is so low that it is barely detectable by both ^1H and ^{13}C NMR. For the complex Y(DOTA-BA), we were supposed to observe only one pair of enantiomers as demonstrated by the ^1H NMR (Figure 16) since the complex does not contain any chiral centers. We also prepared the corresponding ^{90}Y complexes, $^{90}\text{Y}(\text{DOTA-MBA})$ and $^{90}\text{Y}(\text{DOTA-BA})$. Using a reversed phase HPLC method (183), we found that both complexes showed only one radiometric peak in their radio-HPLC chromatograms (Figure 17). This is completely consistent with results obtained by ^1H and ^{13}C NMR and suggests that both complexes exist in solution as only one predominant isomer.

Introduction of a substituent at the ethylenic carbon or one of the four acetate arms will result in an extra chiral center and double the number of isomers. For example, DOTA-pNB has a *p*-nitrophenyl substituent attached to one of the four acetate arms (184). Theoretically, it should form lanthanide complexes with eight possible isomers. However, an NMR study shows that the complex $\text{La}(\text{DOTA-pNB})^-$ exists in solution as only one isomer while complexes $\text{Ho}(\text{DOTA-pNB})^-$ and $\text{Ho}(\text{DOTA-pNB})^-$ exist in two isomeric forms with a relative ratio of 88:12 for $\text{Ho}(\text{DOTA-pNB})^-$ and ~3:1 for $\text{Yb}(\text{DOTA-pNB})^-$. The relative ratio of the two isomers for the lanthanide complexes of DOTA and DOTA-pNB varies

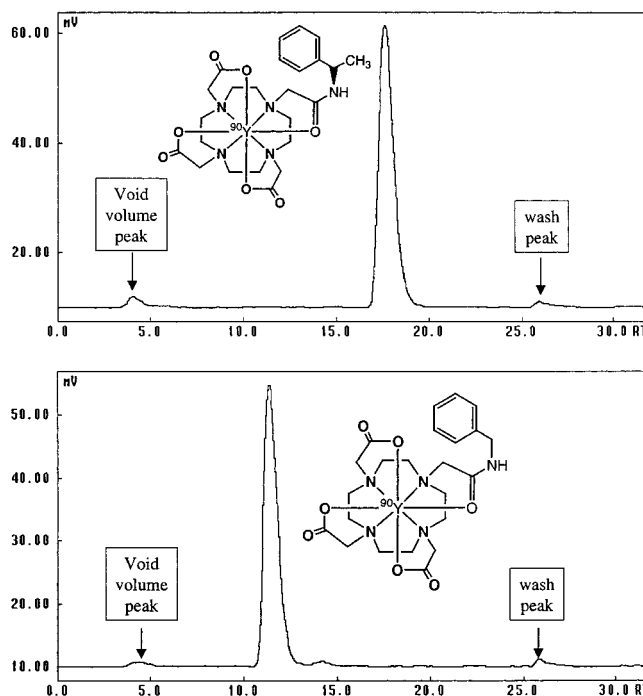


Figure 17. Typical radio-HPLC chromatograms of ^{90}Y -DOTA-MBA (top) and ^{90}Y -DOTA-BA (bottom). The y-axis represents the signal intensity while the x-axis indicates retention time of a radiolabeled compound (data from ref 183).

from metal to metal. There is not a general trend among the lanthanide metals.

DOTP and DOTMP Analogues. Like DOTA, DOTP, and DOTMP also form highly stable lanthanide complexes in which the phosphonate and phosphinate chelating groups are orientated clockwise or counterclockwise. The phosphonate- or phosphinate-P atom is chiral. The coordination of four pendant chelating arms results in a chiral center at each phosphorus. Six diastereomers are possible, RRRR, RRRS, RRSS, RSRS, RSSS, and SSSS, each having two possible wrapping isomers resulting in six enantiomer pairs (185). ^{19}F NMR spectra of the lanthanide complexes of fluorinated phosphonate ester, F-DOTPM, revealed up to 16 resolved ^{19}F NMR resonance signals, consistent with formation of all six possible diastereomers (186). In contrast, the ^1H NMR spectra of Y^{3+} , Yb^{3+} , and Eu^{3+} complexes of the tetraphosphinate DOTBzP showed only one species in solution, with no fluxional behavior observed over a temperature range of 5–80 $^\circ\text{C}$ (187).

Attachment Position of Biomolecules. Generally, there are three possible approaches to attach a biomolecule to DOTA. In the first approach, the attachment is at one of the carbon atoms of the macrocyclic chelator backbone (Figure 18). In principle, this will result in formation of eight possible isomers when it is coordinated to the lanthanide metal. In the second approach, the linker is attached to one of four acetate chelating arms. This may also result in formation of eight possible isomers when it is bonded to the metal center. In both cases, the conjugation of the biomolecule does not lead to a significant change in the thermodynamic stability and kinetic inertness of the metal complex as compared to those of the DOTA complex. In the third approach, the biomolecule is conjugated to one of the four acetate groups via a CO-NH amide bond. Compared to the carboxylate-oxygen, the carbonyl-oxygen is a relatively weak donor for yttrium and lanthanide metal ions. This is consistent with the lower thermodynamic stability of

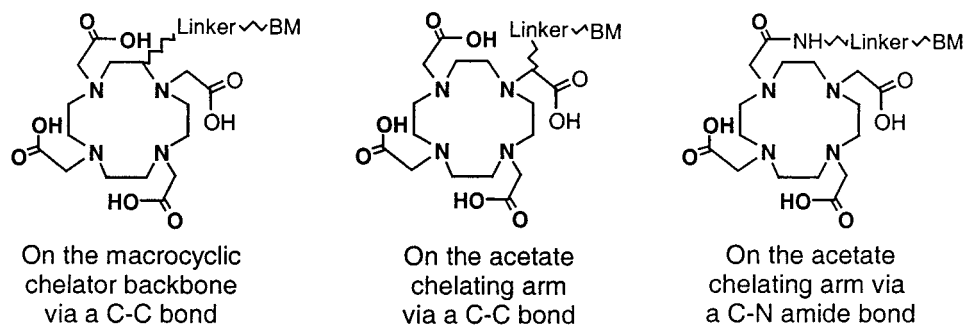


Figure 18. Three possible approaches to attach a biomolecule to a DOTA chelator.

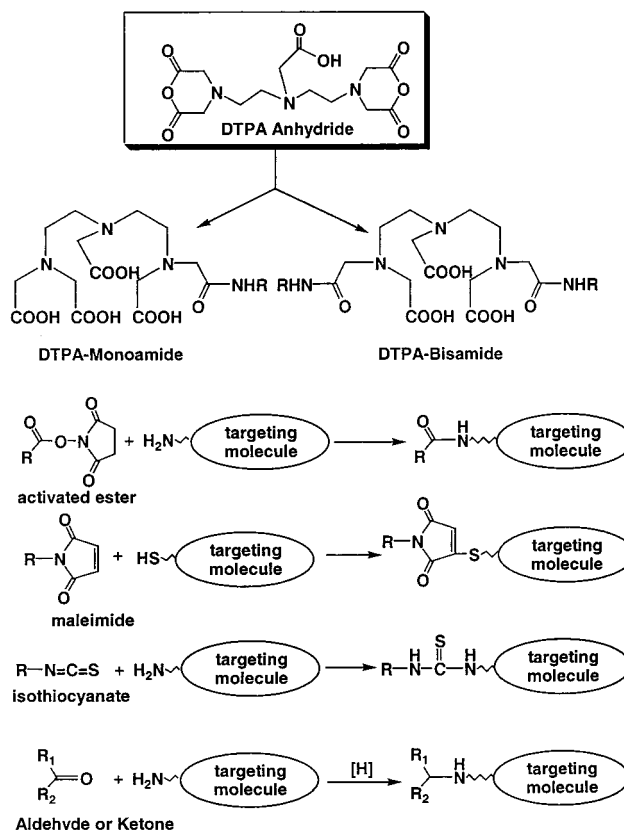
the lanthanide complex (e.g., $K_{Y-DOTA-monoamide} \approx 21.8$; $K_{Y-DOTA} = 23.5$) (60, 188). However, the kinetic inertness of the lanthanide complex remains relatively unchanged as evidenced by the high solution stability of radiolabeled DOTA-BM conjugates.

Conjugation Groups. In the last two decades, a number of conjugation techniques have been developed for modification of proteins, DNA, and other biologically active molecules. There are many conjugation groups useful for attachment of a BFC to a biomolecule. These include the activated disulfide, diazobenzene, acid chloride or anhydride, bromo- or iodoacetamide, isothiocyanate, *N*-hydroxysuccinimide (NHS) ester, aldehyde/ketone, and maleimide. These conjugation groups are electrophiles, which require a nucleophile functionality in the biomolecule. In some instances, the biomolecule of interest contains only electrophilic functionality (e.g., the carboxylic acid), further synthetic elaboration is needed. In these cases, a bis-nucleophilic reagent, such as ethylenediamine or propylenediamine, is often used to convert the electrophilic functionality into a nucleophilic group. These reactive groups, whether they are naturally part of the biomolecule or are artificially introduced, can serve as "handles" for conjugation of a BFC. The selection of the conjugation group is largely dependent on the nature of the handle on the biomolecule. Very often the handle is a primary amine or a thiol group. The functional groups reactive toward primary amines include DTPA dianhydride, NHS-activated esters, isothiocyanates, and aldehyde/ketone, while maleimide is very reactive to the thiol functionality.

DTPA Anhydride. A major advantage of using DTPA as BFC is that DTPA dianhydride is commercially available and reacts readily with primary amines to form the DTPA-biomolecule conjugate. The reaction can be performed in both aqueous and nonaqueous media. There are two possible products: DTPA-monoamide and DTPA-bisamide (Chart 16). The cross-linking between two macromolecules (antibodies and antibody fragments) is disadvantageous, and may have dramatic impact of the biological and immunogenic properties of the DTPA-biomolecule conjugate. For small biomolecules, however, the cross-linking may prove to be beneficial for the improved receptor binding kinetics because simultaneous binding of two biomolecules on adjacent receptor sites will result in a slow dissociation of the receptor ligand. Asymmetric anhydrides of DTPA and DOTA have also been used for the synthesis of the corresponding bioconjugates (188, 189). However, the yield is often very low.

The NHS-activated esters (Chart 16) have intermediate reactivity toward amines, with high selectivity for aliphatic amines. The optimum pH for reaction in aqueous systems is 8.0–9.0. Virtually any molecule that contains a carboxylic group can be converted into its NHS ester, making NHS-activated ester groups among the

Chart 16. Several Important Conjugation Groups

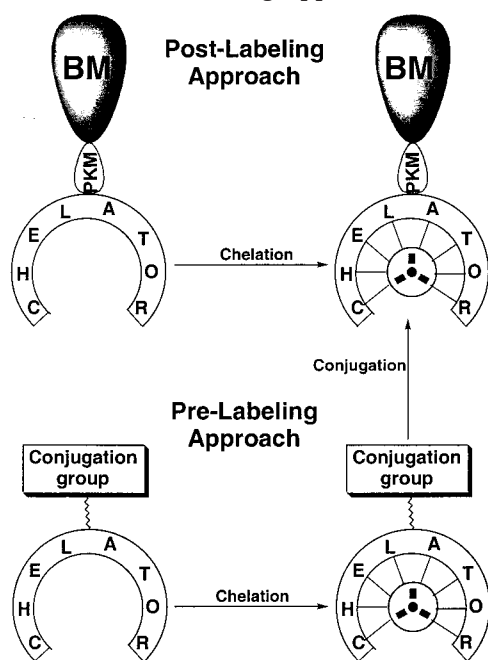


most powerful and the most commonly used conjugation groups for antibodies and their fragments. Water-soluble NHS-activated ester has also been used for conjugation of biomolecules to DOTA (190, 191).

Like NHS esters, isothiocyanates (Chart 16) are amine-reactive groups with an intermediate reactivity, and form thiourea bonds with primary amines of proteins or peptides. They are somewhat more stable in water than the NHS esters and react with amines in aqueous solution optimally at pH 9.0–9.5. Isothiocyanates may not be suitable for modifying proteins that are sensitive to alkaline pH conditions. Aromatic isothiocyanates have been used for conjugation of biomolecules to DTPA and DOTA analogues (117, 154, 192, 193).

Aldehydes and ketones (Chart 16) can react with primary and secondary amines to form Schiff bases. A Schiff base is a relatively labile bond that is readily reversed by hydrolysis in aqueous solution. The formation of Schiff bases is enhanced at basic pH, but they are not completely stable. A number of reducing agents can be used to convert the Schiff base C=N bond into an alkylamine linkage, which is highly stable under both acidic and basic conditions. Virtually any molecule that

Chart 17. Two Radiolabeling Approaches



contains an aldehyde or ketone group can be converted into its Schiff base form. The aldehyde conjugation group has been used for radiolabeling of antibodies and antibody fragments. However, the aldehyde and ketone conjugation groups have not been widely used for conjugation of small biomolecules.

Maleimide (Chart 16) is a thiol-reactive group (191) and reacts selectively with a thiol from an antibody to form a thioether bond without any interference from histidine and other reactive groups. The optimum pH for the reaction is near 7.0. At pH higher than 8.0, maleimides may hydrolyze to form nonreactive maleamic acids. Since many small biomolecules contain no thiol functionality, the use of maleimide as a conjugation group is in some way limited.

Radiolabeling Approaches. The choice of radiolabeling approach depends on the type of biomolecules to be labeled and the purpose of the study. In general, there are two approaches (the prelabeling approach and the postlabeling approach) useful for the radioabeling of biomolecules with lanthanide radionuclides. In the post-labeling approach (Chart 17), a BFC is first attached to the biomolecule either directly or via a linker to form the BFC-BM conjugate. The radiolabeling can be accomplished simply by reacting the BFC-BM with radiometal chloride in a buffer solution in the presence of weak chelating agent (such as citrate), if necessary. DTPA-BM conjugates can be readily radiolabeled within 10 min at room temperature and pH 5–7. The high radiolabeling efficiency (fast and high yield labeling) can be attributed to the linear chelator backbone of DTPA analogues. The labeling kinetics of DOTA-BM conjugates is usually slow. In this case, higher pH and elevated temperature are often needed to achieve fast labeling and high radiolabeling yield. The postlabeling approach is particularly useful for biomolecules that are not sensitive to the sometimes forcing conditions used in the chelation step. For biomolecules, which are sensitive to heating, the prelabeling approach might be the best alternative.

The prelabeling approach (Chart 17) involves formation of the metal complex with a BFC, and conjugation of the M-BFC complex to a biomolecule in a separate step on the tracer level (43). In this approach, the chemistry is

well defined, and the biomolecule is not exposed to the harsh conditions used in the chelation step. For research purposes, this approach is very useful to demonstrate the proof of principle in a short period of time. However, this approach is too complex and time-consuming for routine clinical use. It is also not practical for large-scale production since it involves two steps of chromatographic separation of ^{90}Y -labeled molecules at the tracer level.

Quality Control of the ^{90}Y -Labeled Biomolecules. Before discussing details of ^{90}Y -labeling of biomolecules, it is very important to clarify a misconception on two commonly used terms: radiolabeling yield and radiochemical purity (RCP). In a radiolabeled "kit" mixture, there are a number of possible ^{90}Y -containing species: [^{90}Y]acetate if acetate is used as a buffering agent, [^{90}Y]colloid formed during the radiolabeling, the [^{90}Y]BFC-BM complex, and other impurities. Radiolabeling yield is a term to describe the overall percentage of ^{90}Y -labeled species except [^{90}Y]colloid and [^{90}Y]acetate. Radiochemical purity is the fraction of the total radioactivity in the desired chemical form of a radiopharmaceutical. Radioimpurities arise either from BFC-containing organic impurities or decomposition of the [^{90}Y]BFC-BM complex during the radiolabeling process. If there are no other ^{90}Y -species (such as [^{90}Y]acetate, [^{90}Y]colloid, and ^{90}Y -impurities) formed, the radiochemical purity is the radiolabeling yield of a radiopharmaceutical. However, in most cases, the radiochemical purity of a radiopharmaceutical is *always lower than* its radiolabeling yield.

TLC/ITLC is the most frequently used procedure for the quality control of ^{90}Y -labeled biomolecules. It is simple and quick, usually taking only 5–15 min to complete the whole procedure. Ideally, one TLC paper strip or plate is needed for the separation of [^{90}Y]acetate, [^{90}Y]colloid, and the [^{90}Y]BFC-BM complex. The mobile phase is often comprised of an organic solvent (acetone, ethanol, acetonitrile, methyl ethyl ketone or isopropyl alcohol) and a buffer. The [^{90}Y]colloid and [^{90}Y]acetate remain at the origin and the [^{90}Y]BFC-BM complex migrates to the solvent front. It should be noted that TLC methods typically give only the radiolabeling yield. Rarely can they provide the radiochemical purity (RCP) data for the [^{90}Y]BFC-BM complex, and is not suitable for separation of different isomeric forms.

For the past decade, HPLC has become a routine technique for quality control of various radiopharmaceuticals. The advantage of radio-HPLC is its capability to determine the RCP for the [^{90}Y]BFC-BM complex, and to separate different ^{90}Y species in the kit. It is also a very powerful tool for separation of different isomers such as epimers and diastereomers. The separation of optical isomers requires the use of chiral chromatographic conditions (chiral column or chiral mobile phase). However, radio-HPLC also has its limitations. For example, it cannot be used for the assessment of the amount of [^{90}Y]colloid in the radiolabeled kit. Therefore, a TLC method is needed in combination with radio-HPLC to assess both the radiolabeling yield and the radiochemical purity of the ^{90}Y -labeled biomolecule. In addition, a 4 mM DTPA solution (pH 4–5) is often recommended for accurate determination of unchelated ^{90}Y during the standard quality control by HPLC (17). If DTPA is not used, the unchelated ^{90}Y will be readily bound to the column; thereby giving a false indication of the radiochemical purity of the radiopharmaceutical.

It is very important to emphasize that a single peak in the HPLC chromatogram does not mean that there is only one ^{90}Y -species. This is particularly true when the

Table 4. Trace Metal Contaminants in a Commercial ^{90}Y Stock Solution and the Stability Constants for Their DOTA Complexes.

metal ion	maximum amount ($\mu\text{g}/\text{Ci Y-90}$)	maximum amount ($\mu\text{mol}/\text{Ci Y-90}$)	stability constant for M-DOTA
Y	~ 2.0	~ 0.022	24.3^a
Al	<30.0	<1.11	
Ca	<100	<2.50	17.2^b
Ce	<20	<0.143	23.0^c
Co	<20	<0.339	20.2^b
Cr	<20	<0.385	
Cu	<30	<0.469	22.5^b
Fe	<20	<0.357	29.4^d
La	<20	<0.144	21.7^d
Ni	<20	<0.339	20.5^d
Pb	<20	<0.100	22.7^e
Sr	<20	<0.227	15.22^d
Zn	<40	<0.615	21.05^d
Zr	<20	<0.217	

^a Reference 199. ^b Reference 200. ^c Reference 201. ^d Reference 202. ^e Smith, R. M., and Martell A. E. (1997) *NIST Critical Selected Stability Constants of Metal Complexes Database*, Version 3, Program developed by R. J. Motekaitis.

chromatographic conditions are not optimized. Different chromatographic conditions (such as an isocratic mobile phase or a long and slow gradient mobile phase) are strongly recommended to confirm that the single peak observed in the HPLC chromatogram is really one peak and that there are no other radioimpurities or isomeric forms coeluting with the ^{90}Y -BFC-BM complex.

^{90}Y -Labeling of DTPA-BM Conjugates. As discussed in the previous section, a major advantage of using DTPA analogues as BFCs is fast radiolabeling kinetics under mild conditions. Stimmel et al. studied the ^{90}Y -chelation properties of a series of DOTA and DTPA analogues (194, 195). It was found that the ^{90}Y -chelation efficiency of acyclic chelators (DTPA, nitro-CHX-A-DTPA, nitro-MX-DTPA, and TMT-amine) was much higher than that of macrocyclic chelators, and that trace metal (Ca^{2+} , Fe^{2+} , and Zn^{2+}) contamination had minimal effect on the radiolabeling. The DTPA-BM conjugates can be readily radiolabeled under mild conditions (room temperature and pH 4–7). We studied the ^{90}Y -chelation properties of a DTPA-conjugated cyclic peptide (SQ169), and found that higher pH gives faster radiolabeling kinetics (173). High yield labeling (RCP > 95%) can be readily achieved by reacting 2 μg of SQ169 with 20 mCi of $^{90}\text{YCl}_3$ in the 0.5 M ammonium acetate buffer (pH 8.0). The SQ169:Y ratio is $\sim 4:1$ under these conditions, and chelation is almost instantaneous at room temperature.

^{90}Y -Labeling of DOTA-BM Conjugates. The labeling kinetics of DOTA-BM conjugate is usually slow, and much more dependent on the radiolabeling conditions (195–198). There are many factors influencing the radiolabeling yield. These include the amount of DOTA-BM conjugate, the pH in the reaction mixture, temperature and heating time, buffer agent and buffer concentration, stabilization agent and its concentration, and the presence of other trace metal contaminants, such as Fe^{3+} and Zn^{2+} .

Metal Ion Contamination. Unlike that of acyclic chelators (DTPA, nitro-CHX-A-DTPA, nitro-MX-DTPA, and TMT-amine), the ^{90}Y -chelation efficiency of macrocyclic chelators (nitro-DOTA, nitro-TRITA, and nitro-PADOTA) diminished when the concentration of trace metal contaminants (Ca^{2+} , Fe^{2+} , and Zn^{2+}) was increased (194, 195). Table 4 shows the maximum limits of trace metal contaminants (as of production date) in the ^{90}Y -stock solution from NEN (New England Nuclear) and

Table 5. Half-Times for the ^{90}Y -Labeling of DOTA-MoAb Immunoconjugate in 0.5 M Ammonium Acetate Buffer under Perspective Radiopharmacy Labeling Conditions^a

pH value	half-time (min)	pH value	half-time (min)
4.5	46–410	7.5	0.28–2.5
5.5	4.3–38	8.5	0.06–0.53
6.5	4.2–37		

^a Radiolabeling conditions are 15–45 mg/mL DOTA-MoAb, one to three DOTA chelating groups conjugated per MoAb and 1.5–11 GBq/mL ^{90}Y (data from ref 196).

stability constants of their corresponding DOTA complexes (199–203). Most of the trace metals (Al, Ca, Ce, Co, Cr, Cu, Fe, La, Ni, Pb, Zn, and Zr) in the ^{90}Y -stock solution have the potential to influence the ^{90}Y -labeling efficiency of a DOTA-based BFC. The actual metal contaminant concentration may not be as high as specified by the manufacturer; but results from our lab clearly showed that the concentration of total trace metal contaminants in the ^{90}Y -DOTA-BM reaction mixture is much higher than that of ^{90}Y . It is strongly recommended that the trace metal contamination be minimized during the radiolabeling.

BFC-BM Conjugate Concentration. Radiolabeling efficiency is a term often used to describe the ability of a chelator to form its radiometal complex at a low concentration under mild conditions. The radiolabeling efficiency of a macrocyclic chelator is largely dependent upon radiolabeling conditions (the concentration of trace metal contaminants, pH, heating temperature, and reaction time). It was reported that the radiolabeling efficiency of DOTA analogues was maximal only when the chelator-to-radiometal ratio was greater than 3 (194, 195). It should be noted that the radiolabeling yields and radiochemical purity reported in these studies were determined by only ITLC not a combination of radio-HPLC and ITLC. *The bonding of the radiometal to the BFC does not mean that the desired radiometal complex is formed.* Giving the fact that the radionuclide solution is often contaminated with other trace metals at higher concentrations than that of the radiometal, the data reported for the radiolabeling efficiency of macrocyclic chelators should be used with caution.

Heating Temperature and Reaction Time. Room temperature labeling offers advantages over heating at 100 °C; but it often requires much longer reaction time and a higher concentration of the DOTA-BM conjugate in the reaction mixture to complete the radiolabeling (RCP > 90%). Under the same radiolabeling conditions, higher temperature and longer heating time usually gives better radiolabeling yield provided that the biomolecule is not subjected to thermal decomposition. It has been reported that the rate of ^{90}Y -chelation could be significantly enhanced by increasing temperature from 22 °C to 37 °C (204). For a DOTA-BM conjugate, heating at 100 °C for a short period of time (5–10 min) is preferred to avoid extensive radiolysis during radiolabeling, particularly when a large amount of ^{90}Y (100–200 mCi) is used for radiolabeling.

Buffering Agent and Its Concentration. The choice of a buffering agent depends on the optimum pH value for complexation. Ammonium acetate and ammonium citrate are preferred buffering agents to avoid any competition from other metals with ^{90}Y and to stabilize the Y^{3+} cation by forming a weak ^{90}Y -acetate or ^{90}Y -citrate complex. The buffering agent concentration is normally 0.1–0.5 M. It was reported that the 0.5 M ammonium acetate buffer (pH 7.0–7.5) is ideal for the

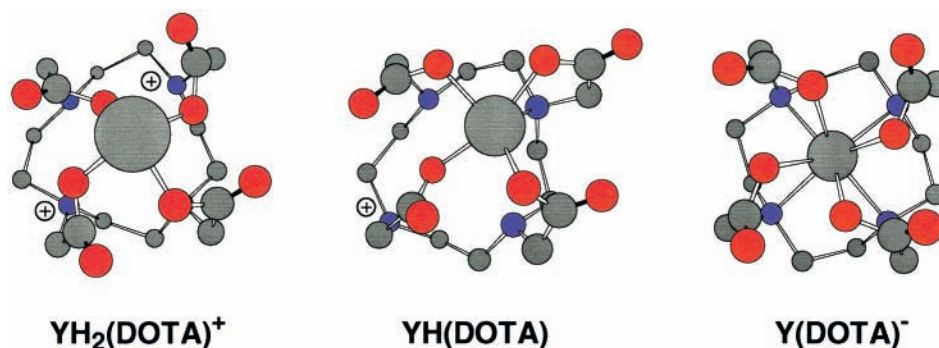


Figure 19. Possible structures of yttrium complexes $\text{YH}_2(\text{DOTA})^+$, $\text{YH}(\text{DOTA})$, and $\text{Y}(\text{DOTA})^-$.

radiolabeling of DOTA-conjugated biomolecules (204). The use of phosphate and carbonate/bicarbonate should be avoided since both phosphate and carbonate readily form a precipitate with Y^{3+} .

Meares and co-workers (204) recently reported optimized conditions for ^{90}Y -chelation of DOTA-immunoconjugates. It was found that the pH of the reaction mixture has a dramatic impact on the rate of chelation. For example, the time required to chelate 94% of ^{90}Y was 17–148 min at pH 6.5, but it was only 1–10 min at pH 7.5 when the concentration of DOTA conjugate was 97–870 μM and ^{90}Y concentration was in the range of 0.83–6.1 μM .

DOTA and its derivatives tend to exist in solution as their zwitterion forms (198–209), and the conformation of the chelator is determined by the degree of protonation of DOTA. For example, the predominant form at pH 3.6–5.0 is $\text{H}_3(\text{DOTA})^-$ while DOTA exists primarily (90%) as $\text{H}_2(\text{DOTA})^{2-}$ at pH 6.0–7.0. The formation of $\text{Y}(\text{DOTA})^-$ complexes proceeds via two intermediate complexes: $\text{YH}_2(\text{DOTA})^+$ and $\text{YH}(\text{DOTA})$. The rate-limiting step is proton transfer from $\text{YH}(\text{DOTA})$ to form the eight-coordinated complex $\text{Y}(\text{DOTA})^-$ since it requires a significant rearrangement of the macrocyclic ring (210), including rotation of C–C and C–N bonds and inversion of the N-atom. Once the proton is removed, chelation of the remaining three N-atoms will be fast. Thus, higher pH results in faster complexation rate.

Different deprotonated intermediates of DOTA form $\text{Y}(\text{III})$ complexes with different structures (210). It has been suggested that in the doubly protonated complex $\text{YH}_2(\text{DOTA})^+$ (Figure 19), $\text{Y}(\text{III})$ is located outside the cage composed of carboxylate-oxygens and ring nitrogen and is coordinated solely by four carboxylate-oxygens. Upon removal of one proton from the ring nitrogen to form $\text{YH}(\text{DOTA})$, the $\text{Y}(\text{III})$ moves toward the ring to become five-coordinated to the four carboxylate oxygens and the one ring nitrogen. Because of static interaction, the N-atom bonding to the metal is most likely to be the one trans to the protonated N-atom. With the removal of the last proton, the expected eight-coordinated complex $\text{Y}(\text{DOTA})^-$ is formed in a square prismatic coordination geometry.

CONCLUSIONS

A target-specific metalloradiopharmaceutical usually contains a targeting biomolecule, a linker, a BFC, and a metallic radionuclide. The targeting biomolecule is only one part of the whole molecule, and the discovery of a new class of biomolecules is just the beginning of the whole drug development process. The development of an efficient BFC and new radiolabeling technologies is equally important. For the receptor-based therapeutic radiopharmaceuticals, it is very important to remember

that the receptor population is often limited. The use of a large amount of unlabeled BFC–BM conjugate may block the binding of the radiolabeled BFC–BM conjugate at the receptor sites. Therefore, the BFC must have very high labeling efficiency (fast and high-yield labeling) and form metal complexes with high specific activity.

The choice of BFC is largely dependent upon the nature and oxidation state of the metal ion and requires a good understanding of the coordination chemistry of any given radiometal. Among various radionuclides, yttrium is of particular importance due to its chemical and nuclear characteristics. The solution stability of a radiopharmaceutical depends on the thermodynamic stability and more importantly the kinetic inertness of the metal chelate. Thus, DOTA derivatives are often used as BFCs for the ^{90}Y -labeling of small biomolecules.

Many acyclic and macrocyclic bifunctional chelators (BFCs) have been synthesized and used for the radiolabeling of antibodies and small peptides. Although formation of isomers in yttrium and lanthanide complexes of DTPA analogues has been studied by various NMR methods, very little attention is paid to the isomerism of the metal chelate (radionuclide and BFC) in radiolabeled DTPA- and DOTA-biomolecule conjugates at the tracer level. For radiolabeled antibodies, the attachment of the BFC and the radionuclide may not have a significant impact on their biological properties due to the fact that the metal chelate is only a small part of the radiolabeled protein. For small biomolecules, however, the metal chelate contributes greatly to the overall size and molecular weight of the radiopharmaceutical. Isomerism may have significant impact on the biological performance (receptor binding and biodistribution). Therefore, it is very important to develop appropriate analytical methods and to study the possible isomerism in the metal chelate in order to understand the biological properties of a new metalloradiopharmaceutical.

A major advantage of using DTPA analogues as BFCs is fast radiolabeling kinetics under mild conditions. However, the kinetic lability of their metal chelates may prove to be problematic when they are used as BFCs for biomolecules having a long blood retention and slow blood clearance. The labeling kinetics of DOTA-based BFCs is usually slow, and much more dependent on the radiolabeling conditions, including the BFC–BM concentration, pH, reaction temperature and heating time, buffer agent and buffer concentration, and presence of other metal ions, such as Fe^{3+} and Zn^{2+} . The ultimate goal is to prepare a therapeutic radiopharmaceutical under the optimized radiolabeling conditions and to retain its chemical and biological integrity during release and transportation.

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CORRECTIONS

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BIFUNCTIONAL CHELATORS FOR THERAPEUTIC LANTHANIDE RADIOPHARMACEUTICALS

The authors sincerely apologize to Dr. Garry E. Kiefer and co-workers for the oversight of their US patent no. 5587451, which was inadvertently omitted in reference to Chart 4 of this manuscript. US patent no. 6013793 disclosed the routes showing condensation of triethylene-tetraamine (TETA) with glyoxal, followed by oxidation with bromine and formation of a dicationic intermediate. In contrast, the monocationic intermediate depicted in Chart 4 is obtained from the condensation of TETA with dimethylformamide dimethyl acetal as described in US patent no. 5587451.

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of potential cancer therapeutics. Until recently, most of the efforts to develop drugs to reduce the activity of oncogenic Ras proteins has focused on the inhibition of protein farnesyltransferase (PFTase). However, PFTase inhibitors may have limited utility against many cancers since some Ras proteins, including oncogenic forms of Ki4B-Ras are geranylgeranylated in a compensatory manner when farnesylation is blocked by inhibition of PFTase (6). Inhibition of Ca_{1a_2}X prenyl protease will disrupt the processing of both types of prenylated Ras proteins and could provide another approach to reducing the activity of oncogenic Ras.

Ca_{1a_2}X prenyl protease (7) activity has been studied in yeast (8, 9), rat (10), and bovine (11) membrane preparations. The rat and bovine enzymes were partially purified (11–13). Prenylated tri-C(farnesyl) a_{1a_2} and tetra-C(farnesyl) a_{1a_2}X peptides are substrates for proteolysis. Recently, the yeast and human genes for Rce1p were expressed in Sf9 insect cells by infection with a recombinant baculovirus (14). The yeast gene was also expressed in a protease-deficient strain of *Saccharomyces cerevisiae*. Biochemical and site-directed mutagenesis studies suggest that yRce1p is a cysteine protease (15).

A variety of assays have been developed for the enzyme. These include HPLC detection of a radioactive cysteine product, a fluorometric HPLC assay (16), and a coupled proteolysis/methylation assay (8, 9, 14). Liu et al. recently reported an assay that relies on the strong affinity of avidin for biotin (17). We were attracted to this assay since it does not require a chromatographic separation or use of a coupling enzyme, is sensitive, and has potential for development as a high throughput system. The peptide synthesized by Liu et al. consisted of a C(S-farnesyl)-VI-[^3H]S tetrapeptide attached to biotin by a hydrophobic spacer. Their synthesis combined a solid-phase assembly of a biotinylated CVI tripeptide with solution phase additions of the farnesyl moiety [^3H]serine. HPLC purifications were required to purify material following each solution phase step. We encountered considerable difficulties with insoluble intermediates and low yields when attempting to adapt these protocols to our system and decided to develop a solid-phase approach for the entire synthesis on a resin whose chemistry was compatible with acid sensitive side chains. In addition, the hydrophobic linker was replaced by a PEG-linker to improve overall solubility of the biotinylated substrate used in the coupling reaction. We now describe the solid-phase synthesis of a radiolabeled, biotinylated, and farnesylated Ca_{1a_2}X peptide and its use to assay for Ca_{1a_2}X proteolytic activity in membranes enriched with recombinant yRce1p.

EXPERIMENTAL PROCEDURES

Amino acids, commercially available *N*-BOC protected amino acids, farnesyl bromide, di-*tert*-butyl dicarbonate, *N,N'*-dicyclohexylcarbodiimide, and *N*-hydroxybenzotriazole, and other chemicals were purchased from Aldrich Chemical Co. Polystyrene resin was purchased from BioRad. [^{14}C]-L-Alanine was obtained from NEN DuPont Corp. Biochemical reagents were purchased from Gibco or Sigma. Kaiser's benzophenone oxime resin was synthesized by the method of DeGrado and Kaiser (18). Avidin resin was purchased from Pierce. THF was dried and distilled from sodium benzophenone. CH_2Cl_2 was distilled from CaH_2 . DMF was vacuum distilled from CaH_2 . Analytical thin-layer chromatography was carried out on either E. Merck or Whatman precoated silica gel

60 (0.2 mm, aluminum support) TLC plates. Preparative TLC was carried out using E. Merck silica gel 60 glass backed TLC plates. C_{18} reverse phase HPLC was carried out using a Vydac Protein and Peptide column with monitoring at 214 nm. Solvents for C_{18} HPLC were a gradient of acetonitrile and water containing 0.1% v/v TFA.

^1H , ^{13}C , and DEPT NMR spectra were obtained using a UNITY Varian 300 MHz NMR and were referenced to either TMS or the residual NMR solvent signal for d_6 -DMSO at 2.50 ppm for ^1H or 39.5 ppm for ^{13}C . Optical rotations were obtained in spectral grade solvents. Infrared spectra were obtained as thin films on NaCl plates. FABMS (positive or negative ion) were obtained from the Mass Spectrometry Service Center in the Chemistry Department. MALDI spectra were provided by the Mass Spectral facility in the Department of Medicinal Chemistry.

Syntheses. *N-tert*-Butoxycarbonyl-L-Valinyl-L-Isoleucinyl Kaiser's Oxime Ester Resin (2). To a solution of *N*-BOC-L-isoleucine (1.64 g, 7.11 mmol) and HOBt (0.96 g, 7.11 mmol) in 20 mL of anhydrous THF at 0 °C under nitrogen was added dropwise DCC (1.47 g, 7.11 mmol) in 10 mL of anhydrous THF. The mixture was stirred for 3 h while it was allowed to warm to room temperature. Dicyclohexylurea was removed by filtration through a plug of Celite, and the plug was washed with ice-cold THF. Solvent was removed at reduced pressure to give L-isoleucine active ester.

The isoleucine active ester was dissolved in 100 mL of anhydrous CH_2Cl_2 and added to 10.0 g of Kaiser's resin. Anhydrous triethylamine (0.91 mL, 6.52 mmol) was added, and the mixture rotated under N_2 overnight. The resin was collected by suction filtration on a medium porosity sintered glass funnel and washed with the following standard protocol: (1) four 100 mL portions of CH_2Cl_2 ; (2) four 100 mL portions of DMF; (3) four 100 mL portions of 2-propanol; and (4) four 100 mL portions of CH_2Cl_2 . The resin was dried overnight under high vacuum.

N-BOC-L-valine (1.54 g, 7.11 mmol) was converted to the HOBt active ester as described previously for *N*-BOC-L-isoleucine. The active ester was isolated as described above.

N-BOC-L-isoleucinyl Kaiser's oxime resin was added to 160 mL of anhydrous CH_2Cl_2 . TFA (40 mL) was added and the suspension rotated for 1 h under nitrogen. The resin was collected by suction on a medium porosity sintered glass funnel, washed with four 100 mL portions of CH_2Cl_2 and added to a solution of L-valine active ester in 100 mL of anhydrous CH_2Cl_2 . Anhydrous triethylamine (2.0 mL, 14.2 mmol) was added, and the suspension was rotated overnight under N_2 . The resin was collected by suction on a sintered glass funnel and washed with the four solvent protocol as described above. The resin was dried under high vacuum overnight to afford 11.32 g of a tan solid.

(*S*-(*E,E*-Farnesyl)-L-cysteinyl)-L-valinyl-L-isoleucinyl Kaiser's Oxime Ester Resin Trifluoroacetate Salt (3). A suspension of *N*-(triphenylmethyl)-(*S*-(*E,E*-farnesyl)-L-cysteine) (4.48 g, 7.89 mmol) (19), HOBt (1.07 g, 7.89 mmol), and 30 mL of anhydrous THF was cooled to 0 °C under N_2 . DCC (1.63 g, 7.89 mmol) in 30 mL of anhydrous THF was slowly added dropwise. The reaction mixture was stirred for 24 h while slowly warming to room temperature. Dicyclohexylurea was removed by filtration through a plug of Celite, and the Celite was washed with 25 mL of ice-cold THF. Solvent was removed in vacuo to afford the *N*-trityl active ester as a thick oil.

A slurry of 10.10 g of *N*-BOC-L-valinyl-L-isoleucinyl Kaiser's oxime ester resin, 100 mL of anhydrous CH_2Cl_2 and 25 mL of TFA were rotated under N_2 for 1.5 h. The resin was collected by suction filtration and washed with four 100 mL portions of CH_2Cl_2 . The deprotected resin was added to a solution of the *N*-trityl active ester in 100 mL of anhydrous CH_2Cl_2 and anhydrous triethylamine (2.2 mL, 15.78 mmol). The suspension was rotated under nitrogen for 24 h. The resin was collected on a sintered glass funnel, washed with the four solvent wash protocol, and dried overnight under high vacuum to afford 10.24 g of a tan solid.

The *N*-trityl Kaiser's oxime ester resin (9.45 g) was suspended in 100 mL of anhydrous CH_2Cl_2 . Ethylmethyl sulfide (5.52 mL, 61 mmol) was added by syringe to scavenge advantageous cations, followed by 1.18 mL (15.3 mmol) of TFA. The resin was rotated for 30 min, collected by suction, washed by mixing with four 125 mL portions of CH_2Cl_2 , and dried in vacuo for 15 min to give the TFA salt of Kaiser's resin **3**.

N-(13-Amino-4,7,10-trioxatridecanyl)biotinamide (**4**) was synthesized by the method of Wilbur et al. (20).

(*N*-(13-*N*-Succinimidyl-4,7,10-trioxatridecanyl)biotinamide-(2,3,5,6-tetrafluorophenyl Active Ester) (**5**). To a solution of 1.0 g (2.24 mmol) of **4** and 0.25 g (2.46 mmol) of succinic anhydride in 20 mL of anhydrous DMF under N_2 , 0.35 mL (2.46 mmol) of anhydrous triethylamine was added by syringe. The reaction was stirred for 16 h before the DMF was removed in vacuo at 40 °C. The residue was dissolved with vigorous stirring in 25 mL of 1 M acetic acid and 25 mL of *n*-butanol. The mixture was extracted with two 25 mL portions of *n*-butanol, and the pooled organic phases were evaporated in vacuo. The residue was repeatedly evaporated from 25 mL portions of CHCl_3 to afford a white solid. The solid was purified by silica column chromatography upon elution with 1:5 MeOH: CHCl_3 , followed by 1:5 MeOH: CHCl_3 containing 5% v/v acetic acid. Fractions that were homogeneous by silica TLC (1:5 MeOH: CHCl_3 containing 5% v/v acetic acid) were pooled and concentrated in vacuo to afford 1.01 g (82%) of a white solid; ^1H NMR (300 MHz, DMSO) δ 1.20–1.34 (m, 2 H), 1.40–1.68 (m, 6 H), 2.04 (t, 2 H, biotin CH_2CO), 2.28 (t, 2 H, succinimidyl CH_2), 2.40 (t, 2 H, succinimidyl CH_2), 2.55 (d, 1 H), 2.73–2.89 (m, 1 H), 3.03–3.12 (m, 5 H), 3.38 (t, 4 H), 3.42–3.52 (m, 8 H), 4.09–4.15 (m, 1 H), 4.28–4.32 (m, 1 H), 6.38 (s, 1 H, biotin NH), 6.46 (s, 1 H, biotin NH), 7.76 (t, 1 H, NH), 7.82 (t, 1 H, NH); ^{13}C NMR (75 MHz, DMSO) δ 173.96, 171.94, 170.87, 162.78, 69.79, 69.56, 68.12, 68.06, 61.07, 59.08, 59.22, 55.47, 39.89, 35.82, 35.74, 35.23, 30.04, 29.43, 29.37, 29.24, 28.26, 28.07, 25.34 ppm. Positive ion FABMS: calcd for $\text{C}_{24}\text{H}_{43}\text{N}_4\text{O}_8\text{S}$ ($\text{M} + \text{H}$) 547; found ($\text{M} + \text{H}$) m/z 547.

To a solution of 1.67 g (3.05 mmol) of *N*-(13-*N*-succinimidyl-4,7,10-trioxatridecanyl)biotinamide and 0.86 mL (6.11 mmol) of anhydrous triethylamine in 32 mL of anhydrous DMF was added dropwise a solution of 1.20 g (4.58 mmol) of 2,3,5,6-tetrafluorophenyltrifluoroacetate (**21**) in 5 mL of anhydrous DMF. The reaction was stirred for 1 h under N_2 . DMF was removed in vacuo with a water bath at ≤ 50 °C to afford a pale yellow oil. Diethyl ether (200 mL) was added with stirring under N_2 . Slowly, over 30 min a white solid formed on the walls of the flask. The ether was removed by suction using a pipet, and a second 200 mL portion of diethyl ether was added. The suspension was stirred for 30 min, and the ether was removed as before. The white solid was dried under high vacuum for 30 min and used immediately.

(1-*N*-Biotinyl-(13-*N*-(succinimidyl-(*S*-(*E*,*E*-farnesyl)-L-cysteinyl)-L-valinyl-L-isoleucinyl)-4,7,10-trioxatridecanediamine) Kaiser's Resin Oxime Ester (**6**). To resin **3** (9.45 g) and newly synthesized tetrafluorophenyl active ester of *N*-(13-*N*-succinimidyl-4,7,10-trioxatridecanyl)biotinamide **5** was added 60 mL of DMF and 60 mL of CH_2Cl_2 , followed by dropwise addition of 1.75 mL (12.51 mmol) of anhydrous triethylamine. The suspension was rotated under N_2 for 48 h.

The slurry was collected on a sintered glass funnel and washed with the four solvent protocol described previously. The resin was dried under high vacuum for 18 h to afford 9.79 g of a tan solid.

(1-*N*-Biotinyl-(13-*N*-succinimidyl-(*S*-(*E*,*E*-farnesyl)-L-cysteinyl)-L-valinyl-L-isoleucinyl-L-alaninyl)-4,7,10-trioxatridecanediamine) Benzyl Ester (**8**). To a suspension of 0.50 g of (1-*N*-biotinyl-(13-*N*-(succinimidyl-(*S*-(*E*,*E*-farnesyl)-L-cysteinyl)-L-valinyl-L-isoleucinyl)-4,7,10-trioxatridecanediamine), Kaiser's resin **6**, were added 0.33 g (0.97 mmol) of L-alanine benzyl ester tosylate and 5 mL of anhydrous CH_2Cl_2 . Glacial acetic acid (56 μL , 0.97 mmol) and anhydrous triethylamine (136 μL , 0.97 mmol) were added. The flask was rotated under N_2 for 24 h.

The resin slurry was poured into a sintered glass funnel that contained 20 mL of 2-propanol and was washed with five 40 mL portions of CHCl_3 and 2-propanol. The CHCl_3 /2-propanol filtrate was concentrated in vacuo and redissolved in 45 mL of CHCl_3 . The solution was washed with 15 mL of saturated NaHCO_3 solution and two 15 mL portions of 1 M acetic acid. The organic layer was dried over anhydrous MgSO_4 , and the solvent was removed at reduced pressure. The last traces of acetic acid were removed by high vacuum overnight.

The residue (0.14 g) was dissolved in CHCl_3 and applied to two preparative silica gel thin-layer chromatography plates. The plates were eluted with 1:10 MeOH: CHCl_3 . The UV active band at $R_f = 0.24$ was removed by scraping and eluted from the silica with a total of 200 mL of 1:5 MeOH: CHCl_3 . Solvent was removed in vacuo to afford **8** (0.11 g, 26% overall) as a white solid; HPLC analysis was performed on a Vydac C_{18} reverse phase column with detection at 214 nm and elution with acetonitrile:water containing 0.1% TFA. The gradient profile was 45% acetonitrile:55% water to 80% acetonitrile:20% water over 20 min. The retention time of **8** under these conditions was 14.82 min. ^1H NMR (300 MHz, DMSO) δ 0.70–0.82 (m, 12 H, valine and isoleucine CH_3), 0.97–1.09 (m, 2 H), 1.27 (d, 3 H, $J = 7.2$ Hz, alanine CH_3), 1.30–1.70 (m, 10 H), 1.54 (s, 6 H, farnesyl CH_3), 1.60 (s, 3 H, farnesyl CH_3), 1.62 (s, 3 H, farnesyl CH_3), 1.86–2.10 (m, 10 H, biotin CH_2CO and farnesyl allylic CH_2), 2.24–2.40 (m, 4 H, succinimidyl CH_2), 2.56 (d, 2 H, $J = 12.3$ Hz, CH_2S), 2.70–2.85 (m, 2 H, SCH_2), 3.00–3.18 (m, 7 H), 3.37 (t, 4 H, $J = 6.3$ Hz, PEG CH_2), 3.42–3.52 (m, 8 H, PEG CH_2), 4.09–4.22 (m, 3 H, cysteine, valine, and isoleucine CH), 4.25–4.34 (m, 2 H, biotin CH), 4.45 (q, 1 H, $J = 7.8$ and 8.1 Hz, alanine CH), 5.00–5.10 (m, 4 H, farnesyl olefinic and benzylic CH_2), 5.15 (t, 1 H, $J = 7.8$ and 8.1 Hz, farnesyl olefinic), 6.47 (br s, 2 H, biotin NH), 7.33 (s, 5 H, aromatic H), 7.71 (d, 1 H, $J = 8.7$ Hz, NH), 7.77 (t, 1 H, $J = 5.4$ Hz, PEG NH), 7.86 (t, 1 H, $J = 5.4$ Hz, PEG NH), 7.89 (d, 1 H, $J = 9.0$ Hz, NH), 8.21 (d, 1 H, $J = 8.1$ Hz, NH), 8.38 (d, 1 H, $J = 6.6$ Hz, NH); ^{13}C NMR (75 MHz, DMSO) δ 172.20, 171.92, 171.64, 171.20, 170.65, 170.42, 170.38, 162.76, 138.30, 135.95, 134.55, 130.65, 128.38, 127.99, 127.75, 124.13, 123.68, 120.27, 69.79, 69.57, 68.12, 65.85, 61.07, 59.23, 57.83, 56.36, 55.47, 52.55, 47.66, 39.88,

39.12, 36.69, 35.85, 35.72, 35.22, 32.69, 30.82, 30.40, 29.43, 29.36, 28.96, 28.25, 28.06, 26.20, 25.93, 25.51, 25.34, 24.18, 19.18, 18.09, 17.56, 16.81, 15.80, 15.07, 10.94 ppm. Positive ion FABMS: calcd for $C_{65}H_{103}N_8O_{12}S_2$ (M + H) 1228; found (M + H) m/z 1228.

1-N-Biotinyl-(13-N-succinimidyl-(S-(E,E-farnesyl)-L-cysteiny)-L-valinyl-L-isoleucinyl-L-alanine))-4,7,10-trioxatridecanediamine (1). To 0.11 g (0.088 mmol) of benzyl ester **8** in 2.2 mL of dry THF was added 0.45 mL (0.110 mmol) of 0.25 M KOH. The mixture was stirred under N_2 for 24 h. TLC analysis on silica gel (1:10 MeOH:CHCl₃) showed the presence of a small amount of starting benzyl ester. A 0.17 mL (0.042 mmol) portion of 0.25 M KOH was added, and the mixture stirred for 4 h. TLC analysis showed no benzyl ester. Solvent was removed at reduced pressure and distilled deionized water (10 mL) was added. The solution was extracted with 40 mL of diethyl ether; the aqueous layer was acidified with 20 mL of 1 M acetic acid, and the resulting milky suspension was extracted with three 20 mL portions of water saturated *n*-butanol. The extracts were combined and the *n*-butanol was removed in vacuo with a water bath at $\leq 40^\circ C$. The residue was dissolved in 1:5 MeOH:CHCl₃ and transferred to a tared flask. Solvent was removed in vacuo to give 0.092 g of a white solid. HPLC analysis as described above gave a retention time of 10.20 min for **1**. The material was purified by silica column chromatography upon elution with 1:5 MeOH:CHCl₃ containing 5% v/v acetic acid. Homogeneous fractions were pooled, and the solvent removed in vacuo to give 0.072 g (72%) of **1** as a white solid, $[\alpha]_D^{20} = -3.80$ (1:5 MeOH:CHCl₃ containing 5.0% v/v glacial acetic acid, $c = 5.0$); IR TF (NaCl) 3353, 3211, 3082, 2961, 2930, 2869, 1707, 1633, 1549, 1454, 1385, 1222, 1115, 755, 709 cm⁻¹; TLC, $R_f = 0.57$ using 1:5 MeOH:CHCl₃ with 5.0% v/v glacial acetic acid on silica; ¹H NMR (300 MHz, DMSO) δ 0.70–0.84 (m, 12 H, valine and isoleucine CH₃), 0.94–1.12 (m, 2 H), 1.16–1.30 (m, 3 H, alanine CH₃), 1.32–1.74 (m, 10 H), 1.51 (s, 6 H, farnesyl CH₃), 1.57 (s, 3 H, farnesyl CH₃), 1.59 (s, 3 H, farnesyl CH₃), 1.84–2.06 (m, 10 H, biotin CH₂CO and farnesyl allylic CH₂), 2.20–2.40 (m, 4 H, succinimidyl CH₂), 2.53 (d, 2 H, $J = 12.3$ Hz, CH₂S), 2.66–2.84 (m, 2 H, S CH₂), 2.94–3.16 (m, 7 H), 3.34 (t, 4 H, $J = 6.3$ Hz, PEG CH₂), 3.38–3.52 (m, 8 H, PEG CH₂), 4.00–4.20 (m, 4 H, cysteine, valine, isoleucine CH, and biotin CH), 4.25–4.34 (m, 1 H, biotin CH), 4.41 (q, 1 H, $J = 7.5$ and 8.1 Hz, alanine CH), 4.96–5.08 (m, 2 H, farnesyl olefinic), 5.12 (t, 1 H, $J = 7.5$ Hz, farnesyl olefinic), 6.35 (s, 1 H, biotin NH), 6.43 (s, 1 H, biotin NH), 7.60–7.96 (m, 4 H, NH), 8.00–8.10 (m, 1 H, NH), 8.17 (d, 1 H, $J = 7.5$ Hz, NH); ¹³C NMR (75 MHz, DMSO) δ 173.92 (carbonyl), 171.90 (carbonyl), 171.60 (carbonyl), 171.18 (carbonyl), 170.40 (carbonyl), 162.76 (urea carbonyl), 138.30 (quaternary), 134.55 (quaternary), 130.65 (quaternary), 124.13 (CH), 123.68 (CH), 120.26 (CH), 69.78 (CH₂), 69.56 (CH₂), 68.10 (CH₂), 61.06 (CH), 59.21 (CH), 57.81 (CH), 56.48 (CH), 55.46 (CH), 52.53 (CH), 47.60 (CH), 39.88 (CH₂), 39.22 (CH₂), 39.12 (CH₂), 36.67 (CH), 35.84 (CH₂), 35.72 (CH₂), 35.22 (CH₂), 32.70 (CH₂), 30.81 (CH₂), 30.41 (CH), 29.42 (CH₂), 29.35 (CH₂), 28.94 (CH₂), 28.24 (CH₂), 28.05 (CH₂), 26.19 (CH₂), 25.93 (CH₂), 25.52 (CH₃), 25.33 (CH₂), 24.16 (CH₂), 19.20 (CH₃), 18.08 (CH₃), 17.57 (CH₃), 17.18 (CH₃), 15.80 (CH₃), 15.21 (CH₃), 11.01 (CH₃) ppm; negative FABMS (glycerol/25 mM NH₄HCO₃): calcd m/z 1135.3 (M – H); found m/z 1135.4 (M – H). The derived isotopic pattern for the M–H signal was found to be identical to the theoretical spectrum: 1135 (100.00%), 1136 (66.87%), 1137 (37.69%), 1138 (16.71%), 1139 (7.87%); MALDI mass spectroscopy: calcd

for $C_{56}H_{97}N_8O_{12}S_2$ (M + H) 1137.6667, found 1137.6810 (4%); calcd for (M + Na) 1159.6487, found 1159.6715 (59%); calcd for (M + K) 1175.6226, found 1175.6484 (37%).

Synthesis of [¹⁴C]1**.** A solution of 116 μ L (3 μ mol, 5 mg/mL) *p*-toluenesulfonic acid in anhydrous THF was evaporated to dryness before 5.0 mL of [¹⁴C]-L-alanine (2.9 μ mol, 0.5 mCi, 174.6 mCi/mmol) in 2:98 ethanol:water was added by syringe. Two 1.0 mL portions of 2:98 ethanol:water were used to rinse the syringe. The solution was flash frozen in a dry-ice/acetone bath and lyophilized to give [¹⁴C]-L-alanine tosylate.

Spectral grade methanol (250 μ L) was added to the residue followed by 30 μ L of 2 M thionyl chloride in anhydrous CH₂Cl₂. The mixture was stirred 4 h at which time 250 μ L of spectral grade methanol and 30 μ L of thionyl chloride/CH₂Cl₂ solution were added. The mixture was stirred for 18 h before addition of third portion of 30 μ L of thionyl chloride/CH₂Cl₂ solution. The reaction was stirred for 2 h before the solvent was removed with a stream of argon. The flask was placed under high vacuum for 15 min.

To [¹⁴C]-L-alanine methyl ester tosylate and 0.020 g of (1-N-biotinyl-(13-N-succinimidyl-(S-(E,E-farnesyl)-L-cysteiny)-L-valinyl-L-isoleucinyl)-4,7,10-trioxatridecanediamine) Kaiser's resin **6** were added a stock solution of glacial acetic acid (5.8 μ mol) in CH₂Cl₂ (200 μ L) and triethylamine (5.8 μ mol) in CH₂Cl₂ (200 μ L). The slurry was vigorously stirred for 96 h. The suspension was filtered through a Pasteur pipet/cotton plug. The resin was washed with four 1.0 mL portions of CHCl₃, three 1.0 mL portions of 2-propanol, and five 1.0 mL portions of 1:5 MeOH:CHCl₃. Solvent was removed from the filtrate with a gentle stream of argon.

The residue was dissolved in a minimal amount of 1:5 MeOH:CHCl₃ and applied to an analytical Merck glass-backed silica gel 60 thin-layer chromatography plate. The plate was eluted with 1:5 MeOH:CHCl₃ containing 5% v/v acetic acid. A faint UV-positive band of R_f value 0.68–0.80 was carefully removed by scraping, and the silica was placed in a Pasteur pipet plugged with cotton. The silica was eluted with twelve 1.0 mL portions of 1:5 MeOH:CHCl₃ containing 0.5% v/v acetic acid. Solvent was removed with a gentle stream of argon followed by high vacuum to remove final traces of acetic acid.

To the residue were added 200 μ L of THF and 200 μ L of 50 mM aqueous KOH. The progress of the reaction was monitored by silica TLC (1:5 MeOH:CHCl₃ containing 5% v/v acetic acid). Two hundred microliter portions of 50 mM aqueous KOH were added every 15 min until TLC analysis indicated the starting methyl ester had been saponified. Acetic acid (50 μ L, 1 M) was added. Solvent was removed with a gentle stream of argon, followed by high vacuum to removed traces of acetic acid and water. DMF (500 μ L) was added, and the solution was divided into portions, which were stored at $-80^\circ C$. The overall radiochemical yield was 45% from [¹⁴C]-L-alanine.

Growth of JDY101/pJel- α Rcemyc (15). Following the procedure of Worland and Wang (22), single colonies from freshly streaked plates were used to inoculate 10 mL cultures of synthetic minimal media lacking uracil and supplemented with 2% (w/v) glucose to repress PGAL1. Cultures were grown to late log phase, and a 3 mL portion was used to inoculate 300 mL of synthetic minimal media lacking uracil and supplemented with 3% (w/v) glycerol and 2% (w/v) lactic acid. The cultures were grown at $30^\circ C$ for 20–24 h (OD₆₀₀ = 0.5–0.7). Galactose was added to a final concentration of 2%, and the cells

were grown for an additional 6 h. Cells were pelleted by centrifugation at 5000*g* for 15 min, washed with cold sterile water, and stored at -80 °C.

Preparation of Membrane Fractions. Alkaline carbonate-leached membranes (AC-P139) were prepared according to the procedure of Ashby and Rine (9), with slight modifications. Briefly, 4 mL of frozen cell paste was thawed on ice and resuspended in 8 mL of SST buffer (0.3 M sorbitol, 0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, pH 8, 1 mM PMSF, 0.5 mM 1,10-phenanthroline). Acid washed glass beads (212–300 μm) were added (8 mL), and the suspension chilled on ice for 5 min prior to vortexing at maximum setting for 1 min and then chilled on ice for 1 min. The vortex-chilling cycle was repeated 10 times. The cellular lysate was clarified by centrifugation at 3500*g* for 15 min at 4 °C. The centrifugation was repeated for the supernatant. The final 3500*g* supernatant was transferred to ultracentrifuge tubes, mixed with 1/10 volume of 2 M Na_2CO_3 , pH 11.5, and spun at 139000*g* (40 000 rpm, TY65 rotor) for 1 h at 2 °C. The P139 pellet was gently washed twice with cold SST buffer (8 mL), resuspended in 8 mL of cold SST buffer with a Dounce homogenizer. The sample was spun again at 139000*g* for 1 h at 2 °C. The pellet was washed as before and was resuspended in 2 mL of cold SST buffer. The AC-P139 membrane preparation was divided into portions, quickly frozen in dry-ice/acetone, and stored at –80 °C.

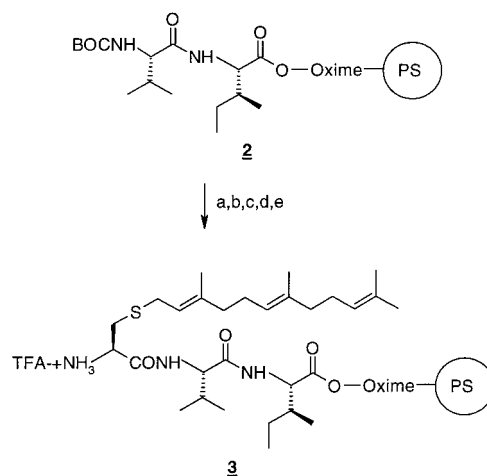
Protease Assays. *Biotin/Avidin-Binding Assay.* Assays (50 μ L) contained AC-P139 membranes (1–5 μ g) and 1-*N*-biotinyl-(13-*N*-succinimidyl-(*S*-(*E*,*E*-farnesyl)-L-cysteinyl)-L-valinyl-L-isoleucinyl-L-alanine)-4,7,10-trioxatridecanediamine ([^{14} C]1) in 100 mM Tris, pH 7.4, 0.5 mM 1,10-phenanthroline, and 1 mM PMSF. Reactions were initiated by the addition of [^{14} C]1 in 2.5 μ L of DMF. After incubation for 15–30 min at 37 $^{\circ}$ C, the enzyme was inactivated by heating at 80 $^{\circ}$ C for 5 min. The incubation mixtures were briefly cooled on ice, avidin resin (150 μ L, Pierce) was added, and the material was allowed to stand at room temperature for 15 min with frequent mixing. Assay buffer (150 μ L) was added, the tubes were centrifuged for 1 min, and radioactivity in 200 μ L of supernatant was measured.

Radioactive HPLC Assay to Confirm Endo-Protease Activity. Assays (50 μ L) contained AC-P139 membranes (1–100 μ g) and [^{14} C]**1** in 100 mM Tris, pH 7.4, 0.5 mM 1,10-phenanthroline, and 1 mM PMSF. Reactions were initiated by the addition of [^{14} C]**1**. Incubation proceeded for 15–30 min at 37 $^{\circ}$ C followed by termination in boiling water for 5 min. Assay tubes were briefly centrifuged, and the supernatants were transferred to fresh tubes. Avidin resin (150 μ L) was added and the tubes were allowed to stand at room temperature for 15 min with frequent mixing. Assay buffer (150 μ L) containing a mixture of cold A, I-A, and V-I-A was added, and the tubes were centrifuged for 1 min. A 200 μ L portion of the peptide/reaction mixture was transferred to fresh tubes and an additional 150 μ L of the mixture containing cold peptide was added. These samples were frozen at -80° C until HPLC analysis. Samples were analyzed on a 4.6 $\times$ 250 mm Vydac reversed phase C₁₈ column with isocratic elution using 3.5% acetonitrile/0.06% TFA. After the first min, 1 min fractions were collected, added to 10 mL of scintillation cocktail, and counted.

RESULTS AND DISCUSSION

Synthesis of Nonradioactive and Radiolabeled Biotinylated Farnesylated Tetrapeptide 1. Biotin-

Scheme 2. Synthesis of S-Farnesylated Kaiser's Resin^a

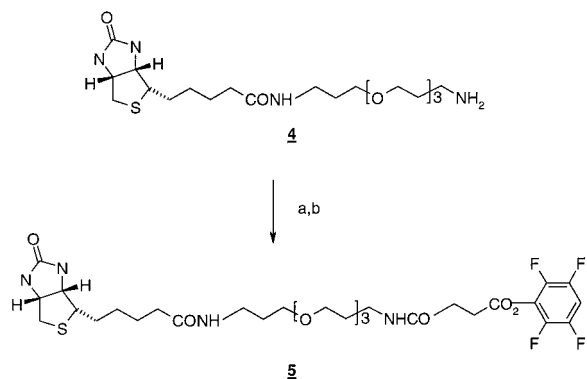


^a Reagents: (a) TFA in CH₂Cl₂, 1.5 h; (b) *N*-trityl-(*S*-farnesyl)-L-cysteine, THF, DCC, HOBT, 0 °C; (c) Et₃N, CH₂Cl₂; (d) wash protocol, dried in vacuo; (e) 2.5 equiv of TFA, 10 equiv of EtSMe, CH₂Cl₂.

ylated **1** was synthesized using solid-phase methods based on Kaiser's oxime resin (*18*) as outlined in Schemes 2, 3, and 4. This approach allowed us to take advantage of solid-phase chemistry to assemble the entire biotinylated, farnesylated, radiolabeled tetrapeptide and to introduce the radioactive unit near the end of the synthetic sequence. The reactions needed to assemble and cleave the peptide from the resin are compatible with the sensitive isoprenoid group attached to the cysteine residue.

Kaiser's benzophenone oxime resin was synthesized on a 100 g scale using the method of DeGrado and Kaiser (18). *N*-BOC-L-valinyl-L-isoleucinyl resin **2** was produced by a standard sequence using HOBt/DCC activation and TFA mediated *N*-BOC deprotection steps. As outlined in Scheme 2, *N*-BOC-L-valinyl-L-isoleucinyl resin **2** was deprotected using TFA and then allowed to react with the HOBt-derived active ester of *N*-triphenylmethyl-S-(*E,E*-farnesyl)-L-cysteine. A standard wash protocol of four 100 mL portions of CH₂Cl₂, four 100 mL portions of DMF, four 100 mL portions of 2-propanol, and finally four 100 mL portions of CH₂Cl₂ was used after all peptide or linker coupling steps. After drying in vacuo, the *N*-trityl resin was suspended in CH₂Cl₂ containing ethylmethyl sulfide as a cation scavenger and deprotected with TFA. The TFA salt of the resin **3** was used immediately in the next step.

Attempts using solution phase chemistry to attach biotin to farnesylated cysteine by an L-alanine linker were unsatisfactory. Although coupling between biotin-L-alanine and S-(*E,E*-farnesyl)-L-cysteine esters appeared to proceed as judged by TLC, the product formed an intractable gel in all solvents we studied, including DMSO-*d*₆, typically used for NMR spectra. Lui et al. (17) had reported a similar synthesis; however, in their case the cysteine was not farnesylated, and the isoprenoid moiety was added in an additional step near the end of the synthesis. To avoid problems with solubility, a PEG linker (20) was used to tether biotin to the tetrapeptide (Scheme 3). The biotinylated-PEG linker (**4**) was synthesized as described by Wilbur et al. (20). The free amine of **4** was converted to the succinate half-amide, and the free carboxylate group was activated using 2,3,5,6-tetrafluorophenyltrifluoroacetate (21). This activating agent was particularly useful, since the biotinylated

Scheme 3. Synthesis of Biotinylated Linker^a

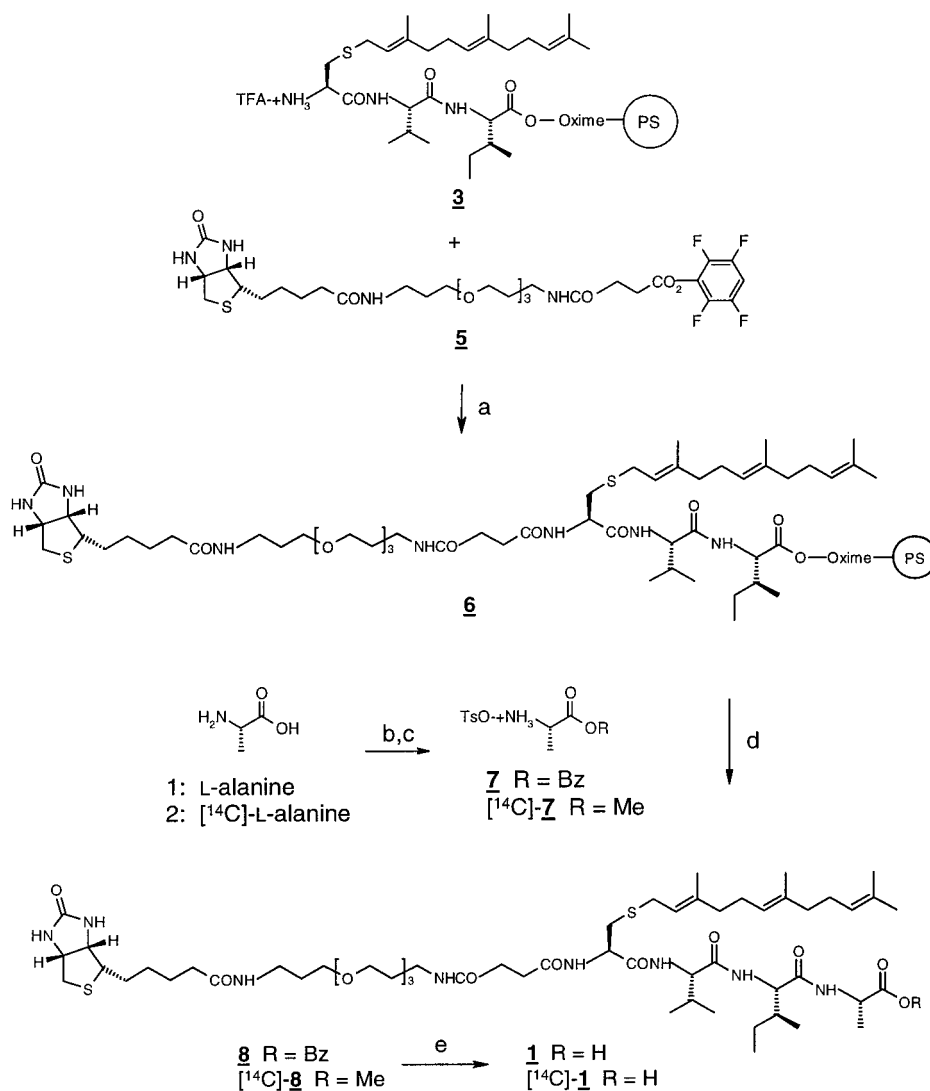
^a Reagents: (a) succinic anhydride, DMF, Et₃N; (b) 2,3,5,6-tetrafluorophenyltrifluoroacetate, DMF, Et₃N.

active ester could easily be purified by precipitation using diethyl ether to remove excess activating agent and triethylamine salts. Active ester **5** was isolated by ether precipitation, dried in vacuo, and used immediately in the next step.

The final reactions for syntheses of both the nonradioactive and radiolabeled forms of the biotinylated sub-

strate **1** are shown in Scheme 4. The TFA salt of the farnesylated tripeptide resin **3** and the biotinylated tetrafluorophenyl active ester **5** were successfully coupled to give the biotinylated farnesylated Kaiser's oxime ester resin **6**. Resin **6** was used to synthesize both the non-radioactive substrate **1** and radioactive [¹⁴C]**1**. The final peptides were cleaved from the resin as also outlined in Scheme 4. The coupling procedures for radioactive and nonradioactive peptides differed slightly with respect to the type of alanine ester used. Initially, L-alanine benzyl ester was used for the nonradioactive synthesis, to take advantage of the UV-active benzyl group during purification. Accordingly, biotinylated farnesylated Kaiser's oxime ester resin **6** was treated with L-alanine benzyl ester tosylate **7** (R = Bz) to give **8** (R = Bz) in 26% yield overall from Kaiser's oxime resin. Saponification of **8** (R = Bz) with aqueous KOH in THF gave nonradioactive **1** in 72% yield.

Methyl ester [¹⁴C]**7** (R = CH₃) was used to synthesize [¹⁴C]**1**. Initial attempts to esterify L-alanine as a benzyl ester on a scale compatible with the synthesis of radioisotopes gave unsatisfactory yields. Accordingly, [¹⁴C]-L-alanine was esterified using *p*-toluenesulfonic acid and thionyl chloride in methanol to afford the tosylate salt of [¹⁴C]**7** (R = CH₃). [¹⁴C]**7** (R = CH₃) was coupled to

Scheme 4. Synthesis, Radiosubstrate Cleavage, and Saponification^a

^a Reagents: (a) 1:1 DMF:CH₂Cl₂, Et₃N; (b) 1.05 equiv of *p*-TsOH, (c) (1) BzOH, PhH, reflux; (2) MeOH, SOCl₂; (d) HOAc, Et₃N, CH₂Cl₂; (e) KOH, THF.

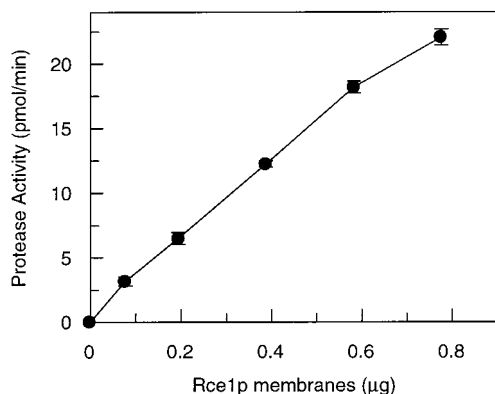


Figure 1. $\text{Ca}_{1.2}\text{X}$ endoprotease activity increases with increasing amounts of alkaline carbonate leached membranes. $[\text{C}^{14}]\text{1}$ ($25\text{ }\mu\text{M}$) was incubated with yRce1p membranes ($0.1\text{--}1.0\text{ }\mu\text{g}$ of protein) at $37\text{ }^{\circ}\text{C}$ for 15 min. The reaction was stopped and the mixture was treated with avidin resin as described in the Experimental Section.

biotinylated farnesylated **6** using conditions similar to those for unlabeled material, followed by aqueous work-up, to afford $[\text{C}^{14}]\text{8}$ ($\text{R} = \text{CH}_3$). The tetrapeptide was purified easily by analytical TLC to remove any unreacted radioactive methyl ester in order to minimize any radioactive contaminants that would contribute to a higher background in the enzymatic assay. The methyl ester was saponified to afford $[\text{C}^{14}]\text{1}$ in an overall radiochemical yield of 45% for the coupling and hydrolysis steps. This material comigrated with unlabeled **1** on reverse-phase HPLC.

Endoprotease Assays. yRce1 activity was measured by release of the radioactive C-terminal tripeptide from $[\text{C}^{14}]\text{1}$ using biotin/avidin binding to separate the radioactive product and substrate. The assay involved incubation of $[\text{C}^{14}]\text{1}$ with yRce1p membranes (15) followed by heat inactivation. Unreacted substrate was separated from the VIA fragment by incubation of the reaction mixture with avidin resin at room temperature. The resin was pelleted by centrifugation, and the radioactivity of $\text{VI}[\text{C}^{14}]\text{A}$ in the supernatant was measured by liquid scintillation counting. A proportional increase in protease activity was observed upon incubation of a saturating concentration of $[\text{C}^{14}]\text{1}$ with increasing amounts of membranes (Figure 1). At saturating substrate concentrations ($>25\text{ }\mu\text{M}$), it was necessary to use $150\text{ }\mu\text{L}$ of avidin resin in order to achieve 10:1 signal-to-noise. Incubation of the assay mixture with avidin resin for 15 min with frequent mixing was sufficient to remove radioactive substrate.

Steady-state kinetic constants were determined for membrane-bound yRce1p. A plot of initial velocity versus $[\text{C}^{14}]\text{1}$ is shown in Figure 2. The experimental data were fit to the Michaelis–Menten equation to give $K_M = 1.3 \pm 0.3\text{ }\mu\text{M}$ and $V_{\text{Max}} = 46.1 \pm 2.7\text{ nmol min}^{-1}\text{ mg}^{-1}$. Addition of biotin and the PEG linker to the farnesylated tetrapeptide apparently had a minimal impact on the ability of yRce1p to hydrolyze the substrate. A K_M of $2.8\text{ }\mu\text{M}$ was obtained with membranes from native yeast using the peptide substrate KWDP(farnesyl)CV-[4,5- ^3H]**1A** (9). In addition, a non-hydrolyzable inhibitor of mammalian $\text{Ca}_{1.2}\text{X}$ endoproteases reported by Ma et al. (23), inhibited membrane-bound recombinant yRce1p with an $\text{IC}_{50} = 103 \pm 9\text{ nM}$ (data not shown).

Although alanine and the IA dipeptide are potential radioactive products from $[\text{C}^{14}]\text{1}$ by nonspecific proteolysis by Rce1p or contaminating proteases in the crude membrane preparation, analysis of the reaction mixture by

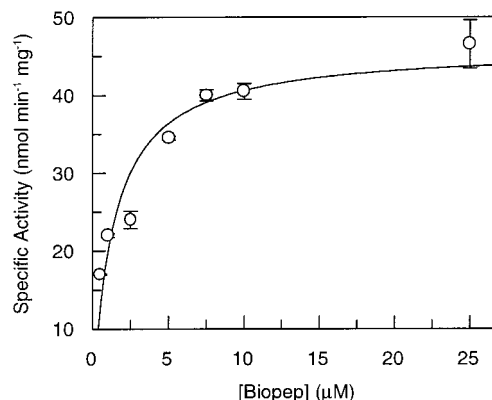


Figure 2. A plot of the initial velocity for proteolysis versus $[\text{C}^{14}]\text{1}$ concentration. yRce1p membranes ($0.29\text{ }\mu\text{g}$) were incubated with $[\text{C}^{14}]\text{1}$ for 15–30 min at $37\text{ }^{\circ}\text{C}$. $\text{VI}[\text{C}^{14}]\text{A}$ was separated from unreacted substrate with avidin resin and counted as described in the Experimental Section.

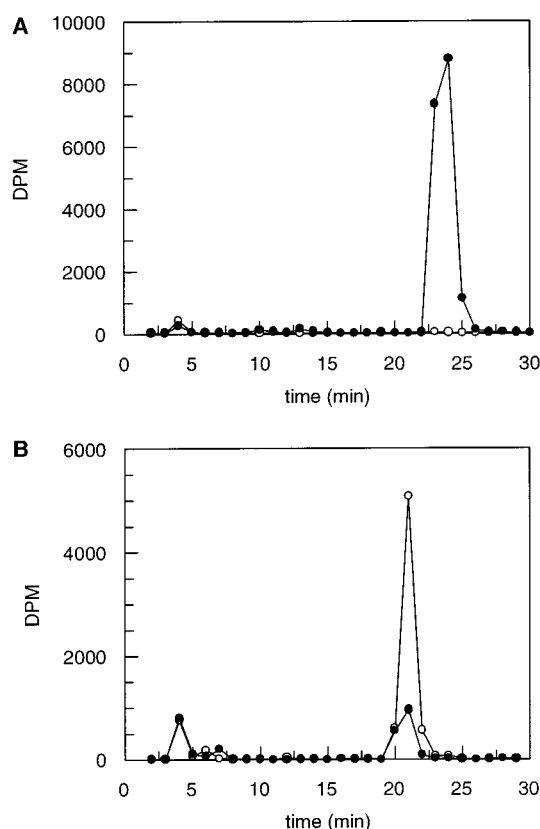


Figure 3. Reversed-phase HPLC analysis of the products from farnesylated peptide, $[\text{C}^{14}]\text{1}$ upon incubation with yRce1p. Yeast membranes ($5\text{--}20\text{ }\mu\text{g}$ of protein) were incubated with $50\text{ }\mu\text{M}$ $[\text{C}^{14}]\text{1}$ for 45 min at $37\text{ }^{\circ}\text{C}$. The reactions were stopped by boiling for 5 min and were treated with avidin resin as described in the Experimental Section. Cold A, IA, and VIA were added to the nonbiotinylated products. The mixture was analyzed by HPLC on a C_{18} reversed-phase column as described in the Experimental section. Fractions were collected in 1 min intervals. Detection of the cold standards was monitored at 214 nm .

reversed phase HPLC confirmed that $\text{VI}[\text{C}^{14}]\text{A}$ was the only radioactive product. After incubating $[\text{C}^{14}]\text{1}$ with Rce1p under standard assay conditions, the avidin-treated supernatant was mixed with cold A, IA, and VIA, and the mixture was analyzed by HPLC to give the results shown in Figure 3. The large peak of radioactivity coeluted with VIA, and no significant radioactivity above background was detected with the fractions correspond-

ing to alanine (Figure 3A). In contrast, prolonged incubation with membranes prepared from a yeast strain deficient in Rce1p activity (15) showed no radioactivity coeluting with VIA and only a small amount of radioactivity coeluting with alanine. A similar profile was seen when no membranes were added. The peak coeluting with VIA decreased when the RPI inhibitor was added to the assay (Figure 3B). In contrast, membranes isolated from rat liver produced tripeptide VI-[³H]S, dipeptide I-[³H]S, and, in some cases, [³H]S (17). The smaller radioactive fragments, released from substrate and the tripeptide product by contaminating protease activities, limited the utility of the assay to precisely measure the activity of the Ras and a-mating factor proteases. Our recombinant membrane preparations were essentially free of activities that compete with yRce1p for the substrate. [¹⁴C]1 and recombinant yRce1p were recently used to assess the sensitivity of the enzyme to metal ion chelators, heavy metals, and a variety of protease inhibitors (15) and to screen a library of *N*-acetyl-S-(*E,E*-farnesyl) Ca₁a₂X tetrapeptides (24).

Spacers separate biotin from bulky groups elsewhere in the molecule that can interfere with its binding avidin and streptavidin (25) and help maintain strong biotin/avidin binding (20). We used a commercially available water soluble spacer, 4,7,10-trioxa-1,13-tridecanediamine and a succinic diamide unit to link biotin to the farnesylated Ca₁a₂X peptide. The PEG spacer improved the solubility of the biotinylated prenyl Ca₁a₂X peptide in common organic solvents and greatly facilitated its synthesis and ease of handling. The tetrafluorophenyl active ester used to couple the biotinylated-PEG linker to the resin bound tripeptide proved to be ideal. The ester was easy to prepare, easy to purify by ether precipitation, and coupled smoothly to resin-bound peptide in good yield.

A completely solid-phase method based on Kaiser's resin provides several advantages over previously reported syntheses of compounds of this kind. Acid-sensitive groups such as the farnesyl and geranylgeranyl moieties found in prenylated proteins can be introduced regiospecifically as modified amino acids (i.e., *S*-farnesyl cysteine) during the peptide elongation process. The mild conditions used to cleave the peptide from the resin are ideal for preserving acid-sensitive side chains. Since the nucleophile employed to cleave the peptide becomes the *N*-terminal amino acid in the released peptide, radiolabel can be introduced into the final product at a late stage in the synthesis in a manner that minimizes the handling of radioactive materials. The high specific activity of [¹⁴C]1, and corresponding high sensitivity of the assay, has proved to be useful for screening and characterization of inhibitors for Ca₁a₂X proteases.

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Synthesis, Characterization, and Biological Properties of Cyanine-Labeled Somatostatin Analogues as Receptor-Targeted Fluorescent Probes

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We present the synthesis and characterization of the somatostatin receptor-specific peptide $\text{H}_2\text{N}(\text{D-Phe})\text{-cyclo}[\text{Cys-Phe}(\text{D-Trp})\text{-Lys-Thr-Cys}]\text{-Thr-OH}$, which is labeled with a carboxylated indodicarbo- and an indotricarbocyanine dye at the N-terminal amino group. The preparation was performed by automated solid-phase synthesis, with subsequent attachment of the cyanine dye and cleavage of the entire conjugate from the resin. The compounds display high molar absorbance and fluorescence quantum yields typical for cyanine dyes and are thus suitable receptor-targeted contrast agents for molecular optical imaging. The ability of these agents to target the somatostatin receptor was demonstrated by flow cytometry in vitro, in which the indotricarbocyanine conjugate led to elevated cell-associated fluorescence on somatostatin receptor-expressing tumor cells. In contrast, the corresponding linearized derivative of the sequence $\text{H}_2\text{N}(\text{D-Phe})\text{-Met-Phe}(\text{D-Trp})\text{-Lys-Thr-Met-Thr-OH}$ produced only minimal cell fluorescence, hence confirming the specificity of the cyclic somatostatin analogue. Intracellular localization could be visualized by near-infrared (NIR) fluorescence microscopy. In conclusion, receptor-specific peptides are promising tools for designing site-directed optical contrast agents for use in molecular optical imaging.

INTRODUCTION

Neuropeptides and their pharmacologically optimized analogues have a unique clinical potential as carrier molecules for diagnostic and therapeutic substances. Based on the observation that many human tumors overexpress receptors for neuropeptides (1, 2), new diagnostic methods have been developed that detect and localize tumors using radiolabeled neuropeptides (3, 4). The clinical diagnostic value of receptor scintigraphy has been demonstrated by the use of [^{111}In]DTPA-octreotide (Pentetreotide, OctreoScan), a radiodiagnostic agent with high binding affinity for somatostatin receptors (5).

To date, five different somatostatin receptor subtypes have been cloned and functionally characterized (6). These receptor subtypes mediate the many physiological effects of somatostatin, for example the regulation of hormone release (7, 8). They are present in both normal (6) and tumor tissues of the central nervous system and peripheral areas including the gastroenteropancreatic system (9, 10). In vitro studies have shown that the majority of these tumors, particularly neuroendocrine tumors, preferentially express the somatostatin receptor subtype 2 (9, 11). This represents the molecular basis for therapeutic and diagnostic applications of somatostatin analogues, such as octreotide.

Octreotide, a synthetic octapeptide with improved metabolic stability and high binding affinity for the somatostatin receptor subtypes 2 and 5, was first used for the symptomatic treatment of patients with neuroendocrine tumors (12). The high density of somatostatin receptor subtype 2 in neuroendocrine tumors has led to the development of octreotide scintigraphy (5). In vivo visualization of somatostatin receptor positive tumors using [^{111}In]DTPA-octreotide, is based on the binding, internalization, and retention of the radioligand in intracellular compartments such as endosomes or lysosomes (13, 14).

The emerging field of biomedical optical imaging (15) has awakened a great deal of interest in developing optical contrast agents capable of engendering disease-specific optical signals within the tissue (16). The detection of site-specific fluorescence provides a notable alternative to radioactive imaging methods, as tissue is relatively transparent for near-infrared light (700–900 nm) (15). Cyanine dyes have proven to be promising contrast agents for the in vivo fluorescence demarcation of tumors (17, 18) and have been successfully applied as fluorescent labels for target-specific carriers (19, 20).

We report on the synthesis and the photophysical and biological properties of novel fluorescently labeled somatostatin receptor-specific peptides of the sequence $\text{H}_2\text{N}(\text{D-Phe})\text{-cyclo}[\text{Cys-Phe}(\text{D-Trp})\text{-Lys-Thr-Cys}]\text{-Thr-OH}$, which are labeled with a carboxylated indodicarbo- or indotricarbocyanine dye at the N-terminal amino group. The compounds represent fluorescent analogues of pentetreotide ([^{111}In]DTPA-cyclo[Cys-Phe(D-Trp)-Lys-Thr-Cys]-Thr(ol)). Using the cyanine-labeled peptides, we intend to show that the advantages of low molecular weight

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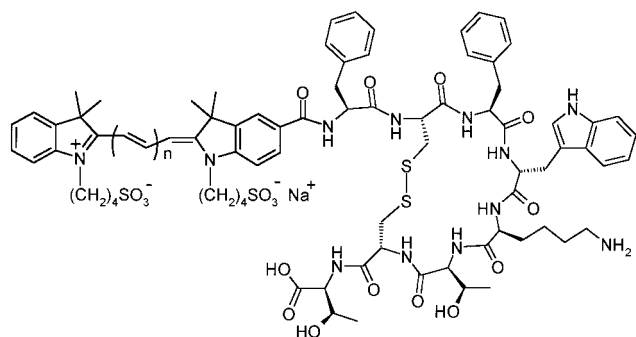


Figure 1. Chemical structures of indodicarbocyanine-peptide conjugate **1** ($n = 2$) and indotricarbocyanine-peptide conjugate **2** ($n = 3$).

peptides are of practical use for other diagnostic techniques and permit the development of site-directed fluorescent *in vivo* contrast agents.

For this purpose, we have synthesized and characterized two monocarboxylated cyanine dyes of different absorption wavelengths employing a carboxylic acid group that permits covalent attachment to peptides during solid-phase preparation. We describe results from the solid-phase synthetic preparation of the two cyanine-labeled octreotide-analogue peptide conjugates **1** and **2** (Figure 1). Results from studies on cellular uptake *in vitro* demonstrate that low molecular weight peptides, structurally based on somatostatin, enable receptor-mediated intracellular delivery of optical probes.

EXPERIMENTAL PROCEDURES

Synthesis of Cyanine Dyes. *Chemicals and Analysis.* 1-(4-Sulfobutyl)-2,3,3-trimethyl-3H-indolenin (**5**) and 5-carboxy-1-(4-sulfobutyl)-2,3,3-trimethyl-3H-indolenin (**6**) were synthesized as described in the literature (21, 22). Glutaconic aldehyde dianilide hydrochloride was purchased from Lancaster Synthesis (Mühlheim, Germany). All other reagents and solvents were purchased from Sigma-Aldrich Co. Reactions were monitored by reversed phase TLC (RP-18 plates) with water/methanol (1:2). Purification of the synthesized dyes was performed by reversed phase chromatography on an MPLC (Kronwald, Sinsheim, Germany) with RP-18 material (Euoprep 60–30 C-18, 60 Å, 30 μ m, Knauer) using water/methanol as eluant. The synthesized dyes were characterized by ^1H NMR and mass spectroscopy. The ^1H NMR spectra were recorded in CD_3OD with a Bruker AC400 (400 MHz). The mass spectra were obtained with a ZAB-E spectrometer (Fisons Scientific Equipment, Loughborough, UK). Measured mass/charge ratios (m/z) and proton shifts (δ) are listed for each compound.

Bis-1,1'-(4-sulfobutyl)indodicarbocyanine-5-carboxylic Acid, Sodium Salt (7). A mixture of **5** (1 g, 3.4 mmol) and malonaldehyde-bis(phenylimine) monohydrochloride (0.78 g, 3.0 mmol) in 12 mL of acetic anhydride was stirred for 30 min at 120 $^\circ\text{C}$. After cooling to room temperature, **6** (1.2 g, 3.6 mmol), sodium acetate (1.0 g, 12 mmol), 12 mL of acetic anhydride, and 5 mL of acetic acid were added. The resultant mixture was heated to 120 $^\circ\text{C}$ under vigorous stirring, cooled to room temperature, and poured into 150 mL of diethyl ether. The precipitate was collected by filtration and then purified by reversed phase chromatography (Euoprep 60–30 C-18, 60 Å, 30 μ m, Knauer; eluents water/methanol 9:1 to 1:1) yielding 1.3 g (72%) **7** as a blue lyophilisate.

^1H NMR: δ 1.75 (s, 12H, $4 \times \text{CH}_3$), 2.0 (m, 8H, $2 \times \text{CH}_2\text{CH}_2\text{CH}_2\text{SO}_3^-$), 2.9 (t, 4H, $2 \times \text{CH}_2\text{SO}_3^-$), 4.1, 4.2 (2t,

4H, $2 \times >\text{NCH}_2$), 6.3, 6.45 (2d, 2H, $\alpha\text{-H}$, $\alpha'\text{-H}$), 6.7 (t, 1H, $\gamma\text{-H}$), 7.3 (dd, 2H, $5'\text{-H}$, $6'\text{-H}$), 7.4 (m, 2H, $4'\text{-H}$, $7'\text{-H}$), 7.5 (d, 1H, 7-H), 8.05 (s, 1H, 4-H), 8.1 (d, 1H, 6-H), 8.2, 8.3 (2dd, 2H, $\beta\text{-H}$, $\beta'\text{-H}$). FAB-MS: 715 [35, (M + Na) $^+$], 693 [70, (M + H) $^+$], 671 [35, (M + 2H-Na) $^+$], 91 (100).

Bis-1,1'-(4-sulfobutyl)indotricarbocyanine-5-carboxylic acid, sodium salt (8). The synthesis and product isolation were performed as described for **7** using glutaconic aldehyde dianilide hydrochloride (0.86 g, 3.0 mmol) with **5** (1 g, 3.4 mmol), **6** (1.2 g, 3.6 mmol), and sodium acetate (1.0 g, 12 mmol), yielding 1.5 g (80%) **8** as a blue lyophilisate. ^1H NMR: δ 1.6, 1.65 (2s, 12H, $4 \times \text{CH}_3$), 1.7–1.9 (m, 8H, $2 \times \text{CH}_2\text{CH}_2\text{CH}_2\text{SO}_3^-$), 2.55 (t, 4H, $2 \times \text{CH}_2\text{SO}_3^-$), 3.95, 4.2 (2 t, 4H, $2 \times >\text{NCH}_2$), 6.25 (d, 1H, $\alpha'\text{-H}$), 6.55 (dd, 1H, $\gamma'\text{-H}$), 6.65 (dd, 1H, $\gamma\text{-H}$), 6.7 (d, 1H, $\alpha\text{-H}$), 7.25 (d, 1H, 7-H), 7.35, 7.45 (2t, 2H, $5'\text{-H}$, $6'\text{-H}$), 7.6, 7.65 (2d, 2H, $4'\text{-H}$, $7'\text{-H}$), 7.75 (m, 2H, $\delta\text{-H}$, $\beta'\text{-H}$), 7.9 (d, 1H, 6-H), 7.95 (s, 1H, 4-H), 8.0 (dd, 1H, $\beta\text{-H}$). FAB-MS: 719 [12, (M + H) $^+$], 697 [25, (M + 2H-Na) $^+$], 135 (100).

Synthesis of Dye–Peptide Conjugates. *Chemicals, Equipment, and Analysis.* Fmoc-protected amino acids were purchased from Orpegen (Heidelberg, Germany) and Nova Biochem GmbH (Bad Soden, Germany) and used with the following side-chain protecting groups: *tert*-butoxycarbonyl (Boc) for Lys and Trp, *tert*-butyl ether for Thr, and Trityl (trt) for Cys. Dimethylformamide (DMF) and acetonitrile were purchased from Proligo GmbH (Hamburg, Germany), and piperidine and diisopropyl ethylamine from Sigma-Aldrich Co. Peptide resins were obtained from Rapp Polymere GmbH (Tübingen, Germany). Assembly of the protected peptide chain was carried out using the stepwise solid-phase method on an automated peptide synthesizer (433A, Perkin-Elmer GmbH, Überlingen, Germany). The mass spectra (MALDI-TOF) were recorded on an Compact Maldi II spectrometer (Shimadzu, Japan). Analytical HPLC analysis was performed using a Nucleosil 300 C18 column (5 μ m particle size; 125 $\times$ 4.6 mm); gradient: 10% B to 100% B for 45 min [(A) 1000 mL of water, 2 mL of TFA; (B) 500 mL of acetonitrile, 100 mL of water, 1 mL of TFA].

Synthesis of Dye–Peptide Conjugates 1 and 2. The peptide sequence (D-Phe)-Cys-(D-Trp)-Lys-Thr-Cys-Thr-OH was built up by Fmoc chemistry using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as the activating agent and 20% piperidine in DMF for deprotection of the Fmoc groups.

In the last step of the synthesis, **7** or **8** were coupled for 18 h on resin in DMF using HBTU, diisopropyl ethylamine (preactivation with 1.1 equiv of HBTU for 15 min). After completion, the resin was washed with DMF and methylene chloride, dried, and then treated with 10 mL of a mixture containing 90% trifluoroacetic acid (TFA), 5% water, and 5% triisopropylsilane. TFA was removed under vacuum and the crude peptide precipitated with diethyl ether. Cys–Cys cyclization was achieved by dissolving the crude peptide in dimethyl sulfoxide (DMSO)/water (15:85), adjusting the pH to 7.5, and stirring the mixture for 18 h. After completion (HPLC control) the peptide solution was lyophilized twice to remove the DMSO. The crude, cyclized peptide was purified by reversed-phase HPLC on a VYDAC C18 column (40 $\times$ 300 mm, 1520 μ m, 300 Å) at a flow rate of 100 mL/min using an acetonitrile gradient of 30% B to 80% B for 50 min [(A) 1000 mL of water, 2 mL of TFA; (B) 500 mL of acetonitrile, 100 mL of water, 1 mL of TFA]. The fractions were analyzed by HPLC. The purified fraction containing the desired product was collected,

freeze-dried, and analyzed by mass spectrometry (MALDI-TOF). (1) Calcd 1705, found 1699; (2) calcd 1731, found 1726.

Synthesis of Control Dye–Peptide Conjugates 3 and 4. The synthesis of the control peptide sequence (D-Phe)-Met-Phe-(D-Trp)-Lys-Thr-Met-Thr-COOH and the conjugation of dyes 7 and 8 were performed analogously. The crude product was directly subjected to chromatographic purification as described above. MALDI-TOF. (3) Calcd 1734, found 1730; (4) calcd 1760, found 1756.

Photophysical Characterization. Absorption measurements were performed with a Lambda 2 photometer (Perkin-Elmer GmbH, Überlingen, Germany). Fluorescence properties were measured in phosphate-buffered saline (PBS) and bovine plasma with a SPEX Fluorolog fluorometer (Instruments S. A. GmbH, Grassbrunn, Germany) (single excitation and emission monochromator, 350 W xenon lamp, PM Hamamatsu R928) with 2 μ M dye solutions in front-face mode. The solutions of the indodicarbocyanine peptides 1 and 3 were excited at 590 nm, those of the indotricarbocyanine peptide conjugates 2 and 4 at 690 nm. To determine fluorescence quantum yields (QY's), the lamp and photomultiplier were calibrated with respect to their spectral sensitivity. The quantum yields obtained are based on the standards indocyanine green (ICG) in DMSO ($\Phi = 0.13$) and NK1967 in DMSO ($\Phi = 0.28$) (23).

Cell Culture Experiments. The cellular uptake of 2 and 4 was examined by fluorescence microscopy and flow cytometry using somatostatin receptor subtype 2 transfected RIN38 tumor cells (RIN38 SSTR2 cells) (24). RIN38 cells were cultured following standard procedures (RPMI 1640 medium; Life Technologies GmbH, Eggenstein, Germany) and using 5% fetal bovine serum and 5% newborn calf serum supplemented with Geneticin (200 μ g/L).

In preparation for flow cytometry, 1×10^5 RIN38 SSTR2 cells were cultured on 24-well microtiter plates for 3 days. Cells were incubated in 300 μ L of buffer containing 50 mM Tris/HCl, pH 7.5, 5 mM MgCl_2 , 1 mM CaCl_2 , 100 mM NaCl, 4% BSA (incubation buffer) in the presence of 100 nM cyanine dye-labeled peptides for 1 h at 37 $^\circ\text{C}$. After incubation, the medium was removed and the cells were washed twice with PBS. The cells were then incubated with 200 μ L of a 0.25% trypsin solution in PBS for 2 min at room temperature. The cell suspension was transferred to a 5 mL polystyrene tube (Becton Dickinson, Heidelberg, Germany) and centrifuged for 5 min at 4 $^\circ\text{C}$ and 400g. The supernatant was removed and the cells were resuspended in 300 μ L of CellFIX (Becton Dickinson, Heidelberg, Germany). FACS analysis was performed using a FACSCalibe flow cytometer (Becton Dickinson, Heidelberg, Germany) with a red laser diode (~ 635 nm) for excitation and FL3 optics for detection. Cell-associated fluorescence was recorded within a defined cell population until the event of 10 000 cells was reached.

For fluorescence microscopic studies RIN38 SSTR2 cells were cultured on cover slides (22 $\times$ 22 mm). After 24 h the cover slides were transferred to a six-well macro plate (Greiner GmbH, Frickenhausen, Germany) containing 0.5 mL of incubation buffer (see above). Peptide conjugate 2 or 4 was added at a final concentration of 1 μ M, and incubation was carried out for 1 h at 4 $^\circ\text{C}$. After incubation, the cells were washed twice in 2 mL of cold PBS, and 0.5 mL of prewarmed incubation buffer was added. The cells were incubated at 37 $^\circ\text{C}$ in the absence of peptides for a further 1 and 60 min. The cells were fixed by the addition of 2 mL of 0.1% CellFIX (Becton

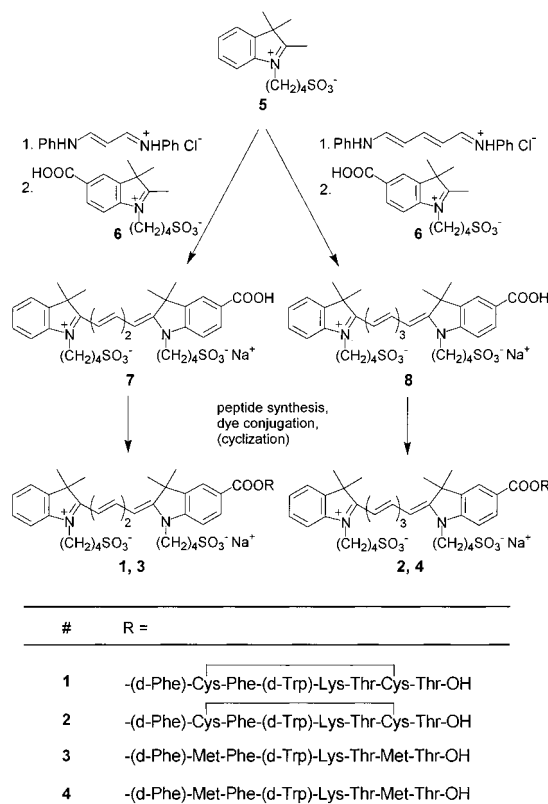


Figure 2. Scheme showing the preparation of the monocarboxylated indodicarbo- (7) and indotricarbocyanine (8), and the structures of the resulting peptide conjugates 1–4.

Dickinson, Heidelberg, Germany) per well for 15 min at room temperature. Cover slides were mounted for microscopic analysis using Roti Histokit (Carl Roth GmbH, Karlsruhe, Germany). The fluorescence signal of the cells was recorded using an Axiovert 135 fluorescence microscope (Carl Zeiss Jena GmbH, Göttingen, Germany) equipped with a Cy 7 filter set (Exciter, HQ 710/75; Emitter, HQ 810/90; Beam splitter, Q750LP). An AttoArc HBO 100 W microscopic illuminator (Carl Zeiss Jena GmbH, Göttingen, Germany) was used as a light source for fluorescence excitation. Images were taken using a thermoelectrically cooled charge-coupled device (CCD) (Micromax, model RTE/CCD-576, Princeton Instruments Inc., Trenton, NJ) and analyzed using WinView Software version 1.6.2 (Princeton Instruments Inc., Trenton, NJ).

RESULTS AND DISCUSSION

Synthesis of Cyanine Dye–Peptide Conjugates.

To enable a directed and uniform labeling of peptides, we synthesized two cyanine dyes bearing a carboxylic acid group as activatable moiety for further linkage. In contrast to dyes described in the literature (25–27), the carboxylic acid group is positioned at one of the benzoic rings of the chromophore, thus resulting in nonsymmetrically substituted compounds with a benzoic acid-like element. The synthesis is easily performed starting from the indolenins 5 (21) and 6 (22), giving bis-1,1'-(4-sulfobutyl)indodicarbocyanine-5-carboxylic acid (7) and bis-1,1'-(4-sulfobutyl)indotricarbocyanine-5-carboxylic acid (8) in 72 and 80% yields, respectively, after chromatographic purification. This synthesis is illustrated in Figure 2. The performance of the reactions allowed the suppression of the formation of the corresponding symmetric byproducts, which were estimated (TLC; data not shown) to be less than 20% in the crude product.

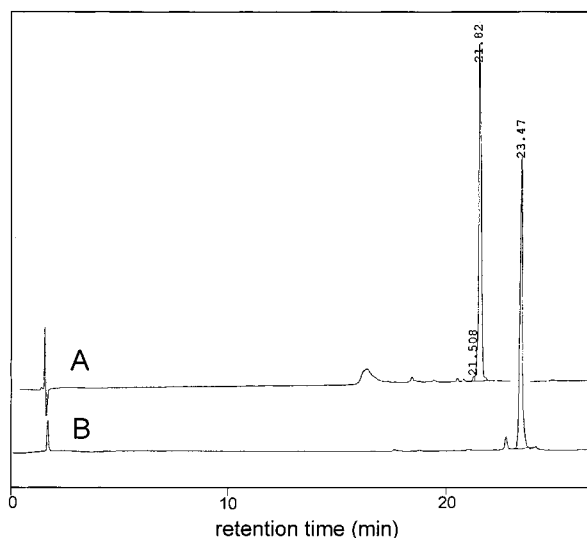


Figure 3. Reversed-phase HPLC chromatograms of **1** (A) and **2** (B). Conditions are stated in the text.

Table 1. Spectral Properties of Indodicarbocyanine and Indotricarbocyanines Peptide Conjugates in Comparison with the Free Dyes

compd ^a	$\lambda_{\text{max,abs}}$ (nm)	$\lambda_{\text{max,em}}$ (nm)	Stokes shift (nm)	ϵ (L mol ⁻¹ cm ⁻¹)	QY ^b
7 (free dye)	647	673	26	255 000	0.30 ^c
1	648	670	22	135 000	0.26 ^c
3	649	675	26	178 000	0.31 ^c
8 (free dye)	747	782	35	190 000	0.09 ^d
2	748	780	32	110 000	0.10 ^d
4	748	784	36	135 000	0.08 ^d

^a Solvent PBS. ^b Fluorescence quantum yields based on ICG as standard (QY = 0.13 in DMSO). ^c Excited at 605 nm. ^d Excited at 685 nm.

The somatostatin receptor-specific peptide sequence (D-Phe)-Cys-Phe-(D-Trp)-Lys-Thr-Cys-Thr-OH was generated by automated solid-phase synthesis using Fmoc chemistry. For N-terminal dye attachment at the resin, **7** and **8** were readily activated using HBTU as activating agent followed by incubation with the dye reaction mixture for 18 h. TFA cleavage of protecting groups and liberation of the conjugate from the resin was possible without noticeable decomposition of the chromophore, so that conjugates **1** and **2** were assessable in good purity (above 95%) after HPLC. In Figure 3 typical chromatograms of these compounds are depicted.

The control peptides, for which we expected a significantly decreased receptor affinity, were obtained by synthesizing the corresponding noncyclic peptides of the sequence (D-Phe)-Met-Phe-(D-Trp)-Lys-Thr-Met-Thr-OH with the two cysteins replaced by methionin. Dye coupling in an analogous manner led to the conjugates **3** and **4**.

Absorption and Fluorescence Properties. We studied the absorption and fluorescence properties of the conjugates and determined the absorption and fluorescence maxima, as well as the molar extinction and fluorescence quantum yields in comparison to the free dyes **7** and **8**.

Carbocyanine dyes are known to have high molar extinction coefficients with ϵ -values between 100 000 and 250 000 L mol⁻¹ cm⁻¹ being less affected by the chemical environment. However, fluorescence is known to be considerably quenched for certain compounds when the dye is attached to biomolecules. A general observation for cyanine dyes is that quenching of fluorescence is

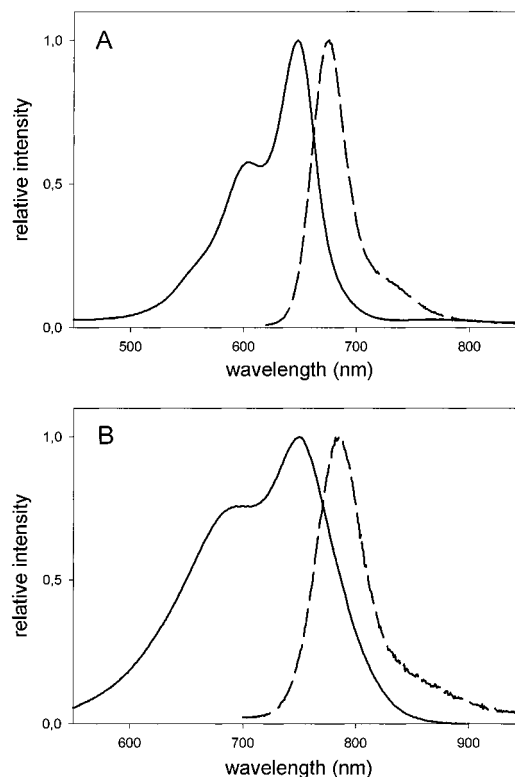


Figure 4. Normalized absorbance (—) and fluorescence emission (---) spectra of **1** (A) and **2** (B) in PBS (pH 7.4). Concentration, 2 μ M.

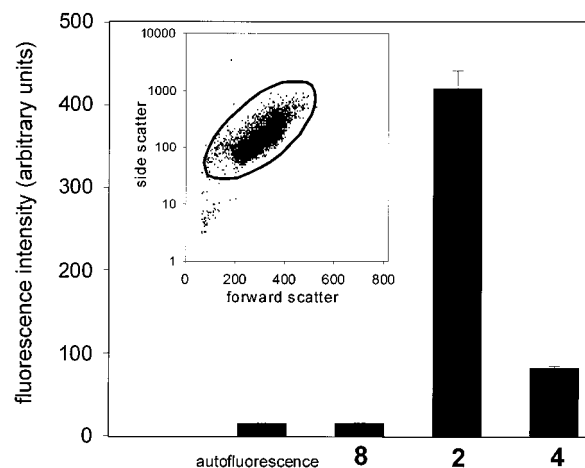


Figure 5. FACS analysis of RIN38 SSTR2 cells after incubation with free dye **8**, indotricarbocyanine dye-peptide conjugate **2**, and the corresponding linear conjugate **4**. The inserted picture represents a FACS plot of the cell population typically used. Fluorescence was recorded for 10 000 cells of the defined gate.

accompanied by deformation of the spectral absorbance profile, with the shorter-wavelength dimer shoulder being more pronounced compared with the monomer peak (28).

Nevertheless, the absorption and fluorescence emission characteristics of the synthesized dye-peptide conjugates are similar to those of the free dyes, as is apparent from the data in Table 1. The molar extinction coefficients of the conjugates exhibit decreased values of around 110 000–180 000 L mol⁻¹ cm⁻¹ compared with those of the free dyes. Within both the indodicarbocyanine (compounds **1**, **3**, and **7**) and indotricarbocyanine groups (compounds **2**, **4**, and **8**) the noncyclic conjugates (**3** and **4**) tended to have higher extinction coefficients than the cyclic conjugates **1** and **2**. In Figure 4 the absorption

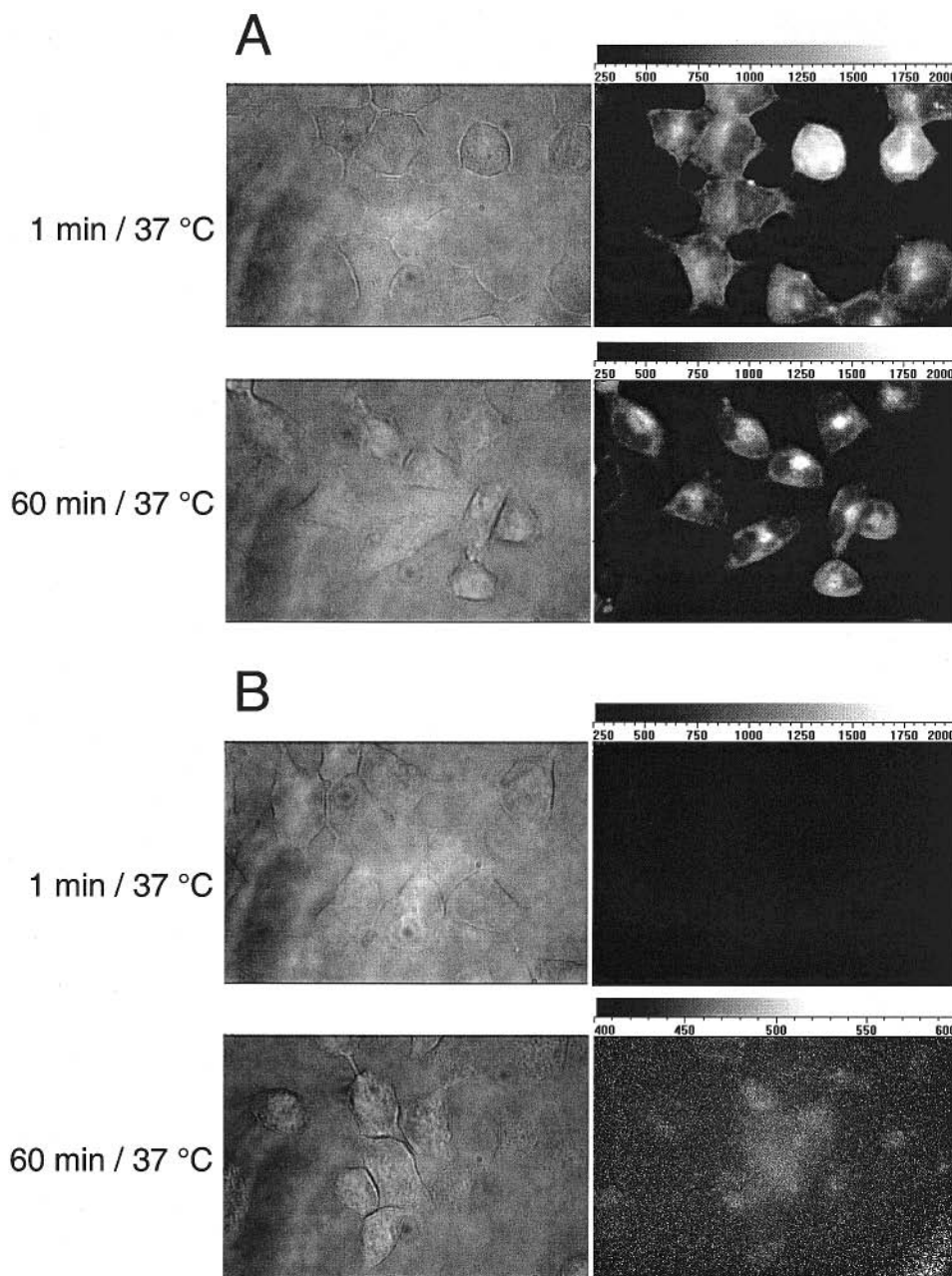


Figure 6. Bright field (left row) and corresponding NIR fluorescence images (right row) of RIN38 SSTR2 cells after 1 and 60 min of incubation in the presence of 1 μ M **2** (set A) and 1 μ M **4** (set B) at 37 °C. The fluorescence image after 60 min at 37 °C for **4** (B) was scaled to a smaller signal range to visualize the poor cell-associated intensity.

spectra and fluorescence emission spectra measured in PBS as solvent are displayed for **1** and **2**. Both the absorbance and the fluorescence spectra are typical in shape compared to known indodi- or indotricarbocyanine-based chromophores (25–27). Only the indotricarbocyanine conjugate **2** showed a more pronounced dimer shoulder (690 nm), indicating that the compound exhibits a greater tendency to form aggregates.

Fluorescence quantum yields (QY) for the indodicarbocyanines **1**, **3**, and **7** were in the range of 30%, whereas the indotricarbocyanines **2**, **4**, and **8** displayed values of about 10% (Table 1).

No remarkable deviations in fluorescence quantum yields were observed in either group, suggesting that the degree of aggregate formation, as supported by the absorbance profile for conjugate **2**, does not influence the fluorescence properties of the dyes when conjugated to the peptide. It has to be underlined, that the extent of

aggregate formation cannot be extracted from the data described herein. Hence, further studies directly analyzing the aggregation behavior are needed.

Cell Culture Studies on Cellular Uptake. To study cellular uptake by means of an overall fluorescence signal derived from a defined population of tumor cells, we used the method of flow cytometry and compared the indotricarbocyanine peptide conjugate **2** with the control conjugate **4** as well as with the free label **8**. Tumor cells were incubated for 1 h at a probe concentration of 100 nM. The mean fluorescence signals obtained from the trypsinated and fixed cell population are displayed in Figure 5. The fluorescence intensity derived from **2** is about 5-fold above that from **4** and 40-fold above the signal generated by autofluorescence or by incubation with the free dye **8**. The data indicate that the underlying binding mechanism of **2** to the cells is a specific receptor–ligand interaction.

To consolidate receptor-mediated internalization into the cell, we performed NIR microscopic studies. Figure 6 shows fluorescence microscopic images of RIN38 SSTR2 tumor cells incubated with **2** (1 μ M) at two different time points as well as the corresponding bright field images. After 7 min, the fluorescence signal is distributed over the whole cell and most likely associated with the plasma membrane, whereas after 30 min of incubation the fluorescence was observed in the perinuclear region and disappeared almost completely from the membrane. The appearance of small fluorescent spots suggests that **2** is localized within intravesicular compartments. Consistent with the results from flow cytometry, only negligible perinuclear signals were detectable after 60 min of incubation at 37 °C using the control probe **4** (Figure 6B), again underlining receptor specificity of **2**.

The receptor affinities (IC_{50} values) of these optical probes remain to be investigated by an appropriate competition assay. Additionally, an important aspect of such compounds is their ability to fluorescently delineate tumors in vivo after systemic administration. This is currently being explored by fluorescence imaging in tumor-bearing animals.

CONCLUSION

We have demonstrated that an octreotide-derived peptide can be conjugated to near-infrared absorbing and fluorescing cyanine dyes, and that the resulting compounds maintain their photophysical properties. Through flow cytometry and fluorescence microscopy using tumor cells we showed that the conjugates specifically bind the somatostatin receptor and are internalized into intracellular compartments.

The developed peptide conjugates structurally related to [^{111}In]DTPA-octreotide exemplify the strength of low molecular weight peptide probes as vehicles for receptor-targeted optical imaging. This principle may be utilized for other clinically more relevant receptor–ligand systems, thus broadening the spectrum of applications for contrast-enhanced optical imaging.

ACKNOWLEDGMENT

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Fluorescence-Labeled Octapeptides as Substrates for Histone Deacetylase

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The determination of histone deacetylase (HDAC) activity and the screening of potential inhibitors is gaining increasing importance due to the involvement of HDAC in transcription regulation. The level of histone acetylation can be modulated by HDAC inhibitors resulting in differentiation and/or apoptosis in cancer cells. We have previously reported the development of a nonisotopic assay for HDAC using a fluorescent derivative of ϵ -acetyl lysine. Here we report fluorescein-labeled octapeptides which are substrates for HDAC that bear closer resemblance to the native substrate. HPLC with fluorescence detection is successfully applied to the analysis of the time- and site-dependent deacetylation. LC-MS analyses are used to confirm the findings. The observed selectivity toward one of two possible deacetylation sites might result from steric hindrance by the label but the methodology presented here could be applied to similar larger peptides which might be improved tools to characterize HDAC site selectivity in vitro.

1. INTRODUCTION

The reversible acetylation of lysine residues near the N-termini of nucleosomal histones by histone deacetylases (HDACs) and histone acetyltransferases (HATs) regulates chromatin structure and transcriptional activity. The importance of the level of histone-acetylation for basic cellular functions such as DNA-replication, transcription, differentiation, and apoptosis is highlighted by a increasing number of experimental studies (1, 2). Most important, HDACs and HATs usually are recruited by other proteins in suppressing or activating transcriptional activity with HDACs usually being co-suppressors and HATs acting as co-activators (3–7). In several cases, deregulated histone acetylation has been correlated with malignant disease, and interactions of HDAC with cancer-related proteins such as mutated retinoic acid receptors PML-RAR α and PLZF-RAR α in acute promyelocytic leukemia (APL) have been identified as the basis of the disease on a molecular level (8, 9). Therefore, HDAC inhibitors are an attractive target for drug discovery and valuable tools for studying the impact of chromatin acetylation on gene regulation. One way toward a better understanding of the function of various deacetylases is the analysis of their reactivity in terms of time- and substrate-dependent turnover. This may also lead to the design of new inhibitors.

The natural substrate, acetylated histones (10), or synthetic oligopeptides which consist of 8 (11) or 24 (12) amino acids, have traditionally been used in activity and inhibition assays. The latter are derived from sequences from the N-termini of histones. In either case, the substrate is labeled with [³H]acetic acid and the liberation of tritiated acid is quantitated by scintillation

counting or a scintillation proximity assay (13). Thus, there are problems with the exposure of operating personnel and the disposal of radioactive waste. The labeled histones are obtained by a procedure involving treatment of chickens with phenylhydrazine over several days and the sacrifice of the animals is unavoidable. The acetylation degree of the prelabeled histones is difficult to standardize resulting in varying substrate properties. There is also a cell-based screening assay that relies on antibody-mediated detection of H4-hyperacetylation (14). The analysis of HDAC-site-selectivity in substrates with multiple lysines has so far been restricted to histones and requires gel electrophoresis and the use of several specific antibodies (15). Our studies deal with the development of fluorescence-based in vitro assays for the characterization of HDAC reactivity and its inhibition. We have previously reported on the first nonradioactive HDAC in vitro screening assay that relies on the use of a fluorescent cumarin-labeled acetyl-lysine **1** (see Figure 1). It can be used to analyze HDAC-activity in protein purification and for the screening of new inhibitors (16, 17) and has become commercially available.

In this report, we describe the enzymatic conversion of an octapeptide **2a** upon incubation with rat liver HDAC. It contains two acetyl-lysine residues and is labeled with fluorescein carboxylic acid at the N-terminus (see Figure 1) and we apply HPLC and fluorescence detection to the question of deacetylation-site preference. One of the lysines is deacetylated readily by a rat liver HDAC whereas only little of the bis-deacetylation product **2d** can be found.

2. EXPERIMENTAL PROCEDURES

2.1. Chemicals. Trichostatin A was purchased from Wako. 5-Aminofluorescein was obtained from Sigma, *tert*-butyl alcohol, 4(5)-carboxyfluorescein, *N,N*-dimethylformamide (DMF), 1-hydroxybenzotriazole, thioanisole, and trifluoroacetic acid from Fluka (Buchs, Switzerland). Fmoc-amino acids were obtained from Alexis (Läufelfin-

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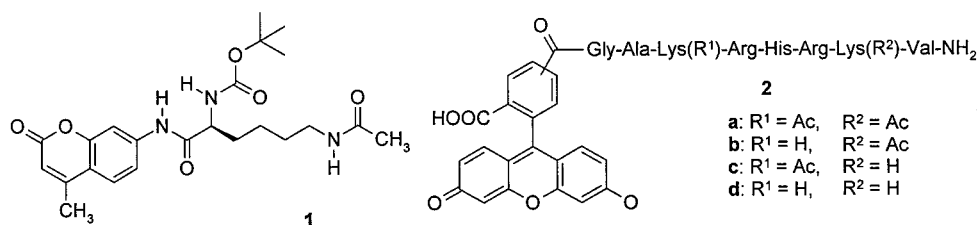


Figure 1. Fluorescent HDAC-substrates.

gen, Switzerland) and 4-(2',4'-dimethoxyphenyl)-Fmoc-aminomethyl)phenoxy (Rink amide) resin from NovaBiochem (Läufelfingen, Switzerland). Diisopropylcarbodiimide and thiocresol were obtained from Aldrich (Buchs, Switzerland), and diethyl ether and acetonitrile from Romil (Cambridge, England). ^3H -Labeled histones were a gift from Prof. Dr. P. Loidl (University of Innsbruck).

2.2. Octapeptides. Peptides were built up by Fmoc-strategy on a Rink amide resin by using a robot system (Syro, MultiSynTech, Bochum) as described previously (18). Trt side-chain protection was used for His, Boc for Lys, and 2,2,5,7,8-pentamethyl-6-chromansulfonyl for Arg. Acetylated Lys was incorporated directly. The peptides were subsequently labeled at the N-terminus with 4(5)-carboxy-fluorescein prior to cleavage from the resin using a 10-fold excess of the acid, diisopropylcarbodiimide, and 1-hydroxybenzotriazol (all 0.25 mM in DMF) at 25 °C in DMF (30 min) (19). They were cleaved with trifluoroacetic acid/thioanisole/thiocresol (20:1:1; v/v) within 3 h, precipitated and washed with cold diethyl ether, collected by centrifugation and lyophilized from water/*tert*-butyl alcohol (3:1).

Cleavage products were investigated by reversed-phase HPLC (Merck-Hitachi, Darmstadt, Germany) on a Nucleosil RP-18 (5 μm) column (125 $\times$ 3 mm, Macherey and Nagel) at a flow rate of 0.6 mL/min. Absorbance was measured at 220 and 437 nm. The solvent system consisted of 0.1% trifluoroacetic acid in water (A) and 0.1% trifluoroacetic acid in acetonitrile (B). A linear gradient from 5 to 55% B in 30 min was applied. Furthermore, the peptides were analyzed by electrospray mass spectrometry (MS) (SSQ710, Finnigan, San Jose, CA). The following analytical data were obtained: (**2a**) HPLC retention time, 14.7 min; MS, $M_{\text{experimental}} = 1392.3$ amu and $M_{\text{theoretical}} = 1392.5$ amu; (**2b**) HPLC retention time, 13.6 min; MS, $M_{\text{experimental}} = 1350.4$ amu and $M_{\text{theoretical}} = 1350.5$ amu; (**2c**) HPLC retention time, 13.8 min; MS, $M_{\text{experimental}} = 1350.3$ amu and $M_{\text{theoretical}} = 1350.5$ amu; (**2d**): HPLC retention time, 13.0 min; MS, $M_{\text{experimental}} = 1308.3$ amu and $M_{\text{theoretical}} = 1308.5$ amu.

2.3. Fluorescence Spectra and Chromatography. Fluorescence spectra of **2** were recorded in a mixture of solvents C and D (80 + 20, v/v, see below) on a Shimadzu RF-540, and the strongest maxima (excitation 475 nm, emission 520 nm) were used for detection in HPLC.

A Shimadzu RF 535 was used as fluorescence detector for HPLC ($\lambda_{\text{ex}} = 475$ nm, $\lambda_{\text{em}} = 520$ nm). A Nucleosil RP-18, 3 μm , 125 $\times$ 4 mm (Macherey-Nagel) was used for analysis of the incubation experiments and a column from the same brand (10 μm , 2 $\times$ 4 mm) served as guard column. The eluent consisted of solvent C (doubly distilled water with 0.06% v/v trifluoroacetic acid) and solvent D (acetonitrile/doubly distilled water 80/20 v/v). A gradient was run increasing the percentage of D from 20% (0 min) to 28% (50 min) and decreasing it back to 20% (70 min) with a flow of 0.8 mL/min. Retention times of the substrates were 26.84/29.33 min (**2a**), 14.69/15.60 min (**2b**), 17.07/19.63 min (**2c**), 7.82/9.03 min (**2d**), and 23.82 min for the internal standard (5-aminofluorescein).

2.4. Enzyme and Radioactive Assay. A partially purified rat liver histone deacetylase was used for the incubation experiments. It is prepared via separation on Q-sepharose big beads (Pharmacia) with an increased gradient of NaCl. The detailed procedure is described elsewhere (17). Radioactive assays were performed as described (20) previously. Shortly, 100 μL of rat enzyme preparation (at 37 °C) were incubated (30 min) with 10 μL of total [^3H]acetate prelabeled chicken reticulocyte histones (1 mg/mL). Reaction was stopped by addition of 36 μL of stop solution (1 M HCl/0.4 M acetate) and 800 μL of ethyl acetate. After centrifugation (10000g, 5 min) an aliquot of 600 μL of the upper phase was counted for radioactivity in 3 mL of liquid scintillation cocktail. **2a** showed no inhibition of HDAC at 80 μM .

2.5. Stock Solutions, Incubation, and Sample Preparation. For qualitative analysis, small amounts of the peptides were dissolved in water. The solutions are stable at -20 °C for about 2 weeks. For the quantitative measurements, solutions of the peptides in water were prepared in the following concentrations (mg/mL): 3.540 (**2a**), 9.740 (**2c**), and 9.469 (**2d**). As the peptides contain between 25 and 30% of salt, a salt content of 25% was assumed for all compounds and accordingly corrected concentrations were used for all calculations. **2b** was not used for quantitative analysis as it is not formed upon incubation. The concentration of the internal standard stock solution was 5.021 mg/mL.

For the calibration, different aliquots of **2a**, **2c**, and **2d** were added to 110 μL of incubation buffer [15 mM tris-HCl, pH 7.9; 0.25 mM EDTA; 10 mM NaCl; 10% (v/v) glycerol; 10 mM 2-mercaptoethanol]. A total of 1.5 μL of internal standard solution was added, and incubation buffer was added to a final volume of 120 μL . The reaction was stopped immediately (200 μL stop solution) and vortexed (2 min). 200 μL of solvent C were added and the mixture was vortexed again (2 min). After centrifugation (10 000 rpm, 5 min) an aliquot was taken from the supernatant. A total of 20 μL was injected in the HPLC system via autosampler ($n = 2$). Linear regression provided the following equations: $f(x) = 0.00783 + 0.02242x$, $r = 0.9992$ (**2a**); $f(x) = 0.01474 + 0.02188x$, $r = 0.9997$ (**2c**); $f(x) = -0.09928 + 0.02512x$, $r = 0.9978$ (**2d**).

For the time-dependent analysis of conversion of **2a**, 15 parallel reactions were set up as above. The mixture was incubated on ice for 15 min and at 37 °C afterward. The reaction was stopped and the samples were prepared as above after 1, 2, 3, 4, 6, 8, 10, 12, 14, 18, 22, 24, 36, and 42 h ($n = 3$, except for $t > 18$ h, $n = 2$).

2.6. Mass Spectrometry. A Finnigan LCQ mass spectrometer with an electrospray interface was used for LC-MS coupling analyses. For identification of the incubation products, the column and solvent conditions were used as above (injection volume was changed to 50 μL), and the elution was monitored by UV detection (210 nm). Full-Scan and Zoom-Scan modes were used for analysis of the MS pattern of the standards and the incubation reactions (12 and 20 h). Spiking of the incubation

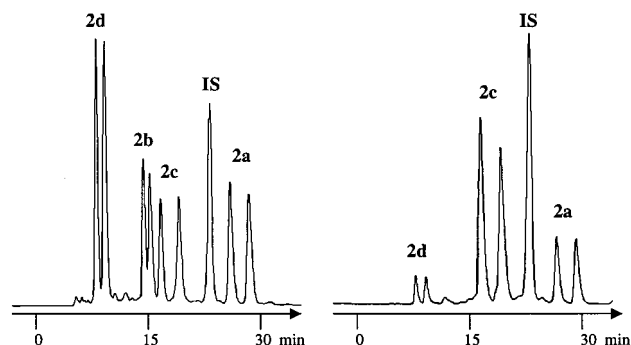


Figure 2. HPLC chromatograms of a standard mixture of **2a–d** and internal standard (IS) (left) and a mixture after incubation with rat liver HDAC for 20 h (right).

reactions with the standards did not lead to other signals. Solutions without substrates or enzyme did not result in signals in the observed range nor in peaks monitored at 210 nm with similar retention times.

RESULTS

The peptides **2** were built up by Fmoc-strategy on a Rink amide resin by using a robot system and subsequently labeled with 4(5)-carboxy-fluorescein prior to cleavage from the resin. The latter reagent had been previously proven to be superior to established derivatization reagents 4(5)-fluoresceinisothiocyanate and 4(5)-carboxyfluorescein-*N*-succinimidylester (19). The labeled peptides were obtained in amounts of 5–10 mg.

A chromatographic system for the separation of the peptides **2a–d** had to be established. Especially the separation of the two mono-acetylated species **2b** and **2c** proved to be difficult. We succeeded applying a system with an increasing gradient of trifluoroacetic acid in water/acetonitrile and the use of a 3 μ m RP-material was crucial to the success. Each of the peptides is showing two peaks due to the isomerism in the commercial starting material used for the labeling procedure. It was not necessary to separate the respective isomers but all eight peptides could be discriminated with the gradient method. Throughout all experiments, there was no indication for a preference of the enzyme for one of the fluorescein isomers. To increase the accuracy and precision of the system, an internal standard was sought and 5-aminofluorescein proved to be suitable. Figure 2 is showing a standard chromatograph of the peptides **2a–d** and the internal standard (IS). We then subjected the peptide **2a** to incubation with a partially purified rat liver HDAC. At first only the mono-deacetylation product **2c** could be detected, while after 3 h also some bis-deacetylation product **2d** was monitored. There was no indication for formation of the mono-deacetylation product **2b** even after 20 h (see Figure 2).

The identity of the incubation products with the synthetic metabolites was not only demonstrated by coelution. LC-MS analyses were performed with the standards and incubation mixtures after 12, 20, and 30 h proved identity of the compounds. Table 1 is showing selected observed mass peaks for a standard mixture and a 30 h incubation mixture obtained by LC-MS coupling. Zoom scan analyses ascertained that monitored signals are indeed $(M + n)/z$ ($n = z = 2$ or 3) peaks for the intact peptides (i.e., protonated species of the highly basic compounds) and not peaks resulting from degradation of the octapeptides by potential residual proteolytic activity in the partially purified rat liver preparation. Spiking of the incubation mixtures with the synthetic

Table 1. LC-MS Signals for Octapeptides **2**

		standard mixture			30 h incubation mixture		
compd	Mr	t_R	$[M + 2]$	$[M + 3]$	t_R	$[M + 2]$	$[M + 3]$
		(min)	$z = 2$	$z = 3$	(min)	$z = 2$	$z = 3$
2a	1392.5	27.06	697.0	465.1	28.05	697.0	465.1
2a	1392.5	26.53	697.0	465.1	25.29	696.9	465.0
2c	1350.5	18.92	675.9	451.1	18.93	675.8	451.0
2c	1350.5	16.81	676.0	451.0	16.69	675.9	451.1
2d	1308.5	10.37	<i>a</i>	437.1	10.20	<i>a</i>	437.1
2d	1308.5	9.44	<i>a</i>	437.1	9.60	<i>a</i>	437.1

a Not observed.

reference materials did not lead to any new signals in the time range monitored. Analysis of samples without the substrate **2a** or solvent blanks did not result in any signals with respective MS signals nor registration of peaks with the retention times of the standards with fluorescence or UV (210 nm) detection.

For quantitative analysis calibrations with standard samples were performed for the substrate **2a** and the two deacetylation products **2c** and **2d**. Figure 3 is showing the calibration curves for the respective peptides. No calibration was performed for **2b** as it is not formed upon incubation with rat liver HDAC.

To monitor the exact time course of the deacetylation reaction we set up 15 parallel experiments in which the enzyme incubation was terminated after increasing periods of time. The results are depicted in Figure 4.

The total area under the curve (AUC) is obtained by multiplying the starting concentration of **2a** and the total incubation time of 42 h and equals 3328.678 h μ mol/L. The measured AUC-values for the three peptides are 384.496 ± 24.163 h μ mol/L (**2a**), 2267.858 ± 140.571 h μ mol/L (**2c**), and 684.861 ± 33.868 h μ mol/L (**2d**). The AUC values in relation to the total AUC are $11.6 \pm 0.7\%$ (**2a**), $68.1 \pm 4.2\%$ (**2c**), and $20.6 \pm 1.0\%$ (**2d**). This adds up to $100.3 \pm 5.9\%$. No additional peaks at 520 nm nor at 210 nm (UV detection) were detected in the time frame of 70 min. Thus, remaining **2a** and all of its biotransformation products are quantitated by the presented method.

DISCUSSION

Fluorescein-labeled octapeptide **2a** is a substrate for histone deacetylase that is gradually deacetylated at the acetyl-lysine near its C-terminus and eventually formation of some bis-deacetylation product **2d** can be observed. This pattern of reactivity may be due to steric hindrance by the N-terminal label but as some deacetylation near the label is monitored this might be an indication for a deacetylation-site preference. Our studies show for the first time that HPLC-chromatography in combination with fluorescence detection and mass spectroscopy is a powerful tool to address questions of regioselective deacetylation in HDAC substrates with several acetyl-lysine residues in-vitro. They may serve as a model for similarly labeled larger peptides that are known to be substrates of histone deacetylase (12) and might not suffer from steric constraint by the label. Similar investigations have just recently been presented with a 20mer-peptide fragment from the N-terminus of histone H3 and the histone acetyltransferase PCAF (p300/CBP-associated factor) (21). Here MALDI/TOF analysis of tryptic digests of the peptidic substrate was used to identify the single acetylation site. Deacetylation site preferences are important for the characterization of various deacetylases and might have an impact on the design of substrate-analogues as HDAC inhibitors. Finally, due to its excitation wavelength of 475 nm our

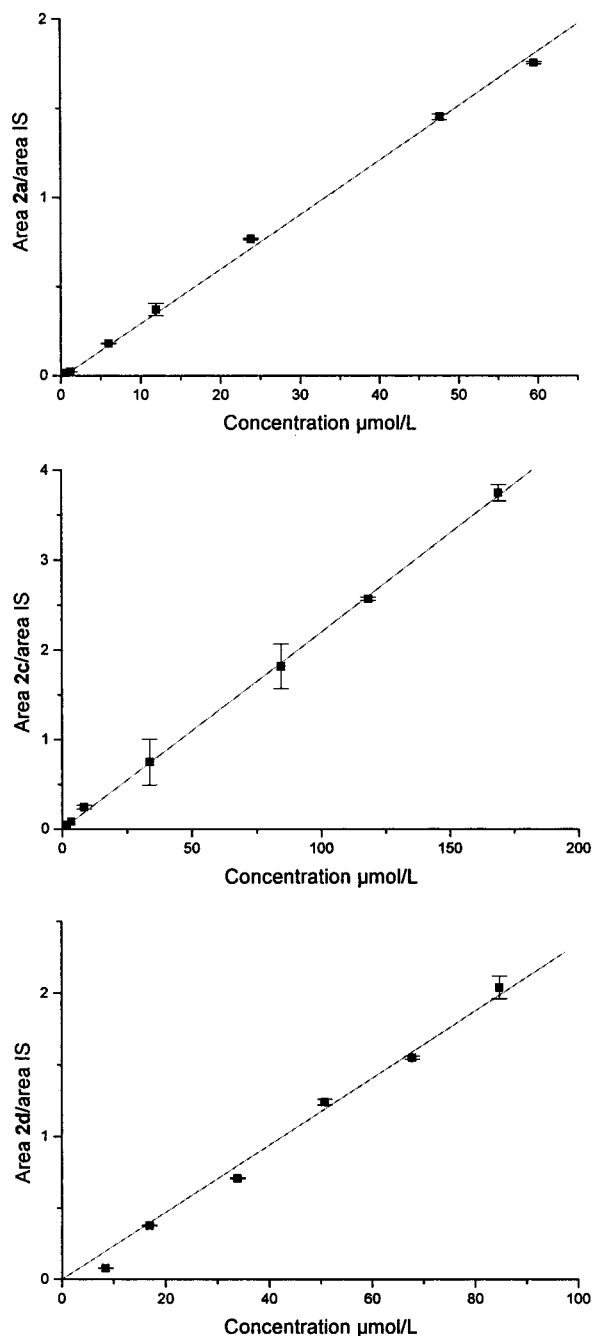


Figure 3. Calibration curves for octapeptides **2a**, **2c**, and **2d**.

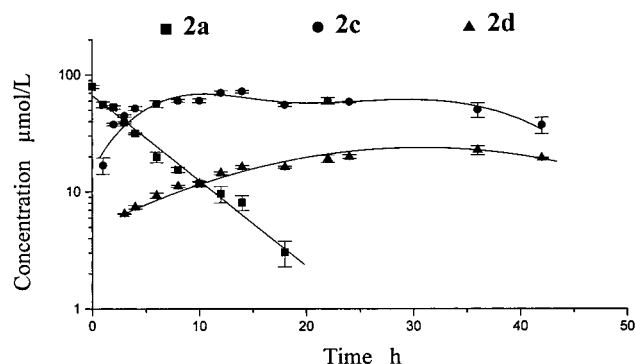


Figure 4. Time-dependent conversion of **2a–2c** and **2d**.

substrate **2a** can be used for applications that are not amenable to the existing coumarin-derived substrate, e.g., fluorescence correlation spectroscopy.

ACKNOWLEDGMENT

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Recognition of Novel Lipopolypeptides with Many Pendent Sugar Residues by Lectin[†]

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A lipid–polypeptide conjugate (lipo-polypeptide) was obtained by the ring-opening polycondensation of *N*- ϵ -Z-L-lysine *N*-carboxyanhydride (NCA) using 3-aminopropyl dioctadecylamine as initiator and subsequent deprotection. Maltose lactone was coupled with the lipo-polypeptide to give novel amphiphiles which carried many maltoamide residues as pendent groups. The sugar group-carrying amphiphiles incorporated in phospholipid liposomes were recognized by a lectin from *Canavalia ensiformis* (Con A), which was proven by the increase in turbidity of the liposome suspension after mixing with the lectin. The recognition was largely affected by the degree of polymerization of lysine residues and the surface density of the amphiphile in the liposomes. The association constant (K_{ass}) of Con A with maltoamide residues on the liposome was much larger than those for small molecular weight sugars due to the “cluster effect”.

INTRODUCTION

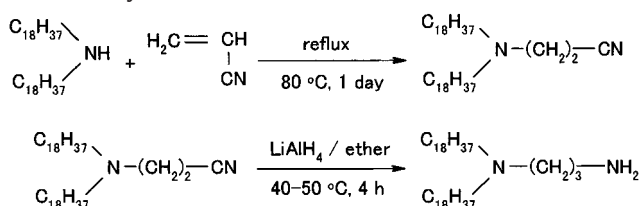
Devices used in drug delivery systems are composed of various components which are designed for targeting, encapsulation, slow release, reduction of immunogenicity, and pharmacological activity, etc. (1). We have been interested in preparation of various liposome-forming hybrid materials having pH- or temperature-responsiveness using a lipophilic radical initiator or lipophilic chain transfer reagent (2, 3), respectively. Copolymerization of polymerizable lipid with pH-responsive vinyl monomer was also carried out to obtain pH-responsive hybrid amphiphiles (4). These materials would be promising candidates as a component in drug delivery systems.

Meanwhile, it has been revealed that oligosaccharides are important mediators for a large number of biological processes, such as cell–cell recognition and adhesion, hormone–cell recognition, and viral or bacterial adhesion to host cells (5–9). Therefore, various diseases and infections can be pharmacologically treated by the administration of molecules which mimic endogenous oligosaccharides or can block the recognition or adhesion processes (9).

We have pointed out that amphiphiles with many pendent sugar residues in their hydrophilic headgroups can be effectively recognized by the sugar binding protein, lectin (10–15). For the preparation of the sugar-carrying amphiphiles (so-called “glycolipids”), the lipophilic radical initiator mentioned above (2) has been mainly used.

In this report, we prepared amphiphilic compounds which have a lipid group, polypeptide chain, and many pendent sugar groups. These compounds can be named “chimeric” amphiphiles because of their hybrid structures. We investigated recognition of the amphiphilic compounds incorporated in phospholipid liposomes by a

Scheme 1. Preparation of 3-Aminopropyl Dioctadecylamine



lectin Concanavalin A (Con A)¹ from *Canavalia ensiformis*. The effects of the length and surface density of the polypeptide (poly-L-lysine in this work) above the liposome surface on the recognition by the lectin were examined.

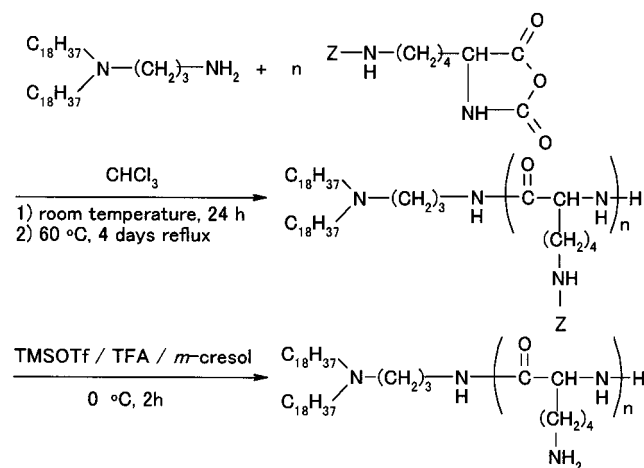
EXPERIMENTAL PROCEDURES

Materials. 3-Aminopropyl dioctadecylamine (DODAAM, Scheme 1) was prepared by the Michael addition of dioctadecylamine (DODA, Fluka, Switzerland) to acrylonitrile at 80 °C and subsequent reduction with LiAlH₄ in dry tetrahydrofuran (16). Maltose lactone was prepared by the oxidation of maltose with iodine, and subsequent lactonization with cation-exchange resins (Amberlite IR-120 B, H⁺-form) and silver carbonate as previously reported (11, 17, 18). Concanavalin A (Con A) from *C. ensiformis*, L- α -dimyristoyl phosphatidylcholine (DMPC), and *N,N'*- α,ϵ -dicarbobenzoxy-L-lysine (di-Z-L-Lys) were from Sigma. Other reagents were commercially

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¹ Abbreviations: Con A, Concanavalin A from *Canavalia ensiformis*; di-Z-L-Lys, *N,N'*- α,ϵ -dicarbobenzoxy-L-lysine; DMPC, L- α -dimyristoyl phosphatidylcholine; DODA, dioctadecylamine; DODAAM, 3-aminopropyl dioctadecylamine; DODA-PLL, dioctadecylamine–poly(L-lysine) conjugate; DODA-PLL-Mal, dioctadecylamine–poly(*N*- ϵ -maltoamido-L-lysine) conjugate; DP, degree of polymerization; DS, degree of substitution; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MeGlc, methyl α -D-glucopyranoside; MeMan, methyl α -D-mannopyranoside; NCA, *N*-carboxyanhydride; PLL-Mal, poly(*N*- ϵ -maltoamido-L-lysine).

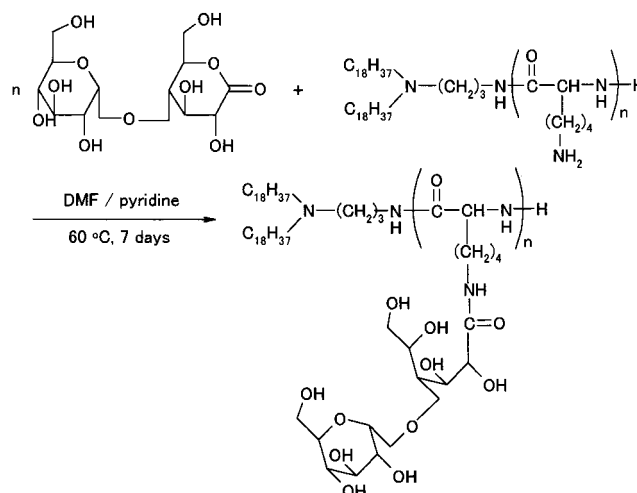
Scheme 2. Preparation of DODA-PLL by Ring-Opening Polymerization

available. A Milli-Q grade water was used for preparation of sample solutions.

Preparation of NCA of ϵ -Z-L-Lysine (*N*- ϵ -Z-L-Lys NCA). Di-Z-L-Lys (4.01 g, 13 mmol) was incubated with phosphorus pentachloride (PCl_5 , 2.41 g) in a dry 1,4-dioxane-ether mixture (10 mL + 15 mL) for 30 min in ice as reported previously (19). The solution mixture was evaporated, and washed with acetonitrile. The solid product was recrystallized from ethyl acetate-*n*-hexane, and dried in vacuo (*N*- ϵ -Z-L-Lys NCA, 2.88 g, 97% yield). IR (KBr): N-H (NCA, Z), ν 3341 cm^{-1} ; C=O (NCA), ν 1814, 1774 cm^{-1} ; C=O (Z), ν 1690 cm^{-1} ; N-H (NCA, Z), δ 1533 cm^{-1} ; CH_2 , ν_{as} 2939 cm^{-1} ; CH_2 , ν_{s} 2866 cm^{-1} ; C(=O)-O, ν 1258 cm^{-1} . Anal. calcd for $\text{C}_{15}\text{H}_{18}\text{N}_2\text{O}_5$: C, 58.82; H, 5.92; N, 9.15. Found: C, 59.05; H, 6.04; N, 9.09.

Preparation of Lipid-Poly(L-Lysine) Conjugate (DODA-PLL, Scheme 2). 3-Aminopropyl dioctadecylamine (20 mg, 34.5 μmol) and *N*- ϵ -Z-L-lysine NCA (0.424 g, 1.4 mmol) were dissolved in dry chloroform (15 mL). After passing N_2 gas for 5 min, the solution mixture was incubated for 24 h at room temperature and afterward refluxed at 60 $^\circ\text{C}$ for 4 days. The solution mixture was condensed by evaporation and purified by washing with ether (0.224 g, 59% yield). The degree of polymerization (DP) of L-lysine residue was estimated to be 25.3 by NMR and elemental analysis. ^1H NMR (400 MHz, CDCl_3): δ 0.88 (t, 6H, CH_3 -), 3.10 (m, 50.6H, $-\text{CH}_2-\text{NH}-\text{Z}$), 4.99 (s, 50.6H, $-\text{O}-\text{CH}_2-\text{C}_6\text{H}_5$), 7.23 (m, 126.5H, $-\text{C}_6\text{H}_5$). Anal. calcd for $\text{C}_{39}\text{H}_{82}\text{N}_2-(\text{C}_{14}\text{H}_{18}\text{N}_2\text{O}_3)_{25.3}$: C, 65.45; H, 7.51; N, 10.21. Found: C, 66.01; H, 7.54; N, 9.61.

The Z groups in the lipid obtained were cleaved with a CF_3COOH (1.40 mL)-*m*-cresol (0.24 mL)-trimethylsilyl trifluoromethanesulfonate (0.40 mL) mixture in ice for 2 h, while N_2 gas was passed through the reaction mixture (20). After evaporation, the oily mixture was dispersed in ether, and the precipitates recovered by decantation were dispersed in water by vortexing. The pH value of the dispersion mixture was adjusted to 5.6 with N/10 HCl and NaOH solutions, and the dispersion was dialyzed (molecular cutoff of the dialysis tube, 1000; Por CE membrane, Spectrum Medical Industries, Houston, TX) against pure water for 7 days. The dispersion was finally lyophilized (DODA-PLL, 0.104 g, 20% yield). ^1H NMR (400 MHz, D_2O): δ 0.61 (t, 6H, CH_3 -), 2.97 (m, 47.8H, $-\text{CH}_2-\text{NH}_2$), 4.30 [m, 23.9H, $-(\text{C}=\text{O})-\text{CH}(\text{NH})-\text{CH}_2-$]. The degree of polymerization (DP) of DODA-PLL became slightly smaller during the deprotection process (25.3 $\rightarrow$ 23.9) due to the partial cleavage of the peptide chain.

Scheme 3. Preparation of Chimeric Compound Which Carries Sugar, Polypeptide, and Lipid Moieties**Preparation of Sugar-Carrying Amphiphile**

(Scheme 3). DODA-PLL (50 mg) and maltose lactone (0.202 g, 0.6 mmol) were dissolved in a water-*N,N*-dimethylformamide mixture (2 mL + 15 mL) in a test tube. Pyridine (50 μL) was added, and the reaction mixture was incubated for 8 days at 70 $^\circ\text{C}$. After evaporation of the solvent, the maltoamide-carrying amphiphile was dissolved in water and purified by ultrafiltration (membrane, Amicon YM-3; molecular cutoff, 3000). The amphiphile, which had been dispersed in water by a vortex mixer and subsequently by an ultrasonifier (Astrason W-385, Heat Systems-Ultrasonics, Inc., NY) for 3 min, was finally lyophilized (21.6 mg, DODA-PLL-Mal-3). IR (KBr): O-H (maltoamide residue), ν 3358 cm^{-1} ; C=O (secondary amide), ν 1609 cm^{-1} ; C=O (tertiary amide), ν 1652 cm^{-1} ; N-H (secondary amide), δ 1540 cm^{-1} ; CH_2 , ν_{as} 2928 cm^{-1} ; CH_2 , ν_{s} 2853 cm^{-1} . The degree of substitution (DS) of the sugar residue was determined as 10.0 by H_2SO_4 -phenol method (21). The degree of polymerization (DP) of amphiphiles estimated by NMR was approximately consistent with that by elemental analyses. Anal. calcd for $\text{C}_{39}\text{H}_{82}\text{N}_2(\text{C}_6\text{H}_{12}\text{N}_2\text{O}_{13.9}(\text{C}_{18}\text{H}_{32}\text{N}_2\text{O}_{12})_{10.0}(\text{CF}_3\text{COOH})_{14.9})$: C, 45.63; H, 6.73; N, 7.98. Found: C, 47.06; H, 7.00; N, 7.55.

By changing the ratio of DODAAM and *N*- ϵ -Z-L-lysine NCA, maltoamide-carrying lipo-polypeptides of smaller degree of polymerization were prepared. Because of the difficulty to purify amphiphilic compounds, the yields of sugar-carrying lipids were in general very low (Table 1).

Acetylation of Lysine Residue in DODA-PLL-Mal.

DODA-PLL-Mal-1 (DP = 5, DS = 4.1, 49.8 mg, 30 μmol) was incubated with acetic acid (0.60 mL, 10 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (20.4 mg, 0.1 mmol) in water at pH 5.0 for 1 h in ice, and afterward at room temperature for 24 h. The solution mixture was purified by ultrafiltration (membrane, Amicon YM-1; molecular cutoff, 1000), and finally lyophilized (26.5 mg, 49.8% yield). The average degree of acetylation of α - and ϵ -amino groups retained in the poly-L-lysine moiety (DP = 5, DS = 4.1) was estimated to be 1.5 by 1,3,5-trinitrobenzenesulfonic acid method (22).

Preparation of Sugar-Carrying Polypeptide. Poly-(L-lysine) hydrobromide salt (DP = 18, 201 mg, 0.96 mequiv) and maltose lactone (811 mg, 2.4 mmol) were dissolved in a water-*N,N*-dimethylformamide-dimethyl sulfoxide mixture (15 mL + 20 mL + 3 mL) in a round-bottomed flask. The reaction mixture was incubated with

Table 1. Preparation of Sugar-Carrying Amphiphiles

amphiphile	DODA-PLL (g)	DP ^a	sugar lactone (g)	solvent (mL)	product (mg) [% yield]	DS ^b
DODA-PLL-Mal-1	0.051	5.0	0.204	10 ^c	75.8 [64]	4.1
DODA-PLL-Mal-2	0.046	8.8	0.096	6 ^d	36.4 [50]	4.0
DODA-PLL-Mal-3	0.050	23.9	0.202	15 ^d	21.6 [28]	10.0

^a Degree of polymerization. ^b Degree of substitution. ^c Dimethyl sulfoxide. ^d *N,N*-Dimethylformamide.

anion-exchange resins (Amberlite IRA 96SB) for 10 days at 70 °C. After filtration of the resins and subsequent evaporation of the solvent, the maltoamide-carrying polypeptide (PLL-Mal) was purified by ultrafiltration (membrane, Amicon YM-1), and finally lyophilized (65.3 mg, 28% yield). The degree of substitution (DS) of the sugar residue was determined as 10.2 by the H₂SO₄-phenol method (21).

Preparation of Liposome. DMPC (16 mg) was dissolved in CHCl₃ (1 mL) in a small round-bottomed flask, and the solvent was evaporated. The thin lipid membrane formed was dispersed into a *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 8.2, 10 mM) in which the sugar-carrying lipid had been dispersed beforehand using a vortex mixer at 60 °C. The dispersion was further ultrasonified for 3 min while N₂ gas was passed through the suspension.

Dynamic Light Scattering Method. Hydrodynamic diameter of liposomes composed of various molar ratios of sugar-carrying lipid and DMPC was determined by the dynamic light scattering method (DLS-7000, Otsuka Electronics, Hirakata, Japan; light source, He-Ne laser 632.8 nm).

Turbidity Measurements. The dispersion of sugar-carrying liposome (0.5 mL) was quickly mixed with a solution of lectin (0.5 mL) using a polyethylene mixing rod in a quartz cell. The lectin-induced agglutination of the liposomes was followed by the increase in decadic absorbance at 350 nm using a UV-vis spectrophotometer (Ubest-35, Japan Spectroscopic Co., Tokyo, Japan). The observation cell was thermostated by a Peltier device. The uncertainties of the rate of turbidity change were within 10%.

Association Constants for Con A to Sugar-Carrying Liposomes. The association constant, K_{ass} , between Con A (L) and the sugar-carrying amphiphile (S) (eq 1) was determined by the Scatchard plot (23).



The ratio of the concentration of free Con A to that of the complex with Con A is given by the eq 2.

$$[L \cdot S]/[L] = K_{\text{ass}}([S]_0 - [L \cdot S]) \quad (2)$$

where $[L \cdot S]$ and $[L]$ refer to the concentration of bound Con A and that of free Con A, respectively, and $[S]_0$ refers to the total concentration of sugar residues. A plot of $[L \cdot S]/[L]$ versus $[L \cdot S]$ should ideally give a straight line with a slope $-K_{\text{ass}}$. The association constant obtained from the plot was expressed in inverse molarity (M⁻¹), assuming a molecular weight of 25 583 for four subunits in the Con A molecules (24).

The sugar-carrying liposome suspension in a test tube (1 mL) was incubated with various concentrations of Con A (1 mL) at 25 °C for 3 h. The Con A bound to the liposome (actually the aggregates of liposomes cross-linked by Con A molecules) was removed with a syringe

filter (Millipore Millex-GV; pore size, 0.22 μm), and the absorbance of the filtrate at 280 nm was measured. Although the filter of low protein binding was used to remove the liposome-Con A aggregates, free Con A was observed to adsorb to the filter a little. Therefore, we at first estimated the relationship between the absorption of filtrate (2 mL) at 280 nm vs the concentration of free Con A before the filtration, and the amount of Con A bound to the liposome was calculated with the help of this calibration curve. The association constant, K_{ass} , between the water-soluble linear polymer and Con A was determined in the same way as the sugar-carrying liposome system.

In the binding experiment of maltose to Con A, a mixture of Con A and maltose dissolved in the HEPES buffer was dialyzed against the same buffer at 25 °C for 24 h using a seamless cellulose membrane (molecular cutoff, 13 000; Wako Pure Chemicals, Osaka, Japan). The concentration of nonbound maltose outside of the membrane was determined by the H₂SO₄-phenol method, and the K_{ass} value for the maltose-Con A system was estimated.

RESULTS AND DISCUSSION

Recognition of Sugar Residues on Liposomes by Lectin. Using the dynamic light-scattering method, diameter of the liposomes composed of DODA-PLL-Mal-3 (DP = 24, DS = 10) and L-α-dimyristoyl phosphatidylcholine (DMPC) was estimated to be 130 nm on average. The diameter of other liposomes was approximately the same value. Turbidity of maltoamide-carrying liposomes (DODA-PLL-Mal-3, DP = 24) at 350 nm was rapidly increased by the addition of lectin (Con A), whereas the glycolipid with a smaller degree of polymerization (DP = 5, 9) showed a slower turbidity change (Figure 1). The rate of turbidity increase was increased with the concentration of Con A, and after passing the maximum, gradually decreased. Similar phenomena were often observed in the lectin-glycolipid-carrying liposome systems (25), lectin-cell systems (26), and antigen-antibody systems (27).

In the presence of methyl α-D-mannopyranoside (Me-Man), which is known to strongly bind to Con A (28), this turbidity increase was largely decelerated, whereas the addition of mannose and glucose decreased the rate of turbidity change less significantly (Figure 2). Furthermore, the addition of MeMan to the suspension of maltoamide-carrying liposome-Con A aggregates induced the prompt reduction of turbidity (Figure 2, inset). In addition, there was no nonspecific lectin-mediated aggregation of DMPC liposomes nor self-aggregation of maltoamide-carrying liposomes (data not shown). These results strongly support that the turbidity change is due to the specific recognition of maltoamide residues (in other words, α-glucoside residues) on the liposomes by Con A, and subsequent aggregation of the liposomes mediated by the tetrameric lectin molecules.

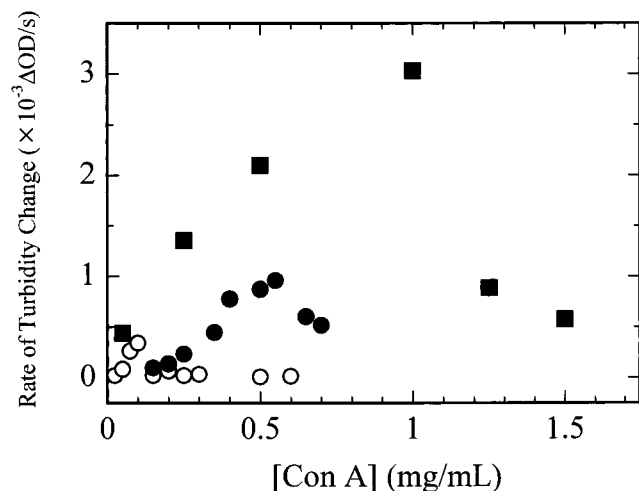


Figure 1. Effect of Con A concentration on the rate of turbidity change at 350 nm after the addition of Con A into the liposome suspension at 25 °C. (○) DODA-PLL-Mal-1 (DP = 5); (●) DODA-PLL-Mal-2 (DP = 9); (■) DODA-PLL-Mal-3 (DP = 24). Content of glycolipid = 2.7 mol %. [liposome] = 0.05 mg/mL.

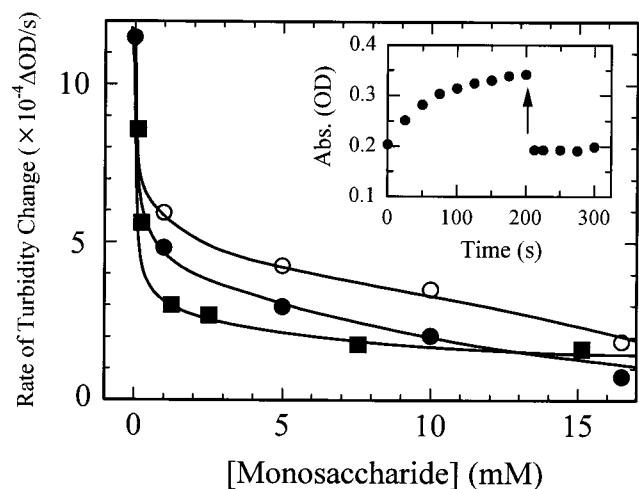


Figure 2. Inhibitory effect of monosaccharides on the rate of turbidity change at 350 nm in the agglutination of sugar-carrying liposomes by Con A. (○) Glucose; (●) mannose; (■) MeMan. Content of DODA-PLL-Mal-3 (DP = 24) = 2.7 mol %. [liposome] = 0.05 mg/mL. (Inset) Time course of turbidity changes at 350 nm after addition of Con A at 25 °C. [DMPC/DODA-PLL-Mal-3] = 0.05 mg/mL. [Con A] = 0.5 mg/mL. MeMan (final concentration, 4 mM) was added at the arrow.

The glycolipid prepared in this work had unmodified ϵ -amino and α -amino groups (at the end of the polypeptide), and these amino groups might increase the hydrophilicity of the sugar-carrying polypeptide chain. There have been many reports that hydrophobic aglycon promotes the recognition of sugars by lectin (29). Actually, by the acetylation of free amino groups, the rate of turbidity change was largely increased as indicated in Figure 3. Furthermore, the inhibitory effect of MeMan on the aggregation of the maltoamide-carrying liposome was affected by the degree of substitution (DS): The more the DS, the more resistant in the agglutinability to the presence of MeMan (data not shown).

Figure 4 shows the effect of weight percent of glycolipid (DODA-PLL-Mal-3, DP = 24, DS = 10) on the rate of turbidity change. There was a prompt increase in the rate of turbidity change at a low content of maltoamide-carrying lipid (1.2 mol %). The presence of threshold value in 5 mol % of lactosyl-ceramide in liposomes at the lectin-induced aggregation was previously reported (25).

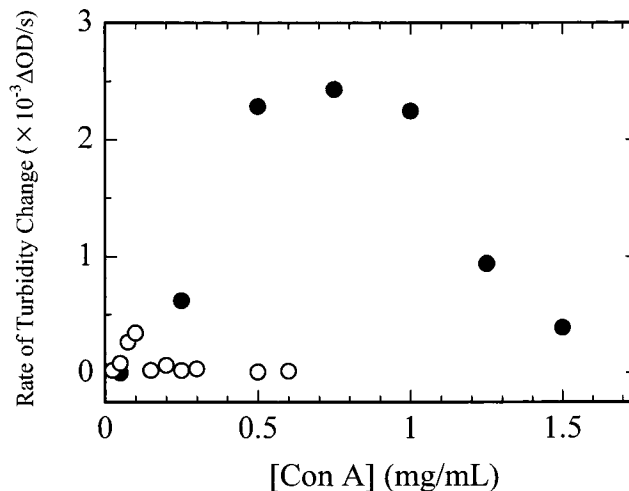


Figure 3. Effect of acetylation of amino groups in the glycolipid on the rate of turbidity change at 350 nm after the addition of Con A into the liposome suspension at 25 °C. (○) DODA-PLL-Mal-1 (DP = 5, DS = 4.1); (●) DODA-PLL-Mal-1 (DP = 5, DS = 4.1, two-thirds of unreacted amino groups were acetylated). [liposome] = 0.05 mg/mL.

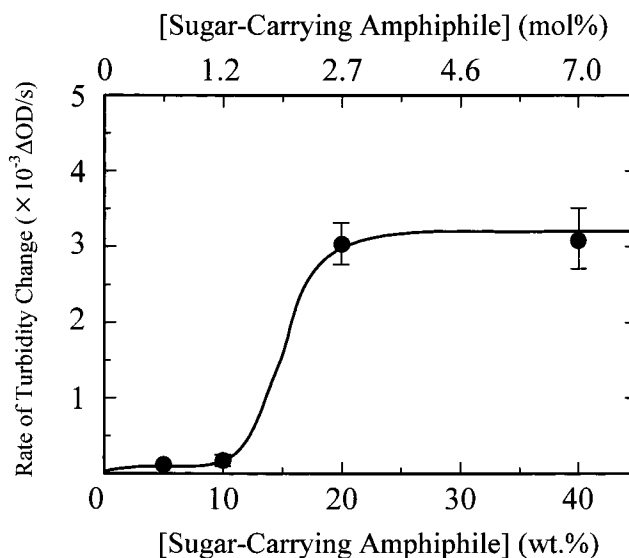


Figure 4. Effect of glycolipid content in the liposome on the rate of turbidity change at 350 nm after the addition of Con A into the liposome suspension at 25 °C. Glycolipid, DODA-PLL-Mal-3. [Con A] = 1 mg/mL. [lipid] = 0.05 mg/mL.

In the case of DODA-PLL-Mal-3 (DS = 10) liposome system, however, the rate of turbidity increase at the very small weight percent was significant, because many sugar residues were present in a polar head region of one lipid molecule, which made it not so largely necessary for the lectin to bind to plural number of sugar lipids on each liposome to bring about the stable aggregation, and consequently, induced the agglutinability of the lectin at the low content of the glycolipid.

Association Constants for Con A to Maltoamide-Carrying Amphiphiles. The association constant (K_{ass}) for Con A with various kinds of sugar derivatives obtained by the Scatchard plot is shown in Table 2. The K_{ass} value for DODA-PLL-Mal-3 was much larger than those of methyl α -D-manopyranoside (MeMan), methyl α -D-glucopyranoside (MeGlc), and maltose, and similar to the value of a branched pentasaccharide (30) shown in the table. The K_{ass} value for the sugar-carrying polypeptide (PLL-Mal) was also much larger than those for small molecular weight sugars. These results clearly

Table 2. Association Constants (K_{ass}) for the Complexation of Con A with Sugar-Carrying Amphiphile and Sugar Derivatives at 25 °C

amphiphile or sugar derivatives	K_{ass} ($\times 10^3 \text{ M}^{-1}$) ^a
DODA-PLL-Mal-3 (DP = 24)	1300
PLL-Mal (DP = 18)	170
methyl α -D-glucopyranoside (MeGlc) ^b	1.96
methyl α -D-mannopyranoside (MeMan) ^b	8.2
maltose	1.2 ^c
(GlcNAc(β -1,2)-Man) ₂ -(α -1,6, α -1,3)Man-OH) ^b	1400

^a In the 10 mM HEPES buffer (pH 8.2) containing 0.1 mM CaCl₂ and 0.1 mM MnCl₂. ^b Reference 30. ^c In ref 30, $1.31 \times 10^3 \text{ M}^{-1}$.

show that sugar residues incorporated in the polymer chain as pendent groups enhance appreciably the affinity for Con A compared with mono- and disaccharides. This is due to the so-called "cluster effect" which enhances the apparent affinity of cluster glycosides toward lectins without significantly changing the sugar concentration (31).

As shown in the table, the K_{ass} value for PLL-Mal was about 1/8 of the DODA-PLL-Mal-3 liposome system. This is probably because sugar-carrying polymer chains stretching from the liposome surface are more advantageous to fix Con A molecules rigidly than the sugar-carrying polymer chain in solution phase. Previously we examined recognition of mannose residue at lipid bilayer-liquid interfaces (14), and observed that the affinity of Con A for the amphiphiles which carried many pendent mannose residues was much higher (10^4 times at the most) than that for MeMan. In addition, we examined catalytic behavior of galactose oxidase at lipid bilayer-liquid interfaces (12, 13) and observed that the affinity of galactose oxidase for the amphiphiles carrying many pendent galactose residues was much higher (10^4 times and 10^2 times at the most) than those for various galactose derivatives with low molecular weight, and those for galactose-carrying water-soluble polymers, respectively. These results might be interpreted by the two-dimensional "cluster effect" in the reaction of glycosides with lectins and other sugar-binding proteins on membrane surfaces.

The number of amino acids and sugar residues in the sugar-carrying lipo-polypeptide can be controlled by the concentration ratio of the initiator (3-aminopropyl diocadecylamine) and lysine NCA, and that of amino group in the lipo-polypeptide and sugar lactone (maltose lactone, lactose lactone (11), and other sugar lactones) at the modification, respectively. Therefore, the compounds prepared here have a possibility to be used as a tool for drug delivery system to cells with sugar-binding receptors (hepatocytes, for example).

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New PEGs for Peptide and Protein Modification, Suitable for Identification of the PEGylation Site

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New PEG derivatives were studied for peptide and protein modification, based upon an amino acid arm, Met-Nle or Met- β Ala, activated as succinimidyl ester. PEG-Met-Nle-OSu or PEG-Met- β Ala-OSu react with amino groups in protein-yielding conjugates with stable amide bond. From these conjugates PEG may be removed by BrCN treatment, leaving Nle or β Ala as reporter amino acid, at the site where PEG was bound. The conjugation of PEG and its removal by BrCN treatment was assessed on a partial sequence of glucagone and on lysozyme as model peptide or protein. Furthermore, insulin, a protein with three potential sites of PEGylation, was modified by PEG-Met-Nle, and the PEG isomers were separated by HPLC. After removal of PEG, as reported above, the sites of PEGylation were identified by characterization of the two insulin chains obtained after reduction and carboxymethylation. Mass spectrometry, amino acid analysis and Edman sequence, could reveal the position of the reporter norleucine that corresponds to the position of PEG binding.

INTRODUCTION

The identification of the PEGylation sites in the primary sequence of peptides and proteins remains an open problem in the characterization of conjugates. This information may be of help to design reaction conditions more suitable to direct PEG toward residues not involved in binding with substrates, ligands, or receptors; furthermore, it is important for characterization of the conjugates as required by FDA.

Indeed, for short peptides, Edman degradation may be sufficient to identify the PEGylation site since the conjugated amino acid appears as a gap in the sequence. This was the case encountered in our laboratory to establish the position of PEG isomers in the 29 amino acid sequence of growth hormone releasing factor (1). However, this procedure is no longer feasible in large peptides and proteins, where a previous enzymatic cleavage must be carried out to get shorter sequence, as was the case of growth hormone or G-CSF (2). In such case, the conjugate was hydrolyzed by trypsin and the site of PEGylation stated on the basis of the missing tryptic peptides (due to PEGylation) in comparison with those obtained from the native protein. Although successful, this method cannot be considered of general suitability, since it is based on a negative information: a missing peptide may come also from incomplete hydrolysis that may occur close to a PEGylated lysine. Despite of their limitations these methods were successfully employed in the characterization of α -interferon also, where only monopegylated isomers, purified by ion exchange chromatography were obtained (3).

A further approach to the problem is based on the use of PEG with a hydrolyzable succinic acid ester arm

between polymer and protein. In this case, a mild basic cleavage removes PEG from the protein, leaving succinic acid bound to the protein as a reporter group. This labeled protein, after being digested with a proteolytic enzyme, is subjected to peptide fractionation and the succinylated peptides identified by Maldi mass spectrometry (4). This original procedure overcomes also the problem of the difficult fractionation of PEGylated peptides due to the hindrance of the polymer, although a limit still remains, i.e., the easy in vivo release of PEG from these conjugates at the level of the labile ester bond (5).

As a contribution to this problem, we describe here a procedure that overcomes the limits of the negative information of one method, the instability of the conjugates obtained with the succinimidyl arm, as well as the general problem connected with the difficult fractionation of PEGylated peptides or proteins. The method is based on the use of PEG-Met-Nle-OSu or PEG-Met- β Ala-OSu for protein modification. The PEG conjugates so obtained are stable in blood stream, but allow the in vitro cleavage at the methionine bond by BrCN, leaving Nle or β Ala bound to protein molecule as reporter group of the PEGylation site (see Figure 1). The peptide or protein, now devoid of PEG hindrance, may undergo any enzymatic and chemical treatment, or chromatographic fractionation, while the presence of the reporter is revealed by amino acid analysis, mass spectroscopy, or Edman degradation.

EXPERIMENTAL PROCEDURES

Methoxypoly(ethylene glycol) (MW 5000) (PEG) was purchased from Shearwater Polymer (Huntsville, AL), PEG-*p*-nitrophenyl carbonate was prepared according to a method previously reported (6), and bovine insulin, methionine, *p*-nitrophenylchloroformate, DL-norleucine methyl ester (Nle-OMe), β -alanine (β Ala), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene-sul-

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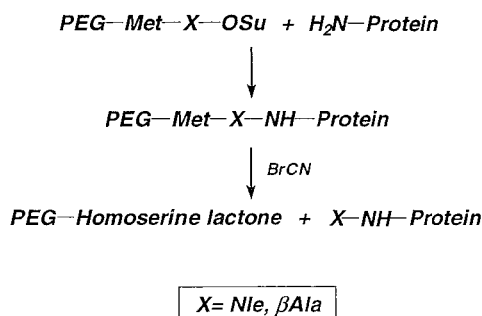


Figure 1. Conjugation of PEG-Met-Nle-OSu or PEG-Met- β Ala-OSu to a protein amino group and its removal by BrCN treatment.

fonate (CMC), and 1-hydroxybenzotriazole (HOBt) were purchased from Sigma (St. Louis, MO). Triethylamine (TEA), *N*-hydroxysuccinimide (HOSu), 1,4-dithio-L-threitol (DTT) and lysozyme from egg white were purchased from Fluka Chemie. Trinitrobenzenesulfonic acid (TNBS) was purchased from Aldrich (Milwaukee, WI), iodoacetamide from Merck (Rahway, NJ), and cyanogen bromide (BrCN) from Pharmacia (Uppsala, SW). The nonapeptide partial sequence of glucagons was from our laboratory.

Proton NMR spectroscopy was performed with a Varian Gemini 200 MHz instrument. Ultraviolet spectroscopy was performed with a Perkin-Elmer λ 5 instrument. Ultrafiltration was performed with an Amicon system and YM3 membrane (cut off 3000 Da). Ionic exchange chromatography was performed on a Pharmacia LKB system using a QAE-Sephadex A50 column. Reversed-phase chromatography was performed with a Shimadzu analytical and semipreparative HPLC system, using a Vydac 218TP54 and Vydac 218TP1022 column, respectively, with UV detector. Gel filtration chromatography was performed on a Pharmacia FPLC system, using a Superose 12TM column and an UV detector. Amino acid analyses were carried out after hydrolysis with HCl in vapor phase and derivatization with phenyl isothiocyanate (Fluka Chemie) (7) using a HPLC column Macherey-Nagel Nucleosil 120-5 C18. Maldi mass spectroscopy was performed in a Kratos Kompact MALDI 1 V5.2.0 instrument. Trinitrobenzenesulfonate (TNBS) was used as reagent for quantitative amino groups determinations following the procedure described by Habeeb (8). PEG content was evaluated colorimetrically using the iodine assay (9).

Synthesis of PEG-methionine-norleucine-hydroxysuccinimidylester (PEG-Met-Nle-OSu) and of PEG-methionine- β -alanine-hydroxysuccinimidylester (PEG-Met- β Ala-OSu). (1) *PEG-Met-Nle-OSu*. PEG-*p*-nitrophenyl carbonate (5 g; 0.97 mmol) was added in small portions to 724 mg (4.8 mmol; 5 equiv) of methionine dissolved in 10 mL of a 50% acetonitrile solution in water and brought to pH 8 with 676 μ L (4.8 mmol; 5 equiv) of triethylamine. After overnight stirring, acetonitrile was removed under reduced pressure and the pH of the reaction mixture was adjusted to 3 using solid citric acid. *p*-Nitrophenol was removed by ether extraction while the product was extracted several times with chloroform. The pooled organic fractions were dried, the volume was reduced to a few milliliters, and the product, precipitated by adding 300 mL of dry diethyl ether, was collected by filtration. For further purification the product was dissolved with acidified water, extracted with chloroform and precipitated by diethyl ether. The yield of the reaction, evaluated by 0.01 N NaOH titration, was almost quantitative. ^1H NMR (chloroform-*d*, 2000 MHz): δ 3.61 (s, PEG backbone -OCH₂-); 3.41 (s, 3H, PEG CH₃O-); 2.55

(t, 2H, Met γ CH₂); 2.07 (s, 3H, Met CH₃). In the following step 523 mg (2.888 mmol; 4 equiv) of norleucine methyl ester and a equimolecular amount of triethylamine (401 μ L) were added to a solution of 10 mL of anhydrous dichloromethane, containing 3.71 g (0.72 mmol) of PEG-Met-OH and mixed at 0 °C with 305 mg (0.72 mmol; 1 equiv) of 1-cyclohexyl-3-(2-morpholino ethyl) carbodiimide metho-*p*-toluene-sulfonate (CMC) and 97 mg (0.72 mmol; 1 equiv) of 1-hydroxybenzotriazole (HOBt). After stirring for 3 days at room temperature, the ureic derivative of CMC was filtered off, the mixture was dropped into 250 mL of dry diethyl ether, and the precipitate collected by filtration and dried. The product was further purified by ionic-exchange chromatography on a QAE Sephadex A50 column, eluted with water followed by 10 mM NaCl. The fractions containing the PEG-dipeptide were collected and lyophilized (yield: 93%). ^1H NMR (chloroform-*d*, 200 MHz): δ 3.70 (s, 3H, OMe CH₃); 3.61 (s, PEG backbone -OCH₂-); 3.41 (s, 3H, PEG CH₃O-); 2.55 (t, 2H, Met γ CH₂); 2.07 (s, 3H, Met CH₃); 1.32 (m, 4H, Nle γ e δ CH₂); 0.88 (t, 3H, Nle CH₃). In the last step, PEG-Met-Nle-OMe (1.41 g; 0.26 mmol) was stirred overnight at room temperature in a solution of methanol (5 mL) containing 20 equiv of 0.1 N NaOH (5.3 mL). The pH of the mixture was brought to 2 using 1 N HCl, and the product extracted with chloroform. The collected organic fractions were dried over anhydrous sodium sulfate, concentrated to a small volume under reduced pressure, and the product, precipitated by addition of dry diethyl ether, was filtered and dried. PEG-Met-Nle-OH was further purified by ionic-exchange chromatography on a QAE Sephadex A50 column, eluted first with water and then with a water solution of 10 mM NaCl. The fractions containing the sodium salt of the PEG-dipeptide were pooled, concentrated, and extracted by chloroform after acidification of the water solution. The purified product was finally obtained by precipitation with dry diethyl ether. Yield, calculated by 0.01 N NaOH titration, was 67%. ^1H NMR (chloroform-*d*, 200 MHz): δ 3.61 (s, PEG backbone -OCH₂-); 3.41 (s, 3H, PEG CH₃O-); 2.55 (t, 2H, Met γ CH₂); 2.07 (s, 3H, Met CH₃); 1.32 (m, 4H, Nle γ e δ CH₂); 0.88 (t, 3H, Nle CH₃).

The 1:1:1 ratio among the integration value of methoxy protons of PEG, methoxy protons of methionine and norleucine demonstrates the correct composition of the product and the complete derivatization of PEG.

The identity of the product was also demonstrated by amino acid analysis following acid hydrolysis (Lys 1.01, Met 0.9, Nle 1.0). In this case, PEG content was evaluated by iodine assay (9). For a mole of PEG (MW 5000) methionine and norleucine corresponded to 0.95 and 1.01 mol, respectively.

For PEG-Met-Nle-OH activation, the product (600 mg 0.11 mmol) was dissolved in 5 mL of dichloromethane, and hydroxy-succinimide (104 mg 0.9 mmol) and DCCI (185 mg, 0.9 mmol) were added. The reaction mixture was left to react for 24 h, and the product, precipitated by ethyl ether, collected by filtration and dried. The activation degree, expressed as percentage of -OSu for PEG-Met-Nleu, was over 95% in three different preparations. The activation degree was determined by the amino groups modification of glycine-glycine as substrate using TNBS for residual NH₂ groups evaluation (10).

(2) *PEG-Met- β Ala-OSu*. This product was prepared according to a procedure different from that followed for the nor-leucine derivative preparation, namely PEG activated as *p*-nitrophenylchloroformate was reacted with H-Met- β Ala-OMe and the product, after alkaline hydrolysis, activated as hydroxysuccinimidylester.

Table 1. Characterization of the Nonapeptide Derived from a Glucagon Sequence Following PEGylation with PEG-Met- β Ala-OSu and Removal of the PEG Chain by BrCN Treatment

	elution volume from HPLC ^a (min)	molecule mass ^b (Da)	Met mol/peptide ^c	β Ala mol/peptide ^c
free monopeptide	16.4	1091.1	0	-
PEGylated form	36.3	11090.0	2	2
following BrCN treatment	16.8	1317.4	0	2

^a A Vydac 218TP54 reverse phase HPLC column was used. ^b The mass was evaluated by Maldi mass spectroscopy. ^c Values obtained by amino acid analysis after acid hydrolysis. These nominal values were calculated from the following amino acid analyses: For PEGylated form, Thr, 0.81; Ser, 1.81; Arg, 1.08; Tyr, 1.77; Leu, 1.03; Arg, 1.02; Met, 1.75; β Ala, 2.20. For the BrCN treated product, Thr, 0.78; Ser, 1.75; Arg, 1.01; Tyr, 1.82; Leu, 0.98; β Ala, 1.04.

The dipeptide was synthesized from BOC-Met-OH (0.7 g, 2.9 mmol) and β -Ala-OMe (0.618 g, 4.4 mmol) in the presence of EDC (0.566 g, 2.9 mmol) and HOBt (0.399 g, 2.9 mmol) in 5 mg of dichloromethane. After extraction with basic and acid aqueous solution, the organic phase has been dried by Na_2SO_4 and evaporated.

¹H NMR (chloroform-*d*, 200 MHz): δ 1.5 (s, 9 H, BOC); 1.9 (m, 2H, $-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_3$); 3.7 (s, 3 H, $-\text{S}-\text{CH}_3$); 2.5 (m, 4 H, $-\text{S}-\text{CH}_2 + \beta\text{Ala}-\text{CH}_2-\text{CO}$); 3.5 (m, $-\text{NH}-\text{CH}_2-\beta\text{Ala}$); 3.7 (s, 3 H, $-\text{COOCH}_3$); 4.2 (m, 1 H, $-\text{CH}-$); 5.25 (d, 1 H, BOCNH); 6.75 (t, 1 H, $-\text{NH}-\text{CO}$). The deprotection has been accomplished by stirring the product in a mixture of 2.5 TFA and 2.5 mL of dichloromethane. H-Met- β Ala-OMe was recovered by ether precipitation. ¹H NMR (chloroform-*d*, 200 MHz): 2.1 (s, 3 H, $-\text{S}-\text{CH}_3$); 2.15 (m, 2 H, $\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_3$); 2.6 (m, 4 H, $-\text{S}-\text{CH}_2 + \beta\text{Ala}-\text{CH}_2-\text{CO}$); 7.6 (t, 1 H, $\text{NH}-\text{CO}$); 7.9 (bs, 2 H, NH_2). The product was coupled to PEG-*p*-nitrophenylchloroformate, the methylester removed and the carboxylic group activated by OSu as previously reported for PEG-Met-Nle-OSu.

The amino acid analysis after acid hydrolysis and PEG evaluation carried out by colorimetric assay demonstrated the identity of the compound. For a mole of PEG (5000 MW), methionine and β -alanine were found to be 0.97 and 1.04, respectively. The activation degree was found to be over 97% in different preparations.

PEG Conjugation, Followed by Its Removal, to a Nonapeptide, Lysozyme, and Insulin. (a) *PEG Conjugation and Removal from H-Thr-Ser-Arg-Tyr-Ser-Lys-Tyr-Leu-Asp-OH*. A total of 41 mg (4.0 μmol) of MPEG-Met- β Ala-OSu was added, in small aliquots, to 0.9 mg (0.8 μmol) of the nonapeptide dissolved in 1 mL of DMSO, at pH 8 with TEA (nonapeptide: MPEG-Met-Nle-OSu molar ratio = 1:5). After 3 days of stirring at room temperature, the pH of the reaction mixture was adjusted to 3 with 1 N HCl, and the product was extracted several times by chloroform. The chloroform was anhydriified with dry Na_2SO_4 and concentrated to dryness. The collected conjugate was further purified by means of a Shimadzu semipreparative HPLC system, using a reversed-phase Vydac 218TP54 column (C18 bonded phase, 0.46×25 cm i.d., $5 \mu\text{m}$ particle diameter). A linear gradient of aqueous 0.05% trifluoroacetic acid and 0.05% trifluoroacetic acid in acetonitrile was used and the fractions of the unique peak revealed by absorption at 280 nm and by iodine assay were pooled and concentrated to dryness. The PEGylation was verified by the disappearance of reactivity of the peptide amino groups and by the presence of two β Ala and Met residues revealed by amino acid analysis after acid hydrolysis (see Table 1). For the removal of bound PEG, 1 mg (85.2 μmol) of PEG-Met- β Ala-nonapeptide was treated with 100 equiv of BrCN in aqueous 70% formic acid (11). After 24 h standing at room temperature, the mixture was poured into 10 vol of water and lyophilized; this procedure was repeated twice. The product was fractionated by a Shi-

madzu analytical HPLC system, using a reversed-phase Vydac 218TP54 column (C18 bonded-phase, 0.46×25 cm i.d., $5 \mu\text{m}$ particle diameter). The eluent was a linear gradient of aqueous 0.05% trifluoroacetic acid and 0.05% trifluoroacetic acid in acetonitrile.

(b) *PEG Conjugation and Removal from Lysozyme*. A total of 42.2 mg (7.8 μmol) of mPEG-Met-Nle-OSu was added, in small aliquots while stirring, to 3.75 mg (0.26 μmol) of lysozyme from egg white dissolved in 1 mL of 0.2 M borate buffer, pH 8.5. The molar ratio of lysozyme amino groups to mPEG was 1:5. After 30 min of stirring at room temperature, the conjugate was purified by means of a semipreparative FPLC system, using a gel filtration Superose 12TM column (1.5×25 cm) eluted with 0.01 M phosphate buffer and 0.15 M NaCl, pH 7.2. The fractions were analyzed for lysozyme elution by absorption at 280 nm and iodine assay for PEG elution. The fractions containing the modified lysozyme were collected and concentrated to a small volume by ultrafiltration with an Amicon system using a YM3 membrane (cut off 3000 Da). The concentrated solution was diluted with water and ultrafiltered. This procedure was repeated and the product was finally lyophilized. For PEG removal, 0.04 μmol of modified lysozyme was treated with 600 equiv of BrCN in aqueous 70% formic acid and left stirring for 24 h at room temperature. The mixture was poured into 50 mL of water and lyophilized; the procedure was repeated twice. The product obtained was analyzed by gel filtration column by a FPLC system as above, to compare the chromatography profiles.

(c) *Insulin Modification by PEG-Met-Nle-OSu Followed by Removal of PEG and Identification of the Sites of PEGylation*. PEG-Met-Nle-OSu was added to 7 mg (1 μmol) of bovine insulin dissolved in 1 mL of DMSO until a 3:1 PEG: insulin molar ratio was reached. The mixture was left stirring for 5 h at room temperature. The conjugate was purified by means of a Shimadzu preparative HPLC system, using a reversed-phase Vydac 218TP1022 column (C18 bonded phase, 2.2×25 cm i.d., $10 \mu\text{m}$ particle diameter) and a linear gradient of aqueous 0.05% trifluoroacetic acid and 0.05% trifluoroacetic acid in acetonitrile, monitoring the absorption at 226 nm wavelength. Three peak fractions of conjugated insulin were collected (at 21.867, 23.275, and 24.158 min of elution) and lyophilized. The product of each peak, called insulin A, insulin B, and insulin C, were separately treated with BrCN in aqueous 70% formic acid to remove PEG. The molar ratio between insulin and BrCN was 1 to 200. After 24 h stirring at room temperature, the mixture was poured into 10 vol of water and freeze-dried; the procedure was repeated twice. The obtained products were analyzed and purified by means of a Shimadzu analytical HPLC system, using a reversed-phase Vydac 218TP54 column (C18 bonded phase, 0.46×25 cm i.d., $5 \mu\text{m}$ particle diameter) with a linear gradient of aqueous 0.05% trifluoroacetic acid and 0.05% trifluoroacetic acid

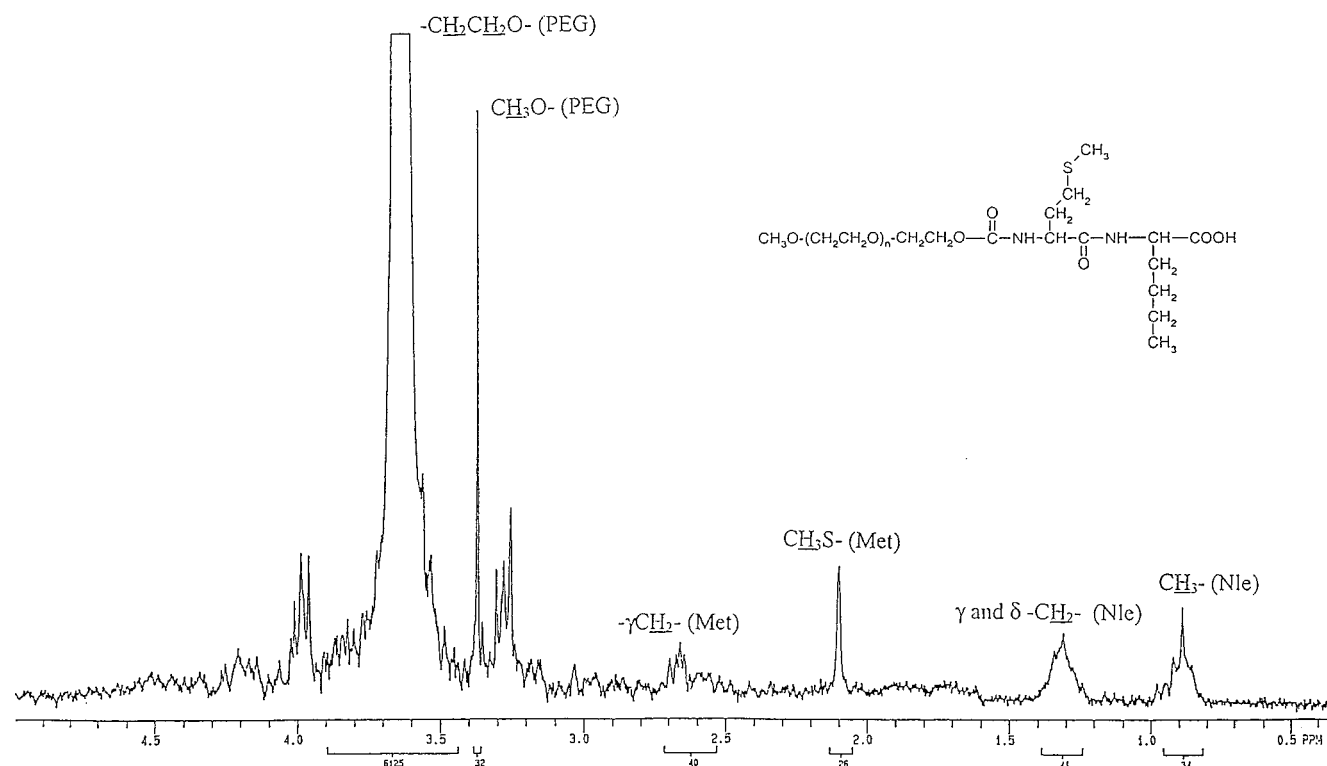


Figure 2. ^1H NMR spectrum (CDCl_3 , 200 MHz) of mPEG-Met-Nle-OH.

in acetonitrile. To determine the position of PEG in the insulin chains, the disulfide bonds were reduced and carboxymethylated. To this aim, 500 μg (0.08 μmol) of each Nle-insulin sample, obtained from PEG-Met-Nle-insulin A, B, and C after BrCN treatment, were dissolved in 250 μL of Tris-HCl buffer, pH 7.5, containing 2 mM EDTA and 6 M Gdn-HCl; 3.8 mg of 1,4-dithio-L-threitol were added, and the mixture was left standing for 2 h at 37 $^\circ\text{C}$. Finally, 9.2 mg of iodoacetamide were added and the mixture was left for 1 h at 37 $^\circ\text{C}$. The solutions were lyophilized, and the products fractionated by means of a Shimadzu semipreparative HPLC system, using a reversed-phase Vydac 218TP54 column as described above. The eluted products monitored by absorption at 226 nm wavelength revealed in all the samples the presence of two main peaks, corresponding to the reduced carboxymethylated α and β chains, respectively. The two polypeptides were separated, recovered, and analyzed by amino acid analysis, mass spectrometry and eventually Edman degradation (12).

RESULTS

Synthesis of PEG-Met-Nle-OSu and PEG-Met- β Ala-OSu. The synthesis of the two new PEG derivatives may be carried out by sequential amino acid addition to PEG according to the polymer supported liquid-phase synthesis procedure (13) or by the linkage of PEG to the dipeptide, followed by the carboxylic groups activation. Here the first method was employed for PEG-Met-Nle, while the second for PEG-Met- β Ala preparation. The identity of the polymer derivatives was verified qualitatively and quantitatively by ^1H NMR spectroscopy (see Figure 2 for PEG-Met-Nle-OH) and by evaluation of the three components: PEG by iodine assay, methionine and nor-leucine (14) or β -alanine by amino acid analysis after acid hydrolysis.

Using glycyl-glycine as model (10), it was verified the degree of activation as hydroxysuccinimidylester. It also demonstrated that the rate of aminolysis of OSu of these

new PEG derivatives was the same of the known simple PEG-Nle-OSu (14).

Protein and Peptide Conjugation with the New PEG-Derivatives Followed by PEG Removal by BrCN. The peptide and protein conjugation of the new PEG-peptide arms, activated as succinimidyl ester, followed by deblocking by BrCN treatment, was verified using three different models: a nonapeptide, the small protein insulin and the 12 kDa protein lysozyme.

(A) The nonapeptide H-Thr-Ser-Arg-Tyr-Ser-Lys-Tyr-Leu-Asp-OH, a partial sequence of glucagon, was chosen since it presents two reactive amino groups. Following treatment with PEG-Met- β Ala-OSu, 5 kDa, the mass of the nonapeptide, evaluated by Maldi mass spectrometry, increased from the 1091.11 kDa of the free peptide to a value of about 11410 kDa. The mass spectrometry peak was broad (spectrum not shown) due to the known polydispersity of PEG. The increase in mass, of approximately 10 kDa, corresponds to the conjugation of two PEG-Met- β Ala chains and is accompanied by the disappearance of amino group reactivity, demonstrated by TNBS reaction 12. Contemporaneously an increase in reversed-phase HPLC elution volume from 16.4 to 36.3 mL was found. Following BrCN treatment, a sharp peak with a mass of 1233.26 kDa, corresponding to the nonapeptide mass plus two β Ala residues could be observed by mass spectrometry. At the same time the HPLC elution dropped to 16.8 mL, a volume close to that of the free nonapeptide (16.4 mL). Furthermore the amino acid analysis showed the presence of two β Ala residues per peptide molecule and no trace of methionine. These values are summarized in Table 1.

(B) Figure 3 reports the elution from a GPC column of three lysozyme species: native, PEGylated and after BrCN treatment. The PEGylated lysozyme (b) was eluted by gel filtration chromatography at the 12 mL, much ahead of the native enzyme, 18 mL, (a) due to its large increase in mass. Six PEG chains were bound as revealed by mass spectrometry, amino acid analysis following acid

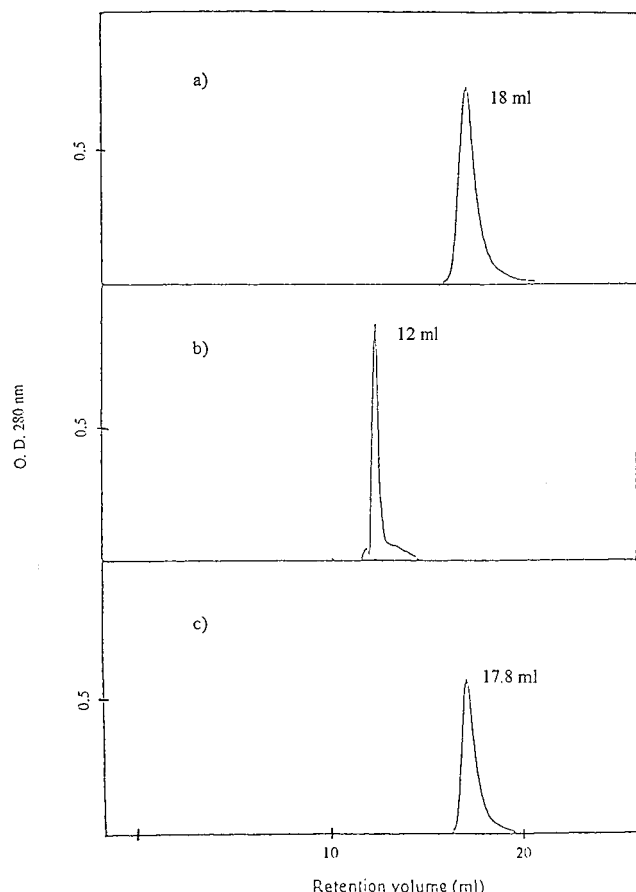


Figure 3. Elution peaks of lysozyme (a), PEGylated lysozyme (b) and the conjugation product after PEG removal by BrCN treatment (c), from a GPC column.

hydrolysis, and amino groups titration by TNBS. The sample obtained after deblocking with BrCN. Nle conjugated lysozyme (c), was eluted 17.8 mL a volume close to that of the native protein (18 mL). The small difference in elution being related to the difference in mass of the two species: 14 388 kDa of native lysozyme, as compared to 15 066 kDa of lysozyme plus the six nor-leucine residues as confirmed by amino acid analysis.

The data regarding a small peptide or a model protein, reported in A and B, are in favor of the possibility to link these novel PEGs to polypeptides as well as of their quantitative removal by BrCN treatment, leaving Nle or β Ala as reporter amino acids bound to the starting molecule.

Localization of Conjugation Site in PEGylated Insulin. Insulin was the third polypeptide model chosen to test the suitability of the proposed methodology, because in this case different PEGylated isomers may be obtained in the conjugation reaction. Figure 4b, shows that when insulin is PEGylated in dimethyl sulfoxide at a 1 to 1 molar ratio of polymer to insulin available amino group, three conjugation products are obtained. They are eluted later as compared to native insulin (18.3 mL, see Figure 4a) since the presence of PEG. Colorimetric amino group titration and amino acid analysis following acid hydrolysis on the basis of methionine and nor-leucine presence, demonstrated that peak A (eluted at 21.9 min) corresponds to mono-PEGylated insulin. Peak B (eluted at 23.3 min) corresponds to an insulin species with two PEG molecules bound. The third peak C (eluted at 24.1 min) is the three-PEGylated product (see Figure 4b).

This last product (c) being modified at all available insulin amino groups (Gly 1 of α chain, Phe 1 and Lys

29 of β chain), did not need further studies for its characterization.

As shown in Figure 5a, when the mono-PEGylated product (peak A of Figure 4) was treated with BrCN, HPLC fractionation yielded a single peak that, by mass spectrometry, showed a mass of 5845.32 Da, corresponding to the mass of one insulin plus 1 residue of norleucine (expected value 5846.68 Da, see Figure 5b). The elution from HPLC, 18.4 mL (see Figure 5a) is close to that of native insulin, 18.3 mL (see Figure 4a), the difference is due to the fact the two products differ for only one amino acid (Nle).

To identify the position of PEGylation, the two insulin chains were separated by reduction followed by carboxymethylation. It was found that only the heavy chain was PEGylated, showing a mass of 3629.81 Da (the expected mass of β chain plus 1 Nle residue is 3627.59 Da, Figure 6).

To identify whether the PEGylation was at Phe 1 or at Lys 29, one step of Edman was carried out. Mass spectrometry demonstrated the release of one phenylalanine and one norleucine residue (expected mass 3367.22, found 3369.23) (see Figure 7). This demonstrated that the α amino group of phenylalanine was free (Phe was in fact released by Edman) and consequently that PEG was bound to ϵ -lysine (to note that Nle also was released by CNBr from the Nle- ϵ -Lys bond).

The di-PEGylated insulin, corresponding to the major peak B of Figure 4, was also purified to homogeneity and PEG was removed (see Figure 8a). The dePEGylated product yielded a mass of 5954.09 that corresponds to insulin plus 2 Nle residues (see Figure 8b, expected value 5959.86). After reduction and carboxymethylation, the two chains were separated by HPLC. They were identified by mass spectrometry: one showed a mass of 3628.95 Da, already found in the peak A of Figure 4, corresponding to mono-PEGylation; the second, of 2689.06 Da (see Figure 9) corresponds instead to a mono-PEGylated light chain (expected value 2681.18).

While for the α chain there is only one position of PEGylation at the level of glycine in position 1 of the peptide, for the β chain two positions are possible, as described above. One step of Edman degradation showed that the α amino group was free since phenylalanine was released demonstrating that the PEG binding involved lysine 29, as for the previous peak B of insulin.

From these experiments, it was therefore possible to assign the position of PEGylation to the mixture of products obtained in insulin conjugation. It was contemporaneously possible to demonstrate that, under these conditions of reaction, the order of reaction is lysine 29 of β chain (the one always found), followed by glycine 1 of α chain, being phenylalanine 1 of β chain the less reactive. These results are in agreement with the known properties of insulin residues in which the low reactivity of Phe 1 of α chain is ascribed to its pK_a (15, 16). Extensive PEGylation of this residue was obtained only by a previous protection by BOC of the other two more reactive amino groups (17–19).

CONCLUSIONS

The data obtained using a nonapeptide, insulin and lysozyme as models, strongly support the use of PEG with Met-X as peptide arm for the modification of proteins. Convenient X are amino acids non natural or non frequent in proteins, norleucine and β -alanine standing as the preferred ones.

This functionalized polymers present unique advantages for the analytical characterization of PEG conju-

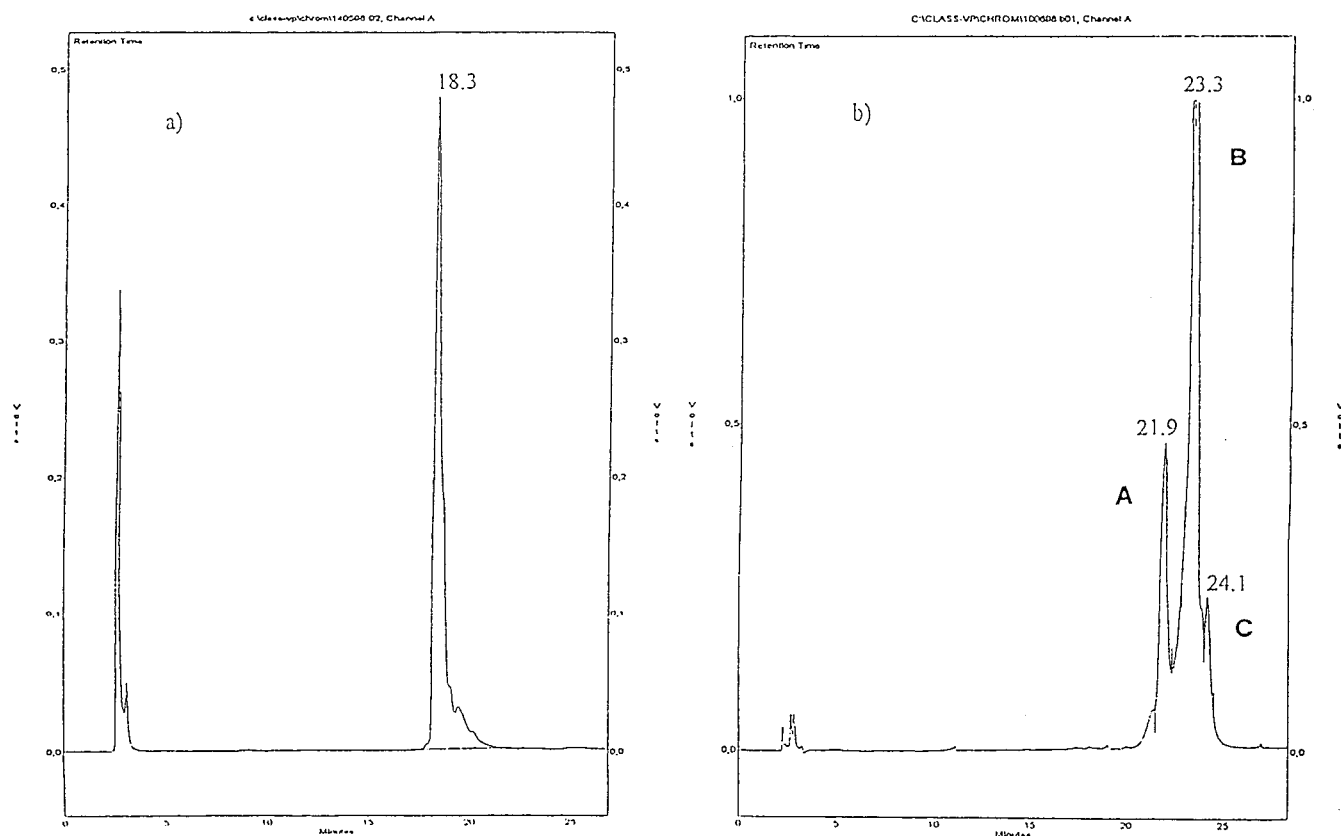


Figure 4. (a) Elution pattern from reversed-phase column of insulin and (b) of the reaction mixture of insulin conjugation with PEG-Met-Nle-OSu. Peaks A, B, and C correspond to insulin with 1, 2, and 3 PEG chains bound per insulin molecule.

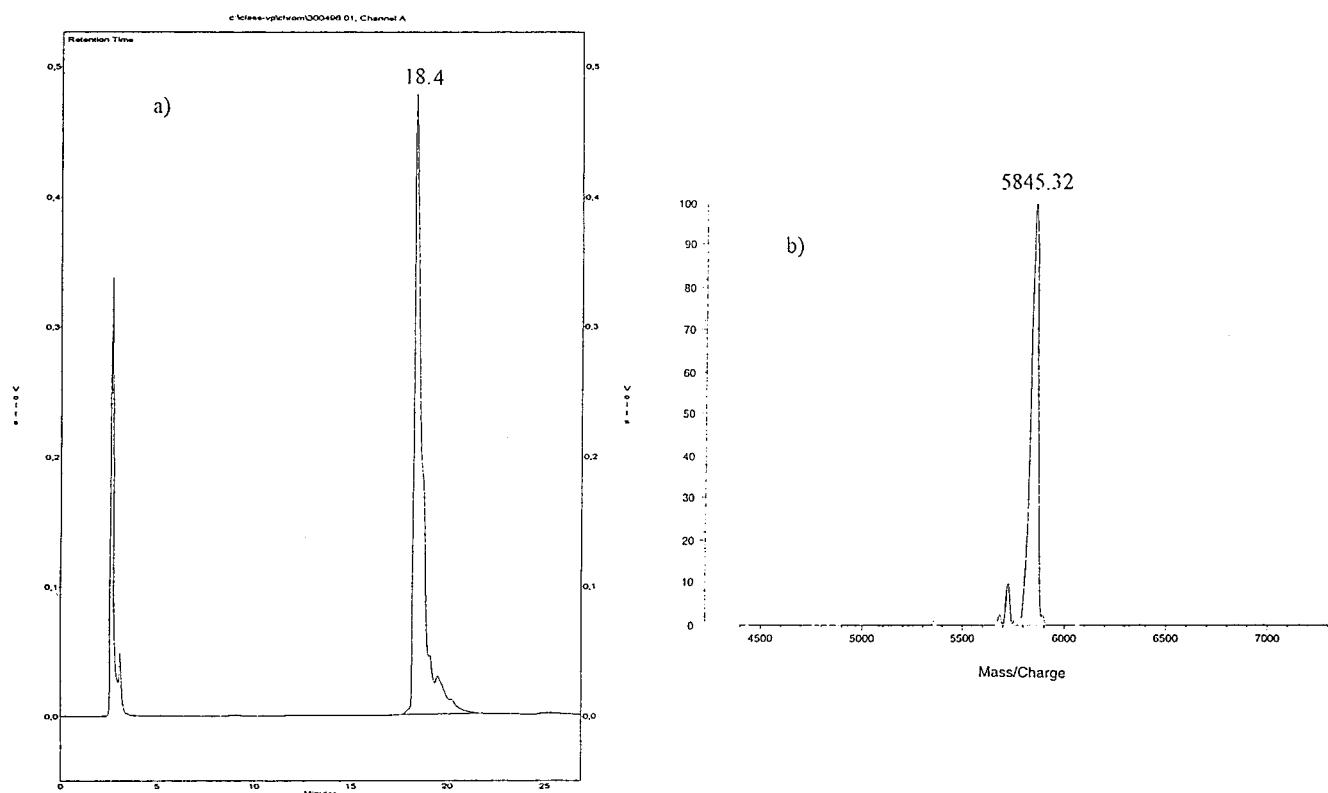


Figure 5. (a) Elution pattern of mono-PEGylated insulin, corresponding to peak A of Figure 4, following purification and treatment with BrCN to release PEG, to leave the reporter Nle only bound to the peptide; (b) mass spectrum of the Nle insulin reported in panel a. Found: 5845.32 Da. Expected: 5846.68 Da.

gates, since the hindered PEG may be specifically removed by BrCN treatment leaving bound norleucine or β -alanine as reporter group bound to the proteins. These

de-PEGylated proteins are more easily fractionated in their constituent isomers of the PEG containing products, since their lower hindrance. This is an important aspect

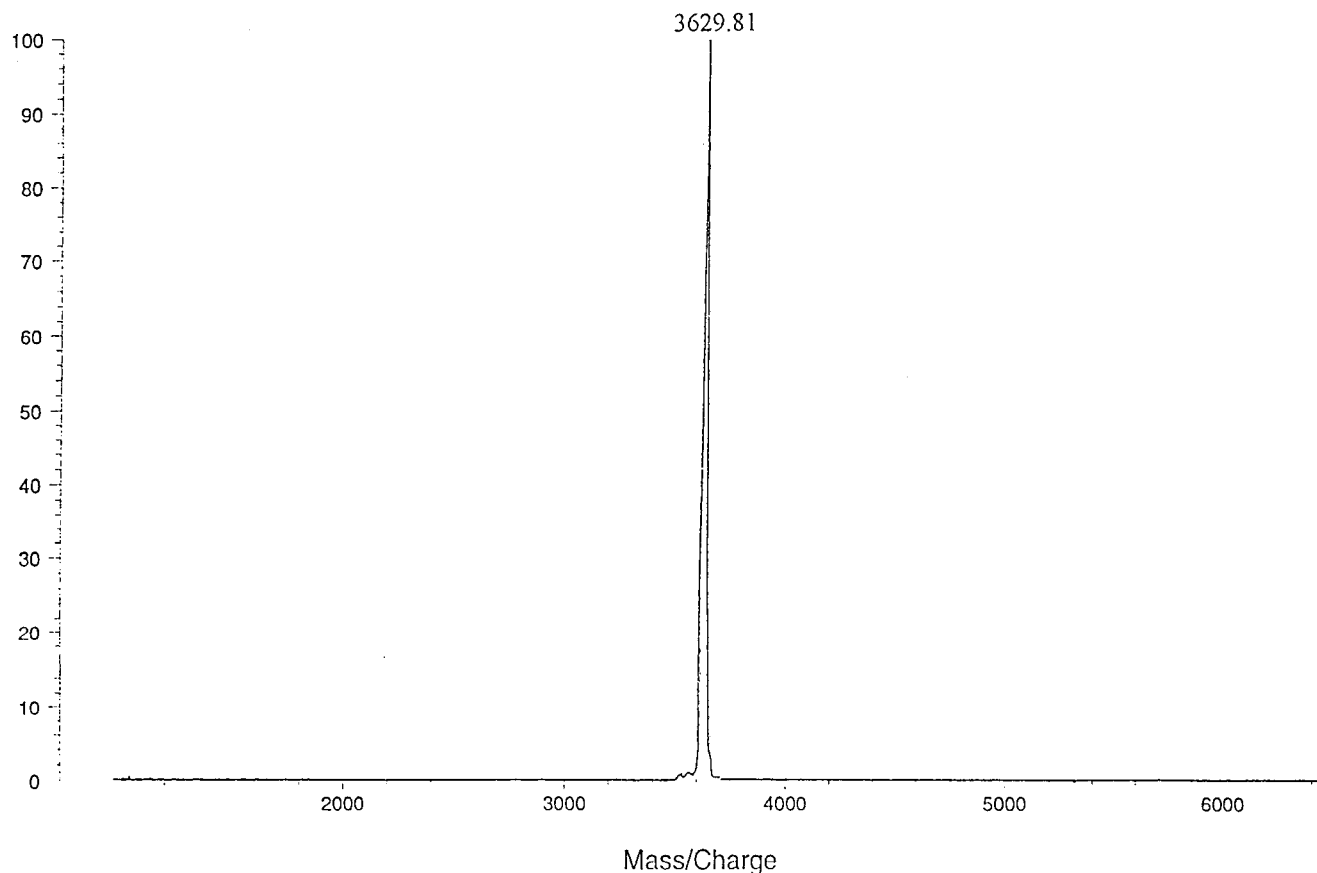


Figure 6. Mass spectrum of the reduced carboxymethylated insulin heavy chain carrying one norleucine residue. The sample comes from the peak A of Figure 4 after purification, dePEGylation (see Figure 5a) and separation of the chains. Found: 3629.81 Da. Expected: 3627.59 Da.

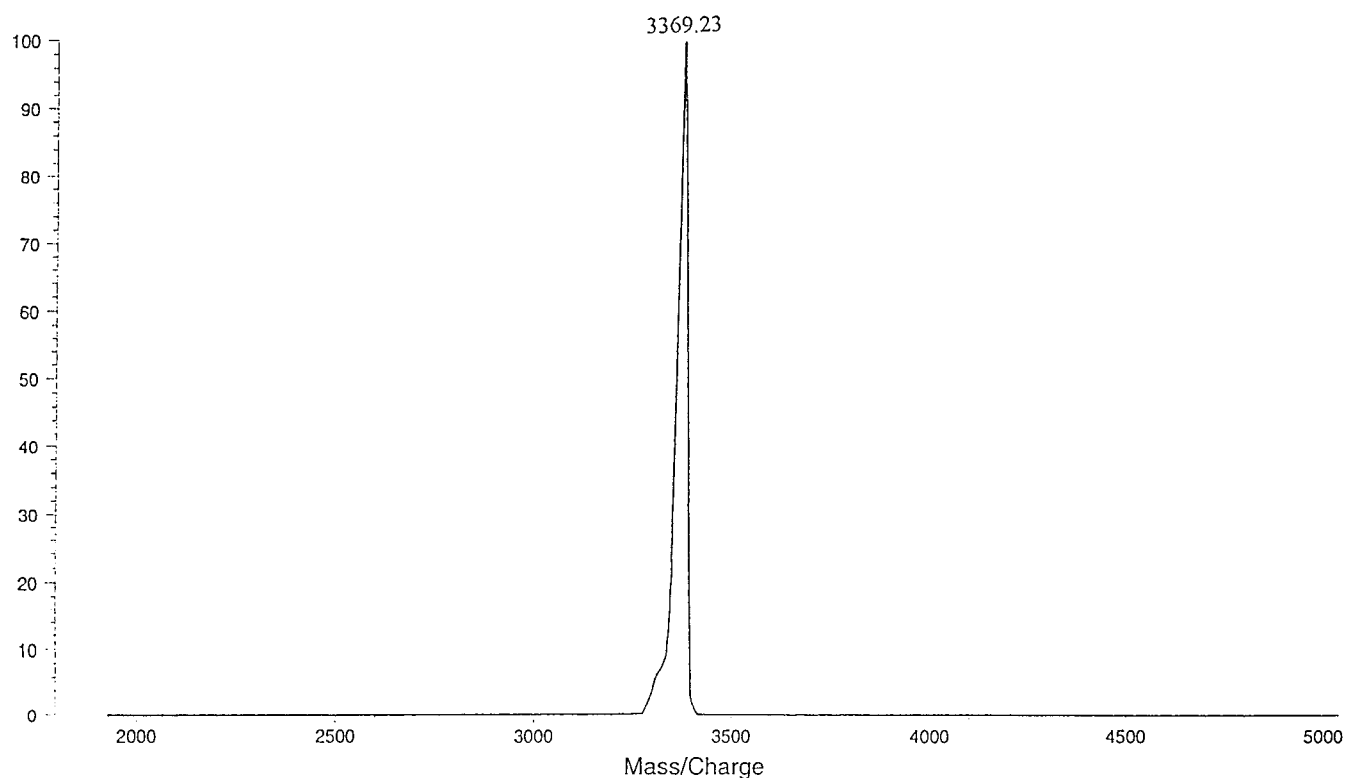


Figure 7. Mass spectrum of heavy chain of insulin of peak A of Figure 4, after dePEGylation reduction, carboxymethylation and one step of Edman degradation. Found: 3369.23 Da. Expected: 3367.22 Da.

because it is known that PEGylation yields heterogeneous mixture, that only in few lucky cases may be separated.

Furthermore the proteins, devoid of PEG, may undergo any chemical or enzymatic cleavage needed for sequence evaluation and the labeled peptides are easily identified

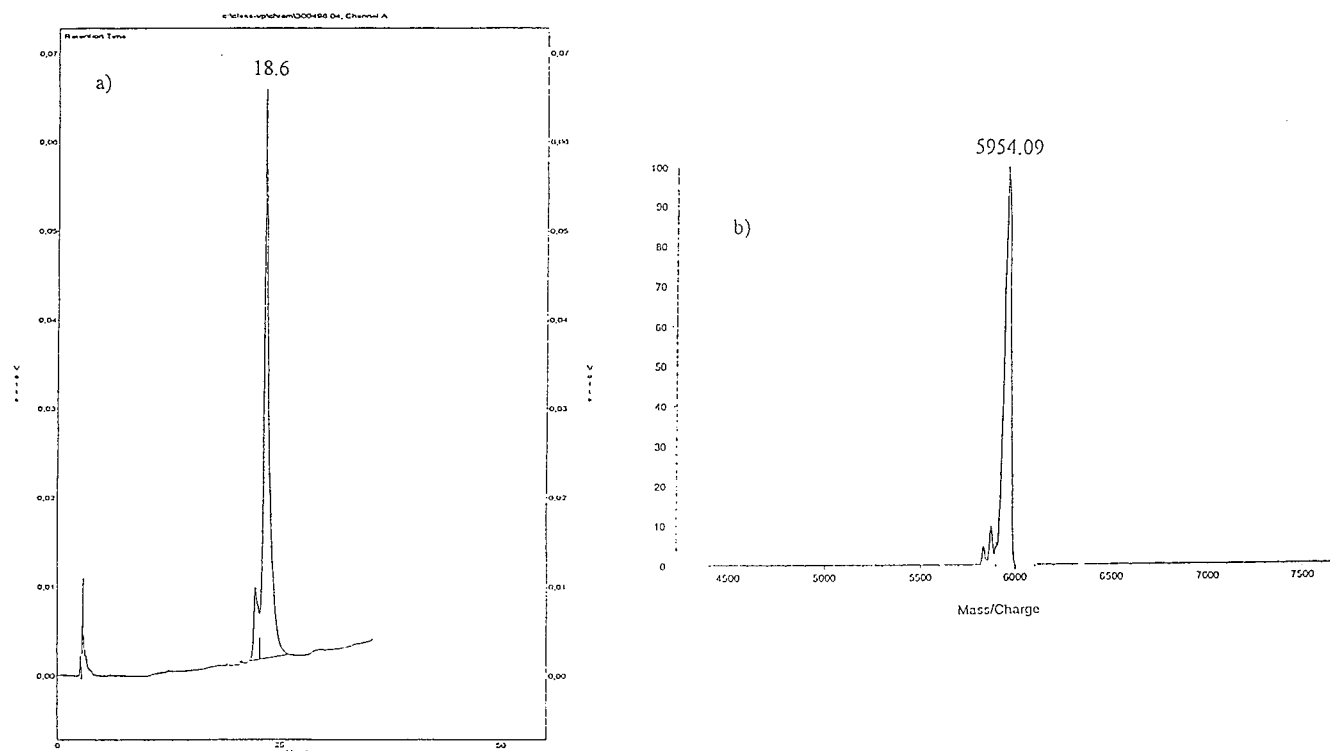


Figure 8. (a) Elution pattern of di-PEGylated insulin, corresponding to peak B of Figure 4, following treatment with BrCN to release PEG. (b) Mass spectrum of Nle insulin reported in panel a, showing that two Nle residues are bound to the protein. Found: 5954.09 Da. Expected: 5959.86 Da.

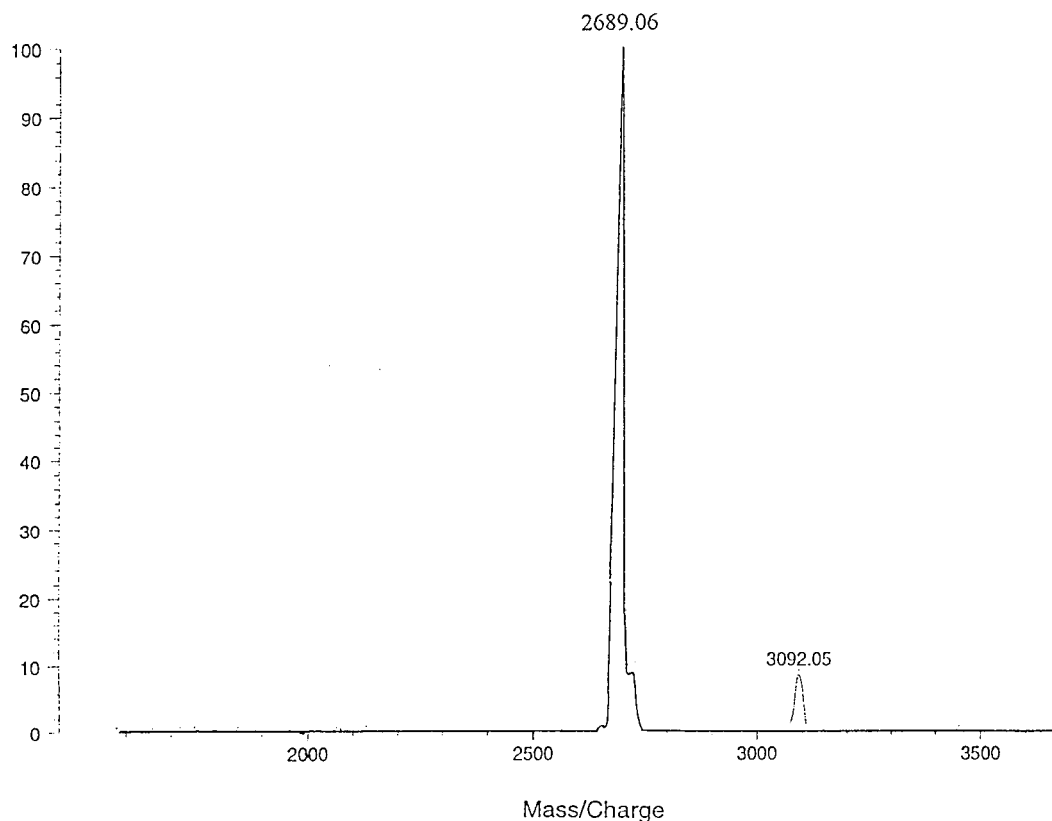


Figure 9. Mass spectrum of light chain found of dePEGylated, reduced and carboxymethylated insulin of peak B in Figure 4. Found: 2689.06 Da. Expected: 2681.18 Da.

on the basis of norleucine or β -alanine detectable by amino acid analysis or mass spectrometry. In this respect norleucine appears ideal as reporter residue in mass spectrometry analysis for its higher molecular weight. β -Alanine is less convenient in this respect, but it

presents the advantage, over norleucine, of not being released by Edman degradation, thereby allowing its localization in a peptide where multiple lysine residues are present (20).

The BrCN based removal of PEG offers an additional

advantage for the identification of the site of PEGylation. As well-known BrCN, in the same step of dePEGylation, cleaves also the protein at the level of methionine, if present in the primary sequence, yielding shorter peptides where the reporter groups may be directly identified. This peptide cleavage did not take place in our models nonapeptide or in insulin, both devoid of methionine. In lysozyme, on the other hand, the cleavage took place, but the peptides remained still linked together by disulfide bridges.

To verify the suitability of these new PEGs for a therapeutic application of their protein conjugates, studies to evaluate the stability in blood of PEG-Met-Nle and PEG-Met- β Ala conjugates are presently under way, and will be reported separately.

ACKNOWLEDGMENT

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Reaction of Nitric Oxide with Synthetic Hemoprotein, Human Serum Albumin Incorporating Tetraphenylporphinatoiron(II) Derivatives

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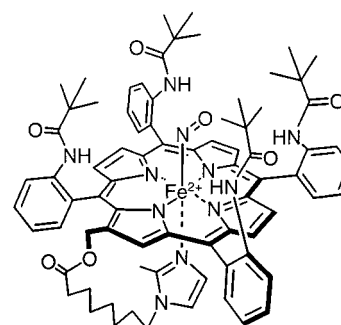
The reaction of nitric oxide (NO) with a synthetic hemoprotein, the recombinant human serum albumin (rHSA) incorporating *eight* tetraphenylporphinatoiron(II) derivatives bearing a covalently linked axial base (FeP) [rHSA-FeP], has been investigated. The UV–vis absorption spectrum of the phosphate buffer solution (pH 7.3) of rHSA-FeP showed maxima at 425 and 546 nm upon the addition of NO. The carbonyl rHSA-FeP, in which FePs are six-coordinate CO-adducts, also moved to the same species after bubbling with NO gas. ESR spectroscopy revealed that the incorporated FePs in the albumin formed six-coordinate nitrosyl complexes; the proximal imidazole moiety does not dissociate from the central iron when NO binds to the trans side. The NO-binding affinity of rHSA-FeP ($P_{1/2}^{\text{NO}}$, 1.7×10^{-6} Torr, pH 7.3, 298 K) was significantly lower than that of FeP itself ($P_{1/2}^{\text{NO}}$, 1.8×10^{-8} Torr in toluene). Kinetically, this arises from the decreased association rate constant ($k_{\text{on}}^{\text{NO}}$, $8.9 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} \rightarrow 1.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$). Since NO-association is diffusion controlled, incorporation of the synthetic heme into the albumin matrix appears to restrict the NO access to the central iron(II).

INTRODUCTION

Several types of hemoglobin (Hb)-based O_2 -carriers are currently being studied in clinical trials (Chang, 1997; Tsuchida, 1998). Administration of these cell-free Hbs as red cell substitutes, however, often induces acute increases in systemic blood pressure by vasoconstriction (Keipert et al., 1993; Moisan et al., 1998; Schultz et al., 1993). This side effect is now recognized to be due to the fact that small Hb molecules extravasate through the vascular endothelium and react with the endothelial-derived relaxation factor (EDRF), *namely nitric oxide* (NO), thus inhibiting its vasorelaxing action. On the other hand, we have recently found that the recombinant human serum albumin (rHSA) incorporating a tetraphenylporphinatoiron(II) derivative with a covalently linked proximal base, 2-[8-{*N*-(2-methylimidazolyl)}octanoyloxy-methyl]-*meso*-tetrakis($\alpha,\alpha,\alpha,\alpha$ -*o*-pivalamido)phenylporphinatoiron(II) (FeP) [rHSA-FeP], can reversibly bind and release dioxygen (O_2) under physiological conditions (in aqueous media, pH 7.3, 37 °C) like Hb and myoglobin (Mb) (Komatsu et al., 1999; Tsuchida et al., 1997, 1999; 2000). A maximum of eight FeP molecules are included inside the albumin structure by hydrophobic interaction, and the O_2 -binding affinity satisfies the initial clinical requirements for an artificial red cell. On the basis of laser flash photolysis experiments, the two-phase kinetics of CO- and O_2 -binding to rHSA-FeP was also carefully studied related to the molecular environment around each FeP in the albumin (Komatsu et al., 2000a). However, to date, much less is known for the reaction of NO to rHSA-FeP. One can anticipate that the injection of this cell-free type O_2 -carrier into the blood vessels may induce unfavorable hypertension, if its NO-binding af-

finity is enough high to react with 1 μM NO in the endothelium cells (Malinski et al., 1996). Hence, it is an urgent necessity to elucidate the NO-binding properties of this albumin-heme hybrid.

On the other hand, from the viewpoint of coordination chemistry, a considerable number of studies have been made on the nitrosyl hemoproteins and synthetic porphyrinatoirons, because NO shows a unique negative trans effect, which is quite different from the other gaseous ligand such as carbon monoxide (CO) and O_2 (Hoffman et al., 1978; Hoshino et al., 1989; Nasri et al., 1997; Moore et al., 1976; Rose et al., 1983; Shimizu et al., 1984; Traylor et al., 1992). The most significant difference is that the axial base coordination to the Fe(II)porphyrin is weakened upon the addition of NO; this often leads to a rupture in the iron(II)–imidazole bond. On the contrary, we have shown that covalently attaching the proximal imidazole directly to the porphyrin periphery provides an incredibly large equilibrium constant for the imidazole bonding and prevents the *base-off* reaction when NO is bound to the trans side. Indeed, the FeP formed a six-coordinate nitrosyl complex in toluene at 298 K (Komatsu et al., 2000b).



Six-coordinate nitrosyl FeP

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This paper describes for the first time the formation of the six-coordinate nitrosyl complex of FeP in rHSA and the equilibrium and kinetic parameters for NO-binding to this albumin-heme hybrid, rHSA-FeP.

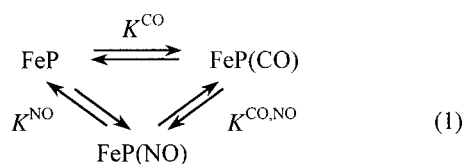
EXPERIMENTAL PROCEDURES

Materials and Preparation of rHSA-FeP Solution. 2-[8-{*N*-(2-Methylimidazolyl)}octanoyloxymethyl]-*meso*-tetrakis($\alpha,\alpha,\alpha,\alpha$ -*p*-ivalamidophenyl)porphinatoiron(II) (FeP) was synthesized as previously described (Tsuchida et al., 1995). A recombinant human serum albumin (rHSA; 25 wt %) was obtained from the WelFide Corporation. The rHSA-FeP solutions [FeP/rHSA = 8 mol/mol; [FeP] = 20 μ M to 1 mM, in 33 mM phosphate buffer, pH 7.3] were prepared according to our previously reported procedure (Tsuchida et al., 1999). The nitric oxide (NO) gas cylinders (1% and 5 ppm) were purchased from Takachiho Chemical Industry Co., Ltd.

Nitrosylation of FeP. (a) *In Toluene Solution.* The iron(III) complex of FeP in toluene was reduced by a heterogeneous two-phase system using aqueous dithionite under an argon atmosphere (Tsuchida et al., 1995). After removing the water phase and dehydration with anhydrous Na_2SO_4 , the UV-vis absorption spectrum [λ_{max} = 440, 541, 559, 608(sh) nm] showed the formation of the five-*N*-coordinated high-spin Fe(II) complex. The UV-vis absorption measurements were performed using a Shimadzu V-570 spectrometer within the range of 350–700 nm. The FeP concentrations of 20 μ M were normally used. Five parts per million NO in N_2 was then bubbled through the solution until the spectrum reached equilibrium. To remove the higher oxides, NO was successively passed through the gas-washing bottles filled with solid KOH and phosphate buffer solution (pH 7.3), respectively, in turn prior to use. A double stopcock was equipped on the quartz cuvette as a barrier to the O_2 diffusion.

(b) *In Aqueous Solution.* The aqueous solution of rHSA incorporating the carbonyl FePs [rHSA-FeP, FeP/rHSA = 8 mol/mol; [FeP] = 20 μ M] was prepared according to our previously reported procedures (Tsuchida et al., 1999). For the UV-vis absorption spectroscopy, we used special glassware, consisting of a two-neck round flask (normally 50 mL) with one neck having a double-stopcock on the top, and the other with the quartz cuvette. CO was gently bubbled through the sample in the flask in which a magnetic stirring bar was continuously stirred to avoid O_2 contamination. One percent NO in N_2 was then introduced into the solution through a stainless steel needle. Three hours of bubbling was normally required to obtain a constant absorbance at room temperature. Bubbling occasionally resulted in the denaturation of rHSA-FeP, but a low flow rate tends to prevent this. After the solution was transferred to the cuvette, the UV-vis absorption measurements were carried out.

NO-Binding Affinity. For determination of the NO-binding affinity (partial pressure at half NO-binding to FeP, $P_{1/2}^{\text{NO}} = 1/K^{\text{NO}}$), we used the ligand exchange method of Romberg and Kassner, which requires titration of the six-coordinate carbonyl complex with NO (Romberg et al., 1977).



The partition coefficient between NO and CO, $N (=K^{\text{CO,NO}})$ is defined by the equation

$$N = \frac{[\text{FeP(NO)}]P^{\text{CO}}}{[\text{FeP(CO)}]P^{\text{NO}}} \quad (2)$$

where P^{CO} and P^{NO} correspond to the partial pressure of CO and NO. When the concentration of the nitrosyl complex becomes 50%, N is equal to $P_{1/2}^{\text{CO}}/P_{1/2}^{\text{NO}}$. The equilibrium constants are written as

$$K^{\text{NO}} = K^{\text{CO}}K^{\text{CO,NO}} \quad (3)$$

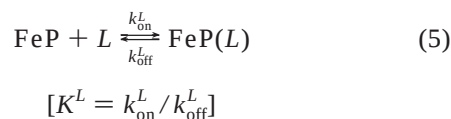
This equation can be expressed in terms of $P_{1/2}$ and N :

$$P_{1/2}^{\text{NO}} = \frac{P_{1/2}^{\text{CO}}}{N} \quad (4)$$

Consequently, the $P_{1/2}^{\text{NO}}$ was calculated from $P_{1/2}^{\text{CO}}$ and the N value determined spectrophotometrically. In the case of rHSA-FeP, however, reproduction of the spectral changes was not constant and had a relatively large error, due to protein denaturation by the long gas bubbling in the solution. Therefore, the $P_{1/2}^{\text{NO}}$ of rHSA-FeP was eventually determined by $k_{\text{on}}^{\text{NO}}/k_{\text{off}}^{\text{NO}}$ (vide infra). A KOFLOC Gas Blender GB-3C was used to prepare the CO/NO gas for the desired partial pressure. The obtained gas was passed through the KOH and water columns before bubbling the solution as already described for elimination of the small amount of higher oxides. The concentrations of NO and CO in toluene and the phosphate buffer were calculated from their partial pressures and the solubilities (NO, 11 mM; CO, 7.41 mM in toluene; and NO, 2 mM; CO, 0.94 mM in water) at 1 atm and 298 K.

ESR Measurements. ESR measurements at the X-band frequency were performed using a JEOL JES-RE2X spectrometer with 100 kHz field modulation at 298 and 77 K. A JEOL ES-SCXA frequency meter calibrated the magnetic field, and the field corrections for the samples were made with MnO_2 . The nitrosyl FeP concentration in toluene or in aqueous rHSA solutions was approximately 1 mM. After confirming the visible absorption spectra, which show the formation of the nitrosyl complex, the samples were carefully transferred to an ESR tube equipped with the double stopcock on the top.

Association and Dissociation Rate Constants. The bindings of gaseous ligands to FeP were expressed by



where L is NO, CO, or O_2 . The association rate constant of NO ($k_{\text{on}}^{\text{NO}}$) was obtained from the rebinding kinetics following NO-photodissociation after the laser flash photolysis [Continuum Surelight I-10 (Nd:YAG laser SHG: 532 nm, 6 ns pulse width)]. The Xenon lamp (150 W) was used as the monitoring light source. The time dependence of the absorption changes accompanied the NO-recombination were detected by a Hamamatsu Photonics R636 photomultiplier, and the relaxation curves were analyzed by the second-order kinetics (Hoshino et al., 1989), affording the association rate constants ($k_{\text{on}}^{\text{NO}}$). The sample cuvette ([FeP] = 20 μ M) was placed in a thermostated holder and the temperature was maintained at 298 K.

On the other hand, studies of the dissociation of NO from rHSA-FeP were accomplished by diluting a nitrosyl rHSA-FeP solution (Moore et al., 1976). The NO-dissociation reaction was initiated by mixing of a CO-saturated aqueous dithionite solution with the nitrosyl rHSA-FeP solution (1/2 v/v). The absorption change at 428 nm was continuously measured for 10^4 s, and its relaxation was analyzed by first-order kinetics. The absorption at infinite time was calculated from the FeP concentration and the extinction coefficient at 428 nm.

RESULTS AND DISCUSSION

Nitrosylation of rHSA-FeP. Upon the addition of NO (5 ppm in N_2) through the toluene solution of the five-coordinate FeP or six-coordinate carbonyl FeP, the UV-vis absorption spectrum immediately changed to the corresponding NO-adduct [$\lambda_{\max} = 415, 479(\text{sh}), 542$, and 610 nm]. The ESR and IR spectroscopies exhibited the formation of the six-coordinate nitrosyl complex in which the intramolecularly bound 2-methylimidazole does not dissociate from the central iron (Komatsu et al., 2000b). We have concluded that covalently attaching the imidazolyl group directly to the porphyrin periphery inhibits the proximal base elimination, which is triggered by the NO-binding to the trans side. The NO gas (1% in N_2) was then exposed to the phosphate buffer solution (pH 7.3) of rHSA-FeP. The UV-vis absorption spectrum showed maxima at 425 and 546 nm (Figure 1). The carbonyl rHSA-FeP, in which the incorporated FePs are six-coordinated carbonyl complex, also moved slowly to the same species after bubbling the NO gas. Further addition of the 100% CO gas to these solutions led to the spectral

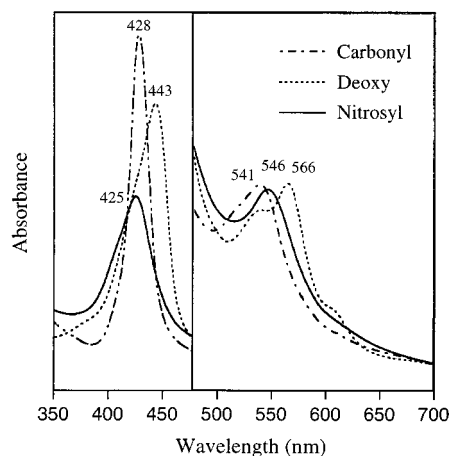


Figure 1. Visible absorption spectral changes of rHSA-FeP in phosphate buffer solution (pH 7.3) at 298 K.

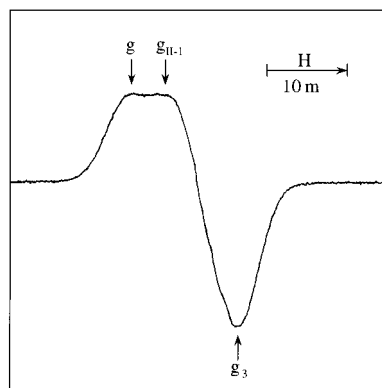


Figure 2. ESR spectrum of nitrosyl rHSA-FeP in frozen phosphate buffer (pH 7.3) at 77 K.

Table 1. ESR Parameters of Nitrosyl rHSA-FeP in Aqueous Solution at 77 K

	solvent	g_1	g_{II-1}	g_2	g_3
FeP	toluene	2.072	2.034	2.002	1.976
Fe(II)TPP(1-MeIm) ^a	toluene	2.072	2.036	2.004	1.968
rHSA-FeP	pb ^b	2.073	2.046		1.987
Hb (T-state) ^c	pb ^d	2.075		2.005	1.975
Mb ^c	pb ^d	2.080	2.030	2.005	1.975

^a Yoshimura et al. (1991). ^b pb: phosphate buffer (30 mM, pH 7.3). ^c Yonetani et al. (1972). ^d pb: phosphate buffer (0.1 M, pH 7.0).

changes in the carbonyl complexes ($\lambda_{\max} = 428, 541$ nm), indicating that (i) the nitrosylation of FeP is reversible and (ii) the central iron was not oxidized during the reaction. ESR spectroscopy was again employed to confirm the coordination structure of this nitrosyl rHSA-FeP. The absorption curve of the frozen solution at 77 K showed the characteristic shape of rhombic symmetry around the paramagnetic center and very similar to those of the nitrosyl Hb and Mb (Figure 2) (Yonetani et al., 1972). The obtained g values (g_1, g_{II-1}, g_3) are also in good agreement with those of the six-coordinate nitrosyl Hb, Mb, as well as FeP (Table 1) (Yonetani et al., 1972; Yoshimura, 1991). It is well-known that an ESR spectrum of the nitrosyl Hb is sensitive to the experimental conditions (pH, presence of IHP, etc.), especially, the spectral pattern directly correlates with the rupture of the iron(II)–imidazole bonding which is often seen during the nitrosylation of the T-state Hb (Scholler et al., 1979). If the imidazolyl moiety is dissociated from the central iron(II) of the FeP, the spectrum of the five-coordinate nitrosyl complexes become a sharp intense triplet associated with g_3 . However, this was not observed. On the basis of these findings, it can be concluded that FeP forms the six-coordinate nitrosyl complex with an intramolecularly bound 2-methylimidazolylalkyl arm in the albumin structure, as in the toluene solution.

NO-Binding Parameters of rHSA-FeP. The NO-binding affinity ($P_{1/2}^{\text{NO}}$) of Fe(II)porphyrin is usually determined by the ligand-exchange procedure, which requires the titration with NO of the carbonyl complex (Romberg et al., 1977). During the measurements of the toluene solution of FeP, the isosbestic points were clearly observed in the visible absorption spectra during the titration (not shown). In the case of the aqueous solution of rHSA-FeP, however, reproductions of the spectral changes were not obtained due to the partial denaturation of the albumin by the long gas bubbling into the solution. Therefore, the $P_{1/2}^{\text{NO}}$ of rHSA-FeP was finally calculated using the ratio of the association and dissociation rate constants of NO: $k_{\text{on}}^{\text{NO}}/k_{\text{off}}^{\text{NO}}$ (1.7×10^{-6} Torr), which was significantly lower than that of FeP itself ($P_{1/2}^{\text{NO}} = 1.8 \times 10^{-8}$ Torr in toluene).

The NO-association rate constants ($k_{\text{on}}^{\text{NO}}$) of rHSA-FeP can be obtained by laser flash photolysis techniques. The time dependence of the absorption decay at 445 nm after the laser flash irradiation showed second-order kinetics, which was independent of the monitoring wavelength and the laser pulse intensity (Figure 3). The $k_{\text{on}}^{\text{NO}}$ was determined to be $1.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. Regarding the CO- and O_2 -rebinding to rHSA-FeP, the kinetics are composed of three phases, in which the long-lived biphasic decay corresponds to the direct recombination of CO and O_2 (Komatsu et al., 2000a). Two of the eight binding sites of FeP in rHSA are estimated to be the *slow sites* for the CO- and O_2 -associations, which is probably due to the steric restriction by the amino acid residues of the protein

interior. In contrast to these results, the kinetics of the NO-rebinding to rHSA-FeP displayed a monophasic decay, which can be fitted to a simple second-order equation (Figure 3 inset). On the other hand, the dissociation rate constant ($k_{\text{off}}^{\text{NO}}$) of NO was determined by the diluting method of a nitrosyl rHSA-FeP solution with sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) (Moore et al., 1976). The addition of excess CO to the dissociation mixture yields a single-exponential process that is substantially independent of the FeP and $\text{Na}_2\text{S}_2\text{O}_4$ concentrations (Figure 4). The obtained NO-binding parameters of rHSA-FeP are summarized in Table 2. Kinetically, the low NO-binding affinity of rHSA-FeP compared to that of FeP arises from the decreased association rate constant. It

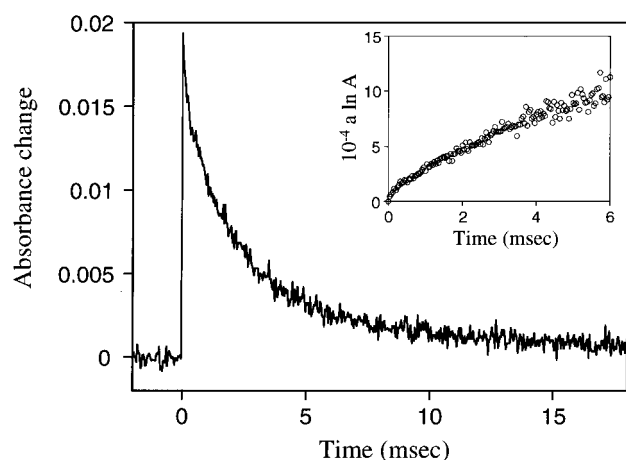


Figure 3. Absorption change accompanied NO-rebinding to rHSA-FeP after the laser flash photolysis (Nd:YAG 532 nm) at 445 nm (phosphate buffer, pH 7.3, 298 K). The inset shows the second-order plots of the absorption decay in which $a = 1/([\text{FeP}]_0 - [\text{NO}]_0)$, $A = ([\text{FeP}][\text{NO}]_0)/([\text{FeP}]_0[\text{NO}])$.

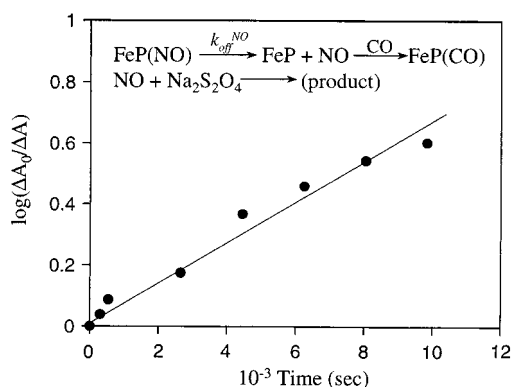


Figure 4. Dissociation of NO from nitrosyl rHSA-FeP under CO atmosphere (phosphate buffer, pH 7.3, 298 K). $[\text{Na}_2\text{S}_2\text{O}_4] = 1\%$, $[\text{CO}] = 253$ Torr.

Table 2. NO-Binding Affinity and Kinetic Parameters of rHSA-FeP in Aqueous Solution at 298 K

	solvent	$10^6 P_{1/2}$ (Torr)	$10^{-8} k_{\text{on}}$ ($\text{M}^{-1} \text{s}^{-1}$)	$10^4 k_{\text{off}}$ (s^{-1})
FeP	toluene	0.018	8.9	2.3 ^a
rHSA-FeP	pb ^b	1.7	0.15	0.67
Fe(II)PP (1-MeIm) ^c	water ^d	0.57	1.8	2.9
Hb (T-state) ^e	ab ^f	15	0.18	40
Mb ^g	pb ^h	2.7	0.17	1.2

^a The dissociation rate of NO was determined by $k_{\text{off}}^{\text{NO}}/K^{\text{NO}}$. ^b pb: phosphate buffer (30 mM, pH 7.3). ^c Fe(II)PP(1-MeIm): Protoporphyrinatoiron(II)(1-methylimidazole) complex, at 22 °C, E. J. Rose et al. (1983). ^d 20% N-Methylimidazole solution (pH 9.0). ^e E. J. Rose et al. (1983). ^f ab: aqueous buffer (pH 8.0). ^g At 20 °C, E. G. Moore et al. (1976). ^h pb: phosphate buffer (50 mM, pH 7.0).

has been widely recognized that the NO-binding to Fe-(II)porphyrin and natural hemoproteins are diffusion controlled (Hoshino et al., 1989; Rose et al., 1983; Traylor et al., 1992). In fact, the $k_{\text{on}}^{\text{NO}}$ value of FeP in toluene ($8.9 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) approaches the value for a diffusion controlled reaction ($\sim 4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) (Amdur et al., 1966), and Hb and Mb have identical $k_{\text{on}}^{\text{NO}}$ values (approximately $1.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$), which are 10-fold lower than that of the naked protoheme-imidazole [Fe(II)-protoporphyrin(1-methylimidazole)] (Rose et al., 1983). Interestingly, a similar decrease is observed in our synthetic hemoproteins, i.e., incorporation of FeP into the albumin matrix restricts the NO access to the central iron which led to 60-fold reduction in its NO-association rate.

In conclusion, synthetic hemoprotein, rHSA-FeP, forms a nitrosyl adduct upon addition of NO at ambient temperature, in which FePs are six-coordinate nitrosyl complexes with a proximal imidazole. The NO-binding affinity of rHSA-FeP at pH 7.3 was significantly lower than that of FeP itself, which is mainly due to the decreased association rate constant. This result demonstrates that incorporation of FePs into the albumin structure retards NO association to the central iron as similarly observed in Hb and Mb. As a red cell substitute, the NO-binding affinity of rHSA-FeP is still 9-fold higher compared to the Hbs. Consequently, there is a concern that an infusion of this cell-free type O_2 -carrier into the blood streams may induce the hypertensive effect. However, the preliminary experiments on the responses to administration of rHSA-FeP solution into anesthetized rats showed negligibly small increases in the systemic blood pressure. For this reason, it is noteworthy that the permeability of serum albumin through the muscle capillary pores is less than 1/100 that of Hb, because of its negative charges on the molecular surface ($\text{pI} = 4.8$) (Guyton, 1986). We now expect that rHSA-FeP can circulate in the blood vessels for a relatively longer time without extravasation, due to the electrostatic repulsion between the albumin and the surface of the glomerular basement membrane. If this is true, the albumin based O_2 -carrier may become a promising material as a new type of red cell substitute. To obtain insight into this hypothesis, further in vivo experimental study is now in progress.

ACKNOWLEDGMENT

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A New Approach to the Synthesis of APTRA Indicators

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We have devised a general synthesis of Mg^{2+} indicators which is based on the aminophenol triacetic acid (APTRA) structure. The key step is a palladium-catalyzed coupling reaction of a precursor of the APTRA ligand with a fluorescent group. This strategy resulted in new ratioable fluorescent APTRA indicators and the finding that the fluorescence response of these indicators is different for Mg^{2+} and Ca^{2+} in some cases. We believe that this represents a generally useful approach for combining fluorophore and chelator functionalities.

INTRODUCTION

Fluorescent probes have proven to be valuable tools for the determination of the concentration of free metal ions in the cell cytosol (1) and have provided important insight into the function of these ions. As an analytical methodology, fluorescence spectroscopy is an attractive technique as it is very sensitive and only minute intracellular amounts of the indicator are necessary to obtain good signal-to-noise ratios. Intracellular loading of a fluorescent probe leaves the cells intact (in contrast to atomic absorption spectroscopy), thereby allowing one to study the functioning cell. Fluorescent probes can be used to study whole cell populations, single cells, or even subcellular domains. Time dependent changes in intracellular ion distribution can be studied with high spatial and temporal resolution. Fluorescent indicators can also be used in conjunction with flow cytometry to sort cells on the basis of their free metal content or other properties.

Magnesium, the most abundant divalent metal ion in tissues, participates in a broad range of biochemical and regulatory processes. Magnesium ions are involved in many important functions in the cell such as enzyme activity, the conformation of DNA, ion transport through the cell membrane, the maintenance of cell shape, and in signaling pathways (2). Magnesium ions are essential cofactors for most of the ATP-dependent phosphorylation reactions which take place in the cell. Furthermore, the distribution of cytosolic magnesium and subcellular magnesium appears to be important in cell cycle control and regulation of cell differentiation (3).

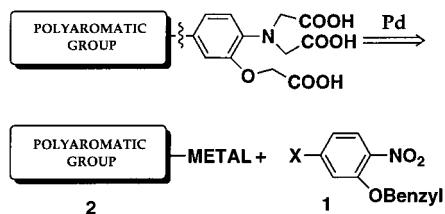
Mag-fura-2 (1) [also known as FURAPTRA (4)], Mag-fura-5 (1), and Mag-indo (1) are three, widely used, commercially available, fluorescent probes for magnesium. A literature search revealed that in the period 1997–1998, over 40 papers have described the use of these indicators. In some recent literature examples, fluorescent indicators have been used to study the mechanism of Mg^{2+} transport across the cell membrane (5–8); to determine the influence of hormones on Mg^{2+} regulation in the cell (9–12); and in the investigation of this ion's role in the fructose-induced activation of

glycogen phosphorylase (13). The use of Mag-fura 2 has revealed the high levels of cytosolic free Mg^{2+} in platelets of patients with essential hypertension (14) and vasospastic angina (15).

An important and desirable feature of Mag-furas and Mag-indo is that they are ratioable indicators. Upon binding Mg^{2+} , the excitation maximum (of all three indicators) and the emission maximum (Mag-indo) of the indicator-cation complex display a significant wavelength shift relative to the free indicator (1, 4, 16–18). This allows the ion concentration to be measured as a function of the ratio of the fluorescence at two different wavelengths, preferably the fluorescence maxima of both species (16). By using a ratioable fluorophore, the measurements of Mg^{2+} are not influenced by variations in the efficiency of the instrument and the dye concentration in the cell (18). Other commercially available fluorescent probes (1), Magnesium Green, Magnesium Orange, and Mag-Fura Red, are not ratioable but have excitation wavelengths in the visible region thereby eliminating the interference from the fluorescence of intracellular chromophores (18).

The chelating moiety of the magnesium probes is based on the aminophenol triacetic acid (APTRA) structure, which was developed for this purpose by Levy et al. (19). At physiological pH, the deprotonated carboxylate groups, the anilino nitrogen atom, and the ether oxygen are all available for complexation (19). The dissociation constants (K_D^{Mg}), determined in vitro, of APTRA indicators fall in the low millimolar range [1.0–3.9 mM at physiological pH (1)]. These values correspond well with the low and submillimolar levels of cytosolic free Mg^{2+} (2), and thus, one can make use of the optimum sensitivity of the fluorophores. Care must be taken in using in vitro determined K_D^{Mg} values as recent publications on the in vivo calibration of APTRA-type indicators, reported higher K_D^{Mg} values [5.2 mM for Mag-indo (20) and 5.4 mM for Mag-fura-2 (21)]. It should also be noted that APTRA chelators have a higher affinity for Ca^{2+} than for Mg^{2+} ; the K_D^{Ca} values were found to be around 30 μM . However, these values are 2 orders of magnitude higher than basal, free Ca^{2+} levels (17), and calcium, found at levels of about 100 nM, does not interfere with magnesium measurements under normal conditions. However, APTRA indicators also have been used as low affinity

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Scheme 1. Retrosynthetic Approach to an APTRA Indicator

Ca^{2+} indicators (22–24). In addition, APTRA-type fluorophores can be applied to measure Zn^{2+} and Cd^{2+} (1). Recently, Prodi et al. has reported a nonratioable fluorescent 1,10-diaza-18-crown-6 with a higher affinity for Mg^{2+} over Ca^{2+} (25).

For the chelator to have fluorescent properties, the APTRA unit is connected to a reporter group, preferably a polyaromatic group, or the APTRA chelator may be incorporated as part of a polyaromatic structure. The development of new fluorescent magnesium indicators has been limited because of the lack of an efficient and general synthesis of these compounds. The present approach of a unique synthesis for each candidate is unattractive because the fluorescent response of an indicator upon metal complexation is unpredictable. From a retrosynthetic perspective, a general approach to the synthesis of new APTRA fluorophores would employ in one step, the joining of an APTRA chelator, or some precursor thereof, with a fluorescent group. The method of choice for the formation of such a carbon–carbon bond between two aryl groups is a palladium catalyzed coupling reaction (26). A coupling reaction, catalyzed by an appropriate palladium catalyst, between triflate **1** ($\text{X} = \text{OTf}$) and an organometallic reaction partner **2**, carrying the fluorescent group, would give the basic structure of a new fluorophore (Scheme 1). Further elaboration of the obtained protected nitrophenol through established chemistry would afford the desired APTRA indicator.

Using the strategy described above, we have synthesized three new fluorescent APTRA chelators carrying a naphth-1-yl, a naphth-2-yl, and a benzo[*b*]fur-2-yl substituent. The fluorescent response of these three fluorophores was found to depend strongly on the nature of the polyaromatic group and on whether the chelated ion is Mg^{2+} or Ca^{2+} .

EXPERIMENTAL PROCEDURES

General Remarks. Tetrahydrofuran (THF) was dried and distilled (Na/benzophenone) prior to use. Dimethylformamide (DMF) was dried over freshly activated (300 °C) molecular sieves (4 Å) and acetonitrile was distilled from P_2O_5 . NaI was dried at 100 °C in vacuo. Ethyl acetate, hexane, diethyl ether, and all other commercially available chemicals were used as received. After extractive workup, the combined organic phases were dried over MgSO_4 . Water used in the UV and fluorescence experiments was filtered through a Hydro Picopure2 filter. Thin-layer chromatography was carried out on Sigma-Aldrich TLC Plates. Chromatographic purifications were done by flash chromatography using Merck Silica Gel, grade 9385, 230–400 mesh, 60 Å. Melting points were measured on a Fisher-Johns melting point apparatus and are uncorrected. ^1H NMR spectra and ^{13}C NMR spectra were recorded on a Nicolet NT 360 MHz NMR Spectrometer and a Varian UNITY plus 400 MHz NMR Spectrometer. Chemical shifts are reported in parts per million (δ), using TMS as an internal standard, and signals are

expressed as an s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). UV spectra were recorded with a Beckman DU 640 Spectrophotometer using Fisherbrand semimicro quartz cells (Fisher Scientific). Fluorescence measurements were recorded with an Aminco-Bowman Series 2 Spectrometer using quartz fluorimeter cuvettes from Sigma.

2-Benzyloxy-4-fluoro-nitrobenzene (4). To a suspension of K_2CO_3 (5.18 g, 38 mmol) in 15 mL of DMF was added 2-nitro-5-fluorophenol (**3**) (3.93 g, 28 mmol) in one portion at room temperature. After gas evolution ceased, the temperature of the bright orange reaction mixture was raised to 70 °C and a solution of benzyl bromide (3.25 mL, 28 mmol) in 10 mL of DMF was added rapidly. After 4 h, TLC (10% EtOAc in hexane) indicated complete conversion of the starting material. The reaction mixture was poured into water and extracted three times with Et_2O . The combined organic layers were washed twice with water. Evaporation of the solvent in vacuo gave a viscous yellow oil (5.96 g, 95%) which slowly solidified at room temperature, mp 58–60 °C. ^1H NMR (CDCl_3) δ 5.22 (s, 2H), 6.72 (ddd, 1H, $J = 2.6$ Hz, $J = 7.0$ Hz, $J = 9.1$ Hz), 6.82 (dd, 1H, $J = 2.6$ Hz, $J = 10.2$ Hz), 7.32–7.48 (m, 5H), 7.95 (dd, 1H, $J = 5.9$ Hz, $J = 10.2$ Hz). ^{13}C NMR (CDCl_3) δ 72.52, 103.88 (d, $J = 26$ Hz), 108.70 (d, $J = 24$ Hz), 128.02, 129.10, 129.22, 129.52, 129.88, 135.87.

3-Benzyloxy-4-nitrophenol (5). To a solution of **4** (4.36 g, 17 mmol) in 50 mL of DMSO was added 11 mL of a 20% NaOH solution which resulted in a mildly exothermic reaction. The orange reaction mixture was stirred overnight and TLC (10% EtOAc in hexane) indicated complete consumption of the starting material. The reaction mixture was poured into 250 mL of water containing 5 mL of concentrated HCl and extracted three times with Et_2O . The combined organic layers were washed two times with water. Evaporation of the solvent gave **5** as a yellow solid (4.04 g, 95%), mp 134–6 °C. ^1H NMR (CDCl_3) δ 5.21 (s, 2H), 6.44 (dd, 1H, $J = 2.6$ Hz, $J = 9.0$ Hz), 6.55 (d, 1H, $J = 2.2$ Hz), 7.22–7.40 (m, 5H), 7.95 (d, 1H, $J = 9.2$ Hz). ^{13}C NMR (CDCl_3) δ 72.00, 102.34, 107.66, 126.94, 128.23, 128.42, 128.71, 135.49, 154.84, 160.97. One quaternary carbon not observed.

2-Benzyloxy-4-(trifluoromethyl)sulfonyl-nitrobenzene (1). A solution of **5** (3.92 g, 16 mmol) in a mixture of 25 mL CH_2Cl_2 , distilled from CaH_2 , and 10 mL of pyridine was cooled with an ice-bath. Over a period of 1 h, a solution of trifluoromethanesulfonic anhydride (5 g, 18 mmol) in 15 mL of CH_2Cl_2 was added. The reaction mixture was allowed to warm to room temperature and stirring was continued overnight. Subsequently, 25 mL of water and 100 mL of CH_2Cl_2 was added. The organic layer was separated and washed twice with 10% HCl. The organic layer was then washed twice with water. Evaporation of the solvent resulted in a yellow oil which was further purified by flash chromatography (30% EtOAc in hexane) to give a pale yellow oil which slowly solidified upon standing at room temperature (4.87 g, 80%), mp 56–8 °C. ^1H NMR (CDCl_3) δ 5.25 (s, 2H), 6.96 (dd, 1H, $J = 2.6$ Hz, $J = 8.9$ Hz), 7.03 (d, 1H, $J = 2.4$ Hz), 7.30–7.50 (m, 5H), 7.96 (d, 1H, $J = 9.1$ Hz). ^{13}C NMR (CDCl_3) δ 72.18, 109.00, 113.26, 118.66 (q, $J = 320$ Hz), 127.14, 127.19, 128.66, 128.84, 134.30, 139.64, 151.97, 153.21.

2-[3-Benzyloxy-4-nitrophenyl]benzo[*b*]furane (7a). A 50 mL three-necked, round-bottomed flask, well-purged with argon, was charged with **1** (1.31 g, 3.70 mmol), benzo[*b*]furan-2-boronic acid (**6a**) (470 mg, 3.0 mmol), $\text{Pd}[\text{P}(\text{C}_6\text{H}_5)_3]_4$ (20 mg, 0.017 mmol), 1 M Na_2CO_3 (5 mL) and

10 mL of DME. The reaction mixture was heated for 4.5 h at 80 °C, cooled, poured into water and extracted three times with CH_2Cl_2 . Evaporation of the solvent gave an orange solid which was crystallized twice from benzene to give **7a** as bright yellow crystals (782 mg, 75%), mp 137–9 °C. ^1H NMR (CDCl_3) δ 5.37 (s, 2H), 7.20–7.70 (m, 12H), 7.99 (d, 1H, J = 8.5 Hz), ^{13}C NMR (CDCl_3) δ 71.77, 104.72, 111.42, 117.03, 121.53, 123.51, 125.65, 126.39, 126.46, 127.18, 128.31, 128.72, 135.52, 135.99, 152.73, 153.32, 155.39. One quaternary carbon not observed. MS: (m/z) 345.0994, required for $\text{C}_{21}\text{H}_{15}\text{NO}_4$ 345.1001.

1-[3-Benzyloxy-4-nitrophenyl]naphthalene (7b).

In a manner similar to the preparation of **7a**, compound **7b** was prepared and isolated pure (91%) as a white powder by flash chromatography (10% EtOAc in hexane), mp 115–8 °C. ^1H NMR (CDCl_3) δ 5.28 (s, 2H), 7.18 (dd, 1H, J = 1.7 Hz, J = 8.1 Hz), 7.30–7.50 (m, 6H), 7.50–7.60 (m, 2H), 7.69 (d, 1H, J = 8.8 Hz), 7.93 (d, 1H, J = 8.1 Hz), 7.94 (d, 1H, J = 8.1 Hz), 8.02 (d, 1H, J = 8.1 Hz). ^{13}C NMR (CDCl_3) δ 71.42, 117.23, 122.33, 125.13, 125.19, 125.62, 126.11, 126.67, 126.76, 127.05, 128.17, 128.46, 128.67, 128.81, 130.97, 133.83, 135.53, 137.95, 147.12, 151.66. MS: (m/z) 355.1233; required for $\text{C}_{23}\text{H}_{17}\text{NO}_3$, 355.1208.

2-[3-Benzyloxy-4-nitrophenyl]naphthalene (7c).

In a manner similar to the preparation of **7a**, compound **7c** was prepared and isolated pure (92%) as an off-white powder by flash chromatography (10% EtOAc in hexane), mp 114–6 °C. ^1H NMR (CDCl_3) δ 5.36 (s, 2H), 7.30–7.46 (m, 5H), 7.50–7.58 (m, 4H), 7.64 (dd, J = 9 Hz, J = 1.5 Hz), 7.84–8.08 (m, 5H). ^{13}C NMR (CDCl_3) δ 71.71, 114.54, 119.69, 124.83, 126.27, 126.59, 126.76, 126.79, 127.15, 127.70, 128.26, 128.34, 128.71, 128.87, 133.28, 133.48, 135.67, 136.38, 139.26, 147.39, 152.44. MS: (m/z) 355.1196; required for $\text{C}_{23}\text{H}_{17}\text{NO}_3$, 355.1208.

2-(3-Hydroxy-4-aminophenyl)benzo[b]furan-*N,N*,*O*-triacetic acid trimethylester (9a). Compound **7a** (345 mg, 1.00 mmol) and 100 mg of 5% Pd on C were suspended in 25 mL of ethanol. This reaction mixture was hydrogenolyzed over 16 h in a Parr apparatus at an initial H_2 pressure of 30 psi. The reaction mixture was filtered and the solvent evaporated in vacuo. TLC (50% EtOAc in hexane) indicated complete consumption of the starting material and ^1H NMR of the crude product confirmed complete removal of the benzyl group. The crude aminophenol **8a** was dissolved in 10 mL of acetonitrile and transferred to a 100 mL oven-dried, three-necked, round-bottomed flask, equipped with a stirring bar and oven-dried condenser and charged with NaI (1.0 g, 6.7 mmol), Proton Sponge (1.7 g, 7.9 mmol), and methyl bromoacetate (1.0 mL, 10 mmol). The reaction mixture was refluxed for 16 h under an argon atmosphere, allowed to cool to room temperature, and poured into 200 mL of Et_2O . The resulting white suspension was filtered and the filtrate was washed with 50 mL portions of buffer (pH 2, Hydriion), until the aqueous layer remained acidic. The ether layer was dried, filtered and 5 g of silica gel was added. After evaporation of the solvent, the silica gel was loaded on a pre-packed column (33% EtOAc in hexane). Triester **9a** was obtained as a colorless oil (210 mg, 47% overall). ^1H NMR (CDCl_3) δ 3.75 (s, 6H), 3.82 (s, 3H), 4.26 (s, 4H), 4.76 (s, 2H), 6.87 (s, 1H), 6.92 (d, 1H, J = 8.5 Hz), 7.20–7.28 (m, 2H), 7.30 (d, 1H, J = 1.5 Hz), 7.42 (dd, 1H, J = 1.8 Hz, J = 8.2 Hz), 7.49 (d, 1H, J = 8.2 Hz), 7.54 (d, 1H, J = 8.7 Hz). ^{13}C NMR (CDCl_3) δ 51.70, 52.07, 53.82, 66.56, 100.29, 110.96, 111.88, 119.80, 119.85, 120.59, 122.84, 123.87, 124.84, 129.40, 140.07, 149.67, 154.75, 155.60, 168.98, 171.35. MS: (m/z) 441.1400; required for $\text{C}_{23}\text{H}_{23}\text{NO}_3$, 441.1424.

1-(3-Hydroxy-4-aminophenyl)naphthalene-*N,N*,*O*-triacetic Acid Trimethylester (9b). In a manner similar to the preparation of **9a**, compound **9b** was obtained as a colorless oil (64% overall yield) by flash chromatography (25% EtOAc in hexane). ^1H NMR (CDCl_3) δ 3.76 (s, 3H), 3.77 (s, 6H), 4.30 (s, 4H), 4.68 (s, 2H), 6.93 (d, 1H, J = 2.0 Hz), 6.98 (d, 1H, J = 8.4 Hz), 7.07 (dd, 1H, J = 2.0 Hz, J = 8.4 Hz), 7.37 (dd, 1H, J = 0.8 Hz, J = 7.0 Hz), 7.41 (ddd, 1H, J = 1.6 Hz, J = 6.8 Hz, J = 8.4 Hz), 7.45–7.50 (m, 2H), 7.82 (d, 1H, J = 8.0 Hz), 7.88 (d, 1H, J = 8.8 Hz), 7.91 (d, 1H, J = 8.8 Hz). ^{13}C NMR (CDCl_3) δ 52.89, 53.16, 54.69, 67.13, 117.47, 120.36, 125.27, 126.40, 126.81, 127.02, 127.07, 127.83, 128.54, 129.30, 132.67, 134.86, 135.87, 139.72, 140.61, 150.12, 170.22, 172.89. MS: (m/z) 451.1621; required for $\text{C}_{25}\text{H}_{23}\text{NO}_7$, 451.1631.

2-(3-Hydroxy-4-aminophenyl)naphthalene-*N,N*,*O*-triacetic Acid Trimethylester (9c). In a manner similar to the preparation of **9a**, compound **9c** was obtained as a colorless oil (51% overall yield) by flash chromatography (25% EtOAc in hexane). ^1H NMR (CDCl_3) δ 3.75 (s, 6H), 3.81 (s, 3H), 4.27 (s, 4H), 4.77 (s, 2H), 7.00 (d, 1H, J = 8.0 Hz), 7.18 (d, 1H, J = 2.0 Hz), 7.31 (dd, 1H, J = 2.0 Hz, J = 8.0 Hz), 7.43–7.51 (m, 2H), 7.65 (dd, 1H, J = 2.0 Hz, J = 8.4 Hz), 7.85 (dd, 1H, J = 9.6 Hz, J = 9.6 Hz), 7.87 (d, 1H, J = 8.8 Hz), 7.93 (s, 1H). ^{13}C NMR (CDCl_3) δ 52.90, 53.24, 54.68, 67.50, 115.34, 121.08, 122.79, 126.19, 126.34, 126.85, 127.36, 128.68, 129.13, 129.45, 133.50, 134.71, 136.55, 138.85, 140.06, 150.89, 170.50, 172.73. MS: (m/z) 451.1618, required for $\text{C}_{25}\text{H}_{23}\text{NO}_7$, 451.1631.

2-(3-Hydroxy-4-aminophenyl)benzo[b]furan-*N,N*,*O*-triacetic Acid (10a). In a 25 mL round-bottomed flask, equipped with a condenser, triester **9a** (60 mg, 0.17 mmol) was suspended in a mixture of 2 mL of methanol and 2 mL of 1 M NaOH. The reaction mixture was refluxed for 4 h and allowed to cool to room temperature. The solvent was evaporated in vacuo and the remaining solid material was dissolved in 2 mL of water to give a clear yellow solution. The aqueous solution was cooled with a ice-bath and acidified with 1 M HCl to pH 3. Stirring was continued for 15 min. The precipitate was collected by filtration and dried in vacuo over P_2O_5 to give 50 mg (89%) of an off-white powder which decomposes above 240 °C. ^1H NMR ($\text{DMSO}-d_6$) δ 3.53 (s, 4 H), 4.17 (s, 3 H), 6.73 (d, 1H, J = 8.4 Hz), 6.76 (s, 1H), 6.90 (d, 1H, J = 1.7 Hz), 7.02–7.10 (m, 3H), 7.28 (d, 1H, J = 8.0 Hz), 7.37 (d, 1H, J = 7.0 Hz). ^{13}C NMR [D_2O (1.0 mL and 50 μL of 40% NaOD)] δ 57.78, 68.67, 100.85, 110.44, 111.94, 118.81, 119.31, 121.86, 123.36, 124.15, 125.03, 130.38, 150.39, 155.27, 156.91, 178.12, 180.42.

1-(3-Hydroxy-4-aminophenyl)naphthalene-*N,N*,*O*-triacetic acid (10b). In a manner similar to the preparation of **10a**, triacid **10b** was obtained as an off-white powder (57%), which decomposes above 230 °C. ^1H NMR [D_2O (1.0 mL, and 50 μL of 40% NaOD)] δ 3.68 (s, 4H), 4.33 (s, 2H), 6.83 (d, 1H, J = 8.3 Hz), 6.95 (s, 1H), 7.08 (d, 1H, J = 7.7 Hz), 7.30–7.48 (m, 2H), 7.55 (d, 1H, J = 8.0 Hz), 7.60–7.80 (m, 3H), 7.83 (s, 1H). ^{13}C NMR [D_2O (1.0 mL and 50 μL of 40% NaOD)] δ 58.13, 68.54, 112.50, 119.65, 121.01, 125.42, 126.00, 127.09, 127.62, 128.59, 129.05, 129.47, 133.07, 134.74, 138.34, 140.55, 150.93, 171.97, 180.33.

2-(3-Hydroxy-4-aminophenyl)naphthalene-*N,N*,*O*-triacetic acid (10c). In a manner similar to the preparation of **10a**, triacid **10c** was obtained as a slightly pink powder (49%), which decomposes above 250 °C. ^1H NMR [D_2O (1.0 mL, and 50 μL of 40% NaOD)] δ 3.86 (s, 4H), 4.44 (s, 2H), 6.87 (s, 1H), 6.92 (d, 1H, J = 8.42 Hz), 6.99

Table 1. Spectroscopic Data and K_D Values of Indicators 10a–c

compd	abs. max (nm)	Mg^{2+}			Ca^{2+}		
		λ_{emiss} (nm) ^b	λ_{excit} (nm) ^{b,c}	K_D (mM)	λ_{emiss} (nm)	λ_{excit} (nm) ^{b,c}	K_D (μ M)
10a	330	416 → 407	340 → 328 (410)	2.3	414 → 411	340 → 328	70
10b	310	499, 358	325 → 295 (499)	1.8	500, 362	325 → 499	17
10c	316	497	328 → 305 (498)	2.1	498	328 → 498	28

^a Absorption maximum of the free indicator. ^b An arrow denotes a shift of the fluorescence maximum upon ion complexation. ^c The emission wavelength used is given in brackets.

(d, 1H, $J = 7.7$ Hz), 7.40–7.58 (m, 4H), 7.87 (d, $J = 8.05$, 1H), 7.90–8.02 (m, 2H). ^{13}C NMR [D_2O (1.0 mL and 50 μ L of 40% NaOD)] δ 57.91, 68.51, 115.75, 118.88, 123.81, 126.77, 126.84, 127.22, 127.49, 127.92, 128.44, 129.37, 132.04, 134.06, 134.62, 140.22, 140.53, 150.33, 178.05, 180.61.

Fluorescence Measurements. The buffered solutions used in all experiments contained 120 mM KCl, 20 mM NaCl, and 15 mM Tris and were brought to pH 7.20 with concentrated HCl. Using sonication, a stock solution of indicator **10b** was prepared by dissolving 3.2 mg (7.8 μ mol, 1.6 mM) in 5 mL of buffer. To record a UV spectrum, the stock solution of **10b** was diluted 40 times (25 μ L in 975 μ L of buffer). For the fluorescence titration experiments, 25 μ L of stock solution was diluted 100 times (to 16 μ M) with 2475 μ L of buffer. A fluorescence emission and a fluorescence excitation spectrum were recorded (for excitation and emission wavelengths, see Table 1). To 2475 μ L of 500 mM $MgCl_2$ solution was added 25 μ L of stock solution of the indicator. To the solution of the free indicator, increasing amounts of the $MgCl_2$ /indicator solution (5, 10, 20, 40, 80, and 160 μ L) were added and after each titration step, a fluorescence emission and a fluorescence excitation spectrum were recorded. In the case of a fluorescence titration with $CaCl_2$, a 1 mM solution was used and portions of 5, 10, 20, 40, 80, 160, 320, and 320 μ L of the $CaCl_2$ /indicator solution were added. Using this procedure, fluorescence titrations of indicators **10a**, and **10c** were performed with stock solutions of these indicators of 0.81 and 0.54 mM, respectively.

The corresponding dissociation constants (K_D^{Mg} and K_D^{Ca}) were determined from the fluorescence/titration experiments as follows. In the case of Mg^{2+} , the free Mg^{2+} concentration, $[Mg^{2+}]$, was approximated by the total Mg^{2+} added, since the correction for bound Mg^{2+} is minimal. The data were fit to a straight line according to

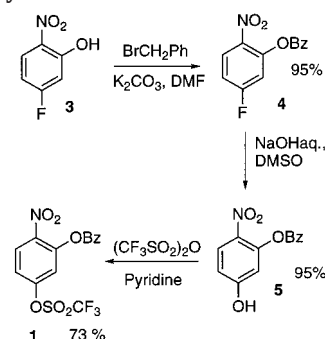
$$[Mg^{2+}] = K_D^{Mg} \left[\frac{(F - F_0)}{(F_{sat} - F)} \right] \quad (1)$$

where F is the fluorescence at a given wavelength (preferably the fluorescence maximum) and at a given $[Mg^{2+}]$, and F_0 and F_{sat} are the fluorescence values for zero and saturating Mg^{2+} . For a ratioable indicator, with fluorescence maxima at λ_1 and λ_2 for the free and metal-bound indicator, respectively, the data were fit to a straight line applying the following equation:

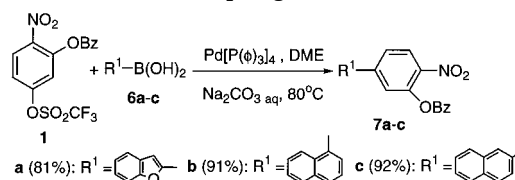
$$[Mg^{2+}] = K_D^{Mg} \left[\frac{(R - R_0)}{(R_{sat} - R)} \right] \left(\frac{F_{0,\lambda_2}}{F_{sat,\lambda_2}} \right) \quad (2)$$

where R is the ratio of the fluorescence values at λ_1 and λ_2 at a given $[Mg^{2+}]$, R_0 and R_{sat} are the ratios at zero and saturating Mg^{2+} , and F_{0,λ_2} and F_{sat,λ_2} represent the fluorescence values of the free and saturated indicator at λ_2 .

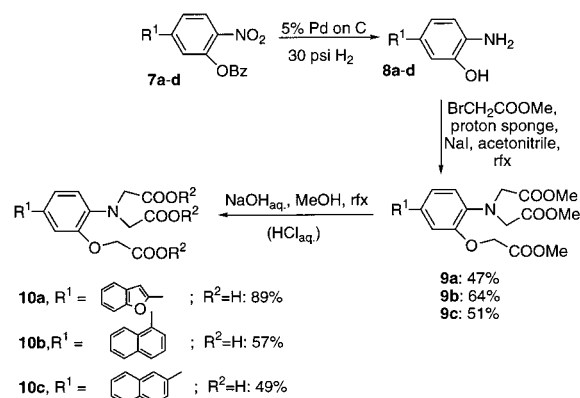
Scheme 2. Synthesis of Triflate 1



Scheme 3. Suzuki Coupling of 1



Scheme 4. Completion of the APTRA Synthesis



Since for Ca^{2+} binding, the Ca^{2+} dissociation constant, K_D^{Ca} , is of the same order of magnitude as the indicator concentration, the data were analyzed using a two parameter fit using Mathematica to fit a nonlinear function according to

$$F_{obs} = F_F(1 - p_B) + F_B p_B \quad (3)$$

where F_{obs} is the observed intensity of the fluorescence at the measured wavelength, F_F and F_B are the indicator fluorescence values of the uncomplexed and Ca^{2+} -complexed forms, and p_B the fraction of indicator bound to Ca^{2+} . The latter were calculated from the equilibrium equation assuming 1:1 binding stoichiometry:

$$p_B = \frac{Ca - I}{I_0} \quad (4)$$

leading to

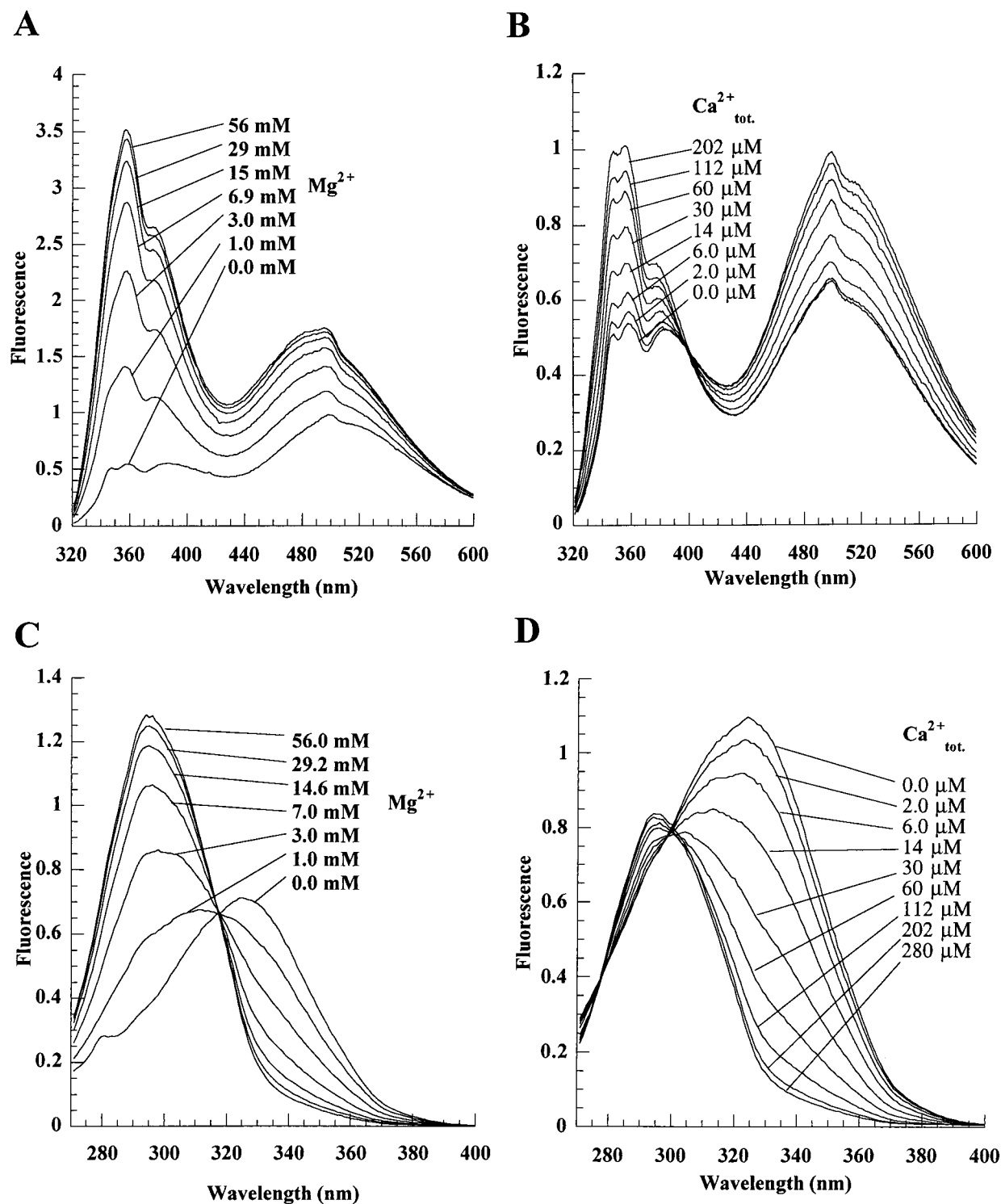


Figure 1. Fluorescent responses of compound 10b to Mg^{2+} and Ca^{2+} . (A) Emission spectra as a function of $[\text{Mg}^{2+}]$ (in mM), excitation at 310 nm; (B) emission spectra as a function of $[\text{Ca}^{2+}]$ (in μM), excitation at 310 nm; (C) Excitation spectra as a function of $[\text{Mg}^{2+}]$ (in mM), emission measured at 499 nm; (D) Excitation spectra as a function of $[\text{Ca}^{2+}]$ (in μM), emission measured at 499 nm. Spectra obtained at 20 °C.

$$p_B = (I_0 + K_D^{\text{Ca}} + [\text{Ca}^{2+}] - \sqrt{(I_0 + K_D^{\text{Ca}} + [\text{Ca}^{2+}])^2 - 4I_0[\text{Ca}^{2+}]})/2 \quad (5)$$

where I_0 is the total indicator concentration, the two parameters to be fit are the fluorescence of the Ca^{2+} -indicator complex, F_B , and the Ca^{2+} -indicator dissociation constant, K_D^{Ca} . The value of F_F is obtained directly from the spectrum of the sample in the absence of added calcium.

SYNTHESIS

Triflate **1** was obtained in three steps (Scheme 2) starting from commercially available 2-nitro-5-fluorophenol **3**. The starting phenol **3** was protected as its benzyl ether **4** by a reaction with benzyl bromide in DMF/ K_2CO_3 . The crude benzyl ether proved to be sufficiently pure for further manipulation. The key step in the synthesis of **1** stems from the realization that the electron-poor aromatic ring of compound **4** readily un-

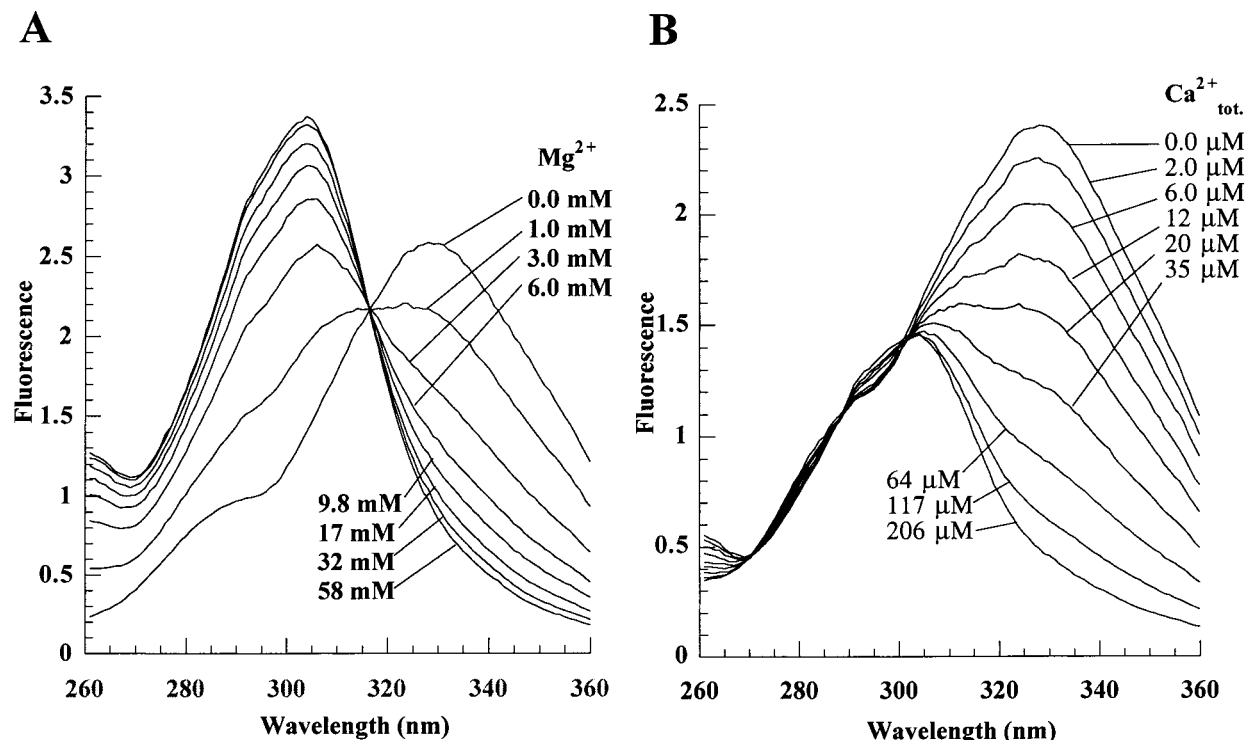


Figure 2. Fluorescent excitation spectra for compound 10c. (A) as a function of $[Mg^{2+}]$ (in mM), emission measured at 498 nm; (B) as a function of $[Ca^{2+}]$ (in μM), emission measured at 498 nm. Spectra obtained at 20 °C.

dergoes a nucleophilic aromatic substitution at the carbon bound to fluorine. Thus, when **4** was subjected to NaOH in DMSO/H₂O at room temperature, the fluorine substituent was replaced by a hydroxyl group to give phenol **5** in quantitative yield. Phenol **5** is then converted to the triflate **1** in good yield. Protection of the phenol **3** as its benzyl ether serves two purposes. First, it prevents deprotonation of the phenol which would prevent the substitution reaction from taking place. Second, it allows a regioselective preparation of triflate **1** via the newly created hydroxyl group *para* to the nitro substituent of **4**.

Standard conditions for the Suzuki-coupling reaction (Scheme 3) of boronic acids **6a–c** with triflate **1** were employed (27). It was found that using the weaker base NaHCO₃ instead of Na₂CO₃ did not result in conversion of **1**. Consumption of **1** was complete after several hours at 80 °C in all cases studied. The coupling products were obtained pure and in excellent yields after extractive workup, followed by flash chromatography. The highly crystalline nature of benzo[*b*]fur-2-yl derivative **7a** allowed its purification by crystallization from benzene.

Reduction of the nitro group and deprotection of the adjacent benzyl ether of compounds **7a–c**, was accomplished in one step by palladium-catalyzed hydrogenation and debenzylation in ethanol. The ¹H NMR spectrum of the crude products showed that complete reduction/deprotection was achieved after 16 h. The use of ethyl acetate, although a better solvent, led to incomplete removal of the benzyl ether. The crude aminophenol derivatives **8a–d** were found to be sufficiently pure for further manipulation. Alkylation of aminophenols **8a–d** with methyl bromoacetate was achieved in refluxing acetonitrile in the presence of Proton Sponge and sodium iodide. After extractive workup and flash chromatography the corresponding triesters **9a–d** were obtained in good overall yields (Scheme 4). It was found that the use of carefully dried acetonitrile and the use of NaI (to form, in situ, the more reactive methyl iodoacetate) were necessary for success of this reaction.

The triesters were readily saponified in refluxing aqueous MeOH/NaOH. After acidic workup, tri-acids **10a–c**, R²=H were isolated in a pure state by filtration.

FLUORESCENCE

The emission and excitation fluorescence spectra of **10a–c** were recorded using 5.4–16 μM solutions of the chelator, buffered at pH 7.2. The solutions also contained 120 mM KCl and 20 mM NaCl to mimic the ionic strength of the intracellular milieu. These solutions were titrated with stock solutions of MgCl₂ or CaCl₂ containing an equimolar concentration of the APTRA chelator to cancel out dilution effects during the titration. After each titration step, both the emission and excitation fluorescence spectra were recorded.

The dissociation constants of indicators **10a–c** for Mg²⁺ were determined as originally described by Grynkiewicz et al. (18). It should be noted that the micromolar range concentrations of the APTRA indicators used here are of the same order of magnitude as the expected *K_D*s for Ca²⁺. We found that when using APTRA concentrations in the nanomolar range for the fluorescence titrations with Ca²⁺, the likely presence of trace metal impurities in the materials used caused nonreproducible fluorescent responses. We therefore chose to apply higher indicator concentrations to minimize the influence of trace metal impurities on the total fluorescence. That the problem was with the adventitious trace metals was confirmed by the fact that addition of EGTA—to capture trace metal impurities—to micromolar solutions of the free indicator did not change the fluorescence intensities. The *K_D* values for Ca²⁺ were determined by applying a nonlinear least-squares module of Mathematica (see Experimental Procedures). Table 1 shows the results of the spectral analysis of indicators **10a–c**. Because of the buffering effect of the indicator on the Ca²⁺ concentration, it is not possible to compare changes in the fluorescence intensities to those observed with Mg²⁺.

RESULTS

Indicator **10a** proved to be a nonratioable fluorescent indicator for Mg^{2+} and Ca^{2+} . The change in fluorescent response to increasing Mg^{2+} concentrations of APTRA chelator **10a**, carrying a benzo[*b*]fur-2-yl group, is small. Upon saturation of the chelator with Mg^{2+} , the fluorescence emission intensity increases 25%. In addition, a small shift of the emission maximum from 407 to 416 nm is observed. The change in fluorescence response to increasing Ca^{2+} concentrations is similar. When saturated with Mg^{2+} , the fluorescence excitation maximum of **10a** at 340 nm displays an overall decrease of its intensity. In addition, a shift of the excitation fluorescence maximum to 328 nm from 340 nm is observed. An identical shift of the excitation fluorescence maximum of **10a** upon an increase of the Ca^{2+} concentration was found.

Figures 1 and 2 show the titration/fluorescence spectra of compounds **10b** and **10c** with Mg^{2+} and Ca^{2+} . The emission fluorescence spectrum of naphth-1-yl-substituted APTRA ligand **10b** shows two maxima at 358 and 499 nm (Figure 1A). Saturation of the chelator with Mg^{2+} results in an increase of the fluorescence intensities at both wavelengths; a 7-fold increase at 358 nm and a 1.8-fold increase at 499 nm are observed. No shifts of either emission maxima were found. Titration of **10b** with Ca^{2+} resulted in a different response in its fluorescence spectrum (Figure 1B). The fluorescence intensity at 362 nm increased, as was observed with Mg^{2+} , but the fluorescence intensity at 500 nm was found to decrease upon increasing Ca^{2+} concentration. An isosbestic point was found at 400 nm. Thus, APTRA chelator **10b** is a ratioable indicator for Ca^{2+} . Titration of **10b** with Mg^{2+} and measurement of its fluorescence excitation spectra shows that this APTRA derivative can be used as a ratioable indicator (Figure 1C). The excitation fluorescence at 325 nm (emission observed at 499 nm), corresponding to the free chelator, decreases upon complexation whereas a peak at 295 nm of the Mg^{2+} complex appears and increases in intensity upon further titration. An isoexcitation point was observed at 318 nm. In contrast, upon titration with Ca^{2+} , the fluorescence excitation spectrum displayed a different behavior. Again, the intensity of the excitation fluorescence at 325 nm decreases with increasing Ca^{2+} concentration (emission observed at 499 nm), while there is a very small increase in intensity of the excitation fluorescence at lower wavelengths (Figure 1D), in contrast with the case of a titration with Mg^{2+} (Figure 1C). This difference between the responses of the fluorescence excitation of **10b** could allow one to make a distinction between changes in Mg^{2+} or Ca^{2+} levels in the cell. In the latter case, a change in fluorescence excitation at 325 nm will not be accompanied by a change in fluorescence at 295 nm.

In contrast to the naphth-1-yl-substituted APTRA chelator, the naphth-2-yl-substituted derivative **10c** shows only one maximum in its emission spectrum at 498 nm. Upon saturation of the chelator with Mg^{2+} , the fluorescence intensity does not change significantly; a shift of fluorescence maximum to 482 nm being observed. Saturation of **10c** with Ca^{2+} results in a reduction of the fluorescence intensity at 498 nm. Titration of **10c** with Mg^{2+} and recording the excitation fluorescence spectra shows that this chelator possesses the spectral characteristics required for a ratioable indicator (Figure 2A). The fluorescence excitation at 328 nm (emission observed at 498 nm) decreases upon increasing Mg^{2+} concentration, whereas the fluorescence at 305 nm increases. An isoexcitation point is found at 313 nm. Titration of **10c**

with Ca^{2+} only results in a decrease of the fluorescence at 328 nm (emission observed at 498 nm), but no response of the fluorescence was observed at lower wavelengths (Figure 2B). Thus, chelator **10c** could also be useful to distinguish changes of intracellular Mg^{2+} levels from "calcium spikes" in the cell.

CONCLUSIONS

The palladium catalyzed coupling reaction has proven to be a useful tool for the synthesis of new fluorescent APTRA indicators. Triflate **1** could be readily coupled with polyaromatic organoboron species and the resulting coupling products could then be easily carried on to the desired APTRA derivatives in good overall yields. The fluorescent response of the indicators on complexation of Mg^{2+} and Ca^{2+} depends greatly on the nature of the polyaromatic group coupled to the chelating APTRA moiety. Benzo[*b*]fur-2-yl-substituted ligand **10a** was found to be a nonratioable indicator for both Mg^{2+} and Ca^{2+} . However, the APTRA-ligand substituted with a naphth-1-yl or a naphth-2-yl group did result in the discovery of two new ratioable fluorescent indicators for Mg^{2+} . Indicators **10b** and **10c** showed an appreciable spectral shift of 30 and 23 nm, respectively in their fluorescent excitation spectra upon complexation of Mg^{2+} and with useful dissociation constants. Thus, these new ratioable indicators may be expected to find use in studying intracellular Mg^{2+} levels by fluorescence microscopy/imaging techniques.

We also found that the nature of the fluorescent response of indicators **10b** and **10c** depends strongly on the metal ion. This is most notable for indicators **10b** and **10c**. When titrated with Mg^{2+} , both indicators displayed the desired ratioable characteristics in their excitation fluorescence spectra. On the other hand, the excitation fluorescence spectra showed only a decrease of the fluorescence intensity upon increasing Ca^{2+} levels. This observation is important because corrections made for (high intracellular levels of) Ca^{2+} when measuring intracellular Mg^{2+} are often based on the assumption that the fluorescent response of an indicator to both metal ions is identical (13).

The dissociation constants for Mg^{2+} found for all three indicators **10a-c**, ranging from 1.8 mM to 2.3 mM, are similar to the values reported for Mag-indo and Mag-fura (1). Analysis of the Ca^{2+} fluorescence/titration data via a two parameter nonlinear fit resulted in K_D^{Ca} values for indicators **10a-c** (70, 17, and 28 μM) which correspond very well with the values found for the commercially available APTRA indicators. The lower Ca^{2+} affinity found for benzo[*b*]fur-2-yl substituted indicator **10a** may be explained by the stronger electron-withdrawing character of this hetero-aromatic system compared to a naphthyl group.

ACKNOWLEDGMENT

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Isomerism and Solution Dynamics of ^{90}Y -Labeled DTPA–Biomolecule Conjugates

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This report describes the synthesis of two DTPA-conjugated cyclic peptides, cyclo(Arg-Gly-Asp-D-Phe-Lys)DTPA (SQ169) and [cyclo(Arg-Gly-Asp-D-Phe-Lys)]₂DTPA (SQ170), and a chromatographic study of their ^{90}Y complexes (RP762 and RP763, respectively). The goal is to study the solution structure and the possible isomerism of ^{90}Y -labeled DTPA–biomolecule conjugates at the tracer level ($\sim 10^{-10}$ M). RP762 was prepared in high radiochemical purity (RCP > 95%) by reacting 2 μg of SQ169 with 20 mCi of $^{90}\text{YCl}_3$ (corresponding to a SQ169:Y ratio of $\sim 4:1$) in the 0.5 M ammonium acetate buffer (pH 8.0) at room temperature. RP763 was prepared in a similar fashion using SQ170. In both cases, the ^{90}Y -chelation was instantaneous. By a reversed-phase HPLC method, it was found that RP762 exists in solution as a mixture of two *detectable* isomers (most likely cis and trans isomers), which interconvert at room temperature. The interconversion of different isomeric forms of RP762 involves a rapid exchange of “wrapping isomers” via the “wagging” of the diethylenetriamine backbone, “shuffling” of the two NO_2 donor sets, and inversion at the terminal amine-nitrogen atom. The inversion at a terminal nitrogen atom requires simultaneous dissociation of the NO_2 donor set. For RP763, the interconversion of different isomers is much faster than that for RP762 due to the weak bonding of two carbonyl-oxygen donors. Therefore, RP763 shows only one broad radiometric peak in its HPLC chromatogram. The rapid interconversion of different isomers is intramolecular via a partial dissociative mechanism. The results obtained in this study are consistent with the lack of kinetic inertness of ^{90}Y - and ^{111}In -labeled DTPA–biomolecule conjugates. Thus, the design of new BFCs should be focused on those which form lanthanide complexes with high thermodynamic stability and more importantly kinetic inertness.

INTRODUCTION

In the past decade, many acyclic and macrocyclic bifunctional chelators (BFCs) have been synthesized and used for the radiolabeling of antibodies and peptides (1–24). Although formation of isomers in yttrium and lanthanide complexes of DTPA analogues has been studied by various NMR methods, very little information is available on isomerism of the radiolabeled DTPA–biomolecule conjugates at the tracer level. In many cases, TLC is the only tool for analysis of the final product. Radio-HPLC chromatograms are not presented nor is the HPLC method for the characterization of the radiolabeled biomolecule. It is not surprising that there has been no detailed discussion about isomerism and the impact on the biological properties of the radiolabeled DTPA–biomolecule conjugates.

Recently, Caravan and co-workers (25) extensively reviewed both the solid state and solution structures of lanthanide complexes of various acyclic and macrocyclic chelators. Solid state structures provide a wealth of information about the coordination chemistry of a specific chelating system. However, the solution structure may be different from the solid state structure because of possible dissociation of certain donor atom(s) or the coordination of water molecules. The isomerism observed in the solid state structure may not be seen in solution

due to interconversion of isomers or fluxionality of the ligand framework. On other occasions, only one isomeric form is found in the solid state, and more isomers are observed in solution. Thus, it is imperative to study the possible isomerism in the metal chelate, particularly at the tracer level, to understand the biological properties of a new metalloradiopharmaceutical.

For radiolabeled antibodies, the attachment of the BFC and the radionuclide normally does not disturb the tertiary structure of the polypeptide; thereby the biological and receptor binding properties are not significantly affected. For small biomolecules, however, the metal chelate (radionuclide and BFC) contributes greatly to the overall size and molecular weight of the radiopharmaceutical. Isomerism may have significant impact on the biological performance (receptor binding and biodistribution) since the distribution of the radiopharmaceutical is dependent on the physical and chemical properties of both the biomolecule and the metal chelate.

In this report, we describe the synthesis of two DTPA-conjugated cyclic peptides (SQ169 and SQ170) and a chromatographic study of their ^{90}Y complexes (RP762 and RP763 in Figure 1). The purpose of the study is to obtain a sufficient understanding of the solution structure of these complexes, and to explore the possible isomerism in ^{90}Y -labeled DTPA–biomolecule conjugates (DTPA–monoamide and DTPA–bisamide) at the tracer level ($\sim 10^{-10}$ M), the kinetics for the formation of isomers, and the dynamics of interconversion between different isomeric forms.

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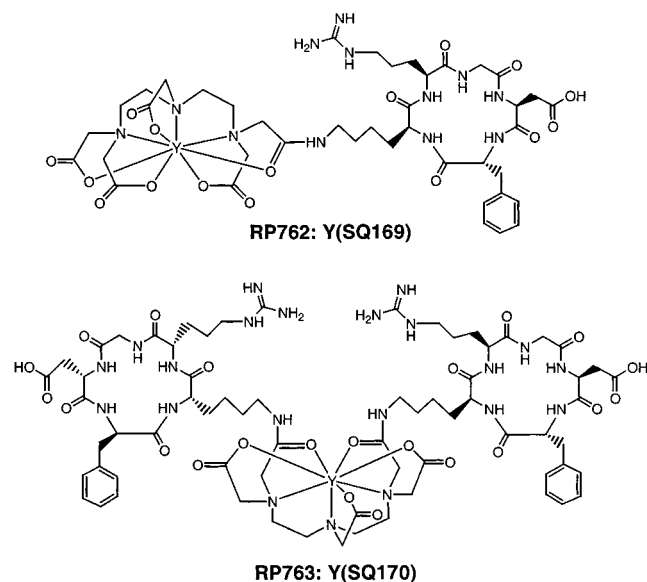


Figure 1. Structures of yttrium complexes of two DTPA conjugates (SQ169 and SQ170).

EXPERIMENTAL SECTION

Materials. Acetic acid (ultrapure), ammonium hydroxide (ultrapure), and diethylenetriaminepentaacetic acid cyclic dianhydride were purchased from either Aldrich or Sigma Chemical Co. and were used as received. ⁹⁰YCl₃ (in 0.05 N HCl) was purchased from New England Nuclear, N. Billerica, MA. The cyclic pentapeptide, cyclo(Arg-Gly-Asp-D-Phe-Lys), as its trifluoroacetic acid (TFA) salt was prepared according to the literature method (26).

Synthesis of Cyclo(Arg-Gly-Asp-D-Phe-Lys)DTPA (SQ169) and [Cyclo(Arg-Gly-Asp-D-Phe-Lys)]₂DTPA (SQ170). To a solution of cyclo(Arg-Gly-Asp-D-Phe-Lys)·2TFA (0.050 g, 0.060 mmol) in DMF (2 mL) was added triethylamine (41.9 μ L, 0.30 mmol). This solution was added dropwise over 4 h to a solution of diethylenetriaminepentaacetic dianhydride (0.107 g, 0.30 mmol) in DMF (2 mL) and dimethyl sulfoxide (2 mL). The reaction mixture was stirred for 16 h, and was then concentrated to give an oily residue, which was purified using a preparative HPLC method, which used a Rainin Rabbit HPLC system, a Knauer VWM UV-vis detector (λ = 230 nm), and a Vydac C₁₈ preparative column (21.2 mm $\times$ 25 cm). The flow rate was 15 mL/min with the mobile phase starting from 98% solvent A (0.1% TFA in H₂O) and 2% solvent B (0.1%TFA in ACN/H₂O = 9:1) to 63.2% solvent A and 36.8% solvent B at 16 min. The two peaks at 12 and 15 min were collected separately. LC-MS data showed that the peak at 12 min was from SQ169 and the peak at 15 min was from SQ170. After lyophilization, SQ169 was obtained in 46% yield (30 mg) and SQ170 in 21% yield (21.5 mg). ES-MS for SQ169: *m/z* 977.5 (M-H)⁻ (C₄₁H₆₂N₁₂O₁₆). ES-MS for SQ170: *m/z* 1562.8 (M-H)⁻ (C₆₈H₁₀₁N₂₁O₂₂).

General Procedure for Synthesis of RP762 and RP763. To a lead-shielded clean 5 mL vial containing 2–100 μ g of SQ169 or SQ170 in 1 mL of 0.5 M ammonium acetate buffer (pH 8.0) was added 10–20 μ L of ⁹⁰YCl₃ stock solution (~20 mCi) in 0.05 N HCl. The reaction mixture was allowed to stand at room temperature for 20 min. A sample of the resulting solution was diluted 20-fold with saline and then analyzed by HPLC (Gradient A for RP762 and Gradient B for RP763) and by ITLC.

The HPLC method used a HP-1100 HPLC system with a UV-vis detector (λ = 215 nm), an IN-US radio-detector, and a Zorbax C₁₈ column (4.6 mm $\times$ 250 mm, 80 Å pore size). The flow rate was 1 mL/min with a gradient mobile phase. For RP762, the gradient was 100% solvent A (95:5 mixture of 25 mM ammonium acetate buffer, pH 6.8, and acetonitrile) to 95% A and 5% solvent B (50:50 mixture of 25 mM ammonium acetate buffer, pH 6.8, and acetonitrile) at 8 min and to 90% of solvent A and 10% solvent B at 24 min. For RP763, the gradient was 85% solvent A and 15% solvent B to 80% of solvent A and 20% solvent B at 18 min. The ITLC method used Gelman Sciences silica gel ITLC paper strips and 1:1 mixture of acetone and saline as eluant. By this method, RP762 and RP763 migrate to the solvent front while [⁹⁰Y]colloid and [⁹⁰Y]acetate remain at the origin.

Interconversion of the Two Isomers of RP762. To a shielded clean 5 mL vial containing 20 μ g of SQ169 or SQ170 in 1 mL of 0.5 M ammonium acetate buffer (pH 8.0) was added 20 μ L of ⁹⁰YCl₃ stock solution (~20 mCi) in 0.05 N HCl. The reaction mixture was allowed to stand at room temperature for 20 min. The two peaks at 16.8 and 20.9 min were separated by HPLC, and were collected into separate 10 mL round-bottom flasks. The activity concentration of two collections (peak A and peak B) was about 0.5 mCi/mL. Each collected fraction was dispensed into two separate vials: one was kept at room temperature while the other was kept in a 54 °C water bath. Samples of these solutions were analyzed by HPLC every 30 min for 6 h.

⁹⁰Y-Chelation Efficiency of SQ169. To a lead-shielded clean 5 mL vial containing 2, 25, 50, and 100 μ g of SQ169 in 1 mL of 0.5 M ammonium acetate buffer (pH 8.0) was added 20 μ L of ⁹⁰YCl₃ stock solution (~20 mCi) in 0.05 N HCl. The reaction mixture was allowed to stand at room temperature for 30 min. A sample of the resulting solution was diluted 20-fold with saline, and then analyzed by HPLC (Gradient A) and by ITLC. The RCP was corrected by subtracting the percentage of [⁹⁰Y]-colloid and [⁹⁰Y]acetate obtained by ITLC from the yield obtained by HPLC.

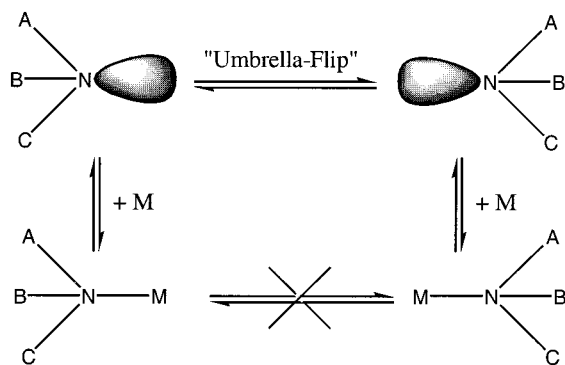
Synthesis of [⁸⁹Y]RP762 for LC-MS and HPLC Concordance Experiment. To a clean 5 mL vial containing 0.5 mg of SQ169 in 0.5 mL of 0.5 M ammonium acetate buffer (pH 8.0) was added 0.2 mL of the Y(NO₃)₃·6H₂O solution (10 mg/mL in 0.5 M ammonium acetate buffer, pH 8.0). The reaction mixture was allowed to stand at room temperature for 30 min. A sample of the resulting solution was analyzed by LC-MS. No further purification was performed for the LC-MS analysis and the HPLC concordance experiment. The LC-MS spectra were collected using a HP1100 LC/MSD system with API-electrospray interface. The LC-MS method used a Zorbax C₁₈ column (4.6 mm $\times$ 150 mm, 3.5 μ m particle size) at a flow rate of 1 mL/min and a gradient mobile phase starting from 92% Solvent A (10 mM ammonium acetate buffer, pH 7.0) and 8% Solvent B (methanol) to 100% B at 23 min. The MSD parameters are found in Table 1.

RESULTS AND DISCUSSION

The pyramidal environment at coordinated amine-nitrogen atoms has been conclusively established by X-ray methods (27). Rapid inversion (Chart 1) at the amine nitrogen atoms is common. However, once the nitrogen atom in a polydentate chelator is bonded to the metal ion, the inversion becomes more difficult and

Table 1

detection mode	positive
gain	1.0
gas temperature	350 °C
nebulizer pressure	60 psig (max)
UV-vis detector	$\lambda = 220$ nm
mass range	0–2000
fragmentor	30 V
drying gas flow	13 L/min
V capillary	4000 V

Chart 1. Inversion at the Coordinated and Uncoordinated Amine-Nitrogen.

involves dissociation and reformation of the coordination bond (Chart 1). Therefore, coordination of a polydentate chelator containing amine-nitrogen donor atoms often results in formation of isomers in its yttrium and lanthanide complexes. For example, DTPA, DTPA-MA (MA = monoamide), and DTPA-BA (BA = bisamide) contain three amine-nitrogen donors. In principle, this will result in formation of eight possible isomers once they are coordinated to a metal ion. In the solid state, only some of them were observed by X-ray crystallography (27–31). In solution, however, all eight isomers of lanthanide complexes have been observed by NMR at low temperature (32–39). Some of them interconvert via a partial dissociation at elevated temperatures. For example, the proton NMR spectra of the Pr^{3+} and Eu^{3+} complexes of DTPA show that at room temperature there are only two main isomers (Figure 2), which undergo rapid exchange in solution at temperatures higher than 95 °C (32–34). For the Lu^{3+} chelate of DTPA-BMA, however, the temperature for the rapid exchange is lower (85 °C), reflecting the weak bonding of carbonyl-oxygen as compared to carboxylate-oxygen donors (35–39). The major feature of the exchange process involves the shuffling of coordinated acetate accompanied by a flip of the backbone ethylene bridges between staggered conformations. This results in a change in helicity of the complex and leads to the equilibration of acetate arms. Inversion at the coordinated nitrogen atoms has been excluded based on ^1H NMR data (35). In all the cases, the concentration of the lanthanide complexes studied by NMR ranged from 0.05 to 0.20 M.

^{90}Y -labeled DTPA-biomolecule conjugates (DTPA-MA and DTPA-BA) are often prepared at low concentration (2–20 mCi/mL corresponding to 4×10^{-9} to 4×10^{-8} M). For HPLC analysis, the amount of injected radioactivity is even lower (1.0 μL corresponding to about 1 μCi). The actual complex concentration in the mobile phase is only $\sim 10^{-15}$ M. Because of this concentration difference, these lanthanide complexes may have different solution characteristics at the tracer level. The purpose of the study is to obtain a sufficient understanding of the solution structure and isomerism of the ^{90}Y -labeled DTPA-

peptide conjugates at the tracer level, and the dynamics of interconversion between different isomeric forms.

Synthesis of SQ169 and SQ170. The DTPA conjugates (SQ169 and SQ170) were prepared according to Chart 2 by reacting DTPA with the cyclic pentapeptide, cyclo(Arg-Gly-Asp-D-Phe-Lys)·2TFA, in a mixture of DMF and DMSO. SQ169 and SQ170 were isolated as their TFA salts, and were repurified by HPLC before being used for radiolabeling.

Synthesis of RP762 and RP763. RP762 was prepared (Chart 2) by reacting SQ169 with $^{90}\text{YCl}_3$ in the 0.5 M ammonium acetate buffer (pH 8.0) at room temperature. The pH is very important for high yield radiolabeling. A higher pH usually gives better radiolabeling yield. Using 0.5 M ammonium acetate buffer (pH 8.0), RP762 could be prepared in high yield (RCP > 95%) by reacting 2 μg of SQ169 with 20 mCi of $^{90}\text{YCl}_3$ corresponding to a SQ169:Y ratio of $\sim 4:1$. RP763 was prepared in a similar fashion using SQ170 substituting for SQ169. In both cases, the ^{90}Y -chelation was instantaneous. This is consistent with the results reported in the literature (40, 41).

HPLC Characterization of RP762. For the past decade, HPLC has become a routine technique for quality control of various radiopharmaceuticals. The advantage of radio-HPLC is its capability to determine the RCP for a specific radiopharmaceutical, and to separate different radiolabeled species in the reaction mixture. It is also a very powerful tool for separation of different isomers such as diastereomers. In this study, we used a reversed-phase HPLC method to analyze reaction mixtures containing RP762. Figure 3 shows typical radio-HPLC chromatogram of RP762. Two radiometric peaks at 16.8 and 20.9 min were detected and there are no other radio-impurities. We also tried several other reversed-phase HPLC methods. In all the cases, RP762 shows only two radiometric peaks, suggesting that the HPLC method is specific. The presence of the two peaks in the HPLC chromatogram also suggests that RP762 exists as two detectable isomers in solution.

It is interesting to note that the peak ratio of peak A and peak B changes over time until the equilibrium is reached. For example, the peak ratio at $t = 0$ h was 50:50 while it was about 48:52 at 12 h and remained relatively constant from that time. This observation suggests that there is a slow conversion from peak A to peak B at room temperature, and the formation of the different isomeric forms is probably kinetically controlled. Since the ^{90}Y -chelation was instantaneous under the conditions described in the experimental, it is not surprising that these isomers were formed at the same time with equal population at the early stage.

We also studied the conversion rate of HPLC purified peak A and peak B. First, RP762 was prepared using 20 mCi of ^{90}Y , and the isomers at 16.8 and 20.9 min were collected into two separate 5 mL vials. The two vials were either kept at room temperature or placed in a water bath at 54 °C. Samples of the collected fractions (activity concentration = 0.5 mCi/mL) were analyzed by the HPLC over 6 h. It was found that the rate of interconversion between these two peaks is very slow at room temperature (Figure 4). That may explain why it takes almost 24 h for these isomers to reach the equilibrium. At a higher temperature (54 °C), the rate of interconversion between different isomers of RP762 is accelerated (Figure 5), and it took about 6.5 h for peak A to reach the 48:52 (peak A/peak B) ratio. However, it took a much longer time (>7 h) for peak B to reach the equilibrium, suggesting that peak B is thermodynamically more stable. It is

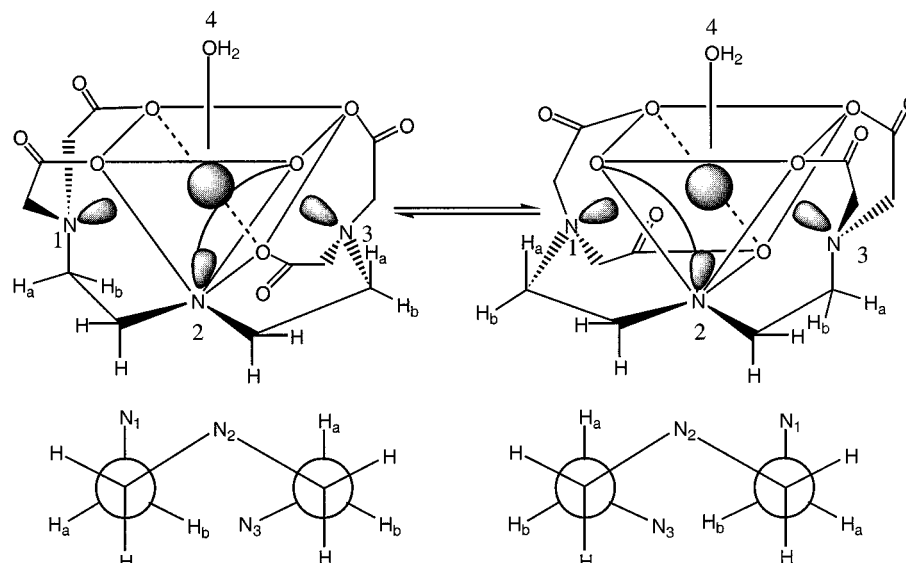
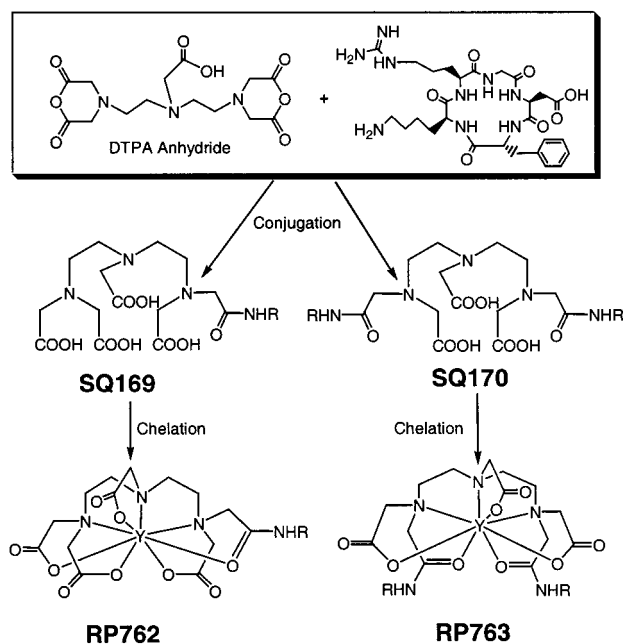


Figure 2. Proposed mechanism for the interconversion between the two wrapping isomers of lanthanide DTPA complexes.

Chart 2. DTPA Conjugation and ^{90}Y -Chelation.



very important to note that both peak A and peak B remained stable in the HPLC mobile phase (containing 10% acetonitrile) at 54 °C for more than 6 h. There is no significant decomposition of the metal chelate, suggesting that interconversion between these isomeric forms is intramolecular.

HPLC Concordance Experiment. We performed a HPLC concordance experiment using RP762 and [^{89}Y]-RP762. [^{89}Y]-RP762 was prepared by reacting SQ169 with yttrium(III) nitrate in 0.5 M ammonium acetate buffer (pH 8.0), and was analyzed without further purification using identical chromatographic conditions to those for RP762. Figure 6 shows HPLC chromatograms of RP762 (top) and [^{89}Y]-RP762 (bottom). It is quite clear that the same complexes were prepared at the tracer (^{90}Y) and macroscopic (^{89}Y) levels. We used LC-MS to determine the composition of [^{89}Y]-RP762. The two peaks of [^{89}Y]-RP762 show identical fragmentation patterns and the same molecular ions at m/z 1065.2 for $(\text{M} + \text{H})^+$ and 533.3 for $(\text{M} + 2\text{H})^{2+}$ (Figure 7). This provides direct evidence to support the conclusion that the two peaks in the radio-

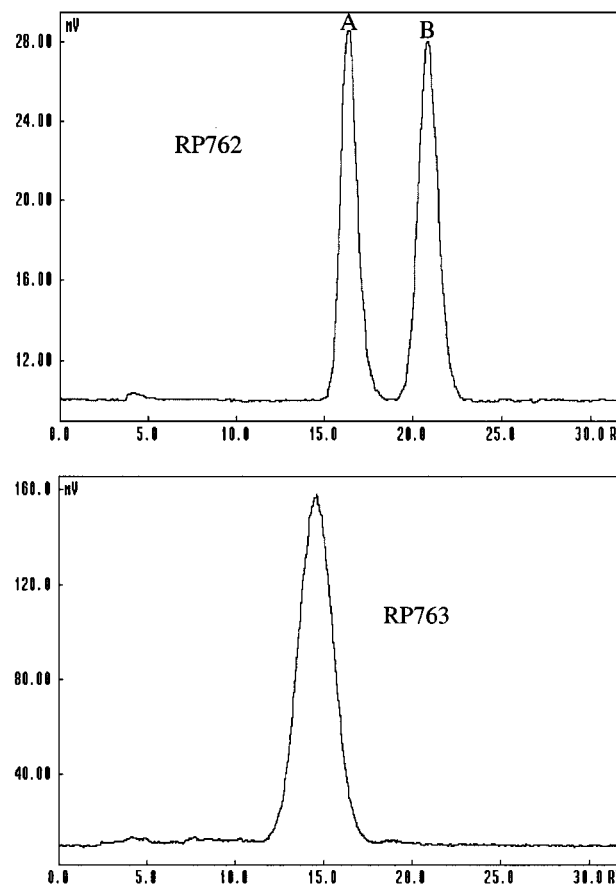


Figure 3. Typical radio-HPLC chromatograms for RP762 (top) and RP763 (bottom).

HPLC chromatogram of RP762 are indeed due to the resolution of two isomers.

HPLC Characterization of RP763. We used a slightly different mobile phase when analyzing RP763 by radio-HPLC due to its higher lipophilicity. Figure 3 shows a typical radio-HPLC chromatogram for RP763. In contrast to RP762, RP763 shows only one broad peak with the half-width of ~3 min. There are no other radio-impurities in the HPLC chromatogram. In general, there are two possibilities for the large half-width of the peak.

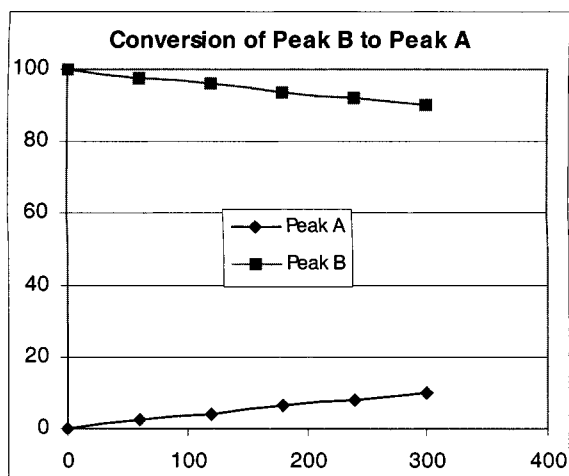
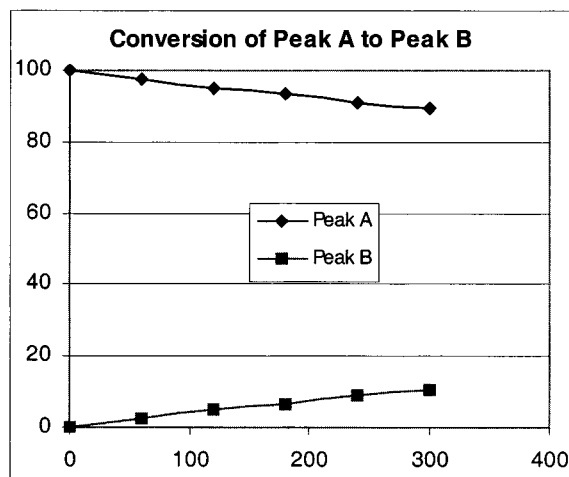


Figure 4. Interconversion kinetics of the two isomers of RP762 at room temperature.

The first possibility is that the peak at ~15 min is a combination of several unresolved peaks from different isomers. If that were true, it should have been possible to separate them using long gradient or isocratic chromatographic conditions. We tried several different gradients, and found that RP763 always shows one broad peak with different half-widths depending upon the HPLC chromatographic conditions. The second possibility is that there is a rapid interconversion between different isomers at room temperature under the conditions used in this study.

In general, the lanthanide complexes of DTPA analogues assume distorted tricapped trigonal prism (TTP) geometries. In the TTP arrangement, the neutral donor atoms with longer bond lengths will prefer to occupy face capping positions rather than a prismatic corner. However, since it is not possible for all three amine-nitrogen donors to occupy capping positions, the central nitrogen atom tends to occupy a prismatic corner (Figure 2) while the two terminal amine-nitrogen atoms occupy capping positions. Because of distortion, the actual coordination geometry is, in most cases, between TTP and monocapped square antiprism (CSAP).

SQ169 is a DTPA-monoamide and bonds to yttrium(III) with one carbonyl-oxygen, three amine-nitrogen, and four acetate-oxygen donor atoms. Theoretically, there are eight possible isomers (four pairs of enantiomers in Chart 3) due to the chirality of three coordinated nitrogen donors. If the biomolecule in the DTPA-peptide conjugate is stereochemically pure, it will form an yttrium

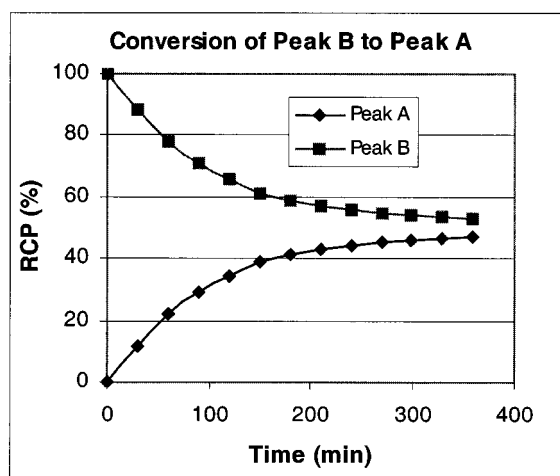
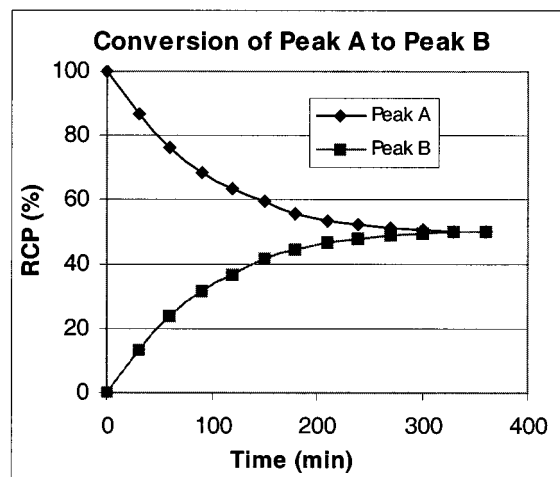


Figure 5. Interconversion kinetics of the two isomers of RP762 at 55 °C.

complex with the same number of isomers as that of the Y-DTPA complex. Previously, ^1H and ^{13}C NMR studies of lanthanide complexes of DTPA and DTPA-bisamide derivatives have revealed a rapid interconversion of two wrapping isomers (26, 32–39). It has been proposed that the rapid exchange process involves “wagging” of the diethylenetriamine backbone and shuffling of coordinated acetate groups. Inversion at the coordinated nitrogen atoms has been excluded due to the fact that the ^1H signals of methylene-hydrogens of the acetate and acetamide groups remain as AB quartets even at 85 °C (34). This strongly suggests that the energy barriers for the inversion at the coordinated amine-nitrogen donors is much higher than that for the rapid interconversion of “wrapping isomers”.

It should be noted that the concentration of the lanthanide complexes for NMR studies ranged from 0.05 to 0.2 M. At this concentration, the dissociation of acetate-oxygen and amine-nitrogen donors is not significant. At the tracer level, however, the concentration of RP762 in the HPLC mobile phase is only $\sim 10^{-10}$ M. At this concentration, it is quite possible for acetate-oxygen, carbonyl-oxygen and amine-nitrogen donors to become dissociated due to the competition from acetate anions in the mobile phase. Partial dissociation of these donor atoms makes the interconversion between “wrapping isomers” much easier. That may account for the different solution characteristics of lanthanide complexes of DTPA-MA and DTPA-BA at different concentration levels.

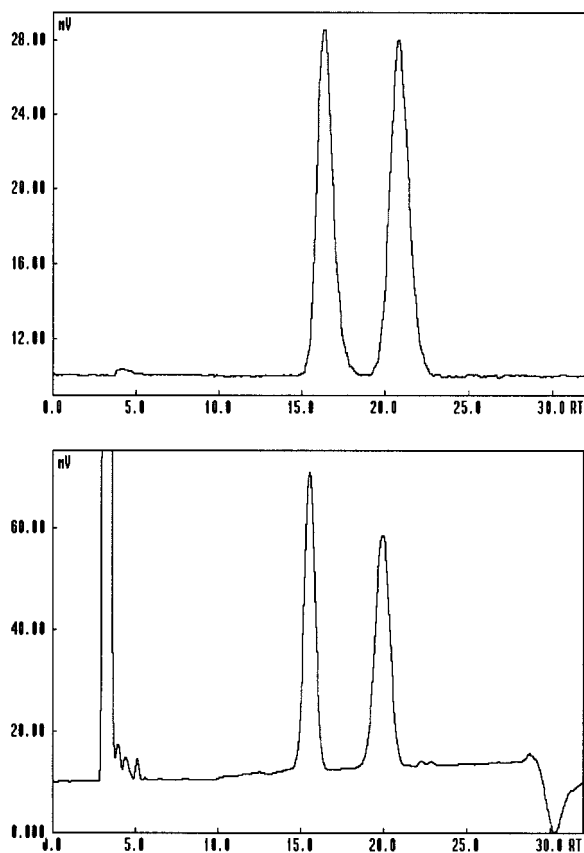
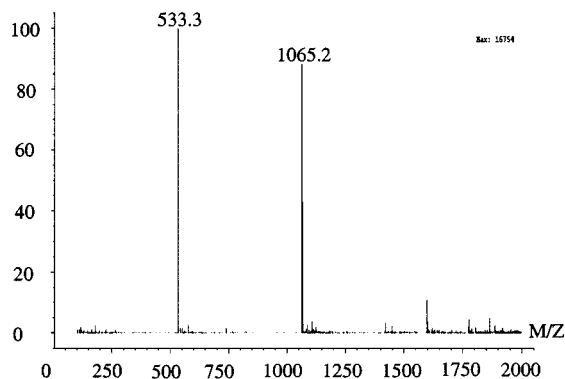


Figure 6. HPLC concordance of RP762 (top) and [⁸⁹Y]RP762 (bottom).

MSD1 SPC, time = 16.32:17.10, API-ES, Positive, Max = 16754



MSD1 SPC, time = 19.32:20.47, API-ES, Positive, Max = 15431

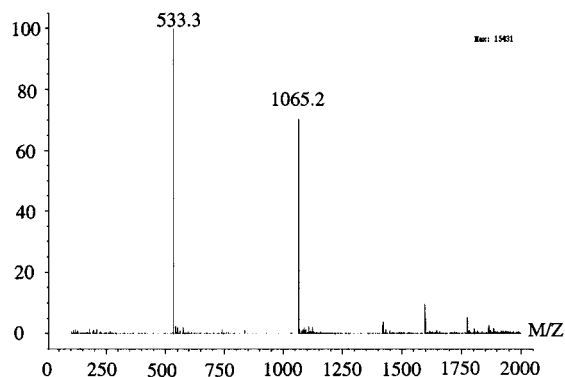
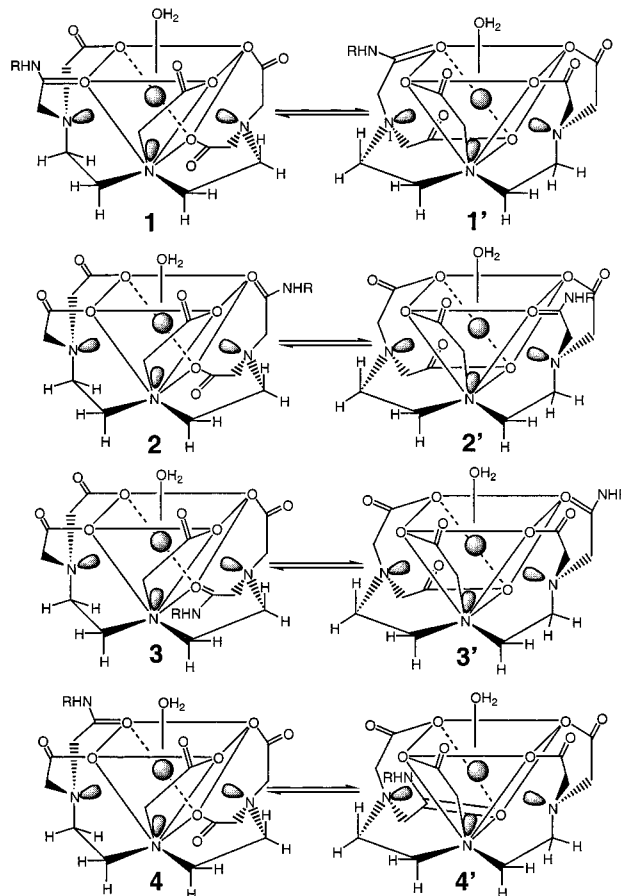


Figure 7. LC-MS spectra of the two isomers of RP762.

RP762 has four possible pairs of enantiomers, which are not separable by the HPLC methods described in this

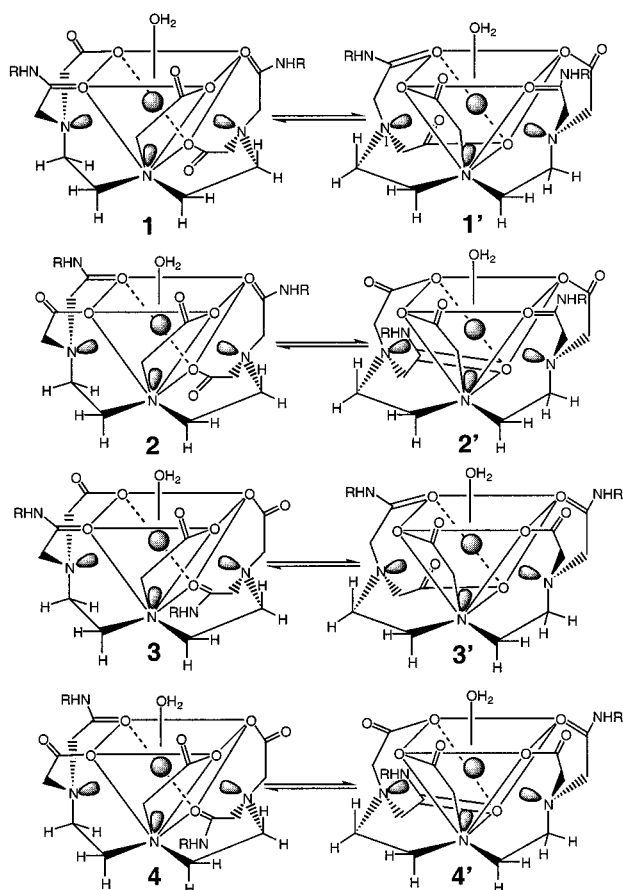
Chart 3. Eight Isomers of the Complex Y(DTPA-MA).



study. If one assumes that the rapid interconversion occurs between “wrapping isomers” at room temperature, isomer **1** is readily converted to **1'** via “shuffling” of the two NO₂ donor sets and “wagging” of the diethylenetriamine backbone. The same is true for **2** and **2'**. Since **1** and **2** are the two enantiomer pairs, these four isomers are not separable by radio-HPLC, and are expected to appear as single peak while the remaining four isomers (**3**, **3'**, **4**, and **4'**) as another peak in the HPLC chromatogram. This is completely consistent with the presence of two radiometric peaks in the HPLC chromatogram of RP762 (Figure 3).

It should be noted that the relative orientation of the acetamide group to the acetate arm at the central nitrogen atom is different (cis and trans, respectively) in isomers **1** and **4** (Chart 3). The same is true for isomers **1'** and **4'**, **2** and **3**, **2'** and **3'**. For cis and trans isomers to interconvert, inversion at the terminal amine-nitrogen atoms has to occur. This suggests that the NO₂ donor set have to be dissociated and accompanied by a rapid “umbrella-type” inversion at the nitrogen atom. That explains the fact that both radiometric peaks of RP762 interconvert and the rate of interconversion is temperature dependent. Since RP762 remains stable for at least 6 h at 54 °C without a significant decomposition, it is reasonable to assume that the remaining part of the DTPA-monoamide conjugate remains firmly bonded to Y(III) when one of the two NO₂ donor sets undergoes a rapid “umbrella-type” inversion at the terminal nitrogen atom.

SQ170 is a DTPA-BA and bonds to yttrium(III) as an octadentate chelator using two carbonyl-oxygen, three amine-nitrogen, and three acetate-oxygen donor atoms. Like DTPA-MA, DTPA-BA forms a Y(III) complex with

Chart 4. Eight Isomers of the Complex Y(DTPA-BA).

eight isomers (Chart 4) due to the chirality of the three coordinated amine-nitrogen atoms. Compared to acetate-oxygen, the carbonyl-oxygen is a weak donor for yttrium(III). Thus, it is easier for the two NO_2 donor sets to be dissociated in solution, which results in faster interconversion between "wrapping isomers" and inversion at coordinated amine-nitrogen atoms. As a result, RP763 shows only one broad radiometric peak in its HPLC chromatogram (Figure 3).

CONCLUSION

In this study, we presented synthesis and characterization of two DTPA-peptide conjugates (SQ169 and SQ170) and the corresponding ^{90}Y complexes (RP762 and RP763, respectively). By a reversed-phase HPLC method, we found that the ^{90}Y -labeled DTPA-monoamide conjugate exists in solution as a mixture of two detectable isomers (cis and trans isomers), which interconvert at room temperature under the chromatographic conditions described in this study. The interconversion of different isomeric forms of RP762 involves a rapid exchange of "wrapping isomers" via the "wagging" of the diethylenetriamine backbone, "shuffling" of the two NO_2 donor sets, and inversion at the terminal amine-nitrogen atom. For the ^{90}Y -labeled DTPA-BA conjugate (RP763), the interconversion of different isomers (particularly cis-cis, cis-trans, trans-cis, and trans-trans isomers) is much faster than that for RP762 due to the weak bonding of two carbonyl-oxygen donors; thereby RP763 shows one broad radiometric peak in its HPLC chromatogram.

The results obtained from this study have significant implications for ^{90}Y -labeled DTPA-biomolecule conjugates. The ^{90}Y -labeled DTPA-biomolecule conjugate exists in solution as a mixture of eight possible isomers,

some of which undergo rapid interconversion at room temperature. Therefore, the presence of different isomeric forms may not impose a significant problem with respect to the difference in biological properties of these isomers. The most critical issue using DTPA as a BFC is its low kinetic inertness. When the two isomers of RP762 were separated by HPLC, the HPLC mobile phase did not contain any significant amount of trace metal ion. The rapid interconversion of different isomers is purely intramolecular. Once the ^{90}Y -labeled DTPA-biomolecule conjugate is injected into a biological system, the presence of biologically important metal ions such as Ca^{2+} , Cu^{2+} , Fe^{2+} , and Zn^{2+} , is expected to have a significant impact on the biological fate of the ^{90}Y -labeled DTPA-biomolecule conjugate. These metal ions can compete with ^{90}Y for the DTPA-biomolecule conjugate due to their high concentration. Once ^{90}Y is dissociated from the DTPA-biomolecule conjugate, it will most likely deposit in bone. It is not surprising that many ^{90}Y - and ^{111}In -labeled DTPA-biomolecule conjugates often show high bone activity. Thus, the design of new BFCs should be focused on those which form lanthanide complexes with high thermodynamic stability and more importantly kinetic inertness.

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Histidylated Polylysine as DNA Vector: Elevation of the Imidazole Protonation and Reduced Cellular Uptake without Change in the Polyfection Efficiency of Serum Stabilized Negative Polyplexes

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We have reported that polylysine substituted with histidyl residues (His) was suited to make complexes with plasmid DNA (pDNA) and to transfect cells *in vitro* in the presence of serum. The present study was performed to determine whether the acetylation of the α -amino group of histidyl residues (AcHis) had an influence on the size and the charge of polyplexes and on their transfection efficiency. We found that the presence of free α -amino groups allowed the formation of smaller polyplexes but did not modify the ζ potential of +17 mV. At a physiological salt concentration, the adsorption of many serum proteins on His- and AcHis-polyplexes reduced their size below 100 nm, inhibited their aggregation, and reversed their ζ potential to –25 mV. The acetylation of the α -amino groups reduced slightly the adsorption of serum proteins. The presence of the α -amino groups increased the pK of the imidazole protonation of histidine bound to polylysine from pH 5.8 to 6.9; in addition, the protonation was further elevated in the presence of pDNA. Serum stabilized negative histidylated polyplexes were less taken up by cells but their transfection efficiency did not decrease; depending on the cell line, His-polyplexes were more efficient than AcHis-polyplexes. The results indicate that (i) the α -amino groups of histidyl residues bound to polylysine favorably influence the size and the transfection efficiency of polyplexes, (ii) the α -amino groups also elevate the imidazole protonation of His-polyplexes, which is suited to destabilize the membrane of early endocytic vesicles in order to favor pDNA delivery in the cytosol, and (iii) the absorption of selective serum proteins on His-polyplexes could be a way for *in vivo* gene targeting.

INTRODUCTION

Soluble interpolyelectrolyte complexes (termed polyplexes) (1) formed between a plasmid DNA (pDNA) and a cationic polymer are a promising approach for a nonviral gene delivery (for reviews, see refs 2 and 3). Their self-assembly in an aqueous solution results from a cooperative system between the polyanionic phosphate backbone of nucleic acids and the positive charge of amino groups of the cationic polymers via electrostatic bonds. Such electrostatic interactions allow pDNA condensation in morphologically variable polyplexes depending on the polymer structure (4). To be efficient, polyplexes must have the capacity (i) to enter into cells of interest, (ii) to transfer a great number of copies into the cytosol in a sufficient number of cells, and (iii) to transport a great number of copies into the cell nucleus. Polylysine and ligand-substituted polylysine are widely used to condense pDNA in toroid particles and short rods that are taken up by cells via nonspecific and receptor-mediated endocytosis, respectively. Polyplexes must escape the endocytic pathway and pDNA reach the cytosol. This is favored by using chloroquine (5–7) glycerol (8) or acidic dependent fusogenic peptides (6, 9–14).

Recently, we have reported that partially histidylated polylysine complexed with pDNA actively transfect cells in culture in the presence of serum and in the absence of chloroquine or a fusogenic peptide (15). We found that the transfection efficiency into cystic fibrosis airway

epithelial cell lines was as great as that obtained with polyethylenimine or lipofectamine as pDNA vectors (16). It was shown with polylysine and polyethylenimine that the polymer/DNA ratio, the chemical substitution of the cationic polymer and conditions of the polyplexes formation largely influenced the morphology, the size, the charge as well as the transfection efficiency of polyplexes (4, 17–22).

This paper was focused on a comparative study of polyplexes made with polylysine substituted with either histidyl or *N*-acetyl-histidyl residues in order to evaluate the influence of the α -amino substitution on the transfection efficiency. The size and the charge of both polyplexes were determined as a function of the DNA/polymer ratio, the salt concentration, the pH medium and the amount of serum. Histidylated polylysines which were designed to favor pDNA delivery into the cytosol after the uptake of polyplexes by the cells, contain several imidazoles which can induce membrane destabilization after their protonation in an acidic medium. We have determined the pH of the protonation of imidazole in both polymers as well as in polyplexes and showed that the protonation of His-polyplexes¹ can occur in the lumen of early endocytic vesicles.

MATERIALS AND METHODS

Chemicals. Chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Poly(L-lysine) and HBr (degree of polymerization = 190, Bachem, Bubendorf, Switzerland) were changed in polylysine *p*-toluenesulfonate salt as previously described (23).

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Histidylated Polylysine (His₁₀₉-pLK). Polylysine *p*-toluenesulfonate salt (50 mg; 0.87 μ mol) in 3 mL of DMSO was reacted for 15 h at 20 °C in the presence of diisopropylethylamine (DIEA) (23 μ L; 143.5 μ mol) with the *N*-succinimidyl derivative of BocHis(Boc)-OH (25.5 mg; 56.5 μ mol) (Bachem). The *N*-protecting Boc groups of histidyl residues were removed by acidic treatment by adding H₂O/trifluoroacetic acid mixture (1:1; v:v) for 12 h at 20 °C. Water and trifluoroacetic acid were removed under reduced pressure. Histidylated polylysine was precipitated by adding 10 vol of 2-propanol and collected by centrifugation (1800*g* for 15 min). The pellet was washed with 2-propanol, collected by centrifugation, solubilized in distilled water, and freeze-dried.

***N*-Acetyl-histidylated Polylysine (AcHis₁₀₀-pLK).** Polylysine *p*-toluenesulfonate salt (50 mg; 0.87 μ mol) in 3 mL of DMSO was reacted in the presence of DIEA (60 μ L; 344 μ mol) for 24 h at 20 °C with *N*-acetyl-His-OH (18.8 mg; 0.96 μ mol) in the presence of 84.5 mg of BOP (1.92 μ mol). The polymer was precipitated by adding 10 vol of 2-propanol and collected by centrifugation (1800*g* for 15 min). The pellet was washed with 2-propanol, collected by centrifugation, solubilized in distilled water, and freeze-dried.

Determination of the Histidyl Polylysine Substitution Level. The average number of histidyl residues (*x*) bound per pLK molecule was determined by ¹H NMR spectroscopy at 300 MHz in D₂O according to $x = 6(h_{8.7}/h_{Lys})DP$, where $h_{8.7}$ was the value of the integration of the signal at 8.7 ppm corresponding to the proton (C2-H) of histidyl as well as of *N*-acetylhistidyl residues and h_{Lys} was the value of the integration, in the range 1.3–1.9 ppm, corresponding to the six methylene protons (β,γ,δ) of lysyl residues and DP was the degree of polymerization of pLK.

Fluoresceinylated Polymers. His₁₀₉-pLK and AcHis₁₀₀-pLK (5 mg in 0.5 mL of DMSO) were reacted for 6 h at 20 °C with 5 equiv of FITC (Molecular Probes, Eugene, OR) in the presence of DIEA (2.3 μ L; 14.3 μ mol). Fluoresceinylated polymers were purified by precipitation in 10 vol of 2-propanol and spun down by centrifugation (800*g*, 10 min). Pellets were washed three times with 5 vol of 2-propanol in order to remove unreacted FITC. The absence of FITC was checked by silica thin-layer chromatography with a chloroform/methanol/water (6:6:1; v:v:v) mixture as solvent.

Plasmids. pSV2Luc (5.0 kb) plasmid was an expression vector encoding the firefly luciferase gene under the control of the SV40 T large antigen promoter (24). pUT650 plasmid (5.15 kb) (CAYLA, Toulouse France) was an expression vector encoding the firefly gene under the control of the human cytomegalovirus promoter.

Polyplexes Formation. A polymer in a total volume of 30 μ L of distilled water containing 5% (w/v) glucose unless otherwise stated was mixed with 10 μ g of pDNA in 70 μ L of distilled water containing 5% (w/v) glucose. The mixture was kept 30 min at 20 °C to allow the formation of polyplexes.

ζ Potential Measurement. The global surface charge of polyplexes was measured by electrophoretic mobility

using a ZetaSizer 3000 (Malvern Instruments, Orsay, France) with the following parameters: viscosity, 0.89 cP; dielectric constant, 79; temperature, 25 °C; Smoluchowsky constant $F(ka)$, 1.50; maximum voltage of the current, 15 V. The system was routinely calibrated using a -50 ± 5 mV standard dts 5050 from Malvern. The measurement was performed five times for 30 s with zero field correction in aqueous dep cell on 1.1 mL samples containing a final DNA concentration of 10 μ g/mL.

Size Measurement. Polyplexes size was determined by quasi-elastic laser light scattering (QELS) using a ZetaSizer 3000 with a sample refractive index of 1.59; viscosity, 0.89 cP. The system was routinely calibrated using a 220 nm standard dts 5050 from Malvern. For serum free polyplexes, measurements were performed 10 times for 12 s on 1 mL of sample. The size corresponds to an average size calculated by using the cumulant analysis mode.

Imidazole Protonation of Histidylated Polymers. The protonation of the imidazole of histidine free base (5 mg/mL) and of histidylated polymers (10 mg/mL) was assessed by measuring the chemical shift of the C2-H of histidyl residues versus the pH medium by using ¹H NMR spectroscopy in D₂O at 300 MHz. Chemical shifts were determined downfield the HDO peak at 4.75 ppm used as the internal standard.

Effect of Serum on the ζ Potential of Polyplexes. Polyplexes prepared as described above were diluted in 1 mL of 5 mM Hepes buffer, pH 7.4, containing 10 or 20% of 0.2 μ m filtered fetal bovine serum (FBS, Gibco, Renfrewshire, U.K.). After 30 min, the solution was loaded on 50% (w/v) simple sucrose cushion and centrifuged at 4 °C for 4 h at 30 000 rpm in a Beckman Ultracentrifuge SW40Ti rotor. The supernatant was carefully removed and the pellet containing polyplexes was resuspended in 200 μ L of distilled water. An agarose gel electrophoresis of the suspension showed that more than 90% of polyplexes was in the pellet. ζ -Potential measurement was performed as described above after dilution in 1 mL of distilled water.

Cells and Cell Culture. Human hepatocarcinoma cells (HepG2 cells, ATCC HB 8056, Rockville, MA) were cultured in Eagle's minimal essential medium (MEM, Gibco) with 10% heat inactivated FBS. The immortalized human tracheal epithelial cell line (Σ CFTE29o- cells) was from a cystic fibrosis patient homozygous for the Δ F508 mutation and was kindly given by D. C. Gruenert (Cardio-vascular Research Institute, San Francisco, CA). Σ CFTE29o- cells were plated on tissue culture plasticware coated with fibronectin, collagen, and bovine serum albumin and grown in MEM supplemented with 10% non de complemented FBS (25). All culture media were supplemented with 2 mM L-glutamax (Gibco) and antibiotics (100 units/mL penicillin and 100 μ g/mL streptomycin from Gibco). Cells were grown at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air. Cells were harvested by treatment with PET (PBS with 0.02% (w/v) EDTA and 2.5 μ g/mL trypsin) at 37 °C for 5 min. Cells were mycoplasma free as evidence by bis-benzimidazole (Hoechst 33258) (26).

Uptake of Histidylated Polyplexes. HepG2 and Σ CFTE29o- cells (4×10^5 cells) were incubated for 3 h at 37 °C in the presence of fluorescein-labeled polyplexes at 10 μ g/mL pDNA made with either *F*-His₁₀₉-pLK (30 μ g) or *F*-AcHis₁₀₉-pLK (40 μ g) and chased 1 h in the presence of the respective unlabeled polyplexes at the same concentration. Cells were washed three time with PBS, trypsinized for 5 min at 37 °C and suspended in cold PBS. The cell-associated fluorescence was analyzed

¹ Abbreviations: AcHis, *N*-acetyl-histidyl residue; BOP, benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate; EDTA, ethylenediaminetetracetic acid; FITC, fluorescein isothiocyanate isomer I; *F*-, fluorescein-labeled; His, histidyl residue; MEBS, 1 mM 3-[*N*-morpholino] propane sulfonic acid, 0.1 mM EDTA, 10 mM NaCl; PBS, phosphate-buffered saline; pLK, poly-L-lysine; QELS, quasi-elastic laser light scattering; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

using a FACSort flow cytometer (Becton Dickinson, Grenoble, France) before and after a postincubation for 15 min at 4 °C in the presence of 50 μ M monensin (27, 28). Data were analyzed using Cell Quest Software (Becton Dickinson).

Polyfection and Luciferase Assay. Two days prior the transfection, cells were seeded at 2×10^5 cells per 4 cm² in a 12 wells plate and incubated at 37 °C in a humidified atmosphere (5% CO₂). The polyplexes were prepared as described above in 100 μ L of water containing 5% (w/v) glucose and then diluted with 900 μ L of culture medium containing either 0, 10, or 20% FBS. Cells were then incubated at 37 °C in 5% CO₂ with polyplexes. After 4 h, the medium containing polyplexes was removed and replaced by culture medium containing 10% FBS without polyplexes. Luciferase gene expression was measured at 72 h by monitoring the luminescence activity according to De Wet et al. (29). The medium was discarded, and cells were washed three times with PBS. The homogenization buffer (200 μ L of 8 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 1% Triton $\times$ 100, 15% glycerol, 25 mM Tris-phosphate buffer, pH 7.8) was poured into each well. After 15 min at 20 °C, the solution was recovered and spun down (5 min at 800 g). Ninety five μ L of a 2 mM ATP solution in the homogenization buffer without Triton $\times$ 100 was added to 60 μ L of supernatant and the solution was shaken with a vortex. The luminescence was recorded for 4 s in a Lumat LB 9501 luminometer (Berthold, Wildbach, Germany) upon addition of 150 μ L of a 167 mM luciferin solution in water. Measurements were done in duplicate. The luciferase activity was expressed as the number of relative light units (RLU) per cell. The number of RLU of 1 pg/mL of luciferase was 2000 under these conditions.

RESULTS

Polyplexes Formation. The condensation of pDNA in the presence of histidylated polylysines at low salt concentration was assessed by agarose gel electrophoresis, ζ potential and size measurements of polyplexes. The migration in the agarose gel showed that 10 μ g of pDNA mixed with 30 μ g of His₁₀₉-pLK or 30 μ g of AcHis₁₀₀-pLK was completely retarded and thus all the plasmid was complexed whereas pDNA was completely retarded with 10 μ g of pLK (Figure 1). At pH 7.4, polyplexes made with 30 μ g of His₁₀₉-pLK and 10 μ g of pDNA at a N:P of 3.4 (ratio of lysyl ϵ -amino plus histidyl α -amino groups to pDNA phosphates) were positively charged and exhibited a ζ potential of $+18 \pm 2$ mV (Table 1). Increasing the amount of polymer did not increase the ζ potential. Polyplexes made with 30 μ g of polylysine at the same N:P of 3.4 (ratio of lysyl ϵ -amino groups to pDNA phosphates) exhibited a similar ζ potential ($+17$ mV) which increased to $+22 \pm 3$ mV with 40 μ g of pLK. Polyplexes made with 40 μ g of AcHis₁₀₀-pLK (N:P, lysyl ϵ -amino groups to DNA phosphates ratio of 2.3) exhibited a ζ potential of $+14 \pm 3$ mV close to that of polyplexes made with His₁₀₉-pLK at a N:P of 3.4 (Table 1). QELS measurements showed that polyplexes made with His₁₀₉-pLK gave homogeneous particles of mean diameter 110 ± 15 nm under maximal condensation which were slightly bigger than those made with pLK (Table 1). AcHis₁₀₀-pLK formed larger polyplexes (165 ± 15 nm) than His₁₀₉-pLK indicating that with the latter the α -amino groups interacted with pDNA phosphates and induced a better condensation. These results indicated that 10 μ g of pDNA was completely condensed and saturated with 30 μ g of His₁₀₉-pLK. In water, the presence of histidyl as well as *N*-acetyl-histidyl residues on

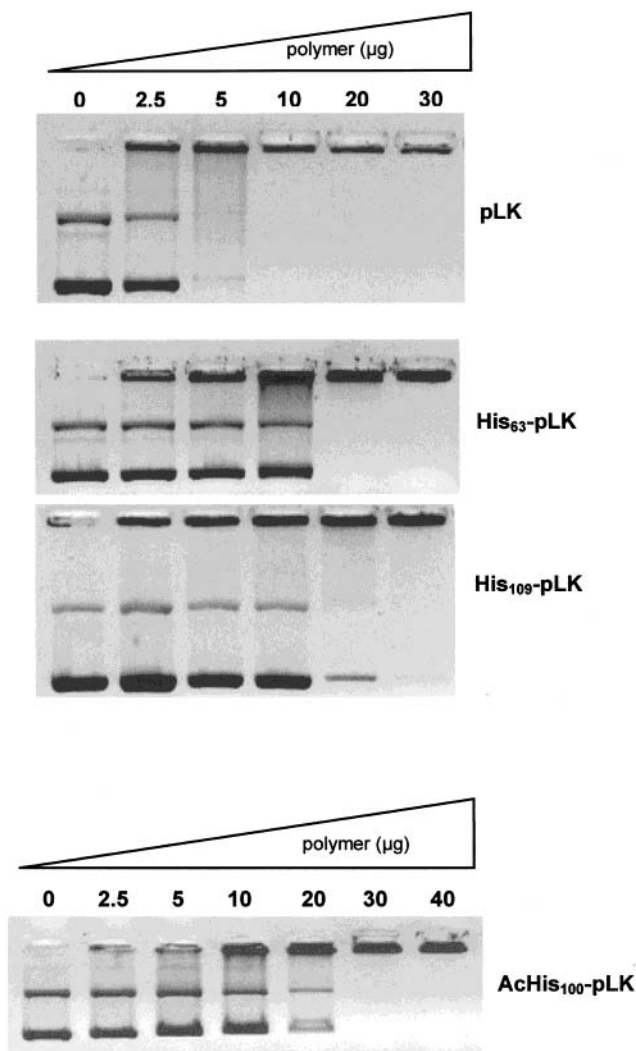


Figure 1. Agarose gel electrophoresis of polyplexes. Various amounts of cationic polymer expressed in micrograms per milliliter (μ g/mL) were mixed with 10 μ g of plasmid in 60 μ L of distilled water. After 30 min incubation at 20 °C, 20 μ L of each sample was analyzed by electrophoresis for 2 h under 80 V/cm, through a 0.6% agarose gel containing ethidium bromide (1 μ g/mL of gel) in Tris-borate-EDTA buffer (95 mM Tris, 89 mM boric acid, and 2.5 mM EDTA), pH 8.6.

polylysine increased 2-fold the global charge of polyplexes ($+33$ mV). The enhancement of the ζ potential due to the presence of protonated imidazoles shows that protonated imidazoles do not interact with pDNA phosphates probably because of their distance between the polymer backbone.

Effect of the Salt Concentration on the Polyplexes Size. We have investigated the influence of the salt concentration on the size of histidylated polyplexes because it is known that at physiological salt concentration (i.e., 0.15 M NaCl) pLK/DNA complexes form aggregates. QELS measurements showed that in distilled water and water containing 5% glucose, the size of polyplexes was 120 ± 10 and 105 ± 15 nm, respectively (Figure 2). Small polyplexes of 100 nm were also obtained after dilution in MEBS, pH 7.2, or 5 mM Hepes, pH 7.0. However, aggregates appeared as soon as the salt concentration was 25 mM NaCl and large aggregates of 800 nm were formed at 0.15 M NaCl. Large aggregates were also observed when the polyplexes were prepared at physiological salt concentration in 0.15 M NaCl serum-free medium.

Table 1. Size and ζ Potential of Histidylated Polyplexes^a

	polymer/pDNA weight ratio ($\mu\text{g}/\mu\text{g}$)					
	ζ potential (mV)			size (nm)		
	2	3	4	2	3	4
His ₁₀₉ -pLK	+17.5 $\pm$ 3 (+32)*	+18 $\pm$ 2 (+33)	+17 $\pm$ 3 (+34)	140 $\pm$ 10	110 $\pm$ 10	110 $\pm$ 10
AcHis ₁₀₀ -pLK	-18 $\pm$ 2 (+32)	-5 $\pm$ 3 (+34)	+14 $\pm$ 3 (+37)	179 $\pm$ 10	170 $\pm$ 10	165 $\pm$ 15
pLK	+10 $\pm$ 3 (+12)	+17 $\pm$ 3 (+18)	+22 $\pm$ 3 (+22)	100 $\pm$ 10	100 $\pm$ 10	90 $\pm$ 10

^a Polyplexes were prepared by mixing 10 μg of pDNA with different amounts of cationic polymers in 100 μL of water containing 5% (w/v) glucose. The mixture was kept for 30 min at 20 $^{\circ}\text{C}$ and diluted with 900 μL of either 5 mM Hepes buffer, pH 7.4, or (*) water containing 5% glucose. Size and ζ potential measurements were performed by using the ZetaSizer 3000.

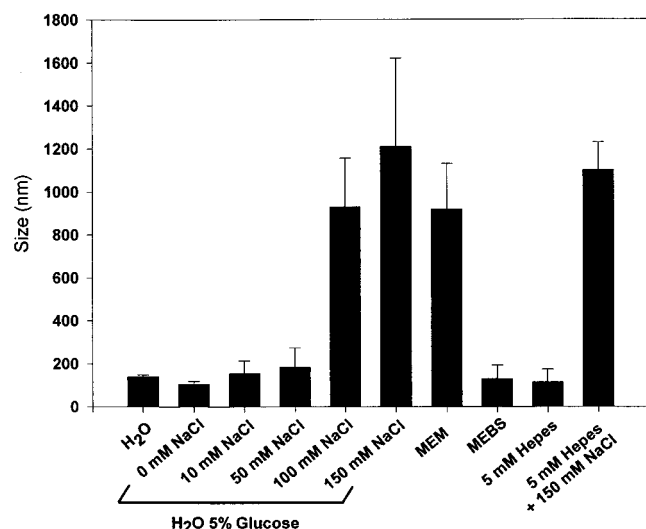


Figure 2. Effect of the salt concentration on the polyplexes size. pDNA/His-pLK polyplexes (10/30; $\mu\text{g}/\mu\text{g}$) were prepared in 100 μL of distilled water. After 30 min at 20 $^{\circ}\text{C}$, they were diluted with 900 μL of either distilled water, water containing 5% glucose and various NaCl concentration, serum-free MEM or other buffers. Polyplexes size was then determined after 1 h by QELS measurements with the ZetaSizer 3000.

Effect of Serum on the Size and the Charge of Polyplexes. As shown above, the size of polyplexes grown up rapidly in the absence of serum and formed aggregates of 1–3 μm within 30 min (Figure 3). In contrast, the size of polyplexes was stabilized at 250 ± 10 and at 100 ± 10 nm when polyplexes prepared at low salt concentration were diluted 10 times in 0.15 M NaCl buffer containing 1 and 10% serum, respectively (Figure 3). The hydrodynamic diameter of polyplexes was further reduced to 70 ± 5 and 50 ± 5 nm in the presence of 20 and 50% serum, respectively. This inhibition of the aggregation and the reduction of the size of polyplexes in the presence of serum was also observed with AcHis-polyplexes but their size reached only 90 ± 10 nm in the presence of 50% serum (Figure 3). These data indicate that serum anionic components bind to the surface of cationic polyplexes and prevent hydrophobic contacts between the particles and/or modify the global surface charge allowing repulsion between particles. First of all, we found that the size of polyplexes decreased and reached a minimum of 375 nm in the presence of 10 mg/mL serum albumin a protein present in large quantity in the serum (Figure 3, inset). The size was bigger than that obtained in the presence of serum suggesting that other proteins interacted with polyplexes to form particles of 70 nm. Then, we have performed SDS-PAGE analysis of polyplexes after incubation in the presence of bovine fetal serum and isolation by ultracentrifugation

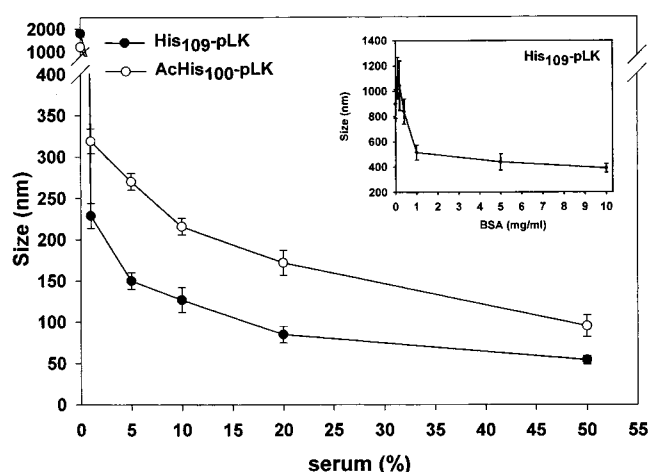


Figure 3. Influence of fetal bovine serum (FBS) on the polyplexes size. pDNA/His₁₀₉-pLK (10/30; $\mu\text{g}/\mu\text{g}$) and pDNA/AcHis₁₀₀-pLK (10/40 $\mu\text{g}/\mu\text{g}$) polyplexes were prepared in 100 μL of H₂O 5% glucose. After 30 min at 20 $^{\circ}\text{C}$, they were diluted with 900 μL of 0.15 M NaCl 5 mM Hepes buffer, pH 7.4 containing various amounts of FBS and the solution was kept 1 h at 20 $^{\circ}\text{C}$. QELS measurements were performed upon 10 times dilution of polyplexes at a final serum concentration less than 2.5% (v/v) in order to prevent any significant light scattering signal coming from serum components. (Inset) Influence of bovine serum albumin (BSA) on the polyplexes size. pDNA/His₁₀₉pLK polyplexes were diluted 10 times in 0.15 M NaCl 5 mM Hepes buffer, pH 7.5, containing various amount of BSA.

on sucrose cushion. We found that several major serum proteins of 66 (probably serum albumin), 59, 52, 45, and 27 kDa molecular mass (Figure 4, lane j) were associated with both histidylated polyplexes (Figure 4, lanes d–g). In addition, there were several other proteins with high, medium (between 30 and 45 kDa) and low (10, 14, and 16 kDa) molecular mass present in low quantity in the serum that were more strongly associated with His-polyplexes (Figure 4, lanes d and e). The acetylation of the α -amino group of histidyl residues reduced drastically the binding of all serum proteins (Figure 4, lanes f and g).

The global surface charge of polyplexes incubated with 10 and 20% serum was also investigated after their isolation by ultracentrifugation on sucrose cushion. His- and AcHis-polyplexes exhibited a negative ζ potential of -25 and -15 mV, respectively (Figure 5).

Influence of pH Medium on Polyplexes Size. The size of His-polyplexes increased from 230 nm at pH 7.5 to 350–430 nm at pH 9.5 due to a decrease of the interactions between His-pLK and pDNA after deprotonation of α -amino groups (pK close to pH 7.0) when the pH of the medium was above 7.5 giving less condensed polyplexes (Figure 6). The size decreased to 100 nm at

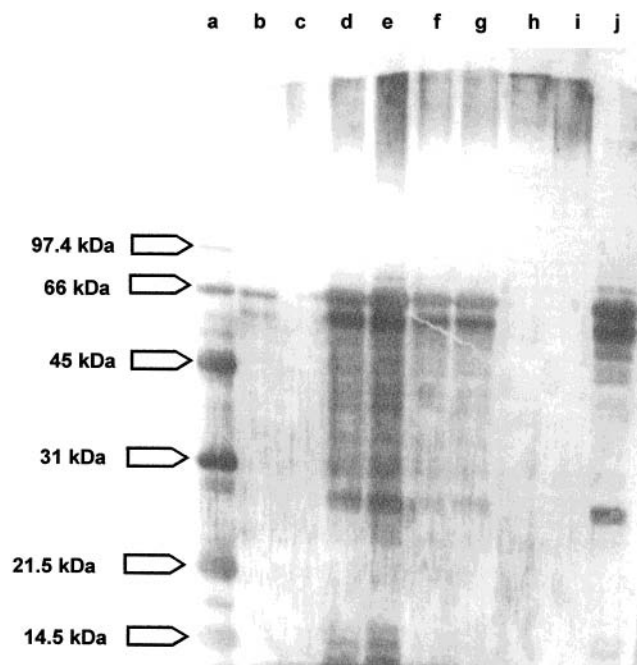


Figure 4. SDS-PAGE analysis of serum proteins bound to His- and AcHis-polyplexes. Polyplexes containing 10 $\mu\text{g/mL}$ pDNA in 100 μL of H_2O 5% glucose were diluted in 5 mM Hepes buffer, pH 7.4, containing filtered bovine fetal serum. After 30 min, polyplexes were isolated by ultracentrifugation on 50% (w/v) sucrose cushion and analyzed under SDS-PAGE with visualization by a silver staining. (Lane a) Molecular weight markers; (lane b) 100 times diluted filtered bovine fetal serum; (lane c) His-polyplexes without serum; (lane d) His-polyplexes with 10% serum; (lane e) His-polyplexes with 20% serum; (lane f) AcHis-polyplexes with 10% serum; (lane g) AcHis-polyplexes with 20% serum; (lane h) histidylated polylysine; (lane i) plasmid DNA; (lane j) serum without polyplexes.

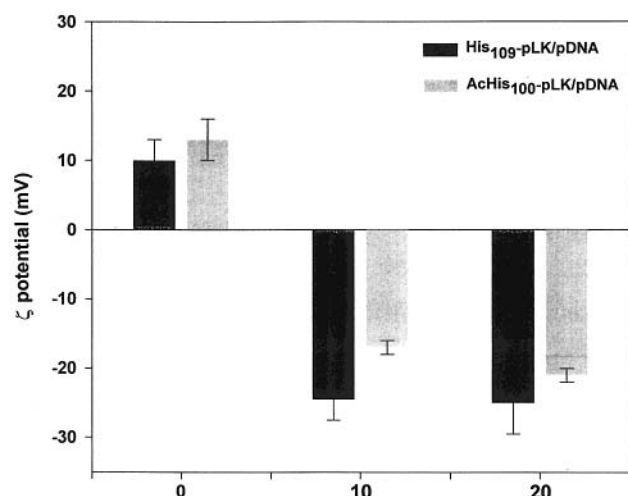


Figure 5. Influence of serum on polyplexes charge. (Dark bar) pDNA/His₁₀₉-pLK and (shaded bar) pDNA/AcHis₁₀₉-pLK complexes were prepared in 100 μL of H_2O . After 30 min at 20 $^{\circ}\text{C}$, polyplexes were diluted in 900 μL of 5 mM Hepes buffer, pH 7.4, in the absence or the presence of bovine fetal serum and the solution kept 30 min at 20 $^{\circ}\text{C}$. Polyplexes were then isolated by ultracentrifugation on 50% (w/v) sucrose cushion. ζ potential measurements were performed on resuspended polyplexes.

pH 6.5 and remained stable at lower pH when the α -amino groups were protonated. In contrast, the size of AcHis-polyplexes (165 nm) did not depend on the pH of the medium.

Imidazole Protonation. The protonation of the imidazole in His₁₀₉-pLK, AcHis₁₀₀-pLK and histidine free base

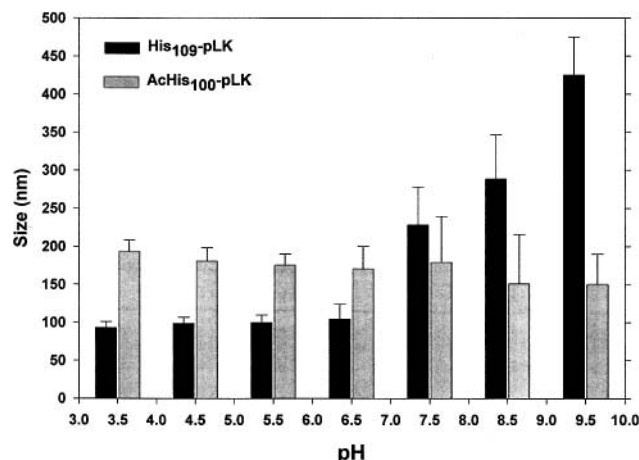


Figure 6. Influence of pH medium on the polyplexes size. pDNA (10 μg) was complexed with either (dark barr) His₁₀₉-pLK (30 μg) or (shaded barr) AcHis₁₀₀-pLK (40 μg) for 30 min in 100 μL of 5 mM Hepes buffer, pH 7.4, and diluted 10 times in 5 mM Hepes buffer, pH 7.4. The pH medium was then adjusted under pH meter by adding aliquots of either a 0.1 M HCl or a 0.1 M NaOH solution and the size of polyplexes was measured.

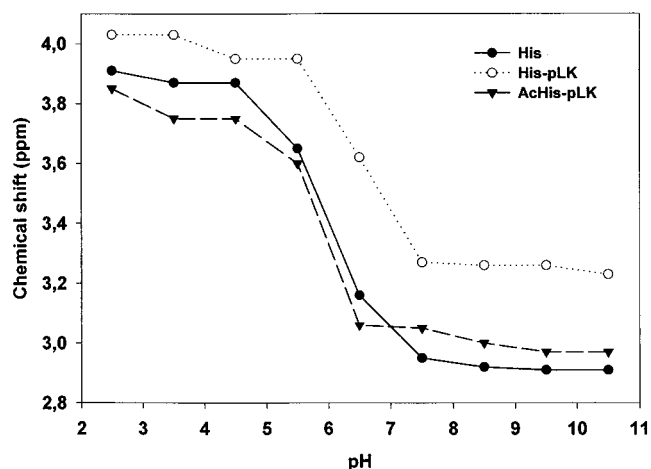


Figure 7. Imidazole protonation in histidylated polylysines. The chemical shifts of imidazole C2-H of either ($\circ$) His-pLK, (∇) AcHis-pLK, or ($\bullet$) histidine reflecting the charge state of the imidazole ring was measured by ^1H NMR spectroscopy in D_2O at 300 MHz as a function of the pH medium. Chemical shifts were determined downfield from the HDO peak at 4.75 ppm used as the internal standard.

was determined by ^1H NMR spectroscopy by monitoring the C2-H peak position versus the pH medium reflecting the charge state of the imidazole ring (30). The pK of the imidazole in histidine free base, His₁₀₉-pLK, and AcHis₁₀₀-pLK was found to be 5.8, 6.9, and 6.0, respectively (Figure 7). The protonation of the imidazole of a *N*-acetyl-histidyl residue bound to polylysine had a pK similar to that of histidine free base. In contrast, the protonation occurred at a 1.1 pH unit higher than in histidine free base when the α -amino groups were free.

The imidazole protonation was also investigated in polyplexes by measuring their ζ potential at various pH. Measurements were performed with polyplexes made with both histidylated polylysines but only data from ζ potential values of those made with AcHis₁₀₀-pLK were used to evidence the protonation of the imidazole. The ζ potential values of polyplexes made with His₁₀₉-pLK did not only correspond to the imidazole protonation but also to that of the α -amino group which occurs between pH 7.0 and 6.5. The ζ potential of AcHis-polyplexes increased

Table 2. Uptake of Histidylated Polyplexes and Luciferase Activity into Σ CFTE290- and HepG2 Cells

polymer	FBS (%)	Σ CFTE290-			HepG2		
		cell fluorescence ^a		luciferase activity ^b	cell fluorescence ^a		luciferase activity ^b
		-Mo	+Mo		-Mo	+Mo	
His ₁₀₉ -pLK	0	902	1572	46	243	638	22
	10	363	643	109	140	248	28
	20	286	414	93	132	233	23
AcHis ₁₀₀ -pLK	0	1386	3867	35	565	2845	17
	10	946	1642	28	250	1642	23
	20	952	1478	24	216	1690	35

^a Mean of the cell-associated fluorescence (arbitrary units) measured by flow cytometry before (-Mo) and after (+Mo) a posttreatment with monensin. The cell-associated fluorescence was expressed relative to that of cells incubated with His₁₀₉-pLK/pDNA polyplexes.

^b Luciferase activity was expressed as the relative light unit (RLU) per cell.

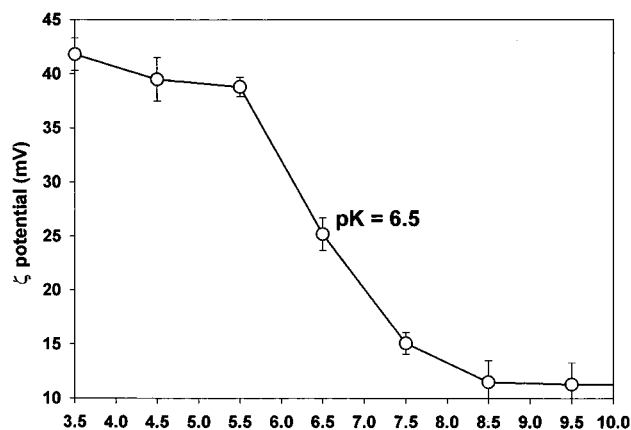


Figure 8. Imidazole protonation in *N*-acetyl-histidylated polyplexes. pDNA (10 μ g) was complexed with AcHis₁₀₉-pLK (40 μ g) in 200 μ L of 5 mM Hepes buffer, pH 7.4, the solution kept 30 min at 20 °C and then diluted 10 times in 5 mM Hepes buffer pH 7.4. The pH was adjusted under pH meter by adding aliquots of either a 0.1 M HCl or 0.1 M NaOH solution and the ζ potential of polyplexes was measured.

from +5 to +40 mV when the pH decreased from pH 7.5 to 5.0 as a consequence of the protonation of the imidazole (Figure 8). In polyplexes, the pK of the imidazole was estimated to 6.5 which was slightly higher than that found for the polymer in the absence of plasmid (pK = 6.0) (Figure 7).

Uptake of Histidylated Polyplexes. HepG2 and Σ CFTE290- cells were incubated with pDNA complexed with either *F*-His-pLK or *F*-AcHis-pLK. After 3 h, the medium was removed and cells were chased 1 h in the presence of unlabeled polyplexes. The cell-associated fluorescence was then analyzed by flow cytometry after a postincubation with the proton/sodium ionophore monensin to evidence the uptake of polyplexes. Monensin induces neutralization of acidic compartments and restores the fluorescence of fluorescein quenched in an acidic medium such as in endosomes and lysosomes (27, 28). The fluorescence intensity of both cell lines incubated in the absence of serum with His-polyplexes and AcHis-polyplexes was 2–3-fold higher in average than that of cells incubated in the presence of serum (Table 2). This showed that polyplexes aggregates generated in the absence of serum were more associated with the cells than the 100 nm stabilized polyplexes in the presence of serum. Indeed, the number of polyplexes molecules was increased on the surface of adherent cells because the aggregates sedimented rapidly in serum-free medium; in addition, their positive ζ potential favored their binding to sulfated proteoglycans of the plasma membrane. Conversely, the binding and the uptake by the cells of small polyplexes which exhibited a negative ζ potential were reduced in the presence of serum (Table 2). AcHis-

polyplexes were more associated with both cell lines than His-polyplexes probably because their size was bigger and their sedimentation faster than His-polyplexes. After a posttreatment with monensin, the cell-associated fluorescence of Σ CFTE290- cells incubated with both polyplexes increased 2-fold showing that some polyplexes were internalized in acidic compartments (Table 2). The cell-associated fluorescence of HepG2 cells incubated with *F*-AcHis-polyplexes was 5–7.8-fold higher after monensin posttreatment. This indicated that these polyplexes were internalized in more acidic compartments than in Σ CFTE290- cells or that a greater amount of polyplexes were internalized in the same compartments as in Σ CFTE290- cells.

Polyfection with Histidylated Polyplexes. The luciferase activity into the cell lines transfected with both histidylated polyplexes was similar in the absence of serum, but the luciferase activity was 2-fold greater in Σ CFTE290- cells (Table 2). The luciferase activity into Σ CFTE290- cells transfected with His-polyplexes in the presence of serum was 2-fold greater than that obtained in the absence of serum; it was also 4–5-fold greater than into cells transfected with AcHis-polyplexes (Table 2). This indicates that small polyplexes are more efficient in transfecting cells and that pDNA condensation is likely to play a role in the uptake and the intracellular processing of polyplexes which could depend on the cell type. Indeed, the luciferase activity into HepG2 cells transfected with both histidylated polyplexes was similar and did not depend on the presence of serum (Table 2).

DISCUSSION

The assembly of pDNA with histidylated polylysine at low ionic strength via both lysyl ϵ -amino and histidyl α -amino groups, gave homogeneous small particles with a positive ζ potential. As reported for polylysine and polyethylenimine (17–22), histidylated polyplexes showed high tendency to aggregate in 0.15 M NaCl and serum-free medium. In contrast, the polyplexes particles remained stable and kept a small size in 10% serum supplemented medium. This is due to the adsorption of serum proteins on the polyplexes surface which then prevents hydrophobic particle/particle interactions. Beside the major proteins of the serum including serum albumin, several other proteins with low molecular masses and molecular masses ranging between 30 and 45 kDa difficult to identify were strongly associated with His-polyplexes. They might be proteins with acidic pI such as α 1-fetoprotein (66 kDa), α 1-acid glycoprotein (41 kDa), apolipoproteins (A-I, 30 kDa; E, 34 kDa; A-II, 8.5 kDa) and α 1-microglobulin (31 kDa). The binding of these serum proteins probably occurred via interactions with protonated imidazole of His-polyplexes. Indeed, in agreement with the respective protonation of imidazole, their

binding at pH 7.4 on AcHis-polyplexes was reduced because the number of protonated imidazole in AcHis-polyplexes was lower than in His-polyplexes. While the ζ potential of both polyplexes was similar, a different distribution of the charge on the surface of polyplexes could also explain a decrease of the binding of serum proteins on AcHis-polyplexes. By using the same protocol but with plasma which instead of serum contains high molecular weight proteins of the coagulation cascade, pLK/DNA complexes were found to bind proteins with molecular masses of 66 (probably serum albumin) and 120 kDa (31, 32). Polyplexes with polyethylenimine were found to bind high molecular mass plasma proteins (up to 150 kDa) such as IgM, fibronectin, fibrinogen, complement C3 and albumin, but in this case the isolation of polyplexes was carried out by filtration (33).

In addition to the inhibition of the polyplexes aggregation, the binding of serum proteins lead to polyplexes with a net negative ζ potential (-25 mV). This reversion of the global charge of polyplexes decreased their uptake by the cells but did not decrease their transfection efficiency; it even increased 2-fold for Σ CFTE29o- cells. This suggests that a preincubation of polyplexes in autologous serum could be a way to decrease the non-specific cell binding via the negatively charged sulfated proteoglycans (34) and might be suited to overcome the activation of the alternative complement pathway by cationic particles (35). Identification of serum proteins that bind strongly to His-polyplexes could be useful for *in vivo* gene targeting.

Histidylated polylysines contain several protonable imidazoles which are supposed to induce membrane destabilization after protonation in an acidic medium. The protonation of the imidazoles appears more sensitive to the presence of the α -amino group of the histidyl residues than that of the ϵ -amino group of the lysyl residues on the polymer. The protonation of 50% of the imidazoles of His-pLK occurs at pH 6.9 whereas that of AcHis-pLK occurs at pH 6.0. The imidazole protonation is also modified when the polymer is complexed with pDNA; in AcHis-polyplexes it occurs at pH 6.5, at a 0.5 pH unit higher than in AcHis-pLK. This elevation of the pK is consistent with that reported for polyhistidine/DNA complexes (36) and for Poly(Lys,His) copolymer/DNA complexes (37). In the latter protonated imidazoles were found to interact with DNA phosphates. Therefore, the imidazole protonation of histidylated polyplexes can surely occur in endocytic vesicles following acidification of their lumen from pH 7.4 to 6.0 and can allow the destabilization of vesicles to deliver pDNA into the cytosol. However, the mechanism leading to pDNA delivery into the cytosol in the presence of His-pLK is not yet known. Membrane could be disrupted via a direct interactions of His-pLK as for H5WYG peptide (14). This peptide (GLFHAIAHFIHGGWHGLIHGWYG) containing five histidines permeabilizes the plasma membrane of cells at pH 6.1 with a midpoint at pH 6.8. The involvement of the imidazole protonation in the pDNA delivery in the cytosol is also supported by confocal microscopy data showing that histidylated oligolysines induce a cytosolic delivery of antisense oligonucleotides after their uptake by cells via acidic vesicles (38). In addition, the effect of H5WYG, histidylated oligolysines and histidylated polylysines are inhibited in the presence bafilomycin A1 an inhibitor of the ATPase proton pump of endosomes. However, the imidazole protonation of histidylated polyplexes could also buffer the lumen of vesicles containing polyplexes and induce swelling and

destabilization of vesicles as for chloroquine and polyethylenimine.

In conclusion, this study shows that the presence of free α -amino group on the histidyl residues on His-pLK allows the formation of small and soluble polyplexes. It shifts the imidazole protonation up to a neutral pH which is in favor for a destabilization of very early endocytic vesicles. The adsorption of serum proteins especially on His-polyplexes which (i) renders the particles negatively charged, (ii) reduces their nonspecific binding on the cells, (iii) stabilizes particles with a small size, and (iv) does not affect their transfection efficiency, points out that selective proteins recognized by a given cell type could be used for *in vivo* gene targeting.

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Comparison of the Macromolecular MR Contrast Agents with Ethylenediamine-Core versus Ammonia-Core Generation-6 Polyamidoamine Dendrimer

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Two novel macromolecular MRI contrast agents based upon generation-6 polyamidoamine dendrimers (G6) of presumed similar molecular size, but of different molecular weight, were compared in terms of their blood retention, tissue distribution, and renal excretion. Two G6s with either ammonia core (G6A) or with ethylenediamine core (G6E), which possessed 192 and 256 exterior primary amino groups, respectively, were used. These dendrimers were reacted with 2-(*p*-isothiocyanatobenzyl)-6-methyl-diethylenetriaminepentaacetic acid (1B4M). The G6–1B4M conjugates were reacted with ¹⁵³Gd for studying biodistribution and blood clearance or Gd(III) for the MRI study. 3D-micro-MR angiography of the mice were taken with injection of 0.033 mmol of Gd/kg of G6A–(1B4M-Gd)₁₉₂ or G6E–(1B4M-Gd)₂₅₆ using a 1.5-T superconductive MRI unit. Although numerous fine vessels of ~100 μm diameter were visualized on subtracted 3D-MR-angiography with both G6A–(1B4M-Gd)₁₉₂ and G6E–(1B4M-Gd)₂₅₆, ¹⁵³Gd-labeled saturated G6E–(1B4M)₂₅₆ remained in the blood significantly more than ¹⁵³Gd-labeled saturated G6A–(1B4M)₁₉₂ at later than 15 min postinjection (*p* < 0.01). In addition, G6E–(1B4M-Gd)₂₅₆ visualized these finer vessels longer than G6A–(1B4M-Gd)₁₉₂. The G6A–(1B4M-Gd)₁₉₂ showed higher signal intensity in the kidney on the dynamic MR images and brighter kidney images than G6E–(1B4M-Gd)₂₅₆. In conclusion, the G6A–(1B4M-Gd)₁₉₂ was observed to go through glomerular filtration more efficiently than G6E–(1B4M-Gd)₂₅₆ resulting faster clearance from the blood and higher renal accumulation, even though both of G6–1B4M conjugates have almost similar molecular size and same chemical structure. In terms of the ability of intravascular contrast agents, G6E–(1B4M-Gd)₂₅₆ was better due to more Gd(III) atoms per molecule and longer retention in the circulation than G6A–(1B4M-Gd)₁₉₂.

INTRODUCTION

The starburst polyamidoamine (PAMAM)¹ dendrimers are a new class of highly branched spherical polymers that are highly soluble in aqueous solution and have a unique surface of primary amino groups (1, 2). Compared with many other types of dendritic macromolecules that recently have been synthesized, PAMAM dendrimers are the only class of macromolecules that are undispersed and show high positive charge densities restricted to the surface of the molecule (3). The defined structure and large number of available surface amino groups of PAMAM dendrimers have led to their use as substrates for the attachment of many chelating agents to single

antibody molecule (2, 4–6) and in the preparation of novel MRI contrast agents (7, 8).

Two types of macromolecular contrast agents for MRI have been reported. One was albumin conjugated with diethylenetriaminepentaacetic acid (DTPA) [albumin-(DTPA-Gd)_{30–34}], originally described by Ogan et al. (9). The other was fundamentally a polyamine conjugated with multiple chelates [DTPA derivatives or 1,4,7,10-tetraazacyclododecanetetraacetic acid (DOTA) derivatives]. Although polylysine has been used for the core of contrast agents among the various polyamines (10, 11), PAMAM dendrimers have recently been preferred as an alternative to polylysine (10–12). Wiener et al. and we reported the preferable use of generation-6 PAMAM dendrimers (G6) with ammonia core (G6A) conjugated with DTPA derivative as an intravascular macromolecular MR contrast agent (7, 13). G6 with an ethylenediamine core (G6E) have almost similar molecular size and possesses more amines than G6A (256 for G6E compared with 192 for G6A) (Figure 1). Therefore, G6E fully conjugated with DTPA can potentially complex and sequester 1.33 times as many metal atoms as G6A.

In this study, the *in vivo* pharmacokinetics of ¹⁵³Gd-labeled G6E–(1B4M)_x were evaluated in the mice and compared with those of ¹⁵³Gd-labeled G6A–(1B4M)_x. In addition, the differences between G6E–(1B4M-Gd)₂₅₆

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¹ Abbreviations: PAMAM, polyamidoamine dendrimer; G6, generation-6 PAMAM dendrimer; 1B4M, 2-(*p*-isothiocyanatobenzyl)-6-methyl-diethylenetriamine-pentaacetic acid; DOTA, 1,4,7,10-tetraazacyclododecane tetraacetic acid; MIP, maximum intensity protection.

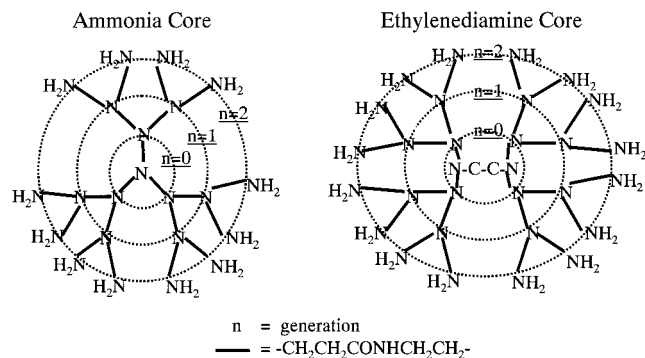


Figure 1. Scheme of the two core types of PAMAM dendrimers.

and G6A-(1B4M-Gd)₁₉₂ as MR contrast agents were evaluated.

EXPERIMENTAL PROCEDURES

PAMAM Dendrimers. The generation-6 PAMAM dendrimer with an ammonia core (G6A) (Polysciences, Warrington, PA) that possesses 192 amino groups and has a molecular mass of 43 451 Da and the generation-6 PAMAM dendrimer (G6E) (Aldrich Chemical Co., Milwaukee, WI) with an ethylenediamine core that possesses 256 reactive amino groups and has a molecular mass of 57 991 Da were used.

Conjugation of Chelates to G6 Dendrimers. The dendrimers were concentrated to ~5 mg/mL and diafiltered against 0.1 M phosphate buffer at pH 9. The G6A and G6E dendrimers were reacted with a 192 or a 256-fold molar excess of 2-(*p*-isothiocyanatobenzyl)-6-methyldiethylenetriaminepentaacetic acid (1B4M) at 40 °C, respectively, and maintained at pH 9 with 1 M NaOH for 24 h. Another additional equal amount of the 1B4M was added to each after 24 h as a solid. The resulting preparations were purified by diafiltration using a Centricon 30 (Amicon Co., Beverly, MA) for either G6A or G6E. This resulted in 99.2 ± 1.1 and 99.8 ± 1.5% of the amine groups on the G6A and G6E dendrimers reacting with the 1B4M for four different preparations of each core, respectively, as determined by ¹¹¹In (Medipysics, Takarazuka, Japan) labeling of the reacted samples as previously described (14). In brief, ~500 000 cpm of ¹¹¹In acetate was added to ~100 µg/10 µL of the conjugate samples before diafiltration and incubated in 0.2 M acetate buffer for 15 min at room temperature. Then, the bound fractions to the conjugates and free 1B4M were separated using a PD-10 column (Pharmacia) and counted. After which, the numbers of 1B4M chelates bound to both G6A and G6E were calculated, respectively, from the count of the radioactivity in the bound fraction to the conjugates.

To evaluate the effect of fully saturating the amino groups on the surface of G6s with 1B4M chelates on their biodistribution properties, G6A and G6E were each reacted with a 4-fold molar excess of 1B4M under the same conditions described above. Finally, after purification as related above we isolated the G6A-1B4M₂ and G6E-1B4M₂ conjugates. Assaying again by ¹¹¹In confirmed saturation of the surface of the dendrimers with the 1B4M chelating agent.

Radiolabeling of G6-1B4Ms. Carrier-free ¹⁵³GdCl₃ was purchased from NEN DuPont (Boston, MA) and ~750 µg (1 µmol) of each G6-1B4M conjugate was reacted with 20 µCi of ¹⁵³Gd citrate in 0.3 M citrate buffer at pH 5 for 30 min at room temperature. To remove any nonincorporated free radiometal, 10 µL of 0.5 M EDTA

(Nakarai, Tokyo, Japan) was added and the product was purified by PD-10 column (Pharmacia, Uppsala, Sweden), which was eluted with PBS, (pH 7.4). The resulting purified materials were referred to as "unsaturated" because on the average they had one radiometal (¹⁵³Gd) per total molecule.

To evaluate the effect on biodistribution by saturation of the 1B4M chelating agents with metals, the prepared G6-1B4M conjugates were each saturated with nonradioactive gadolinium. The preparation method was the same as described above, except that a 3-fold molar excess of nonradioactive Gd(III) citrate (Nakarai, Tokyo, Japan) (3 µmol) to conjugated 1B4M was added to the reaction mixture and incubated for 1 h before addition of EDTA and subsequent column purification. The radiolabeling yields were 95–97% and from ~40%, respectively, for the unsaturated and saturated preparations.

Preparation of Contrast Agent for MRI with nonRadioactive Gd(III). Approximately ~3 mg of each G6-1B4M conjugate (containing 4 µmol of 1B4M) were mixed with 6 µmol of nonradioactive Gd(III) citrate in 0.3 M citrate buffer for 2 h at 40 °C. The excess Gd in each preparation was removed by diafiltration using the Centricon 30 (Amicon Co.) for G6A and G6E while simultaneously changing the buffer to 0.05 M PBS. The purified samples were diluted to 0.5 mL with 0.05 M PBS and 100 µL was used as the MRI contrast agent per mouse. A replacement assay for three different preparations of each conjugate using ¹⁵³Gd determined that 81.9 ± 1.9 and 82.5 ± 1.8% of the 1B4M on the G6D-1B4M conjugates were indeed chelating Gd(III) atoms, respectively. In brief, ~100 000 cpm of ¹⁵³Gd was added with 0.1 µmol of nonradioactive Gd(III) to 5 µL of the injected samples and incubated in 0.5 M citrate buffer for 2 h at 40 °C, after which the bound and unbound fractions to the conjugates were separated using a PD-10 column (Pharmacia) and counted. After which, the number of Gd atoms replaced from the gadolinium citrate to 1B4M was calculated from the count of the radioactivity in the bound fraction. Then, the number of available 1B4M chelates for binding Gd atoms on the conjugates was calculated.

Biodistribution of ¹⁵³Gd-Labeled G6-1B4Ms. Six groups of nude mice (*n* = 4 in each group) were each injected with one of the following preparations: 0.8 µCi/200 µL of ¹⁵³Gd-labeled unsaturated G6A-(1B4M)₁₉₂ or G6E-(1B4M)₂₅₆; 0.5 µCi/200 µL of ¹⁵³Gd-labeled saturated G6A-(1B4M)₁₉₂ or G6E-(1B4M)₂₅₆; 0.5 µCi/200 µL of ¹⁵³Gd-labeled saturated G6A-(1B4M)₂ or G6E-(1B4M)₂. All studies were approved by the Animal Care Committee of Kyoto University. The mice were euthanized 15 min after the injection of ¹⁵³Gd-labeled preparation. Biodistribution studies were carried out, and the data were expressed as the percent injected dose per gram (% ID/g) of tissue and blood-to-normal tissue ratio. The blood was drawn (~0.7 mL) by the cardiac puncture. In the case of bone, the bone marrow was included.

Blood Clearance of ¹⁵³Gd-Labeled Saturated G6-1B4Ms. Two groups of nude mice (*n* = 4 in each group) were carefully injected with 3.5 µCi/200 µL of ¹⁵³Gd-labeled saturated G6A-(1B4M)₁₉₂ or G6E-(1B4M)₂₅₆ from the proximal portion of the left tail vein without any subcutaneous leakage. All mice received a small cut on their distal or middle portion of the right tail vein and blood samples were collected with a 5-µL micropipet 2, 6, 15, 30, 60, 120, and 240 min postinjection of ¹⁵³Gd-labeled preparations. The data were expressed as the

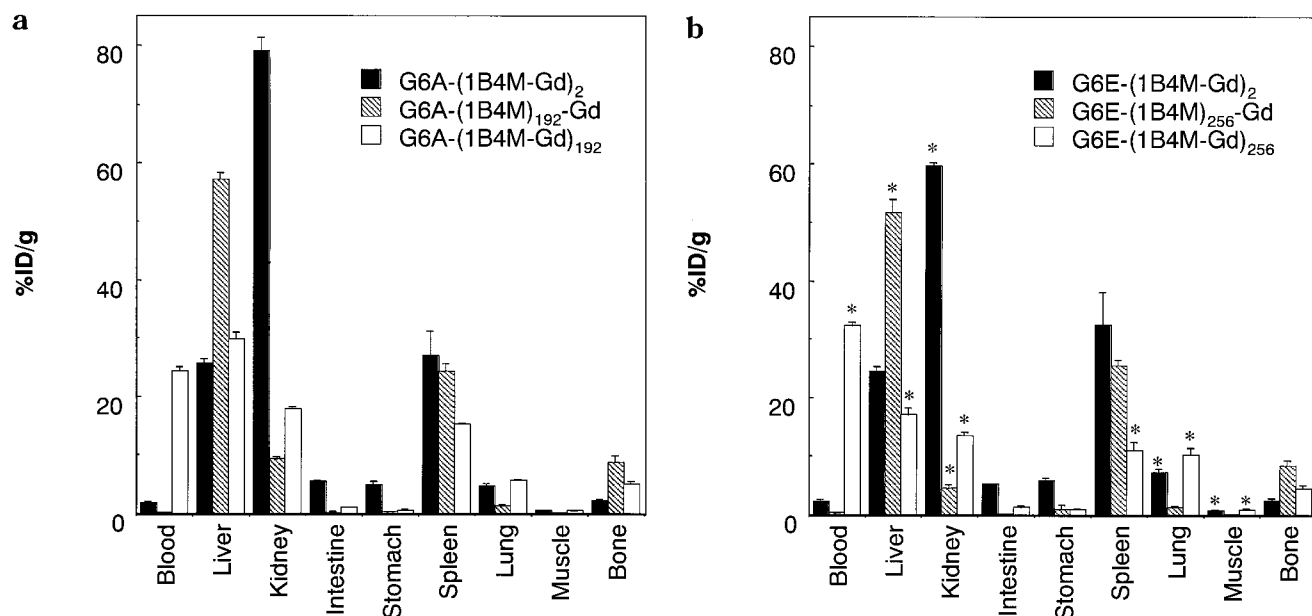


Figure 2. Biodistribution of ^{153}Gd -labeled unsaturated or saturated preparations of G6A [G6A-(1B4M-Gd)₂ (■), G6A-(1B4M)₁₉₂-Gd (cross-hatched bar), or G6A-(1B4M-Gd)₁₉₂ (□)] (a) and ^{153}Gd -labeled unsaturated or saturated preparations of G6E [G6E-(1B4M-Gd)₂ (■), G6E-(1B4M)₂₅₆-Gd (cross-hatched bar), or G6E-(1B4M-Gd)₂₅₆ (□)] (b) in normal nude mouse at 15 min after injection. The data are expressed as the mean percentages of injected dose per gram of normal tissues and standard deviation ($n = 4$ or 5). The asterisks indicate significant differences ($p < 0.01$) compared with G6A.

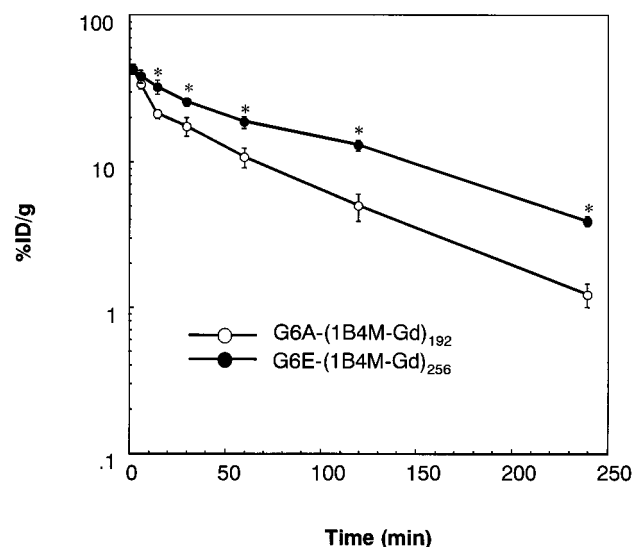


Figure 3. Blood clearance of ^{153}Gd -labeled saturated G6A-(1B4M-Gd)₁₉₂ (open circle) or G6E-(1B4M-Gd)₂₅₆ (closed circle) in normal nude mouse 2, 6, 15, 30, 60, 120, and 240 min postinjection. The data are expressed as the mean percentages of injected dose per gram of blood and standard deviation ($n = 4$). The asterisks indicate significant differences ($p < 0.01$) compared with G6A.

percent injected dose per gram (% ID/g) of blood, and then the data of each mouse were fitted by the single-exponential curve, and the half-life of each preparation from the blood was calculated.

Excretion and Body Retention of ^{153}Gd -Labeled Saturated G6D-1B4M Conjugates. Two groups of nude mice ($n = 3$ in each group) were injected with 2 $\mu\text{Ci}/200 \mu\text{L}$ of either ^{153}Gd -labeled saturated G6A-(1B4M)₁₉₂ or G6E-(1B4M)₂₅₆. The mice were put in a metabolic cage for 2 days and their urine and feces were serially collected 3, 10, 24, and 48 h postinjection. The mice were euthanized and the amount of ^{153}Gd in the carcass was measured. The data were expressed as percentage of injected dose (% ID).

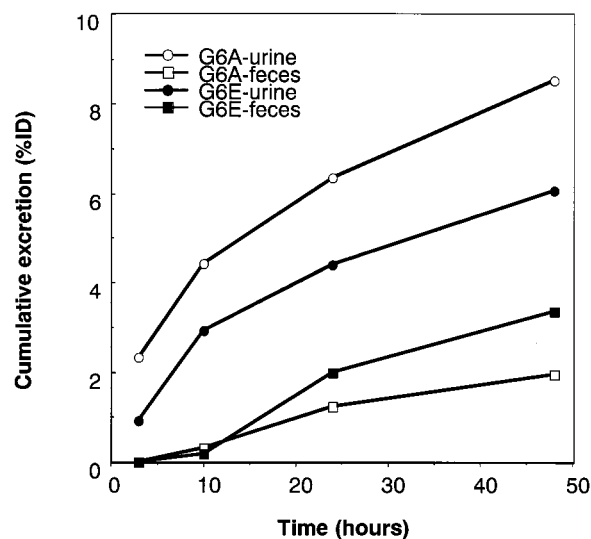


Figure 4. Urinary (circle) and fecal (square) excretion of ^{153}Gd -labeled G6A-(1B4M-Gd)₁₉₂ (open circle/square), or G6E-(1B4M-Gd)₂₅₆ (closed circle/square). The data are expressed as the mean percentages of injected dose of three mice, which put in a metabolic cage together.

3D-micro-MR Angiography of Mice. MR angiography of the mice were taken with injection of 0.033 mmol of Gd/kg of G6D-A-(1B4M-Gd)₁₉₂ (175 kDa) or G6D-E-(1B4M-Gd)₂₅₆ (240 kDa) using a 1.5-T superconductive magnet unit (Signa, General Electric Medical System, Milwaukee, WI). Six female 8 week-old balb/c nu/nu mice of 18–21 g body weight in each group were used and each contrast agent was prepared at least four separate times for these imaging studies. All images were obtained with dual phased-array 3-in. round surface coils fixed at 3-cm intervals by an in-house constructed mouse and coil holder. The mice were anesthetized with 1.15 mg of sodium pentobarbital (Dainabot, Osaka, Japan) and placed at the center of the coils. The 3D-fast spoiled gradient echo (FSPGR; TR/TE 150/4.2; flip angle 60°; scan time 1 min, 48 s) with chemical fat-suppression

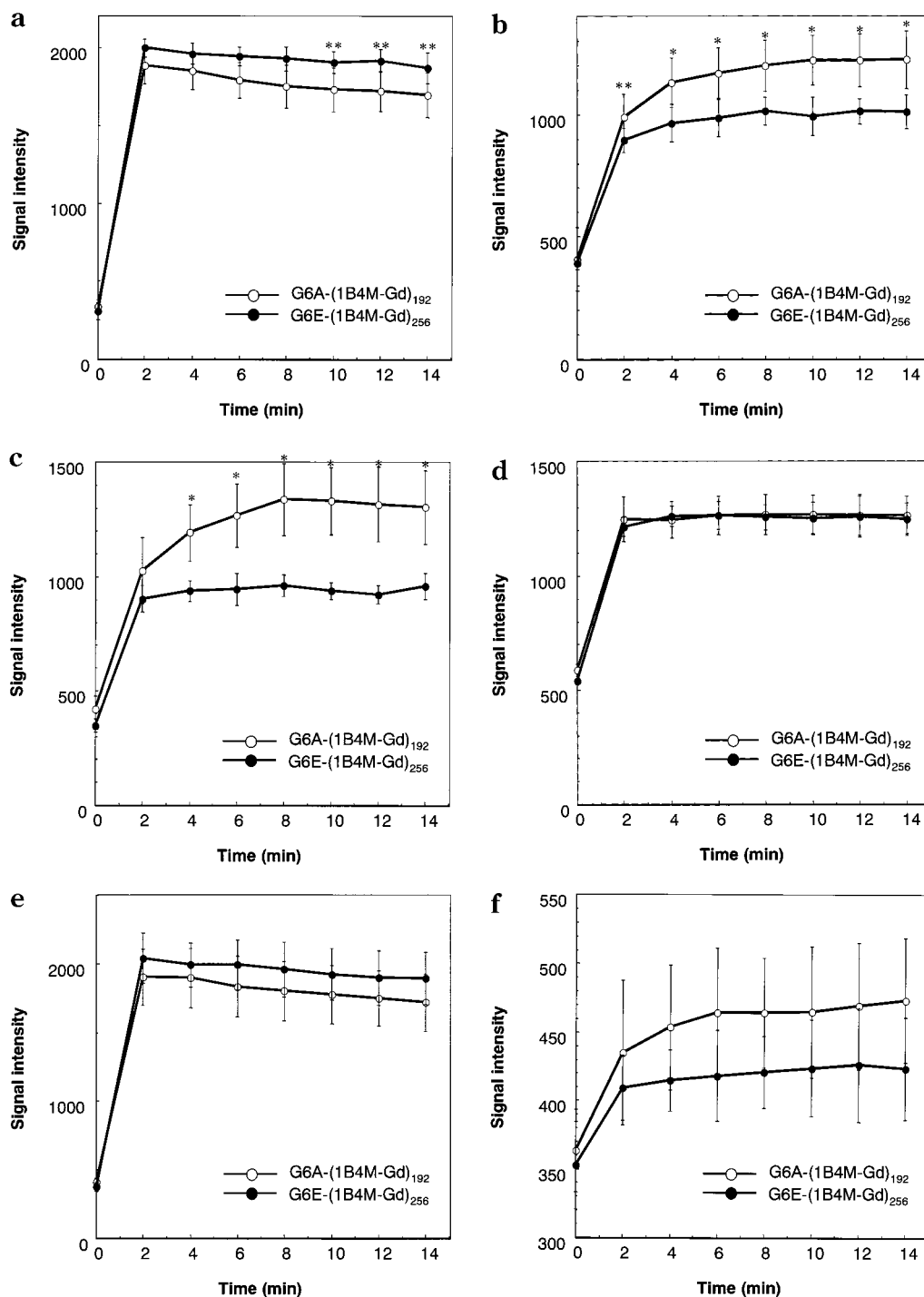


Figure 5. Plots of the signal intensity in the left ventricle of the heart (a), the cortex (b) and medulla (c) of the kidney, the inferior vena cava (d), the liver (e), and the muscle (f) obtained from the contrast-enhanced dynamic MRI of mice with 0.033 mmol/Kg of G6A-(1B4M-Gd)₁₉₂ (open circle) or G6E-(1B4M-Gd)₂₅₆ (closed circle). The single and double asterisks indicate significant differences ($p < 0.01$ and $p < 0.05$, respectively).

technique were used for all mice from 0 to 14 min after injection of the contrast agents for all mice. The coronal images were reconstructed with 2-mm section thickness without gaps. The FOV was 8×6 cm and the size of matrix was 256×192 . The regions of interest were set to be the ventricles, inferior vena cava, the cortex and medulla of the kidney, and other organs and analyzed the time-intensity curve. In addition, the slice data were processed subtracting from postcontrast images to pre-contrast images and constructing into 3D images with the maximum intensity projection (MIP) method using

a work station (Advantage Windows, General Electric Medical System).

Statistical Analysis. Statistical analysis was performed using either student-*t* test or the one-way analysis of variance (ANOVA), with pair wise comparison using the Bonferroni method (Sigmastat, Jandel Scientific, San Rafael, CA).

RESULTS

Biodistribution of ¹⁵³Gd-Labeled G6-1B4Ms. Saturated G6E vs G6A. The ¹⁵³Gd-labeled saturated G6E-

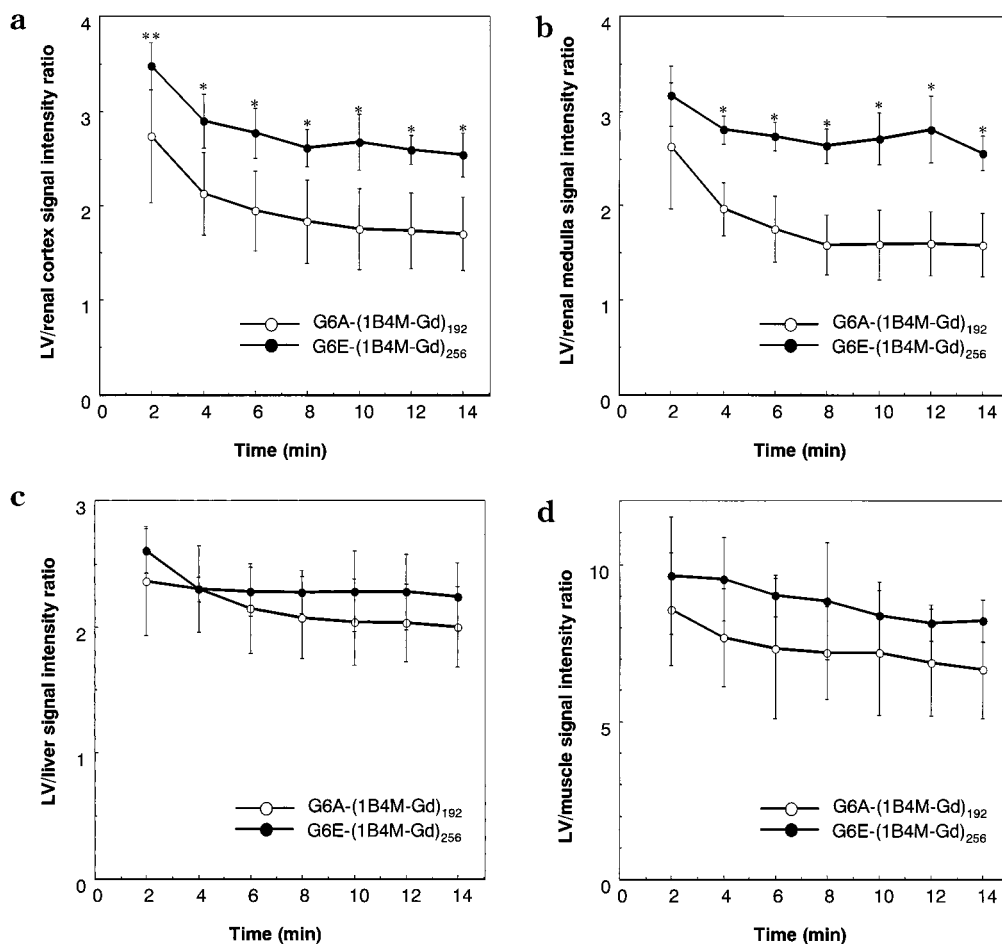


Figure 6. The values of the enhancement effects ratio in the cortex (a) and the medulla (b) of kidney, the liver (c), and the muscle (d) to the blood in the left ventricle of the heart were plotted. (enhancement effects ratio) = $\{[(\text{the postcontrast signal intensity of the organs}) - (\text{the precontrast signal intensity of the organs})] / [(\text{the postcontrast signal intensity of the left ventricle}) - (\text{the precontrast signal intensity of the left ventricle})]\}$. The single and double asterisks indicate significant differences ($p < 0.01$ and $p < 0.05$, respectively).

(1B4M)₂₅₆ ($32.4 \pm 0.6\%$ ID/g) remained in the blood significantly more than the ^{153}Gd -labeled saturated G6A-(1B4M)₁₉₂ ($24.2 \pm 1.1\%$ ID/g) ($p < 0.01$) and accumulated significantly less in the liver (17.1 ± 1.2 vs $28.9 \pm 1.2\%$ ID/g) and in the kidney (13.6 ± 0.5 vs $17.8 \pm 0.4\%$ ID/g) than all other preparations ($p < 0.01$) (Figure 2).

Saturated G6E/G6A vs Unsaturated G6E/G6A. Both ^{153}Gd -labeled unsaturated G6-(1B4M)_x preparations accumulated significantly more in the liver (51.6 ± 2.4 and $57.2 \pm 1.2\%$ ID/g for G6E and G6A) and less in the blood (0.4 ± 0.1 and $0.3 \pm 0.1\%$ ID/g for G6E and G6A) than the saturated preparations ($p < 0.01$) (Figure 2).

G6-(1B4M)₂ Preparation. Both ^{153}Gd -labeled saturated G6-(1B4M)₂ preparations which have much smaller molecular masses (60 and 45 kDa for G6E and G6A), accumulated significantly less in the liver (24.4 ± 0.5 and $25.7 \pm 0.9\%$ ID/g for G6E and G6A) and more in the kidney (59.8 ± 0.5 and $78.7 \pm 2.7\%$ ID/g for G6E and G6A) than both the ^{153}Gd -labeled unsaturated and saturated G6E-(1B4M)₂₅₆ and ^{153}Gd -labeled G6A-(1B4M)₁₉₂ ($p < 0.01$) (Figure 2). In this case, ^{153}Gd -labeled saturated G6E-(1B4M)₂ accumulated significantly less in the kidney than ^{153}Gd -labeled saturated G6A-(1B4M)₂ ($p < 0.01$).

Blood Clearance of ^{153}Gd -Labeled Saturated G6-1B4Ms. The blood retention of ^{153}Gd -labeled G6E-(1B4M)₂₅₆ was significantly greater than that of ^{153}Gd -labeled G6A-(1B4M)₁₉₂ at all time points later than 15 min postinjection (Figure 3; $p < 0.01$). The blood half-life of the ^{153}Gd -labeled saturated PAMAM-1B4Ms was

132 ± 29 and 51 ± 17 min for G6E-(1B4M-Gd)₂₅₆ and G6A-(1B4M-Gd)₁₉₂, respectively.

Excretion and Body Retention of ^{153}Gd -Labeled Saturated G6D-1B4M Conjugates. The cumulative urinary excretion of ^{153}Gd -labeled G6E-(1B4M)₂₅₆ (6.0% ID) was less than that of ^{153}Gd -labeled G6A-(1B4M)₁₉₂ (8.5% ID) within 48 h postinjection (Figure 4). The cumulative fecal excretion of ^{153}Gd -labeled G6E-(1B4M)₂₅₆ (3.4% ID) was more than that of ^{153}Gd -labeled G6A-(1B4M)₁₉₂ (1.9% ID) within 48 h postinjection (Figure 4). The whole body retention of ^{153}Gd -labeled G6E-(1B4M)₂₅₆ and ^{153}Gd -labeled G6A-(1B4M)₁₉₂ was 80.9 ± 2.8 and $79.7 \pm 3.2\%$ ID 48 h postinjection, respectively ($p = 0.6$).

3D-micro-MR Angiography of Mice. G6E-(1B4M-Gd)₂₅₆ showed slightly higher signal intensity in the left ventricle of the heart and the inferior vena cava at some of the later time points and significantly lower signal intensity than G6A-(1B4M-Gd)₁₉₂ in the both cortex and medulla of the kidney at all time points that were examined ($p < 0.01$; Figure 5). No significant difference in the signal intensity was observed in the liver and the muscle. Therefore, left ventricle/kidney signal intensity ratio of G6E-(1B4M-Gd)₂₅₆ was significantly lower than that of G6A-(1B4M-Gd)₁₉₂ ($p < 0.01$; Figure 6).

The subtraction MR-angiography with G6A-(1B4M-Gd)₁₉₂ showed apparently brighter kidneys on both early and delayed images (Figure 7).

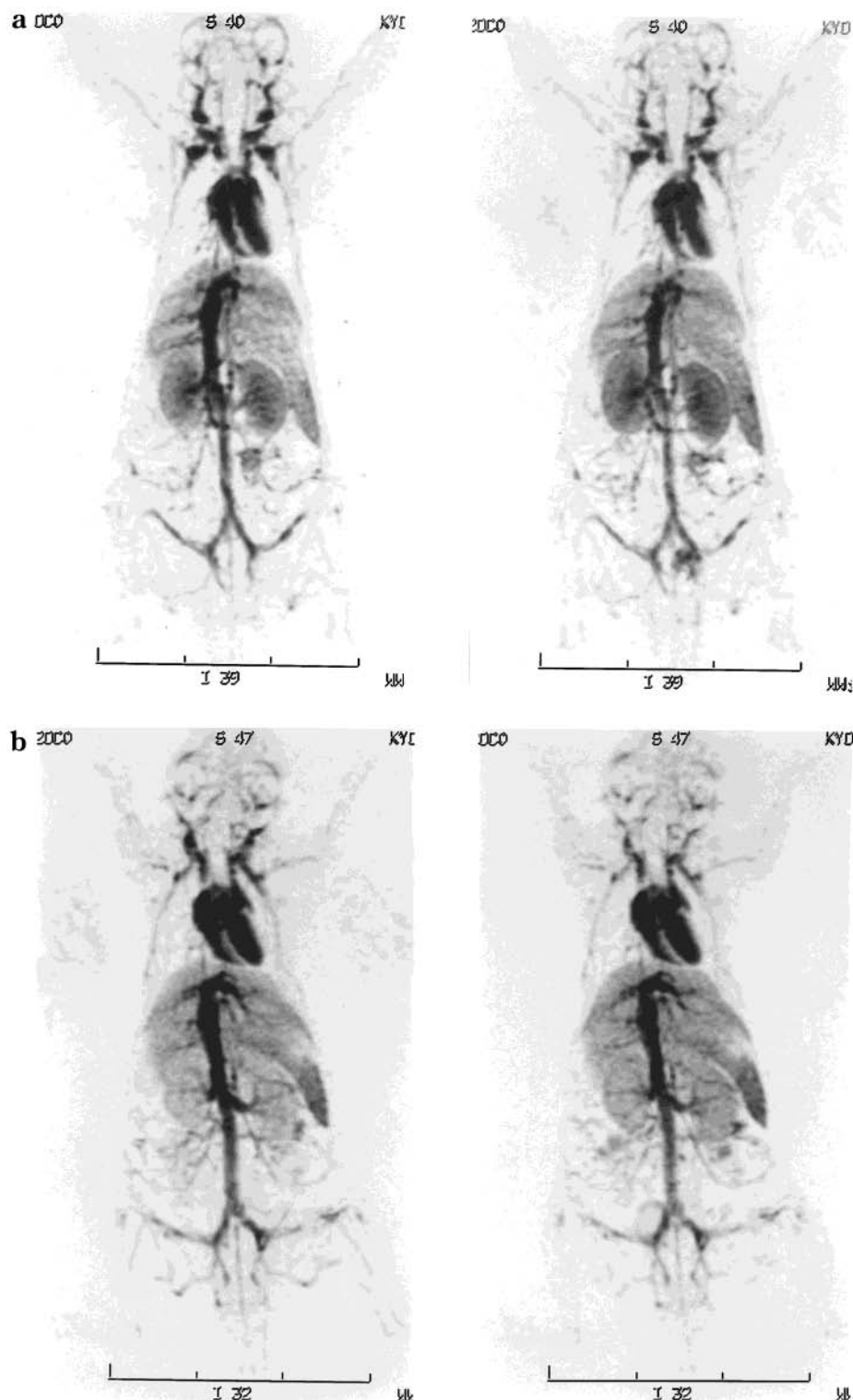


Figure 7. A pair of the whole body 3D-micro-subtraction-MR-angiography of a mouse, which were constructed with the MIP method with immediately after (left) and 8 min after (right) injection of 0.033 mmol/kg of G6A-(1B4M-Gd)₁₉₂ (a) or G6E-(1B4M-Gd)₂₅₆ (b) are shown. The brighter kidney was shown on panel a than on panel b. The scale shown at the bottom indicates 1 cm.

DISCUSSION

The difference in molecular diameter between G6E-(1B4M-Gd)₂₅₆ and G6A-(1B4M-Gd)₁₉₂ was theoretically expected to be very small because the only difference between those molecules were the core portions (ammonia versus ethylenediamine). Both of these molecules were expected then to have arms of the same length and thus similar spherical shape. When the 1B4M was conjugated with all amino-groups on the surface of the

molecule, the chemical properties of both molecules were then expected to remain similar. However, not only G6E-(1B4M-Gd)₂₅₆ versus G6A-(1B4M-Gd)₁₉₂, but also G6E-(1B4M-Gd)₂ versus G6A-(1B4M-Gd)₂, showed different pharmacokinetics in vivo, especially in the kidney (glomerular filtration). The G6A was predicted to have sparser arms than G6E simply on a numerical basis. Therefore, we speculated that G6A-(1B4M-Gd)₁₉₂ would be more flexible for changing shape than G6E-(1B4M-Gd)₂₅₆,

resulting in increasing the possibility to pass through glomerular filtration.

In the previous and current studies, supporting data was obtained to explain that not only the molecular weight, but also the size and shape of the molecule is an important contributing criteria for glomerular filtration (13). In brief, a hypothesis that the PAMAM-(1B4M-Gd)_x might be filtered through the glomerulus more readily than a nonspherical protein with a comparable molecular weight was formed. Previous results indicated that the G5A-(1B4M-Gd)₉₆ (88 kDa) was partially filtered through the glomerulus, even though G5A-(1B4M-Gd)₉₆ had a greater molecular mass than albumin, thus supporting this hypothesis. In addition, although G4E-(1B4M-Gd)₆₄ (57 kDa) had greater molecular mass and smaller molecular size than G6A-(1B4M-Gd)₂ (45 kDa), the G4E-(1B4M-Gd)₆₄ was significantly filtered more from the glomerulus than G6A-(1B4M-Gd)₂. From these results, not only molecular mass, but also shape, size or charge of the molecule affects the glomerular filtration of these molecules.

A second aspect of this study was that differences in the surface charge density of these molecules might also be an important criteria influencing both clearance rate and route. G6E should have higher surface density charge than G6A simply due to the greater number of 1B4Ms on the exterior of a similar size spherical surface. We have previously reported that the charge of two molecules of similar molecular size, ranging from 40 to 70 kDa, can strongly affect their glomerular filtration (15). However, in this case, this explanation would not be appropriate. The unsaturated G6E-(1B4M-Gd)₂₅₆, which possesses a greater negative charge and G6E-(1B4M-Gd)₂, which has a greater positive charge, both exhibited lower renal accumulation than their respective G6A counterparts. The differences in glomerular filtration influenced the blood clearance rate for both molecules as well, which was reflected in their ability to function as macromolecular intravascular MR contrast agent.

Macromolecular contrast agents for magnetic resonance imaging (MRI) have been reported to be excellent methods to evaluate microvasculature and histological capillary density in tumor tissues (16, 17). Although postcontrast signal changes in the tumors have shown better correlation with histological capillary mass, only in our previous report was the intratumoral microvasculature directly evaluated with MRI using macromolecular MR contrast agents (Kobayashi et al. in submission to *Magn. Reson. Med.*)

In terms of prolongation of contrast-enhancement and large number of Gd(III) atoms containing in a molecule, G6E-(1B4M-Gd)₂₅₆ is superior to G6A-(1B4M-Gd)₁₉₂. Additionally, when using the G6A-(1B4M-Gd)₁₉₂ to visualize microvasculature clearly, the dose of Gd(III) atoms could be decreased to 33% of the current clinical dose for Gd-DTPA (13). Thus, use of G6E-(1B4M-Gd)₂₅₆ would possibly decrease the dose of Gd(III) minimizing toxicity.

In conclusion, G6E-(1B4M-Gd)₂₅₆ demonstrated longer blood retention and lower renal accumulation than the G6A-(1B4M-Gd)₁₉₂, although both of these molecules were expected to theoretically have approximately the same molecular diameter. G6E-(1B4M-Gd)₂₅₆ may be a potential candidate as an intravascular MR contrast agent with a maximum number of Gd(III) atoms and minimal size so as not to be excreted through the kidney and perform as a superior contrast agent for vascular imaging.

ACKNOWLEDGMENT

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New Cationic Liposomes for Gene Transfer into Mammalian Cells with High Efficiency and Low Toxicity

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Novel cationic amphiphiles, based on hydrophobic cholesterol linked to L-lysineamide or L-ornithineamide, were designed and tested as nonviral gene transfer vectors. Each amide form of amino acid was conjugated to cholesterol by a carbamate ester bond to facilitate efficient degradation in animal cells. Cytotoxicity tests were performed for some cell lines. The transfection efficiency of the amphiphiles on different cell lines was evaluated as a liposomal solution in the presence of the fusogenic helper lipid, dioleoyl phosphatidylethanolamine (DOPE). The efficiency was also compared with other generally used gene carriers, such as lipofectin, 3 β [N-(N'-N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) liposome, and polyethylenimine (PEI).

INTRODUCTION

Gene therapy is generally considered as a promising approach not only in the treatment of diseases with genetic defects but also in the development of strategies for treatment and prevention of chronic diseases such as cancer, cardiovascular diseases, and rheumatoid arthritis. Intensive investigation has been conducted during the past decade into methods for transferring genes to cells (1–4). The methods used so far make use of either viral systems (retroviruses or adenoviruses) or nonviral systems (polymers, electroporation, calcium-phosphate precipitation, peptides, and liposomes). Even though some viral systems showed remarkable levels of expression, they are limited for general use in vivo due to their low capacity for large-sized therapeutic genes as well as short and long-term risks such as generation of host immune responses and the possibility of inserted genes combining with endogenous viruses or activation of oncogenes (2, 4).

Consequently, there has been increasing attention focused on the development of safe and efficient nonviral gene transfer vectors (5), which are either polycationic polymers or cationic lipids. Polycationic polymers, e.g., poly-L-lysine, poly-L-ornithine, and polyethylenimine (6, 7), that could interact with DNA forming polyionic complexes, have been introduced for gene delivery. Various cationic lipids have also been proven to form lipoplexes with DNA and induce efficient transfection of a large number of eukaryotic cells (5, 8, 9). Among such kinds of synthetic vectors, cationic lipids are attractive and widely used because it is possible to design and synthesize various kinds of derivatives that are outstanding in the aspects of transfection efficiency, biodegradability, and low toxicity (10). Some cationic lipids are commercially available, and several lipids have already been used in the clinical setting. Among them, cationic cholesterol derivatives are known to be very useful for their high transfection efficiency and low toxicity (11–17). However, there are still many issues to be tackled

such as the gene transfer potency, the half-life in the blood stream, and the biocompatibility deserving less cytotoxicity.

In light of the above considerations, we have prepared and described in this paper new promising DNA delivery agents composed of L-lysineamide, L-ornithineamide and cholesterol, which are designed to be readily susceptible to metabolic degradation after incorporation into animal cells. 3 β [L-lysineamide-carbamoyl] cholesterol (K-Chol)¹ and 3 β [L-ornithineamide-carbamoyl] cholesterol (O-Chol) were synthesized by the solid-phase synthesis method which has several advantages such as simplification of reaction procedures, easy separation of the supported products, and possibility of application to automatic systems.

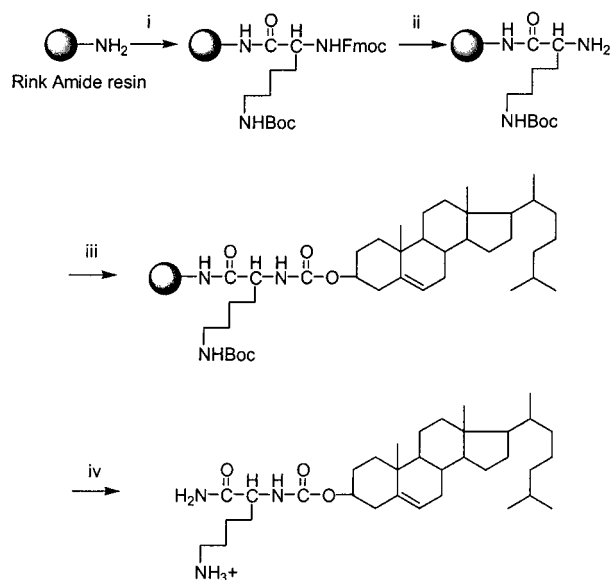
It was demonstrated that the newly designed cationic cholesterol derivatives were less toxic and more efficient in transfection than lipofectin and DC-Chol. The results for the biocompatible lipids suggest their potential use as general reagents for transfection of mammalian cells and in vivo applications for gene therapy.

MATERIALS AND METHODS

Materials. N- α -Fmoc-N- ϵ -tBOC-L-lysine, N- α -Fmoc-N- ϵ -tBOC-L-ornithine, N-hydroxybenzotriazole (HOBt), and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from AnaSpec, Inc. (San Jose, CA), and cholesteryl chloroformate, N,N-diisopropylethylamine (DIPEA), N,N-dimethylformamide (DMF), piperidine, dichloromethane, and polyethylenimine (PEI, 25 kDa) were from Aldrich (St. Louis, MO). N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), ethidium bromide (EtBr), o-nitrophenyl- β -D-galactopyranoside (ONPG), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Rink amide

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¹ Abbreviations: K-Chol, 3 β [L-lysineamide-carbamoyl] cholesterol; O-Chol, 3 β [L-ornithineamide-carbamoyl] cholesterol; DC-Chol, 3 β [N-(N'-N'-dimethylaminoethane)-carbamoyl] cholesterol; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine; PEI, polyethylenimine; ONPG, o-nitrophenyl- β -D-galactopyranoside.

Scheme 1. Outline of Step-wise Solid-Phase Synthesis of K-Chol^a

^a (i) Fmoc-Lys(Boc)-OH, HOBt, HBTU, DIPEA, DMF. (ii) 30% piperidine. (iii) Cholesteryl chloroformate, DIPEA, dichloromethane. (iv) TFA/DCM (50:50).

resin was from Novabiochem (San Diego, CA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and Dulbecco's phosphate-buffered saline (DPBS) were purchased from GIBCO (Gaithersburg, MD). Minimal essential medium (MEM) was from Hyclone (Logan, UT). 5× Reporter lysis buffer, lipofectin, and pSV- β -gal plasmid (EMBL accession no. X65335) were all purchased from Promega (Madison, WI). DC-Chol was synthesized according to Gao and Huang (12).

Synthesis of K-Chol and O-Chol. As shown in Scheme 1, L-lysine was attached to the rink amide resin by using fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry (18, 19). Coupling of amino acid to the resin was performed with 6 equiv of HOBt, HBTU, *N*- α -Fmoc-N- ϵ -tBOC-L-lysine (AnaSpec, Inc., San Jose, CA), and DIPEA, respectively, in anhydrous DMF. Piperidine (30%) was used for deprotection of the Fmoc group of lysine. Cholesteryl chloroformate was added to the resin-bound lysine (6 equiv, DCM, 25 °C, 4 h). The progress of each reaction was monitored by ninhydrin test until completion. After treating TFA (50:50 TFA-DCM v/v, 4 °C, 1.5 h), the final product was precipitated in ethyl ether and washed with excess ether. The product was solubilized in water and collected by freeze-drying yielding colorless powder (85% yield based on the initial loading level of rink amide resin). The compound gave a single spot on a TLC plate (ethyl acetate:*n*-butanol:acetic acid:H₂O = 2:1:1:1 v/v/v/v, *R_f* = 0.7). ¹H NMR, (300 MHz, *d*₆-DMSO) δ (ppm) 0.65–2.26 (m, skeleton of cholesterol, -(CH₂)₃- of Lys), 2.75 (br, s, ϵ -CH₂ of Lys), 4.3 (br, s, α -CH of Lys) 7.2 (br, -NH₂ of Lys) 7.7 (br, -CO-NH- of Lys). Matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectra, *m/z* 576 [M + Na]⁺. The product was resuspended in water and stored in refrigerator before use. The compound was stable in aqueous solution over 6 months at 4 °C.

The overall synthesis scheme for O-Chol was the same as described above. *N*- α -Fmoc-N- ϵ -tBOC-L-ornithine was used for the preparation of O-Chol. The compound also gave a single spot on a TLC plate (*R_f* = 0.63). ¹H NMR (300 MHz, *d*₆-DMSO) δ (ppm) 0.65–2.26 (m, skeleton of cholesterol, -(CH₂)₂- of Orn), 2.75 (br, s, -CH₂ of Orn),

4.3 (br, s, -CH of Orn) 7.2 (br, -NH₂ of Orn) 7.7 (br, -CO-NH- of Orn). MS (MALDI) *m/z* 560.2 [M + Na]⁺.

Liposome Preparation. DOPE in chloroform was evaporated under a stream of N₂ gas and further dried under vacuum. Each cationic lipid solubilized in water was added to the dried DOPE and vortex mixed (weight ratio of cationic lipid: DOPE = 1:1). After overnight incubation at 4 °C for hydration, the mixture was sonicated using a bath-type sonicator. The shape and size distribution of the liposome was determined by atomic force microscopy as described below.

Plasmid Preparation. Plasmid pSV- β -gal, where the β -galactosidase reporter gene is expressed was amplified in *Escherichia coli* and prepared as described previously (20).

Agarose Gel Electrophoresis. Lipoplexes were formed at different charge ratios between K-Chol and pSV- β -gal plasmid by incubation in serum-free DMEM at room temperature for 30 min. Each sample was then analyzed by electrophoresis on a 0.7% agarose gel containing ethidium bromide (0.5 μ g/mL of gel).

Atomic Force Microscopy. Atomic force microscopy (Nanoscope IIIa system, Digital Instruments, Inc., Santa Barbara, CA) was used for imaging the shape and particle size of the liposomes and liposome-DNA complexes, as previously reported (21). Briefly, 2 μ L aliquots of either liposome solution or aqueous liposome-DNA complex solutions were loaded onto the center of a freshly split mica disk. The charge ratio of liposome/DNA was set to 1.7, at which the transfection efficiency was optimal. After adsorption for 1–2 min at room temperature, excess complex solution was removed by absorption onto filter paper and the mica surface was further dried at room temperature before imaging. The image mode was set to tapping mode and the average scanning speed was 5 Hz.

Cell Culture. Human embryonic kidney 293 and mouse embryonic fibroblast NIH3T3 cells (ATCC, Rockville, MD) were grown in DMEM with 10% FBS. Human liver carcinoma HepG2 cells (Korean Cell Line Bank) were propagated in MEM supplemented with 10% FBS. All cells were routinely maintained on plastic tissue culture dishes (Falcon) at 37 °C in a humidified 5% CO₂/95% air containing atmosphere.

Determination of Cell Toxicity. Each of the cell lines was seeded in 96 well plates (1–2 $\times$ 10⁴ cells/well) and incubated for 1 day prior to experiment. Lipofectin, PEI, and cationic liposomes were introduced to the cells and incubated for 48 h. The cytotoxicity was determined by comparing the amount of MTT reduced by the cells treated with carriers to that reduced by control cells (22).

Transfection Procedures. For transfection, (1–1.5) $\times$ 10⁵ cells/well were seeded in 24 well plates 1 day prior to transfection experiments, and grown in the appropriate medium with 10% FBS. The cell lines were 60–70% confluent at the time of transfection. Complexes were prepared by mixing each reagent with plasmid DNA (2.0 μ g/well) in FBS-free medium. Each complex solution was further incubated for 30 min at room temperature and added to the cells. Transfection was performed in serum-free medium for 4 h. Each medium was replaced with a fresh complete medium and gene expression was assayed 48 h post-transfection. Control transfections were performed by using DC-Chol liposome and commercially available transfection reagents, such as lipofectin and PEI.

Transfection Assay. The expressed β -galactosidase activity was measured by a standard method as recommended by the manufacturer (23). Briefly, each cell in

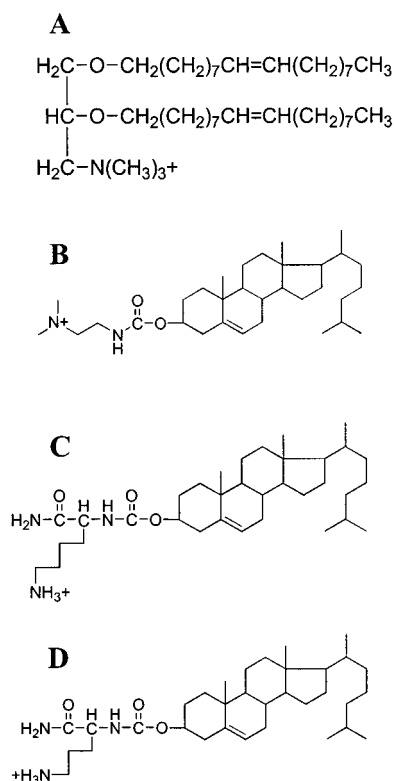


Figure 1. The structures of the cationic lipids. (A) DOTMA (lipofectin). (B) DC-Chol. (C) K-Chol. (D) O-Chol.

the 24 well plates was washed with DPBS and lysed with Reporter lysis buffer. The cell lysates were analyzed using the colorimetric ONPG assay in a 96-well plate format. The protein concentration was determined by the Pierce Micro BCA Protein Assay Reagent Kit, using BSA as standard. β -Galactosidase activity is expressed in total milliunits per mg of protein. One milliunit is defined as the amount of β -galactosidase that hydrolyzes 1 nmol of ONPG/min at pH 7.5 at 37 °C.

RESULTS AND DISCUSSION

Syntheses and Features of K-Chol and O-Chol.

The cationic lipids, K-Chol and O-Chol, were prepared in good yields by the solid-phase synthesis method and their structures are presented in Figure 1. The amphiphiles are composed of naturally occurring lipid and amino acid that could be metabolized after administration into animal cells. The amphiphiles are structurally related to the various cationic cholesterol derivatives previously reported, such as ChoSC or ChoTB (11), DC-Chol (12), spermine-cholesterol (13), cholesteryl-spermidine (14), BGSC or BGTC (15), and some DC-Chol derivatives (16, 17). As the primary amino group is abundant in poly-L-lysine and PEI, which are generally used in polymer-mediated gene transfer, that of lysinamide or ornithinamide is considered to be capable of interacting electrostatically with polyphosphate anions of plasmid DNA. The amphiphiles contain a carbamate ester bond that could be efficiently degraded in the cells (12).

Charge Ratio-Dependent Complex Formation and Transfection Activity. To assess the complex formation of K-Chol with DNA, agarose gel electrophoresis of cationic lipid:DNA complexes was performed at various charge ratios. As shown in Figure 2A, plasmid DNA became completely saturated at a charge ratio of around 2 ($\pm$). In addition, some completely retarded

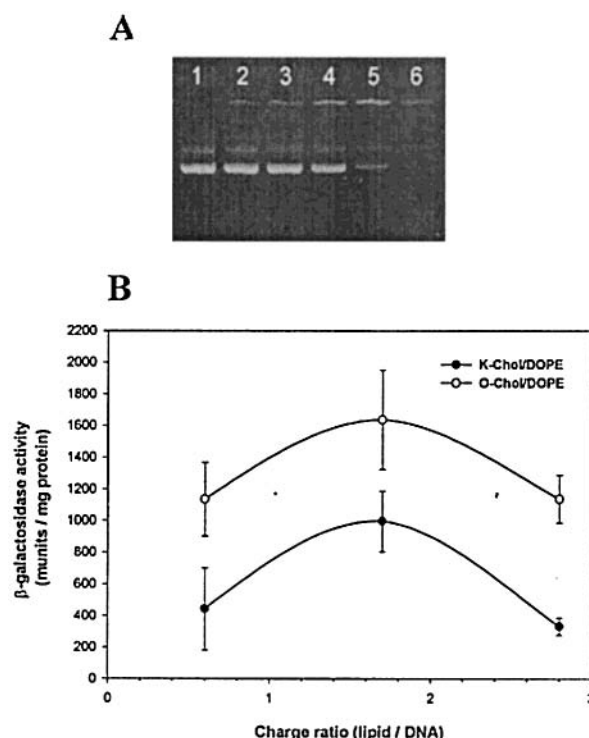


Figure 2. (A) Agarose gel retardation of K-Chol/DNA complexes as a function of cationic lipid to plasmid DNA charge ratio. pSV- β -gal plasmid DNA (1.0 μ g) only (lane 1), charge ratio of K-Chol/DNA = 0.2, 0.3, 0.6, 1.7, and 2.8 (lanes 2, 3, 4, 5, and 6, respectively). (B) β -Galactosidase expression as a function of the mean ionic charge ratio of the lipid/DNA complexes (ϵ -NH₃⁺ group of the cationic lipid to DNA phosphate anion). HepG2 cells were transfected at a fixed amount of DNA (2.0 μ g/well) with various amounts of cationic liposome. Data are shown as mean $\pm$ SD ($n = 3$).

complexes were observed at charge ratios even below 1, which indicates that the mode of K-Chol binding to DNA is cooperative (24).

The results shown in Figure 2B demonstrate the dose dependent transfection activity of K-Chol and O-Chol liposomes for HepG2 cells, with an optimal charge ratio of 1.7 ($\pm$). It was apparent that the transfection efficiency showed a bell-shape dependence on the liposome concentration. Low transfection efficiency at charge ratios below 1 is generally presumed to be due to reduced cell membrane binding, and decreased efficiency at high charge ratios seems to be due to the toxicity of the cationic lipid (15).

AFM Images of the Liposomes and the Liposome-Plasmid DNA Complexes. To examine the shape and size distribution of liposomes and liposome-DNA complexes, atomic force microscopy was introduced. As shown in Figure 3, panels A and B, K-Chol/DOPE liposome gave small vesicles with a diameter of 40–110 nm and O-Chol/DOPE liposome was somewhat larger in size, ranging from ca. 60 to 150 nm. When these liposomes were complexed with plasmid DNA, larger heterogeneous particles were observed and their shapes varied from spherical vesicles to amorphous aggregates (Figure 3, panels C and E). Uncomplexed free liposomes were also observed. As for K-Chol/DOPE-DNA complexes, the particles were 0.3–0.6 μ m in size and O-Chol/DOPE-DNA complexes gave larger particles in the range 0.4–1.2 μ m. As presented in Figure 3, panels C and E, fiberlike structures were also observed surrounding spherical vesicle-like structures. Some sections of Figure 3, panels C and E, are enlarged and presented in panels

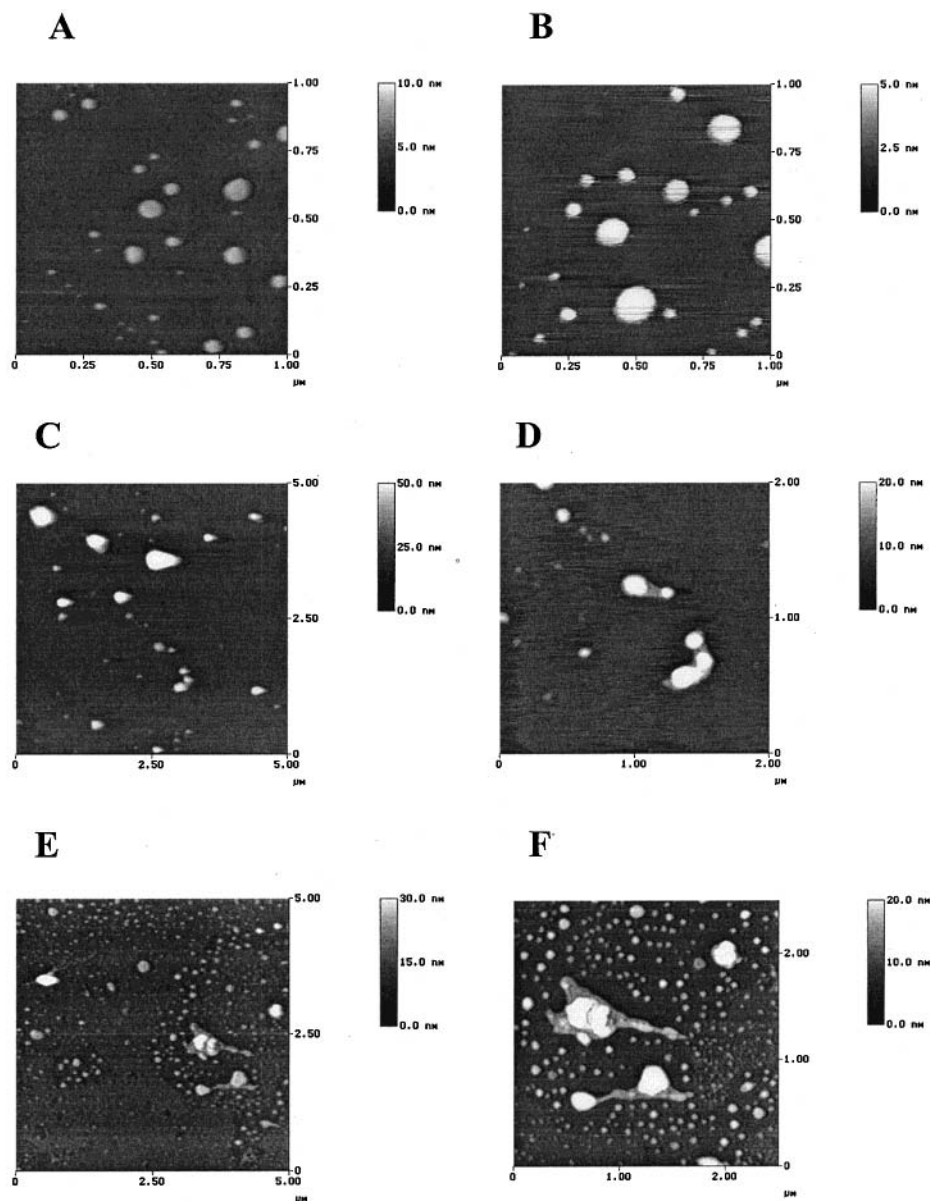


Figure 3. AFM images of liposomes and liposome-DNA complexes. (A) K-Chol/DOPE liposome; (B) O-Chol/DOPE liposome; (C), (D) K-Chol/DOPE liposome-DNA complex; (E), (F) O-Chol/DOPE liposome-DNA complex. The white color indicates a height in excess of the designated *nm* above the mica surface. The *x* and *y* dimensions are scaled as shown.

D and F, respectively. These complexes showed aggregates connected in rows by fibrous structures, and this result conforms to previous electron microscopy studies that have reported large aggregates surrounded by thin fibers (25). Even though the structure and location of complexed DNA could not be definitely distinguished in the presented images, the fibrous structures are presumed to result from the interaction of DNA with the cationic liposomes.

Effects of the Liposomes on the Viability of Cells.

The cytotoxicity of our cationic liposomes was compared with that of lipofectin, DC-Chol liposome, and PEI, all are widely used as gene transfer reagents. As shown in Figure 4, the viability of the cells decreased abruptly accordingly as the amount of PEI or lipofectin increased. Cationic cholesterol liposomes showed a lower toxicity level for 293 cells compared to lipofectin and PEI (Figure 4A). In the case of NIH3T3 cells, toxicity was also observed for all the reagents at concentrations much higher than those required for optimal transfection, even though K-Chol and O-Chol liposomes showed much

reduced toxicity toward the cells at the same concentration ranges (Figure 4B). For HepG2 cells, K-Chol and O-Chol liposomes were noticeably less toxic to the cells than other reagents (Figure 4C). The IC_{50} values of the transfection reagents are shown in Table 1.

An important feature of our cationic liposomes is their relatively low toxicity toward the cells at concentrations required for optimal transfection, for cytotoxicity is one of the major hurdles in the application of many cationic amphiphiles. The toxicity of some of the commercially available cationic lipids and synthetic polycationic polymers, such as lipofectin and PEI, has been attributed to their nonnatural, nonbiodegradable nature.

Transfection Efficiency on Different Cell Lines.

To investigate the effectiveness of K-Chol and O-Chol for in vitro transfection of mammalian cells, we performed in vitro transfection tests with some cell lines routinely used in laboratories. It already has been shown that some cell types, especially NIH3T3 cells, are not susceptible to transfection with lipofectin (8, 26, 27) or DC-Chol (27). It was therefore of interest to examine the versatility in

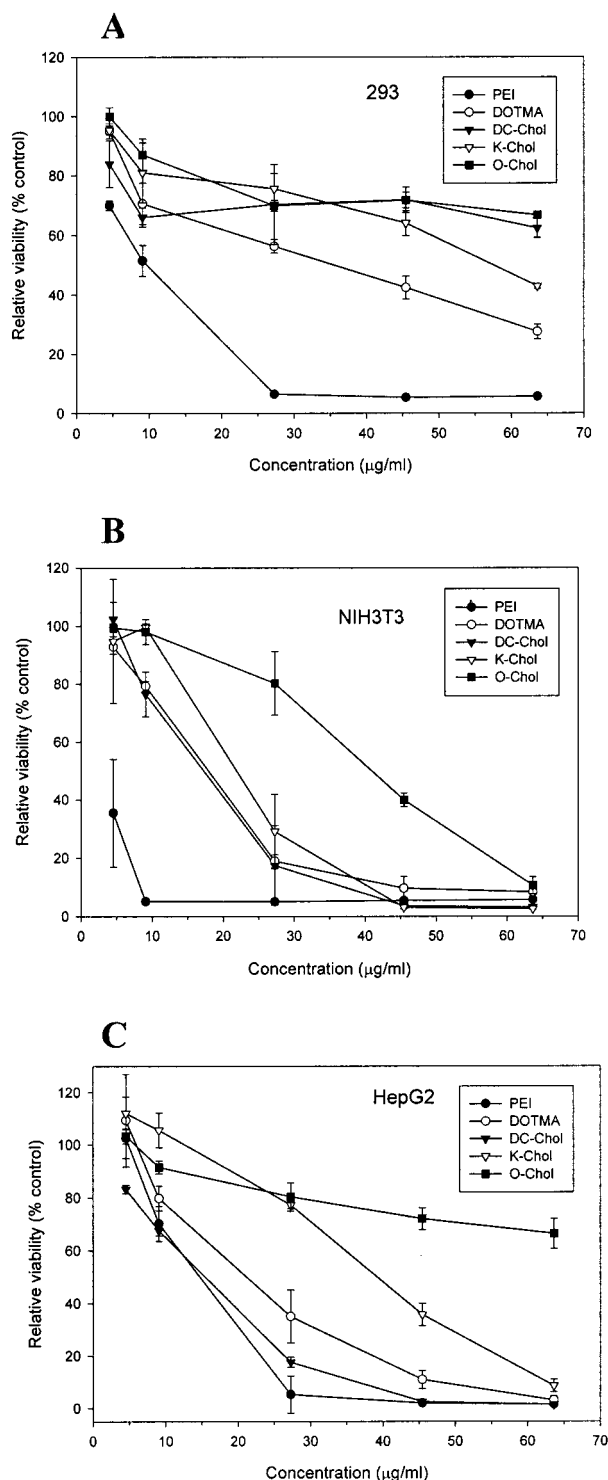


Figure 4. Effect of PEI, lipofectin, DC-Chol/DOPE, K-Chol/DOPE, and O-Chol/DOPE liposome on the cell viability. Relative cell viability was calculated, assuming the absorbance at 570 nm of intact control cells to be 100%. (A) 293 cells. (B) NIH3T3 cells. (C) HepG2 cells. Each data point is the average $\pm$ SD of three different measurements.

transfection efficacy of K-Chol and O-Chol liposomes. To determine the efficiency of the liposomes, they were compared directly with PEI, lipofectin and DC-Chol liposome on the three cell lines. The amounts of lipofectin and DC-Chol liposome were adjusted to the optimum transfection condition. PEI (25 kDa) is known to have the greatest transfection efficiency among PEI derivatives (7, 28). In this experiment, PEI was also tested and

Table 1. Comparison of Cytotoxicity for Different Transfection Reagents^a

transfection reagents	IC ₅₀ (μ g/mL)		
	293	NIH3T3	HepG2
polyethylenimine	9.45	<4.54	14.7
lipofectin	35.5	17.9	21.2
DC-Chol/DOPE	ND	17.2	15.5
K-Chol/DOPE	57.5	21.9	39.2
O-Chol/DOPE	ND	40.9	ND

^a The concentration in which relative viability reached 50% comparing to the control was determined as IC₅₀. When the viability did not reach to the half value even the concentration was over 60 μ g/mL, it was represented as ND (not determined).

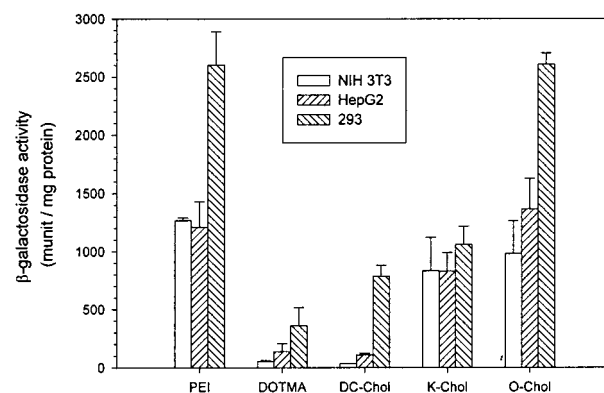


Figure 5. Transfection activity of PEI, lipofectin, DC-Chol/DOPE, K-Chol/DOPE, and O-Chol/DOPE liposome. Each value is the mean $\pm$ SD of three different experiments.

used at its optimal condition as previously reported (29, 30). As shown in Figure 5, PEI and O-Chol liposome were the most effective carriers in transfection efficiency for all cells at the routine transfection condition, i.e., for 4 h transfection in the serum free medium. In addition, NIH3T3 and HepG2 cells, which were somewhat refractory to transfection with lipofectin and DC-Chol liposome, displayed high transfection susceptibility toward K-Chol and O-Chol liposome. Our cationic liposomes were more effective than lipofectin and DC-Chol liposome in transfection efficacy for all the cell lines tested. Moreover, the efficiency of O-Chol liposome-mediated transfection was remarkable and was comparable to that of PEI reagent.

In conclusion, we have described the development of some novel cholesterol-based amphiphiles, which were named as K-Chol and O-Chol, respectively. These cationic liposomes self-assembled with plasmid DNA and displayed a relatively high efficient capability to transfect some mammalian cells in vitro. Their transfection efficiency with relatively low cytotoxicity and ease of preparation would make them further promising candidates for general gene therapy experiments both in vitro and in vivo.

ACKNOWLEDGMENT

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Highly Fluorinated Lipospermines for Gene Transfer: Synthesis and Evaluation of Their in Vitro Transfection Efficiency

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Fluorinated double-chain lipospermines (one or both of these chains being ended by a highly fluorinated tail of various length) which are close analogues of DOGS (Transfectam) were designed as synthetic vectors for gene delivery. For *N/P* ratios (*N* = number of amine functions of the lipid; *P* = number of DNA phosphates) from 0.8 to 10, these lipospermines condensed DNA, with or without the use of DOPE, to form fluorinated lipoplexes. The efficiency of the fluorinated lipoplexes to transfect lung epithelial A549 cells was significantly higher than that of the DOGS lipoplexes. No specific cell toxicity was evidenced for the fluorinated lipoplexes as compared to that of the DOGS ones. The palette of structural elements explored allowed to determine those required for efficient transfection, highlighting the importance of highly fluorinated chains, the unique properties of unsaturated double-chain lipids and of the use of DOPE as helper lipid on transfection.

INTRODUCTION

Gene transfer has become an increasingly important technique and powerful research tool for gene therapy which is applicable to inherited deficiency diseases (cystic fibrosis, dystrophies, haemophilias, ...) as well as to acquired diseases (cancer, AIDS, multiple sclerosis, ...). Successful gene therapy depends on the efficient delivery of genetic material to cells and its effective expression within these cells.

Synthetic nonviral gene transfer vectors have gained wide acceptance over the past decade. Until now, a large variety of (poly)cationic lipids, liposomes, and (lipo)-polymers, eventually associated to molecular conjugates for improving cell targeting, cytoplasmatic delivery and/or nuclear transport, have been tested, in vitro and/or in vivo, as gene delivery systems (Felgner et al., 1987; Remy et al., 1998; Gao and Huang, 1991, 1993; Byk et al., 1998; Miller, 1998; and references therein). These (poly)cationic systems are capable of condensing or compacting DNA into small-sized complexes (e.g., lipoplexes or polyplexes). Although at present, the in vivo expression levels of such "artificial viruses" are lower than for viral vectors and duration of gene expression is relatively short, the former systems present several advantages with respect to low-cost and large-scale production, safety, cell targeting, lower immunogenicity, and capacity to deliver large fragments of DNA.

Several studies have shown that the transfer efficiency of the DNA complexes, and subsequent DNA expression, especially in vivo, vary in function, among others, of the cationic components, the size of the complexes, their stability in biological fluids and their interactions with the cell membranes. Little is also known concerning the intracellular traffick of DNA to the nucleus, and the ongoing research remains highly empirical. Conse-

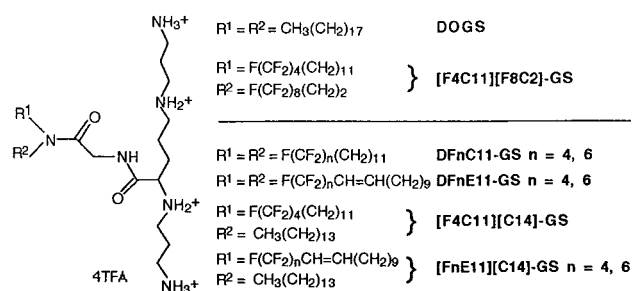


Figure 1. Chemical structure and code name of the fluorinated and reference lipospermines described in this study.

quently, there is a need to develop new compounds, especially (poly)cationic compounds, with characteristics and properties that are substantially different from those already described. Lipopolyamines with highly fluorinated hydrophobic and lipophobic chains belong potentially to this class of components. It was shown indeed that liposomes made from highly fluorinated lipids offer some of the specific features that make up the uniqueness of fluorinated material, including their unique high hydrophobic and lipophobic character (Vierling et al., 1995). Such "fluorinated" liposomes demonstrated meaningfully improved physicochemical and biological properties as compared to conventional liposomes, including increased stability in biological fluids (Vierling et al., 1995; Gadras et al., 1999; and references therein), extended in vivo blood circulation times comparable to those of some stealth liposomes (Vierling et al., 1995; Santaella et al., 1993).

We report herein on the synthesis of a series of new highly fluorinated lipopolyamines (Figure 1) and on their abilities of compacting and transferring DNA into cells as well as on their cellular compatibility. These compounds are close analogues of DOGS or Transfectam which is, among the cationic lipids tested so far, one of the most efficient synthetic gene transfer agent (Remy et al., 1998, 1994).

The synthesis of a fluorinated analogue of DOGS (e.g., [F4C11][F8C2]-GS in Scheme 1) was described in the

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past by some of us (Remy et al., 1994). Although [F4C11]-[F8C2]-GS and DOGS demonstrated similar transfection properties of NIH-3T3 murine fibroblasts with a chloramphenicol-acetyltransferase reporter plasmid, it has proven to be difficult to formulate lipoplexes with this fluorinated lipospermine, owing to its high degree of fluorination.

We have now extended this series to new fluorinated lipopolyamines. Several studies have shown a close correlation of the transfection capability with hydrophobicity and/or physical state of the bilayers that theoretically the lipids form. A modular molecular structure was therefore selected for these fluorinated lipospermines in order to explore the influence of the fluorocarbon moiety on lipoplex formulation, transfection ability and cell viability. Their framework consists of a spermine moiety connected to a hydrophobic part, which includes two saturated (e.g., **DFnCm-GS** and **[FnCm][Cm']-GS**), or unsaturated hydrophobic chains (e.g., **DFnEm-GS** and **[FnEm][Cm']-GS**), one (e.g., **[FnCm][Cm']-GS** and **[FnEm][Cm']-GS**) or both (e.g., **DFnCm-GS** and **DFnEm-GS**) being ended by a highly fluorinated tail of variable length ($n = 4$ or 6). This is also aimed at modulating the hydrophobic/lipophobic character and consequently the biological properties (biodistribution, bioavailability, in vivo fate) of the "fluorinated" lipoplexes that they are able to form with DNA. As the use of DOPE as co-lipid was found to enhance the transfection activity of DOGS (Thierry et al., 1997), we report also on the transfection efficacy of the fluorinated lipospermines used in conjunction with DOPE. Such an approach was further worth to be tested, as we show very recently, and in parallel to this study, that the formulation of "fluorinated" lipoplexes with a fluorinated analogue of DOPE and a conventional (nonfluorinated) lipopolyamine led to a significant in vitro and in vivo transfection improvement (Boussif et al., 2000).

EXPERIMENTAL PROCEDURES

General Experimental and Analytical Conditions. Most of the reactions were performed in anhydrous solvents under dry and oxygen-free nitrogen. Anhydrous solvents were prepared by standard methods. The purifications by column chromatography were carried out using silica gel 60 (Merck, 70–230 mesh) and chloroform (CHCl_3), dichloromethane (CH_2Cl_2), methanol (MeOH), or mixtures thereof as indicated. Unless noted otherwise, the ratios describing the composition of solvent mixtures represent relative volume. Advancing of the reaction was followed by thin-layer chromatography (TLC) on silica plates F_{254} (Merck). The following developing systems were used: UV light, KMnO_4 , $\text{H}_2\text{SO}_4/\text{EtOH}$, Dragendorff reagent (Sigma), ninhydrin reagent (Sigma).

The perfluoroalkylated acid chlorides were synthesized according to Santaella et al. (1991). The perfluoroalkyl iodides were purified over neutral alumina before use. DOGS (or Transfectam) was prepared as described in Remy et al. (1994). DOPE was purchased from Sigma. All other organic chemicals were from Aldrich, Fluka or Novabiochem.

IR spectra were recorded using a Bruker FT-ITS 45 spectrometer. Liquids were analyzed as thin film between two KBr blocks. ^1H , ^{13}C and ^{19}F NMR spectra were recorded at 200, 50.3, and 188.3 MHz, respectively, on a Bruker AC-200. Chemical shifts were measured relative to CHCl_3 (δ 7.27 ppm) or CH_3OD (δ 3.35 ppm) for ^1H , relative to CDCl_3 (δ 76.9 ppm) for ^{13}C and expressed indirectly in relation to TMS, and relative to CCl_3F as internal reference for ^{19}F . The following abbreviations are

used to describe the signal multiplicities: s (singlet), d (doublet), t (triplet), q (quadruplet), and m (multiplet). Chemical shifts are expressed in parts per million (ppm) and listed as follows: (multiplicity, coupling, integration, and attribution). Elemental analyses were carried out by the Service Central de Microanalyses du CNRS.

Synthesis of the di-11-(F-alkyl)-undec-10-enylamines, 2a,b. Di-undec-10-enylamine 1. Cyanamide (0.34 g, 0.8 mmol; Fluka) was added to a stirred 50% aqueous sodium hydroxide (6 mL) at room temperature. Aliquot 336 (50 mg; Fluka) and 11-bromo-1-undecene (4.7 g, 20 mmol; Fluka) were added, and the resulting mixture was stirred at 80 °C for 2 h, then extracted with CH_2Cl_2 . The organic extract was washed with water, dried, and evaporated. The residue was chromatographed twice on silica gel (CH_2Cl_2 /hexane, 8/2) to afford 1.3 g (3.8 mmol, 47%) of di-undec-10-enylcyanamide. TLC (CH_2Cl_2 , H_2SO_4): $R_f = 0.70$. ^1H NMR (CDCl_3): δ 1.18–1.45 (s large, 24H, $(\text{CH}_2)_6(\text{CH}_2)_2\text{N}$); 1.52–1.70 (m, 4H, $\text{CH}_2\text{CH}_2\text{N}$); 1.94–2.08 (m, 4H, $=\text{CHCH}_2$); 2.93 (t, $^3J = 7.2$ Hz, 4H, CH_2N); 4.88–5.09 (m, 2H, $\text{CH}_2=$); 5.70–5.95 (m, 1H, $=\text{CH}$). ^{13}C NMR (CDCl_3): δ 26.5, 27.7, 28.9, 29.1, 29.2, 29.4 ($(\text{CH}_2)_7\text{CH}_2\text{N}$); 33.8 ($=\text{CHCH}_2$); 51.5 (CH_2N); 114.2 ($\text{CH}_2=$); 117.8 (CN); 139.1 ($=\text{CH}$). A solution of di-undec-10-enylcyanamide (1.3 g, 3.8 mmol) in 10 mL of concentrated H_2SO_4 was refluxed for 24 h. After cooling to room temperature, the solution was brought to pH 12 with 1 M NaOH, then extracted with CH_2Cl_2 . The organic phase was then washed with H_2O until neutrality, dried and evaporated. The residue was purified by silica gel chromatography (CH_2Cl_2 /MeOH from 100/0 to 95/5) to afford 1.05 g (3.3 mmol, 86%) of **1**. TLC (CH_2Cl_2 /MeOH 9/1, ninhydrine): $R_f = 0.63$. ^1H NMR (CDCl_3): δ 1.12–1.41 (s large, 24H, $(\text{CH}_2)_6(\text{CH}_2)_2\text{N}$); 1.48–1.72 (m, 4H, $\text{CH}_2\text{CH}_2\text{N}$); 1.87–2.04 (m, 4H, $=\text{CHCH}_2$); 2.66 (t, $^3J = 7.6$ Hz, 4H, CH_2N); 4.88–5.09 (m, 2H, $\text{CH}_2=$); 5.70–5.95 (m, 1H, $=\text{CH}$); 5.91–6.14 (s large, 1H, NH). ^{13}C NMR (CDCl_3): δ 27.2, 28.1, 28.9, 29.1, 29.3, 29.4, 29.5 ($(\text{CH}_2)_7\text{CH}_2\text{N}$); 33.8 ($=\text{CHCH}_2$); 49.0 (CH_2N); 114.2 ($\text{CH}_2=$); 139.1 ($=\text{CH}$).

Di-11-(F-butyl)-undec-10-enylamine 2a. A solution of **1** (0.5 g, 1.56 mmol), perfluorobutyl iodide (6.8 g, 19.7 mmol), 2-aminoethanol (0.6 mL; Fluka), and CuCl (0.2 g) in *t*-BuOH (3 mL) was stirred under reflux during 2 days. After extraction with CH_2Cl_2 , the combined organic phases were washed, dried, and evaporated and the residue purified by silica gel chromatography (CH_2Cl_2 /MeOH 100/0 to 95/5) to afford 380 mg of **2a** as a pale yellow oil (0.5 mmol, 33%). TLC (CH_2Cl_2 /MeOH 9/1, ninhydrine): $R_f = 0.54$. ^1H NMR (CDCl_3): δ 1.18–1.50 (s large, 24H, $(\text{CH}_2)_6(\text{CH}_2)_2\text{N}$); 1.50–1.70 (m, 4H, $\text{CH}_2\text{CH}_2\text{N}$); 2.09–2.49 (m, 4H, $=\text{CHCH}_2$); 2.66 (t, $^3J = 7.6$ Hz, 4H, CH_2N); 5.39–5.72 (m, 1H, $\text{CF}_2\text{CH} = \text{CH}$); 6.30–6.52 (m, 1H, $\text{CF}_2\text{CH} = \text{CH}$). ^{13}C NMR (CDCl_3): δ 27.2, 27.9, 28.5, 29.2, 29.4 ($(\text{CH}_2)_7\text{CH}_2\text{N}$); 32.0 ($=\text{CHCH}_2$); 49.4 (CH_2N); 116.7 (t, $^2J_{\text{CF}} = 23$ Hz, $\text{CF}_2\text{CH} = \text{CH}$); 143.3 (t, $^3J_{\text{CF}} = 9$ Hz, $\text{CF}_2\text{CH} = \text{CH}$). ^{19}F NMR (CDCl_3): δ -81.5 (3F, CF_3); -107.2 (~0.2F, CF_2CH , Z); -111.9 (~1.8F, CF_2CH , E); -125.0 (2F, $\text{CF}_3\text{CF}_2\text{CF}_2$); -126.1 (2F, CF_3CF_2).

Di-11-(F-hexyl)-undec-10-enylamine, 2b. The same process as described for **2a**, when applied to **1** and perfluorohexyl iodide, afforded **2b** (69%) as a pale yellow oil. TLC (CH_2Cl_2 /MeOH 95/5, ninhydrine): $R_f = 0.48$. ^1H and ^{13}C NMR (CDCl_3) identical to those of **2a**. ^{19}F NMR (CDCl_3): δ -81.5 (3F, CF_3); -107.2 (~0.2F, CF_2CH , Z); -111.9 (~1.8F, CF_2CH , E); -122.3, -123.6, -124.2 (2F, 2F, 2F, $\text{CF}_3\text{CF}_2(\text{CF}_2)_3$); -126.8 (2F, CF_3CF_2).

Note: both **2a** and **2b** consist in a mixture of *E/E*, *E/Z*, and *Z/Z* isomers, as shown by ^{19}F NMR (*E/Z* +

Z/Z representing $\leq 10\%$). They are also accompanied by the $\text{CF}_2\text{-CH}_2\text{-CH=CH-}$ isomers (less than 5%; see text).

Synthesis of the di-11-(F-alkyl)-undecylamines 3a,b. The synthesis of di-11-(F-butyl)-undecylamine and di-11-(F-hexyl)-undecylamine has been performed by condensation of the respective 11-(F-alkyl)undecanoyl chloride and 11-(F-alkyl)undecylamine, then reduction of the amide thus obtained with LiAlH_4 (50–70% overall yields) according to Remy et al. (1994).

Di-11-(F-butyl)-undecylamine, 3a and di-11-(F-hexyl)-undecylamine, 3b (white powder). TLC ($\text{CHCl}_3/\text{MeOH}$ 95/5, ninhydrine): $R_f = 0.28$ and $R_f = 0.62$, respectively. These two derivatives display identical ^1H and ^{13}C NMR spectra. ^1H NMR (CDCl_3): δ 1.15–1.35 (s large, 28H, $\text{CF}_2(\text{CH}_2)_2(\text{CH}_2)_7$); 1.30–1.70 (m, 8H, $\text{CF}_2\text{-CH}_2\text{CH}_2$, $\text{CH}_2\text{CH}_2\text{NH}$); 1.98 (tt, $^3J = 7.5$ Hz, $^3J_{\text{HF}} 18.8$ Hz, 4H, CF_2CH_2); 2.50 (t, $^3J = 7.5$ Hz, 4H, CH_2NH). ^{13}C NMR (CDCl_3): δ 20.2 (t, $^3J_{\text{CF}} = 4$ Hz, $\text{CF}_2\text{CH}_2\text{CH}_2$); 27.5 ($\text{CH}_2\text{CH}_2\text{NH}$); 29.2, 29.3, 29.5, 29.6, 30.3 ($\text{CF}_2(\text{CH}_2)_2\text{-(CH}_2)_7$); 31.0 (t, $^2J_{\text{CF}} = 22$ Hz, CF_2CH_2); 50.3 (CH_2NH). ^{19}F NMR (CDCl_3) of **3a**: δ -81.3 (3F, CF_3); -115.1 (2F, CF_2CH_2); -124.9 (2F, $\text{CF}_3\text{CF}_2\text{CF}_2$); -126.5 (2F, CF_3CF_2). ^{19}F NMR (CDCl_3) of **3b**: δ -81.3 (3F, CF_3); -114.9 (2F, CF_2CH_2); -122.4, -123.4, -124.1 (6F, $\text{CF}_3\text{CF}_2(\text{CF}_2)_3$); -126.6 (2F, 2F, 2F, CF_3CF_2).

Synthesis of N-Tetradecyl-11-(F-alkyl)undec-10-enamine, 4a,b. N-Tetradecyl-undec-10-enamine. The procedure described elsewhere (Remy et al., 1994) when applied to undec-10-enoyl chloride and tetradecanamine afforded N-tetradecyl-undec-10-enamide (white powder, 99%). ^1H NMR (CDCl_3): δ 0.88 (t, $^3J = 6.5$ Hz, 3H, CH_3); 1.14–1.43 (s large, 32H, $(\text{CH}_2)_5(\text{CH}_2)_2\text{C(O)N(H)(CH}_2)_2\text{-(CH}_2)_{11}$); 1.43–1.71 (m, 4H, $\text{CH}_2\text{CH}_2\text{C(O)NHCH}_2\text{CH}_2$); 1.97–2.11 (m, 2H, $=\text{CHCH}_2$); 2.16 (t, $^3J = 7.5$ Hz, 2H, CH_2CO); 3.18–3.32 (m, 2H, CH_2NH); 4.87, 4.94 (m, 2H, $\text{CH}_2=$); 5.75 (m, 1H, $=\text{CH}$); 5.35–5.55 (s large, 1H, NH). ^{13}C NMR (CDCl_3): δ 14.1 (CH_3); 22.7, (CH_3CH_2); 25.9, 27.0, 28.9, 29.1, 29.4, 29.6, 29.7 ($(\text{CH}_2)_6\text{CH}_2\text{C(O)NHCH}_2\text{-(CH}_2)_{10}$); 32.0 ($\text{CH}_3\text{CH}_2\text{CH}_2$); 33.8 ($=\text{CHCH}_2$); 37.0, 39.5 ($\text{CH}_2\text{C(O)NHCH}_2$); 114.2 ($=\text{CH}_2$); 139.2 (CH=); 173.0 (CO).

N-Tetradecyl-11-(F-butyl)undec-10-enamine, 4a. Reduction of N-tetradecyl-undec-10-enamide with LiAlH_4 as described in Remy et al. (1994) gave N-tetradecyl-undec-10-enamine as a white powder (96%). TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 92/8, ninhydrine): $R_f = 0.52$. ^1H NMR (CDCl_3): δ 0.88 (t, $^3J = 6.5$ Hz, 3H, CH_3); 1.20–1.38 (s large, 34H, $(\text{CH}_2)_6(\text{CH}_2)_2\text{N(H)(CH}_2)_2(\text{CH}_2)_{11}$); 1.38–1.56 (m, 4H, $\text{CH}_2\text{-CH}_2\text{N}$); 1.96–2.12 (m, 2H, $=\text{CHCH}_2$); 2.58 (t, $^3J = 7.1$ Hz, 4H, CH_2N); 4.87, 4.94 (m, 2H, $\text{CH}_2=\text{CH}$); 5.75 (m, 1H, $\text{CH}_2=\text{CH}$). ^{13}C NMR (CDCl_3): δ 14.1 (CH_3); 22.7 ($\text{CH}_3\text{N(H)}$); 27.5, 29.0, 29.1, 29.4, 29.5, 29.7, 30.3 ($(\text{CH}_2)_7\text{-CH}_2\text{N(H)CH}_2(\text{CH}_2)_{10}$); 32.0 ($\text{CH}_3\text{CH}_2\text{CH}_2$); 33.8 ($=\text{CHCH}_2$); 50.2 (CH_2N); 114.2 ($=\text{CH}_2$); 139.2 (CH=). Then, the same process as described for the synthesis of **2a**, when applied to N-tetradecyl-undec-10-enamine and perfluorobutyl iodide, afforded **4a** as a yellow oil (58%); TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9/1, ninhydrine): $R_f = 0.63$. ^1H NMR (CDCl_3): δ 0.88 (t, $^3J = 6.5$ Hz, 3H, CH_3); 1.20–1.38 (s large, 34H, $(\text{CH}_2)_6(\text{CH}_2)_2\text{NH(CH}_2)_2(\text{CH}_2)_{11}$); 1.38–1.56 (m, 4H, $\text{CH}_2\text{-CH}_2\text{N}$); 2.10–2.28 (m, 2H, $=\text{CHCH}_2$); 2.58 (t, $^3J = 7.1$ Hz, 4H, CH_2N); 5.44–5.71 (m, 1H, $\text{CF}_2\text{CH=}$); 6.27–6.48 (m, 1H, $\text{CF}_2\text{CH=CH}$). ^{13}C NMR (CDCl_3): δ 14.1 (CH_3); 22.7 (CH_3CH_2); 27.5, 29.0, 29.1, 29.4, 29.5, 29.7, 30.3 ($(\text{CH}_2)_7\text{-CH}_2\text{N(H)CH}_2(\text{CH}_2)_{10}$); 32.0, 32.1 ($\text{CH}_3\text{CH}_2\text{CH}_2$, $=\text{CHCH}_2$); 50.2 (CH_2N); 116.7 (t, $^2J_{\text{CF}} = 23$ Hz, $\text{CF}_2\text{CH=}$); 143.0 (t, $^3J_{\text{CF}} = 9$ Hz, $\text{CF}_2\text{CH=CH}$). ^{19}F NMR (CDCl_3) identical to that of **2a**.

N-Tetradecyl-11-(F-hexyl)undec-10-enamine, 4b. The same process as described for the synthesis of **2a**, when applied to N-tetradecyl-undec-10-enamine and perfluorohexyl iodide, afforded **4b** as a yellow oil (45%). TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9/1, ninhydrine): $R_f = 0.66$. ^1H and ^{13}C NMR (CDCl_3) identical to those of **4a**, respectively. ^{19}F NMR (CDCl_3): identical to that of **2b**.

Note: both **4a** and **4b** consist into a mixture of the E- and Z-isomers, the E-isomer representing $\sim 90\%$. They are also accompanied by the $\text{CF}_2\text{-CH}_2\text{-CH=CH-}$ isomers (less than 3%, see text).

Synthesis of the Unsaturated [DFnE11]-GlyNH₂ and [FnE11][C14]-GlyNH₂ Glycinamines. The synthesis of these derivatives has been performed from their respective perfluoroalkylated amines **2a,b** or **4a,b** and benzyloxycarbonylglycine p-nitrophenyl ester (Z-Gly-ONP, Novabiochem) using the procedure described in ref 10 (yields ranging from 50 to 80%), followed by the Z-deprotection in the [DFnE11]-GlyNH-Z or [FnE11]-[C14]-GlyNH-Z derivatives thus obtained. This deprotection was achieved with 40–80% yields, by placing these Z-derivatives in a 30% HBr/AcOH solution for 1 h. After extraction with CH_2Cl_2 , the organic phase was washed with 1 M NaOH, dried and evaporated. The residue was chromatographed on silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 100/0 to 95/5) to afford the respective [DFnE11]-GlyNH₂ or [FnE11][C14]-GlyNH₂ glycinamines as colorless liquids.

N',N'-di-11-(F-butyl)-(resp. F-hexyl)-undec-10-enyl-N-benzyloxycarbonyl-glycinamine, DF4E11-GlyNH-Z (resp. DF6E11-GlyNH-Z). TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 99/1, UV and ninhydrine): $R_f = 0.83$ and 0.86, respectively. These two derivatives display identical ^1H and ^{13}C NMR spectra. ^1H NMR (CDCl_3): δ 1.09–1.41 (s large, 24H, $(\text{CH}_2)_6(\text{CH}_2)_2\text{N}$); 1.41–1.70 (m, 4H, $\text{CH}_2\text{-CH}_2\text{N}$); 1.96–2.41 (m, 4H, $=\text{CHCH}_2$); 3.17, 3.33 (t, $^3J = 7.6$ Hz, 4H, CH_2N); 4.06 (d, $^3J = 4.0$ Hz, 2H, CH_2NH); 5.15 (s, 2H, CH_2Ph); 5.48–5.76 (m, 1H, $\text{CF}_2\text{CH=}$); 5.81–5.93 (m, 1H, NH); 6.30–6.55 (m, 1H, $\text{CF}_2\text{CH=CH}$); 7.20–7.35 (m, 5H, Ph). ^{13}C NMR (CDCl_3): δ 26.9, 27.0, 27.6, 28.0, 28.8, 28.9, 29.2, 29.3, 29.4 ($(\text{CH}_2)_7\text{CH}_2\text{N}$); 32.0 ($=\text{CHCH}_2$); 42.7 (CH_2NH); 46.3, 47.0 (CH_2N); 66.9 ($\text{CH}_2\text{-Ph}$); 117.0 (t, $^2J_{\text{CF}} = 23$ Hz, $\text{CF}_2\text{CH=CH}$); 128.1, 128.2, 136.7 (Ph); 143.4 (t, $^3J_{\text{CF}} = 9$ Hz, $\text{CF}_2\text{CH=CH}$); 156.4 (NHCO); 167.5 (COCH_2). ^{19}F NMR (CDCl_3) identical to that of **2a** and **2b**, respectively.

N'-[11-(F-butyl)-(resp. F-hexyl)-undec-10-enyl]-N'-tetradecyl-N-benzyloxy-carbonyl-glycinamine, [F4E11][C14]-GlyNH-Z (resp. [F6E11][C14]-GlyNH-Z). TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 99/1, UV and ninhydrine): $R_f = 0.38$ and 0.43, respectively. These two derivatives display identical ^1H and ^{13}C NMR spectra. ^1H NMR (CDCl_3): δ 0.88 (t, $^3J_{\text{HH}} = 6.5$ Hz, 3H, CH_3); 1.20–1.38 (s large, 34H, $(\text{CH}_2)_6(\text{CH}_2)_2\text{N(CH}_2)_2(\text{CH}_2)_{11}$); 1.38–1.56 (m, 4H, $\text{CH}_2\text{-CH}_2\text{N}$); 2.10–2.28 (m, 2H, $=\text{CHCH}_2$); 3.15, 3.32 (tt, 4H, $^3J = 7.3$ Hz, CH_2N); 4.00 (d, $^3J = 4.0$ Hz, CH_2NH); 5.08 (s, 2H, OCH_2Ph); 5.44–5.71 (m, 1H, $\text{CF}_2\text{CH=}$); 5.88 (m, 1H, NH); 6.27–6.48 (m, 1H, $\text{CF}_2\text{CH=CH}$); 7.20–7.35 (m, 5H, Ph). ^{13}C NMR (CDCl_3): δ 14.1 (CH_3); 22.7 (CH_3CH_2); 26.9, 27.0, 27.6, 28.0, 28.9, 29.0, 29.4, 29.6, 29.7 ($(\text{CH}_2)_7\text{-CH}_2\text{NCH}_2(\text{CH}_2)_{10}$); 31.9, 32.0 ($\text{CH}_3\text{CH}_2\text{CH}_2$, $=\text{CHCH}_2$); 42.7 (CH_2NH); 46.2, 46.9 (CH_2N); 66.8 (CH_2Ph); 116.9 (t, $^2J_{\text{CF}} = 23$ Hz, $\text{CF}_2\text{CH=}$); 128.0, 128.5, 136.8, (Ph); 143.3 (t, $^3J_{\text{CF}} = 9$ Hz, $\text{CF}_2\text{CH=CH}$); 156.3 (NHCO); 167.3 (CH_2CO). ^{19}F NMR (CDCl_3) identical to that of **2a** and **2b**, respectively.

N',N'-di-11-(F-butyl)-(resp. F-hexyl)-undec-10-enyl glycinamine, DF4E11-GlyNH₂, (resp. DF6E11-GlyNH₂). TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95/5, ninhydrine): $R_f =$

0.58 and 0.68, respectively. These two derivatives display identical ^1H and ^{13}C NMR spectra. ^1H NMR (CDCl_3): δ 1.04–1.31 (s large, 24H, $(\text{CH}_2)_6(\text{CH}_2)_2\text{N}$); 1.31–1.62 (m, 4H, $\text{CH}_2\text{CH}_2\text{N}$); 1.95–2.36 (m, 4H, $=\text{CHCH}_2$); 3.10, 3.28 (t, $^3J = 7.6$ Hz, 4H, CH_2N); 3.52 (s large, 2H, CH_2NH_2); 5.30–5.68 (m, 1H, $\text{CF}_2\text{CH}=\text{CH}$); 6.22–6.48 (m, 1H, $\text{CF}_2\text{CH}=\text{CH}$). ^{13}C NMR (CDCl_3): δ 27.0, 27.1, 27.8, 28.1, 28.9, 29.0, 29.4, 29.5 ($(\text{CH}_2)_7\text{CH}_2\text{N}$); 32.0 ($=\text{CHCH}_2$); 42.5 (CH_2NH_2); 46.6, 47.0 (CH_2N); 117.0 (t, $^2J_{\text{CF}} = 23$ Hz, $\text{CF}_2\text{CH}=\text{CH}$); 143.4 (t, $^3J_{\text{CF}} = 9$ Hz, $\text{CF}_2\text{CH}=\text{CH}$); 165.5 (CO). ^{19}F NMR (CDCl_3) identical to that of **2a** and **2b**, respectively.

***N'*-[11-(*F*-butyl)-(resp. *F*-hexyl)-undec-10-enyl]-*N'*-tetradecyl glycinamine, [F4E11][C14]-GlyNH₂, (resp. [F6E11][C14]-GlyNH₂).** TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9/1, ninhydrine): $R_f = 0.50$ and 0.54 , respectively. These two derivatives display identical ^1H and ^{13}C NMR spectra. ^1H NMR (CDCl_3): δ 0.87 (t, $^3J = 6.5$ Hz, 3H, CH_3); 1.09–1.38 (s large, 34H, $(\text{CH}_2)_6(\text{CH}_2)_2\text{N}(\text{CH}_2)_2(\text{CH}_2)_{11}$); 1.38–1.69 (m, 4H, $\text{CH}_2\text{CH}_2\text{N}$); 1.96–2.42 (m, 2H, $=\text{CHCH}_2$); 3.12, 3.30 (t, 4H, $^3J = 7.3$ Hz, CH_2N); 3.45 (s, 2H, CH_2NH_2); 5.38–5.73 (m, 1H, $\text{CF}_2\text{CH}=\text{CH}$); 6.28–6.50 (m, 1H, $\text{CF}_2\text{CH}=\text{CH}$). ^{13}C NMR (CDCl_3): δ 14.1 (CH_3); 22.7 (CH_3CH_2); 26.9, 27.0, 27.7, 28.0, 28.8, 28.9, 29.3, 29.4, 29.7 ($(\text{CH}_2)_7\text{CH}_2\text{NCH}_2(\text{CH}_2)_{10}$); 31.9, 31.9 ($\text{CH}_3\text{CH}_2\text{CH}_2$, $=\text{CHCH}_2$); 43.0 (CH_2NH_2); 46.4, 46.9 (CH_2N); 117.0 (t, $^2J_{\text{CF}} = 23$ Hz, $\text{CF}_2\text{CH}=\text{CH}$); 143.3 (t, $^3J_{\text{CF}} = 9$ Hz, $\text{CF}_2\text{CH}=\text{CH}$); 171.9 (CO). ^{19}F NMR (CDCl_3) identical to that of **2a** and **2b**, respectively.

Synthesis of the Saturated [DFnC11]-GlyNH₂ and [F4C11][C14]-GlyNH₂ Glycinamines. The synthesis of the [DFnC11]-GlyNH₂ glycinamines has been performed in two steps using the procedures described in reference 10 from their respective perfluoroalkylated amines **3a**, **b**, and Z-Gly-ONP, giving derivatives [DFnC11]-GlyNH-Z (yields ranging from 50 to 80%), followed by Z-deprotection using $\text{H}_2/\text{Pd/C}$ (yields ranging from 90 to 95%). The [F4C11][C14]-GlyNH₂ glycinamine has been obtained by the simultaneous Z-deprotection and double bond hydrogenation of [F4E11][C14]-GlyNH-Z using $\text{H}_2/\text{Pd/C}$ (60%).

***N,N'*-di-11-(*F*-butyl)-(resp. *F*-hexyl)-undecyl-*N*-benzyloxycarbonylglycin-amine, DF4C11-GlyNH-Z (resp. DF6C11-GlyNH-Z).** TLC (CHCl_3 , UV and ninhydrine): $R_f = 0.20$ and TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 99/1, UV and ninhydrine): $R_f = 0.59$, respectively. These two derivatives display identical ^1H and ^{13}C NMR spectra. ^1H NMR (CDCl_3): δ 1.15–1.40 (s large, 28H, $\text{CF}_2(\text{CH}_2)_2(\text{CH}_2)_7$); 1.40–1.78 (m, 8H, $\text{CF}_2\text{CH}_2\text{CH}_2$, $\text{CH}_2\text{CH}_2\text{N}$); 2.05 (tt, $^3J = 7.5$ Hz, $^3J_{\text{HF}} = 19.1$ Hz, 4H, CF_2CH_2); 3.15, 3.32 (t, 4H, $^3J = 7.3$ Hz, CH_2N); 4.00 (d, $^3J = 4.0$ Hz, CH_2NH); 5.08 (s, 2H, OCH_2Ph); 5.88 (m, 1H, NH); 7.20–7.35 (m, 5H, Ph). ^{13}C NMR (CDCl_3): δ 20.1 (t, $^3J_{\text{CF}} = 4$ Hz, $\text{CF}_2\text{CH}_2\text{CH}_2$); 26.9, 27.0, 27.6, 28.8, 29.1, 29.2, 29.4, 29.5, $(\text{CF}_2(\text{CH}_2)_2(\text{CH}_2)_8)$; 30.9 (t, $^2J_{\text{CF}} = 22$ Hz, CF_2CH_2); 42.7 (CH_2NH); 46.2, 46.9 (CH_2N); 66.8 (CH_2Ph); 128.0, 128.5, 136.8, (Ph); 156.4 (NHCO); 167.5 (CH_2CO). ^{19}F NMR (CDCl_3) identical to that of **3a** and **3b**, respectively.

***N,N'*-di-11-(*F*-butyl)-(resp. *F*-hexyl)-undecyl glycinamine, DF4C11-GlyNH₂ (resp. DF6C11-GlyNH₂).** TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95/5, ninhydrine): $R_f = 0.53$ and 0.62 , respectively. These two derivatives display identical ^1H and ^{13}C NMR spectra. ^1H NMR (CDCl_3): δ 1.15–1.40 (s large, 28H, $\text{CF}_2(\text{CH}_2)_2(\text{CH}_2)_7$); 1.40–1.78 (m, 8H, $\text{CF}_2\text{CH}_2\text{CH}_2$, $\text{CH}_2\text{CH}_2\text{N}$); 2.05 (tt, $^3J = 7.5$ Hz, $^3J_{\text{HF}} = 19.1$ Hz, 4H, CF_2CH_2); 3.15, 3.32 (t, 4H, $^3J = 7.3$ Hz, CH_2N); 3.47 (s large, 2H, CH_2NH_2). ^{13}C NMR (CDCl_3): δ 20.1 (t, $^3J_{\text{CF}} = 4$ Hz, $\text{CF}_2\text{CH}_2\text{CH}_2$); 26.9, 27.0, 27.6, 28.8, 29.1, 29.2, 29.4, 29.5, $(\text{CF}_2(\text{CH}_2)_2(\text{CH}_2)_8)$; 30.9 (t, $^2J_{\text{CF}} = 22$ Hz,

CF_2CH_2), 42.3 (CH_2NH_2); 46.2, 46.9 (CH_2N); 170.7 (CO). ^{19}F NMR (CDCl_3) identical to that of **3a** and **3b**, respectively.

***N'*-[11-(*F*-butyl)-undecyl]-*N'*-tetradecyl glycinamine, [F4C11][C14]-GlyNH₂.** TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9/1, ninhydrine): $R_f = 0.31$. ^1H NMR (CDCl_3): δ 0.88 (t, $^3J = 6.5$ Hz, 3H, CH_3); 1.12–1.42 (s large, 36H, $(\text{CH}_2)_7(\text{CH}_2)_2\text{N}(\text{CH}_2)_2(\text{CH}_2)_{11}$); 1.42–1.80 (m, 6H, $\text{CF}_2\text{CH}_2\text{CH}_2$, $\text{CH}_2\text{CH}_2\text{N}$); 2.06 (tt, $^3J = 7.5$ Hz, $^3J_{\text{HF}} = 19.1$ Hz, 4H, CF_2CH_2); 3.14, 3.30 (t, 4H, $^3J = 7.3$ Hz, CH_2N); 3.48 (s, 2H, CH_2NH_2). ^{13}C NMR (CDCl_3): δ 14.1 (CH_3); 20.1 (t, $^3J_{\text{CF}} = 4$ Hz, $\text{CF}_2\text{CH}_2\text{CH}_2$); 22.7 (CH_3CH_2); 26.9, 27.0, 27.7, 28.9, 29.1, 29.2, 29.3, 29.5 ($(\text{CH}_2)_8\text{CH}_2\text{NCH}_2(\text{CH}_2)_{10}$); 30.8 (t, $^2J_{\text{CF}} = 22$ Hz, CF_2CH_2); 31.9 ($\text{CH}_3\text{CH}_2\text{CH}_2$); 43.2 (CH_2NH_2); 46.3, 46.8 (CH_2N); 171.3 (CO). ^{19}F NMR (CDCl_3) identical to that of **3a**.

Synthesis of Tetra-*tert*-butoxycarbonylspermine-5-carboxylic Acid (Boc₄SperCOOH). This compound was synthesized in three steps as described previously with some modifications related to the first step (Remy et al., 1994). The addition of acrylonitrile (in MeOH) was performed onto the L-ornithine tetramethylammonium salt in MeOH, in place of DMF. In our hands, when we performed this synthesis in DMF, it failed in most cases. After stirring for 1 h at room temperature, the solvent was removed. The residue was dissolved in MeOH and the *N,N'*-biscyanoethylated derivative was precipitated by addition of HCl 12 N and isolated as its HCl salt by filtration (white powder; 60%). ^1H NMR (D_2O): δ 1.60–2.10 (m, 4H, CHCH_2CH_2); 2.82–3.01 (m, 4H, CH_2CN); 3.01–3.22 (m, 2H, $\text{CH}(\text{CH}_2)_2\text{CH}_2$); 3.22–3.55 (4H, $\text{CH}_2\text{CH}_2\text{CN}$); 3.58–3.80 (m, 1H, CH). ^{13}C NMR (D_2O): δ 14.8, 15.0 (CH_2CN); 21.5, 26.5 (CHCH_2CH_2); 42.2, 42.7, 47.1 (CH_2N); 62.0 (CH); 117.5 (CN); 172.4 (COO).

Synthesis of the [R¹][R²]-GS Lipospermines. The syntheses of these lipospermines were carried out in two steps from their respective [R¹][R²]-GlyNH₂ glycinamine and Boc₄SperCOOH, followed by Boc-deprotection. Boc₄SperCOOH was condensed with the glycinamines in the presence of 1.1 equiv of a coupling agent, such as DCC, DCC/HOSu, EDC, BOP, and PyBrop, in CH_2Cl_2 . When the condensation was performed with DCC/HOSu, the acid was preactivated for about 12 h before addition of the glycinamines. In all the other cases, the coupling agent was added to the solution containing the acid and the glycinamine. After 12–24 h of reaction, the organic phase was washed, dried and evaporated and the residue purified by silica gel chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 100/0 to 95/5) to afford the [R¹][R²]-GS(Boc) derivatives as colorless liquids (yields ranging from 20 to 70%). The Boc-deprotection was quantitatively achieved by dissolving and keeping the [R¹][R²]-GS(Boc) derivatives in a large excess of TFA at room temperature for 1 h. The excess TFA was removed by coevaporation in the presence of cyclohexane. Then, the product was triturated in CH_2Cl_2 to give the desired [R¹][R²]-GS lipospermines, as their tetra-TFA salts.

Tetra-*tert*-butoxycarbonylspermine-5-carboxyglycine *N,N*-di-[11-(*F*-butyl)-(resp. *F*-hexyl)-undec-10-enyl]amide, DF4E11-GS(Boc) (resp. DF6E11-GS(Boc)). TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9/1, ninhydrine): $R_f = 0.80$. TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 96/4, ninhydrine): $R_f = 0.48$, respectively. These two derivatives display identical ^1H and ^{13}C NMR spectra. ^1H NMR (CD_3OD): δ 1.17–1.37 (s large, 24H, $(\text{CH}_2)_6(\text{CH}_2)_2\text{N}$); 1.37–1.83 (m, 48H, $\text{CH}_2\text{CH}_2\text{N}$, $\text{CH}_2\text{CH}_2\text{NH}(\text{Boc})$, CHCH_2CH_2); 2.13–2.35 (m, 4H, $=\text{CHCH}_2$); 2.91–3.38 (m, 14H, CH_2N , CH_2NBoc , $\text{CH}_2\text{NH}(\text{Boc})$); 3.95–4.08 (m, 3H, COCH_2NH , CH); 5.58–5.85 (m, 1H, $\text{CF}_2\text{CH}=\text{CH}$); 6.34–6.54 (m, 1H, $\text{CF}_2\text{CH}=\text{CH}$). ^{13}C NMR (CD_3OD):

OD): δ 25.6, 26.4 (CHCH_2CH_2 , $\text{CH}_2\text{CH}_2\text{NHBoc}$); 27.8, 28.0, 28.6, 28.7, 29.2, 29.8, 30.1, 30.4, 30.6 ($(\text{CH}_2)_7\text{CH}_2\text{N}$); 28.8 (CH_3 Boc); 33.0 ($=\text{CHCH}_2$); 39.0, 39.2, 43.0, 46.0 (CH_2NBoc , CH_2NHBoc); 42.1 (COCH_2NH); 47.5, 48.4 (CH_2N); 59.6 (CH); 80.0, 81.2, 82.0 ($\text{C}(\text{CH}_3)_3$); 118.0 (t, $^2J_{\text{CF}} = 23$ Hz, $\text{CF}_2\text{CH}=\text{CH}$); 145.5 (t, $^3J_{\text{CF}} = 9$ Hz, $\text{CF}_2\text{CH}=\text{CH}$); 157.6, 158.5 (CO Boc); 169.5 (NCOCH_2); 173.7 (NCOCH). ^{19}F NMR (CD_3OD): identical to that of **2a** and **2b**, respectively.

Tetra-tert-butoxycarbonylspermine-5-carboxyglycine N,N-di-11-(F-butyl)-(resp. F-hexyl)-undecylamide DF4C11-GS(Boc) (resp. DF6C11-GS(Boc)). TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9/1, ninhydrine): $R_f = 0.75$. TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95/5, ninhydrine): $R_f = 0.78$, respectively. These two derivatives display identical ^1H and ^{13}C NMR spectra. ^1H NMR (CDCl_3): δ 1.20–1.40 (s large, 28H, $\text{CF}_2(\text{CH}_2)_2(\text{CH}_2)_7$); 1.40–1.87 (m, 52H, $\text{CF}_2\text{CH}_2\text{CH}_2$, $\text{CH}_2\text{CH}_2\text{N}$, $\text{CH}_2\text{CH}_2\text{NHBoc}$, CHCH_2CH_2); 2.05 (tt, $^3J = 7.5$ Hz, $^3J_{\text{HF}} = 19.1$ Hz, 4H, CF_2CH_2); 2.98–3.58 (m, 14H, CH_2N , CH_2NBoc , CH_2NHBoc); 3.96–4.12 (m, 3H, COCH_2NH , CH). ^{13}C NMR (CDCl_3): δ 20.1 (t, $^3J_{\text{CF}} = 4$ Hz, $\text{CF}_2\text{CH}_2\text{CH}_2$); 25.0, 25.1 ($\text{CH}_2\text{CH}_2\text{NHBoc}$); 26.9, 27.0, 27.6, 28.8, 29.1, 29.2, 29.3, 29.5 ($\text{CF}_2(\text{CH}_2)_2(\text{CH}_2)_8$); 28.4 (CH_3 Boc); 30.8 (t, $^2J_{\text{CF}} = 22$ Hz, CF_2CH_2); 38.1, 42.6, 46.3, (CH_2NBoc , CH_2NHBoc); 41.3 (COCH_2NH); 46.2, 46.9 (CH_2NCO); 78.9, 79.7, 81.0, ($\text{C}(\text{CH}_3)_3$); 156.1 (CO Boc); 167.0 (NCOCH_2); 171.2 (NCOCH). The resonances corresponding to the CHCH_2CH_2 carbons are not detectable. ^{19}F NMR (CDCl_3) identical to that of **3a** and **3b**, respectively.

Tetra-tert-butoxycarbonylspermine-5-carboxyglycine N-[11-(F-butyl)-undec-10-enyl]-N-tetradecylamide [F4E11][C14]-GS(Boc) (resp. [F6E11][C14]-GS(Boc)). TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 97/3, ninhydrine): $R_f = 0.45$. TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 96/4, ninhydrine): $R_f = 0.62$, respectively. These two derivatives display identical ^1H and ^{13}C NMR spectra. ^1H NMR (CDCl_3): δ 0.88 (t, $^3J = 6.5$ Hz, 3H, CH_3); 1.07–1.37 (s large, 34H, $(\text{CH}_2)_6(\text{CH}_2)_2\text{N}(\text{CH}_2)_2(\text{CH}_2)_{11}$); 1.37–2.08 (m, 48H, $\text{CH}_2\text{CH}_2\text{N}$, $\text{CH}_2\text{CH}_2\text{NHBoc}$, CHCH_2CH_2); 2.08–2.30 (m, 2H, $=\text{CHCH}_2$); 2.94–3.47 (m, 14H, CH_2N , CH_2NBoc , CH_2NHBoc); 3.93–4.06 (m, 3H, COCH_2NH , CH); 5.45–5.73 (m, 1H, $\text{CF}_2\text{CH}=\text{CH}$); 6.28–6.50 (m, 1H, $\text{CF}_2\text{CH}=\text{CH}$). ^{13}C NMR (CDCl_3): δ 14.1 (CH_3); 22.7 (CH_3CH_2); 25.5, 26.4 (CHCH_2CH_2 , $\text{CH}_2\text{CH}_2\text{NHBoc}$); 26.9, 27.0, 27.6, 27.9, 28.4, 28.7, 28.9, 29.3, 29.6 ($(\text{CH}_2)_7\text{CH}_2\text{NCH}_2(\text{CH}_2)_{10}$); 28.4 (CH_3 Boc); 31.9, 31.9 ($\text{CH}_2\text{CH}_2\text{CH}_2$, $=\text{CHCH}_2$); 37.5, 37.9, 43.1, 46.4 (CH_2NBoc , CH_2NHBoc); 41.2 (COCH_2NH); 46.1, 46.9 (CH_2N); 59.8 (CH); 78.5, 78.9, 80.9 ($\text{C}(\text{CH}_3)_3$); 116.6 (t, $^2J_{\text{CF}} = 23$ Hz, $\text{CF}_2\text{CH}=\text{CH}$); 143.3 (t, $^3J_{\text{CF}} = 9$ Hz, $\text{CF}_2\text{CH}=\text{CH}$); 156.0 (CO Boc); 167.0 (NCOCH_2); 170.9 (NCOCH). ^{19}F NMR (CDCl_3) identical to that of **2a** and **2b**, respectively.

Tetra-tert-butoxycarbonylspermine-5-carboxyglycine N-[11-(F-butyl)-undec-10-enyl]-N-tetradecylamide [F4C11][C14]-GS(Boc). TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 96/4, ninhydrine): $R_f = 0.63$. ^1H NMR (CDCl_3): δ 0.88 (t, $^3J = 6.5$ Hz, 3H, CH_3); 1.11–1.39 (s large, 36H, $(\text{CH}_2)_7(\text{CH}_2)_2\text{N}(\text{CH}_2)_2(\text{CH}_2)_{11}$); 1.39–1.87 (m, 50H, $\text{CF}_2\text{CH}_2\text{CH}_2$, $\text{CH}_2\text{CH}_2\text{N}$, $\text{CH}_2\text{CH}_2\text{NHBoc}$, CHCH_2CH_2); 2.06 (tt, $^3J = 7.5$ Hz, $^3J_{\text{HF}} = 19.1$ Hz, 4H, CF_2CH_2); 2.94–3.47 (m, 14H, CH_2N , CH_2NBoc , CH_2NHBoc); 3.93–4.09 (m, 3H, COCH_2NH , CH). ^{13}C NMR (CDCl_3): δ 14.1 (CH_3); 20.1 (t, $^3J_{\text{CF}} = 4$ Hz, $\text{CF}_2\text{CH}_2\text{CH}_2$); 22.7 (CH_3CH_2); 25.7, 26.5 (CHCH_2CH_2 , $\text{CH}_2\text{CH}_2\text{NHBoc}$); 26.9, 27.0, 27.6, 28.8, 29.1, 29.2, 29.3, 29.6 ($(\text{CH}_2)_8\text{CH}_2\text{NCH}_2(\text{CH}_2)_{10}$); 28.4 (CH_3 Boc); 30.8 (t, $^2J_{\text{CF}} = 22$ Hz, CF_2CH_2); 31.9 ($\text{CH}_3\text{CH}_2\text{CH}_2$); 37.9, 38.5, 43.3, 44.2, 46.8 (CH_2NBoc , CH_2NHBoc); 41.3 (COCH_2NH); 46.2, 47.0 (CH_2N); 60.2 (CH); 79.2, 79.7, 81.0

($\text{C}(\text{CH}_3)_3$); 156.2 (CO Boc); 167.0 (NCOCH_2); 170.0 (NCOCH). ^{19}F NMR (CDCl_3) identical to that of **3a**.

Spermine-5-carboxyglycine N,N-di-[11-(F-butyl)-(resp. F-hexyl)-undec-10-enyl]amide DF4E11-GS (resp. DF6E11-GS) (isolated as their tetra-TFA ammonium salt). These two derivatives display identical ^1H and ^{13}C NMR spectra. ^1H NMR (CD_3OD): δ 1.17–1.41 (s large, 24H, $(\text{CH}_2)_6(\text{CH}_2)_2\text{N}$); 1.41–1.73 (m, 4H, $\text{CH}_2\text{CH}_2\text{N}$); 1.73–2.18 (m, 8H, CHCH_2CH_2 , $\text{CH}_2\text{CH}_2\text{NH}_3^+$); 2.18–2.40 (m, 4H, $=\text{CHCH}_2$); 2.89–3.44 (m, 14H, CH_2N , CH_2N^+); 3.90–4.08 (m, 1H, CH); 4.18 (s, 2H, COCH_2NH); 5.58–5.88 (m, 1H, $\text{CF}_2\text{CH}=\text{CH}$); 6.35–6.60 (m, 1H, $\text{CF}_2\text{CH}=\text{CH}$). ^{13}C NMR (CD_3OD): δ 22.7, 28.5 (CHCH_2CH_2); 25.3, 25.3 ($\text{CH}_2\text{CH}_2\text{NH}_3^+$); 27.8, 28.0, 28.6, 29.0, 29.7, 30.0, 30.2, 30.3, 30.4, 30.5, 30.6 ($(\text{CH}_2)_7\text{CH}_2\text{N}$); 32.9 ($=\text{CHCH}_2$); 37.8, 37.9, 48.3 (CH_2N^+); 42.0 (COCH_2NH); 44.9, 45.9 (CH_2N); 61.2 (CH); 118.0 (t, $^2J_{\text{CF}} = 23$ Hz, $\text{CF}_2\text{CH}=\text{CH}$); 145.5 (t, $^3J_{\text{CF}} = 9$ Hz, $\text{CF}_2\text{CH}=\text{CH}$); 169.0 (NCOCH_2); 169.6 (NCOCH). ^{19}F NMR (CD_3OD) of **DF4E11-GS,4TFA**: δ -75.7 (12F, 4TFA); -81.5 (6F, CF_3); -107.2 (0.4F, CF_2CH , Z); -111.9 (3.6F, CF_2CH , E); -125.0 (4F, $\text{CF}_3\text{CF}_2\text{CF}_2$); -126.1 (4F, CF_3CF_2). ^{19}F NMR (CD_3OD) of **DF6E11-GS,4TFA**: δ -75.7 (12F, 4TFA); -81.5 (6F, CF_3); -107.2 (0.4F, CF_2CH , Z); -111.9 (3.6F, CF_2CH , E); -122.3, -123.6, -124.2 (4F, 4F, $\text{CF}_3\text{CF}_2(\text{CF}_2)_3$); -126.8 (4F, CF_3CF_2). **DF4E11-GS,4TFA**: Anal. ($\text{C}_{51}\text{H}_{72}\text{F}_{30}\text{N}_6\text{O}_{10}$) H, N; C: calcd, 40.86; found, 41.81. **DF6E11-GS,4TFA**: Anal. ($\text{C}_{55}\text{H}_{72}\text{F}_{38}\text{N}_6\text{O}_{10}$) N; C: calcd, 38.88; found, 40.75; H calcd, 4.27; H found, 4.81.

Note: both **DF4E11-GS** and **DF6E11-GS** consist in a mixture of E/E, E/Z, and Z/Z isomers, as shown by ^{19}F NMR (E/Z + Z/Z representing $\leq 10\%$). The $\text{CF}_2\text{CH}_2\text{CH}=\text{CH}$ isomers (less than 3%) are detected by ^{19}F NMR.

Spermine-5-carboxyglycine N,N-di-11-(F-butyl)-(resp. F-hexyl)-undecylamide DF4C11-GS (resp. DF6C11-GS) (isolated as their tetra-TFA ammonium salt). These two derivatives display identical ^1H and ^{13}C NMR spectra. ^1H NMR (CD_3OD): δ 1.27–1.48 (s large, 28H, $(\text{CH}_2)_7(\text{CH}_2)_2\text{N}$); 1.51–1.81 (m, 8H, $\text{CH}_2\text{CH}_2\text{N}$, $\text{CF}_2\text{CH}_2\text{CH}_2$); 1.76–2.30 (m, 12H, CF_2CH_2 , CHCH_2CH_2 , $\text{CH}_2\text{CH}_2\text{NH}_3^+$); 2.92–3.43 (m, 14H, CH_2N , CH_2N^+); 3.96–4.07 (m, 1H, CH); 4.15 (s, 2H, COCH_2NH). ^{13}C NMR (CD_3OD): δ 21.0 (t, $^3J_{\text{CF}} = 4$ Hz, $\text{CF}_2\text{CH}_2\text{CH}_2$); 22.5, 28.2 (CHCH_2CH_2); 25.0, 25.1, ($\text{CH}_2\text{CH}_2\text{NH}_3^+$); 27.7, 27.9; 28.5, 29.5, 29.9, 30.1, 30.3, 30.4 ($\text{CF}_2(\text{CH}_2)_2(\text{CH}_2)_8$); 31.5 (t, $^2J_{\text{CF}} = 22$ Hz, CF_2CH_2); 37.6, 37.7, 48.4 (CH_2N^+); 41.9 (COCH_2NH); 44.6, 45.7 (CH_2N); 60.9 (CH); 168.8 (NCOCH_2); 170.2 (NCOCH). ^{19}F NMR (CD_3OD) of **DF4C11-GS,4TFA**: δ -75.9 (12F, 4TFA); -81.3 (6F, CF_3); -115.1 (4F, CF_2CH_2); -124.9 (4F, $\text{CF}_3\text{CF}_2\text{CF}_2$); -126.5 (4F, CF_3CF_2). ^{19}F NMR (CD_3OD) of **DF6C11-GS,4TFA**: δ -75.9 (12F, 4TFA); -81.3 (6F, CF_3); -114.9 (4F, CF_2CH_2); -122.4, -123.4, -124.1 (4F, 4F, 4F, $\text{CF}_3\text{CF}_2(\text{CF}_2)_3$); -126.6 (4F, CF_3CF_2). **DF4C11-GS,4TFA**: Anal. ($\text{C}_{51}\text{H}_{76}\text{F}_{30}\text{N}_6\text{O}_{10}$) $\cdot \text{H}_2\text{O}$, C, H, N. **DF6C11-GS,4TFA**: Anal. ($\text{C}_{55}\text{H}_{76}\text{F}_{38}\text{N}_6\text{O}_{10}$) $\cdot \text{H}_2\text{O}$, C, H, N.

Spermine-5-carboxyglycine N-[11-(F-butyl)-(resp. F-hexyl)-undec-10-enyl]-N-tetradecylamide, [F4E11]-[C14]-GS (resp. [F6E11][C14]-GS) (isolated as their tetra-TFA ammonium salt). These two derivatives display identical ^1H and ^{13}C NMR spectra. ^1H NMR (CD_3OD): δ 0.90 (t, $^3J = 6.5$ Hz, 3H, CH_3); 1.12–1.42 (s large, 34H, $(\text{CH}_2)_6(\text{CH}_2)_2\text{N}(\text{CH}_2)_2(\text{CH}_2)_{11}$); 1.42–1.77 (m, 4H, $\text{CH}_2\text{CH}_2\text{N}$); 1.77–2.20 (m, 8H, CHCH_2CH_2 , $\text{CH}_2\text{CH}_2\text{NH}_3^+$); 2.20–2.36 (m, 4H, $=\text{CHCH}_2$); 2.87–3.43 (m, 14H, CH_2N , CH_2N^+); 3.95–4.07 (m, 1H, CH); 4.15 (s, 2H, COCH_2NH); 5.58–5.88 (m, 1H, $\text{CF}_2\text{CH}=\text{CH}$); 6.35–6.60 (m, 1H, $\text{CF}_2\text{CH}=\text{CH}$). ^{13}C NMR (CD_3OD): δ 14.4 (CH_3);

22.5, 28.4 (CHCH₂CH₂); 23.7 (CH₃CH₂); 25.1, 25.2 (CH₂-CH₂NH₃⁺); 27.7, 27.9, 28.6, 29.0, 29.6, 29.8, 30.4, 30.5, 30.6, 30.7 ((CH₂)₇CH₂NCH₂(CH₂)₁₀); 32.9, 33.0 (CH₃-CH₂CH₂, =CHCH₂); 37.8, 38.0, 48.2 (CH₂N⁺); 41.9 (COCH₂NH); 44.7, 45.7 (CH₂N); 61.2 (CH); 116.6 (t, ²J_{CF} = 23 Hz, CF₂CH=CH); 143.3 (t, ³J_{CF} = 9 Hz, CF₂CH=CH); 169.2 (NCOCH₂); 169.5 (NCOCH). ¹⁹F NMR (CD₃-OD) of [F4E11][C14]-GS,4TFA: δ -75.7 (12F, 4TFA); -81.5 (3F, CF₃); -107.2 (~0.2F, CF₂CH, Z); -111.9 (~1.8F, CF₂CH, E); -125.0 (2F, CF₃CF₂CF₂); -126.1 (2F, CF₃CF₂). ¹⁹F NMR (CD₃OD) of [F6E11][C14]-GS,4TFA: δ -75.7 (12F, 4TFA); -81.5 (3F, CF₃); -107.2 (0.05F, CF₂CH, Z); -111.9 (1.95F, CF₂CH, E); -122.3, -123.6, -124.2 (2F, 2F, 2F, CF₃CF₂(CF₂)₃); -126.8 (2F, CF₃CF₂). [F4E11][C14]-GS,4TFA: Anal. (C₅₀H₈₁F₂₁N₆O₁₀) C, H, N. [F6E11][C14]-GS,4TFA: Anal. (C₅₂H₈₁F₂₅N₆O₁₀) C, H, N.

Note: both [F4E11][C14]-GS and [F6E11][C14]-GS consist in a mixture of the E and Z isomers, as shown by ¹⁹F NMR (E representing ~90%). The CF₂-CH₂-CH=CH- isomer (less than 3%) is detected by ¹⁹F NMR.

Spermine-5-carboxyglycine N-[11-(F-butyl)-undec-10-enyl]-N-tetradecyl-amide, [F4C11][C14]-GS (isolated as its tetra-TFA ammonium salt). ¹H NMR (CD₃OD): δ 0.88 (t, ³J = 6.5 Hz, 3H, CH₃); 1.17–1.48 (s large, 36H, (CH₂)₇(CH₂)₂N(CH₂)₂(CH₂)₁₁); 1.48–1.76 (m, 6H, CH₂CH₂N, CF₂CH₂CH₂); 1.76–2.30 (10H, CF₂CH₂, CHCH₂CH₂, CH₂CH₂NH₃⁺); 2.94–3.44 (m, 14H, CH₂-NCO, CH₂N⁺); 3.95–4.08 (m, 1H, CH); 4.15 (s, 2H, COCH₂NH). ¹³C NMR (CD₃OD): δ 14.4 (CH₃); 21.2 (t, ³J_{CF} = 4 Hz, CF₂CH₂CH₂); 22.7, 28.4 (CHCH₂CH₂); 23.6 (CH₃CH₂); 25.2, 25.4 (CH₂CH₂NH₃⁺); 27.8, 28.1, 28.7, 29.7, 30.2, 30.4, 30.5, 30.6, 30.7 ((CH₂)₈CH₂NCH₂(CH₂)₁₀); 31.7 (t, ²J_{CF} = 22 Hz, CF₂CH₂); 33.1 (CH₃CH₂CH₂); 38.0, 48.5 (CH₂N⁺); 42.1 (COCH₂NH); 45.0, 46.0 (CH₂N); 61.2 (CH); 169.5 (NCOCH₂); 170.0 (NCOCH). ¹⁹F NMR (CD₃-OD): δ -75.7 (12F, 4TFA); -81.3 (3F, CF₃); -115.1 (2F, CF₂CH₂); -124.9 (2F, CF₃CF₂CF₂); -126.5 (2F, CF₃CF₂). Anal. (C₅₀H₈₃F₂₁N₆O₁₀) C, H, N.

Preparation of Complexes Composed of the Lipospermines and Plasmid pTG11033. The plasmid pTG11033 was produced by Transgene SA. The endotoxin content of the plasmid preparation was checked using a Limulus Amebocyte Lysat kit (Biogenic, France). This value was below 1 endotoxin unit(eu)/mg of plasmid, hence below the 5 eu/mg of DNA recommended for in vivo protocols. The quantities of compound used were calculated according to the desired DNA concentration of 0.1 mg/mL, the N/P ratio, the molar weight, and the number of positive charges in the selected lipospermine. The lipospermine/DNA complex is formulated by adding a desired volume of lipospermine preparation at a concentration of 10 mg/mL (in 20 mM HEPES buffer, pH 7.5, containing 10% DMSO and 10% EtOH) to the desired volume of DNA solution to reach desired DNA concentration (for example, 0.1 mg/mL). Thus, for the preparation of the DOGS/DNA complex at N/P ratio 5 and 0.1 mg/mL DNA, 50 μL of a DOGS solution (10 mg/mL in EtOH) was transferred to a borosilicate glass tube (16 × 100 mm). The solvent was evaporated in Rotavap evaporation system (45 °C, 30 pm, 0.2 bar, 40 min). A total of 5 μL of DMSO, 5 μL of EtOH, and 40 μL of HEPES 20 mM, pH 7.5, were added to the film obtained. The preparation was vortexed for 12 h. Then, 47.9 μL of this preparation was added to 952.1 μL of DNA solution [(100 μL of DNA (1 mg/mL) diluted with 852.1 μL of HEPES buffer)]. This preparation can be used for further experiments.

When the composition further comprises DOPE, the desired volume of 10 mg/mL chloroform solution (to get mole/mole DOGS/DOPE) was added to the volume of

DOGS solution (10 mg/mL in EtOH), and then the mixture was transferred to a borosilicate glass tube. The following steps were as described above.

Measurement of the Size of the Lipoplexes. The average sizes were measured by photon correlation spectroscopy using a Coulter N4Plus particle size analyzer. The analyses were performed at 25 °C, after equilibrating the sample at 25 °C for 20 min. One aliquot of the sample was aspirated and discharged several times prior to pipetting. The sample was diluted with 20 mM HEPES in the measurement tube and homogenized. These analyses were thus carried out on lipoplexes having a 10 μg/mL concentration of DNA. After waiting 180 s, the light diffraction at 90° was measured for 180 s. The range was 3 nm to 10 μm, using 31 bins. The size measurement is valid if the sample gives between 50 000 and 1 000 000 counts/s. The formulations and analyses were reproduced twice.

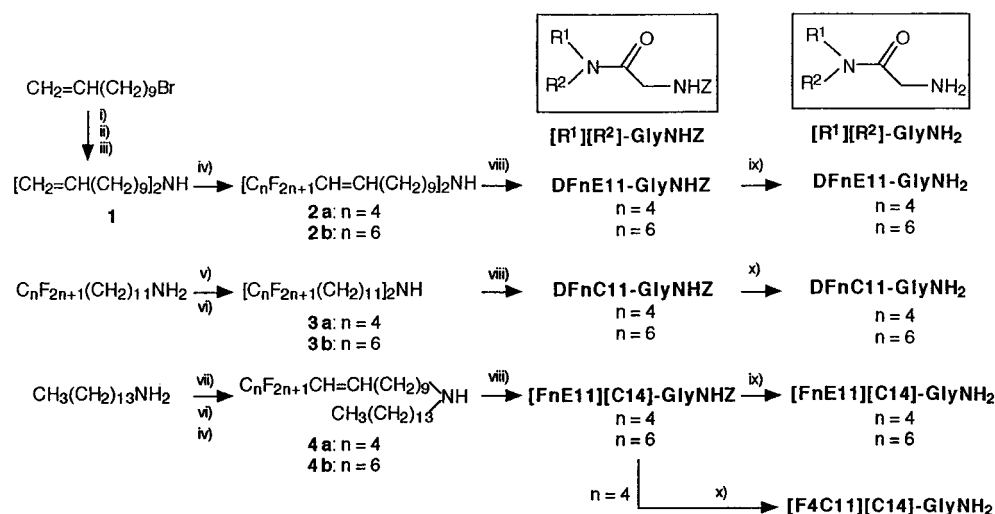
Agarose Gel Electrophoresis. Each sample (0.5 μg of plasmid) was analyzed by electrophoresis for about 90 min under 75 V/cm, through a 0.8% agarose gel in TAE 1× (Tris-acetate-EDTA) buffer. The DNA was visualized in 200 mL of TAE buffer containing 20 μL of ethidium bromide (Sigma, 10 mg/mL). The plasmid integrity in each sample (0.25 μg) was confirmed by electrophoresis after decomplexing the lipoplex (10 μL of a plasmid 0.05 mg/mL) with sodium dodecyl sulfate, SDS (10 μL, 80 g/l).

In Vitro Transfection of A549 Cells. Twenty-four hours before transfection, A549 cells (epithelial cells derived from human pulmonary carcinoma) were grown in Dulbecco-modified Eagle culture medium (DMEM) (GIBCO-BRL), containing 10% fetal calf serum, FCS (SIGMA), in 96-well plates (2 × 10⁴ cells/well), in a wet (37 °C) and 5% CO₂/95% air atmosphere. Volume of DNA/lipospermine lipoplex (20, 10, 5, and 1 μL, respectively) was diluted to 100 μL in DMEM supplemented with 10% FCS in order to obtain various amounts of DNA (2, 1, 0.5, and 0.1 μg, respectively) in the preparation. The culture medium was removed and replaced by 100 μL of DMEM supplemented with 10% FCS and containing the desired amount of DNA. After 4 and 24 h, 50 and 100 μL of DMEM supplemented with 30 and 10% FCS, respectively, were added. Forty-eight hours after transfection, the culture medium was discarded and the cells were washed twice with 100 μL of PBS and then lysed with 50 μL of lysis buffer (Promega). The lysates were frozen at -40 °C awaiting analysis of luciferase activity. This measurement was done for 10 s on 10 μL of the lysis mixture in a Berthold LB96P luminometer in dynamic mode, using the "Luciferase" determination system (Promega) in 96-well plates. The total protein concentration per well was determined using conventional techniques (BCA test, Pierce). For cells grown in the absence of lipoplexes, a well contains around 30–50 μg of proteins. Each DNA/lipospermine complex was formulated twice and each formulation was tested twice.

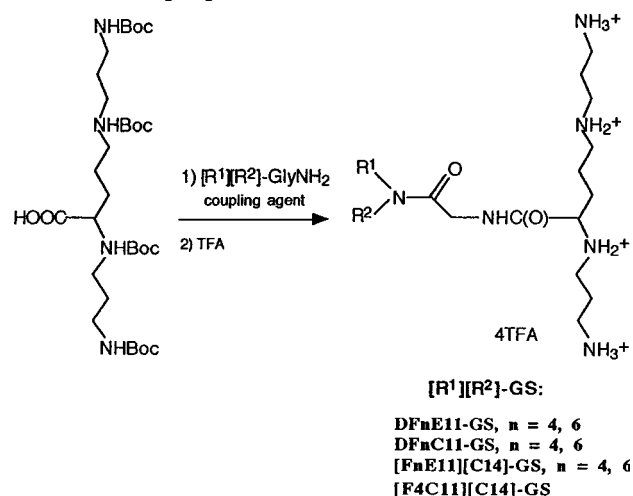
Statistical Analyses. Statistical analyses were performed using a Student test. The difference between two means was considered as significant when the probability *p* is ≤ 0.05.

RESULTS AND DISCUSSION

Synthesis. The syntheses of the fluorinated lipospermines have been performed using the procedures described for their hydrocarbon DOGS analogue and are shown in Schemes 1 and 2 (Remy et al., 1994). Their specificity lies in the use of perfluoroalkylated secondary amines which are not commercially available. The most

Scheme 1. Synthetic Route to the [R¹][R²]-GlyNH₂ Amines^a

^a (i) NC-NH₂; (ii) H₂SO₄; (iii) base; (iv) C_nF_{2n+1}I/CuCl/t-BuOH/ethanolamine; (v) C_nF_{2n+1}(CH₂)₁₀COCl/NEt₃; (vi) LiAlH₄/THF; (vii) CH₂=CH(CH₂)₈COCl/NEt₃; (viii) Z-Gly-ONP; (ix) HBr/AcOH, then base; (x) H₂/Pd/C.

Scheme 2. Synthetic Route to the Fluorinated [R¹][R²]-GS Lipospermines.

straightforward synthesis of the “unsaturated” symmetrical secondary amines **2** (Scheme 1) was performed by alkylation under phase-transfer-catalysis conditions of cyanamide using 11-bromo-1-undecene followed by hydrolysis (Jonczyk et al., 1978), then grafting of the perfluoroalkyl segment onto the terminal double bonds of **1**. The addition of a linear perfluoroalkyl iodide C_nF_{2n+1}I on a terminal CH₂=CH- double bond may be performed with high yields by various processes. An attractive way is the modified method of Burton and Kehoe (1966) consisting of the one-pot two-step reaction, i.e., radical addition of C_nF_{2n+1}I initiated by CuCl/2-aminoethanol and α,β -dehydroiodination in the presence of a base, which yields the perfluoroalkenyl derivative (mainly of (*E*)-configuration). When this method was applied to **1**, the addition of the C_nF_{2n+1} group to both its C=C bonds and subsequent HI elimination, led to **2**. Under these conditions, no HF elimination was observed. However, according to ¹⁹F NMR which showed more particularly the presence of two resonances for the CF₂ in α to the C=C bonds, the isolated amines **2** consisted in a mixture of isomers differing in the configuration of their two C=C bonds. The most abundant one was the *E,E*-isomer (at least 85%). These products were contaminated by compound(s) (less than 5%) which possess(es) a CF₂-CH₂-

CH=CH- sequence in one and/or in both hydrophobic chains, as a result of a β,γ -HI elimination during the perfluoroalkylation step (Burton and Kehoe, 1966). This was evident from the ¹H-, ¹³C- and ¹⁹F NMR spectra of **2** (dt, ³J_{HH} = 7 Hz, ³J_{HF} = 18 Hz) at 2.7 ppm, t (²J_{CF} = 23 Hz) at 34.7 ppm for CH₂ and a CF₂ resonance at -115.8 ppm).

Although a mixture of alkenyl isomers are obtained, this reaction sequence remains nevertheless the best way for the synthesis of “unsaturated” fluorinated di-alkenylamines such as **2**. One of its advantages lies also in the alkylation step which is not performed with a high cost fluorinated material. Another advantage of this strategy is its versatility as the same synthon **1** can be used for the preparation of di-alkenylamines differing in the length of their perfluoroalkyl tails (such as **2a** and **2b**), and even of saturated ones such as **3** (after hydrogenolysis, vide infra).

The more demanding synthetic route to the saturated di-alkylamines **3** is depicted in Scheme 1. It requires the synthesis of the fluorinated acid chlorides (using a three-step procedure starting from 10-undecenoic acid) and of the fluorinated primary amines which are obtained in two steps from their corresponding fluorinated acid chlorides (amidation with NH₃, then reduction). We used this strategy as both the fluorinated primary amines and acid chlorides were available from this laboratory. Although a more easier way to these amines would have been to perform the hydrogenolysis of the fluorinated di-alkenylamines **2**, this strategy can be recommended for the preparation of secondary amines possessing two different fluorinated alkyl chains.

The synthesis of the mixed perfluoro-alkenyl/hydrocarbon secondary amines **4** (Scheme 1) has been performed using tetradecylamine and 10-undecenoic acid, reduction of the amide followed by the radical addition of the appropriate perfluoroalkyl group and HI elimination (vide supra).

The [R¹][R²]-GlyNH₂ glycinamines were obtained by condensing the amines **2**, **3**, or **4** with Z-Gly-ONP followed by Z-deprotection using either HBr/AcOH for the C=C derivatives or hydrogenolysis for the saturated ones. The mixed saturated perfluoroalkyl/hydrocarbon derivative [F4C11][C14]-GlyNH₂ was prepared by hydrogenolysis of the Z-protected glycinamide deriving from the perfluoro-alkenyl/hydrocarbon secondary amine **4a**. This

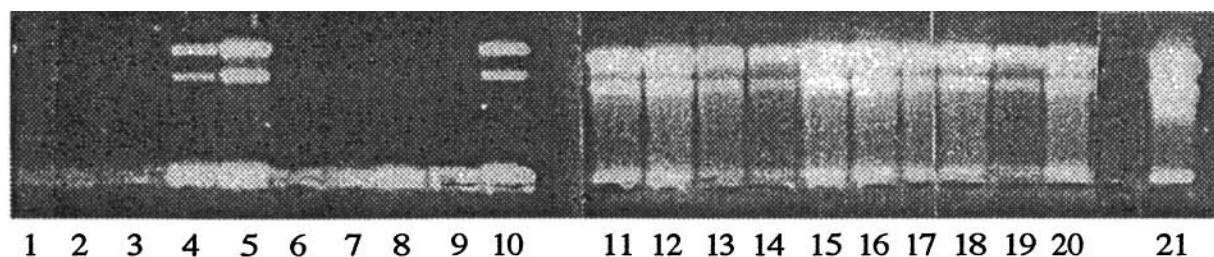


Figure 2. Gel electrophoresis of the DF4C11-GS/DOPE (1/1) lipoplexes (lanes 1–5: N/P = 10, 5, 2.5, 1.25, and 0.8, respectively) and of the DF4C11-GS lipoplexes (lanes 6–10: N/P = 10, 5, 2.5, 1.25, and 0.8, respectively). Lanes 11–20 are identical to lanes 1–10 but with SDS. Lane 21: naked plasmid pTG11033. For more details, see Experimental Section.

procedure resulted in the simultaneous Z-deprotection and hydrogenolysis of the C=C bond. That hydrogenolysis of the C=C bond in α to a perfluoroalkyl group occurred was quiet surprising as it has been shown elsewhere that reduction of such de-activated C=C bonds could be obtained only under high H_2 pressure (Ravily et al., 1996).

The synthesis of the fluorinated $[R^1][R^2]$ -GS lipospermines (isolated as their tetra-TFA salt, as attested by ^{19}F NMR) was achieved by coupling the $[R^1][R^2]$ -GlyNH₂ glycinamines with tetra-*tert*-butoxycarbonylspermine-5-carboxylic acid in the presence of a coupling agent, such as DCC, DCC/HOSu, EDC, BOP, and PyBrop, followed by the quantitative Boc-deprotection with TFA. The characterization and purification of these derivatives was best performed on the Boc-protected derivatives rather than on their highly hygroscopic tetra-TFA ammonium salts.

Lipoplex Formation and Characterization. The capability of the various fluorinated $[R^1][R^2]$ -GS lipospermines to condense DNA and to form lipoplexes was analyzed with or without an equimolar amount of DOPE. The size of the lipoplexes (which is an important parameter to control when in vivo uses are contemplated or when their in vitro transfection efficiency is evaluated (Thierry et al., 1997; Boussif et al., 1996)) was analyzed by light scattering spectroscopy (LSS). These studies were performed with pTG11033 plasmid, also used for the in vitro transfection assays (see below). The procedure applied for the lipoplex preparation relies on the dilution from DMSO/EtOH solution in buffer, using N/P ratios of 10, 5, 2.5, 1.25, and 0.8 (N = number of lipid cations; P = number of DNA phosphates). Table 1 collects the mean particle sizes measured for the different lipoplexes. These formulations were also analyzed by gel electrophoresis (Figure 2) which showed the absence of “free” plasmid for N/P ratios from 10 to 1.25, the plasmid being accessible to ethidium bromide in the case of N/P 0.8 formulations. When DOPE was used as co-lipid, N/P 0.8 and 1.25 formulations displayed free plasmid while from N/P 2.5 and up to 10, the DNA was totally protected from ethidium bromide interaction.

For large excesses of cationic charges, such as N/P ratios of 10, 8, and 5, all the fluorinated lipospermines were able to condense DNA into cationic lipoplexes with mean particle sizes in the 115–180 nm range. When an equimolar amount of DOPE was used for the formulation of the lipoplexes, it results in most cases in a slight lipoplex size increase (150–300 nm), although in a few cases larger sized particles (350 to 2700 nm) are formed.

For a N/P ratio of 2.5 and 1.25, and with or without the addition of DOPE, one observed often precipitates. For a N/P ratio of 0.8, the fluorinated lipospermines condensed DNA into “anionic” lipoplexes of a mean size in the 280–470 nm range. The addition of an equimolar amount of DOPE had no significant effect on lipoplex size (320–550 nm).

Table 1. Mean Size of the Lipoplexes Formed between Plasmid pTG11033 and Various Fluorinated Lipospermines, as Determined by Light Scattering Spectroscopy

lipospermine	N/P ratio ^a	mean diameter in nm (SD) ^{b,c,d}	
		lipoplexes	lipoplexes with DOPE
DOGS	10	130 (50)	200 (70)
DF4C11-GS	10	130 (50)	180 (60)
DF6C11-GS	10	120 (40)	170 (60)
DF4E11-GS	10	130 (45)	180 (100)
DF6E11-GS	10	150 (45)	195 (80)
[F4E11][C14]-GS	10	115 (25)	2700 (broad)
[F6E11][C14]-GS	10	180 (55)	240 (80)
[F4C11][C14]-GS	10	130 (50)	180 (65)
DOGS	8	150 (50)	185 (65)
DF4C11-GS	8	115 (40)	150 (broad)
DF6E11-GS	8	140 (80)	nt
[F4C11][C14]-GS	8	nt	220 (80)
DOGS	5	380 (170) 20% 1400 (500) 80%	300 (120)
DF4C11-GS	5	125 (40)	280 (80)
DF6C11-GS	5	120 (40)	250 (90)
DF4E11-GS	5	125 (45)	180 (65)
DF6E11-GS	5	135 (50)	230 (80)
[F4E11][C14]-GS	5	125 (40)	1600 (500)
[F6E11][C14]-GS	5	175 (55)	365 (broad)
[F4C11][C14]-GS	5	120 (35)	450 (100)
DOGS	2.5–1.25	p	p
DF4C11-GS	2.5–1.25	p	p
DF6C11-GS	2.5–1.25	p	p
DF4E11-GS	2.5	200 (70)	p
	1.25	p	p
DF6E11-GS	2.5–1.25	p	p
[F4E11][C14]-GS	2.5	135 (40)	p
	1.25	p	
[F6E11][C14]-GS	2.5	p	p
	1.25	320 (110)	320 (100)
[F4C11][C14]-GS	2.5–1.25	p	p
DOGS	0.8	650 (600)	340 (140)
DF4C11-GS	0.8	340 (120)	340 (120)
DF6C11-GS	0.8	320 (110)	330 (120)
DF4E11-GS	0.8	470 (broad)	430 (160)
DF6E11-GS	0.8	400 (150)	350 (115)
[F4E11][C14]-GS	0.8	300 (110)	550 (190)
[F6E11][C14]-GS	0.8	280 (100)	320 (110)
[F4C11][C14]-GS	0.8	350 (broad)	400 (broad)

^a N = lipid cation equivalents; P = plasmide phosphate equivalents. ^b % of the overall population. ^c p = precipitate. ^d nt = not tested.

Altogether, these results in terms of impact of N/P ratio on lipoplex stability, i.e., formation of precipitates for N/P ratios corresponding to neutrally charged lipoplexes ($1 < N/P \leq 3$) and stable dispersions for N/P ratios outside this range (negatively and positively charged lipoplexes), are close to those reported for other lipopolyamines (Pitard et al., 1997).

As compared to DOGS, significantly smaller lipoplexes are formed with the fluorinated lipospermines for a N/P ratio of 5 or 0.8. While mixing DOGS with pTG11033

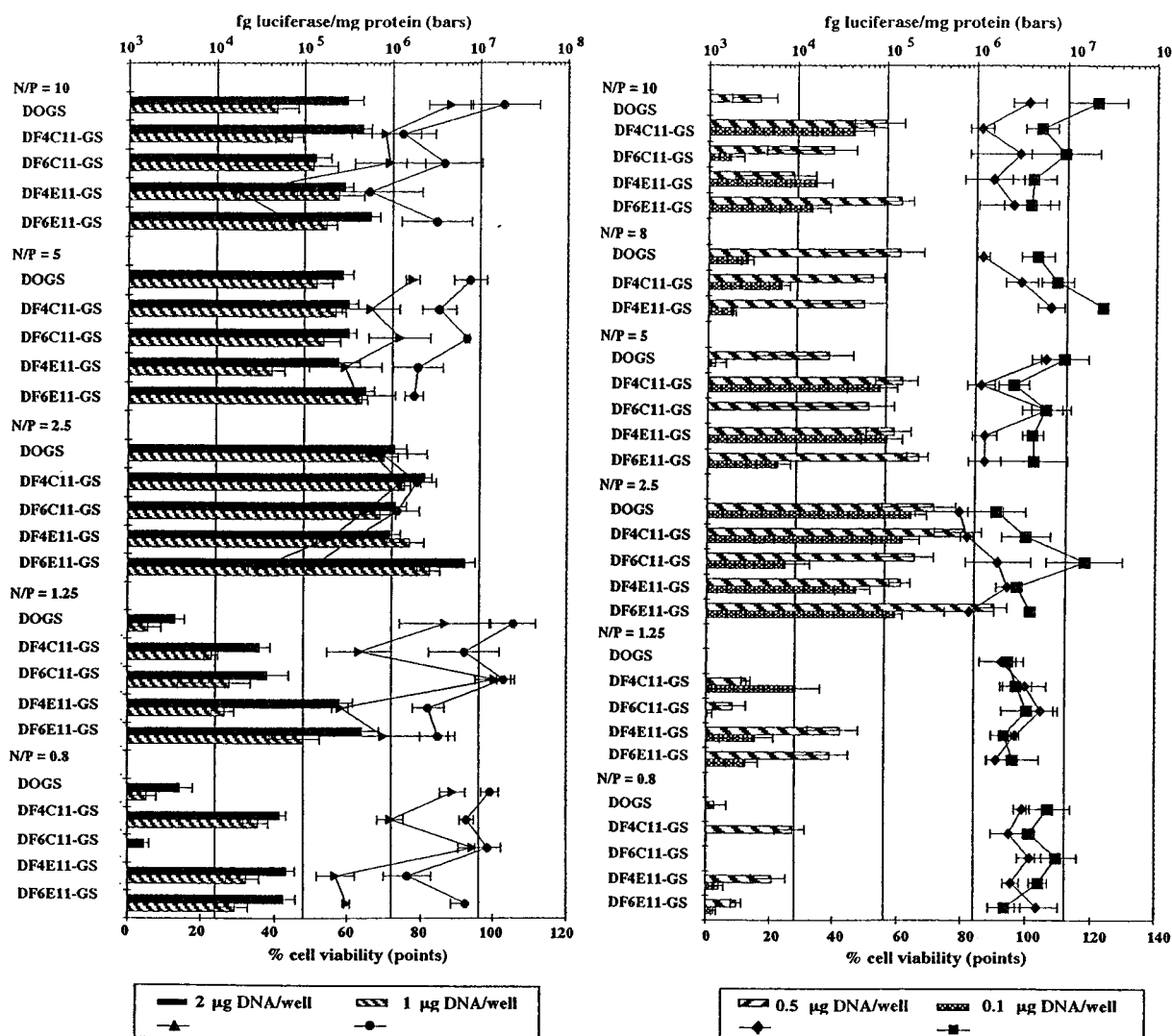


Figure 3. Luciferase expression (bars) and cell viability (points) of the fluorinated lipoplexes made of pTG11033 and **DFnC11-GS** or **DFnE11-GS** lipospermines, as compared to DOGS in A549 cells. Means $\pm$ SEM are given.

plasmid at *N/P* ratios of 2.5 and 1.25 led to aggregates, formation of small-sized DNA particles with some fluorinated lipospermines was observed. These results suggest that the fluorinated lipospermines are more effective than DOGS, in terms of compacting DNA into small-sized particles.

No impact of the nature of the lipospermine hydrophobic chains on lipoplex size could be evidenced. Indeed, the fluorinated lipospermines whether containing a saturated or an olefinic chain (e.g., **DFnC11-GS** vs **DFnE11-GS**), a fluorinated tail on each or only one hydrophobic chains (e.g., **DFnE11-GS** vs **[FnE11][C14]-GS** or **DFnC11-GS** vs **[FnC11][C14]-GS**) led to complexes of comparable sizes. Neither could we detect an effect of the length of the fluorinated tail on the lipoplex mean diameter, the F4 and their corresponding F6 lipospermines behaving very similarly.

In Vitro Transfection. The transfection potential of the fluorinated lipospermines, with an equimolar amount of DOPE and without DOPE, was assayed in vitro on lung epithelial A549 cells, from human pulmonary carcinoma. These assays were performed using plasmid pTG11033 (pCMV-intronHMG-luciferase-SV40pA) in the presence of 10% fetal calf serum for 24 h. pTG11033 is a plasmid of 9572 bps. All lipoplex formulations, even those that precipitated were tested. The transfection efficiency

of the lipoplexes (expressed in fg of luciferase/mg of protein) was evaluated for gradual *N/P* ratios (0.8, 1.25, 2.5, 5, 8 and 10) and various amounts of DNA (2, 1, 0.5, and 0.1 μ g/well) as compared to naked DNA and to the lipoplexes based on DOGS and DOGS/DOPE (1/1 mol). Cells treated with naked DNA under equivalent conditions showed expression levels of about 10² fg of luciferase/mg of protein. The cell viability of the lipoplexes was also checked by determining the total protein amount per well of the transfected cells relative to that measured for untreated cells (for which the total protein amount per well is in a 30–60 μ g/well range). The transfection and toxicity results are illustrated in Figures 3, 4, 5, and 6 for the lipoplexes formulated with the fluorocarbon/fluorocarbon lipospermines (without and with DOPE) or the mixed fluorocarbon/hydrocarbon ones (without and with DOPE), respectively, as compared to DOGS or DOGS/DOPE. These results indicate that all the fluorinated lipospermines, even without the use of the helper DOPE lipid, do enable transfection of the plasmid into the cells. All the fluorinated lipoplexes tested so far showed indeed a higher efficiency to transfect A549 cells than naked DNA and even than those based on DOGS (*vide infra*).

It is particularly noticeable that for the lowest amount of plasmid used (i.e., 0.1 μ g/well) which is a most

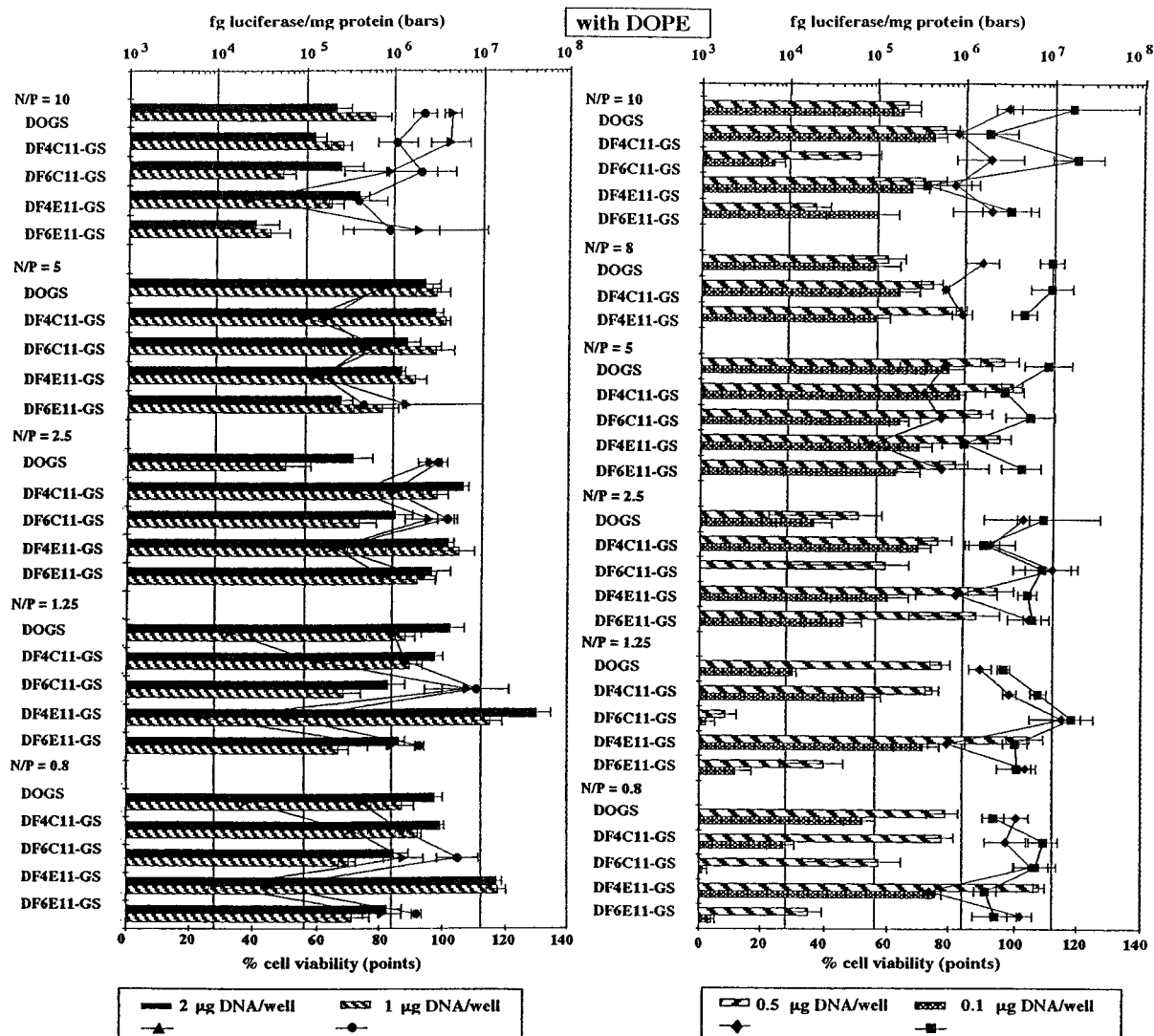


Figure 4. Luciferase expression (bars) and cell viability (points) of the fluorinated lipoplexes made of pTG11033 and **DFnC11-GS/DOPE** (1/1 mol) or **DFnE11-GS/DOPE** (1/1 mol) lipids, as compared to DOGS/DOPE (1/1 mol) in A549 cells. Means $\pm$ SEM are given.

important factor to consider when the *in vivo* use of lipoplexes is intended, some of the fluorinated lipospermines displayed a higher transfection potential than DOGS. Indeed, the “fluorinated” lipoplex formulations based on [DF4C11]-GS or [DF4E11]-GS and for a *N/P* ratio of 5 already peaked out at 10^5 fg of luciferase/mg of protein while for DOGS and such a low DNA amount, the luciferase expression levels stood around 10^3 fg of luciferase/mg of protein ($p < 0.001$).

A closer analysis of the results shows that the transfection efficiency of the lipospermines was statistically increasing when replacing the hydrocarbon chains of DOGS by fluorinated ones. Figure 7 displays the luciferase level ratios (LLR) measured for the double fluorocarbon/fluorocarbon lipospermine formulations vs that measured for DOGS. Considering that the effect of the number of fluorocarbon chains is significant if this ratio is out of the 0.2–5 range and if its associated p value is ≤ 0.05 , we found that, as compared to the respective DOGS formulation and irrespectively of the nature of the fluorocarbon chain, the *N/P* ratio and DNA amount, higher transfection levels were obtained with the formulations based on the double fluorocarbon/fluorocarbon derivatives (e.g., **DFnC11-GS** or **DFnE11-GS**) for 29% of the 84 formulations investigated while it was compa-

table for almost 70% of these lipoplexes. For the complexes based on the mixed fluorocarbon/hydrocarbon lipospermines and as compared to those based on DOGS (Figure 8), we measured higher and comparable luciferase expression levels for 20 and 65% of the 60 formulations, respectively, and lower ones for the remaining 15% formulations.

Where the effect on the A549 cell viability of the lipoplexes is concerned, no specific cell toxicity was evidenced for the fluorinated ones as compared to that of the DOGS lipoplexes, at least for DNA amounts ≤ 1 μ g/well.

All these results together indicate that the fluorinated lipoplexes are appealing candidates for *in vivo* purposes, highlighting the diversity of lipids leading to efficient transfection.

Where the impact on transfection of an increase of the fluorinated tail length is concerned (see Figure 9), we found that in the single fluorinated-chain lipospermine series (e.g., [F6E11][C14]-GS vs [F4E11][C14]-GS) this was accompanied by a significant increase of the transfection efficiency. Indeed and as shown in Figure 9, the fluorinated tail lengthening from F4 to F6 resulted in a luciferase expression increase in 60% of the cases while the level remained unchanged for 35% of the cases and

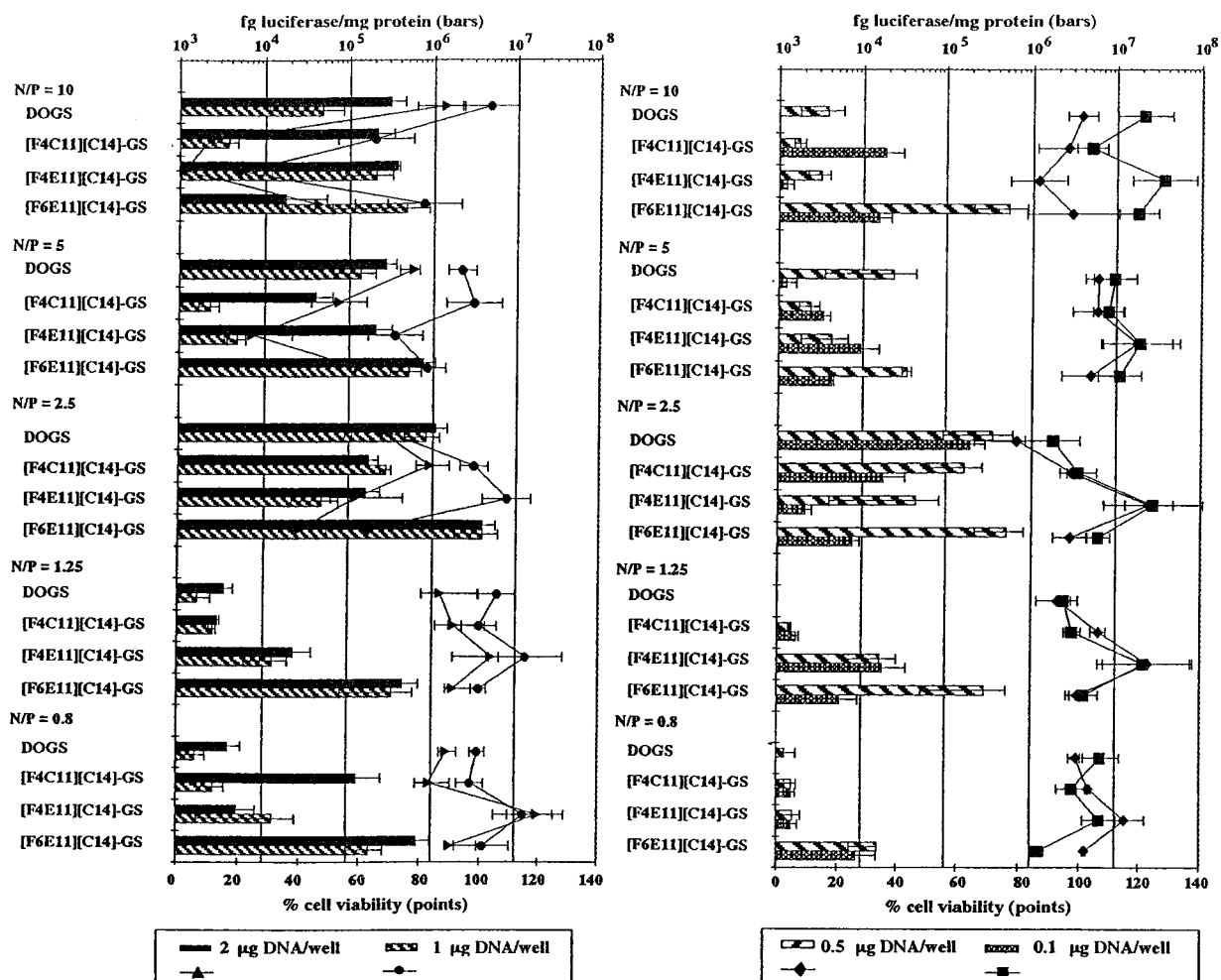


Figure 5. Luciferase expression (bars) and cell viability (points) of the fluorinated lipoplexes made of pTG11033 and [F4C11]-[C14]-GS or [F6E11][C14]-GS lipospermines, as compared to DOGS in A549 cells. Means $\pm$ SEM are given.

in only 5% a transfection decrease was observed. A similar tendency could be evidenced for the unsaturated fluorinated double-chain series (e.g., **DF6E11-GS** vs **DF4E11-GS**, Figure 9), although at a lower extent: for 25% of the formulations, we measured a transfection increase, while the luciferase expression remained at a comparable level for 70% and decreased for the remaining 5%.

By contrast, the opposite tendency was found for some formulations of the saturated series (Figure 9). For 20% of the cases, we observed a significant transfection decrease when going from the F4C11 to the F6C11 series and a comparable level for the remaining 80%.

The opposite tendencies resulting from lengthening the fluorinated tail in the unsaturated and saturated series could be rationalized in terms of lipid thermotropism. Previous works have shown that optimal transfection activity was achieved when using cationic lipids containing hydrophobic chains organized, at the incubation temperature, as "fluid" lamellar phases rather than "gel" lamellar ones (Balasubramaniam and Malone, 1996; Byk et al., 1998) or as inverted hexagonal phases where chains are in a fluid state as well (Koltover et al., 1998). The exact mechanism governing lipoplex endocytic uptake and cytosol DNA release from endosomes is still unclear. Although speculative, the improvement of gene expression resulting from the use of lipids which are in a "fluid" physical state, is presumably due to their larger miscibility with endogenous ones, and consequently, to their greater ability to promote membrane fusion with

and destabilization of the endosome membrane allowing optimal DNA release in the cytosol, thus preventing internalized DNA from endosome and lysosome degradation. Concerning fluorinated lipids, it has been shown that phosphatidylcholines containing two saturated F4C11 or F6C11 chains display a lamellar phase transition temperature below or above 37 °C, respectively, while those containing unsaturated F4E11 or F6E11 chains have melting temperatures far below 37 °C (Vierling et al., 1995). Therefore, the transfection decrease when going from F4C11 to F6C11 in the saturated series most likely may be attributed to a rigidifying effect which accompanies this fluorinated tail length increase (see also next section). On the other hand, in the unsaturated series when going from F4E11 to F6E11, the transfection increase which accompanies this tail length increase, may be related to an increase of the hydrophobic and lipophobic character of the lipospermine, thus better preventing the fluorinated lipoplexes from interactions with the serum components.

The effect of the C=C bond on transfection is illustrated in Figure 10 which displays the luciferase level ratios measured for the formulations with **DFnE11-GS** (each being a mixture of trans/trans (mainly), trans/cis and cis/cis isomers) or [F4E11][C14]-GS vs that measured for the corresponding **DFnC11-GS** or [F4C11]-[C14]-GS homologues, respectively. Assuming that the effect of the C=C bond is significant if this ratio is out of the 0.2–5 range and if p is ≤ 0.05 , we found that this structural element improved the transfection efficiency

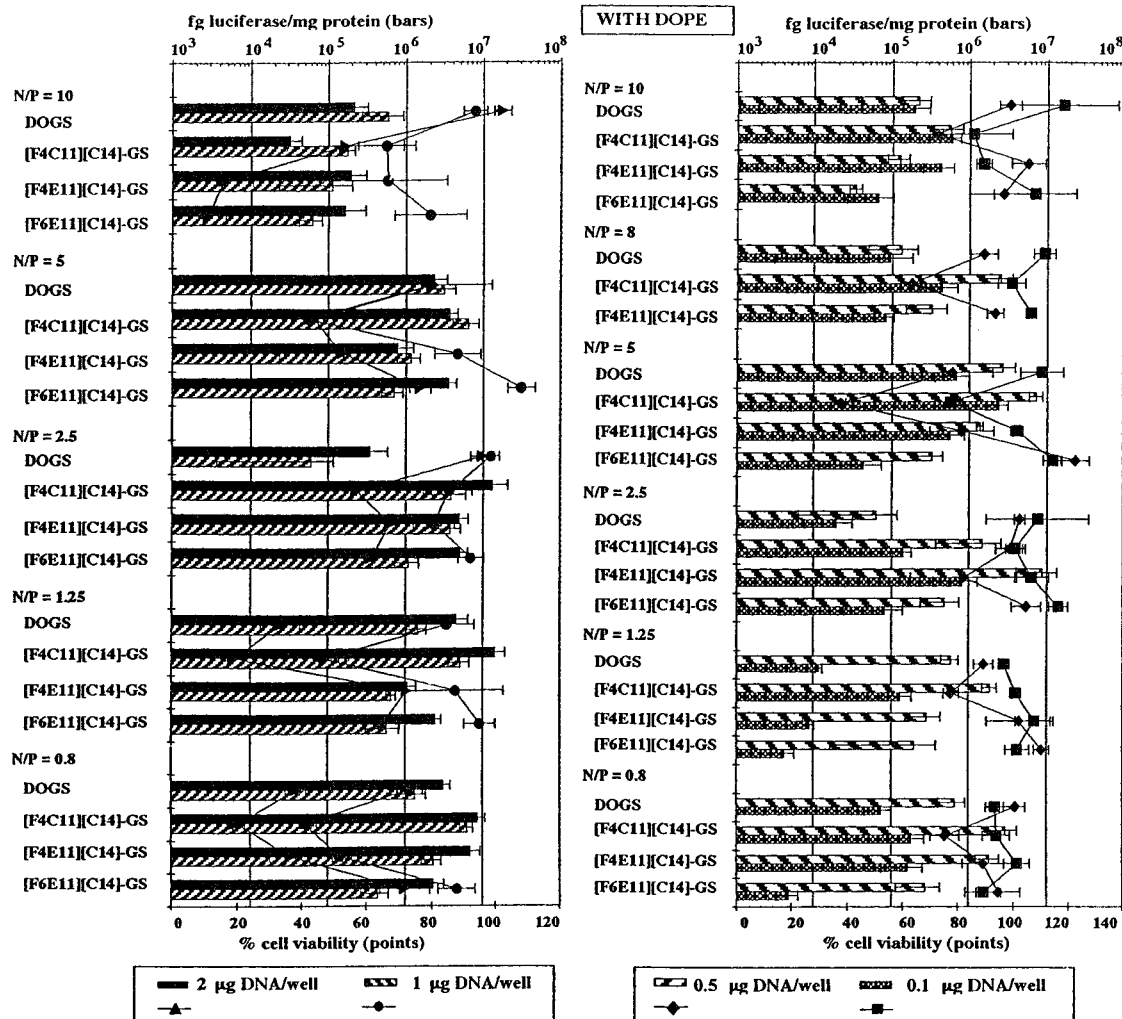


Figure 6. Luciferase expression (bars) and cell viability (points) of the fluorinated lipoplexes made of pTG11033 and [F4C11]-[C14]-GS/DOPE (1/1 mol) or [F6E11][C14]-GS/DOPE (1/1 mol) lipids, as compared to DOGS/DOPE (1/1 mol) in A549 cells. Means $\pm$ SEM are given.

only for the double fluorinated-chain lipospermines which contained F6 tails. Indeed, 40% of the **DF6E11-GS** formulations display a higher transfection level than their respective **DF6C11-GS** ones and a comparable level for the remaining 60%. As discussed above, this is likely to be related to the "fluidification" effect of the C=C bonds when going from **DF6C11-GS** to **DF6E11-GS**. This is further supported by the fact that no influence of the C=C bond on transfection was found with the F4E11 vs F4C11 (single or double chain) containing lipospermines, all these lipids being expected to have chain melting temperatures below 37 °C (Vierling et al., 1995).

All these data suggest that the physical state of the fluorinated lipospermine along the cell entry pathway and the intracellular traffick up to DNA integration into the nucleus is of importance. That transfection increases with lipophobicity (fluorinated amphiphiles are usually not miscible with their hydrocarbon analogues (Rolland et al., 1997)) indicates further that the mixing of the cationic lipids with cellular lipids is not determinant for the gene transfer and expression process. It is rather attributable to some protection effects of DNA resulting from its formulation as lipophobic and hydrophobic lipoplexes with fluorinated lipospermines, thus preventing DNA from interactions with lipophilic and hydrophilic biocompounds.

The transfection efficiency and the cell tolerance of the fluorinated lipoplexes depends as well on the amount of

DNA, on the *N/P* ratio and on the lipoplex formulation (DOPE added or not) as for the conventional (poly)-cationic lipids tested so far. Some of these parameters are linked and will be discussed together.

Thus, the transfection efficiency of the fluorinated lipoplexes usually increased on raising the DNA concentration from 0.1 to 2 $\mu\text{g}/\text{well}$, and in most cases a plateau of luciferase expression was attained for a DNA concentration of 0.5 $\mu\text{g}/\text{well}$. Whatever the amount of plasmid used, luciferase expression levels usually grew when *N/P* ratio increased from 0.8 to 10. Concomitantly, cell toxicity appeared but remained however frequently at an acceptable level, comparable to the DOGS formulation. The lowest cell tolerance was observed (when observed) for the highest DNA concentration (i.e., 2 $\mu\text{g}/\text{well}$) and *N/P* ratio (i.e., 10) tested.

The transfection efficiency and toxicity increase when raising the cationic charge of the lipoplexes is most probably related to an increase of the lipoplex cellular uptake which remains at a level compatible with cell growth providing the DNA amount remained below 2 $\mu\text{g}/\text{well}$, whatever their charge ratio. The lipoplexes became more toxic for the cells, likely as a result of a high level of cellular uptake, and/or of cell membrane disruption, and/or as a result of the presence of a high amount of "free" uncomplexed lipids.

Concerning the effects on transfection resulting from the formulation of the fluorinated lipoplexes with DOPE,

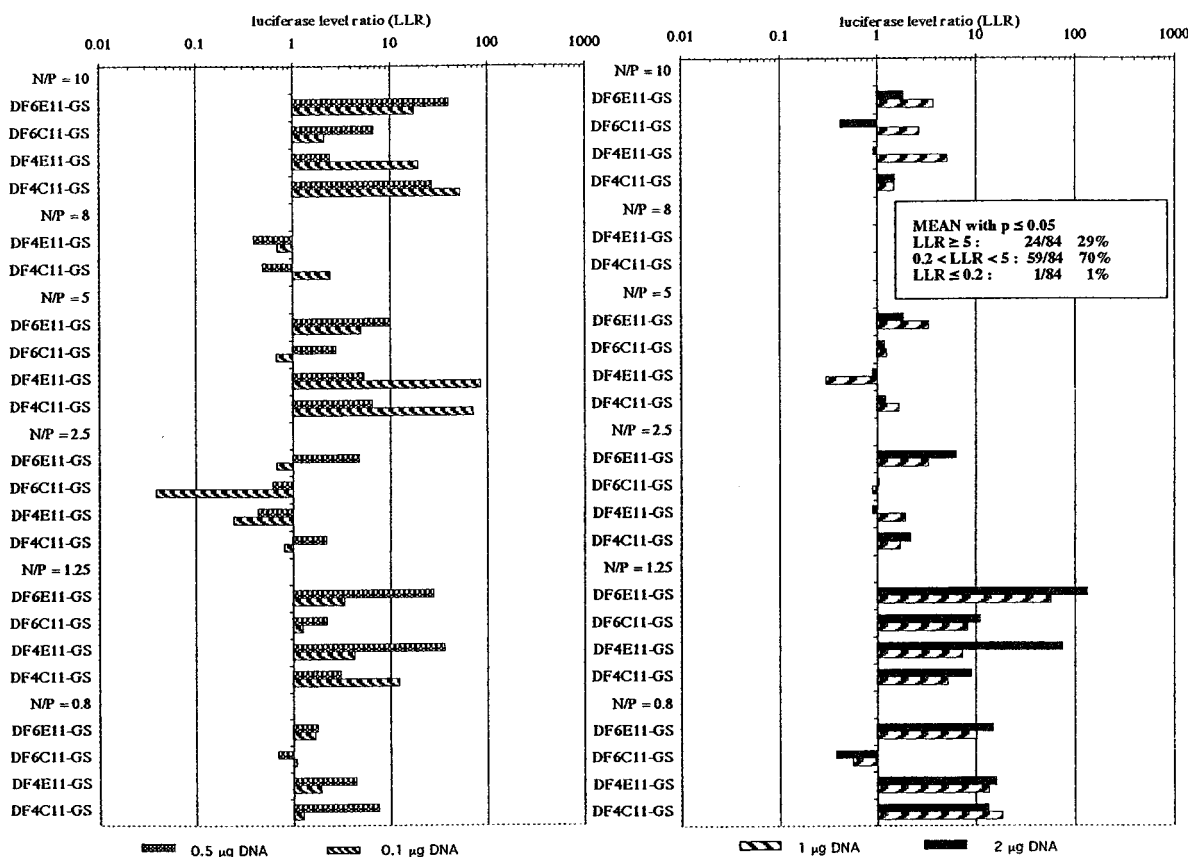


Figure 7. Transfection efficiency of the fluorinated lipoplexes vs that of their corresponding DOGS lipoplexes. The luciferase level ratio LLR is the ratio of luciferase amount measured for the fluorinated lipoplexes vs that measured for the corresponding DOGS-lipoplexes. The efficiency is significantly higher if LLR is ≥ 5 , significantly lower if $0.2 < \text{LLR} \leq 0.2$, or comparable if $0.2 < \text{LLR} < 5$ ($p \leq 0.05$). The ratio and percentage value given in the insert for each LLR condition represents the number of formulations vs the total number of formulations and the corresponding percentage, respectively, which satisfy the given LLR condition. The fluorinated lipoplexes are made from the double-chain fluorinated DF6E11-GS or DF6C11-GS lipospermines.

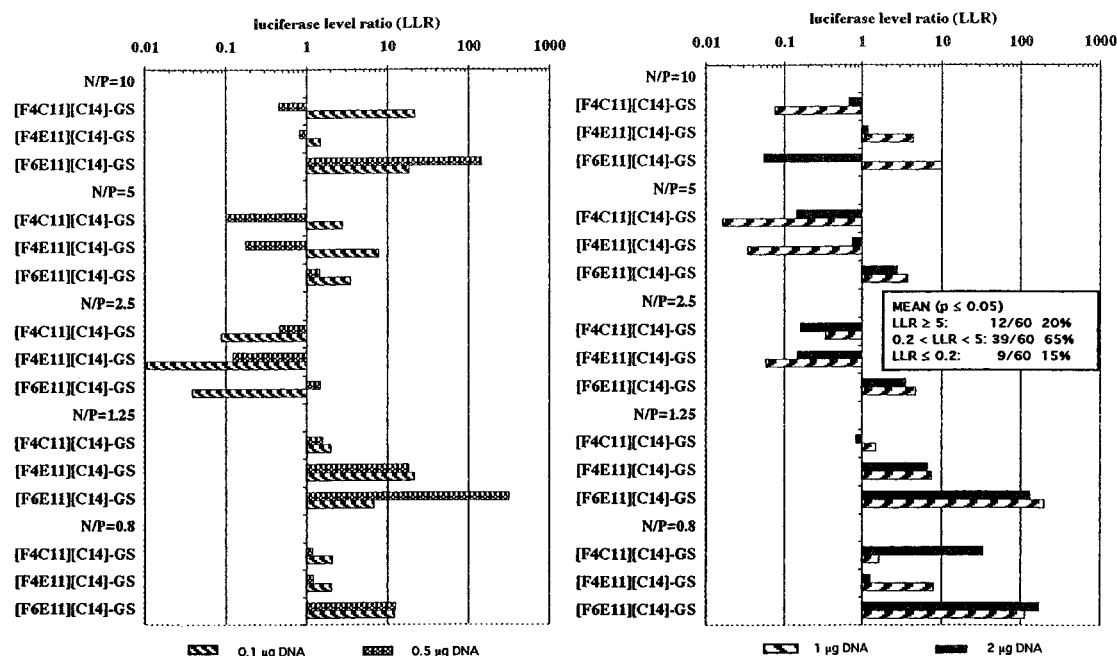


Figure 8. Transfection efficiency of the fluorinated lipoplexes vs that of their corresponding DOGS-lipoplexes. The luciferase level ratio LLR is the ratio of luciferase amount measured for the fluorinated lipoplexes vs that measured for the corresponding DOGS-lipoplexes. The fluorinated lipoplexes are made from the mixed-chain [F6E11][C14]-GS or [F6C11][C14]-GS lipospermines. For more details concerning the significance of the results and the meaning of the ratio and percentage values given in the insert, see caption of Figure 7.

a subsequent improvement (≥ 5 -fold increase, $p \leq 0.05$) is observed for 55% of the 142 formulations investigated,

highlighting again the unique properties of DOPE as helper lipid (results not shown). A 3, 2, and 1 order of

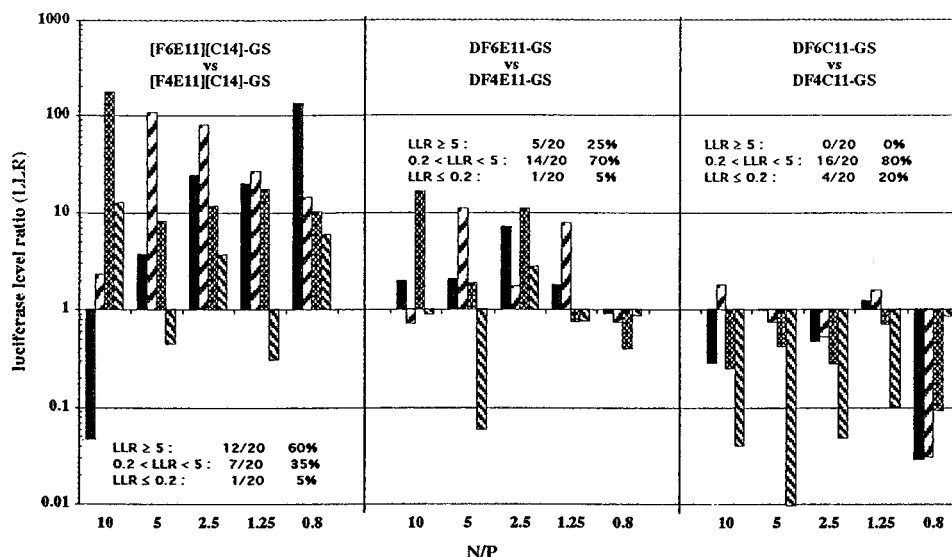


Figure 9. Effect on transfection of the fluorinated tail lengthening. The luciferase level ratio LLR is the ratio of luciferase amount measured for the F4E11 (resp. F4C11) lipoplexes vs that measured for their corresponding F6E11 (resp. F6C11) ones. The efficiency is significantly higher if LLR is ≥ 5 , significantly lower if LLR ≤ 0.2 , or comparable if $0.2 < \text{LLR} < 5$ ($p \leq 0.05$). The ratio and percentage value given for each LLR condition and in each section of the graph represents the number of formulations vs the total number of formulations and the corresponding percentage, respectively, which satisfy the given LLR condition.

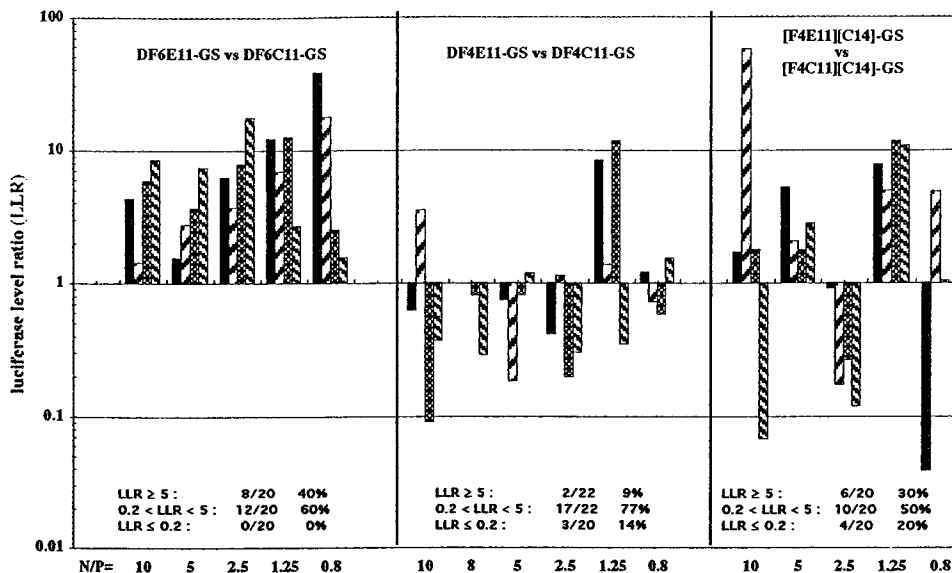


Figure 10. Effect on transfection of the double-bond. The luciferase level ratio LLR is the ratio of luciferase amount measured for the F4E11 lipoplexes vs that measured for their corresponding F4C11 ones ($n = 4$ or 6). For more details concerning the significance of the results and the meaning of the ratio and percentage values given in each section of the graph, see caption of Figure 9.

magnitude increase is indeed measured for about 8, 25, and 51% of these formulations, respectively. For 44% of the 142 formulations, a comparable luciferase expression level is attained with or without DOPE. Finally, for only about 1% of the formulations investigated, a decrease of transfection is measured by adding DOPE. A closer analysis shows that adding DOPE to the formulations increased the transfection levels for 65% formulations made of mixed-chain lipospermines and for 48% of the formulations made of lipospermines having two fluorinated chains. In these two series, respectively, 87% and 70% of the formulations made of lipospermines with F4C11 or F4E11 moieties showed a synergistic effect with DOPE while only 27 and 24% of the formulations with F6C11 or F6E11 lipospermines were improved by the addition of this helper. This behavior is likely to be attributable to the miscibility between DOPE and the fluorinated lipospermines, which is expected to be higher (i) with the mixed-chain rather than with the double-

chain fluorinated ones or (ii) with those having the shortest fluorinated tail.

One should further underline that the cell viability of the fluorinated lipoplexes remained unaffected by their co-formulation with DOPE.

CONCLUSION

The present work suggests that the new fluorinated lipospermines which were synthesized appear as promising synthetic vectors for gene delivery. The palette of structural elements explored allowed to determine those required for efficient transfection, highlighting the importance of highly fluorinated chains, and (again) the unique properties of unsaturated double-chain lipids and of the use of DOPE as helper lipid on transfection. Although encouraging, the *in vitro* results obtained here with the fluorinated lipospermines lead to a rather modest improvement over the first generation of lipo-

spermines such as DOGS, and do not compete with the efficiency of viral vectors. This conclusion does not however prejudice their *in vivo* behavior.

The *in vitro* (resistance to and gene expression in the presence of detergents, serum and nucleases to other cell types) and *in vivo* potential of these fluorinated lipospermines are currently under investigation.

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Supporting Information Available: One figure showing the effect of DOPE on the transfection efficiency. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Modification of Cyclosporin A and Conjugation of Its Derivative to HPMA Copolymers

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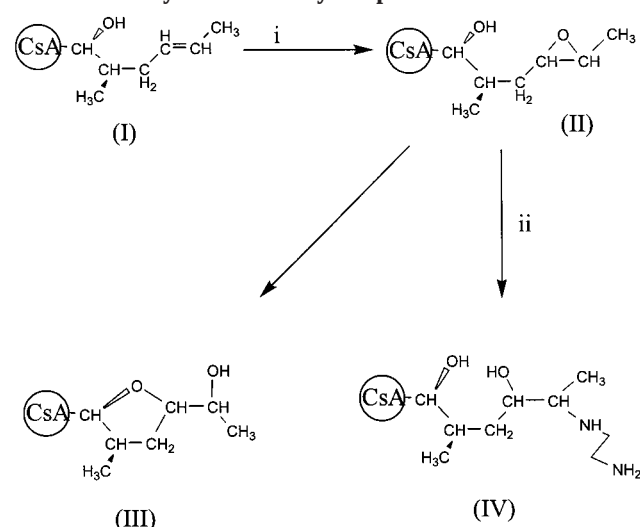
Cyclosporin A (CsA) was epoxidized with *m*-chloroperoxybenzoic acid in the presence of sodium carbonate or with *tert*-butyl hydroperoxide in the presence of dioxomolybdenum iminodiethanoxide. The CsA epoxide was not stable and rearranged into a compound with a more stable five-member ring structure. An amino group containing cyclosporin A derivative (CsA amine) was obtained by the reaction of CsA epoxide with excess ethylenediamine. The yield of the CsA amine was 30–40% based on the CsA. An HPMA copolymer–CsA conjugate was prepared by the reaction of the CsA amine with an HPMA and MA-Gly-Phe-Leu-Gly-ONp copolymer. The content of CsA amine in the conjugate was 8.7 wt %. The CsA amine was released from the copolymer by enzymatic hydrolysis with papain.

INTRODUCTION

Cyclosporin A (CsA)¹ is a neutral, hydrophobic cyclic undecapeptide (structure 1), isolated from the fungal species *Tolypocladium inflatum* Gams (1). The compound is widely used in organ transplantation and was also found to be effective in the treatment of diseases related to immunoregulatory dysfunctions, such as inflammatory bowel diseases, type I diabetes, etc. (2), as well as in depressing multidrug resistance in cancer chemotherapy (3). However, its clinical applications are associated with significant nephrotoxicity and other toxic side effects. Its poor aqueous solubility may impair its therapeutic efficacy (4). Chemical modification of cyclosporin A may increase its hydrophilicity, reduce its side effects, and improve its therapeutic efficacy. However, because of the chemical inertness of CsA, few modifications of CsA have been reported (5–9).

CsA has two functional groups that can be used for chemical modification, the hydroxyl group and the double bond on the 2-*N*-methyl-*R*-((*E*)-2-butenyl)-4-methyl-L-threonine (MeBmt) residue. Modification on the hydroxyl group requires severe reaction condition because of steric hindrance (5, 6). There are few reports on the modification of the double bond. The double bond was oxidized to form an aldehyde (7) or a lactone (8). The CsA lactone can be produced in good yield, but the yield of CsA aldehyde was very low, possibly because the aldehyde formed reacted further with the hydroxyl group in the MeBmt residue to form a more stable five-membered ring

Scheme 1. Synthesis of Cyclosporin Amine^a



^a (i) *m*-Chloroperoxybenzoic acid, anhydrous Na₂CO₃, methylene chloride, overnight, r.t.; or *tert*-butyl hydroperoxide, MoO₂(OCH₂CH₂)₂NH, benzene, refluxing, 2 h. (ii) Ethylene diamine, THF, refluxing, 24 h.

structure (structure 2). The oxidation reactions damaged the integrity of the MeBmt by cleaving the double bond. The MeBmt structure can be maintained by modifying the double bond in an addition reaction. For example, hydroformylation of the double bond introduced an aldehyde functional group (9) to CsA, and the functional group was used to conjugate CsA to HPMA copolymer–antibody conjugates (10). The biological investigation suggested that the modified CsA maintained its activity with reduced side effects after conjugation to the HPMA copolymer (10).

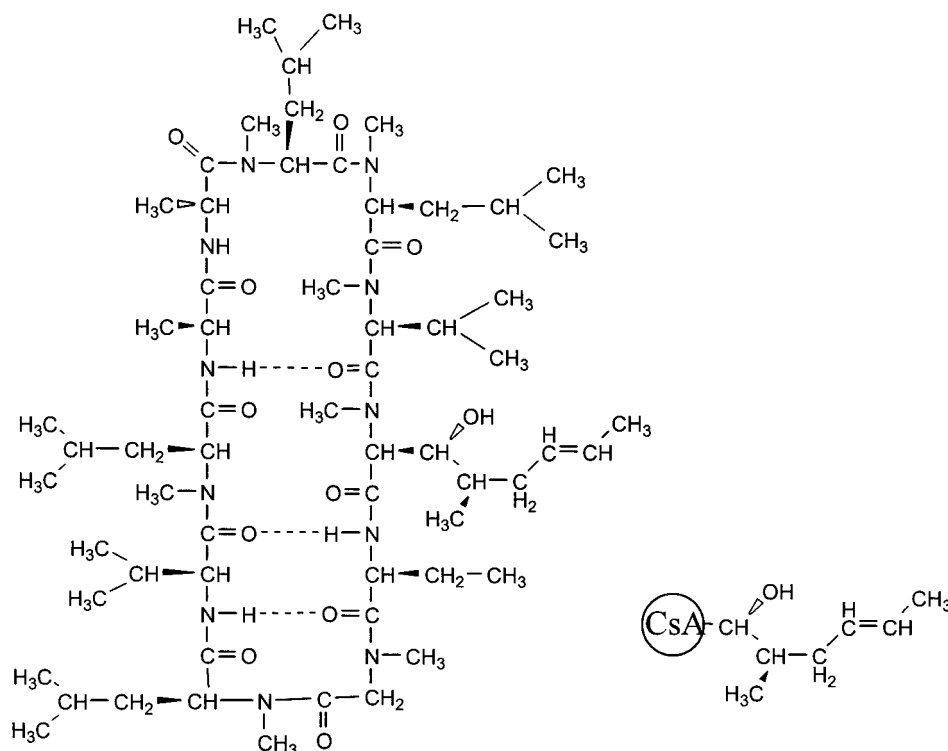
Here we report on the direct epoxidation of cyclosporin A and the synthesis of an amino group functionalized CsA from its epoxide, Scheme 1. The new CsA derivative was conjugated to an HPMA copolymer via tetrapeptide (Gly-Phe-Leu-Gly) side-chain termini. The release of the CsA derivative from the HPMA copolymer conjugate was investigated by enzymatic cleavage with papain.

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¹ Abbreviations: CsA, cyclosporin A; DMSO, dimethyl sulfoxide; ESI MS, electrospray ionization mass spectrometry; HPMA, *N*-(2-hydroxypropyl) methacrylamide; MA, methacryloyl; MeBmt, 2-*N*-methyl-*R*-((*E*)-2-butenyl)-4-methyl-L-threonine; *M*_n, number average molecular weight; *M*_w, weight average molecular weight; ONp, *p*-nitrophenoxyl; PBS, phosphate-buffered saline; SEC, size-exclusion chromatography; TBHP, *tert*-butyl hydroperoxide; THF, tetrahydrofuran; wt %, weight percentage.



Structure 1: Cyclosporin A

RESULTS AND DISCUSSION

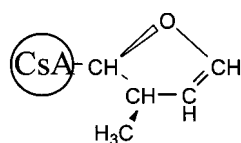
The epoxidation of CsA was performed by reaction with *m*-chloroperoxybenzoic acid in the presence of sodium carbonate at room temperature. The reaction with *m*-chloroperoxybenzoic acid gave very high conversion (almost 100%) of the double bond of CsA. However, the CsA epoxide (II) is not stable in acidic solution, and it may rearrange to a more stable structure (III) with a five-membered ring by an acidic ring opening reaction of the epoxide with the γ -hydroxyl group on the MeBmt residue (Scheme 1). To minimize the formation of this side product (III), sodium carbonate was added to neutralize the *m*-chlorobenzoic acid formed during the reaction. The reaction was carried out overnight at rt. Because of the low stability of the CsA epoxide, its mixture with compound III was used for the reaction with ethylenediamine immediately after their isolation from the reaction mixture.

Reaction of the CsA epoxide with ethylenediamine produced an amino group-containing CsA derivative (IV). The use of a large excess of primary amine could facilitate the complete conversion of the CsA epoxide to a CsA amine (IV). The CsA amine (IV) was formed because the nucleophilic ring opening reaction mainly occurred at the site with the least steric hindrance. The CsA amine (IV) and compound (III) were separated from the reaction mixture by silica gel chromatography. The yield of the CsA amine (IV) was 33 and 30% (based on CsA) for compound III after separation. The epoxidation products

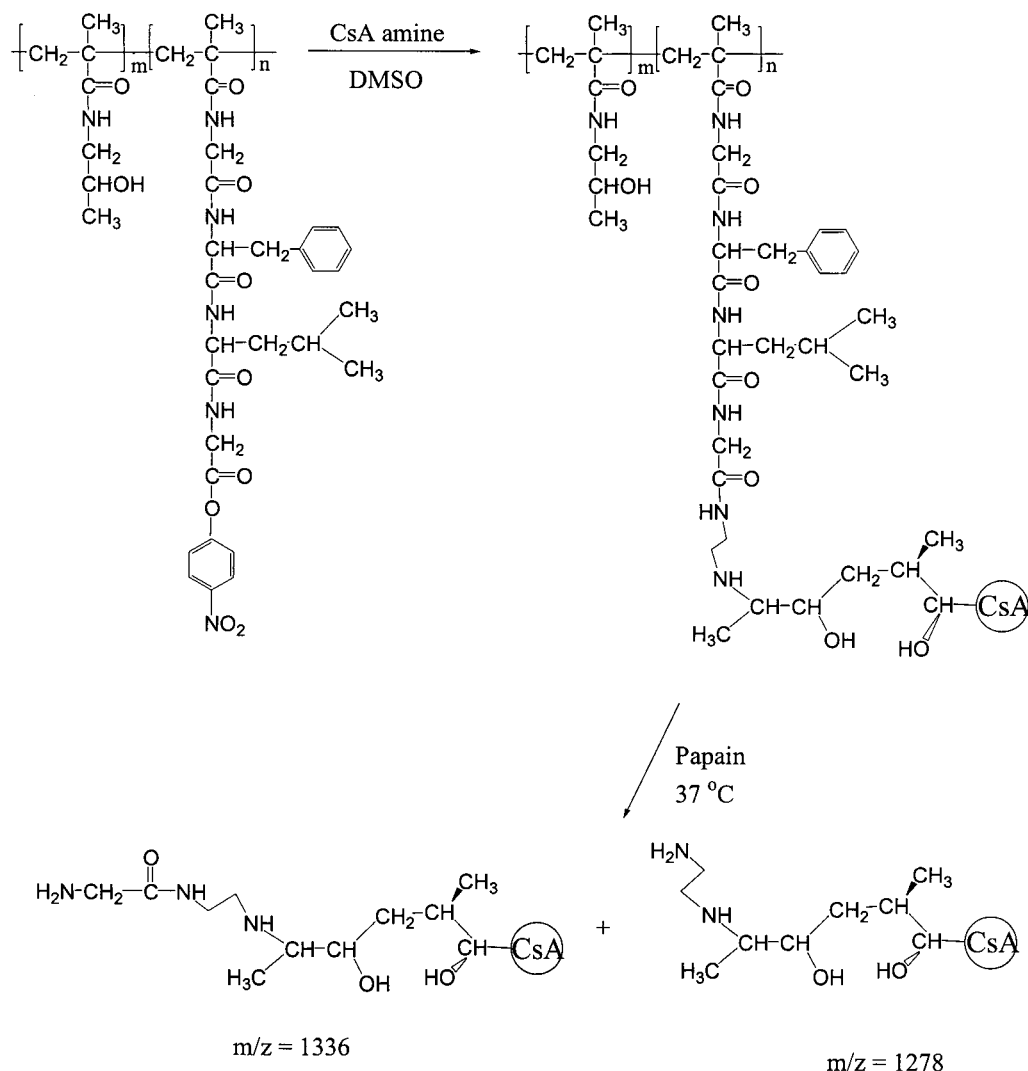
and CsA derivatives III and IV were characterized by electrospray ionization mass spectrometry. Both the CsA epoxide and compound III had the m/z of 1219 ($+H^+$) and the CsA amine IV had a m/z of 1279 ($+H^+$). The compounds III and IV were also characterized by proton NMR. Chemical shifts of the *N*-methyl groups in the CsA amine were not as distinct as those of CsA. The peaks of the *N*-methyl groups overlapped around 2.99 ppm. This might be caused by the addition of two amino groups and one more hydroxyl group in the structure of CsA. These additional functional groups might disturb the hydrogen bonding of the cyclic peptide by forming new hydrogen bonds. The formation of new hydrogen bonds may change the conformation of the CsA and steric orientation of the *N*-methyl groups.

The yield of CsA amine or CsA epoxide was significantly affected by the presence of sodium carbonate and the reaction temperature. If CsA and *m*-chloroperoxybenzoic acid were refluxed in chloroform without the presence of sodium carbonate, the yield of CsA amine was very low, less than 10%. This was because of the acid-catalyzed transformation of the CsA epoxide to compound III by the byproduct *m*-chlorobenzoic acid. Sodium carbonate was added to neutralize the *m*-chlorobenzoic acid, and an optimum yield of CsA amine was obtained with reaction overnight at rt.

We tried to avoid acidic epoxidation conditions by using the catalytic epoxidation with *tert*-butyl hydroxyperoxide (TBHP) with a molybdenum complex, dioxomolybdenum iminodiethanoxide (11). This catalytic epoxidation is a very efficient process with a conversion of more than 90% for alkenes and TBHP and without formation of acidic byproduct. The catalytic epoxidation of CsA with TBHP was performed in benzene with refluxing for 2 h. After the benzene was removed, the residue was immediately refluxed with excess ethylenediamine in THF. The final reaction mixture was analyzed by electrospray ionization



Structure 2

Scheme 2. Synthesis and the Degradation of the HPMA Copolymer-CsA Amine Conjugate^a

^a (i) CsA amine, DMSO, r.t., overnight. (ii) Papain, 37 °C, 24 h

mass spectrometry. The results indicated that the CsA was completely converted (CsA epoxide and compound III). The yield of CsA amine was 40% as estimated from the mass spectrum. There was still a considerable amount of compound III (m/z 1219, $M^+ + 1$) formed. This compound was formed by the rearrangement of the CsA epoxide at a high temperature. An unknown side product (m/z 1248) was also identified in the spectrum.

As a result of the introduction of a hydroxyl group and an ethylenediamine moiety to CsA, the CsA amine exhibits a better water solubility than CsA. The primary amino group in the CsA amine can be used for further modification, such as conjugation to an antibody or a polymeric drug carrier for targeted drug delivery.

Water soluble *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers were developed as polymeric drug carriers for cancer chemotherapy (reviewed in ref 12). The conjugation of anticancer drugs to the HPMA copolymer reduced the side effects of drugs and improved the specific accumulation of macromolecular therapeutics in tumor tissue as a result of the enhanced permeability and retention (EPR) effect (12). The conjugation of CsA derivatives to HPMA copolymers may also improve the therapeutic efficacy of CsA with reduced side effects. It has been reported that the conjugation of a CsA derivative to a water-soluble copolymer maintained the thera-

peutic activity of CsA and increased its specificity with reduced side effects (10).

Previously, attempts to conjugate CsA to HPMA copolymers via the hydroxyl group of CsA did not succeed because of steric hindrance. With the new CsA derivative, CsA amine IV, the conjugation was achieved by reacting compound IV with a *p*-nitrophenol active ester at the tetrapeptide side chain termini in an HPMA copolymer. It is possible that the bifunctionality (primary and secondary amines) of the CsA amine IV may cause cross-linking. However, no obvious cross-linking was observed in the size-exclusion chromatographic (SEC) analysis of the HPMA copolymer-CsA conjugate. This suggested that the CsA amine was mainly conjugated via the primary amine, probably due to the steric hindrance of the secondary amine. The content of CsA in the copolymer was 8.7 wt % as determined by amino acid analysis.

The CsA amine was attached to an HPMA copolymer via a Gly-Phe-Leu-Gly tetrapeptide spacer, Scheme 2. The tetrapeptide spacer is susceptible to cathepsin B-catalyzed hydrolysis in the lysosomal compartment of a cell (13, 14). The release of the CsA amine was investigated by the incubation of an HPMA copolymer-CsA amine conjugate with papain at 37 °C (papain has a similar specificity to cathepsin B). After 24 h of incubation, the reaction mixture was extracted with methylene

dichloride. The extracts were analyzed by electrospray ionization (ESI) mass spectrometry. CsA amine (m/z 1278) and glycylated CsA amine (Gly-CsA) (m/z 1336) were identified in the mass spectrum. The release of a Gly-CsA amine indicated that the bond originating in the leucine residue was one of the cleavage sites. It is known that papain prefers a phenylalanine residue in the S_2 subsite of its active site (15). Despite the steric hindrance of the polymeric chain on the formation of the enzyme–substrate complex (16), such an arrangement of the polymeric substrate in papain's active site may be energetically favorable. With increasing the incubation time, the amount of Gly-CsA decreased eventually to a level nondetectable by ESI mass spectrometry. One can hypothesize that the bond originating in the glycine residue is also a cleavage site for papain, but slower. A detailed degradation study is needed to prove the hypothesis on the structure of the enzyme–substrate complex. However, *in vivo*, the lysosomal aminopeptidases would probably convert any Gly-CsA amine into the CsA amine, which would be the main product at longer time intervals. On another note, the identification of the glycylated CsA amine as one of the papain cleavage products confirmed the conjugation of CsA amine to the HPMA copolymer.

In summary, a new CsA derivative containing amino groups has been synthesized by the epoxidation of CsA and subsequent aminolysis with ethylenediamine. The compound was water soluble, and the primary amino group could be used for the conjugation to polymers or proteins. The CsA amine was conjugated to HPMA copolymers via a Gly-Phe-Leu-Gly spacer and was released from the copolymers by the action of papain.

EXPERIMENTAL SECTION

Chemicals. An HPMA copolymer with Gly-Phe-Leu-Gly-ONp side chains was prepared as previously described (13). CsA was provided by Galena (Czech Republic). Mo catalyst, $\text{MoO}_2(\text{OCH}_2\text{CH}_2)_2\text{NH}$, was synthesized according to method described (11). *tert*-Butyl hydroperoxide (5.5 M in decane, Fluka, Ronkonkoma, NY) and *m*-chloroperoxybenzoic acid (Aldrich, Milwaukee, WI) were used without further purification. Solvents and all other reagents were of reagent grade or better. Proton NMR spectra were recorded on a NR/200 FTNMR spectrometer (IBM Instruments). Electrospray ionization mass spectrometry was performed on a Trio 2000 single quadrupole mass spectrometer (Micromass). The amino acid analysis of the HPMA copolymer–CsA amine conjugate was performed by J. Strohalm in the Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague.

Epoxidation of Cyclosporin A. Cyclosporin A (100 mg, 83 μmol), *m*-chloro-peroxybenzoic acid (16 mg, 93 μmol), and anhydrous Na_2CO_3 (20 mg, 190 μmol) in 10 mL of methylene chloride was stirred overnight at rt. The reaction mixture was washed with 20% sodium bisulfite and 10% sodium carbonate. The organic layer was dried over anhydrous sodium carbonate. The solvent was removed under vacuum producing a crystalline product. Yield: 96 mg. The product was characterized by ESI mass spectrometry, m/z 1219 ($M^+ + 1$). The product was immediately used for next reaction without further purification.

Preparation of Cyclosporin Amine IV. The product (96 mg) from the above reaction was refluxed with an excess of ethylenediamine (200 μL) in 10 mL of THF for 24 h. The solvent was removed under vacuum, and the

reaction product was isolated from the solid residue using silica gel column chromatography. Compound III was first eluted out with hexane/acetone (1:2), then the cyclosporine amine was eluted with methanol. The solvent of the first fraction was removed by a rotavapor leaving a colorless crystalline product (31 mg, 30%, based on CsA). The solvent of the second fraction was removed under vacuum leaving a yellowish solid product (38 mg, 33%, based on CsA). Both fractions were characterized by ESI mass spectrometry. The first fraction with m/z 1219 ($M^+ + 1$) was identified as compound III, and the second fraction with m/z 639 [$(M^+ + 2)/2$], 1278 ($M^+ + 1$) was characterized as the CsA amine. ^1H NMR: compound III (δ , ppm, CDCl_3), 7.96, 7.75, 7.45, 7.14 (d, 1H, CONH), 3.49 (s, 3H, NCH_3), 3.38 (s, 3H, NCH_3), 3.03 (s, 6H, NCH_3), 2.67 (s, 6H, NCH_3); CsA amine (δ , ppm, DMF-d_7), 8.25 (m, 4H, CONH), 2.99 (m, 21 H, NCH_3).

Preparation of Cyclosporin Amine (Method 2). Cyclosporin A (5 mg, 0.4 μmol) and *tert*-butyl hydroperoxide (5.5 M, 20 μL) were dissolved in 5 mL of benzene, and $\text{MoO}_2(\text{OCH}_2\text{CH}_2)_2\text{NH}$ (1 mg) was added. The reaction mixture was refluxed for 2 h. The solvent was removed under vacuum. The residue was dissolved in 10 mL THF with 100 μL of ethylenediamine (excess) and refluxed with stirring for 24 h. The reaction mixture was analyzed with ESI mass spectrometry. The yield of the cyclosporin amine (40%) was estimated from the peak intensities of the CsA derivatives in the mass spectrum.

Conjugation to HPMA Copolymers. A copolymer of HPMA and MA-Gly-Phe-Leu-Gly-ONp ($M_n = 15\,900$, $M_w = 21\,400$, ONp content = 5.1 mol %, 40 mg) was dissolved in 1 mL of DMSO and mixed with the cyclosporin amine (12 mg) in 0.5 mL of DMSO. The reaction mixture was stirred overnight at rt. The yellow solution (color of *p*-nitrophenolate) was diluted to 5 mL with methanol and was purified on a Sephadex LH-20 column and eluted with methanol. The Sephadex LH-20 purification was repeated once more. The fractions corresponding to the polymer–drug conjugate were pooled and the solvent was removed. The residue was dissolved in water and lyophilized. Yield: 35 mg, 85%. The content of cyclosporin amine in the HPMA copolymer conjugate was 8.7 wt % as determined by amino acid analysis.

Degradation of the Polymer–Drug Conjugate. The HPMA copolymer–CsA amine conjugate (10 mg) was incubated with papain (4.5 mg) in PBS buffer (5.0 mL) with 1 mM DTT at 37 °C for 24 h. The incubation mixture was extracted with methylene dichloride. The extract was characterized by ESI mass spectrometry. CsA amine (m/z 1279, $M^+ + 1$) and glycylated CsA amine (m/z 1336, $M^+ + 1$) were identified in the spectrum of the extract.

ACKNOWLEDGMENT

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COMMUNICATIONS

A New Strategy for Site-Specific Protein Modification: Analysis of a Tat Peptide–TAR RNA Interaction

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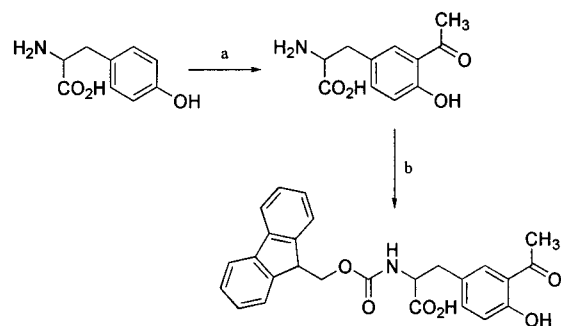
Site-specific modification of proteins and peptides with reporter molecules provides a powerful research tool in chemistry and biology. We report the synthesis and application of a tyrosine analogue, *N*- α -*Fmoc*-3-acetyl-L-tyrosine, for selective modification of proteins. As a model system, we synthesized the human immunodeficiency virus type 1 (HIV-1) Tat peptide (amino acids 47–56) containing the arginine rich RNA-binding region and replaced the Tyr-47 with 3-acetyl-tyrosine. The acetyl-Tyr-Tat peptide was subsequently labeled with a fluorescein derivative to study RNA–protein interactions by fluorescence energy transfer experiments. Our results showed that the Tat peptide binds to the rhodamine labeled TAR RNA with a dissociation constant (K_D) of 1.0 ± 0.5 nM. This strategy of selective protein modification offers a versatile new procedure for labeling peptides of biological interest at a desired site when several nucleophilic side chains of lysine and cysteine are present. These methods would provide tools for postsynthetic peptide modification and introducing biophysical probes for structural and functional analysis of proteins.

Site-specific modification of proteins and peptides with reporter molecules provides a powerful research tool in chemistry and biology. Selective chemical modification of proteins involves covalent derivatization of the functional group (e.g., $-NH_2$ and $-SH$) of a single, unique amino acid residue in the protein without causing any effect on other functional groups (1). However, the attachment of a reporter molecule at a specific site in a protein containing multiple lysine and cysteine residues is difficult to achieve and in most cases requires some orthogonal protection of these side chains. Since the reaction of ketone and aldehyde with a hydrazide is

orthogonal to the functional groups present in proteins, this chemistry has been used to label peptides and proteins by the oxidation of N-terminal serine to the corresponding aldehyde and subsequent coupling with an appropriate hydrazide (2). Schultz and co-workers have introduced a ketone handle into a unique site in a protein by unnatural amino acid mutagenesis which was subsequently modified with a fluorescein derivative (3). Amino acid analogues preconjugated with structural probes have also been used in the solid-phase peptide synthesis, (4–7) however, this methodology has a limited application because once introduced into peptides, this specified site is no longer available for modification with other reporter molecules. Thus, the development of novel amino acid analogues with selective reactivity other than with the

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Scheme 1



^a CH_3COCl - AlCl_3 /nitrobenzene, 100 °C, 6 h. ^b Fmoc-Osu- Na_2CO_3 /dioxane- H_2O .

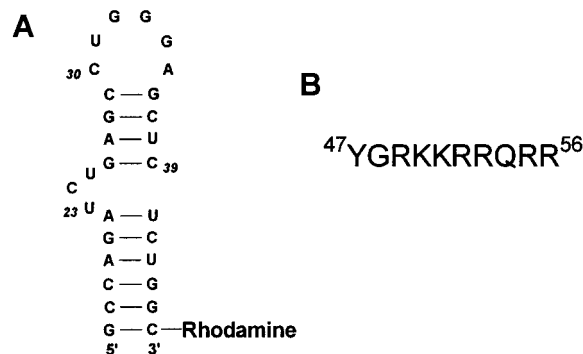


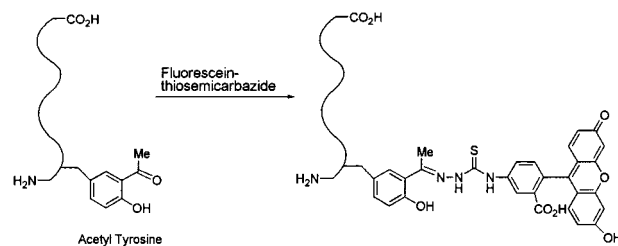
Figure 1. (A) Secondary structure of TAR RNA used in this study. TAR RNA spans the minimal sequences that are required for Tat responsiveness *in vivo* (21) and for *in vitro* binding of Tat-derived peptides (34). Chemical syntheses were performed on ABI synthesizer model 392 using standard protocols. All the monomers of (2-cyanoethyl)phosphoramidites were obtained from Glen Research (Sterling, VA). TAR RNA was chemically synthesized on a rhodamine-containing CPG 500 support. Synthesis of RNA, deprotection, characterization, and purification were performed as described earlier (35, 36). (B) The Tat-derived peptide, amino acids 47 to 56, contains the RNA-binding region of Tat protein. Acetyl-Tyr was incorporated at position 47 and modified with fluorescein (12).

lysine or cysteine side chains ($-\text{NH}_2$ and $-\text{SH}$ functional groups) would be very useful for selective postsynthetic labeling of proteins. Such amino acid analogues would especially be useful for incorporation of labels into a protein without modification of cysteine and lysine residues involved in protein function (e.g., nucleic acid binding proteins requiring lysine side chains for nucleic acid recognition and cysteine side chains for metal coordination such as zinc finger structures).

Here, we report the synthesis and application of a tyrosine analogue, *N*- α -Fmoc-3-acetyl-L-tyrosine, for selective modification of proteins. We have modified the side chain of tyrosine with a keto functional group without disrupting its potential phosphorylation and metal coordination site (8) and used its Fmoc derivative in standard solid-phase peptide synthesis (Scheme 1). As a model system, we synthesized the human immunodeficiency virus type 1 (HIV-1) Tat peptide (amino acids 47–56) containing the arginine rich RNA-binding region and replaced the Tyr-47 with 3-acetyl-tyrosine (Figure 1). The acetyl-Tyr-Tat peptide was subsequently labeled with a fluorescent dye molecule (Scheme 2) to study RNA–protein interactions.

3-Acetyl-tyrosine was synthesized from tyrosine by Friedel–Crafts reaction (8–11) and converted to its Fmoc derivative by Fmoc-Osu reaction (Scheme 1) (12). Tat-derived peptide (from amino acids 47–56) was synthesized on an Applied Biosystems 431A peptide synthesizer

Scheme 2



using standard FastMoc protocols. Cleavage and deprotection of the peptide was carried out in 2 mL of Reagent K for 6 h at room temperature. Reagent K contained 1.75 mL of TFA, 100 μL of thioanisole, 100 μL of water, and 50 μL of ethanedithiol (13). After cleavage from the resin, peptide was purified by HPLC on a Zorbax 300 SB-C₈ column. The mass of fully deprotected and purified peptide was confirmed by MALDI mass spectrometry (12). The presence of this 3-acetyl-tyrosine in a peptide can easily be detected by monitoring the absorption spectrum because 3-acetyl-tyrosine gives a unique UV spectrum with absorption maxima at 254 nm ($\epsilon = 5915 \text{ cm}^{-1}\text{M}^{-1}$) and 330 nm ($\epsilon = 1643 \text{ cm}^{-1}\text{M}^{-1}$).

To site-specifically label the 3-acetyl-Tyr containing Tat peptide with a dye, we used a semicarbazide derivative of fluorescein that modified acetyl-Tyr side chain through a semicarbazone linkage (Scheme 2) (12). This reaction was carried out by the addition of 2 equiv of 30 mM solution of fluorescein-5-thiosemicarbazide in DMF to 1 mM solution of the peptide in conjugation buffer (100 mM potassium phosphate, pH 7.0, 50% DMF), and incubating the reaction mixture at 4 °C overnight. The peptide-fluorescein conjugate was purified by reversed-phase HPLC and characterized by MALDI/TOF mass spectrometry (12). The conjugation reaction of the dye to acetyl-Tyr peptide was very efficient and resulted in ~80% yields. Although this Tat peptide contains two lysine residues in its sequence, these NH_2 -groups do not react with acetyl-tyrosine to form the imine linkage because the reaction is highly reversible under the conjugation conditions.

Our main interest in developing this methodology was to achieve site-specific peptide labeling with reporter molecules to probe protein–RNA interactions. In contrast to DNA, RNA molecules can fold into extensive structures containing regions of double-stranded duplex, hairpins, internal loops, bulged bases, and pseudoknotted structures (14). Three-dimensional structures of RNA create binding sites for specific interactions with proteins. One example of such interactions is the mechanism of *trans*-activation of human immunodeficiency virus type 1 (HIV-1) gene expression that requires the interaction of Tat protein with the *trans*-activation responsive region (TAR) RNA, a 59-base stem-loop structure located at the 5'-end of all nascent HIV-1 transcripts (15–18). TAR RNA contains a six-nucleotide loop and a three-nucleotide pyrimidine bulge which separates two helical stem regions (Figure 1) (15, 19–21). The trinucleotide bulge is essential for high affinity and specific binding of the Tat protein (22, 23). Due to difficulties in Tat purification, small Tat peptides that specifically bind TAR RNA with high affinity have been used in many Tat-TAR studies (24–30). Since Tat-derived peptides which contain the basic arginine-rich region of Tat are able to form *in vitro* complexes with TAR RNA (24, 26, 27), these peptides provide a well-characterized system to test new probes to study RNA–protein interactions. Although Tat peptides-TAR-binding affinities have been well studied by

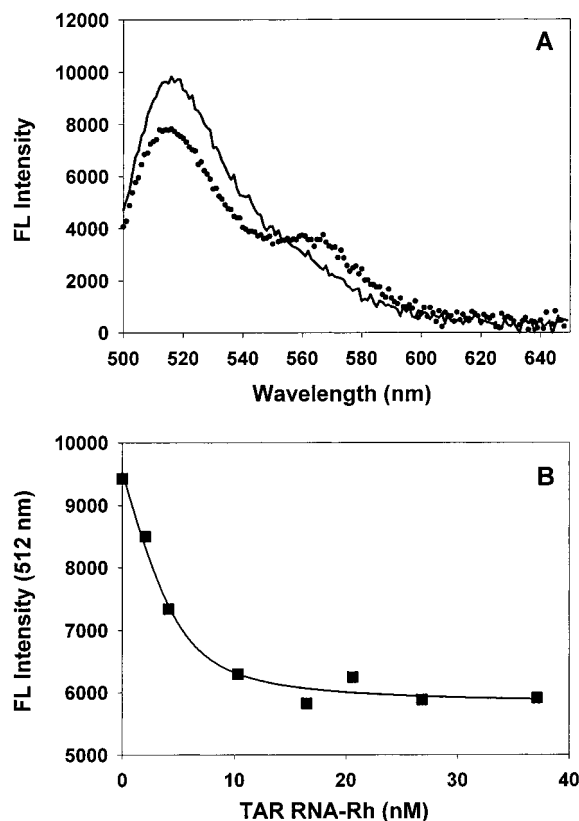


Figure 2. (A) Fluorescence spectra of Tat-Fl (solid line) and Tat-Fl with TAR RNA-Rh (dotted circles). The concentrations of TAR RNA-Rh, Tat-Rh and Tat-Fl were 3 and 5 nM, respectively. (B) Fluorescence intensity of Tat-Fl (5 nM) at 512 nm in the presence of increasing TAR-Rh concentrations. The solid line represents the best fits of the data by nonlinear regression to quadratic eq 1. The fluorescence measurements were performed on PTI fluorescence spectrophotometer controlled by Felix software. The excitation wavelength was 492 nm and slits width was set 3.5 nm for both excitation and emission. All samples were corrected for the light source excitation effect and for the background intensity of buffer fluorescence as well as for dilution factors. Buffer conditions: 50 mM Tris-HCl, pH 7.4, at 25 °C, 20 mM KCl.

electrophoretic mobility shift assays, there is no available data reporting Tat peptide-TAR affinities in solution under physiological conditions.

To determine the binding affinities of a Tat peptide under physiological conditions and test the application of 3-acetyl-Tyr, we developed a fluorescence resonance energy transfer (FRET) system containing TAR RNA and a Tat peptide uniquely labeled with donor and acceptor dye molecules (Figure 1). FRET, in which a fluorescent donor molecule transfers energy via a nonradiative dipole-dipole interaction to an acceptor molecule (which is usually also a fluorescent molecule) is a standard spectroscopic technique for measuring distances in the 10–70 Å range (31, 32). The donor's lifetime and quantum yield are reduced upon energy transfer, and the acceptor fluorescence is increased or sensitized. Quantification of the efficiency of energy transfer allows determination of the distance between the two fluorophores. We used a well-characterized donor-acceptor dye-pair, fluorescein-rhodamine, for FRET experiments.

We examined FRET between the fluorescein labeled Tat peptide (Tat-Fl) and rhodamine labeled TAR RNA (TAR-Rh) (Figure 1). Emission scans from 500 to 650 nm of Tat-Fl (solid line) and Tat-Fl with TAR-Rh (dotted circles) were recorded (Figure 2A). Tat-Fl has an emission maximum around 512 nm (solid line), whereas upon

adding TAR RNA-Rh (dotted circles), this peak is decreased and another peak at 575 nm is increased. This reduction in fluorescein emission and the corresponding increase in rhodamine emission were due to the resonance energy transfer from Tat-Fl to TAR-Rh. To determine the binding affinity between the Tat peptide and TAR RNA, we examined fluorescence quenching of Tat-Fl at several different TAR-Rh concentrations (Figure 2B). K_D values were calculated by fitting data to quadratic eq 1.

$$F = F_{\min} - \{(F_{\max} - F_{\min})[(R + P_0 + K) - ((R + P_0 + K)^2 - 4RP_0)^{1/2}]\}/2P_0 \quad (1)$$

where F is the relative fluorescent intensity, $F_{\min}$ is the fluorescent intensity at the start of the titration, $F_{\max}$ is the fluorescent intensity at saturating concentration of RNA, R , P_0 is the total concentration of Tat-Fl. K is the dissociation constant of the RNA and Tat peptide and can be obtained by fitting data to eq 1 (33). Our results showed that Tat-Fl binds to the TAR-Rh with a dissociation constant (K_D) of 1.0 ± 0.5 nM.

This strategy of selective protein modification offers a versatile new procedure for labeling peptides of biological interest at a desired site when several nucleophilic side chains of lysine and cysteine are present. These methods would provide tools for postsynthetic peptide modification and introducing biophysical probes for structural and functional analysis of proteins.

ACKNOWLEDGMENT

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Supporting Information Available: Synthesis of N - α -Fmoc-3-acetyl-L-tyrosine, and Figures 3S–6S showing structures and MALDI mass spectra of the Tat (amino acids 47–56) peptides containing 3-acetyl-tyrosine and 3-acetyl-tyrosine conjugated with fluorescein. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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A Chemoselective Method for Site-Specific Immobilization of Peptides via Aminoxy Group

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Site-specific modification of peptides and proteins is an important area of basic research for preparation of well-defined biosensors and probes. The unique properties of aminoxy group present an opportunity for chemoselective site-specific immobilization of peptides to prepare well-defined biosensors. We have prepared FLAG peptide derivatives containing L- ϵ -aminoxylysine (L- ϵ -AOLys, **1a**) and L-lysine units in their sequence at the C- and N-terminals via solid-phase synthesis. Site-specific modification of peptides through aminoxy group was demonstrated in the preparation of biosensors and selective conjugation in the preparation of biotinylated probes. Effect of the incorporation of L- ϵ -AOLys (**1a**) into the peptide sequence and its subsequent labeling on the FLAG epitopic character was measured using a surface plasmon resonance detector. It was found that incorporation of L- ϵ -AOLys (**1a**) into the FLAG peptide and site-specific immobilization through aminoxy group preserved the integrity of FLAG epitope.

INTRODUCTION

Recent advances in solid-phase chemistry and molecular biology have spurred the construction of larger peptides and small proteins through stepwise synthesis using automated instruments. A variety of biophysical probes have been produced from peptides and proteins with carefully engineered properties for applications in medicinal and biotechnology fields (1–8). In contrast to the synthesis of low molecular weight natural products, which is aided by plethora of specific reactions and protective groups, site-specific modification of unprotected peptides and proteins to prepare well-defined bioconjugates is challenging. The complexity becomes even more apparent in the preparation of solid supported probes and biosensors, for example, in applications using surface plasmon resonance technology (6, 9). The observed binding properties of a peptide or protein in solution could be compromised upon immobilization depending on its site of attachment to the solid support in a biosensor. A key requirement for site-specific conjugation is the chemoselectivity. It is desired that only the intended site in a peptide or protein be modified in the presence of other functional groups and thus leading to the formation of a well-defined bioconjugate. It is critical to use such conjugates for implementation of quality control (10, 11) and reliable interpretation of biological results. We and others have been exploring the utility of aminoxy group for a variety of applications such as detection of abasic sites in DNA (12, 13), development of reagents for immunoassays (14, 15), preparation of artificial proteins (16–20) and peptide dendrimers (21), and selective fluorescent labeling of *N*-methylaminoxy peptides (22). The unique properties of aminoxy group suggest that peptides incorporated with an unnatural amino acid bearing this functionality

could be chemoselectively conjugated to a solid support of interest (Figure 1) to provide well-defined biophysical probes. In this paper, we describe an efficient method for chemoselective site-specific immobilization of peptides **12** and **14** via primary aminoxy group ($-\text{ONH}_2$) incorporated as an L- ϵ -aminoxylysine residue (L- ϵ -AOLys, **1a**) (23) to a carboxymethyl dextran chip to form a biosensors. Binding properties of these peptides **12** and **14** and a biotinylated conjugate **16** to anti-FLAG MAb using surface plasmon resonance (BIAcore) are presented.

RESULTS AND DISCUSSION

Before attempting the site-specific immobilization of peptides through the aminoxy group, competitive reactivity of aminoxy and amino groups in model amino derivatives **6** and **7** at different pH values was studied. Thus, a mixture of **6** (1.0 M equiv) and **7** (1.0 M equiv) (Scheme 1) was treated with biotin succinimidyl ester **8** (1.0 M equiv) in DMSO and sodium phosphate buffer at pH 2.0, 3.0, 3.8, 5.0, 6.0, 7.0, 8.2, and 10.3. Any difference in the reactivity of **6** and **7** in the biotinylation to form **9** or **10** under identical conditions must arise from aminoxy or amine groups. As expected (Figure 2), **7** was found to be unreactive at a pH below 6.0, however, **6** was reactive in acidic buffers down to pH 3.0. When the pH of medium was decreased to 2.0, the rate of conjugation of **6** dropped dramatically, presumably due to the protonation of the aminoxy group or hydrolysis of **8**. Under more basic conditions (pH > 6.0), compound **7** competed with **6** for the active ester **8**. When the pH of the medium increased to 10.3, the amine **7** reacted completely and the aminoxy compound **6** remain unmodified. The competitive reactivity of aminoxy and amine groups was then studied by immobilization of compounds **6** and **7** on a carboxymethyl dextran chip and quantitated by streptavidin binding using surface plasmon resonance (SPR) (6, 9, 24). Thus, **6** and **7** were immobilized (2.0 mM, 150 μL , at pH 3.8) onto separate channels of the preactivated chip surface (35 μL of 0.25 M NHS/EDAC in water over 7 min

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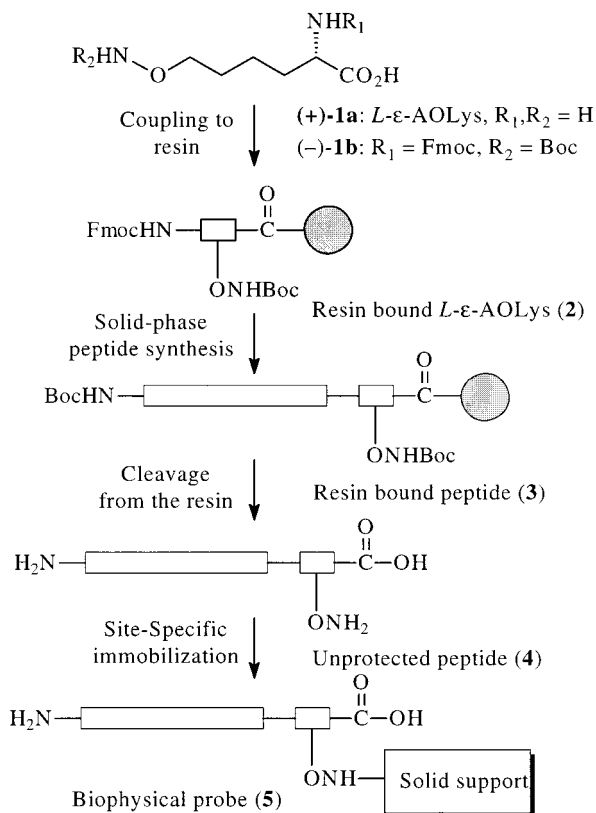
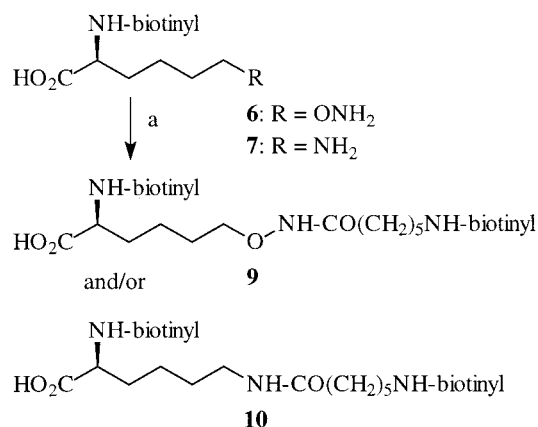


Figure 1. General strategy for construction of peptides containing L-ε-AOLys (**1a**) residue in their sequence via phase synthesis and preparation of biophysical probes by site-specific immobilization through aminoxy (-NH₂) group.

Scheme 1. Biotinylation of 6 and 7 at Different pH Values^a



^a Reagents and conditions: (a) biotinamidocaproate-*N*-hydroxysuccinimide ester (**8**, 1.0 M equiv), DMSO, sodium phosphate buffer (4:96 ratio, v/v), 23 °C, 16 h at 2.0, 3.0, 3.8, 5.0, 6.0, 7.0, 8.2, 10.3 pH.

at 5.0 μ L/min). Efficiency of the immobilization process was determined by passing a solution of streptavidin (10 μ g/mL in HBS buffer) over both the channels. The signal from the channel immobilized with **6** was 2500 RU, while the signal for channel with **7** was 20 RU (<0.8%), indicating that only **6** with aminoxy group was immobilized.

We then proceeded to prepare peptides, which had L-ε-AOLys (**1a**) unit incorporated on the C-terminal and N-terminal, **12** and **14**, respectively (Figure 1, Scheme 2). These model peptides are the derivatives of the "FLAG peptide" **11** for which specific antibodies are commercially available. Thus, α -Fmoc-L-Lys(ONH-*t*-Boc) (**1b**) was ac-

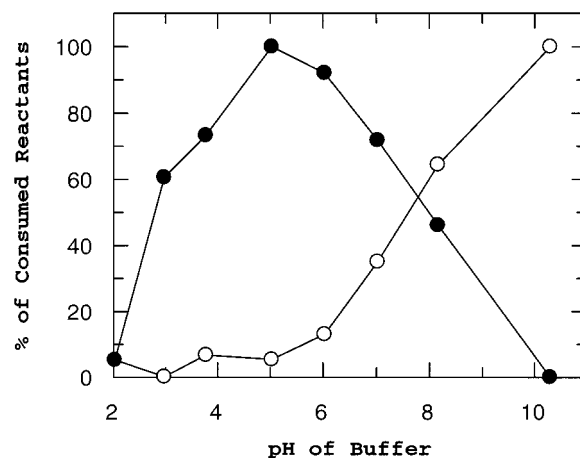


Figure 2. Competitive reaction of compounds **6** (dark circle) and **7** (normal circle) with succinimidyl ester **8** in DMSO, sodium phosphate buffer (4:96 ratio), 23 °C, 16 h at different pHs (2.0, 3.0, 3.8, 5.0, 6.0, 7.0, 8.2, and 10.3) as shown in Scheme 1.

Scheme 2: Solid-Phase Synthesis of Peptides with Incorporation of L-ε-AOLys (12**, **14**) and L-Lys (**13**, **15**) in Their Sequence, and the Site-Specific Conjugation of Peptide **14** and **15** with Active Ester **8**^a**

Native FLAG peptide: Asp-Tyr-Lys-Asp-Asp-Asp-Lys (**11**)

Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-Lys(ε-ONH₂) (**12**)

Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-Lys (**13**)

Lys(ε-ONH₂)-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (**14**)

Lys-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (**15**)

14 $\xrightarrow{a}$ Lys(ε-ONH-B)-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (**16**)

15 $\xrightarrow{b}$ Lys(B₂)-Asp-Tyr-Lys(B)-Asp-Asp-Asp-Asp-Lys(B) (**17**)

^a Reagents and conditions: (a) active ester **8** (8.0 equiv), DMSO, sodium phosphate buffer (pH 3.2) 23 °C, 19 h 58%; (b) **8** (8.0 equiv), DMSO, sodium phosphate buffer (pH 8.2) 23 °C, 19 h 55%. B = -CO(CH₂)₅NH-biotinyl.

tivated (0.5 M HATU–1.0 M Et₃N) and coupled to PAL-PEG-PS resin via double coupling to ensure high loading efficiency, and the peptide **12** was then assembled on a Pioneer Peptide Synthesizer. Similarly, peptide **14** with L-ε-AOLys unit at the N-terminus was assembled starting from α -Fmoc-L-Lys(NH-*t*-Boc). Finally, peptides **12** and **14** were cleaved from the resin by treatment with TFA-water (90:10 ratio) and isolated by reversed phase preparative HPLC in 40–61% yield. In addition, the peptides **13** and **15**, which contain a L-Lys residue in the place of L-ε-AOLys, were also prepared for control experiments. The site-specific immobilization of peptides bearing an aminoxy group in the presence of primary amines (α and ϵ -amines of Lys) was then demonstrated by passing a solution of **12–15** (2.0 mM, 150 μ L) in sodium phosphate buffer (100 mM) at pH 3.8 over four dextran surfaces (channels). The chip in each channel was pre-activated using NHS-EDAC solution (35 μ L of 0.25 M NHS/EDAC in water over a period of 7 min at 5.0 μ L/min flow rate). The resulting biosensors were compared for their ability to bind to anti-FLAG MAb. The saturated response from sensors immobilized with peptides **12** and **14** were 4260 and 6950 RU, respectively. Whereas the sensors for peptides **13** and **15** generated only 75 and 60 RU respectively (<2% reactivity) indicating negligible immobilization of **13** and **15**. To confirm the selectivity in the immobilization process, a site-specific conjugation of peptide **14** with biotin active ester **8** (Scheme 2) was explored. Accordingly, a solution of **14** in sodium phosphate buffer at pH 3.8 was treated with a DMSO solution

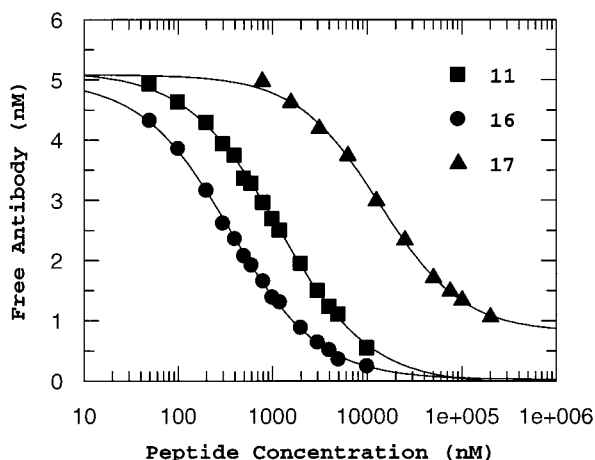


Figure 3. Measurement of inhibition constants (IC_{50}) for native FLAG peptide **11** and conjugates **16** and **17**. Anti-FLAG MAb (5 nM) was mixed with peptide solutions of different concentrations. After equilibrium, the free MAb concentration was quantitated on a sensor immobilized with **14**. The sensor was calibrated with a range of known concentrations of anti-FLAG MAb (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 3.0, 4.5, 5.0, and 5.5 nM). Grafit 3.0 was used to fit data to obtain IC_{50} values. (■) Native FLAG peptide (**11**); (●) conjugate **16**; (▲) conjugate **17**.

of **8** to afford the conjugate **16**. Analysis of conjugate **16** by mass spectrometry (ThermoQuest LCQ, ESI-MS) indicated that, only one biotin-label was present [1496.4 (MH) $^+$, 748.9 (MH $_2$) $^{2+}$]. The MS-MS analysis of **16** further confirmed its amino acid sequence and more importantly the location of the single biotin-label, which was found to be exclusively on the AOLys residue. As a control, the peptide **15**, which had a Lys residue in the place of AOLys, was treated with the active ester **8** (8.0 equiv) under identical conditions (pH 3.8), and no conjugation was observed even after 19 h. However, treatment of **15** with **8** at pH 8.2 resulted in all amines being conjugated with biotin (conjugate **17**). The structure of **17** was confirmed by MS analysis.

The binding properties of native FLAG peptide **11**, its derivatives **14** and **15** and biotinylated conjugates **16** and **17** with anti-FLAG MAb were investigated using SPR. The biosensor generated by site-specific immobilization of peptide **14** (antibody binding capacity: 6950 RU) was calibrated with anti-FLAG MAb (range 0.5–5.5 nM) under mass transport limiting condition. Calibration curves were then generated by plotting the slope of each sensorgram vs concentration of MAb. Then, solution competition experiments were conducted by mixing anti-FLAG MAb (5 nM) with FLAG peptide or conjugate solutions of different concentrations (Figure 3). Peptides **14** and **15** with AOLys and Lys residues had an IC_{50} value of $346 (\pm 32)$ nM and $361 (\pm 46)$ nM, respectively, a ~ 3.5 -fold affinity gain when compared to the native FLAG peptide **11** [IC_{50} 1217 (± 103) nM]. Importantly, the affinity of conjugate **16** [IC_{50} 345 (± 11) nM] was not changed, thus, demonstrating that site-specific biotinylation did not affect the epitopic character. However, conjugate **17**, which had four biotin labels, lost antibody binding affinity dramatically [IC_{50} 13 518 (± 260) nM]. Thus, incorporation of L- ϵ -AOLys (**1a**) into the FLAG peptide and site-specific conjugation through aminooxy group preserved the integrity of FLAG epitope. In summary, peptides **12** and **14** were assembled by incorporation of L- ϵ -AOLys (**1a**) unit in their sequence via solid-phase synthesis. Site-specific immobilization of unprotected peptides **12** and **14** through an aminooxy group was demonstrated in the preparation of carboxymethylated

dextran biosensors. The effect of the incorporation of L- ϵ -AOLys (**1a**) into the peptide sequence and its subsequent immobilization on the FLAG epitopic character was found to be minimal as measured by surface plasmon resonance.

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Polymeric Prodrug for Release of an Antitumoral Agent by Specific Enzymes

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The clinical usefulness of antitumor chemotherapy has been strongly limited by the lack of specificity of most anticancer drugs, which act also against healthy cells. The aim of this work was to design, synthesize, and evaluate a macromolecular prodrug of Cytarabine, a known antitumor drug, which is a specific substrate for plasmin enzyme whose concentration is high in various kinds of tumor mass as a result of plasminogen activator secretion. α,β -Poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA), a known synthetic and biocompatible polyamino acid, was used as a drug carrier, and Cytarabine was linked to PHEA by D-Val-Leu-Lys spacer synthesized beginning from Cbz-D-Val-LeuOH dipeptide and *N*⁶-CbzLys methyl ester. The content of Cytarabine in the purified PHEA-D-Val-Leu-Lys-Cytarabine conjugate was equal to 3% w/w. In vitro experiments in the presence of plasmin evidenced the ability of this enzyme to strongly increase drug release from the macromolecular prodrug, as well as plasma incubation shows high stability of drug–polymer linkage. The direct linkage of Cytarabine to PHEA was also performed and, like PHEA-D-Val-Leu-Lys-Cytarabine conjugate, the obtained PHEA–Cytarabine conjugate showed high stability in plasma, but no release of Cytarabine was revealed in the presence of plasmin.

INTRODUCTION

The use of therapeutic systems to obtain a controlled drug release in cancer therapy is a more and more exploited approach in the modern pharmaceutical technology (1, 2). Growing interest in this field rises on one side from the often unsuccessful research of effective and nontoxic new drugs and on the other side from the new knowledge on the biochemical events around and inside tumor tissue that allow to propose novel therapeutic strategies. Many drug delivery systems have been assayed and among them drug–antibody conjugates (immunoconjugates) and conjugates obtained by linking drugs to natural or synthetic polymers (macromolecular prodrugs) (2–5). Unfortunately immunoconjugates have often disappointed starting promising expectations (6, 7) mainly because of the limited access of these relatively big macromolecules into tumor mass, the heterogeneity of tumor cells and the different humoral answer among patients.

For the past several years in anticancer therapy, strong interest has been aroused, for their potential, by macromolecular prodrugs. They are systems obtained by covalent linking of active agents to a water-soluble macromolecule (carrier) with suitable functional groups, directly or by a chemical spacer arm (8–10).

The coupling of low-molecular weight drugs to water-soluble carrier can involve: (a) the improvement of drug movement in the body prolonging its time residence in circulation (11, 12); (b) a slow release of the drug from polymeric conjugate by chemical or enzymatic hydrolysis (13, 14); (c) the promotion of drug incorporation into cells via endocytosis process (15); (d) the site-specific transport of drug by using moieties recognizing target cells (16);

(e) the exploitation of enhanced permeability and retention (EPR) effect of tumor mass (17).

In addition to these advantages the use of macromolecular prodrugs in common chemotherapeutic programs can strongly improve tumor localization of drug (17). In the recent years a lot of investigations has been performed in order to clarify the biochemical events concerning tumor growth and formation of metastasis (18–20) and to identify the sole properties of neoplastic cell that can be exploited to concentrate drug into tumor mass. These studies have demonstrated that the ability of the tumor cells to invade surrounding tissues involves an increased activity of proteolytic enzymes able to degrade the basal membrane and the extracellular matrix (18–20). These enzymes can be secreted by tumor cells or remain bound to their cellular surface (21). Although they are also produced by normal cells, their activity is normally tightly regulated by hormonal controls and by specific inhibitors (22, 23). It seems that the normal regulating mechanism is disturbed for tumor cells and they use these enzymes to activate degradation cascades close to cell surface in order to clear a path for the invading cancer cells (24). Most of these enzymes belong to the families of extracellular matrix metalloproteinases (25), cathepsins (26), and plasminogen activators (Pas), which catalyze the conversion of inactive plasminogen to plasmin, the active enzyme in extracellular matrix degradation (20, 27). The levels of Pas are high in many types of malignant cells and human tumors such as malignant lung (28) and colon (29) showing that tumor-associated plasmin activity is highly localized (20, 27, 28–31).

This phenomenon suggested to design macromolecular prodrugs by using proper peptidic spacers (usually tri- or tetra-peptides) able to release drug inside or near tumor by hydrolytic activity of this enzyme.

The chosen peptidic spacer has to be able to give rise a macromolecular prodrug resistant to the serum pepti-

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dase activity but always susceptible to enzymes present around tumor mass. Since it is well-known from the literature that plasmin is a protease with specificity for arginine or lysine as amino acids participating to bond cleaved (32) and the examination of the preferred sites for plasmin cleavage of the fibrinogen molecule (physiological function of plasmin) shows that the preferred sites involves lysine linked to an hydrophobic amino acid, the choice of which peptidic sequence to use has been focused on a peptide having an hydrophobic amino acid linked to lysine; therefore, D-Val-Leu-Lys tripeptide, already proposed by literature as a possible good substrate for plasmin (33), was so chosen.

Among antitumor compounds, the cytotoxic S-phase-specific antimetabolite Cytarabine (Ara-C) (34) is the most frequently used drug for the treatment of patients with acute myelogenous leukemias though its application has a few disadvantages. First, it is not very specific, and this compound affects also healthy cells causing many toxic side effects. Second, the amino group of the heterocycle in the drug is highly sensitive to the action of deaminase enzymes; this process leads to the inactivation of the drug by formation of an ineffective uridine derivative (35). Therefore, effective blood levels of Cytarabine can be achieved by continuous infusion or saturating drug metabolizing enzyme with potentially very toxic massive doses.

Various derivatives monosubstituted at the N-4 position of the cytosine ring have been prepared, and their hydrolytic stability has been evaluated in vitro and in vivo (36). It has been demonstrated that the derivatization of this molecule by using the amino group seems to be a valid method to increase drug stability to deaminases and to obtain less toxic derivatives (33, 36, 37).

The aim of this work is to design, synthesize, and characterize a macromolecular prodrug of Cytarabine by using a peptidic spacer, which is an ad hoc substrate for plasmin enzyme, such as D-Val-Leu-Lys, and involving labile amine group of cytosine ring; this rational has the objective to improve chemical stability and then the bioavailability of Cytarabine, as well as specificity and action selectivity.

α,β -Poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA), a synthetic macromolecule at protein-like structure, freely water-soluble, nontoxic, nonantigenic, and nonimmunogenic, already proposed as plasma substitute and drug carrier of antiinflammatory (38, 39) and antiviral drugs (12, 14, 40) has been used.

The obtained PHEA-D-Val-Leu-Lys-Cytarabine conjugate properly purified and characterized has been subjected to in vitro plasma and plasmin activity to verify its potential in cancer therapy.

The direct linkage of Cytarabine to PHEA has been also performed and the obtained PHEA-Cytarabine conjugate was subjected to the same in vitro hydrolysis experiments in order to evaluate its stability in these media and its possible ability to drug release in the presence of plasmin.

EXPERIMENTAL PROCEDURES

Apparatus. Infrared spectra were obtained using a Perkin-Elmer 1720 IR Fourier Transform Spectrophotometer in potassium bromide disks. Ultraviolet (UV) spectra were recorded by using a Perkin-Elmer 330 Instrument equipped with a 3600 Station.

Elemental analysis (C, H, N) was carried out on a Carlo Erba model 1106 analyzer.

The mass spectra were obtained using a Hybrid Quadrupole-TOF LC/MS/MS Mass Spectrometer (manufactured by Perkin-Elmer Sciex Instruments) equipped with an Apple Macintosh System 8.6 Hardware operating with TOFTune 1.5b37 software by using the Electron Spray Technique.

The ^1H NMR spectra were obtained with a Bruker AC-250 instrument operating at 250.13 MHz. A Brookhaven Bi-200 SM Laser Spectra Physics Spectrometer was used for determination of weight average of molecular weight.

Centrifugations were performed using a Centra MP4R IEC centrifuge. Low-temperature reactions were performed by using a Haake F3 Digital thermostat.

TLC analyses were performed on silica gel plate 60 F₂₅₄ (Merck) and on RP-18 silica gel plate F₂₅₄^S (Merck).

HPLC analyses were carried out on a system consisting of a Varian 9012 Liquid Chromatograph equipped with a Rheodyne Injector 7125 (fitted with a 10 μL loop) and a Kontron HPLC Detector 432 on line with a computerized HP workstation. In the method HPLC, a reversed-phase C₁₈ column (μ Bondapak; 10 μm of 250 mm $\times$ 4.6 mm i.d., obtained from Waters) equipped with a direct-connect guard column C₁₈ (Waters) was used.

Materials. Cytarabine (1- β -arabinofuranosylcytosine) was obtained from Upjohn as a gift; Cbz-L-lysine methyl ester hydrochloride, hydroxybenzotriazole (HOBt), and *N*-methylmorpholine (NMeMor) were purchased from Sigma (Italy); Cbz-D-Val-LeuOH was purchased from Bachem (Switzerland); DL-aspartic acid, ethanolamine, 1,1'-carbonyldiimidazole (CDI), dicyclohexylcarbodiimide (DCC), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCI-HCl), 10% Pd/C, Triton X-100, and acetone were purchased from Fluka (Switzerland); acetone was dried by using CaCl_2 and distilled before using; black palladium, *N*-hydroxysuccinimide (N-HSI), 4-(dimethylamino)pyridine (4-DMAP), anhydrous pyridine, *N*-methylpyrrolidone (NMP), anhydrous diethyl ether, and colloidal silica for column chromatography (60, 70–230 mesh), 60 Å were purchased from Aldrich (Italy); 4-nitrophenylchloroformate (PNFC), methanol, chloroform, and formic acid were purchased from Merck (Germany); methanol was dried by using Mg and I₂ and distilled before using *N,N*-dimethylformamide (DMF) (Hoechst), dried using P₂O₅, and distilled under reduced pressure before use. All other chemicals were reagent grade.

PHEA was prepared and purified according to a previously reported procedure (41). The weight-average molecular weight of PHEA, measured by light scattering, was 51 800 ($M_w/M_n = 1.79$).

Human plasma was obtained from voluntary healthy blood donors.

Plasmin was purchased by Boehringer Mannheim (Italy).

Synthesis of Cbz-D-Val-Leu-N⁶-Cbz-Lys-OMe (3). To a solution of 454 mg (1.372 mmol) of Cbz-L-Lysine-OMe-HCl (1) in 1.06 mL of dry pyridine and 0.17 mL of *N*-methylmorpholine at 0 °C were added 500 mg (1.372 mmol) of Cbz-D-Val-Leu-OH (2), 298 mg (2.20 mmol) of HOBt, and 454 mg (2.20 mmol) of DCC in 2.2 mL of dry pyridine.

The reaction mixture was kept under stirring at 0 °C for 4 h and at room temperature for 36 h. After this time, the reaction mixture was filtered to separate produced dicyclohexylurea and solvent was removed under pressure. The obtained residue was dissolved in CHCl_3 , and the organic solution was treated by subsequent steps with alkaline solution, water, and acid solution, and again with water, extracting aqueous phases by CHCl_3 . The

organic fractions were collected, dried over Na_2SO_4 at room temperature for some hours, and then filtered and evaporated. The obtained product was purified by column chromatography by using silica gel 60 and $\text{CHCl}_3/\text{AcOEt}$ (2:8) as mobile phase; the yield was 76% based on **2**.

The purity of product was verified by TLC on silica gel plate 60 F₂₅₄ and using $\text{CH}_3\text{COOC}_2\text{H}_5/\text{CHCl}_3$ (4/1, v/v) as mobile phase; the R_f value was 0.77.

The purified product was characterized by elemental analysis, melting point (mp), mass spectrum, IR, and ^1H NMR spectroscopies. Anal. ($\text{C}_{34}\text{H}_{48}\text{N}_4\text{O}_8$) C, 63.73; H, 7.55; N, 8.74; mp, 176–178 °C. The mass spectrum obtained by ESY Technique shows the MH^+ on at m/z 641 and mean peaks at 406 and 293 m/z . IR (KBr): 3309 cm^{-1} (ν -NH-), 3066 cm^{-1} (ν -CH- aromatic), 2958 cm^{-1} (ν -CH- aliphatic), 1735 cm^{-1} (ν C=O ester), 1691 cm^{-1} (ν C=O urethanic), 1640 cm^{-1} (ν C=O amidic), 1541 cm^{-1} (δ -NH- amide). ^1H NMR ($\text{DMSO}-d_6$): δ 0.82 (m, broad, 12H, 4CH₃ isopropyl), δ 1.2–2 (m, broad, 10H, 3CH₂ *n*-butyl of lysine, 2H isopropyl, CH₂ isobutyl), δ 2.95 (m, 2H, CH₂ *n*-butyl of lysine), δ 3.58 (s, 3H, -OCH₃), δ 3.85 (m, 1H, 1CH α of lysine), δ 4.18 (m, 1H, 1CH α), δ 4.34 (m, 1H, 1CH α), δ 4.99 (s, 4H, 2CH₂ benzyl), δ 7.34 (m, 12H, 10H aromatic, 2H -CH-NH-COOCH₂-Ph), δ 8.04 (d, 1H, -CH-NH-CO-), δ 8.14 (d, 1H, -CH-NH-CO-).

Synthesis of Cbz-D-Val-Leu-N⁶-Cbz-Lys-OH (4). To a solution of 200 mg (0.31 mmol) of Cbz-D-Val-Leu-N⁶-Cbz-Lys-OMe (**3**) in 3.0 mL of dioxane was added 1.5 mL of NaOH 1 N. Reaction mixture was kept under stirring at 0 °C for 2 h and after this time added with HCl 0.1 N until pH 3, then treated several times with CHCl_3 ; organic fractions were collected and evaporated. The purity of **4** was verified by TLC on silica gel plate 60 F₂₅₄ and using $\text{CHCl}_3/\text{CH}_3\text{OH}$ (4/1, v/v) as mobile phase; the R_f value was 0.66.

The dried product (**4**) obtained with a yield of 85% based on **3** was characterized by elemental analysis, melting point (mp), mass spectrum, IR, and ^1H NMR spectroscopies. Anal. ($\text{C}_{33}\text{H}_{46}\text{N}_4\text{O}_8$) C, 63.24; H, 7.40; N, 8.94; mp, 215–217 °C.

The mass spectrum obtained by ESY technique shows the MH^+ on at m/z at 627 and mean peaks at 536, 446, and 301 m/z . IR (KBr): 3323 cm^{-1} (ν -NH-), 3065 cm^{-1} (ν -CH- aromatic), 2958 cm^{-1} (ν -CH- aliphatic), 1738 cm^{-1} (ν C=O acid), 1688 cm^{-1} (ν C=O urethanic), 1632 cm^{-1} (ν C=O amidic), 1540 cm^{-1} (δ -NH- amide). ^1H NMR ($\text{DMSO}-d_6$): δ 0.85 (m, broad, 12H, 4CH₃ isopropyl), δ 1.2–2.1 (m, broad, 10H, 3CH₂ *n*-butyl of lysine, 2H isopropyl, CH₂ isobutyl), δ 2.96 (m, 2H, CH₂ *n*-butyl of lysine), δ 3.86 (m, 1H, 1CH α of lysine), δ 4.12 (m, 1H, 1CH α), δ 4.35 (m, 1H, 1CH α), δ 4.99 (s, 4H, 2CH₂ benzyl), δ 7.33 (m, 12H, 10H aromatic, 2H -CH-NH-COOCH₂-Ph), δ 8.04 (d, 1H, -CH-NH-CO-), δ 8.14 (d, 1H, -CH-NH-CO-).

Synthesis of Cbz-D-Val-Leu-N⁶-Cbz-Lys-Cytarabine (5). A total of 513 mg (0.81 mmol) of Cbz-D-Val-Leu-N⁶-Cbz-Lys-OH (**4**), 138 mg (1.20 mmol) of N-HSI, and 158 mg (0.82 mmol) of EDCI-HCl was dissolved in 19.7 mL of anhydrous DMF. Reaction mixture was kept under stirring at room temperature for 4 h than, at 4 °C for 12 h. After this time, 197 mg (0.85 mmol) of cytarabine were added, and the reaction mixture was kept under stirring at 45 °C for 20 h. After this time, solvent was removed under low pressure and crude residue was purified by column chromatography by using a mixture $\text{CHCl}_3/\text{CH}_3\text{OH}$ (4:1) as mobile phase.

The purity of **5** was verified by TLC on silica gel plate 60 F₂₅₄ and using $\text{CHCl}_3/\text{CH}_3\text{OH}$ (4/1, v/v) as mobile phase; the R_f value was 0.26.

The purified product obtained with a yield of 65% based on **4**, was characterized by elemental analysis, melting point (mp), mass spectrum, IR, and ^1H NMR spectroscopies. Anal. ($\text{C}_{42}\text{H}_{57}\text{N}_7\text{O}_{12}$) C, 59.21; H, 6.74; N, 11.51; mp, 139–140 °C. The mass spectrum obtained by ESY technique shows the MH^+ on at m/z 852 and mean peaks at 446 and 301 m/z . IR (KBr): 3308 cm^{-1} (ν -NH- and -OH of arabinose), 3066 cm^{-1} (ν -CH- aromatic), 2959 cm^{-1} (ν -CH- aliphatic), 1701 cm^{-1} (ν C=O urethanic), 1653 cm^{-1} (ν C=O amidic and C=O pyrimidinic ring), 1541 cm^{-1} (δ -NH- amide). ^1H NMR ($\text{DMSO}-d_6$): δ 0.85 (m, broad, 12H, 4CH₃ isopropyl), δ 1–2 (m, 10H, 3CH₂ *n*-butyl of lysine, 2H isopropyl, CH₂ isobutyl), δ 2.98 (m, 2H, CH₂ *n*-butyl of lysine), δ 3.61 (m, 2H, CH₂ C5'), δ 3.83–4.07 (m, 4H, 3H C2' C3' C4', CH α of lysine), δ 4.37 (m, 2H, 2CH α), δ 5.05 (m, 5H, 2CH₂ benzylic, OH C5'), δ 5.49 (m, 2H, (OH, C2' C3')), δ 6.05 (d, 1H C1'), δ 7.15 (d, 1H C5), δ 7.25–7.34 (m, 12H, 10H aromatic, 2NH), δ 7.90 (m, 1H, NH), δ 8.06 (d, 1H, C6), δ 8.18 (d, 1H, NH), δ 10.89 (s, 1H, NH).

Synthesis of D-Val-Leu-Lys-Cytarabine (6). To a solution of 50 mg (0.058 mmol) of Cbz-D-Val-Leu-N⁶-Cbz-Lys-Cytarabine (**5**) in 5 mL of anhydrous $\text{CH}_3\text{OH}/\text{HCOOH}$ (15/85 v/v) mixture were added 20 mg of black Pd and 30 mg of Pd/C (10%). Reaction mixture was kept under stirring and under inert atmosphere at room temperature for 20 h monitoring the progress of reaction by TLC on silica gel plate 60 F₂₅₄ and using $\text{CHCl}_3/\text{CH}_3\text{OH}$ (4/1, v/v) as mobile phase through the progressive disappearance of spot at R_f = 0.26 (related to **5**) and the appearance of a spot with R_f = 0. Reaction mixture was filtered and solvent was removed under low pressure at controlled temperature (no more than 40 °C). The obtained residue was treated several times with acetone (~50 mL), and the dried solid product obtained with a yield of 60% was characterized by elemental analysis, melting point (mp) and ^1H NMR spectroscopy. Anal. ($\text{C}_{26}\text{H}_{45}\text{N}_7\text{O}_8$) C, 53.68; H, 7.45; N, 16.85; mp, 130–132 °C. ^1H -NMR ($\text{DMSO}-d_6$): δ 0.85 (m, 12H, 4CH₃ isopropyl), δ 1–2 (m, 10H, 3CH₂ *n*-butyl of lysine, 2CH isopropyl, CH₂ isobutyl), δ 2.98 (m, 2H, CH₂ *n*-butyl of lysine), δ 3.61 (m, 2H, CH₂ C5'), δ 3.83–4.07 (m, 4H, 3H C2' C3' C4', CH α of lysine), δ 4.37 (m, 2H, 2CH α), δ 4.6–5 (m, 7H, 2-NH₂, 3H, OH C5' C2' C3'), δ 6.05 (d, 1H C1'), δ 7.15 (d, 1H C5), δ 7.70 (m, 1H, NH), δ 8.06 (d, 1H, C6), δ 8.25 (d, 1H, NH), δ 10.89 (s, 1H, NH).

Nitrophenyl Chloroformate Activation of PHEA (7). To a solution of 100 mg (0.001 93 mmol) of freshly lyophilized PHEA in 2.1 mL of NMP/pyridine (3.6:1 v/v) mixture, were added 21 mg of DMAP (0.171 mmol) and 228 mg (1.132 mmol) of PNFC at the temperature of -10 °C; the reaction mixture was kept under stirring for 1 h at the same temperature then left to go to room temperature and precipitated dropwise into an anhydrous diethyl ether/acetone (6:4 v/v) mixture (about 20 mL) kept to 0 °C. The obtained suspension was centrifuged at 4 °C for 6 min at 10 000 rpm and the residue was treated several times with the same precipitant mixture and dried.

Determination of 4-Nitrophenyl Carbonate Content of (7). The activation percentage of PHEA was evaluated by UV spectroscopy. A total of 10 mg of **7** were added to 25 mL of 0.1 N NaOH and the absorbance of solution was measured at 400 nm. The 4-nitrophenyl carbonate content of activated polymer (**7**) was equal to about 12.2% w/w, corresponding to an activation degree of polymer of 13.2 mol %.

Determination of Total Carbonate Content of 7. A total of 50 mg of activated polymer was dissolved in 7

mL of 0.1 M barium hydroxide. The mixture was kept to reflux for 1 h, preventing uptake of CO₂ from the air. After cooling the reflux condenser was washed with 5 mL of CO₂-free water and the aqueous solution was titrated with 0.1 M HCl using phenolphthalein as indicator.

Synthesis of PHEA-D-Val-Leu-Lys-Cytarabine Conjugate (8). To a solution of 114 mg of activated PHEA (7) in 2.1 mL of an anhydrous DMF/pyridine (9:1 v/v) mixture, was added 100 mg (0.171 mmol) of D-Val-Leu-Lys-Cytarabine (6); the reaction mixture was kept under stirring at 20 °C for 24 h, and after this time, it was added dropwise to an anhydrous diethyl ether/acetone (6:4 v/v) mixture (20 mL). The obtained suspension was centrifuged at 4 °C for 6 min and 10 000 rpm, then treated once with the same precipitant mixture, centrifuged, and dried under low pressure. The obtained residue was dissolved in distilled water and purified by exhaustive dialysis by using Visking Dialysis 18/32 in. (with a molecular weight cutoff of 12000–14000) then lyophilized. The purity of obtained polymeric conjugate was evaluated by HPLC analysis.

The amount of free drug in the conjugate was evaluated equal to 0.01% w/w.

PHEA-D-Val-Leu-Lys-Cytarabine conjugate (8) was characterized by elemental analysis, UV and ¹H NMR spectroscopies. Anal. calcd (C_{6.57}H_{10.87}N_{2.15}O_{3.19}) (relative to 3% w/w of substitution) C, 46.15; H, 6.41; N, 17.59. Found: C, 47.05; H, 7.15; N, 17.89. UV: the UV spectrum in the range between 230 and 350 nm shows an absorption maximum at 278 nm. ¹H NMR (DMSO-*d*₆/D₂O): δ 0.95 (m, broad, 12H, 4CH₃ isopropyl), δ 1–2 (m, 10H, 3CH₂ *n*-butyl of lysine, 2CH isopropyl, CH₂ isobutyl), δ 2.79 (m, broad, 2H -CH-CH₂-CO-NH-), δ 2.98 (m, 2H, CH₂ *n*-butyl of lysine), δ 3.36 (m, 2H -NH-CH₂-CH₂-OH), δ 3.6 (m, 4H, 2H CH₂-CH₂-OH, 1CH₂ C5'), δ 3.8–4.5 (m, 6H, 3H C2' C3' C4', 1CH_α of lysine, 2H 2CH_α), δ 4.72 (1H -NH-CH(CO)CH₂-), δ 6.1 (d, 1H, C1'), δ 6.98 (d, 1H C5), δ 8.02 (d, 1H, C6).

Synthesis of PHEA–Cytarabine Conjugate (9). To a solution of 115 mg of activated PHEA (7) in 2.1 mL of an anhydrous DMF/pyridine (9:1, v/v) mixture, was added 145 mg (0.59 mmol) of Cytarabine; the reaction mixture was kept under stirring at 40 °C for 24 h, and after this time, it was added dropwise to an anhydrous diethyl ether/acetone (6:4, v/v) mixture (15 mL). The obtained suspension was centrifuged at 10 °C for 6 min and 10 000 rpm, then washed several times with the same precipitant mixture, centrifuged, and dried under low pressure. The obtained residue was dissolved in distilled water and purified by exhaustive dialysis by using Visking Dialysis Tubing 18/32 in. The purity of obtained polymeric conjugate was calculated by HPLC analysis. The occurred linkage between Cytarabine and PHEA was verified by UV and ¹H NMR spectroscopies. The content of linked drug in the conjugate was evaluated by elemental analysis, alkaline hydrolysis followed by HPLC analysis and confirmed by ¹H NMR spectroscopy. Anal. calcd (C_{10.87}H_{16.32}N_{3.52}O_{5.75}) (relative to 4.6% w/w of substitution) C, 45.28; H, 5.71; N, 17.10. Found: C, 46.05; H, 6.05; N, 17.58. UV: the UV spectrum in the range between 230 and 350 nm shows an absorption maximum at 292 nm. ¹H NMR (D₂O): δ 2.59 (m, broad, 2H -CH-CH₂-CO-NH-), δ 3.13 (m, 2H -NH-CH₂-CH₂-OH), δ 3.37 (m, 2H, CH₂-CH₂-OH), δ 3.56 (m, 2H, CH₂ C5'), δ 3.72 (m, 1H, C4'), δ 3.98 (m, 2H, C2' and C3'), δ 4.66 (1H -NH-CH(CO)CH₂-), δ 6.20 (d, 1H, C5), δ 7.22 (d, 1H, C1'), δ 8.2 (d, 1H, C6).

Plasmatic Hydrolysis Studies of 8 and 9. PHEA-D-Val-Leu-Lys-Cytarabine and PHEA–Cytarabine conjugates were subjected to plasmatic hydrolysis by using

human plasma at 37 ± 0.1 °C. The hydrolyses were initiated by adding 50 μL of a buffered phosphate solution of 8 or 9 at pH 7.4 (at a concentration of 2 g/100 mL) to samples (1 mL) of preheated plasma. The samples were kept in a water bath at 37 ± 0.1 °C, under continuous stirring and at suitable intervals, 2 mL of 10% trifluoroacetic acid (v/v) were added in order to precipitate plasma proteins. After immediate mixing and centrifugation for 5 min at 10 000 rpm at 4 °C was analyzed 10 μL of clear supernatant by HPLC to determine released species. Every experiment was repeated in triplicate. As control, analogous experiments were performed in the absence of plasma.

After 24 h of incubation samples were added with 2 mL of carbonate buffer at pH 11.5, kept at 37 ± 0.1 °C and analyzed by HPLC at suitable time intervals.

Hydrolysis of 8 and 9 by Plasmin. The hydrolytic activity of plasmin toward PHEA-D-Val-Leu-Lys-Cytarabine and PHEA–Cytarabine conjugates was studied in 0.05 M Tris buffer solution and 0.1 M NaCl at pH 7.4 with a 1.2 × 10⁻⁴ M conjugate concentration in Cytarabine. To samples of 1 mL of 8 or 9 solutions, 85 μL of a plasmin 5.9 × 10⁻⁷ M solution in Tris buffer at pH 7.4 were added.

Solutions were incubated at 37 ± 0.1 °C and sampled at suitable interval time by HPLC analysis. After 2 h from the beginning of the experiment the same volume of fresh plasmin solution was added. As control, analogous experiments were performed by adding the proper volume of Tris buffer without enzyme to the conjugate solutions.

HPLC Analysis of Cytarabine. A single chromatographic method was used to detect Cytarabine released from 8 and 9 conjugates during in vitro hydrolysis studies in the presence of plasmin; C₁₈ column was eluted at room temperature with CH₃OH:0.1% H₃PO₄ (2:98) using a flow rate of 1 mL/min and monitoring the eluate at 280 nm. The HPLC conditions used for plasmatic hydrolysis studies were different because of the presence of an interfering endogenic peak in blank plasma chromatogram, i.e., acetonitrile: pH 7.0 phosphate buffer (1:99, v/v) at a flow of 1 mL/min; preliminary experiments showed that this method allows a recovery of Cytarabine from plasma of about 98.6% w/w. Quantification of Cytarabine was done by measuring the peak area in relation to standard solutions of drug.

RESULTS AND DISCUSSION

The ability of tumor cells to invade tissues and metastasize is thought to involve an increased expression of proteinases including cathepsins and plasminogen activators (27). The presence of these last enzymes at high concentration around and inside tumor causes an increased plasmin activity in ill tissues (42). This biochemical event can be properly exploited to achieve a targeted drug delivery. In fact the synthesis of a macromolecular prodrug of an antitumor drug by using a peptidic spacer in which the drug-spacer linkage is the ad hoc substrate for plasmin enzyme, such as D-Val-Leu-Lys, should seem to be a suitable method to achieve a high concentration of drug in target site also exploiting EPR effect that allows blood circulating macromolecules to be accumulated inside tumor mass (Figure 1).

To this aim the polymeric conjugate in which Cytarabine is linked to α,β-poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA) by D-Val-Leu-Lys spacer has been synthesized.

The synthesis of PHEA-D-Val-Leu-Lys-Cytarabine conjugate involves following subsequent phases: (1) synthe-

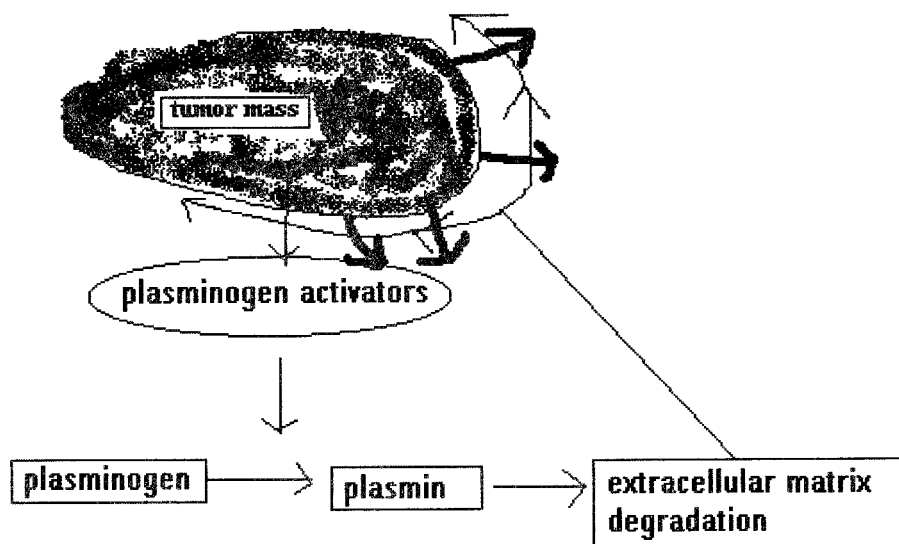
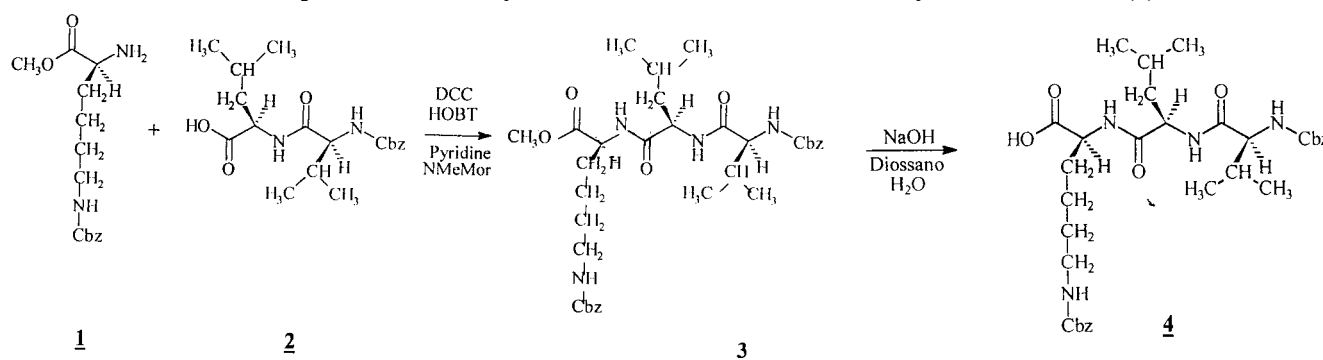


Figure 1. Schematic representation of targeted drug release in tumor mass.

Scheme 1. Schematic Representation of Synthesis of Cbz-D-Val-Leu-N⁶-Cbz-Lys-OH Derivative (4)



sis of protect tripeptidic spacer Cbz-D-Val-Leu-N⁶-Cbz-Lys-OH; (2) linking of Cytarabine to this spacer and deprotection of amine groups of peptidic derivative; (3) coupling of D-Val-Leu-Lys-Cytarabine derivative to PHEA.

The synthesis of Cbz-D-Val-Leu-N⁶-Cbz-Lys-OH (**4**) was performed from methyl ester of Cbz-L-Lysine (**1**) and Cbz-D-Val-Leu-OH (**2**) dipeptide by using as condensing agent DCC and in the presence of HOBT (Scheme 1). In effect we are not able to reproduce literature conditions of synthesis of this compound (33) then we had to set up different experimental conditions.

The product (**3**) purified by chromatographic column and characterized (see Experimental Section) was subsequently subject to alkaline hydrolysis in order to hydrolyze methyl ester of lysine and to make available carboxylic group for coupling of Cytarabine.

The Cbz-D-Val-Leu-N⁶-Cbz-Lys-OH (**4**) so obtained was kept to react with N-HSI in the presence of EDCI-HCl (Scheme 2), to obtain the reactive *N*-hydroxysuccinimide ester. The used experimental condition for time and temperature allow to obtain high yield in the activation process, that was confirmed by the high yield in the following conjugation reaction with Cytarabine (Scheme 2). The product (**5**) isolated and purified by silica gel column chromatography was characterized by elemental analysis, FAB mass spectrum, IR and ¹H NMR analyses (see Experimental Section).

The deprotection of amine groups of Cbz-D-Val-Leu-N⁶-Cbz-Lys-Cytarabine derivative was afterward carried out (Scheme 2) since these amine groups allow tripeptide-Cytarabine derivative to be linked to polymeric backbone.

If on one side the alone hydrolysis reaction of Cbz groups is not difficult and numerous reaction conditions

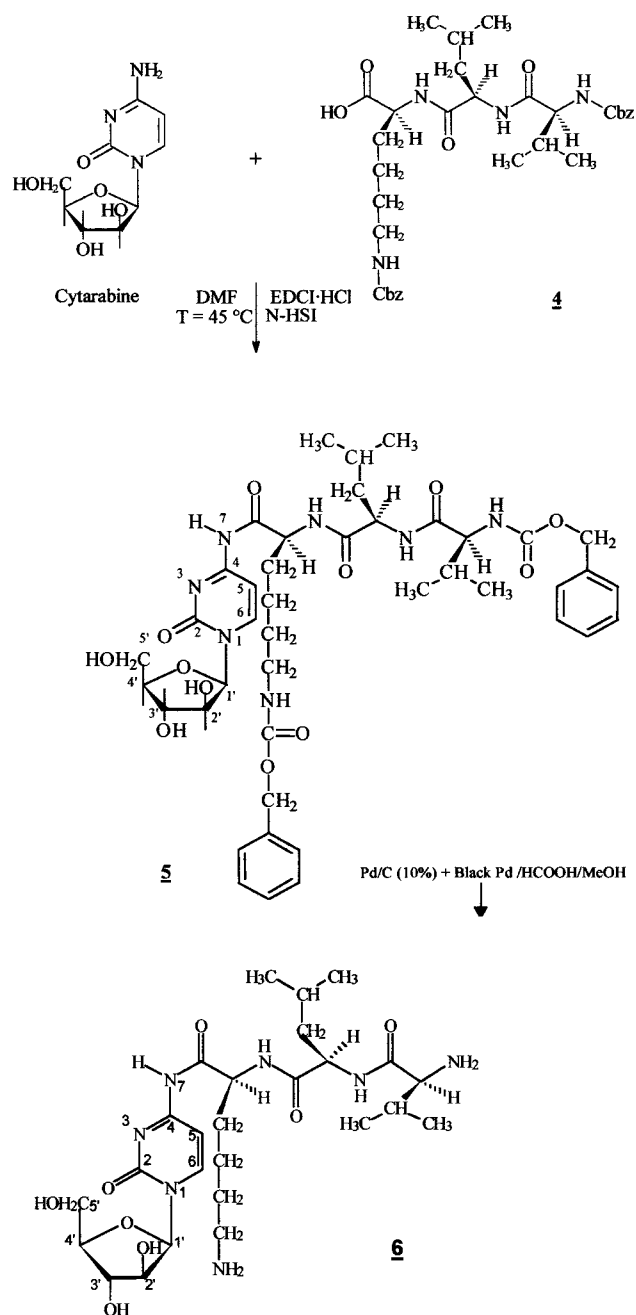
are reported in the literature, on the other side, the complexity of **5**, but above all the weakness of linking between Cytarabine and tripeptidic spacer in the presence of metallic catalysts, in aqueous acid medium, makes difficult to set better reaction conditions. Many experimental conditions were tested and some of them include the use of H₂ at atmospheric pressure or greater in the presence of methanol or THF as solvent and Pd/C as catalyst or the use of HBr and CH₃COOH at room temperature or lower. The complete deprotection of **5** was obtained by using Pd/C and black Pd mixture and high concentration of anhydrous HCOOH.

The D-Val-Leu-Lys-Cytarabine derivative (**6**) so obtained was characterized by elemental analysis, FAB mass spectrum, IR and ¹H NMR analyses (see Experimental Section).

Subsequently experimental conditions to link D-Val-Leu-Lys-Cytarabine derivative to PHEA were set up.

For the coupling of drug molecules to functional groups of a macromolecular carrier a suitable preactivation reaction is generally required. This reaction can involve either chemical groups of the drug molecules or often slightly reactive groups of macromolecular chains (43). If polymer contains hydroxyl groups an attractive activation procedure commonly reported in the literature is the chloroformate activation method (43, 44). By the reaction of activated polymers with molecules bearing amine groups, conjugates with urethane linkages between these molecules and the macromolecular chain, can be obtained.

Therefore the chemical activation of hydroxyl groups of PHEA by 4-nitrophenyl chloroformate (PNFC) was

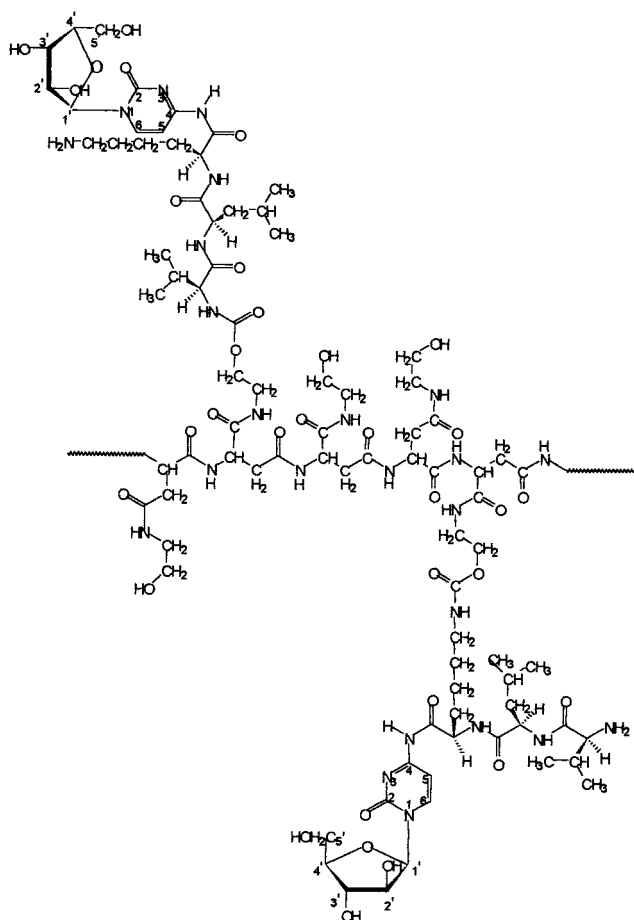
Scheme 2. Schematic Representation of Synthesis of D-Val-Leu-Lys-Cytarabine Derivative (6)

performed (see Experimental Section) and the activation percentage of **7** was evaluated by UV spectrophotometry.

Since it was demonstrated that the content of 4-nitrophenyl carbonate is equal to the present total carbonate content, the concentration of 4-nitrophenolate was used to determine the percentage of hydroxyl groups of PHEA activated by PNFC, and it was evaluated equal to 13.2 mol %.

However, it was verified that the degree of activation of PHEA is capable of being modulated, properly modifying reaction conditions such as moles of PNFC/PHEA repeating units ratio, and temperature. It was also shown that the presence of water in the reaction medium causes a strong decrease of polymer activation degree.

The synthesis of PHEA-D-Val-Leu-Lys-Cytarabine conjugate (**8**) (Scheme 3) was carried out by the reaction of activated PHEA (**7**) with D-Val-Leu-Lys-Cytarabine derivative (**6**).

Scheme 3. Schematic Representation of PHEA-D-Val-Leu-Lys-Cytarabine Conjugate (8)

The presence of unlinked drug derivative in **8**, purified by dialysis, was evaluated by HPLC analysis and estimated equal about 0.01% w/w of Cytarabine.

The occurred linkage between drug derivative and carrier was confirmed by UV and ^1H NMR spectroscopies. The UV spectrum of **8** showed an absorption band in the range 230–350 nm with a maximum of absorption at 278 nm due to Cytarabine linked to PHEA. The drug linkage to the carrier did not cause any shift in the absorption maximum. Besides, ^1H NMR spectrum of **8** (see Experimental Section) evidenced the presence of signals assignable to protons belonging to tripeptide-Cytarabine derivative (**6**) linked to PHEA.

The content of linked Cytarabine in this polymeric conjugate, determined by alkaline hydrolysis at experimental conditions that did not alter Cytarabine (carbonate buffered solution at pH 11.5, 40 °C for 24 h), followed by HPLC analysis, was evaluated equal to 3% w/w.

This result was confirmed by ^1H NMR evaluation; in fact by comparing the integral of the peaks related to protons at δ 0.95 assignable to isopropyl group of **6** with the integral of the peaks related to protons at δ 2.79 assignable to $-\text{CH}-\text{CH}_2-\text{CO}-\text{NH}-$ of polymer carrier, an average percent value of linked drug of about 2.12% mol was found, corresponding to about 3% w/w.

To evaluate the stability of this conjugate in plasma and its real ability to carry Cytarabine in plasma until action site, i.e., tumor mass, **8** was subjected to in vitro incubation with plasma.

Results are reported in Figure 2.

As it can be seen in Figure 2, the amounts of Cytarabine released from **8** both in plasma and at pH 7.4 were very similar and just about 2.5% (w/w) of linked Cytara-

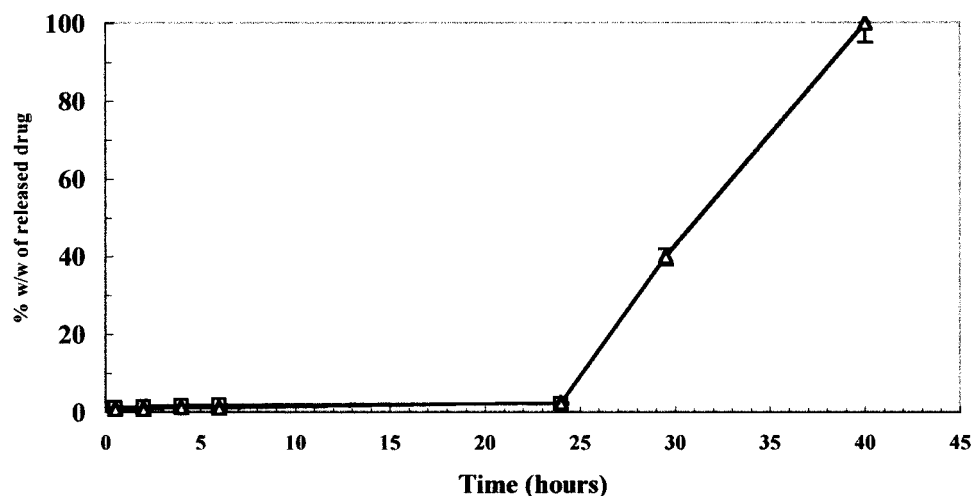


Figure 2. Release of Cytarabine from PHEA-D-Val-Leu-Lys-Cytarabine conjugate in human plasma (Δ) and in phosphate-buffered solution at pH 7.4 ($\square$) at 37 ± 0.1 °C.

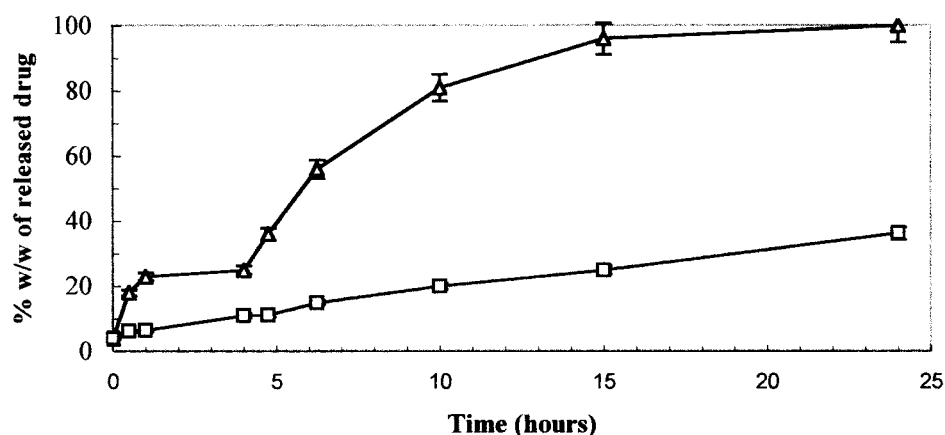


Figure 3. Release of Cytarabine from PHEA-D-Val-Leu-Lys-Cytarabine conjugate in the presence (Δ) and in the absence of plasmin ($\square$) at 37 ± 0.1 °C in Tris-buffered solution at pH 7.4.

bine was released from the polymeric conjugate within 24 h in both conditions. This result suggests the possibility that this drug-polymer conjugate could really carry drug to target since it is quite stable in plasma. After 24 h, to evidence the actual presence of linked Cytarabine in **8** in the intact form, samples were alkalized at pH 11.5 and then analyzed by HPLC. The complete release of Cytarabine in the intact form was then evidenced from **8**.

The possibility that the polymeric conjugate obtained can be ad hoc substrate for plasmin, proteolytic enzyme present at high concentration in the tumor mass and able to catalyze the hydrolysis of amide bonds at the level of lysine or arginine residues, has been evaluated subjecting PHEA-D-Val-Leu-Lys-Cytarabine conjugate at hydrolysis in the presence of this enzyme.

Results are reported in Figure 3.

As it can be seen from the Figure 3, about 23% (w/w) of linked drug was released from the polymeric conjugate in the presence of plasmin in the intact form after 1 h, while in the absence of enzyme just 6% (w/w) of linked drug was released at the same experimental conditions. After 2 h from the beginning of the hydrolysis experiment the drug release rate in the presence of plasmin was not different than the blank, then fresh and active enzyme solution was added. Within about 10 h from the second addition of enzyme solution the release of drug from the polymeric conjugate was complete. On the contrary in the absence of enzyme a low amount of Cytarabine was released at the same time.

The amounts of drug released from the polymeric conjugate versus time at pH 7.4 in phosphate buffer solution (Figure 2) and in Tris buffer (Figure 3, at the same pH) are different probably for the presence of different buffer species in two media. Drug release rate in Tris buffer is greater than that obtained in phosphate buffer reasonably for the occurring of a catalytic effect via Tris buffer species (45).

The stability of PHEA-D-Val-Leu-Lys-Cytarabine conjugate in plasma and its ability to release drug in the presence of plasmin suggest the actual ability of this conjugate to carry drug in the intact form to the action site and to release it inside tumor mass in the presence of plasmin.

To verify if also the direct linkage of Cytarabine to PHEA could give a macromolecular prodrug with some potential in anticancer therapy, although the presence of urethane linkage between carrier and drug, which is very stable in biological medium, we synthesized PHEA-Cytarabine conjugate (**9**) by the reaction of PHEA activated with PNFC directly with Cytarabine (Scheme 4), and this conjugate was submitted to the same in vitro hydrolysis experiments of **8**.

The absence of free drug was verified by HPLC analysis. The occurred linkage between Cytarabine and activated PHEA was evaluated by UV and ^1H NMR. UV spectrum shows an absorption band in the range 230–350 nm in which alone polymeric carrier does not absorb, with an absorption maximum at 292 nm indicating the presence of linked Cytarabine.

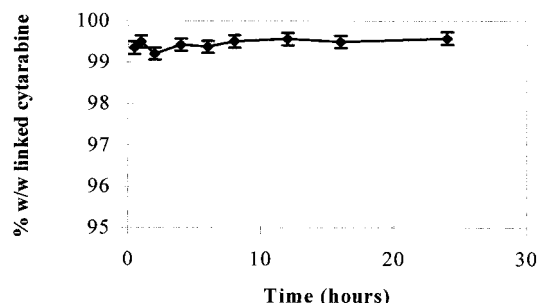
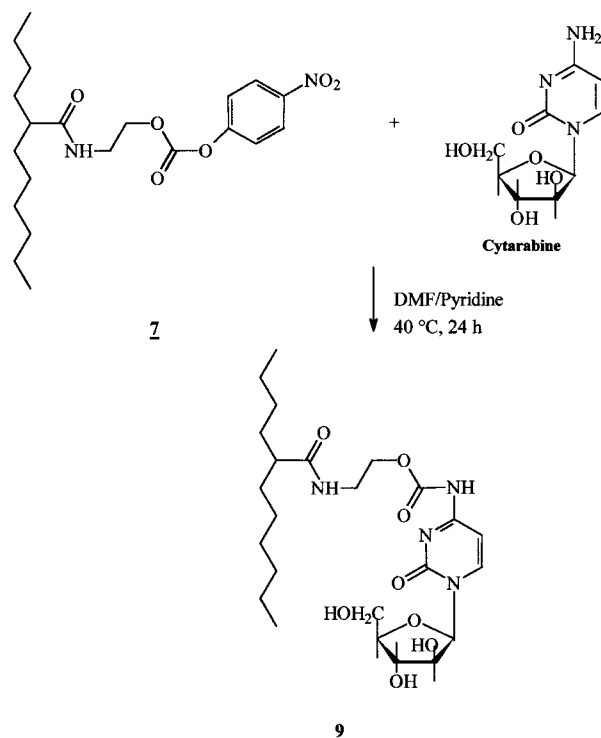


Figure 4. Stability of PHEA-Cytarabine conjugate in plasma.

Scheme 4. Schematic Representation of Coupling of Cytarabine to Activated PHEA (7)



The percentage of linked drug, evaluated by alkaline hydrolysis followed by HPLC, was estimated equal to about 4.6% (w/w) corresponding to 3.16 mol %. This result was confirmed by ^1H NMR spectroscopy. By comparing the integral peaks related to protons at δ 6.1 assignable to one proton of Cytarabine (d, 1H, C5) with the integral of a peak at δ 4.66 related to polymer (m, 1H, -NH-CH-CO-), the average value of linked drug was estimated equal to 4.6% w/w.

Therefore, the conjugate (**9**) was subjected to *in vitro* hydrolysis studies in plasma at 37 °C and obtained results were compared with that of plasmatic hydrolysis of free Cytarabine. The results of both experiments were reported in Figures 4 and 5 respectively; as it can be seen, while the concentration of free Cytarabine quickly decreases probably for degradation phenomena in plasma by cytosine deaminase (35) (Figure 5), PHEA-Cytarabine conjugate was quite stable and no cytarabine release was shown within 24 h from this conjugate (Figure 4). These results confirm the high hydrolytic resistance of urethane bounds in physiological environment.

After 24 h from the beginning of plasma treatment the addition of strong bases causes the complete release of Cytarabine in the intact form, evidencing a remarkable effect of protection on Cytarabine through linking to polymer.

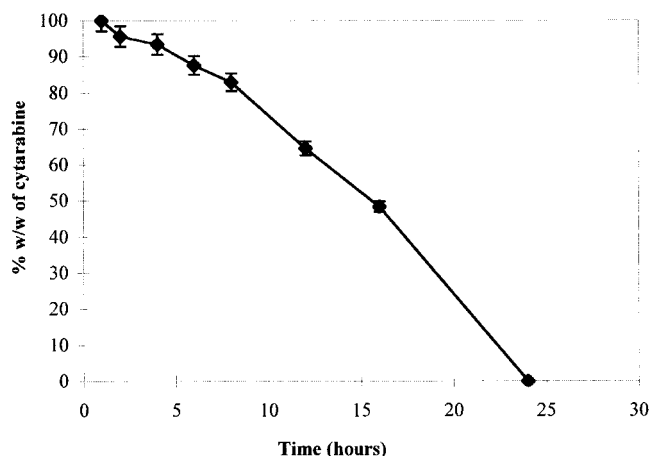


Figure 5. Stability of Cytarabine in plasma.

This result, once again, suggests the strong protective effect of PHEA toward Cytarabine toward enzymatic inactivation but, of course, since the drug is not released from this conjugate, more studies should be necessary to evaluate the antitumor activity of Cytarabine linked to PHEA.

CONCLUSIONS

The macromolecular prodrug obtained linking Cytarabine molecules to PHEA via the D-Val-Leu-Lys spacer showed some interesting peculiarities which make it attractive in the drug delivery for anticancer therapy. It was very stable in plasma, but it was able to release drug in the presence of plasmin. The excellent result confirms the rational of our work. In fact, by the proper design of the spacer arm to be used between drug molecules and macromolecular carrier, it seems possible actually to achieve a specific and targeted drug release at the action site in the antitumor chemotherapy. Besides the Cytarabine in the linked form was protected from the deamination process by cytosine deaminase enzyme and this should allow *in vivo* a decrease of drug dose so decreasing also side effects.

The macromolecular conjugate obtained directly linking Cytarabine molecules to PHEA was very stable in plasma, but it was not able to release drug in the presence of plasmin.

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Biotinylated Indoles as Probes for Indole-Binding Proteins

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Biotinylated indoles were prepared for application as bifunctional probes for the detection of indole-binding proteins which participate in the life processes of humans, animals, plants, and bacteria. The indole nucleus was functionalized, at ring positions 3, 5, or 6, by attachment of a 2-aminoethyl group, which was then coupled to the carboxyl moiety of biotin, via a spacer composed of 3 or 4 concatenated β -alanine residues. The constructs thus obtained were able to inhibit tryptophanase activity, similarly to indole in a concentration-dependent manner. They also bound strongly to lysozyme and weakly to bovine and human serum albumins, in accordance with the known affinities of these proteins for indole and 3-(2-aminoethyl)indole (tryptamine). The biotin end of the protein-bound bifunctional probes could then be detected by coupling to (strept)avidin conjugated to alkaline phosphatase or horseradish peroxidase, followed by incubation with substrates which are converted by these enzymes to intensely colored or chemiluminescent products.

INTRODUCTION

In bacteria, indole is an intermediate in the biogenesis and metabolism of tryptophan. Humans and vertebrates cannot synthesize tryptophan and also circumvent indole in its catabolism. Nevertheless, they have to cope with the indole produced by intestinal microorganisms and thus evolved special detoxification mechanisms resulting in products which are excreted via the urinary tract. Still, the main metabolite, indoxyl sulfate, appears to retain sufficient toxicity to contribute significantly to the progress of kidney disease (1). In plants, indole is formed by the α -subunit of tryptophan synthase to be immediately condensed with serine by the β -subunit. Under certain conditions, the indole appears to escape from being incorporated into tryptophan. Moreover, maize encodes a tryptophan synthase α that appears to synthesize indole independently of the β -subunit. The indole produced by this "indole synthase" is utilized in the production of at least one secondary metabolite: the defense compound 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (2, 3). Specific indole oxidases (EC 1.13.11.17) have been isolated from phylogenetically remote species such as *Zea mays* (4), *Jasminum grandiflorum* (5), and *Tecoma stans* (6–8), thus suggesting widespread distribution of this group of enzymes in plants. In humans and mammals, related enzymes (also classified as EC 1.13.11.17) oxidize indolealkylamines, such as serotonin and melatonin (9, 10) and may be part of a signaling cascade (11) triggered, for example, by pathogen attack (12).

Indole is now also receiving attention as one of the possible intermediates in the biosynthesis of the endogenous plant growth regulator, indole-3-acetic acid (IAA)¹

(13, 14). According to traditional concepts, IAA is made from tryptophan (15), but at least one additional, tryptophan-independent, route is suggested by tracer studies (reviewed in refs 13 and 14). Moreover, Arabidopsis *trp2* and maize *orp* mutants defective in tryptophan synthase β subunit, contain more IAA (free and conjugated) than wild-type seedlings (16, 17). Indole accumulates in these mutants and became the suspected precursor of the IAA. Indeed, stable-isotope labeled indole is incorporated into IAA by *orp* maize (18).

Here we present biotinylated indoles **3A–D** (Figure 1) as tools for the detection, isolation, and intracellular localization of the above enzymes, provided the metabolic processing of the enzyme-bound indole moiety can be prevented by withholding necessary cosubstrate(s), or by appropriate inhibitors. Compounds **3A–D** should also label proteins which, like serum albumin, bind indole without metabolizing it (19, 20), as well as enzymes which bind indole as an inhibitor (e.g., ref 21). Indeed, compounds **3A**, **3C**, and **3D** inhibited the tryptophanase-catalyzed conversion of serine to pyruvic acid in a similar fashion as previously shown for indole (22). Constructs **3A–D** also bound immobilized lysozyme, one of the readily available indole-binding proteins, and a key player in the defense of the human and animal organism against pathogenic microorganisms (23) and, at least according to some authors, against neoplasia (24). One of the probes (**3A**) was also tested with immobilized bovine and human serum albumins (BSA and HSA) and showed similar moderate to weak binding as reported for indole and tryptamine (19, 20). The oligopeptide spacer and indole moieties did not interfere with the binding of the biotin moiety to avidin. The biotin moiety of the

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¹ Abbreviations: BSA, bovine serum albumin; FAB, fast-atom bombardment; HSA, human serum albumin; IAA, indole-3-acetic acid; PEG, poly(ethylene glycol); in the abbreviated names used for peptide moieties Bal stands for a β -alanine residue, Boc for *t*-butoxycarbonyl, Cbz for carbobenzoxy (= benzyloxycarbonyl), and OSu for *N*-hydroxysuccinimide ester.

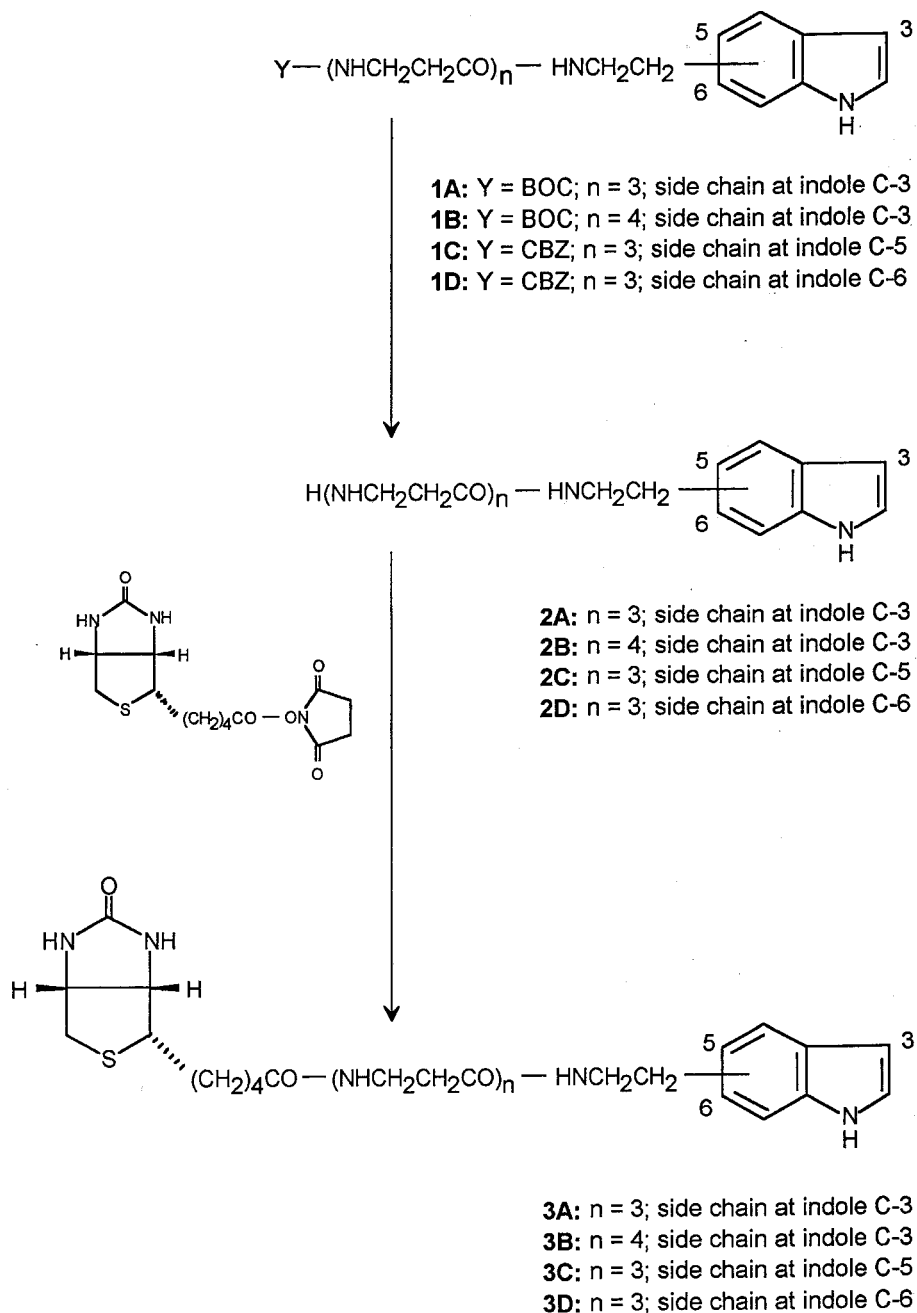


Figure 1. Final steps in the preparation of biotinylated indoles **3A–D**.

bifunctional probes was thus detected using either NeutrAvidin-linked alkaline phosphatase or Streptavidin-linked horseradish peroxidase and either chemiluminescent or chromogenic substrates.

EXPERIMENTAL PROCEDURES

General. Melting points were determined in open capillaries and were not corrected. NMR spectra were recorded (at 20 °C unless stated otherwise) on a Varian Gemini 300 spectrometer operating at 300 MHz for ^1H and at 75 MHz for ^{13}C . Chemical shifts are reported in parts per million downfield from tetramethylsilane. The signals of the biotin moiety were assigned according to Ikura and Hikichi (25). For combined high-pressure liquid chromatography–mass spectrometry with ionization by fast-atom bombardment (FAB HPLC-MS) (26), samples were passed through a 250 × 0.32 mm i.d. capillary column (custom-packed by LC Packings, Am-

sterdam, The Netherlands) with reversed-phase silica gel (Waters Symmetry C₁₈), particle size 3.5 μm, eluted (5 μL/min) with 20–80% (gradient elution) aqueous methanol containing 1% each of acetic acid and glycerol (matrix for FAB). The effluent was introduced, via a frit-FAB HPLC-MS interface, into the ion source (kept at 56 °C) of a JEOL JMS SX-102 mass spectrometer (low resolution) or a JEOL SX/SX-102A tandem instrument (high resolution). Ions were generated with a beam of 5 keV xenon atoms at an emission current of 20 mA, and positive-ion mass spectra were acquired. For exact mass measurements, poly(ethylene glycol) (PEG 300 + PEG 600) was added to the HPLC solvent (0.001%) to furnish the referent ions, and the resolution was set to $R = 5000$. Notably, the ion subjected to exact mass determination was the protonated molecular ion $[MH]^+$. Analytical HPLC was on reversed-phase (octadecylsilane, particle size of 5 μm) columns using the following protocols. For

general purity check and determination of the retention times listed below: Waters Symmetry (15 cm $\times$ 3.9 mm i.d.) using solvent mixtures composed of water/trifluoroacetic acid (99.9:0.1, solvent D), acetonitrile/trifluoroacetic acid (99.9:0.1, solvent E), water/acetic acid (99:1, solvent F), and acetonitrile/acetic acid (99:1, solvent G), i.e., protocol I, 0–5 min, 90% D + 10% E; 5–30 min, exponential gradient, 90 $\rightarrow$ 0% D, 10 $\rightarrow$ 100% E; protocol II, same but substituting solvent F for solvent D, and solvent G for solvent E. *For quantification of 3A–D in stock solutions for bioassays:* Phenomenex (Torrance, CA; 50 $\times$ 4.6 mm i.d.) using a linear gradient from 18 to 50% methanol added within 15 min to 5% aqueous acetic acid. *For the final purity check for compounds 3A–D:* Nucleosil (Macherey & Nagel; 25 cm $\times$ 4.6 mm i. d.) eluted isocratically with 50% aqueous methanol. Preparative HPLC was on a C₁₈ Radial-PAK cartridge (Millipore Waters; 10 cm $\times$ 8 mm i.d., particle size 10 μ m) eluted with 50% aqueous methanol. Flow rates were 1 mL/min (analytical) and 1.5–2 mL/min (preparative). The effluent was monitored for absorbance at 215 and 278/280 nm. Column chromatography was on silica gel 60 (Merck), particle diameter: 0.063–0.2 mm. TLC was on silica gel GF₂₅₄ (Merck) developing with dichloromethane/methanol/acetic acid (90:10:1, solvent A₁; 95:5:1, solvent A₂; 80:20:1, solvent A₃), 2-propanol/ethyl acetate/25% ammonia(aq) (35:45:20, solvent B) or 2-propanol/water/25% ammonia(aq) (8:1:1, solvent C). In addition to detection by UV absorption, the following spray reagents were used: ninhydrin (0.2% in ethanol) for free amino groups, the Ehrlich reagent (1% *p*-dimethylaminobenzaldehyde in a 1/1 mixture of ethanol and 35% HCl) for indolic compounds, and *p*-dimethylaminocinnamaldehyde (0.2% in ethanol, mixed before use with an equal volume of 2% H₂SO₄ in ethanol) for the biotin moiety (27). Chromatograms of *N*-(*t*-butyloxycarbonyl)- and *N*-(benzyloxycarbonyl)-amino acids and -peptides were first sprayed with 35% HBr in acetic acid (diluted with the 4-fold volume of ethanol), and the thus deprotected amino functions were detected with ninhydrin. Preparative TLC was on silica gel PF₂₅₄ (Merck); zones were detected by UV absorption. Of the solvents used in the synthetic procedures, *N,N*-dimethylformamide, dioxane, and tetrahydrofuran (the latter two free of peroxides) must be anhydrous; for optimal results, (dry) tetrahydrofuran was distilled over sodium immediately before use. Commercial analytical grade chemicals were used, with the exception of 5- and 6-(2-aminoethyl)indole (28), *N*-(*t*-butyloxycarbonyl)- β -alanine [Boc-Bal-OH (29, 30)], β -alanyl- β -alanine (H-Bal₂-OH) as its trifluoroacetate salt (36), as well as the *N*-hydroxysuccinimide esters of *d*-biotin (31, 32) and *N*-(*t*-butyloxycarbonyl)- β -alanine (Boc-Bal-OSu) (33) which were prepared according to published procedures. To speed up the preparation of some *N*-hydroxysuccinimide esters, the parent amino acid or peptide was reacted with an excess of *N*-hydroxysuccinimide and/or 1,3-dicyclohexylcarbodiimide (38).

Syntheses. *Cbz-Bal-OSu.* Small aliquots of 1,3-dicyclohexylcarbodiimide (495 mg, 2.4 mmol) were added to a stirred solution of commercial (Sigma) Cbz-Bal-OH (446 mg, 2 mmol) and *N*-hydroxysuccinimide (253 mg, 2.2 mmol) in tetrahydrofuran (10 mL), through 45 min, at -10°C . After a further 15 min, the temperature was raised to 0°C for 2 h, and then kept at 4°C for 48 h, with a further aliquot of 1,3-dicyclohexylcarbodiimide (495 mg, 2.4 mmol) added after 24 h. The 1,3-dicyclohexylurea formed was filtered off, and the filtrate was evaporated. The residue was dissolved in 3 mL of 2-propanol at 80°C . Cooling to -5°C afforded the title

compound (558 mg, 87%), mp $58\text{--}61^{\circ}\text{C}$ [lit. (34) $83\text{--}84^{\circ}\text{C}$]. Homogeneous by TLC, $R_f = 0.8$ (solvent A₁), 0.6 (solvent A₂), except for the, well-known, minor hydrolysis during sample application. ¹H NMR (CD₃COCD₃) δ 3.00 (4H, s), 3.03 (2H, t), 3.65 (2H, quart), 5.20 (2H, s), 6.66 (1H, broad s), 7.48 (5H, m). ¹³C NMR (CD₃COCD₃) δ 25.6, 31.5, 36.7, 66.1, 128.2, 128.7, 137.8, 156.7, 167.8, 170.1.

Boc-Bal₂-OH. To an efficiently stirred solution of β -alanine (544 mg, 6.10 mmol) in 5% NaHCO₃(aq) (20 mL) was added dropwise a solution of Boc-Bal-OSu (1734 mg, 6.06 mmol) in dioxane (12 mL) through 5 min, at room temperature. When the reaction was complete (ca. 1 h), the mixture was diluted with an equal volume of water, saturated with NaCl, acidified to pH 3.0 by adding crystalline citric acid, and partitioned against ethyl acetate. The extract was washed with brine, dried (Na₂SO₄), and evaporated. The crude product (1476 mg) was dissolved in boiling ethyl acetate and crystallized by adding 30–40% (by volume) of heptane to yield pure Boc-Bal₂-OH (35) (1344 mg, 85%), mp $124\text{--}127^{\circ}\text{C}$, homogeneous by TLC, $R_f = 0.3$ (solvent A₁). Purity by HPLC (protocol I; retention time, 25.3 min) 100.0% (215 nm). FAB HPLC-MS m/z 261.1442 ([MH]⁺). C₁₁H₂₁N₂O₅ requires 261.1450. ¹H NMR [(CD₃)₂SO] δ 1.36 (9H, s), 2.20 (2H, t), 2.36 (2H, t), 3.10 (2H, quart), 3.22 (2H, quart), 6.72 (1H, t), 7.94 (1H, t). ¹³C NMR [(CD₃)₂SO] δ 28.4, 34.0, 34.9, 35.9, 36.9, 77.9, 155.9, 170.9, 173.4.

Boc-Bal₂-OSu. Boc-Bal₂-OH (1344 mg, 5.16 mmol), *N*-hydroxysuccinimide (653 mg, 5.68 mmol), and 1,3-dicyclohexylcarbodiimide (1277 mg, 6.19 mmol) in a mixture of dioxane (91 mL) and ethyl acetate (82 mL) were processed in essentially the same way as described for Cbz-Bal-OSu, except that the total amount of 1,3-dicyclohexylcarbodiimide was added within 45 min. The crude product was dissolved in a minimal volume of boiling tetrahydrofuran. An unidentified precipitate which formed on cooling was discarded. Addition of a 2-fold volume of heptane afforded the crystalline title compound (969 mg, 53%), mp $124\text{--}127^{\circ}\text{C}$, homogeneous by TLC, $R_f = 0.6$ (solvent A₁), except as detailed for Cbz-Bal-OSu. FAB HPLC-MS m/z 358.1620 ([MH]⁺). C₁₅H₂₄N₃O₇ requires 358.1614. ¹H NMR [(CD₃)₂SO] δ 1.38 (9H, s), 2.22 (2H, t), 2.82 (4H, s), 2.85 (2H, t), 3.12 (2H, quart), 3.35 (2H, quart), 6.73 (1H, t), 8.10 (1H, t). ¹³C NMR [(CD₃)₂SO] δ 25.5, 28.3, 30.6, 34.2, 35.8, 36.7, 77.7, 155.7, 167.7, 170.4, 170.9.

Boc-Bal₃-OH. A solution of Boc-Bal-OSu (1430 mg, 5 mmol) in dioxane (8 mL) was added dropwise, at room temperature, into a vigorously stirred solution of H-Bal₂-OH trifluoroacetate (1370 mg, 5 mmol) in 8% NaHCO₃(aq) (25 mL). After a reaction time of 1 h, and workup as for Boc-Bal₂-OH, recrystallization from ethyl acetate (250 mL) afforded the title compound (1200 mg, 73%), mp $135\text{--}137^{\circ}\text{C}$, homogeneous by TLC, $R_f = 0.2$ (solvent A₁), 0.3 (solvent B). Purity by HPLC (protocol I; retention time, 24.1 min) 97.4% (215 nm). FAB HPLC-MS m/z 332.1793 ([MH]⁺). C₁₄H₂₆N₃O₆ requires 332.1822. ¹H NMR [(CD₃)₂SO] δ 1.37 (s, 9H), 2.19 (t, 2H), 2.20 (t, 2H), 2.37 (t, 2H), 3.10 (q, 2H), 3.21 (q, 2H), 3.23 (q, 2H), 6.74 (t, 1H), 7.88 (t, 1H), 7.96 (t, 1H). ¹³C NMR [(CD₃)₂SO] δ 28.4, 34.0, 34.8, 35.4, 35.5, 35.9, 36.8, 77.9, 155.9, 170.8, 170.9, 173.3.

Cbz-Bal₃-OH. Crude H-Bal₂-OH trifluoroacetate, prepared by deprotection of Boc-Bal₂-OH (234 mg, 0.90 mmol) as described (36), was dissolved in 10% aqueous NaHCO₃ (2 mL) at room temperature, and a solution of Cbz-Bal-OSu (288 mg, 0.90 mmol) in dioxane (1.5 mL) was added dropwise, with efficient stirring, through 15 min. After a reaction time of 2 h, the slightly turbid

solution was filtered, acidified with crystalline citric acid to pH 3.0, and left overnight at +4 °C. The precipitate was collected by centrifugation, washed with small aliquots of cold water and recrystallized from water to yield the title compound (173 mg, 53%), mp 185–187 °C [lit. (37) 194–195 °C], R_f = 0.1 (solvent A₁), 0.3 (solvent B). ¹H NMR [(CD₃)₂SO] δ 2.21 (2H, t), 2.23 (2H, t), 2.37 (2H, t), 3.21 (6H, m), 5.01 (2H, s), 7.23 (1H, t), 7.35 (5 H, m), 7.90 (1H, t), 7.95 (1H, t). ¹³C NMR [(CD₃)₂SO] δ 34.0, 34.8, 35.39, 35.42, 35.8, 37.2, 65.4, 128.0, 128.6, 137.5, 156.3, 170.5, 170.8, 173.3.

Boc-Bal₄-OH. Boc-Bal₂-OSu (619 mg, 1.73 mmol) in methanol (4.5 mL) was added dropwise into a stirred solution of H-Bal₂-OH trifluoroacetate (517 mg, 1.89 mmol) in 5% aqueous NaHCO₃ (7 mL). Stirring was continued overnight maintaining a pH range of 7–8 by repeated addition of small aliquots of solid NaHCO₃. The mixture was centrifuged; the supernatant was acidified to pH 3.0 using crystalline citric acid, and extracted with *n*-butanol (3 × 25 mL). The combined *n*-butanol extracts were washed with small aliquots of water and evaporated. The residue was dissolved in methanol (5 mL). After filtration, precipitation with ethyl acetate gave the crude title compound. Purification by preparative TLC (solvent B) afforded Boc-Bal₄-OH (310 mg, 45%) as a white solid. The latter decomposed on heating (no sharp mp). Homogeneous by TLC, R_f = 0.1 (solvent A₁), 0.3 (solvent B). Purity by HPLC (protocol I; retention time, 24.0 min) 97.9% (215 nm). FAB HPLC-MS m/z 403.2145 ([MH]⁺). C₁₇H₃₁N₄O₇ requires 403.2193. ¹H NMR [(CD₃)₂SO] δ 1.37 (9 H, s), 2.22 (6H, t), 2.32 (2H, t), 3.10 (2H, quart), 3.21 (4H, m), 3.23 (2H, m), 6.74 (1H, t), 7.91 (2H, t), 7.93 (1H, t). ¹³C NMR [(CD₃)₂SO] δ 28.3, 34.0, 34.9, 35.4, 35.9, 36.9, 77.8, 155.8, 170.7 (2C), 170.8, 173.3.

Boc-Bal₃-OSu. To a solution of Boc-Bal₃-OH (702 mg, 2.1 mmol), *N*-hydroxysuccinimide (496 mg, 4.3 mmol), and *N*-methylmorpholine (234 μL, 2.1 mmol) (38) in *N,N*-dimethylformamide (6 mL), stirred at 4 °C, 1,3-dicyclohexylcarbodiimide (989 mg, 4.8 mmol) was added, in small aliquots, i.e., 1.6 mmol/day. When the reaction was complete (3 days), the 1,3-dicyclohexylurea formed was filtered off and the filtrate was evaporated to dryness. The residue was recrystallized from ethyl acetate to yield the title compound (646 mg, 71%), mp 169–173 °C, homogeneous by TLC, R_f = 0.5 (solvent A₁), 0.7 (solvent B), except as detailed for Cbz-Bal-OSu. FAB HPLC-MS m/z 429.1975 ([MH]⁺). C₁₈H₂₉N₄O₈ requires 429.1985. ¹H NMR [(CD₃)₂SO] δ 1.38 (9H, s), 2.20 (2H, t), 2.23 (2H, t), 2.82 (4H, s), 2.86 (2H, t), 3.11 (2H, quart), 3.23 (2H, quart), 3.35 (2H, quart), 6.73 (1H, m), 7.88 (1H, t), 8.10 (1H, t). ¹³C NMR [(CD₃)₂SO] δ 25.5, 28.3, 30.6, 34.3, 35.3, 35.4, 35.8, 36.8, 77.7, 155.8, 167.7, 170.5, 170.7, 171.0.

Boc-Bal₄-OSu. To a suspension of Boc-Bal₄-OH (264 mg, 0.66 mmol), *N*-hydroxysuccinimide (153 mg, 1.33 mmol), and *N*-methylmorpholine (146 μL, 1.32 mmol) (38) in dry *N,N*-dimethylformamide (14 mL), stirred at 4 °C, 1,3-dicyclohexylcarbodiimide was added (180 mg, 0.87 mmol within the first hour and a further 122 mg, 0.59 mmol after 24 h). After 8 days of stirring, the 1,3-dicyclohexylurea formed was collected by centrifugation. The supernatant was evaporated and the residue was extracted with diethyl ether (5 × 30 mL) followed by ethyl acetate (3 × 30 mL) leaving the title compound (264 mg, 81%) as a white solid (no definite melting point). Homogeneous by TLC, R_f = 0.3 (solvent A₁), 0.6 (solvent B), except as detailed for Cbz-Bal-OSu. FAB HPLC-MS m/z 500.2368 ([MH]⁺). C₂₁H₃₄N₅O₉ requires 500.2357. ¹H NMR [(CD₃)₂SO] δ 1.37 (9H, s), 2.17–2.23 (6H, m),

2.82 (4H, s), 2.86 (2H, t), 3.10 (2H, quart), 3.21, 3.23 (4 H, 2 overlapping quart), 3.36 (2H, quart), 6.74 (1H, m), 7.90, 7.91 (2 H, 2 overlapping t), 8.13 (1H, t). ¹³C NMR [(CD₃)₂SO] δ 25.5, 28.3, 30.6, 34.3, 35.3, 35.4, 35.8, 36.8, 77.8, 155.8, 167.8, 170.5, 170.7, 171.1.

Cbz-Bal₃-OSu. To a solution of Cbz-Bal₃-OH (279 mg, 0.76 mmol), *N*-hydroxysuccinimide (193 mg, 1.68 mmol), and *N*-methylmorpholine (92 μL, 0.84 mmol) in dry *N,N*-dimethylformamide (6 mL), stirred at –10 °C, was added 1,3-dicyclohexylcarbodiimide (189 mg, 0.91 mmol) in small aliquots, through 30 min. After a further 30 min at –10 °C, stirring was continued at 0 °C, for 2 h, and at +4 °C, for 3 days. The 1,3-dicyclohexylurea formed was filtered off, and the filtrate was evaporated in vacuo. The residue was recrystallized from ethyl acetate/2-propanol (40:1) to yield the title compound as a reddish solid (312 mg, 88%), mp 141–145 °C, homogeneous by TLC, R_f = 0.5 (solvent A₁), 0.3 (solvent A₂), except as detailed for Cbz-Bal-OSu. FAB HPLC-MS m/z 463.1858 ([MH]⁺). C₂₁H₂₇N₄O₈ requires 463.1829. ¹H NMR [(CD₃)₂SO] δ 2.23 (4H, t), 2.81 (4H, s), 2.85 (2H, t), 3.19 (2H, quart), 3.23 (2H, quart), 3.35 (2H, quart), 5.01 (2H, s), 7.23 (1H, t), 7.36 (5 H, m), 7.91 (1H, t), 8.10 (1H, t). ¹³C NMR [(CD₃)₂SO] δ 25.5, 30.6, 34.3, 35.3, 35.4, 35.7, 37.2, 65.3, 128.0, 128.6, 137.5, 156.3, 167.8, 170.5, 171.0.

Boc-Bal₃-Tryptamine (1A). A solution of Boc-Bal₃-OSu (750 mg, 1.75 mmol), tryptamine [3-(2-aminoethyl)indole; 279 mg, 1.74 mmol], and *N*-methylmorpholine (193 μL, 1.75 mmol) in methanol (20 mL) was stirred at room temperature for 60 min. The mixture was evaporated to dryness, and the residue was dissolved in *n*-butanol (140 mL). The solution was partitioned against 0.1 M sodium citrate buffer, pH 3.0 (5 × 35 mL), 5% NaHCO₃(aq) (3 × 35 mL), and water (3 × 5 mL). The organic phase was evaporated to dryness. The residue was dissolved in dichloromethane/methanol (9:1) and passed through a column (72 × 2.5 cm) of silica gel (Merck), particle size 0.063–0.2 mm to yield tryptamine conjugate **1A** (624 mg, 76%), mp 176–178 °C, homogeneous by TLC, R_f = 0.5 (solvent A₁), 0.8 (solvent B). Purity by HPLC (protocol II; retention time, 25.6 min) 98.3% (280 nm). FAB HPLC-MS m/z 474.2679 ([MH]⁺). C₂₄H₃₆N₅O₅ requires 474.2716. ¹H NMR [(CD₃)₂SO] δ 1.37 (9H, s), 2.20 (2H, t), 2.21 (2H, t), 2.24 (2H, t), 2.82 (2H, t), 3.11 (2H, quart), 3.24 (2H, quart), 3.27 (2H, quart), 3.33 (2H, quart), 10.82 (1H, s), 6.75 (1H, t), 6.98 (1H, dd), 7.07 (1H, dd), 7.15 (1H, s), 7.34 (1H, d), 7.53 (1H, d), 7.90 (1H, t), 7.94 (1H, t), 8.01 (1H, t). ¹³C NMR [(CD₃)₂SO] δ 25.3, 28.3, 35.4, 35.5, 35.6, 35.9, 36.9, 39.6, 77.8, 111.6, 112.1, 118.5, 121.1, 122.9, 127.5, 136.5, 155.8, 170.6, 170.7.

Boc-Bal₄-Tryptamine (1B). To a solution of tryptamine (103 mg, 0.64 mmol) in methanol (1 mL), stirred at room temperature, a solution of Boc-Bal₄-OSu (320 mg, 0.64 mmol) in methanol (15 mL) was added dropwise, through 5 min, followed by *N*-methylmorpholine (70 μL, 0.64 mmol), and stirring was continued for 3.5 h. After solvent partition as described for compound **1A**, the crude product was dissolved in dichloromethane/methanol (1:1; 30 mL); silica gel for column chromatography (5 g) was added and the solvent was evaporated. The dried residue, suspended in dichloromethane/methanol (20:1), was placed on top of a column (64 × 2.5 cm) of silica gel (140 g) packed in the same solvent. Elution with 3 L of dichloromethane/methanol, changing the solvent ratio stepwise from 20:1 to 3:1, afforded the title compound (199 mg, 57%) as a white solid which decomposed on heating before melting. Homogeneous by TLC, R_f = 0.3 (solvent A₁), 0.5 (dichloromethane/methanol, 5:1), 0.8 (solvent B). Purity by HPLC (protocol II; retention time, 25.0 min)

99.8% (280 nm). FAB HPLC-MS m/z 545.3100 ($[MH]^+$). $C_{27}H_{41}N_6O_6$ requires 545.3088. ^1H NMR $[(CD_3)_2SO]$ δ 1.36 (9H, s), 2.17–2.23 (6H, m), 2.24 (2H, t), 2.81 (2H, t), 3.10 (2H, quart), 3.17–3.28 (6H, m), 3.32 (2H, quart), 6.75 (1H, t), 6.97 (1H, ddd), 7.06 (1H, ddd), 7.15 (1H, d), 7.34 (1H, broad d), 7.53 (1H, broad d), 7.92 (1H, t), 7.94 (1H, t), 7.97 (1H, t), 8.05 (1H, t), 10.85 (1H, broad s). ^{13}C NMR $[(CD_3)_2SO]$ δ 25.3, 28.3, 35.4, 35.5, 35.8, 36.8, 39.6, 77.8, 111.6, 112.1, 118.4, 121.1, 122.9, 127.5, 136.5, 155.8, 170.6, 170.7.

Cbz-Bal₃-5-(2-Aminoethyl)indole (1C). A solution of 5-(2-aminoethyl)indole (121 mg, 757 μ mol), Cbz-Bal₃-OSu (350 mg, 757 μ mol), and *N*-methylmorpholine (83 μ L, 757 μ mol) in dry *N,N*-dimethylformamide (7.5 mL) was stirred at room temperature for 2 h. After evaporation, the residue was extracted with ethyl acetate (8 $\times$ 10 mL) to remove *N*-hydroxysuccinimide. The remaining solid (375 mg) was dissolved in dichloromethane/methanol (2:1, 45 mL) and mixed with silica gel (5 g) which was, after evaporation of the solvent and drying, layered on top of a column (64 $\times$ 2.5 cm) of silica gel (140 g) packed in dichloromethane/methanol (20:1). Elution with 2 L of dichloromethane/methanol, changing the solvent ratio stepwise from 20:1 to 4:1, afforded the title compound as a white solid (250 mg, 65%), mp 202–205 °C, R_f = 0.4 (solvent A₁), 0.8 (solvent B). Purity by HPLC (protocol II; retention time, 25.8 min) 96.3% (280 nm). Instead of the chromatographic purification, impurities in the crude product could also be extracted with methanol (4 $\times$ 5 mL) and the small amount of coextracted **1C** recovered by preparative TLC (dichloromethane/methanol, 4:1), to yield a product with a mp of 195 $\pm$ 3 °C, pure enough for use in the subsequent syntheses. FAB HPLC-MS m/z 508.2563 ($[MH]^+$). $C_{27}H_{34}N_5O_5$ requires 508.2559. ^1H NMR $[(CD_3)_2SO]$ δ 2.22 (6H, m), 2.75 (2H, t), 3.25 (8H, m), 5.00 (2H, s), 6.35 (1H, broad s), 6.92 (1H, d), 7.24 (1H, t), 7.28–7.35 (8H, m), 7.92 (3H, m), 10.98 (1H, s). ^{13}C NMR $[(CD_3)_2SO]$ δ 35.4, 35.5, 35.6, 35.8, 37.3, 41.1, 65.3, 100.9, 111.4, 119.7, 122.3, 125.6, 128.0, 128.1, 128.6, 129.8, 134.9, 137.5, 156.3, 170.52, 170.46, 170.6.

Cbz-Bal₃-6-(2-Aminoethyl)indole (1D). Cbz-Bal₃-OSu (362 mg, 780 μ mol), 6-(2-aminoethyl)indole (126 mg, 780 μ mol), and *N*-methylmorpholine (86 μ L, 780 μ mol) in dry *N,N*-dimethylformamide (6 mL) were reacted in the same way as described for compound **1C**. The crude product was extracted with ethyl acetate and methanol to yield the title compound (289 mg) as a white solid, mp 234–236 °C, R_f = 0.4 (solvent A₁), 0.8 (solvent B). Purity by HPLC (protocol II; retention time, 25.7 min) 96.3% (280 nm). A further 15 mg was recovered from the methanol extracts using preparative TLC. Overall yield, 304 mg (77%). FAB HPLC-MS m/z 508.2592 ($[MH]^+$). $C_{27}H_{34}N_5O_5$ requires 508.2559. ^1H NMR $[(CD_3)_2SO]$ δ 2.24 (6H, m), 2.78 (2H, t), 3.23 (6H, m), 3.27 (2H, t), 5.01 (2H, s), 6.37 (1H, broadened s), 6.87 (1H, dd), 7.20 (1H, broadened s), 7.25 (1H, t), 7.27 (1H, m), 7.35 (5 H, m), 7.45 (1H, d), 7.92 (2H, t), 7.98 (1H, t), 10.97 (1H, s). ^{13}C NMR $[(CD_3)_2SO]$ δ 35.4, 35.5, 35.8, 37.3, 41.1, 65.3, 101.0, 111.3, 120.1, 120.4, 125.1, 126.3, 128.0, 128.6, 132.4, 136.5, 137.5, 156.3, 170.5, 170.6, 170.7.

H-Bal₃-Tryptamine (2A). Compound **1A** (320 mg, 0.68 mmol) was *N*-deprotected using a mixture of 90% aqueous trifluoroacetic acid (1.2 mL) and anisole (1.2 mL) (39, 40). Precipitation with diethyl ether afforded the title compound as the trifluoroacetate salt. Further purification was by preparative TLC (solvent C). The zone containing peptide conjugate **2A** (as the free base due to the ammonia content of the solvent system used) was collected and extracted with methanol/25% aq. ammonia

(10:1; the addition of ammonia is essential). The extract was evaporated to dryness. The residue was dissolved in methanol/aqueous ammonia as above and filtered to remove contaminant silica gel. Evaporation of the combined filtrates afforded 194 mg (76%) of the pure title compound, mp 178–181 °C. Homogeneous by TLC, R_f = 0.0 (solvent A₁), 0.5 (solvent B), 0.6 (solvent C). Purity by HPLC (protocol II; retention time, 24.3 min) 99.3% (280 nm)/98.5% (215 nm). FAB HPLC-MS m/z 374.2190 ($[MH]^+$). $C_{19}H_{28}N_5O_3$ requires 374.2192. ^1H NMR (D_2O) δ 2.11 (2H, t), 2.21 (2H, t), 2.25 (2H, t), 2.78 (2H, t), 2.86 (2H, t), 3.18 (2H, t), 3.19 (2H, t), 3.41 (2H, t), 7.04 (1H, dd), 7.11 (1H, s), 7.13 (1H, dd), 7.38 (1H, d), 7.55 (1H, d). ^{13}C NMR (D_2O) δ 24.9, 35.9, 36.1, 36.6, 36.7, 37.3, 40.7, 112.7, 112.8, 119.4, 120.0, 122.7, 124.2, 128.0, 137.2, 174.4, 174.6, 174.8.

H-Bal₄-Tryptamine (2B). Compound **1B** (97 mg, 0.18 mmol) was deprotected in the same way as its analogue **1A** to yield the title compound as a white solid (65 mg, 82%) homogeneous by TLC, R_f = 0.0 (solvent A₁), 0.5 (solvent B), 0.6 (solvent C), mp 215–219 °C. Purity by HPLC (protocol II; retention time, 24.4 min) 100.0% (280 nm)/100% (215 nm). FAB HPLC-MS m/z 445.2560 ($[MH]^+$). $C_{22}H_{33}N_6O_4$ requires 445.2563. ^1H NMR (D_2O , 30 °C) δ 2.09 (2H, t), 2.19 (2H, t), 2.23 (2H, t), 2.31 (2H, t), 2.83 (2H, t), 2.87 (2H, broad s), 3.15 (2H, t), 3.18 (2H, t), 3.25 (2H, t), 3.37 (2H, t), 7.01 (1H, dd), 7.08 (1H, s), 7.10 (1H, dd), 7.36 (1H, d), 7.52 (1H, d). ^{13}C NMR (D_2O , 30 °C) δ 24.9, 35.9, 36.0, 36.7, 40.7, 112.7, 112.8, 119.4, 120.0, 122.7, 124.2, 128.0, 137.2, 174.7, 174.6.

H-Bal₃-5-(2-Aminoethyl)indole (2C). Compound **1C** (123 mg, 243 μ mol) was suspended in a mixture of methanol (32 mL) and water (14 mL) and, after addition of 10% palladium-on-charcoal (139 mg) hydrogenated at room temperature and atmospheric pressure, for 1 h. The catalyst was collected by centrifugation and exhaustively rinsed with methanol/water (2:1). The combined supernatant and rinsings were evaporated and the residue was purified as described for compound **2A**, to yield **2C** (63 mg, 69%) homogeneous by TLC, R_f = 0.0 (solvent A₁), 0.5 (solvent B). Purity by HPLC (protocol II; retention time, 23.7 min) 100.0% (280 nm)/98.6% (215 nm). The compound decomposed before melting. FAB HPLC-MS m/z 374.2191 ($[MH]^+$). $C_{19}H_{28}N_5O_3$ requires 374.2192. ^1H NMR (CD_3OD) δ 2.38 (2H, t), 2.43 (2H, t), 2.44 (2H, t), 2.95 (2H, t), 3.00 (2H, t), 3.47 (4H, t), 3.53 (2H, t), 6.46 (1H, d), 7.07 (1H, dd), 7.28 (1H, d), 7.40 (1H, d), 7.47 (1H, s). ^{13}C NMR (CD_3OD) δ 37.0, 37.3, 37.5, 38.7, 39.0, 43.1, 102.4, 112.5, 121.4, 123.8, 126.3, 131.1, 131.2, 137.0, 174.2, 174.3, 174.6.

H-Bal₃-6-(2-Aminoethyl)indole (2D). A suspension of *N*-protected peptide conjugate **1D** (110 mg, 218 μ mol) in a mixture of methanol (30 mL) and water (13 mL) containing 10% palladium-on-charcoal (112 mg) was hydrogenated, and the product worked up as described for compound **2C** to yield 44 mg (54%) of the title compound homogeneous by TLC, R_f = 0.0 (solvent A₁), 0.5 (solvent B), 0.6 (solvent C). Purity by HPLC (protocol II; retention time, 26.1 min) 100.0% (280 nm)/97.8% (215 nm). The compound decomposed before melting. FAB HPLC-MS m/z 374.2177 ($[MH]^+$). $C_{19}H_{28}N_5O_3$ requires 374.2192. ^1H NMR (D_2O) δ 2.02 (2H, t), 2.18 (2H, t), 2.27 (2H, t), 2.77 (2H, t), 2.81 (2H, t), 3.13 (2H, t), 3.16 (2H, t), 3.36 (2H, t), 6.39 (1H, d), 6.87 (1H, d), 7.20 (1H, d), 7.24 (1H, s), 7.48 (1H, d). ^{13}C NMR (D_2O) δ 35.3, 35.8, 36.0, 36.6, 36.8, 37.1, 41.6, 101.9, 112.3, 121.3, 121.5, 126.5, 127.1, 133.6, 137.1, 173.9, 174.7, 174.8.

d-Biotin-Bal₃-Tryptamine (3A). A suspension of peptide conjugate **2A** (78 mg, 210 μ mol), *d*-biotin *N*-hydroxysuc-

cinimide ester (88 mg, "260 μmol ", ca. 90% pure), and *N*-methylmorpholine (23 μL , 210 μmol), in *N,N*-dimethylformamide (8 mL), was stirred at room-temperature overnight. As reactant **2A** dissolved, product **3A** separated as a flaky precipitate. The reaction mixture was evaporated to dryness, and the residue was extracted with ethyl acetate (3 $\times$ 20 mL) to remove *N*-hydroxysuccinimide and left-over reactants. Crystallization of the residue from water afforded the title compound (46 mg, 37%) as white crystals, mp 213–215 $^{\circ}\text{C}$, R_f = 0.1 (solvent A₁), 0.3 (solvent A₃), 0.6 (solvent B), with a purity (HPLC, protocol II; retention time, 26.0 min) of 97.2% (280 nm)/91.0% (215 nm). Further purification by preparative HPLC afforded a purity of 100.0% (280 nm)/98.5% (215 nm). FAB HPLC-MS m/z 600.2960 ($[\text{MH}]^+$). $\text{C}_{29}\text{H}_{42}\text{N}_7\text{O}_5\text{S}$ requires 600.2968. ^1H NMR (CD_3OD) δ 1.47 (2H, m), 1.58–1.80, (4H, m), 2.26 (2H, t), 2.41 (2H, t), 2.47 (4H, t), 2.76 (1H, d), 2.98 (1H, dd), 3.04 (2H, t), 3.23 (1H, ddd), 3.47, 3.48 (6H, 2t), 3.58 (2H, t), 4.35 (1H, dd), 4.56 (1H, dd), 7.12 (1H, dd), 7.21 (1H, dd), 7.22 (1H, s), 7.47 (1H, d), 7.67 (1H, d). ^{13}C NMR (CD_3OD) δ 25.9, 26.6, 29.2, 29.5, 36.8, 37.2, 41.0, 41.4, 56.8, 61.6, 63.4, 112.9, 113.5, 119.7, 120.2, 122.9, 124.2, 128.8, 138.1, 166.5, 174.7, 174.8, 177.3.

d-Biotin-Bal₄-Tryptamine (**3B**). Peptide conjugate **2B** (79 mg, 178 μmol), *d*-biotin *N*-hydroxysuccinimide ester (67 mg, "196 μmol ", ca. 90% pure), and *N*-methylmorpholine (19.6 μL , 178 μmol) in *N,N*-dimethylformamide (18 mL) were processed as described for the synthesis of compound **3A** to yield crude **3B** (101 mg, 84%) as a white solid. For further purification, the compound was dissolved in boiling 70% aqueous methanol and precipitated by concentrating the solution to one-third its original volume. Mp 232–234 $^{\circ}\text{C}$, R_f = 0.1 (solvent A₁), 0.6 (solvent B), with a purity (HPLC, protocol I; retention time, 25.7 min) of 94.1% (280 nm)/84.3% (215 nm). Further purification by preparative HPLC afforded a purity of 100.0% (280 nm)/100.0% (215 nm). FAB HPLC-MS m/z 671.3317 ($[\text{MH}]^+$). $\text{C}_{32}\text{H}_{47}\text{N}_8\text{O}_6\text{S}$ requires 671.3339. ^1H NMR ($\text{CD}_3\text{OD}/\text{D}_2\text{O}$ $\approx$ 4:3, 45 $^{\circ}\text{C}$) δ 1.44 (2H, m), 1.53–1.80 (4H, m), 2.26 (2H, t), 2.40 (2H, t), 2.43–2.48 (6H, m), 2.79 (1H, d), 2.99 (1H, dd), 3.04 (2H, t), 3.25 (1H, m), 3.45–3.49 (8H, m), 3.58 (2H, t), 4.37 (1H, dd), 4.59 (1H, dd), 7.16 (1H, dd), 7.25 (1H, s), 7.25 (1H, dd), 7.51 (1H, d), 7.69 (1H, d). ^{13}C NMR ($\text{CD}_3\text{OD}/\text{D}_2\text{O}$ $\approx$ 4/3, 45 $^{\circ}\text{C}$) δ 25.8, 26.6, 29.2, 29.5, 36.7, 36.8, 37.2, 41.0, 41.4, 56.8, 61.6, 63.4, 112.9, 113.5, 119.7, 120.3, 123.0, 124.3, 128.8, 138.0, 166.5, 174.7, 174.9, 177.5.

d-Biotin-Bal₃-5-(2-Aminoethyl)indole (**3C**). Compound **2C** (86 mg, 230 μmol), *d*-biotin *N*-hydroxysuccinimide ester (98 mg, "287 μmol ", ca. 80% pure), and *N*-methylmorpholine (25 μL , 230 μmol) in dry *N,N*-dimethylformamide (20 mL) were processed as described for the synthesis of compound **3A**. The crude product (133 mg) was recrystallized from water (30 mL) to yield the pure title compound (76 mg) as a white solid, mp 214–215 $^{\circ}\text{C}$, R_f = 0.2 (solvent A₃), 0.6 (solvent B) with a purity (HPLC, protocol I; retention time, 26.0 min) of 100.0% (280 nm)/93.9% (215 nm). Recrystallization of the evaporated mother liquors afforded a second crop (14 mg), mp 212–213 $^{\circ}\text{C}$. Overall yield, 90 mg (66%). Further purification of the main fraction by preparative HPLC afforded a purity of 100.0% (280 nm)/98.5% (215 nm). FAB HPLC-MS m/z 600.2797 ($[\text{MH}]^+$). $\text{C}_{29}\text{H}_{42}\text{N}_7\text{O}_5\text{S}$ requires 600.2968. ^1H NMR (CD_3OD) δ 1.50 (2H, m), $\sim$ 1.7 (4H, m), 2.26 (2H, t), 2.38 (2H, t), 2.44 (2H, t), 2.45 (2H, t), 2.77 (1H, d), 2.95 (2H, t), 2.98 (1H, dd), 3.25 (1H, m), 3.47 (2H, t), 3.51

(4H, t), 3.54 (2H, t), 4.34 (1H, dd), 4.54 (1H, dd), 6.46 (1H, d), 7.07 (1H, d), 7.29 (1H, d), 7.40 (1H, d), 7.48 (1H, s).

d-Biotin-Bal₃-6-(2-Aminoethyl)indole (**3D**). Peptide conjugate **2D** (86 mg, 230 μmol), *d*-biotin *N*-hydroxysuccinimide ester (96 mg, "280 μmol ", ca. 80% pure), and *N*-methylmorpholine (30 μL , 272 μmol) in *N,N*-dimethylformamide (12 mL) solution were processed as described for compound **3A** to yield, after recrystallization from water, 57 mg of pure **3D** as a white solid, mp 223–225 $^{\circ}\text{C}$, R_f = 0.1 (solvent A₁), 0.6 (solvent B) with a purity (HPLC, protocol II; retention time, 26.0 min) of 97.0% (280 nm)/94.1% (215 nm). A further 17 mg, mp 216–220 $^{\circ}\text{C}$, was recovered by recrystallization of the evaporated mother liquors. Overall yield, 74 mg (54%). Further purification of the main fraction by preparative HPLC afforded a purity of 99.6% (280 nm)/98.9% (215 nm). FAB HPLC-MS m/z 600.2958 ($[\text{MH}]^+$). $\text{C}_{29}\text{H}_{42}\text{N}_7\text{O}_5\text{S}$ requires 600.2968. ^1H NMR (CD_3OD) δ 1.48 (2H, m), 1.58–1.84 (4H, m), 2.26 (2H, t), 2.38 (2H, t), 2.44, (2H, t), 2.45 (2H, t), 2.77 (1H, d), 2.97 (2H, t), 2.98 (1H, dd), 3.23 (1H, ddd), 3.47 (4H, t), 3.50 (2H, t), 3.54 (2H, t), 4.34 (1H, dd), 4.54 (1H, dd), 6.47 (1H, d), 6.98 (1H, d), 7.26 (1H, d), 7.32 (1H, s), 7.55 (1H, d).

Binding Assays. Materials. Tryptamine, *d*-biotin, lysozyme (EC 3.2.1.17; lyophilized, from chicken egg white), bovine serum albumin (BSA; 1 $\times$ crystallized and fraction 5 powder), human serum albumin (HSA; 99%, essentially globulin free, approximately 0.005% fatty acids), tryptophanase (EC 4.1.99.1), pyridoxal 5-phosphate, L-serine, β -nicotinamide adenine dinucleotide, reduced form (β -NADH), and polyoxyethylenesorbitan monolaurate (Tween 20) were obtained from Sigma Chemicals (St. Louis MO). Lactate dehydrogenase (EC 1.1.1.27) was obtained from Sigma Chemicals and Worthington Biochemical Corporation (Freehold, NJ). Methanol (HPLC grade) and methyl sulfoxide were obtained from VWR Scientific Products (Bridgeport, NJ). Alkaline phosphatase-conjugated NeutrAvidin (average of 3 alkaline phosphatases/NeutrAvidin), disuccinimidyl suberate, *p*-nitrophenyl phosphate, Reacti-Bind biotin coated microtiter plates and SuperBlock blocking buffer were from Pierce Chemicals (Rockford IL). CovaLink Immuno Module plates were from Nalge Nunc International Corp. (Naperville, IL). Hybond-C nitrocellulose membrane, ECL chemiluminescent detection reagents, streptavidin-horseradish peroxidase conjugate (average of 3–4 peroxidases/streptavidin) and disodium 2-chloro-5-(4-methoxy-*spiro*-[1,2-dioxetane-3,2'-(5'-chloro)-dicyclo[3,3,1,^{3,7}]decan]-4-yl)phenyl phosphate; CDP-star were from Amersham Pharmacia Biotech (Arlington Heights, IL). Biomax X-ray film was from Eastman Kodak Corp. (Rochester, NY). Biotin-free casein was obtained from Schleicher and Schuell (Keene, NH). The binding assays were performed using buffers A (20 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20), B_{7.4} (50 mM Tris/HCl, pH 7.4, 100 mM NaCl), and B_{8.0} (same components as in B_{7.4}, but pH 8.0).

The bifunctional probes **3A–D** were dissolved in methanol/water (2:1) and the respective concentrations determined by comparing the UV absorbance (278 nm) from the HPLC elution profile (see General section for

details) to the UV absorbance profile of known amounts of tryptamine.²

Tryptophanase Assays. Tryptophanase activity was assayed by measuring the velocity of pyruvate formation from serine in a coupled reaction with β -NADH and lactate dehydrogenase, essentially as described by Morino and Snell (22), but using a smaller reaction volume (300 μ L) and 5 units of lactate dehydrogenase/sample. The stock solutions (31 μ M) of compounds **3A**, **3C**, and **3D** contained 66% methanol. Thus methanol was added to the corresponding controls to compensate for its effect on enzyme activity. Tryptophanase (0.3 units, 30 μ L of stock solution) was added to start the reaction and a 100 μ L aliquot of the reaction mixture was monitored for a ten-minute period to follow the decrease of absorbance at 340 nm. The reaction proceeded linearly with time. Assays were performed in triplicate.

Avidin Binding Assays. All procedures were carried out at room temperature unless otherwise indicated. Biotin and **3A–D** were diluted to varying concentrations (10 pM to 30 μ M) in buffer A, mixed with 276 pM alkaline phosphatase-conjugated NeutrAvidin and allowed to equilibrate for 30 min. These mixtures were added to the wells of Reacti-Bind biotin-coated microwell plates and incubated with gentle shaking (75 rpm on a rotary shaker). After 30 min the wells were emptied and washed with buffer A ($3 \times 200 \mu$ L). Phosphatase substrate buffer (1.9 mM *p*-nitrophenyl phosphate, disodium salt in diethanolamine, pH 9.8) was made fresh according to the manufacturer's instructions and 100 μ L were added to the wells. After 30 min the reaction was stopped with 50 μ L of 2 M NaOH and the absorbance at 405 nm was measured. Assays were performed in triplicate.

Covalent Attachment of Lysozyme, BSA, and HSA to Microwell Plates and Assay for Biotin Conjugate Binding. CovaLink Immuno Module plates were treated with 100 μ L of disuccinimidyl suberate (339 μ M disuccinimidyl suberate in 50% methyl sulfoxide, 7.5 mM Na₂CO₃, 17.5 mM NaHCO₃, pH 9.6) for 1 h. The wells were emptied and washed with distilled water ($3 \times 100 \mu$ L). Lysozyme was made 40 μ g/mL in carbonate buffer, pH 9.6 (15 mM Na₂CO₃, 35 mM NaHCO₃), 100 μ L were added to the wells of the treated CovaLink plates and left overnight. BSA and HSA (80 μ g/mL in carbonate buffer) were covalently attached to the CovaLink plates in a similar manner. The wells were emptied and washed twice with 200 μ L of SuperBlock blocking buffer (SuperBlock reagent in buffer B_{7.4}). A fresh 200 μ L aliquot of blocking buffer was added to the wells and left for 15 min. The wells were emptied and allowed to dry for 2 h, then stored desiccated at +4 °C until further use.

The lysozyme-coated wells were washed three times with buffer A and 100 μ L of varying concentrations (1 μ M to 1 mM) of compounds **3A–D** (diluted in buffer A) were added. The plates were incubated for 1 h with gentle shaking, and then washed three times with buffer A. Next the wells were incubated with 100 μ L of alkaline phosphatase conjugated-NeutrAvidin solution (552 pM in buffer A) for 1 h with gentle shaking. The wells were emptied and washed with buffer A ($3 \times 200 \mu$ L). Substrate was added as above, the reaction stopped after 24 min and absorbance at 405 nm was measured. Assays

were performed in triplicate. The BSA- and HSA-coated wells were treated with SuperBlock as above, washed three times with buffer B_{8.0}, and 100 μ L of compound **3A** diluted to a range of concentrations (buffer B_{8.0}) were added. All subsequent steps were the same as for the lysozyme-coated wells except that buffer B_{8.0} was used in place of buffer A and all incubations were conducted at 37 °C.

Binding of Biotinylated Indoles to Proteins Immobilized on Nitrocellulose Membranes. Lysozyme (lyophilized, 95% protein), BSA (fraction 5 and 1 $\times$ crystallized), and tryptophanase were made 10 mg/mL in buffer A and 5–20 μ g were spotted on to a 0.45 μ m Hybond-C nitrocellulose membrane and allowed to air-dry. Four replicate membranes were generated in this manner. The membranes were incubated in SuperBlock blocking buffer for 20 min with gentle agitation, washed three times with buffer A and then incubated with 100 μ M of **3A**, **3C**, or **3D** (diluted in buffer A). The fourth membrane was incubated in buffer A only. After a 1-h incubation with gentle agitation, the membranes were washed three times with buffer A. Alkaline phosphatase-conjugated NeutrAvidin (552 pM in buffer A) was added to each membrane and incubated for 1 h as above. The membranes were washed as above and excess buffer allowed to drain off the surface of the membranes. CDP-star chemiluminescent substrate for alkaline phosphatase was added according to the manufacturer's instructions and the membranes were exposed to X-ray film for 1 h. In a separate experiment, 5–20 μ g of BSA (fraction 5) and tryptophanase were spotted on to a nitrocellulose membrane, incubated with a biotin-free casein blocking solution (5% casein in buffer A) for 1 h, washed with buffer A and then incubated with 100 μ M **3A** for an additional hour. The membrane was washed with buffer A and then incubated with horseradish peroxidase-conjugated streptavidin (833 pM in buffer A) for 30 min. The membrane was washed with buffer A and incubated with ECL chemiluminescent substrate according to the manufacturer's instructions and exposed to X-ray film for 1 h.

RESULTS AND DISCUSSION

Design of Biotinylated Indoles. To permit the detection of biotinylated indoles by (strept)avidin-based indicator modules, it has to be taken into account that their biotin-binding sites are located in pockets which extend deeply below the surface of the protein molecule (41–43). The spacer separating the biotin moiety from the indole ring must thus be appropriate in length. In compounds **3A–D**, we use a spacer composed of two units: an aminoethyl group attached to positions 3, 5, or 6 of the indole nucleus, and a peptide containing three to four concatenated β -alanine residues. The indole is thus separated from the biotin moiety by 16–20 aliphatic bonds. These dimensions for the spacer were suggested by published work, such as the results of Green et al. (44), who coupled biotin to both ends of α,ω -diamino-*n*-alkanes and correlated the length of the alkyl chain to the capacity of the conjugates to bind simultaneously to two avidin molecules. The oligo- β -alanine we use in our spacers is more hydrophilic than the more commonly employed ϵ -aminocaproic acid-based peptides. This appeared necessary to compensate for the low water solubility of both the biotin and the indole moieties.

The protected β -alanine oligopeptides used as starting materials were constructed in a stepwise fashion, using *N*-hydroxysuccinimide esters for the extension of the

² The respective UV spectra exhibit flat-topped absorbance maxima ($\log \epsilon = 3.7$) centered at 282 ± 2 nm with negligible drop in absorbance towards 278 nm, a wavelength passed by a standard filter available for the UV monitor used in the HPLC quantification of compounds **3A–D** in stock solutions for bioassays.

peptide chain. The sequence in which the *d*-biotin and aminoethylindole headgroups were attached to the spacer peptide was of practical significance. If the biotin was introduced first, the resulting *N*-substituted peptides were insoluble in standard solvents and thus technically inconvenient for further processing to **3A–D**. Intermediates with somewhat better (but still challenging) solubility properties were obtained when the indolic components were coupled to *N*-Boc- or *N*-Cbz- β -alanine tri- or tetrapeptides to yield compounds **1A–1D** (Figure 1). The subsequent *N*-deprotection proved to be another critical step. While the Boc-groups in compounds **1A** and **1B**, with the C-terminal end of the peptide attached to 3-(2-aminoethyl)indole (tryptamine), could be removed smoothly by the customary treatment with trifluoroacetic acid, the corresponding derivatives containing 6-(2-aminoethyl) moieties afforded mostly polymeric material (details not shown), a complication apparently due to the exceptional acid sensitivity of indole derivatives with an unsubstituted 3-position (45). *N*-Cbz- β -alanine peptides **1C–D** were thus employed in the preparation of compounds **2C–D**, the deprotection being accomplished by catalytic hydrogenation. Condensation of intermediates **2A–D** with *d*-biotin *N*-hydroxysuccinimide ester completed the synthesis of the bifunctional probes **3A–D**.

The identity of the compounds prepared was confirmed by NMR and by FAB-MS including fragmentation patterns and exact masses of the pseudomolecular ($[MH]^+$) ions. Purity was checked by TLC and, as far as possible, by HPLC. TLC was preferred for *N*-hydroxysuccinimide esters which are hydrolyzed by the aqueous solvent systems required in reversed-phase HPLC (only minor decomposition is known to occur in TLC). Intermediates **1C** and **1D** could not be recrystallized and were thus used in a purity of 96.3% (**1C**) and 93.3% (**1D**). While preparative HPLC afforded compounds **3A–D** in a purity of 98.5+%, the less rigorously purified material obtained by recrystallization proved to be sufficient for the binding assays described herein.

Binding Studies. General. A surprisingly small number of the proteins known to interact with indole and its derivatives (see Introduction) have been studied in sufficient detail to be suitable for gauging the efficacy of compounds **3A–D** as probes for indole-binding proteins. Readily available representatives of this group of proteins comprise 1. the enzyme, tryptophanase, which interacts with indole in the presence of pyridoxal phosphate and aliphatic cosubstrates and 2. the serum albumins of higher vertebrates (19, 20) and lysozyme from chicken eggwhite (46), which bind indolic compounds without mediating cofactors and cosubstrates.

Interaction of Indole and Compounds 3A, 3C, and 3D with Tryptophanase. When supplied with pyridoxal phosphate, tryptophanase converts serine to pyruvic acid. This reaction is inhibited by indole, as studied in detail by Morino and Snell (22). We attempted to replicate their assay as closely as possible in order to determine if compounds **3A**, **3C**, and **3D** mimicked indole in the inhibition of tryptophanase activity. The presence of methanol in the reaction mixtures (because stock solutions of compounds **3A**, **3C**, and **3D** were in 66% methanol) complicated enzyme kinetics and parameters such as constants of inhibition (K_i) could not be derived. However, qualitative comparison of the data presented in Table 1 clearly demonstrates inhibition of tryptophanase by biotinylated indoles. Relative to an equimolar concentration (0.031 mM) of indole, compounds **3A**, **3C**, and **3D** were more inhibitory at the lowest serine concentration (20 mM) tested, about equally inhibitory

Table 1. Inhibitory Effect of Indole and Indole-biotin Conjugates 3A, 3C, and 3D on the Velocity of Tryptophanase-Catalyzed Pyruvate Formation from Serine

serine concentration (mM)	inhibitor (0.031 mM) added	initial velocity (pmol/min)
20	none	57
	indole	36
	3A	25
	3C	16
	3D	11
60	none	63
	indole	39
	3A	48
	3C	47
	3D	42
100	none	93
	indole	68
	3A	72
	3C	71
	3D	79
200	none	130
	indole	82
	3A	106
	3C	117
	3D	105

at intermediate serine levels (60 and 100 mM), and somewhat less inhibitory at the highest serine concentration (200 mM) examined.

Binding of Biotinylated Indoles to Avidin. *d*-Biotin and the biotinylated indoles **3A–D** exhibited comparable binding in the avidin binding assay (Figure 2). A constant amount of alkaline phosphatase conjugated NeutrAvidin was mixed with varying concentrations of biotin or **3A–D** and allowed to equilibrate. The mixture was then added to microwells containing immobilized biotin. Absorbance values at 405 nm correlated inversely with the ability of the biotin or **3A–D** in solution to compete with the immobilized biotin. Compounds **3A–D** competed with the immobilized biotin similarly to *d*-biotin ($\pm 20\%$) over a range of concentrations (μM to pM). From these assays we conclude that the indole moiety and the oligopeptide spacer do not interfere, to a practically relevant extent, with the avidin binding site. The ability of **3A–D** to bind certain proteins via their indole moieties and, simultaneously, avidin via their biotin moieties, was addressed in the following experiments.

Simultaneous Binding of Biotinylated Indoles to Selected Proteins and Avidin. When compound **3A** was incubated at neutral pH with BSA that had been immobilized to a nitrocellulose membrane, we could detect the biotin moiety via chemiluminescent reaction with horseradish peroxidase-conjugated streptavidin. The signal was reproducibly faint but above background, indicating weak binding (data not shown). Tryptophanase, which was applied as a control did not afford a signal, in accord with the fact that the enzyme does not bind indole unless supplied with pyridoxal phosphate and an appropriate aliphatic cosubstrate (22). Using a slightly different protocol, we examined the ability of compounds **3A**, **3C**, and **3D** to bind lysozyme and BSA that had been immobilized on a nitrocellulose membrane. Again, tryptophanase was applied as a control. Four replicate membranes were incubated separately with 100 μM **3A**, **3C**, **3D**, or buffer A. Unbound **3A**, **3C**, or **3D** was washed off and alkaline phosphatase conjugated NeutrAvidin was added. A chemiluminescent substrate for alkaline phosphatase allowed detection of the avidin-bound biotin conjugates. Under these conditions, only lysozyme gave a positive signal above background for all three biotinyl-

Table 2. Affinity of Immobilized Bovine Serum Albumin (BSA) and Human Serum Albumin (HSA) for the Indole Moiety of Compound 3A, as Detected by Coupling the Biotin End of the Albumin-Bound 3A to Neutravidin-Conjugated Alkaline Phosphatase Followed by Incubation with *p*-Nitrophenyl Phosphate^a

concentration (M) of compound 3A	normalized absorbance ^b at 405 nm for samples containing	
	HSA	BSA
5×10^{-6}	0.019 ± 0.033	0.034 ± 0.035
1×10^{-5}	0.138 ± 0.020^c	0.062 ± 0.064
5×10^{-5}	0.080 ± 0.023^c	0.062 ± 0.026^c
1×10^{-4}	0.083 ± 0.038^c	0.081 ± 0.070
1×10^{-3}	0.221 ± 0.083^c	0.236 ± 0.088^c

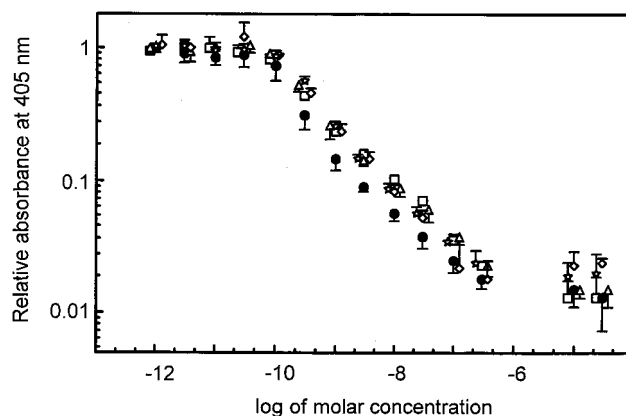
^a The levels of immobilized BSA and HSA were kept constant and the samples were zeroed (normalized) against a control sample not preincubated with **3A**. ^b Arithmetic means $\pm$ SD ($n = 3$).

^c Statistically significant binding (i.e., mean absorbance significantly larger than zero), as judged by Student's *t*-test ($p = 0.05$).

lated indoles tested, indicating that they were able to bind to lysozyme (data not shown).

We also monitored binding of compound **3A** to BSA and HSA that had been covalently attached to the wells of microwell plates. Initially the incubation of **3A** with the immobilized proteins and all subsequent steps were performed over a range of pH and temperature (data not shown). We observed optimal binding at 37 °C and pH 8.0 and performed the binding assay in triplicate. Binding of **3A** (monitored by alkaline phosphatase activity) increased with increasing **3A** concentration (Table 2). The BSA and HSA concentrations in each well were kept constant, so the observed increases in absorbance at 405 nm were indicative of more biotin conjugate binding. In a similar fashion, lysozyme was covalently attached to microwell plates and binding assays were performed with compounds **3A–D**. In all four cases, binding increased as the concentrations of the biotin-indole conjugates increased (Table 3).

Concluding Remarks. The data presented support the suitability of biotinylated indoles (**3A–D**) as probes for indole-binding proteins. Compounds **3A**, **3C**, and **3D** inhibited the tryptophanase-catalyzed conversion of serine to pyruvic acid to a similar extent as indole itself. The weak affinity of **3A** for BSA and HSA that were covalently bound to microwell plates is in accord with the results of previous indole-binding studies in solution (19, 20). For lysozyme immobilized in the same fashion, statistically significant binding was observed at ligand concentrations as low as 10^{-5} to 10^{-6} M. Reliable binding constants for proteins attached to microwell plates could not be determined, because the proteins were linked at

**Figure 2.** Competition between immobilized biotin, and biotin or biotinylated indoles **3A–D** in solution, for alkaline phosphatase-conjugated NeutrAvidin binding. The phosphatase coupled to the immobilized biotin was assayed using a colorogenic substrate (see Materials and Methods) and the resulting absorbance at 405 nm was measured. The relative absorbance at this wavelength, as shown in the Figure, was determined by dividing the absorbance for the mixtures of enzyme-linked NeutrAvidin with biotin or compounds **3A–D** by the absorbance obtained for enzyme-linked NeutrAvidin alone. The data points are presented by the following symbols: filled circles, biotin; squares, compound **3A**; diamonds, compound **3B**; triangles, compound **3C**; stars, compound **3D**.

random amino groups and we could not accurately determine the fraction of protein molecules that had available indole binding sites. Even in solution, binding kinetics is highly complex and the affinity for many indole derivatives exceeds that for indole itself (19, 20, 46). It is thus barely surprising that experiments aimed at demonstrating specific binding of compounds **3A–D**, by showing competition with indole, did not afford conclusive results. Also, what is conventionally termed the "indole-binding site" of BSA and HSA is a group-specific site which will as well accommodate a number of nonindolic drugs (47, 48), while certain indole derivatives bind to a separate site (the "warfarin site") (49) and do not directly compete with ligands accommodated by the "indole-binding site proper". In lysozyme, indole derivatives bind to the center of the hydrophobic core of the protein (50), causing a conformational change and loss of hydrolytic activity toward bacterial cell wall peptidoglycan (46). This may also suggest a group-specific binding site rather than selective indole binding.

Do compounds **3A–D**, in addition to indole-binding sites, also recognize other hydrophobic sites? Only a few proteins have so far been checked for their inability to

Table 3. Affinity of Immobilized Lysozyme for the Indole Moieties of Compounds 3A–D, as Detected by Coupling of the Biotin Ends of Protein-Bound 3A–D to Neutravidin-Conjugated Alkaline Phosphatase Followed by Incubation with *p*-Nitrophenyl Phosphate^a

concentration (M) of biotinylated indoles	normalized absorbance ^b at 405 nm for samples containing			
	3A ^c	3B ^{b,c}	3C	3D
1×10^{-6}	0.160 ± 0.182	$0.107 \pm 0.026^{d,e}$	0.127 ± 0.046^e	0.112 ± 0.008^e
5×10^{-6}	0.216 ± 0.166	0.268 ± 0.045^e	0.138 ± 0.066^e	0.244 ± 0.023^e
1×10^{-5}	0.265 ± 0.107^e	0.287 ± 0.085^e	0.211 ± 0.071^e	0.380 ± 0.074^e
5×10^{-5}	0.368 ± 0.032^e	0.390 ± 0.060^e	$0.301 \pm 0.013^{d,e}$	0.404 ± 0.081^e
1×10^{-4}	0.364 ± 0.084^e	0.387 ± 0.083^e	0.268 ± 0.024^e	0.438 ± 0.087^e
5×10^{-4}	0.380 ± 0.112^e	nd ^f	0.489 ± 0.005^e	0.505 ± 0.103^e
1×10^{-3}	0.344 ± 0.101^e	nd ^f	0.698 ± 0.029^e	0.598 ± 0.100^e

^a The level of immobilized lysozyme was kept constant and absorbance was zeroed (normalized) against a control sample not preincubated with **3A–D**. ^b Arithmetic means $\pm$ SD ($n = 3$ unless explicitly stated otherwise). ^c As far as determined, the absorbance values in samples containing equal concentrations of compounds **3A** and **3B** were not significantly different (unpaired *t*-test, $p = 0.05$). ^d $n = 2$ for these particular dilutions. ^e Statistically significant binding (i.e., mean absorbance significantly larger than zero) as judged by Student's *t*-test ($p = 0.05$). ^f nd = not determined.

bind indole under strictly defined conditions; tryptophanase appears to be the only commercially available representative. In the absence of pyridoxal phosphate and a suitable aliphatic cosubstrate, the protein does not bind indole (22) and, when immobilized on a nitrocellulose membrane, did not bind biotin conjugates **3A**, **3C**, and **3D** (see above). These data indicate that compounds **3A–D** exhibit at least some specificity for indole-binding sites. Even if that specificity should not be absolute (a property which will only become evident after extensive bench testing), the indole-biotin conjugates could still be useful for selective tagging of a restricted number of components in complex mixtures of proteins.

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Supporting Information Available: FAB HPLC-MS fragmentation patterns for biotinylated indoles **3A–D** and for essential intermediates in their synthesis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Drug Delivery Systems Employing 1,6-Elimination: Releasable Poly(ethylene glycol) Conjugates of Proteins

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Using lysozyme as a representative protein substrate that loses its activity when PEGylation takes place on the ϵ -amino group of lysine residues, various amounts of a novel releasable PEG linker (rPEG) were conjugated to the protein. rPEG–lysozyme conjugates were relatively stable in pH 7.4 buffer for over 24 h. However, regeneration of native protein from the rPEG conjugates occurred in a predictable manner during incubation in high pH buffer or rat plasma, as demonstrated by enzymatic activity and structural characterization. The rates of regeneration were also correlated with PEG number: native lysozyme was released more rapidly from the monosubstituted conjugate than from the disubstituted conjugate, suggesting possible steric hindrance to the approach of cleaving enzymes. Recovery of normal activity and structure for the regenerated native lysozyme was shown by a variety of assays.

INTRODUCTION

Conjugation of proteins with poly(ethylene glycol) (PEG),¹ a process that has been termed PEGylation (1), imparts important pharmacological properties to the modified protein. The advantages of PEGylation include increased circulatory half-life, reduced immunogenicity, reduced loss of protein to protease cleavage, and increased water solubility.

It is generally observed that protein functions, such as catalysis and receptor binding, are compromised to varying degrees following PEGylation, usually resulting in diminished activity; this may be due to the presence of the PEG substituent on or near the protein's active or regulatory sites. Early attempts to improve this situation led to the use of differently activated PEG linkers, all producing conjugates with hydrolysis-resistant permanent bonds; no one particular linker has provided consistently superior results. Therefore, it seems likely that the most effective linker for maintaining activity will vary for different proteins and will have to be determined empirically for each case (2, 3). PEG mass and the degree of protein modification have also been explored as a means of preserving activity; the use of fewer PEG strands of higher molecular weight has been reported to produce conjugates with less loss of activity (4, 5).

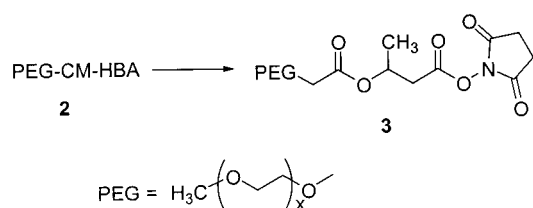
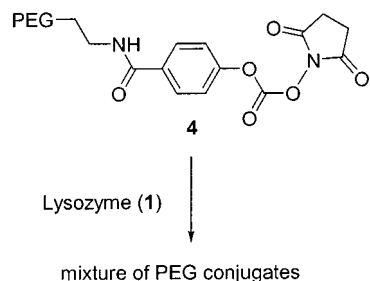
A novel approach for maintaining maximum activity in a conjugate would be to design a functional PEG linker that can predictably break down by enzymatic or pH directed hydrolysis. Such a releasable PEG (rPEG) would provide rPEGylated protein conjugates which are impermeant and could act as a depot or reservoir, continuously discharging native protein with full, albeit potentially short-acting, activity.

Coupling of proteins to substituted maleic anhydrides at the ϵ -amino group of lysine residues results in the

formation of maleic acid half amides which are labile at acidic pH (6, 7). By employing this property, it was demonstrated that conjugation of proteins through ϵ -amino groups with citraconic anhydride or 2,3-dimethylmaleic anhydride (8–11) led to conjugates that could be deacylated at pH <6. Modification of the amino groups of lysozyme by various acylating reagents has been reported to result in complete loss of activity. Therefore, lysozyme was effectively used as a model protein for which, after modification by maleic anhydride, 83% of enzymatic activity was recovered at pH 4.2 by a deacylation mechanism (12). The first application that combined PEG chemistry with anhydride linkers was reported by Garman in 1987 (13). The synthesis of a 5000 molecular weight (mw) PEG substituted maleic anhydride and its conjugation to tissue plasminogen activator (tPA) and urokinase (uPA) were described. Both of the resulting conjugates, unexpectedly, regenerated native protein under physiological conditions (pH 7.4, 37 °C), as shown by gel permeation chromatography (GPC). In the case of tPA, a half-life ($t_{1/2}$) of 42 min was reported, but it was stated that an accurate rate of release could not be determined due to the instability of the conjugate. For uPA a $t_{1/2}$ of 6.1 h was reported. The PEG number (molar ratio of PEG to protein) was not determined for either conjugated species, but it was stated that GPC measurements of the tPA conjugate gave a higher molecular mass than native protein. For uPA, it was reported that an implied deacylation (release) should restore full activity to the protein from the 44% observed initially for the rPEG conjugate. In another example, a novel rPEG linker, PEG (mw 5000) 4-pyridyl disulfide, was synthesized and conjugated with papain, which contains seven cysteine residues, through a disulfide linkage (14). The resulting conjugate was characterized by MALDI MS, and the major component demonstrated to be about 5000 mw larger than native papain. It was concluded that active native protein could be released from the conjugates using mild reducing conditions, but no actual parameters for this transformation were provided. A recent successful attempt (15) at rPEG design was

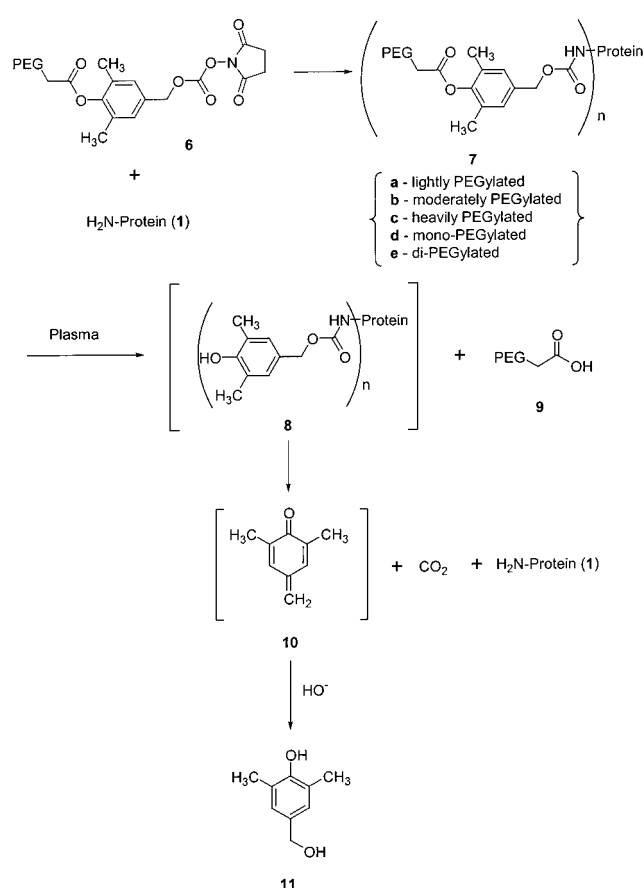
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¹ References to PEG conjugates in this paper will be restricted to methoxy PEG with a molecular weight of 5000.

Scheme 1**Scheme 2**

accomplished by a modification of lysozyme (1) at the α - and ϵ -amino positions with PEG (mw 5000) CM-HBA (2), an ester of 3-hydroxybutyric acid and carboxymethyl PEG, activated on the butyric terminus as the *N*-hydroxysuccinimide (NHS) ester (3, Scheme 1). Lysozyme highly modified by 2 showed complete inactivation, but, apparently, treatment of the conjugate with pH 2.6 phosphate buffer yielded enzyme with about 60% recovered activity. It should be noted that using this type of approach, the PEG ester, when cleaved from the bifunctional spacer, leaves an amide "tag" attached to the amino moiety, thus generating a modified enzyme. Another recent communication concerned with PEG release (16) also employs lysozyme with rPEG linkers such as 4 (Scheme 2). The resulting mixture of mono, di, and tri-PEG conjugates are phenolic carbamates, a class of compounds known to be more labile than aliphatic carbamates (17). After 10 days, hydrolysis at pH 7 and 37 °C, 82% of lysozyme bioactivity was reported to be recovered from these conjugates.

We recently developed a general methodology for synthesizing PEG prodrugs of amino-containing compounds that constitutes the basis for solubilizing insoluble drugs and extending plasma circulating half-lives of the conjugates. This approach utilized a double or tripartate prodrug (18) strategy that relies on enzymatic cleavage of bound PEG, followed by a classical 1,6-benzyl elimination reaction releasing the amine (drug) bound in the form of a carbamate (Scheme 3). The prodrug trigger was composed of ester, carbonate, carbamate, or amide bonds in order to secure predictable rates of hydrolysis. Further refinement of the controlled hydrolysis was accomplished by the introduction of steric hindrance through the use of ortho substituents on the benzyl component of the prodrug. This modification led to longer circulating plasma half-lives of the final tripartate form. The "ortho" effect also had the beneficial effect of directing nucleophilic attack by ϵ -amino groups of lysine residues almost exclusively to the activated benzylic carbonate of the heterobifunctional intermediates. These releasable PEG derivatives should also be applicable to protein and peptide modification at free α - and ϵ -amino functions in the same fashion, providing predictably unstable conjugates. Application of a non-PEGylated 1,6-benzyl elimination prodrug strategy to N-Ras lipopeptides has been reported (19), but the conjugates appear to lack adequate water solubility and addition of

Scheme 3

cosolvents was found necessary in order to perform plasma experiments.

In this work, we demonstrate the application of releasable PEG technology to polypeptides using lysozyme as a model protein and rPEG linkers of mw 5000. Lysozyme, whose structure and function are well understood, also has the advantage that activity is totally lost upon permanent PEGylation. The presence of even a single PEG on its surface results in complete loss of enzymatic activity. This provides an opportunity to demonstrate unambiguously that PEG can be completely removed by regenerating the fully functional enzyme. The releasable PEG linker 6, an *N*-hydroxysuccinimidyl activated carbonate, was designed to react with amines specifically at the alkyl terminus. Reaction of the opposite acyl phenolic terminus is prevented or minimized by the strategic placement of ortho methyl groups on the aromatic ring. This modification also served to slow the rate of enzymatic cleavage of the PEG ballast and thus allows a controlled release of native protein. The rate of release was designed to be compatible with the circulating half-lives of permanently PEGylated proteins, which typically range from a few hours to several days.

EXPERIMENTAL SECTION

Materials. Chicken egg white lysozyme (EC 3.2.1.17), lysozyme substrate bacteria (*Micrococcus lysodeikticus*), and trypsin were obtained from Sigma Inc., St. Louis, MO. The SDS-PAGE precasted gel was from Novex, San Diego, CA.

Enzyme Activity Assay. In a typical lysozyme activity assay, 40–80 μg of lysozyme or rPEG-lysozyme conjugate were added to 0.015% (w/v) of the substrate *M. lysodeikticus* in 250 mL of buffer containing 66 mM

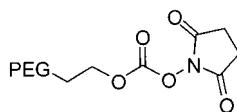


Figure 1. Structure of SC-PEG (**12**).

potassium phosphate, pH 6.2 and 0.01% (w/v) BSA. The absorbance at 450 nm was followed for 5 min. The rate of decrease in absorbance was used as a measure of enzyme activity. One unit of enzyme activity produces a change of 0.001/min at 25 °C at 450 nm. Free PEG or rat plasma had no effect on this assay.

SC-PEG-5000 (**12**) was synthesized according to published procedures (20).

Modification of Lysozyme by PEG. In a typical reaction, activated PEG (**6** or **12**) was added to lysozyme (5 mg/mL), at the indicated molar ratio, in 0.1 M phosphate buffer, pH 7.8. The reaction was quenched by lowering the pH to below 6 with acetic acid; free PEG was removed by ultrafiltration. The amount of the modification was adjusted by changing the molar ratios of PEG to protein in the reaction. Fractionation of individual species of PEG–lysozyme with varying degrees of modification was carried out by HPLC using a cation exchange column (Poros, 1-3 359-06) and a solvent system of 20 mM sodium-phosphate at pH 5.1 and a NaCl gradient.

Tryptic Peptide Mapping. Purified samples were oxidized with a performic reagent (0.912% formic acid, 0.015% hydrogen peroxide) (21), then lyophilized. The residual dry powder was resuspended at 1 mg/mL in 50 mM NaHCO₃, pH 8, 1 mM CaCl₂. The protease trypsin was added at a weight ratio of 1:100 of sample and digestion was conducted at 37 °C for 2 h. Fresh trypsin (1:50) was added and the reaction was continued for another 16 h. The digest was analyzed by reversed-phase HPLC, using a C18 column (Vydac, 218TP54) with a linear gradient of 0.1% TFA (trifluoroacetic acid) in water to 0.1% TFA in acetonitrile over 40 min at a flow rate of 1 mL/min.

Other Analytical Methods. SDS–polyacrylamide gel electrophoresis (PAGE) was used to distinguish conjugate species with different amounts of PEG. The electrophoresis was conducted using a polyacrylamide gel of 14% cross-linking and running buffer consisting of 0.29% Tris base, 1.44% glycine, 0.1% SDS at pH 8.3.

Compound 6. (A) A solution of 3,5-dimethyl-4-hydroxybenzaldehyde (5 g, 0.03 mol) in methanol (75 mL) was cooled to 0 °C and sodium borohydride (1.9 g, 0.05 mol) was added in several portions. The mixture was stirred for 30 min at 0 °C, allowed to warm to room temperature, and then stirred an additional 30 min HCl (4 mL) was added to the reaction solution and stirring continued for 10 min. The solvent was removed in vacuo and the residue dissolved in methanol (100 mL) and filtered. The filtrate was concentrated in vacuo and the residue dissolved in chloroform (1 L) and filtered. Removal of solvent gave the product, 3,5-dimethyl-4-hydroxybenzyl alcohol (3.69 g, 81%), as a pale-yellow solid.

(B) A solution of T-PEG 5000 (**25**) (18 g, 3.6 mmol), 3,5-dimethyl-4-hydroxybenzyl alcohol (2.2 g, 14.5 mmol) and 4-(dimethylamino)pyridine (1.8 g, 14.5 mmol) in anhydrous methylene chloride (90 mL) was refluxed overnight. Solvent was removed by distillation in vacuo, and the residue recrystallized from 2-propanol to yield 15 g (83%) of 3,5-dimethyl-4-hydroxybenzyl alcohol PEGylated exclusively on the phenolic OH: ¹³C NMR (67.80 MHz, CDCl₃) δ 15.75, 58.33, 63.38, 67.59, 68.73–71.31 (PEG), 126.45, 129.17, 138.77, 146.05, 167.66.

Table 1. PEG–Lysozyme Conjugates

compd	PEG type	reactant ratio ^a	fractionation	SDS–PAGE
12	SC-PEG	6:1	none	Figure 2c, lane 1
7a	rPEG	3:1	none	Figure 2a, lane 5
7b	rPEG	10:1	none	Figure 2a, lane 1
7c	rPEG	30:1	none	Figure 2a, lane 2
7d	rPEG	3–6:1	yes; 1-PEG	Figure 2b, lane 2
7e	rPEG	3–6:1	yes; 2-PEG	Figure 2b, lane 1

^a Moles of activated rPEG to moles of protein amino groups.

(C) The product from **(B)** (2.0 g, 0.4 mmol) and disuccinimidyl carbonate (0.2 g, 0.8 mmol) were dissolved in anhydrous methylene chloride (25 mL). The reaction flask was sealed with a rubber septum and cooled to 0 °C. Pyridine (32 μL, 0.4 mmol) was added to the mixture, which was stirred at 0–4 °C overnight. Cold ethyl ether was added to the solution at 0 °C and the solid filtered. The crude product was dissolved in anhydrous methylene chloride (10 mL) and the solution dried over anhydrous MgSO₄. The product was recrystallized from chilled methylene chloride–ether, two times, to give **6** (1.6 g, 80%): ¹³C NMR (67.80 MHz, CDCl₃) δ 15.99, 25.07, 58.63, 67.84, 67.93–71.79 (PEG), 77.19, 128.59, 130.39, 130.66, 147.92, 151.20, 167.77, 168.32.

RESULTS

Lysozyme was PEGylated to different degrees in order to characterize the release of PEG from a range of conjugates under various conditions (Table 1). First, lysozyme was conjugated with rPEG linker **6** (Scheme 3) and permanent linker (**12**, Figure 1) for comparative purposes. When rPEG was used at a 3–6-fold molar ratio with respect to lysozyme, the protein was lightly PEGylated (**7a**), with the majority of the protein having 1–3 rPEGs attached. When rPEG was used at a 10-fold molar ratio, lysozyme was moderately modified (**7b**) with 2–4 rPEGs attached. With a 20–30 molar excess of rPEG linker, the protein was highly modified (**7c**), with the majority of the protein possessing 4–6 rPEG strands. Figure 2a shows the mobility patterns in SDS–PAGE of different preparations of lysozyme, which were PEGylated lightly (**7a**), moderately (**7b**), or heavily (**7c**) using rPEG **6**. Figure 2b shows the single stranded rPEG–lysozyme (**7d**), and two-stranded rPEG–lysozyme (**7e**) used in some of the following studies. The reaction conditions, reaction rate, and SDS–PAGE patterns were similar for conjugates prepared with either rPEG or SC–PEG (Figure 2c). The reaction product typically consisted of a mixture of PEGylated species which were separated by HPLC (see Experimental Section). To more closely examine the details of release, purified subpopulations of rPEG–lysozyme with one or two PEG molecules were isolated (Figure 2b).

As conjugate **7** releases rPEG, its size is expected to diminish. Thus, the size distribution of rPEG–lysozyme conjugates can be used as evidence of the release of PEG. When all PEG molecules are cleaved from **7**, the residual protein should have the same size as native, unmodified lysozyme. This was evident from SDS–PAGE where the completely hydrolyzed rPEG–lysozyme resulted in a material that had the same molecular weight as native lysozyme. The rate of release of PEG for the particular rPEG-linker employed in this study was dependent on several factors, including pH, temperature, and the number of PEGs attached to the protein. As shown in Figure 3, release of PEG, as expected, was faster at higher pH. Lightly rPEGylated lysozyme **7a** released a small amount of PEG under physiological

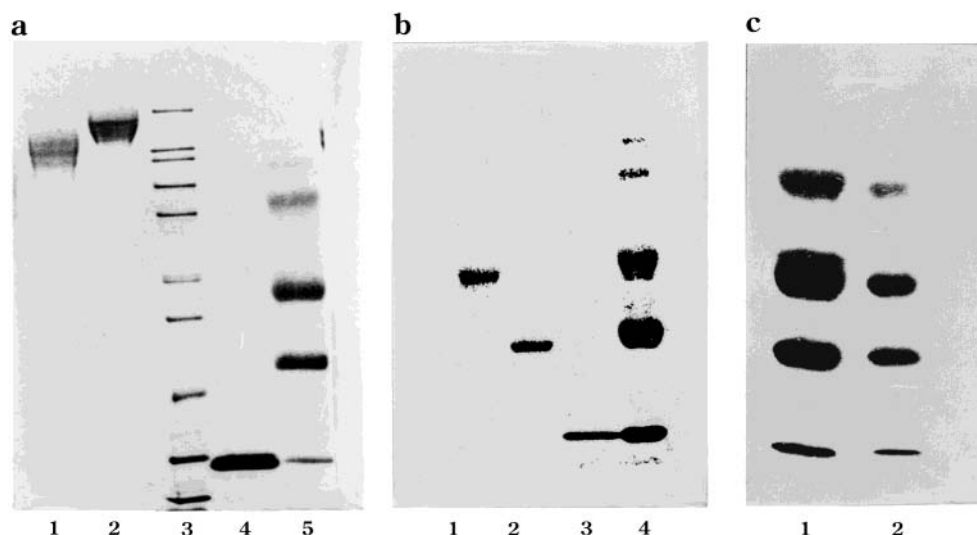


Figure 2. PEGylation of lysozyme with **6**. (a) Lysozyme was modified by the rPEG as described in the Experimental Section. Lanes are 1, **7b**; 2, **7c**; 3, molecular weight marker; 4, native lysozyme; 5, **7a**. (b) Fractionation of rPEG-lysozyme. Lanes are 1, **7e**; 2, **7d**; 3, native; 4, reaction mixture of rPEG lysozyme. (c) Comparison of reaction products of rPEG-lysozyme and PEG-lysozyme using same reaction conditions that generated lightly PEGylated products. (Loading amount in the gel was different). Lane 1: reaction product with SC-PEG. Lane 2: reaction product with rPEG.

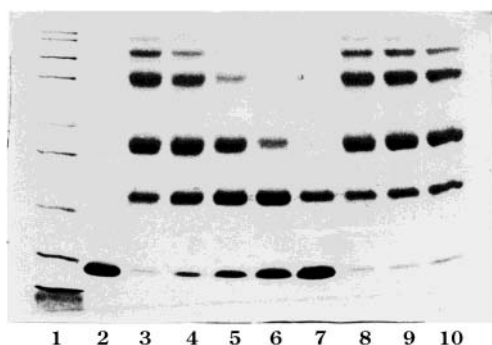


Figure 3. Release of PEG from **7a** in buffer. Lane 1, molecular weight marker; lane 2, native lysozyme; lanes 3 and 8, **7a** before release; lanes 4–7, **7a** in borate buffer pH 8; lane 4, 1 h; lane 5, 3 h; lane 6, 7 h; lane 7, 24 h; lanes 9 and 10, **7a** in phosphate-buffered saline, pH 7.4; lane 9, 3 h; lane 10, 24 h.

conditions (PBS buffer at pH 7.4, 37 °C), while moderately rPEGylated-lysozyme **7b** was completely stable under the same conditions (not shown). When lightly rPEGylated-lysozyme **7a** was incubated in buffer at 37 °C and pH 9, all species originally present were reduced to a mixture of singly rPEGylated and native enzyme over a 24 h period. PEG was released significantly more rapidly at higher temperature (Figure 4).

The release of lysozyme from rPEG conjugates in rat plasma was also studied. SDS-PAGE patterns were difficult to interpret due to interference by rat plasma proteins, so lysozyme enzymatic activity was used to follow PEG release. Specifically, there was no activity initially observed for all samples of **7** that were free of native lysozyme (see time zero in Figure 5 for **7a**). As mentioned previously, lightly rPEGylated lysozyme (**7a**) when incubated in PBS at 37 °C produced only trace amounts of native enzyme, amounting to <3.5% recovery of the original enzyme activity after 24 h. On the other hand, 64% of the activity was recovered upon incubation in pH 9 buffer, over the same time period (Figure 5). With mono-PEGylated rPEG-lysozyme (conjugate **7d**), 50% of the enzyme activity was recovered after incubation in rat plasma at 37 °C for 20 h, and 100% of the activity was recovered after 96 h (Figure 6). When incubation of di-PEGylated rPEG-lysozyme (**7e**) was carried out under

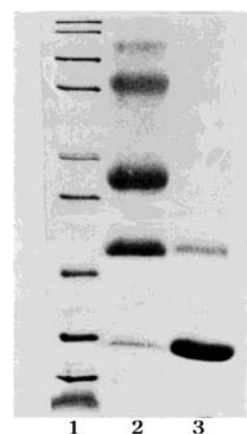


Figure 4. Fast release of rPEG from **7a** at high pH and temperature. **7a** was treated with pH 9 borate buffer at 50 °C. Lane 1, molecular weight marker; lane 2, **7a**; lane 3, hydrolysis of **7a** after 22 h.

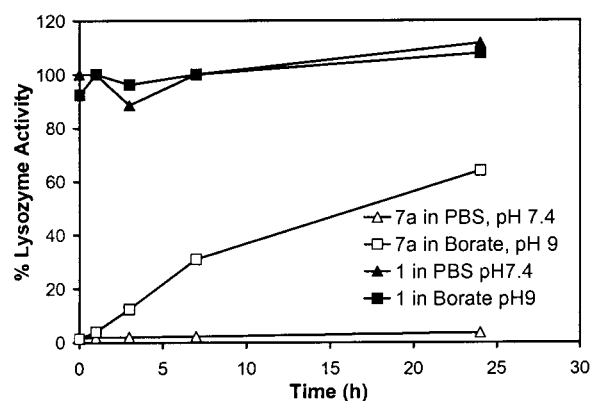


Figure 5. Effect of PEG attachment and release on the enzymatic activity. The release of native lysozyme from **7a** was monitored in terms of its activity. Activity is measured as % activity of native lysozyme (**1**) at time zero.

similar conditions, only 10% of the enzyme activity was recovered after 20 h, 50% after 57 h, and 100% of the activity was observed after 96 h (Figure 7). Note that during this same period, there was almost no recovery of activity from conjugate **7d** in PBS buffer. Since the

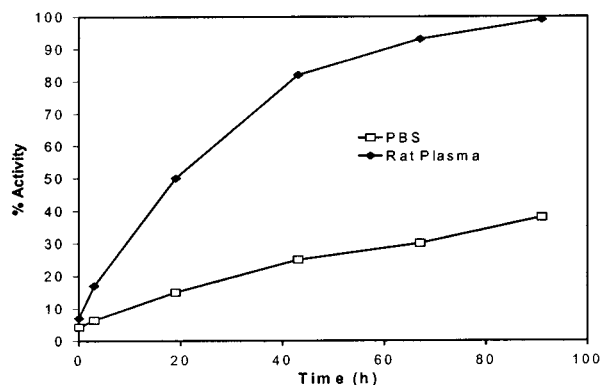


Figure 6. Regeneration of lysozyme from **7d** (rPEG1-lysozyme) in plasma. Compound **7d** was incubated in rat plasma and the activity regenerated was compared with the same sample incubated in phosphate buffered saline.

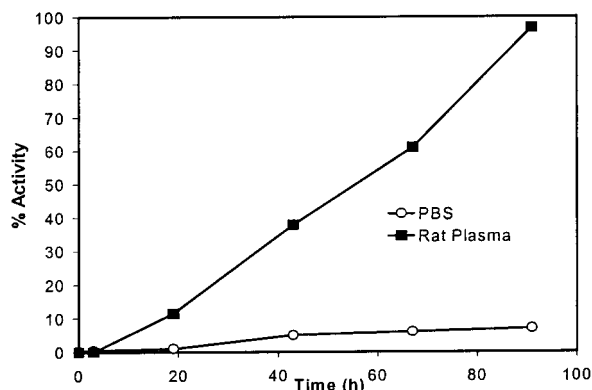


Figure 7. Regeneration of lysozyme from **7e** (rPEG2-lysozyme) in plasma. Compound **7e** incubated in rat plasma and the activity regenerated was compared with the same sample incubated in phosphate buffered saline.

hydrolysis rate is much slower in buffer than in plasma, the release is likely due to nonspecific enzymatic cleavage in plasma of the ester trigger of the conjugate.

In summary, after 22 h at 50 °C in pH 9 buffer, heavily PEGylated rPEG-lysozyme conjugate **7c** exhibited an SDS-PAGE pattern (not shown) that still contained more PEGylated species than was observed after similar treatment of the lightly PEGylated conjugate, **7a** (Figure 4). These results demonstrated that more heavily rPEGylated conjugate released PEG more slowly than the lower conjugate. This phenomenon was studied with the well-defined compounds **7d** and **7e**. The release kinetics of **7d** was much faster than that of **7e** (Figures 6 and 7).

In order for an rPEG protein conjugate to demonstrate effective drug delivery, activity must be regenerated before the protein is eliminated from the body by renal and hepatic clearance pathways. Published results indicate that the circulating $t_{1/2}$ of PEGylated proteins of similar molecular weight as lysozyme is about 10–30 h (3, 22). As can be seen from the data, PEG release from lysozyme **7d** in rat plasma will be in excess of 50% after 20 h which fits the appropriate time frame for delivery of native protein in a controlled fashion.

In plasma, recovery of 100% of enzyme activity was observed from **7d** after PEG was completely removed from the rPEG-lysozyme conjugate, which strongly implies that the released enzyme was identical to **1**. To prove this hypothesis, peptide mapping was used to verify the structural integrity of the regenerated enzyme.

Tryptic digestion followed by reversed-phase HPLC generates a characteristic pattern of fragments from the

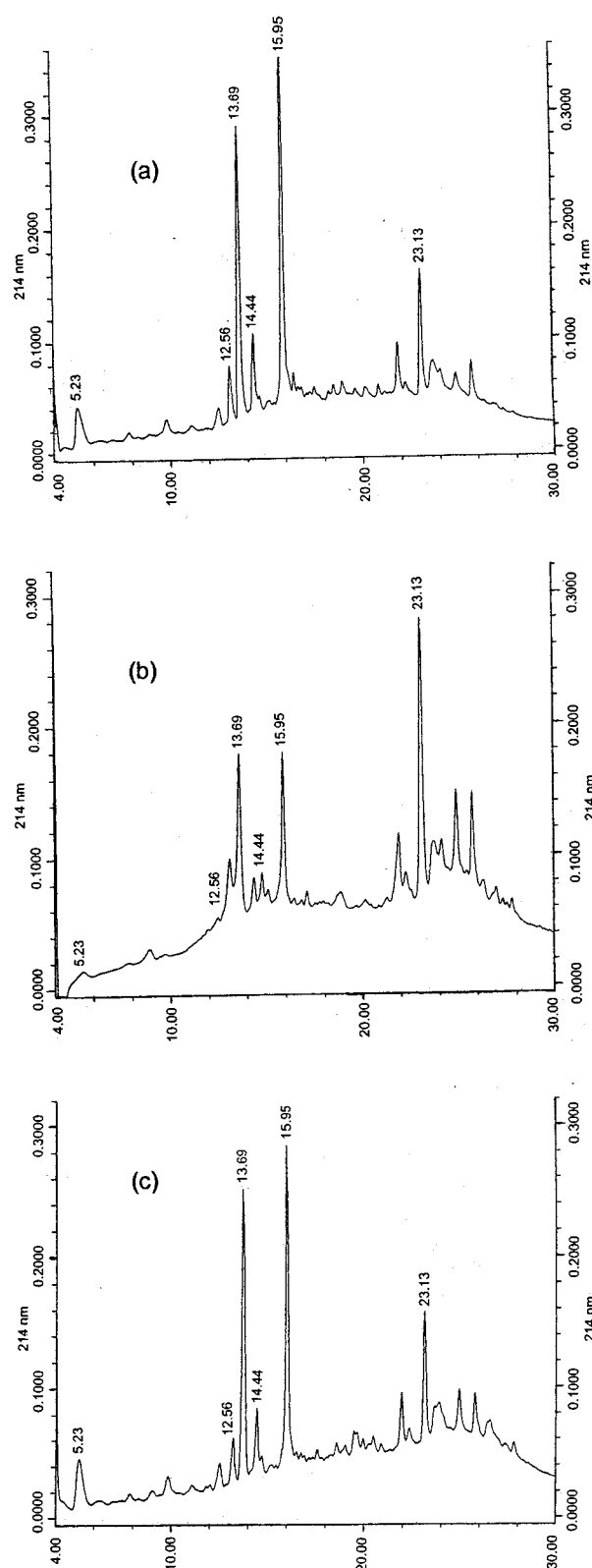


Figure 8. Comparison of peptide map of released lysozyme and native lysozyme analyzed by HPLC at 214 nm. (a) Native lysozyme, (b) **7a**, (c) Regenerated lysozyme from **7a**.

native lysozyme. Fragmentation allowed the PEGylation and the recovery process after release of rPEG to be monitored (Figure 8). A similar approach has been used to characterize PEG-lysozyme (23).

In our system, the size of the group of peaks eluted at 5.23, 12.56, 13.69, 14.44, and 15.95 relative to the peak eluted at 23.13 was particularly useful in characterizing

PEGylation and release. The peak at 15.95 was drastically reduced after PEGylation. After the release of PEG, this peak returned to its original size, as shown in Figure 8, panels a–c. In fact, the peptide map of the regenerated lysozyme was the same as that of the original native enzyme, indicating the equivalence of the proteins.

Several additional analytical methods were used to compare unmodified lysozyme with lysozyme released from the rPEG–lysozyme conjugate. The isoelectric focusing pattern of the regenerated lysozyme was shown to be the same as that of native lysozyme. The amount of free amine as measured by the trinitrobenzenesulfonic acid assay was also found to increase proportionally during release of lysozyme from the rPEG conjugate. Re-PEGylation of the regenerated lysozyme with **6** resulted in newly conjugated species similar to the original rPEG-medium fraction, demonstrating restoration of PEGylation sites. Furthermore, the pH profile of the enzymatic activity of **1** was the same as that of the regenerated lysozyme, indicating that the microenvironment of the catalytic site on the enzyme was restored. In addition, the amino acid composition of the regenerated protein and that of the native protein were found to be identical. From these data, we conclude that there was a quantitative release of unstable intermediate **8**, which rapidly regenerated protein that was identical to the native species.

DISCUSSION

The degree of loss of activity when PEG is attached to a protein typically depends on the number of PEG strands attached to the protein. This loss of activity after PEGylation also depends on the site of PEGylation, the protein structure, and the protein charge (1, 2). A high PEG number may produce a longer plasma half-life, but often at the expense of protein activity. Releasable PEG, exemplified by the linker **6**, allows a high degree of PEGylation of protein with the temporary masking of activity, followed by restoration of activity by a defined mechanism.

We observed that attachment of even one PEG is sufficient to abolish the cell-wall degradation activity of lysozyme. Since the cell wall material is considerably larger than lysozyme, the contact of the enzyme with its substrate should be a relatively flat surface, rather than a deep groove. Thus, PEG covalently attached to lysozyme may prevent the proper docking of the substrate to the enzyme resulting in inhibition of activity. So et al. (23), reported 38% residual activity in lysozyme conjugated to mPEG via a different linker, while data reported by others (15) are consistent with almost total loss of activity for another PEG–lysozyme conjugate. The nature of the linking group is therefore of paramount importance in determining the enzymatic activity of PEG–lysozyme conjugates.

In addition to its cell wall degradation activity, lysozyme has weak esterase activity (24, 25). If this esterase effect was involved in cleavage of rPEG–lysozyme conjugates, then accelerating release kinetics would be expected as accumulating free lysozyme catalyzed cleavage of the remaining conjugates. However, we observed linear release kinetics for the rPEG conjugates, suggesting that the lysozyme esterase activity had little effect on the PEG release rate. Therefore, regeneration of lysozyme is most likely the result of encountering other enzyme systems in plasma.

The observed release rate of protein from conjugates **7d** and **7e** is slower than release rates of small molecules

such as daunorubicin from similar rPEG linkers (18), which generally occurs with a $t_{1/2}$ of about 2 h in rat plasma. Since **7e** had $t_{1/2}$ of hydrolysis approximately three times longer than **7d** in rat plasma, this would seem to indicate that steric factors must also be considered in the enzymatic cleavage.

The clearance $t_{1/2}$ of a protein in blood can be controlled by changing the apparent molecular size of the protein. Attachment of PEG to the protein, which increases the apparent molecular weight, results in a longer clearance $t_{1/2}$ while providing reduced immunogenicity for the conjugate. While this has generally been accomplished using activated linkers such as SC–PEG and T–PEG (25) providing permanently bonded PEG–protein conjugates, rPEG-linkers provides an alternative to these methods. While there are other means of delivering proteins in vivo, such as liposomes or hydrogels, these formulations are difficult to implement due to problems in loading of the protein and administration of the dosage form. rPEG conjugates of proteins derived from activated rPEG **6**, act as a circulating depot or carrier for the protein drug and should thus be a very useful tool for drug delivery.

Our data supports the concept of releasable PEG that predictably generates native protein from the PEG conjugate. Application of this (and similar) technology (26) to other therapeutic proteins, and their in vivo results, will be the subject of future publications. Typically, this involves rPEG-linkers that react with free amine groups present in the protein. In addition to serving as a releasable carrier, rPEG may also be used as an aqueous derived amino-protecting group on or near the active site of the protein. This can be followed by the attachment of permanently bound PEG at other sites of the protein where PEGylation is less critical to the biological activity of the protein to produce what can be termed a “PEG-hybrid”. Once permanently bonded PEG is attached, the releasable PEG can then be removed during processing, leaving a nonimmunogenic conjugate with fewer strands at less active sites and therefore, greater activity. Alternatively, the hybrid conjugate can be used directly in vivo (27), further enhancing the duration of activity of the protein pharmaceutical. Proof of this concept has been demonstrated using dimethyl maleic anhydride to block the active lysines of TNF- α , followed by PEGylation. Pretreatment in this fashion gives conjugates with 20–40% higher specific activity in vitro than directly conjugated PEG–TNF- α (28).

CONCLUSIONS

Our results show that the use of rPEG-linker **6** with the protein lysozyme produces conjugates of various degrees of PEGylation whose pH and enzymatic breakdowns are easily measurable. Lysozyme activity is completely recovered compared to the 50–60% reported by others (15), which indicates that there is no denaturation of the protein during PEGylation and the subsequent hydrolysis of rPEG conjugate **7**. In addition to the controlled release observed for rPEG conjugates, one needs to consider other properties generally required for clinically acceptable pharmaceutical preparations. For long term storage, rPEG–protein conjugates can be lyophilized and reconstituted prior to use. Since protein drugs are normally prepared in aqueous solutions, rPEG conjugates must be stable in physiological buffer solution for up to 24 h: this has been demonstrated for rPEG–protein **7**. In addition, we have shown that **7d** has a regeneration rate comparable to the blood circulation half-life of protein that was modified by a permanently

bound PEG. Thus, rPEG **6** has the potential to be used in practical ways by extension of this system to other proteins such as enzymes and cytokines, which should provide a depot of protein drug in the rPEG form. The depot effect should benefit the treatment of cancer, thrombosis, cardiovascular diseases, and autoimmune diseases with protein drugs. It should also help to reduce side effects due to the spiking caused by large amounts of native protein drug delivered in a short period of time.

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Synthesis, Characterization, and Relaxivity of Two Linear Gd(DTPA)–Polymer Conjugates

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Two linear polyamide conjugates of $\text{Gd}(\text{DTPA})^{2-}$ were synthesized and characterized by high-resolution nuclear magnetic resonance (NMR) spectroscopy and size exclusion chromatography (SEC). DTPA was copolymerized with two different diamines, 1,6-hexanediamine and *trans*-1,4-cyclohexanediamine, yielding the polymers DTPA-HMD and DTPA-CHD, with low polydispersity. Their molecular flexibility in solution was studied using ^{13}C spin–lattice relaxation time measurements, indicating that the cyclohexanediamine linking moiety of the DTPA-HMD polymer is more rigid than that of DTPA-CHD. The influence of the flexibility of the linking functionalities on the relaxivity of the Gd^{3+} -DTPA-polymer conjugates was studied by water nuclear magnetic relaxation dispersion (NMRD). The relaxivity of the $\text{Gd}(\text{DTPA-CHD})$ polymer was only slightly higher than that of the $\text{Gd}(\text{DTPA-HMD})$ polymer, and only two times higher than the usual values for small Gd -DTPA-like chelates. The low relaxivities obtained for both polymers, much lower than expected from the polymer apparent molecular weights, result from their substantial residual flexibility, and also from a too long, nonoptimal, value of the inner-sphere water exchange rate. These polymeric compounds are also cleared very quickly from the blood of rats, indicating that they are of limited value as blood pool contrast agents for MRA.

INTRODUCTION

Contrast agents (CAs) for magnetic resonance imaging (MRI) are mainly chelates of Gd^{3+} , due to its high magnetic moment and long electronic relaxation time (1–4). A $\text{Gd}(\text{III})$ -bound water which exchanges with the bulk water of the body, determines via the enhancement of the relaxation (T_1 and T_2) of the water protons, the intensity of the water signal and thus the contrast of the images. The relaxation theory predicts that higher relaxation rates are obtained upon increase of the rotational correlation time of the Gd^{3+} complexes (1–4), which may be achieved by noncovalent or covalent binding of low-molecular weight Gd^{3+} chelates to macromolecules or polymers (3, 4). These conjugates might have prolonged residence time in the cardiovascular system, with potential applications in MRI Angiography (MRA) (5, 6).

In fact, in the past few years there has been a great interest in the development of efficient, nontoxic, blood pool CAs for MRA examinations. For this purpose, low molecular weight Gd^{3+} -chelate complexes, such as $\text{Gd}(\text{DTPA})^{2-}$ or $\text{Gd}(\text{DOTA})^-$, have been conjugated to natural or synthetic polymeric materials, like polysaccharides (7–

16), albumin (17,18), polyamino acids such as polylysine and polyornithine (19–25) or dendrimers (26). Some linear polymers of DTPA have also been prepared in which the chelate is incorporated directly in the polymeric chain (27–29). These macromolecules lead to an increase in blood pool lifetime and should be more efficient in respect to their relaxivity, as a consequence of their increased rotational correlation time resulting from their high molecular weight. However, the observed increase of their relaxivity was not as high as expected, which was attributed to a combination of two effects: the rapid internal rotational motion of the linking moieties between the Gd^{3+} -chelate in the polymeric chain and too long inner-sphere water exchange rates. The fact that dendrimer-based contrast agents show higher relaxivities compared to other systems confirms that more rigid structures lead to more efficient macromolecular contrast agents (26).

Here we report the synthesis, physicochemical characterization and in vivo pharmacokinetics in Wistar rats of two linear polyamide conjugates of $\text{Gd}(\text{III})$ -DTPA, $\text{Gd}(\text{DTPA-HMD})$, and $\text{Gd}(\text{DTPA-CHD})$ (see their structures in Figure 1), obtained by copolymerization of DTPA with two different diamines, the linear and conformationally flexible 1,6-hexanediamine and the cyclic and conformationally more rigid *trans*-1,4-cyclohexanediamine. The influence of the molecular flexibility of the respective functionalities on the relaxivity of the $\text{Gd}(\text{III})$ -DTPA-polymer conjugates is also reported and compared with other systems from the literature. As macromolecular polyamides, they should be biodegradable and biocom-

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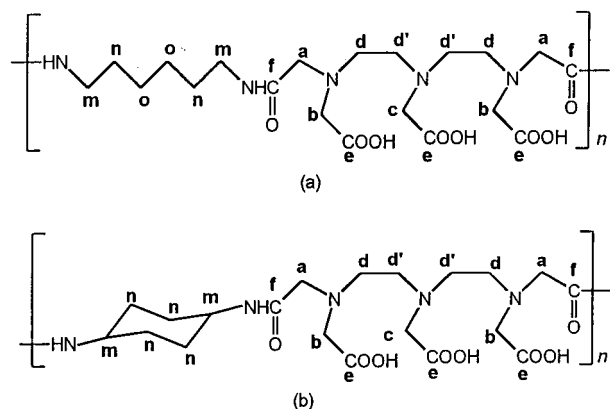


Figure 1. Structure of the repeating unit of the DTPA linear polymers: (a) DTPA-HMD; (b) DTPA-CHD.

patible, and their Gd^{3+} complexes should in principle be promising as NMR angiography (MRA) contrast agents.

EXPERIMENTAL PROCEDURES

Reagents. Diethylenetriamine pentaacetic acid (DTPA) 97% purity, $GdCl_3 \cdot 6H_2O$, *trans*-1,4-cyclohexanediamine (CHD) and 1,6-hexanediamine (HMD) were purchased from Aldrich and used as supplied. Acetic anhydride, pyridine and dimethyl sulfoxide, also from Aldrich, were dried over 3 Å molecular sieves before use.

Synthesis and Characterization of DTPA Copolymers and Gd^{3+} Conjugates. *Preparation of DTPA-bis-Anhydride (DTPA-BA).* A modification of procedure described in the literature (28, 30) was applied: 0.125 mol of DTPA was mixed with 0.558 mol of dried acetic anhydride and 0.770 mol of dried pyridine. The resulting suspension was stirred for 24 h at 65 °C. Then the solid obtained was filtered, washed with dried diethylether and dried under vacuum, until constant weight at 25 °C. The DTPA-bis-anhydride was characterized by NMR. ^1H NMR ($DMSO-d_6$) δ (ppm) 3.7 (s,8H), 3.29 (s,2H), 2.43 (t,4H), 2.58 (t,4H); ^{13}C NMR ($DMSO-d_6$) δ (ppm) 171.9, 165.7, 54.5, 52.5, 51.7, 50.7. This product was considered sufficiently pure to be used in the preparation of the polymers.

Polymerization of DTPA-BA with HMD. In a round-bottom flask, 6 mmol of DTPA-BA were dissolved in 30 mL of DMSO and an equimolar amount of HMD was added. The mixture was heated at 70 °C under N_2 bubbling. Over a period of 3 h a large part of the solvent was slowly distilled under N_2 at reduced pressure (70 °C). The product was precipitated in acetone, filtered, and dissolved in 20 mL of distilled water. The solution was dialyzed for 3 days, using a MWCO 12–14000 Membrane purchased from Medical, London, U.K., and lyophilized to give 0.33 g of polymer (11.6%).

Polymerization of DTPA-BA with CHD. The same procedure as described above was followed except that 5.6 mmol of DTPA-BA and of CHD were used. After lyophilisation, 0.63 g of polymer was obtained (24%).

Preparation of the Gd^{3+} Conjugates. The polymers were dissolved in a small amount of distilled water and equimolar amounts of an aqueous solution of $GdCl_3$ were added dropwise with stirring, for the period of 1 h. The pH was maintained at 5.8 by the addition of 0.1M NaOH. The solutions were dialyzed until no free Gd^{3+} was detected, using xylenol-orange as indicator. The samples were then lyophilized.

Determination of Gd^{3+} . The metal content (%) in the polymer conjugates was determined by inductively coupled

plasma optical emission spectroscopy (ICP-OES), using a Jobin Yvon-Jy 70 Plus spectrometer.

The metal binding to the polymers was also determined by titration of aqueous solutions (D_2O) of those polymers with solid aliquots of $GdCl_3$ while maintaining neutral pH by addition of aqueous NaOH. The formation of the complex was monitored by measuring the shift of the ^{17}O NMR signal of water at 348 K (2, 16). The presence of unbound Gd^{3+} was readily detected by a dramatic increase in the chemical shift and width of the signal. Then, evaluation of the titration data allowed for determination of the percent of DTPA binding moieties of the polymer complexed to Gd^{3+} .

NMR Measurements. ^1H (499.824 MHz), ^{13}C (125.697 MHz), and ^{17}O (67.760 MHz) NMR spectra were recorded on a Varian Unity 500 spectrometer (at an external field of 11.8 T) in D_2O (99.8% D from Sigma Chem. Co.) solutions. For the ^1H NMR spectra, the water signal was used as internal reference, set at δ 4.75 ppm (at 298 K) and was suppressed by a presaturation pulse. ^{13}C NMR spectra were measured with broad-band proton decoupling, using the methyl resonance of TSP (sodium-3-trimethylsilylpropionate-2,2–3,3- d_4) as internal reference, set at δ = 0 ppm. ^{13}C NMR spin–lattice relaxation time (T_1) values were measured using the inversion–recovery technique. Assignments of the ^1H and ^{13}C NMR spectra were based on literature data for similar systems (28, 31) and on the results of two-dimensional homonuclear correlation spectra (COSY). The pH of the solutions was adjusted with DCl and CO_2 -free NaOD (from Sigma Chem. Co.) using a Crison MicropH 2002 pH-meter with an Ingold 405-M5 combined electrode. The temperature precision of the experiments was ± 0.5 °C.

SEC Measurements. The apparent molecular weights of the polymers were determined by size-exclusion chromatography (SEC) using two series of Pl-Aquagel-OH-30 columns (from Polymer Laboratories) coupled to a LC-25 RI detector. The eluent was 0.2 M NaCl. Systematic errors in the molecular weight determinations, leading to apparent values result from the charges of the polymers. The weight-average molecular weight (M_w) and number-average molecular weight (M_n) of the polymers are given by the expressions $M_w = (SN_i M_i^2)/(SN_i M_i)$ and $M_n = (SN_i M_i)/(SN_i)$, where N_i is the number of molecules of molecular weight M_i . M_w and M_n were calculated using a calibration curve of poly(ethylene glycol) standards obtained using a software program, EzChrom Chromatography V6.6, Scientific Software, Inc. The M_w/M_n ratio, named polydispersity, which is a measure of the width of the molecular weight distribution, was also calculated.

Relaxometry Studies. The chelates were dissolved in ultrapure H_2O and the water T_1 relaxation times of the solutions were measured at 9 MHz and 298 K using an MRS-4 relaxometer (Jozef Stefan Institute, Ljubljana, Slovenia), using an inversion recovery pulse sequence (180° – t – 90°) with eight delay times. The relaxivity was determined by calculating the slope of the curve of relaxation rate ($1/T_1$) versus Gd^{3+} concentration (mmol/L).

Proton nuclear magnetic relaxation dispersion (NMRD) profiles were recorded at 310 K on a field cycling relaxometer (Field Cycling Systems, Honesdale, PA) working between 0.02 and 50 MHz. Additional points at 60 and 300 MHz were obtained on a Bruker Minispec mq60 (Bruker, Karlsruhe, Germany) and on a Bruker AMX 300 spectrometer (Bruker, Karlsruhe, Germany). Fitting of the ^1H NMRD curves was performed with a previously described software using minimization routines (Minuit, CERN Library) (32, 33).

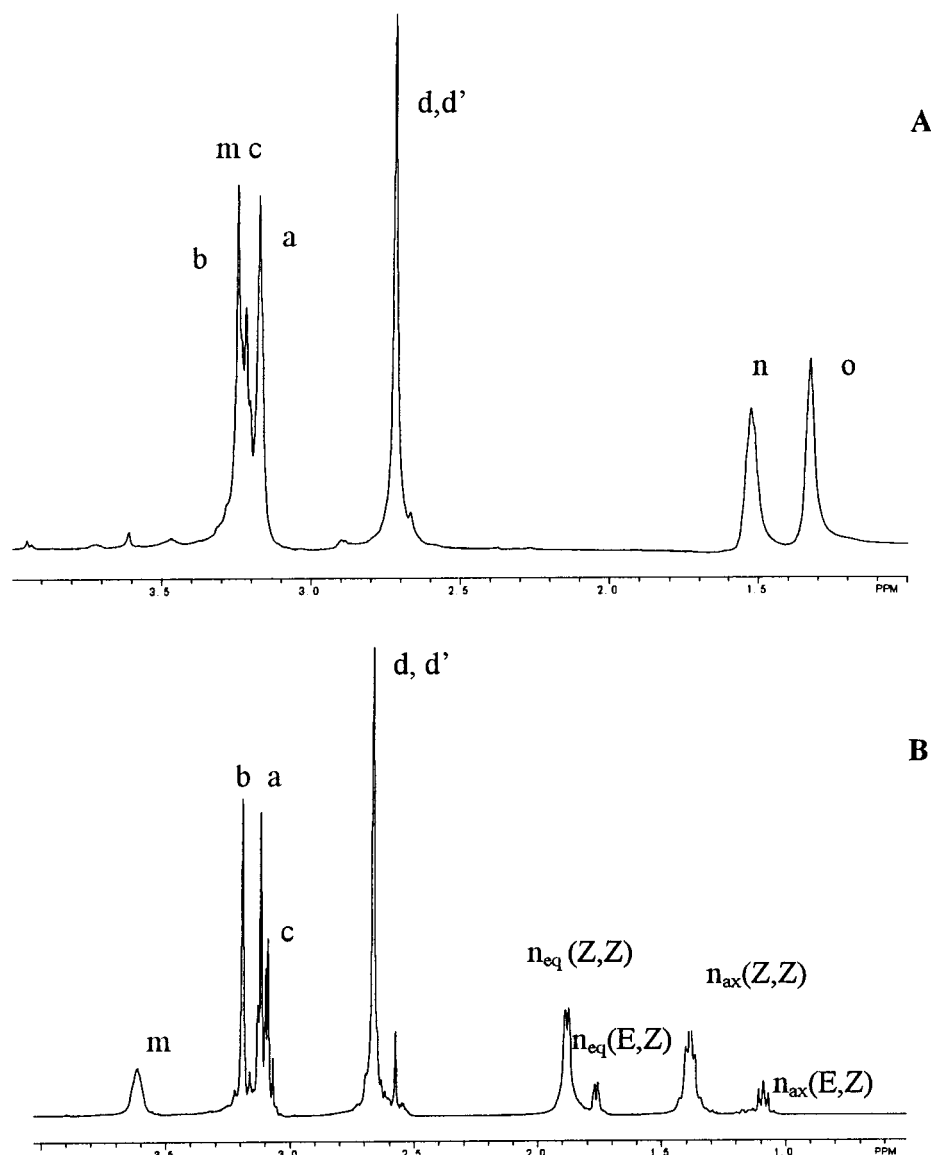


Figure 2. ^1H NMR spectra of the copolymers: (A) DTPA-HMD polymer obtained in $\text{D}_2\text{O} + \text{NaOD}$ (57.2 mg/mL) at 25 $^\circ\text{C}$, pH 10.71; (B) DTPA-CHD polymer obtained in $\text{D}_2\text{O} + \text{NaOD}$ (41.6 mg/mL) at 25 $^\circ\text{C}$, pH 11.2

Pharmacokinetics. The pharmacokinetic studies were carried out according to the following protocol: the Wistar rats were anesthetized by i.p. injection of Nembutal (100 $\mu\text{L}/100$ g b.w.), the carotid artery was catheterized for rapid blood collection, 200 μL of contrast agent were injected in the blood through the femoral vein; blood samples (± 300 $\mu\text{L}/\text{sample}$) were collected 1, 2, 5, 10, 15, 30, 45, and 60 min. afterward; the longitudinal relaxation rate R_1 was measured at 39 $^\circ\text{C}$ on a spin analyzer PC120 (Bruker, Rheinstetten, Germany) on each sample.

RESULTS AND DISCUSSION

The copolymers of DTPA obtained were synthesized by an easy method that leads to polyamides. After purification, these polymers were found to be water soluble. The structure of both polymers is represented in Figure 1. Their ^1H NMR spectra, shown in Figure 2, have narrow peaks suggesting that the polymers present are rather flexible. The assignments of the ^1H signals were based on their intensities, multiplicities and chemical shifts, and confirmed by ^1H -COSY spectra. The assignments of the methylene protons of the DTPA moiety were obtained from the previously described proton NMR titration

curves of DTPA derivatives (31). In spectrum B, we can observe the polymeric species corresponding to two conformations. The substitution groups in cyclohexane are mainly in diequatorial configuration because of the effect of 1,3-diaxial interactions. However, due to the partial double bond character of the amide $\text{N}-\text{C}$ bonds, the repeated unit in the polymer can be in three conformations, which are E,Z, E,E, and Z,Z (Figure 3). Only two sets of signals, with intensity ratios 4.9:1, were observed in the ^1H NMR spectrum. An inspection of molecular models suggests that the steric strain in the three possible configurations of the disubstituted cyclohexane moieties follows the order $\text{E,E} > \text{E,Z} > \text{Z,Z}$. Therefore, we assign the two sets of signals to the E,Z and Z,Z configurations, and we assume that the population of the E,E configuration is negligible. In the DTPA-HMD polymer only a single set of broad signals was observed, which can be explained by the fast exchange between the isomers on the NMR time scale.

Figure 4 shows the proton decoupled ^{13}C NMR spectra of the two polymers, which were assigned on the basis of the pH dependence of their chemical shifts and previous work on small DTPA-bisamides (34). For the DTPA-CHD

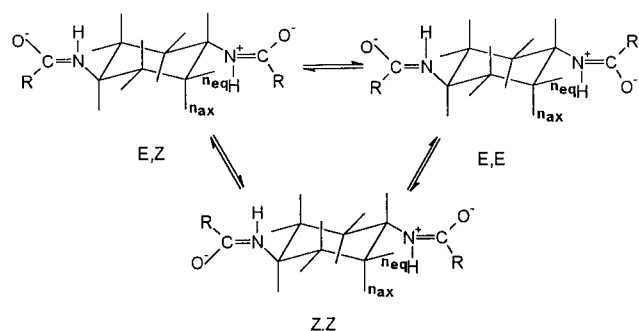


Figure 3. E,Z, E,E, and Z,Z conformations of the *trans*-1,4-cyclohexanediamine in the DTPA-CHD polymer.

polymer, we see two sets of resonances for carbons a–f. This is in agreement with the ^1H NMR data.

Table 1 shows the ^{13}C NMR T_1 values, obtained for the two polymers. These values are rather long for the ester and carboxylate carbons, due to the absence of efficient dipolar relaxation from neighboring protons. It is more instructive to compare the T_1 values for the CH_2 carbons, where the ^{13}C relaxation rates ($1/T_1$) are proportional to an effective local rotational correlation time, which modulates the dipolar interaction between the ^{13}C and the directly bound protons, reflecting the local mobility of the molecule (35). This relationship is valid for local rotational correlation times smaller than 10^{-9} s (36) and can be applied to polymers that have fast internal motions. Here we see that, for both polymers, the

Table 1. ^{13}C T_1 Values (s) for the Copolymers DTPA-HMD and DTPA-CHD in D_2O at 25 $^\circ\text{C}$, pH 11.0

^{13}C nucleus (see Figure 1)	polymer	
	DTPA-HMD	DTPA-CHD
e	3.18 ± 0.09	2.76 ± 0.24
f	1.76 ± 0.08	1.49 ± 0.13
a	0.23 ± 0.01	0.24 ± 0.03
b	0.21 ± 0.01	0.20 ± 0.04
c	^a	^a
d	0.21 ± 0.02	0.18 ± 0.02
d'	0.20 ± 0.01	0.19 ± 0.04
m	0.27 ± 0.02	0.40 ± 0.03
n	0.30 ± 0.02	0.19 ± 0.01
o	0.37 ± 0.03	

^a Not determined.

methylene carbons of the DTPA moieties have the same mobility. This mobility is smaller than that of the linking chain for the DTPA-HMD polymer, but the same for the DTPA-CHD polymer. This surprising result may be a consequence of the slower overall tumbling rate of the second polymer, with its larger molecular weight, rather than of the local mobility of the linking moieties. However, to correlate these data with the relaxivity of the macromolecular complexes we have to take into account the mobility of the DTPA moiety and the correlation time of the water proton.

The SEC chromatograms of our samples of the two polymers and of their Gd^{3+} complexes, obtained using an eluent of high ionic strength, are shown in Figure 5. The results of their analysis (Table 2) show that the DTPA-

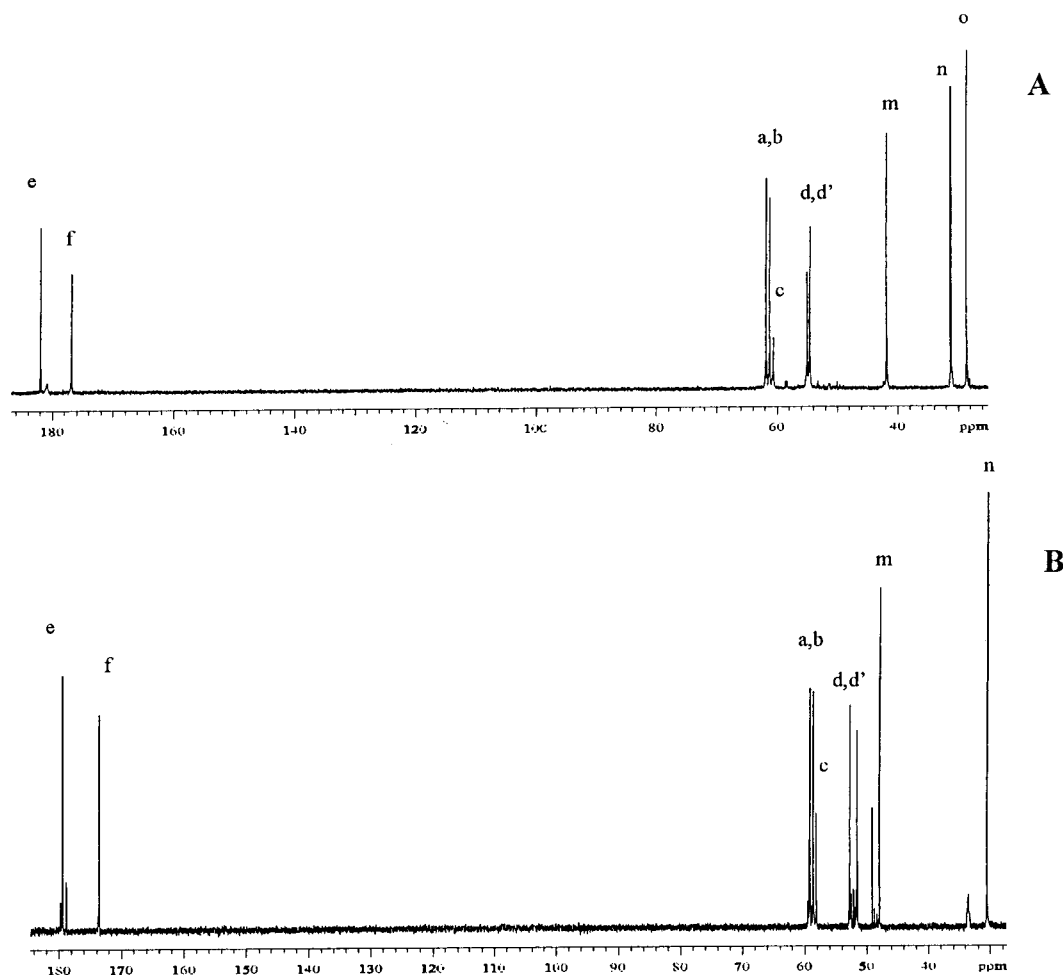


Figure 4. ^{13}C NMR spectra of the copolymers: (A) DTPA-HMD polymer obtained in D_2O + NaOD at 25 $^\circ\text{C}$, pH 10.7; (B) DTPA-CHD polymer obtained in D_2O + NaOD at 25 $^\circ\text{C}$, pH 11.2.

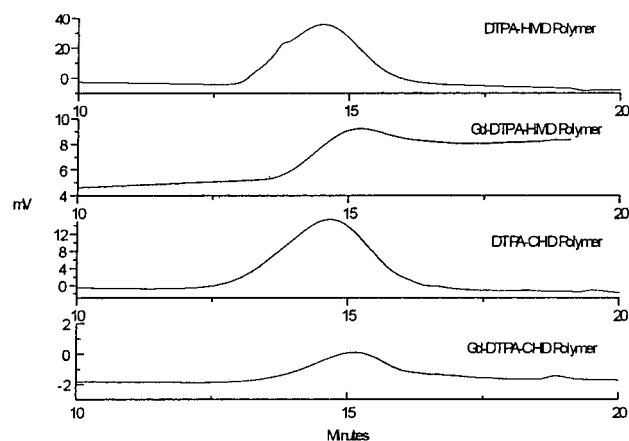


Figure 5. SEC chromatograms of the polymers DTPA-HMD and DTPA-CHD, and of the respective Gd^{3+} complexes.

Table 2. Comparison of the Apparent Molecular Weights of the Different Polymers Studied in this Work with Data from the Literature

polymer	M_w	M_n	polydispersity	ref
DTPA-HMD	7900	6200	1.27	<i>a</i>
Gd(DTPA-HMD)	4900	3200	1.53	<i>a</i>
DTPA-CHD	8000	5800	1.37	<i>a</i>
Gd(DTPA-CHD)	6400	4250	1.50	<i>a</i>
DTPA-HMD		2700		26
Gd(DTPA-HMD)	19 400	13 300	1.46	27

^a Present work.

HMD and DTPA-CHD polymers have very similar apparent molecular weights and low polydispersities, indicating that they are quite homogeneous, as we can observe in the SEC chromatograms in Figure 5. A reduction in the apparent molecular weight values of both polymers can be observed after Gd^{3+} complexation, and their low polydispersity slightly increases. The molecular weights of DTPA-HMD and DTPA-CHD must be lower than those of the corresponding Gd^{3+} complexes, due to the contribution of the cation. Thus, the observed reduction of the SEC-measured apparent values for the complexed polymers should be a result of the neutralization of charge of the DTPA polymers when bound to Gd^{3+} . In fact, even using an high ionic strength eluent (0.2 M NaCl), the repulsion forces between the negatively charged polymer carboxylate groups increase the hydrodynamic volumes of the polymeric chains, leading to lower elution volumes and over-evaluated molecular weights. This is confirmed by the observation that using a low ionic strength eluent (0.01 M NaCl), the apparent molecular weights of the of DTPA-HMD and DTPA-CHD polymers increased by a factor of 6, while those of their Gd^{3+} complexes increased only by a factor of 2 (data not shown). Thus, the determination of molecular weights for this type of compounds remains a difficult problem, casting some doubt on the reliability of some of the literature data.

The Gd^{3+} contents in the two polymers, as measured by ICP, are shown in Table 3. Similar Gd^{3+} contents were measured by Kellar et al. (29) for the Gd(DTPA-HMD) polymer. The Gd^{3+} yields obtained show that ca. 20 and 30% of the DTPA units in Gd(DTPA-CHD) and Gd(DTPA-HMD), respectively, are uncomplexed.

The Gd^{3+} yields, which give the percent of DTPA binding moieties of the polymers complexed to Gd^{3+} , were also determined by measuring the shift of the ^{17}O NMR signal of water at 348 K while titrating D_2O solutions of those polymers with solid aliquots of GdCl_3 at neutral

Table 3. Gd^{3+} Content in the Polymer Complexes and Their Relaxivities at 9 MHz and 298 K

polymer	$\text{Gd}^{3+}(\text{mol})/\text{sample (g)}$	Gd^{3+} yield ^a (%)	relaxivity ($\text{s}^{-1} \text{mM}^{-1}$)	ref
Gd(DTPA-CHD)	1.7×10^{-3}	79	8.1	<i>b</i>
Gd(DTPA-HMD)	1.4×10^{-3}	68	7.8	<i>b</i>
Gd(DTPA-HMD)	1.4×10^{-3}		9 ^c	27

^a Gd^{3+} yield defined as $[\text{Gd}^{3+}(\text{mol})/\text{repeating unit of polymer}(\text{mol})] \times 100$. ^b Present work. ^c Estimated from NMRD curve (27).

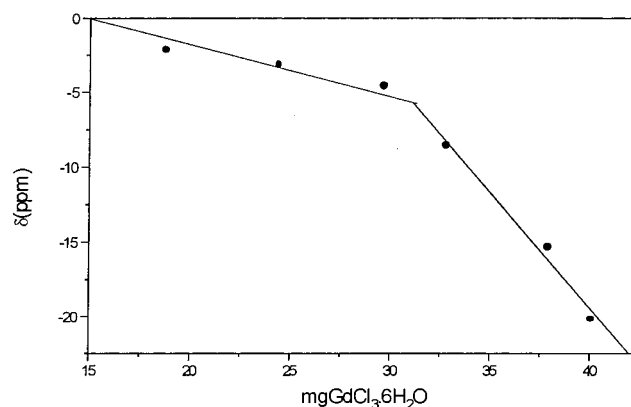


Figure 6. Plot of Gd(III) induced ^{17}O chemical shift of water (δ) versus the amount of $\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$ added for Gd^{3+} complexation titration of 44.8 mg of DTPA-CHD polymer in 0.6 mL of D_2O at 348 K.

pH (2). Figure 6 shows the plot resulting from a typical experiment with the DTPA-CHD polymer. The position of the break of the plot, indicating the presence of unbound Gd^{3+} gave a Gd^{3+} yield of 87% for the Gd(DTPA-CHD) polymer, a value in reasonably good agreement with that obtained for ICP data.

The relaxivities of the two Gd^{3+} polymers at 9 MHz and 298 K are also shown in Table 3. The relaxivities for the polymers were less than twice the value of $5.6 \text{ s}^{-1} \text{mM}^{-1}$, observed for the $\text{Gd}(\text{DTPA})^{2-}$ chelate itself in the same conditions. The relaxivity of Gd(DTPA-HMD) is quite similar to the value reported in the literature (29). As CHD has a more rigid structure than HMD, allowing only chain-chain conformational interconversions but no single C–C bond rotational motions, it should hinder the rotational motion of the DTPA-linking moieties in the polymeric chain. As can be seen in Table 3, the relaxivity of the Gd(DTPA-CHD) polymer was not as high as expected, being only slightly higher than that of the Gd(DTPA-HMD) polymer. This could result from the substantial residual flexibility of that polymer, and also from a nonoptimal value of the inner-sphere water exchange rate.

To be able to better understand the factors determining the relaxivity of these polymers, we undertook a full proton nuclear magnetic relaxation dispersion (NMRD) study of these systems. The proton NMRD profiles recorded at 310 K (see Figure 7) show a hump between 10 and 100 MHz characteristic of slowly tumbling paramagnetic complexes. The analysis of the proton relaxivity takes into account the outersphere (37) and the innersphere magnetic interactions (38, 39). For Gd(DTPA-HMD), the values evaluated from the NMRD profiles analysis at 310 K for the rotational correlation time τ_R , for the electronic parameters (τ_{SO} , the electronic relaxation time at low field, and τ_V , the correlation time modulating the electronic relaxation) and the residence time of the inner-sphere water molecule (τ_M) (see Table

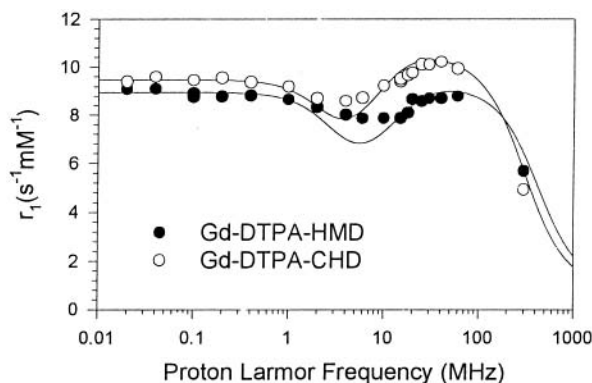


Figure 7. Longitudinal proton relaxivity of Gd(DTPA-HMD) (0.45 mM) and Gd(DTPA-CHD) (0.424 mM) at 310 K.

Table 4. Parameters Obtained from the Fitting of the Proton NMRD Data Obtained at 310 K^a

	τ_{SO} (ps) ^b	τ_V (ps) ^c	τ_R (ps) ^d	τ_M (μ s) ^e
Gd(DTPA-HMD)	120	21	580	1.33
Gd(DTPA-CHD)	140	32	997	1.45

^a The following parameters were fixed: the distance between Gd³⁺ and the inner-sphere water proton ($r = 0.31$ nm), the distance of closest approach ($d = 0.36$ nm), the relative diffusion constant ($D = 3.5 \times 10^{-9}$ m² s⁻¹) and the number of inner-sphere water molecules ($q = 1$). ^b τ_{SO} = electronic relaxation time at low field. ^c τ_V = correlation time modulating the electronic relaxation. ^d τ_R = rotational correlation time. ^e τ_M = residence time of the inner-sphere water molecule.

4) are comparable to those reported by Toth et al. (27) (from this publication, a τ_M value of 1.03 ms can be calculated at 310 K for the compound with $n = 6$). The observed constancy of τ_M is in line with the general observation that derivatization has hardly any effect on the water exchange rate of bisamides, which limit the relaxivity values. The main difference between both compounds is the rotational correlation time, which is longer for Gd(DTPA-CHD) than for Gd(DTPA-HMD). The τ_R values obtained by the fitting of the proton NMRD curves are shorter than those expected for such polymers and are indicative of internal motion. Therefore, the unexpectedly low relaxivity of these polymers can be explained by their relatively short τ_R and the long residence time of their inner-sphere water molecules.

The present results show that introducing short, conformationally rigid DTPA-linking moieties does not prevent the fast local motions in the extended polymeric chain, which dominate τ_R and keeps the relaxivity low. Other studies with this type of DTPA copolymers, containing as linking units long hydrophilic poly(ethylene-glycol) (PEG) (40), or hydrophobic alkyl chains with a varying number (n) of methylenes (29), showed that higher relaxivities were obtained only for long hydrophobic chains with $n \geq 10$. For these polymers, hydrophobic interactions between different chains lead to intramolecular aggregates and the formation of more rigid compact, globular structures, with slower local motions and global rotation, leading to higher relaxivity (27, 29). Studies with other polymeric carriers (15, 16) have also shown that higher relaxivities are obtained for more rigid compact, globular structures, for example by increasing the degree of substitution of the carrier.

The results of pharmacokinetic studies of the two polymeric compounds Gd(DTPA-HMD) and Gd(DTPA-CHD) injected in Wistar rats are shown in Figure 8. This shows that both compounds exhibit a fast exponential clearance from the blood. With a blood half-life shorter

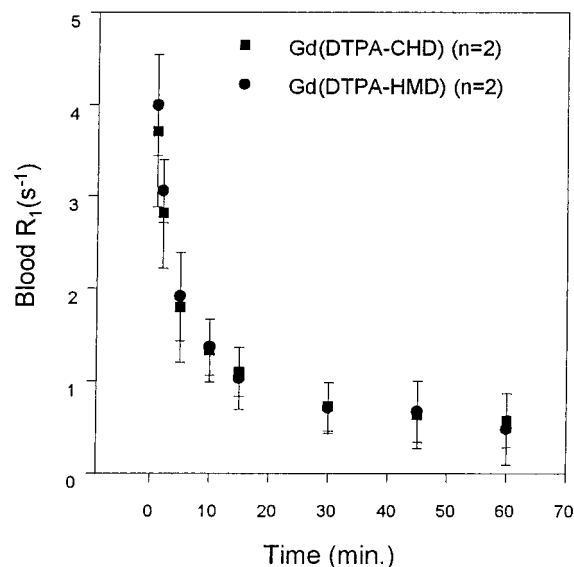


Figure 8. Pharmacokinetics of Gd(DTPA-HMD) and Gd(DTPA-CHD) in Wistar rats.

than 10 min, they show the same pharmacokinetic behavior as Gd(DTPA)²⁻, the extravascular/extracellular parent molecule.

CONCLUSIONS

We have obtained two main chain DTPA polymers by copolymerization of DTPA with two diamines of different structures and internal flexibilities. Despite the derivatization of two terminal carboxylate groups of each DTPA residue, with a somewhat reduced Gd³⁺ binding ability of the resulting amide moieties, the stability of the polymeric complexes should still be high enough to be used in vivo. This is supported by the observation that the reduced thermodynamic stability of small Gd³⁺ chelates of DTPA-bis amides relative to Gd(DTPA)²⁻ is compensated by their comparable or even favorable conditional stability constants and selectivity factors for Gd³⁺ relative to other biologically relevant cations such as Zn²⁺, leading to nonincreased in vivo dissociation of Gd³⁺ (31, 41, 42).

The Gd³⁺ complexes of the two polymers were obtained with Gd³⁺ yields of about 80 and 70% for Gd(DTPA-CHD) and Gd(DTPA-HMD), respectively, as measured by ICP. Solvent water ¹⁷O NMR shift monitoring of titration experiments of the polymers with solid GdCl₃ gave comparable results. The incomplete loading of the polymers with Gd³⁺, resulting in the presence of ca. 20–30% of uncomplexed, negatively charged DTPA units, while increasing their solubility and SEC-determined apparent molecular weights due to the negative charges present, will also contribute to an increased internal mobility and reduced relaxivities.

The Gd³⁺ macromolecular complexes show somewhat higher relaxivities than the small parent Gd³⁺ chelate, Gd(DTPA)²⁻. The rigidity of the cyclic diamide linking moiety did improve the relaxivity of the complexes in water, but not as much as expected. This may result from the short length of the linking groups, which prevents the hydrophobic interaction between polymeric chains and the formation of a compact polymer, as happens with similar polymers with longer chains in the linking groups (29). A substantial residual flexibility of those polymers may still be present. Alternatively, the local motions located at the linking groups in the polymer chains might be less important than those of the DTPA moieties in

determining the correlation time of the water molecule bound to the Gd^{3+} ion.

The rotational correlation time is longer for $Gd(DTPA-CHD)$ than for $Gd(DTPA-HMD)$, indicating a substantial decrease of the residual flexibility of the first polymer. However, the rotational correlation times obtained for these polymers are shorter than expected, indicating that internal motion is present. The low relaxivity of these polymers can thus be explained by their relatively short τ_R and the long residence time of the inner-sphere water molecule.

With a blood half-life shorter than 10 min., both compounds exhibit the same pharmacokinetic behavior as $Gd(DTPA)^{2-}$, the extravascular/extracellular parent molecule. Molecules with molecular weights lower than about 15 kDa pass freely through glomerular filtration in the kidneys. Large size polymers are usually cleared slowly from the blood because of a restricted glomerular filtration. However, the clearance has to be related not only to the molecular weight of the product but also to its spatial conformation. In the present case, the polymers are made of linear and flexible chains and are characterized by a molecular weight not large enough to prevent the glomerular filtration. Thus, although the polymers are stable in vivo for the short time they stay in circulation, the fast excretion properties severely restrict the applicability of this type of Gd^{3+} polymeric complexes as MRA contrast agents.

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Renal Metabolism of 3'-Iodohippuryl *N* ϵ -Maleoyl-L-lysine (HML)-Conjugated Fab Fragments

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Renal localization of radiolabeled antibody fragments constitutes a problem in targeted imaging and radiotherapy. Recently, we reported use of a novel radioiodination reagent, 3'-[¹³¹I]iodohippuryl *N* ϵ -maleoyl-L-lysine (HML), that liberates *m*-iodohippuric acid before antibody fragments are incorporated into renal cells. In mice, HML-conjugated Fab demonstrated low renal radioactivity levels from early postinjection times. In this study, renal metabolism of HML-conjugated Fab fragments prepared by different thiolation chemistries and by direct radioiodination were investigated to determine the mechanisms responsible for the low renal radioactivity levels. Fab fragments were thiolated by 2-iminothiolane modification or by reduction of disulfide bonds in the Fab fragments, followed by conjugation with radioiodinated HML to prepare [¹³¹I]HML-IT-Fab and [¹²⁵I]HML-Fab, respectively. In biodistribution studies in mice, both [¹³¹I]HML-IT-Fab and [¹²⁵I]HML-Fab demonstrated significantly lower renal radioactivity levels than those of [¹²⁵I]Fab. In subcellular distribution studies, [¹²⁵I]Fab showed migration of radioactivity from the membrane to the lysosomal fraction of the renal cells from 10 to 30 min postinjection. On the other hand, the majority of the radioactivity was detected only in the membrane fraction at the same time points after injection of both [¹³¹I]HML-IT-Fab and [¹²⁵I]HML-Fab. In metabolic studies, while [¹²⁵I]Fab remained intact at 10 min postinjection, both HML-conjugated Fab fragments generated *m*-iodohippuric acid as a radiometabolite at the same postinjection time. [¹³¹I]HML-IT-Fab registered two radiometabolites (intact [¹³¹I]HML-IT-Fab and *m*-iodohippuric acid), whereas additional radiometabolites were observed with [¹²⁵I]HML-Fab. This suggested that metabolism of both HML-conjugated Fab fragments would occur in the membrane fractions of the renal cells. The findings of this study reinforced our previous hypothesis that radiochemical design of antibody fragments that liberate radiometabolites that are excreted into the urine by the action of brush border enzymes would constitute a useful strategy to reduce renal radioactivity levels from early postinjection times.

INTRODUCTION

Antibody fragments such as Fab and single-chain Fv fragments show rapid targeting to tumor and uniform distribution in tumor tissues (1). Such properties render antibody fragments useful to deliver radioactivity for both targeted imaging and radiotherapy. However, radiolabeled antibody fragments show persistent localization of radioactivity in the kidneys, which impairs diagnostic accuracy in the kidney region and limits therapeutic potential (2–8). Thus, radiolabeled antibody fragments would become much more useful in diagnosis and targeted radiotherapy if the renal radioactivity could be reduced without impairing the radioactivity levels in the target.

Recent studies indicated that the persistent localization of the radioactivity after administration of radiolabeled antibody fragments and peptides was attributable

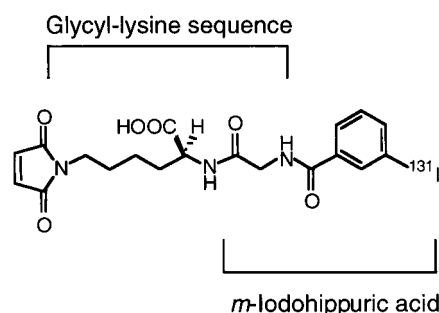


Figure 1. Chemical structure of [¹³¹I]HML.

to slow elimination rates of radiometabolites generated after lysosomal proteolysis of the parental Fab fragments in renal cells (7, 9–11). There have been a number of attempts to facilitate elimination of radiometabolites generated after lysosomal proteolysis of glomerularly filtered antibody fragments through the use of cleavable linkages between antibody fragments and radiolabeled compounds (12–14) (Figure 2A). Although the rationale behind the radiochemical design is well supported by recent metabolic studies as cited above, this approach may also impair target radioactivity levels when applied

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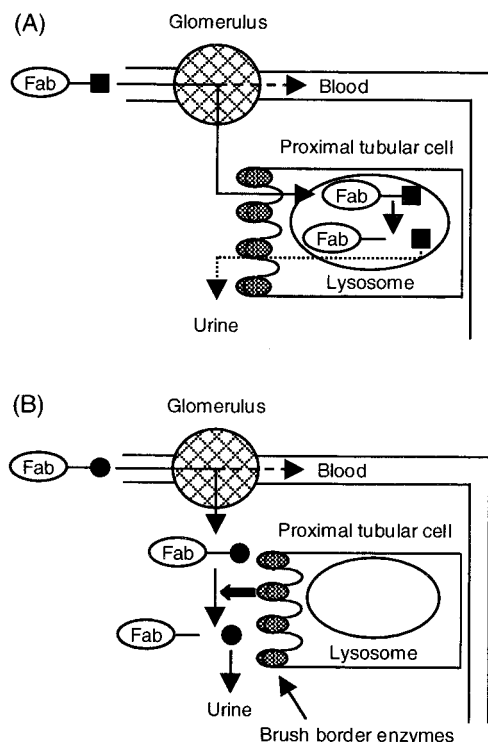


Figure 2. (A) Potential mechanism of renal radioactivity localization of radiolabeled Fab fragments. Slow elimination rates of radiometabolites (■) from the lysosomal compartment would be responsible for the long residence time of the radioactivity in the kidney. (B) Proposed mechanism of renal metabolism of [¹³¹I]HML-IT-Fab. [¹³¹I]HML-IT-Fab would release *m*-[¹³¹I]iodohippuric acid (●) before the antibody fragments are incorporated into the renal cells by the action of the brush border enzymes.

to antibody fragments or peptides that are incorporated into target cells.

We recently reported that radioiodinated Fab fragments labeled with 3'-[¹³¹I]iodohippuryl *N*-maleoyl-L-lysine ([¹³¹I]HML, Figure 1) demonstrated markedly low renal radioactivity levels with kidney-to-blood ratios of 1 from 10 min to 1 h after injection into mice due to rapid release of *m*-[¹³¹I]iodohippuric acid. In addition, HML-conjugated Fab did not impair the radioactivity levels in the tumor when compared with directly radioiodinated Fab in nude mice model (15, 16). On the other hand, radioiodinated Fab via direct radioiodination reached its peak kidney-to-blood ratios of 7.3 at 1 h postinjection. Since the two simultaneously administered radioiodinated Fab fragments showed similar radioactivity levels in the blood, we hypothesized that [¹³¹I]HML-conjugated Fab fragments would release *m*-[¹³¹I]iodohippuric acid before the antibody fragments were incorporated into the renal cells (Figure 2B). That is, the glycyl-lysine sequence in HML would be cleaved by the action of the brush border enzyme carboxypeptidase M (17) present on the lumen of renal tubules (18, 19). Since high-level and persistent localization of the radioactivity is also observed with radiolabeled peptides, further investigation of the mechanism responsible for the low renal radioactivity levels of [¹³¹I]HML-conjugated Fab fragments would provide an insight for designing radiolabeled antibody fragments and peptides much more useful for both targeted imaging and radiotherapy.

In the present study, we investigated renal metabolism of [¹³¹I]HML-conjugated Fab fragments. Since esterase-mediated cleavage of the ester bonds is affected by the distance between the parental protein and the substrate

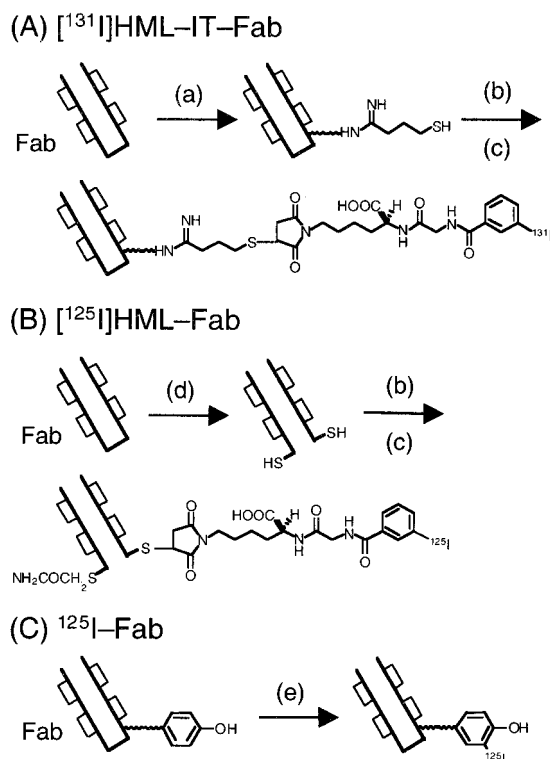


Figure 3. Preparation of radioiodinated Fab fragments. (A) [¹³¹I]HML-IT-Fab, (B) [¹²⁵I]HML-Fab, (C) [¹²⁵I]Fab. Reagents: (a) 2-IT, 0.16 M borate buffer/2 mM EDTA (pH 8.0); (b) [¹³¹I]-HML, 0.1 M phosphate buffer/2 mM EDTA (pH 6.0); (c) iodoacetamide, 0.1 M phosphate buffer/2 mM EDTA (pH 6.0); (d) 2-ME, 0.1 M phosphate buffer/2 mM EDTA (pH 7.0); (e) chloramine T, Na[¹²⁵I], 0.3 M phosphate buffer (pH 7.3).

(20, 21), radioiodinated HML was conjugated to Fab fragments via different thiolation chemistries to locate the glycyl-lysine sequence in HML at a position distal or close to the parental Fab fragments; Fab fragments were thiolated by reacting 2-iminothiolane (2-IT) with amine residues of the protein ([¹³¹I]HML-IT-Fab, Figure 3A) or by reducing the disulfide bonds with 2-mercaptoethanol (2-ME) ([¹²⁵I]HML-Fab, Figure 3B). Fab fragments were also directly radioiodinated by the chloramine T method ([¹²⁵I]Fab, Figure 3C) as a control. Subcellular localization and chemical forms of the radioactivity remaining in the kidney at early time points after injection of the three radioiodinated Fab fragments were investigated and the mechanism of renal metabolism of each radioiodinated Fab fragment will be discussed. The findings of this study provided further evidence that the low renal radioactivity levels of HML-conjugated Fab fragments are due to rapid release of *m*-iodohippuric acid before incorporation of the fragments into the renal cells.

MATERIALS AND METHODS

Reagents and Chemicals. Na[¹³¹I] and Na[¹²⁵I] were obtained from Daiichi Radioisotope Laboratories (Tokyo, Japan) and Daiichi Kagaku (Tokyo), respectively, and were diluted with 0.1 M phosphate buffer (pH 7.4) to 37 MBq (1 mCi)/2–10 μ L. Size-exclusion high-performance liquid chromatography (SE-HPLC) was performed with a Cosmosil Diol-120 column (7.5 $\times$ 600 mm, Nacalai Tesque, Kyoto, Japan) eluted with 0.1 M phosphate buffer (pH 6.8) at a flow rate of 1 mL/min. Reversed-phase HPLC (RP-HPLC) was performed with a Cosmosil 5C₁₈-AR-300 column (4.6 $\times$ 150 mm, Nacalai Tesque) eluted with a mixture of 0.1% aqueous phospho-

ric acid and acetonitrile (3:1) at a flow rate of 1 mL/min. The eluent was collected with a fraction collector (Redi-Frac; Pharmacia Biotech, Tokyo) at 30-s intervals, and the radioactivity levels in each fraction (500 μ L) were determined with an auto well counter (ARC-2000; Aloka, Tokyo). TLC analyses were performed with silica plates (Merck Art 5553). To facilitate collection of urine and feces after administration of radiolabeled Fab, mice were housed in metabolic cages (Metabolica, MM type; Sugiyama-Gen Iriki Co. Ltd., Tokyo). Other reagents were of reagent grade and were used as received.

Monoclonal Antibody (mAb). The mAb against osteogenic sarcoma (OST7, IgG₁), generated by the standard hybridoma technique, was purified by ammonium sulfate precipitation with subsequent protein A affinity chromatography (Pharmacia Biotech Co. Ltd., Tokyo), as reported previously (22). The Fab fragment was prepared by the standard procedure using papain (23).

Preparation of Radioiodinated Fab Fragments. [^{131/125}I]HML was prepared as described previously (21). Conjugation of [^{131/125}I]HML with the Fab fragment of OST7 was performed as illustrated in Figure 3.

[¹³¹I]HML-IT-Fab was prepared by treating the Fab fragment with 2-IT, followed by conjugation reaction between the thiol groups of Fab and the maleimide groups of [¹³¹I]HML (Figure 3A). Briefly, a solution of Fab (200 μ L, 2 mg/mL) in well-degassed 0.16 M borate buffer (pH 8.0) containing 2 mM EDTA was allowed to react with 7.2 μ L of 2-IT solution (1 mg/mL) prepared in the same buffer. After gentle agitation of the reaction mixture for 60 min at room temperature, excess 2-IT was removed by a centrifuged column procedure (24) using Sephadex G-50 (Pharmacia Biotech Co. Ltd.) equilibrated and eluted with 0.1 M phosphate buffer (pH 6.0) containing 2 mM EDTA. Aliquots of this mixture were sampled for estimation of the number of thiol groups with 2,2'-dipyridyl disulfide (25). The filtrate (100 μ L) was then added to a reaction vial containing crude [¹³¹I]HML. After gentle agitation of the reaction mixture for 1.5 h at room temperature, 14.8 μ L of iodoacetamide (10 mg/mL) in 0.1 M phosphate buffer (pH 6.0) containing 2 mM EDTA was added. The reaction mixture was further incubated for 30 min to alkylate the unreacted thiol groups. [¹³¹I]HML-IT-Fab was subsequently purified by the centrifuged column procedure, equilibrated and eluted with 0.1 M acetate buffer (pH 6.0).

[¹²⁵I]HML-Fab was prepared by reducing disulfide bonds in the Fab fragment with 2-ME, followed by conjugation between Fab and [¹²⁵I]HML (Figure 3B). Briefly, a solution of Fab (200 μ L, 2 mg/mL) in well-degassed 0.1 M phosphate buffer (pH 7.0) containing 2 mM EDTA was allowed to react with 5.6 μ L of 10-fold diluted 2-ME solution using the same buffer and stirred gently for 60 min at room temperature. The experimental procedures for preparation of [¹²⁵I]HML-Fab after introduction of thiol groups were similar to those used for [¹³¹I]-HML-IT-Fab except that [¹²⁵I]HML was used in place of [¹³¹I]HML.

Direct radiodination of Fab was performed by the chloramine T method (Figure 3C) (15). To 100 μ L aliquots of Fab (2.5 mg/mL) in 0.3 M phosphate buffer (pH 7.3) was added 1 μ L of Na[¹²⁵I]. Chloramine T (0.1 mg/mL, 12.5 μ L), freshly prepared in the same buffer, was then added. After incubation of the mixture for 5 min at room temperature, [¹²⁵I]Fab was purified by the centrifuged column procedure, as described above.

Radiochemical purities of the radioiodinated Fab fragments were determined by SE-HPLC and TLC developed with a mixture of methanol and water (4:1).

Biodistribution of Radioiodinated Fab Fragments. In vivo studies were performed after simultaneous administration of a mixed solution of [¹³¹I]HML-IT-Fab and [¹²⁵I]HML-Fab or after injection of a solution of [¹²⁵I]Fab. Three mL of 0.1 M phosphate buffer (pH 7.4) containing 600 μ g of unmodified Fab were mixed with 10 μ L of each radioiodinated Fab fragment (12–13 μ Ci) prior to administration. The biodistribution of radioactivity after i.v. administration of each mixture in 6-week-old male ddY mice (27–30 g) (26) was monitored at 10 and 30 min and 1, 3, 6, and 24 h postinjection. Groups of five mice, each receiving 20 μ g of Fab fragments, were used for the experiments. Organs of interest were removed and weighed, and the radioactivity was determined with a well counter (ARC 2000). A window from 29 to 97 keV was used for counting ¹²⁵I, whereas a window from 280 to 440 keV was used for ¹³¹I. Correlation factors to eliminate crossover of ¹³¹I activity into ¹²⁵I were determined by counting ¹³¹I standard in each window. The crossover of ¹²⁵I into the ¹³¹I channel was negligible. To determine the amounts and routes of excretion of radioactivity from the body, mice were housed in metabolic cages for 24 h after administration of the respective preparation. Urine and feces were collected for 6 and 24 h postinjection, and the radioactivities were determined.

Subcellular Distribution of the Radioactivity. The subcellular distribution of the radioactivity in the kidney was determined 10 and 30 min after administration of 20 μ g of [¹³¹I]HML-IT-Fab, [¹²⁵I]HML-Fab, or [¹²⁵I]Fab, according to the procedures described previously (27), with slight modifications. At the indicated times, murine kidneys were isolated, minced, and homogenized in 4 vol of ice-cold 0.25 M sucrose buffered with 10 mM phosphate buffer (pH 7.4) with a Dounce homogenizer by hand (20 strokes). This was followed by two final strokes in an ice-cold Potter–Elvehjem homogenizer with a Teflon pestle at 800 rpm. The resultant 20% homogenate was centrifuged twice for 5 min each time at 340g at 4 °C. The isolated supernatant was layered on top of iso-osmotic (0.25 M sucrose) Percoll (Pharmacia Biotech Co. Ltd.) at a density of 1.08 g/mL. After centrifugation at 20000g (RP 30 rotor, Hitachi Co. Ltd., Tokyo) for 90 min at 4 °C, the gradient (1.02–1.14 g/mL) was collected in 14 fractions before analyses of β -galactosidase (28) and alkaline phosphodiesterase I (29) activities, density, and the radioactivity of each fraction.

Radiolabeled Species in the Kidney. The radio-labeled species remaining in the kidney at 10 and 30 min postinjection of each preparation were analyzed according to the procedure described previously (27). Briefly, each [^{131/125}I]-labeled Fab (20 μ g) was administered intravenously into mice. At 10 and 30 min postinjection, both kidneys were perfused in situ with cold 0.1 M Tris-citrate buffer (pH 6.5) containing 0.15 M NaCl, 0.02% sodium azide, 1 TIU/mL aprotinin, 2 mM benzamidine-HCl, 2 mM iodoacetamide, 1 mM phenylmethanesulfonyl fluoride, and 5 mM diisopropyl fluorophosphate before being removed. Each tissue sample was placed in a test tube and subjected to three cycles of freezing (dry ice–acetone bath) and thawing. After addition of 5 vol of the same buffer containing an additional 35 mM β -octyl-glucoside, the samples were homogenized with a Polytron homogenizer (PT10-35, Kinematica GmbH Littau, Switzerland) at full speed with three consecutive 30-s bursts on ice before centrifugation at 48000g for 20 min at 4 °C (Himac CS-120 Centrifuge, Hitachi Co. Ltd.). The supernatant

was separated from the pellet, and the radioactivity was counted. The kidney supernatant was analyzed immediately by SE-HPLC after filtration through a polycarbonate membrane with a pore diameter of 0.45 μm (Nacalai Tesque). Each sample was also analyzed immediately by RP-HPLC and TLC after ultrafiltration with a 10 kDa cutoff membrane (Microcon-10, Amicon Grace, Tokyo). TLC plates were developed with a mixture of chloroform, methanol, and water (15:8:1). Under these conditions, protein-bound radioactivity, free iodine, *m*-iodohippuric acid, and *m*-iodobenzoic acid had R_f values of 0, 0.1, 0.45, and 0.65, respectively.

Radiolabeled Species in the Urine. The radio-labeled species excreted in the urine for 6 h postinjection of each $^{131}\text{I}/^{125}\text{I}$ -labeled Fab were analyzed by SE-HPLC after filtration through a polycarbonate membrane with a pore diameter of 0.45 μm (Nacalai Tesque). After ultrafiltration with a 10 kDa cutoff membrane (Microcon-10), the urine samples were also analyzed by RP-HPLC and TLC developed with a mixture of chloroform, methanol, and water (15:8:1) for ^{131}I HML-IT-Fab and ^{125}I HML-Fab and by TLC developed with (a) a mixture of methanol and acetic acid (100:1) or (b) a mixture of methanol and 10% ammonium acetate (10:1) for ^{125}I Fab (30). In point a, monoiodotyrosine and free iodine had R_f values of 0.5 and 0.75, whereas in point b, R_f values were 0.55 and 0.80, respectively.

Radiolabeled Species in the Plasma. Blood samples were taken at 10 and 30 min after injection of ^{131}I HML-IT-Fab, ^{125}I HML-Fab, or ^{125}I Fab. Each sample was centrifuged at 800g for 15 min at 4 °C. The supernatant of each sample was analyzed by SE-HPLC after filtration through a polycarbonate membrane with a pore diameter of 0.45 μm (Nacalai Tesque).

RESULTS

Preparation of Radioiodinated Fab Fragments.

Treatment of Fab fragments with 2-IT or 2-ME introduced 3.65 or 3.67 thiol groups per molecule of Fab, respectively, as determined by 2,2'-dipyridyl disulfide. Conjugation of $^{131}\text{I}/^{125}\text{I}$ HML with Fab fragments was performed by reacting the thiolated Fab fragments with maleimide groups of $^{131}\text{I}/^{125}\text{I}$ HML. After purification by the centrifuged column procedure, ^{131}I HML-IT-Fab was obtained with a radiochemical purity and specific activity of 98.5% and 62.5 $\mu\text{Ci}/\text{mg}$, respectively, as determined by SE-HPLC. Radiochemical purities and specific activities were 99.3% and 192 $\mu\text{Ci}/\text{mg}$ for ^{125}I HML-Fab, and 94.9% and 640 $\mu\text{Ci}/\text{mg}$ for ^{125}I Fab, respectively.

Biodistribution of Radioiodinated Fab Fragments. Two independent biodistribution studies were performed to achieve the results shown in Figure 4. One was performed after simultaneous administration of a mixed solution of ^{131}I HML-IT-Fab and ^{125}I HML-Fab and the other was performed after injection of directly radioiodinated Fab (^{125}I Fab). ^{125}I Fab showed the lowest radioactivity levels in the blood among the three, whereas the two HML-conjugated Fab fragments registered similar radioactivity levels. In the kidney, the two HML-conjugated Fab fragments showed much lower radioactivity levels than those of ^{125}I Fab. Although ^{125}I Fab showed the highest radioactivity levels (65% injected dose per gram; ID/g) in the kidney at 30 min postinjection, ^{131}I HML-IT-Fab and ^{125}I HML-Fab had no radioactivity peaks. ^{125}I HML-Fab demonstrated significantly higher radioactivity levels than those of ^{131}I HML-IT-Fab in the kidney at 10 and 30 min postinjection. Notable differ-

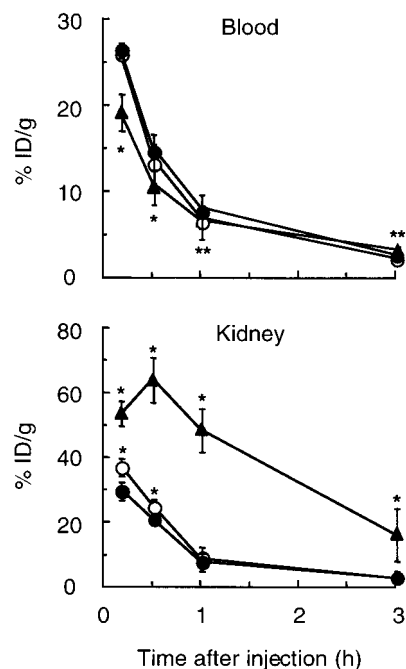


Figure 4. Biodistributions of radioactivity in mice after injection of ^{131}I HML-IT-Fab (●), ^{125}I HML-Fab (○), and ^{125}I Fab (▲). Significance was determined by unpaired Student's *t*-test [(*) $p < 0.01$; (**) $p < 0.05$ compared to ^{131}I HML-IT-Fab].

ences were not observed in other tissues, except for the uptake in the stomach observed with ^{125}I Fab (data not shown).

Subcellular Distribution of the Radioactivity.

Percoll density gradient centrifugation profiles of the radioactivity at 10 and 30 min after injection of ^{131}I HML-IT-Fab, ^{125}I HML-Fab, and ^{125}I Fab are shown in Figure 5. Both ^{131}I HML-IT-Fab and ^{125}I HML-Fab indicated major radioactivity peaks only at the density of 1.04–1.05 mg/mL at both 10 and 30 min postinjection. On the other hand, ^{125}I Fab showed migration of the radioactivity peak from the fraction with the density of 1.04–1.05 mg/mL to the fraction with that of 1.10 mg/mL from 10 to 30 min postinjection, respectively. The radioactivity peak at a density of 1.04–1.05 mg/mL was copurified with alkaline phosphodiesterase I activity, whereas that at a density of 1.10 mg/mL was copurified with β -galactosidase activity.

Radiolabeled Species in the Kidney. The supernatant of each kidney homogenate was extracted with efficiencies of over 90% for all experiments. The radio-labeled species in the kidney at 10 and 30 min postinjection of radioiodinated Fab fragments were analyzed by SE-HPLC (Figure 6). Although the kidney supernatants at both 10 and 30 min after injection of ^{131}I HML-IT-Fab showed only two radioactivity peaks at retention times of 16–17 and 31 min, the kidney supernatants of ^{125}I HML-Fab showed numerous radioactivity peaks. The majority of the radioactivity in the kidney supernatant after administration of ^{125}I Fab was detected at a retention time similar to that of intact ^{125}I Fab at both postinjection time points. Figure 7 shows RP-HPLC and TLC radioactivity profiles of the kidney supernatant at 10 min after injection of ^{131}I HML-IT-Fab or ^{125}I HML-Fab. The majority of the radioactivity in the kidney had a retention time of 8 min on RP-HPLC and R_f value of 0.45 on TLC, which were identical to those of *m*-iodohippuric acid (Figure 7, panels A and B). On the other hand, the kidney supernatants of ^{125}I HML-Fab showed

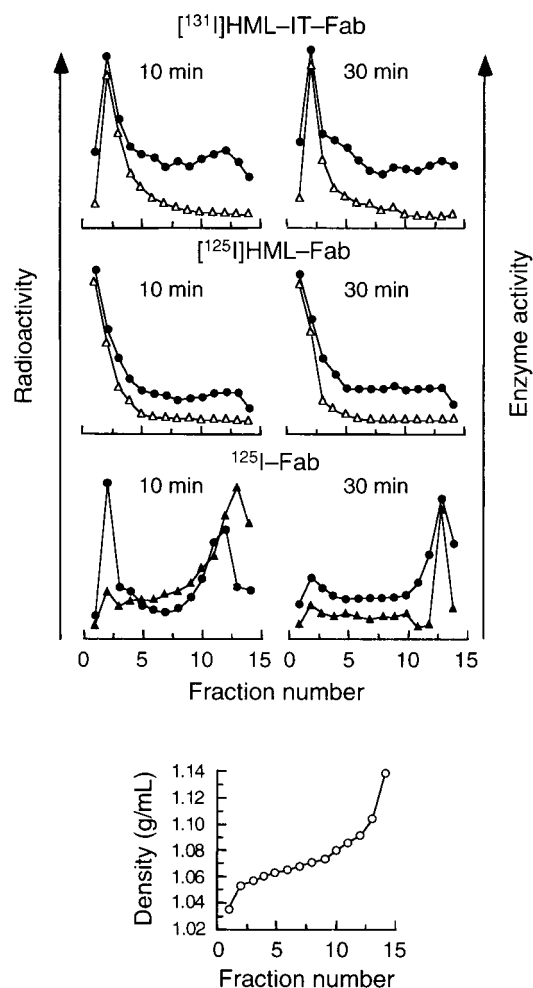


Figure 5. Subcellular fractionation of the radioactivity (●) in the kidney at 10 and 30 min after injection of radioiodinated Fab fragments. Gradients were collected in 14 fractions from the top of the gradient, assayed for the lysosomal enzyme β -galactosidase (▲) and the plasma membrane enzyme alkaline phosphodiesterase I (Δ), and density was measured (○).

two radioactivity peaks at retention times of 6 and 8 min on RP-HPLC and R_f values of 0.1 and 0.45 on TLC, respectively (Figure 7, panels C and D). Similar radiochromatograms were obtained when the kidney supernatant obtained 30 min after injection of radioiodinated Fab fragments was analyzed (data not shown).

Radiolabeled Species in the Urine. Figure 8 shows radiochromatograms of urine samples obtained for 6 h postinjection of $[^{131}\text{I}]\text{HML-IT-Fab}$, $[^{125}\text{I}]\text{HML-Fab}$, or $[^{125}\text{I}]\text{-Fab}$. On SE-HPLC analyses, the majority of the radioactivity was observed in the low molecular weight fraction with a retention time of 31 min for both $[^{131}\text{I}]\text{HML-IT-Fab}$ and $[^{125}\text{I}]\text{HML-Fab}$ (Figure 8, panels A and C). On RP-HPLC, most of the radioactivity in the urine samples was observed with a retention time (8 min) identical to that of *m*-iodohippuric acid for both $[^{131}\text{I}]\text{HML-IT-Fab}$ and $[^{125}\text{I}]\text{HML-Fab}$ (Figure 8, panels B and D). This was confirmed by TLC analyses of the urine samples where most of the radioactivity was observed with an R_f value identical to that of *m*-iodohippuric acid (data not shown). On the other hand, two radioactivity peaks were observed at retention times of 16–17 and 24 min on SE-HPLC analysis for $[^{125}\text{I}]\text{Fab}$ (Figure 8E). Most of the radioactivity was detected in the fraction with an R_f value (0.75) identical to that of free iodine (Figure 8F) on TLC analysis developed with solvent system a. TLC analysis using solvent system b also indicated that most

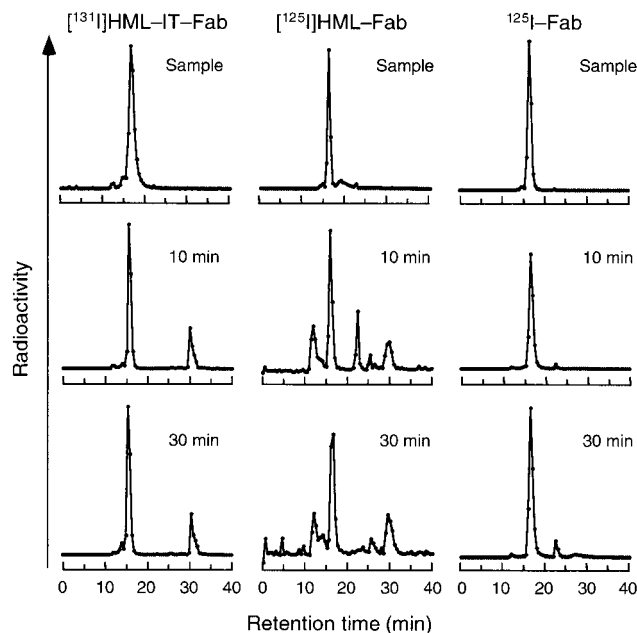


Figure 6. SE-HPLC radioactivity profiles of kidney homogenates at 10 and 30 min postinjection of radioiodinated Fab fragments. Under these conditions, Fab (50 kDa), cytochrome C (13 kDa), aprotinin (6 kDa), and *m*-iodohippuric acid (305 Da) had retention times of 16, 21, 28.5, and 31 min, respectively.

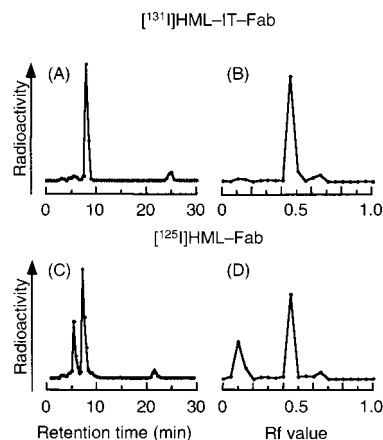


Figure 7. Radioactivity profiles of kidney homogenates at 10 min after injection of HML-conjugated Fab fragments by RP-HPLC (A and C) and TLC (B and D). Samples were analyzed after filtration through a 10 kDa cutoff membrane. Under these conditions, *m*-iodohippuric acid had a retention time and R_f value of 8 min and 0.45, respectively.

of the radioactivity was present in the fraction with an R_f value identical to that of free iodine (data not shown). The radiochromatograms of urine samples obtained for 24 h after injection of radioiodinated Fab fragments were similar to those obtained for 6 h after injection (data not shown). For 24 h after injection of radioiodinated Fab fragments, only 2–3% of the injected radioactivity was excreted in the feces, whereas the majority of the radioactivity excreted from the body was recovered in the urine (60–70% of the injected radioactivity).

Radiolabeled Species in the Plasma. Figure 9 shows SE-HPLC radiochromatograms of plasma at 10 and 30 min after injection of radioiodinated Fab fragments. Both HML-conjugated Fab fragments showed a new radioactivity peak in the high molecular weight fraction along with intact Fab fragments. The radiochromatogram of $[^{125}\text{I}]\text{Fab}$ showed a small increase in the radioactivity at higher and lower molecular weight fractions at 30 min.

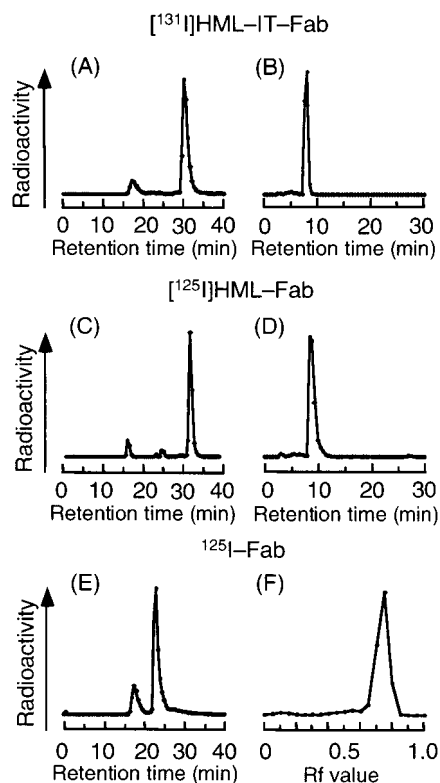


Figure 8. Radioactivity profiles of urine collected 6 h after injection of radiolabeled Fab fragments by SE-HPLC (A, C, and E), RP-HPLC (B and D), and TLC (F). Urine samples were analyzed after filtration through a polycarbonate membrane with a pore diameter of $0.45\ \mu\text{m}$ (A, C, and E) and after filtration through a 10 kDa cutoff membrane (B, D, and F). Under these conditions, $^{125}\text{I}^-$ had a retention time and R_f value of 23 min and 0.75, respectively.

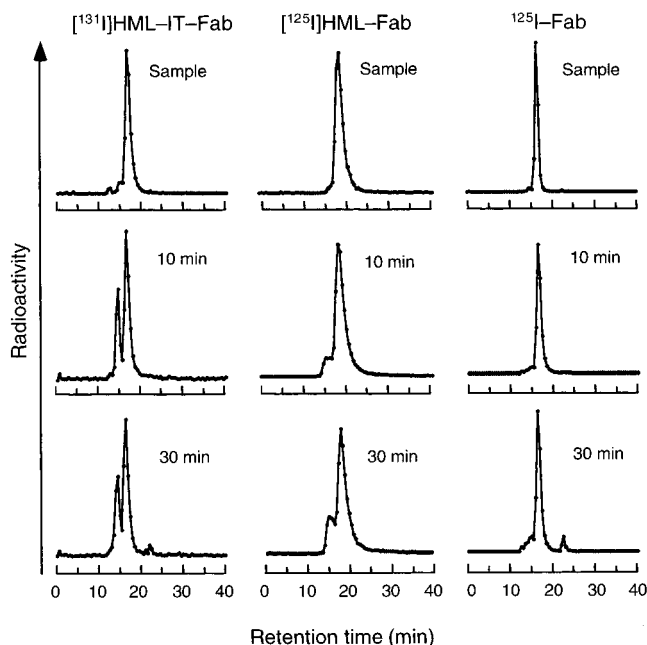


Figure 9. SE-HPLC radioactivity profiles of mouse plasma at 10 and 30 min after injection of radiolabeled Fab fragments.

DISCUSSION

In this study, renal metabolism of HML-conjugated Fab fragments prepared by different thiolation chemistries was compared to understand the mechanisms responsible for the low renal radioactivity levels of HML-conjugated Fab fragments. Our previous study showed

that the spacer structure between the parental antibody molecule and the substrate (ester bonds) plays an important role in the esterase-mediated hydrolysis of ester bonds in radioimmunoconjugates (20). HML-conjugated glycoproteins prepared by different thiolation chemistries were also shown to liberate *m*-iodohippuric acid as the sole radiometabolite at similar rates after lysosomal proteolysis in hepatic parenchymal cells (21). On the basis of these findings, if both $[^{131}\text{I}]\text{HML-IT-Fab}$ and $[^{125}\text{I}]\text{HML-Fab}$ were metabolized primarily in the lysosomes of the renal cells, both radioiodinated Fab fragments would liberate *m*-iodohippuric acid as the sole radiometabolite at similar rates. If either $[^{131}\text{I}]\text{HML-IT-Fab}$ or $[^{125}\text{I}]\text{HML-Fab}$ liberates *m*-iodohippuric acid on the membrane fractions of renal cells by the action of brush border enzymes, different renal radioactivity levels and radiometabolites would be observed between the two HML-conjugated Fab fragments in the kidneys after administration. $[^{125}\text{I}]\text{Fab}$ fragments produced by direct radioiodination were used as controls to estimate lysosomal proteolysis of the antibody molecules since radiometabolites from radioiodinated proteins are rapidly eliminated from lysosomes (31–33).

In biodistribution studies, $[^{125}\text{I}]\text{Fab}$ reached its maximum renal radioactivity levels at 30 min postinjection (Figure 4). The radioactivity in the renal cells migrated from the membrane to the lysosomal fraction from 10 to 30 min after injection of $[^{125}\text{I}]\text{Fab}$ (Figure 5). In addition, SE-HPLC analyses of the kidney homogenates after injection of $[^{125}\text{I}]\text{Fab}$ showed that while most of the radioactivity was detected as intact $[^{125}\text{I}]\text{Fab}$ at 10 min postinjection, small amounts of lower molecular weight radiometabolites were observed at 30 min postinjection (Figure 6). These findings suggested that Fab fragments would be transported and metabolized in the lysosomes of murine renal cells 30 min after injection into mice.

Both $[^{125}\text{I}]\text{HML-Fab}$ and $[^{131}\text{I}]\text{HML-IT-Fab}$ showed significantly lower renal radioactivity levels than those of $[^{125}\text{I}]\text{Fab}$ as early as 10 min postinjection, as shown in Figure 4. The majority of the renal radioactivity was observed in the membrane fractions at both 10 and 30 min after injection of the two HML-conjugated Fab fragments (Figure 5). HPLC analyses of the kidney homogenates at 10 min postinjection indicated generation of *m*-iodohippuric acid as the major radiometabolite following administration of the two HML-conjugated Fab fragments (Figures 6 and 7). The absence of *m*-iodohippuric acid in the blood at 10 min after injection of both $[^{125}\text{I}]\text{HML-Fab}$ and $[^{131}\text{I}]\text{HML-IT-Fab}$ (Figure 9) indicated that the radiometabolite would be generated not in the blood but in the kidneys, as shown by our previous study (15). These findings supported our previous hypothesis that HML-conjugated Fab fragments released *m*-iodohippuric acid at the membrane before being reabsorbed and transported to the lysosomal compartment of the renal cells (Figure 2B).

However, renal analyses demonstrated generation of different radiometabolites between the two HML-conjugated Fab fragments in the kidneys. While $[^{131}\text{I}]\text{HML-IT-Fab}$ registered both intact Fab and *m*- $[^{131}\text{I}]\text{iodohippuric acid}$ at 10 min postinjection, generation of additional radiometabolites was observed with $[^{125}\text{I}]\text{HML-Fab}$ at the same postinjection time point (Figure 7, panels C and D). Since *m*-iodohippuric acid was detected as the major radiometabolite in the urine after injection of both $[^{125}\text{I}]\text{HML-Fab}$ and $[^{131}\text{I}]\text{HML-IT-Fab}$ (Figure 8), the unidentified radiometabolites derived from $[^{125}\text{I}]\text{HML-Fab}$ would include intermediate species containing the HML moiety.

The majority of the radioactivity was present in the membrane fraction of the renal cells at this postinjection time point of the two HML-conjugated Fab fragments (Figure 5). Thus, while [^{131}I]HML-IT-Fab would liberate *m*-iodohippuric acid selectively, a few sequential reactions would be required to release the final radiometabolite, *m*-iodohippuric acid, from [^{125}I]HML-Fab, which was reflected in the small but significant differences in renal radioactivity levels between [^{131}I]HML-IT-Fab and [^{125}I]HML-Fab at early postinjection time points (Figure 4). The glycyl-lysine sequence in [^{125}I]HML-Fab probably hindered enzyme access to a greater extent compared with that in [^{131}I]HML-IT-Fab due to steric interference of the parental Fab fragments as also reported previously using a radioiodination reagent with an ester bond to release *m*-iodohippuric acid from polypeptides (13, 20). Generation of the intermediate radiometabolites from [^{125}I]HML-Fab suggested that brush border enzymes may have cleaved some amino acid sequences that were exposed by reducing and alkylating the disulfide bonds in the Fab fragments. These findings also indicated that it is necessary to consider the potential differences in metabolism between the two commonly used thiolation chemistries when conjugating radiolabels or other chemicals to antibodies.

In conclusion, the findings of this study provided further evidence that the low renal radioactivity levels of HML-conjugated Fab from early postinjection times are attributable to rapid release of *m*-iodohippuric acid by the action of brush border enzymes before the fragments are incorporated into renal cells. The findings of this study also reinforced the suggestion that chemical design of radiolabeling reagents that liberate radiometabolites showing urinary excretion by the action of brush border enzymes would constitute an attractive strategy to reduce renal radioactivity levels from early postinjection times. This study suggested that chemical design of radiolabeling agents that liberate radiometabolites that are excreted into the urine from renal tubules by renal brush border enzymes but are retained in the lysosomes by lysosomal proteolysis would be useful to achieve long-term and selective target radioactivity levels using antibody fragments or peptides as vehicles regardless of whether the parental molecules are internalized into target cells.

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Chemistry of Sulforhodamine–Amine Conjugates

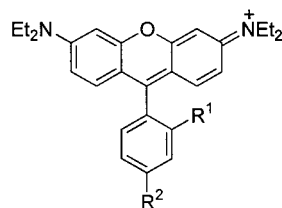
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National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K. Received July 6, 2000; Revised Manuscript Received November 30, 2000

Commercially-available sulforhodamine sulfonyl chlorides contain two isomeric monosulfonyl chlorides. Conjugates of these isomers with amines have different properties because the sulfonamide formed from one isomer can undergo ring-closure to a colorless sultam. This chemistry has been examined for a model conjugate with methylamine and for a bioconjugate with 2'(3')-O-[N-(2-aminoethyl)-carbamoyl]ATP. The interaction of each isomer of the latter conjugates with myosin subfragment 1 has been characterized. Significant differences between the two isomers are observed in these interactions.

INTRODUCTION

Rhodamine dyes are used extensively for conjugation with biomolecules because of their excellent fluorescence properties. Among the most commonly used reactive dyes for amine labeling are the sulforhodamine chlorides, principally Sulforhodamine B¹ sulfonyl chloride **1a** and Texas Red sulfonyl chloride **2a**. We became interested



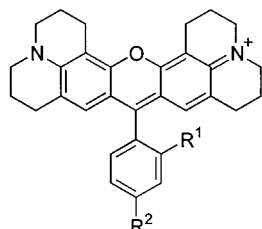
1a R¹, R² = SO₂Cl, SO₃⁻

1b R¹, R² = SO₃⁻

1c R¹ = SO₂Cl, R² = SO₃⁻

1d R¹ = SO₃⁻, R² = SO₂Cl

1e R¹, R² = SO₂Cl

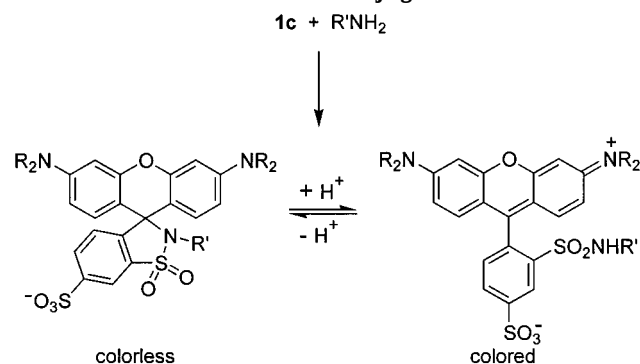


2a R¹, R² = SO₂Cl, SO₃⁻

2b R¹, R² = SO₃⁻

in these dyes for preparation of ribose-modified fluorescent nucleotides (1, 2) but were intrigued to observe that treatment of commercial SRB–SC² **1a** with an excess of a primary amine reduced the intensity of the visible absorption band by ~50%. The color change was reversible upon acidification. For the product obtained from reaction with methylamine, the color was highest at pH 4 and lowest at pH 9. To explain this observation, it is necessary to consider the structure of the starting sulfonyl chloride. Compounds **1a** and **2a** are prepared from the corresponding bis-sulfonates **1b** and **2b**, typically by treatment with POCl₃ or PCl₅. The original report (3) of Texas Red sulfonyl chloride recognized the probability that two isomeric monosulfonyl chlorides were present. SRB–SC dates from a 1958 paper (4) in which isomerism

Scheme 1. Formation and Ring-Chain Tautomerism of Sulforhodamine–Amine Conjugates



was not considered, although more recent reports (5, 6) have discussed the presence of two isomers.

The presence of a sulfonyl chloride ortho to the xanthylium ring system, as in isomer **1c**, presents the opportunity for spiro-sultam formation upon reaction with a primary amine (Scheme 1) and the spiro-sultam (as shown), in which the extended rhodamine chromophore is absent, is expected to be colorless. However, these rhodamine spiro-sultams are capable of ring-opening under appropriate pH conditions. In fact, these properties of sulforhodamine spiro-sultams were first recorded in qualitative terms 100 years ago (7), and the color change in either direction has been exploited in patents related to photographic (8) and thermal (9) imaging. Despite these precedents, the origin of this pH-dependent behavior continues to be subject to misinterpretation (e.g., ref 10), evidently based on an assumption that amine conjugation takes place only on the sulfonyl group para to the xanthylium ring, i.e., reaction with isomer **1d**. We now describe a detailed study of these phenomena, with particular focus on the properties of amine conjugates derived from the different isomers and the consequences of the different structures for biological applications of these dyes. The investigation covers a conjugate of SRB–SC with methylamine as a convenient model, a full characterization of the chemistry of the recently described Rhodamine Red-X reagent **7** (11), and studies of ribose-modified ATP conjugates derived from the two different isomers of SRB–SC. The results with the ATP conjugates (and with similar GTP conjugates not reported

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¹ Sulforhodamine B **1b** is also known as Lissamine Rhodamine B.

² Abbreviation: SRB–SC, sulforhodamine B sulfonyl chloride; ED–ATP, 2'(3')-O-[N-(2-aminoethyl)carbamoyl]ATP.

here) are particularly relevant to our previous work in which these analogues were used in fluorescence energy transfer measurements (1, 2). In the earlier of those studies (1), we had assumed that only the conjugate formed from isomer **1d** was present in the material used for fluorescence studies (i.e., that it was homogeneous and not subject to variations of fluorescence induced by pH changes). The present work confirms that two isomers are formed in the synthesis but are well separated by the purification protocol. The interactions reported here of the ATP conjugates with myosin subfragment 1 reveal differences in fluorescence responses for the two isomers. We note that the ring-chain tautomerization chemistry described here is relevant only to conjugates with primary amines, as similar conjugates with secondary amines cannot cyclize.

MATERIALS AND METHODS

SRB–SC **1a** (lots 1141-1 and 1161-2) and Rhodamine Red-X succinimidyl ester **7** (lot 4462) were from Molecular Probes, Eugene OR. ED-ATP² **11** was prepared as described (12). Myosin subfragment 1 was prepared by the method of Weeds and Taylor (13). Reduced Triton X-100 and DEAE-cellulose anion exchanger were from Sigma. Silica gel for flash chromatography was Merck type 9385. All other materials were from Aldrich and used without further purification. TLC was performed on Merck 60F silica gel plates.

NMR spectra were recorded on a Bruker AM400 spectrometer for solutions in CDCl₃ (unless otherwise specified) and referenced to internal tetramethylsilane. Electrospray mass spectra were determined at low resolution on a VG Platform instrument and the FAB mass spectrum at high resolution was determined on a VG ZAB–SE instrument. UV–Vis spectra were determined on a Beckman DU70 instrument. Analytical reversed-phase HPLC was carried out on a Novapak C18 column (3.9 × 150 mm) eluted isocratically with 100 mM potassium phosphate, pH 6.8, containing 25% acetonitrile (v/v) at 0.5 mL min⁻¹. Steady-state fluorescence intensity measurements were made on an SLM 8000S fluorimeter. Excitation was at 572 nm and emitted light was monitored through a monochromator at 585 nm. Fluorescence anisotropy titrations were carried out on the same instrument but with the use of Glan-Thompson polarizers. The data were fitted to eq 1:

$$A_{\text{obs}} = A_L + (A_{\text{EL}} - A_L) \frac{[\text{EL}]}{[\text{L}_T]} \quad (1)$$

where $[\text{EL}] = 0.5[(K + [\text{E}_T] + [\text{L}_T]^2) \pm \sqrt{(K + [\text{E}_T] + [\text{L}_T]^2)^2 - 4[\text{E}_T][\text{L}_T]}]$, A_{obs} = observed anisotropy, A_L = anisotropy of free ligand, A_{EL} = anisotropy of enzyme-ligand complex, $[\text{E}_T]$ = total enzyme concentration, $[\text{L}_T]$ = total ligand concentration, and K = equilibrium dissociation constant.

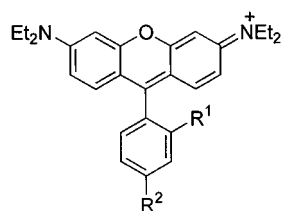
Stopped-flow measurements were made on a Hi-Tech Scientific MX61 instrument operating in absorbance mode for studies of pH changes with compound **3** (wavelength 567 nm) or fluorescence mode for studies with the conjugates **12–15** (excitation at 530 nm; emission monitored through a Wratten 21 cutoff filter that passed light above 540 nm). For experiments on the interaction of myosin subfragment 1 with **12–15**, all concentrations quoted are those after mixing so the syringe concentrations were twice these values. Data were analyzed using Hi-Tech software. Mean values of rate constants and amplitudes with their standard deviations

for stopped-flow data were obtained from analysis of 4–6 reactions but single data sets are shown in the Figures.

Buffer solutions for measurements in aqueous ethanol were prepared from water-ethanol (3:1 v/v) solutions of appropriate acids at the molarities specified and adjusted to the required pH values by addition of NaOH. pH values were determined with a glass electrode referenced to buffer solutions in 100% aqueous solution.

NMR Spectroscopic Characterization of SRB–SC (1a). Two batches of commercial material were examined. In the first sample (lot 1141-1), two major families of signals were distinguishable because their relative intensity ratios were ~6:4. Selected single frequency decoupling experiments confirmed the assignment to one or other set for some of the signals. The first set of signals (A) were as follows (multiplicities are consistent within each set): δ 9.05 (d, J = 2.1 Hz, 1H), 8.12 (dd, J = 8.0 and 2.1 Hz, 1H), 7.36 (d, J = 8.0 Hz, 1H), 7.22 (d, J = 9.6 Hz, 2H), 6.83 (dd = 9.6 and 2.4 Hz, 2H), 6.74 (d, J = 2.4 Hz, 2H), 3.58 (q, J = 6.5 Hz, 8H) and 1.29 (t, J = 6.5 Hz, 12H). The second set of signals (B) had δ 9.00 (d, J = 1.5 Hz, 1H), 8.55 (dd, J = 7.8 and 1.5 Hz, 1H), 7.41 (d, J = 7.8 Hz, 1H), 7.08 (d, J = 9.5 Hz, 2H), 6.90 (dd, J = 9.5 and 2.3 Hz, 2H), 6.79 (d, J = 2.3 Hz, 2H), 3.64 (q, J = 6.5 Hz, 8H) and 1.35 (t, J = 6.5 Hz, 12H). A third, minor set of signals (C) was present in this sample, but was much more prominent in a second batch of material (lot 1161-2). These signals, for which the line shapes were broader than those of the other two sets, were at δ 8.93 (d, J = 1.6 Hz, 1H), 8.77 (dd, J = 8.0 and 1.6 Hz, 1H), 8.27 (d, J = 8.0 Hz, 1H), 7.04 (d, J = 9.2 Hz, 2H), 6.97 (dd, J = 9.2 and 2.1 Hz, 2H) and 6.87 (d, J = 2.1 Hz, 2H). The signals from the ethyl side chains were not resolved from those of the other two components. In this sample the relative intensity of the signals in sets A, B, and C was approximately 18:44:38.

Reaction of SRB–SC with Methylamine: Compounds 3 and 4. A solution of SRB–SC (lot 1141-1; 100



3 $R^1 = \text{SO}_2\text{NHMe}$, $R^2 = \text{SO}_3^-$

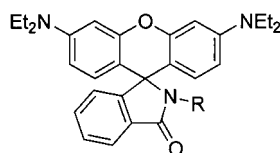
4 $R^1 = \text{SO}_3^-$, $R^2 = \text{SO}_2\text{NHMe}$

mg, 0.17 mmol, in acetone (100 mL) was mixed with 40% aq. methylamine (20 mL) and concentrated under reduced pressure to remove most of the acetone. The remaining solution was diluted with water to ~50 mL, filtered to remove some insoluble material and lyophilized. The residue was dissolved in 50% aqueous ethanol (20 mL) and quantified by spectrophotometry of a solution at pH 4. The total rhodamine concentration was 7.08 mM (83% recovery) but fell to 4.12 mM after storage for 48 h at 4 °C because part of the solute precipitated. In this mixture the absorbance ratio at 567 nm in solutions at pH 4 and 9 was 3.6:1, which was taken to indicate that the relative proportion of compounds **3** and **4** present in solution was 2.6:1. This material was used without further processing for most of the experiments described below. For experiments in later work where pure **3** was required, a portion of the stock solution, containing 45 μmol mixed rhodamines, was lyophilized and the residue

was dissolved in MeOH and absorbed onto silica gel (1 g). This was added to a packed flash chromatography column, that was eluted with a step gradient of CHCl₃-MeOH (from 97.5:2.5 to 92:8; total volume 1200 mL). Fractions were analyzed by UV-vis spectroscopy at pH 4 and 9 and the main pH-responsive fraction (26 μ mol) was combined and evaporated. This material retained a trace contaminant ($\sim$ 3%; λ_{max} 558 nm) that was not decolorized at pH 9 and interfered with the required measurements. It was removed by repeated preparative TLC (silica gel; CHCl₃-MeOH 85:15) to give pure **3** (16.5 μ mol); ¹H NMR (MeOH-*d*₄-NaOD) δ 8.18 (d, *J* = 1.3 Hz, 1H), 7.90 (dd, *J* = 8.2 and 1.3 Hz, 1H), 6.99 (d, *J* = 8.2 Hz, 1H), 6.65 (d, *J* = 8.5 Hz, 2H), 6.32–6.35 (m, 4H), 3.29 (q, *J* = 7.0 Hz, 8H), 2.32 (s, 3H), and 1.07 (t, *J* = 7.0 Hz, 12H). The addition of NaOD was made to drive the compound into its colorless sultam form. In MeOH-*d*₄ alone the spectrum showed a mixture of the open and closed forms in $\sim$ 1:1 ratio. ES-MS, *m/e* (*M* + *H*) calcd for C₂₈H₃₃N₃O₆S₂ + *H*, 572; found, 572.

Ring Opening and Closing Kinetics of 3. For stopped-flow measurements starting from high pH, the **3**+**4** mixture was diluted to 9 μ M (i.e., 6.5 μ M **3**, 2.5 μ M **4**) in 5 mM sodium borate, pH 9.0, and for measurements in the opposite direction it was diluted in 5 mM sodium citrate, pH 4.0. In each case, these starting solutions were mixed in the stopped-flow apparatus with an equal volume of 100 mM MOPS, pH 7.0. All concentrations are premixing values. Buffer solutions were prepared in water-EtOH (3:1 v/v) as described above and experiments were conducted at 4 °C. The observation light traversed a 1-cm path length. Transmission data were converted to absorbance and analyzed as the best fit to single exponentials.

3',6'-Bis(diethylamino)-2-(2,3-dihydroxypropyl)-spiro[1*H*-isoindole-1,9'-[9*H*]xanthen]-3(2*H*)-one (6).



5 R = Me

6 R = CH₂CH(OH)CH₂OH

A mixture of Rhodamine B (330 mg) and SOCl₂ (5 mL) was kept at room temperature for 18 h and excess reagent was removed under reduced pressure. The residue was dissolved in CHCl₃ (50 mL), cooled in ice and treated with a solution of 1-aminopropane-2,3-diol (1 g) and triethylamine (1.5 g) in DMF (50 mL). The solution was stirred at room temperature for 2 h, by which time most of the color was discharged. Solvents were evaporated in vacuo and a solution of the residue in CHCl₃ was washed with water, dried and evaporated. Flash chromatography (EtOAc-hexanes 80:20) and crystallization ($\times$ 3 from benzene-hexanes) gave lactam **6** (104 mg), mp 222–224 °C; UV (EtOH) λ /nm (ϵ /M⁻¹ cm⁻¹) 240 (56 900), 273 (33 400), and 314 (13 100); ¹H NMR (CDCl₃ after D₂O exchange) δ 7.88–7.92 (m, 1H), 7.36–7.50 (m, 2H), 7.11–7.13 (m, 1H), 6.36–6.40 (m, 4H), 6.25 and 6.28 (2 $\times$ dd, *J* = 8.8 and 2.4 Hz for each, 2 $\times$ 1H), 3.40 (dd, *J* = 12.2 and 3.2 Hz, 1H), 3.29–3.37 (m, 10H), 3.15 (dd, *J* = 14.4 and 6.1 Hz, 1H), 3.04–3.09 (m, 1H) and 1.16 and 1.17 (2 $\times$ t, *J* = 9 Hz, 12H); FAB-MS, *m/z* (*M* + *H*) calcd for C₃₁H₃₈N₃O₄ + *H*, 516.2880; found, 516.2880.

Spectroscopic Titrations. For sultam **3** the mixture of **3** + **4**, prepared as described above, was diluted to a

total rhodamine concentration of 8.3 μ M in various buffers. Aqueous ethanolic buffers (each 25 mM in buffer salt) were acetate, pH 4, phosphate, pH 7, and borate, pH 10. For measurements in the presence of detergent, the buffers (each 25 mM) were citrate, pH 2.1, and borate, pH 9, each containing 0.1% reduced Triton X-100 but without ethanol. In each case, solutions were mixed in various proportions to generate $\sim$ 20 solutions at different pH values. The pH of each solution was measured and the absorbance spectrum was recorded (10 mm path length). The 567 nm absorbance values were fitted to a single p*K* equation over the pH range 4–9. For the rhodamine-ATP conjugates **12** and **13** (see below), measurements were performed similarly in either aqueous or aqueous ethanol solutions, using 0.1 M buffers prepared from MES (pH 6.5), Tris (pH 8 and 8.5), CHES (pH 9.0, 9.5 and 10.0), CAPS (pH 10.5), and sodium phosphate (pH 12.0). Absorbance data were fitted to a single p*K* equation.

For the titration of **6**, 16.7 μ M solutions of the lactam were prepared in either hydrochloric acid, pH 1 or in 25 mM sodium phosphate, pH 7, each of which contained 25% ethanol (v/v) and $\sim$ 30 solutions were prepared by mixing the two stock solutions in varying proportions. The pH values were determined as above and absorbance spectra were recorded over the range 220–400 nm. Absorbance values at 275 and 320 nm were least-squares fitted to eq 2, using the Marquardt algorithm with software written in-house by Dr. S. R. Martin.

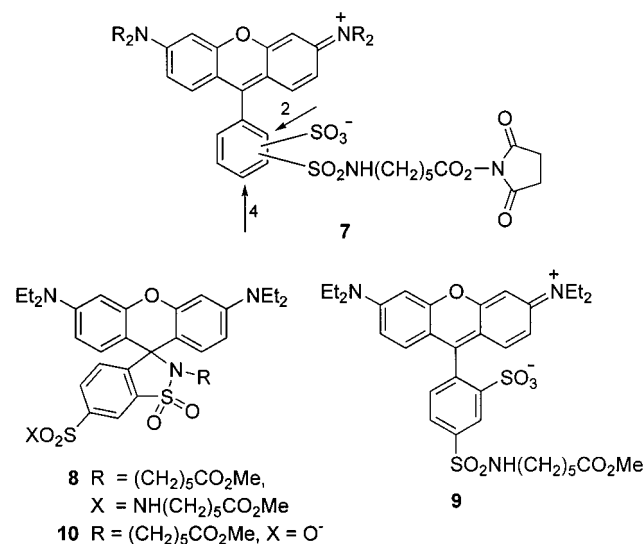
$$\text{Absorbance} = \epsilon_{\text{H}_2\text{L}}[\text{H}_2\text{L}^{2+}] + \epsilon_{\text{HL}}[\text{HL}^+] + \epsilon_{\text{L}}[\text{L}] \quad (2)$$

where $\epsilon_{\text{H}_2\text{L}}$, ϵ_{HL} , and ϵ_{L} are absorption coefficients for the diprotonated (H₂L²⁺), monoprotanated (HL⁺), and unprotonated (L) forms, respectively. The concentrations were calculated using eq 3:

$$[\text{L}] = \frac{L_{\text{Total}}}{1 + K_{\text{L}}[\text{H}^+] + K_{\text{L}}K_{\text{HL}}[\text{H}^+]^2} \quad (3)$$

where $K_{\text{L}} = [\text{HL}^+]/([\text{L}][\text{H}^+])$ and $K_{\text{HL}} = [\text{H}_2\text{L}^{2+}]/([\text{HL}^+][\text{H}^+])$.

Reaction of SRB-SC with Methyl 6-Aminohexanoate: Compounds 8, 9, and 10. A mixture of methyl



6-aminohexanoate hydrochloride (80 mg, 0.44 mmol) and triethylamine (0.12 mL) in chloroform (8 mL) was stirred in an ice-bath and SRB-SC (lot 1161–2; 200 mg, 0.35 mmol) was added in portions over 5 min. The solution

was allowed to warm to room temperature and stirred overnight, then diluted to 50 mL with chloroform. For initial analysis, an aliquot was diluted in ethanol ($\times 50$) and further diluted into buffers at pH 4 and 9. The absorbance ratio (567 nm) at low and high pH was 1.54.

The chloroform solution was washed with water (3×30 mL) and dried, and the solvent was removed. TLC analysis (CHCl_3 -MeOH 95:5) showed three main spots, R_f 0.70, 0.25, and 0.10. Flash chromatography with a step gradient from 0 to 7.5% MeOH in CHCl_3 (total volume 850 mL) gave the first two components essentially pure (TLC), while the third was contaminated with a trace of slightly less polar material. Visible spectroscopy at pH 4 showed recoveries of 56, 33, and 24 μmol for the highest, middle and lowest mobility compounds, respectively. The total recovery based on the starting rhodamine was 32%. At pH 9, the 567 nm absorption of the highest and lowest mobility compounds was essentially zero, while that of the middle fraction was the same as at pH 4.

The highest mobility fraction was 3',6'-bis(diethylamino)-2-(5-methoxycarbonyl-1-pentyl)-6-[(5-methoxycarbonyl-1-pentyl)amino]sulfonyl]spiro[1,2-benzisothiazole-3(2H)-9'-[9H]xanthene]-1,1-dioxide **8**; ^1H NMR δ 8.34 (d, $J = 1.8$ Hz, 1H), 7.88 (dd, $J = 8.2$ and 1.8 Hz, 1H), 7.13 (d, $J = 8.2$ Hz, 1H), 6.82 (d, $J = 9.7$ Hz, 2H), 6.35–6.37 (m, 4H), 4.58 (t, $J = 6.7$ Hz, 1H), 3.66 (s, 3H), 3.60 (s, 3H), 3.35 (q, $J = 7.1$ Hz, 8H), 3.01 (q, $J = 6.7$ Hz, 2H), 2.91 (t, $J = 7.3$ Hz, 2H), 2.30 (t, $J = 7.3$ Hz, 2H), 2.11 (t, $J = 7.6$ Hz, 2H), 1.49–1.63 (m, 6H), 1.31–1.41 (m, 6H) and 1.18 (t, $J = 7.1$ Hz, 12H); ES-MS, m/z ($M + H$) calcd for $\text{C}_{41}\text{H}_{56}\text{N}_4\text{O}_9\text{S}_2 + \text{H}$, 813; found 813.

The middle fraction was 3,6-bis(diethylamino)-9-[(4-[(5-methoxycarbonyl-1-pentyl)amino]sulfonyl]-2-sulfophenyl)xanthylum, inner salt **9**; ^1H NMR (CDCl_3 -MeOH- d_4 9:1) δ 8.68 (d, $J = 1.8$ Hz, 1H), 8.04 (dd, $J = 7.9$ and 1.8 Hz, 1H), 7.29 (d, $J = 7.9$ Hz, 1H), 7.19 (d, $J = 9.4$ Hz, 2H), 6.87 (dd, $J = 9.4$ and 2.4 Hz, 2H), 6.75 (d, $J = 2.4$ Hz, 2H), 3.69 (s, 3H), 3.57–3.64 (m, 8H), 3.02 (t, $J = 7.0$ Hz, 2H), 2.35 (t, $J = 7.5$ Hz, 2H), 1.66 (quintet, $J = 7.7$ Hz, 2H), 1.60 (quintet, $J = 7.6$ Hz, 2H), 1.38–1.45 (m, 2H) and 1.33 (t, $J = 7.1$ Hz, 12H); ES-MS, m/z ($M + H$) calcd for $\text{C}_{34}\text{H}_{43}\text{N}_3\text{O}_8\text{S}_2 + \text{H}$, 686; found, 686.

The least mobile fraction was 3',6'-bis(diethylamino)-2-(5-methoxycarbonyl-1-pentyl)spiro[1,2-benzisothiazole-3(2H)-9'[9H]xanthene]-1,1-dioxide-6-sulfonic acid **10**; ^1H NMR δ 8.68 (d, $J = 1.7$ Hz, 1H) 8.35 (dd, $J = 7.8$ and 1.7 Hz, 1H), 7.24 (d, $J = 7.8$ Hz, 1H), 7.19 (d, $J = 9.4$ Hz, 2H), 6.88 (dd, $J = 9.4$ and 2.1 Hz, 2H), 6.72 (dd, $J = 2.1$ Hz, 2H), 3.60 (s, 3H) superimposed on 3.57–3.66 (m, 8H), 2.89 (t, $J = 6.5$ Hz, 2H), 2.22 (t, $J = 7.4$ Hz, 2H), 1.50 (quintet, $J = 7.4$ Hz, 2H), 1.43 (quintet, $J = 7.0$ Hz, 2H), 1.32 (t, $J = 7.0$ Hz, 12H) and 1.18–1.26 (m, 2H); ES-MS, m/z ($M + H$) calcd for $\text{C}_{34}\text{H}_{43}\text{N}_3\text{O}_8\text{S}_2 + \text{H}$, 686; found, 686.

Rhodamine-ED-ATP Conjugates 12 and 13. The procedure here amplifies that given previously (2) and describes in detail the isolation of isomers **12** and **13**. A solution of ED-ATP (0.25 mmol) in 50 mM NaHCO_3 (25 mL) was mixed with a solution of SRB-SC (lot 1141-1; 0.5 mmol) in acetone (25 mL) and kept for 2 h at 20 $^\circ\text{C}$. A control reaction without ED-ATP was set up in parallel, and, at the end of the reaction time, each solution was analyzed by TLC [2-propanol-water- $\text{NH}_3(\text{aq})$ 7:2:1]. The control reaction had a single red spot, R_f 0.9, also present in the reaction mixture, but the latter had two additional spots, one at the origin and the other with R_f 0.2. The latter spot only became colored as ammonia evaporated from the TLC plate. The reaction mixture was concentrated under reduced pressure to remove the acetone, then diluted with 1.5 vol of water and applied to a DEAE-

cellulose column (34×150 mm, bicarbonate form). The column was eluted with a linear gradient of 0–1 M triethylammonium bicarbonate (total volume 3 L) and the eluate was monitored at 254 nm. Unreacted ED-ATP eluted at 0.27 M followed by two well-resolved peaks at 0.36 and 0.60 M that both contained the rhodamine fluorophore. On reverse-phase HPLC each peak eluted as partly resolved doublets because of isomerism arising from linkage through the 2'- or 3'-hydroxyl group (12, 21). The peak that eluted at 0.60 M TEAB (later assigned as **12**) had t_R 10.2 and 12.0 min while the peak at 0.36 M TEAB (later assigned as **13**) had t_R 7.9 and 8.6 min. The 2'/3'-isomers were not separated on a preparative scale in this work. Fractions containing the resolved components **12** and **13** were separately pooled and concentrated by rotary evaporation followed by further evaporation from methanol (3×30 mL) to remove buffer salts. Each residue was redissolved in water (5 mL) and the solutions were analyzed by TLC as above. The first and second fluorescent peaks eluted from the DEAE-cellulose column had R_f 0 and 0.2, respectively, and the latter spot was reversibly bleached on the TLC plate by exposure to ammonia vapor. By comparison with the behavior of the methylamine conjugate **3**, this was assigned structure **12** while the spot that remained at the origin was assigned as structure **13**. The yields were 13.5 and 66 μmol for **12** and **13** respectively (overall yield 32% based on ED-ATP). For ^1H NMR characterization, the triethylammonium salts were converted to Na^+ salts (Dowex 50) and the spectra were interpreted with the aid of 2D TOCSY and COSY spectra to correlate overlapping spin systems that arise from the presence of unresolved 2'- and 3'-isomers, together with previous data for related ED-ATP conjugates with Cy3 (21). However, complete assignment for each isomer was not possible. Reported intensities are consistent within the data for a particular isomer. For **13** (500 MHz, D_2O , acetone ref.), the 2'-isomer had assignable signals at δ 8.56 (d, $J = 1.8$ Hz, 1 H, xanthene H-3), 8.34 (dd, $J = 7.5$ and 1.8 Hz, 1 H, xanthene H-5), 7.92 (d, $J = 7.5$ Hz, 1 H, xanthene H-6), 6.04 (d, $J = 2.0$ Hz, 1 H, H-1'), 5.24 (dd, $J = 5.6$ and 2.0 Hz, 1 H, H-2'), 4.71–4.73 (m, 1 H, H-4'), 4.21–4.28 (m, 2 H, H-5'). The signal for H-3' was obscured under the HOD peak. The 3'-isomer had δ 8.55 (d, $J = 1.8$ Hz, 1 H, xanthene H-3), 8.29 (dd, $J = 8.0$ and 1.8 Hz, 1 H, xanthene H-5), 7.59 (d, $J = 8.0$ Hz, 1 H, xanthene H-6), 5.78 (d, $J = 6.0$ Hz, 1 H, H-1'), 5.14–5.16 (m, 1 H, H-3'), 4.81–4.84 (m, 1 H, H-2'), 4.25–4.45 (m, 1 H, H-4'), 4.13–4.26 (m, 2 H, H-5'). Signals not assignable to a specific isomer were at δ 8.33 and 8.18 (purine H), 6.60–6.99 (m, xanthene H-1' to H-8'), 3.50–3.66 (m, NCH_2CH_3), 3.20–3.46 (m, NCH_2CH_2), and 1.21–1.29 (NCH_2CH_3).

The spectrum of isomer **12** in pure D_2O was complex. This was shown to be due to noncovalent rhodamine dimerization at the concentration of the NMR sample, as revealed by absorption spectroscopy (0.1 mm path length) in the range 500–600 nm, that showed a spectrum typical for rhodamine dimers (22). Dimerization was not detected by absorption spectroscopy of **13**. Addition of 50% methanol to the aqueous solution of **12** abolished the dimer absorption spectrum, and therefore, the ^1H NMR spectrum was run in D_2O -methanol- d_4 (1:1 v/v). The 2'-isomer had assignable signals at δ 6.09 (d, $J = 2$ Hz, 1 H, H-1'), 5.31 (dd, $J = 5$ and 2 Hz, 1 H, H-2'), 4.71 (t, $J = 5$ Hz, 1 H, H-3'). The 3'-isomer had δ 6.02 (d, $J = 5$ Hz, 1 H, H-1'), 5.21 (d, $J = 4$ Hz, 1 H, H-3'), 4.94 (dd, $J = 5$ and 4 Hz, 1 H, H-2'). Signals not specifically assignable to either isomer were at δ 4.05–4.18 (m, H-4',5'), 3.35–3.60 (m, NCH_2CH_3), 3.05–3.30 (m, NCH_2 -

CH_2N), and 1.10–1.13 (m, NCH_2CH_3). The xanthene protons appeared as broad envelopes in the ranges δ 7.3–8.8 (H-3,5 and 6) and 6.4–7.1 (H-1' to H-8'), evidently because of exchange broadening between the open and closed forms of the rhodamine sultam, while sharp singlets at δ 8.62, 8.47, 8.19, and 8.14 were assignable to the purine protons, that in this case were distinct in each isomer.

These ATP analogues were converted to the corresponding ADP analogues by incubating solutions of 34 μM analogue with 3.4 μM myosin subfragment 1 in 50 mM Tris·HCl, pH 7.5, 100 mM KCl, 5 mM MgCl_2 , and 1 mM DTT for 5 min at 20 °C. HPLC analysis showed quantitative conversion of both analogues to the ADP derivatives **14** (t_R 12.1 and 16.6 min; see above for the cause of double peaks) and **15** (t_R 8.8 and 10.1 min).

RESULTS

The ^1H NMR spectrum of one commercial sample of SRB–SC **1a** indicated the presence of two major components, presumed to be isomeric species **1c** and **1d**. The spectrum of a second sample used in later work revealed an additional component, inferred from the products of its reaction with an amine to be the bis-sulfonyl chloride **1e** (see below). The 567 nm absorbance of the mixture obtained by treatment of the first sample of **1a** with excess methylamine was ~ 2 -fold greater at pH 4 than at pH 9. This result is consistent with the presence of the pH-responsive compound **3** and its isomer **4** in $\sim 1:1$ ratio. The absorbance (or fluorescence) of **4** is not expected to vary with pH changes in this range, as the sulfonamide at the *para*-position cannot interact with the xanthylium system. Marchesini et al. (5) have previously reported the separation of **3** and **4** and described their ^1H NMR spectra. Our studies were mostly conducted on a mixture of **3** + **4**, in which **3** and **4** were present in $\sim 2.6:1$ ratio because a proportion of **4** selectively precipitated from the stock solution after initial preparation. Later measurements not described here required pure **3**, which was isolated from the mixture by repeated chromatography.

The colorless form of **3** was favored at alkaline pH and the apparent pK_a for ring-chain tautomerization was 7.37, determined from 567 nm absorbance measurements in 25% ethanol–75% aqueous buffer. Solutions could not be accurately manipulated without this concentration of ethanol, presumably because of losses by surface adsorption of the more hydrophobic ring-closed form. Nevertheless, an estimate of the apparent pK_a in fully aqueous solution was obtained by measuring absorbance values immediately after dilution into appropriate aqueous buffers. This gave a value of approximately 6.95, suggesting that the presence of 25% ethanol did not greatly perturb the measurement. In contrast, the presence of detergent (5) had a significant effect and with 0.1% reduced Triton X-100 in fully aqueous solution, the measured pK_a was 5.36. This difference is considered in the Discussion.

Rates of interchange between the colored and colorless forms were investigated with pH changes imposed by rapid mixing in stopped-flow experiments. At 4 °C and in the region of pH 7, observed rate constants for the color change were $\sim 200 \text{ s}^{-1}$ whether approached from the colored or colorless form (data not shown). A full data set covering the pH range 0–9.5 will be described elsewhere with an account of the detailed mechanism operating in the ring-opening and ring-closing reactions.

We considered whether an amine conjugate of a carbhorhodamine would show analogous ring-chain tautom-

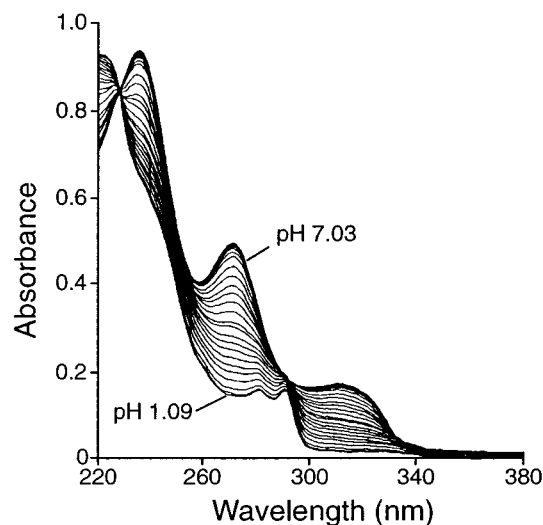


Figure 1. UV absorption spectra of lactam **6** as a function of pH. The figure shows superimposed traces from 30 individual solutions at various pH values between the extremes specified.

erism. The *N*-methyl lactam **5** derived from Rhodamine B has been reported to remain colorless “to about pH 4” (14) but was insufficiently soluble in water-ethanol (3:1) for accurate investigation. However, the more hydrophilic diol **6** was sufficiently soluble for accurate titration. This compound had no visible chromophore at any pH in the range 2–7, but its UV spectrum showed significant changes (Figure 1) associated with sequential protonation of the two diethylamino groups. The mean fitted pK_a values (using separate fits to data at 240, 275, and 320 nm where the absorbance changes were greatest) were 2.97 and 4.52.

Our clarification of the chemistry of **3** and **4** enabled us to reinvestigate aspects of a recent report that describes amine conjugates of **1a** and **2a** as improved reagents for fluorescent labeling of proteins (11). For the Rhodamine Red-X conjugate derived from **1a**, the structure was shown as **7**, leaving open the possibility that the sulfonamide and sulfonate groups were present as a mixture at positions 2 and 4. However, visible spectra of aqueous solutions of a commercial sample of Rhodamine Red-X succinimidyl ester were identical at pH 4 and 9, implying that the compound was a single isomer with the sulfonamide *para* to the xanthylium system. This result was unexpected in view of other results presented here, so the first stage of preparation of the Rhodamine Red-X reagent was repeated, i.e., reaction of **1a** with methyl 6-aminohexanoate (11). This experiment used a batch of **1a** for which the NMR spectrum showed three main species. TLC analysis of the crude reaction mixture also showed three major products, that were isolated by flash chromatography and characterized by visible, mass, and NMR spectroscopy. The least polar spot (50% of total rhodamines isolated by chromatography) was sulfonamide sultam **8**, the middle spot (29% of recovered rhodamines) was monosulfonamide **9**, and the most polar spot (21%) was sultam **10**. Both **8** and **10** showed pH dependence of their visible spectra, similarly to the other sultams investigated here, while the spectrum of **9** was unaffected in the pH range 4–9. Isolation of the doubly derivatized compound **8** confirms the presence of bis-sulfonyl chloride **1e** in the starting material used for this experiment. Consequences of these results are considered in the Discussion.

Last, the influence of structural isomerism in sulforhodamine derivatives was examined for the conjugates

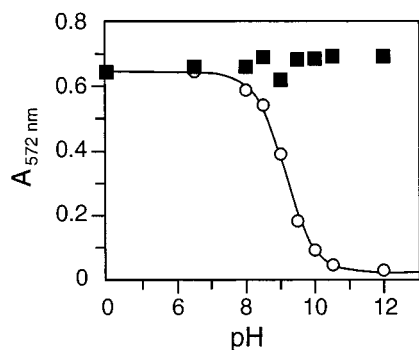
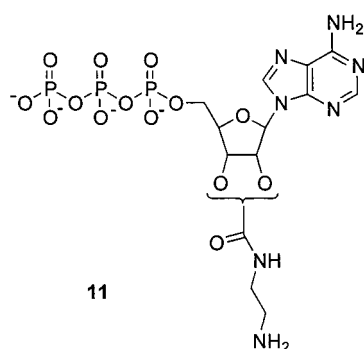
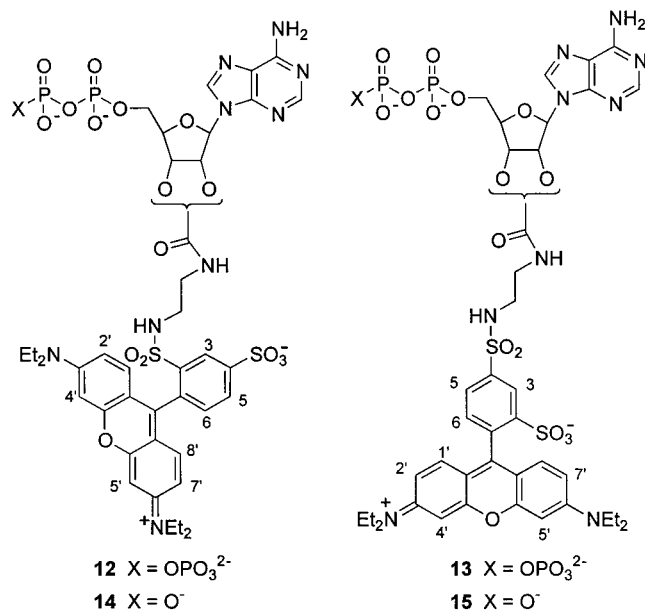


Figure 2. Titration of the rhodamine-ED-ATP analogues **12** (○) and **13** (■) in aqueous solution as a function of pH. The solid line is a least-squares fit to the data of **12** for a single pK_a .

12 and **13** derived by coupling SRB–SC **1a** with ED-ATP **11**. The reaction produced two species that were readily



separated analytically by reverse-phase HPLC and preparatively by anion exchange chromatography. Structures were assigned by inference from the known starting materials, the reactivity with myosin subfragment 1 (see below), the ^1H NMR spectra and the response of each compound to pH changes. The visible absorption of **12**



was pH dependent as for the methylamine conjugate **3**, with apparent pK_a 9.12 in fully aqueous solution (Figure 2), that reduced to 8.24 in solutions containing 25% ethanol (v/v). The absorption spectrum of **13** was invariant over the pH range 4–12 (Figure 2).

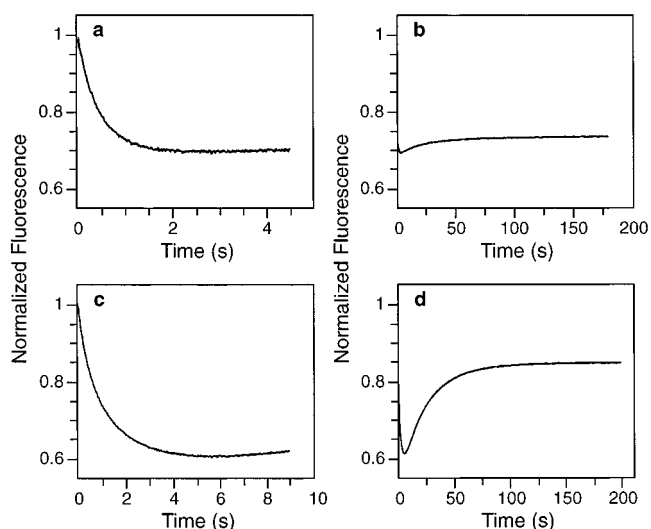


Figure 3. Stopped-flow fluorescence records of the interaction of the rhodamine-ATP analogues **12** (panels a and b) and **13** (panels c and d) with excess myosin subfragment 1. The reactions were monitored over two time scales.

The interactions of ATP conjugates **12** and **13** with myosin subfragment 1 were investigated under single turnover conditions (i.e., $[\text{protein}] > [\text{ATP analogue}]$) at pH 7.5, 20 °C in 50 mM Tris·HCl, 100 mM KCl, 5 mM MgCl_2 , 1 mM DTT. A 0.5 μM solution of either isomer was rapidly mixed with 2.5 μM subfragment 1 in a fluorescence stopped-flow instrument and the fluorescence was recorded with time (Figure 3). With both isomers, there was a fast initial decrease in intensity followed by a slower recovery. These phases correspond to binding of the analogue to subfragment 1 and the rate-limiting release of inorganic phosphate after hydrolysis (see Discussion). With **12** the initial fluorescence decrease was to $69.6 \pm 0.6\%$ of the starting value with subsequent recovery to $73.4 \pm 0.4\%$ of the starting value. For **13**, the initial decrease was to $57.6 \pm 0.6\%$ of the starting value followed by recovery to $81.5 \pm 1.3\%$ of the starting value. Thus, both isomers give a similar fluorescence decrease on binding to subfragment 1 but the fluorescence recovery is smaller for the pH-responsive isomer **12** than for **13**. Rate constants of the above two processes were 2.0 ± 0.1 and $0.050 \pm 0.003 \text{ s}^{-1}$ for **12** and 1.07 ± 0.08 and $0.042 \pm 0.001 \text{ s}^{-1}$ for **13**. Although a concentration dependence of the binding reactions were not made, the rate constants of the faster processes represent second-order association constants of 8.0×10^5 and $4.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for **12** and **13**, respectively. These calculations depend on the subfragment 1 being 100% active and so represent lower limits of these rate constants. These single turnover experiments were repeated at pH 8.5, i.e., closer to the pK value for the color change of **12**, so perturbation of this pK on binding to subfragment 1 might have been observed. In practice, neither isomer showed differences between pH 7.5 and 8.5 in the amplitudes of either phase of the fluorescence changes (data not shown).

Dissociation rate constants of the isomeric ADP conjugates **14** and **15** from subfragment 1 were measured in displacement experiments by rapid mixing of a solution containing 2.5 μM subfragment 1 and 0.5 μM **14** or **15** with a solution containing 100 μM ATP (Figure 4). With **14** there was $41.4 \pm 0.9\%$ exponential increase in fluorescence with a rate constant of $1.15 \pm 0.07 \text{ s}^{-1}$ while **15** showed a $27.2 \pm 0.9\%$ exponential increase in fluorescence with a rate constant of $0.75 \pm 0.10 \text{ s}^{-1}$. Association rate constants, determined by mixing 0.5 μM **14** or

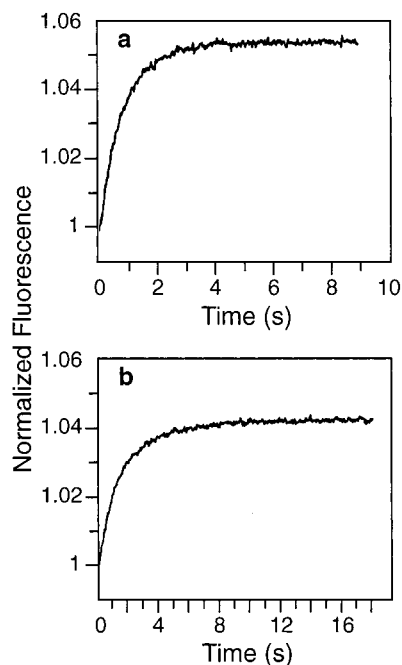


Figure 4. Stopped-flow fluorescence records of the displacement of rhodamine-ADP analogues **14** (panel a) and **15** (panel b) from myosin subfragment 1 by excess ATP.

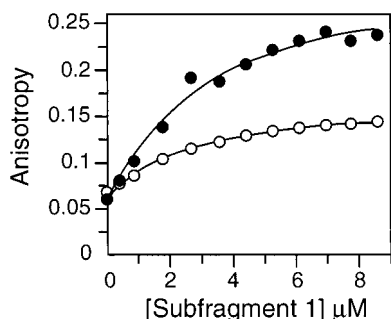


Figure 5. Equilibrium binding of rhodamine-ADP analogues **14** (○) and **15** (●) to myosin subfragment 1, monitored by fluorescence anisotropy. The solid lines are best fits to eq 1, with values for K_d and limiting anisotropy as shown in the Results.

15 with $2.5 \mu\text{M}$ subfragment 1, were 1.9 s^{-1} and 1.3 s^{-1} , respectively (data not shown).

The equilibrium dissociation constants of **14** and **15** from subfragment 1 were investigated by fluorescence anisotropy titrations. Increasing concentrations of subfragment 1 were added to a solution of $1 \mu\text{M}$ **14** or **15**. The anisotropies of **14** and **15** free in solution were 0.070 and 0.060, respectively, and, as the subfragment 1 concentration increased, both isomers showed a hyperbolic increase in anisotropy (Figure 5). Fitting these data to eq 1 gave values for the limiting anisotropy of 0.168 and 0.294, and equilibrium dissociation constants of 2.62 ± 0.26 and $2.27 \pm 0.60 \mu\text{M}$ for **14** and **15**, respectively.

DISCUSSION

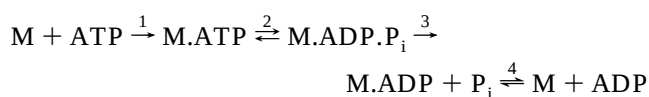
The pH-dependent reversible cyclization of rhodamine sultams has consequences for experiments that use sulforhodamine dyes. First, the apparent pK_a for the colored to colorless transition of **3** varies markedly with changes in solution conditions. In contrast to the value of 7.37 determined here by absorbance measurements of water-ethanol solutions, Marchesini et al. (5) reported an apparent pK_a of ~ 4.6 from fluorescence measurements in fully aqueous solutions that contained 0.1% reduced

Triton X-100. In our hands, absorbance measurements under the latter conditions gave a value of 5.36. Thus, the apparent pK_a is substantially lower in the presence of reduced Triton X-100. The critical micellar concentration of this detergent is 0.25 mM (15), substantially below the 0.1% level used by Marchesini et al. [1.6 mM based on an average molecular weight of 625 (16)]. We suggest the lipophilic sultam form of **3** would partition preferentially into micelles and the resultant stabilization of the conjugate base is responsible for enhanced acidity of the open form. The earlier proposal (5) that **3** could be used as a pH indicator for cellular compartments of acidic pH is therefore problematic. However, introduction of hydrophilic substituents on one or more of the nitrogen atoms in **3** could generate a new family of fluorescent pH indicators. Modulation of the pK_a by variable substitution of the aromatic amino groups in compounds related to **3** has been qualitatively described in the patent literature (8a). In the present work, the sensitivity of the apparent pK_a to changes in substituents is demonstrated by the values obtained for the ATP conjugate **12**, i.e., 9.12 in fully aqueous solution and 8.24 in solutions containing 25% ethanol. The overall shift to a higher value can be rationalized by the inductive effect of the β -nitrogen in the substituent on the sulfonamide, that reduces the nucleophilicity of the sulfonamide nitrogen, i.e., the driving force for ring closure is reduced. The apparent pK_a values for **3** and **12** appear to change in opposite directions when measured in water or ethanol–water. Complex solvation effects are well-known to influence acid and base strengths (17) and probably underlie these differences.

A second point is that the kinetics of the ring opening and closing reactions of the model sultam **3** at pH values near neutrality are in the millisecond time domain, i.e., comparable to rates of many biological processes. Thus small variations of pH could themselves generate rapid signals from probes that incorporate the sultam structure. Since the kinetics of the color change are slow compared to most indicators, these rhodamine sultams would not be suitable to measure very rapid pH changes.

Third, the ability to separate compounds **9** and **10** means that rhodamine probes of this type, insensitive or sensitive respectively to the effects of pH and suitable for covalent attachment to biomolecules, can be considered as accessible. In particular, the Rhodamine Red-X reagent (11) has been shown to be insensitive to pH changes and therefore must have a structure derived from isomer **9**.

The interactions of the isomeric ATP conjugates **12** and **13** with myosin subfragment 1 demonstrate the potential for the different isomers of these and other sulforhodamine conjugates to report differently on underlying biological processes. In single turnover experiments when **12** and **13** were mixed with excess subfragment 1, both isomers showed an initial exponential decrease in fluorescence followed by a slower increase in fluorescence. Such behavior has previously been observed when ATP was mixed with subfragment 1 and intrinsic tryptophan fluorescence monitored (18) or when fluorescent analogues of ATP were mixed with subfragment 1 (19–23). The results can be interpreted in light of a simplified scheme for the subfragment 1 ATPase mechanism (24).



The initial process is associated with the essentially irreversible binding of ATP (or an analogue thereof) to subfragment 1 (step 1) to form the M·ATP state, that is in rapid equilibrium with M·ADP·P_i (step 2). This is followed by the rate-limiting inorganic phosphate release step of the overall hydrolysis process (step 3), that is also essentially irreversible, and the cycle terminates with the release of ADP (step 4).

The rate constants given in the Results section for the initial fluorescence decrease when **12** or **13** were mixed with subfragment 1 show that **12** binds at over twice the rate of **13**. However, for the subsequent slow increase of fluorescence that corresponds to the rate-limiting step of the overall hydrolysis (step 3), rate constants for the two isomers were identical within experimental error and similar to that of the subfragment 1 ATPase (18).

As shown in Figure 3, the amplitudes of the slow fluorescence recovery, that corresponds to step 3 above, were markedly different for the two isomers. The much smaller amplitude of the slow phase for analogue **12** could result from its ADP form **14** binding more tightly to subfragment 1 than the isomeric ADP analogue **15** and/or that the enhancement of fluorescence on binding to subfragment 1 is greater for isomer **14**. The displacement of these ADP analogues from subfragment 1 by excess ATP gave a larger increase in fluorescence for **14** than for **15**, which again might be due to tighter binding or higher fluorescence change of **14**. The rate constant for dissociation of **14** was almost twice that for **15**. However, the equilibrium titrations based on fluorescence anisotropy measurements show that, within experimental error, the two ADP analogues bind to subfragment 1 with the same affinity, suggesting that **14** has a faster second-order association rate constant than **15**, as reported in the Results. Thus the ATP and ADP analogues are similar in that in each case the pH-responsive isomer shows more rapid binding. The differences highlight the importance of using defined isomers of these rhodamine conjugates. A particular source of potential error could have arisen in our previous FRET-based distance measurements (1, 2) using these conjugates if the pH-responsive isomer were present and underwent some degree of cyclization upon binding to a protein. The results now reported and related data not shown confirm that sulforhodamine conjugates with both GTP and ATP used in previous work were solely the nonresponsive isomers because the pH-responsive isomers were well-resolved by preparative anion exchange chromatography.

The results described in this work put the chemistry of sulforhodamine-amine conjugates on a sound foundation and clarify some previous reports. Future challenges are to define in detail the mechanism of the ring-chain tautomerization process and explore uses of these conjugates, particularly the pH-responsive forms, as probes in biophysical research.

ACKNOWLEDGMENT

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Rational Design of a Potent, Long-Lasting Form of Interferon: A 40 kDa Branched Polyethylene Glycol-Conjugated Interferon α -2a for the Treatment of Hepatitis C

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A potent, long-lasting form of interferon α -2a mono-pegylated with a 40 kilodalton branched poly(ethylene glycol) was designed, synthesized, and characterized. Mono-pegylated interferon α -2a was comprised of four major positional isomers involving Lys³¹, Lys¹²¹, Lys¹³¹, and Lys¹³⁴ of interferon. The *in vitro* anti-viral activity of pegylated interferon α -2a was found to be only 7% of the original activity. In contrast, the *in vivo* antitumor activity was severalfold enhanced compared to interferon α -2a. Pegylated interferon α -2a showed no immunogenicity in mice. After subcutaneous injection of pegylated interferon α -2a, a 70-fold increase in serum half-life and a 50-fold increase in mean plasma residence time concomitant with sustained serum concentrations were observed relative to interferon α -2a. These preclinical results suggest a significantly enhanced human pharmacological profile for pegylated interferon α -2a. Results of Phase II/III hepatitis C clinical trials in humans confirmed the superior efficacy of pegylated interferon α -2a compared to unmodified interferon α -2a.

INTRODUCTION

Interferon α is comprised of a family of closely related, species-restricted proteins that exhibit anti-viral, anti-tumor, and immunomodulatory activities (1). The recombinantly produced human interferon α -2a and interferon α -2b (1) have undergone extensive clinical investigations and have become useful drugs for both viral and oncological indications (2–6).

The reported terminal half-life of interferon α ranges from 4 to 8 h, with peak serum concentrations occurring at 3–8 h following intravenous (i.v.) or subcutaneous (s.c.) administration (7). Little or no interferon α is detected 24 h following i.v. or s.c. administration (7, 8), necessitating frequent dosing of interferon α to achieve sustained efficacy. The frequency of interferon α treatment is dependent upon the disease being treated. For example, certain oncological indications require daily dosing, while viral indications may require only three times weekly dosing. Interferon α treatment for these diseases at the above-mentioned intervals may range from periods of several months to a year or longer. Furthermore, frequent treatment with interferon α produces several dose-dependent side effects (9).

In recent years, poly(ethylene glycol) (PEG) conjugated biomolecules have been shown to exhibit superior clinically useful properties than their corresponding unmodified parent molecules (10–12). These include better physical and thermal stability, greater protection against proteolytic degradation, higher solubility, longer *in vivo*

circulating half-lives, and lower clearance, thus enhancing efficacy. Additional qualities of pegylated proteins are reduced immunogenicity and antigenicity, as well as reduced toxicity.

Hepatitis C virus (HCV) infection is a blood-borne disease of the liver. In the United States alone, an estimated four million Americans are infected with HCV and up to 180 000 new infections occur each year. Currently, the only available treatment for HCV is with interferon, injected three times per week. However, the patient population experiencing sustained response of undetectable levels of HCV is only 10–15%.

In the early 1990s, an attempt was made to develop a pegylated form of interferon α -2a for hepatitis C. The desired target profile was once a week dosing with comparable efficacy to interferon α -2a given three times weekly. Interferon α -2a was pegylated with a linear 5K PEG via urea linkage (13). In 1994, development of first generation PEG-interferon α -2a with once a week dosing was discontinued during Phase II clinical trial because efficacy equivalent to interferon α -2a given three times weekly was not achieved.

Since then, many new insights have been gained on the pegylation of protein therapeutics. Among them are (1) *in vitro* activity (cell-based) of a number of cytokines, whose pharmacological effects involve receptor interactions, is shown to be inversely proportional to the attached PEG mass and is not predictive of *in vivo* biological activity (12, 14, unpublished data); (2) one large PEG at a single site is preferable to several small PEGs attached at multiple sites (12); (3) linear PEG is distributed throughout the body with larger distribution volume (15); (4) branched PEG is distributed with a smaller distribution volume and early on delivered to the liver

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and spleen (15); (5) branched PEG protein conjugates exhibit increased pH and thermal stability, as well as greater stability toward proteolytic digestion (16); (6) smaller linear PEGs may deposit in the kidney vacuoles (17).

Another major development was the elucidation of a model, which predicts efficacy based upon combined pharmacokinetic and pharmacodynamic analyses (8, 18). Of great importance, the model predicts a correlation between efficacy and the trough levels of interferon-induced surrogate marker protein, 2',5'-oligoadenylate synthetase (2',5'-OAS) in peripheral blood mononuclear cells (PBMNs), but not with peak levels or area under the curve of 2',5' OAS. Pharmacokinetic data obtained from animal studies can also be used to develop simulations that will indicate what might occur in patients who receive various doses and regimens of PEG-interferon α -2a.

The ability to predict the efficacy of PEG-interferon α -2a, coupled with the latest advancements in pegylation technology convinced us to pursue the development of a second generation PEG-interferon α -2a with hepatitis C virus (HCV) as the first target indication.

A 40K branched PEG was selected because of the possibility that it would facilitate the delivery of PEG-interferon α -2a to the liver and its ability to circulate for a long period of time in the blood. Thus, interferon α -2a conjugated with a branched 40K PEG (PEG₂-IFN) would have a better chance of eradicating hepatic and extra-hepatic HCV infection.

EXPERIMENTAL PROCEDURES

Materials. Interferon α -2a (IFN) and 5K PEG-IFN were obtained from the Biopharmaceuticals Department of Hoffmann-La Roche Inc. A branched 40K succinimidyl derivative of PEG (PEG₂-NHS) was purchased from Shearwater Polymers Inc. (Huntsville, AL). Fractogel EMD CM 650(M) was supplied by EM Separations (Gibbstown, NJ). Precast SDS-PAGE gels and electrophoresis units were purchased from Novex (San Diego, CA). Titrisol, concentrated iodine solution, was from EM Science (Gibbstown, NJ). Anti-PEG AGP3 monoclonal antibody was acquired from Academia Sinica (Taipei, Taiwan). Super Signal ULTRA Western blotting kit was provided by Pierce (Rockford, IL). The LAL endotoxin kit was obtained from Associates of Cape Cod (Woods Hole, MA). All other reagents used were of highest quality available. The cannulated rats and BDF-1 mice were supplied by Charles River Laboratories (Wilmington, MA).

Preparation of PEG₂-IFN. Pegylation reaction mixture was comprised of interferon α -2a and 40K PEG₂-NHS reagent at 1:3 molar ratio in 50 mM sodium borate buffer, pH 9.0. The final protein concentration was ~5 mg/mL. Reaction mixture was stirred for 2 h at 4 °C. Reaction was stopped by adjusting the pH of the mixture to 4.5 with glacial acetic acid.

Reaction mixture was diluted 10-fold with water and applied onto a column packed with Fractogel EMD CM 650(M) resin, previously equilibrated with 20 mM sodium acetate, pH 4.5, at a linear velocity of 1.3 cm/min. Protein loading onto the column was 2 mg/mL gel. Column was washed with the equilibration buffer to remove excess

PEG reagent, reaction byproducts and PEG₂-IFN oligomers. The desired mono-pegylated PEG₂-IFN was then eluted with 200 mM sodium chloride in the equilibration buffer. Unmodified interferon α -2a still adsorbed onto the column was removed by washing with 750 mM sodium chloride in the equilibration buffer. Mono-pegylated PEG₂-IFN eluate was further concentrated to approximately 1 mg/mL and diafiltered into the final storage buffer, 20 mM sodium acetate, pH 5.0, containing 150 mM sodium chloride. Concentrated PEG₂-IFN was sterile filtered with a 0.2 μ m filter and stored at 4 °C.

Protein Determination. Protein concentrations were determined using an A_{280} value of a 1 mg/mL solution of PEG₂-IFN as 1.05.

SDS-PAGE Analysis. Reaction mixture and the purified PEG₂-IFN was subjected to sodium dodecyl (lauryl) sulfate/polyacrylamide (8–16%) gel electrophoresis according to the methods of Laemmli (19) and stained for protein using Coomassie blue dye.

PEG moieties in the PEG₂-IFN conjugates were specifically stained using Titrisol iodine solution (EM Science, Gibbstown, New Jersey) according to a modified procedure of Kurfurst (20). The SDS-PAGE gel was rinsed with distilled water and placed in 5% barium chloride solution. After 10 min, the above gel was washed with distilled water and placed in 0.1 N Titrisol iodine solution for another 10 min. Titrisol was washed off with distilled water. The PEG stained (orange brown bands) SDS-PAGE gel containing PEG₂-IFN samples was stored in distilled water in a heat-sealed Kapak/Scotchpak bag.

Western Blotting with Anti-PEG AGP3 Monoclonal Antibody. PEG₂-IFN was transferred from SDS-PAGE gel to nitrocellulose membrane using standard techniques. An anti-PEG monoclonal antibody, denoted AGP3 (21), was used as the primary antibody. Development was performed according to the manufacturer's (Pierce) instructions using SuperSignal ULTRA Western blotting kit for the detection of mouse IgG (Pierce). Secondary antibody used was goat anti-mouse IgG conjugated with horseradish peroxidase. An ultra sensitive chemiluminescent compound provided as part of the Western blotting kit (Pierce) was used as the enzyme substrate.

Amino Acid Analysis. N-Terminal sequence and amino acid composition analyses were performed with a postcolumn ninhydrin amino acid analyzer (22).

Mass Spectrometry. Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI TOF MS) was used to determine the true molecular weight of PEG₂-IFN (13).

Determination of Pegylation Sites. Positional isomers of PEG₂-IFN were separated as described (13) using cation exchange HPLC. Separated positional isomers of PEG₂-IFN were then analyzed by a combination of analytical methods including, peptide mapping, sequencing and mass spectrometry, as described (13).

Determination of Endotoxin Levels. Endotoxin level in PEG₂-IFN preparation was determined using the lumulus ameocyte lysate (LAL) method according to the manufacturer's (Associates of Cape Cod) instructions.

In vitro Anti-Viral Activity. The in vitro anti-viral activities of interferon α -2a and PEG₂-IFN were determined in a cell culture bioassay employing Mardin-Darby bovine kidney cells challenged with vesicular stomatitis virus as described (23).

In Vivo Antitumor Activity. Athymic nude mice (Harlan) received a subcutaneous implant of 2×10^6 human renal A498 cells or human renal ACHN cells or human renal G402 cells under the rear flank. Three to

¹ Abbreviations: IFN, interferon; PEG, poly(ethylene glycol); HCV, hepatitis C virus; PEG₂-NHS, branched succinimidyl ester derivative of PEG; PEG₂-IFN, 40K PEG conjugated interferon α -2a; PEGASYS, registered name of 40K branched PEG-conjugated IFN- α -2a.

six weeks were allowed for the tumors to get established. The size criteria for acceptance into the study was $0.05-0.50$ cubic centimeters ($\text{Vol.} = L \times W^2$). Mice were treated once weekly (Monday) with each of the dosages of 30, 60, 120, or $300 \mu\text{g}$ of PEG₂-IFN. In the case of interferon α -2a the mice were treated thrice weekly (Monday, Wednesday, and Friday) with 10, 20, 40, or $100 \mu\text{g}$ of interferon α -2a. The duration of treatment was 4–5 weeks depending on tumor growth. Tumor volumes were measured every Monday prior to treatments.

Pharmacokinetics. Female Sprague Dawley rats, surgically implanted with jugular cannulas with an average body mass of 240–260 g were housed individually, allowed free access to food and water and maintained in a 12 h light/dark cycle. Within 4–6 h after arrival, jugular cannulas were flushed with PBS (phosphate buffered saline). The following day, after flushing with $0.15-0.2$ mL PBS, 2×10^6 units of interferon α -2a and PEG₂-IFN were injected, followed by injection of $0.15-0.2$ mL of PBS to ensure that all drug was washed into the animal. Thus, each animal received a dosage of 8×10^6 interferon units/kg body weight.

Blood samples were drawn at 0.5, 1, 3, 5, 12, and 24 h after injection of the drugs. At all time points, after discarding the first $0.15-0.2$ mL of blood, an aliquot of 0.5 mL of blood was withdrawn using a fresh syringe via the jugular cannula. Samples were discharged into serum separator tubes at room temperature and centrifuged at $14000g$ in a refrigerated Eppendorf centrifuge for 10 min. The separated sera were then transferred into 1.5 mL microfuge tubes and kept frozen at -80°C until ready for the determination of anti-viral activity. Serum samples were diluted appropriately and the anti-viral activity was determined as described previously (23).

Neutralizing Antibodies. Normal BDF-1 mice (10/group) were injected intraperitoneally once per day five times a week with interferon α -2a, aggregates of interferon α -2a, 5K PEG-IFN and PEG₂-IFN, each having 300 000 units of anti-viral activity. Blood samples were taken 19 days following the last injection and the serum was evaluated for neutralizing antibodies as described (24).

RESULTS

Description of Branched Pegylation Reagent.

PEG₂-NHS (Figure 1) consists of two monomethoxy poly(ethylene glycol) chains each having an average molecular weight of 20K, linked to a lysine molecule via urethane bonds, one at the α -amino group and the other at the ϵ -amino group of lysine. The carboxyl group of lysine in turn is derivatized to an *N*-hydroxysuccinimide ester (16).

Synthesis of Pegylated Interferon α -2a. PEG₂-IFN was formed as a result of the reaction between an *N*-hydroxysuccinimide ester derivative of a 40K branched PEG molecule and the free amino group of interferon α -2a forming an amide bond. The reaction mechanism is illustrated in Figure 1.

Under the experimental conditions used, pegylation reaction products were a mixture containing approximately 45–50% monomer, 5–10% oligomers (predominantly dimer), and 40–50% unmodified interferon α -2a, as determined by densitometric measurements and by mass spectrometry (data not shown). Purified PEG₂-IFN was ~95–99% mono-pegylated, as determined by SDS-PAGE analysis (both protein and PEG staining, Figure 2, respectively) and mass spectrometry, as well as by Western blotting with a newly discovered high-affinity

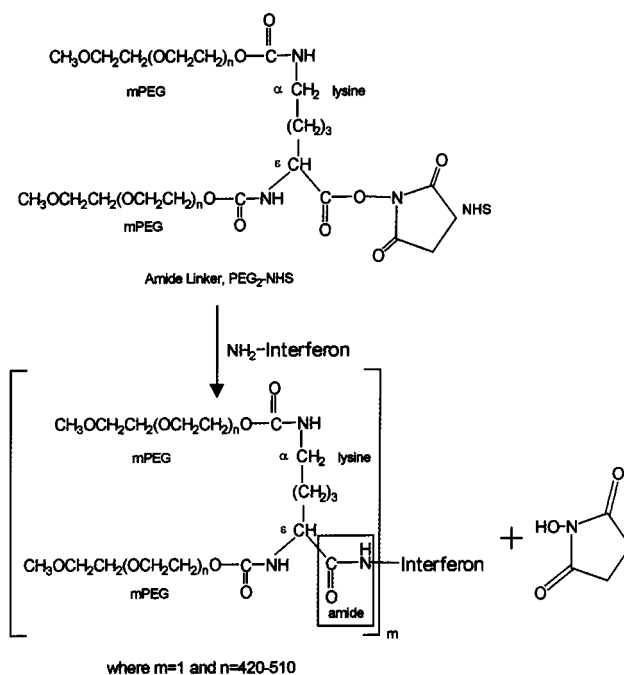


Figure 1. Reaction mechanism of the synthesis of PEG₂-IFN. The free amino groups of interferon α -2a react with the *N*-hydroxysuccinimide ester derivative of a 40K branched poly(ethylene glycol) forming an amide bond between the two.

monoclonal antibody which recognizes PEG (21) (Figure 3). PEG₂-IFN preparation contained <2.5 endotoxin units/mg.

Characterization of PEG₂-IFN. Upon the basis of the 40K average molecular weight of the branched PEG moiety and the 19.2K molecular weight of interferon α -2a, it is estimated that the molecular weight of PEG₂-IFN is ~59.2K. However, the actual molecular weight of PEG₂-IFN is dependent upon the average molecular weight (39–43K) of PEG₂-NHS reagent used in the pegylation reaction.

Figure 2 illustrates the SDS-PAGE profiles of the pegylation reaction mixture, purified PEG₂-IFN and interferon α -2a as determined by protein specific and PEG-specific staining. In addition to the various PEG₂-IFN conjugates (mono-, di-, tri-, etc.), reaction mixture contains unmodified interferon α -2a and excess PEG reagent (Figure 2). PEG₂-IFN's electrophoretic mobility is considerably slowed because of PEG's large hydrodynamic volume (3 mol of water form an adduct with each ethylene oxide subunit of PEG) (25). In addition, branched PEG could get trapped into the polyacrylamide gel matrix, further slowing down the electrophoretic mobility. Thus, PEG₂-IFN appears to have a higher molecular weight (~96K, Figure 2A) than the calculated molecular weight when compared to molecular weight marker proteins. However, in Figure 2B, PEG₂-IFN has a molecular weight corresponding to its expected molecular weight of 59–60K when compared to standard PEG molecular weight markers. This result is consistent with that obtained by MALDI-TOF mass spectrometry (data not shown).

Western blotting with AGP3 antibody directed against PEG (21) demonstrated that the purified PEG₂-IFN is authentic, predominantly mono-pegylated and did not contain fragments of PEG or significant amounts of oligomeric forms of PEG₂-IFN (Figure 3).

Both interferon α -2a and PEG₂-IFN have identical amino acid sequence and amino acid composition (data not shown). The only difference in the composition of

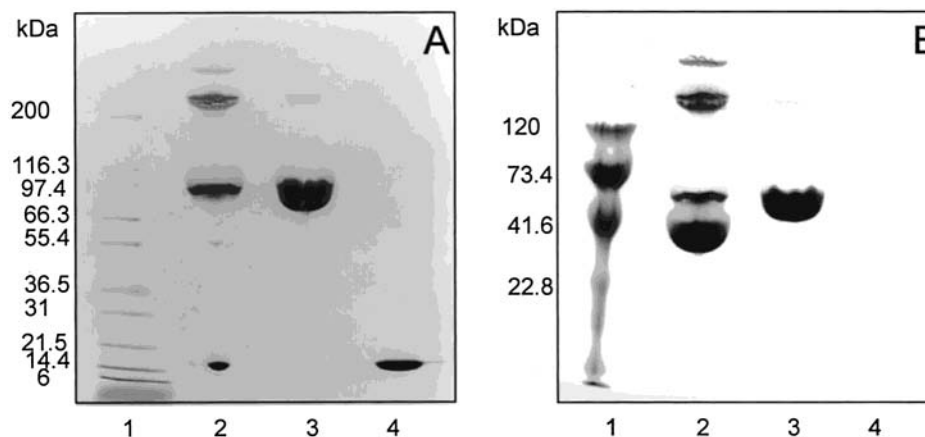


Figure 2. SDS-PAGE analysis. (A) Specifically stained for protein with Coomassie blue. Lanes: 1, molecular weight marker proteins; 2, pegylation reaction mixture; 3, purified PEG₂-IFN; and 4, interferon α -2a. (B) Specifically stained for PEG with iodine. Lanes: 1, molecular weight marker PEGs; 2–4, same as in Figure 2A. Note that lane 4 containing interferon α -2a in gel B is not stained by iodine.

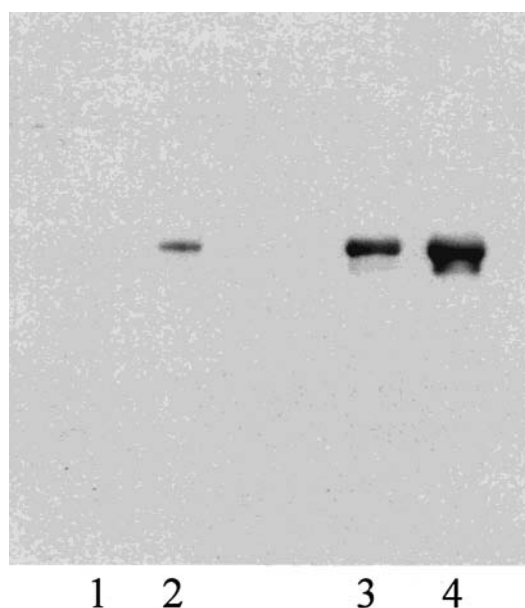


Figure 3. Western blotting of PEG₂-IFN with AGP3 monoclonal antibody directed against PEG. Five second exposure of the film. Lanes: 1, interferon α -2a (1 μ g), as expected, no recognition by AGP3 antibody; 2, PEG₂-IFN (0.1 μ g); 3, PEG₂-IFN (0.5 μ g); and 4, PEG₂-IFN (1 μ g).

native and modified proteins is due to the formation of an amide bond between the amino group of interferon α -2a and the PEG molecule at the point of attachment (Figure 1).

A combination of high performance cation exchange chromatography, peptide mapping, amino acid sequencing and mass spectrometric analyses were performed to identify the pegylation sites (data not shown). A single unit of branched poly(ethylene glycol) was found to be attached to either one of Lys³¹, Lys¹²¹, Lys¹³¹, or Lys¹³⁴. Collectively, ~94% of the PEG attachment takes place in these four sites. The remaining ~6% of the pegylation takes place at Lys⁷⁰ and Lys⁸³. The N-terminal cysteine, which is involved in a disulfide bond (Cys¹–Cys⁹⁸), does not appear to be a pegylation site.

In Vitro Anti-Viral Activity. The anti-viral activities of interferon α -2a and PEG₂-IFN are compared in a cell culture bioassay utilizing Mardin-Darby bovine kidney (MDBK) cells challenged with vesicular stomatitis virus. This assay measures interferon's inhibitory effect on virus induced cell lysis (cytopathic effect). Procedure

Table 1. In Vitro Antiviral Activities of Interferon α -2a and PEG₂-IFN^a

protein	anti-viral activity (IU/mg)	residual activity (%)
interferon α -2a	2×10^8	100
PEG ₂ -IFN	1.4×10^7	7

^a Antiviral activity determined in MDBK cells infected with vesicular stomatitis virus.

involves the addition of serially diluted interferon samples MDBK cells followed by a challenge with virus. The endpoint of the assay is approximately a 50% protection of MDBK cells. The quantity of interferon present is determined relative to a reference preparation of interferon. The unit of anti-viral activity is an arbitrarily established value by the World Health Organization. PEG₂-IFN had an anti-viral specific activity of approximately 7% of that of interferon α -2a. Results are listed in Table 1.

In Vivo Antitumor Activity. The in vivo antitumor activity of PEG₂-IFN and interferon α -2a were evaluated by studying their ability to reduce the size of various human tumor cells implanted subcutaneously into mice. PEG₂-IFN showed a marked reduction in human renal A498 tumor size (Figure 4, bottom panel) compared to interferon α -2a (Figure 4, top panel) for all dosage levels tested, at 7, 14, 21, and 28 days after the beginning of treatment. After 4 weeks of treatment and 7 days after treatment was discontinued, three mice in each group were sacrificed. In the three mice treated with PEG₂-IFN, no residual tumor was observed. In the mice treated with interferon α -2a, tumor weight was 1.28, 0.62, and 1.6 g, respectively. In the three control mice, the A498 tumor weights were 2.32, 2.37 and 1.94 g, respectively. Eighty days following termination of PEG₂-IFN treatment, all seven remaining mice were free of tumor as determined by palpation in the 4-week treatment group. Similarly, PEG₂-IFN effected a significant reduction in the size of ACHN and G402 human renal tumors (data not shown).

Pharmacokinetic Parameters. Pharmacokinetic parameters of PEG₂-IFN were determined after subcutaneous injection into female Sprague–Dawley rats. Figure 5 illustrates the mean serum activity versus time. Summary and individual pharmacokinetic parameters are found in Tables 2 and 3. Serum activity of interferon α -2a peaked 1 h after the subcutaneous injection, whereas the peak activity of PEG₂-IFN occurred 24 h after the injection. PEG₂-IFN's peak activity was 3-fold greater

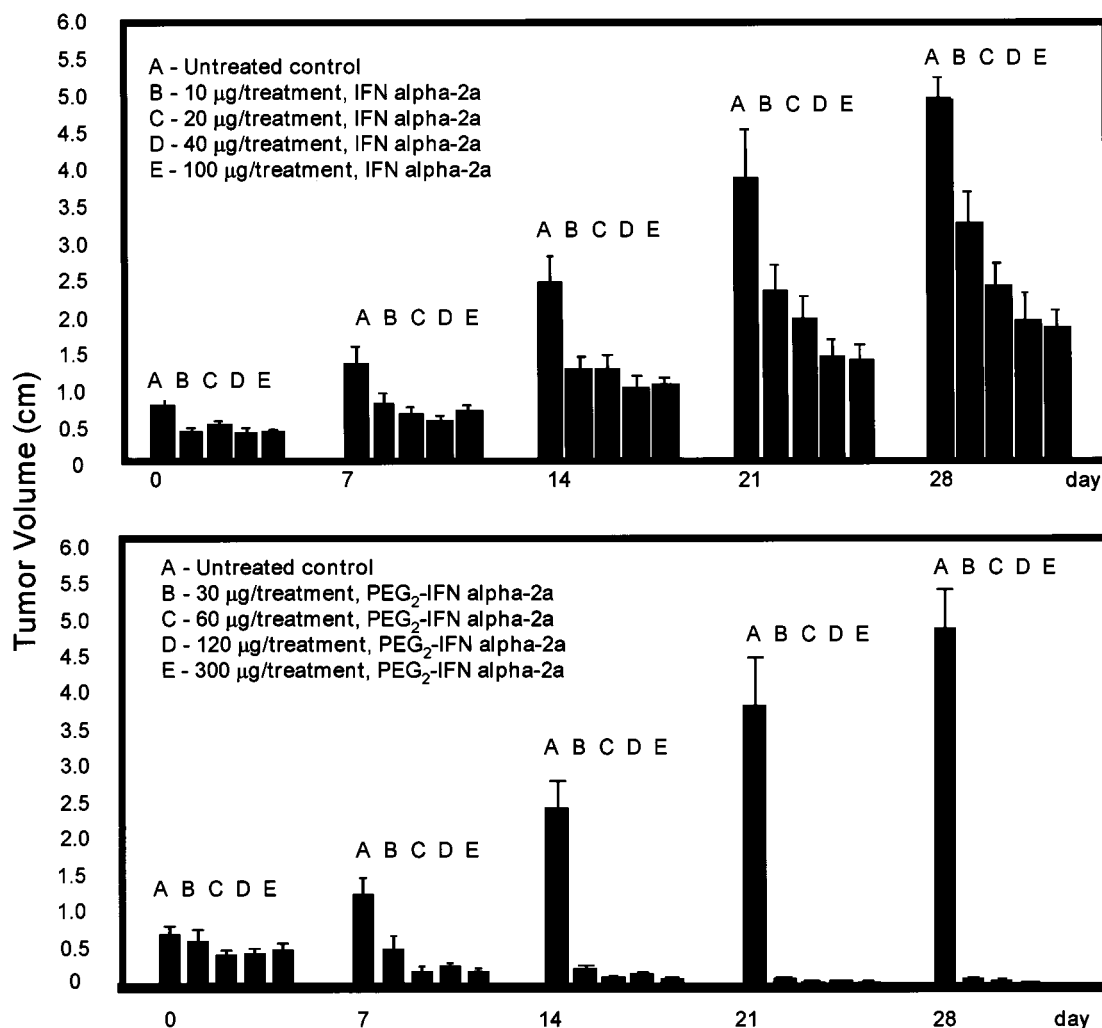


Figure 4. In vivo antitumor activity of interferon α -2a (top panel) and PEG₂-IFN (bottom panel) in athymic nude mice subcutaneously implanted with human renal A498 cells. Insert shows the amount of interferon α -2a and PEG₂-IFN used in the treatment of the mice implanted with the tumor. x- and y-axes indicate the days and the corresponding tumor volumes, respectively.

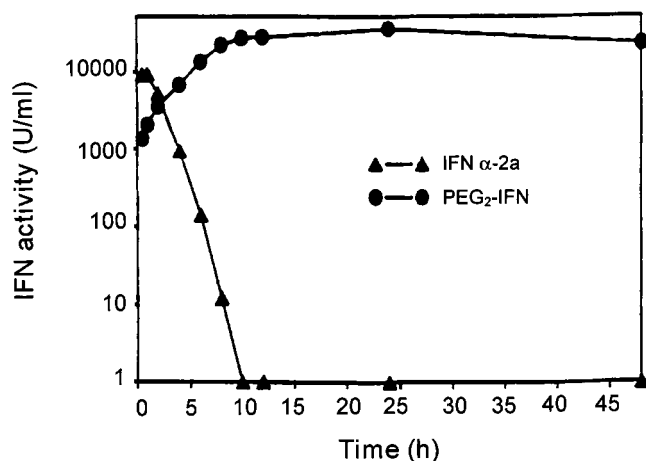


Figure 5. Mean serum activity versus time of interferon α -2a and PEG₂-IFN after subcutaneous injection in rats. PEG₂-IFN has a 51-h half-life compared to 0.7-h for interferon α -2a concomitant with mean plasma residence time of 80 and 1.6 h, respectively. Logarithmic regression analyses were used to calculate the pharmacokinetic parameters.

than that of interferon α -2a. Area under the curve, up to the last measurable time point of PEG₂-IFN, was 6-fold greater than that of interferon α -2a. After subcutaneous injection of PEG₂-IFN, a 70-fold increase in half-life and a 50-fold increase in mean residence time (MRT) were

observed. C_{max} and AUC of the PEG₂-IFN were 3.5- and 59-fold increased, respectively compared to those of interferon α -2a. Consequently, PEG₂-IFN has a significantly increased plasma exposure.

Neutralizing Antibodies. As seen in Table 4, mice injected with PEG₂-IFN produced no detectable amount of neutralizing antibodies in 9 of the 10 animals tested. In contrast, mice injected with interferon α -2a produced neutralizing antibodies and this response was greatly pronounced in mice injected with interferon α -2a aggregates. A much-reduced response was observed with the first generation PEG-interferon α -2a in which the PEG moiety was linear and had a molecular weight of 5K.

DISCUSSION

To take full advantage of the clinically useful properties of branched poly(ethylene glycol) and to meet the minimum threshold PEG mass requirement, we chose a 40K branched poly(ethylene glycol) moiety for the pegylation of interferon α -2a. PEG₂-IFN was well-characterized using various analytical methods including immunoblotting with a newly discovered high-affinity AGP3 monoclonal antibody directed against PEG. The discovery and availability of this antibody opens whole new avenues to follow the metabolic fates of PEG₂-IFN and other pegylated protein therapeutics.

Table 2. Summary of Interferon Pharmacokinetic Parameters

interferon pharmacokinetic parameters									
rat no.	$T_{\max}$ (h)	$C_{\max}$ (units/mL)	T_{last} (h)	C_{last} (units/mL)	AUC_{last} (units h/mL)	half-life (h)	AUC_{inf} (units h/mL)	$\text{AUC}_{\%}$ extrap	MRT (h)
1	1	10 726	6	113	22 202	0.7	22 318	0.5	1.5
2	0.5	12 299	4	401	20 840	0.7	21 250	1.9	1.4
3	1	11 786	6	28	22 833	0.5	22 854	0.1	1.4
4	1	5888	8	44	18 402	0.7	18 448	0.2	2.2
mean	0.9	10 175	6	147	21 069	0.7	21 217	0.7	1.6
SD	0.3	2932	1.6	174	1963	0.1	1963	0.8	0.4

Table 3. Summary of PEG₂-IFN Alfa-2a Pharmacokinetic Parameters

PEG ₂ -IFN alfa-2a pharmacokinetic parameters									
rat no.	$T_{\max}$ (h)	$C_{\max}$ (units/mL)	T_{last} (h)	C_{last} (units/mL)	AUC_{last} (units h/mL)	half-life (h)	AUC_{inf} (units h/mL)	$\text{AUC}_{\%}$ extrap	MRT (h)
1	24	42 220	48	21 110	1 530 853	45	2 911 253	47	66
2	24	36 200	48	27 215	1 277 506	58	3 566 959	64	94
3	24	27 190	48	19 225	912 794.5	48	2 243 867	59	81
mean	24	35 203	48	22 517	1 240 384	51	2 907 360	57	80
SD	0	7564	0	4177	310 697	7	661 555	9	14

Table 4. Summary of Immunogenicity Data

protein	antibody (INU/mL) ^a	
	median	range
interferon α -2a	2400	217–8533
interferon α -2a aggregates	42 667	8000–768533
first generation 5 kDa PEG ₂ -IFN α -2a	75	50–750
PEG ₂ -interferon α -2a	0	0–1133 ^b

^a Interferon neutralizing units/mL. INU/mL = (units of interferon neutralized $\times$ serum dilution)/(final test volume). ^b One of 10 animals in the group produced neutralizing antibody.

PEG₂-IFN is comprised of mono-pegylated interferon α -2a (1 PEG/mol of interferon α -2a) but distributed among four major pegylation sites. In addition to the N-terminus, there are 11 lysines available for pegylation. It should be noted that when a low molecular weight poly(ethylene glycol) (e.g., 5K) was used for pegylation of interferon α -2a all 11 lysines were pegylated (13), thus more heterogeneous than the PEG₂-IFN in terms of positional isomers. Not all pegylation sites are readily accessible to the 40K branched PEG due to its larger size and shape. The end-result is fewer sites are pegylated. Thus PEG₂-IFN is less heterogeneous than the linear low molecular weight PEG-IFN.

It is postulated that the chemical attachment of PEG moieties to the polypeptide backbone of interferon α -2a produces some form of conformational perturbances, which may adversely affect receptor/ligand interactions and thus lower binding affinity (12). This is one reason in vitro anti-viral activity is considerably decreased compared to interferon α -2a. In addition, the relatively short incubation time (hours) of the in vitro assay is probably insufficient for the low affinity PEG₂-IFN to reach peak activity. On the other hand, in vivo bioassays in mice for example, are much longer (days) and are terminated after several days after the injection of the drug. The extended exposure time afforded by the longer half-life of the PEG₂-IFN (Tables 2 and 3) compensates for any slack in the binding interactions due to pegylation. The end-result is maximum biological effect. A sustained release prodrug mechanism is ruled out because the amide bond between PEG and interferon α -2a is stable.

Fish (26) and Klaus et al. (27) define amino acid domains Cys²⁹–Asp³⁵ and Phe¹²³–Trp¹⁴⁰ in interferon α -2a as responsible for receptor binding interactions, as well as constituting the receptor recognition site. These

two domains are located on the surface of interferon α -2a and are in close spatial proximity. The four major pegylation sites in PEG₂-IFN also fall within these domains. In the cell-based in vitro assay with the relatively short incubation time combined with the sites of pegylation plays a major role in how many chances PEG₂-IFN gets to interact with its receptor. However, in vivo biological effect is not expected to be affected in a similar fashion. Though there may be some interference in receptor binding due to pegylation, the very large circulating half-life combined with the flexibility of poly(ethylene glycol) provides enough opportunities for interferon α -2a to come into contact with the receptor to exert its biological effect.

Contrary to the reduced in vitro anti-viral activity of PEG₂-IFN, a significantly enhanced in vivo antitumor activity was observed in various transplanted human tumors. Reduced in vitro activity accompanied with increased in vivo biological activity has been observed with other pegylated protein therapeutics. Examples are G-CSF (28), IL-2 (29), TNF- α (30), IL-6 (31), and CD4-IgG fusion protein (32), among others. The enhanced antitumor activity suggests that PEG₂-IFN is potentially a far superior antitumor agent than interferon α -2a. Consequently, there are a number of clinical trials ongoing or planned for the treatment of several forms of cancer, such as renal carcinoma, malignant melanoma, chronic myelogenous leukemia, among others.

PEG₂-IFN provides extraordinary protection against production of antibodies against interferon α -2a in mice compared to the linear low molecular weight first generation PEG-interferon α -2a. Since interferons are species-restricted, human interferons are generally immunogenic in animals. Therefore, the lack of immunogenicity in mice is a good indication that it will be less immunogenic in humans.

A Phase II clinical trial was initiated in 1998 with PEG₂-IFN in noncirrhotic patients. The Phase II clinical trial results (33) demonstrated that hepatitis C patients treated with PEG₂-IFN once weekly showed undetectable levels of the virus in 76% of the patients, 12 weeks after treatment. Another 36% of patients had similar response, 24 weeks following completion of treatment. On the other hand, only 17% of the patients who had undergone the standard interferon α -2a treatment experienced undetectable levels of hepatitis C virus after 12 weeks of

treatment and only 3% maintained undetectable levels of virus for 24 weeks following the completion of treatment.

A Phase II/III clinical trial of PEG₂-IFN in cirrhotic hepatitis C patients had shown that 29% of the patients experienced a sustained response of nondetectable levels of hepatitis C virus, compared to only 6% of patients treated with interferon α -2a. Sustained response is measured for 24 weeks following 48 weeks of treatment (34).

Finally, in a large Phase III clinical trial (35), PEG₂-IFN exhibited superior efficacy than standard interferon. Highlights of the trial include (1) at the end of treatment virological response was 69% for PEG₂-IFN compared to only 29% for interferon treated group, (2) sustained virologic response was observed in 39% of patient population, (3) in the toughest to treat patient population; those with cirrhosis, 39% experienced sustained virological response, (4) histology was significantly improved, (5) tolerability was similar to standard interferon, and (6) dosing regimen included convenient once weekly dosing compared to thrice weekly dosing of standard interferon.

It is remarkable that although PEG₂-IFN used in the clinical trials had only 5–10% of the original in vitro antiviral activity of interferon α -2a, yet it was significantly more effective in treating HCV infection in humans than the standard interferon. Thus PEG₂-IFN, with the registered name of PEGASYN is a well-tolerated and effective treatment for hepatitis C patients, including those with difficult to treat liver cirrhosis.

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4-Oxo-4*H*-quinolizine-3-carboxylic Acids as Mg^{2+} Selective, Fluorescent Indicators

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In this paper, we present the synthesis of a new series of substituted 4-oxo-4*H*-quinolizine-3-carboxylic acids and the evaluation of their fluorescent response upon complexation with Mg^{2+} and Ca^{2+} . This has led to the development of the first Mg^{2+} -selective, ratioable fluorescent indicators. We found that 4-oxo-4*H*-quinolizine-3-carboxylates are excellent fluorophores which show a strong fluorescent response to Mg^{2+} but not to Ca^{2+} ; the latter metal ion often causing interference during Mg^{2+} measurements with previously described indicators. The dissociation constants of the studied fluorophores—around 1.0 mM—are of the same order of magnitude as is usually observed for intracellular Mg^{2+} .

INTRODUCTION

Magnesium ions play a fundamental role in cellular biochemical processes (1–7), and epidemiological studies have suggested correlations between plasma or cellular Mg^{2+} levels and various chronic diseases, including ischaemic heart disease (8), hypertension (9), atherosclerosis (10), osteoporosis (11), and migraines (12). It is therefore surprising that methods for the determination of both intracellular and plasma Mg^{2+} are fairly limited. In particular, recent studies by Elin and co-workers have documented significant problems with determinations based on Mg^{2+} -selective electrodes (13–15). Fluorine or phosphorus-containing NMR indicators have proven useful, but have limited sensitivity (16–23). Fluorescent indicators for Ca^{2+} and other cations provide a high sensitivity means for the determination of intracellular ion levels (18, 24). Most currently used fluorescent indicators for cellular Mg^{2+} are based on the aminophenol triacetic acid (APTRA) structure (16, 25, 26). While this class of indicators possesses many desirable characteristics as a fluorescent indicator, most particularly a fluorescence emission or excitation shift (depending on the derivative) upon Mg^{2+} complexation, these indicators suffer from the important limitation that they have a higher affinity for Ca^{2+} than for Mg^{2+} (16). This behavior significantly limits the application of APTRA derivatives for some types of measurements; in fact, in some systems APTRA indicators have been used as low affinity Ca^{2+} indicators (27–29). We note here that a number of Mg^{2+} -selective chelators and even fluorescent indicators that have been described (e.g., refs 30 and 31) are not directly applicable to measurements in biological systems due to limited water solubility, mismatch of the K_D to physiological Mg^{2+} levels or other limiting factors.

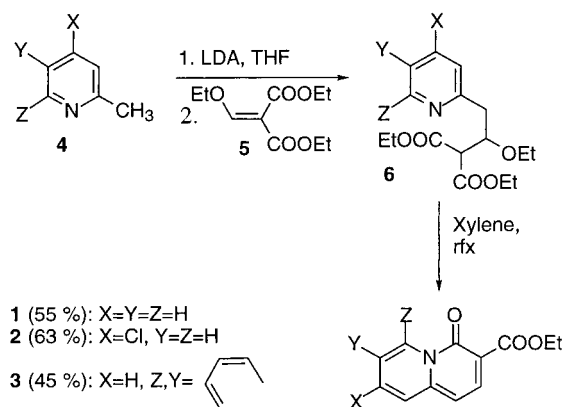
In our search for a more Mg^{2+} selective indicator, we became aware of the fluorinated quinolones, widely used as antibiotics, which are known to chelate Mg^{2+} (32–34). The dissociation constants for Mg^{2+} , K_D^{Mg} are typically close to 1 mM, well suited for determinations of either intra- or extracellular Mg^{2+} concentrations, while the K_D^{Ca} values are typically an order of magnitude greater (32, 34). Although the K_D values for other cations are in some cases lower than the K_D^{Mg} value, these ions are

typically not present at concentrations which would be expected to compromise the measurement of Mg^{2+} . Sensitivity of the spectroscopic properties of the fluoroquinolones to ion complexation has previously been noted (32–34). Chapman et al. (35) have reported increases in the fluorescence of the benzoquinolones fleroxacin, norfloxacin, and pefloxacin in the presence of Mg^{2+} with no concomitant shift in the wavelength of the resulting complex. In contrast, the fluorescence spectra of these molecules were found to be insensitive to 1 mM Ca^{2+} .

A recent report on the synthesis and biological activity of the isomeric 4-oxo-4*H*-quinolizine-3-carboxylic acids (36) encouraged us to explore their utility as possible fluorescent chelators. In this study we describe the synthesis (Scheme 1) of a series of new 4-oxo-4*H*-quinolizine-3-carboxylic acid derivatives and the evaluation of their potential as fluorescent indicators for Mg^{2+} . Our synthetic efforts were aimed at preparing indicators with appropriate dissociation constants and, ideally, an excitation or emission shift in order to allow a determination based on a fluorescence ratio, to introduce polyvalency into the 4-oxo-4*H*-quinolizine-3-carboxylic acids, and to extend the conjugation of the parent nucleus so as to achieve 500+ nm fluorescence emission maxima. Esters of 4-oxo-4*H*-quinolizine-3-carboxylic acids were conveniently synthesized by a thermal ring closure reaction of the Michael adduct of a 2-picoline and diethyl ethoxymethylenemalonate. Further elaboration of the heterocyclic ring system was achieved by a regioselective, electrophilic aromatic substitution at the 1-position of **1**, or by a nucleophilic substitution of the Cl substituent at the 8-position of **2**. Our search has led to the finding that 4-oxo-4*H*-quinolizine-3-carboxylic acids are excellent fluorophores that respond to changing Mg^{2+} levels. The dissociation constants for Mg^{2+} were found to be in the low or submillimolar range. In addition, and most importantly, these fluorophores are hardly responsive to Ca^{2+} concentrations well above basal intracellular and even extracellular (millimolar) levels.

EXPERIMENTAL SECTION

General Remarks. Tetrahydrofuran (THF) was dried (Na/benzophenone) and distilled prior to use. Dimethyl-

Scheme 1. Synthesis of the Ethyl Esters of 4-Oxo-4H-quinolizine-3-carboxylic Acids 1, 2, and 3

formamide (DMF) was dried over molecular sieves (4 Å). Ethyl acetate, hexane, and diethyl ether and all other commercially available chemicals were used as received. After extractive workup, the combined organic phases were dried over MgSO_4 . Water used in the spectrophotometric experiments was filtered through a Hydro Picopure2 filter. Thin-layer chromatography was carried out on Sigma-Aldrich TLC Plates (silica gel on polyester). Chromatographic purifications were done by flash chromatography using Merck Silica Gel, grade 9385, 230–400 mesh, 60 Å. Melting points were measured on a Fisher-Johns melting point apparatus and are uncorrected. ^1H NMR spectra were recorded on a Nicolet NT 360 MHz NMR Spectrometer and a Varian UNITY plus 400 MHz NMR spectrometer. Chemical shifts are reported in parts per million (δ), using TMS as the internal standard, and signals are expressed as an s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). High resolution mass spectra were obtained from the Washington University Resource for Biomedical and Bio-organic Mass Spectrometry on samples which were chromatographically pure. Compounds **9a** and **21a** were analyzed by FABMS using NBA Li matrix. UV absorption spectra were recorded with a Beckman DU 640 spectrophotometer using Fisherbrand semimicro quartz cells (Fisher Scientific).

Fluorescence Measurements. Fluorescence measurements were recorded with an Aminco-Bowman Series 2 Spectrometer using quartz fluorimeter cuvettes from Sigma. The buffered solutions used in all experiments contained 120 mM KCl, 20 mM NaCl, and 15 mM TRIS, and were brought to pH 7.20 with concentrated HCl. A stock solution of 2 mg of the indicator in 5 mL of buffer was prepared. To record a UV spectrum, the stock solutions were diluted 40 times (25 μL in 975 μL buffer). For the fluorescence titration experiments, 25 μL of the stock solution was diluted 100 times with 2475 μL of buffer. Emission fluorescence and excitation fluorescence spectra were recorded using the appropriate excitation wavelength derived from the UV spectrum of the free indicator. To 2475 μL of a MgCl_2 solution [100 mM, 250 mM, and 500 mM (**2a**)] was added 25 μL of the stock solution of the indicator. Increasing amounts of the MgCl_2 /indicator solution were added to the solution of the free indicator; after each titration step, fluorescence emission and fluorescence excitation spectra were recorded. To determine the pK_a of **21a**, 250 μL of the stock solution of this fluorophore was added to 25 mL, 10 mM MOPS at pH 4.29. A fluorescence emission spectrum was recorded. A 0.1 M NaOH solution, containing an equal concentration of the fluorophore, was added dropwise and

after each pH increase of 0.2 an emission spectrum was recorded. At pH 6.31, no change of the fluorescence emission was observed. The fluorescence emission was plotted against the pH to determine the pK_a which was found to be 5.6.

Ethyl 8-Chloro-4-oxo-4H-quinolizine-3-carboxylate (2). A 250 mL oven-dried (140 $^\circ\text{C}$), three-necked, round-bottomed flask, equipped with an oven-dried 100 mL addition funnel and a pentane thermometer was purged with argon. The flask was charged with 50 mL of dry THF and diisopropylamine (7.2 mL, 50 mmol, dried over NaOH). The addition funnel was charged with a solution of 4-chloro-2-picoline (5.0 g, 40 mmol) in 50 mL of dry THF. The THF/amine solution was cooled to -50 $^\circ\text{C}$, and 20 mL of a 1.6 M solution of *n*-BuLi in hexanes was added rapidly via syringe. Stirring was continued for 30 min. The reaction mixture was then cooled to -78 $^\circ\text{C}$, and the picoline solution was added over a 15 min period while the temperature of the reaction mixture was maintained below -60 $^\circ\text{C}$. The reaction mixture was stirred for an additional 30 min, during which time a bright red suspension formed. The addition funnel was charged with a solution of diethyl ethoxymethylenemalonate (9.5 g, 45 mmol) in 25 mL of dry THF, and the solution was added slowly to the reaction mixture while maintaining the temperature below -60 $^\circ\text{C}$. A pale yellow, clear solution formed which was allowed to warm to -20 $^\circ\text{C}$ over a period of 2 h. The reaction mixture was poured into 500 mL of water and extracted three times with 300 mL of CH_2Cl_2 . Subsequent evaporation of the organic solvent gave a yellow oil (13.30 g, 96%) which was dissolved in 60 mL of xylene. The reaction mixture was refluxed for 16 h, allowed to cool to room temperature, and the orange crystalline residue collected by filtration. The xylene in the filtrate was evaporated in vacuo, the remaining orange solid was stirred overnight with 30 mL of Et_2O and filtered. The combined filtration residues were dried in vacuo over KOH to give pure **2** (6.28 g, 63%), mp 161–4 $^\circ\text{C}$. ^1H NMR (CDCl_3): δ 1.42 (t, $J = 7$ Hz, 3H), 4.41 (q, $J = 7$ Hz, 2H), 6.55 (d, $J = 8$ Hz, 1H), 7.09 (dd, $J = 2$ Hz, $J = 7$ Hz, 1H), 7.53 (d, $J = 2$ Hz, 1H), 8.40 (d, $J = 8$ Hz, 1H), 9.29 (d, $J = 7$ Hz, 1H). MS: m/z 251.0360; required for $\text{C}_{12}\text{H}_{10}\text{ClNO}_3$; 251.0349.

Ethyl 4-Oxo-4H-quinolizine-3-carboxylate (1). Using the procedure described for **2**, compound **1** was obtained as an orange crystalline solid (55%) by flash chromatography (EtOAc), mp 105–9 $^\circ\text{C}$. ^1H NMR (CDCl_3): δ 1.43 (t, $J = 7.0$ Hz, 3H), 4.42 (q, $J = 7.0$ Hz, 2H), 6.65 (d, $J = 8.4$ Hz, 1H), 7.16–7.24 (m, 1H), 7.52–7.66 (m, 2H), 8.41 (d, $J = 8.4$ Hz, 1H), 9.40 (d, $J = 7.3$ Hz, 1H). MS: m/z 217.0735; required for $\text{C}_{12}\text{H}_{11}\text{NO}_3$, 217.0738.

Ethyl Benzo[6,7]-4-oxo-4H-quinolizine-3-carboxylate (3). Using the procedure described for **1**, compound **3** was obtained as an orange powder (45%) by flash chromatography (EtOAc :hexanes 7:3), mp 106–8 $^\circ\text{C}$. ^1H NMR (CDCl_3): δ 1.42 (t, $J = 7.0$ Hz, 3H), 4.42 (q, $J = 7.0$ Hz, 2H), 6.49 (d, $J = 9.7$ Hz, 1H), 7.14 (d, $J = 9.2$ Hz, 1H), 7.48–7.64 (m, 4H), 8.27 (d, $J = 7.7$ Hz, 1H), 9.87 (d, $J = 8.8$ Hz, 1H). MS: m/z 267.0908; required for $\text{C}_{16}\text{H}_{13}\text{NO}_3$, 267.0895.

Ethyl 1-Nitro-4-oxo-4H-quinolizine-3-carboxylate (7). Nitric acid (1.08 g, 17 mmol) was added slowly to acetic anhydride (7 mL) at 5 $^\circ\text{C}$. Stirring was continued for 5 min and the reaction mixture was cooled to -10 $^\circ\text{C}$ (ice/salt bath). Compound **1** (920 mg, 4.2 mmol) was added in one portion. After 5 min an orange precipitate formed, and after 15 min of total reaction time the reaction was judged to be complete by TLC (EtOAc). The

reaction mixture was poured on to 250 g of crushed ice and stirred for 30 min to give an orange suspension which was extracted three times with 100 mL of CH_2Cl_2 . The combined organic layers were washed three times with 100 mL of water. Evaporation of the solvent gave a deep red solid which was further purified by flash chromatography to give 497 mg (50%) of a bright yellow solid, mp 169–70 °C. ^1H NMR (CDCl_3): δ 1.44 (t, J = 7.0 Hz, 3H), 4.45 (q, J = 7.0 Hz, 2H), 7.55, (dd, J = 7.0 Hz, J = 7.0 Hz, 1H), 8.15 (dd, J = 9.7 Hz, 7.0 Hz, 1H), 9.31 (d, J = 9.1 Hz, 1H), 9.47 (s, 1H), 9.60 (d, J = 7.0 Hz, 1H). MS: m/z 262.0601; required for $\text{C}_{12}\text{H}_{10}\text{N}_2\text{O}_5$: 262.0590.

Ethyl 1-[N,N-Di(carbomethoxymethyl)]-4-oxo-4H-quinolizine-3-carboxylate (11). A suspension of **7** (540 mg, 2.0 mmol) and 5% Pd on C in 25 mL of ethanol was stirred under an atmosphere of hydrogen until 50 mL of H_2 had been consumed. TLC (EtOAc) confirmed complete conversion of the starting material. The reaction mixture was filtered, and evaporation of the solvent in vacuo gave 400 mg (86%) of a red solid. The crude product was dissolved in 9 mL of dry acetonitrile and methyl bromoacetate (1.0 mL, 11 mmol), NaI (1.5 g, 10 mmol), and Proton Sponge (2.6 g, 12 mmol) were added. The reaction mixture was refluxed for 17 h, allowed to cool to room temperature, and poured into 200 mL of EtOAc. The resulting suspension was stirred for 1 h and filtered, and the red filtrate was washed three times with a pH 2 buffer solution. Evaporation of the solvent and purification of the crude product by flash chromatography (EtOAc) yielded 410 mg (54%) of a yellow solid, mp 145–8 °C. ^1H NMR (CDCl_3): δ 1.43 (t, J = 7 Hz, 3H), 3.88 (s, 6H), 4.08 (s, 4H), 4.42 (q, J = 7 Hz, 4H), 7.23 (dd, J = 7 Hz, J = 7 Hz, 1H), 7.72 (dd, J = 7 Hz, J = 7 Hz, 1H), 8.50 (d, J = 8 Hz, 1H), 8.62 (s, 1H), 9.43 (d, J = 7 Hz). MS: m/z 376.1252; required for $\text{C}_{18}\text{H}_{20}\text{N}_2\text{O}_7$: 376.1270.

Ethyl 1-Formyl-4-oxo-4H-quinolizine-3-carboxylate (8). **1** (1 g, 4.0 mmol) was dissolved in 2 mL of DMF followed by the addition of POCl_3 (1.0 mL, 11 mmol). The reaction mixture was stirred for 1 h, poured into 100 mL of water and extracted three times with 100 mL of CH_2Cl_2 . Evaporation of the organic solvent gave a sticky yellow solid which was further purified by flash chromatography (EtOAc), followed by overnight stirring with Et_2O . Filtration of the suspension gave a bright yellow powder (920 mg, 93%), mp 179–81 °C. ^1H NMR (CDCl_3): δ 1.43 (t, J = 7 Hz), 4.42 (q, J = 7 Hz), 7.51 (dd, J = 7 Hz, J = 7 Hz, 1H), 8.06 (dd, J = 7 Hz, J = 7 Hz, 1H), 8.80 (s, 1H), 9.57 (d, J = 7 Hz), 9.90 (s, 1H). MS: m/z 245.0683; required for $\text{C}_{13}\text{H}_{11}\text{NO}_4$: 245.0688.

Ethyl 1-Bromo-4-oxo-4H-quinolizine-3-carboxylate (9). To a solution of **1** (2.33 g, 10.7 mmol) in 20 mL of HOAc was rapidly added a solution of Br_2 (1.9 g, 12 mmol) in 10 mL of HOAc. After 5 min, TLC (EtOAc) indicated complete consumption of the starting material. The solvent was evaporated in vacuo and the remaining yellow solid was dissolved in 20 mL of CH_2Cl_2 . To this solution, 3 g of silica gel was added and the solvent was evaporated in vacuo. The silica gel was poured on a prepacked column and the eluted with EtOAc to give **9** (2.1 g, 71%) as a bright yellow solid, mp 156–8 °C. ^1H NMR (CDCl_3): δ 1.43 (t, J = 7 Hz, 3H), 4.43 (q, J = 7 Hz, 2H), 7.03 (dd, J = 7 Hz, J = 7 Hz, 1H), 7.30 (dd, J = 7 Hz, J = 7 Hz, 1H), 7.80 (dd, J = 7 Hz, J = 7 Hz, 1H), 8.63 (s, 1H), 9.49 (d, J = 7 Hz). MS: m/z 294.9839; required for $\text{C}_{12}\text{H}_{10}\text{BrNO}_3$: 294.9843.

Ethyl 1-(2,2-Dicarbethoxyvinyl)-4-oxo-4H-quinolizine-3-carboxylate (12). A 100 mL round-bottomed flask, equipped with a Dean–Stark trap was charged with 50 mL of benzene, **8** (1.22 g, 5 mmol), acetic acid

(5 g, 83 mmol), NH_4OAc (5 g, 65 mmol), and diethyl malonate (2.3 g, 9 mmol). The reaction mixture was refluxed for 48 h. The reaction mixture was allowed to cool to room temperature and washed three times with 50 mL of water. The solvent was evaporated in vacuo, and the crude product was purified by flash chromatography (EtOAc) to yield **12** as a yellow powder (1.58 g, 82%), mp 149–151 °C. ^1H NMR (CDCl_3): δ 1.31 (t, J = 7 Hz, 3H), 1.36 (t, J = 7 Hz, 3H), 1.42 (t, J = 7 Hz, 3H), 4.34 (q, J = 7 Hz, 2H), 4.36 (q, J = 7 Hz, 2H), 4.41 (q, J = 7 Hz, 2H), 7.34 (dd, J = 7 Hz, J = 7 Hz, 1H), 7.82 (dd, J = 8 Hz, 1H), 7.96 (d, J = 8 Hz, 1H), 8.05 (s, 1H), 8.60 (s, 1H), 9.50 (d, J = 7 Hz). MS: m/z 387.1331; required for $\text{C}_{20}\text{H}_{21}\text{NO}_7$: 387.1318.

Ethyl 1-(2,2-Dicarbethoxyethyl)-4-oxo-4H-quinolizine-3-carboxylate (13). To a bright yellow suspension of **12** (750 mg, 1.94 mmol) in 5 mL of ethanol was added NaBH_3CN (68 mg, 1.08 mmol). The reaction mixture turned red, and all solid material dissolved. Stirring was continued for 1 h and the reaction mixture was poured into 50 mL of water. Ethanol was added to the aqueous suspension until a clear solution was obtained which was extracted three times with 100 mL of CH_2Cl_2 . The organic solvent was evaporated, and the crude product was purified by flash chromatography (EtOAc) to give **13** as a bright yellow solid (275 mg, 36%), mp 105–8 °C. ^1H NMR (CDCl_3): δ 1.22 (t, J = 7 Hz, 6H), 1.41 (t, J = 7 Hz, 3H), 3.41 (d, J = 7 Hz, 2H), 3.65 (t, J = 7 Hz, 1H), 4.10–4.25 (m, 4H), 4.41 (q, J = 7 Hz, 2H), 7.23 (m, 1H), 7.70 (m, 1H), 7.84 (d, J = 8 Hz, 1H), 8.33 (s, 1H), 9.50 (d, J = 7 Hz, 1H). MS: m/z 389.1466; required for $\text{C}_{20}\text{H}_{23}\text{NO}_7$: 389.1475.

Ethyl 8-Azido-4-oxo-4H-quinolizine-3-carboxylate (14). An oven-dried 25 mL round-bottomed flask was purged with Ar and charged with **2** (250 mg, 1 mmol), NaN_3 (300 mg, 4.6 mmol), and 5 mL of DMF. The reaction mixture was stirred at 40 °C for 1 h, allowed to cool to room temperature, and poured into 100 mL of water. The aqueous layer was extracted three times with 100 mL of CHCl_3 , and the combined organic layers were washed three times with 100 mL of water. Evaporation of the organic solvent, followed by flash chromatography (EtOAc), gave a deep red crystalline solid (218 mg, 85%), mp 145–150 °C. ^1H NMR (CDCl_3): δ 1.42 (t, J = 7 Hz, 3H), 4.41 (q, J = 7 Hz, 2H), 6.53 (d, J = 8 Hz, 1H), 6.84 (dd, J = 2 Hz, J = 7 Hz, 1H), 7.08 (d, J = 2 Hz, 1H), 8.37 (d, J = 8 Hz, 1H), 9.36 (d, J = 7 Hz, 1H). MS: m/z 258.0751; required for $\text{C}_{12}\text{H}_{10}\text{N}_4\text{O}_3$: 258.0753.

Ethyl 8-Amino-4-oxo-4H-quinolizine-3-carboxylate (15). A suspension of **14** (253 mg, 1.0 mmol) and 20 mg of 5% Pd on C in 15 mL of ethanol was stirred under a hydrogen atmosphere. The color of the reaction mixture rapidly changed from deep red to dark yellow. After 30 mL of H_2 had been absorbed by the reaction mixture, the catalyst was filtered off and the solvent was evaporated to give pure **15**, 221 mg (94%), as a yellow/green powder, mp > 250 °C. ^1H NMR ($\text{DMSO}-d_6$): δ 1.23 (t, J = 7 Hz, 3H), 4.13 (q, J = 7 Hz, 2H), 6.23 (d, J = 7 Hz, 1H), 6.53 (d, J = 2 Hz, 1H), 6.79 (dd, J = 2 Hz, J = 7 Hz, 1H), 7.32 (s, 2H), 7.81 (d, J = 8 Hz, 1H), 8.94 (d, J = 7 Hz, 1H). MS: m/z 232.0837; required for $\text{C}_{12}\text{H}_{12}\text{NO}_3$: 232.0848.

Ethyl 8-(3-Nitrophenoxy)-4-oxo-4H-quinolizine-3-carboxylate (16). An oven-dried, 50 mL three-necked, round-bottomed flask was purged with argon and charged with NaH (200 mg, 60% suspension in oil, 5 mmol and washed with hexane,) and 10 mL of dry DMF. To this suspension, *m*-nitrophenol (626 mg, 4.5 mmol) was added in one portion. After gas evolution had ceased, **2** (1.00 g, 4 mmol) was added in one portion and stirring was

continued at room temperature for 2 h. TLC (EtOAc) indicated complete consumption of the starting material, and the reaction mixture was poured into 250 mL of water. The aqueous layer was extracted three times with 200 mL of CH_2Cl_2 , and the combined organic layers were washed three times with 200 mL of water. The organic solvent was evaporated and the crude product was stirred overnight with 30 mL of Et_2O . Filtration gave 950 mg (67%) of a bright yellow solid, mp 161–3 °C. ^1H NMR (CDCl_3): δ 1.42 (t, $J = 7$ Hz, 3H), 4.41 (q, $J = 7$ Hz, 2H), 6.39 (d, $J = 8$ Hz, 1H), 6.70 (d, $J = 2$ Hz, 1H), 6.99 (dd, $J = 0.5$ Hz, $J = 7$ Hz, 1H), 7.55 (d, $J = 7$ Hz, 1H), 7.72 (dd, $J = 8$ Hz, $J = 8$ Hz, 1H), 8.07 (s, 1H), 8.24 (d, $J = 8$ Hz, 1H), 8.32 (d, $J = 8$ Hz, 1H), 9.43 (d, $J = 7$ Hz, 1H). MS: m/z 354.0850; required for $\text{C}_{18}\text{H}_{14}\text{N}_2\text{O}_6$: 354.0852.

Ethyl 8-[3-(*N,N*-Dicarbomethoxymethyl)phenoxy]-4-oxo-4*H*-quinolizine-3-carboxylate (18). A suspension of **16** (710 mg, 2 mmol) and 200 mg of 5% Pd on C in 25 mL of ethanol was stirred under an atmosphere of hydrogen for 6 h. The resulting deep yellow solution was filtered from the catalyst, and the solvent was evaporated to give 650 mg of the crude amine as a golden brown crystalline solid. The amine was dissolved in 9 mL of acetonitrile and NaI (1.5 g, 10 mmol), methyl bromoacetate (1 mL, 10 mmol), and Proton Sponge (2.6 g, 12 mmol) were added. The reaction mixture was refluxed for 16 h, allowed to cool to room temperature and poured into 200 mL of EtOAc. The organic layer was washed with 200 mL portions of buffer (pH 2) until an acid reaction on litmus appeared. The solvent was evaporated and the crude product was purified with flash chromatography (EtOAc) to give a bright yellow powder (310 mg, 30% over two steps), mp 161–3 °C. ^1H NMR (CDCl_3): δ 1.41 (t, $J = 7$ Hz, 3H), 3.76 (s, 6H), 4.16 (s, 4H), 4.39 (q, $J = 7$ Hz, 2H), 6.29–6.45 (m, 2H), 6.51–6.64 (m, 2H), 6.71 (d, $J = 2$ Hz, 1H), 6.97 (dd, $J = 2$ Hz, $J = 7$ Hz, 1H), 7.32 (dd, $J = 8$ Hz, $J = 8$ Hz, 1H), 8.26 (d, $J = 8$ Hz, 1H), 9.38 (d, $J = 8$ Hz, 1H). MS: m/z 468.1535; required for $\text{C}_{24}\text{H}_{24}\text{N}_2\text{O}_8$: 468.1532.

Ethyl 8-[Dicarbomethoxymethyl]-4-oxo-4*H*-quinolizine-3-carboxylate (19). A solution of $\text{Na}[\text{CH}(\text{CO}_2\text{Me})]$ in DMSO was prepared by adding dimethylmalonate (5.6 g, 42 mmol) in 10 mL of DMSO to a suspension of NaH (2.0 g of a 60% suspension in oil, 50 mmol and washed with hexane) in 10 mL of DMSO. An oven-dried, 250 mL, three-necked, round-bottomed flask equipped with a 100 mL dropping funnel containing the freshly prepared malonate anion solution, was charged with **2** (3.76 g, 15.3 mmol) and 20 mL of DMSO. The malonate anion solution was added rapidly to the reaction mixture and stirring was continued overnight. The reaction mixture was poured into 500 mL of water and extracted three times with 400 mL of CH_2Cl_2 . The organic solvent was evaporated and the crude product stirred overnight with 20 mL of EtOAc. Filtration and drying in vacuo gave 3.22 g (63%) of a yellow powder, mp 195–7 °C. ^1H NMR (CDCl_3): δ 1.42 (t, $J = 7$ Hz, 3H), 3.82 (s, 6H), 4.41 (q, $J = 7$ Hz, 2H), 4.73 (s, 1H), 6.64 (d, $J = 8$ Hz, 1H), 7.22 (d, $J = 8$ Hz, 1H), 7.59 (s, 1H), 8.41 (d, $J = 8$ Hz, 1H), 9.34 (d, $J = 7$ Hz, 1H). MS: m/z 347.1021; required for $\text{C}_{17}\text{H}_{17}\text{NO}_7$: 347.1004.

Ethyl 8-[1,1-Dicarbomethoxy-2-carbethoxyethyl]-4-oxo-4*H*-quinolizine-3-carboxylate (20). An oven-dried, 50 mL, three-necked, round-bottomed flask equipped with a reflux condenser was charged with NaH (160 mg, 60% suspension in oil, 4 mmol and washed with hexane) and 10 mL of THF. Compound **19** (700 mg, 2 mmol) was added in three portions and stirring was continued for 1 h, followed by the addition of ethyl bromoacetate (0.5 mL,

5.3 mmol). The reaction mixture was refluxed overnight, allowed to cool to room temperature, and poured into 100 mL of water. The aqueous layer was extracted three times with 100 mL of CH_2Cl_2 . The organic solvent was evaporated and the crude product was purified by flash chromatography (EtOAc) to give a yellow viscous oil (630 mg, 74%). ^1H NMR (CDCl_3): δ 1.24 (t, $J = 7$ Hz, 3H), 1.42 (t, $J = 7$ Hz, 3H), 3.39 (s, 2H), 3.84 (s, 6H), 4.14 (q, $J = 7$ Hz, 2H), 4.41 (q, $J = 7$ Hz, 2H), 6.63 (d, $J = 8$ Hz, 1H), 7.14 (dd, $J = 2$ Hz, $J = 7$ Hz, 1H), 7.61 (d, $J = 2$ Hz, 1H), 8.40 (d, $J = 8$ Hz, 1H), 9.29 (d, $J = 8$ Hz, 1H). MS: m/z 433.1363; required for $\text{C}_{21}\text{H}_{21}\text{NO}_9$: 433.1372.

Ethyl 1-Bromo-8-[1,1-dicarbomethoxy-2-carbethoxyethyl]-4-oxo-4*H*-quinolizine-3-carboxylate (21). Using the bromination procedure described for the synthesis of **9**, compound **21** was isolated as an orange mass in a yield of 75% by flash chromatography (EtOAc). ^1H NMR (CDCl_3): δ 1.26 (t, $J = 7$ Hz, 3H), 1.42 (t, $J = 7$ Hz, 3H), 3.45 (s, 2H), 3.85 (s, 6H), 4.16 (q, $J = 7$ Hz, 2H), 4.41 (q, $J = 7$ Hz, 2H), 7.33 (d, $J = 8$ Hz, 1H), 8.06 (s, 1H), 8.61 (s, 1H), 9.36 (d, $J = 8$ Hz, 1H). MS: m/z 511.0473; required for $\text{C}_{21}\text{H}_{22}\text{BrNO}_9$: 511.0477.

Ethyl 8-(4-Methoxyphenyl)-4-oxo-4*H*-quinolizine-3-carboxylate (22). An oven-dried, 50 mL, round-bottomed flask, equipped with a reflux condenser, was purged with Ar and charged with **2** (500 mg, 2 mmol), 4-methoxyphenylboronic acid (372 mg, 2.4 mmol), CsF (600 mg, 3.9 mmol), $\text{PdCl}_2[\text{P}(\text{c-Hex})_3]_2$ (80 mg, 0.1 mmol), and 6 mL of DMF. The reaction mixture was stirred at 110 °C for 12 h and allowed to cool to room temperature. TLC (EtOAc) indicated that the starting material had been completely consumed. The reaction mixture was poured into 100 mL of water and extracted three times with 100 mL of CHCl_3 . The combined organic layers were washed three times with 50 mL of water. The organic solvent was evaporated and the crude product was further purified by column chromatography (5% MeOH in EtOAc) to give 340 mg (50%) of a bright yellow solid, mp 205–9 °C. ^1H NMR (CDCl_3): δ 1.43 (t, $J = 7$ Hz, 3H), 3.90 (s, 3H), 4.42 (q, $J = 7$ Hz, 2H), 6.65 (d, $J = 8$ Hz, 1H), 7.05 (d, $J = 9$ Hz, 2H), 7.43 (dd, $J = 2$ Hz, $J = 7$ Hz, 1H), 7.58–7.80 (m, 3H), 8.37 (d, $J = 8$ Hz, 1H), 9.41 (d, $J = 7$ Hz, 1H). MS: m/z 323.1159; required for $\text{C}_{19}\text{H}_{17}\text{NO}_4$: 323.1157.

Ethyl 8-(Naphth-1-yl)-4-oxo-4*H*-quinolizine-3-carboxylate (23). Using the procedure described for **22**, compound **23** was isolated (51% yield) by flash chromatography (EtOAc), mp 245–7 °C. ^1H NMR (CDCl_3): δ 1.48 (t, $J = 7$ Hz, 3H), 4.48 (q, $J = 7$ Hz, 2H), 6.73 (d, $J = 8$ Hz, 1H), 7.40 (dd, $J = 2$ Hz, $J = 8$ Hz, 1H), 7.55–7.64 (m, 4H), 7.72 (s, 1H), 7.91 (d, $J = 8$ Hz, 1H), 7.98–8.06 (m, 2H), 8.48 (d, $J = 8$ Hz, 1H), 9.52 (d, $J = 8$ Hz, 1H). MS: m/z 343.1213; required for $\text{C}_{22}\text{H}_{17}\text{NO}_3$: 343.1208.

Ethyl 8-(Benzo[6,7]fur-2-yl)-4-oxo-4*H*-quinolizine-3-carboxylate (24). Using the procedure described for **22**, compound **24** was isolated (48% yield) by flash chromatography (EtOAc), mp 195–7 °C. ^1H NMR (CDCl_3): δ 1.43 (t, $J = 7$ Hz, 3H), 4.42 (q, $J = 7$ Hz, 2H), 6.72 (d, $J = 8$ Hz, 1H), 7.22–7.54 (m, 4H), 7.57 (d, $J = 8$ Hz, 1H), 7.67 (d, $J = 8$ Hz, 1H), 7.96 (d, $J = 1$ Hz, 1H), 8.40 (d, $J = 8$ Hz, 1H), 9.37 (d, $J = 8$ Hz, 1H). MS: m/z 333.1011; required for $\text{C}_{20}\text{H}_{15}\text{NO}_4$: 333.1000.

4-Oxo-4*H*-quinolizine-3-carboxylic Acid (1a). To a suspension of **1** (100 mg, 0.46 mmol) in 4 mL of MeOH was added 300 μL of 3 M NaOH. The reaction mixture was stirred overnight followed by evaporation of the solvent in vacuo. The remaining solid was dissolved in 4 mL of water, and the clear solution was acidified with 1 M HCl to pH 1. The resulting suspension was filtered

and the residue dried in vacuo over P_2O_5 to give **1a** as yellow plates (63 mg, 72%), mp 235–40 °C. ^1H NMR (DMSO- d_6): δ 7.26 (d, J = 8 Hz, 1H), 7.64 (dd, J = 7 Hz, J = 7 Hz, 1H), 8.03 (dd, J = 7 Hz, J = 7 Hz, 1H), 8.14 (d, J = 9 Hz, 1H), 8.39 (d, J = 9 Hz, 1H), 9.27 (d, J = 7 Hz), 14.13 (s, 1H). MS: m/z 189.0433; required for $C_{12}H_{11}NO_3$: 189.0428.

4-Oxo-8-chloro-4H-quinolizine-3-carboxylic Acid (2a). Using the procedure described for **1a**, compound **2a** was isolated in 52% yield, mp >250 °C. ^1H NMR (DMSO- d_6): δ 7.13 (d, J = 9 Hz, 1H), 7.62 (d, J = 7 Hz, 1H), 8.34 (s, 1H), 8.40 (d, J = 8 Hz, 1H), 9.16 (d, J = 7 Hz, 1H). MS: m/z 223.0028; required for $C_{10}H_6NO_3$: 223.0036.

[6,7]Benzo-4-oxo-4H-quinolizine-3-carboxylic Acid (3a). Using the procedure described for **1a**, compound **3a** was obtained as a red powder in 50% yield, mp >250 °C. ^1H NMR ($CDCl_3$): δ 6.90 (d, J = 8 Hz, 1H), 7.33 (d, J = 9 Hz, 1H), 7.60–7.83 (m, 4H), 8.68 (d, J = 8 Hz, 1H), 9.96 (d, J = 9 Hz, 1H), 14.38 (s, 1H). MS: m/z 239.0575; required for $C_{14}H_9NO_3$: 239.0592.

1-Bromo-4-oxo-4H-quinolizine-3-carboxylic Acid (9a). Using the procedure described for **1a**, compound **9a** was obtained as a bright yellow powder in 57% yield, mp > 250 °C. ^1H NMR (DMSO- d_6): δ 7.76 (dd, J = 7 Hz, J = 7 Hz, 1H), 8.20–8.32 (m, 2H), 8.53 (s, 1H), 9.41 (d, J = 7 Hz, 1H). MS: m/z 273.9693; required for $C_{10}H_6BrNO_3Li$: 273.9691.

1-[N,N-Di(carboxymethyl)]-4-oxo-4H-quinolizine-3-carboxylic Acid (11a). Using the procedure described for **1a**, compound **11a** was obtained as a bright yellow powder in 50% yield, mp 231–4 °C. ^1H NMR (D_2O): δ 3.80 (s, 4H), 7.66 (dd, J = 7 Hz, J = 7 Hz, 1H), 8.07 (dd, J = 7 Hz, J = 7 Hz, 1H), 8.32 (s, 1H), 8.63 (d, J = 8 Hz, 1H), 9.33 (d, J = 7 Hz, 1H). MS: m/z 276.0745 (M- CO_2); required for $C_{13}H_{12}N_2O_5$: 276.0746.

1-(2,2-Dicarboxyvinyl)-4-oxo-4H-quinolizine-3-carboxylic Acid (12a). Using the procedure described for **1a**, compound **12a** was obtained as a bright yellow powder in 61% yield, mp >250 °C. ^1H NMR (DMSO- d_6): δ 7.72 (dd, J = 7 Hz, J = 7 Hz, 1H), 7.90 (s, 1H), 8.13 (dd, J = 7 Hz, J = 7 Hz, 1H), 8.26 (d, J = 9 Hz, 1H), 8.57 (s, 1H), 9.36 (d, J = 7 Hz). MS: m/z 215.0591 (M-2 CO_2); required for $C_{12}H_9NO_3$: 215.0582.

1-(2,2-Dicarboxyethyl)-4-oxo-4H-quinolizine-3-carboxylic Acid (13a). Using the procedure described for **1a**, compound **13a** was obtained as a bright yellow powder in 49% yield, mp >250 °C. ^1H NMR (D_2O , 1% NaOD): δ 3.32 (d, J = 7 Hz, 2H), 3.42 (t, J = 7 Hz, 1H), 7.37 (dd, J = 7 Hz, J = 7 Hz, 1H), 7.74 (dd, J = 7 Hz, J = 7 Hz, 1H), 8.01 (s, 1H), 8.02 (d, J = 7 Hz, 1H), 9.22 (d, J = 7 Hz, 1H). MS: m/z 261.0627 (M- CO_2); required for $C_{13}H_{11}NO_5$: 261.0637.

8-Amino-4-oxo-4H-quinolizine-3-carboxylic Acid (15a). Using the procedure described for **1a**, compound **15a** was obtained as a bright yellow powder in 61% yield, mp >250 °C. ^1H NMR (DMSO- d_6): δ 6.61 (d, J = 9 Hz, 1H), 6.71 (d, J = 2 Hz, 1H), 6.96 (dd, J = 2 Hz, J = 8 Hz, 1H), 7.6 (br), 7.82 (d, J = 9 Hz, 1H), 8.95 (J = 7 Hz, 1H). MS: m/z 160.0634 (M - CO_2); required for $C_9H_8N_2O$: 160.0636.

8-[3-(N,N-di(carboxymethyl)phenoxy)-4-oxo-4H-quinolizine-3-carboxylic Acid (18a). Using the procedure described for **1a**, compound **18a** was obtained as a bright yellow powder in 71% yield, mp = 231–4 °C. ^1H NMR (D_2O): δ 3.98 (s, 4H), 6.38 (s, 1H), 6.52 (d, J = 7 Hz, 1H), 6.57 (d, J = 8 Hz, 1H), 6.75 (d, J = 9 Hz, 1H), 7.08 (s, 1H), 7.23–7.47 (m, 2H), 8.06 (d, J = 8 Hz, 1H), 9.12 (d, J = 8 Hz, 1H). MS: m/z 350.0160 (M - CO_2 - H_2O); required for $C_{19}H_{14}N_2O_5$: 350.2359.

8-[Carboxymethyl]-4-oxo-4H-quinolizine-3-carboxylic Acid (19a). Using the procedure described for **1a**, compound **19a** was obtained as a yellow powder in 68% yield, mp > 250 °C. ^1H NMR (D_2O): δ 3.77 (s, 2H), 7.07 (d, J = 8 Hz, 1H), 7.50 (d, J = 7 Hz, 1H), 7.78 (s, 1H), 8.26 (d, J = 8 Hz, 1H), 9.13 (d, J = 7 Hz). MS: m/z 203.0575 (M - CO_2); required for $C_{11}H_9NO_3$: 203.0582.

8-(1,2-Dicarboxyethyl)-4-oxo-4H-quinolizine-3-carboxylic Acid (20a). Saponification of **20** was done as described for **1**. After completion of the reaction, the solvent was evaporated in vacuo, and the resulting yellow solid was dissolved in water. The aqueous layer was acidified to pH 1 and extracted three times with ethyl acetate. Evaporation of the organic solvent gave **20a** as a yellow solid in 48% yield. ^1H NMR (DMSO- d_6): δ 2.72–2.76 (m, 2H), 3.02–3.06 (m, 1H), 7.14 (d, J = 9 Hz, 1H), 7.60 (dd, J = 2 Hz, J = 9 Hz, 1H), 7.99 (s, 1H), 8.34 (d, J = 9 Hz, 1H), 9.20 (d, J = 9 Hz, 1H). MS: m/z 261.0601 (M - CO_2); required for $C_{13}H_{11}NO_5$: 261.0637.

1-Bromo-8-(1,2-dicarboxyethyl)-4-oxo-4H-quinolizine-3-carboxylic Acid (21a). Using the procedure described for **1a** and heating the reaction mixture to 60 °C, compound **21a** was obtained as a yellow powder in 42% yield. ^1H NMR (D_2O , 1% NaOD): δ 2.74 (m, 1H), 2.94 (m, 1H), 4.05 (m, 1H), 7.35 (d, J = 7 Hz, 1H), 8.01 (s, 1H), 8.33 (s, 1H), 9.17 (d, J = 7 Hz, 1H). MS: m/z 389.9819; required for $C_{14}H_{10}BrNO_7Li$: 389.9801.

8-(4-Methoxyphenyl)-4-oxo-4H-quinolizine-3-carboxylic Acid (22a). Using the procedure described for **1a**, compound **22a** was obtained as a yellow powder in 49% yield, mp > 250 °C. ^1H NMR (D_2O): δ 3.62 (s, 3H), 6.40–6.62 (m, 3H), 7.11 (d, J = 8 Hz, 1H), 7.16–7.32 (m, 3H), 7.94 (d, J = 8 Hz, 1H), 8.64 (d, J = 8 Hz, 1H). MS: m/z 278.0826 (M - OH); required for $C_{17}H_{12}NO_3$: 278.0817.

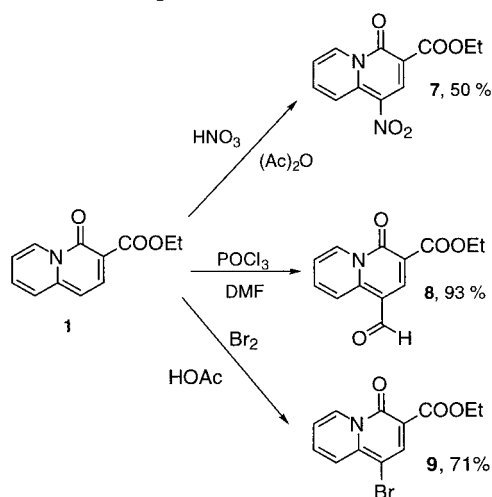
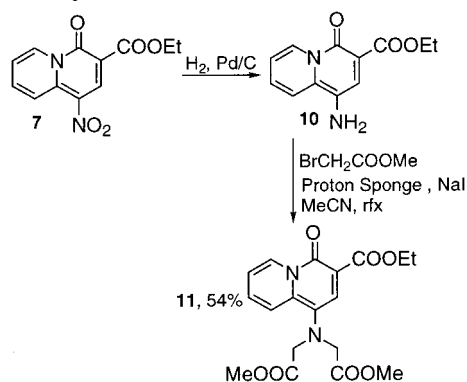
8-(Naphth-1-yl)-4-oxo-4H-quinolizine-3-carboxylic Acid (23a). Using the procedure described for **1a**, compound **23a** was obtained as a yellow powder in 67% yield, mp = 245–7 °C. ^1H NMR (DMSO- d_6): δ 7.33 (d, J = 8 Hz, 1H), 7.55–7.74 (m, 4H), 7.81 (dd, J = 2 Hz, J = 7 Hz, 1H), 8.04–8.17 (m, 2H), 8.35 (s, 1H), 8.45 (d, J = 8 Hz, 1H), 9.36 (d, J = 7 Hz, 1H). MS: m/z 315.0905; required for $C_{20}H_{13}NO_3$: 315.0895.

8-(Benzo[6,7]furyl)-4-oxo-4H-quinolizine-3-carboxylic Acid (24a). Using the procedure described for **1a**, compound **24a** was obtained as a yellow powder in 47% yield, mp > 250 °C. ^1H NMR (DMSO- d_6): δ 7.26–7.40 (m, 2H), 7.48 (dd, J = 8 Hz, J = 8 Hz, 1H), 7.70 (d, J = 8 Hz, 1H), 7.79 (d, J = 8 Hz, 1H), 8.02 (s, 1H), 8.05 (dd, J = 2 Hz, J = 7 Hz, 1H), 8.38 (d, J = 8 Hz, 1H), 8.52 (s, 1H), 9.24 (d, J = 8 Hz, 1H). MS: m/z 305.0674; required for $C_{18}H_{11}NO_4$: 305.0687.

RESULTS

The ethyl esters of 4-oxo-4H-quinolizine-3-carboxylic acids **1**, **2**, and **3** were synthesized in two steps starting from the commercially available 2-picolines or 2-methylquinoline **4** (Scheme 1) (26). The first step is a Michael reaction of the lithiated 2-picoline anion **4-Li**, generated in situ with LDA, with diethyl ethoxymethylenemalonate **5**. Subsequent ring closure of the Michael adducts **6** was brought about in refluxing xylene. The desired ethyl esters of 4-oxo-4H-quinolizine-3-carboxylic acids **1**, **2**, and **3** were obtained as crystalline materials in moderate to good yields.

Electrophilic Aromatic Substitution. The introduction of functionality into compound **1** is readily achieved by regioselective electrophilic aromatic substitution at the

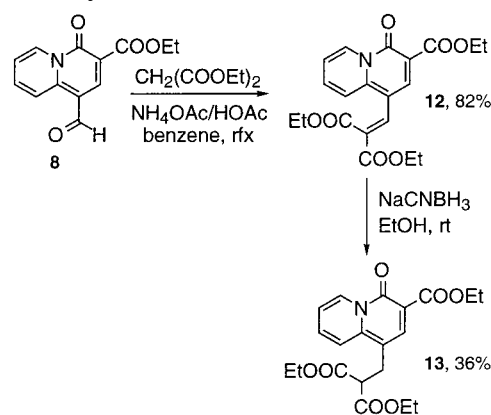
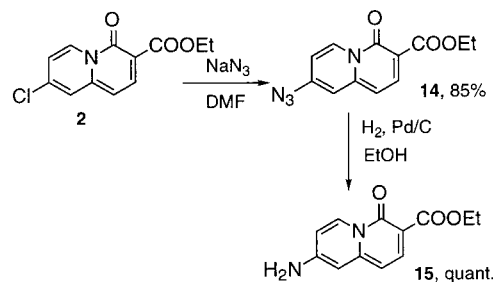
Scheme 2. Electrophilic Aromatic Substitution of 1**Scheme 3. Synthesis of 11**

1-position (37). Thus, treatment of **1** with HNO₃ in acetic anhydride resulted in the isolation of ethyl 1-nitro-4-oxo-4H-quinolizine-3-carboxylate (**7**) in a yield of 50%. Similarly, formylation of **1** with a Vilsmeier–Haack reaction gave ethyl 1-formyl-4-oxo-4H-quinolizine-3-carboxylate (**8**) in 93% yield. The bromination of **2** was achieved by treatment with Br₂ in acetic acid to give **9** in good yield (Scheme 2).

The nitro group and the formyl group in compounds **7** and **8**, respectively, are excellent starting points for the introduction of two extra carboxylic acid groups in the 4-oxo-4H-quinolizine-3-carboxylic acid structure to give a polyvalent fluorophore. The reduction of the nitro group of **7** was accomplished by Pd-catalyzed hydrogenation to give amine **10**. Alkylation of **10** with methyl bromoacetate in refluxing acetonitrile, in the presence of NaI and Proton Sponge, resulted in the formation of triester **11** in a 54% overall yield from **7** (Scheme 3).

An aldol condensation of aldehyde **8** with diethyl malonate led to the preparation of diethyl methylenemalonate triester **12**. The saturated derivative **13** was obtained by a selective reduction of the double bond in **12** with NaCNBH₃ in ethanol (Scheme 4).

Nucleophilic Substitution of the Cl Substituent of 1. The Cl substituent of ethyl 8-chloro-4-oxo-4H-quinolizine-3-carboxylate (**2**) is easily substituted with a variety of nucleophiles and undergoes palladium catalyzed coupling reaction with boronates (Suzuki coupling). This reactivity of **2** offers additional possibility to introduce functionality into the 4-oxo-4H-quinolizine-3-acid structure and to tune its physical and fluorescent properties. Treatment of **2** with NaN₃ in DMF at 40 °C resulted in clean replacement of the Cl substituent by an azido

Scheme 4. Synthesis of 12 and 13**Scheme 5. Synthesis of 15**

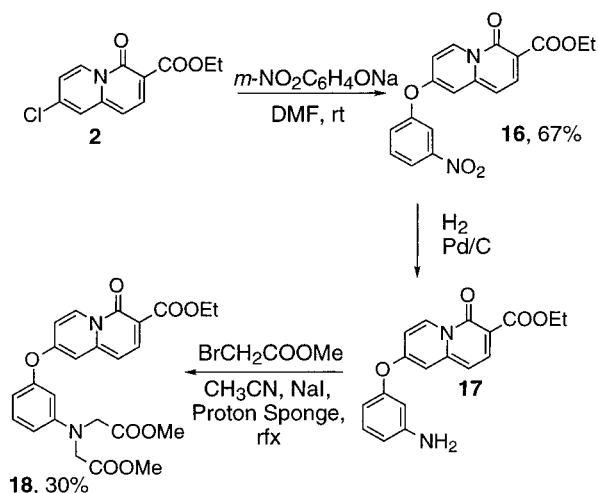
group to give **14** in 85% yield (Scheme 5). Reduction of the azido group by palladium-catalyzed hydrogenation afforded the corresponding amine **15** in almost quantitative yield. Alkylation of **15** with methyl bromoacetate, analogous to the preparation of **10** was not successful. It is believed that the strong electron deficient nature of this heterocyclic system, which facilitated the replacement of the Cl substituent in **2**, actually lowers the nucleophilic character of the amine substituent. An alternative approach, the substitution of the Cl substituent in **2** with diethyl iminoacetate [HN(CH₂COOMe)₂], also failed. Presumably, this was due to a lack of sufficient nucleophilicity of the amine.

The reaction of **2** with the anion of *m*-nitrophenol, generated in situ by deprotonation of the phenol with NaH, resulted in substitution of the Cl substituent with an oxygen nucleophile to give ether **16** in a yield of 67%. Reduction of the nitro group of **16** afforded amine **17**. A subsequent alkylation of the crude amine with methyl bromoacetate resulted in the isolation of triester **18** in 30% yield over two steps (Scheme 6).

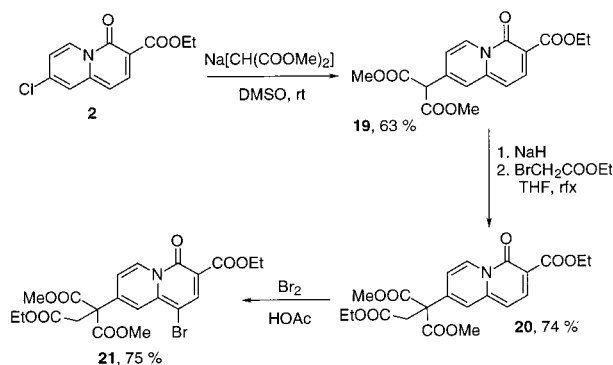
The Cl substituent of **2** can also be replaced by a carbon nucleophile. Thus, the reaction of **2** with the anion of diethyl malonate in DMSO led to the formation of triester **19** which was isolated in 63% yield (Scheme 7). It was found that at least two equivalents of the malonate anion were required for complete consumption of **2**, most likely as a result of a deprotonation of the more acidic substitution product **19** by a malonate anion. The remaining acidic proton of **19** was readily abstracted by NaH in THF and alkylation of the resulting anion with ethyl bromoacetate afforded tetraester **20** in a yield of 74%. Bromination of **20** was achieved by the reaction with Br₂ in acetic acid to give **21** in 75% yield (Scheme 7).

The extension of the pi-system of the 4-oxo-4H-quinolizine-3-carboxylic acid structure as in **3** was found to shift the fluorescence emission maximum to longer wavelengths. It was thought that the introduction of other conjugated aromatic substituents might extend the

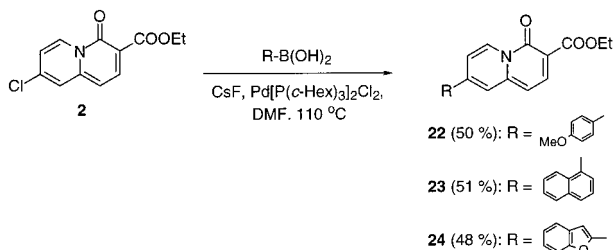
Scheme 6. Synthesis of 18



Scheme 7. Synthesis of 19, 20, and 21



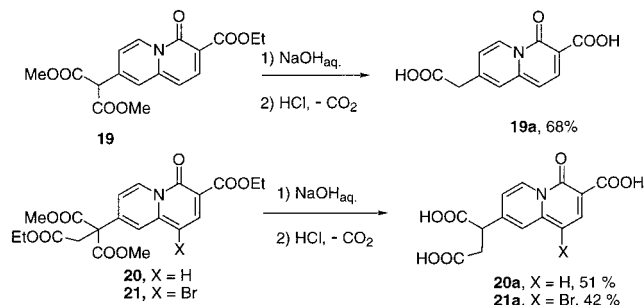
Scheme 8. Suzuki Coupling Reaction of 2



emission maximum further, perhaps into the visible light range. A Pd-catalyzed coupling reaction of the electron poor aryl chloride **2** with an organoboron species—a Suzuki reaction—is one way of substituting the Cl atom at the 8-position of **2** with an aromatic carbon nucleophile. The two phase system, consisting of an organic solvent and an aqueous base, that is usually applied for the Suzuki coupling could not be used because of the ester group in **2**. We therefore chose to use the base free reaction conditions described by Shen (38) which has proven to be efficient for a Suzuki coupling reaction of electron poor aryl chlorides, and here gave us good yields of the coupled products. Using this procedure, a *p*-methoxyphenyl, a naphth-1-yl, and a benzo-[6,7]-furyl substituent were substituted at the 8-position of the parent 4-oxo-4H-quinolizine-3-carboxylate structure (Scheme 8).

Saponification. Compounds **1**, **2**, **3**, **9**, **11**, **12**, **13**, **15**, **18**, **19**, **20**, **21**, **22**, **23**, and **24** were saponified, followed by acidic workup to isolate the (poly)acids **1a**, **2a**, **3a**, **9a**, **11a**, **12a**, **13a**, **15a**, **18a**, **19a**, **20a**, **21a**, **22a**, **23a**, and **24a** in the protonated form. The saponification products

Scheme 9. Synthesis of 19a, 20a, and 21a

Table 1. Spectroscopic Data and K_D Values

	A (nm) ^a	λ_{emiss} (nm) ^b	K_D (mM)		A (nm) ^a	λ_{emiss} (nm) ^b	K_D (mM)
1a	385	435 → 408	1.1	18a	370	412 → 402	1.0
2a	391	445 → 418	5.0	19a	380	432 → 410	1.1
3a	410	485 → 467	4.6	20a	390	432 → 411	0.4
9a	403	460 → 442	1.5	21a	407	463 → 437	0.7
11a	410	552 → 548	0.5	22a	406	470 → 444	1.2
12a	400	466 → 461	0.4	23a	386	490 → 456	1.5
13a	395	456 → 429	0.5	24a	440	496 → 459	1.3
15a	385	406 → 396	1.1				

^a Absorption maximum of the free indicator. ^b An arrow denotes a shift of the fluorescence maximum upon saturation of the indicator.

of triester **19** and tetraesters **20** and **21** decarboxylated rapidly at low pH and at 5 °C, and the resulting diacid **19a** and triacids **20a** and **21a** were isolated (Scheme 9).

Fluorescence. The emission and excitation fluorescence spectra of **1a**, **2a**, **3a**, **9a**, **11a**, **12a**, **13a**, **15a**, **18a**, **19a**, **20a**, **21a**, **22a**, **23a**, and **24a** were recorded using 0.5–1.0 μM solutions of the chelator, buffered at pH 7.2. The solutions also contained 120 mM KCl and 20 mM NaCl to mimic the ionic composition of the intracellular milieu. These solutions were titrated with stock solutions of MgCl_2 containing an equimolar concentration of the 4-oxo-4H-quinolizine carboxylic acids to cancel out dilution effects during the titration. After each titration step, both the emission and excitation fluorescence spectra were recorded. The absorption maxima and the fluorescence emission maxima of the studied 4-oxo-4H-quinolizine carboxylic acids are presented in Table 1. The apparent dissociation constants of indicators for Mg^{2+} were determined from the fluorescence spectra as originally described by Gryniewicz et al. (39) and are also given in Table 1. Solutions of the fluorophores in the buffer proved to be stable for at least 9 months at 4 °C.

In most cases, a bathochromic shift of the fluorescence emission occurs upon binding Mg^{2+} by the indicator. The observed shifts range from 4 nm for **11a** to 37 nm for **24a**. The parent compound **1a** displays a bathochromic shift of 23 nm when saturated with Mg^{2+} and an isosbestic point was found at 437 nm (Figure 1a). This compound thus fulfills the criterion for a ratioable indicator. Figure 1b presents the fluorescent response of **1a** to increasing Ca^{2+} concentrations, and it is clear that intracellular Ca^{2+} levels in the nanomolar to submillimolar range will not effect a change in the fluorescence emission of **1a**. For most other 4-oxo-4H-quinolizine-3-carboxylic acids, the Mg^{2+} induced fluorescence shift is not sufficient for these chelators to serve as ratioable indicators as the fluorescence emission spectra of the free indicator and the Mg^{2+} -bound indicator largely overlap. The fluorescent response of the alkyl-substituted indicator **13a**, a triacid in which a 2,2-dicarboxyethyl substituent has been introduced at the 1-position to enhance

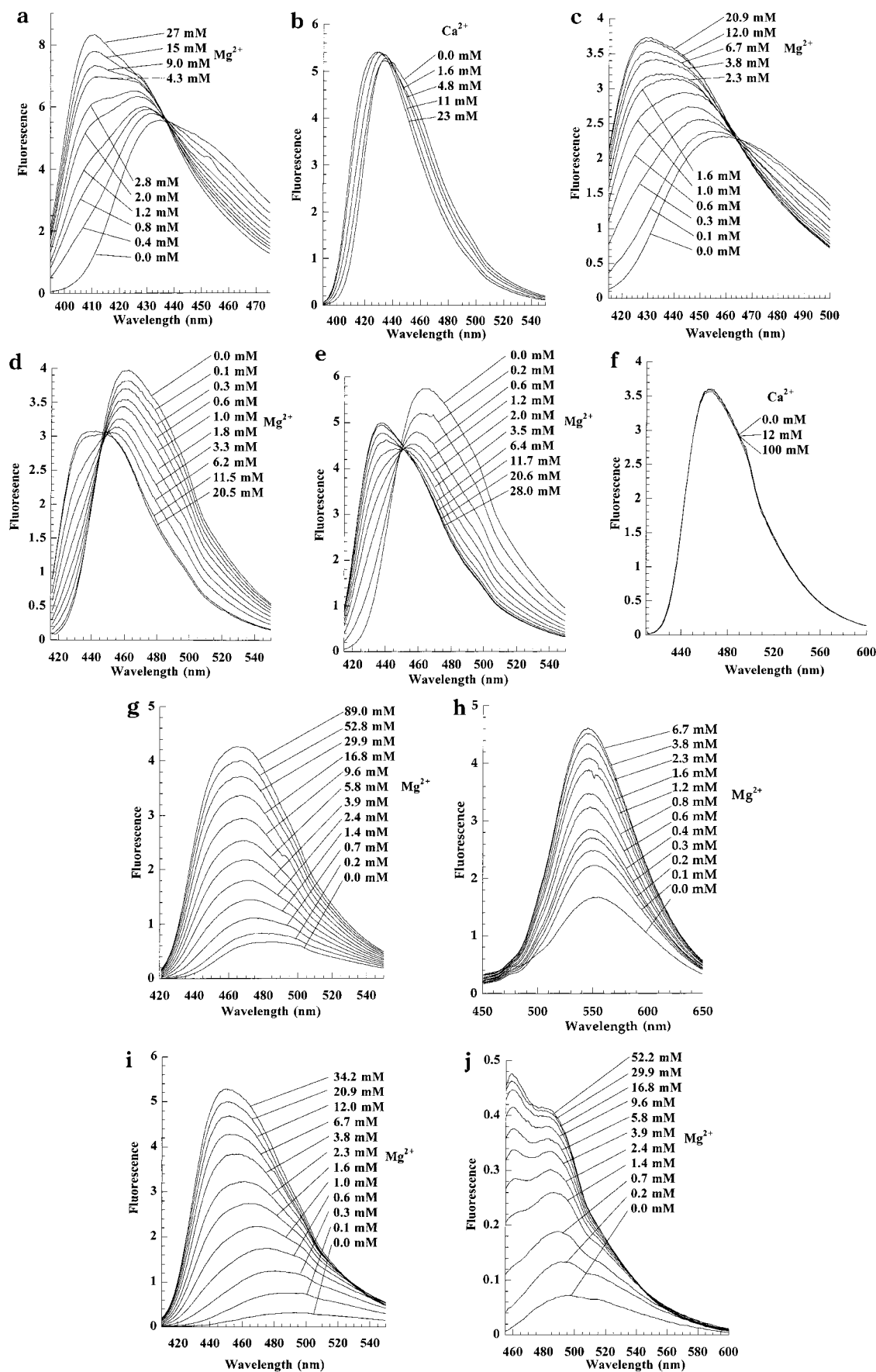


Figure 1. Fluorescence emission spectra of the indicated compounds as a function of $[\text{Mg}^{2+}]$ or $[\text{Ca}^{2+}]$ (in mM), as indicated. Excitation in each case was at the absorption maximum indicated in the Table. Spectra were recorded at 20 °C. (a) **1a** as a function of $[\text{Mg}^{2+}]$; (b) **1a** as a function of $[\text{Ca}^{2+}]$; (c) **13a** as a function of $[\text{Mg}^{2+}]$; (d) **9a** as a function of $[\text{Mg}^{2+}]$; (e) **21a** as a function of $[\text{Mg}^{2+}]$; (f) **21a** as a function of $[\text{Ca}^{2+}]$; (g) **3a** as a function of $[\text{Mg}^{2+}]$; (h) **11a** as a function of $[\text{Mg}^{2+}]$; (i) **23a** as a function of $[\text{Mg}^{2+}]$; (j) **24a** as a function of $[\text{Mg}^{2+}]$.

water solubility, is almost identical to that observed for **1a** (Figure 1c). Alkylation at the 8-position such as in **19a** and **20a**, however, results in overlap of the fluorescence spectra of both the free and complexed forms.

The presence of a Br substituent at the 1-position of fluorophores **9a** and **21a** results in a bathochromic shift of 18 and 26 nm, respectively, when binding Mg^{2+} (Figure 1, panels d and e). These fluorophores both display well-separated fluorescence maxima for the free and Mg^{2+} -bound state with isosbestic points at 446 and 451 nm, respectively. Both fluorophores show no fluorescent response to increasing Ca^{2+} concentrations up to 100 mM as shown for **21a** in Figure 1f. The pK_a of the carboxylate group at position **3** on the ring system of **21a**, which is involved in forming the metal ion complex, was determined to be 5.6 by a fluorescence/pH titration. Thus, all of the indicator is present in the deprotonated form and available for complexation at physiological pH.

Fluorophores **3a**, **11a**, **23a**, and **24a** show an increase of the fluorescence emission upon binding to Mg^{2+} (Figure 1, panels g–j) and were found to be very sensitive to changing Mg^{2+} levels. An ideal fluorescent indicator displays an emission signal above 500 nm such as triacid **11a** which has a fluorescence maximum at 552 nm. The presence of the amino group at the 1-position gave a hypsochromic shift of 117 nm with respect to the parent molecule **1a**. As expected, the introduction of an aromatic moiety at the 8-position (compounds **22a–24a**), or extension of the pi-system as in **3a** also resulted in a hypsochromic shift to longer wavelengths, albeit smaller than found for **11a**.

CONCLUSIONS

4-Oxo-4H-quinolizine-3-carboxylic acids are a new class of Mg^{2+} -selective, fluorescent indicators. The dissociation constants ranging from 0.4 to 5.0 mM correspond well with commonly observed intracellular Mg^{2+} levels, a requirement for optimum sensitivity. The fluorophores show very little if any change of the fluorescence emission in the presence of Ca^{2+} . The observed changes of the fluorescence emission with increasing Mg^{2+} levels depends on the substitution pattern of the 4-oxo-4H-quinolizine-3-carboxylic acid.

Visible light-excitable, nonratioable APTRA-type indicators which undergo a large increase of their fluorescence emission with increasing Mg^{2+} levels have found practical application (40). Similar fluorescent behavior was observed for 4-oxo-4H-quinolizine-3-carboxylic acids **3a**, **11a**, **23a**, and **24a** which proved to be very sensitive fluorophores. Fluorophore **11a**—a triacid and thus potentially loadable into the cell and with a fluorescence emission maximum at 552 nm—is a promising candidate for an ion-selective indicator for intracellular Mg^{2+} with a 500+ nm emission wavelength.

The presence of a Br substituent at the 1-position of a 4-oxo-4H-quinolizine-3-carboxylic acid proved to be a key structural element in order to obtain a ratioable fluorescent indicator. It may be expected that other electron withdrawing/halogen substituents at this position will have the same effect on the fluorescent behavior of 4-oxo-4H-quinolizine-3-carboxylic acids. Fluorophores **9a** and **21a** both display two fully separated fluorescence maxima for the free indicators and their complex with Mg^{2+} , respectively, which is ideal for a ratioable indicator. Compound **21a** is the first ratioable, loadable, Mg^{2+} -selective, fluorescent indicator and is currently being investigated in biophysical studies in our laboratories.

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Size Optimization of Synthetic Graft Copolymers for in Vivo Angiogenesis Imaging

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Angiogenesis is a critical step in tumor development and more than 25 angiogenesis inhibitors are currently in clinical trials. Noninvasive in vivo imaging of angiogenesis represents a unique opportunity of repeatedly quantitating microvascular parameters prior to and during anti-angiogenic treatments. While several imaging tracers have been proposed for MR and nuclear imaging, there does not exist any consensus of what constitutes an ideal size of an imaging agent. A series of synthetic pegylated DOTA derivatized graft copolymers (30, 60, 120 kDa) were synthesized and their in vivo behavior tested in two breast cancer models differing in vascular endothelial growth factor (VEGF) expression. Polymers were labeled with different lanthanides (Eu, Gd, Dy) and absolute blood and tumor concentrations were determined by ICP-AES measurements. DOTA and the 30 kDa polymers underwent renal clearance resulting in low plasma levels. Slow leakage across neovasculature into tumor interstitium was clearly dependent on the molecular mass of all tested agents in MCF-7 tumors. However, a cutoff was observed with minimal extravasation occurring at and above 120 kDa in well differentiated MCF-7 tumors. VEGF overexpression caused detectable differences in extravasation of all polymers, including the 120 kDa compound. We conclude that large molecular weight contrast agents with a molecular mass of <120 kDa extravasate from experimental tumor neovasculature and may not be an accurate marker for measuring true blood volume fractions when in vivo imaging is performed in the steady state.

INTRODUCTION

Angiogenesis, the formation of new blood vessels from an existing vascular network, is a critical step in carcinogenesis and metastagenesis (1–3). Currently, there are more than 25 inhibitors of angiogenesis in clinical trials while many other compounds await testing. New anti-angiogenic agents include compounds with different mechanism of action including (1) drugs that inhibit endothelial cell proliferation directly (e.g., TNP-470, endostatin), (2) drugs that block angiogenic activators [e.g., Anti-vascular endothelial growth factor (VEGF) monoclonal antibody, SU6668], (3) drugs that inhibit matrix breakdown (e.g., AG3340, Marimastat) and drugs with nonspecific mechanism (e.g., Interleukin-12). The main rationale for developing anti-angiogenic treatments is severalfold: (1) tumors do not grow beyond a certain size unless supplied by vasculature, (2) a single vessel supplies large tumor areas, and (3) drug resistance is unlikely to develop because endothelial cells are genetically stable. Cytostatic anti-angiogenic agents may prove particularly useful in combination with cytotoxic chemotherapeutic regimen. Irrespective of a specific regimen, antiangiogenic treatments are typically long-term and ideally require periodic assessment of treatment efficacy. For this reason and because phenotypic changes can take weeks to months to manifest (4), other surrogate endpoints of treatment efficacy are required for the clinical testing of new agents.

At present, the most widely used method of assessing angiogenesis in solid tumors is still the determination of intratumoral microvessel density (MVD) in areas of the most active neovascularization (5, 6). There are obvious limitations of using this technique repeatedly and longitudinally in the same patient. For this reason, imaging techniques have been developed to quantitate tumoral angiogenic burden using different approaches, most of them relying on the administration of a contrast agent and measuring certain microvascular parameters during first pass (small molecular weight contrast agents) or during steady-state (large molecular weight contrast agents). It is now well accepted from intravital microscopy and physiology studies that a reasonable surrogate marker of angiogenesis is the functional vascular volume fraction, i.e., the fraction of tissue that carries oxygen and nutrients to tumors (7, 8). A number of excellent in vivo angiogenesis imaging studies have been published on this subject (9–11). However, there is lack of agreement on what molecular weight (or corresponding hydrodynamic radius) an “ideal” agent should have for accurately measuring neovascular volume in tumor microcirculation. Even knowledge of approximate cutoff sizes and/or size ranges would be useful for further development of clinical contrast agents. While some quantitative data is available for certain experimental tumor models and compounds (12), this work has not been extended to clinically viable contrast agents.

The goal of the current study was to synthesize structurally identical, large molecular weight synthetic graft copolymers that could be used for imaging (either nuclear, MR, or CT imaging). The rationale for choosing polymers was the ability to control the synthesis to obtain different sized products. The developed polymers had

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mean molecular masses of 30 kDa ("P30"), 60 kDa ("P60"), and 120 kDa ("P120"). Comparison compounds included a structurally related graft copolymer of 525 kDa ("PGC") (13) and 0.5 kDa DOTA. The PGC graft copolymer had previously been validated as a marker of intravascular volume in experimental tumors (14–16). To compare the compounds in different environments a slow growing well differentiated estrogen dependent human breast cancer (MCF-7) was chosen and compared to a VEGF overproducing matched MCF-7 transgenic clone (MV-165) (16). The experimental polymers were each labeled with different lanthanides (Eu, Dy, and Gd for P30, P60 and P120, respectively), mixed and then injected intravenously into orthopic tumor bearing animals. Blood and tumor concentrations were then measured at different time points by inductive coupled plasma atomic emission spectrometry (ICP-AES).

MATERIALS AND METHODS

Synthesis of Graft Copolymers. Three differently sized graft copolymers (P30, P60, and P120) were synthesized using three poly-L-lysine (PL) hydrochloride preparations (molecular mass of 3, 6, or 13 kDa respectively; Figure 1). The following is a description of P120 synthesis but was essentially similar for the smaller PL. MPEG-SPA (795 mg, MW = 5000, Shearwater Polymers, Huntsville, AL) was added slowly to an ice-cold solution of PL (100 mg, 13 kDa, Schering AG, Berlin) in bicarbonate (0.1 M, pH 8.0, 25 mL). The pH was adjusted to pH 7.7 and the mixture was stirred at room temperature for 3 h. The solution was then extensively dialyzed (cutoff 8–10 kDa) in deionized water to remove small molecular weight byproducts. The product (**I** in Figure 1) was recovered by lyophilization (774 mg). The degree of PEGylation was calculated based on the difference of free amino groups before and after the reaction using a TNBS assay.

Three hundred mg of (**I**) was dissolved in water and reacted with 1,4,7,10-tetraazacyclododecane-(1,4,7-tris-acetic acid *tert*-butyl ester)-10-(1-methyl-acetyl-glycyl)-*p*-nitrophenyl ester (Bu-DOTA-PNP, Schering Ag, Berlin; 315 mg, MW = 867.7) in DMSO at room temperature for 24 h. The reaction mixture was diluted with an additional 20 mL of PBS, dialyzed in water using 12–14 kDa cutoff membrane and lyophilized to obtain MPEG-PL-tBu-DOTA (**II**).

The protected MPEG-PL-Bu-DOTA (265 mg; **II**) was dissolved in 1.2 mL of trifluoroacetic acid/triisopropylsilane (2/1) and stirred at room temperature to remove *tert*-butyl protecting groups. The final product MPEG-PL-DOTA (**III**) was then precipitated with methyl-*tert*-butyl ether, washed with methyl-*tert*-butyl ether, redissolved in water and lyophilized overnight.

Product (**III**; P120) was labeled with gadolinium citrate at pH 4.5, 80 °C for 3 h. The Gd labeled (**III**) was then dialyzed in water using a 12–14 kDa cutoff membrane and lyophilized. The same protocol was used to label (**III**) with Eu (P30) with Dy (P60). The metal content of the labeled compounds were analyzed by ICP-AES.

To obtain a fluorescent derivative of (**III**), a 10-fold molar excess of tetramethyl rhodamine isothiocyanate (TRITC, Molecular Probes, Eugene, OR) was used (0.1 M sodium bicarbonate, pH 8.5, 1 h). The rhodamine labeled conjugate was purified by dialysis. Unreacted amino groups on (**III**) were further acetylated by treatment with acetic anhydride.

Characterization. The above synthesized compounds were subjected to size exclusion high-pressure liquid

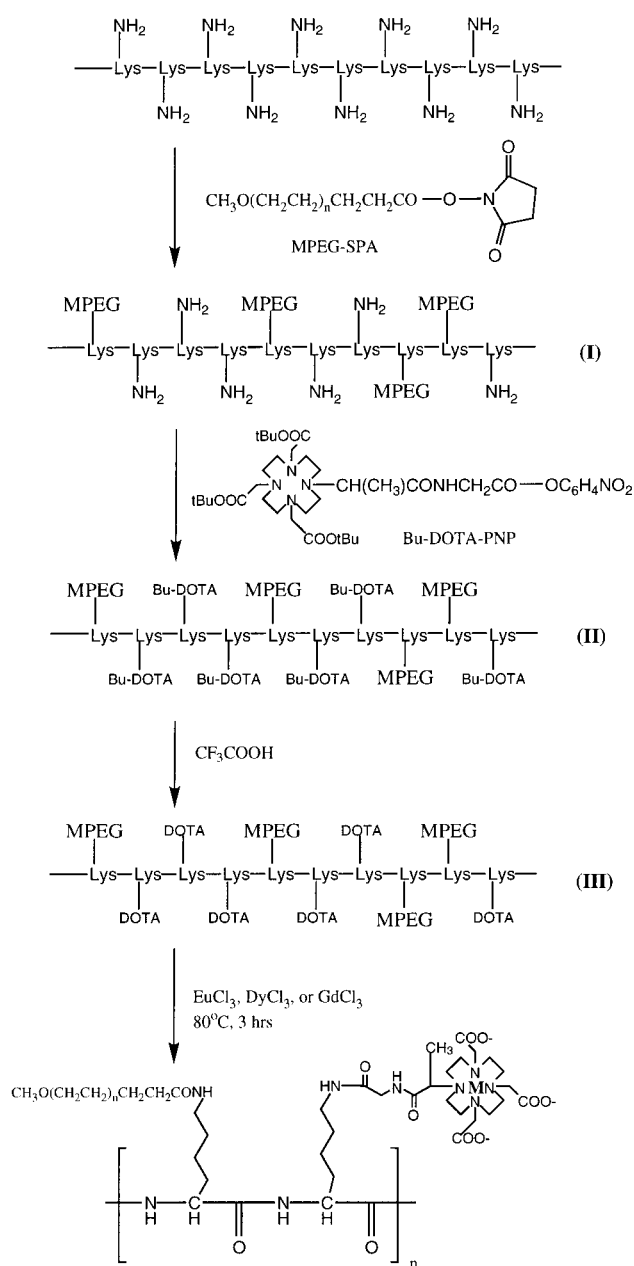


Figure 1. Synthesis of experimental graft copolymers. The only difference among the synthesized compounds was the chain length (n) of the PL backbone. Roman numerals on the right refer to intermediary reaction products. M = Eu, Dy, Gd, In.

chromatography analysis (TSK-GEL G3000SWXL, Toso Haas, Montgomeryville, PA), measurement of molecular weight distribution (photon correlation spectrometer PCS 4700c, Malvern Instruments), elemental analysis and determination of metal content to verify that the synthesized compounds corresponded to theory.

The stability of the compounds was tested under different conditions including (1) incubating them in plasma and/or whole blood for up to 24 h, (2) lyophilization and storage for 4 months, and (3) heating at 80 °C for 3 h. Stability was tested using HPLC as described above.

Control Compounds. Two other contrast agents were also tested in the MCF-7 tumor model (Table 1). The first contrast agent was another PL-MPEG graft copolymer (PGC) of larger molecular mass (525 kDa) and containing DTPA as a chelate (rather than DOTA) and Eu as the lanthanide (13). The graft copolymer has a mean blood

Table 1. Overview and Characteristics of Compounds^a

compd	MW of PL (kDa)	% MPEG modification	MW (kDa) ^b	chelator	metal label	tumor model tested
experimental graft copolymers						
P30	3	25	30	DOTA	Eu	MCF-7/MV165
P60	6	25	60	DOTA	Dy	MCF-7/MV165
P120	13	22	120	DOTA	Gd	MCF-7/MV165
control/comparison compounds						
PGC	35	29	525	DTPA	Eu	MCF-7
DOTA	NA	NA	0.5	DOTA	Dy	MCF-7

^a See Figure 1 for the synthesis and structure of the experimental graft copolymers. ^b Calculated mean molecular mass.

half-life of 36 h in rats and of 20 h in humans (17). The second control compound was Dy labeled DOTA (MW 0.5 kDa) (18).

Tumor Model. MCF-7 breast carcinoma cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in minimal essential medium (MEM, Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum and antibiotics. The MV165 cells (a gift from Dr. Francis Kern) are a subclone of MCF-7 cells that were stably transfected with the 165 isoform of human vascular endothelial growth factor (VEGF165) resulting in VEGF overexpression (16). MV165 cells were grown in DMEM with 10% serum and antibiotics. All cells were cultured in a humidified, 37 °C, 5% CO₂ incubator. Both cell lines were tested for VEGF expression as described in more detail elsewhere (16).

Animals. A cohort of over 50 female nu/nu athymic mice, 3–4 weeks old were obtained from Charles River Laboratories (Wilmington, MA) for tumor implantations. Three days before injection of the cells, a pellet of 17 β -estradiol (0.72 mg/pellet, 60 days release time, Innovative Research of America, Sarasota, FL) was implanted subcutaneously on the animal's back. Approximately 10⁷ cells were inoculated in each mouse. A total of 27 animals developed tumors 5–10 mm in diameter and only these animals were used for the in vivo studies (MCF-7 *n* = 18; MV165 *n* = 9). Animals received an IV injection of a mixture containing all three experimental compounds (P30-Eu, P60-Dy, and P120-Gd (group 1, *n* = 18) or PGC-Eu and Dy-DOTA (group 2, *n* = 9); 1.8 μ mol of lanthanide/mouse corresponding to approximately 0.1 mmol of lanthanide/kg). Animals were sacrificed at 3, 6, and 24 h after IV administration and tumor and other organs were removed for lyophilization and ICP-AES measurements. Blood-half-lives and biodistribution in nontumor bearing mice (*n* = 9) was determined with ¹¹¹In labeled compounds.

Determination of Metal Content in Tissue Samples. The lanthanide concentrations were analyzed by means of inductively coupled plasma atomic emission spectrometry (ICP-AES, Plasma 1000, Perkin-Elmer, Überlingen, Germany). The measurement was performed at the element-specific wavelengths for Gd (342.25 nm), Eu and Dy. Scandium was used as an internal standard and was measured at 424.6 nm by a separate optical subunit at the same time.

Histology. Tumor specimen were frozen in liquid nitrogen, stored at –86 °C and then cut into 8 μ m-thick sections (2800 Frigocut-E; Leica Instruments, Nussloch, Germany). Some sections were stained with hematoxylin/eosin. Other, sections were analyzed by fluorescence microscopy following IV administration of rhodaminated P120. Images were obtained using a cooled charge coupled device (Photometrics, Tuscon, AZ).

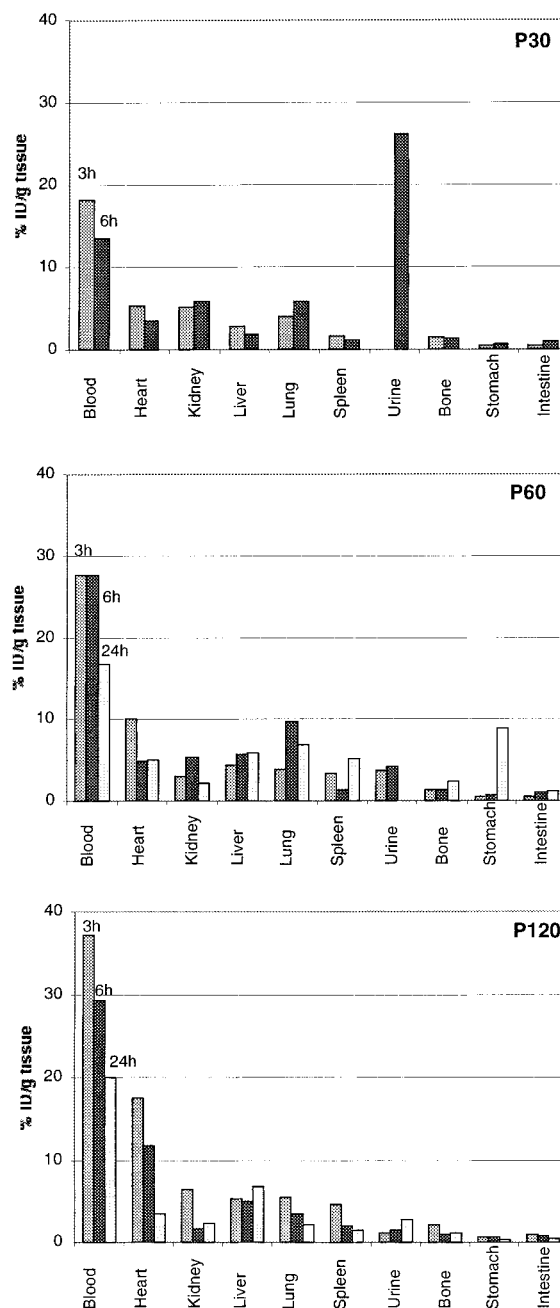


Figure 2. Biodistribution of the three experimental graft copolymers in normal mice (3, 6, or 24 h after IV administration). All polymers were labeled with ¹¹¹In (*n* = 3 per compound).

Analysis. Tissue concentrations of graft copolymers were expressed as % injected dose/g tissue in normal animals using gamma counting. In the remainder of the animals, total tumor, blood and muscles concentrations were expressed as nmol M/g wet weight. The amount of polymer extravasated into tumor interstitium (*C_{Ti}*) was

Table 2. Lanthanide Concentrations (nmol/g tissue) of Whole Tumors (CT), Blood (CV), and Muscle Tissue^a

organ	P30			P60			P120			PGC		
	3 h	6 h	24 h	3 h	6 h	24 h	3 h	6 h	24 h	3 h	6 h	24 h
MCF-7												
tumor	5 ± 2	8 ± 3	11 ± 2	3 ± 1	4 ± 0	7 ± 0	3 ± 0	7 ± 0	11 ± 1	29 ± 11	40 ± 10	40 ± 10
blood	66 ± 19	66 ± 16	24 ± 0	41 ± 30	56 ± 17	16 ± 0	71 ± 44	107 ± 24	58 ± 4	612 ± 67	537 ± 49	415 ± 117
muscle	7 ± 5	6 ± 2	4 ± 0	BD	BD	BD	5 ± 0	5 ± 1	5 ± 1	16 ± 11	12 ± 1	12 ± 1
MV-165												
tumor	11 ± 10	7 ± 5	20 ± 6	8 ± 3	6 ± 3	11 ± 3	6 ± 5	12 ± 6	16 ± 4	ND	ND	ND
blood	104 ± 12	64 ± 26	18 ± 5	88 ± 22	58 ± 20	13 ± 4	117 ± 39	83 ± 32	41 ± 10	ND	ND	ND
muscle	6 ± 0	5 ± 1	6 ± 1	BD	BD	BD	5 ± 1	4 ± 0	7 ± 1	ND	ND	ND

^a Data are shown for the MCF-7 (wild-type) and VEGF overexpressing (MV165) Breast cancers in mice. BD = below detection threshold; ND = not determined.

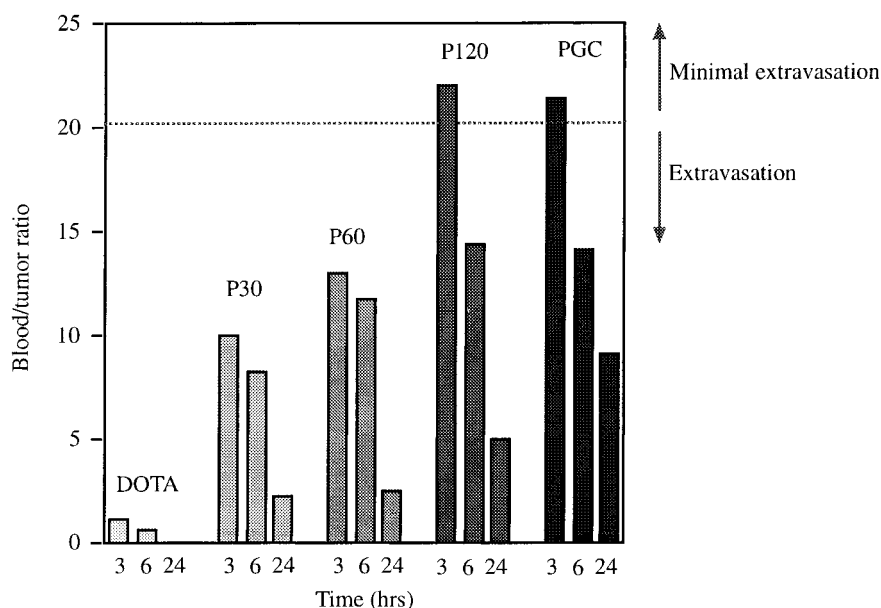


Figure 3. Effect of molecular mass of graft copolymers on blood/tumor ratios, a measure of extravasation into MCF-7 tumor interstitium (DOTA, 0.5 kDa; P30, 30 kDa; P60, 60 kDa; P120, 120 kDa; PGC, 525 kDa). Note the plateau with larger polymers (P120 and PGC) where incremental size increase does not result in higher ratios (arrows on right indicate arbitrary cutoff values as corroborated by histology).

calculated by subtracting vascular contributions from measured tumor samples (C_T), taking into account different blood concentrations (C_B) and vascular volume fractions (VVF) of MCF-7 (VVF = 0.017) and MV165 (VVF = 0.089) tumors (16) according to the following formula

$$C_{TI} = [C_T - (C_B \times VVF)] / 1 - VVF$$

Means and standard error of means were calculated from groups of at least three animals. Blood/tumor ratios, a measure of extravasation were calculated as

$$BT \text{ ratio} = C_B / C_{TI}$$

Differences among groups were calculated by nonparametric testing using the Wilcoxon test.

RESULTS

Characterization. Table 1 summarizes the characteristics of the different synthetic graft copolymers. The degree of MPEG modification (20–30%) and DOTA/lanthanide attachment (1:1 ratio) was within the expected range (2–3% w/w of lanthanide). There was no nonspecific lanthanide attachment to the polymer itself. Prior to testing the compounds in vivo, stability tests were performed to ensure that the attached DOTA groups would not be hydrolyzed in vivo. Results from these size

exclusion HPLC studies show that the polymers were stable without detectable degradation products after 24 h of incubation in plasma. Likewise, there was no degradation after incubation with whole blood or after heat treatment at 80 °C for 3 h.

Figure 2 summarizes the different biodistribution profiles of the three experimental compounds in nontumor bearing animals. Blood half-lives were 2 h for P30, 3 h for P60, and 5.2 h for P120. The smallest compound, PL3 had the highest urinary excretion with over 25% ID/g at the 6 h time point. Urinary excretion was lower for P60 and P120 (<5% ID/g). The organ distribution pattern was roughly similar, presumably reflecting differences in blood volume fraction in different organs.

Tumoral Behavior. For the further biological characterization of the compounds, mixtures of three compounds (Eu-P30, Dy-P60, and Gd-P120) were injected intravenously. Table 2 summarizes the absolute tissue concentrations for the different lanthanides in tumor interstitium (C_{TI}), blood (C_B) and muscle compartments. Blood tumor ratios were also calculated and are summarized in Figure 3. Extravasation from blood into tumor interstitium was dependent on (1) molecular weight, (2) time after injection, and (3) tumor environment.

Size Dependence. The blood/tumor ratio (MCF-7) increased with increasing molecular weights of the tested polymers reflecting lower extravasation and higher blood concentrations with larger polymers (Figure 3). For

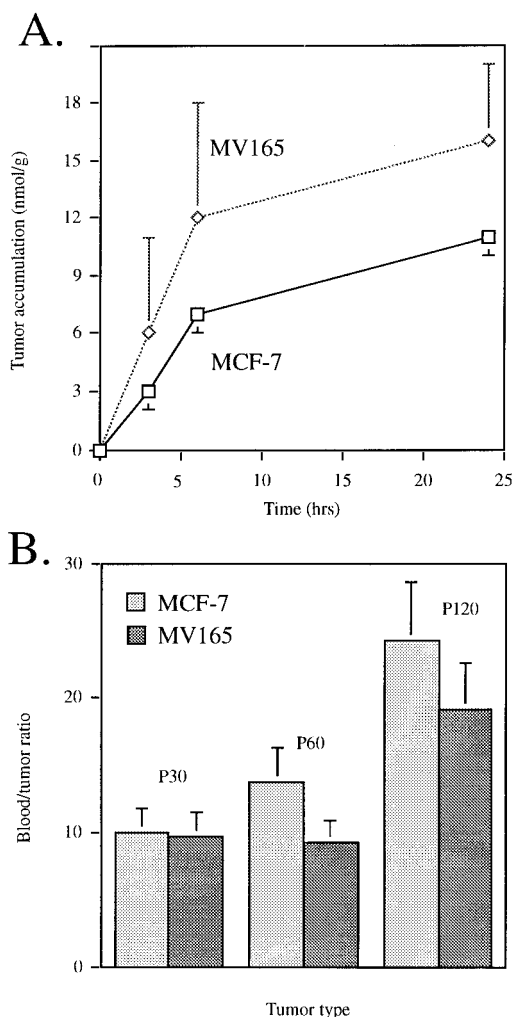


Figure 4. (A) Accumulation of P120 in tumor interstitium as a function of time. Compared to MCF-7 there is higher extravasation and also larger variability in these tumors with more chaotic vasculature. (B) Comparison of blood-tumor ratios for different time points among the two tumor models: estrogen dependent moderately differentiated breast adenocarcinoma (light gray) and the same tumor cell lines but stable transfected with human VEGF (dark gray).

DOTA this ratio was <1 consistent with rapid extravasation. At 3 h after injection the blood/tumor ratios were 10:1 for P30, 14:1 for P60 and 24:1 for P120. Interestingly, further increases in molecular mass above 120 kDa did not result in higher ratios (22.5 for the 525 kDa PGC) in the MCF-7 tumor model.

Time Dependence. All of the tested polymers showed a decrease in blood/tumor ratios over 24 h, reflecting slow clearance and extravasation into tumor interstitium during the observation time. At 6 h after administration, the blood/tumor ratios were still over 13:1 for P60 and P120 and PGC. After 24 h after administration, the highest ratio (10:1) was observed for PGC.

Effect of Tumor Environment. To determine whether VEGF overexpression would alter the above distributions and ratios, we also tested the three experimental compounds in the matched MV-165 control tumors that had been transfected with VEGF (16). Figure 4A juxtaposes the tumoral interstitial concentrations for P120. In case of the MCF-7 tumors, there was slow accumulation with an initially faster component and then a slower one. The standard deviations were relatively narrow. In contra-

distinction, MV165 tumors showed higher accumulations at any time point investigated as well as larger standard deviations.

Figure 4B compares the 3 h blood/tumor ratios for the different experimental compounds in MCF-7 and MV-165 tumors. With P30, the ratios were similar, reflecting indiscriminate extravasation. For the P60 and the P120 polymer however, the differences were more marked. These data indicate that VEGF overexpression can have pronounced effects on extravasation, increasing the vascular pore cutoff to above 120 kDa.

Microscopic Evaluation. Figure 5 summarizes histologic features of the two different tumors. Hematoxylin/eosin staining of MCF-7 and MV165 tumors shows a more aggressive behavior of the latter tumor with higher vascular density.

To determine the fate of P120 in the tumor we also performed additional fluorescence microscopy experiments (Figure 5, panels C and D). At the 3 h time point, the polymer was almost exclusively found in small blood vessels consistent with a high blood/tumor ratio determined with ICP-AES (Figure 5C). At the 24-hour time point, the polymer was more homogeneously distributed throughout tumor. At higher magnifications (see Figure 5D insert), it became evident that a significant portion of the polymer was present within tumor cells as well as tumor associated macrophages. Within cells, the polymer appeared in tubular and round intracellular vesicles.

DISCUSSION

Imaging techniques such as MRI and nuclear techniques have the potential to measure a number of different microvascular parameters during angiogenesis and may thus be clinically useful in assessing the efficacy of antiangiogenic treatments. While some of these measurements can be made with first pass bolus techniques [e.g., dynamic Gd-DTPA enhanced MR imaging (10)] others require the use of contrast agents that have a more limited intravascular distribution (so-called "long-circulating", "blood-pool", or "macromolecular contrast agents"). The latter approach allows imaging of multiple tumors and multiple sections throughout a given tumor since there are fewer timing constraints than for first pass techniques. A number of different macromolecular contrast agents have been described including Gd labeled albumin (70–90 kDa) (9, 19), dendrimers (35 kDa) (20), and pegylated graft copolymers (525 kDa) (16). Despite these studies there is little consensus as to what molecular weight a given "blood pool agent" should have, to measure functional vascular volume fractions. Furthermore, little information exists on how heterogeneous such microvascular parameters are in tumors in vivo and what effect angiogenic factors such as VEGF have on the in vivo distribution of different compounds. Some prior studies have investigated capillary permeability in tumor microvessels using size markers such as fluorescent dextrans, albumins (bovine serum albumin, ovalbumin), immunoglobulins and their fragments (Fc, Fab, Fab₂) or liposomes (12, 21). Most of these experimental studies have been limited to a few human tumor lines in orthopic location (22).

In the current study, we have synthesized differently sized graft copolymers ultimately to be utilized for in vivo imaging of tumor microvasculature. The rationale for designing these types of graft copolymers has recently been laid forward, while clinical studies show acceptable tolerance (17). On the basis of results of prior studies we attempted to cover molecular masses (30–525 kDa)

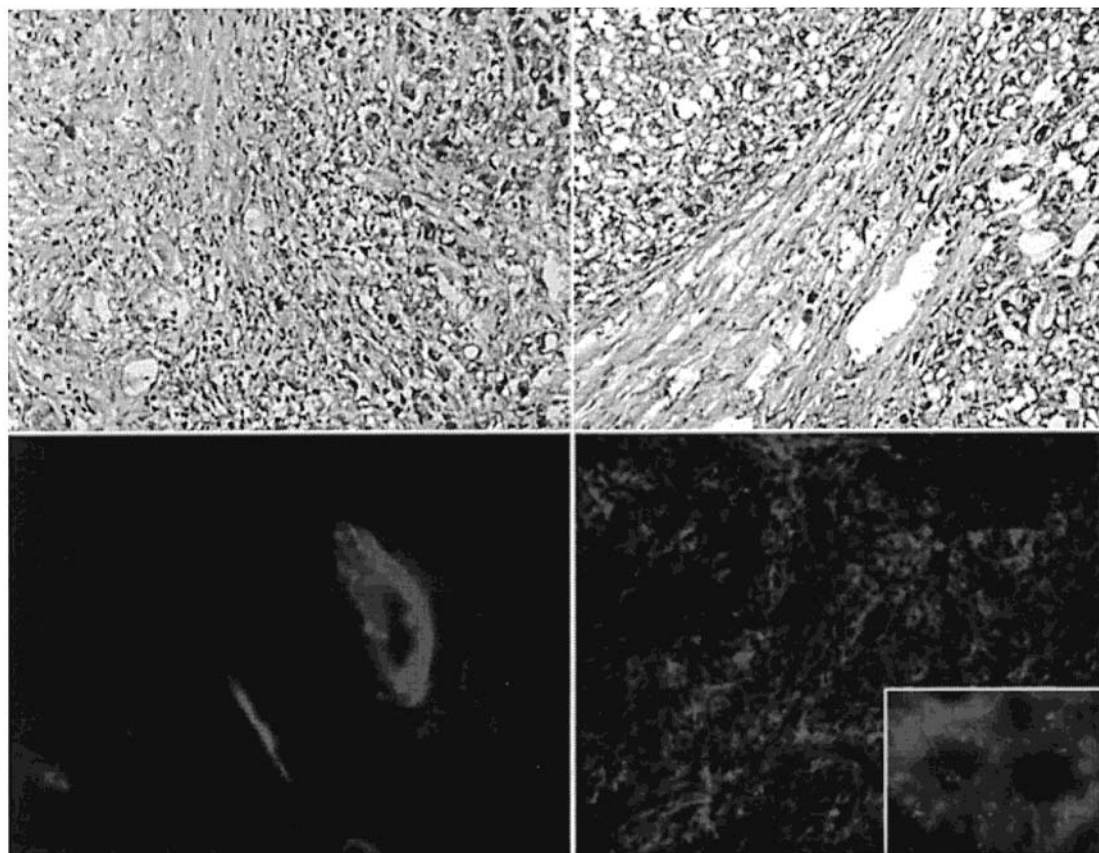


Figure 5. Light (A, B) and immunofluorescence (C, D) microscopy. (A) Hematoxylin/eosin stain of MCF-7. (B) Hematoxylin/eosin stain of MV165. (C) Immunofluorescence microscopy with rhodaminated P120 at 3 h after IV administration of the compound. Note the predominant distribution within small vessels (indicated by asterisk; magnification 400 $\times$). (D) At 24 h after administration, a larger number of cells are now stained indicating slow extravasation and cellular uptake (magnification 200 $\times$). At higher magnification (insert, 600 $\times$), the rhodaminated compound is seen within small vesicular intracellular locations, presumably lysosomes and endosomes. The round signal voids represent cell nuclei at this magnification.

which are synthetically feasible. Our data show a clear size dependence on extravasation into tumors with a detectable cutoff in MCF-7 human breast adenocarcinomas. Blood/tumor ratios were highest (24:1) for the 120 kDa polymer and did not further increase with the 525 kDa polymer. As expected, the 30 and 60 kDa polymers showed much lower ratios as they extravasated into tumor interstitium. On the basis of these results a 120 kDa polymer would suffice for imaging of certain tumors such as well differentiated MCF-7, a finding which was also corroborated by fluorescence microscopy (Figure 5).

To determine the effects of VEGF on the distribution of the polymers, we performed additional experiments using a parental MCF-7 clone that had been stable transfected with human VEGF (16). The effects of VEGF as a regulator of vascular physiology are well reported while more recently some of the modes of action and consequences have been identified (4). Our results confirm prior observations on the effects of VEGF on vascular permeability of large molecular mass size markers (12, 23). MV165 tumors showed faster and higher extravasation of the 120 kDa polymer than did the MCF-7 tumors. Blood/tumor ratios were also significantly lower at 3 h after administration (19 vs 24 for P120). These results suggest that VEGF production (and likely other agonists or antagonists) can have pronounced effect on the pharmacology of a prospective imaging agents. These results suggest that a molecular cutoff size above 120 kDa is required for an intravascular agent used in the evaluation of angiogenesis.

Fluorescence microscopy confirmed the more quantitative ICP-AES measurements. While P120 was almost exclusively present in vessels in MCF-7 tumors within 3 h after IV administration, it had clearly extravasated at 24 h. Our data furthermore indicate that the polymer was partially taken up by tumor cells and also by tumor-associated macrophages. Although the percentage of uptake into either cell type was not quantitated, such studies have been performed for dextran coated particles (24) and PGC derivatives (25). The implications of such cellular uptake following extravasation is that long circulating graft copolymers could theoretically be used for tumoral drug delivery.

The current study was set out to quantitate differences among the macromolecular agents, reason for which we investigated relatively late time points following intravenous injection but not early ones where the expected changes would have been less pronounced. In a clinical scenario however, imaging would most likely be performed within the first hour after administration of a given agent, when blood-to-tumor interstitium ratios are higher. Assuming that permeability does not change significantly over time, one could roughly extrapolate ratios for earlier time points (see Figure 3). For the MCF-7 tumor, a well differentiated adenocarcinoma, the blood/tumor ratios could thus be 10–20% higher within 1 h after administration.

The current class of polymers was designed to be stable, while having DOTA chelating groups to minimize the possibility of de-chelation. This design builds on earlier version of other graft copolymers but has the

added benefits of better-controlled chemistry (e.g., non-cross-linking through DTPA and stability of PEG-PL anchorage sites). All polymers were labeled with lanthanides at high efficiency while ^{99m}Tc and ^{111}In labels could be envisioned for nuclear imaging (Figure 2). The choice of imaging modality will likely be governed by additional prerequisites such as the need to map spatial heterogeneity of microvascular parameters (high-resolution MR imaging) within a lesion or the need for quantitation (nuclear vs MR imaging).

It should be noted that the described polymers have a preferential vascular distribution and will selectively enhance macro- and microvasculature. Because leakage of larger contrast agents into tumor interstitium can be limited, these agents are unlikely to be as useful for tumor detection as low molecular weight agents. However, it is very likely that these contrast agents will be used to characterize tumors and more importantly to test the efficacy of novel antiangiogenic treatments (15).

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Tumor Targeting of Radiometal Labeled Anti-CEA Recombinant T84.66 Diabody and T84.66 Minibody: Comparison to Radioiodinated Fragments

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Recombinant antibody fragments offer potential advantages over intact monoclonal antibodies in the radioimmunoscintigraphy (RIS) of solid tumors. Due to their smaller molecular size, antibody fragments have shown rapid tumor targeting and blood clearance, a more uniform tumor distribution and a lower potential to elicit a human immune response. Previously, we have expressed two genetically engineered antibody fragments, the T84.66 diabody (scFv dimer) and the T84.66 minibody (scFv-CH3 dimer), specific to carcinoembryonic antigen (CEA). When radioiodinated, both antibody fragments exhibited rapid tumor targeting and rapid blood clearance in xenografted mice. To extend and optimize their future clinical RIS utility with radiometals, these antibody fragments were conjugated with the macrocycle 1,4,7,10-tetraazacyclododecane *N,N',N'',N'''*-tetraacetic acid (DOTA) and labeled with ¹¹¹In. Tumor targeting and biodistribution studies were carried out in athymic mice xenografted with a human colorectal tumor cell line, LS174T. The [¹¹¹In]T84.66 diabody (55 kDa) exhibited very rapid tumor targeting with 12.5 ± 0.4% injected dose per gram (% ID g⁻¹ ± standard error) at 2 h and reached a maximum of 13.3 ± 0.9% ID g⁻¹ at 6 h. However, kidney uptake was observed to reach a peak of 183.5 ± 21.0% ID g⁻¹ at 6 h, a result similar to that reported by others for other low molecular weight fragments labeled with radiometals. Preadministration of an oral dose of D-lysine resulted in a 59% lowering of the renal accumulation at 6 h, but was accompanied by a 31% reduction of tumor uptake to 9.2 ± 1.2% ID g⁻¹. The second recombinant antibody fragment, the [¹¹¹In]T84.66 minibody (80 kDa), displayed rapid tumor targeting of 14.2 ± 6.1% ID g⁻¹ at 2 h, and reached a maximum activity of 24.5 ± 6.1% ID g⁻¹ by 12 h. Renal uptake achieved a plateau of 12–13% ID g⁻¹ which cleared to 7.2% ID g⁻¹ at 72 h. However, hepatic uptake was elevated and reached a maximum of 26.0 ± 1.0% ID g⁻¹ at 12 h in these xenograft-bearing mice. Experiments in nontumor bearing mice showed a reduction of hepatic activity at 12 h to 16.6 ± 1.5% ID g⁻¹, indicative of an intrinsic hepatic accumulation of the [¹¹¹In]DOTA-T84.66 minibody or metabolites. While the anti-CEA [¹¹¹In]DOTA-T84.66 diabody and T84.66 minibody retain the rapid tumor targeting properties of the radioiodinated form, the normal organ accumulation (kidneys and liver, respectively) of the [¹¹¹In]DOTA forms appeared problematic for RIS and RIT applications. Development of alternative blocking strategies or new metabolizable chelates are under investigation to enhance the utility of the radiometal form of these and other promising recombinant antibody fragments.

INTRODUCTION

Radiolabeled monoclonal antibodies (Mabs)¹ have been under continued development for the detection and imaging of primary and metastatic disease and have been extensively reviewed (1–3). However, intact Mabs have not proven to be ideal imaging agents due to their slow tumor targeting and extended blood clearance, the inability to uniformly penetrate large tumors, and the

elicitation of anti-murine or anti-chimeric human antibody responses (4). Improvements to the pharmacokinetics have come from the generation of antibody fragments by *in vitro* enzymatic digestion or the more easily produced single-chain Fv recombinant fragments (5). These antibody derived fragments have shown rapid tumor targeting and swift blood clearance. However, initial work has demonstrated that these truncated molecules may still require additional development (4, 6, 7).

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¹ Abbreviations: Mab, monoclonal antibody; RIS, radioimmunoscintigraphy; scFv, single-chain Fv protein; DOTA, 1,4,7,10-tetraazacyclododecane *N,N',N'',N'''*-tetraacetic acid; CEA, carcinoembryonic antigen; DTPA, diethylenetriaminepentaacetic acid; HSA, human serum albumin; ANOVA, two way analysis of variance; CT computed tomography; pI, isoelectric point; microPET, micro positron emission tomography; RIT, radioimmunotherapy.

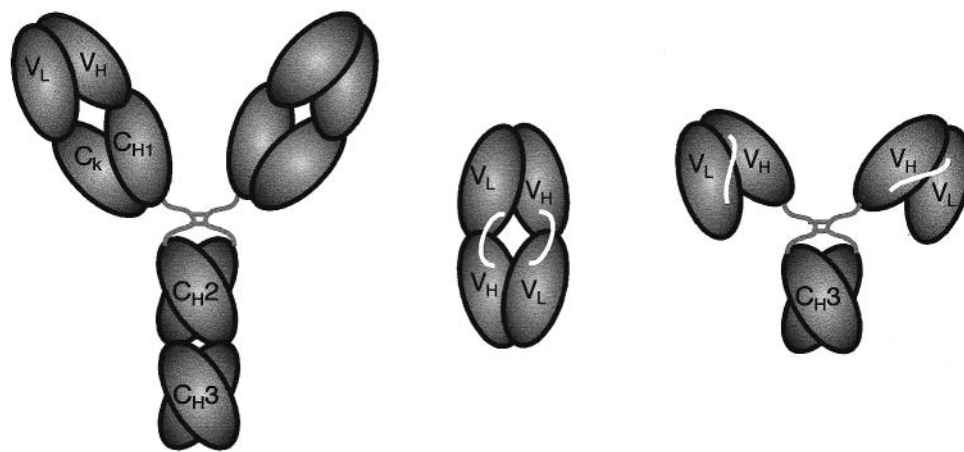


Figure 1. Schematic drawing shows the domain structure of the parental intact T84.66 antibody (left), T84.66 diabody (center), and the T84.66 minibody (right). In mammalian cell culture, the T84.66 diabody (scFv) is secreted as noncovalent cross-paired dimer (55 kDa), while the T84.66 minibody (scFv-CH₃) self-assembles into a covalent dimer (80 kDa).

Table 1. List of Potential Radionuclides for Radioimmunoscinigraphy^a

radionuclide	half-life	$E_{\beta \text{ max}}$ (MeV)	E_{γ} (MeV)
¹²³ I	13.2 h	0.13	0.159 (97%)
¹³¹ I	8.1 d	0.61	0.365 (81%)
^{99m} Tc	6 h	0.12	0.140 (90%)
⁶⁴ Cu	12.7 h	0.57 (β^-) 0.66 (β^+)	0.510 (38%)
⁶⁷ Cu	61 h	0.57	0.180 (40%)
¹⁰⁵ Rh	36 h	0.96	0.310 (24%)
¹¹¹ In	2.8 d	0.24	0.174 (89%) 0.250 (94%)
^{117m} Sn	13.6 d	0.13 0.16	0.158 (87%)
¹⁵³ Sm	1.9 d	0.81	0.103 (29%)
¹⁷⁷ Lu	6.8 d	0.50	0.21 (6%)
¹⁸⁶ Re	3.8 d	1.07	0.137 (9%)
¹⁸⁸ Re	17 h	2.12	0.16 (10%)

^a A table of potential radionuclides for imaging based on half-life and emission spectra. Those above the horizontal break are negative ions; those below are positive ions. All β emissions are for electrons except in the case of ⁶⁴Cu which emits both electrons and positrons. Percentages listed are the probability of a photo-emission.

The development of antibody fragments for radioimmunoscinigraphy (RIS) can be categorized in terms of selection of radionuclide, metal chelate, production of sufficient quantities of protein, and reduction of the nontargeted uptake in normal tissues. Radiolabeling with ¹³¹I is the most commonly used radionuclide for RIS but its usage in cancer imaging is restricted by a high energy photon (360 keV), a long physical half-life ($T_{1/2} = 8$ days), high energy beta emission and a significant loss of signal due to dehalogenation (3). Use of ¹²³I has been a second possible isotope, but its relatively high cost, availability and short (13.2 h) half-life may restrict its clinical usage. ^{99m}Tc has been used for RIS, but with an even shorter half-life of 6 h, there is a substantial reduction of the amount of radioactivity deposited to the tumor tissue by the time of imaging (1). To overcome some of these limitations, a number of radiometals may be considered for RIS such as ⁶⁴Cu, ⁶⁷Cu, ¹⁰⁵Rh, ¹¹¹In, ^{111m}Sn, ¹⁵³Sm, ¹⁷⁷Lu, ¹⁸⁶Re, and ¹⁸⁸Re. These radionuclides are listed in Table 1, along with their half-lives and emission details.

Developing new recombinant antibodies for RIS, two genetically engineered antibody fragments from the highly specific, anti-CEA murine antibody T84.66 have been previously expressed (Figure 1). The T84.66 diabody (55 kDa) is a single chain variable region (scFv) dimer

which when radioiodinated exhibited rapid tumor targeting and blood clearance in tumor bearing xenografted mice (8). The second construct, the T84.66 minibody, is a scFv-CH₃ fusion protein that self-assembles into a covalent 80 kDa dimer, and when radioiodinated exhibited higher tumor targeting with a longer biological half-life in xenograft-bearing mice (9).

To investigate the radiometal-labeled utility of these recombinant antibody fragments, the antibodies were conjugated with the macrocyclic chelating agent, 1,4,7,10-tetraazacyclododecane *N,N',N'',N'''*-tetraacetic acid (DOTA) (10). This allowed labeling with ¹¹¹In for biodistribution studies in tumor-bearing xenografted mice. To determine the merits and limitations of the [¹¹¹In]form, a statistical analysis was made comparing the tumor targeting and biodistributions properties between the ¹¹¹In and previous radioiodinated forms of these two promising antibody fragments.

MATERIALS AND METHODS

Antibody Development. The T84.66 diabody previously expressed in *Escherichia coli* and characterized in radioiodinated biodistribution studies (8), has now been produced in one hundred milligram quantities from mammalian cell culture (Yazaki et al., manuscript submitted). The T84.66/212 Flex minibody (scFv-CH₃) was expressed in Sp2/0 mammalian cells, radioiodinated, and characterized in animal biodistribution studies (9). A second minibody version, the T84.66/GS18 Flex minibody, was constructed from the T84.66/212 Flex minibody DNA template using the polymerase chain reaction/splice overlap extension as previously described (9). With an 18 amino acid linker (GSTSGGGSGGGSGGGSS in single letter code), the GS18 Flex minibody was expressed in NS0 murine myeloma cells (Yazaki et al., manuscript submitted) (11). The expressed proteins were purified by hydrophobic interaction chromatography (Source ISO, Amersham Pharmacia Biotech, Piscataway, NJ) followed by anion-exchange chromatography (HQ50, PE Biosystems, Foster City, CA) using a BioCAD 700E chromatography system (PE Biosystems, Foster City, CA) (Yazaki et al., manuscript submitted). Purity was determined by electrophoresis on 10% acrylamide Ready Gels (Bio-Rad Laboratories, Hercules, CA) under nonreducing conditions on SDS-PAGE (12) with Kaleidoscope protein standards (Bio-Rad Laboratories, Hercules, CA) and staining with Coomassie Blue R-250. CEA-binding activity was determined by ELISA using microtiter plates

coated with a recombinant N-A3 protein (N and A3 domains of CEA) (13) and an anti-human Fc alkaline phosphatase antibody for detection (Jackson Immuno-research, West Grove, PA). Chimeric T84.66 (cT84.66) antibody was used as the standard (14).

Conjugation and Radiolabeling. For radiometal studies, purified T84.66/GS18 Flex minibody and the T84.66 diabody were conjugated to DOTA using the water-soluble *N*-hydroxysuccinimide method (10). In a typical experiment, 400 μ g of protein was reacted with a 1000:1 molar ratio of DOTA to protein for 18–24 h at 4 °C at pH 7.0. Following conjugation, the protein was dialyzed extensively in 0.2 M NH_4OAc , pH 7.2, and concentrated to greater than 5 mg/mL. The DOTA:antibody conjugation ratio was determined by incubation of the DOTA-conjugated antibody with a labeling solution containing known quantities of ^{111}In (Mallinckrodt, Hazelwood, MO) and cold indium (Aldrich, Milwaukee, WI). ^{111}In labeling was analyzed by thin-layer chromatography using Monoclonal Antibody ITLC strips (Biodex Medical Systems, Shirley, NY). Percent bound indium was determined, and the DOTA:antibody ratio calculated. DOT-A:antibody ratios ranged from 1:1 to 6:1 chelate per minibody while for the diabody the ratio was 0.5:1. DOTA-minibody or DOTA-diabody (400 μ g of protein) were incubated with 1.3 mCi of pure ^{111}In chloride (Mallinckrodt, Hazelwood, MO) in 0.25 M NH_4OAc pH 5.0 for 1 h at 43 °C. The reaction was stopped by the addition of DTPA to a final concentration of 1 mM. Conjugated monomeric proteins were purified by HPLC size-exclusion chromatography using a TSK-G2000SW column (30 cm $\times$ 7.5 mm ID; Tosoh, Montgomeryville, PA) for the minibody and a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech, Piscataway, NJ) for the diabody.

Radioiodination of the T84.66 minibody was by the Iodogen method as previously described (15). Radio-labeling efficiency was determined by integrating areas on the size-exclusion HPLC trace and determining the percentage of radioactivity associated in the 80 kDa peak for the minibody and the 55 kDa peak for the diabody. Immunoreactivity was determined by incubation of the labeled protein with a 20-fold excess (w/w) of either purified CEA or the recombinant N-A3 fragment of CEA (13) in 0.15 mL of PBS/1% HSA. Analysis by size-exclusion HPLC was on tandem Superose 6 HR 10/30 columns (Amersham Pharmacia Biotech, Piscataway, NJ) to assess the formation of antibody:antigen complexes.

Animal Studies. Groups of 7–8-week old female athymic mice (Charles River Laboratories, Wilmington, MA) were injected subcutaneously in the flank region with 10^6 LS174T human colon carcinoma cells obtained from ATCC (Manassas, VA). After 10 days, when tumor masses were in the range of 100–300 mg, 1–3 μ Ci of ^{111}In DOTA-antibodies/animal (2–3 μ g of protein) were injected into the tail vein. Time points of analysis for the T84.66 diabody were 0, 2, 4, 6, 12, and 24 h while for the T84.66 minibody 0, 2, 6, 12, 24, 48, and 72 h were selected. For individual experiments, groups of five to six mice at the selected time points were euthanized, necropsy performed and organs weighed and counted for radioactivity. Data from several individual experiments of the same construct and radionuclide were compared and when there was not a significant statistical difference ($p > 0.01$), the data were combined. This resulted in pooled data sets of up to 16 mice/time point. All data are mean values and have been corrected for radiodecay back to the time of injection, allowing organ uptake to be reported as percent of the injected dose per gram (% ID/

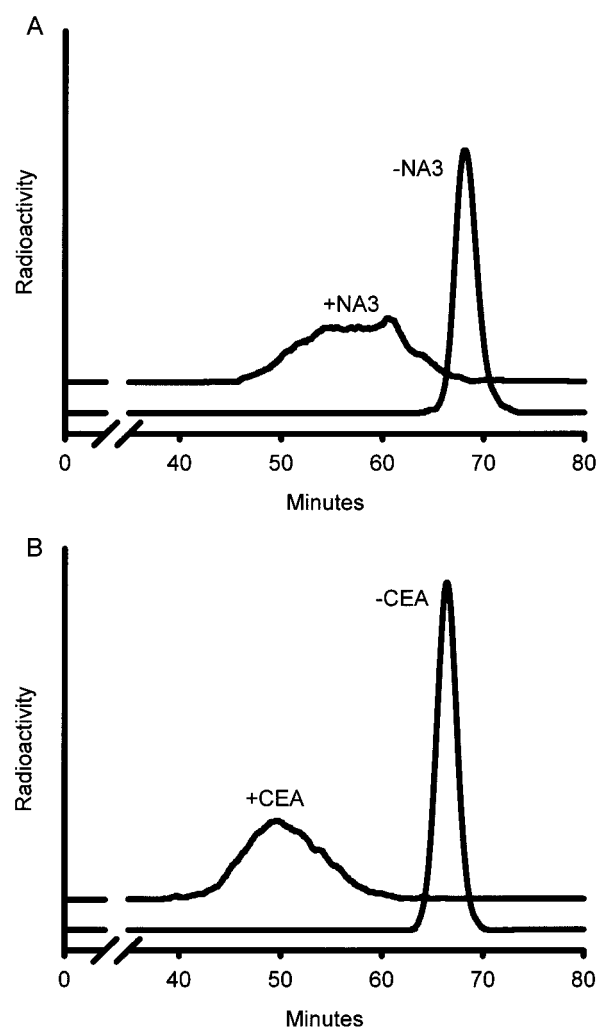


Figure 2. Radiochromatograms of size-exclusion HPLC analysis of ^{111}In DOTA-T84.66 diabody (A) and ^{111}In DOTA-T84.66 minibody (B). Lower trace was the starting sample used for evaluation of immunoreactivity. Upper trace, following incubation with CEA or N-A3 (a CEA mimetic) the bulk of the ^{111}In DOTA-T84.66 diabody and ^{111}In DOTA-T84.66 minibody are found in antibody:antigen complexes.

g^{-1}) with standard errors. Blood curves were calculated using the ADAPT II software (16).

D-Lysine renal-blocking experiments were carried out according to the protocol of Behr et al. (17). Briefly, D-lysine (Sigma, St. Louis, MO) was dissolved in PBS at a concentration of 200 mg/mL and 100 mg was administered by an oral intubation catheter, 30 min prior to the injection of the ^{111}In T84.66 diabody.

Biostatistical Analysis. To compare changes in percent of the injected dose per gram over time among antibody constructs and radionuclides, two-way analysis of variance (ANOVA) was performed (16). Time (h), construct or radionuclide, and the interaction between these two factors were included in the statistical model. Dependent variables compared using this model included the percent of the injected dose per gram for organs blood, liver, spleen, kidney, lung, and intact bone as well as tumor and carcasses. Tumor-to-blood and tumor-to-liver ratios as well as tumor masses were recorded. To compare differences in mean percent of the injected dose per gram between antibody constructs at specific time points, the independent *t*-test was used. All significance testing was done at the 0.01 level, using the SAS/STAT software (SAS Inc., Cary NC).

Table 2. Biodistribution of [¹¹¹In]T84.66 Diabody in Athymic Mice Bearing LS174T Xenografts^a

	time (h)					
	0	2	4	6	12	24
organ						
tumor	1.94 (0.53)	12.53 (1.00)	12.12 (1.60)	13.30 (2.06)	12.28 (3.09)	7.73 (1.01)
blood	49.50 (4.42)	2.70 (0.38)	0.64 (0.14)	0.34 (0.06)	0.11 (0.03)	0.04 (0.01)
liver	7.92 (0.70)	4.59 (0.59)	4.51 (0.78)	5.45 (1.61)	3.61 (0.45)	3.55 (0.51)
spleen	6.97 (0.57)	1.32 (0.09)	1.41 (0.40)	1.30 (0.40)	1.38 (0.35)	1.08 (0.30)
kidney	26.34 (10.74)	134.57 (19.29)	162.29 (17.66)	183.52 (20.97)	145.66 (16.43)	125.76 (11.63)
lung	12.48 (0.85)	2.08 (0.30)	1.23 (0.18)	1.07 (0.13)	0.87 (0.22)	0.51 (0.06)
bone	3.27 (0.70)	1.51 (0.47)	0.95 (0.20)	1.18 (0.35)	1.11 (0.32)	0.56 (0.09)
carcass	2.46 (0.06)	1.75 (0.18)	1.40 (0.14)	1.37 (0.14)	1.03 (0.22)	0.88 (0.12)
ratios						
tumor/blood	0.04 (0.00)	4.7 (0.4)	19.4 (1.6)	39.5 (3.4)	112 (11.2)	196 (11.1)
tumor/liver	0.24 (0.03)	2.8 (0.2)	2.8 (0.3)	2.6 (0.4)	3.5 (0.6)	2.2 (0.1)
tumor						
tumor mass (g)	0.49 (0.13)	0.20 (0.04)	0.15 (0.04)	0.30 (0.10)	0.27 (0.08)	0.32 (0.10)

^a [¹¹¹In]T84.66 diabody was injected into xenograft-bearing mice and tumor targeting and biodistribution studies were carried out. Groups of five mice were analyzed at each time point. Tumor and normal organ uptake are expressed as percent injected dose per gram (% ID/g). Standard errors of the mean (SE) are given in parentheses. The ratios presented are the averages of the tumor/blood and tumor/liver ratios for the individual mice. Tumor masses are in grams.

RESULTS

[¹¹¹In]DOTA-T84.66 Diabody. The T84.66 diabody was conjugated with the macrocycle DOTA to allow labeling of the protein with a variety of radiometals. The chelate-to-diabody ratio was 0.5:1 and when radiolabeled resulted in 67–100% incorporation of ¹¹¹In into the T84.66 diabody. Size-exclusion chromatography displayed a single radiolabeled peak with a specific activity that ranged from 1.4 to 3.2 $\mu\text{Ci}/\mu\text{g}$. The radiolabeled diabody displayed high immunoreactivity to N-A3 (CEA mimetic) with over 95% immunoreactivity (Figure 2A). For biodistribution studies, the tissues (tumor, blood, liver, spleen, kidney, lung, intact bone, and carcass) from groups of five mice per time point (0, 2, 4, 6, 12, and 24 h) were counted for radiolabel uptake and are shown in Table 2. The [¹¹¹In]T84.66 diabody exhibited very rapid tumor uptake in the CEA expressing LS 174T xenografts. There was $12.5 \pm 0.4\%$ ID g^{-1} at 2 h and the deposition of the radiolabel was persistent with $12.3 \pm 1.4\%$ ID g^{-1} at 12 h. The diabody was found to clear quickly from the blood circulation with only $2.7 \pm 0.2\%$ ID g^{-1} remaining at 2 h with a terminal phase $T_{1/2\beta}$ of 3.04 h (Table 3). Excellent tumor-to-blood ratios were observed with 39.5:1 at 6 h rising to 196:1 at 24 h. The initial hepatic accumulation at T_0 was low measuring $7.9 \pm 0.3\%$ ID g^{-1} and decreased to $3.6 \pm 0.2\%$ ID g^{-1} by 24 h. Other normal tissues showed low accumulation except for the kidney where a high level of accumulation was observed. This renal uptake reached a maximum of $183.5 \pm 21.0\%$ ID g^{-1} at 6 h with slow clearance during the 24 h time course of the experiment.

Preadministration of cationic amino acids has been shown to block the renal proximal tubular cell reabsorption of mAbs and antibody fragments without an effect on tumor uptake (18). Comparisons of the kidney and tumor uptake of the [¹¹¹In]DOTA-T84.666 diabody with and without the administration of D-lysine are shown in Figure 3. Oral administration of D-lysine resulted in a

Table 3. Comparison of Parameters of the Two-Exponential Fit of the Radiolabeled Forms of the T84.66 Diabody and T84.66 Minibody Blood Data^a

radiolabel and construct	A ₁	A ₂	k ₁	k ₂	T _{1/2α} (h)	T _{1/2β} (h)
[¹²³ I]T84.66	33.0	6.4	2.7	0.2	0.25	2.89
diabody (8)	1.3	1.2	0.2	0.1		
[¹¹¹ In]T84.66	48.2	1.3	1.6	0.2	0.43	3.04
diabody	0.3	0.3	0.03	0.1		
[¹³¹ I]T84.66	28.3	14.6	0.58	0.1	1.19	6.99
minibody	2.0	2.0	0.1	0.01		
[¹¹¹ In]T84.66	23.9	22.9	1.6	0.15	0.42	4.51
minibody	0.9	0.9	0.3	0.01		

^a The T84.66 minibody and the T84.66 diabody were labeled with radioiodine or ¹¹¹In. Current and previous (8) biodistribution studies were carried out in xenografted mice. Animal blood curves were calculated using the ADAPT II software (16) and the standard deviations are shown below the results. A₁ and A₂ are the amplitudes of the faster and slower clearance components. T_{1/2} are related to the inverse of k₁ and k₂ by the relationship: $T_{1/2} = 0.693/k$.

59.2% reduction at the 6 h time point where renal uptake reached the maximum (183.5 ± 21.0 vs $75.0 \pm 7.1\%$ ID g^{-1}). However at 6 h, there was a 31% reduction of tumor uptake, lowering the tumor activity to $9.2 \pm 1.2\%$ ID g^{-1} .

[¹¹¹In]DOTA-T84.66 Minibody. As was performed for the diabody, the T84.66 minibody was conjugated with DOTA for radiolabeling with ¹¹¹In. In three conjugations, the chelate-to-minibody ratio ranged from 1:1 to 6:1 and following radiolabeling resulted in 24–85% incorporation of ¹¹¹In. Size-exclusion chromatography displayed a single radiolabeled peak with a specific activity that ranged from 2.3 to 4.7 $\mu\text{Ci}/\mu\text{g}$. The [¹¹¹In]DOTA-T84.66 minibody retained high immunoreactivity to CEA with a range of 70 to 100% immunoreactivity (Figure 2B). Two experimental studies of the biodistributions of the [¹¹¹In]T84.66/GS18 Flex minibody in xenograft-bearing mice were carried out with groups of five mice per time point. All tissues (see above) were counted for radionuclide uptake

Table 4. Biodistribution of [¹¹¹In]T84.66 Minibody in Athymic Mice Bearing LS174T Xenografts^a

	time (h)						
	0	2	6	12	24	48	72
organ							
tumor	1.70 (0.17)	14.19 (0.98)	20.67 (1.47)	24.47 (6.10)	18.84 (1.17)	11.33 (0.95)	8.33 (0.90)
blood	46.69 (1.51)	17.74 (0.79)	9.14 (0.68)	3.61 (0.59)	0.60 (0.04)	0.17 (0.03)	0.08 (0.01)
liver	7.64 (0.46)	17.82 (0.77)	20.73 (1.00)	18.95 (0.74)	25.97 (0.99)	20.22 (1.04)	17.21 (1.22)
spleen	6.18 (0.28)	5.52 (0.24)	5.81 (0.39)	6.05 (0.74)	6.05 (0.29)	5.34 (0.43)	3.89 (0.26)
kidney	10.96 (0.44)	13.26 (0.90)	12.47 (0.62)	11.58 (0.93)	12.48 (0.69)	7.59 (0.85)	7.16 (0.57)
lung	14.92 (1.27)	6.88 (0.43)	5.11 (0.60)	3.11 (0.37)	2.06 (0.12)	1.34 (0.06)	1.09 (0.08)
bone	3.13 (0.14)	3.48 (0.17)	3.87 (0.19)	3.88 (0.31)	3.50 (0.35)	2.72 (0.14)	2.33 (0.10)
carcass	2.08 (0.10)	2.70 (0.12)	2.84 (0.09)	2.60 (0.07)	2.37 (0.11)	1.75 (0.05)	1.58 (0.05)
ratios							
tumor/blood	0.04 (0.00)	0.81 (0.06)	2.30 (0.13)	7.19 (1.24)	32.17 (2.06)	109.48 (25.70)	140.38 (32.05)
tumor/liver	0.23 (0.03)	0.81 (0.06)	1.02 (0.09)	1.36 (0.40)	0.73 (0.05)	0.57 (0.04)	0.49 (0.06)
tumor							
tumor mass (g)	0.15 (0.03)	0.22 (0.05)	0.16 (0.02)	0.16 (0.02)	0.21 (0.03)	0.36 (0.06)	0.36 (0.06)

^a [¹¹¹In]T84.66 minibody was injected into xenograft-bearing mice and tumor targeting and biodistribution studies were carried out. Groups of 10 mice were analyzed at each time point. Tumor and normal organ uptake are expressed as percent injected dose per gram (% ID/g). Standard errors of the mean (SE) are given in parentheses. The ratios presented are the averages of the tumor/blood and tumor/liver ratios for the individual mice. Tumor masses are in grams.

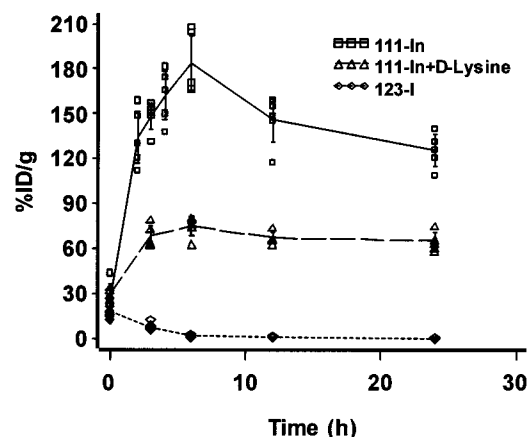


Figure 3. Comparison of renal uptake of the [¹²³I]T84.66 diabody and the [¹¹¹In]T84.66 diabody, with and without preadministration of D-lysine. The [¹²³I]T84.66 diabody and [¹¹¹In]T84.66 diabody with or without preadministration of D-lysine were injected into xenograft-bearing mice. Groups of five or more mice were sacrificed at each time point, and the tissues counted for radioactivity. Activity was expressed as percent injected dose per gram (% ID/g).

at 0, 2, 6, 12, 24, and 48 h. Statistical analysis showed no significant difference between the respective tissues from both studies and the ¹¹¹In biodistributions were combined (Table 4). Tumor uptake of the [¹¹¹In]T84.66 minibody achieved $14.2 \pm 6.1\%$ ID g⁻¹ at 2 h and reached a maximum of $24.5 \pm 6.1\%$ ID g⁻¹ at 12 h. Activity remained at the tumor site with $11.3 \pm 0.9\%$ ID g⁻¹ at 48 h but decreased to $8.3 \pm 0.1\%$ ID g⁻¹ by 72 h. The [¹¹¹In]T84.66 minibody was cleared quickly from the circulation with a $T_{1/2\beta}$ of 4.51 h. It was found that the renal uptake was low, reaching a plateau of 12–13% ID g⁻¹ over the first 24 h and clearing to 7.2% by 72 h. Intact bone activity reached a level of 3.9% ID g⁻¹ during the first 24 h but was reduced to 2.3% ID g⁻¹ by 72 h. The liver showed the highest normal organ accumulation,

reaching $26.0 \pm 1.0\%$ ID g⁻¹ at 24 h with slow clearance to $17.2 \pm 1.2\%$ ID g⁻¹ at 72 h.

High hepatic uptake had been observed for the intact [¹¹¹In]chimeric T84.66 (cT84.66) antibody in xenograft bearing mice (19). This accumulation had been determined to be the result of trafficking of CEA-antibody immune complexes into the hepatocytes (20). To investigate whether similar uptake of the antibody–antigen complex was occurring for the [¹¹¹In]T84.66 minibody, biodistribution studies were also carried out in normal athymic mice. The only tissues that showed a significant difference compared to the tumor bearing animals were the liver and the spleen. The corresponding activity curves are shown in Figure 4, along with the activity curve of the radioiodinated form in tumor bearing mice. While the [¹¹¹In]T84.66 minibody showed reduced hepatic uptake in the normal mice, the hepatic activity remained elevated in particular when compared to the radioiodinated form. For the spleen, normal athymic mice had a higher accumulation of the ¹¹¹In form than the tumor bearing mice but again the radioiodinated form was much lower. No statistical significant difference was observed for blood ($p = 0.83$) or kidney distributions ($p = 0.94$).

Indium vs Iodine Analysis. To understand the biodistribution properties of radiometal labeled fragments, the ¹¹¹In data obtained was compared to the radioiodinated biodistribution data. A two way statistical comparison was made between the [¹¹¹In]T84.66 diabody data given here and previously published [¹²³I]T84.66 diabody data (8). Uptake in the tumor of the two radionuclides reached comparable levels within the first 4 h but after 6 h a higher level of activity remain for ¹¹¹-In labeled material ($p < 0.0007$). Initial blood uptake for the indium label was marginally greater ($49.5 \pm 4.4\%$ ID g⁻¹ vs $41 \pm 2.8\%$ ID g⁻¹) with the overall blood clearance slightly slower for the indium labeled material. Calculations of the effective half-lives were made and are shown in Table 3. The ¹¹¹In form showed a slower alpha

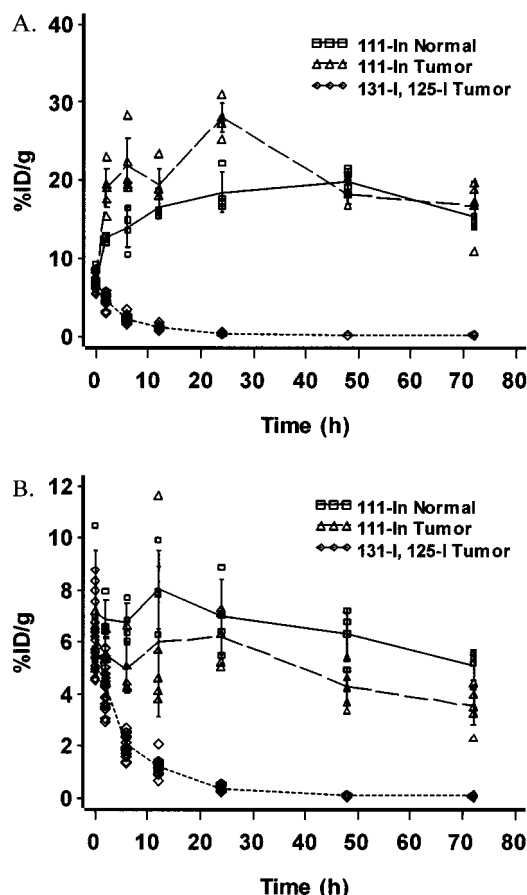


Figure 4. Hepatic and splenic uptake of the [^{111}In]T84.66 minibody in xenograft-bearing and normal mice and the radioiodinated T84.66 minibody in xenograft-bearing mice. [^{111}In]T84.66 minibody and [^{123}I] and [^{125}I]T84.66 minibody were injected into tumor-bearing mice as well as the [^{111}In]T84.66 minibody into normal athymic mice. Groups of five or more mice were sacrificed at each time point. Biodistribution of liver uptake (A) and spleen uptake (B) are shown.

phase but comparable beta phase. Hepatic uptake was significantly higher for the ^{111}In -diabody with a slower clearance pattern compared to the radioiodinated form ($p < 0.0001$). Renal uptake was substantially higher for the indium form over all but the initial time point ($p < 0.0001$). Blocking of renal absorption by D-lysine substantially lowered the [^{111}In]T84.66 diabody accumulation but the level of renal uptake was still highly elevated over the levels observed for the radioiodinated diabody (8).

Two versions of the T84.66 minibody, the GS18 Flex and the 212 Flex were expressed differing only in the length of the scFv linker. Both versions exhibited identical *in vitro* biochemical properties and antigen binding activities (Yazaki et al., manuscript submitted) and when radioiodinated displayed similar biodistributions in xenograft-bearing animals (7). The data from these two studies plus a third minibody experiment were pooled, resulting in a data set of 16 mice/time point (Table 5). Tumor uptake was $14.1 \pm 1.5\%$ ID g^{-1} at 2 h, and reached a maximum of $26.2 \pm 4.6\%$ ID g^{-1} at 12 h. Blood clearance resulted in a $T_{1/2\beta}$ of 6.99 h (Table 3). The radioiodinated T84.66 minibody exhibited some initial uptake or blood pool activity into normal tissue, but there was rapid clearance of the radiolabel. Levels immediately after injection were the following: lung ($13.6 \pm 1.1\%$ ID g^{-1}),

kidney ($9.9 \pm 0.3\%$ ID g^{-1}), liver ($7.2 \pm 0.3\%$ ID g^{-1}), and spleen ($6.2 \pm 0.3\%$ ID g^{-1}), and all were below 1% ID g^{-1} by 24 h.

In an identical manner as to the T84.66 diabody, a comparison was made between the ^{111}In and the radioiodinated forms of the T84.66 minibody. Activity in the tumor ($p = 0.18$) and blood ($p = 0.11$) showed no significant difference. There was significantly faster clearance of radioiodinated form from the liver and kidneys compared to the ^{111}In form ($p < 0.0001$).

DISCUSSION

Radiolabeled antibody fragments offer potential advantages over intact Mabs for the imaging of solid tumors. Due to their smaller molecular size, antibody fragments have exhibited rapid tumor targeting and blood clearance, increased tumor penetration and a lower potential of eliciting an immune response (4). Clinical studies with [$^{99\text{m}}\text{Tc}$]Fab' (21) and [^{123}I]scFv (22) antibody fragments have shown promise as cancer imaging agents, particularly in complement with CT scanning.

Previously, we have expressed two anti-CEA recombinant antibody fragments, the T84.66 diabody (8) and the T84.66 minibody (9), which when radioiodinated, displayed rapid tumor targeting and rapid blood clearance in xenograft-bearing mice. To extend the clinical utility of the T84.66 diabody and T84.66 minibody, labeling with one of the radiometals available (Table 1) can arguably offer many potential advantages over the radioiodinated form. These include the lack of dehalogenation, a more efficient emission spectrum for γ camera imaging, and a potentially more suitable physical half-life. In addition, radiometals compared to ^{131}I are less injurious to the patient due to a lack of 600 keV β particles produced in the decay scheme (3).

In this present work, the biodistribution properties of the [^{111}In]DOTA-T84.66 diabody displayed high level rapid tumor targeting of $12.5 \pm 0.4\%$ ID g^{-1} at 2 h and a rapid clearance from the circulation ($T_{1/2\beta} = 3.04$ h). This resulted in high tumor-to-blood ratios which reached 196:1 at 24 h compared to the 4.6:1 ratio observed for the parental cT84.66 at 24 h (23). Unfortunately, high renal uptake was observed for the [^{111}In]T84.66 diabody as has been documented for other radiometal labeled antibody fragments (4, 6, 24).

To overcome the renal accumulation problem, the T84.66 minibody was designed to have a molecular weight that exceeded the renal threshold (~ 60 kDa) (18). Two versions of the minibody were expressed, the 212 Flex and GS18 Flex, which differed in the length of the scFv peptide linker (14 vs 18 amino acids, respectively). When radioiodinated both minibody versions showed comparable animal biodistributions in xenograft-bearing mice (7) as well as identical biochemical and affinity characteristics (Yazaki et al., manuscript submitted). For all subsequent studies, the T84.66/GS18 Flex minibody was used due to the ability to produce grams of crude material from mammalian cell culture production. The [^{111}In]T84.66 minibody displayed rapid tumor targeting of $24.5 \pm 6.1\%$ ID g^{-1} at 12 h with considerable activity remaining at 72 h ($8.3 \pm 0.9\%$ ID g^{-1}) when the tumor-to-blood ratio reached 140:1. While the ^{111}In form had higher tumor uptake at 24 and 48 h compared to the radioiodinated form, overall the curves were not statistically different. Where a difference was noted between the radionuclide forms was in the normal tissue uptake. The hepatic uptake of the ^{111}In form was comparable to the level of tumor activity. This hepatic accumulation may

Table 5. Biodistribution of Radioiodinated T84.66 Minibody in Athymic Mice Bearing LS174T Xenografts^a

	time (h)						
	0	2	6	12	24	48	72
organ							
tumor	2.35 (0.83)	14.05 (1.49)	18.21 (1.42)	26.23 (4.57)	12.58 (1.77)	5.64 (0.85)	6.82 (1.67)
blood	42.83 (0.84)	20.82 (1.11)	8.68 (0.55)	4.83 (0.31)	1.00 (0.17)	0.13 (0.01)	0.09 (0.02)
liver	7.15 (0.25)	4.62 (0.19)	2.25 (0.13)	1.18 (0.07)	0.45 (0.03)	0.16 (0.01)	0.18 (0.02)
spleen	6.20 (0.34)	4.34 (0.23)	2.05 (0.10)	1.22 (0.08)	0.38 (0.03)	0.09 (0.01)	0.09 (0.01)
kidney	9.91 (0.32)	6.55 (0.27)	2.75 (0.14)	1.68 (0.08)	0.44 (0.04)	0.12 (0.01)	0.15 (0.01)
lung	13.63 (1.06)	8.85 (0.43)	4.02 (0.24)	2.77 (0.21)	0.61 (0.07)	0.12 (0.01)	0.11 (0.02)
bone	7.01 (3.77)	2.99 (0.24)	1.35 (0.11)	0.84 (0.07)	0.48 (0.12)	0.12 (0.07)	0.27 (0.15)
carcass	1.95 (0.16)	2.94 (0.09)	1.79 (0.07)	1.20 (0.09)	0.58 (0.03)	0.29 (0.02)	0.48 (0.08)
ratios							
tumor/blood	0.05 (0.02)	0.69 (0.07)	2.12 (0.12)	5.50 (0.78)	16.08 (2.13)	53.30 (7.28)	73.49 (9.09)
tumor/liver	0.34 (0.13)	3.05 (0.30)	8.47 (0.79)	22.20 (3.12)	30.28 (4.82)	40.40 (7.52)	34.83 (7.48)
tumor							
tumor mass (g)	0.22 (0.48)	0.22 (0.52)	0.23 (0.04)	0.21 (0.06)	0.36 (0.08)	0.39 (0.08)	0.52 (0.08)

^a Two versions of the T84.66 minibody were labeled with ¹³¹I and ¹²⁵I, and tumor targeting and biodistribution studies were carried out in xenograft-bearing mice. Groups of 16 mice were analyzed at each time point except at 72 h where the group size was 11 mice. Tumor and normal organ uptake are expressed as percent injected dose per gram (% ID/g). Standard errors of the mean (SE) are given in parentheses. The ratios presented are the averages of the tumor/blood and tumor/liver ratios for the individual mice. Tumor masses are in grams.

be a result of circulating CEA which can complex with anti-CEA antibodies resulting in uptake by the hepatic immunoglobulin receptor system (20). Hepatic uptake with the intact [¹¹¹In]T84.66 antibody in mice had been observed to reach 9.8% ID g⁻¹ at 96 h as the result of trafficking of CEA-antibody immune complexes into the hepatocytes (19). However, with only a slight reduction of hepatic accumulation noted in normal mice as compared to tumor-bearing mice, the possibility that a circulating CEA–minibody complex was the cause of the hepatic activity was excluded. Thus, the accumulation of the majority of the hepatic activity must be presumed to be due to a protein-radiometal-chelate effect of the [¹¹¹In]T84.66 minibody and/or ¹¹¹In labeled metabolites. It should be noted that similar tumor and hepatic uptake was observed for [⁶⁴Cu]DOTA-T84.66 minibody by microPET imaging (25). However, hepatic accumulation was not observed with the radioiodinated minibody, but because of dehalogenation of the radiolabel, we do not know if the protein or metabolite was present.

Several approaches have been reported to lower the uptake of the radiolabeled antibody fragments in normal organs (4, 6, 24). To lower the renal uptake these efforts have focused on the following areas: (1) charge modification of the protein by lowering the pI, (2) blocking of the renal reabsorption by administration of cationic amino acids, and (3) use of metabolizable linkers. Since the pI of the T84.66 diabody was predicted by cDNA analysis to be acidic (pI 4.9) and verified by isoelectric focusing gels (data not shown), we attempted to lower the renal uptake by blocking the renal proximal tubular cell reabsorption process. Behr et al. (17) had reported the administration of the nonmetabolizable amino acid, D-lysine, before injection of a [⁹⁰Y]Fab in xenografted mice resulted in a 5-fold reduction in renal uptake with no effect on tumor uptake. For the [¹¹¹In]T84.66 diabody, this resulted in a 2.4-fold reduction of the kidney uptake, however this was accompanied by a 1.4-fold reduction of

the tumor uptake. This is the first report of a reduction of tumor activity with D-lysine using an immunoconjugate, although it had been noted for L-lysine in ¹¹¹In- and ¹⁶¹Tb-labeled octreotide in neuroendocrine tumors (18). This conflicting result could be due to a number of factors, the main difference being the antibody and tumor system. While this lowered tumor activity (9.2 ± 1.2% ID g⁻¹ at 6 h) still provided sufficient deposition of radionuclide for tumor imaging, a further reduction of the renal accumulation levels must be achieved. Alternative administration schedules of cationic amino acids in mice have been reported using multiple administrations (26).

This high lysine dosage when translated to human studies would appear limited due to the induction of proteinuria by blocking renal tubular reabsorption (27). One clinical study was carried out in which five patients were infused with 2 L of a commercially available nutritive solution, which contained 2.25 g/L lysine-glutamate, 2.5 g/L arginine, salts and xylitol, before injection of a [^{99m}Tc]anti-CEA Fab' (28). In this small group of patients, this much lower dose had a 38% reduction of renal activity compared to the control group who was infused with saline. Another commercially available solution is Aminosyn, which showed a 78.5% lower renal activity of a [¹⁸F] fluoromethylbenzoyl disulfide stabilized Fv in baboons (29).

In the case of the T84.66 minibody, while the renal levels appear acceptable, the elevated hepatic activity limits the use of the T84.66 minibody for RIS of CEA positive hepatic disease and subsequent radioimmunotherapy (RIT). However, this hepatic uptake should not preclude the scintigraphy of CEA-positive lesions in the colon, rectum, breast, bone, lung and other organs. Systematic mutagenesis could produce modified minibodies with further optimized pharmacokinetics.

Alternatively, the largest advances for the use of radiometal labeled antibody fragments may come from the development of metabolizable chelate chemistry.

Intervention at two points in the metabolic process have been targeted for cleavage of the radiometal/chelate complex to be excreted from the body. The first approach would rely on the cleavage of a peptide linker before cellular uptake by the catabolic enzymes that are found at the lumen of the brush border region of the renal proximal tubule cells (30) and speculatively at the surface of hepatic cells. The second approach focuses on the design of chelates that will be cleaved by lysosomal proteases to liberate radiometabolites which would be exported from the cell and rapidly excreted in the urine (24). New chelate peptide linkers and administration schemes are currently under development in our laboratory and elsewhere. Such metabolizable chelates alone, or in combination with a clinically acceptable dose of a blocking solution, may reduce the normal organ uptake of the radiometal labeled antibody fragments to acceptable levels for highly sensitive RIS and RIT in all tissues.

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Phenylboronic Acid–Salicylhydroxamic Acid Bioconjugates.

1. A Novel Boronic Acid Complex for Protein Immobilization

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A chemical affinity system exhibiting antibody-like properties is described. The system exploits bioconjugates with appended phenylboronic acid (PBA) moieties and a support-bound phenylboronic acid complexing reagent derived from salicylhydroxamic acid (SHA) for protein immobilization on a chromatographic support. The structure of the PBA·SHA complex was characterized by ¹¹B NMR and mass spectrometry and compared with complexes derived from model compounds. Protein modification reagents were synthesized from 3-aminophenylboronic acid and utilized to prepare bioconjugates from alkaline phosphatase (AP) and horseradish peroxidase (HRP). AP obtained from one source afforded PBA bioconjugates exhibiting significant loss of enzymatic activity, whereas AP obtained from a second source afforded PBA bioconjugates exhibiting only a modest loss of enzymatic activity. Conversely, HRP afforded PBA bioconjugates exhibiting no loss of enzymatic activity. SHA-modified Sepharose was prepared by reaction of methyl 4-[(6-aminohexanoylamino)methyl]salicylate with CNBr-activated Sepharose 4B, followed by treatment with aqueous alkaline hydroxylamine. PBA–AP and PBA–HRP conjugates were efficiently immobilized on SHA–Sepharose at pH 8.3. PBA–AP conjugates were retained after washing with acidic buffers at pH 6.7, 4.2, and 2.5, whereas PBA–HRP conjugates were retained after washing with buffer at pH 6.7, but were eluted to some extent at and below pH 4.2. The results are interpreted in terms of multivalent interactions involving boronic acid complex formation between the enzyme bioconjugates and immobilized complexing reagent.

INTRODUCTION

Immobilized phenylboronic acids have been utilized to prepare chromatographic supports that selectively retain polar molecules having certain requisite functionalities from diverse biological samples. Many important biological molecules including carbohydrates, catecholamines, prostaglandins, ribonucleosides, and steroids have been either analyzed or purified in this manner. The preparation and use of phenylboronic acid chromatographic supports has been the subject of several reviews (1–5). Carbohydrates having cis or coaxial 1,2-diol and 1,3-diol moieties as well as catecholamines complex with immobilized phenylboronate anions under alkaline conditions to form cyclic phenylboronic acid esters. Acidification of diol-boronate complexes to neutral pH is known to release the diol containing species, presumably due to hydrolysis of the cyclic esters.

Some years ago, in an effort to characterize the performance of a new silica phenylboronic acid (PBA) chromatographic support, an investigation was undertaken to identify compounds that would exhibit reproducible retention times when injected into PBA columns under standardized conditions. Since the columns were optimized for the analysis of catecholamines by high-

performance liquid affinity chromatography, structurally related compounds were sought as chromatographic probes of column performance. With this in mind, the retention characteristics of a series of ortho-substituted phenols with substituents thought to potentially complex with PBA were systematically determined. Phenylboronic acid and diphenylboronic acid complexes involving ortho-substituted phenols reported in the literature have included those derived from catechol and L-dopa (6–9), 4,5-dihydroxynaphthalene-2,7-disulfonic acid and 2,3-dihydroxynaphthalene-6-sulfonic acid (10–12), salicylamide, *N*-phenylsalicylamide and disalicylamide (13), salicylaldehyde (14, 15), salicylaldehyde (16, 17), and salicylic acid (10, 18).

One of the compounds investigated, salicylhydroxamic acid (SHA), exhibited retention times under mildly acidic, neutral, and alkaline conditions that were of a greater magnitude than routinely observed for structurally related ortho-substituted phenols. This was a remarkable observation, in that most PBA complexes exhibit aqueous stability only under alkaline or mildly acidic conditions. Because the PBA·SHA complex was stable under a broad range of conditions, it was proposed that the complex itself might prove useful as a chemical affinity system (12) for protein immobilization. A system was envisioned wherein PBA would function in a manner analogous to that of a hapten and SHA in a manner analogous to that of an *anti*-hapten antibody. This system would potentially exhibit many of the positive attributes associated with biological recognition systems, while circumventing many of the limitations associated with the use of macromolecules. This article, and the following paper in this issue,

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describes our initial efforts toward the development of the envisioned chemical affinity system.

In a few instances, phenylboronic acids have been previously proposed as linkers for bioconjugation. 2-Acetamidophenylboronic acids have been proposed for selective conjugation via the vicinal diol moieties of the carbohydrate residues associated with glycoproteins (19). 3-Aminophenylboronic acids have been covalently appended to proteins by a variety of chemical methods and the resulting conjugates evaluated with respect to their ability to complex with mannose, sorbitol, and glycated hemoglobin (20). Unfortunately, the interactions proved to be reversible and of very low affinity, rendering the bioconjugates of limited practical use. Similarly, a PBA-alkaline phosphatase conjugate utilized in an enzyme-linked assay for glycated hemoglobin failed to detect the presence of glycoprotein. Radioactive technetium dioxime complexes have been conjugated to monoclonal antibodies with either 3-isothiocyanatophenylboronic acid (21) or 3-carboxy-5-isothiocyanatophenylboronic acid (22) and have proven useful for medical imaging.

MATERIALS AND METHODS

General. Reagents used in the syntheses were purchased from Aldrich (Milwaukee, WI) and Fluka (Ronkonkoma, NY), and utilized without further purification. CNBr-activated Sepharose 4B and Sephadex G25 were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Alkaline phosphatase (EC 3.1.3.1) was purchased from Sigma (St. Louis, MO) and Calzyme Laboratories (San Luis Obispo, CA). Horseradish Peroxidase (EC 1.11.1.7) was purchased from Calzyme Laboratories. An IgG fraction of rabbit *anti*-alkaline phosphatase and ChromPure Proteins from normal Human serum were purchased from Rockland (Gilbertsville, PA).

^1H , ^{11}B , and ^{13}C NMR spectra were recorded at 300, 121, and 75 MHz, respectively, on a Varian Gemini 2000 spectrometer with a broadband probe. Internal referencing was employed and chemical shifts are reported as δ values. Coupling constants are reported in hertz. High-resolution mass spectra of synthetic intermediates and reagents were recorded on a PE Biosystems Mariner ESI-TOF mass spectrometer in the positive ion mode. Mass spectra of phenylboronic acid complexes were recorded on a Hewlett-Packard Series 1100 LC/MSD by direct injection negative ion electrospray mass spectrometry with a fragmentation power setting of 60.

The purity of synthetic products was determined by analytical HPLC on a Hewlett-Packard 1050Ti system equipped with a diode array detector. A 2.1×220 mm C4 column (Aquapore Butyl, PE Biosystems) equilibrated with 0.1 M triethylammonium acetate, pH 6.5, was eluted at a flow rate of 0.5 mL/min after an initial period of 5 min with a linear gradient of from 0 to 100% methanol over 30 min. Product purity exceeded 95% in every instance. Protein bioconjugates were characterized with respect to incorporation ratio on a Hewlett-Packard 8453 diode array UV-vis spectrophotometer.

N-Hydroxy(2-hydroxyphenyl)-N-methylcarboxamide (4). To a stirred solution of 860 mg (10.3 mmol) of *N*-methylhydroxylamine hydrochloride in 15 mL of water cooled in an ice-water bath were added 8.00 mL of 6.25 N NaOH and 1.27 g (10.1 mmol) of Na_2SO_3 . After stirring for 5 min, 2.00 g (10.1 mmol) of 2-(chlorocarbonyl)phenyl acetate was added and the reaction was stirred for 24 h at room temperature. The solution was cooled and stirred in an ice-water bath, and concentrated

HCl added dropwise until a precipitate formed. The precipitate from the solution was filtered, and the filtrate extracted with ethyl acetate (3×15 mL). Extracts were combined, washed with brine, and dried over MgSO_4 and evaporated solvent. Crystallization from 1:1 ethyl acetate:hexane gave 420 mg (25%) of **4** as a white solid. ^1H NMR ($\text{DMSO}-d_6$): δ 3.16 (s, 3H), 6.82 (m, 2H), 7.21 (m, 1H), 7.30 (d, $J = 7.0$ Hz, 1H), 10.0 (br s, 2H). ^{13}C NMR ($\text{DMSO}-d_6$): δ 37.3, 116.2, 118.7, 122.1, 128.9, 131.0, 155.3, 167.0. HRMS (ES^+) calcd for $\text{C}_8\text{H}_{10}\text{NO}_3$: 168.0661. Obsd: 168.0661.

(2-Hydroxyphenyl)-N-methoxycarboxamide (5).

To a stirred solution of 842 mg (10.1 mmol) of methoxylamine hydrochloride in 15 mL of deionized water cooled in an ice-water bath were added 8.10 mL of 6.25 N NaOH and 1.27 g (10.1 mmol) of Na_2SO_3 . After stirring for 5 min, 2.00 g (10.1 mmol) of 2-(chlorocarbonyl)phenyl acetate was added and the reaction was stirred for 24 h at room temp. The solution was cooled and stirred in an ice-water bath, and concentrated HCl added dropwise until a precipitate formed. The precipitate from the solution was filtered, and the filtrate extracted with ethyl acetate (3×15 mL). Extracts were combined, washed with brine, and dried over MgSO_4 and evaporated solvent. The product was purified by silica gel chromatography with 1:1 ethyl acetate:hexane. The product fraction was collected, and the solvent was evaporated to give 590 mg (34%) of **5** as a white solid. ^1H NMR ($\text{DMSO}-d_6$): δ 3.71 (s, 3H), 6.88 (m, 2H), 7.38 (m, 1H), 7.62 (dd, $J = 1.6$ Hz, $J = 7.9$ Hz, 1H), 11.73 (s, 1H), 11.78 (s, 1H). ^{13}C NMR ($\text{DMSO}-d_6$): δ 63.6, 114.6, 117.4, 119.0, 127.8, 133.8, 159.1, 166.3. HRMS (ES^+) calcd for $\text{C}_8\text{H}_{10}\text{NO}_3$: 168.0661. Obsd: 168.0659.

3-[N-(3-Dihydroxyborylphenyl)carbamoyl]propanoic Acid (8). To a stirred suspension of 100 g (535 mmol) of 3-aminophenylboronic acid hemisulfate in 240 mL of pyridine cooled in an ice-water bath was added 56.5 g (565 mmol) of succinic anhydride. After 15 min, the reaction was removed from the ice-water bath and continued to be stirred for 12 h at room temperature. The solvent was evaporated to afford an amber syrup that was dissolved in 100 mL of water and concentrated by rotary evaporation to again afford an amber syrup. Syrup was dissolved in 700 mL of water, cooled by stirring in an ice-water bath, and pH adjusted to ~ 1 by adding 1 mL aliquots of concentrated HCl. The suspension was stirred in an ice-water bath for 1 h, and white precipitate was collected by filtration and washed with 50 mL of ice-cold water. Precipitate was dissolved in 700 mL of water by stirring while heating on a hot plate to a gentle boil. Solution was cooled to room temperature, and a white precipitate was allowed to form. The resulting suspension was stirred for 1 h at room temperature, placed in an ice-water bath, and stirred for an additional 1 h. The product was collected by filtration, washed with 50 mL of ice-cold water, and dried on the filter. The product was dried overnight in vacuo over NaOH pellets to give 100 g (80%) of **8** as a white solid, mp: 171–172 °C (uncorrected). ^1H NMR ($\text{DMSO}-d_6$): δ 12.07 (b s, 1H), 9.83 (s, 1H), 7.95 (s, 2H), 7.67 (s, 1H), 7.65 (d, $J = 7.9$ Hz, 1H), 7.41 (d, $J = 7.0$ Hz, 1H), 7.20 (t, $J = 7.8$ Hz, 1H), 2.60–2.40 (m, 4H). ^{13}C NMR ($\text{DMSO}-d_6$): δ 174.2, 170.2, 138.7, 129.0, 127.8, 125.2, 121.2, 31.0, 28.9. HRMS (ES^+) calcd for $\text{C}_{10}\text{H}_{12}\text{BNO}_5\text{Na}$: 260.0706. Obsd: 260.0707.

2,5-Dioxopyrrolidinyl 3-[N-(3-(1,3,2-Dioxaboran-2-yl)phenyl)carbamoyl]propanoate (9). To a stirred suspension of 28.4 g (120 mmol) of **8** in 120 mL of 1,4-dioxane in a 1 L round-bottom flask was added 8.7 mL (120 mmol) of 1,3-propanediol. The flask was fitted with

a West condenser, placed in boiling-water bath, and stirred for 10 min until all solids dissolved. The solution was concentrated by rotary evaporation to give either a light yellow syrup or an off-white solid. The product was dissolved in 50 mL of 1,4-dioxane, concentrated by rotary evaporation, and the product was again dissolved in 50 mL of 1,4-dioxane and again concentrated by rotary evaporation. The product was dissolved in 450 mL of stirred 1,4-dioxane, and 14.9 g (129 mmol) of *N*-hydroxysuccinimide added followed by 26.3 g (127 mmol) of DCC. The reaction was stirred under nitrogen for 4 h, white precipitate from solution was filtered and washed with 50 mL of 1,4-dioxane. The filtrate was combined, washed, and concentrated by rotary evaporation to ~100 mL. To the vigorously stirred concentrate was slowly added 400 mL of diethyl ether to produce a white precipitate. The suspension was stirred in an ice–water bath for 30 min, and the product was collected by filtration. The product on the filter was washed with 30 mL of diethyl ether, and the filter cake was dried by maintaining a vacuum for at least 5 min. The product was dried overnight in vacuo over NaOH pellets to give 42.5 g (95%) of **9** as a white solid. ^1H NMR (DMSO- d_6): δ 9.99 (s, 1H), 7.87 (s, 1H), 7.65 (d, J = 8.0 Hz, 1H), 7.32 (d, J = 7.3 Hz, 1H), 7.22 (t, J = 7.6 Hz, 1H), 4.09 (t, J = 5.4 Hz, 4H), 2.95 (t, J = 6.7 Hz, 2H), 2.79 (s, 4H), 2.69 (t, J = 6.7 Hz, 2H), 1.98 (q, J = 5.4 Hz, 2H). ^{13}C NMR (DMSO- d_6): δ 174.4, 169.0, 168.9, 138.7, 128.4, 128.1, 124.3, 121.4, 61.6, 30.3, 26.9, 25.7, 25.4. HRMS (ES^+) calcd for $\text{C}_{14}\text{H}_{15}\text{BN}_2\text{O}_7\text{Na}$ (**9** minus 1,3-propanediol): 357.0870. Obsd: 357.0876.

***N*'-Amino-*N*-(3-dihydroxyborylphenyl)butane-1,4-diamide (**10**, PBA-X-hydrazide).** To a stirred suspension of 9.0 g (24 mmol) of **9** in 125 mL of 1,4-dioxane in an ice–water bath was added 6 mL (124 mmol) of hydrazine monohydrate, and the reaction was stirred at room temperature for 1 h, during which time all solids dissolved. Solution was concentrated by rotary evaporation to afford a white solid that was taken up in 100 mL of water and filtered through a coarse porosity glass sinter. The insoluble residue on the filter was washed with 10 mL of water. The filtrate was combined, washed, and stirred in an ice–water bath for 10 min, and then the pH was adjusted to ~3 by adding 1 mL aliquots of concentrated HCl. A white precipitate formed upon addition of each aliquot of acid. The resulting suspension was stirred in an ice–water bath for 1 h, and the precipitate was collected by filtration and washed on the filter with 15 mL of ice-cold water. The product was dried overnight in vacuo over NaOH pellets to give 4.9 g (80%) of **10** as a white solid. ^1H NMR (DMSO- d_6): δ 9.86 (s, 1H), 9.02 (s, 1H), 7.98 (s, 2H), 7.81 (s, 1H), 7.68 (d, J = 8.2 Hz, 1H), 7.44 (d, J = 7.0 Hz, 1H), 7.23 (dd, J = 8.2 and 7.3 Hz, 1H), 4.27 (br s, 2H), 2.54 (t, J = 7.3 Hz, 2H), 2.33 (t, J = 7.3 Hz, 2H). ^{13}C NMR (DMSO- d_6): δ 171.1, 170.4, 138.6, 128.9, 127.8, 125.2, 121.2, 31.6, 28.6. HRMS (ES^+) calcd for $\text{C}_{10}\text{H}_{14}\text{BN}_3\text{O}_4\text{Na}$: 274.0975. Obsd: 274.0978.

6-{3-[*N*-(3-Dihydroxyborylphenyl)carbamoyl]propanoylamino}hexanoic acid (11**).** To a stirred suspension of 5.75 g (44 mmol) of 6-aminocaproic acid and 15 mL of diisopropylethylamine in 300 mL of 1,4-dioxane in an ice–water bath was added 15.0 g (40 mmol) of **9**. After stirring for 5 min, 300 mL of methanol was added, and the reaction was removed from the ice–water bath and stirred for 1 h at room temp. solvents were removed by rotary evaporation to afford a pale yellow syrup. The product was dissolved in 40 mL of water, and water was removed by rotary evaporation. Again, the product was dissolved in 40 mL of water, and again, water was

removed by rotary evaporation. The product was dissolved in 120 mL of water, stirred in an ice–water bath and adjusted pH to ~1 by dropwise addition of concentrated HCl. A white precipitate formed upon addition of each aliquot of acid. The resulting suspension was stirred in an ice–water bath for 1 h, and the precipitate was collected by filtration and washed on the filter with 15 mL of ice-cold water. The product was dried overnight in vacuo over NaOH pellets to give 10.0 g (70%) of **11** as a white powder, mp: 174–177 °C (uncorrected). ^1H NMR (DMSO- d_6): δ 11.98 (s, 1H), 9.84 (s, 1H), 7.97 (s, 2H), 7.83 (t, J = 5.4 Hz, 1H), 7.81 (s, 1H), 7.68 (d, J = 8.2 Hz, 1H), 7.44 (d, J = 7.3 Hz, 1H), 7.22 (t, J = 7.8 Hz, 1H), 3.00 (q, J = 6.3 Hz, 2H), 2.52 (t, J = 7.1 Hz, 2H), 2.36 (t, J = 7.1 Hz, 2H), 2.17 (t, J = 7.3 Hz, 2H), 1.50–1.41 (m, 2H), 1.39–1.32 (m, 2H), 1.28–1.23 (m, 2H). ^{13}C NMR (DMSO- d_6): δ 174.7, 171.3, 170.6, 138.7, 128.9, 127.8, 125.2, 121.2, 8.4, 33.6, 31.7, 30.4, 28.9, 26.0, 24.2. HRMS (ES^+) calcd for $\text{C}_{16}\text{H}_{23}\text{BN}_2\text{O}_6\text{Na}$: 373.1547. Obsd: 373.1542.

2,5-Dioxopyrrolidinyl 6-{3-[*N*-(3,3,2-Dioxaboran-2-yl)phenyl]carbamoyl}propanoyl-amino}hexanoate (12**, PBA-XX-NHS).** To a suspension of 6.3 g (18 mmol) of **11** in 60 mL of 1,4-dioxane was added 1.37 g (18 mmol) of 1,3-propanediol. The suspension was stirred in a boiling-water bath until a solution was obtained. The solvent was evaporated to afford either a colorless syrup or an off-white solid. The product was dissolved in 30 mL of 1,4-dioxane, and the solvent was removed by rotary evaporation. Again, product was dissolved in 30 mL of 1,4-dioxane, and solvent was removed by rotary evaporation. The product was dissolved in 225 mL of 1,4-dioxane, and 2.3 g (20 mmol) of *N*-hydroxysuccinimide (2.3 g, 20 mmol) was added followed by 4.1 g (20 mmol) of DCC. The reaction was stirred gently for 6 h, during which time a white precipitate formed. The precipitate was removed by filtration and washed with 50 mL of 1,4-dioxane. The precipitate was discarded, and the filtrate was combined and washed, and the solvent was removed by rotary evaporation until a colorless syrup was obtained (do not evaporate to dryness). Triturated product under 200 mL of diethyl ether to produce a white precipitate. Collected precipitate by vacuum filtration and washed with 30 mL of diethyl ether. The product was dried overnight in vacuo over NaOH pellets to give 8.0 g (91%) of **12** as a white powder, mp: 111–117 °C (uncorrected). ^1H NMR (DMSO- d_6): δ 9.85 (s, 1H), 7.85 (s, 1H), 7.82 (t, J = 5.7 Hz, 1H), 7.64 (d, J = 7.9 Hz, 1H), 7.29 (d, J = 7.3 Hz, 1H), 7.22 (dd, J = 7.6 Hz and J = 7.6 Hz, 1H), 4.07 (t, J = 5.4 Hz, 4H), 3.05–2.95 (m, 2H), 2.79 (s, 4H), 2.62 (t, J = 7.3 Hz, 2H), 2.50 (t, J = 6.7 Hz, 2H), 2.36 (t, J = 6.7 Hz, 2H), 1.50–1.41 (m, 2H), 1.41–1.30 (m, 4H). ^{13}C NMR (DMSO- d_6): δ 171.3, 170.6, 170.5, 169.2, 138.9, 128.1, 128.0, 124.3, 121.4, 61.5, 38.2, 31.7, 30.4, 30.1, 28.6, 26.8, 25.4, 23.9, 15.1. HRMS (ES^+) calcd for $\text{C}_{20}\text{H}_{27}\text{BN}_3\text{O}_8\text{Na}$ (**13** minus 1,3-propanediol): 448.1891. Obsd: 448.1887.

Methyl 4-Methylsalicylate (13**).** To 100 g (658 mmol) of 4-methylsalicylic acid dissolved in 500 mL of anhydrous methanol was added 25 mL of concentrated H_2SO_4 . The solution was heated under reflux for 18 h and concentrated to 150 mL, and the product was extracted into 250 mL of ethyl acetate. Ethyl acetate was washed with saturated aqueous NaHCO_3 (2 $\times$ 250 mL) and brine (100 mL). Ethyl acetate solution was dried over Na_2SO_4 and concentrated by rotary evaporation to afford a clear, reddish-brown liquid. The crude ester was vacuum distilled (oil pump) to give 98.1 g (90%) of **13** as a clear viscous liquid. ^1H NMR (CDCl_3): δ 2.32 (s, 3H), 3.91 (s, 3H), 6.67 (d, J = 8 Hz, 1H), 6.78 (s, 1H), 7.69 (d, J = 8

Hz, 1H), 10.71 (s, 1H). ^{13}C NMR (CDCl_3): δ 21.8, 52.1, 110.0, 117.9, 120.6, 129.9, 147.3, 161.9, 170.9. HRMS (ES^+) calcd for $\text{C}_9\text{H}_{10}\text{O}_3\text{Na}$: 189.0528. Obsd: 189.0528.

Methyl 4-(Bromomethyl)salicylate (14). To 98.1 g (590 mmol) of **13** dissolved in 600 mL of carbon tetrachloride was added 105.0 g (590 mmol) of *N*-bromosuccinimide followed by 0.7 g (3 mmol) of benzoyl peroxide. The solution was heated for 2 h under reflux, and an additional 0.7 g (3 mmol) of benzoyl peroxide was added and heated an additional 6 h under reflux. The reaction mixture was cooled to room temperature, during which time some solid formed. The solid was removed by filtration, and the filtrate evaporated to afford a viscous yellow syrup that solidified on standing. A total of 500 mL of hexanes was added to solid and heated at near boiling until almost all solids dissolved. The hot solution was filtered and concentrated until the solid just began to precipitate. The solution was heated again until the solids dissolved, and the resulting solution was allowed to cool slowly to room temperature, during which time pale yellow crystals formed. The mixture was cooled in an ice-water bath for 2 h to complete crystallization. The product was filtered, washed with 100 mL of cold hexanes, and dried in vacuo to give 83.5 g (58%) of **14** as off-white needles, mp: 73–75 °C (uncorrected). ^1H NMR (CDCl_3): δ 3.95 (s, 3H), 4.40 (s, 2H), 6.90 (d, J = 8 Hz, 1H), 7.00 (s, 1H), 7.80 (d, J = 8 Hz, 1H), 10.78 (s, 1H). ^{13}C NMR (CDCl_3): δ 32.1, 52.5, 112.4, 118.2, 120.1, 130.7, 145.8, 162.0, 170.5. HRMS (ES^+) calcd for $\text{C}_9\text{H}_9\text{O}_3\text{NaBr}$ 266.9633. Obsd: 266.9635.

Methyl 4-(Azidomethyl)salicylate (15). To a stirred solution of 83.5 g (341 mmol) of **14** dissolved in 150 mL of dry DMF was added 25.0 g (380 mmol) of sodium azide. The resulting solution turned brown, and a precipitate of NaBr formed. The suspension was stirred for 16 h, and the precipitate was filtered from solution and concentrated by rotary evaporation to afford a brown oil. The oil was dissolved in 100 mL of a mixture of 1:1 ethyl acetate:hexanes and adsorbed onto 25 g of silica gel (flash chromatography grade). Silica gel was washed on a glass sinter funnel with 1:1 ethyl acetate–hexanes (3×50 mL). The washes were combined, and the solvents evaporated to afford a colorless oil (**15**). Crude **15** was utilized for subsequent reaction without further purification. ^1H NMR (CDCl_3): δ 3.94 (s, 3H), 4.32 (s, 2H), 6.82 (d, J = 8 Hz, 1H), 6.93 (s, 1H), 7.83 (d, J = 8 Hz, 1H), 10.80 (s, 1H). ^{13}C NMR (CDCl_3): δ 52.5, 54.3, 112.3, 117.0, 118.7, 130.8, 143.9, 162.1, 170.5.

Methyl 4-(Aminomethyl)salicylate Hydrochloride (16). Crude **15** was dissolved in 750 mL of methanol and transferred to a Parr hydrogenation flask, and 3.8 g of palladium on carbon catalyst (10% w/w) was added in 25 mL of water followed by 35 mL of concentrated HCl. The mixture was shaken on a Parr apparatus for 16 h at room temperature under 40 psi of hydrogen gas. The reaction mixture was filtered through a 0.45 mm nylon filter, and the retained solid washed with 150 mL of methanol, 100 mL of water, and 150 mL of methanol. The filtrates were combined and concentrated by rotary evaporation to afford a tan solid. The solid was dissolved in 150 mL of hot denatured ethanol, and the solution was allowed to cool to room temperature, during which time white crystals quickly formed. The suspension was cooled for 18 h at 4 °C to complete crystallization. The solid was filtered, washed with 50 mL of cold ethanol, and 150 mL of diethyl ether. The product was dried in vacuo over KOH pellets to give 51.5 g (65% yield for two steps) of **16** as an off-white powder, mp: 225–227 °C (uncorrected). ^1H NMR ($\text{DMSO}-d_6$): δ 3.87 (s, 3H), 4.00 (s, 2H),

7.06 (d, J = 8 Hz, 1H), 7.13 (s, 1H), 7.77 (d, J = 8 Hz, 1H), 8.59 (br s, 3H), 10.55 (s, 1H). ^{13}C NMR ($\text{DMSO}-d_6$): δ 41.6, 52.6, 113.0, 117.7, 119.8, 130.4, 142.1, 160.0, 169.1. HRMS (ES^+) calcd for $\text{C}_9\text{H}_{12}\text{NO}_3$: 182.0817. Obsd: 182.0820.

Methyl 4-([6-[(*tert*-Butoxy)carbonylamino]hexanoylamino)methyl)salicylate (17). A total of 2.25 g (10.3 mmol) of **16** was suspended in 30 mL of anhydrous DMF, and 3.6 mL of diisopropylethylamine (20.7 mmol) was added followed by 3.38 g (10.3 mmol) of 2,5-dioxopyrrolidinyl 6-[(*tert*-butoxy)carbonylamino]hexanoate. The mixture was stirred gently under dry nitrogen for 18 h, during which time all solids dissolved. The solvent was removed by rotary evaporation to afford a light brown syrup that was partitioned between 100 mL of ethyl acetate and 100 mL of 1 N HCl. The layers were separated, and ethyl acetate was washed with 100 mL of saturated aqueous NaHCO_3 and 100 mL of brine. Ethyl acetate was dried over Na_2SO_4 , filtered, and concentrated by rotary evaporation to afford an amorphous off-white solid. The product was crystallized from 1:1 ethyl acetate:hexanes, filtered, and dried in vacuo to give 3.25 g (80%) of **17** as an off-white powder, mp: 120–121 °C (uncorrected). ^1H NMR ($\text{DMSO}-d_6$): δ 1.22 (m, 2H), 1.36 (m, 2H), 1.36 (s, 9H), 1.51 (m, 2H), 2.13 (t, J = 7 Hz, 2H), 2.87 (q, J = 6 Hz, 2H), 3.87 (s, 3H), 4.24 (d, J = 6 Hz, 2H), 6.75 (t, J = 6 Hz, 1H), 6.80 (d, J = 8 Hz, 1H), 6.82 (s, 1H), 7.72 (d, J = 8 Hz, 1H), 8.35 (t, J = 6 Hz, 1H), 10.53 (s, 1H). ^{13}C NMR (CDCl_3): δ 25.0, 26.0, 28.2, 29.3, 35.3, 41.7, 52.4, 77.4, 111.2, 115.5, 118.3, 130.1, 148.7, 155.8, 160.5, 169.5, 172.6. HRMS (ES^+) calcd for $\text{C}_{20}\text{H}_{31}\text{N}_2\text{O}_6$: 395.2182. Obsd: 395.2178.

Methyl 4-[(6-Aminohexanoylamino)methyl]salicylate Hydrochloride (18). A total of 3.00 g (7.6 mmol) of **17** was dissolved in 100 mL of ethyl acetate, and dry HCl gas was slowly bubbled through the solution. After 5 min, the gas stirred reaction was discontinued for 30 min at room temperature, during which time a white precipitate formed. The reaction mixture was cooled for 2 h in an ice-water bath. The precipitate was filtered, washed with 50 mL of diethyl ether, and dried in vacuo over KOH pellets to give 2.50 g (99%) of **18** as an off-white powder, mp: 158–160 °C (uncorrected, decomposes with effervescence). ^1H NMR ($\text{DMSO}-d_6$): δ 1.28 (m, 2H), 1.54 (m, 4H), 2.16 (t, J = 8 Hz, 2H), 2.71 (m, 2H), 3.85 (s, 3H), 4.22 (d, J = 6 Hz, 2H), 6.80 (d, J = 8 Hz, 1H), 6.83 (s, 1H), 7.70 (d, J = 8 Hz, 1H), 8.10 (br s, 3H), 8.54 (t, J = 6 Hz, 1H), 10.35 (br s, 1H). ^{13}C NMR (CDCl_3): δ 24.8, 25.6, 26.7, 35.1, 41.7, 111.5, 115.6, 118.4, 130.2, 148.6, 160.4, 169.4. HRMS (ES^+) calcd for $\text{C}_{15}\text{H}_{23}\text{N}_2\text{O}_4$: 295.1658. Obsd: 295.1656.

Preparation of SHA-Sepharose. A total of 15.0 g of CNBr-activated Sepharose 4B was suspended in 150 mL of ice-cold 1 mM HCl, collected in a medium porosity glass sinter funnel, and washed with 850 mL of ice-cold 1 mM HCl. The liquid was removed by vacuum filtration to afford a moist cake that was quickly transferred into a 100 mL wide-mouth screw cap plastic bottle containing a solution of 0.3 g of **18** dissolved in 50 mL 0.2 M NaHCO_3 . A total of 5 mL of 1 M NaHCO_3 was added, and the bottle was attached to a rotator, and the reaction was allowed to proceed overnight. Methyl salicylate-modified Sepharose intermediate (**19**) was collected in a medium porosity glass sinter funnel, washed with 1 L of deionized water, and dried on the funnel to a moist cake. A solution containing 0.5 M NH_2OH and 50 mM Na_2SO_3 was prepared by dissolving 3.4 g of hydroxylamine hydrochloride and 0.62 g of sodium sulfite in 80 mL of deionized water. 10 N NaOH was added dropwise to the

hydroxylamine solution until pH $\sim$ 8.5, after which added 10 mL of 1 M NaHCO₃, pH 10.0. The final volume of hydroxylamine solution was adjusted to 100 mL, and the pH was checked to ensure that it was in the range 9.8–10.0. Modified Sepharose was transferred into a 100 mL wide-mouth screw cap bottle, 50 mL of the hydroxylamine solution was added, the bottle was attached to a rotator, and the reaction was allowed to proceed for 2 h. SHA-Sepharose (**20**) was collected in a medium porosity glass sinter funnel, washed with 1 L of deionized water, and stored as a slurry in 20% (v/v) ethanol.

Capacity of SHA-Sepharose. The capacity of the SHA-Sepharose was estimated by first suspending 1 mL of gel in 2 mL of 50% (v/v) glycerol, removing a 0.5 mL aliquot of the suspension, and further diluting with 2.5 mL of 50% (v/v) glycerol. Finally, the SHA content of the gel was determined by comparing the absorbance at 320 nm ($\epsilon_{320} = 3200$) of SHA-Sepharose to that of unmodified Sepharose 4B.

Preparation of PBA–Alkaline Phosphatase (PBA–AP) Conjugates. Alkaline phosphatase (Type VII-T, 50,000 DEA units, Sigma, St. Louis, MO) was dialyzed against 1 L of 0.1 M NaHCO₃ buffer, pH 8.3, for 72 h at 4 °C (Slide-A-Lyzer; 3500 MWCO; Pierce). Determined concentration of dialyzed enzyme from the absorbance at 280 nm ($\epsilon_{280} = 107\,500$). A stock solution of PBA-XX-NHS (**12**) was prepared by dissolving 47.5 mg in 1 mL of DMF. The reactivity of the stock solution was checked by adding 1 μ L of stock solution to 700 μ L of TBS buffer [10 mM tris(hydroxymethyl)aminomethane, 140 mM NaCl, pH 8.0] and monitoring the increase in absorbance at 260 nm over time corresponding to the hydrolysis of the 2,5-dioxopyrrolidiny ester. A total of 2.5 mg (450 μ L of 5.6 mg/mL) of alkaline phosphatase was conjugated by adding an appropriate volume of PBA-XX-NHS stock solution to afford a 5-, 10-, and 50-fold molar excess of reagent. Reactions were incubated on ice for 1 h. Conjugates were dialyzed against 1 L of 0.1 M NaHCO₃ buffer, pH 8.3, for 48 h at 4 °C. Incorporation ratios were determined by comparing the ratio of the absorbance at 260 nm ($\epsilon_{260} = 4500$) for PBA to the absorbance at 280 nm ($\epsilon_{280} = 107\,500$) for AP. Enzymatic activity was determined of each of the PBA–AP conjugates employing *p*-nitrophenyl phosphate as substrate (pNPP, Sigma). A pNPP stock solution was prepared by dissolving a 15 mg pNPP tablet in 15 mL of DEA buffer (1 M diethanolamine, 1 mM MgCl₂, 0.1 mM ZnSO₄, pH 10.2). In a multiwell plate, 100 μ L of each of the conjugate solutions diluted to 1.0 μ g/mL with TBS buffer was added to each of six wells and assayed for AP activity by adding 100 μ L of pNPP stock solution to each well and incubating at 37 °C for 10 min. Enzymatic activity of the PBA–AP conjugates was determined relative to unmodified AP from the average absorbance at 405 nm obtained from each of six determinations. Multiwell plates were analyzed on a Bio-Tek Instruments ELx808 plate reader.

Preparation of PBA–Horseradish Peroxidase (PBA–HRP) Conjugates. A total of 25 mg of Horseradish Peroxidase (HRP) was weighed into each of three 15 mL conical tubes and dissolved in 2.5 mL of 0.1 M sodium acetate, 0.1 M sodium chloride buffer, pH 5.5. A total of 70 μ L of 350 mM sodium periodate was added to each of the tubes, and solutions were incubated in the dark for 30 min at 4 °C. A total of 130 μ L of 0.4 M Na₂SO₃ was added to each tube to quench the oxidation reaction. A stock solution of PBA-X-hydrazide was prepared by first dissolving 24.1 mg of **10** in 800 μ L of DMSO and then adding 800 μ L of 0.1 M sodium acetate, 0.1 M sodium chloride buffer, pH 5.5. Horseradish peroxidase was

conjugated by adding an appropriate volume of PBA-X-hydrazide stock solution to afford a 5-, 10-, and 50-fold molar excess of reagent and incubating the solution in the dark for 4 h at 4 °C. Enzyme conjugates were dialyzed against 2 L of 0.1 M NaHCO₃, pH 8.3, for 72 h at 4 °C (Slide-A-Lyzer; 3500 MWCO; Pierce). Incorporation ratios were determined by comparing the absorbance at 260 nm ($\epsilon_{260} = 4500$) to the absorbance at 404 nm ($\epsilon_{404} = 97\,700$). Enzymatic activity was determined of each of the PBA–HRP conjugates employing 2,2'-azine-di(3-ethylbenzthiazoline sulfonate) as HRP substrate (1-Step ABTS, Pierce). In a multiwell plate, 100 μ L of each of the conjugate solutions (0.05 μ g/mL) was added to each of six wells and assayed for HRP activity by adding 100 μ L of ABTS stock solution to each well and incubating at 37 °C for 10 min. The multiwell plate was read on a Bio-Tek Instruments ELx808 plate reader at 405 nm. Enzymatic activity of the PBA–HRP conjugates was determined relative to unmodified HRP from the average A_{405} value obtained from each of six determinations.

Chromatography of PBA–HRP and PBA–AP Conjugates on SHA-Sepharose. Equilibrated a 0.5 $\times$ 15 cm column containing 1 mL of SHA-Sepharose with 50 mL of 0.1 M NaHCO₃, pH 8.3. Protein concentrations of the PBA–HRP and PBA–AP conjugates prepared above were determined from the absorbance at 404 and 280 nm, respectively, and adjusted to 0.2 mg/mL by addition of 0.1 M NaHCO₃, pH 8.3. Conjugates (5 mL, 0.2 mg/mL) were applied to SHA-Sepharose column and eluted by addition of 5 mL of 0.1 M NaHCO₃, pH 8.3. A fraction of 10 mL was subsequently collected and stored at 4 °C. The column effluent was continuously monitored by measuring the absorbance at 280 nm with an EM-1 Econo UV Monitor equipped with a 280 nm filter (Bio-Rad). During collection of each 10 mL fraction, the absorbance at 280 nm first increased, indicating the elution of unretained conjugate, and then returned to baseline. Aliquots (10 mL) of each of three buffers of progressively lower pH were subsequently applied to the column, collected, immediately adjusted to pH 8.3 by addition of 1 N NaOH, and stored at 4 °C. The buffers included 0.1 M phosphate buffer, pH 6.7; 0.1 M phosphate buffer, pH 4.2; and 0.1 M glycine hydrochloride buffer, pH 2.5. This wash protocol was undertaken to explore the potential impact of an acidic elution gradient on retention of PBA conjugates. Unconjugated HRP and AP controls were applied to the SHA-Sepharose column to determine the extent of nonspecific binding. The relative quantity of protein in each of the fractions collected was determined by assaying the enzymatic activity of the controls and conjugates as described above, taking into account the reduced enzymatic activity associated with some of the conjugates.

RESULTS AND DISCUSSION

Structural Elucidation of PBA Complexes. Figure 1 illustrates the ¹¹B NMR spectra obtained for the complexes prepared from phenylboronic acid (PBA, **1**) and each of salicylhydroxamic acid (SHA, **2**), (2-hydroxyphenyl)-*N*-methoxycarboxamide (**5**), and *N*-hydroxy-(2-hydroxyphenyl)-*N*-methylcarboxamide (**4**) at pH 5.0, 7.0, 9.0, and 11.0 and ambient temperature. Samples were dissolved in 85:15 (v/v) deuterium oxide/methanol at a concentration of 5 mg/mL, and the pH adjusted as appropriate with concentrated hydrochloric acid or aqueous sodium hydroxide. The chemical shift(s) of the ¹¹B nucleus of **1** alone and of **1** complexed with **2**, **4**, and **5** as a function of pH are given in Table 1.

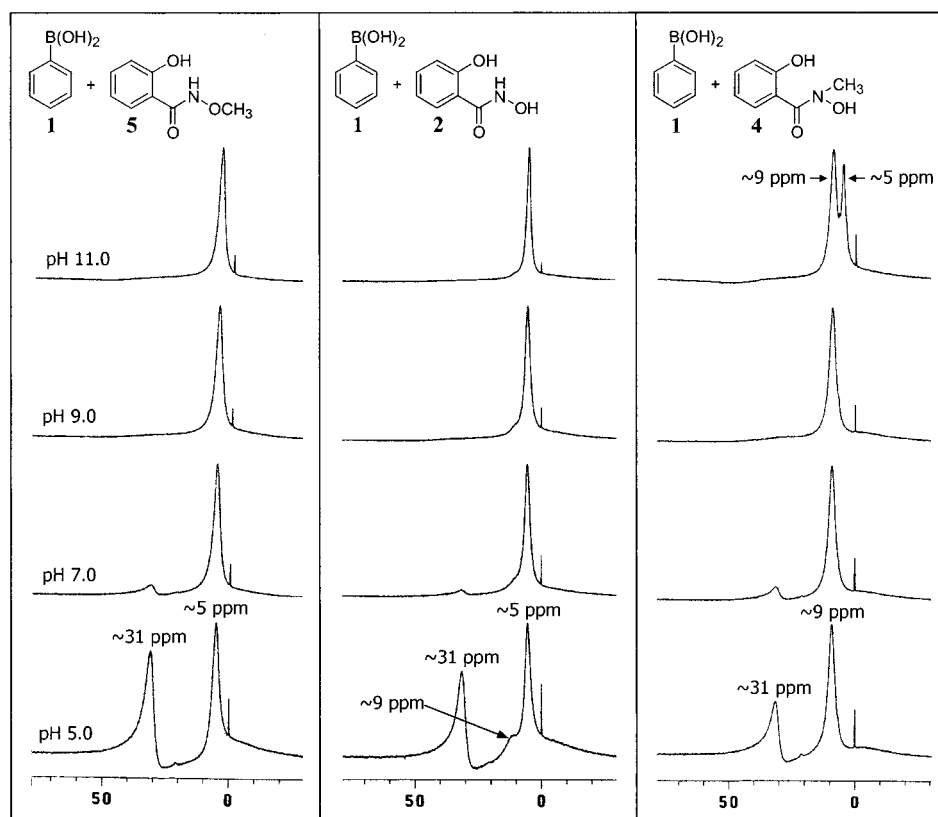


Figure 1. ^{11}B NMR spectra of phenylboronic acid complexes recorded at pH 5.0, 7.0, 9.0, and 11.0. Complexes were prepared from (left), phenylboronic acid and (2-hydroxyphenyl)-*N*-methoxycarboxamide (**1**·**5**); (center), phenylboronic acid and salicylhydroxamic acid (**1**·**2**); and (right), phenylboronic acid and *N*-hydroxy-(2-hydroxyphenyl)-*N*-methylcarboxamide (**1**·**4**).

Table 1. ^{11}B NMR Chemical Shifts^a of Phenylboronic Acid (**1**) and Complexes Prepared from PBA with Salicylhydroxamic Acid (**1**·**2**), *N*-Hydroxy-(2-hydroxyphenyl)-*N*-methylcarboxamide (**1**·**4**), and (2-Hydroxyphenyl)-*N*-methoxycarboxamide (**1**·**5**)

pH	1	1·2 complex	1·4 complex	1·5 complex
11.0	5.1	ND, ^b 4.9	9.0, 5.1	5.3
9.0	22.3	ND, ^b 5.3	9.1	5.5
7.0	30.7	ND, ^b 5.4	9.1	5.6
5.0	30.7	30.6, ND, ^b 5.1	31.2, 9.1	31.3, 5.4

^a Chemical shifts reported above, measured relative to NaBF_4 at 0.0 ppm, represent estimates due to difficulties encountered when phasing spectra containing broad signals. ^b The chemical shift of the signal recorded as a shoulder downfield of the signal associated with the predominant PBA·SHA complex cannot be precisely determined due to its low intensity, but is estimated to be in the range 9–10 ppm.

It is known that the chemical shift of the ^{11}B NMR signal from boronic acids is dependent on the hybridization state of the boron nucleus (trigonal or tetrahedral). As can be seen in the data for PBA, at pH 5.0 (approximately four pH units below the pK_a of 8.9), the chemical shift is 30.7 ± 0.1 ppm (characteristic of trigonal boronic acid); while at pH 11.0 (approximately two units above the pK_a), the chemical shift is 5.1 ± 0.1 ppm (characteristic of tetrahedral boronate anion). Only one signal is observed in the ^{11}B NMR spectrum of PBA at all three pH values, indicating that the exchange reaction involving the interconversion of boronic acid and boronate anion is rapid on the NMR time scale. These data are consistent with previous reports regarding the ^{11}B NMR spectrum of PBA (8, 23–25).

The center panel of Figure 1 shows the ^{11}B NMR spectrum of PBA in the presence of 1 equiv of SHA (**2**) as a function of pH. At pH 5.0, three signals are

observed: a large signal at about 31 ppm, a large signal at about 5 ppm, and a small signal at 9–10 ppm (observed as a shoulder on the 5 ppm signal). The signal at 31 ppm can be assigned to uncomplexed PBA by comparison with the spectrum of PBA alone at this pH. We assign the other two signals to different structural forms of the PBA·SHA complex, as these signals are absent in the spectrum of PBA alone at pH 5.0. Both of these signals appear at chemical shift values characteristic of tetrahedral phenylboronate. We thus conclude that the interaction of PBA and SHA results in the conversion of trigonal boronic acid to tetrahedral boronate anion in both of the observed structural forms. It appears that the two PBA·SHA complexes are in slow exchange with uncomplexed PBA as well as with each other, based on the observation of three distinct signals.

When the pH of the PBA and SHA mixture is raised to 7.0 (approximately two units below the pK_a of PBA), the amount of free PBA diminishes to near zero as the complex becomes dominant. At pH 9.0, no signal arising from free PBA is observed (it would appear at about 22 ppm; see Table 1), indicating that complex formation is complete at this pH. It is not possible to determine the extent of complex formation at pH 11.0 from the NMR data, since both the complex and any free phenylboronate anion would give rise to signals around 5 ppm. It is interesting to note that amount of the minor structural form of the complex decreases relative to the major structural form as the pH increases.

Figure 2 presents two possible structures of the PBA·SHA complex (**6** and **7**). Structures **6** and **7** are proposed based on the known propensity of phenylboronic acid to form five-membered and six-membered ring complexes with 1,2- and 1,3-diols, catechols, 2-aminoethanol, and

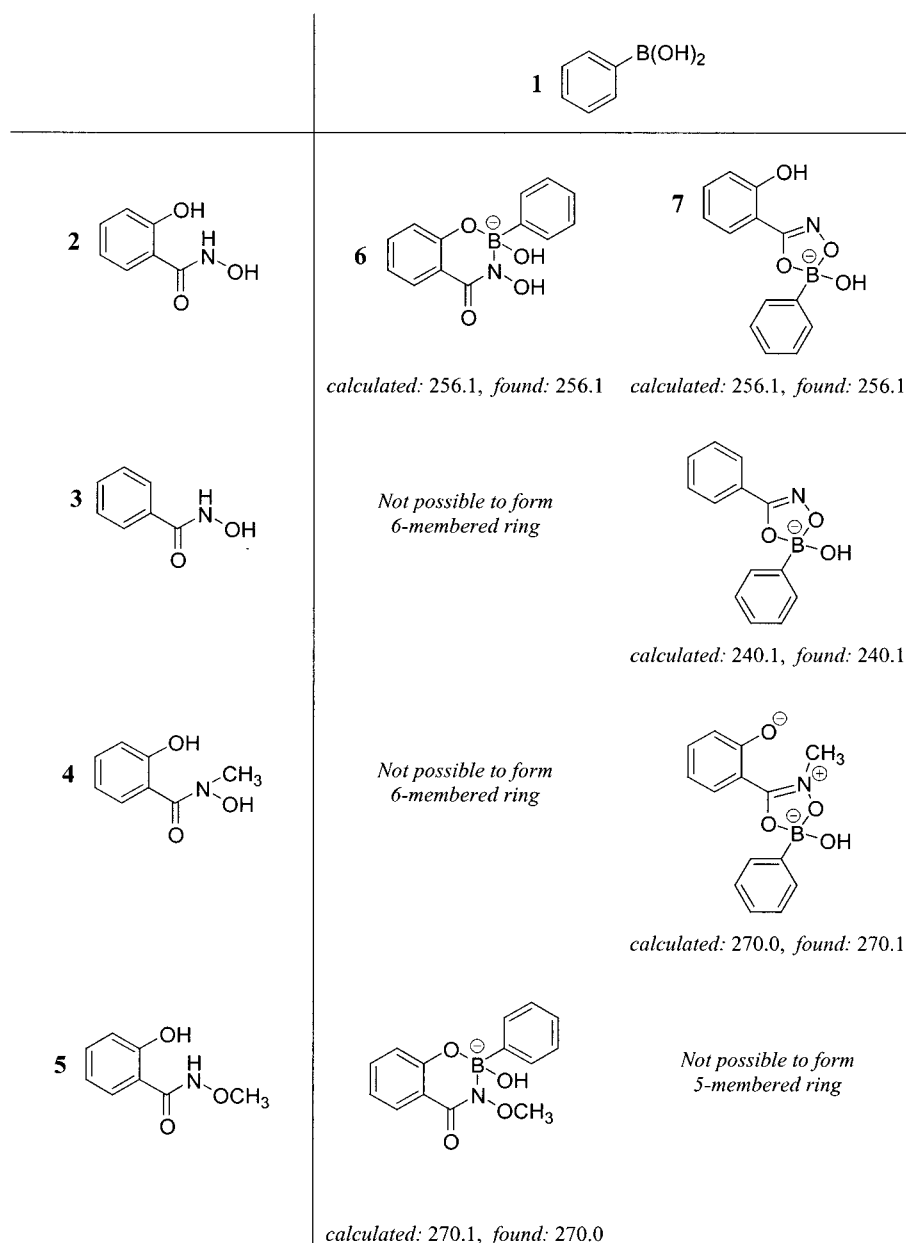


Figure 2. Proposed structures consistent with phenylboronic acid complexes prepared from salicylhydroxamic acid (**1**·**2**), benzenecarbohydroxamic acid (**1**·**3**), *N*-hydroxy-(2-hydroxyphenyl)-*N*-methoxycarboxamide (**1**·**4**), and (2-hydroxyphenyl)-*N*-methoxycarboxamide (**1**·**5**).

similar compounds. They are compatible with the ^{11}B NMR results discussed previously in that each structure contains a tetrahedral boronate anion.

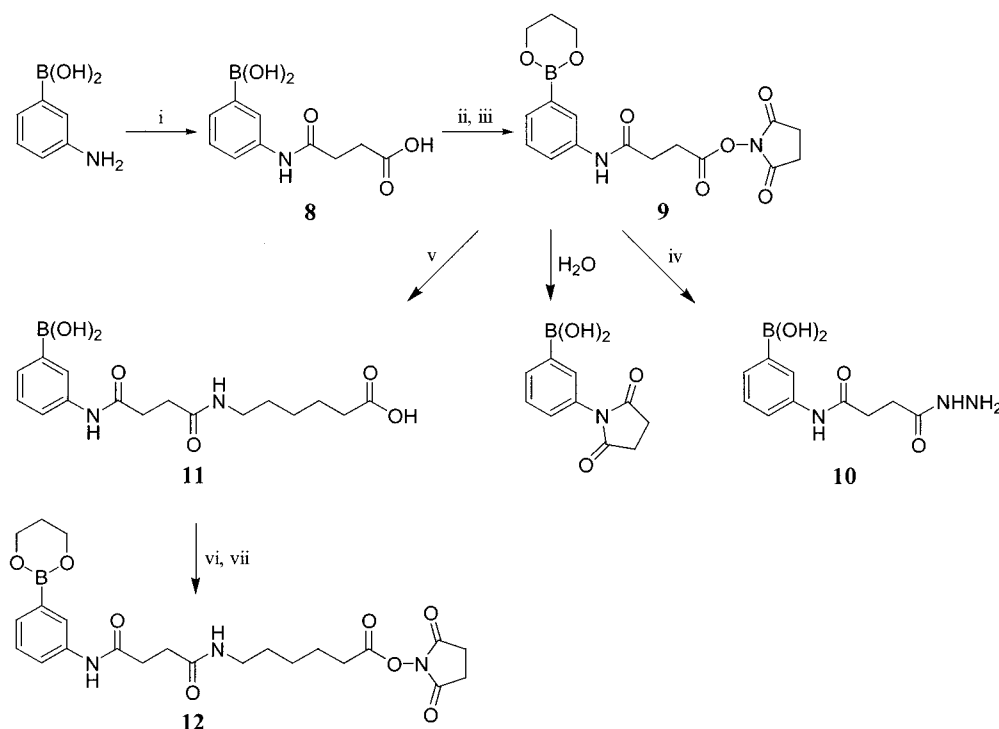
Compounds **4** and **5** were chosen to facilitate the assigning of structures to the two forms of the PBA·SHA complex observed in the ^{11}B NMR spectra. As indicated in Figure 2, **4** can form a five-membered ring complex with PBA similar to PBA·SHA complex **7**, but cannot form a six-membered ring complex similar to **6** by virtue of the replacement of the hydrogen on the nitrogen of the hydroxamic acid with a methyl group. Conversely, **5** can form a six-membered ring complex with PBA but not a five-membered ring complex, again due to the substitution of a methyl group for hydrogen.

The ^{11}B NMR spectra of PBA in the presence of 1 equiv of **4** or **5** as a function of pH are shown in the first and third panels of Figure 1. In both cases, two peaks are observed at pH 5.0, assignable to free PBA and complexed PBA. Both systems mimic the PBA·SHA system, in that at pH 7.0 the respective complex dominates the spectrum

and complex formation is complete at pH 9.0. However, the signal assigned to the PBA·**5** complex (a six-membered ring) is observed at 5 ppm, while that assigned to the PBA·**4** complex (a five-membered ring) appears at 9 ppm.

It is again not possible to determine the extent of complex formation between PBA and **5** at pH 11.0 from the NMR data, since both the complex and any free phenylboronate anion would give rise to signals around 5 ppm. However, in the case of PBA and **4**, the appearance of two signals in the pH 11.0 spectrum, one at 9 ppm and one at 5 ppm, indicates that at least this complex is somewhat unstable at this pH, producing some free phenylboronate anion (hence the signal at 5 ppm).

Complexes prepared from PBA and **2**, **4**, **5**, or benzenecarbohydroxamic acid (**3**) proved to be sufficiently stable for analysis by electrospray mass spectrometry when ionized at low fragmentation power. The data from the mass spectral analysis of these complexes is given in Figure

Scheme 1. Synthesis of Phenylboronic Acid Reagents^a

^a (i) Succinic anhydride in pyridine; (ii) 1,3-propanediol; (iii) *N*-hydroxysuccinimide and DCC; (iv) hydrazine monohydrate in 1,4-dioxane; (v) 6-aminocaproic acid and diisopropylethylamine in 1,4-dioxane; (vi) 1,3-propanediol; and (vii) *N*-hydroxysuccinimide and DCC.

2. In all cases, only the mass values predicted by the structures in Figure 2 were observed in the mass spectra. Since structures **6** and **7** are isomeric, it is not possible to determine the relative amounts of each from the mass data.

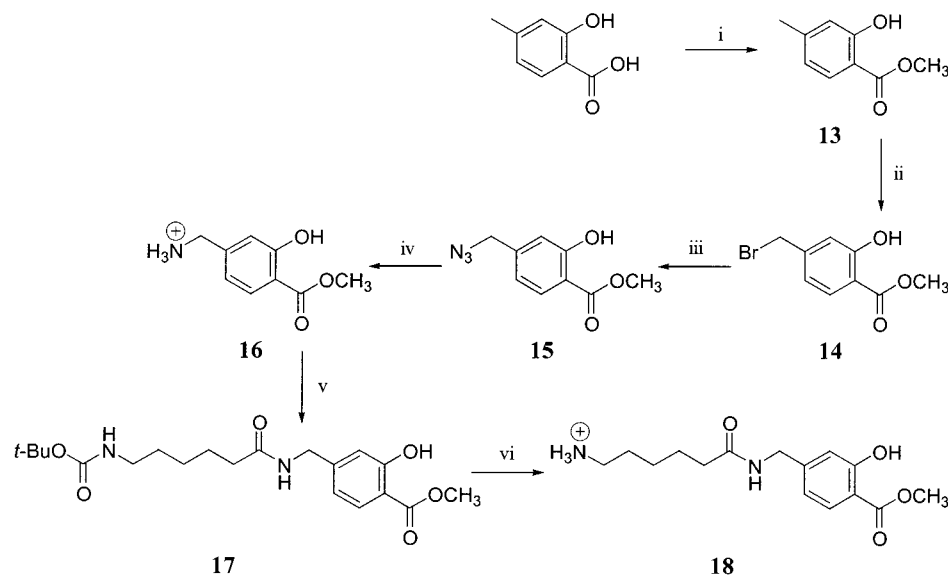
On the basis of these observations, we can thus assign the structural form **6** of the PBA-SHA complex to the major peak at 5 ppm and the structural form **7** to the minor peak at 9–10 ppm observed in the ¹¹B NMR spectra of this complex. These assignments are further supported by previously reported observations that six-membered tetrahedral phenylboronate complexes exhibit greater stability than five-membered tetrahedral phenylboronate complexes (presumably due to reduced ring strain) and that ¹¹B NMR signals for six-membered tetrahedral phenylboronate complexes generally appear upfield of signals for five-membered membered tetrahedral phenylboronate complexes (**7**, **8**). Additionally, complexes analogous to **6**, prepared from PBA and salicylamide or *N*-phenylsalicylamide, have been reported (**13**), as have complexes analogous to **7**, prepared from PBA and *N*-substituted hydroxamic acids (**26**).

Reagent Syntheses. Phenylboronic acid reagents **10** and **12** were prepared by 3- and 4-step optimized syntheses, respectively, as outlined Scheme 1. The starting material, 3-aminophenylboronic acid, was first reacted with succinic anhydride in pyridine to afford the succinamic acid **8** in 80% yield. Protection of the boronic acid moiety of **8** with 1,3-propanediol and subsequent reaction with *N*-hydroxysuccinimide in the presence of 1,3-dicyclohexylcarbodiimide next produced the *N*-hydroxysuccinimidyl ester **9** in 95% yield. Reaction of **9** with hydrazine monohydrate in 1,4-dioxane produced the hydrazide reagent **10** (PBA-X-hydrazide) in 80% yield. Attempts to employ **9** as a protein conjugation reagent failed owing to rapid intramolecular cyclization to the corresponding succinimide upon introduction into alkaline aqueous buffer. To circumvent this limitation, reac-

tion of **9** with 6-aminocaproic acid in 1,4-dioxane in the presence of diisopropylethylamine produced **11** in 70% yield. Finally, protection of the boronic acid moiety of **11** with 1,3-propanediol and subsequent reaction with *N*-hydroxysuccinimide in the presence of 1,3-dicyclohexylcarbodiimide afforded the *N*-hydroxysuccinimidyl ester **12** (PBA-XX-NHS) in 90% yield. Compounds **4** and **5** were prepared in small quantities without optimization of yields for use as model compounds for ¹¹B NMR and mass spectrometric studies involving their phenylboronic acid complexes.

Reagent **18** was prepared by a 6-step synthesis as outlined in Scheme 2. The starting material, 4-methylsalicylic acid, was esterified by refluxing with methanol and sulfuric acid to afford **13** in 90% yield. Reaction of **13** with *N*-bromosuccinimide in carbon tetrachloride in the presence of benzoyl peroxide and UV light next produced **14** in 58% yield. A small amount (≤10%) of α,α-dibrominated material as well as minor amounts of ring bromination products are also produced, leading to the modest yield of desired product. Alkylation of **14** with sodium azide in DMF produced crude **15**, which was hydrogenated in acidified aqueous methanol with palladium on carbon to produce **16** as the hydrochloride salt (65% yield for two steps). Reaction of **16** with 2,5-dioxopyrrolidinyl 6-[(*tert*-butoxy)carbonylamino]hexanoic acid in DMF in the presence of diisopropylethylamine produced **17** in 80% yield. Finally, deprotection of **17** by hydrogen chloride gas in ethyl acetate afforded **18** in 99% yield as the hydrochloride salt.

SHA-Sepharose was prepared by a two-step synthesis as outlined in Figure 3. Methyl 4-[(6-aminohexanoylamino)methylsalicylate] hydrochloride (**18**) was neutralized with sodium bicarbonate and reacted with cyanogen bromide activated Sepharose 4B in the pH range 9.8–10.0 to produce an immobilized intermediate (**19**), which was subsequently reacted with excess aqueous hydroxylamine at pH 10 in the presence of an antioxidant (Na₂-

Scheme 2. Synthesis of Methyl 4-[(6-Aminohexanoylamino)methyl]salicylate Hydrochloride^a

^a (i) Sulfuric acid and methanol; (ii) *N*-bromosuccinimide and benzoyl peroxide in carbon tetrachloride; (iii) sodium azide in DMF; (iv) hydrogen gas and palladium on carbon in acidified aqueous methanol; (v) 2,5-dioxopyrrolidinyl 6-[(*tert*-butoxy)carbonylamino]hexanoic acid in DMF; and (vi) hydrogen chloride gas in ethyl acetate.

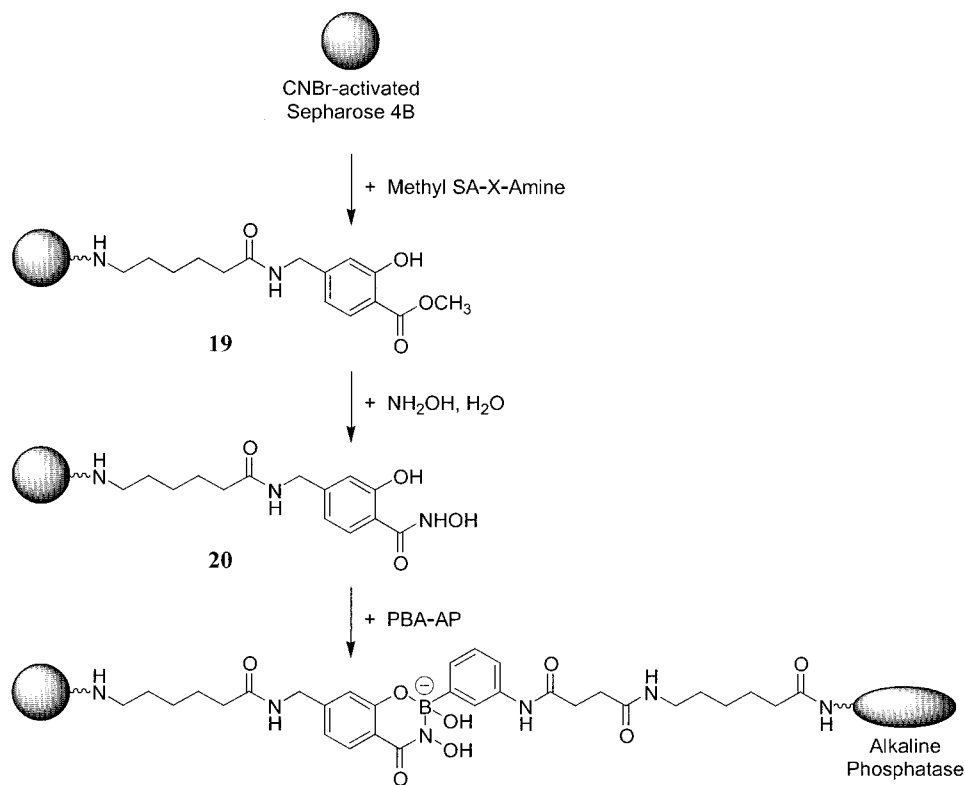


Figure 3. Preparation of SHA-Sephacrose. CNBr-activated Sepharose 4B is reacted with **18** under alkaline aqueous conditions to afford synthetic intermediate **19**. Reaction of **19** with excess alkaline aqueous hydroxylamine affords SHA-Sephacrose (**20**). PBA-AP, prepared by conjugation of AP with PBA-XX-NHS, is immobilized on SHA-Sephacrose by formation of the PBA-SHA complex. The illustration represents an oversimplification in that immobilization results from a multivalent interaction involving several boronic acid complexes.

SO₃) to produce SHA-Sephacrose (**20**). Capacity was determined to be 1.21 μmol SHA/mL settled bed volume by comparing the absorbance at 320 nm to that of unmodified Sepharose 4B. The methodology employed for determining the capacity does not differentiate between the immobilized intermediate (**19**) and immobilized SHA (**20**). Consequently, some percentage of the reported capacity may not be available for complex formation with PBA.

Characterization of PBA-HRP and PBA-AP Conjugates. Phenylboronic acid enzyme bioconjugates were prepared from alkaline phosphatase and horseradish peroxidase and characterized with respect to retention of enzymatic activity. Enzymes were conjugated with PBA at molar input ratios of 5-, 10-, or 50-fold. Alkaline phosphatase was conjugated through the lysine amino acid side-chains by reaction with a PBA-XX-NHS at pH 8.3, whereas the glycoprotein horseradish peroxidase was

Table 2. Incorporation Ratio, Enzymatic Activity and Chromatographic Retention of PBA–Enzyme Conjugates on SHA–Sephacrose at pH 8.3

conjugate	input ratio ^a	incorporation ratio ^b	enzymatic activity (%)	retention (%)
PBA-AP ^c	5:1	4.7:1	57	65
PBA-AP ^c	10:1	6.2:1	49	80
PBA-AP ^c	50:1	18.8:1	28	87
PBA-AP ^d	5:1	2.0:1	95	ND
PBA-AP ^d	10:1	5.9:1	82	ND
PBA-AP ^d	50:1	11.8:1	60	ND
PBA-HRP	5:1	2.4:1	100	13
PBA-HRP	10:1	3.9:1	100	30
PBA-HRP	50:1	9.6:1	100	91

^a Input ratio is defined as the ratio of moles of reagent introduced per mole of protein modified. ^b Incorporation ratio is defined as the ratio of moles of reagent incorporated per mole of protein modified. ^c Sigma Catalog no. P6774 from bovine intestine. ^d Calzyme Laboratories Catalog no. 235B4500 from calf intestine (molecular biology grade).

conjugated through carbohydrate residues by periodate oxidation and subsequent reaction with a PBA-X-hydrazide. Table 2 summarizes the incorporation ratios obtained corresponding to each molar input ratio as well as the enzymatic activities associated with each of the bioconjugates. Horseradish peroxidase proved particularly tolerant with respect to PBA conjugation in that the activity of the enzyme was unaffected even after appending an average of 9.6 PBA moieties. This tolerance presumably results from the site-specific nature of the conjugation reaction, wherein the modified carbohydrate residues are remote from the active site of the enzyme. Alkaline phosphatase (Sigma, catalog no. P6774 from bovine intestine) proved less tolerant with respect to PBA conjugation; however, in that appending an average of 6.2 PBA moieties to the enzyme was accompanied by the loss of nearly 50% of the enzymatic activity. Similar results have been reported for biotinylation of alkaline phosphatase when employing an *N*-hydroxysuccinimidyl ester (27). A sample of alkaline phosphatase obtained from a different source (Calzyme Laboratories, catalog no. 235B45400 from calf intestine) was conjugated in an identical manner. In this instance, appending an average of 5.9 PBA moieties to the enzyme was accompanied by the loss of less than 20% of the enzymatic activity.

Retention of PBA–AP and PBA–HRP Conjugates on SHA–Sephacrose. Table 2 also summarizes the retention of PBA–AP and PBA–HRP bioconjugates on SHA–Sephacrose at pH 8.3. The alkaline phosphatase conjugate was immobilized at an efficiency of $\geq 80\%$ when the incorporation ratio exceeded six PBA moieties. However, the horseradish peroxidase conjugate was efficiently immobilized only at the highest incorporation ratio obtained (9.6:1). When the horseradish peroxidase conjugate was first prepared and the excess reagent removed by dialysis, the formation of an insoluble aggregate was observed. In retrospect, we now speculate that aggregation resulted from intermolecular complex formation involving conjugated PBA moieties and carbohydrate residues. In this instance, aggregation results from the well-known interaction involving the phenylboronate anion and vicinal diol containing compounds. Accordingly, we conclude that the reduced immobilization efficiency of PBA–HRP on SHA–Sephacrose observed at low incorporation ratios results from competitive interactions involving carbohydrate residues. This complication can be potentially mitigated by adjusting the pH to neutral or slightly acidic conditions prior to immobilization so as to minimize the availability of the phenylbo-

Table 3. Elution of PBA–Enzyme Conjugates from SHA–Sephacrose Attributed to Washing with Buffers at Low pH

conjugate	incorporation ratio ^a	pH 6.7 buffer ^b as eluent (%)	pH 4.3 buffer ^c as eluent (%)	pH 2.5 buffer ^d as eluent (%)
PBA-AP ^e	4.7:1	<1	2	5
PBA-AP ^e	6.2:1	<1	<1	<1
PBA-AP ^e	18.8:1	<1	<1	<1
PBA-HRP	2.4:1	2	2	5
PBA-HRP	3.9:1	3	5	18
PBA-HRP	9.6:1	1	3	32

^a Incorporation ratio is defined as the ratio of moles of reagent incorporated per mole of protein modified. ^b 0.1 M phosphate buffer, pH 6.7. ^c 0.1 M phosphate buffer, pH 4.3. ^d 0.1 M glycine hydrochloride buffer, pH 2.5. ^e Sigma Catalog no. P6774 from bovine intestine.

ronate anion ($pK_a = 8.9$), thereby minimizing the potential for diol interaction. It is interesting to note that the principal site of phenylboronate interaction with carbohydrate residues, the vicinal diol groups, is also the site of the periodate oxidation reaction that was exploited for the purpose of conjugating PBA moieties to the enzyme. Consequently, one might assume that periodate oxidation would represent a preferred mechanism of glycoprotein conjugation with PBA, in that it eliminates a potential site of secondary interaction while simultaneously affording a site of conjugation. However, such an approach would be subject to considerable optimization and may only prove viable for glycoproteins with modest carbohydrate content. In the case of Horseradish Peroxidase, the persistent potential for phenylboronate–carbohydrate interaction under alkaline conditions may be attributable to the high carbohydrate content of the enzyme, which is 20 wt %.

Careful examination of Table 2 reveals that the efficiency with which both PBA–AP and PBA–HRP are immobilized is directly related to the incorporation ratio, in that only conjugates having on average six or more PBA moieties are immobilized with efficiencies of $\geq 80\%$. In light of the limited capacity of the SHA–Sephacrose ($\leq 1.2 \mu\text{mol}$ of SHA/mL of gel), we conclude that immobilization of PBA conjugates on SHA–Sephacrose results from multivalent complex formation involving several PBA moieties. From the available data it is impossible to estimate the minimum number of interactions required for efficient immobilization, but we can conclude that for the system described herein, that number appears to be greater than one and less than six. Further details regarding these phenomena and their implications will be described in a future manuscript.

Table 3 summarizes the release of immobilized PBA conjugates from SHA–Sephacrose attributable to washing with solutions of progressively lower pH. When the incorporation ratio exceeded six PBA moieties, the PBA–AP conjugates were insensitive to acidic washes of pH 6.7, 4.2, and 2.5. Only in the instance of the lowest incorporation ratio (4.7:1) was a modest amount of enzyme eluted, and then only in the most acidic wash. The PBA–HRP conjugate was more sensitive to acidic washes, in that modest amounts of enzyme were eluted in the pH 6.7 and pH 4.2 washes. At pH 2.5, significant elution of enzyme conjugate was observed (18% for the 3.9:1 conjugate and 32% for the 9.6:1 conjugate). This trend is opposite that observed for PBA–AP, and may represent a further artifact of competitive carbohydrate interaction. Specifically, at low pH, phenylboronate–carbohydrate complexes are completely dissociated, resulting in elution of that percentage of the enzyme that

was initially retained on SHA-Sepharose by intermolecular phenylboronate-carbohydrate complex formation, rather than through specific formation of the PBA-SHA complex.

CONCLUSION

The complexes derived from phenylboronic acid and salicylhydroxamic acid were characterized by ^{11}B NMR and mass spectrometry and structures were assigned based on model complex studies. Subsequently, conjugation reagents incorporating the PBA moiety (PBA-XX-NHS and PBA-X-hydrazide) were synthesized and shown to be useful for the preparation of protein conjugates from alkaline phosphatase and horseradish peroxidase. PBA-enzyme conjugates retained enzymatic activity after conjugation, and were efficiently immobilized on pre-packed columns of SHA-Sepharose at pH 8.3 by simple gravity-flow application. The protocol employed was analogous to one involving immobilization of a biotinylated or hapten-conjugated protein on either an Avidin or anti-hapten antibody column, respectively. Immobilized PBA-AP was retained on SHA-Sepharose even after washing with buffers of increasing acidity at pH 6.7, 4.2, and 2.5. Immobilized PBA-HRP was retained on SHA-Sepharose at pH 6.7, but significant elution of the conjugate was observed at lower pH. Retention of the HRP conjugate at low pH apparently suffers from the presence of acid labile intermolecular complexes involving phenylboronate moieties and the carbohydrate residues associated with the glycosylated enzyme.

Owing to the encouraging results we have thus far obtained and the relative simplicity of the chemical affinity system described herein, we have begun the process of further optimizing the requisite reagents to better characterize the utility of this approach.

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Phenylboronic Acid–Salicylhydroxamic Acid Bioconjugates.

2. Polyvalent Immobilization of Protein Ligands for Affinity Chromatography

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Phenylboronic acid bioconjugates prepared from alkaline phosphatase by reaction with either 2,5-dioxopyrrolidinyl 3-{*N*-[3-(1,3,2-dioxaboran-2-yl)phenyl]carbamoyl}propanoate (PBA-XX-NHS) or 2,5-dioxopyrrolidinyl 6-{[3,5-di-(1,3,2-dioxaboran-2-yl)phenyl]carbonylamino}hexanoate (PDBA-X-NHS) were compared with respect to the efficiency with which they were immobilized on salicylhydroxamic acid-modified Sepharose (SHA-X-Sepharose) by boronic acid complex formation. When immobilized on moderate capacity SHA-X-Sepharose (5.4 μ mol of SHA/mL of gel), PDBA–alkaline phosphatase conjugates were shown to be stable with respect to both the alkaline (pH 11.0) and acidic (pH 2.5) buffers utilized to recover *anti*-alkaline phosphatase during affinity chromatography. Boronic acid complex formation was compared to covalent immobilization of alkaline phosphatase on Affi-Gel 10 and Affi-Gel 15. PDBA–AP–SHA-X-Sepharose was shown to afford superior performance to both Affi-Gel 10 and Affi-Gel 15 with respect to immobilization of alkaline phosphatase, retention of *anti*-alkaline phosphatase and recovery of *anti*-alkaline phosphatase under alkaline conditions. High capacity SHA-X-Sepharose (≥ 7 μ mol of SHA/mL of gel) was shown to afford superior performance to moderate capacity SHA-X-Sepharose (4.5 μ mol of SHA/mL of gel) with respect to stability at pH 11.0 and pH 2.5 when a PDBA– α Human IgG conjugate with a low incorporation ratio of only 1.5:1 was immobilized on SHA-X-Sepharose and subsequently utilized for affinity chromatography of Human IgG. The results are interpreted in terms of either a bivalent or trivalent interaction involving boronic acid complex formation.

INTRODUCTION

In the previous paper (1) we introduced a small molecule polyvalent system that exploits the interaction between phenylboronic acid (PBA) and salicylhydroxamic acid (SHA) for protein immobilization. The utility of this system was demonstrated by preparing a PBA-modified alkaline phosphatase conjugate (PBA–AP), immobilizing the conjugate on a SHA-modified chromatographic support (SHA-Sepharose), and utilizing the resulting chromatographic support for affinity chromatography of *anti*-alkaline phosphatase. Initial results were promising, although a potential limitation was identified relating to the need to append several PBA moieties when preparing conjugates. The present paper describes the further optimization of the PBA–SHA system with respect to the immobilization of protein ligands for affinity chromatography. Our efforts were directed toward optimizing the reagent components of the system so as to minimize the requisite number of phenylboronic acid moieties while concomitantly increasing both the efficiency and stability with which PBA conjugates are immobilized on SHA chromatographic supports. We report here the development of a new boronic acid reagent that incorporates two equivalents of boronic acid per site of conjugation, PDBA-X-NHS, as well as the development of a new high capacity chromatographic

support, SHA-X-Sepharose. The performance of PDBA-X-NHS and SHA-X-Sepharose is compared to that of PBA-XX-NHS and SHA-Sepharose, respectively.

In nature, polyvalent interactions involving the simultaneous binding of multiple ligands on one biological entity (a macromolecule or cell surface) to multiple receptors on another are often collectively much stronger than the corresponding monovalent interactions. A recent review of polyvalent systems states, “The possibility that multiple simultaneous interactions have unique collective properties that are qualitatively different from properties displayed by their constituents, which interact monovalently, suggests new strategies for the design of drugs and research reagents for biochemistry and biology” (2). Although polyvalent systems usually involve protein receptors, dimeric derivatives prepared from vancomycin and peptides containing D-Ala-D-Ala have recently provided small molecule model systems for investigating polyvalency (3–5). Trimeric derivatives prepared from vancomycin and L-Lys-D-Ala-D-Ala were reported to bind to one another 25 times tighter than avidin-biotin, one of the strongest biological interactions known (6).

MATERIALS AND METHODS

Materials. Reagents used in the syntheses were purchased from Aldrich (Milwaukee, WI) and Fluka (Ronkonkoma, NY) and utilized without further purification. 2,5-Dioxopyrrolidinyl 6-(3-{*N*-[3-(1,3,2-dioxaboran-2-yl)phenyl]carbamoyl}propanoylamino)hexanoate (PBA-XX-NHS), methyl 4-aminomethylsalicylate hydrochloride, and SHA-Sepharose were prepared as previously re-

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ported (1). 2,5-Dioxopyrrolidinyl 6-[[3,5-di(1,3,2-dioxaboran-2-yl)phenyl]carbonylamino}hexanoate (PDBA-X-NHS) and SHA-X-Sepharose were prepared as described below and are now available from Prolinx (Bothell, WA). EAH Sepharose 4B and Sephadex G25 were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Affi-Gel 10 and Affi-Gel 15 were obtained from Bio-Rad (Hercules, CA). Alkaline phosphatase (EC 3.1.3.1) was obtained from Calzyme Laboratories (San Luis Obispo, CA) and rabbit *anti*-alkaline phosphatase (IgG fraction) was obtained from Rockland (Gilbertsville, PA). Goat *anti*-human IgG and ChromPure proteins (purified whole molecule immunoglobulins from normal Human serum) were obtained from Jackson (West Grove, PA).

^1H , ^{11}B , and ^{13}C NMR spectra were recorded at 300, 121, and 75 MHz, respectively, on a Varian Gemini 2000 spectrometer with a broadband probe. Internal referencing was employed and chemical shifts are reported as δ values. Coupling constants are reported in hertz. High-resolution mass spectra of synthetic intermediates and reagents were recorded on a PE Biosystems Mariner ESI-TOF mass spectrometer in the positive ion mode.

The purity of synthetic products was determined by analytical HPLC on a Hewlett-Packard 1050Ti system equipped with a diode array detector. A 2.1 cm $\times$ 0.22 cm C4 column (Aquapore Butyl, PE Biosystems) equilibrated with 0.1 M triethylammonium acetate, pH 6.5, was eluted at a flow rate of 0.5 mL/min after an initial period of 5 min with a linear gradient of from 0 to 100% methanol over 30 min. Product purity exceeded 95% in every instance. Protein bioconjugates were characterized with respect-to-incorporation ratio on a Hewlett-Packard 8453 diode array UV-vis spectrophotometer.

3,5-Di(dihydroxyboryl)benzoic Acid (1). A total of 5.0 g (17.9 mmol) of 3,5-dibromobenzoic acid was dissolved in 300 mL of THF and cooled to -78°C . A total of 12.7 mL (17.8 mmol) of methyllithium was added over 2 min and stirred for 5 min, and 58.0 mL (98.6 mmol) *tert*-butyllithium was added over 5 min. The solution was stirred at -78°C for 15 min, and the solution was allowed to warm to -45°C , stirred for 45 min, and then again cooled to -78°C . A total of 27.0 mL (117 mmol) of triisopropylborate was added, stirred for 15 min at -78°C , and then allowed to warm to room temperature. The solution was stirred at room temperature for 2 h, and the bulk of solvent was removed by rotary evaporation and residual solvent under high vacuum while warming for 1 h to 60°C . The solids were dissolved in 60 mL of water, cooled to 0°C for 1 h and then filtered off solids (borate salts). The filtrate was cooled to 0°C and adjusted pH to ~ 4 by dropwise addition of concentrated HCl. The solution was allowed to stand overnight at 4°C , and solids were collected by filtration, washed with cold water, and dried under high vacuum. The volume of filtrate was reduced to ~ 70 mL, and again the solution was allowed to stand at 4°C overnight. Finally, solids were collected by filtration, washed with cold water, and dried under high vacuum to give 2.23 g (60%) of **1** as a white powder. ^1H NMR (DMSO- d_6): δ 8.18 (s, 4H), 8.41 (s, 3H), 12.65 (br s, 1H). ^{13}C NMR (DMSO- d_6): δ 129.1, 133.5, 137.0, 144.9, 168.4. ^{11}B NMR (DMSO- d_6): δ 27.7. HRMS (ES^+) calcd for $\text{C}_7\text{H}_8\text{B}_2\text{O}_6\text{Na}$: 233.0405. Obsd: 233.0410.

6-[[3,5-Di(dihydroxyboryl)phenyl]carbonylamino}-hexanoic Acid (3). A total of 3.2 g (15.2 mmol) of **1** was suspended in 300 mL of 1,4-dioxane, 2.2 mL (30.4 mmol) of 1,3-propanediol and heated to near reflux while stirring until the solution became homogeneous. The solvent was removed by rotary evaporation, the solid was

dissolved by heating in 150 mL of 1,4-dioxane. Again, the solvent was removed by rotary evaporation, the solid was dissolved by heating in 150 mL of 1,4-dioxane, and then the solvent was removed by rotary evaporation. The solid was dissolved with heating in 400 mL of 1,4-dioxane, the solution was allowed cool to room temperature, and then 1.84 g (16.0 mmol) of *N*-hydroxysuccinimide was added followed by 3.30 g (16.0 mmol) of DCC. The solution was stirred gently overnight at room temperature, during which time a white precipitate formed, the precipitate was filtered from solution, and the filtrate was concentrated by rotary evaporation. The intermediate was dissolved (**2**, colorless solid) in 100 mL of methanol, 3.99 g (30.4 mmol) of 6-aminohexanoic acid (3.99 g, 30.4 mmol) and 10.6 mL (60.9 mmol) of diisopropylethylamine were added. The solution was stirred for 24 h at room temperature, the solvent was concentrated by rotary evaporation, and then the product was dissolved in 75 mL of 0.25 N NaOH. The solid was filtered off, and the filtrate cooled to 0°C and then acidified to pH ~ 4 with 1 N HCl. The product was allowed to stand for 1 day at 4°C . Finally, the precipitate was collected by filtration, washed with cold water, and dried under high vacuum to give 3.59 g (73%) of **3** as a white solid. ^1H NMR (DMSO- d_6): δ 1.29 (m, 2H), 1.51 (m, 4H), 2.19 (t, $J = 7.3$ Hz, 2H), 3.22 (q, $J = 6.3$ Hz, 2H), 8.05 (br s, 4H), 8.19 (s, 2H), 8.28 (s, 1H), 8.47 (t, $J = 11.1$ Hz, 1H), 11.92 (br s, 1H). ^{13}C NMR (CD_3OD): δ 24.0, 25.7, 28.3, 33.1, 39.0, 132.7, 133.3, 141.5, 169.5, 176.1. HRMS (ES^+) calcd for $\text{C}_{13}\text{H}_{19}\text{B}_2\text{NO}_7\text{Na}$: 346.1245. Obsd: 346.1254.

2,5-Dioxopyrrolidinyl 6-[[3,5-Di(1,3,2-dioxaboran-2-yl)phenyl]carbonylamino}hexanoate (4, PDBA-X-NHS). A total of 5.67 g (17.6 mmol) of **3** was suspended in 200 mL of 1,4-dioxane, 2.55 mL (35.3 mmol) of 1,3-propanediol and heated stirred suspension for 15 min under reflux. Decanted solution into another flask leaving behind an oily material. The solvent was removed by rotary evaporation, the solid was dissolved in 150 mL of 1,4-dioxane. Again, the solvent was removed by rotary evaporation, the solid was dissolved in 150 mL of 1,4-dioxane and the solvent was removed by rotary evaporation. The solid was dissolved in 200 mL of 1,4-dioxane, 2.18 g (18.9 mmol) of *N*-hydroxysuccinimide and 3.80 g (18.4 mmol) of DCC and stirred at room temperature overnight, during which time a white precipitate formed. The precipitate was filtered from solution, and the filtrate was concentrated by rotary evaporation. The product was dissolved in a minimum volume of 1,4-dioxane and added to 250 mL of rapidly stirred methyl *tert*-butyl ether. The product initially precipitates as an oil but affords a white solid after 24 h of rapid stirring. The product was collected by filtration and washed with methyl *tert*-butyl ether to give 6.52 g (74%) of **4** as a white powder. ^1H NMR (DMSO- d_6): δ 1.38 (m, 2H), 1.53 (m, 2H), 1.64 (m, 2H), 1.99 (p, $J = 5.1$ Hz, 4H), 2.66 (t, $J = 7.3$ Hz, 2H), 2.79 (s, 4H), 3.22 (q, $J = 6.4$ Hz, 2H), 4.10 (t, $J = 5.4$ Hz, 8H), 8.13 (s, 1H), 8.16 (s, 2H), 8.49 (t, $J = 5.5$ Hz, 1H). ^{13}C NMR (DMSO- d_6): δ 24.0, 25.4, 25.5, 26.9, 28.6, 30.1, 38.9, 61.6, 131.8, 133.4, 134.5, 141.6, 166.7, 169.2, 170.5. HRMS (ES^+) calcd for $\text{C}_{17}\text{H}_{22}\text{B}_2\text{N}_2\text{O}_9\text{Na}$ (**4** minus two 1,3-propanediols): 443.1409. Obsd: 443.1429.

Methyl 4-[[*tert*-Butoxy]carbonylamino]methyl}-salicylate (5). A total of 10.9 g (50 mmol) of methyl 4-aminomethylsalicylate hydrochloride was suspended in 200 mL of anhydrous methanol, and 8.7 g (50 mmol) of di-*tert*-butyl dicarbonate and 7.0 mL (50 mmol) of Et_3N

were added. The solid rapidly dissolved with the slow evolution of gas. The reaction mixture was stirred under dry N₂ for 18 h at room temperature and then evaporated to dryness to afford an amorphous white solid. The solid was partitioned between 200 mL of ethyl acetate and 100 mL of water, the layers were separated, ethyl acetate was dried over Na₂SO₄, the solution was filtered, and the filtrate was evaporated to a white solid. The solid was crystallized from 1:1 ethyl acetate:hexanes, and the product was filtered and dried in vacuo to give 13.7 g (97%) of **5** as a white solid, mp: 95–96 °C (uncorrected). ¹H NMR (CDCl₃): δ 1.42 (s, 9H), 3.90 (s, 3H), 4.26 (d, *J* = 6 Hz, 2H), 4.99 (t, *J* = 6 Hz, 1H), 6.75 (d, *J* = 8 Hz, 1H), 6.84 (s, 1H), 7.73 (d, *J* = 8 Hz, 1H), 10.72 (s, 1H). ¹³C NMR (CDCl₃): δ 28.5, 44.4, 52.4, 80.0, 111.5, 115.9, 118.2, 130.5, 150.0, 156.3, 162.1, 170.8. HRMS (ES⁺) calcd for C₁₄H₁₉NO₅Na: 304.1161. Obsd: 304.1152.

4-[(*tert*-Butoxy)carbonylamino]methyl]salicylic Acid (6**).** A total of 8.7 g (30.9 mmol) of **5** was dissolved in 100 mL of dry THF, and 4.4 g (30.9 mmol) of potassium trimethylsilanolate (90% pure) was added and heated for 24 h under reflux, during which time a tan precipitate formed. The mixture was evaporated to dryness, and the solid was dissolved in 100 mL of water. The stirred solution was cooled in an ice–water bath, and the pH was adjusted to ~2 by dropwise addition of saturated aqueous KHSO₄, thereby producing an off-white precipitate. The precipitate was filtered, washed with ice-cold water, and dried in vacuo over KOH pellets to give 6.7 g (81%) of **6** as a white solid, mp: 141–144 °C (uncorrected, decomposes with effervescence). ¹H NMR (CDCl₃): δ 1.47 (s, 9H), 4.33 (d, *J* = 6 Hz, 2H), 5.07 (t, *J* = 6 Hz, 1H), 6.80 (d, *J* = 8 Hz, 1H), 6.88 (s, 1H), 7.81 (d, *J* = 8 Hz, 1H), 10.72 (br s, 2H). ¹³C NMR (CDCl₃): δ 28.5, 44.4, 80.5, 111.1, 115.9, 118.3, 131.5, 148.5, 156.6, 162.6, 174.1. HRMS (ES⁺) calcd for C₁₃H₁₇NO₅Na: 290.1004. Obsd: 290.1005.

Cyanomethyl 4-[(*tert*-Butoxy)carbonylamino]methyl]salicylate (7**).** A total of 8.2 g (30.6 mmol) of **6** was suspended in 25 mL of chloroacetonitrile, 4.3 mL (30.6 mmol) of Et₃N was added, and the mixture was stirred under dry N₂ for 8 h at 50 °C. The mixture was cooled to room temperature, the solvent was evaporated, and the residue was partitioned between 250 mL of ethyl acetate and 250 mL of water. The layers were separated, and ethyl acetate was washed with 100 mL of saturated aqueous NaHCO₃ and 100 mL of saturated aqueous NaCl. The solution was dried over Na₂SO₄ and filtered, and the filtrate was evaporated to dryness. The pale tan solid was dissolved in 100 mL of ethyl acetate, and the produce was adsorbed onto 10 g of silica gel. Silica gel on a glass frit was collected by vacuum filtration and washed with 100 mL of ethyl acetate, and the filtrate was evaporated to dryness. The residue was crystallized from 1:1 ethyl acetate:hexanes to give 7.9 g (88%) of **7** as an off-white solid, mp: 144–146 °C (uncorrected). ¹H NMR (CDCl₃): δ 1.45 (s, 9H), 4.30 (d, *J* = 6 Hz, 2H), 5.00 (s, 2H), 5.05 (t, *J* = 6 Hz, 1H), 6.83 (d, *J* = 8 Hz, 1H), 6.91 (s, 1H), 7.77 (d, *J* = 8 Hz, 1H), 10.12 (s, 1H). ¹³C NMR (CDCl₃): δ 28.4, 44.3, 49.0, 80.1, 109.6, 114.2, 116.1, 118.7, 130.5, 149.7, 156.2, 162.5, 168.5. HRMS (ES⁺) calcd for C₁₅H₁₈N₂O₅Na 329.1113. Obsd: 329.1111.

Cyanomethyl 4-(Aminomethyl)salicylate Hydrochloride (8**).** A total of 7.7 g (26.2 mmol) of **7** was dissolved in 150 mL of THF, and dry HCl gas was slowly bubbled through the solution. After 5 min, the gas was discontinued, and the reaction was stirred for 30 min at room temperature, during which time a creamy white precipitate formed. The reaction mixture was cooled for

2 h in an ice–water bath. The precipitate was filtered, washed with 100 mL of diethyl ether, and dried in vacuo over KOH pellets to give 5.8 g (91%) of **8** as a white powder. ¹H NMR (DMSO-*d*₆): δ 4.00 (s, 2H), 5.20 (s, 2H), 7.05 (d, *J* = 8 Hz, 1H), 7.15 (s, 1H), 7.75 (d, *J* = 8 Hz, 1H), 8.62 (br s, 3H), 10.38 (s, 1H). ¹³C NMR (DMSO-*d*₆): δ 41.6, 49.7, 113.0, 116.1, 118.0, 119.7, 131.1, 142.3, 159.5, 166.1. HRMS (ES⁺) calcd for C₁₀H₁₁N₂O₃: 207.0770. Obsd: 207.0768.

4-[N-({4-[(Cyanomethyl)oxycarbonyl]-3-hydroxyphenyl)methyl}carbamoyle]butanoic Acid (9**).** A total of 1.22 g (5.0 mmol) of **8** was suspended in 100 mL dry DCM and stirred in an ice–water bath, and a solution of 0.57 g (5.0 mmol) of glutaric anhydride and 0.7 mL (5.0 mmol) of Et₃N was added in 25 mL of dry DCM over 15 min. The reaction was stirred for 18 h at room temperature. The mixture was evaporated to dryness, and the solid was triturated under 50 mL of ice-cold 0.1 N HCl. The solid was collected by filtration, washed with cold water, and dried in vacuo over KOH pellets to give 1.43 g (89%) of **9** as a white solid, mp: 125–126 °C (uncorrected). ¹H NMR (DMSO-*d*₆): δ 1.75 (q, *J* = 7 Hz, 2H), 2.19 (t, *J* = 7 Hz, 2H), 2.22 (t, *J* = 7 Hz, 2H), 4.24 (d, *J* = 6 Hz, 2H), 5.17 (s, 2H), 6.81 (d, *J* = 8 Hz, 1H), 6.85 (s, 1H), 7.70 (d, *J* = 8 Hz, 1H), 8.39 (t, *J* = 6 Hz, 1H), 10.5 (br s, 1H), 11.7 (br s, 1H). ¹³C NMR (DMSO-*d*₆): δ 20.7, 33.1, 34.5, 41.8, 49.7, 111.2, 115.9, 116.2, 118.5, 130.9, 149.1, 160.2, 166.6, 172.3, 174.5. HRMS (ES⁺) calcd for C₁₅H₁₆N₂O₆Na: 343.0906. Obsd: 343.0909.

2,5-Dioxopyrrolidinyl 4-[N-({4-[(Cyanomethyl)oxycarbonyl]-3-hydroxyphenyl)methyl}carbamoyle]butanoate (10**).** A total of 1.00 g (3.1 mmol) of **9** was dissolved in 50 mL of dry THF, 0.36 g (3.1 mmol) of *N*-hydroxysuccinimide and 0.64 g (3.1 mmol) of DCC were added, and the mixture was gently stirred under dry N₂ for 1 h at room temperature, during which time a white precipitate formed. The reaction was stirred for 24 h at room temperature and then cooled for 4 h at –20 °C. The precipitate was filtered from cold solution and washed with 10 mL of THF. The filtrate and wash were combined and evaporated to dryness. The residue was crystallized from 1:1 2-propanol:hexanes and filtered and dried in vacuo to give 1.04 g (80%) of **10** as white needles, mp 116–119 °C (uncorrected). ¹H NMR (DMSO-*d*₆): δ 1.88 (q, *J* = 7 Hz, 2H), 2.30 (t, *J* = 7 Hz, 2H), 2.71 (t, *J* = 7 Hz, 2H), 2.80 (s, 4H), 4.26 (d, *J* = 6 Hz, 2H), 5.18 (s, 2H), 6.82 (d, *J* = 8 Hz, 1H), 6.86 (s, 1H), 7.71 (d, *J* = 8 Hz, 1H), 8.44 (t, *J* = 6 Hz, 1H), 10.18 (br s, 1H). ¹³C NMR (DMSO-*d*₆): δ 20.4, 25.5, 29.7, 33.6, 41.8, 49.6, 111.2, 115.9, 116.2, 118.4, 130.9, 148.9, 160.1, 166.5, 169.0, 170.5, 171.7. HRMS (ES⁺) calcd for C₁₉H₁₉N₃O₈Na: 440.1070. Obsd: 440.1068.

Preparation of SHA-X-Sepharose. Collected EAH Sepharose 4B (50 mL slurry) by vacuum filtration in a Buchner funnel fitted with filter paper (Whatman #1) and washed with 100 mL of deionized water followed by 100 mL of 0.1 M NaHCO₃ buffer, pH 9.8. The moist cake was suspended in 12.5 mL of 0.1 M NaHCO₃ buffer, pH 9.8, and transferred into a 50 mL wide-mouth screw cap plastic bottle. A total of 1.252 g (3.0 mmol) of **10** was dissolved in 3 mL of dry DMF and added to the reaction bottle. The bottle was rotated for 1 h at room temperature. Sepharose was collected on a Buchner funnel and washed with 200 mL of deionized water. The moist cake was transferred to a 200 mL wide-mouth screw cap plastic bottle. A total of 8.686 g (0.125 mmol) of hydroxylamine hydrochloride in 12.5 mL of 1 M NaHCO₃, pH 10.0, was added to 100 mL of deionized water. The pH of hydroxylamine solution was adjusted to 10.0 by

dropwise addition of 10 N NaOH, and then the final volume of the solution was adjusted to 125 mL by adding deionized water. A total of 125 mL of freshly prepared hydroxylamine solution was added to the reaction bottle and the bottle rotated for 16 h at room temperature. Finally, SHA-X-Sepharose was collected on a Buchner funnel, washed with 250 mL of deionized water, and stored as a slurry in 20% (v/v) ethanol/water.

Determined SHA concentration of modified Sepharose by first suspending 1 mL of gel in 2 mL of 50% (v/v) glycerol/water, removing a 0.5 mL aliquot of the suspension, and further diluting with 2.5 mL of 50% (v/v) glycerol/water. Finally, the SHA concentration was estimated by comparing the absorbance of the suspension of SHA-modified gel at 320 nm ($\epsilon_{320} = 4900$) to that of unmodified EAH Sepharose 4B.

Enzymatic Activity of PBA- and PDBA-AP Conjugates. Alkaline phosphatase (AP, 50 000 units; Sigma) was dialyzed in a Slide-A-Lyzer (3500 MWCO, Pierce) against 2 L of 0.1 M NaHCO₃, pH 8.3, for 48 h at 4 °C. Determined concentration of AP dialysate by measuring the absorbance at 280 nm ($\epsilon_{280} = 107\,500$). Conjugated six aliquots of AP dialysate (~4.3 mg/mL) by adding a 5-, 10-, or 50-fold molar excess of either PBA-XX-NHS (100 mM stock solution in DMF) or PDBA-X-NHS (100 mM stock solution in DMF). Reactions were incubated on ice for 1.5 h, and conjugates were transferred to Slide-A-Lyzers (3500 MWCO, Pierce) and dialyzed against 2 L of 0.1 M NaHCO₃, pH 8.3, at 4 °C over 48 h. Incorporation ratios (to within 0.5:1) were estimated by comparing the ratio of the absorbance at 260 nm ($\epsilon_{260} = 4500$ for PBA and $\epsilon_{260} = 4000$ for PDBA) to the absorbance at 280 nm. Enzymatic activity was assayed with 4-nitrophenyl phosphate (pNPP) as substrate. A stock solution of pNPP was prepared by dissolving 15 mg of pNPP in 15 mL of DEA buffer (1 M diethanolamine, 1 mM MgCl₂, 0.1 mM ZnSO₄, pH 10.2). Diluted 5 aliquots (100 μ L) of each of the six conjugates to 0.05 μ g/mL with TBS buffer (10 mM tris(hydroxymethyl)aminomethane hydrochloride, 140 mM NaCl, pH 8.0), in a 96-well plate, and assayed for AP activity by adding 100 μ L of pNPP stock solution to each well and incubating for 10 min at 37 °C. Enzymatic activity was determined of conjugates relative to unmodified AP from the average absorbance at 405 nm obtained from each of five aliquots. A_{405} values were determined on a Bio-Tek Instruments EL_X808 plate reader.

Immobilization of PBA- and PDBA-AP Conjugates on SHA-X-Sepharose. Each of six SHA-X-Sepharose columns (0.5 mL in 0.5 $\times$ 15 cm column; 5.4 μ mol of SHA/mL of gel) were equilibrated with 50 mL of 0.1 M NaHCO₃, pH 8.3. Aliquots of 0.5 mL of each of the conjugates prepared above were diluted to 5 mL total volume with 0.1 M NaHCO₃, pH 8.3. Loaded conjugates on SHA-X-Sepharose columns by simple gravity-flow, and washed columns with 10 mL of 0.1 M NaHCO₃, pH 8.3 (a sufficient quantity of buffer for the A_{280} to return to baseline). Determined the quantity of conjugate immobilized on each column by subtracting the quantity of conjugate in each of the eluents (as determined by the absorbance at 280 nm) from the quantity of conjugate loaded onto each column.

Affinity Chromatography of α AP on PBA-AP-SHA-X-Sepharose and PDBA-AP-SHA-X-Sepharose. Reconstituted 25 mg of rabbit *anti*-alkaline phosphatase Ab (α AP, Rockland) in 5 mL of water and dialyzed in a Slide-A-Lyzer (3500 MWCO; Pierce) against 2 L of 0.1 M NaHCO₃, pH 8.3, for 48 h at 4 °C. Aliquots of 4 mg of α AP were diluted with 0.1 M NaHCO₃, pH 8.3, to a final volume of 5 mL and loaded by simple

gravity-flow onto each of the six columns prepared as described above containing 0.5 mL of either PBA-AP-SHA-X-Sepharose or PDBA-AP-SHA-X-Sepharose. Columns were washed with 15 mL of 0.1 M NaHCO₃, pH 8.3, collecting the first 10 mL fraction of each eluent. Determined protein concentration in each of the fractions from the absorbance at 280 nm ($\epsilon_{280} = 210\,000$) and calculated the quantity of α AP retained on each column by subtracting the quantity of α AP in each of the fractions from the quantity loaded onto each column. Initially, α AP was eluted from each column with 15 mL of 50 mM phosphate buffer, pH 11.0 (high pH eluent), collecting a 10 mL fraction into a tube containing 1 mL of 1 M phosphate buffer, pH 5.75. Subsequently, additional α AP was eluted from each column with 15 mL of 100 mM glycine hydrochloride buffer, pH 2.5 (low pH eluent), collecting a 10 mL fraction. Low pH of the eluent fraction was adjusted to pH 8–9 by addition of 1 drop of 10 N NaOH. Determined protein concentration in each of the fractions from the absorbance at 280 nm and calculated recovery of α AP.

High pH eluent fractions and low pH eluent fractions for affinity purified α AP were analyzed by SDS-PAGE on 7.5% gels according to Laemmli (7). Aliquots of 25 μ L of each fraction were diluted with 25 μ L of reducing application buffer [63 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS; 0.05% (v/v) 2-mercaptoethanol, and 0.001% (w/v) bromophenol blue] and heated for 3 min at 95 °C prior to loading 10 μ L/lane. PAGE was run at 200 V until bromophenol blue reached the bottom of the plate. Visualized proteins by staining gel with Silver stain (Bio-Rad).

Immobilization of AP on SHA-X-Sepharose, Affi-Gel 10, and Affi-Gel 15. Six alkaline phosphatase samples (300 000 units each, Sigma) were dialyzed in six Slide-A-Lyzers (3500 MWCO; Pierce) against 2 L of 0.1 M NaHCO₃, pH 8.3, over 48 h at 4 °C. The concentration of each AP dialysate was determined from the absorbance at 280 nm ($\epsilon_{280} = 107\,500$). A total of 2.0 mg of AP dialysate was conjugated by adding a 15-fold molar excess of PDBA-X-NHS (100 mM stock solution in DMF). The reaction was incubated on ice for 1.5 h, and then the conjugate was dialyzed in a Slide-A-Lyzer (3500 MWCO; Pierce) against 2 L of 0.1 M NaHCO₃, pH 8.3, over 24 h at 4 °C. The extent of PDBA incorporation was determined from the ratio of the absorbance at 260 nm ($\epsilon_{260} = 4000$) to the absorbance at 280 nm. A SHA-X-Sepharose column (0.25 mL in 0.5 $\times$ 15 cm column; 5.3 μ mol of SHA/mL of gel) was equilibrated with 50 mL of 0.1 M NaHCO₃, pH 8.3. PDBA-AP conjugate was loaded on the column by simple gravity-flow, the column was washed with 50 mL of 0.1 M NaHCO₃, pH 8.3, and the first 10 mL fraction of eluent was collected. Protein concentration in the eluent was determined from the absorbance at 280 nm ($\epsilon_{280} = 107\,500$), and the quantity of PDBA-AP immobilized on the column was calculated by subtracting the quantity of PDBA-AP in the eluent from the quantity loaded onto the column.

A total of 0.25 mL of Affi-Gel 10 (Bio-Rad) and 0.25 mL of Affi-Gel 15 (Bio-Rad) were transferred to each of two 1.5 mL micro test tubes. Gels were washed three times each by vortexing with 1 mL of cold deionized water, centrifuging at low speed to pellet the gel, and then removing the supernatant. A total of 2.0 mg of AP dialysate was added to each of the gel suspensions. Rotated Affi-Gel 10 suspension for 2 h at room temperature and Affi-Gel 15 suspension for 4 h at 4 °C. A total of 0.25 mL of a 1 M solution of glycine ethyl ester was

added, pH 8.0, to each of the gel suspensions to consume excess reactive sites and rotated for 1 h at room temperature. Gel suspensions were transferred to 0.5×15 cm columns and washed with 50 mL of 0.1 M NaHCO_3 , pH 8.3, collecting the first 10 mL fraction from each column. Determined the protein concentrations of the two fractions from the absorbance at 280 nm ($\epsilon_{280} = 107\,500$), and calculated the quantity of AP immobilized on each of the columns by subtracting the quantity of AP in each of the fractions from the quantity loaded onto each of the columns.

Affinity Chromatography of α AP on PDBA-AP-SHA-X-Sepharose, AP-Affi-Gel 10, and AP-Affi-Gel 15. A total of 50 mg of rabbit *anti*-alkaline phosphatase Ab (α AP, Rockland) was reconstituted in 5 mL of deionized water and dialyzed in a Slide-A-Lyzer against 2 L of 0.1 M NaHCO_3 , pH 8.3, for 48 h at 4 °C. Aliquots of 2.0 mg of α AP dialysate were diluted with 0.1 M NaHCO_3 , pH 8.3, to a final volume of 5 mL and loaded by simple gravity-flow onto each of the three 0.5×15 cm columns prepared as described above, containing 0.25 mL of either PDBA-AP-SHA-X-Sepharose (5.3 μ mol of SHA/mL of gel), AP-Affi-Gel 10, or AP-Affi-Gel 15. The columns were eluted by gravity-flow sequentially applying 15 mL of 0.1 M NaHCO_3 , pH 8.3, 15 mL of 50 mM sodium phosphate buffer, pH 11.0 (high pH eluent) and 15 mL of 100 mM glycine hydrochloride buffer, pH 2.5 (low pH eluent). The first 10 mL fraction of the high pH eluent and low pH eluent was collected from each of the columns. The protein concentration of each of the fractions was determined from the absorbance at 280 nm ($\epsilon_{280} = 210\,000$) and calculated the recovery of α AP associated with each fraction by subtracting the quantity of protein in each fraction from the quantity loaded onto the corresponding column. Analyzed high pH fractions and low pH fractions for affinity purified α AP by SDS-PAGE as described above.

Purification of Human IgG on PDBA- α Human IgG-SHA-X-Sepharose. A total of 10.0 mg of goat *anti*-human IgG (α Human IgG, Jackson) was dialyzed in a Slide-A-Lyzer (3500 MWCO; Pierce) overnight against 2 L of 0.1 M NaHCO_3 , pH 8.3, at 4 °C. The protein concentration of dialysate was determined from the absorbance at 280 nm ($\epsilon_{280} = 210\,000$). A total of 8.0 mg of α Human IgG dialysate was conjugated by adding a 10-fold molar excess of PDBA-X-NHS (100 mM stock solution in DMF). The reaction was incubated on ice for 1 h, and then the conjugate was desalted on a Sephadex G25 column (13 cm $\times$ 1.5 cm) eluted with 0.1 M NaHCO_3 , pH 8.3. The column effluent was monitored at 280 nm, and the PDBA- α Human IgG fraction was collected. The extent of PDBA incorporation was determined from the ratio of the absorbance at 260 nm ($\epsilon_{260} = 4000$) to the absorbance at 280 nm. Approximately 1.5 mg of PDBA- α Human IgG was diluted to a total volume of 5.0 mL with 0.1 M NaHCO_3 , pH 8.3, and loaded onto a SHA-X-Sepharose column (0.25 mL in 0.5×15 cm column; 7.1 μ mol of SHA/mL of gel). The column was washed with 10 mL of 100 mM glycine hydrochloride buffer, pH 2.5, to remove PDBA- α Human IgG which was unstable with respect to the low pH eluent and then equilibrated column with 50 mL of 0.1 M NaHCO_3 , pH 8.3. A total of 25.0 mg of ChromPure proteins (Jackson) was reconstituted in 10 mL of 0.1 M NaHCO_3 , pH 8.3, and then loaded by simple gravity-flow onto a PDBA- α Human IgG-SHA-X-Sepharose column. Human IgG was eluted with 15 mL of 50 mM phosphate buffer, pH 11.0, collecting a 10 mL fraction into a tube containing 1 mL of 1 M phosphate buffer, pH 5.75. Additional Human IgG was eluted with

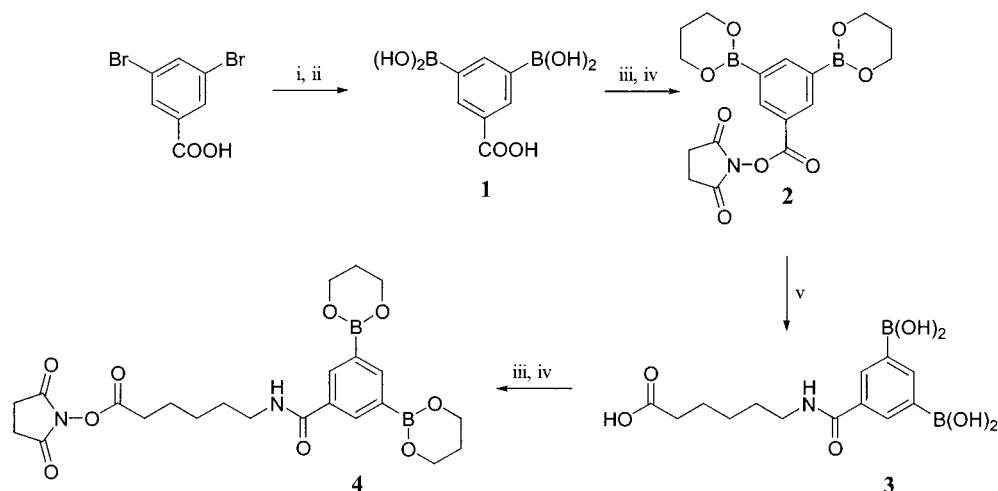
15 mL of 100 mM glycine hydrochloride buffer, pH 2.5, collecting a 10 mL fraction. The pH of the low pH eluent fraction was adjusted to pH 8–9 by addition of 1 drop of 10 N NaOH. The protein concentration of each fraction was determined from the absorbance at 280 nm ($\epsilon_{280} = 210\,000$), and the recovery of Human IgG was calculated by subtracting the quantity of protein in each fraction from the quantity loaded onto the column.

Fractions for Human IgG were analyzed by SDS-PAGE on 12.5% gels according to Laemmli (7). Aliquots of 25 μ L of each fraction were diluted with 25 μ L of reducing application buffer and heated for 3 min at 95 °C prior to loading 10 μ L/lane. PAGE was run at 200 V until bromophenol blue reached the bottom of the plate. Proteins were transferred onto ProBlott membranes (Applied Biosystems) with a Mini Trans-Blot Cell (Bio-Rad) by electroblotting at 50 V for 2 h at room temperature in 10 mM CAPS buffer, pH 11, containing 10% methanol. Nonspecific binding to ProBlott membranes was blocked by incubating overnight at room temperature in freshly prepared TBS buffer [10 mM tris(hydroxymethyl)aminomethane hydrochloride, 140 mM NaCl, pH 7.4], containing 5% (w/v) nonfat dried milk followed by washing three times for 5 min with TBST (TBS containing 0.5% (v/v) Tween 20). Membranes were incubated with either monoclonal *anti*-human IgG-alkaline phosphatase conjugate (1:50000 dilution, Sigma) in TBST or rabbit *anti*-goat IgG-alkaline phosphatase conjugate (1:50000 dilution, Sigma) in TBST for 2 h at room temperature with constant agitation. Membranes were washed three times for 5 min with TBST and developed by incubating with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT FAST Tablets, Sigma). Finally, after sufficient color developed, membranes were rinsed briefly in methanol and dried.

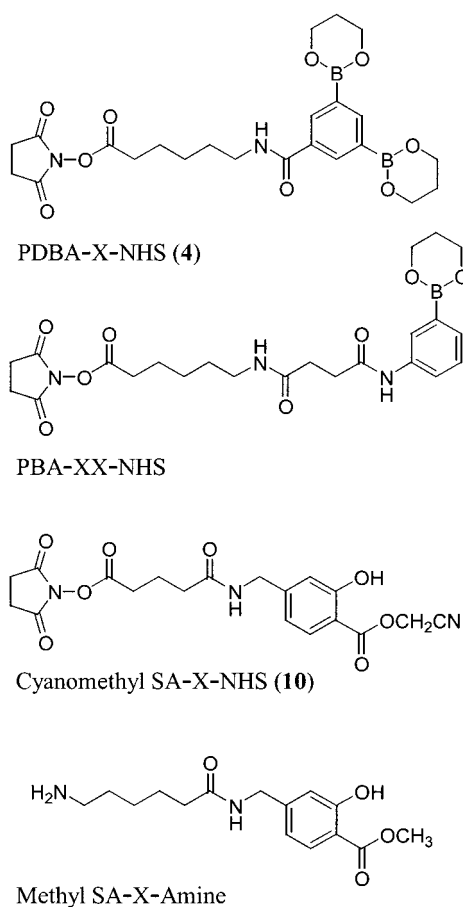
RESULTS AND DISCUSSION

The structures of the target reagents 2,5-dioxypyrrolidinyl 6-[[3,5-di(1,3,2-dioxaboran-2-yl)phenyl]carbonylamino]hexanoate (**4**, PDBA-X-NHS) and 2,5-dioxypyrrolidinyl 4-[*N*-({4-[(cyanomethyl)oxycarbonyl]-3-hydroxyphenyl}methyl)carbamoyl]butanoate (**10**, cyanomethyl SA-X-NHS) are illustrated in Figure 1. Also illustrated, for comparison, are the related reagents 2,5-dioxypyrrolidinyl 6-{3-[*N*-(3-(1,3,2-dioxaboran-2-yl)phenyl)carbamoyl]propanoylamino}hexanoate (PBA-XX-NHS) and methyl 4-[(6-aminohexanoylamino)methyl]salicylate hydrochloride (methyl SA-X-Amine) which were previously prepared (1). The structures of the chromatographic supports SHA-X-Sepharose (**12**) and SHA-Sepharose are illustrated in Figure 2. The results that follow compare the performance of the reagents and chromatographic supports described herein to those previously prepared with respect to the immobilization of protein ligands for affinity chromatography.

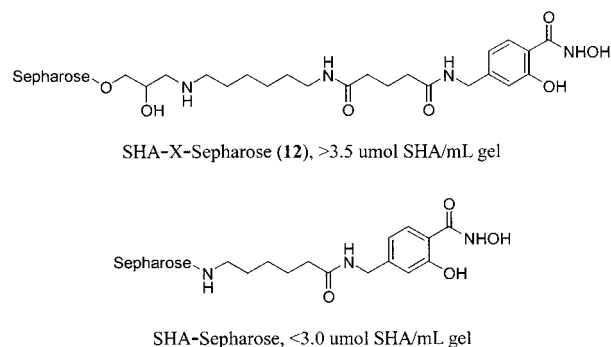
Syntheses of Reagents. Reagent **4** was prepared by the synthesis outlined Scheme 1. The starting material, 3,5-dibromobenzoic acid, was sequentially reacted with methyllithium, *tert*-butyllithium, and triisopropylborate in THF to afford 3,5-di(dihydroxyboryl)benzoic acid (**1**) in 60% yield. Protection of the boronic acid moieties of **1** with 1,3-propanediol in 1,4-dioxane and subsequent reaction with *N*-hydroxysuccinimide and DCC next produced the intermediate NHS ester **2**. Reaction of **2** with 6-aminohexanoic acid and diisopropylethylamine in methanol produced the long-chain carboxylic acid **3** in 73% yield. Finally, protection of the boronic acid moieties of **3** with 1,3-propanediol in 1,4-dioxane and subsequent

Scheme 1. Synthesis of 2,5-Dioxopyrrolidinyl 6-[[3,5-Di(1,3,2-dioxaboran-2-yl)phenyl]carbonylamino]hexanoate (4, PDBA-X-NHS)^a

^a (i) Methyllithium, *tert*-butyllithium, and triisopropylborate in THF; (ii) aqueous HCl (pH <4); (iii) 1,3-propanediol in 1,4-dioxane; (iv) *N*-hydroxysuccinimide and DCC in 1,4-dioxane; and (v) 6-aminohexanoic acid and diisopropylamine in methanol.

**Figure 1.** Reagents utilized to prepare PDBA conjugates (PDBA-X-NHS), PBA conjugates (PBA-XX-NHS), SHA-X-Sepharose (cyanomethyl SA-X-NHS), and SHA-Sepharose (methyl SA-X-amine).

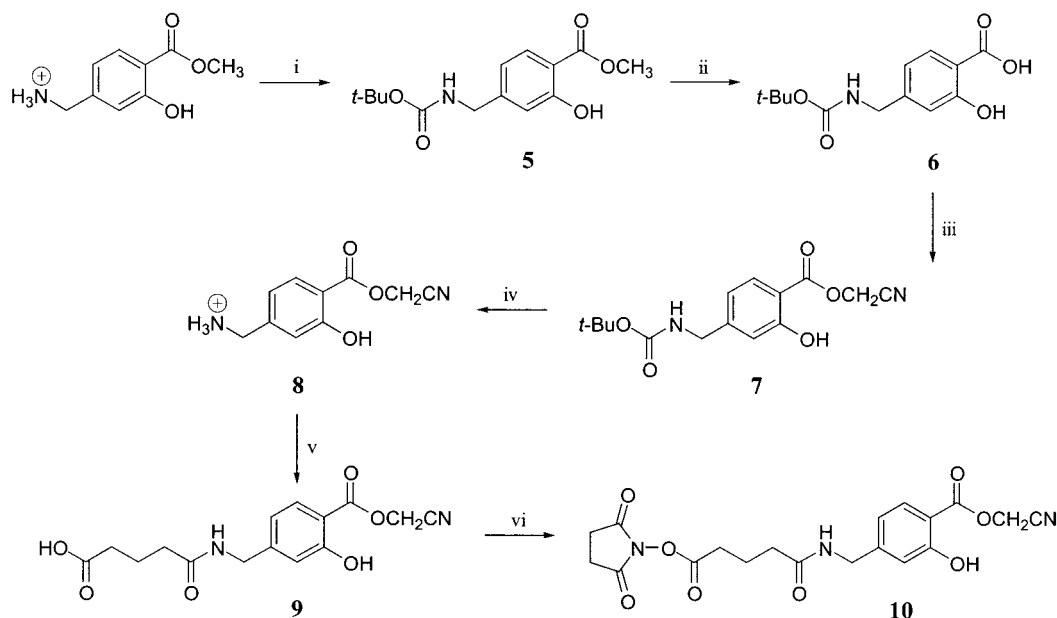
reaction with *N*-hydroxysuccinimide in the presence of DCC afforded the target *N*-hydroxysuccinimidyl ester **4** in 74% yield. Reagent **10** was prepared by the synthesis outlined in Scheme 2. The starting material, methyl 4-aminomethylsalicylate hydrochloride was prepared as described in the previous paper in this issue (*1*) and protected by reaction with di-*tert*-butyl dicarbonate and Et₃N in anhydrous methanol to produce the *tert*-butyl-carbamate (**5**) in 97% yield. Reaction of **5** with potassium

**Figure 2.** Salicylhydroxamic acid-modified Sepharose for immobilization of protein ligands for affinity chromatography.

trimethylsilanolate in THF next produced the salicylic acid (**6**) in 81% yield. Reaction of **6** with chloroacetonitrile in the presence of Et₃N produced the cyanomethyl salicylate (**7**) in 88% yield. Deprotection of **7** with hydrogen chloride gas in THF next produced the amine (**8**) as the hydrochloride salt in 91% yield. Reaction of **8** with glutaric anhydride in the presence of Et₃N in DCM produced the long-chain acid **9** in 89% yield. Finally, reaction of **9** with *N*-hydroxysuccinimide and DCC in THF afforded the target *N*-hydroxysuccinimidyl ester (**10**) in 80% yield.

SHA-X-Sepharose. The preparation of SHA-X-Sepharose is outlined in Figure 3. A suspension of EAH Sepharose 4B in bicarbonate buffer, pH 9.8, was reacted with **10** to produce the immobilized synthetic intermediate **11**, which was subsequently reacted with excess aqueous hydroxylamine, pH 10.0, to produce SHA-X-Sepharose (**12**). The SHA concentration of the modified Sepharose was estimated from the absorbance at 260 nm. Unfortunately, the analytical method does not differentiate among immobilized cyanomethyl salicylate intermediate (**11**), immobilized salicylic acid (potentially resulting from alkaline hydrolysis of cyanomethyl salicylate), and immobilized SHA (**12**). Consequently, some percentage of the estimated SHA concentration may not be available for PBA-SHA complex formation.

The preparation of SHA-X-Sepharose from EAH Sepharose 4B by reaction with **10** affords an affinity chromatographic support with an average of from 3.5 to 7.0 μ mol of SHA/mL of gel, whereas, the preparation of SHA-Sepharose from CNBr-activated Sepharose 4B by

Scheme 2. Synthesis of 2,5-Dioxypyrrolidinyl 4-[N-({4-[(Cyanomethyl)oxycarbonyl]-3-hydroxyphenyl}methyl)-carbamoyl]butanoate (10, cyanomethyl SA-X-NHS)^a

^a (i) Di-*tert*-butyl dicarbonate and TEA in methanol; (ii) potassium trimethylsilanolate in THF; (iii) chloroacetonitrile and TEA; (iv) anhydrous HCl gas in THF; (v) glutaric anhydride and TEA in DCM; and (vi) *N*-hydroxysuccinimide and DCC in THF.

Table 1. Incorporation Ratios and Enzymatic Activity of Alkaline Phosphatase Conjugates Prepared from Either PBA-XX-NHS or PDBA-X-NHS

reagent	input ratio ^a	incorporation ratio ^b	enzymatic activity (%) ^c
PBA-XX-NHS	5:1	4:1	61
PBA-XX-NHS	10:1	6:1	47
PBA-XX-NHS	50:1	18.5:1	18
PDBA-X-NHS	5:1	3:1	54
PDBA-X-NHS	10:1	4.5:1	44
PDBA-X-NHS	50:1	11.5:1	29

^a Input ratio is defined as the ratio of moles of reagent introduced per mole of protein modified. ^b Incorporation ratio is defined as the ratio of moles of reagent incorporated per mole of protein modified. ^c Alkaline phosphatase activity of conjugates determined with *p*-nitrophenyl phosphate substrate relative to unconjugated alkaline phosphatase and reported as the average value obtained for five determinations.

reaction 12 affords an affinity chromatographic support with an average of from 1.5 to 3.0 μmol of SHA/mL of gel. In neither instance does the analytical method differentiate between the immobilized synthetic intermediates and salicylhydroxamic acid itself. Consequently, in neither instance is it possible to exactly determine the extent to which the intermediates are amidated. The cyanomethyl ester was selected to supersede the methyl ester as an immobilized intermediate due to the greater reactivity exhibited by cyanomethyl esters with respect to amidation by α -nucleophiles such as hydroxylamine as well as their resistance toward alkaline hydrolysis (8–12).

Enzymatic Activity of PBA- and PDBA-AP Conjugates. An alkaline phosphatase known to be particularly sensitive with respect to conjugation was intentionally selected to investigate the impact of either PBA or PDBA conjugation on biological activity in a model system. Table 1 summarizes the incorporation ratios obtained (moles of reagent incorporated per mole of protein modified) corresponding to each molar excess of reagent introduced as well as the enzymatic activity associated with each of the conjugates. At identical input ratios PBA-XX-NHS affords somewhat higher incorpora-

tion ratios than PDBA-X-NHS. At similar incorporation ratios the PBA moiety appears somewhat less detrimental than the PDBA moiety with respect to retention of enzymatic activity. In neither instance is the difference particularly pronounced. These observations may be attributable to the longer spacer associated with the PBA reagent, which is 12 atoms in length, as compared to the PDBA reagent, which is only 8 atoms in length (see Figure 1).

Immobilization of PBA-AP and PDBA-AP Conjugates on SHA-X-Sepharose. Table 2 summarizes the immobilization efficiency of alkaline phosphatase conjugates on SHA-X-Sepharose. Immobilization of PDBA-AP on SHA-X-Sepharose by boronic acid complex formation is illustrated in Figure 3. In the previous paper in this issue, we concluded that incorporation of a minimum of six PBA moieties was necessary to ensure efficient immobilization and subsequent stability of the immobilized species with respect to both alkaline and acidic washes (1). For PBA, the results reported here support that conclusion. However, incorporation of only three PDBA moieties affords performance comparable to that of six PBA moieties. Since each PDBA moiety is comprised of two boronic acids, this observation suggests that six boronic acids, either in the form of three PDBA moieties or six PBA moieties remains the requisite number of boronic acids to ensure ideal conjugate performance. In all probability, this conclusion is somewhat dependent upon the SHA concentration of the chromatographic support (see below). The question arises as to how many of the boronic acids incorporated into a conjugate are complexed, on average, with immobilized SHA. Since it is unlikely that both of the boronic acids associated with a single PDBA moiety are simultaneously complexed with SHA as model complexes of the type $\text{PDBA} \cdot (\text{SHA})_2$ have not been detected by either ^{11}B NMR or mass spectrometry, and since a PDBA conjugate with an incorporation ratio of only 3:1 affords efficient immobilization and subsequent stability, we conclude that on average either two or three of the boronic acid moieties associated with the conjugate must be simultaneously

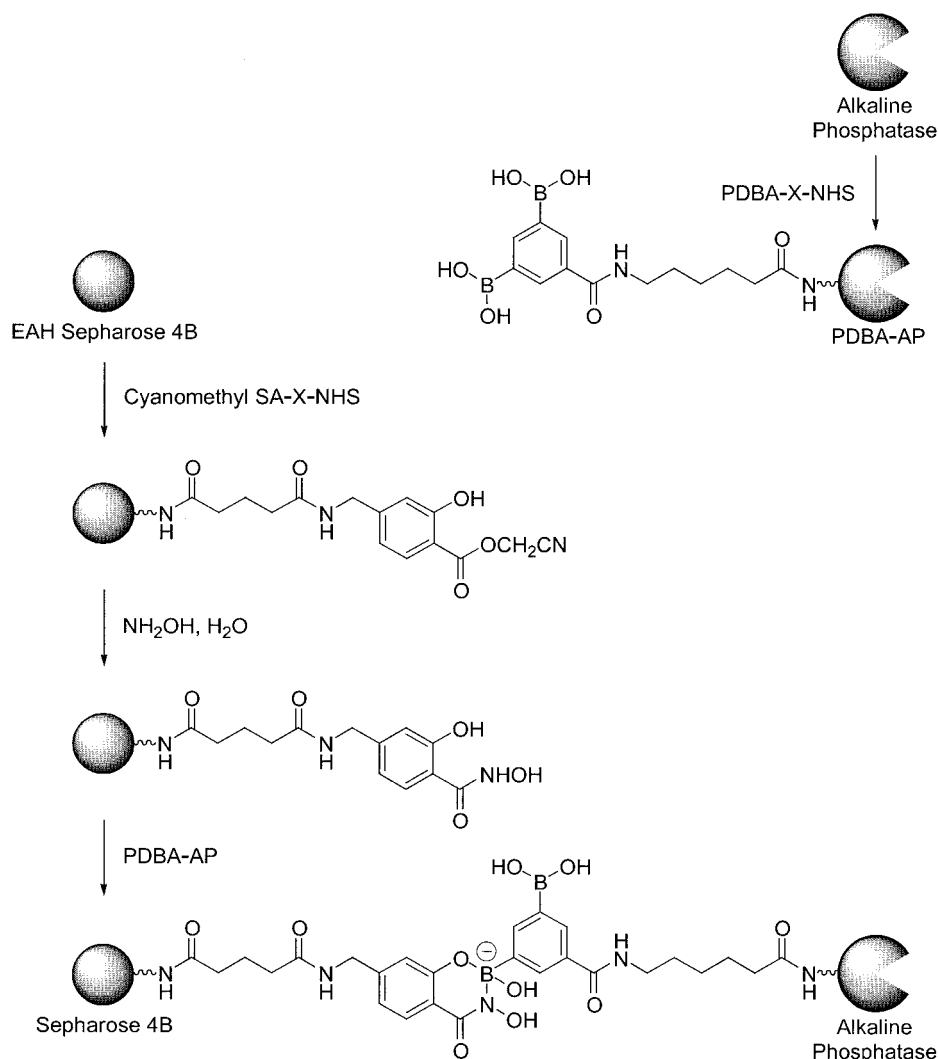


Figure 3. Preparation of SHA-X-Sepharose and AP-PDBA·SHA-X-Sepharose. EAH Sepharose 4B is reacted with cyanomethyl SA-X-NHS (**10**) under alkaline aqueous conditions to afford a synthetic intermediate (**11**). Reaction of **11** with excess alkaline aqueous hydroxylamine affords SHA-X-Sepharose (**12**). PDBA-AP, prepared by conjugation of the lysine amino acid side-chains of alkaline phosphatase, is immobilized on SHA-X-Sepharose by formation of the PBA·SHA complex. The illustration may represent an oversimplification in that indirect evidence suggests that immobilization results from a polyvalent interaction involving at a minimum formation of either two or three PBA·SHA complexes.

Table 2. Immobilization of PBA-AP and PDBA-AP on SHA-X-Sepharose and Affinity Chromatography of anti-Alkaline Phosphatase on PBA-AP·SHA-X-Sepharose and PDBA-AP·SHA-X-Sepharose

AP conjugate (incorporation ratio ^a)	AP immobilized (mg) ^b	αAP retained (mg) ^c	pH 11 αAP recovered (mg) ^d	pH 2.5 αAP recovered (mg) ^e	total αAP recovered (mg)
PBA (4:1)	1.76 (88%)	1.42	0.93 (66%) ^f	0.43 (30%) ^f	1.36 (96%) ^f
PBA (6:1)	1.81 (90%)	1.42	0.68 (48%) ^f	0.27 (19%) ^f	0.95 (67%) ^f
PBA (18.5:1)	1.94 (97%)	1.22	0.38 (31%)	0.11 (9%)	0.49 (40%)
PDBA (3:1)	(>95%)	1.40	0.42 (30%)	0.43 (31%)	0.85 (61%)
PDBA (4.5:1)	(>95%)	1.37	0.38 (28%)	0.15 (11%)	0.53 (39%)
PDBA (11.5:1)	(>95%)	1.03	0.31 (30%)	0.08 (8%)	0.39 (38%)

^a Incorporation ratio is defined as the ratio of moles of reagent incorporated per mole of protein modified. ^b AP conjugates (2.0 mg) in 5 mL of 100 mM sodium bicarbonate buffer, pH 8.3, were immobilized on 0.5 mL of SHA-X-Sepharose (5.5 μmol of SHA/mL of gel). ^c Crude αAP (5 mg) in 5 mL of 100 mM sodium bicarbonate buffer, pH 8.3, was applied to either PBA-AP·SHA-X-Sepharose or PDBA-AP·SHA-X-Sepharose. ^d Recovered in 10 mL of 50 mM sodium phosphate buffer, pH 11.0. ^e Recovered in 10 mL of 100 mM glycine hydrochloride buffer, pH 2.5. ^f SDS-PAGE revealed the presence of contaminating PBA-AP in recovered αAP.

complexed with immobilized SHA. Alternatively, these results could be interpreted to imply at any time only a single boronic acid complex need be present to retain the immobilized species but that as many as six boronic acid moieties may be required to ensure that on average at least one boronic acid complex is always present.

Affinity Chromatography of anti-Alkaline Phosphatase on PBA-AP·SHA-X-Sepharose and PDBA-AP·SHA-X-Sepharose. Results obtained for purification

of αAP on PBA-AP·SHA-X-Sepharose and PDBA-AP·SHA-X-Sepharose are also summarized in Table 2. In every instance, AP columns efficiently retained αAP at pH 8.3, which was subsequently recovered in either an alkaline (pH 11.0) or acidic (pH 2.5) eluent. However, the results involving PBA-AP·SHA-X-Sepharose are misleading in that they represent an overestimate of from about 1 to 10% with respect to protein recovery owing to the presence of trace amounts of contaminating PBA-

Table 3. Affinity Chromatography of *anti*-Alkaline Phosphatase on PDBA-AP-SHA-X-Sepharose, Alkaline Phosphatase Affi-Gel 10 and Alkaline Phosphatase Affi-Gel 15

	AP immobilized (mg) ^a	α AP retained (mg) ^b	pH 11 α AP recovered (mg) ^c	pH 2.5 α AP recovered (mg) ^d	total α AP recovered (mg) ^e
Sepharose ^f	2.9 (73%)	5.2 (24%)	2.5 (48%)	0.72 (14%)	3.22 (62%)
Affi-Gel 10 ^g	1.5 (38%)	3.9 (18%)	0.66 (17%)	0.45 (12%)	1.11 (29%)
Affi-Gel 15 ^h	1.0 (25%)	1.6 (8%)	0.48 (30%)	0.28 (18%)	0.76 (48%)

^a In each instance, 4.0 mg of AP was applied to 0.25 mL of gel. ^b In each instance, 21.6 mg of crude α AP was applied to the column. ^c Recovered in 10 mL of 50 mM phosphate buffer, pH 11.0. ^d Recovered in 10 mL of 100 mM glycine hydrochloride buffer, pH 2.5. ^e Additional α AP can be recovered by increasing the ionic strength of the eluent by adding 200 mM NaCl. ^f PDBA-AP (8.7:1, 4.0 mg) was applied to a column of SHA-X-Sepharose (0.25 mL of 6.24 μ mol of SHA/mL of gel). ^g AP reacted with Affi-Gel 10 for 2 h at room temperature. ^h AP reacted with Affi-Gel 15 for 4 h at 4 °C.

Table 4. Affinity Chromatography of Human IgG on PDBA- α Human IgG-SHA-X-Sepharose

SHA in Sepharose (μ mol/mL of gel)	PDBA- α Human IgG retained (mg) ^a	protein retained (mg) ^b	protein recovered, pH 11.0 (mg) ^c	protein recovered, pH 2.5 (mg) ^d	total protein recovered (mg)
4.1	1.35 (90%)	1.35	0.23 (17%) ^f	0.43 (32%) ^e	0.66 (49%)
4.5	1.41 (94%)	1.37	0.19 (14%) ^f	0.24 (18%) ^e	0.43 (31%)
7.1	1.38 (92%)	0.86	0.26 (30%)	0.16 (19%)	0.42 (49%)

^a PDBA- α Human IgG (1.5 mg) was immobilized on SHA-X-Sepharose (0.25 mL). ^b Large excess of ChromPure proteins (purified whole molecule immunoglobulins from normal Human serum) applied so that the amount retained was proportional to the amount of immobilized PDBA- α Human IgG. ^c Recovered in 10 mL of 50 mM sodium phosphate buffer, pH 11.0. ^d Recovered in 10 mL of 100 mM glycine hydrochloride buffer, pH 2.5. ^e PDBA- α Human IgG detected in Western blot with enzyme-linked detection employing α Goat IgG-AP for detection of (Goat) PDBA- α Human IgG. ^f Trace amounts of PDBA- α Human IgG detected in Western blot with enzyme-linked detection employing α Goat IgG-AP for detection of (goat) PDBA- α Human IgG.

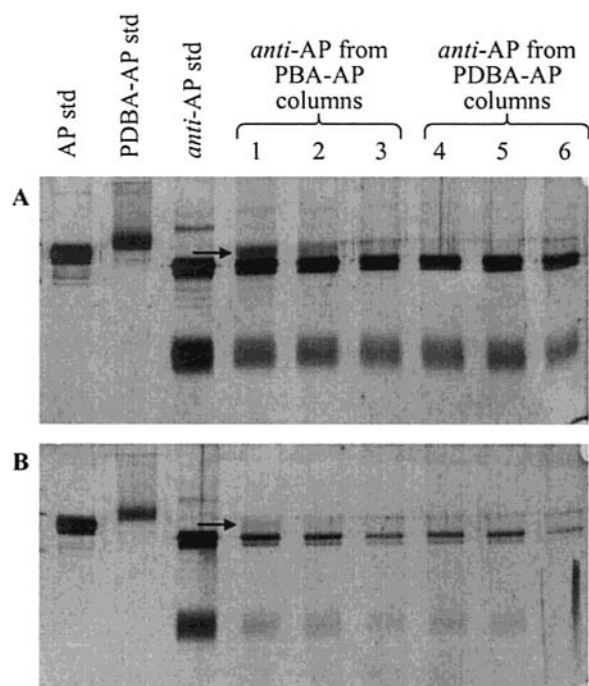


Figure 4. (A) SDS-PAGE of fractions containing α AP recovered from SHA-X-Sepharose in pH 11.0 eluent; (B) SDS-PAGE of fractions containing α AP recovered from SHA-X-Sepharose in pH 2.5 eluent; (1) α AP recovered from PBA-AP (4:1 incorporation ratio) column; (2) α AP recovered from PBA-AP (6:1 incorporation ratio) column; (3) α AP recovered from PBA-AP (18.5:1 incorporation ratio) column; (4) α AP recovered from PDBA-AP (3:1 incorporation ratio) column; (5) α AP recovered from PDBA-AP (4.5:1 incorporation ratio) column; (6) α AP recovered from PDBA-AP (11.5:1 incorporation ratio) column. Note that the arrows indicate the presence of contaminating PBA-AP in fractions recovered from PBA-AP-SHA-X-Sepharose columns. The mobility of PBA and PDBA conjugates is related to the incorporation ratio with low incorporation ratios affording the least reduction in electrophoretic mobility and high incorporation ratios affording the greatest reduction in electrophoretic mobility.

AP that was revealed by SDS-Page. Figure 4 illustrates SDS-PAGE analyses of α AP fractions recovered from both PBA-AP-SHA-X-Sepharose and PDBA-AP-SHA-

X-Sepharose columns at pH 11.0 and 2.5. PBA-AP conjugates with average incorporation ratios of 4:1 and 6:1 were found to be present as contaminants in both high pH and low pH eluent fractions, indicating that some portion of the immobilized conjugate was unstable with respect to the elution conditions. On the other hand, SDS-PAGE revealed that both the high pH and low pH eluent fractions recovered from PDBA-AP-SHA-X-Sepharose columns were essentially free of contaminating conjugate.

Affinity Chromatography of *anti*-Alkaline Phosphatase on PDBA-AP-SHA-X-Sepharose, AP-Affi-Gel 10, and AP-Affi-Gel 15. The performance of PDBA-AP-SHA-X-Sepharose was compared to that of AP-Affi-Gel 10 and AP-Affi-Gel 15 with respect to affinity chromatography of *anti*-alkaline phosphatase so as to compare the approach described herein with covalent immobilization of a protein ligand. Affi-Gel 10 and Affi-Gel 15 are activated immunoaffinity supports for coupling ligands with primary amino groups by reaction with support-bound *N*-hydroxysuccinimidyl esters (7). Similar to Sepharose in many respects, Affi-Gels are prepared from beaded cross-linked agarose and are reported by the manufacturer (Bio-Rad) to have 15 μ mol of *N*-hydroxysuccinimidyl ester/mL of gel. Affi-Gel 10 contains a hydrophilic 10-atom spacer, whereas Affi-Gel 15 contains a hydrophilic 15-atom spacer with a central quaternary amine. Results for affinity chromatography of α AP on immobilized AP are summarized in Table 3. PDBA-AP-SHA-X-Sepharose proved significantly more efficient than both AP-Affi-Gel 10 and AP-Affi-Gel 15 with respect to immobilization of alkaline phosphatase, retention of *anti*-alkaline phosphatase, and recovery of *anti*-alkaline phosphatase at high pH. PDBA-AP-SHA-X-Sepharose proved comparable to both AP-Affi-Gel 10 and AP-Affi-Gel 15 with respect to recovery of *anti*-alkaline phosphatase at low pH. From a practical standpoint, immobilization of PDBA-AP on SHA-X-Sepharose proved far less laborious and time consuming (70 min, including preparation and desalting of PDBA-conjugate) than immobilization of AP on either Affi-Gel 10 (210 min) or Affi-Gel 15 (330 min).

Purification of Human IgG on PDBA- α Human IgG·SHA-X-Sepharose. The purification of Human IgG on PDBA- α Human IgG·SHA-X-Sepharose was employed to investigate the impact of immobilized SHA concentration on the performance of the PDBA·SHA system. Goat *anti*-human IgG was conjugated with PDBA-X-NHS at an input ratio of 10:1 to afford a conjugate (PDBA- α Human IgG) with an average incorporation ratio of only 1.5:1. PDBA- α Human IgG was then immobilized on each of three SHA-X-Sepharose columns with SHA concentrations of 7.1, 4.5, and 4.1 μ mol of SHA/mL of gel. The columns were washed with low pH eluent to remove any loosely complexed conjugate prior to equilibration. A protein fraction from normal human serum comprised primarily of whole molecule immunoglobulins was applied to each of the columns and Human IgG was recovered by eluting the columns with high pH eluent followed by low pH eluent. The results are summarized in Table 4. The fractions were reduced, separated by SDS-PAGE, blotted onto membranes, and analyzed by enzyme-linked immunological detection employing either monoclonal *anti*-human IgG-alkaline phosphatase conjugate (α Human IgG-AP) for detection of human IgG or rabbit *anti*-goat IgG-alkaline phosphatase conjugate (α Goat IgG-AP) for detection of PDBA- α Human IgG (Figure 5). The Western blot detected with *anti*-goat IgG-AP confirmed the immunoreactivity of the conjugate with respect to PDBA- α Human IgG and revealed the presence of trace amounts of PDBA- α Human IgG in the low pH fractions obtained from each of the columns (although PDBA- α Human IgG is barely visible in the low pH fraction recovered from the SHA-X-Sepharose column having 7.1 μ mol of SHA/mL of gel). Qualitatively, the amount of contaminating conjugate detected appears to be inversely proportional to the SHA concentration of the column. Owing to the low incorporation ratio of the PDBA- α Human IgG conjugate (only 1.5:1) it is somewhat remarkable that more contaminating conjugate was not detected.

We concluded above that for efficient immobilization and subsequent stability with respect to both high pH and low pH eluents, either two or three of the boronic acid moieties associated with PDBA conjugates must be available for simultaneous complex formation with immobilized SHA. The results described immediately above imply that, in this instance, only two boronic acid moieties need be available for complex formation with immobilized SHA to confer stability when the effective SHA concentration is relatively high. Since the reported incorporation ratio (1.5:1) represents an average value (implying on average three boronic acid moieties per α Human IgG), the contaminating conjugate found in the low pH fractions may have resulted from that fraction of the conjugates having only a single PDBA moiety.

It is interesting to note that empirical studies involving the purification of recombinant C-terminal polyhistidine fusion proteins on immobilized metal ion affinity chromatography columns indicate that six is the requisite number of C-terminal histidine residues needed to routinely enable efficient retention of fusion proteins on columns charged with nickel ions. This observation is particularly remarkable in light of the fact that nickel ion complexation is known to involve the participation of only two adjacent histidine residues (12–16).

CONCLUSIONS

Alkaline phosphatase conjugates prepared from either PDBA-X-NHS or PBA-XX-NHS were directly compared

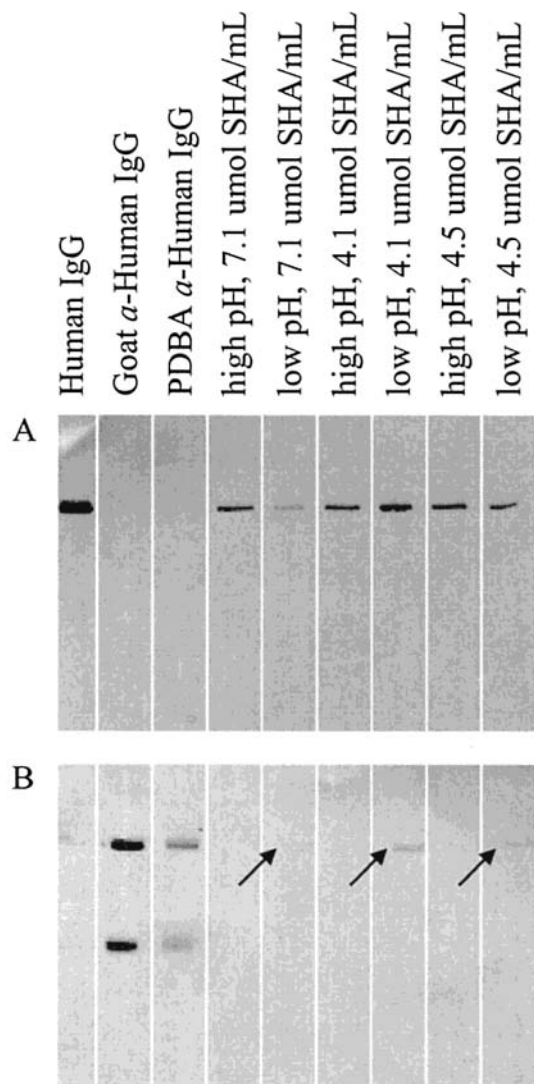


Figure 5. (A) Western blot of Human IgG fractions obtained at high pH (11.0) and low pH (2.5) from PDBA- α Human IgG·SHA-X-Sepharose columns (7.1, 4.1, and 4.5 μ mol of SHA/mL of gel). Blot prepared to detect the presence of residual PDBA- α Human IgG. Stained with *anti*-human IgG-alkaline phosphatase and BCIP/NBT substrate; (B) Western blot of Human IgG fractions obtained at high pH (11.0) and low pH (2.5) from PDBA- α Human IgG·SHA-X-Sepharose columns (7.1, 4.1, and 4.5 μ mol of SHA/mL of gel). Blot prepared to detect the presence of residual PDBA- α Human IgG. Stained with rabbit *anti*-goat IgG-alkaline phosphatase and BCIP/NBT substrate.

with respect to the efficiency with which they were immobilized on SHA-X-Sepharose by PBA·SHA complex formation. For efficient immobilization, it was necessary to prepare conjugates having a minimum of either three appended PDBA moieties or six appended PBA moieties. When immobilized on high capacity SHA-X-Sepharose, alkaline phosphatase conjugates were shown to be stable with respect to both the alkaline (50 mM phosphate buffer, pH 11.0) and acidic (100 mM glycine HCl buffer, pH 2.5) buffers that were utilized to recover *anti*-alkaline phosphatase during affinity chromatography. From indirect evidence we conclude that for efficient immobilization and subsequent stability, on average either two or three boronic acid moieties associated with a protein conjugate must be available for complex formation with immobilized SHA.

Boronic acid complex formation was compared to direct covalent immobilization of alkaline phosphatase on Affi-Gel 10 and Affi-Gel 15 for affinity chromatography of

anti-alkaline phosphatase. PDBA-AP-SHA-X-Sepharose was shown to afford superior performance to both AP-Affi-Gel 10 and AP-Affi-Gel 15 with respect to immobilization of alkaline phosphatase, retention of *anti*-alkaline phosphatase at pH 11.0. Furthermore, immobilization of PDBA-AP on SHA-X-Sepharose proved both less laborious and less time-consuming than immobilization of alkaline phosphatase on either Affi-Gel 10 or Affi-Gel 15.

High capacity SHA-X-Sepharose ($\geq 7 \mu\text{mol}$ of SHA/mL of gel) was shown to afford superior performance to moderate capacity SHA-X-Sepharose (4.1–4.5 μmol of SHA/mL of gel) with respect to immobilized ligand stability at pH 11.0 and pH 2.5 when a PDBA- α Human IgG conjugate with an incorporation ratio of only 1.5:1 was immobilized.

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Cationic Lipid Polymerization as a Novel Approach for Constructing New DNA Delivery Agents

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In vivo gene delivery mediated by cationic lipids is often compromised by aggregation due to complexation with proteins in the blood. To improve the stability of cationic lipid–DNA complexes, the present study aimed to develop a novel approach in which a poly(cationic lipid) (PCL) is utilized to form stable cationic polyplexes for gene transfection. Hydrogenation of the acrylamide analogue of β AE-DMRI, the polymerizable precursor of PCL, provided a monomeric lipid derivative (MHL) which was used for direct comparison of corresponding lipoplex stability, toxicity, and transfection activity. Various formulations of cationic liposomes, such as MHL, MHL-cholesterol (Chol), PCL, PCL-Chol, DOTAP-Chol, and commercially available lipofectamine were generated and examined in this study. The new poly(cationic lipid) did not display any significant toxicity to rat hepatocytes or Hep G₂ cells as indicated by an LDH leakage assay. Furthermore, PCL was significantly less toxic than MHL, DOTAP-Chol or lipofectamine. Suspensions of PCL were resistant to aggregation even after 24 h of exposure to solutions containing 50 and 100% fetal bovine serum (FBS). In contrast, suspensions of lipofectamine extensively aggregated after 24 h of exposure to 50% FBS. To examine the influence of lipid polymerization on gene transfer activity, liposome-mediated transfections of a luciferase vector (pGL₃) were performed in Hep G₂ and Alexander cell lines. The luciferase activity of the PCL formulations in Hep G₂ cells were similar to those of the MHL, DOTAP-Chol and lipofectamine formulations, demonstrating that lipid polymerization does not compromise transfection activity. In comparison to the monomeric precursor MHL and to the industry transfection standards DOTAP and lipofectamine, the novel poly(cationic lipid) exhibited the lowest cytotoxicity, was the most resistant to serum-induced aggregation and had comparable transfection activity when coformulated with cholesterol. This novel polymerization approach for the development of stable and active polyplexes may prove a valuable alternative for in vivo gene delivery.

INTRODUCTION

The prospect for improving nonviral methods of DNA complexation and transfer to mammalian cells continues to motivate researchers in a variety of disciplines. Among the many constructs that have been used to facilitate the intracellular delivery of DNA, cationic lipids and cationic polymers have received the greatest attention (1, 2), and consequently, the technology has since advanced to clinical trials (3, 4). Over the course of the past several years it has become clear that the use of cationic lipid systems for in vivo DNA transfection is compromised by substantial problems that are uniquely dependent on the route of lipid–DNA complex (lipoplex) administration. For example, the systemic administration of lipoplex preparations results in their rapid clearance due to destabilization arising from interactions with serum proteins (5). This obstacle prompted the development of stabilized plasmid lipid particles wherein poly(ethylene glycol) (PEG) chains are conjugated to the surface of lipoplex preparations to provide a measure of steric protection (6). Issues of practicality also point toward the development of more stable lipoplex preparations. For

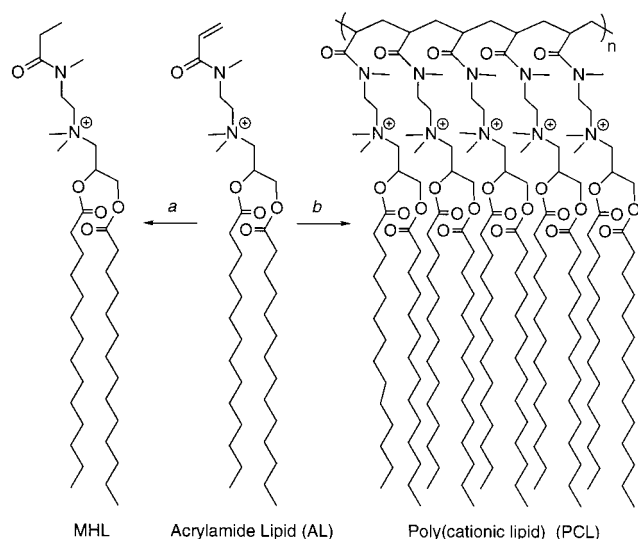
instance, the majority of transfection protocols require that lipoplexes are freshly prepared prior to use to avoid problems associated with lipoplex aggregation (7). Given these and other challenges, we have focused much of our attention on developing new lipid-based constructs to improve lipoplex transfection activity (8, 9).

One approach that has been used to enhance stability and prevent aggregation of lipid-based assemblies is liposome polymerization (10, 11). We have recently developed methods for the polymerization of a novel cationic acrylamide lipid and reconstitution of the resultant poly(lipid) to yield stable cationic vesicles (12). We now report our observations on the ability of this cationic polymer to mediate gene delivery. Although cationic polymers such as poly(L-lysine) (13, 14), poly(ethyleneimine) (15, 16), poly(methacrylate) (17), and polyamidoamine dendrimers (18, 19) have been well studied, to our knowledge the use of a poly(cationic lipid) as a transfection agent remains unexplored. We report herein the first examples of poly(lipid)-mediated DNA transfection and the results of stability and toxicity studies using the new cationic polymer. The attributes of lipid polymerization are defined by comparison of the poly(cationic lipid) (PCL)¹ with lipoplex preparations derived from structurally analogous monomeric cationic lipids.

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Scheme 1^a

^a H₂, 10% Pd/C, EtOAc, room temperature, 6 h. ^b Polymerization: (1) H₂O, dodecylmercaptan (10% w/w), sonication, 50 °C, 20 min; (2) AAPH (0.1 eq), 80 °C, 15 h.

MATERIALS AND METHODS

General Experimental Procedures. CH₂Cl₂ was distilled from CaH₂ prior to use. After reaction workup, solutions were dried using Na₂SO₄ and solvents were subsequently removed by rotary evaporation. NMR spectra were recorded with a General Electric QE-300 spectrometer (¹H at 300 MHz, ¹³C at 75 MHz). Infrared spectra were recorded with a Mattson Genesis II FTIR spectrometer. Melting points are uncorrected. Mass analysis was performed by UCR Mass Spectrometry (Riverside, CA). All sonications were performed in a bath sonicator (Laboratory Supplies Inc., Hicksville, NY).

Lipids. The cationic liposomal formulation lipofectamine (DOSPA-DOPE, 3:1 w/w) was obtained from Gibco Life Technologies (Gaithersburg, MD). *N*-(2,3-(dioleoyloxy)propyl)-*N,N,N*-trimethylammonium chloride (DOTAP) was purchased as a chloroform solution from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol) was purchased from Sigma Chemical Company (St. Louis, MO).

Synthesis of the polymerizable acrylamide lipid (AL) (Scheme 1) proceeded from commercially available 3-bromo-1,2-diol by bis(esterification) with myristoyl chloride (12). Bromide displacement using *N,N'*-dimethylethylenediamine (15 equiv, DMF, 70 °C, 45 min) installed the amine-linker required for subsequent attachment of the polymerizable acrylamide group. Direct treatment of the crude displacement product with acryloyl chloride (1.5 equiv, CH₂Cl₂, room temperature, 2 h) followed by chromatographic purification afforded the corresponding acrylamide lipid. Methyl iodide quaternization and subsequent chloride counterion exchange using an established protocol (20) gave cationic acrylamide lipid AL.

Synthesis of PCL (Scheme 1) was accomplished by first preparing a 5 mL aqueous liposome suspension containing AL (50 mg, 73.0 μmol) and *n*-dodecylmercaptan (5 mg, 25.0 μmol). The lipid mixture was vigorously mixed for 30 s and then sonicated for 20 min at 50 °C to afford

a clear suspension. To the liposome suspension was added 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) (2.0 mg, 7.3 μmol) as a solution in 0.5 mL of H₂O. The resultant mixture was degassed using nitrogen and then placed in a constant-temperature bath heated to 80 °C. After 15 h, the solvent was removed and the polymerized product was analyzed by IR, ¹H, and ¹³C NMR spectroscopy; IR (neat) 2917, 2856, 1747, 1638, 1169 cm⁻¹; ¹H NMR (CDCl₃) δ 0.83 (t, *J* = 6.5 Hz, 6H), 1.25 (m, 40H), 1.57 (m, 4H), 2.32 (m, 4H), 3.39 (m, 3H), 3.53 (m, 6H), 3.87 (m, 4H), 4.10 (m, 2H), 4.31 (m, 1H), 4.53 (m, 1H), 5.66 (m, 1H); ¹³C NMR (CDCl₃) δ 14.0, 22.6, 24.7, 29.0–29.6 (four signals) 31.9, 33.8, 34.1, 36.6, 42.3, 51.7, 62.1, 63.1, 64.0, 65.5, 172.7, 173.1. In all cases examined, the vinyl proton signals of monomer AL (δ 6.57, 6.29, and 5.73 ppm) were absent in the product spectra, suggesting at least a 95% conversion of monomer to polymer. The poly(cationic lipid) was stored at 4 °C without detectable decomposition for periods up to six months.

AL was hydrogenated to obtain a more representative monomer for direct comparison with PCL. A solution of AL (50 mg, 79 μmol) in ethyl acetate (2 mL) at room temperature was treated with 10% palladium on carbon (20 mg). The suspension was placed under an atmosphere of hydrogen and stirred 6 h at which time the suspension was diluted with ethyl acetate and filtered through a short pad of Celite. Removal of the solvent afforded the monomeric hydrogenated lipid (MHL) (46 mg, 92%) as an amorphous white solid, mp = 109–110 °C; TLC, *R*_f = 0.32 (9:1, CH₂Cl₂:MeOH); IR (neat) 2921, 2853, 1747, 1635, 1172 cm⁻¹; ¹H NMR (CDCl₃) δ 0.83 (t, *J* = 6.2 Hz, 6H), 1.06 (t, *J* = 6.7 Hz, 3H), 1.22 (m, 40H), 1.54 (m, 4H), 2.29 (m, 6H), 3.16 (s, 3H), 3.38 (s, 3H), 3.41 (s, 3H), 3.86 (m, 2H), 4.02 (m, 4H), 4.34 (d, *J* = 13.5 Hz, 1H), 4.46 (dd, *J* = 1.5, 13.2 Hz, 1H), 5.57 (m, 1H); ¹³C NMR (CDCl₃) δ 5.0, 13.9, 22.5, 24.7, 25.7, 29.5–29.0 (four signals), 31.8, 33.8, 34.1, 35.5, 42.3, 51.0, 61.5, 63.3, 63.9, 65.8, 171.5, 172.7, 175.0; HRMS (C₃₉H₇₇O₅N₂, M⁺) calcd, 653.5832; found, 653.5865.

Liposome Formulations. Monomer MHL and polymer PCL were hydrated separately using 10 mM Tris-HCl buffer containing NaCl (150 mM) at a concentration of 1 mg/mL (1.5 mM cationic construct). The hydrated lipid suspensions were vigorously mixed and then sonicated for 5 min at 50 °C to afford turbid liposome suspensions. To generate the cholesterol-containing liposome formulations of MHL, PCL, or DOTAP, the lipids were dissolved separately in chloroform and mixed with cholesterol at a 1:1 molar ratio, except for DOTAP, which was mixed with cholesterol in a 3:1 molar ratio. The chloroform was evaporated and the thin lipid films were dried using a stream of nitrogen followed by drying under vacuum for 30 min. The MHL-Chol and DOTAP-Chol mixtures were subsequently hydrated using 10 mM Tris-HCl buffer containing NaCl (150 mM) at a concentration of 1 mg/mL (1.5 mM cationic lipid) and then briefly sonicated at 50 °C. The PCL-Chol mixture was hydrated in identical fashion and at the same concentration, but was sonicated 20 min at 50 °C. Lipofectamine was purchased from Gibco Life Technologies and used as received.

Cytotoxicity Study. Sprague–Dawley rats were purchased from Harlan Laboratory, Inc. (Indianapolis, IN), kept at 12-h light/dark cycles and fed a commercial chow diet. The protocol for rat hepatocyte isolation was approved by the UCD Institutional Animal Use and Care Administrative Advisory Committee. Rat hepatocytes were isolated by two-step collagenase digestion according

¹ Abbreviations: AL, acrylamide lipid; Chol, cholesterol; DOPE, 1- α dioleoyl phosphatidylethanolamine; DOTAP, *N*-(2,3-(dioleoyloxy)propyl)-*N,N,N*-trimethylammonium chloride; FBS, fetal bovine serum; LDH, lactate dehydrogenase; MHL, monomeric hydrogenated lipid; PCL, poly(cationic lipid).

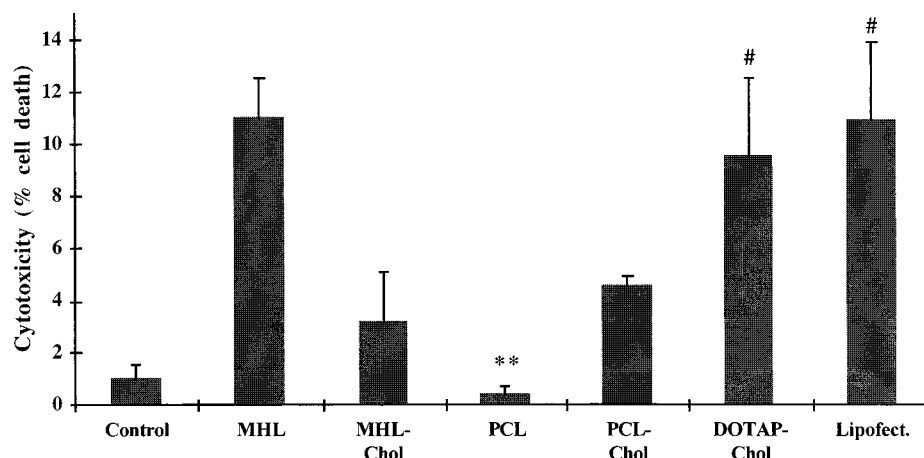


Figure 1. Cytotoxicity of liposomal formulations to isolated rat hepatocytes. Rat hepatocytes were exposed to cationic liposomes (final concentration of lipofectamine = 14 $\mu\text{g}/\text{mL}$, final concentration of all other lipid agents = 20 $\mu\text{g}/\text{mL}$) for 24 h. LDH release from the cells, an indication of cytotoxicity, expressed as percentage of cell death based on the control levels. Each data point represents the mean value of $n = 4$ and the standard deviation from the mean. (**) $p < 0.01$ compared to MHL; (#) $p < 0.05$ compared to PCL. Chol = cholesterol, DOTAP = *N*-(2,3-(dioleoyloxy)propyl)-*N,N,N*-trimethylammonium chloride, LDH = lactate dehydrogenase, Lipofect. = lipofectamine, PCL = poly(cationic-lipid), MHL = monomeric hydrogenated lipid.

to a method reported previously (21) and cultured at 37 °C in 5% CO₂ and air with Williams' medium E with 10% fetal bovine serum (FBS). The cells were cultured overnight and washed with 10 mM PBS, pH 7.4, before being subjected to the cytotoxicity experiments. Hep G₂ cells were cultured in MEM medium with 10% FBS, and the cell passage was performed one or 2 days before analysis in Costar 12-well plates (250 000 cells/well) until the cell culture was 80–90% confluent.

All liposome formulations except lipofectamine were added directly to the cultured rat hepatocytes to reach a final cationic lipid concentration of 20 $\mu\text{g}/\text{mL}$. In the case of lipofectamine, the liposome formulation was added directly to the cultured rat hepatocytes to achieve a final concentration of 14 $\mu\text{g}/\text{mL}$. Experiments in Hep G₂ cells were performed using several concentrations of cationic lipids (10, 20, and 40 $\mu\text{g}/\text{mL}$). The culture medium was sampled 24 h after the cells were exposed to the lipid agents. Lactate dehydrogenase (LDH) leakage from the cells was determined by a commercially available kit from Roche Molecular Biochemicals (Indianapolis, IN), and the cytotoxicity was calculated based on a formula provided by the manufacturer and expressed as percentage of cell death based on the control levels.

Particle Size Determination: Stability Study. The particle sizes of liposome formulations were measured using a laser-based, submicron particle size analyzer from Beckman Coulter, Inc as previously described (22). To test the stability of these liposome suspensions against culture medium containing serum, 200 μL of each liposome suspension was added to 0.5 mL of Williams' medium E containing 10% FBS and then diluted with Tris-HCl buffer to a total volume of 3.5 mL. After equilibration for 2 min, the particle size was measured. To test the stability of MHL, PCL and lipofectamine at higher serum concentrations and longer time intervals, a series of three FBS solutions were prepared containing 10, 50, and 100% FBS by diluting FBS with MEM medium to the appropriate concentration. To these solutions was added a 50–100 μL aliquot of the cationic lipid suspensions. Particle size measurements and size distributions were recorded at 5, 30, 50, 75, and 120 min. A measurement was also taken at 24 h to examine the long-term effect of serum exposure.

Transfection Protocols. Plasmid vector pGL₃-Control which encodes firefly luciferase was purchased from

Promega, Inc. (Madison, WI) and transformed (amplified) into competent *Escherichia coli* cells from Gibco Life Science Technologies (Grand Island, NY). The plasmid DNA was extracted from overnight cultures of the competent cells and purified by affinity chromatography with an Endofree plasmid extraction kit from Qiagen, Inc. (Valencia, CA). The quality of the DNA was determined by UV spectroscopy and agarose gel electrophoresis (1.0% agarose gel) with 0.5 mg/mL ethidium bromide after cleavage by specific restriction endonucleases (23). The DNA concentration was quantitated spectrophotometrically. The plasmid DNA was frozen at –20 °C until immediately prior to use.

The protocol for transfection followed that recommended by the manufacturer for the use of lipofectamine. Hep G₂ cells and Alexander cells were plated at 250 000 cells/well on a standard Costar 12-well plate 48 h prior to transfection. Cells were approximately 60–70% confluent at the time of transfection. Solutions of plasmid DNA in 100 μL of Opti-MEM were mixed gently with 100 μL Opti-MEM solutions containing cationic agents, and then the lipid–DNA suspensions were diluted to a final volume of 0.5 mL. Typically (e.g., using PCL and MHL), 20 μg of the cationic lipid was used to complex 2.0 μg of the plasmid DNA, representing a cationic:DNA phosphate charge ratio of 5:1. Lipofectamine was used at the lipid:DNA weight ratio of 14 μg of lipofectamine to 2 μg of DNA, the ratio determined for maximum expression in Hep G₂ cells. Cationic lipid–DNA complexes were incubated for 15–25 min. The cells were washed twice with 2 mL of 10 mM PBS, pH 7.4, and then the 0.5 mL lipid–DNA solutions were added to the cells. Six hours after adding the lipid–DNA complexes to the cell culture, the Opti-MEM medium was replaced with MEM containing 10% FBS. The transfected cells were lysed following two washes with PBS 48 h after transfection. The luciferase activity in the lysates was measured with a luciferase kit from Promega Inc. and a luminometer from EG&G Wallac (Gaithersburg, MD).

Statistical Analysis. Particle size data were taken directly from the laser light scattering instrument. LDH leakage data and luciferase activity were evaluated by means of the one-way variance test and Newman-Keuls test for multiple comparisons between groups. A p -value of less than 0.05 was considered as statistically significant.

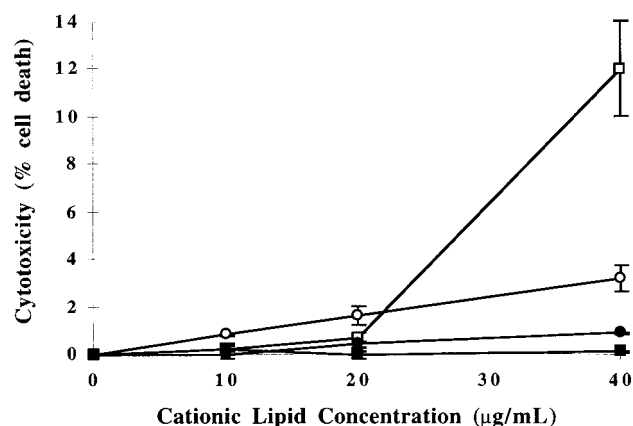


Figure 2. Cytotoxicity of liposomal formulations to Hep G₂ cells. Hep G₂ cells were exposed to cationic liposomes of final cationic lipid/polymer concentrations ranging from 0–40 μg/mL. LDH release from the cells, an indication of cytotoxicity, was measured after 24 h and is expressed as a percentage of cell death based on the control levels. (○) PCL; (●) PCL-Chol; (□) MHL; (■) MHL-Chol. Each data point represents the mean value of *n* = 4, and the standard deviation from the mean.

RESULTS AND DISCUSSION

To examine the influence of cationic lipid polymerization on cytotoxicity, we compared the liposome formulations of monomer MHL, MHL-cholesterol, polymer PCL, PCL-cholesterol, DOTAP-cholesterol, and lipofectamine in a cytotoxicity assay using rat hepatocytes (Figure 1). We examined the influence of cholesterol in these formulations since the addition of cholesterol to cationic liposome preparations such as DOTAP has been reported to improve both transfection activity (24) and resistance to serum (25). Isolated rat hepatocytes in primary cultures were treated with the cationic agents at the concentrations indicated in Figure 1, which are the concentrations that were found to be optimal for DNA transfection (vide infra). The culture medium was sampled after 24 h and LDH activity in the medium was assayed using a commercially available kit. Relative cytotoxicity was calculated based on the control experiment and expressed as the percentage of cell death.

It is clear from Figure 1 that MHL alone caused significantly greater cell death compared to the control, and that the polymer PCL did not display obvious cytotoxicity to isolated hepatocytes at 20 μg/mL. Comparison of PCL with its monomeric analogue MHL reveals that lipid polymerization significantly reduced cytotoxicity to rat hepatocytes. This result is notable since cationic polymers are generally believed to elicit greater cytotoxic responses (26, 27). The cytotoxicity of the popular transfection formulations DOTAP-Chol (20 μg/mL) and lipofectamine (14 μg/mL) also led to significant LDH leakage from the isolated hepatocytes, and their toxicity was more profound than PCL or PCL-Chol.

To further examine the influence of cationic lipid polymerization on cytotoxicity, we exposed Hep G₂ cells to liposome formulations of MHL, MHL-Chol, PCL, and PCL-Chol at varying concentrations (Figure 2). In each case, the culture medium was sampled at 24 h and LDH activity in the medium was assayed as previously described. All formulations were relatively nontoxic (<2% cell death) at 20 μg/mL, which is the concentration that was used for the transfection of Hep G₂ cells. However, at the 40 μg/mL dose, MHL caused significantly greater cell death than its polymerized analogue PCL. The addition of cholesterol to MHL and PCL formulations significantly reduced their respective cytotoxicity in Hep

Table 1. Particle Size Distribution of PCL Liposomes

time (min)	no serum	10% serum	50% serum	100% serum
5	333 (±76)	347 (±60)	309 (±45)	372 (±66)
30	406 (±160)	300 (±25)	331 (±52)	375 (±66)
50	349 (±130)	291 (±36)	348 (±40)	458 (±72)
75	420 (±170)	413 (±48)	328 (±53)	376 (±66)
120	301 (±37)	359 (±49)	312 (±54)	390 (±72)
1440	341 (±61)	363 (±58)	277 (±25)	314 (±26)

Table 2. Particle Size Distribution of MHL Liposomes

time (min)	no serum	10% serum	50% serum	100% serum
5	180 (±69)	273 (±46)	<30 nm	<30 nm
30	170 (±49)	290 (±32)	153 (±13)	235 (±20)
50	159 (±48)	295 (±27)	228 (±46)	240 (±20)
75	190 (±61)	1480 (±250)	644 (±54)	1146 (±198)
120	193 (±57)	1190 (±160)	1040 (±163)	1200 (±231)
1440	159 (±30)	1510 (±250)	1836 (±299)	368 (±66)
		2870 (±340)	1720 (±646)	1180 (±150)
				708 (±102)

G₂ cells. The cytotoxicity results show that polymerization of a cationic acrylamide lipid does not impart cytotoxicity, as has been observed with other polymeric constructs, but rather significantly reduces cytotoxicity in hepatocytes and Hep G₂ cells.

We next investigated the susceptibility of PCL formulations to serum-induced aggregation. The change in particle size of a liposome formulation is an important index of stability. Lipoplex aggregation with serum components has been shown to be detrimental to *in vivo* transfection activity (5). We examined the size distribution of PCL and the representative cationic lipid standards by dispersion in Tris-HCl buffer (Figure 3). PCL and its cholesterol formulation PCL-Chol exhibit a narrow range of particle size distribution (275 ± 50 nm) similar to lipofectamine (340 ± 110 nm) and slightly larger than monomeric MHL and its cholesterol formulation MHL-Chol (180 ± 61 nm). On exposure to medium containing 10% fetal bovine serum (FBS), the sizes of the MHL and MHL-Chol liposomes were increased 213 and 167%, respectively, relative to those in Tris-HCl buffer. When the DOTAP-Chol formulation was dispersed in serum-containing medium, the average particle size was more than 10-fold larger than its original size suggesting extensive aggregation. However, exposure of the PCL and PCL-Chol formulations to the medium containing serum minimally increased the average particle size by 25 and 18%, respectively. Resistance to aggregation under this short period of exposure also was observed when lipofectamine was subjected to the serum conditions, which is not unexpected since resistance to 10% serum was noted previously (28).

To test the stability of PCL and MHL liposomes at higher serum concentrations with longer time intervals, a series of three FBS solutions were prepared containing 10, 50, and 100% FBS. Particle size measurements and size distributions were recorded at 5, 30, 50, 75, and 120 min. A measurement was also taken at 24 h to examine the long-term effect of serum exposure. The liposomal formulations of PCL were remarkably stable to all serum concentrations and resisted aggregation even when exposed to serum for up to 24 h (Table 1). In contrast, the liposome formulations of MHL were dramatically affected, even with exposure to only 10% serum, producing an erratic distribution of lipid aggregates (Table 2).

For additional comparison, exposure of lipofectamine to an elevated serum level also revealed that the PCL

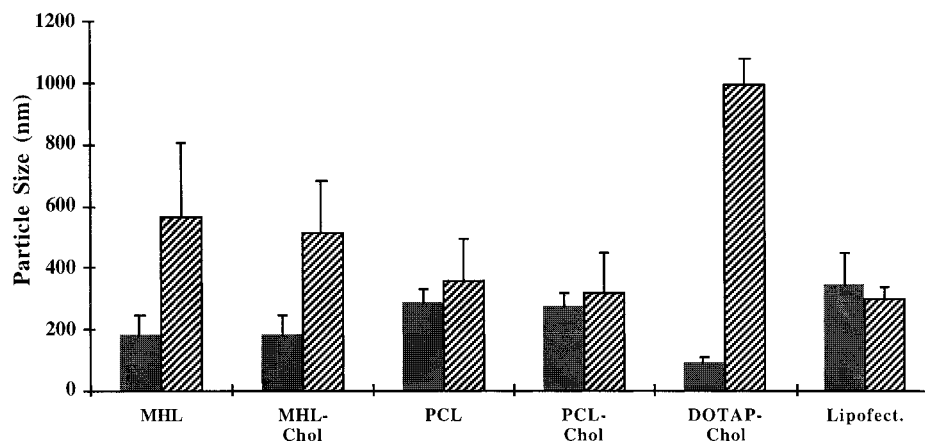


Figure 3. Particle size distribution of cationic lipid formulations and the results of exposure to serum. Solid bars represent particle size of lipid formulations in Tris-HCl buffer. Hashed bars represent particle size of lipid formulations in Williams' medium E containing 10% FBS and Tris-HCl buffer wherein 200 μ L of each lipid suspension was added to 0.5 mL Williams' medium E containing 10% FBS and then diluted with Tris-HCl buffer to a total volume of 3.5 mL. The error bars represent the polydispersity.

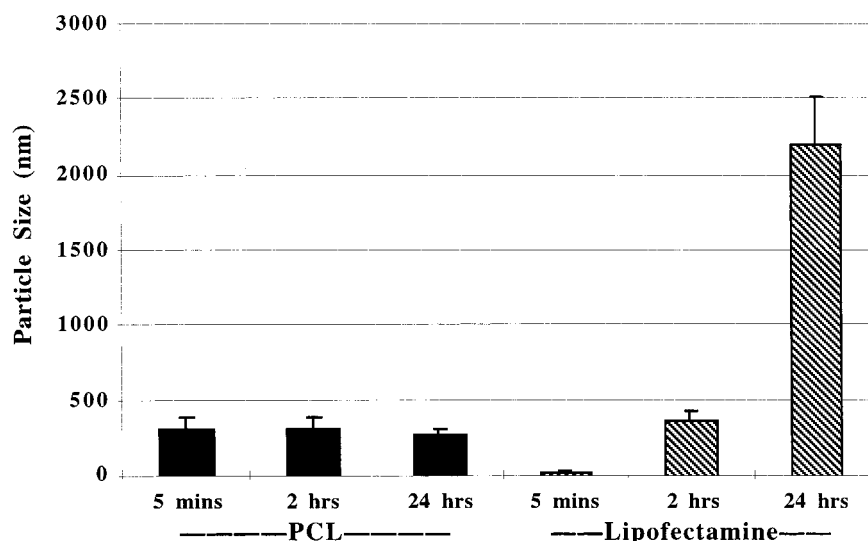


Figure 4. Influence of prolonged exposure to 50% FBS on particle size. PCL and lipofectamine suspensions were exposed 50% FBS. A 100 μ L aliquot of the cationic lipid suspension was added to 3.5 mL of 50% FBS in a cuvette. Particle size measurements were recorded at the times indicated. The error bars represent the polydispersity.

formulation is superior in its ability to resist serum-induced aggregation. When lipofectamine was exposed to 50% FBS, the mean particle size increased from 358 ± 7 nm at 120 min to 2206 ± 311 nm by 24 h (Figure 4). In contrast, the PCL formulation showed no significant increase in particle size, maintaining a size range from 309 ± 45 to 277 ± 25 nm.

Having established that PCL has low cytotoxicity and is resistant to serum-induced aggregation, we examined PCL formulations as transfection agents in two hepatoma cell lines (Figure 5). Transfection experiments were performed on Hep G₂ and Alexander cells using the pGL₃-Control vector encoding firefly luciferase. For purposes of direct comparison, lipid- and polymer-DNA complexes were prepared at a 5:1 cationic:DNA phosphate charge ratio. Preliminary transfection experiments (data not shown) had established that the 5:1 charge ratio was effective for MHL- and PCL-mediated transfections, and the 5:1 charge ratio also had been recommended for DOTAP-mediated transfection (29). Our preliminary transfection experiments determined that lipofectamine was most effective in Hep G₂ cells at a lipid/DNA ratio of 14/2 μ g. The data shows that cationic lipid polymerization does not compromise transfection activity (Figure 5). Comparison of monomer MHL and polymer PCL

revealed that these agents possess similar transfection efficacies in both cell lines. The 1000-fold increase in luciferase expression for MHL relative to naked DNA (control) in Hep G₂ cells was not unexpected since MHL is structurally analogous to the highly efficacious transfection agent γ AP-DLRIE (30). The corresponding cholesterol formulations of MHL and PCL also displayed similar luciferase activity in Alexander cells, each apparently unchanged by co-formulation with cholesterol. However, the influence of cholesterol was more pronounced in Hep G₂ cells where the activity of the PCL-Chol formulation was >10-fold higher than the MHL-Chol formulation. Moreover, the transfection activity of the PCL-Chol was similar to those of the well-established transfection lipids DOTAP-Chol and lipofectamine in Hep G₂ cells.

To probe the influence of charge ratio on the transfection activity of PCL formulations, we transfected Hep G₂ cells using PCL polyplexes having PCL:DNA and PCL-Chol:DNA charge ratios of 1.5:1, 2.5:1, 5:1, and 10:1. The data shows that PCL formulations efficiently deliver DNA to Hep G₂ cells at most charge ratios examined (Figure 6). No significant differences in transfection activity were noted for the activity of PCL from the 1.5:1 to 5:1 range. At the highest charge ratio of 10:1, a slight

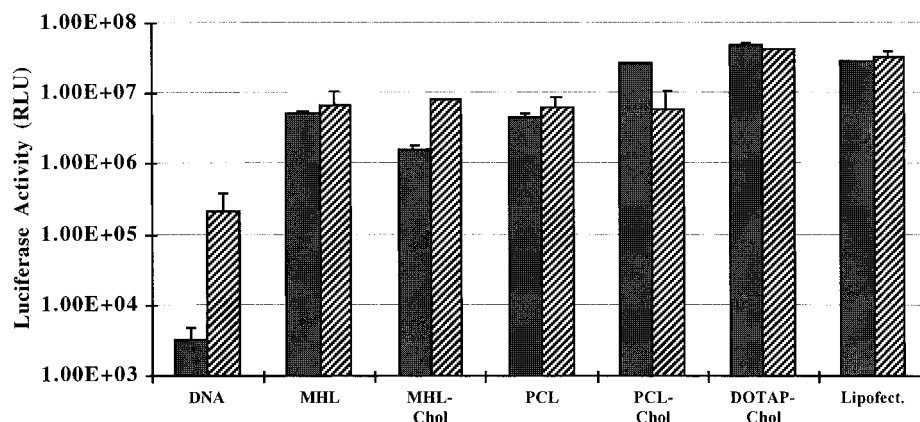


Figure 5. Transfection of Hep G₂ cells (solid bars) and Alexander cells (hashed bars). Cationic vesicles were prepared from the cationic lipid panel and poly(cationic lipid) with and without equimolar cholesterol and were used to deliver 2 μ g DNA/well. All lipoplex and polyplex preparations were formulated at a 5:1 cationic lipid:DNA phosphate charge ratio except lipofectamine, which was used at a lipid:DNA ratio of 14:2 μ g. Luciferase activity in the cell lysates was determined 48 h after transfection and expressed as relative light units (RLU). Each data point reflects the mean value from $n = 6$, and the standard deviation from the mean.

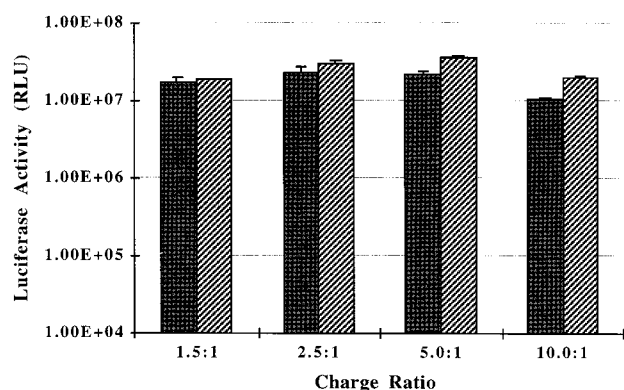


Figure 6. Influence of cationic lipid:DNA phosphate charge ratio on transfection of Hep G₂ cells using PCL (solid bars) and PCL-Chol (hashed bars). Cationic vesicles were prepared from the lipid polymer with and without equimolar cholesterol, and were used to deliver 2 μ g of DNA/well. All polyplex preparations were formulated at charge ratios ranging from 1.5:1 to 10:1. Luciferase activity in the cell lysates was determined 48 h after the transfection and expressed as relative light units (RLU). Each data point represents the mean value from $n = 4$, and the standard deviation from the mean.

drop in PCL-mediated transfection was noted. Coformulation of PCL with cholesterol at a 1:1 molar ratio appears to slightly improve the transfection activity at all ratios examined. We were gratified to note efficient delivery of DNA at the low charge ratio of 1.5:1 using both the PCL and PCL-Chol formulations since most cationic polymers for gene transfer typically require higher charge ratios (2).

In summary, we have presented the first examples of poly(lipid)-mediated DNA transfection. The absolute transfection activity of the novel poly(lipid) PCL, and certainly the improvement in the transfection activity over its monomeric form MHL, documents that the lipid polymerization process may be a useful method for converting transfection-active agents into polymers which have lower cytotoxicity and improved serum resistant properties with comparable transfection activity. In comparison to the monomeric precursor MHL and to the industry transfection standards DOTAP and lipofectamine, the novel cationic polymer PCL exhibited the lowest cytotoxicity, was the most resistant to serum-induced aggregation and had comparable transfection activity when co-formulated with cholesterol. More importantly, this work points toward the potential of lipid polymeri-

zation as a promising new approach for developing biocompatible materials for gene transfer.

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Diquaternary Ammonium Compounds as Transfection Agents

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Diquaternary ammonium salts constitute a new class of reagent for mediating transfection of DNA in mammalian cell lines. *N,N'*-dioleoyl-*N,N,N',N'*-tetramethyl-1,2-ethanediamine (TmedEce), *N,N'*-dioleoyl-*N,N,N',N'*-tetramethyl-1,3-propanediamine (PropEce), *N,N'*-dioleoyl-*N,N,N',N'*-tetramethyl-1,6-hexanediamine (HexEce), and their corresponding *N,N'*-dicetyl saturated analogues (TmedAce, PropAce and HexAce) have all been synthesized and characterized. They were prepared via a bis-Menschutkin reaction of the corresponding tetramethyldiamine with 2.2 M equiv of a long-chain alkyl halide (saturated or unsaturated). The reaction was run in anhydrous acetonitrile for ca. 3 days at 60 °C, which produced the diquaternary ammonium halides in good to nearly quantitative yields for most derivatives. DNA transfection comparable to commercially available reagents such as Lipofectin, Lipofectace, Lipofectamine, and *O*-ethyl dioleoylphosphatidylcholine triflate has been achieved in vitro with these new reagents. There was no need to use a colipid for effective transfection, but serum did significantly inhibit transfection. The saturated and the unsaturated derivatives differed with respect to hydration behavior. The saturated derivatives appeared to retain a lamellar-type crystalline array structure upon hydration, whereas the unsaturated versions formed micelles and/or liposomes, depending on the ionic strength: HexEce was micellar in both water and saline; PropEce was micellar in water but lamellar in saline; and TmedEce was lamellar in both. Despite these different hydration patterns, all of these unsaturated derivatives formed productive transfection complexes with DNA. Varying the distance between the quaternary sites affected transfection efficacy in the order HexAce > TmedAce = PropAce for the saturated derivatives and in the order PropEce = HexEce > TmedEce, with a smaller spread, for the unsaturated derivatives.

INTRODUCTION

The in vitro introduction of plasmid DNA into mammalian cells (transfection) for transient expression of a variety of desired proteins has become a standard technique in molecular biology. Earlier methods involved the use of DNA coprecipitation with calcium phosphate, soluble DEAE or Polybrene resins (1), as well as electroporation (2). More recently, the use of cationic amphiphiles or more simply "cationic lipids" for mediating DNA transfection has become widespread. This trend was initiated by the demonstration of Felgner et al. that the cationic lipid, DOTMA (dioleolxypropyl-trimethylammonium bromide), was much faster, simpler, cheaper, and generally more effective than alternative methods in transfecting DNA into cultured cells (3). Since that time, there have been additional reports of different cationic lipids with transfection activity (e.g., refs 4–11).

One of the potentially most important applications of cationic lipids is as DNA delivery agents for gene therapy (12). Another potential clinical application is delivery of antisense oligonucleotides (13). Other molecules can be delivered to cells as cationic complexes, such as a transcription regulator protein (14), a viral trans-activator protein (15), and a mushroom toxin (16) and it is likely that additional applications will be developed.

Our investigation was prompted by the fact that dimethyldioctadecylammonium bromide, when formulated with an equimolar amount of dioleoylphosphatidylethanolamine (DOPE), was shown to be quite an

effective DNA transfection agent (6). (This mixture is marketed by Life Technologies under the trade name Lipofectace.) It appeared possible that introduction of a second quaternary ammonium group could increase the strength of interaction with DNA and perhaps generate an improved transfection agent. We thus sought a synthetic route to generate a molecule with two dimethylammonium groups, and two long-chain alkyl groups. The route we chose involved alkylating tetramethyldiamines with long-chain alkyl halides; it was found to provide convenient and efficient access to dialkyltetramethyldiquaternary ammonium transfection agents.

EXPERIMENTAL PROCEDURES

Materials. Chemical reagents and solvents were normally purchased either from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO) and were used without further purification. BHK-21 cells (Syrian or Golden Hamster) were obtained from American Type Culture Collection (Rockville, MD). Tissue culture reagents were purchased from Life Technologies (Grand Island, NY). The DNA plasmid with the β -galactosidase gene driven by the cytomegalovirus promoter (pCMV- β -gal) was also purchased from Life Technologies. It was propagated in DH5 cells and purified with a Plasmid Mega Kit from Qiagen (Valencia, CA).

Synthetic Methods for Diquaternary Ammonium Salts. To an oven-dried and desiccated 1.2 $\times$ 2.5 cm screw cap vial was added anhydrous acetonitrile (1 mL), followed by either tetramethylethylenediamine, tetramethyl-1,3-propanediamine, or tetramethyl-1,6-hexanediamine (300 μ mol), and finally by the long-chain alkyl halide (cetyl iodide or oleyl bromide, 665 μ mol), and the

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vial was sealed tightly. The reaction vial was then placed in a dry heating block at 60 °C, covered with aluminum foil, and allowed to react for ca. 3 days. In the case of the saturated analogues TmedAce and HexAce, significant precipitation of product was observed when the reaction mixture was heated, while PropAce only precipitated after it cooled. After cooling, the solid was triturated with absolute ethanol and then suction filtered. The solid was dried under high vacuum and weighed. Yields were 60–84% for TmedAce and HexAce and 17% for PropAce. The unsaturated analogues did not precipitate in the reaction mixture until they cooled to RT. Their workup was modified due to their different solubility characteristics (soluble in absolute ethanol and chloroform). The acetonitrile above the solid was first decanted away, and then the residue dissolved in 1–2 mL of chloroform. The latter solution was transferred to a culture tube, and the bulk of the volatiles were removed with a stream of argon under warming. The solid residue was redissolved in 3–6 mL of chloroform and subjected to silica gel chromatography. Typically, the CHCl_3 solution containing the crude product was applied to a dry silica gel column of ≥ 2.5 mL. The column was washed with ca. 5 vol of CHCl_3 , followed by 9:1 CHCl_3 : CH_3OH , at which point the bulk of the product eluted from the column. After collection of six 6-mL fractions, the remaining product was eluted with 5:1 and then 4:1 CHCl_3 : CH_3OH (three fractions each). The desired product has an R_f value of ca. 0.57 when run on an analytical silica gel TLC plate (in CHCl_3 : CH_3OH :acetic acid, 65:25:4). The yield for TmedEce was 19%, while the yields for PropEce and HexEce were essentially quantitative.

Synthesis of Dimethyl-2-dimethylaminoethylcetyl-ammonium Iodide (Quatamine-1). To an oven-dried and desiccated 1.2×2.5 cm screw cap vial was added anhydrous acetonitrile (1 mL) followed by tetramethylethylenediamine (35 mg, 302 μmol) and finally cetyl iodide (106 mg, 301 μmol), and the vial was sealed tightly. The vial was then placed in a dry heating block at 60 °C, covered with aluminum foil, and then allowed to react overnight. After this time, the sample was cooled to room temperature, and a small precipitate was removed by gravity-filtration through Celite (ca. 2 cm). The bulk of the volatiles were evaporated away with a gentle stream of argon (while warming in a water bath), yielding a light orange-yellow residue. Unreacted tetramethylethylenediamine was removed by extraction as follows. The mixture was dissolved in chloroform (5 mL), which solution was then transferred to a 50 mL graduated cylinder. Methanol (2.5 mL) was added, followed by 10% HCl (3 mL). After mixing, the lower organic layer was removed and gravity-filtered through granular sodium sulfate. It was then transferred to a tared vial, and the bulk of the volatiles removed as described above. The sample was then placed under high vacuum for 35 min and weighed. Fifty milligrams (35% Yield) of very pure monoquaternized salt (as determined by ^1H NMR) was obtained.

Product Characterization. Liquid secondary ion mass spectra were obtained on a VG70 mass spectrometer (VG Analytical) with Cs ion as a primary source. Proton NMR spectra were obtained on a Varian Gemini 300 MHz spectrometer, using tetramethylsilane as an internal reference, and chemical shifts are given in δ (ppm).

By TLC and proton NMR, all of the diquaternary ammonium compounds were found to be pure. No evidence of starting materials or monoquaternary interme-

diate was observed in the proton NMR spectra or analytical TLC of the purified products.

Spectral Data (all ^1H NMR spectra were obtained at 300 MHz). *TmedAce*, in deuteriochloroform:methanol- d_4 [1:1]: δ 4.21 (singlet, 4H, internal methylenes adjacent to quaternary nitrogens), 3.57 (broad triplet, $J = 8$ Hz, 4H, external methylenes adjacent to quaternary nitrogens), 3.32 (singlet, 12H, quaternary methyl groups), 1.84 (broad singlet, 4H), 1.5–1.2 (multiplet, 52H), 0.89 (triplet, $J = 7$ Hz, 6H, terminal methyl groups). MS: 552 ($\text{M}^+ - \text{methyl}$).

PropAce, in deuteriochloroform:methanol- d_4 : δ 3.57 (broad triplet, $J = 8$ Hz, 4H, internal methylenes adjacent to quaternary nitrogens), 3.46 (broad triplet, $J = 8$ Hz, 4H, external methylenes adjacent to quaternary nitrogens), 3.23 (singlet, 12H, quaternary methyl groups), 2.41 (broad singlet, 2H, central internal methylene), 1.83 (broad singlet, 4H), 1.5–1.2 (multiplet, 52H), 0.89 (triplet, $J = 7$ Hz, 6H, terminal methyl groups). MS: 708 ($\text{M}^+ + \text{I}^-$), 566 ($\text{M}^+ - \text{methyl}$).

HexAce, in deuteriochloroform:methanol- d_4 : δ 3.44 (broad triplet, $J = 8$ Hz, 4H, internal methylenes adjacent to quaternary nitrogens), 3.36 (broad triplet, $J = 8$ Hz, 4H, external methylenes adjacent to quaternary nitrogens), 3.14 (singlet, 12H, quaternary methyl groups), 1.95–1.7 (multiplet, 8H), 1.55 (broad singlet, 4H), 1.48–1.2 (multiplet, 52H), 0.89 (triplet, $J = 7$ Hz, 6H, terminal methyl groups). MS: 750 (M^+), 608 ($\text{M}^+ - \text{methyl}$).

TmedEce, in deuteriochloroform: δ 5.38–5.3 (overlapping triplets, $J = 6$ Hz, 4H, vinyl), 4.77 (singlet, 4H, internal methylenes adjacent to quaternary nitrogens), 3.71 (broad triplet, $J = 8$ Hz, 4H, external methylenes adjacent to quaternary nitrogens), 3.50 (singlet, 12H, quaternary methyl groups), 2.06–1.92 (multiplet, 8H, allylic), 1.92–1.70 (multiplet, 12H), 1.5–1.2 (multiplet, 36H), 0.88 (triplet, $J = 7$ Hz, 6H, terminal methyl groups). MS: 697/699 ($\text{M}^+ + \text{Br}^-$), 604 ($\text{M}^+ - \text{methyl}$).

PropEce, deuteriochloroform: δ 5.38–5.3 (overlapping triplets, $J = 6$ Hz, 4H, vinyl), 3.92 (broad triplet, $J = 8$ Hz, 4H, internal methylenes adjacent to quaternary nitrogens), 3.48 (broad triplet, $J = 8$ Hz, 4H, external methylenes adjacent to quaternary nitrogens), 3.36 (singlet, 12H, quaternary methyl groups), 2.83–2.7 (multiplet, 2H, central internal methylene), 2.06–1.92 (multiplet, 8H, allylic), 1.92–1.70 (multiplet, 12H), 1.46–1.2 (multiplet, 36H), 0.88 (triplet, $J = 7$ Hz, 6H, terminal methyl groups). MS: 711/713 ($\text{M}^+ + \text{Br}^-$), 618 ($\text{M}^+ - \text{methyl}$).

HexEce, deuteriochloroform: δ 5.38–5.3 (overlapping triplets, $J = 6$ Hz, 4H, vinyl), 3.80–3.65 (multiplet, 4H, internal methylenes adjacent to quaternary nitrogens), 3.50–3.29 (overlapping broad triplet and singlet, 16H, external methylenes adjacent to quaternary nitrogens and quaternary methyl groups), 2.06–1.92 (multiplet, 8H, allylic), 1.81–1.55 (multiplet, 12H), 1.42–1.20 (multiplet, 44H), 0.88 (triplet, $J = 7$ Hz, 6H, terminal methyl groups). MS: 753/755 ($\text{M}^+ + \text{Br}^-$), 660 ($\text{M}^+ - \text{methyl}$).

Quatamine-1, deuteriochloroform: δ 4.40–4.30 (broad singlet, 2H, internal methylene adjacent to quaternary nitrogen), 4.20–4.10 (broad singlet, 2H, external methylene adjacent to quaternary nitrogen), 3.60–3.50 (multiplet, 2H, methylene adjacent to dimethylamino-group), 3.45 (broad singlet, 6H, quaternary methyl groups), 3.13 (broad singlet, 6H, dimethylamino-group), 1.88–1.74 (broad singlet, 2H), 1.40–1.16 (multiplet, 26H), 0.88 (triplet, $J = 7$ Hz, 3H, terminal methyl group). MS: 341 (M^+).

Storage of Diquaternary Ammonium Salts and their Dispersal. Diquaternary ammonium salts were

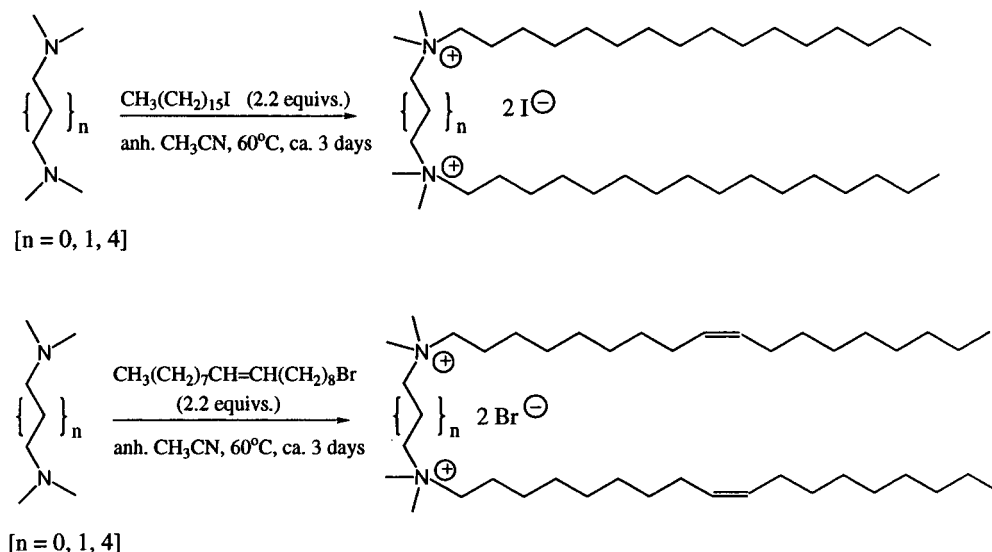


Figure 1. Reactions used for the synthesis of the diquaternary ammonium compounds. The upper scheme describes the synthesis of the saturated series and the lower scheme describes that of the unsaturated derivatives. In each series, compounds with 2, 3, and 6 carbons separating the nitrogen atoms were prepared.

normally stored at -20°C as 10–40 mg/mL $\text{CHCl}_3\text{:CH}_3\text{OH}$ [1:1] or CHCl_3 solutions for the dicetyl and dioleyl derivatives, respectively. An age-dependent reduction of transfection activity was observed with the unsaturated derivatives, and therefore, we recommend that they be used within 1–2 months after final purification and solution preparation.

Formation of Aqueous Dispersions of Diquaternary Ammonium Salts. Aliquots of stock were transferred to vials where the bulk of the solvent was removed under a stream of argon. The vial was then placed under an oil pump vacuum for at least 30 min/mg to remove residual solvent. Next, the appropriate amount of phosphate-buffered saline (PBS) was added in order to obtain a 1 mg/mL solution, and the tube was vortexed or bath-sonicated briefly.

Transfection Protocol. BHK cells were transfected with pCMV- β gal plasmid in 96 well microplates. Complete medium was GMEM containing 5 or 10% fetal bovine serum, 2 mM glutamine, 2% tryptose phosphate, and 1/100 PenStrep. The cells were incubated at 37°C in 5% CO_2 . Cells were seeded on the day before transfection at densities such that they would be approximately 70% confluent when transfected. Cells were prepared for transfection by removal of complete medium and replacing it with medium lacking serum, tryptose, and antibiotics. Amphiphiles, suspended in PBS at 1 mg/mL, were added to the plasmid DNA in PBS at 0.1 mg/mL with gentle mixing and allowed to incubate for 20–30 min. The DNA–amphiphile complex was then added to the cells and incubated 4–6 h at 37°C . After this time, the cells were returned to complete medium, either following washing with PBS or simply upon addition of 1/10 or 1/20 volume of fetal bovine serum. Twenty hours after treatment with the DNA–amphiphile complex, the cells were assayed for β -galactosidase activity using a microplate fluorometric assay (17). Briefly, the cells were washed with PBS and permeabilized by adding 100 μL of PBS containing 0.03% Triton X-100. Ten microliters of 0.1 mM fluorescein digalactoside was added to the wells (final volume was 100 μL); 30 and 60 min after addition of substrate, the plate was read in a microplate fluorometer (Packard Instruments). The rate of reaction was linear in this time range and the fluorescence intensity difference between the two times was propor-

tional to β -galactosidase activity. Standards of purified β -galactosidase (Sigma) were run on each plate to validate the assay reaction. We did not routinely quantify the number of cells or protein in transfected cultures, so we normally express the galactosidase activity in terms of light units. To date, optimum conditions yield approximately 10 milliunits of enzyme activity/well of a 96 well plate.

RESULTS

All of the diquaternary ammonium salts were prepared via a bis-Menshutkin reaction of their corresponding tetramethyldiamines with 2.2 M equiv of a long-chain alkyl halide (saturated or unsaturated). The reaction was run in anhydrous acetonitrile for 3 days at 60°C in the dark, which provides the diquaternary ammonium halides in good to nearly quantitative yields for most derivatives (see Figure 1). An example of a monoquaternarized version of TmedAce, Quatamine-1, was produced through an analogous procedure, with the exception that only 1 M equiv of the cetyl iodide was used, and the reaction was only run overnight. For more details, see Materials and Methods.

The diquaternary ammonium halides were characterized by both ^1H NMR and mass spectral analysis. As can be seen in the Spectral Data section of Materials and Methods, the NMR resonances are consistent with the diquaternary structure assigned for each derivative. The mass spectral data consistently show both a parent ion minus one halide, as well as a parent ion minus one methyl group, for each derivative.

Most cationic amphiphiles described in the literature as transfection reagents are more effective in the presence of DOPE (4, 11, 15), although there are exceptions (7, 18). In the case of the diquaternary ammonium salts, transfection efficiency was actually reduced by DOPE (see Figure 2 for a representative example), therefore, most of our studies were done without colipid.

The monoquaternary Quatamine-1 was found to be highly toxic, even at low doses (data not shown) and underscores the need to isolate pure diquaternary ammonium salt for transfection purposes. Generally, investigators have not examined the effects of small amounts

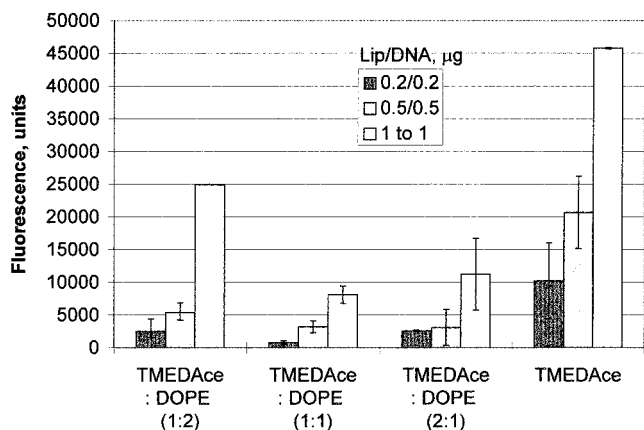


Figure 2. Transfection activity of TmedAce/DOPE at different ratios of TmedAce to DOPE. Transfection complexes were prepared in DPBS according to the standard protocol. Then lipid/DNA complexes were applied to BHK cells in the amounts: 0.2/0.2, 0.5/0.5, and 1.0/1.0 μg (first, second, and third bars, of each set, respectively). Error bars indicate high and low values of replicates.

of side products that may be carried over in the final product as impurities. Given the high toxicity of some monoalkylamines, small variations in the purity of even highly pure materials could generate errors in evaluation of transfection agents and perhaps also account for some of the unexplained variations in the response of different cells to different compounds.

The diquaternary compounds exhibited a variety of hydration behavior. The saturated derivatives, according to freeze–fracture electron microscopy, formed stacked, planar lamellae. The unsaturated compounds were observed by light microscopy to form liposomes at high ionic strength. At low ionic strength, two of the unsaturated compounds generated clear solutions, implying the formation of a micellar phase. Specifically, HexEce was micellar in both water and saline, PropEce was micellar in water but lamellar in saline, and TmedEce was lamellar in both aqueous phases.

All of the diquaternary ammonium salts exhibited good to excellent transfection activity in BHK cells. Many transfection lipids are more active when formulated with equimolar DOPE, although this is not the case with the diquaternary compounds. Transfection activity of TmedAce formulated with DOPE at several ratios is shown in Figure 2. The TmedAce without colipid was most active and a similar pattern was found with the other diquaternary compounds (data not shown).

The most efficient reagent of the saturated derivatives was HexAce (Figure 3). TmedAce and PropAce were 3–4 times less effective. This was observed under a variety of doses and lipid to DNA ratios (Figure 4). In the case of the unsaturated derivatives, PropEce and HexEce were both found to be highly efficient and essentially equivalent (Figures 5 and 6, respectively), with TmedEce only slightly less effective (Figure 7). In all cases, the presence of 10% serum was found to significantly reduce if not completely inhibit the transfection efficacy of these reagents.

The diquaternary ammonium salts were, at optimal lipid:DNA ratio and dose, at least as effective transfection agents as *O*-ethyl-dioleoylphosphatidylcholine (EDOPC), the cationic lipid with which we have had the most experience. EDOPC, a phospholipid derivative, was previously shown to be as effective as one of the current

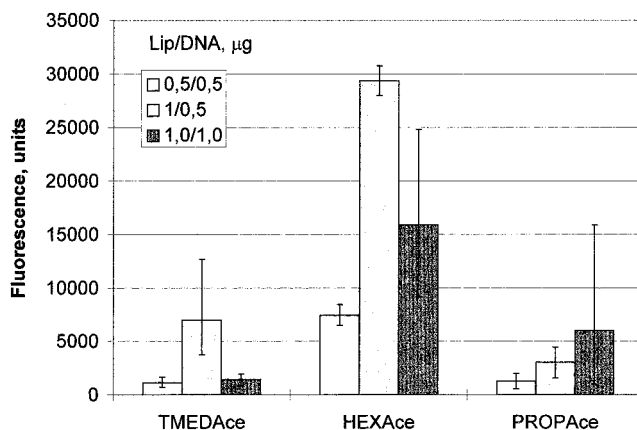


Figure 3. Comparison of TmedAce, PropAce and HexAce in transfection of BHK cells. Transfection complexes were formed in DPBS and applied to cells in the amounts: 0.5/0.5, 1.0/0.5, and 1.0/1.0 μg (first, second, and third bars, of each set, respectively). Error bars indicate high and low values of replicates.

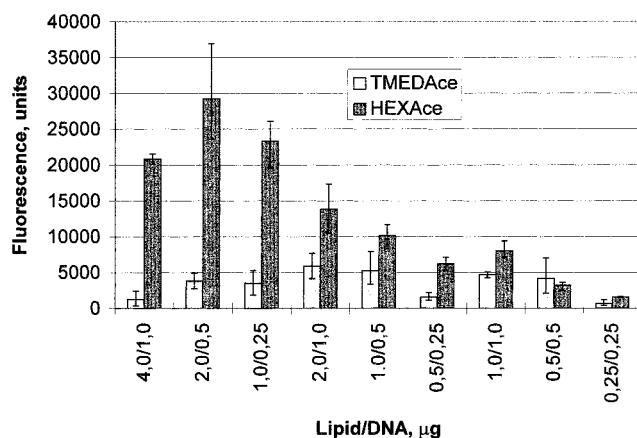


Figure 4. Comparison of TmedAce and HexAce transfection activities for different lipid/DNA ratios and doses. BHK cells were treated with plasmid DNA complexed with either TmedAce (first bar of each pair) or HexAce (second bar of each pair) in the amounts ($\mu\text{g}/\text{well}$) shown. Bars indicate high and low values of replicates.

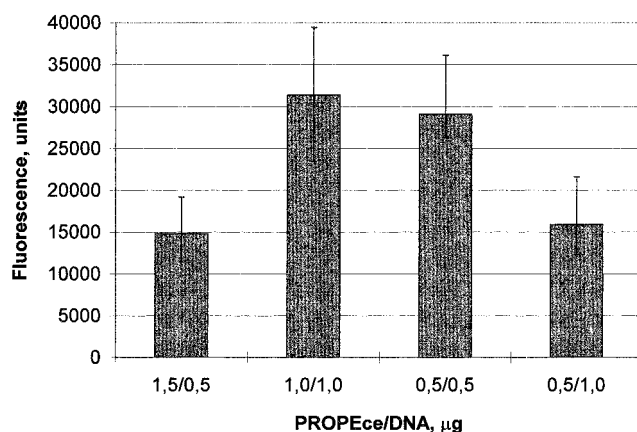


Figure 5. Transfection activity of PropEce as a function of the lipid/DNA ratio and dose. BHK cells were treated with plasmid DNA complexed in DPBS with PropEce in the amounts ($\mu\text{g}/\text{well}$) shown. The data of transfection with EDOPC are presented for comparison. Bars indicate high and low values of replicates.

benchmarks of transfection reagents, Lipofectamine, in mediating transfection of BHK cells (19, 20). Its activity was also similar to that of Lipofectace and considerably superior to that of Lipofectin (unpublished).

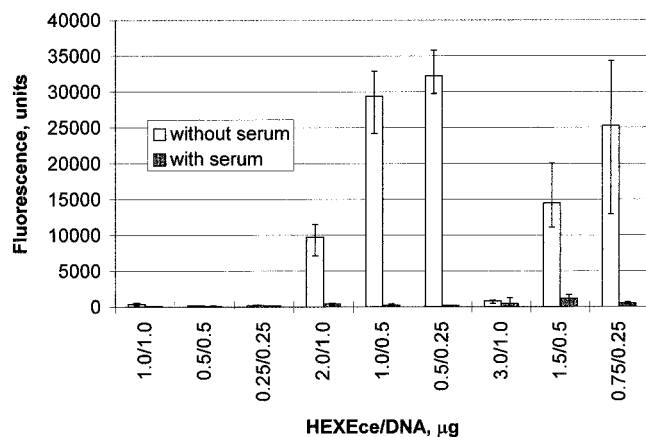


Figure 6. Transfection efficiency of HexEce with and without serum. BHK cells were transfected with HexEce/DNA complexes prepared in DPBS at different lipid/DNA ratios. Transfection was performed in medium without supplements (first and open bar of each group) and in medium containing 10% FBS (second and filled bar of each group) in the amounts ($\mu\text{g}/\text{well}$) shown. Bars indicate high and low values of replicates.

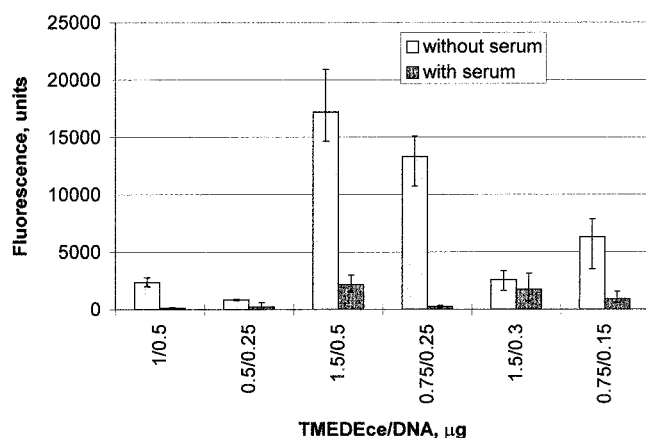


Figure 7. Transfection efficiency of TmedEce with and without serum. BHK cells were transfected with TmedEce/DNA complexes prepared in DPBS at different lipid/DNA ratios. Transfection was performed in medium without supplements (first bar of each group) and in medium containing 10% FBS (second bar of each group) in the amounts ($\mu\text{g}/\text{well}$) shown. Bars indicate high and low values of replicates.

DISCUSSION

The diquatary ammonium salts are conveniently synthesized in a single step, via a bis-Menschutkin reaction of their corresponding tetramethyldiamines with long-chain alkyl halides (either saturated or unsaturated). Yields ranged from good to nearly quantitative. Such straightforward chemistry as well as the commercial availability of a range of various tetramethyldiamines as well as long-chain alkyl halides, provides the opportunity to explore many permutations through solution phase combinatorial chemistry.

The observation that DOPE is not necessary for highly efficient transfection contrasts with the requirement of LipofectAce for DOPE. A number of other transfection lipid mixtures also contain DOPE as an integral component. Therefore, it appears that the addition of a second permanent positive charge in the diquatary compounds somehow obviates the requirement for DOPE that is exhibited by the monoquatary dimethyldioctadecylammonium bromide component of LipofectAce.

The low transfection efficacy observed in the presence of 10% serum may preclude the *in vivo* usage of the

diquatary ammonium salts as vectors for systemic gene therapy, although procedures to reduce or eliminate the serum sensitivity of other cationic lipid formulations (18, 21) may well be applicable to the diquatary compounds.

The hydration behavior of the unsaturated derivatives follows a logical pattern, given the length of the alkyl chain that separates the two quatary sites and the known effects of molecular shape on polymorphic phase preferences (22). TmedEce remains lamellar in both water and saline since the ethyl bridge between the two quatary sites geometrically precludes anything but a parallel orientation of the two oleyl chains, which thus leads to a lamellar phase in both media. PropEce, possessing an extra carbon in its tether group, has enough space to allow ionic strength to affect its phase stability; it assumes a lamellar configuration in saline, wherein chloride anions screen the repulsion between the two positive sites and allow them to come close enough that the oleyl chains may be parallel, whereas in water, the two quatary sites must move further away due to electrostatic repulsion, which consequently causes the oleyl chains to assume a wedge shape, favoring a micellar phase. Finally, HexEce was micellar in both water and saline since the two quatary sites are held apart by the hexyl chain tether, the steric effect of the oleyl chain is independent of ionic strength, so the molecule is wedge-shaped and hence micellar in both media.

The fact that efficient transfection was obtained with both HexEce and TmedEce shows that their different initial hydration behavior does not preclude either from forming a productive complex with DNA [in analogy to what has been observed with *O*-alkyl-dioleoylphosphatidylcholine compounds (23)].

In conclusion, diquatary ammonium salts constitute a new class of reagent for mediating transfection of DNA. Some of the derivatives we studied do so with high efficiency without a requirement for colipid. At least in the absence of serum, diquatary compounds exhibit transfection activity that is at least as effective, if not superior to that of a number of popular, commercially available agents. Their low cost, extreme ease of synthesis and suitability to varying their chemical (and hence also physical) properties in a very straightforward manner allows for optimizing activity for particular cell and transfection conditions.

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Metabolism and Renal Clearance of ^{111}In -Labeled DOTA-Conjugated Antibody Fragments

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Radiometal-labeled antibody fragments are promising reagents for radioimmunotherapy due to their high tumor uptake and rapid pharmacokinetics, but their therapeutic potentials are limited by high uptake and retention in the kidney. Identification of metabolic products is a first step in designing rationale approaches to lower kidney uptake. Previous studies in rats have shown that ^{111}In -labeled DTPA-conjugated antibody fragments (via lysine residues) were degraded to an DTPA- ϵ -amino-lysine derivative and retained in the lysosomal compartments of the liver and kidney [Rogers et al. (1995) *Cancer Res.* 55, 5714s–5720s]. To determine the metabolic profile of another widely used metal-chelate, [^{111}In]DOTA conjugated to lysines in antibody fragments via active ester chemistry, we analyzed kidney homogenates from nude mice injected with an [^{111}In]DOTA-Fab generated enzymatically from the anti-lymphoma intact antibody Rituxan. The major kidney metabolite was identified as [^{111}In]DOTA- ϵ -amino-lysine by comparison to an authentic synthetic standard. This end product was also identified in the urine, along with relatively small amounts of [^{111}In]DOTA-Fab. Since injection of [^{111}In]DOTA- ϵ -amino-lysine into nude mice resulted in rapid clearance into the urine without kidney retention, it is likely that the renal retention observed was due to kidney uptake of [^{111}In]DOTA-Fab, followed by lysosomal degradation to [^{111}In]DOTA- ϵ -amino-lysine, which is only slowly cleared from this compartment. This observation is supported by autoradiographs of the kidney showing rapid localization of radioactivity into the distal regions of the kidney cortex. To extend this analysis to clinical trials, we have also analyzed urine taken from a patient injected with the intact antibody [^{111}In]DOTA-cT84.66. In that example, we found that the major radioactive species was also [^{111}In]DOTA- ϵ -amino-lysine.

INTRODUCTION

Antibodies can be used to deliver radionuclides (Klein et al., 1989), toxins (Spitler, 1987), or cytotoxic drugs (Starling et al., 1988) to tumors due to their high specificity for tumor surface antigens. Radiometals that are conjugated to antibody via chelates are a good choice for radioimmunotherapy (RIT),¹ as exemplified by clinical studies with radiometal labeled anti-lymphoma (Press, 1999), anti-colon cancer (Wong et al., 1995), and anti-breast cancer antibodies (DeNardo et al., 1997). In RIT, the use of intact immunoglobulin conjugates lead to a long circulation time in blood, thus exposing normal tissues to unwanted radiation (LoBuglio et al., 1989). Due to its large size (150 kDa), the IgG molecule also shows a relatively slow diffusion from the vasculature into tumors (Yokota et al., 1992; Blumenthal et al., 1995). While IgG fragments exhibit a faster biodistribution and better tumor penetration than intact IgG (Yokota et al.,

1992), this advantage is offset by nonspecific organ uptake, especially in the kidney (Andrew et al., 1988). Although this problem may be partially corrected by using metabolically labile isotopes such as radioiodine (Andrew et al., 1988), this is attained at the price of reduced tumor retention, thus requiring higher amounts of radioactivity to achieve the same radiation absorbed dose achieved with lower amounts of radiometal. Thus, although radiometal labeled antibody fragments have faster blood clearance and better tumor penetration than their intact IgG counterparts, their uptake and retention in the radiation sensitive kidney has limited their therapeutic application.

The first step to lowering kidney uptake is to identify the radiolabeled metabolic products. A series of studies with [^{111}In]DTPA-conjugated (via lysine residues) glycoproteins (Duncan and Welch, 1993; Arano et al., 1994) or antibody fragments (Anderson et al., 1995; Rogers et al., 1995) in animal models revealed that these conjugates were metabolized in the lysosomal compartment at both tumor and nontumor sites. For example, Duncan and Welch (Duncan and Welch, 1993) found that [^{111}In]DTPA-labeled glycoproteins targeted to cell surface receptors were degraded to [^{111}In]DTPA- ϵ -amino-lysine, which was then slowly released from cells and recovered intact in both urine and feces. Rogers et al. (1995) identified [^{111}In]DTPA- ϵ -amino-lysine as the major metabolites of DTPA-conjugated (via lysines) antibodies and antibody fragments. These findings suggest that lysosomal enzymes are unable to cleave the amide bond of [^{111}In]DTPA- ϵ -

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¹ Abbreviations: DOTA, 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; RIT, radioimmunotherapy; IgG, immunoglobulin; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; sulfo-NHS, *N*-hydroxysulfosuccinimide; CEA, carcinoembryonic antigen; BFC, bifunctional chelate; TFA, trifluoroacetic acid; BFC, bifunctional chelate.

amino-lysine and the end product is only slowly cleared from this compartment.

Although DTPA is an excellent chelate for radiometal-based immunoconjugates, its radiometal stability constants, especially for the imaging and therapeutic radiometals $^{111}\text{In}^{3+}$ and $^{90}\text{Y}^{3+}$, respectively, are such that significant amounts of radionuclide escape into nontarget organs such as the bone (Williams et al., 1996; Govindan et al., 1998). For this reason we have begun to utilize the more stable macrocycle chelate DOTA (Li et al., 1994) which can be conveniently linked to lysines in proteins via simple active ester chemistry (Lewis et al., 1994). Using the metabolic studies on [^{111}In]DTPA-lysine based conjugates as a guide, we speculated that DOTA-lysine-based conjugates would be degraded to [^{111}In]DOTA- ϵ -amino-lysine and retained in the lysosomal compartment. Therefore, we synthesized this derivative, studied its biodistribution in nude mice, the usual preclinical model for RIT, and showed that it is rapidly cleared into the urine with no net organ retention. However, when [^{111}In]DOTA-Fab fragments are injected into nude mice, high kidney retention of [^{111}In]DOTA- ϵ -amino-lysine is observed, suggesting that kidney filtration of the fragment results in cellular uptake, followed by metabolism and retention in the lysosomal compartment.

EXPERIMENTAL PROCEDURES

Preparation and Radiolabeling of Antibody Fab Fragment Conjugate. Rituxan (75 mg, IDEC) was digested with immobilized papain (Pierce) and purified on a protein A column (Bio-Rad) according to manufacturer's protocols. Purified Rituxan-Fab (70% yield) gave a single band at 50 kDa on a 10–15% nonreducing SDS–polyacrylamide gel. The Fab was conjugated to DOTA using the water-soluble EDC/sulfo-NHS method according to Lewis et al. (1994) with a DOTA-ester-to-antibody ratio of 100:1. Following conjugation, the protein was dialyzed against 0.2 M NH_4OAc , pH 7.2, and concentrated to 3 mg/mL. The antibody was radiolabeled with In-111 at a ratio of 2.5–3 mCi/mg. Indium-111 chloride in 0.05 N HCl was buffered with an equal volume of 0.2 M NH_4OAc at pH 7 prior to addition of antibody. The reaction mixture was incubated at 43 °C for 1 h, then 10 mM DTPA solution was added to give a final concentration of 1 mM DTPA. This was followed by a 15 min incubation at room temperature, after which purification was performed by HPLC using a Superdex 75 HR 10/30 gel filtration column (Pharmacia) eluted with normal saline at a flow rate of 0.5 mL/min. Fractions of 0.5 mL each were collected into tubes containing 1 drop 25% human serum albumin. Labeling efficiency, as determined during HPLC purification, ranged from 84 to 96%.

Synthesis of DOTA- ϵ -amino-lysine and Radiolabeling. DOTA active ester was prepared as follows: DOTA (trisodium salt, 100 mg, 213 μmol), EDC (81 mg, 425 μmol), and sulfo-NHS (92 mg, 425 μmol) in were reacted in 150 μL of water at RT at pH 7.0 for 1 h. α -Boc-Lys (104 mg, 425 μmol) dissolved in 1 mL of water was reacted with 0.5 equiv of the DOTA active ester for 22 h at RT. The solution was cooled to 4 °C and acidified with 1 mL of HCl and passed over a Bio-Rad Ag50W-X8 column (6 mL in the ammonium form). After extensive washing with water, the product was eluted with stepwise 10 mL aliquots of 0.5, 1.0, 2.0, and 4.0 M NH_4OH . The fractions (1 mL) were dried and the product was identified by ESI/MS (Finnigan LCQ ion trap). The product was loaded onto a Dowex AG1-X4 (6 mL in the formate form), washed with water, and eluted stepwise with 10 mL each of 0.1, 0.2, 0.5, 1.0, and 2.0 M formic acid. The fractions (1 mL) were dried, and the product

identified as before. The product (29 mg of white powder) had the correct mass by ESI/MS (MH^+ of 532.6 expected, 532.9 observed) and the following proton NMR: (D_2O , 500 MHz) δ 1.32–1.46 (m, 2H, γ - CH_2 of lysine), 1.52–1.57 (m, 2H, β - CH_2 of lysine), 1.80–1.94 (m, 2H, δ - CH_2 of lysine), 2.92–3.04 (m, 4H, ring protons of DOTA), 3.06–3.20 (m, 6H, ϵ - CH_2 of lysine plus ring protons of DOTA), 3.18–3.45 (m, 8H, ring protons of DOTA), 3.46 (s, 2H, acetate protons of DOTA), 3.52 (s, 2H, acetate protons of DOTA), 3.74–3.78 (m, 2H, α - CH_2 of lysine), 3.80 (s, 2H ring protons of DOTA), 3.83 (s, 2H, ring protons of DOTA). Unreacted DOTA (60 mg) was recovered and recycled for further reactions. The product (15 μL of a 37.5 mM solution in water) was radiolabeled at 43 °C with 531 μCi of $^{111}\text{InCl}_3$ (an equal volume of 0.2 M 7.0 NH_4OAc buffer of pH 7.0 was added to 35 μL of $^{111}\text{InCl}_3$ in 0.04 M HCl).

Biodistributions of [^{111}In]DOTA-Rituxan-Fab and [^{111}In]DOTA- ϵ -amino-lysine. Groups of five nontumor-bearing nude mice (Charles River) per time point were injected with 5.0 μCi (2.0 μg) of ^{111}In -labeled DOTA-Rituxan Fab via tail vein. Animals were sacrificed at 0, 1, 2, 4, 6, and 12 h after injection. Major organs and blood were weighed and radioactivity measured and expressed as percentage injected dose per gram of tissue. In a separate experiment, groups of two nontumor-bearing nude mice per time point were injected with 10 μCi (4.0 μg) of [^{111}In]DOTA-Rituxan-Fab or 50 μCi (30 μg) of [^{111}In]DOTA- ϵ -amino-lysine. Kidneys, blood, and urine samples were collected at 1, 5, and 9 h after injection and the radioactivity measured.

Kidney Cryostat-Sections. Kidney tissues were dissected from the mice injected with 5.0 μCi (2.0 μg) Rituxan Fab at 0, 4, and 12 h after injection. Renal tissues were embedded in O. C. T. (Miles Inc.), quick frozen on dry ice, cut into 4 μm thick slices, and were then mounted on slides. Frozen section slides were fixed in cold acetone for 10 min, and then exposed to a phosphor imager (Molecular Dynamics) for 2 weeks.

Kidney Metabolites of [^{111}In]DOTA-Rituxan Fab. Kidneys were removed from mice injected with 5 μCi (2.0 μg) or 10 μCi (4.0 μg) of [^{111}In]DOTA-Rituxan Fab. The organs were rinsed with PBS, frozen and thawed, and homogenized in an equal volume of PBS using a Bounce homogenizer. The PBS insoluble fractions were boiled and solubilized in 1% SDS. Samples were then centrifuged in an Eppendorf centrifuge at 12 000 rpm for 60 min, passed through a 0.22 μm filter, and were analyzed by SDS–PAGE 10 to 15% gradient gels (Pharmacia, Phast gel), gel filtration chromatography (Pharmacia, tandem Superose 12 columns, each 1 $\times$ 30 cm, flow rate 0.5 mL/min in PBS) and anion-exchange chromatography (Perceptive Biosystems, Poros HQ, 4.6 $\times$ 100 mm, flow rate 1 mL/min in 20 mM Tris, pH 8.5. Gradient started 5 min after injection and increased to 0.02 M Tris, pH 7.0, 0.5 M NaCl over 35 min).

Analysis of Patient Urine Sample. Urine was collected from a patient enrolled in a phase I therapy trial involving pretherapy imaging with the anti-CEA intact antibody (Williams et al., 1996) [^{111}In]DOTA- chT84.66 . The urine sample was collected between 24 and 48 h after administration of the pretherapy [^{111}In]DOTA- chT84.66 antibody (5 mCi, 5 mg of antibody). Aliquots (200 μL) were applied to the anion-exchange column as described above.

RESULTS

[^{111}In]DOTA-Rituxan Fab Biodistribution. DOTA was conjugated to the lysine residues of purified Fab fragments of Rituxan using active ester chemistry and

Table 1. Biodistribution of ^{111}In -labeled DOTA-Rituxan Fab in Non-Tumor-Bearing Nude Mice^a

organ	0 (h) ^b	1 (h)	2 (h)	4 (h)	6 (h)	12 (h)
blood	45.21(3.59)	1.44(0.28)	1.00(0.34)	0.76(0.10)	0.53(0.04)	0.30(0.10)
liver	7.11(0.52)	1.65(0.38)	1.70(0.36)	1.79(0.47)	1.43(0.23)	1.29(0.10)
spleen	7.40(0.51)	1.31(0.43)	1.03(0.20)	1.10(0.26)	0.98(0.14)	1.01(0.20)
kidney	58.82(7.91)	45.36(13.70)	43.20(13.88)	70.28(68.69)	79.14(60.15)	95.87(65.42)
lung	13.23(1.33)	1.57(0.20)	1.18(0.26)	1.01(0.08)	0.82(0.16)	0.62(0.10)
bone	2.90(0.38)	0.96(0.41)	0.77(0.20)	0.71(0.07)	0.65(0.03)	0.66(0.23)
carcass	2.10(0.14)	1.46(0.56)	1.05(0.28)	1.08(0.07)	0.89(0.12)	0.82(0.17)

^a Groups of five nontumor-bearing mice were injected with 5.0 μCi (2.0 μg) of ^{111}In -labeled DOTA-Rituxan Fab. Mean values ($\pm\text{SD}$) are in units of percent injected dose per gram (% ID/g) for each organ at each time point. ^b Approximately 20 min after injection.

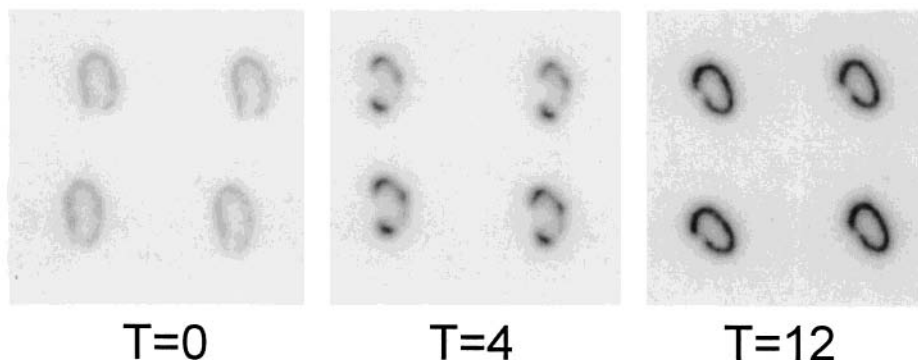


Figure 1. Kidney cryostat-sections. Kidneys excised from four mice injected with 5.0 μCi (2.0 μg) [^{111}In]DOTA-Rituxan Fab at $t = 0, 4$, and 12 h were cut into 4 μm thickness and then exposed onto a phosphor imager for 2 weeks. Little movement of the activity was observed in these specimens over the 12 h interval.

radioabeled with ^{111}In (5.0 μCi , 2.0 μg). Biodistribution studies in tumor-free nude mice showed rapid blood clearance ($<2\%$ ID/g at 1 h) and high kidney accumulation (up to 95% ID/g at 12 h), as shown in Table 1. Accumulation in all other organs, including spleen and liver were at the order of 2% ID/g after 1 h.

Localization of [^{111}In]DOTA Rituxan Fab in the kidney. To determine the anatomical location of the radioactivity in the kidneys, kidneys were harvested from the nude mice injected with [^{111}In]DOTA-Rituxan-Fab at 0, 4, and 12 h. Frozen sections were exposed on a phosphor imager. The results show that the radioactivity accumulated in the cortical region of the kidney, corresponding to the tubules (Figure 1).

Comparative Biodistributions of [^{111}In]DOTA- ϵ -amino-lysine and [^{111}In]DOTA-Rituxan Fab. Since it was suspected that the metabolic end product containing the radioactivity was [^{111}In]DOTA- ϵ -amino-lysine, the parent compound was synthesized and radiolabeled with ^{111}In . To determine if this compound is directly retained by the kidneys, 50 μCi of [^{111}In]DOTA- ϵ -amino-lysine was injected into one group of nontumor-bearing nude mice and compared to a second group injected with 10 μCi (4.0 μg) of [^{111}In]DOTA-Rituxan-Fab, and analyzed at 1, 5, and 9 h. Kidney, blood, and urine samples were collected and the percentage injected dose per gram of tissue (% ID/g) calculated (Table 2). In the group injected with [^{111}In]DOTA- ϵ -amino-lysine, accumulation of radioactivity in blood and kidney was negligible ($<2\%$) compared to the group injected with [^{111}In]DOTA-Rituxan-Fab ($>200\%$ ID/g in the kidney at 9 h). The higher renal uptake in this group (Table 1 vs Table 2) is attributed to the higher dose injected and the fact that we have not reached saturation. While we did not collect total urine excreted by mice during the course of the experiment, substantial counts were recovered at 1 h for the [^{111}In]DOTA- ϵ -amino-lysine group compared to the [^{111}In]DOTA-Rituxan-Fab group. These data demonstrated that free [^{111}In]DOTA- ϵ -amino-lysine passed quickly from blood, did not accumulate in kidney and was rapidly excreted into the urine. The radiolabeled Fab fragment, on the other hand, was taken up, metabolized and retained in renal cells.

Table 2. Biodistribution of ^{111}In -Labeled DOTA-Rituxan Fab and [^{111}In]DOTA- ϵ -amino-lysine in Non-Tumor-Bearing Nude Mice^a

organs	1 (h)	5 (h)	9 (h)
kidney (DOTA Rituxan Fab)	262.0 (4.2)	191.5 (96.9)	258.5 (2.1)
kidney (DOTA-Lys)	1.7 (0.4)	1.5 (0.2)	1.5 (0.0)
blood (DOTA Rituxan Fab)	1.1 (0.2)	0.6 (0.4)	0.3 (0.0)
blood (DOTA-Lys)	0.2 (0.1)	0.2 (0.0)	0.1 (0.0)

^a Groups of two nontumor-bearing mice were injected with either 10 μCi (4.0 μg) of [^{111}In]DOTA-Rituxan Fab or 50 μCi (30 μg) of [^{111}In]DOTA- ϵ -amino-lysine. Kidneys were excised and blood samples drawn at 1, 5, or 9 h after injection. Mean values ($\pm\text{SD}$) are in units of percent injected dose per gram (% ID/g) for each tissue at each time point.

Gel Filtration Chromatography of Kidney Homogenates and Urine Samples. Kidneys from mice injected with 7.5 μCi [^{111}In]DOTA-Rituxan-Fab were collected at 12 h and the homogenates analyzed on tandem Superose 12 columns. More than 90% of the counts found in the kidney could be extracted into a fraction soluble in PBS. [^{111}In]DOTA and DOTA-Rituxan Fab were run as markers. In this analysis, the kidney metabolites coelute with [^{111}In]DOTA as a low molecular weight species (Figure 2A). The elution profile of kidney homogenate at 12 h showed mostly low molecular weight peaks, although there is a trace of a higher molecular weight species, corresponding to undegraded [^{111}In]DOTA-Rituxan-Fab.

Urine was collected at 1 h and analyzed by gel filtration. At this time point, the urine sample contained about 50% of the counts as intact [^{111}In]DOTA-Rituxan-Fab and 50% as two low molecular weight species, one of which corresponds to the [^{111}In]DOTA standard (Figure 2B). These results demonstrate that appreciable amounts of intact [^{111}In]DOTA-Rituxan-Fab can be filtered into the urine without degradation and that low molecular weight metabolic end products are also excreted into the urine. However, these amounts are insufficient to account for the total accumulation of the low molecular species retained in the kidney ($<10\%$). Thus, the low molecular species are only excreted at a low rate. Interestingly, the

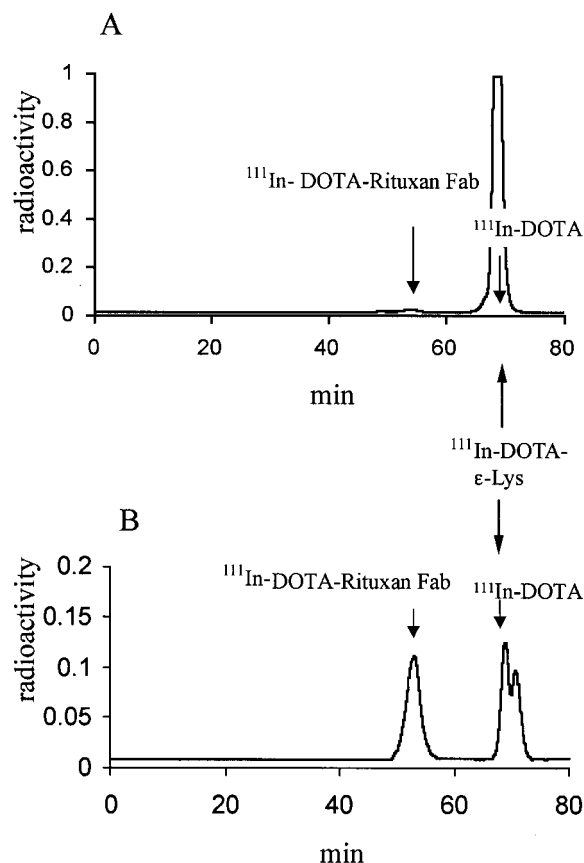


Figure 2. Gel filtration chromatography of kidney metabolites and urine samples. (A) Kidney homogenates made from mice injected with 5.0 μCi (2.0 μg) of [^{111}In]DOTA-Rituxan Fab at 12 h were analyzed on tandem Superose 12 columns, equilibrated in PBS and eluted at 0.5 mL/min. The elution positions of DOTA-Rituxan Fab, [^{111}In]DOTA- ϵ -amino-lysine, and [^{111}In]DOTA are indicated by arrows. (B) Urine samples collected from mice injected with 5.0 μCi (2.0 μg) of [^{111}In]DOTA-Rituxan Fab at 1 h were analyzed on tandem Superose 12 columns.

low molecular species in the urine separates into two peaks by this analysis. Possibilities include separation of [^{111}In]DOTA from [^{111}In]DOTA- ϵ -amino-lysine. Since we cannot distinguish [^{111}In]DOTA from [^{111}In]DOTA- ϵ -amino-lysine (or other low molecular species) by this analysis, further studies were required.

SDS-PAGE Analysis of Kidney Metabolites. Kidney homogenates generated from nude mice injected with [^{111}In]DOTA-Rituxan-Fab were also analyzed on SDS gradient gels (10–15%) and exposed to X-ray film for 1 week (Figure 3). Although both the PBS-soluble and SDS-solubilized fractions were analyzed, the PBS-soluble fractions contained >90% of the counts. [^{111}In]DOTA and $^{111}\text{InCl}_3$ standards ran anomalously at an apparent mass of 20 kDa while [^{111}In]DOTA- ϵ -amino-lysine failed to enter the gel. While we cannot explain the anomalous behavior of these compounds on SDS gel electrophoresis, the migrations were reproducible and served to uniquely identify them. [^{111}In]DOTA-Rituxan-Fab ran as expected at 48 kDa. By this analysis, the kidney homogenates at 0 h contain a small amount of intact [^{111}In]DOTA-Rituxan-Fab. By 6 h, the kidney homogenates containing the radioactive metabolites have the same pattern as that of [^{111}In]DOTA- ϵ -amino-lysine, suggesting that they are completely degraded to this end product.

Anion-Exchange Chromatography of Kidney Metabolites. Kidneys harvested from mice injected with 10 μCi of [^{111}In]DOTA-Rituxan-Fab were analyzed on a strong anion-exchange column (Figure 4). [^{111}In]DOTA-

ϵ -amino-lysine eluted as a sharp peak at 2 min (Figure 4A) and [^{111}In]DOTA eluted as a broad peak at about 4.5 min (Figure 4B). The major kidney metabolites showed a single sharp peak that coeluted with [^{111}In]DOTA- ϵ -amino-lysine at all time points analyzed (Figure 4C showing $t = 5$). The urine sample taken at 5 h eluted as a sharp peak at 2 min (Figure 4D) corresponding to the [^{111}In]DOTA- ϵ -amino-lysine standard. We conclude that [^{111}In]DOTA- ϵ -amino-lysine is the ultimate end product of kidney metabolism of [^{111}In]DOTA-Rituxan-Fab, and that this product is then excreted into urine. Unlike the gel filtration analysis, there was no evidence of a second metabolite. We conclude that the peak splitting of the low molecular weight metabolite on the gel filtration analysis was probably an artifact.

Anion-Exchange Chromatography of Patient Urine Sample. Since we had access to urine samples from patients being imaged with an [^{111}In]DOTA conjugated intact antibody, we hypothesized that their urine would contain the same radioactive end product. The rationale is that DOTA was conjugated to lysines of intact antibody by the same active ester chemistry. Indeed, the urine of these patients contained substantial amounts of a peak corresponding to [^{111}In]DOTA- ϵ -amino-lysine when analyzed by anion-exchange chromatography (Figure 5). In this study, urine from a patient receiving [^{111}In]DOTA-chT84.66 (an anti-CEA antibody) in a phase I trial involving a preimaging dose of [^{111}In]DOTA-chT84.66 to be followed by a therapy dose of [^{90}Y]DOTA-chT84.66 was analyzed by anion-exchange chromatography (Figure 5). As shown in the figure, approximately 80% of the detected urine activity corresponded to [^{111}In]DOTA- ϵ -amino-lysine. We have not identified the minor peak eluting at 11 min. Nonetheless, using both murine and human data, we conclude that [^{111}In]DOTA- ϵ -amino-lysine is the common and ultimate end product for kidney metabolism of active ester DOTA-conjugated antibody and antibody fragments, and that there is no obvious difference in this metabolism between mice and men. This preliminary analysis only describes the final metabolic product and does not address the difference in kinetics between the two protein species.

DISCUSSION

The routine use of radiolabeled antibody fragments for tumor therapy is limited by their rapid kidney uptake and metabolism within the lysosomal compartment of kidney tubule cells. Thus, it appears that antibody fragments are not simply filtered by the kidney into the urine, but are actively degraded to recycle their constitutive amino acids. If the attendant radiolabel is scavenged and/or cleared, as in the case of radioiodine on tyrosine residues, there is no net radiolabel accumulation. However, in the case of chelate-conjugated antibodies labeled with radiometals, there is a net accumulation in the lysosomal compartment, suggesting a slow clearance mechanism for the ultimate radiolabeled end product. The relative abundance of lysine residues in proteins and the nucleophilic reactivity of the ϵ -amino group of lysine has led to a variety of approaches for attaching bifunctional chelates (BFC) to these residues (Meares et al., 1984; Deshpande et al., 1990). The advantage of this approach is to produce conjugates that can be radiolabeled just prior to use. For example, first generation chelates based on EDTA contained a benzyl-isothiocyanate group for coupling to lysine residues (Meares et al., 1984; Deshpande et al., 1990). Arano and co-workers (1994) showed that neoglycoproteins (a deliberate model system for inducing high liver uptake) labeled with this [^{111}In]EDTA BFC had high liver uptake (65–95% ID/g at 1 h), and identified an [^{111}In]EDTA- ϵ -amino-lysine

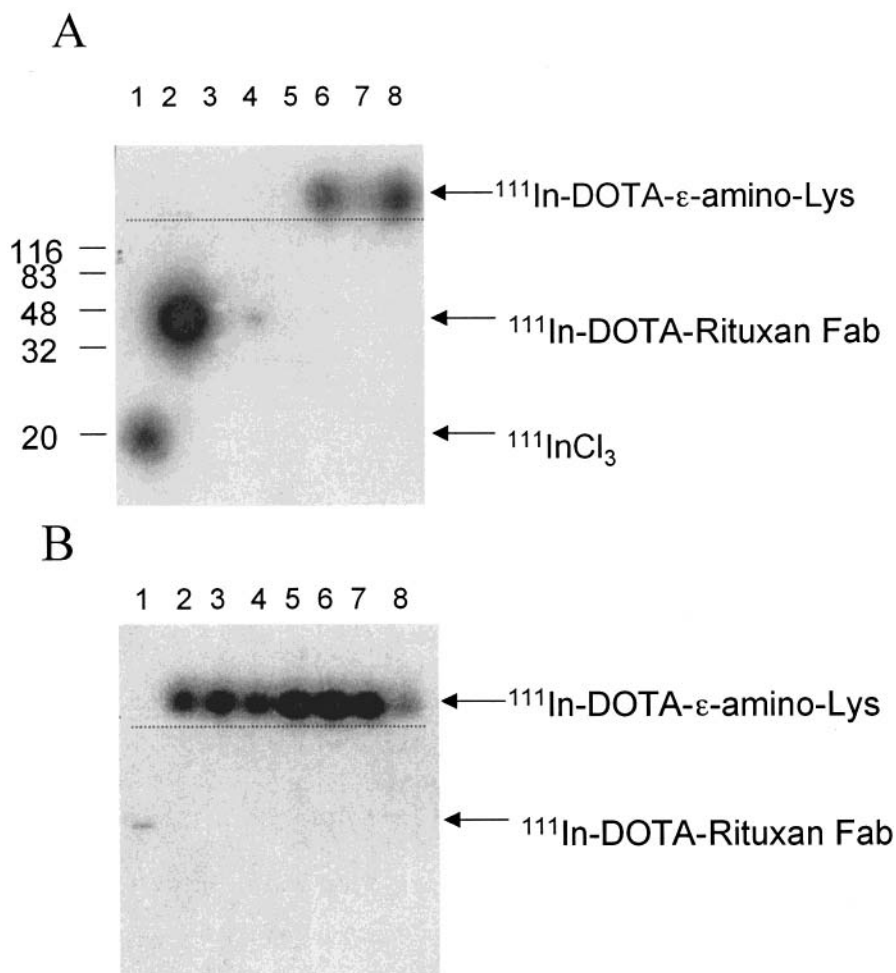


Figure 3. SDS-PAGE analysis of kidney homogenates. Kidney homogenates from mice injected with 5.0 μCi (A) or 10 μCi (B) [^{111}In]DOTA-Rituxan Fab and markers were analyzed on 10–15% gradient SDS gels under nonreducing conditions. (A) Lane 1, [^{111}In]DOTA. Lane 2, [^{111}In]DOTA Rituxan Fab. Lane 3, prestained MW markers. Lanes 4–5, $t = 0$ h, PBS soluble and SDS solubilized fractions. Lanes 6–7, $t = 12$ h, PBS soluble and SDS solubilized fractions. Lane 8, $t = 6$ h, PBS soluble fraction. The elution positions of $^{111}\text{InCl}_3$ and [^{111}In]DOTA- ϵ -amino-lysine were indicated by arrows. (B) Lane 1, [^{111}In]DOTA-Rituxan Fab. Lanes 2–4, $t = 9, 5$, and 1 h SDS solubilized fractions. Lanes 5–7, $t = 9, 5$, and 1 h PBS soluble fractions. Lane 8, [^{111}In]DOTA- ϵ -amino-lysine.

derivative as the main metabolic end product in the lysosomal compartment. In the case of the second generation chelates based on DTPA (the bicyclic anhydride of DPA conjugated to lysines in neoglycoproteins), Duncan and Welch (Duncan and Welch, 1993) also found a low molecular weight ^{111}In -labeled metabolite in the lysosomal compartment of the liver. Subsequently, this group identified the metabolite as [^{111}In]DTPA- ϵ -amino-lysine (Franano et al., 1994). Significantly, radioactivity cleared more slowly from the DTPA-based BFC than from the EDTA-based BFC. In the case of antibody fragments labeled with [^{111}In]DTPA BFC, high kidney uptake and retention were observed in the lysosomal compartment, and the major metabolic end product was again identified as [^{111}In]DTPA- ϵ -amino-lysine (Rogers et al., 1995).

More recently, many groups have turned their attention to third generation chelate, DOTA, which has stability constants $>10^{30} \text{ M}^{-1}$ and negligible off rates for trivalent metals such as In^{3+} and Y^{3+} (Craig et al., 1989; Deshpande et al., 1990; Moi et al., 1990; Peterson et al., 1999). As with previous generation BFCs, lysines have been the major target residue for coupling to proteins (Moi et al., 1990). In our own work, we have shown that DOTA can be coupled to lysines in proteins using a simple activated ester chemistry (Lewis et al., 1994). In the case of intact antibodies labeled with [^{111}In]DOTA, liver retention in the nude mouse model is moderate ($\approx 10\%$ ID/g at 1–48 h) and kidney retention low ($\approx 12\%$

ID/g at 1–48 h) (Williams et al., 1998). However, the same BFC coupled to a Fab antibody fragment and labeled with ^{111}In resulted in 45–95% ID/g in the kidneys (Table 1). This exceptionally high uptake was similar to that observed for [^{111}In]DTPA labeled F(ab')_2 fragments (Kuhlmann and Steinstrasser, 1988; Massuger et al., 1991; Yemul et al., 1993). On the basis of the results for EDTA and DTPA based BFCs, we examined that possibility that the major metabolic end product would be [^{111}In]DOTA- ϵ -amino-lysine.

Our analysis of the kidney homogenates by three methods (gel filtration, SDS-PAGE, and ion-exchange chromatography) revealed [^{111}In]DOTA- ϵ -amino-lysine as the ultimate metabolic end product with only trace amounts of intact [^{111}In]DOTA-Fab found at the earliest time points. Analysis of the urine shows appreciable amounts of [^{111}In]DOTA-Fab is filtered without degradation at early time points, but later on only [^{111}In]DOTA- ϵ -amino-lysine is found. Additionally, analysis of urine samples from a patient injected with [^{111}In]DOTA-chT84.66 showed the identical end product, thus confirming that this conclusion can be extended to other species and intact antibodies.

In earlier studies, Schott et al. (1992) conjugated a benzyliothiocyanato derivative of DOTA to a recombinant scFv version of the antitumor antibody CC49, radio-labeled it with ^{177}Lu and compared its biodistribution to an ^{125}I -labeled version. At 1 h the kidney uptake was

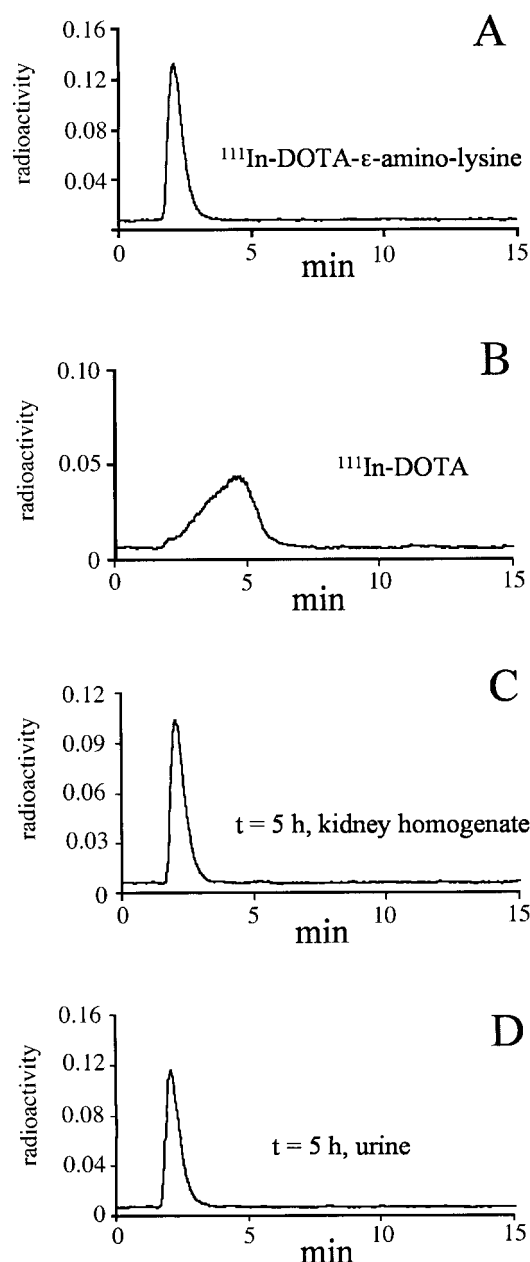


Figure 4. Anion-exchange chromatography analysis of kidney homogenates and urine samples. Kidney homogenates and urine samples taken at 5 h from mice injected with 10 μCi of [^{111}In]DOTA-Rituxan Fab were analyzed on a Poros-HQ column and compared with markers. (A) [^{111}In]DOTA- ϵ -amino-lysine. (B) [^{111}In]DOTA. (C) Kidney homogenate, 5 h. (D) Urine, 5 h.

>200% ID/g for the [^{177}Lu]DOTA-scFv and slowly dropped to >150% ID/g by 48 h. As expected, the ^{125}I -labeled version had low kidney uptake (10% ID/g at 1 h and <2% ID/g at 48 h). More recently, Govindan et al. (1998) studied the biodistribution of ^{88}Y -labeled DOTA-F(ab')₂ fragments of two antibodies (anti-CEA and anti-lymphoma). High retention was observed in the kidneys at 4, 24, and 48 h (70–104% ID/g). Although the Fab fragment used in our study has a smaller molecular size and we utilized ^{111}In rather than ^{88}Y as the radiometal, the biodistributions were quantitatively similar. In addition, the presence of tumor in their study did not affect the high kidney uptake. This study suggests the possibility of in vivo breakdown of their F(ab')₂ to Fab' fragments which are small enough to be cleared by the kidney. In agreement with this possibility Lamki et al.

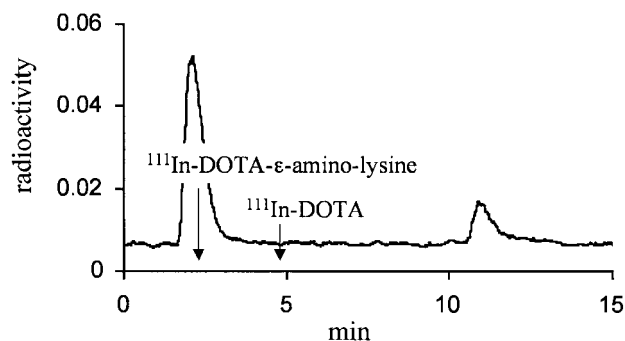


Figure 5. Anion-exchange chromatography analysis of patient urine sample. Urine sample from a patient being imaged with [^{111}In]DOTA-chT84.66 was analyzed on a Poros-HQ column. The urine sample corresponded to 24–48 h after administration of the pre-therapy [^{111}In]DOTA-chT84.66 antibody. An aliquot (200 μL) was applied to the column. The elution positions of [^{111}In]DOTA and [^{111}In]DOTA- ϵ -amino-lysine are indicated by arrows.

(Lamki et al., 1990) showed that chemically stabilized ^{111}In -labeled F(ab')₂ fragments have low kidney uptake.

An alternative approach to lower renal uptake has been to attach chelates to antibody fragments via a metabolically labile linker such as cleavable ester linkers (Meares et al., 1988; Paik et al., 1989; Weber et al., 1990; Arano et al., 1996). However, the results have been uniformly disappointing, giving only a modest reduction in kidney retention, and sometimes leading to cleavage within the blood.

While our study showed that [^{111}In]DOTA-Fab has high kidney uptake, injection of the metabolite [^{111}In]DOTA- ϵ -amino-lysine, results in excretion directly into the urine. These data suggest that [^{111}In]DOTA- ϵ -amino-lysine produced by metabolism of radiolabeled DOTA-antibody in other tissues would clear rapidly from the blood and not be retained in the kidney. Thus, the accumulation in kidney occurs at the level of the antibody fragment and failure to excrete the majority of the metabolic product produced there (e.g., see Figure 1).

On the basis of these results, we are now designing DOTA-based chelates that are expected to have enhanced kidney clearance. Our strategy is based on the peptide approach of Arano and co-workers (Wakisaka et al. 1997; Arano 1998; Arano, Fujioka et al. 1999) and Meares and co-workers (Li and Meares 1993; Peterson et al. 1999) who have appended their radiolabeled moiety to peptides. This approach promises to decrease renal uptake and/or increase the rate of metabolism in the kidney.

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Carbohydrate-Directed Conjugation of Cobra Venom Factor to Antibody by Selective Derivatization of the Terminal Galactose Residues

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Cobra venom factor (CVF) can cause cell death by complement-mediated bystander cell lysis. Several studies have investigated CVF for application in cancer therapy by conjugating CVF to antibodies against tumor cell surface-specific antigens via the side-chain amino acid residues. In most cases, the activity of CVF was markedly impaired, presumably by modification of the factor B binding domain due to random derivatization. Since CVF is a glycoprotein and its oligosaccharide chains are distal to the factor B binding domain, coupling of CVF to antibodies through its oligosaccharide chains is expected to yield immunoconjugates with retention of CVF activity and elimination of the immunoreactivity of the terminal α -galactosyl residues. In this study, we investigated the carbohydrate site-directed conjugation of CVF to a monoclonal IgG specific to a cell-surface antigen of human ovarian cancer cells. The terminal galactosyl residues of CVF were selectively modified at C-6 by treatment with galactose oxidase, and the generated aldehyde groups were derivatized in situ with hydrazides containing either protected thiol or maleimide functional groups. The CVF derivatives were allowed to react with thiol groups introduced to the antibody by derivatization with 2-iminothiolane to yield carbohydrate site-directed CVF–antibody conjugates. In both cases, 30–40% of the antibody cross-linked to CVF to yield predominantly monovalent CVF–antibody conjugates. The purified immunoconjugates retained 70–75% of CVF activity and significant level of antigen-binding capacity. This is the first study to exploit the oligosaccharide chains of CVF for the preparation of active immunoconjugates.

INTRODUCTION

Cobra venom factor (CVF),¹ a nontoxic complement-activating glycoprotein in cobra venom, is a functional analogue of mammalian complement component C3b. When added to mammalian serum, CVF forms CVF,Bb, a C3/C5 convertase of the alternative pathway of the complement (1, 2). CVF,Bb can activate the complement in a manner similar to C3b,Bb, the physiologic C3/C5 convertase. However, CVF,Bb is completely resistant to the action of complement regulatory factors H and I. Therefore, when administered to animals, CVF can deplete the complement and build up membrane-attaching complexes; the latter can cause bystander lysis of cells (1). This property of CVF has been used for selective killing of tumor cells by conjugating to antibodies against cancer-specific cell surface antigens (1, 3–10).

CVF consists of three polypeptide chains, designated α , β , and γ , and three N-linked oligosaccharides; two in

the α -chain, one in the β -chain, and no carbohydrates in the γ -chain (11, 12). More than 80% of the oligosaccharide chains of CVF terminate with the unusual α -galactosylated Lewis X structures (12–14). CVF is immunoreactive to natural human anti- α -Gal antibody because of the presence of terminal Gal α 1–3Gal residues in its oligosaccharide chains (12, 15). Therefore, therapeutic application of CVF requires the abolition of this immunoreactivity. Although the anti- α -Gal antibody immunoreactivity of CVF can be abolished by the removal of terminal galactosyl residues, the exposed Lewis X structures are recognized by liver cell lectins, leading to rapid clearance of CVF from the circulation (16).

The complement-activating activity of CVF is not affected by either the removal of its oligosaccharide chains or the binding of the terminal α -galactosyl residues with lectin or antibodies (12). This implies that the oligosaccharide chains of CVF are distal to the factor B binding domain. Therefore, coupling of CVF through the terminal galactosyl residues to antibodies should yield immunoconjugates with the retention of the CVF activity and simultaneously eliminating the anti- α -Gal immunoreactivity due to masking of the terminal α -galactosyl residues. In addition, this mode of cross-linking of CVF to antibodies should avoid the rapid, asialoglycoprotein receptor-dependent, hepatic clearance of CVF immunoconjugates from the circulation. We have recently shown that the terminal galactosyl residues of CVF can be modified conveniently at C-6 by galactose oxidase treatment and in situ derivatization with hydrazides (17) and that this modification completely abolishes the anti- α -Gal immunoreactivity without affecting CVF activity (17).

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¹ Abbreviations: CVF, cobra venom factor; PDPH, 3-(2-pyridyldithio)propionic acid hydrazide; MPBH, 4-(4-N-maleimidophenyl)butyric acid hydrazide; M₂C₂H, 4-(maleimidomethyl)-cyclohexane-1-carboxylic acid hydrazide; PDPH-CVF, PDPH derivative of CVF; M₂C₂H-CVF, M₂C₂H derivative of CVF; MPBH-CVF, MPBH derivative of CVF; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); SDS–PAGE, sodium dodecyl sulfate-polyacrylamide electrophoresis; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; DTT, dithiothreitol; OVB-3, mouse monoclonal IgG_{2b} directed against a cell surface antigen of human ovarian cancer cells.

In this study, we used this site-specific derivatization procedure to conjugate CVF to a monoclonal antibody (OVB-3) directed against a cell surface antigen of human ovarian cancer cells and assessed the purified immunokonjugates for CVF and antibody activities.

EXPERIMENTAL PROCEDURES

Materials. CVF was isolated from *Naja naja kaouthia* venom as described previously (18). 3-(2-Pyridyldithio)propionic acid hydrazide (PDPH) and 4-(4-*N*-maleimidophenyl)butyric acid hydrazide (MPBH) were synthesized as reported previously by Zara et al. and Chamow et al., respectively (19, 20). 4-(Maleimidomethyl)-cyclohexane-1-carboxylic acid hydrazide (M_2C_2H), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and 2-iminothiolane hydrochloride were purchased from Pierce Chemical Co. (Rockford, IL). Galactose oxidase (25 units/mg of protein) and coffee bean α -galactosidase (10 units/mg of protein) were from Boehringer Mannheim (Indianapolis, IN). HRP (type VIA, 1000 units/mg) was from Sigma Chemical Co. (St. Louis, MO). Centricon 30 tubes were from Amicon (Danvers, MA). FITC-conjugated goat anti-mouse IgG was from Southern Biotechnology Associates, Inc. (Birmingham, AL). RPMI 1640 medium, insulin, penicillin/streptomycin were from Life Technologies (Gaithersburg, MD). Guinea pig erythrocytes were obtained by femoral vein puncture. OVB-3, a mouse monoclonal antibody (IgG_{2b}, ref 21) was a generous gift from Dr. Ira Pastan, National Cancer Institute, NIH.

Carbohydrate Analysis. OVB-3 antibody (50 μ g) was hydrolyzed with 400 μ L of 2.5 M trifluoroacetic acid at 100 °C for 5 h. The hydrolysates were evaporated to dryness in a Speed Vac, dissolved in water and then analyzed for neutral sugars and hexosamines using a Dionex HPLC (Dionex Corp., Sunnyvale, CA) with pulsed amperometric detection on a CarboPac PA1 column (4 $\times$ 250 mm) (22). Elution was with 20 mM NaOH at a flow rate of 0.9 mL/min.

Sialic acids from OVB-3 antibody (20 μ g) were quantitatively released either by hydrolysis with 0.1 M sulfuric acid (40 μ L) at 80 °C for 50 min or by treatment with *Clostridium perfringens* sialidase (20 milliunits) in 50 μ L of 50 mM sodium acetate, pH 5.5, at 37 °C for 24 h. The released sialic acids were analyzed on a CarboPac PA1 high-pH anion-exchange column (4 $\times$ 250 mm) with 100 mM NaOH/150 mM NaOAc at a flow rate of 0.8 mL/min (22).

Derivatization of the Terminal Galactosyl Residues of CVF by Acid Hydrazides. Derivatization of the primary hydroxyl groups of the terminal galactosyl residues of CVF was carried out by a modification of the published procedures (23, 24). CVF (2–2.5 mg) in 1 mL of 100 mM sodium phosphate, pH 6.5, was incubated with galactose oxidase (10 units, added in two equal aliquots at 0 and 8 h), HRP (30 units/mL) and PDPH (50–100 mol/mol CVF) at 37 °C for 30 h (17). To minimize the hydrolysis of reactive maleimide groups to maleic acid, derivatization of the enzyme-oxidized CVF with MPBH or M_2C_2H was performed at the end of enzyme incubation for 2 h at room temperature. Uncoupled hydrazides were removed by filtration using Centricon 30 tubes or by gel filtration on Sephadex G-25 columns (0.9 $\times$ 15 cm) in 50 mM sodium phosphate, 150 mM NaCl, 2 mM EDTA, pH 6.5, 7.2 or 8.0.

To determine the extent of derivatization, an aliquot of PDPH-CVF (150 μ g in PBS, pH 7.2) was reduced with 50 mM DTT at room temperature for 20 min (25). The released pyridine-2-thione was determined by measuring

the absorbance of the solution at 343 nm and by using the molar extinction coefficient of $8.08 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (25).

To determine the amount of M_2C_2H or MPBH coupling to CVF, M_2C_2H -CVF, and MPBH-CVF were reduced with sodium borohydride and treated with coffee bean α -galactosidase. The released galactose was measured by HPLC using high-pH anion-exchange column (see below).

Stability of M_2C_2H and MPBH in Buffer Solutions. Stock solutions of M_2C_2H or MPBH (28 mM in *N,N*-dimethylformamide) were diluted to 350 nM with 100 mM NaOAc (pH 5.5) or 100 mM sodium phosphate (pH 6.5, 7.0, 7.5, or 8.0). The hydrolysis of the maleimide moiety of M_2C_2H and MPBH was monitored at intervals by measuring the increase in absorbance at 320 nm for M_2C_2H or the decrease in absorbance at 300 nm for MPBH (20).

Derivatization of OVB-3 with 2-Iminothiolane. OVB-3 (1.2 mg) in 800 μ L of 50 mM sodium phosphate, 150 mM NaCl, 2 mM EDTA, pH 8.0, was incubated in a nitrogen atmosphere with 2-iminothiolane (104 μ g) at room temperature for 1 h (26). The unreacted 2-iminothiolane was removed by gel filtration on Sephadex G-25 columns (0.9 $\times$ 15 cm) in 150 mM NaCl, 2 mM EDTA, pH 8.0. The amount of 2-iminothiolane that coupled to OVB-3 was determined by treating the derivatized antibody with DTNB and measuring spectrophotometrically the released 2-nitro-5-thiol-benzoic acid (26).

Coupling of Hydrazide-Derivatized CVF to 2-Iminothiolane Derivative of OVB-3. PDPH-CVF, M_2C_2H -CVF and MPBH-CVF (0.5 mg each) were separately mixed with 2-iminothiolane-derivatized OVB-3 (0.5 mg) in 400 μ L of 150 mM NaCl, 2 mM EDTA, pH 6.5, 7.2, or 8.0, and incubated overnight at 25 °C with gentle rocking in a nitrogen atmosphere. Unreacted thiol groups in the derivatized OVB-3 and maleimide groups in M_2C_2H -CVF and MPBH-CVF were blocked with cysteine at 25 °C for 1 h. Aliquots of the solutions were analyzed by SDS-PAGE under nonreducing conditions (27).

The reaction mixtures (200–250 μ g protein) were chromatographed on Bio-Silect SEC 400-5 gel filtration HPLC columns (300 $\times$ 7.8 mm) (Bio-Rad, Hercules, CA) using a Dionex Bio-LC HPLC in PBS, pH 7.2. The elution was with 50 mM sodium phosphate, 150 mM NaCl, pH 7.2, at a flow rate of 0.9 mL/min. Proteins were monitored by on-line UV detection at 280 nm using a Dionex variable wavelength detector. Fractions corresponding to CVF-IgG conjugates were pooled and analyzed for CVF activity.

Derivatization of the Oligosaccharide Chains of OVB-3 with PDPH. OVB-3 (2 mg/mL) in 50 mM sodium phosphate, 150 mM NaCl, pH 6.5, was treated with 5 mM sodium periodate (1 mL) in the above buffer at 4 °C for 10 min. Excess periodate was allowed to react with glycerol (25 μ L) at 4 °C for 30 min. The solution was dialyzed against 50 mM sodium phosphate, 150 mM NaCl, pH 6.5, and then incubated with 50-fold molar excess PDPH at 25 °C for 5 h. The amount of PDPH coupled to the antibody was determined as described above (25).

Coupling of CVF to OVB-3 via the Carbohydrate Moieties of Both Proteins. The PDPH-derivatized OVB-3 (0.5 mg) was treated with 20 mM DTT in 200 μ L of 100 mM NaOAc, pH 4.5, at 25 °C for 20 min. The solution was chromatographed on Sephadex G-25 (0.9 $\times$ 15 cm) in 50 mM sodium phosphate, 150 mM NaCl, 2 mM EDTA, pH 8.0. The void volume fractions were concentrated in Centricon 30 tubes and allowed to react with PDPH-CVF (0.5 mg) in 250 μ L of 50 mM sodium

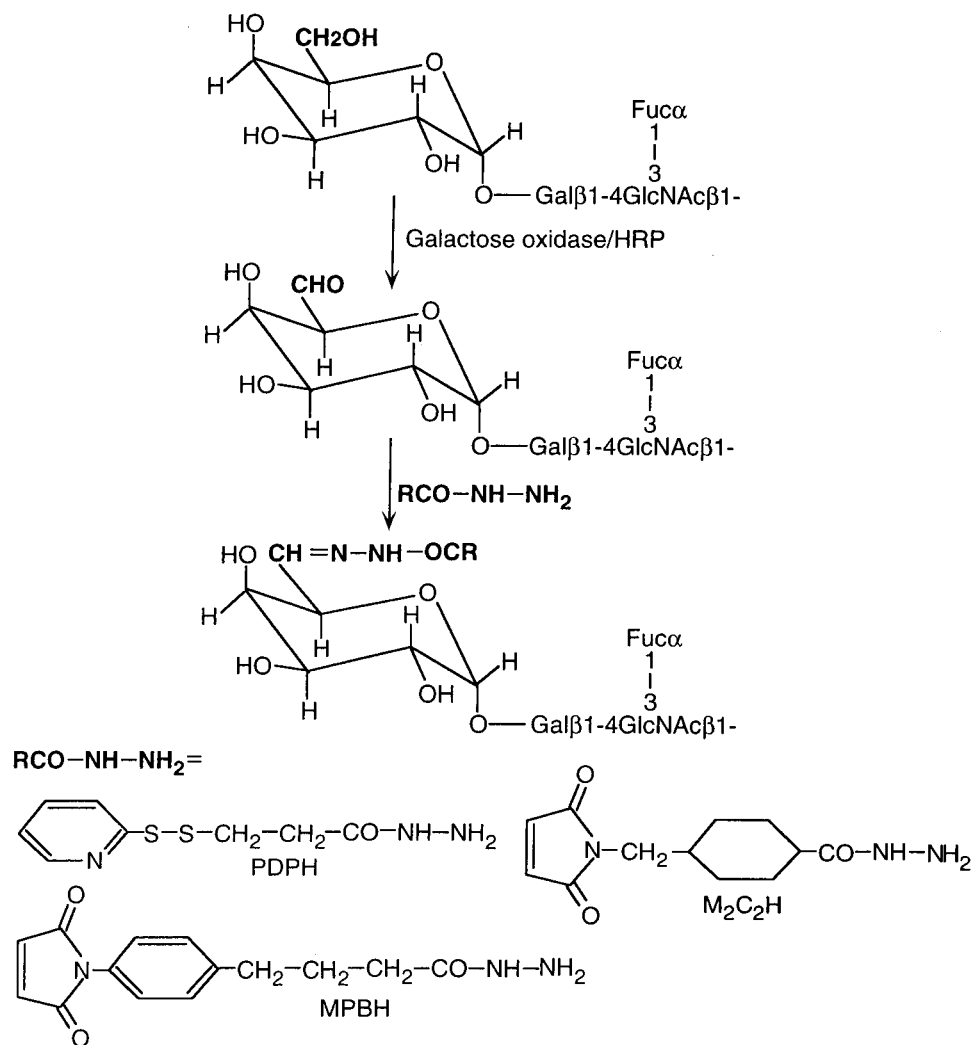


Figure 1. Scheme for the derivatization of the terminal galactosyl residues of the oligosaccharide chains (partial structure is shown) of CVF with heterobifunctional hydrazides. Oxidation of CVF with galactose oxidase in the presence of HRP generates aldehyde groups at the C-6 positions of the terminal galactose residues. The derivatization of the aldehyde groups with protected thiol group- or maleimide group-containing hydrazides yielding stable hydrazones offers a convenient approach to couple CVF to antibodies.

phosphate, 150 mM NaCl, 2 mM EDTA, pH 8.0, overnight at 25 °C in a nitrogen atmosphere. Unreacted thiol groups were blocked with cysteine at 25 °C for 1 h. Aliquots of the solutions were analyzed on 5% SDS-polyacrylamide gel under nonreducing conditions.

Hemolytic Assay for CVF Activity. CVF, hydrazide-derivatized CVF, or CVF-OVB-3 conjugates (8–2000 ng CVF protein in each case) in PBS, 1 mM CaCl_2 , 0.5 mM MgCl_2 , and 0.1% (w/v) gelatin, pH 7.2 (20 μL) were mixed with guinea pig serum (20 μL) and guinea pig erythrocytes (10×10^6 in 20 μL of the above buffer) and incubated at 37 °C for 40 min (12). Cold PBS, pH 7.2 (1 mL), was added, the unlysed erythrocytes were removed by centrifugation and the released hemoglobin was measured at 412 nm.

OVB-3 Binding to Ovarian Cancer Cell Surface Antigen. Human ovarian cancer cells (NIH:OVCAR-3 cells obtained from ATCC, Rockville, MD) were cultured as monolayers in RPMI 1640 medium with 10% fetal calf serum, insulin (10 $\mu\text{g}/\text{mL}$), penicillin (100 units/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$), according to Pirker et al. (28). Cells were detached by gentle scraping, washed with serum-free medium and suspended in PBS, pH 7.2, containing 2% bovine serum albumin. The cells were treated with OVB-3, 2-iminothiolane-derivatized OVB-3 or CVF-OVB-3 conjugates (5 μg antibody/mL in PBS, pH

7.2) at 4 °C for 2 h. After washing with PBS, pH 7.2, the cells were treated with FITC-labeled goat anti-mouse IgG at 4 °C for 1 h, washed, fixed with 2% paraformaldehyde, and surface fluorescence was measured using a Becton Dickinson FACS II analyzer at the Georgetown University Lombardi Cancer Center core facility.

RESULTS

Derivatization of Terminal Galactosyl Residues of CVF with Hydrazides. CVF contains approximately 5.6 terminal galactosyl residues (~ 4.1 α -linked and ~ 1.5 β -linked) (17). The C-6 hydroxyl groups of the terminal galactosyl residues were oxidized with galactose oxidase in the presence of HRP and the generated aldehyde groups were derivatized with three different hydrazides, which contain electrophilic centers for cross-linking of CVF to antibodies as depicted in Figure 1 (17). Treatment of PDPH-CVF with DTT released about 5.5 mol of pyridyl-2-thione, indicating that $>95\%$ of the terminal galactosyl residues of CVF were derivatized. In agreement with this result, treatment of sodium borohydride-reduced PDPH-CVF, $\text{M}_2\text{C}_2\text{H}$ -CVF, or MPBH-CVF with coffee bean α -galactosidase did not release detectable amounts of galactose. The hemolytic activity of CVF derivatized with all three hydrazides, as measured by the

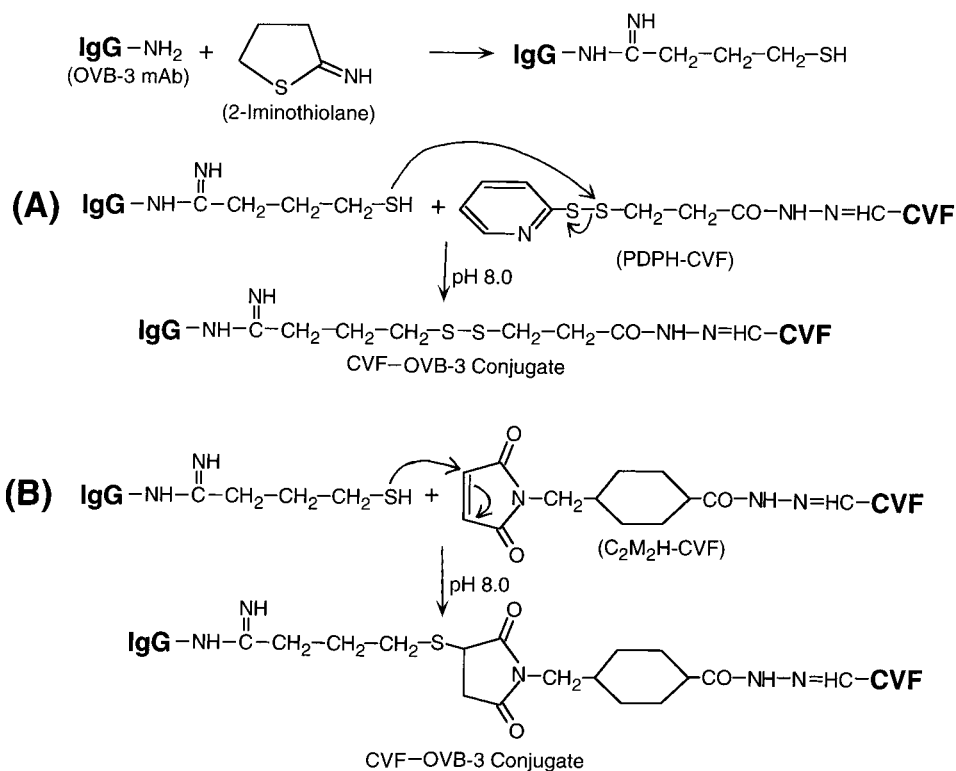


Figure 2. Scheme for the conjugation of OVB-3 to the oligosaccharide chains of CVF. The derivatization of the OVB-3 with 2-iminothiolane introduces thiol groups to the antibody. When the derivatized antibody allowed to react with PDPH-CVF (A) or M₂C₂H-CVF (B) under nitrogen atmosphere yields disulfide- or thioether-linked CVF-OVB-3 conjugates.

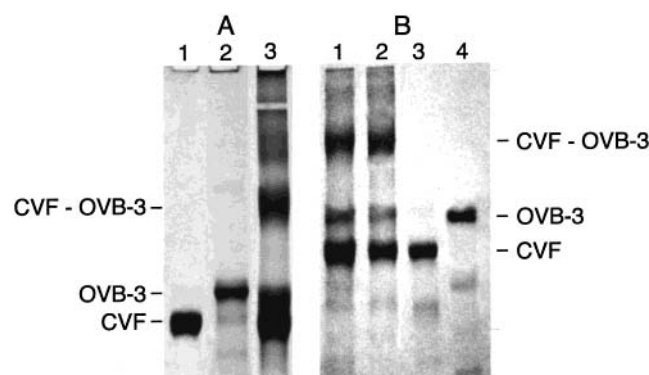


Figure 3. SDS-polyacrylamide gel electrophoresis of CVF-OVB-3 conjugates. The hydrazide-derivatized CVF (5–10 μ g), 2-iminothiolane derivatized OVB-3 (5–10 μ g) and CVF-OVB-3 conjugates (20–30 μ g), prepared as outlined in Figure 2, were electrophoresed on 5% (panel A) and 6% (panel B) SDS-polyacrylamide gel under nonreducing conditions. The gels were stained with Coomassie blue. (A) Lane 1, PDPH-CVF; lane 2, 2-iminothiolane derivative of OVB-3; lane 3, CVF-OVB-3 conjugates prepared from PDPH-CVF. Panel B: Lane 1, CVF-OVB-3 prepared from MPBH-CVF; lane 2, CVF-OVB-3 prepared from M₂C₂H-CVF; lane 3, MPBH-CVF; lane 4, 2-iminothiolane derivatized OVB-3.

bystander lysis of guinea pig erythrocytes in guinea pig serum, was similar to that of untreated CVF (see Figure 5).

Carbohydrate Moieties of OVB-3. In view of coupling hydrazide-derivatized CVF to carbohydrate moieties of the monoclonal antibody OVB-3, the nature of the carbohydrate moieties of the antibody was examined. More than 90% of the carbohydrate moiety of OVB-3 was released by *N*-glycanase, an enzyme that removes asparagine-linked oligosaccharides in glycoproteins (29). Carbohydrate compositional analysis of the enzyme-released carbohydrates revealed sialic acid, galactose,

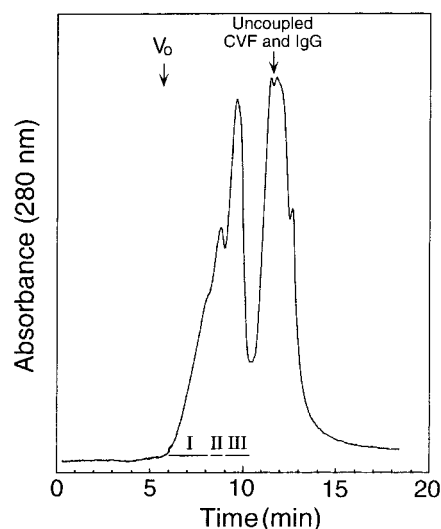


Figure 4. HPLC fractionation of CVF-OVB-3 conjugates. The CVF-OVB-3 conjugates prepared as shown in Figure 2 were fractionated by HPLC gel filtration. Shown is the separation of CVF-OVB-3 prepared from the conjugation of 2-iminothiolane-derivatized OVB-3 to PDPH-CVF. PDPH-CVF monovalent conjugates (fraction III, $M_r \approx 300$ kDa) and multivalent conjugates (fractions II and I, $M_r \approx 450$ and 600 kDa, respectively) were pooled as indicated. The immunoconjugates prepared by cross-linking 2-iminothiolane derivatized OVB-3 to MPBH-CVF and M₂C₂H-CVF were also fractionated by HPLC (not shown).

N-acetylglucosamine, mannose, fucose, *N*-acetylgalactosamine in the approximate molar ratios of 2.1:2.8:4.4:3.0:0.9:0.3. Sialic acid was found to be a mixture of 60% *N*-acetylneuraminic acid and 40% *N*-glycolylneuraminic acid. The results suggest that the carbohydrate moieties of OVB-3 are predominantly complex type *N*-linked oligosaccharides, present most likely in the Fc region of

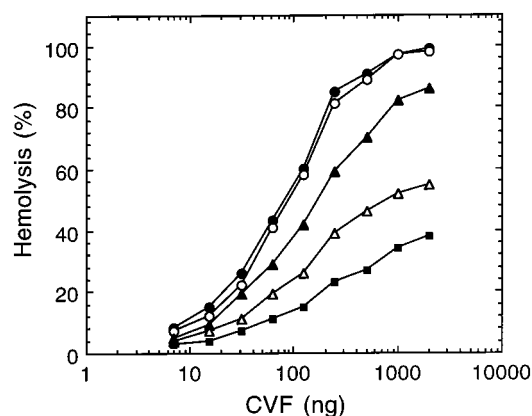


Figure 5. Effect of hydrazone derivatization and conjugation of the antibody on CVF activity. CVF activity was measured by complement-mediated lysis of unsensitized guinea pig erythrocytes. The indicated amounts of the hydrazone derivatives of CVF and CVF-antibody conjugates were incubated with unsensitized guinea pig erythrocytes in guinea pig serum. The absorbance of the released hemoglobin was measured at 412 nm. (●) CVF; (○) PDPH-CVF; (▲) CVF-OVB-3 conjugate fraction III in Figure 4; (△) CVF-OVB-3 conjugate fraction II in Figure 4; (■) CVF-OVB-3 conjugate fraction I in Figure 4.

the antibody. IgGs contain two *N*-linked oligosaccharides in their Fc regions; one oligosaccharide per polypeptide chain.

Derivatization of OVB-3 with 2-Iminoethanol and Conjugation to Hydrazone Derivatives of CVF.

The OVB-3 antibody was derivatized with 2-iminoethanol, a widely used reagent for the introduction of thiol groups to proteins for cross-linking (26, 30–33). Treatment of OVB-3 with ~90-fold molar excess of 2-iminoethanol introduced 4–5 thiol groups/molecule of the antibody. The derivatization procedure did not significantly affect the antigen-binding activity of the antibody (see legend to Figure 6).

OVB-3 derivatized with 2-iminoethanol was allowed to react separately with the PDPH-, M_2C_2H -, and MPBH-derivatives of CVF at various pH according to the scheme outlined in Figure 2. SDS-PAGE analysis of the reaction mixtures showed that the derivatized OVB-3 efficiently conjugated to PDPH-CVF at pH 8.0, yielding a major product at ~300 kDa and minor products at >450 kDa; approximately 40% of OVB-3 was coupled to CVF (Figure 3). The cross-linking of OVB-3 to PDPH-CVF was 5–10% at pH 6.5 or 7.2 (not shown). In the case of M_2C_2H -CVF and MPBH-CVF, the coupling efficiency at pH 8.0 was <10% (not shown), which is likely due to the rapid hydrolysis of maleimide groups compared with the rate of conjugation under the basic reaction conditions employed (see below).

To determine appropriate conditions for cross-linking OVB-3 to MPBH-CVF and M_2C_2H -CVF, we studied the rate of hydrolysis of maleimide groups in MPBH and M_2C_2H by incubating these compounds in aqueous buffers at pH 5.5, 6.5, 7.0, 7.5, and 8.0, as described previously (20). The maleimide groups in these compounds were relatively stable at pH ≤ 7, but hydrolyzed significantly at pH 8.0 (not shown). These results agree with the previously reported property of maleimide functional group-containing compounds (20, 32, 33). Therefore, we carried out the conjugation of 2-iminoethanol-derivatized OVB-3 to M_2C_2H -CVF and MPBH-CVF at pH 7.2. Under this condition, approximately 30–35% of OVB-3 was coupled to CVF, yielding immunoconjugates at levels similar to those formed in the case of PDPH-CVF (Figure 3).

HPLC separated the immunoconjugates from the unreacted compounds as multiple peaks; in each case, a major conjugate corresponding to peak at ~300 kDa, a minor partially resolved peak at ~450 kDa, and a minor unresolved shoulder at ~600 kDa (Figure 4). The CVF-antibody conjugate fractions (fractions I, II, and III) were pooled as shown in Figure 4. The conjugates obtained from the cross-linking OVB-3 to PDPH-CVF were treated with DTT and analyzed by SDS-PAGE under reducing conditions. Fractions I and III contained approximately equal amounts of CVF and OVB-3, whereas fractions II had about 30–40% CVF and 60–70% OVB-3. These results together with the observed molecular weights of the conjugate fractions suggest that fraction III is a monovalent conjugate (one molecule each of CVF and OVB-3), fraction II is CVF₂-IgG and CVF-IgG₂, and fraction I is CVF₂-IgG₂.

The HPLC-fractionated CVF-OVB-3 conjugates (see Figure 4) were analyzed for CVF activity and OVB-3 antigen-binding capacity. CVF in the CVF-OVB-3 monovalent conjugate (fraction III) exhibited 70–75% hemolytic activity compared with that of the unmodified or the PDPH-, MPBH-, or M_2C_2H -derivatized CVF (Figure 5), whereas CVF in conjugate fractions I and II showed ~20–30% and 40–50% activity, respectively (Figure 5). The antigen-binding activity of OVB-3 in CVF-OVB-3 conjugate fractions was assessed by measuring cell surface immunofluorescence using NIH:OVCAR-3 cells cultured as monolayers. OVB-3 in the immunoconjugate fraction III showed a significant level of antigen-binding capacity, whereas the antibody in conjugate fractions I and II exhibited only marginal activity (Figure 6).

Cross-Linking of OVB-3 to CVF via the Carbohydrate Moieties of Both Glycoproteins. Since 40% of the sialic acid residues in OVB-3 are *N*-glycolylneuraminic acid, which is not expressed in man, therapeutic use of OVB-3 requires the removal or masking of these residues. Cross-linking of CVF to the sialic acid residues of OVB-3 is expected to mask the possible adverse effects of *N*-glycolylneuraminic acid. Besides, this approach should yield immunoconjugates with retention of the CVF and antibody activities because the factor B-binding site in CVF and antigen-binding site in OVB-3 are not modified. Therefore, we studied the coupling of PDPH-CVF to the carbohydrate moieties of OVB-3. Aldehyde groups were generated on the sialic acid residues of the oligosaccharides of OVB-3 by mild oxidation with sodium periodate and they were then derivatized with PDPH. This procedure introduced 3–3.5 residues of PDPH to OVB-3. The PDPH derivative of OVB-3 was treated with DTT, and the generated thiol groups were allowed to react with PDPH-CVF as outlined in Figure 7. SDS-PAGE analysis showed that the coupling efficiency of the reaction was very low; <5% CVF and the antibody were coupled to form CVF-OVB-3 conjugates (Figure 8). This low coupling efficiency was surprising considering that OVB-3 and CVF contained 3.5 and 5.5 reactive groups per mol of protein, respectively. The reason for this low coupling efficiency could be due to the limited accessibility of the carbohydrate moieties of OVB-3 because of their localization in the sterically hindered hinge region of the antibody. This is consistent with the results of a previous study in which the reactive groups on the side-chain amino groups of an antibody were better accessible than those on the carbohydrate chains in the Fc regions of the antibody for coupling with CVF containing protected thiol groups (34). Another reason for the low coupling efficiency could be that the presence of reactive functional groups on the flexible oligosaccharide chains of both proteins

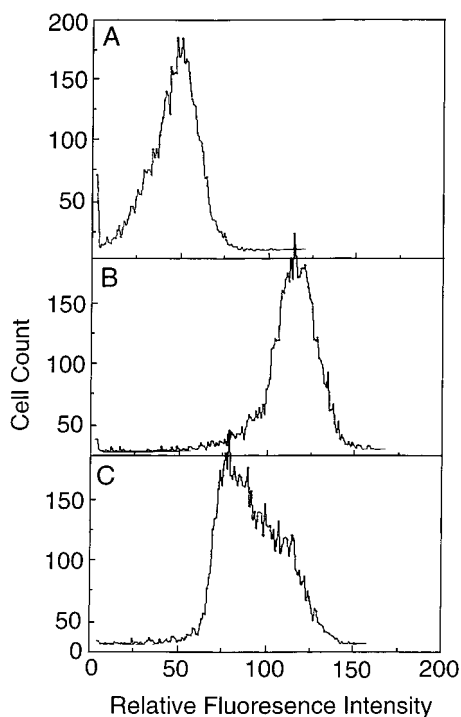


Figure 6. Effect of coupling of OVB-3 to CVF on the antigen-binding activity of the antibody. NIH:OVCAR-3 cells were incubated with nonspecific mouse IgG (A), OVB-3 (B), or CVF-OVB-3 conjugate (fraction III in Figure 4) (C). After washing, the cells were treated with FITC-conjugated goat anti-mouse IgG, washed, fixed with paraformaldehyde, and the intensity of cell surface immunofluorescence was measured by flow cytometry. 2-Iminothiolane-treated OVB-3 showed a similar immunofluorescence profile (not shown) compared with that of the untreated OVB-3. The binding of CVF-OVB-3 conjugate fractions I and II to NIH:OVCAR-3 cells was also examined; both fractions exhibited only marginal activity (not shown).

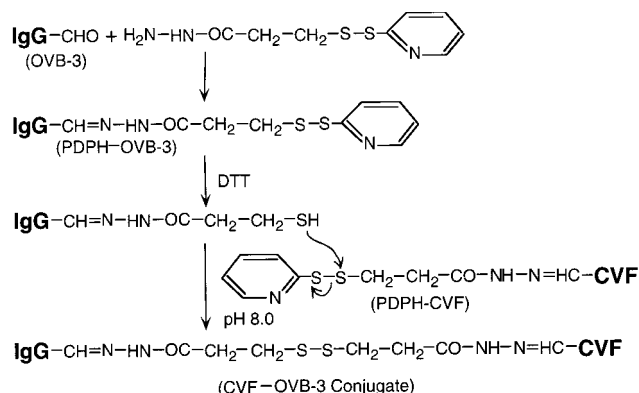


Figure 7. Scheme for the conjugation of the antibody OVB-3 to CVF through the carbohydrate moieties of CVF and OVB-3. Treatment of OVB-3 with sodium periodate under mild conditions forms aldehyde groups at C-7 or C-8 of the terminal sialic acid residues of OVB-3. Derivatization of the aldehyde groups with PDPH and treatment with DTT generates free thiol groups in OVB-3. Incubation of the derivatized OVB-3 overnight at room temperature with PDPH-CVF under nitrogen atmosphere yields CVF-OVB-3 conjugates.

markedly limits the probability of the functional groups to come in close proximity of one another for efficient coupling.

DISCUSSION

CVF immunoconjugates offer several advantages over conjugates of toxins (35, 36) or drugs (38) for the targeted killing of tumor cells. First, CVF is nontoxic, and cell

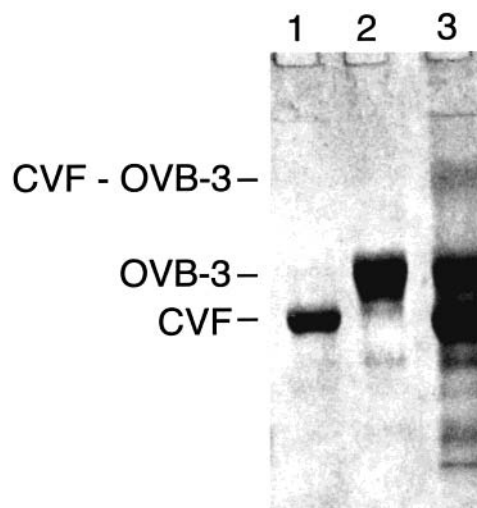


Figure 8. SDS-polyacrylamide gel electrophoresis of CVF-OVB-3 conjugates. The CVF-OVB-3 conjugates (20–30 μg) prepared as shown in Figure 7 were electrophoresed on 6% SDS-polyacrylamide gel under nonreducing conditions. The gels were stained with Coomassie blue. Lane 1, PDPH-CVF; lane 2, PDPH-OVB-3; lane 3, CVF-OVB-3 conjugates.

killing is mediated by the host effector mechanism, namely the complement activation (1). Second, unlike toxins or drugs, CVF does not require internalization by the target cells. Third, since CVF is not a drug or a mutagen, it does not cause complications such as induction of secondary malignancies or resistance to cytotoxicity (36). However, the immunoreactivity of the carbohydrate moieties of CVF to natural anti- α -Gal antibodies in humans limits the therapeutic application of CVF. Carbohydrate site-directed conjugation of CVF can mask this immunoreactivity.

Previously, CVF-antibody conjugates have been prepared by coupling CVF to various tumor-cell surface specific antigens and studied the ability of conjugates to kill tumor cells by complement-mediated cell lysis (1, 3–10). In those studies, CVF was coupled to the antibodies through the ϵ -amino groups of lysine residues using heterobifunctional reagents containing amino group-reactive succinimidyl esters (33, 37–39). The activity of CVF was markedly impaired because the random derivatization of the amino acid side chains of CVF resulted in the conjugation of antibodies at or in the proximity of the factor B binding domain, presumably blocking factor B binding. However, the oligosaccharide chain-directed coupling of CVF to antibodies should yield immunoconjugates with retention of CVF activity while simultaneously masking the anti- α -Gal immunoreactivity (12). In this study, we pursued the carbohydrate site-directed cross-linking of CVF to OVB-3, a monoclonal antibody specific to a cell surface antigen of human ovarian cancer cells, and assessed the complement-activating activity of CVF and the cell surface antigen-binding capacity of the antibody (21).

Carbohydrate-directed cross-linking of glycoproteins to proteins is commonly accomplished by derivatization of terminal sugar residues with heterobifunctional hydrazides. Oxidation with sodium periodate under mild conditions to generate aldehyde groups has been used extensively for the hydrazide-derivatization of the terminal sugar residues of glycoproteins (40–42). When this approach was used for the derivatization of terminal galactosyl residues of CVF, the complement-activating activity of CVF was markedly decreased due to the oxidation of critical methionine residue(s) (43). To overcome this problem, we sought a derivatization method

for the facile introduction of functional groups into the carbohydrate moiety of CVF that are amenable to conjugation. Recently, we reported that oxidation of CVF with galactose oxidase in the presence of acid hydrazides introduces functional groups on the C-6 position of the terminal galactosyl residues of CVF that can be used for conjugation to antibodies (17). This procedure selectively and quantitatively derivatizes the terminal galactosyl residues of CVF with complete retention of functional activity and simultaneous elimination of the anti- α -Gal immunoreactivity (17). In the present study, we used this strategy for the conjugation of CVF to the OVB-3 antibody. Three different heterobifunctional hydrazides, PDPH, M_2C_2H , and MPBH, which are amenable to cross-linking to antibodies, were coupled to the aldehyde groups formed at the C-6 positions of the terminal galactosyl residues of CVF (17). The derivatization was quantitative irrespective of the hydrazide used. As reported previously, this procedure eliminated the anti- α -Gal immunoreactivity without affecting CVF activity (17).

When PDPH-CVF, M_2C_2H -CVF, and MPBH-CVF were allowed to react at pH 8.0 with OVB-3 derivatized with 2-iminothiolane, a significant difference in coupling efficiency was apparent. Whereas about 40% of OVB-3 coupled to PDPH-CVF at pH 8.0, only about 10% of the antibody coupled to M_2C_2H -CVF or MPBH-CVF under this condition. The low antibody coupling efficiency to M_2C_2H -CVF and MPBH-CVF was likely due to the rapid inactivation of maleimide groups by base-catalyzed hydrolysis at pH 8.0. The maleimide functional group-containing heterobifunctional reagents have been widely and successfully used for protein-protein or protein-hapten conjugation (20, 33). Although the maleimide group is relatively stable under acidic pH, it undergoes rapid hydrolysis at pH >8.0. Therefore, protein-protein cross-linking involving nucleophilic addition of thiol group to maleimide moiety have been performed in most cases at pH between 6.5 and 7.0 (20, 33). When the thiol group-containing OVB-3 was allowed to couple to M_2C_2H -CVF or MPBH-CVF at pH 6.5, the efficiency was poor; only 5–10% of the antibody conjugated to CVF. However, when the reaction was carried at pH 7.2, the yield of the CVF-OVB-3 conjugates was 30–35%. Thus, the results of this study demonstrate that the hydrazides containing either protected thiol group or reactive maleimide group are amenable, under appropriate conditions, for the carbohydrate site-directed conjugation of CVF to antibodies. The results also demonstrate that the hydrazides containing protected thiol groups are superior reagents compared with those containing maleimide function for the preparation of CVF immunoconjugates. One major advantage of using protected thiol group-containing hydrazides is their stability during enzymatic oxidation of galactose residues of CVF. Thus, efficient hydrazide derivatization of the aldehyde formed on the oligosaccharide chains by the action of galactose oxidase can be achieved in situ during enzyme incubation. However, use of such a procedure for the maleimide group-containing hydrazides is likely inactivate significant amount of the maleimide moiety, despite slow hydrolysis at pH 6.5, because of long incubation period with the enzyme. Although this disadvantage can be surmounted by the addition of the hydrazides at the end of the enzyme reaction, the possibility of further oxidation of some aldehyde groups to carboxylic groups could adversely affect the derivatization of hydrazides (44). Over oxidation would be particularly significant when HRP was not added to the reaction mixture.

2-Iminothiolane is a widely used reagent to introduce reactive thiol groups into proteins for protein-protein conjugation (26, 30–33). The relatively reduced reactivity of 2-iminothiolane compared with open chain methyl 3-mercaptopropionimides allows for the better control of the degree of protein modification (31). This is important when biological activity of proteins has to be retained. Therefore, in this study, we chose to use 2-iminothiolane. While derivatization of OVB-3 with 2-iminothiolane had little or no effect on the antigen-binding capacity of OVB-3, coupling of the derivatized antibody to CVF appreciably reduced the antibody activity. Since the antigen-binding activity of 2-iminothiolane derivatized OVB-3 was similar to that of the untreated OVB-3, the results suggest that the side-chain amino acid residues in the antigen-binding sites of the antibody were not modified to a significant level. However, it appears that some of the amino acid residues proximal to the antigen-binding sites were derivatized, since coupling of OVB-3 to bulky CVF molecules caused an appreciable level of steric blockage to antigen binding.

To overcome this problem, we explored the carbohydrate site-directed coupling of OVB-3 to PDPH-CVF. OVB-3 was treated with sodium periodate under mild conditions to selectively oxidize the side-chain carbons of the terminal sialic acid residues to form aldehyde groups, which were then derivatized with PDPH. Reactive thiol groups were generated on the terminal sugar residues of oligosaccharide chains by treating PDPH-OVB-3 with DTT under conditions that specifically reduced the disulfide bonds of PDPH moiety, but not those of OVB-3. The thiol groups on the oligosaccharide chains of OVB-3 were then allowed to react with PDPH-derivative of CVF. Although this carbohydrate-to-carbohydrate coupling approach was expected to yield antibody-CVF conjugates with maximal retention of both antibody and CVF activity while simultaneously abolishing the immunoreactivities due to the α -galactosyl residues, the low coupling efficiency (<5%) limits the use of this approach for the preparation of CVF immunoconjugates.

The low coupling efficiency of carbohydrate-to-carbohydrate site-directed cross-linking of CVF to OVB-3 may be due in part to the limited accessibility of the OVB-3 carbohydrate chains, which are localized most likely in the sterically hindered hinge regions (34). This conclusion agrees with the reported very low coupling efficiency of SPDP-derivatized CVF to the hydrazide-derivatized carbohydrate moieties of an IgM (34). Additionally, the placement of reactive functional groups on highly extended, flexible oligosaccharide chains of CVF and antibody appears to drastically limit the probability of the functional groups to come in close enough proximity for conjugation.

In summary, the results presented in this paper demonstrate for the first time that the carbohydrate site-directed conjugation of CVF to the side-chain amino acid residues of antibodies is a viable approach for the preparation of CVF immunoconjugates. The conjugates obtained by this approach retained high levels of CVF and antibody activities while eliminating the anti- α -Gal immunoreactivity of CVF. The results presented here also show that derivatization of terminal sugar residues of CVF with the protected thiol-containing hydrazides offers significant advantage over the maleimide-containing hydrazides for efficient in situ derivatization of the enzyme-generated aldehyde groups. Conjugation via the carbohydrate moieties of both CVF and antibody is not practical because of very low coupling efficiency.

ACKNOWLEDGMENT

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Effects of Structure of β -Cyclodextrin-Containing Polymers on Gene Delivery

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Linear cationic β -cyclodextrin-based polymers (β CDPs) are capable of forming polyplexes with nucleic acids and transfecting cultured cells. The β CDPs are synthesized by the condensation of a diamino-cyclodextrin monomer A with a diimide comonomer B. In this paper, the effects of polymer structure on polyplex formation, in vitro transfection efficiency and toxicity are elucidated. By comparison of the β CDPs to polyamides lacking cyclodextrins, the inclusion of a cyclodextrin moiety in the comonomer A units reduces the IC₅₀s of the polymer by up to 3 orders of magnitude. The spacing between the cationic amidine groups is also important. Different polymers with 4, 5, 6, 7, 8, and 10 methylene units (β CDP4, 5, 6, 7, 8, and 10) in the comonomer B molecule are synthesized. Transfection efficiency is dependent on comonomer B length with up to 20-fold difference between polymers. Optimum transfection is achieved with the β CDP6 polymer. In vitro toxicity varied by 1 order of magnitude and the lowest toxicity is observed with β CDP8. The LD₄₀ of the β CDP6 to mice is 200 mg/kg, making this polymer a promising agent for in vivo gene delivery applications.

INTRODUCTION

The development of polyplexes [cationic polymer + nucleic acid (1)] for gene delivery has grown rapidly to include cationic polymers such as poly-L-lysine (PLL) (2), chitosan (3), pAMAM dendrimers (4), polyethylenimine (PEI) (5), and many other polycations. A recent review of cationic polymer based delivery systems is presented by De Smedt et al. (6). Cationic polymers are able to deliver plasmid DNA into cells by self-assembling with the anionic DNA via electrostatic interactions with subsequent condensation into small particles that are readily endocytosed.

While cationic polymers share a similar mechanism of delivery, their transfection efficiencies differ greatly from polymer to polymer. Indeed, significant transfection and toxicity variations have been noted with the use of various molecular weight fractions of the same polymer. Ziady et al. observed both in vitro and in vivo behavioral differences with the use of short (36-mer) and long (256-mer)-chain PLL (7). Additionally, polyplexes formed with these polymers had different physicochemical characteristics. The transfection efficiency of PEI is also highly dependent on molecular weight and structure (8, 9). Remy et al. compared in vitro gene delivery for various branched and linear PEIs and found significant variations with PEI structure (10). Malik et al. also found that the dendrimer generation influences the efficacy of pAMAM dendrimers (11).

Plank et al. studied peptide derivatives within a low molecular weight range by synthesizing branched cationic peptides with variations in number and type of amino acids within the peptides (12). They found DNA binding affinity to be mainly a function of the number of cationic residues, and the transfection efficiency to be

influenced by the type of cationic residues used. It is evident from these reports that polycation structure impacts delivery efficiency. Additionally, it is clear that further investigation into the features of polycations that influence their behavior in gene delivery is merited. Here, we perform a structure–property study of this type.

We have reported the synthesis of a new family of β -cyclodextrin-based polymers (β CDPs) that are prepared by the condensation of difunctionalized CD monomers (comonomer A) with other difunctionalized comonomers (comonomer B) (13). The modular synthesis of the β CDPs allows for the systematic study of the effects of polymer structure on polyplex formation and polyfection. Previously, the importance of a spacer between the cyclodextrin and amine functional groups in the comonomer A units has been discussed (13). The spacer was shown to be required for DNA binding of comonomer A, most likely by relieving the steric hindrance of the bulky cyclodextrin cups. Here, we study the effect of comonomer B structure on DNA delivery and toxicity, since variations in comonomer B result in changes in polymer charge density and hydrophobicity. To better understand the origin of the functional differences, the polyplexes are characterized by comparing particle size, DNase protection ability, and relative DNA binding constants.

MATERIALS AND METHODS

β -Cyclodextrin-Polymers. β -Cyclodextrin polymers (β CDPs) were prepared by the polymerization of a difunctionalized β -CD monomer (A) with a difunctionalized comonomer (B) to give an ABAB product as described previously (13). The synthesis procedure involves the preparation of the difunctionalized β -CD (6A,6D-dideoxy-6A,6D-di(2-aminoethanethio)- β -cyclodextrin; denoted dicysteamine- β -cyclodextrin) and the synthesis of the difunctionalized comonomer B.

Synthesis of the Comonomer A (**3**, Figure 1). β -CD (Wacker Biochem Corp, Adrian, MI) was dried in vacuo (<0.1 mTorr) at 120 °C for 14 h before use. Biphenyl-

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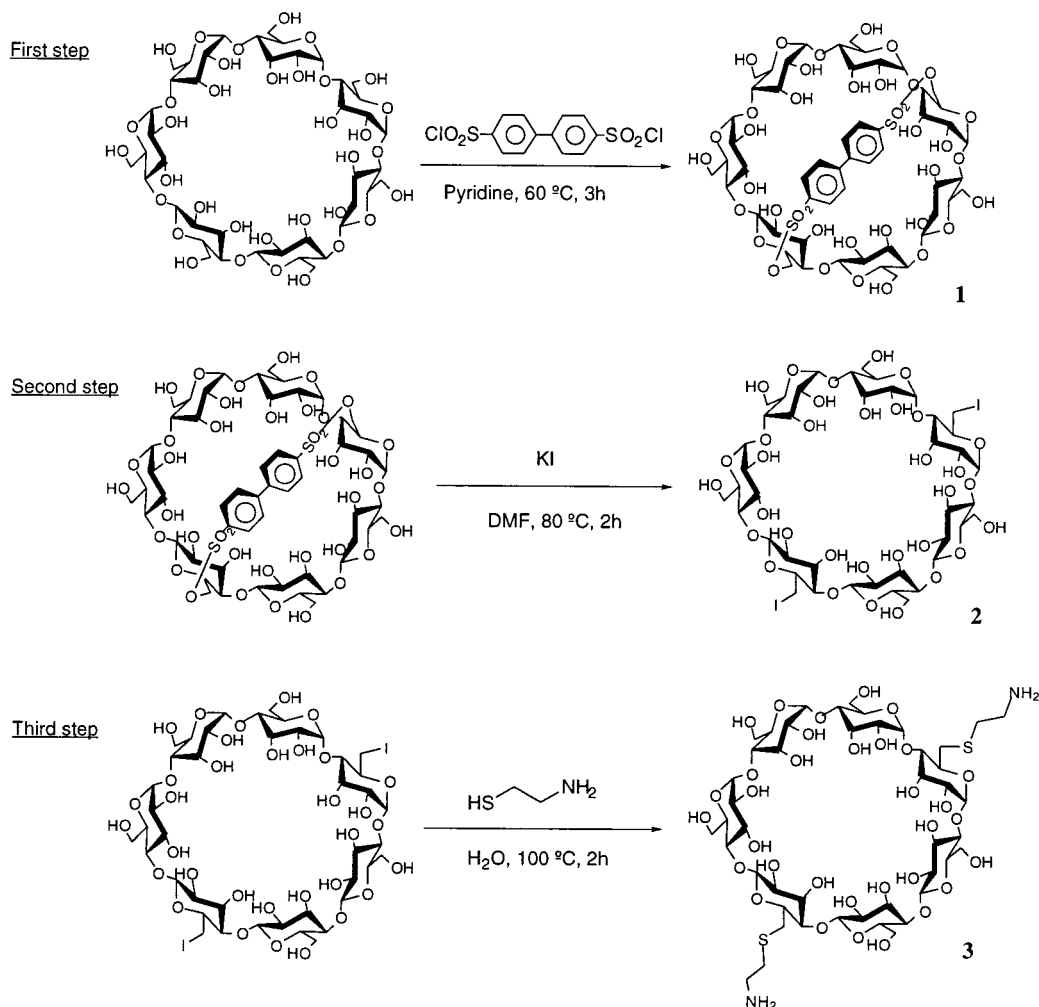


Figure 1. Synthesis of comonomer A (dicysteamine- β -cyclodextrin).

4,4'-disulfonyl chloride (Aldrich, Milwaukee, WI) was recrystallized from chloroform/hexane. Potassium iodide was powdered with a mortar and pestle and dried in an oven at 200 °C. Cysteamine (Fluka, Milwaukee, WI) was sublimed at 40 °C under reduced pressure before use. All other reagents were obtained from commercial suppliers and were used as received without further purification.

First step: synthesis of biphenyl-4,4'-disulfonate-capped β -CD **1**. The capped β -CD was synthesized according to a modified procedure of Tabushi et al. (14). To a solution of dried β -CD (10 g, 8.81 mmol) in 250 mL of freshly distilled anhydrous pyridine (Aldrich) was added 2.78 g (7.93 mmol) of biphenyl-4,4'-disulfonyl chloride in four equal portions at 15 min intervals. The resulting solution was stirred at 60 °C under nitrogen for an additional 3 h and the solvent was removed to dryness in vacuo at room temperature. The residue was subjected to C8 reversed-phase column chromatography using a gradient elution of 0–40% acetonitrile in water. Fractions were analyzed by HPLC and the appropriate fractions were combined. After removing the acetonitrile on a rotary evaporator, the resulting aqueous suspension was lyophilized to dryness. This procedure gave 5.42 g (40% yield) of **1**. ¹³C NMR (Bruker 500 MHz, Me₂SO-d₆) δ ppm 60.3 (C6), 70.9, 71.6, 72.7 (C2, C3, C5), 82.4 (C4), 102.0 (C1), 128.4–143.2 (phenyl).

Second step: synthesis of diiodo β -CD **2** (15). To a solution of 5.42 g (3.52 mmol) of **1** in 100 mL of anhydrous DMF (Aldrich) was added 17.53 g (0.106 mol) of dry powdered potassium iodide. The solution was kept at

80 °C for 2 h with stirring. After the mixture cooled to room temperature, insoluble materials were removed by filtration and the solution was evaporated to dryness in vacuo. The residue was then dissolved in 60 mL of water and 6 mL of tetrachloroethylene was added at 0 °C with vigorous stirring. The precipitate formed was collected and dried in vacuo to give 4.69 g (90% yield) of a slightly yellow colored solid. ¹³C NMR (Bruker 500 MHz, Me₂SO-d₆) δ ppm 10.4 (C6 adjacent to I), 60.3 (C6 adjacent to OH), 70.9, 71.6, 72.7 (C2, C3, C5), 82.4 (C4), 102.0 (C1).

Third step: synthesis of dicysteamine β -CD **3**. To a solution of 4.69 g (3.17 mmol) of **2** in 100 mL of degassed water was added 0.489 g (6.34 mmol) of freshly sublimed cysteamine. The solution was stirred under reflux for 2 h. After cooling to room temperature and acidifying with 1 N HCl, the solution was applied to a Toyopearl SP-650M ion-exchange column (NH₄⁺ form) and the product was eluted with a 0 to 0.2 M ammonium bicarbonate gradient. Appropriate fractions were combined and lyophilized to dryness. This procedure gave 1.87 g (39% yield) of a white solid. The solid was characterized by TLC [silica gel, *n*-PrOH-AcOEt-H₂O-NH₃(aq) 5/3/3/1, detection by ninhydrin] and exhibited a major spot corresponding to **3** and a minor spot corresponding to free cysteamine. This unreacted cysteamine was eliminated on a rotary evaporator after dissolution of the solid in water with monitoring by Ellman's reagent (16). Matrix-assisted laser desorption/ionization (MALDI) time-of flight (TOF) mass spectrum was recorded on 2 m ELITE instrument supplied by PerSeptive Biosystems,

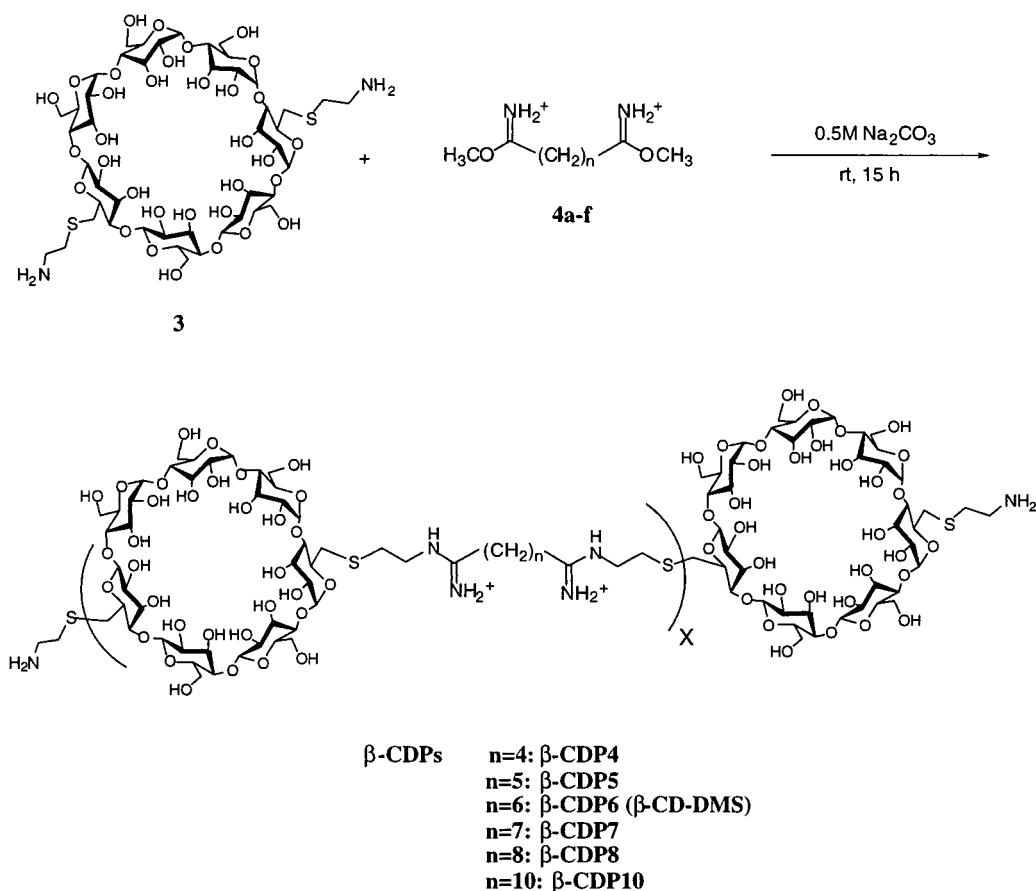
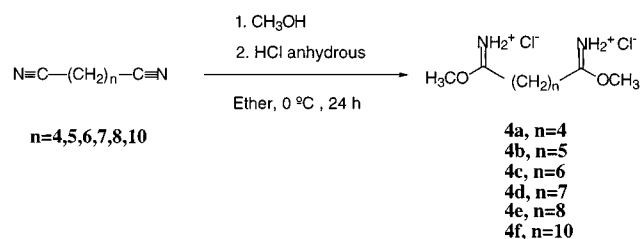


Figure 2. Polymerization scheme for β -cyclodextrin polymers.

Inc. MALDI-TOF m/z calcd for **3**, 1252; found, 1253.5 [$M + H$]⁺, 1275.5 [$M + Na$]⁺, 1291.4 [$M + K$]⁺. ¹³C NMR (Bruker 500 MHz, D₂O) δ ppm 32.1 (S-CH₂) and 38.8 (CH₂-NH₂), 32.9 (C6 adjacent to S), 60.2 (C6 adjacent to OH), 70.8, 71.4, 72.5 (C2, C3, C5), 81.8 (C4), 101.7 (C1).

Synthesis of Comonomer B [4a–f (17)].



Dimethyladipimide·2HCl, **4a**; dimethylpimelimate·2HCl, **4b**; and dimethylsuberimide·2HCl, **4c** (DMS) were purchased from Pierce Chemical Co. and were used as received.

Synthesis of Dimethylazelimate·2HCl, 4d. Azelani-trile (1.08 g, 7.2 mmol) (Aldrich) was dissolved in an ice-cold mixture of anhydrous methanol (4 mL) and anhydrous diethyl ether (30 mL). Anhydrous hydrogen chloride was passed through the solution for 60 min and the reaction mixture was left at 0 °C for 24 h. Dry diethyl ether (50 mL) was added to precipitate the product that was washed twice with 100 mL of dry methanol/diethyl ether [mixture (volume ratio 1:3)] under anhydrous conditions. The solid was dried under vacuum for 2 h. This procedure gave 1.64 g (79% yield) of **4d**. Calcd for C₁₁H₂₄O₂N₂Cl₂: C, 45.96; H, 8.36; N, 9.75. Found: C, 45.48; H, 8.30; N, 9.71. ¹³C NMR (Bruker 500 MHz, CD₃-

OD) δ ppm 26.1, 29.4, 29.5, 33.9 (4 CH₂), 59.9 (OCH₃), 183.2 (C=NH).

Synthesis of Dimethylsebacimide·2HCl, 4e. This compound was obtained from 1.16 g (7.1 mmol) of sebaconitrile (Aldrich) by the same procedure used to prepare **4d** and gave 2.04 g (96% yield). Calcd for C₁₂H₂₆O₂N₂Cl₂: C, 47.81; H, 8.63; N, 9.30. Found: C, 47.68; H, 8.51; N, 9.23. ¹³C NMR (Bruker 500 MHz, CD₃OD) δ ppm 26.2, 29.6, 29.7, 33.9 (4 CH₂), 59.9 (OCH₃), 183.2 (C=NH).

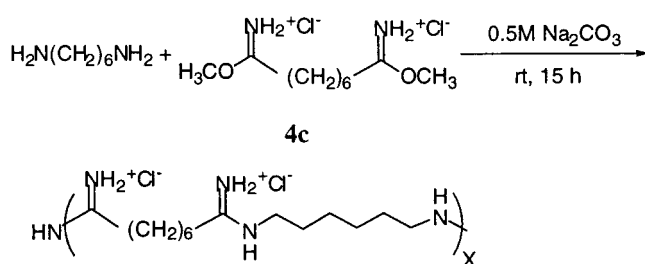
Synthesis of Dodecanediimide·2HCl, 4f. This compound was obtained from 1.35 g (7.0 mmol) of dodecanedinitrile (TCI America Organic Chemicals, Portland, OR) by the same procedure used to prepare **4d** and gave 1.85 g (80% yield). Calcd for C₁₄H₃₀O₂N₂Cl₂: C, 51.03; H, 9.11; N, 8.51. Found: C, 50.96; H, 9.08; N, 8.44. ¹³C NMR (Bruker 500 MHz, CD₃OD) δ ppm 26.3, 29.8, 30.0, 30.3, 33.9 (5 CH₂), 60.0 (OCH₃), 183.4 (C=NH).

Polymerization (Figure 2). β CDPs were synthesized as described previously (13). In a typical experiment, a 25 mL vial was charged with a solution of the bis-(hydrogen carbonate) salt of dicysteamine β -CD **3** (399.6 mg, 0.269 mmol) dissolved in 500 μ L of 0.5 M Na₂CO₃. The comonomer B, e.g., **4c** (73.5 mg, 0.269 mmol) was added and the solution was centrifuged briefly to dissolve the components. The resulting mixture was stirred at 25 °C for 15 h. The mixture was then diluted with 10 mL of water and the pH brought below 4 with the addition of 1 N HCl. This solution was then dialyzed against a Spectra/Por 7 MWCO 3500 dialysis membrane (Spectrum) in dH₂O for 24 h. The dialyzed solution was lyophilized to dryness. ¹³C NMR (Bruker 500 MHz, D₂O) δ ppm 25.8, 26.0, 27.0, 28.7, 29.9, 32.2, 37.5, 38.1, 41.1, 60.0, 71.6, 72.3, 72.6, 80.8, 101.4, 167.9.

Polyamidine Polymers. Polyamidine polymers were synthesized as described for β CDPs except methylenediamines were used as the comonomer A instead of dicysteamine- β -CD. These non- β -CD-containing polymers were prepared for comparison to the β CDPs in order to ascertain the influence of the β -CD in the polymer backbone upon gene delivery.

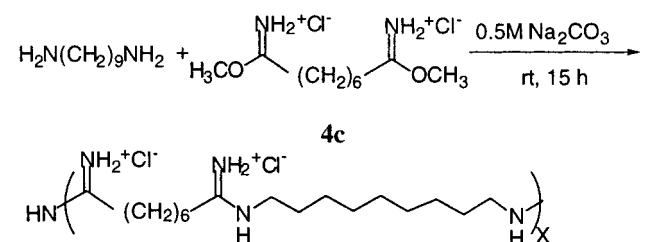
C6-C6 Polyamidine. An 8 mL glass vial was charged with 80 mg of hexamethylenediamine (comonomer A, 0.690 mmol, Aldrich) and 195 mg of dimethylsuberimidate-2HCl (comonomer B, 0.714 mmol, Pierce). The solids were dissolved by the addition of 0.25 mL of 0.5 M Na_2CO_3 . The mixture was stirred at 25 °C for 15 h, yielding a viscous, clear solution. The mixture was diluted with water and pH brought below 4 with the addition of 1N HCl. The solution was transferred to a Spectra/Por 7 MWCO 1000 dialysis membrane and dialyzed for 24 h in dH_2O . The dialyzed material was lyophilized to dryness, yielding 30 mg of a white, fluffy solid (13% yield).

C6-C6 Polyamidine:



C9-C6 Polyamidine. The C9-C6 polyamidine polymer was synthesized as described above for the C6-C6 polyamidine polymer except 1,9-diaminononane (comonomer A, Aldrich) was reacted with dimethylsuberimidate-2HCl (comonomer B) to result in a 21% yield of a white, fluffy solid. The C9 distance is a reasonable approximation to that of the β -CD.

C9-C6 Polyamidine:



Light Scattering and Molecular Weight Determination. β CDPs were analyzed on a Hitachi D6000 HPLC system equipped with a ERC-7512 RI detector, a Precision Detectors PD2020/DLS light scattering detector and a Progel-TSK G2000 SW_{XL} column using 0.3M NaCl as eluant at a 0.7 mL/min flow rate as described previously (13).

End Group Analysis by Amine Quantification. β CDPs were dissolved in 0.1 M NaHCO_3 at a concentration of 0.25 mg/mL, and the amine concentration of these solutions was determined by reaction with TNBS (16). In brief, 250 μL of 0.01% TNBS in 0.1 M NaHCO_3 were added to 500 μL of polymer solutions and resulting mixture placed in a 37 °C water bath. After 2 h, 250 μL of 10% sodium dodecyl sulfate and 125 μL of 1N HCl was added to each sample. The absorbance at 335 nm was determined and compared to a dicysteamine- β -CD standard curve.

Plasmids. Plasmid pGL3-CV (Promega, Madison, WI), that contains the luciferase gene under the control of the SV40 promoter, and plasmid pCA-EGFP (generously donated by Dr. Rusty Lansford and Prof. Scott Fraser at the California Institute of Technology), that contains the EGFP gene under a chick actin promoter, were amplified by *Escherichia Coli* strain DH5 α and were purified using Qiagen's Endotoxin-free Megaprep kit (Valencia, CA).

Complex Formation and Characterization. An equal volume of cationic polymer dissolved in dH_2O was added to plasmid DNA (0.1 mg/mL in dH_2O) at the appropriate charge ratios (cationic to anionic, denoted as "+/-").

Dynamic Light Scattering. For particle sizing by dynamic light scattering (DLS), 2 μg of pCA-EGFP was complexed with polymers at 50+/- and allowed to stand for 10 min before diluting with 1.2 mL of dH_2O . Particle size was measured using a ZetaPals dynamic light scattering instrument (Brookhaven Instruments Corporation, Holtsville, NY).

Transmission Electron Microscopy. One microgram of DNA was complexed with cationic polymer at 50+/- charge ratio and applied to Formvar carbon-coated grids for 45 s before removing excess liquid. Samples were then stained with 2% uranyl acetate for 45 s before blotting. Images were recorded using a Philips 201 Electron Microscope operated at 80V.

DNase Protection Assay. The DNase protection assay was modified from a procedure by Gao et al. (18). One microgram of DNA (pCA-EGFP) was complexed with cationic polymer at 5+/- . The complexes were then incubated with 20 μL of fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD) for 30 min. Five microliters of 10% SDS was added to the complexes to displace the polymers from the DNA. Twenty microliters of FBS were added to "control" complexes followed by immediate addition of SDS for the "0 min" timepoint. The samples were then applied to a 0.8% agarose gel containing 6 μg of ethidium bromide/100 mL of TAE buffer (40 mM Tris-acetate, 1 mM EDTA) after the addition of 1 μL of loading buffer and electrophoresed.

Competitive Displacement by Heparan Sulfate. Cationic polymer was complexed with 0.5 μg of pGL3-CV at a 20+/- charge ratio. Heparan sulfate (5 mg/mL in water, from porcine intestinal mucosa, Sigma, St. Louis, MO) was then added to the complexes. After a 5 min incubation, 1 μL of loading buffer was added and samples were electrophoresed in an agarose gel as described for the DNase protection assay.

Cell Culture and Polyfections. BHK-21 cells were purchased from ATCC (Rockville, MD) and maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 100 units/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 0.25 $\mu\text{g}/\text{mL}$ amphotericin at 37 °C and 5% CO_2 . Media and supplements were purchased from Gibco BRL (Gaithersburg, MD). For transfections, BHK-21 cells were plated at 50 000 cells/well in 24-well plates. Cells were transfected with 1 μg of pGL3-CV complexed with β CDPs at various charge ratios in serum-free media. Lysates were analyzed for luciferase activity as described previously (13). The toxicities associated with the transfections were assessed by the Lowry protein assay, also described previously (13).

IC₅₀ Determination. BHK-21 cells were plated at 5000 cells/0.1 mL/well in 96 well plates and incubated at 37 °C for 24 h. β CDPs (16 mg/mL in complete media) were then added to the third column of cells and diluted serially across the rows. The cells were incubated with the polymers for 24 h at 37 °C. The media was then

Table 1. Effect of the Length of Comonomer B on the Polymerization

polymer	yield (%)	M_w (kDa) ^a	M_w/M_n ^a	average degree of polymerization	M_n (kDa) ^b
β CDP4	18	6.1	1.13	4	5.3
β CDP5	21	5.8	1.12	4	4.8
β CDP6	20	5.8	1.12	4	5.1
β CDP7	29	6.9	1.14	4	4.6
β CDP8	37	7.6	1.16	5	6.2

^a Determined by static light scattering. ^b Estimated by end group analysis.

removed from the cells and cells were washed once with PBS. Fifty microliters of MTT (2 mg/mL in PBS) was added to each well followed by 150 μ L of complete media. The cells were incubated for an additional 24 h before the media was removed and blue formazan crystals formed were dissolved in 150 μ L of DMSO. The absorbances were read at 562 nm in a microplate reader (EL-312e Biokinetics Reader, Biotek Instruments, Winooski, VT).

Intravenous Toxicity of β CDP6 in Mice. Groupings of five outbred male mice were injected intravenously with a single dose of β CDP6 (8.33 μ L/g mouse). Stock dosing solutions of β CDP6 were prepared in 0.9% NaCl, sterilized by passing through a 0.2 μ m filter, and diluted with 0.9% NaCl as needed. Mice were observed for 3 days postinjection.

RESULTS

β CDPs Synthesis and Characterization. β CDPs were synthesized using comonomer Bs with 4, 5, 6, 7, 8, and 10 methylene units. The length of the comonomer B was found to influence the polymerization as shown by the data given in Table 1. The molecular weight M_w and the polydispersity index M_w/M_n as determined by light scattering are provided in Table 1 for each polymer. Molecular weight M_n was also estimated by end group analysis. The two possible terminal functional groups for the β CDPs are carboxylic acids or amines. Attempts to conjugate a fluorescent tag (Alexa Hydrazide from Molecular Probes) to the polymer by DCC coupling assuming carboxylic end groups resulted in no observed reaction. However, the conjugation of TNBS (reacts with primary amines) resulted in the detection of molecular weights M_n similar to those detected by SLS (Table 1). These molecular weights give an average degree of polymerization of 4–5 for all β CDPs studied. The yield of polymer increased with the number of methylene groups within comonomer B. The yield of β CDP10 was 56%; however, this polymer was synthesized from a different batch of comonomer A than all the other polymers. Therefore, direct comparison to the other β CDPs may not be appropriate.

Polyplex Characterization. β CDPs were complexed with plasmids at various charge ratios and polyplex size was measured by dynamic light scattering (DLS) and TEM. Particle size depended on polymer and DNA concentrations during polyplex formulations. However, above 3 $\pm$, polyplex size remained largely independent of charge ratio. When measured by DLS, particle sizes of polyplexes formulated at DNA concentrations of 50 μ g/mL ranged from 124 to 152 nm (Figure 3). β CDP4, β CDP5, and β CDP10-based polyplexes gave the largest particles, with diameters of 148, 141, and 144 nm, respectively. β CDP6, β CDP7, and β CDP8-based particles had smaller diameters of 127, 128, and 124 nm, respectively. Electron micrographs of all β CDP-polyplexes

prepared in water showed uniform particles that appeared spherical in shape. β CDP6-based polyplexes are shown in Figure 4 as an example.

DNase Protection. The ability of the β CDPs to protect plasmid DNA from DNase degradation was assessed. Plasmid DNA was complexed with β CDPs and incubated with an equal volume of fetal bovine serum for 30 min at 37 $^{\circ}$ C. The DNA was released from the complexes by the addition of sodium dodecyl sulfate and run on an agarose gel (Figure 5). β CDPs with 4, 6, 8, and 10 methylene units completely protected DNA, while β CDP5 and β CDP7 offered only partial protection.

In Vitro Transfection and Toxicity of β CDP Polyplexes. BHK-21 cells were plated in 24-well plates and transfected under serum-free conditions with 1 μ g of pGL3-CV complexed with the β CDPs at various charge ratios. Transfection efficiencies were determined by assaying for luciferase protein activity, with results reported in relative light units (RLUs). Several polymer to DNA charge ratios were tested (Figure 6) and a comparison of relative transfection efficiencies and cell survival are reported here for 50+/- (Figure 7). At this charge ratio, the transfection efficiencies have reached their maximum and the toxicity differences between the polymers become evident. The β CDP with six methylene units, β CDP6, gives the highest transfection efficiency: 2.5×10^{10} RLU/mg protein. The transfection efficiencies of all the polymers are normalized to the β CDP6 transfection achieved at 50+/- and reported as a fraction of β CDP6 transfection (Figure 7). β CDP7 and β CDP8 transfected at 50 and 64%, respectively, of the β CDP6 levels while β CDP4, β CDP5, and β CDP10 compared even less favorably at 22, 6, and 10%, respectively.

The toxicity of the β CDPs associated with the transfections at 50+/- was measured by determining the total protein concentration in cell lysates 48 h after transfection and comparing with protein levels of untransfected cells. The fraction cell survival in the presence of the polyfection mixture was also dependent on comonomer B length (Figure 7). No toxicity was observed for β CDP8. However, toxicity in general increased for all comonomer B lengths differing from that used in β CDP8.

The IC_{50} s of the β CDPs to BHK-21 cells were determined by plating the cells in 96-well plates, exposing the polymers to the cells in serial dilutions for 24 h and monitoring cell viability by the MTT assay. The polymer IC_{50} s show the same trend as the toxicities associated with polyplex transfection (Figure 8). β CDP8 had the highest IC_{50} (2200 μ M, reported as cyclodextrin concentration), followed in order of decreasing IC_{50} s by β CDP7 (1800 μ M), β CDP6 (1100 μ M), β CDP4 (380 μ M), β CDP5 (350 μ M), and β CDP10 (330 μ M).

Polyamide polymers were synthesized by the condensation of methylenediamines (hexylmethyldiamine and 1,9-diaminononane) as comonomer A with dimethylsuberimidate (DMS, comonomer B with 6 methylene units) to give C6–C6 polyamide and C9–C6 polyamide, respectively. The IC_{50} s of the polyamide polymers to BHK-21 cells were determined to be 4.6 μ M (reported per repeat unit) for C6–C6 polyamide and 34.0 μ M for C9–C6 polyamide.

Polymer Displacement by Heparan Sulfate. Polyplexes can be unpackaged by the addition of an anionic competitor such as heparan sulfate to bind to the cationic polymer and release the DNA (19). The relative binding constants of the β CDPs were determined by complexing the polymers with plasmid DNA at a 20+/- charge ratio and then adding increasing amounts of heparan sulfate. The solutions were loaded onto an ethidium bromide-

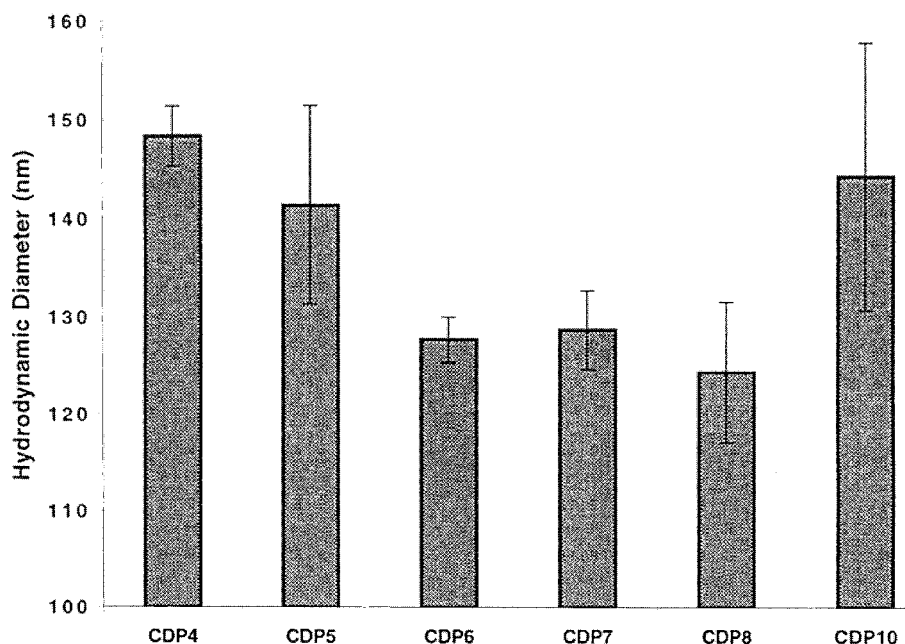


Figure 3. Hydrodynamic diameter of β CDP-based polyplexes as measured by dynamic light scattering. Polyplexes were prepared with 2 μ g of plasmid DNA at a DNA concentration of 0.05 mg/mL and then diluted with dH₂O to 1.2 mL final volume for measurements. The results shown are mean $\pm$ SD for three experiments, each measured in triplicate.

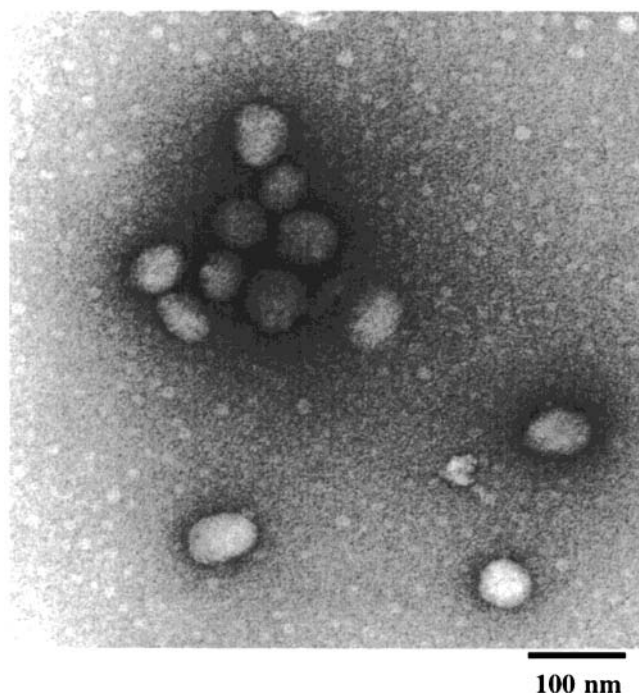


Figure 4. Electron micrograph depicting polyplexes of β CDP6 and pGL3-CL at a 50+/- charge ratio.

containing agarose gel and electrophoresed. Complexed DNA remained in the loading well. Successful polymer displacement by heparan sulfate released the DNA, that migrated in the electric field and was visualized by ethidium bromide intercalation. A comparison of the amount of heparan sulfate required to release the DNA from the β CDPs reflected the relative binding constants. β CDP6 demonstrated the highest DNA binding constant, requiring the addition of 22 μ g of heparan sulfate (heparan sulfate to β CDP6 charge ratio of 3+/-) before any DNA release could be observed (Figure 9). A total of 14 μ g (1.8+/-) of heparan sulfate were necessary to

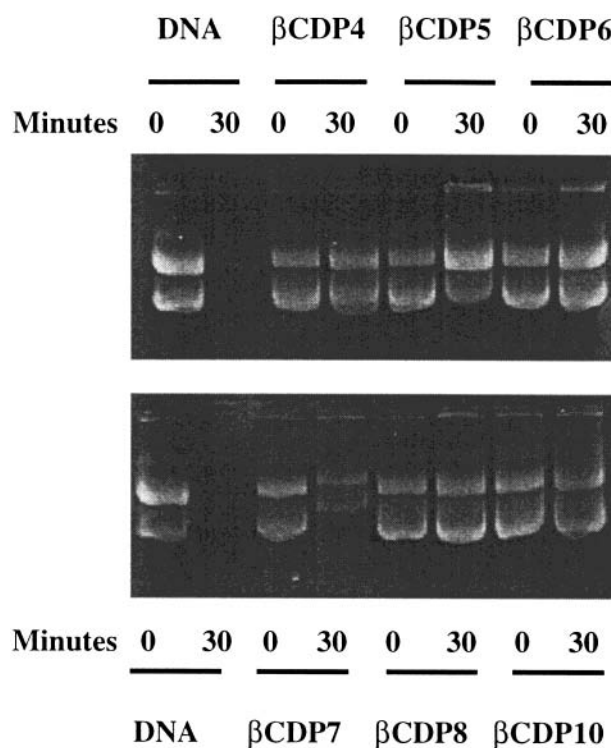


Figure 5. DNase protection of plasmid DNA by β CDP complexation. Polyplexes of DNA complexed with the various β CDPs were incubated with an equal volume of fetal bovine serum for 0 or 30 min. β CDPs were then displaced by SDS and DNA visualized by electrophoresis in an agarose gel.

displace β CDP4 while 16 μ g (2.1+/-) were required to displace β CDP5. The polymers with comonomer B longer than 6 methylene units showed a different behavior. For β CDP7, β CDP8, and β CDP10, the addition of heparan sulfate, even as low as 6 μ g (0.8+/-), resulted in the visualization of some uncomplexed DNA. For β CDP8 and β CDP10, the DNA release remains only partial, even with the addition of 32 μ g of heparan sulfate.

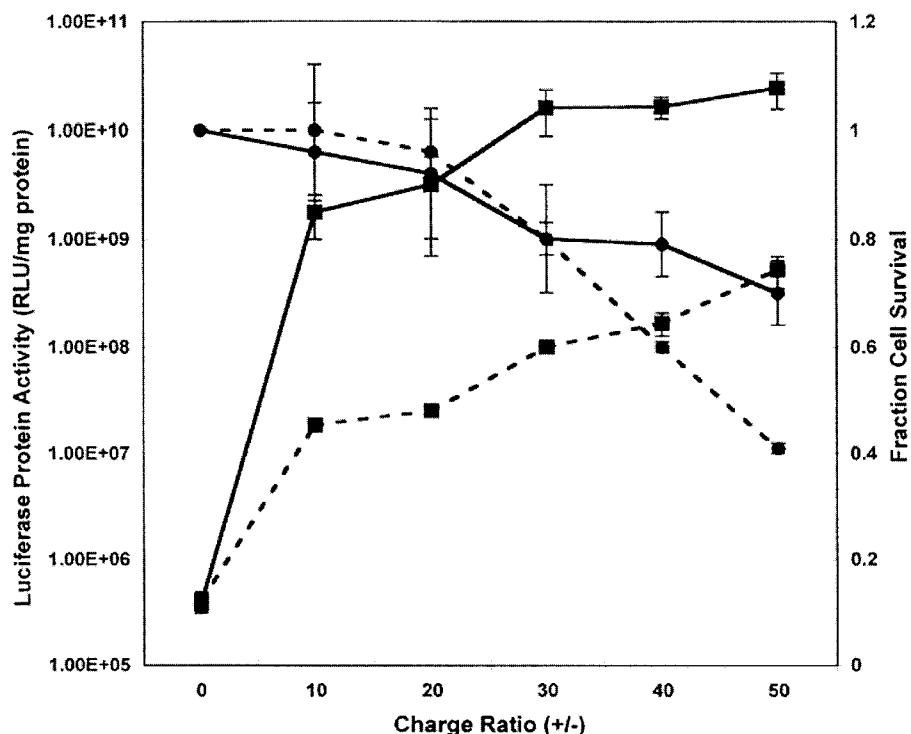


Figure 6. Transfection efficiencies (■) and cell survival (●) for BHK-21 cells transfected with 1 μ g of pGL3-CV complexed with β CDP5 (dashed lines) and β CDP6 (solid lines) at charge ratios ranging from 10 to 50+/- . Luciferase protein activity is reported as relative light units (RLU) per milligram of total cellular protein, as determined by Lowry protein assay. Cell survival was calculated by dividing cellular protein levels of transfected cells by untransfected cells. Data are reported as the mean $\pm$ SD of three samples.

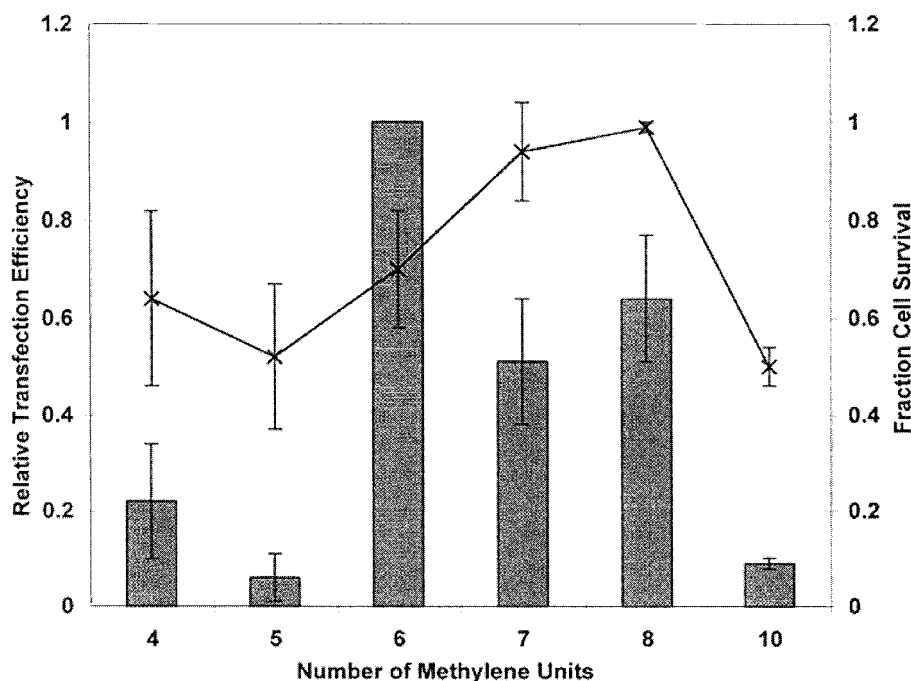


Figure 7. Comparison of luciferase transfection and cell survival for β CDP-based polyplexes at 50+/- charge ratios. The data reported are the mean $\pm$ SD of three experiments, each performed in triplicate. Transfection results for the different polymers are normalized with the average transfection achieved by β CDP6-based polyplexes for each experiment. Cell survival (X) was determined by assaying for total protein concentrations and normalizing each sample with protein levels for untransfected cells.

DISCUSSION

The objective of this study is to investigate the structure-property relationships between a family of β -cyclodextrin containing, cationic polymers and their interaction with and ability to deliver to cells plasmid DNA. To accomplish these goals a series of β CDPs were prepared using comonomer Bs with 4, 5, 6, 7, 8, and 10 methylene

units. The polymer yields were proportional to the comonomer B length with β CDP10 $\approx$ β CDP8 $>$ β CDP7 $>$ β CDP6 $>$ β CDP5 $>$ β CDP4. Higher reaction yield with longer diimide **4a-f** linkers was also noted by Denking et al. (17) who cross-linked poly(allylamine) with esters of diimide acids such as those used here (**4a-f**) to form hydrogels. The β CDP molecular weights, detected

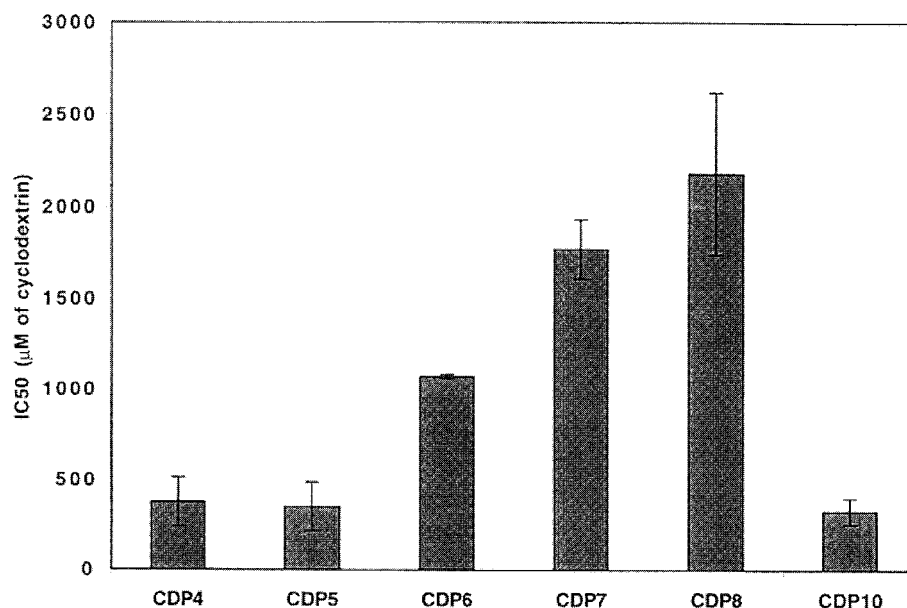


Figure 8. IC₅₀s of β CDPs to BHK-21 cells. β CDPs were added in serial dilutions to BHK-21 cells plated in 96-well plates. Cell viability was determined 24 h after polymer exposure by MTT assay. The IC₅₀s, reported as cyclodextrin concentration, were determined by interpolation on a best-fit line and are reported as the mean $\pm$ SD for three replicates.

by both static light scattering and end group analysis, ranged from 5800 to 7600, corresponding to a average degree of polymerization of 4–5 and giving 8–10 cationic charges/chain. Therefore, differences in polymer performance should be a result of polymer structure and not molecular weight since they all have similar molecular weights and polydispersities.

The β CDPs form polyplexes with DNA by rapid self-assembly in aqueous solutions. Polyplexes formed in water are relatively monodisperse and uniform in size, as evidenced by DLS and TEM. The size of the polyplexes depends on the formulation conditions, as has been described previously by Duguid et al. (20). Particles formed with β CDP6 have an average diameter of 60 nm at 2 μ g/mL final DNA concentration but an average diameter of 130 nm at 50 μ g/mL as measured by DLS. The latter particles were used for the DLS, TEM and transfection studies presented here. Electron micrographs of β CDP6-based polyplexes show particles smaller than 100 nm in diameter. DLS measurements, due to calculations that are based on weighting by particle light scattering intensity, are biased toward larger particles. DLS also determines the hydrodynamic diameter and hence DLS size measurements of particles are larger than the sizes of the polyplexes pictured by electron micrographs.

Polyplexes formed from β CDP4, β CDP5, and β CDP10 are larger in diameter than polyplexes formed from the other β CDPs (Figure 3). These larger particles transfected cultured cells less efficiently (Figures 6 and 7). A correlation between particle size and transfection efficiency has been described for other systems (6), although it is normally observed that the larger the particle size the greater the transfection. Here, the differences in the particle size are not large and likely not the reason for the large differences in transfection. In addition, the cationic polyplexes are prone to rapid aggregation under physiological salt conditions. Thus, the variations in particle size are more likely to be indicative of differences in the polymer–DNA binding interactions rather than directly affecting particle uptake.

For effective gene delivery, the polycation must protect the DNA from nuclease activity. While β CDP4, β CDP6,

β CDP8, and β CDP10 completely protected plasmid DNA from DNase degradation, β CDP5 and β CDP7 offered only partial protection (Figure 5). Yoshikawa et al. observed a difference in binding of various methylenediamines to DNA; however, they noticed greater DNA condensation with odd numbers of methylene units and larger particles with even numbers of methylene units (21). Thus, small differences in charge separation can produce dramatic effects. It is interesting to note that the two β CDPs with odd numbers of methylenes are the only ones to not completely protect the DNA.

All of the synthesized β CDPs are able to transfect BHK-21 cells, although with varying efficiencies. Transfection increases with charge ratios up to 50+/- (Figure 6). This high optimal charge ratio is probably due to the short nature of the polymers, a phenomenon also noticed by Plank et al. (12) for short peptides. The transfection profiles as a function of polymer to DNA charge ratio are similar for all of the polymers; however, the magnitude of the profiles differs greatly between polymers. To illustrate differences in transfection and toxicity, we chose to compare delivery performance between the polymers at 50+/-, as displayed in Figure 7. The highest transfection efficiency, as determined by luciferase protein activity, is consistently achieved by the β CDP6-based polyplexes. The transfection efficiency is found to be dependent on the comonomer B length. For example, when compared to β CDP6, luciferase transfections with β CDP5 result in a 20-fold reduction of luciferase protein activity, while the difference between the two comonomer B lengths is only 1.8 Å. Therefore, a few Å change in comonomer B length reduces the transfection by up to 1 order of magnitude (Figure 7).

A possible rationalization for the large decrease in transfection is that the spacing between the cationic amidine groups in β CDP6 is optimal for DNA binding. In an attempt to study β CDP–DNA interactions, polymer binding kinetics were monitored via a quartz crystal microbalance and DNA condensation kinetics by DLS. It was found that binding and condensation were complete within one second for all the polymers. Because of the speed of these processes, the procedures used here were unable to provide useful information. Thus, only

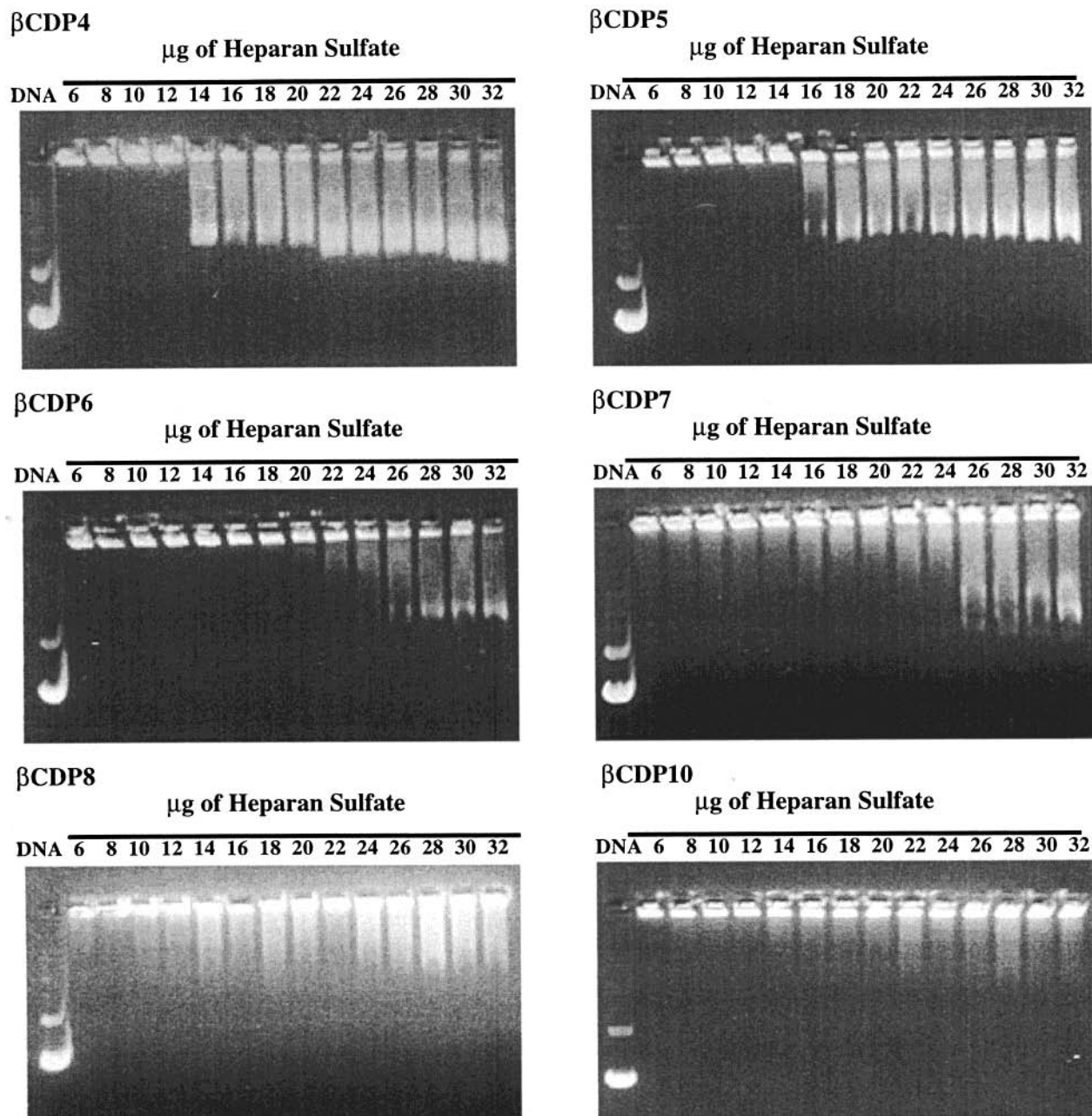


Figure 9. Determination of relative binding constants by heparan sulfate displacement. The amount of heparan sulfate (in μg) required to free DNA from βCDP -based polyplexes (prepared with 1 μg of DNA at 20 $\pm$ –). The first lane of each gel is uncomplexed DNA. The uncomplexed and freed DNA was visualized by ethidium bromide in an agarose gel.

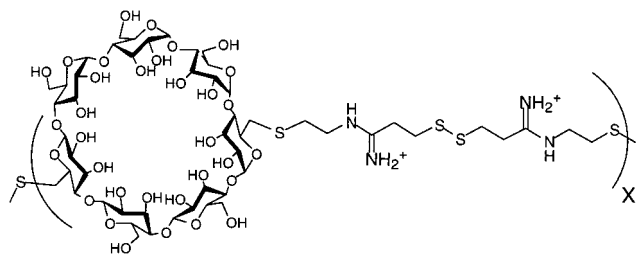
relative binding constants were determined by heparan sulfate displacement. The amount of heparan sulfate required to release DNA from the polyplexes should be related to the DNA binding constant of the displaced polymer. The heparan sulfate displacement studies (Figure 9) reveal that βCDP6 has a significantly higher binding constant than the other βCDPs . The polymers synthesized from shorter comonomer Bs (βCDP4 , βCDP5 , and βCDP6) also seem to bind differently from βCDP7 , βCDP8 , and βCDP10 . The former set of polymers bound tightly to the DNA at low heparan sulfate concentrations but are mostly displaced from the DNA at a critical heparan sulfate concentration that increases with increasing distance between the charges. The latter polymers are slightly displaced from the DNA even at low heparan sulfate concentrations, with a higher intensity of free DNA visualized with increased heparan sulfate

concentration. Therefore, the DNA binding characteristic of βCDP6 likely contributes to its gene delivery properties.

The toxicity of the βCDPs also depends on comonomer B length. βCDP8 reveals no cell death under the tested transfection conditions while βCDP7 is only slightly toxic. Because the comonomer Bs for these polymers were synthesized in our lab while comonomer Bs for βCDP4 , βCDP5 , and βCDP6 were purchased from commercial sources, it is possible that an impurity in the commercial samples could be the origin of toxicity. The βCDP6 was therefore synthesized from three different comonomer Bs: (i) purchased DMS, (ii) purchased and recrystallized DMS, and (iii) freshly synthesized in our labs DMS. The transfection and toxicity of the three βCDP6s were very similar, thus confirming cell toxicity to be caused by the βCDPs and not reaction impurities.

Although no toxicity was detected for β CDP8 at a charge ratio of 50+/-, cell death was observed for the other β CDPs at charge ratios above 20+/- with the toxicity differences between the polymers becoming evident by 50+/- (Figure 7). At this high charge ratio, most of the polymer is free in solution and not bound to DNA. The noted toxicity is therefore probably due mostly to the free polymer in solution. The IC_{50} s of β CDPs alone to BHK-21 cells were determined by the MTT assay. The trends in the IC_{50} s for the β CDPs correlated nicely with the toxicities detected at the 50+/- transfection conditions (Figure 8). Again, the IC_{50} s cover nearly an order of magnitude range. The IC_{50} s increase as the charge density decreases in going from β CDP4 to β CDP8. Additionally, as the comonomer B lengthens, the corresponding polymer becomes less soluble. Therefore, it is possible that the lower solubilities may contribute to the toxicity observed for β CDP10. If such is the case, then there is a tradeoff between charge density and solubility and for the polymers investigated here, the β CDP8 appears to be the optimal material with regard to toxicity.

Wadhwa et al. (22) and Fisher et al. (8) reported reduced cytotoxicity for low molecular weight fractions of PLL and PEI, respectively. Although the β CDPs presented here are low molecular weight polymers, we tested the cytotoxicity of a β CDP capable of being further degraded in the cell. To this end, we synthesized a disulfide-containing β CDP (β CDP-disulfide), as reported



β CDP-Disulfide

previously (13).

The disulfide was incorporated in a comonomer of length 11.9 Å between the charges; that is, 0.9 Å longer than β CDP6 but shorter than β CDP7. β CDP-disulfide should be reduced to monomeric units in the cellular environment. The β CDP-disulfide polymer was able to bind to DNA at the same charge ratio as the β CDP6 and transfect cells with efficiencies between those observed for β CDP6 and β CDP7. However, the toxicity of β CDP-disulfide associated with these transfections was higher than β CDP6 toxicity (62% cell survival versus 70%). These results indicate that the β CDP toxicity will not be decreased further with a polymer that can be reduced to molecules within the cell.

The idea behind the synthesis of the β CDPs was to ascertain whether polymers incorporating relatively non-toxic building units, i.e., cyclodextrins, could maintain the low toxicity of the cyclodextrin while simultaneously providing the properties necessary for gene delivery (13). The influence of cyclodextrin incorporation in the polymer backbone was tested by comparing β CDP polymer behavior to those having methylenediamines as comonomer As rather than CD-dicysteamine. Because the noncyclodextrin polymers were also synthesized by an A-B condensation, the average degree of polymerization was comparable to those achieved for β CDPs (average degree of polymerization of 5 for C6-C6 polyamidine and 8 for C9-C6 polyamidine). These polyamidine polymers are

able to bind to plasmid DNA by charge ratios of 1.5+/-, form polyplexes with hydrodynamic diameters of ~140 nm, and transfect cultured cells as well as the β CDPs. However, their IC_{50} s are 2-3 orders of magnitude lower than the IC_{50} s for β CDP8. These results indicate that cyclodextrin incorporation into the backbone of the cationic polymers greatly reduces the polymer toxicity.

On the basis of the in vitro results, the β CDP6 polymer was chosen for in vivo toxicity testing. This polymer gives the highest transfection efficiency with a reasonably high IC_{50} . Groupings of five mice were injected intravenously with a single dose of β CDP6 ranging from 2 to 200 mg/kg. No toxicity was observed at 100 mg/kg and the LD_{40} was found to be 200 mg/kg. Previously, we have tested a disulfide-containing β CDP for in vivo toxicity and determined it to be well tolerated at 200 mg/kg (13). However, the previous disulfide-containing β CDP displayed lower DNA binding affinity and transfection efficiency than the β CDP6. The in vivo toxicity parallels the in vitro studies in that increased charge density appears to yield higher toxicities. Thus, the in vivo toxicity limit should increase when the β CDP are complexed with DNA.

Here, it is demonstrated that the structure of the β CDPs critically determines performance in DNA delivery and cell toxicity. The difference of a few Å in comonomer B length can affect transfection and polymer IC_{50} values by orders of magnitude. In addition, the incorporation of β -cyclodextrin in the polymer backbone is shown to significantly decrease polymer toxicity. The low in vitro and in vivo toxicity of these β CDPs make them promising and attractive agents for in vivo gene delivery applications.

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Steric Stabilization of Fusogenic Liposomes by a Low-pH Sensitive PEG–Diortho Ester–Lipid Conjugate

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We describe the synthesis and characterization of a pH-sensitive poly(ethylene glycol)-diortho ester-distearoyl glycerol conjugate (POD). POD was prepared by a one-step synthesis, and its acid sensitivity characterized by TLC. The conjugate was found to be stable at neutral pH for greater than 3 h but degraded completely within 1 h at pH 5. Liposomes composed of 10% of POD and 90% of a fusogenic lipid, dioleoyl phosphatidylethanolamine (DOPE) were readily prepared and remained stable for up to 12 h in neutral buffer as shown by photon correlation spectrometry and a liposome contents leakage assay. However, when POD/DOPE liposomes were incubated in acidic pH as mild as 5.5, they aggregated and released most of their contents within 30 min. The kinetics of content release from POD/DOPE liposomes consisted of two phases, a lag phase, and a burst phase. The lag phase is inversely correlated with pH and the logarithm of the length of lag phase showed a linear relationship with the buffer pH. When the POD/DOPE liposomes were incubated in 75% of fetal bovine serum at 37 °C, they remained as stable as traditional PEG-grafted liposomes for 12 h but released 84% of the encapsulated ANTS in the following 4 h. Upon intravenous administration into mice, liposomes composed of 10% POD and 90% DOPE were cleared from circulation by a one-compartment kinetics with a half-life of about 200 min. POD is an example for the design of a novel category of pH sensitive lipids composed of a headgroup, an acid-labile diortho ester linker and a hydrophobic tail. The uniquely fast degradation kinetics of POD at pH 5–6 and its ability to stabilize liposomes in serum make the conjugate suitable for applications for triggered drug release systems targeted to mildly acidic bioenvironments such as endosomes, solid tumors, and inflammatory tissues.

INTRODUCTION

Diacyl chain lipids represent an important class of amphipathic molecules that contain both a polar headgroup and a large hydrophobic moiety. In aqueous phase, such lipids self-assemble into colloidal particles such as liposomes, micelles and hexagonal phase. Liposomes are widely used as models of membrane bilayers and carriers of a variety of diagnostics and medicines (1). Examples of their use in drug delivery include PEG stabilized liposomes that carry cytotoxic drugs to leaky tumor tissues (2) and cationic liposomes that improve gene transfection (3).

Ideally, a targeted drug delivery system, including liposomes, should remain stable until it reaches the target site to minimize the premature loss of its payload (2, 4). Upon accumulation at the target site, drug release needs to be at a high enough level for an effective therapeutic response. Since the decrease of pH is implicated in many physiological and pathological progressions such as endosome processing (5), tumor growth (6), inflammation (7), and myocardial ischemia (8), it has been extensively exploited to trigger the release of drugs from liposomal delivery systems in the past 20 years (9–11). Most of the reported pH-sensitive liposomes are based on the neutralization of excess negative charges on their surface upon protonation, which reduces the hydrodynamic diameter of lipid headgroups and triggers the change of lipid bilayers to hexagonal phases (10).

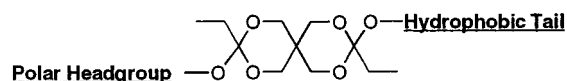
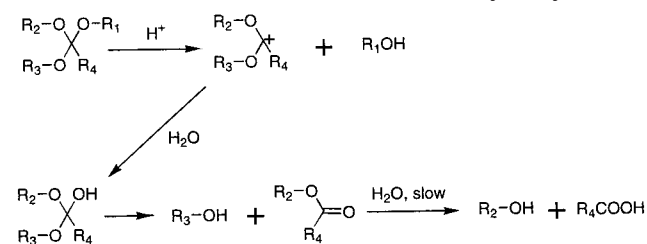


Figure 1. General structure of the diortho ester conjugates.

However, at neutral pH, these excess negative charges induce undesired interactions with serum proteins and fixed macrophages, leading to rapid elimination of the liposomes from circulation (12, 13). Efforts to circumvent this difficulty and provide a nonionic pH-sensitive lipid have employed a neutral monosaccharide as the headgroup, which is attached to long hydrophobic chains via an acetal moiety (14). However, these acetal analogues hydrolyzed relatively slowly at pHs 5 to 7 as found in physiological environment and need a pH less than 4 for rapid degradation. Recently, Thompson and co-workers have reported a number of acid- and light-sensitive lipids containing vinyl ether moieties (15–17). Although vinyl ethers represent a promising approach, their rate of hydrolysis at pH 5 is not optimal for rapid drug release. To develop a versatile strategy to prepare biocompatible liposomes, which are sensitive to small pH decrease, we propose a novel class of lipids (Figure 1) that possess an acid-labile diortho ester linker between the hydrophilic headgroup and the hydrophobic tail. In this case, a variety of headgroups and tails could be conjugated together to render pH-sensitive lipids of different properties.

Ortho esters are relatively stable under basic and neutral conditions but hydrolyze quickly at acidic pH due to a stabilized dialkoxy carbocation intermediate (Scheme

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Scheme 1. Mechanism of Ortho Ester Hydrolysis

1) (18–20). Heller and co-workers (21) have prepared a series of polymers based on the diortho ester moiety and exploited their pH-sensitive properties to develop sustained drug release systems. As an example of the proposed diortho ester lipidic conjugates, we report in this paper the design, preparation, and characterization of a novel lipid (**1**, POD, Scheme 2)¹ that consists of a PEG headgroup, a diortho ester linker, and a distearoyl glycerol hydrophobic tail. We show that POD can be used to prepare long circulating and acid-triggerable liposomes in combination with DOPE, a lipid that by itself does not form stable liposomes, but hexagonal phases at neutral pH.

EXPERIMENTAL PROCEDURES

General Techniques. The diketene acetal, 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5,5]undecane was received as a generous gift from Dr. Jorge Heller at Advanced Polymer Systems (Redwood City, CA). Monomethyl ether of PEG (MW 2000) was purchased from Shearwater Polymers, Inc (Huntsville, AL). Distearoyl glycerol was purchased from Genzyme (Cambridge, MA). Triethylamine was purchased from Aldrich and redistilled under Ar before use. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-oleoylphosphatidylglycerol sodium salt (POPG), dioleoylphosphatidylethanolamine (DOPE), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)-2000] (DSPE-PEG) were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesteryl hemisuccinate (CHEMS) was purchased from Sigma. 8-Aminonaphthalene-1,2,3-trisulfonic acid (ANTS) and *p*-Xylenebis(pyridinium) bromide (DPX) were from Molecular Probes, Inc. (Junction City, OR). MilliQ water, which had a pH of approximately 8 when freshly prepared, was used to prepare all the aqueous buffers. All other chemical reagents and solvents were purchased from Aldrich or Fisher. Ratios of components in chromatography solvent systems are in volume unless stated otherwise. Ratios of lipid components in liposomes are in mole units.

¹H NMR spectra were recorded on an Oxford AS 400. Electrospray mass spectra (ESIMS) were recorded on a Sciex (PE, Foster City, CA) at the Mass Spectrometry Facilities, University of California at San Francisco. Fourier-transformed infrared spectra were measured

with a Nicolet Impact 400. The FT-IR samples were applied as a CHCl₃ solution onto the surface of a NaCl crystal, dried under Ar, and subsequently analyzed. Elemental analysis was carried out by Microanalytical Lab at College of Chemistry, University of California at Berkeley.

3,9-Diethyl-3-(2,3-distearoyloxypropyloxy)-9-(methoxypoly(ethylene glycol)2000-1-yl)-2,4,8,10-tetraoxaspiro[5,5]undecane (POD, **1).** One gram of PEG (2000) monomethyl ether (0.5 mmol) and 312.5 mg of distearoyl glycerol (0.5 mmol) were dissolved in 5 mL of anhydrous THF under Ar. A heat gun was used to melt 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5,5]undecane and 100 μ L (110 mg, 0.5 mmol, $d = 1.1$) of the melted compound was taken by a dry syringe and injected into the THF solution. One drop (~ 40 μ L) of 0.6 mg/mL *p*-toluenesulfonic acid in anhydrous THF was added and the reaction mixture was stirred at 40 $^\circ$ C under Ar for 2 h. The reaction was stopped by adding 200 μ L of triethylamine followed by quenching the reaction into 30-fold volume excess of 1% volume of triethylamine in methanol. Four grams of silica gel were added to the solution and the mixture was evaporated under reduced pressure. The residue was poured onto a silica gel column (45 g) equilibrated with the eluting solvent system (triethylamine/chloroform = 1/50). The column was eluted and fractions corresponding to the product were pooled, evaporated, and dried in high vacuum to give 275 mg (20%) purified product. TLC = R_f 0.4 in CHCl₃/MeOH/30% w/w NH₄OH (100:15:1); FTIR 2910 cm⁻¹ (CH₂ and CH₃), 2850 cm⁻¹ (CH₂ and CH₃), 1743 cm⁻¹ (ester C=O), 1109 cm⁻¹ (PEG and ortho ester C–O); ¹H NMR (400 MHz, CDCl₃, chemical shifts relative to TMS signal) δ 5.22 (1H, m, glycerol methine), 3.7–4.2 (4H, m, glycerol methylene), 3.0–3.7 (~ 170 H, m, OCH₂ and OCH₃), 2.2–2.4 (4H, m, CH₂COO), 1.65–1.76 (4H, m, CH₂CH₂COO), 1.54–1.65 (4H, m, CH₂CH₃ on spiro rings), 1.04–1.36 (56H, m, CH₃(CH₂)₁₄CH₂CH₂COO), 0.82–0.97 (12H, m, CH₂CH₃); ESIMS, calcd for [M + Na]⁺ with 36–48 CH₂-CH₂O units from PEG: C₁₂₃H₂₄₀O₄₆Na 2476.6, C₁₂₅H₂₄₄O₄₇-Na 2520.7, C₁₂₇H₂₄₈O₄₈Na 2564.7, C₁₂₉H₂₅₂O₄₉Na 2608.7, C₁₃₁H₂₅₆O₅₀Na 2652.7, C₁₃₃H₂₆₀O₅₁Na 2696.8, C₁₃₅H₂₆₄O₅₂-Na 2740.8, C₁₃₇H₂₆₈O₅₃Na 2784.8, C₁₃₉H₂₇₂O₅₄Na 2828.8, C₁₄₁H₂₇₆O₅₅Na 2872.9, C₁₄₃H₂₈₀O₅₆Na 2916.9, C₁₄₅H₂₈₄O₅₇-Na 2960.9, C₁₄₇H₂₈₈O₅₈Na 3004.9, found 2478.3 (33%), 2521.1 (47%), 2565.3 (62%), 2609.3 (77%), 2654.2 (94%), 2697.3 (100%), 2741.4 (98%), 2785.3 (93%), 2830.4 (85%), 2874.4 (60%), 2917.5 (46%), 2962.4 (37%), 3006.4 (26%). Anal. calcd for C₁₃₅H₂₆₄O₅₂, C 59.62, H 9.78; HC₁₃₇H₂₆₈O₅₃, C 59.54, H 9.77; C₁₃₉H₂₇₂O₅₄, C 59.46, H 9.76; C₁₄₁H₂₇₆O₅₅, C 59.39, H 9.76; C₁₄₃H₂₈₀O₅₆, C 59.31, H 9.75; C₁₄₅H₂₈₄O₅₇, C 59.24, H 9.74; C₁₄₇H₂₈₈O₅₈, C 59.17, H 9.73; found C 59.16, H 9.57, N < 0.2.

Thin Layer Chromatography (TLC) Analysis of POD Degradation in Buffers of Different pH at 37 $^\circ$ C. Conical polystyrene microcentrifuge tubes (0.5 mL) containing 20 μ L of 100 mM sodium phosphate buffer of different pH were prewarmed at 37 $^\circ$ C for 30 min and silica gel TLC plates (Whatman from Whatman Ltd, Maidstone, Kent, England) were pre-equilibrated with the solvent system (CHCl₃/MeOH/ NH₄OH = 100:15:1) by running the plates without samples followed by brief air-drying. Eight milligrams of POD was dissolved into 100 μ L of water by vortexing and 20 μ L of the solution was added into each of the prewarmed tubes and mixed thoroughly. The tubes were incubated at 37 $^\circ$ C and 10 μ L of the solutions were aliquoted at different time points and mixed with 10 μ L of 10% concentrated NH₄OH (30% w/w) in acetone to stop the hydrolysis. Five microliters

¹ Abbreviations: ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; BPE, *p*-hydroxybenzamidinium phosphatidylethanolamine; CHEMS, cholesteryl hemisuccinate; Chol, cholesterol; DOPE, dioleoylphosphatidylethanolamine; DPX, *p*-xylenebis(pyridinium) bromide; DSG, 1,2-distearoyl glycerol; DSPE-PEG, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)-2000]; HEPES, (hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid; mPEG, PEG(2000) monomethyl ether; PEG, poly(ethylene glycol); POD, poly(ethylene glycol) 2000-diortho ester-distearoyl glycerol conjugate; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol sodium salt; SD, standard deviation; TLC, thin-layer chromatography.

of the mixture was spotted onto preequilibrated TLC plates. Three microliters of distearoyl glycerol, monomethyl ether of PEG2000 and POD in 10 mg/mL CHCl_3 solution were also spotted as standards. TLC plates were developed, heated at 150 °C for 5 min and stained in I_2 chamber for 1 h to observe the locations of POD and its degradation products.

Liposome Preparation for Aggregation and Leakage Assays. Reversed-phase vesicles (REV) were prepared as described previously (22) in 50 mM ANTS, 50 mM DPX, and 5 mM HEPES at pH 8.5. The vesicles were extruded five times through a 0.2- μm polycarbonate membrane (Nucleopore Corp., Pleasanton, CA) through a hand held extrusion device (Avestin, Ottawa, Ontario, Canada). A Sephadex G-75 column was used to separate vesicles from unencapsulated material with an elution buffer composed of 5 mM HEPES and 145 mM NaCl, pH 8.5. All freshly prepared liposomes had mean diameters ranging from 180 to 200 nm (cumulant results) and a polydispersity index of less than 0.2 as measured by a Malvern Zeta1000 Dynamic Light Scattering Instrument using the PCS 1.32a software. The encapsulated volume was 0.25–0.3 $\mu\text{L}/\mu\text{mol}$ total lipid. Lipid concentrations were determined based on lipid phosphorus by a modification of the Bartlett method (23).

Liposome Aggregation Assay. Conical polystyrene microcentrifuge tubes (0.5 mL) containing 300 μL of buffers of various pH (50 mM NaOAc/HOAc and 100 mM NaCl for pH <6; 50 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ and 100 mM NaCl for pH 6 and above) were incubated at 37 °C for 30 min. The liposome preparation was warmed to room temperature and an aliquot (100 μL) was added to each tube and mixed thoroughly. The tubes were incubated at 37 °C for different time periods and 30–40 μL of the mixtures were aliquoted and transferred into disposable polystyrene fluorimeter cuvettes containing 1.5 mL of 100 mM NaHCO_3 /50 mM NaCl buffer at pH 9. The samples were mixed by gently inverting the cuvettes 2–3 times.

Photon correlation spectrometry of the samples was then measured with a Malvern apparatus (Zeta 1000) to determine the size distribution of the liposomes. Typically, three measurements of 2–5 min were made for each sample, using the automatic algorithm mode for data analysis. The average of the three cumulant results of each sample that agreed to within 10% is reported in the paper.

ANTS/DPX Leakage Studies. The ANTS/DPX assay (24) was used to monitor leakage of ANTS from liposomes. Fluorescence measurements were made on a Spex Fluorolog photon counting instrument (Edison, NJ) using a 150-W xenon light source. Excitation was at 370 nm (1.25 mm slit). The 90° emission signal at 550 nm (5 mm, 5 mm slits) resulting from the dequenching of ANTS released out of liposomes was observed through a Corning 3-68 nm cutoff filter (>530 nm). The raw fluorescent data were converted into ASC data files and processed mathematically by Microsoft Excel. The residual fluorescence of the liposomes at the starting time (t_0) of leakage experiments, F_0 , was set as 0% release. At the end of each leakage experiment the liposomes were lysed with the detergent dodecyloctaethylene glycol monoether (C_{12}E_8) and the maximal fluorescence thus obtained, F_{100} , was taken as 100% release. The leakage of ANTS at a particular time point was then determined by the formula: percentage of leakage = $(F_t - F_0)/(F_{100} - F_0) \times 100$, where F_t corresponds to the fluorescence intensity observed at the time point.

In leakage experiments of POD/DOPE liposomes in aqueous buffers of different pHs, the assays were started

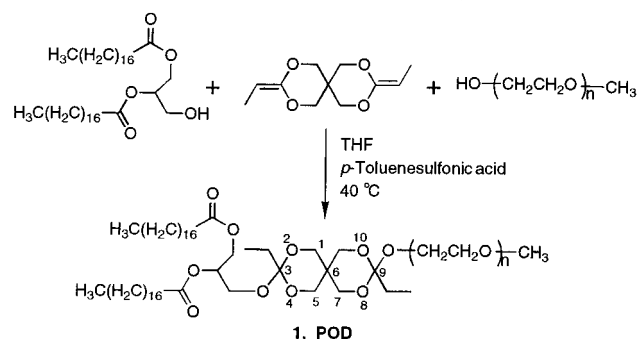
by injecting small volumes (5–10 μL , using a Hamilton syringe) of concentrated liposome suspensions into a magnetically stirred quartz cuvette containing 2 mL of the buffer (50 mM NaOAc/HOAc and 100 mM NaCl, pH <6; 50 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ and 100 mM NaCl, pH 6 and above) at 37 °C. The starting time, t_0 was set 5–10 s after the injection when the fluorescence signal first became stable.

In serum stability assays, aliquots of fetal bovine serum (180 μL in each tube) in 0.6 mL tubes were prewarmed at 37 °C for 30 min. An aliquot of stock liposome solution was diluted to 2 mM of total lipid by phosphate buffered saline (50 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ and 100 mM NaCl, pH 7.4), which had been prewarmed at 37 °C for 30 min. An aliquot of the diluted liposome solution (60 μL) was then mixed with 180 μL of prewarmed fetal bovine serum to give a 75% final serum concentration. At different time points, a 20 μL aliquot was added into a polystyrene fluorimeter cuvette, containing 2 mL of HEPES-buffered saline (5 mM HEPES and 145 mM NaCl, pH 8.5) at room temperature. The cuvette was covered with Parafilm and inverted gently for 3–4 times. The fluorescence thus measured subtracted by the fluorescence of the buffer before the addition of the sample was taken as F_t . The F_t of an aliquot extracted and measured immediately after mixing a liposome sample with fetal bovine serum was taken as F_0 . At the end of each assay an aliquot of the liposome-serum mixture was pipetted into 2 mL of HEPES buffered saline (5 mM HEPES and 145 mM NaCl buffer, pH 8.5), followed by adding the detergent dodecyloctaethylene glycol monoether (C_{12}E_8). The fluorescence measured after adding the detergent minus the fluorescence of the buffer was taken as F_{100} . Each data point of the leakage assay in fetal bovine serum represents the average and standard deviation of three independent samples.

Animals. Blood clearance, distribution and excretion studies were conducted on 4-week-old female ICR mice (about 25 g in weight) purchased from Simonsen (Gilroy, CA). All animals were handled in accordance with protocols established by the National Institute for Health Guidelines for the Care and Use of Laboratory Animals and with the approval of the Committee for Animal Research at the University of California, San Francisco. Animals were sacrificed at stated times with a sodium pentobarbital overdose.

Preparation of ^{125}I -Labeled Liposomes for Animal Studies. *p*-Hydroxybenzamidinium phosphatidylethanolamine (BPE) was synthesized and radiolabeled with Na^{125}I as previously described (25) and the purity assessed by thin-layer chromatography. The labeled lipid contained less than 0.5% of free ^{125}I . The labeled lipid, in chloroform solution, was added to POD/DOPE (1/9) or DSPE-PEG/DOPE (1/9) mixture in chloroform to yield 8.3×10^{11} dpm/mol of total lipid. Chloroform was evaporated under reduced pressure and the resultant lipid film was placed under high vacuum for 2 h to remove the remaining solvent. The dried lipid film was hydrated with HEPES buffered saline (10 mM HEPES and 145 mM NaCl, pH 7.4) over 20 min by intermittent agitation on a vortex mixer and the suspension extruded three times through a 0.1 μm polycarbonate membrane. The diameter of the extruded liposomes, as measured by photon correlation spectrometry, was 140–160 nm. Each mouse received a tail-vein injection of 150 μL of the extruded liposomes containing 0.9 μmol total lipid.

Blood Clearance Assay. At various time points following administration of ^{125}I -labeled liposomes, ani-

Scheme 2. Synthesis of PEG–Diortho Ester–Distearoyl Glycerol Conjugate (POD, 1)

imals were anesthetized with inhalation of isoflurane. Approximately 50 μ L of blood was collected with a glass pasteur pipet from the orbital sinus vein, weighed and its γ -activity measured in Beckman Gamma 8000 (Fullerton, CA). The γ -activity in whole blood and hence, the percentage of dose remaining in the blood was calculated assuming 0.07 g of blood/gram of animal weight. The average and standard deviation of samples collected from three animals were presented.

Tissue Distribution Assay. At various time points following administration of ^{125} I labeled liposomes, animals were anesthetized with an intraperitoneal (i.p.) injection of 100 μ L of anesthetics cocktail (44 mg/kg ketamine, 2.5 mg/kg xylazine, and 0.75 mg/kg acepromazine). Approximately 1 mL of blood was removed *via* intracardiac puncture. One milliliter of phosphate-buffered saline (containing 0.2 g/L KH_2PO_4 , 2.16 g/L $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g/L KCl, and 8.0 g/L NaCl) was perfused through the right cardiac ventricle. The heart, lungs, liver, spleen, kidneys, stomach, intestines, head, mid section, and tail were collected and weighed, and the radioactivity counted in the Beckman Gamma 8000. The γ -activity in whole blood and hence the percentage of dose remaining in the blood was calculated assuming 0.07 g of blood/gram of animal weight. The average and standard deviation of samples from three animals were reported.

RESULTS

Design and Synthesis of PEG–Diortho Ester–Distearoyl Glycerol Conjugate (POD, 1). To devise a lipid that degrades specifically in a low-pH environment but is inert under neutral physiological conditions, we chose PEG as the headgroup since it is one of the most stable synthetic polymers *in vivo* and liposomes coated by PEG have a prolonged circulation period (26). The monomethyl ether of PEG-2000 was used for synthesis to preclude cross-linking or polymerization. The 3,9-diethyl-2,4,8,10-tetraoxaspiro[5,5]undecane moiety was chosen as the diortho ester linker, based on previous research by Heller and co-workers (27), who showed its pH sensitivity and biocompatibility in polymeric drug delivery systems. Distearoyl glycerol, with two saturated hydrocarbon side chains, is attached to the diortho ester linker as the hydrophobic tail to anchor the conjugate into lipid bilayers (28, 29). If the conjugate is incorporated into liposomes of unsaturated phosphatidylethanolamine, distearoyl glycerol would be regenerated in the bilayers upon hydrolysis, and would favor the formation of hexagonal phases due to its conical structure (30).

POD was prepared by a one-step synthesis as illustrated in Scheme 2. The monomethyl ether of PEG2000 and distearoyl glycerol were dissolved in dry THF under

atmospheric Ar. The solid diketene acetal, 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5,5]undecane was melted with a heat gun under atmospheric Ar and added with a syringe to the THF solution to avoid contact with air. The reaction was then initiated by adding a trace amount of *p*-toluenesulfonic acid dissolved in dry THF. The mixture was stirred at 40 $^{\circ}\text{C}$ under Ar for 2 h and the reaction was stopped by addition of triethylamine. Silica gel chromatography with a solvent system containing 2% triethylamine as a stabilizer afforded the purified conjugate (20% synthetic yield) that appears homogeneous on TLC. Although the yield of the reaction was modest, we chose this synthetic route since the method is quick and the reagents are relatively inexpensive.

It should be noted that POD is actually a mixture of conjugates with the mPEG headgroup comprising of a narrow distribution of molecular weights around 2000, due to the polydisperse nature of the commercially available PEG2000 monomethyl ether. The electrospray mass spectrometry showed a distribution of peaks centered on 2697 Da, corresponding to monosodiumated molecular cations with different numbers of ethylene glycol units. The heterogeneity of mPEG as well as the stereochemistry of the conjugate also resulted in NMR peaks of POD appearing as multiplets, a feature which has been previously reported in the literature (31).

Acid Sensitivity of POD. To quickly estimate the sensitivity of the conjugate toward mildly acidic conditions relevant to biological scenarios, we employed a simple TLC assay to monitor the degradation of POD after it was incubated for different time periods at 37 $^{\circ}\text{C}$ in buffers of pH ranging from 4 to 7.4. As shown in Figure 2, within 1 h of incubation at 37 $^{\circ}\text{C}$, POD was completely degraded in buffers of pH 4 and 5 as indicated by the disappearance of the compound spot at $R_f \approx 0.4$. However, in the buffer of neutral pH 7.4, most of the conjugate remained intact over 3 h. In the buffer of pH 6, POD degraded with an approximate half-life of 2.5 h. As expected from the acid-catalyzed hydrolysis illustrated in the Introduction, the degradation of POD yielded both more hydrophilic ($R_f \approx 0.3$) and more hydrophobic ($R_f \approx 0.8$) materials, presumably derivatives of mPEG and distearoyl glycerol, respectively. The hydrophilic degradation products gave more intensely stained spots than the hydrophobic derivatives, because mPEG derivatives are much more sensitive to iodine staining than are the saturated acyl chains. Similar results were obtained when the TLC plates were stained with Molybdate reagent, which clearly showed the hydrophobic degradation products (Data not shown).

Liposome Formation. Encouraged by the TLC results on POD degradation, we proceeded to study whether POD incorporated into a high percentage of DOPE, a fusogenic lipid, can stabilize a lamellar structure in neutral and alkaline solutions, and yet induces destabilizing phase changes upon cleavage of its mPEG headgroup by hydrolysis at low pH. ANTS and DPX (24) were encapsulated into liposomes by reverse phase evaporation (22) and their leakage from the vesicles were used to monitor pH triggered release of liposomal contents. Liposomes of defined size were prepared by extruding five times through polycarbonate membranes of 200 nm pore diameter. Unencapsulated ANTS/DPX was then removed by size-exclusion chromatography. Liposomes composed of 10% POD and 90% DOPE were readily prepared and remained stable for up to 2 weeks in an alkaline HBS buffer (5 mM HEPES, 145 mM NaCl, pH 8.5) at 4 $^{\circ}\text{C}$ with no significant increase in particle size or in residual ANTS fluorescence.

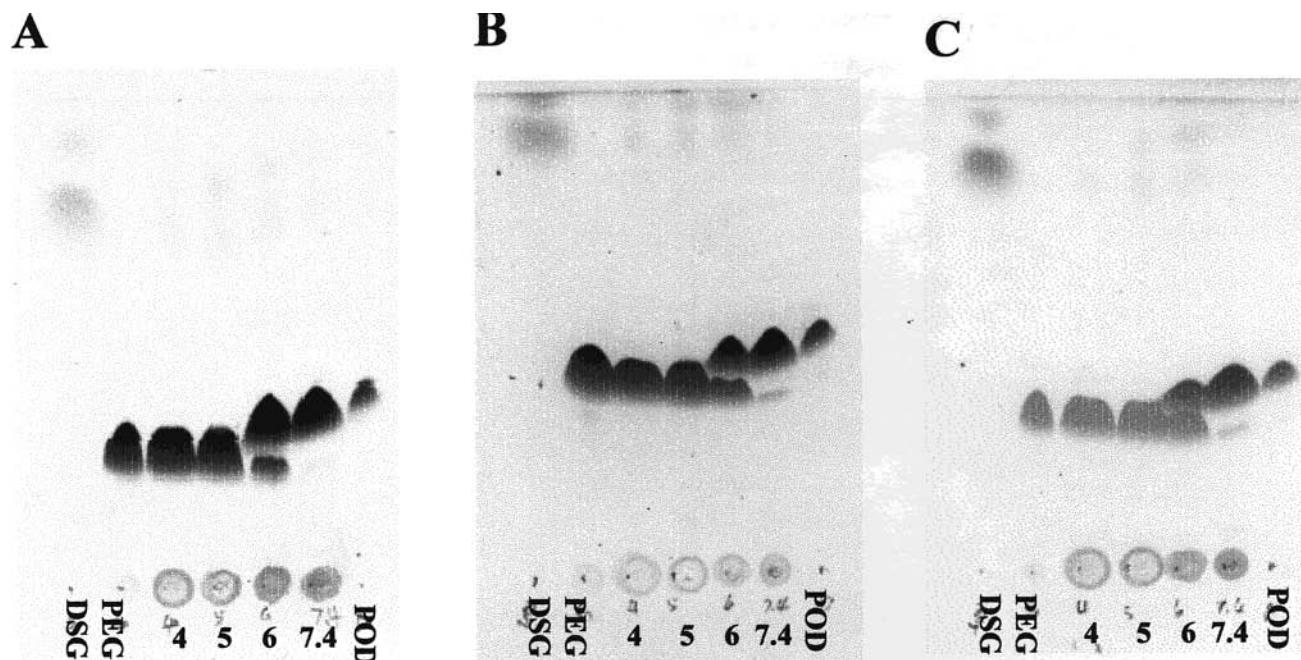


Figure 2. TLC monitored degradation of POD. Lipids were incubated for the period of 1 h (A), 2 h (B), and 3 h (C) in Na_2HPO_4 buffers of the pH indicated under the application origin. POD, mPEG, and DSG in CHCl_3 solution were spotted alongside as standards.

Acid-Triggered Liposome Aggregation. When pH sensitive liposomes composed of phosphatidylethanolamine (PE) are subjected to low pH, the PE lipids undergo a contact-induced aggregation and a phase transition from the lamellar to the hexagonal phase (24). PEG lipids at a high enough concentration on the liposome surface can shield the PE lipids of one liposome from contacting those of another liposome, thus preventing aggregation and membrane-destabilizing phase changes. Thus one can follow the loss of the PEG coating from the liposome surface as a result of POD hydrolysis by monitoring the aggregation of POD/DOPE liposomes at different pHs with photon correlation spectroscopy (PCS). However, as indicated by the TLC assays, the kinetics of the aggregation, particularly at pH below 5.5, may be too fast to be monitored by PCS, which takes about 10–20 min for a triplicate measurement. To circumvent this problem, we elected to “freeze” the aggregation process by quenching aliquots of samples in excess buffer of pH 9. The quenching stopped the acid-catalyzed POD hydrolysis and prevented the liposomes/lipid particles from contacting each other by inducing negative charges to their surfaces via deprotonation of the amine in DOPE. We found “base-freezing” to be a valid method to prevent size increases in the aggregation products. To determine if quenching of the reaction to pH 9 resulted in dispersion of aggregates into smaller particles, we took aliquots of liposomes incubated at pH 6 and diluted them in the same pH 6 buffer. The particle size of the liposomes was then immediately determined by a single PCS measurement of 2 min. The size data thus obtained were not significantly different from the diameters measured after “base-freezing”. Furthermore, the samples quenched at pH 9 gave the same PCS readings after being left overnight at room temperature. Thus, the base quenching yielded a reproducible value that reflected the particle size distribution at the quench time. Therefore, the light scattering of the aliquots quenched at pH 9 was recorded and the particle diameter from the cumulant results was plotted against incubation time in Figure 3.

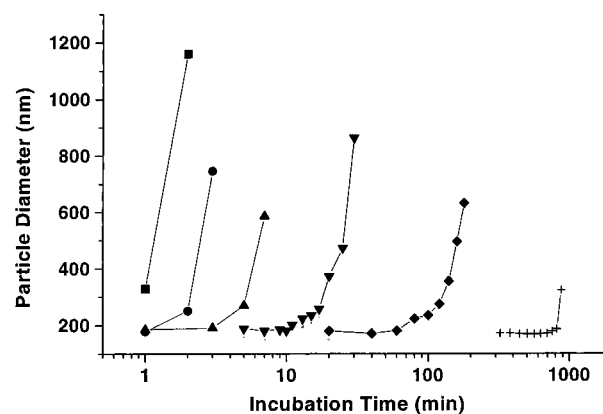


Figure 3. Aggregation of POD/DOPE (1/9) liposomes in buffers of different pH. (■) pH 4; (●) pH 4.5; (▲) pH 5; (▼) pH 5.5; (◆) pH 6.2; (+) pH 7.5.

The aggregation process showed a pH-dependent lag phase followed by a rapid increase in diameter (Figure 3). At pH 7.5, the POD/DOPE liposomes were relatively stable and the aggregation did not occur after more than 10 h of incubation at 37 °C. In buffers of lower pH, the lag phase was much shorter. At pH 6.2, the liposome size started to increase after 1 h and when the pH was decreased to 5, extensive aggregation was observed within 10 min. Thus, the kinetics of the liposome aggregation correlated with that of pH-dependent POD degradation shown by the TLC studies. These results demonstrate the ability of POD to protect the DOPE-containing liposomes from aggregating and converting into hexagonal phases until the mPEG headgroups are removed from the surface of the liposomes at low pH.

Acid-Triggered Content Leakage. Since POD was designed for potential applications in liposomal drug delivery, we used POD/DOPE liposomes containing ANTS/DPX as a model system to examine if the hydrolysis of POD can trigger the release of the contents from DOPE liposomes in response to a low pH environment. The ANTS/DPX method is often used to study pH sensitive liposomal leakage since the fluorescence of ANTS is

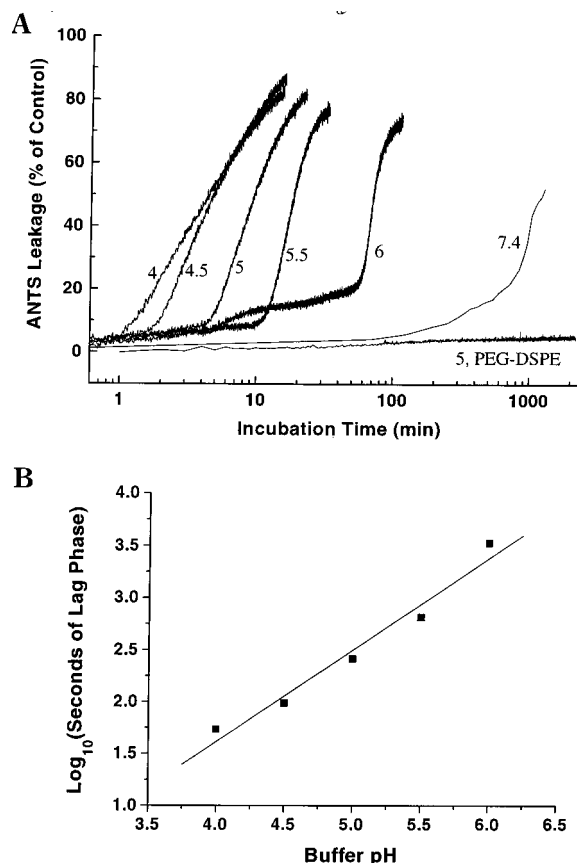


Figure 4. Acid-triggered content release from POD/DOPE (1/9) liposomes. (A) ANTIS leakage in buffers of different pH. The pH is indicated adjacent to the corresponding trace. DSPE-PEG/DOPE (1/9) liposomes were incubated at pH 5 as a control. (B) Length of lag phase in buffers of different pH. The logarithm of the length of lag phase in seconds is regressed versus the buffer pH to give $\log_{10}(\text{seconds of lag phase}) = -1.92 + 0.883 \times \text{pH}$, $r = 0.984$.

virtually unchanged from pH 4 to 8 (24). When both compounds remain encapsulated, the fluorescence of ANTIS is quenched by DPX (24). Upon leakage from the liposomes, ANTIS is no longer quenched by DPX and gives an increase of fluorescent signal at 550 nm (excitation wavelength = 370 nm). As shown in Figure 4A, the release of ANTIS at different pH also occurs in two distinct phases, a lag phase, where a small portion of ANTIS slowly leaks out of the liposomes, followed by a burst phase, when most of the ANTIS is quickly released. The burst phase correlates well with the aggregation of the POD/DOPE liposomes in Figure 3 and the collapse of the lamellar phase. As the liposomes are subjected to more acidic environments, the lag phase shortens. At pH 7.4, the lag phase lasts approximately 12 h; at pH 6, the lag phase is reduced to 60 min; at pH 5, the lag phase is less than 5 min. As a control, ANTIS/DPX was encapsulated into liposomes composed of 90% DOPE and 10% of a pH insensitive PEG lipid, DSPE-PEG. Less than 5% of the encapsulated ANTIS was released over 12 h when the vesicles were treated in the same way as the POD/DOPE liposomes even at the lowest pH. This confirms the role of acid-catalyzed hydrolysis of the PEG-diortho ester-DSG conjugate in the leakage of the POD/DOPE liposomes.

In Figure 4B, we plot the logarithm of the length of lag phase in Figure 4A against buffer pH from 4 to 6 and observe a clear linear relationship ($r = 0.984$). The lag time at pH 7.4 was not plotted in the graph since the

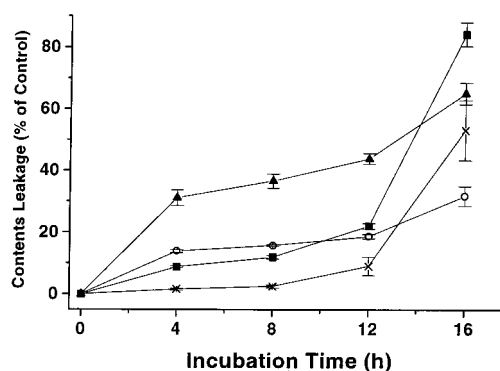


Figure 5. Contents leakage from liposomes of various compositions in 75% fetal bovine serum. ANTIS leakage was monitored after the liposomes were incubated in 75% fetal bovine serum at 37 °C for different time periods. (■) POD/DOPE (1/9); (○) DSPE-PEG/DOPE (1/9); (▲) CHEMS/DOPE (3/7); (×) POPG/POPC/Chol (1/9/8).

transition is too slow to allow a precise determination. The slope of the regressed line is 0.883. This linear relationship demonstrates that the buffer pH plays a pivotal role on the kinetics of liposomal leakage. This phenomenon is discussed in detail in the discussion section, where we propose a mathematical model to account for the data.

Stability of POD Containing Liposomes in Serum. To test the idea that POD should help stabilize liposomes in circulation, we carried out an in vitro stability test in fetal bovine serum. POD/DOPE (1/9) liposomes containing ANTIS/DPX were incubated with 75% fetal bovine serum at 37 °C and the release of ANTIS was measured every 4 h for 16 h (Figure 5). For comparison, ANTIS/DPX was also encapsulated into liposomes composed of DSPE-PEG/DOPE (1/9), CHEMS/DOPE (3/7), POPG/POPC/Chol (1/9/8), and the ANTIS leakage was monitored in the same manner.

POD/DOPE liposomes were found to be relatively stable over 12 h, when less than 25% of the ANTIS leaked out. However, after 16 h of incubation, 84% of the ANTIS was released, indicating a transition from the lag phase to the burst phase. The POD/DOPE liposomes were as stable as the pH-insensitive, PEG-stabilized DSPE-PEG/DOPE liposomes for the first 12 h, but released ANTIS much faster for the next 4 h, indicating a liposome destabilization process specifically mediated by POD hydrolysis. In the first 12 h of incubation, the POD/DOPE liposomes released 2–3-fold less ANTIS than CHEMS/DOPE liposomes, whose excess negative charges from CHEMS tend to induce more interactions with serum components (12). The stable liposome formulation composed of POPG/POPC/Chol released less than 10% of the contents after 12 h of incubation with serum (32). Thus, POD is able to stabilize liposomes in fetal bovine serum as effectively as traditional PEG-derived lipids for up to 12 h, which is consistent with our findings on ANTIS leakage of these liposomes in HEPES buffered saline. (Figure 4).

Stability of Liposomes in Blood Circulation. The steric-stabilizing effect of POD on liposomes in vivo was evaluated by tail-vein injection of radiolabeled liposomes composed of 10% POD and 90% of DOPE into female ICR mice and measuring their rate of elimination from the blood (Figure 6). For comparison, we also administered radiolabeled liposomes composed of 10% DSPE-PEG, a pH-insensitive lipid-PEG conjugate, and 90% DOPE. Liposomes composed of pure DOPE are not stable enough at pH 7.4 to allow their injection into animals. For both

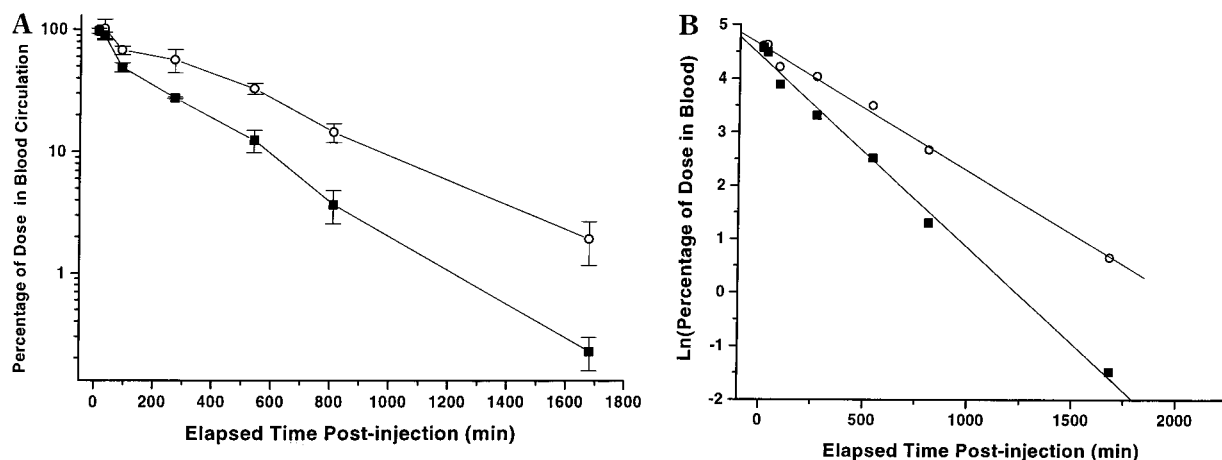


Figure 6. Clearance of [^{125} I]BPE POD/DOPE (1/9) liposomes (■) and [^{125} I]BPE DSPE-PEG/DOPE (1/9) liposomes (○) from circulation. (A) The percentage of the injected dose remaining in blood ($n = 3$, mean $\pm$ SD) is plotted against the elapsed time postinjection. (B) The natural logarithm of the percentage of dose remaining in blood is plotted against elapsed time postinjection and regressed to obtain the liposomal half-life. For POD/DOPE (1/9) liposomes, $\ln(\text{percentage of dose in blood}) = 4.41 - 0.00358 \times (\text{min})$, $r = 0.997$, $T_{1/2} = \ln 2 / 0.00358 = 193.6$ min. For DSPE-PEG/DOPE (1/9) liposomes, $\ln(\text{percentage of dose in blood}) = 4.62 - 0.00235 \times (\text{min})$, $r = 0.997$, $T_{1/2} = \ln 2 / 0.00235 = 295.0$ min.

POD/DOPE and DSPE-PEG/DOPE liposome formulations, the percentage of injected dose remaining in circulation showed a single log-linear decay with a correlation coefficient constant greater than 0.995, indicating a one-compartment clearance kinetics. The blood clearance profile of the POD/DOPE liposomes is similar to the pH-insensitive, sterically stabilized DSPE-PEG/DOPE liposomes than to conventional liposomes, which usually exhibit a two-compartment elimination kinetics (33). The half-life of the POD/DOPE liposome and the DSPE-PEG/DOPE liposome is about 194 and 295 min, respectively. This reasonably close correspondence of the elimination rate between the two formulations is strong evidence that the mPEG headgroup in the pH-sensitive ortho ester conjugate provides a steric-stabilizing effect on the fusogenic DOPE liposomes in circulation.

Distribution and Excretion of Liposomes. To investigate the distribution of radiolabeled POD/DOPE liposomes in more detail, we measured the radioactivity associated with different organs of mice at 270 min, 810 min and 28 h following liposome injection. In previous studies with the ^{125} I-labeled BPE (25), it is known that this radioactive lipid label is metabolized only after being endocytosed together with the liposomes. The urine and the feces were also collected at the 28-h time point and their radioactivity measured to determine the routes of ^{125} I excretion. At 270 min postinjection (Figure 7A), about 20% of POD/DOPE liposomes and 45% of DSPE-PEG/DOPE liposomes remained in circulation. For both formulations, the liver and intestine were the two major organs of radioactivity disposition.

At later time points (810 min and 28 h postinjection), most of the radioactivity of both formulations was excreted from the animals (Figure 8); the liver and intestine were the major organs for the disposition of the remaining radioactivity in the body (Figure 7B, 7C). Twenty-eight h after injection, the residual radioactivity from POD/DOPE liposomes was distributed preferentially to the head (Figure 7C), probably due to an accumulation of the iodine in the thyroid followed by a slow clearance of ^{125} I from this organ. For both formulations at 28 h postinjection, about 70% of radioactivity was excreted into urine and about 20% was found in feces (Figure 9). Overall, POD/DOPE liposomes were eliminated from circulation about 50% faster than DSPE-PEG/DOPE

liposomes but the patterns of distribution and excretion of the two formulations were very similar.

DISCUSSION

The novel pH-sensitive ortho ester lipid derivative introduced here is a useful addition to the other reported pH-sensitive linkages for modifying liposome surfaces. The synthesis is simple and versatile. Although we illustrate the process with monomethyl ether of PEG, the chemistry could be used to attach various other headgroups to diacyl/steryl hydrophobic groups. For instance, anionic groups, cationic groups and even targeting ligands can be attached to a lipid anchor to create a triggerable headgroup for liposome formulations, micelles and other particulates. Modified glycerols, dendrons (34), or poly(vinylpyrrolidone) (35) could also be conjugated to lipids using this chemistry.

Regarding to the synthesis of POD, it should be noted that the acid-catalyzed conjugation does not produce POD as the unique product. Our preliminary TLC observations suggested that the side-products of the reaction include the conjugate of two mPEG or two distearoyl glycerol molecules on both ends of the diortho ester linker. The presence of side reactions, along with the loss of POD during its chromatography purification, may account for the moderate yield of the synthesis.

POD showed a remarkably quick degradation even at a mildly acidic pH of 5–6. Such sensitivity to acidic pH is significantly higher than that of acetals, vinyl ethers, and polyortho esters. We attribute the higher sensitivity of POD to the following two factors. First, the dialkoxy carbocation intermediate of ortho ester hydrolysis has four lone pairs of electrons over which to distribute the positive charge from the carbon, and hence is much more stable (Scheme 1) than the monoalkoxy carbocation intermediate, stabilized by two lone pairs of electrons, in the case of acetals and vinyl ethers. Second, the PEG headgroup of POD is more hydrophilic than the functional groups of the reported polyortho esters, allowing better hydration and faster proton transfer to the diortho ester linkage (36). When POD was incorporated into liposomes composed of a high percentage of DOPE, POD hydrolysis at pH 5–6 triggered the extensive aggregation and leakage of the liposomes in 10–100 minutes.

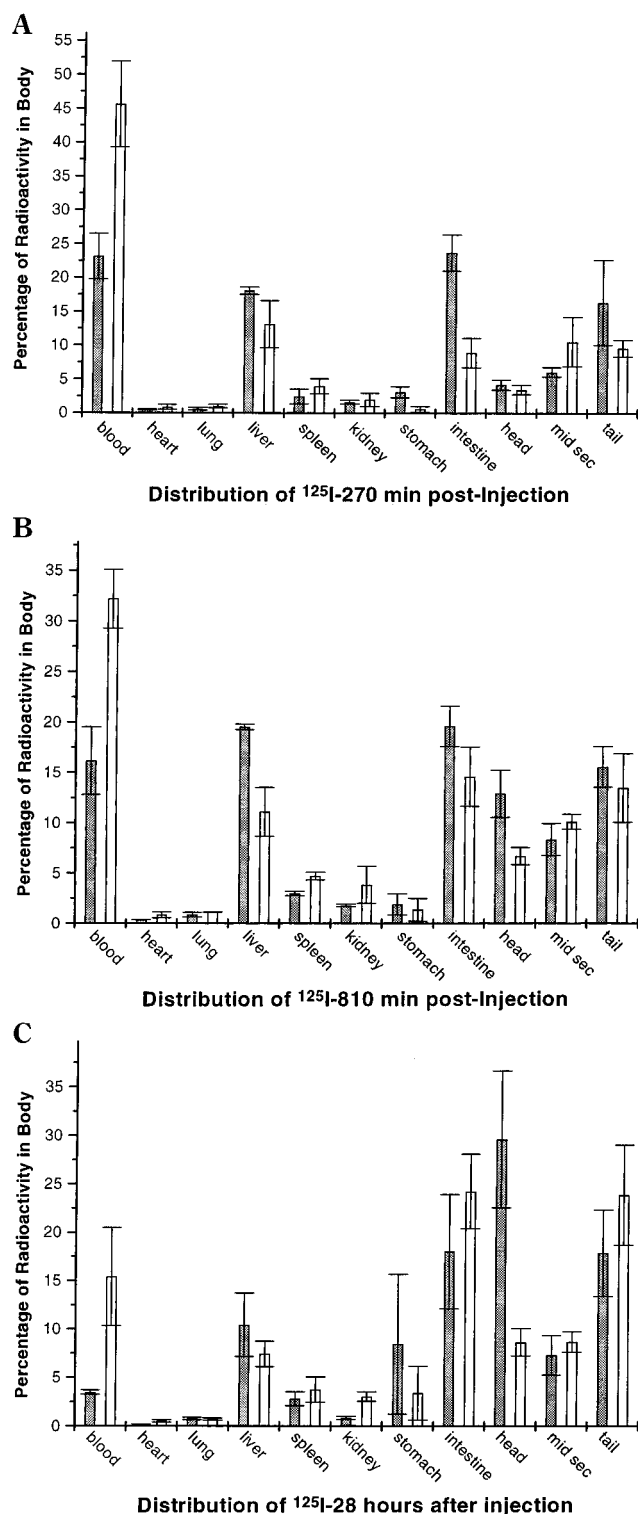


Figure 7. Distribution of [^{125}I]radioactivity of POD/DOPE (1/9) (solid bars) and DSPE-PEG/DOPE (1/9) (open bars) liposomes in female ICR mice at 270 min (A), 810 min (B), and 28 h (C) postinjection. ($n = 3$, mean $\pm$ SD).

We believe that the rapid hydrolysis of POD at mildly acidic pH is a very useful property for drug/gene delivery systems because the decrease of pH at potential therapeutic sites may be only one pH unit or less. For example, the transit through the endosomes in cells occurs in about 10 to 30 min with pH in the range of 5–6 before the endosomal contents are delivered into the lysosome (37, 38). Therefore, it is important for pH sensitive liposomes

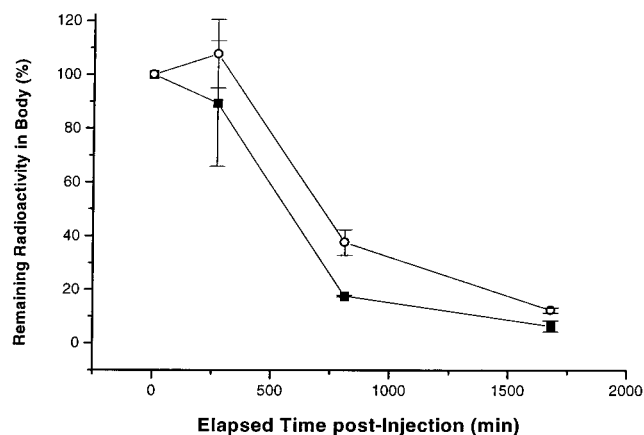


Figure 8. Excretion kinetics of [^{125}I]radioactivity of POD/DOPE (1/9) (■) and DSPE-PEG/DOPE (1/9) (○) liposomes.

to respond quickly to the initial drop in pH and release their contents prior to trafficking into the lysosomal compartment. The pH-dependent release profile of POD/DOPE liposomes may also be important for triggered release at inflammatory tissues (7, 40) and solid tumors (6, 39), where the pH is only 0.5–1 unit more acidic than that of the circulation.

The lag phase of ANTS leakage (Figure 4) corresponds quite closely to the lag time needed for liposome aggregation as measured by the increase of particle diameter (Figure 3). On the basis of these observations, we propose the following model of acid-catalyzed destabilization of POD/DOPE liposomes. Once the liposomes are subjected to an aqueous phase of mildly acidic or neutral pH, the mPEG headgroups are continuously cleaved off the liposome surface as shown in Scheme 1. However, the lipids remain in the lamellar phase until the amount of mPEG on the liposome surface decreases to a critical level, at which point the bilayers of two vesicles can come in contact. The bilayer contact is what triggers the aggregation of liposomes, the transition to the hexagonal phase and the release of liposome contents (24). The model is consistent with the known properties of PEG coated liposomes (41) and the requirement of bilayer contact to trigger the lamellar-hexagonal transition in PE liposomes (24). Assuming complete hydration and unaltered local pH at the liposome surface, the following equation holds:

$$dA/dt = -kA[\text{H}^+] \quad (1)$$

where A is the number of mPEG groups on the surface of a liposome of certain size, $[\text{H}^+]$ is the proton concentration of the aqueous medium, and k is the rate constant for ortho ester hydrolysis. In a buffer of constant pH, the equation can be integrated to give

$$A = A_0 \exp(-k[\text{H}^+]t) \quad (2)$$

where A_0 is A at an arbitrary starting point of POD hydrolysis and t is the incubation time from the starting point. At the transition point between the lag phase and the burst phase of liposomal leakage, we have

$$A_c = A_0 \exp(-k[\text{H}^+]t_c) \quad (3)$$

where A_c is the critical amount of mPEG on the surface of the liposomes necessary to stabilize the lamellar structure and t_c is the length of the lag phase. If the same liposome preparation is incubated in buffers of different pH, both A_c and A_0 should be constant and we can write

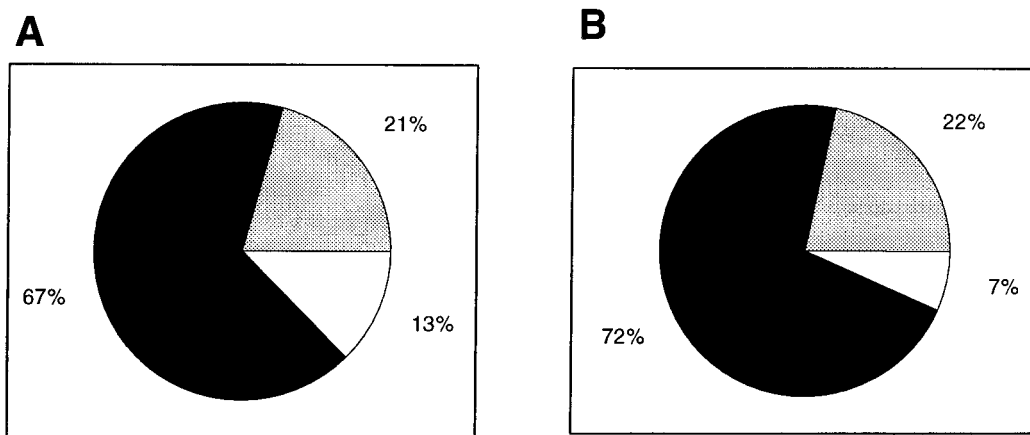


Figure 9. Mass balance of injected radiolabeled liposomes. [^{125}I]Radioactivity of POD/DOPE (1/9) (A) and DSPE-PEG/DOPE (1/9) (B) liposomes from all parts of body (white), urine (black), and feces (gray) were measured 28 h postinjection and plotted in percentage of the total radioactivity recovered. The percentage of injected dose recovered is 110% for POD/DOPE liposomes and 93% for DSPE-PEG liposomes.

the following equation:

$$[\text{H}^+]t_c = C \quad (4)$$

where C is a composite constant composed of A_c , A_o , and k . After taking the logarithm of eq 4 followed by rearrangement, the length of the lag time and the pH of the buffer would have the following relationship:

$$\log t_c = \text{pH} + C' \quad (5)$$

where C' is a constant.

Equation 5 fits closely to the observed leakage data in Figure 4B. The linear relationship between the logarithm of observed lag time and buffer pH, as well as the slope of the regressed line in Figure 4B (0.883 versus 1 in eq 5), support this mechanistic model of acid triggered phase changes of POD/DOPE liposomes. Further biophysical studies are required to confirm this model but the simple model is consistent with previous studies on lamellar-hexagonal transition as well as the data presented in Figure 2, 3, 4, and 5.

When incubated in 75% fetal bovine serum, POD was able to stabilize POD/DOPE (1/9) liposomes for up to 12 h, a finding consistent with the lag time for ANTS leakage of POD/DOPE liposomes in HEPES buffered saline at the same pH. Thus the stability characteristics vis-à-vis drug retention of this two component formulation are compatible for targeted delivery of encapsulated molecules given the observed intravenous elimination rate ($T_{1/2} = 194$ min) of the formulation. Based on the distribution and the excretion data, we propose that both formulations were cleared mainly by liver from circulation, after which the [^{125}I]BPE labels were degraded and excreted into urine and bile.

The half-life of the POD/DOPE formulation in circulation is similar to other formulations that consist primarily of DOPE and that are stabilized by PEG (42, 43). It is not surprising that such formulations have a shorter serum half-life than the traditional sterically stabilized formulations that consist of saturated phosphatidylcholine/Chol/PEG-DSPE (44). Such composition yields an inherently more rigid bilayer that is capable of resisting the penetration of serum components that might contribute to a more rapid elimination of a liposome even when it has a steric coat. Thus, we suspect that further improvements of the serum half-life and drug release characteristics can be achieved by modification of the

lipid composition such as by the inclusion of cholesterol in the formulation (32).

CONCLUSION

In conclusion, a PEG-diortho ester-distearoyl glycerol conjugate (POD) was designed and prepared by a one-step synthesis. POD was characterized for its pH sensitivity and ability to stabilize liposomes in serum and in blood circulation. POD was found to be highly sensitive to acidic conditions but relatively stable at neutral pH. Liposomes composed of 10% POD and 90% DOPE aggregated and released their contents at a mildly acidic pH similar to that found in an endosome. The destabilization of POD/DOPE liposomes consists of two phases, a lag phase where encapsulated contents slowly leaked from the vesicles, and a burst phase that was associated with liposome aggregation and rapid release of contents. Liposomes composed of 10% POD and 90% DOPE remained stable in 75% fetal bovine serum at 37 °C for 12 h and had a half-life of about 200 min in blood circulation of female ICR mice. The fast kinetics of acid-catalyzed POD hydrolysis and its ability to stabilize liposomes in serum and in blood circulation may provide important advantages for the lipid to be used for drug and gene delivery.

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Enzyme Conjugation to the Polysaccharide Chitosan: Smart Biocatalysts and Biocatalytic Hydrogels

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Laccase from *Coriopsis gallica* was conjugated to the renewable biopolymer chitosan using carbodiimide chemistry. The laccase–chitosan conjugate was observed to offer three unique properties. First, the laccase–chitosan conjugate displayed pH-responsive behavior such that the conjugate was soluble and active under acidic conditions, but precipitated when the pH was raised toward neutrality. Second, the laccase–chitosan conjugate was more stable than free laccase at extreme pHs. At pH 1, the inactivation rate constant (k_{in}) for the soluble laccase–chitosan conjugate was 20-fold less than that for free laccase. At pH 13, k_{in} for the insoluble laccase–chitosan conjugate was nearly 3-fold less than that for free laccase. Finally, the laccase–chitosan conjugate could be cross-linked under mild conditions to create biocatalytic hydrogels. Potential benefits for enzyme–chitosan conjugates are discussed.

INTRODUCTION

There are a variety of reasons for coupling polymers with enzymes. Polymer conjugation has been reported to reduce the autolysis of proteolytic enzymes (1) and to allow enzymes to dissolve in nonaqueous solvents (2–4). “Smart” biocatalysts have been prepared by conjugating enzymes to stimuli-responsive polymers that respond to changes in pH (5, 6), ionic strength (7), temperature (8–10), redox potential (6), and light (11). Biocatalytic composites have been created by grafting olefinic functionality to enzymes and then incorporating these enzyme-monomers into synthetic polymer matrices through standard free radical polymerization reactions (12, 13). Similarly, biocatalytic composites have been created with polyurethane-based (14–16) and silicon-based materials (17). Advantages of these biocatalytic composites are that the matrix can be selected to enhance performance (18) and the composites can be cast or formed into various shapes and sizes including foams (14–16) and monoliths (17). Finally, there are numerous examples in which enzymes bound to sugars, or sugar-based polymers are stabilized. Although the stabilization mechanism is not understood it may be due to; a reduction of autolysis (for proteases) (19, 20), multipoint attachment that may limit enzyme distortions (21, 22), or microenvironmental effects (23).

Here we describe the use of chitosan as our polymer for enzyme conjugation. Chitosan is a biopolymer derived from chitin and, as shown in Figure 1, chitosan is made primarily of glucosamine repeating units. Because of its

high amino content, chitosan is soluble in mildly acidic aqueous solutions but is insoluble near and above its pK_a of 6.3 (24, 25). As indicated in the upper path of Figure 1, we examined whether conjugation to chitosan could confer pH-responsive behavior to an enzyme. Additionally, chitosan's amino groups are nucleophilic and reactive at higher pHs. The reactivity of these amino groups allows chitosan to be cross-linked under mild conditions to create gel matrixes (26) of various shapes and sizes including beads (27), membranes (28–30), and fibers (31–33). The lower path in Figure 1 shows that we examined whether cross-linking could be used to incorporate a conjugate into a chitosan hydrogel. It should be noted that cross-linked chitin and chitosan have been extensively studied as insoluble supports for enzyme immobilization (34, 35).

As a model enzyme, we selected the fungal laccase from *Coriopsis gallica*. Laccases are oxidative enzymes that function under acidic conditions (36, 37) and are actively being studied for applications in the pulp and paper industry (38) and for environmental remediation (39). The laccase from *C. gallica* has also been tested for the decolorization of textile effluents (40, 41), and has been observed to be capable of oxidizing polycyclic aromatic hydrocarbons (42). Because of the interest in laccase, several groups have examined its immobilization (43 and references therein) and one group recently reported the immobilization and cross-linking of laccase and chitosan (44).

MATERIALS AND METHODS

Chitosan, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)(ABTS), and buffer salts were obtained from Sigma Chemicals Co. (St. Louis, MO). *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (ECD) was purchased from Fluka Chemie AG (Buchs, Switzerland). Laccase was obtained from the white-rot fungus *C. gallica* and purified as described previously (40).

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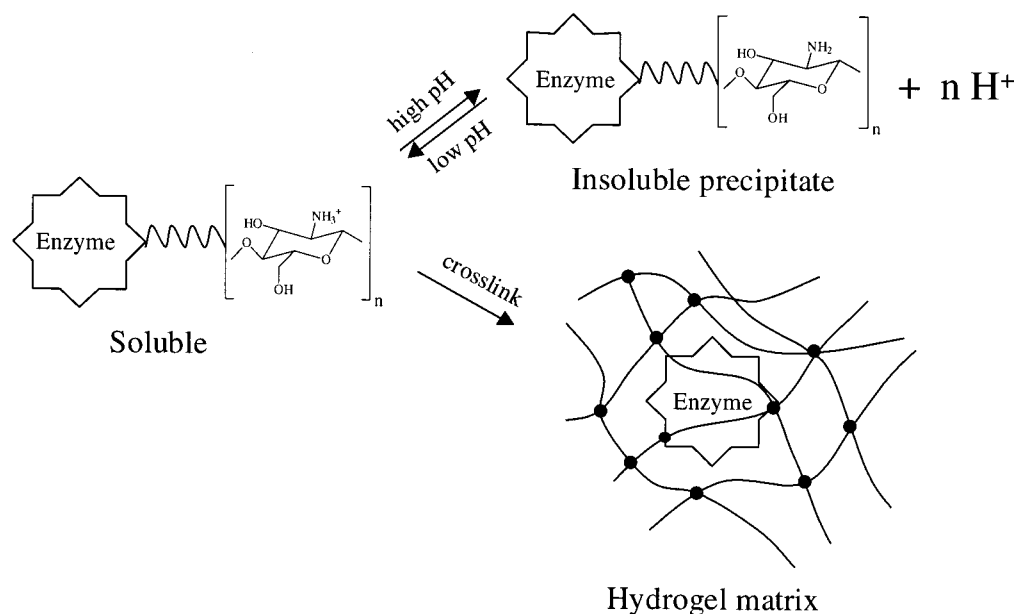


Figure 1. Enzyme-chitosan conjugate for pH-responsive solubility (upper path) and biocatalytic hydrogels (lower path).

The laccase–chitosan conjugate was prepared by mixing laccase (33 units) and chitosan (final concentration 1.0 w/w %) at pH 4.0 to obtain a solution with a molar ratio of glucosamine monomer/protein of 8000. To this solution, 1.6 mM of ECD was added (200 molar excess of relative to laccase). The reaction mixture was kept at room temperature for 2 h, and then the pH was increased to 8.0 by adding 10 volumes of 100 mM Tris buffer pH 8.0 and the suspension was cooled on ice. The precipitated laccase–chitosan conjugate was separated by centrifugation at 5000 rpm for 5 min. The pellet was then dissolved into 3 mL of 60 mM phosphate buffer and the pH was adjusted to 4.0 by adding 0.2 M HCl. When the laccase–chitosan conjugate was prepared for determining the pH-activity profile, for measuring stability at extreme pHs, and for preparing biocatalytic hydrogels, the preparation was precipitated and solubilized three additional times to remove nonconjugated laccase. The laccase–chitosan conjugate was stored in solution (pH 4) at 4 °C.

Enzymatic activities for free and conjugated laccase were determined colorimetrically using ABTS as the substrate. Assays were conducted in 1 mL reaction mixture containing 1 mM ABTS in a 60 mM acetate buffer (pH 4.5). Reactions were initiated by adding free or conjugated enzyme and laccase activity was estimated from the absorbance increase at 436 nm using an extinction coefficient of $23\,900\text{ M}^{-1}\text{ cm}^{-1}$ (45). Laccase units are defined as 1 μmol of substrate oxidized/min at 25 °C.

The stability of free and conjugated laccase at extreme pHs was compared by incubating 100 μL of each preparation in buffered solutions of different pH. An acetate-HCl buffer (60 mM) was used to achieve a pH of 1, while a sodium carbonate-NaOH buffer (60 mM) was used for pHs of 12 and 13. After incubation, the mixture was diluted to 1 mL with acetate buffer, pH 4.5, and enzyme activity was assayed at pH 4.5 as described above. The inactivation rate constants (k_{in}) were determined from a first-order fit of the loss of enzyme activity over time.

Biocatalytic gels were prepared by glutaraldehyde cross-linking. A solution containing laccase–chitosan conjugate (5.19 units/mL) was mixed with solution containing nonconjugated chitosan to obtain a solution with a laccase activity of 0.5 units/mL and a chitosan

concentration of 1 w/w %. To initiate cross-linking, differing amounts of a concentrated glutaraldehyde solution were added to these solutions to obtain glutaraldehyde concentrations between 3.1 and 31 mM (glutaraldehyde/glucosamine monomer molar ratios ranging from 0.05 to 0.5). Cross-linking was allowed to occur overnight at room temperature. The enzyme activity for the cross-linked chitosan gels could not be determined by the colorimetric procedure because the gels absorbed the colored product of the ABTS reaction. To determine the laccase activity of the biocatalytic gels, the consumption of dissolved oxygen (Microelectronics Inc., Bedford, NH) was measured for the reaction of catechol (20 mM) in 60 mM acetate buffer, pH 4.5. The reactions were carried out in a sealed container and initiated by adding catechol.

The relative stiffness (i.e., “strength”) of the cross-linked hydrogels was determined by a penetration method described by Gregson et al. (46). For this measurement, gels were formed in a cylindrical container (42.3 mm diameter $\times$ 9.0 mm high) by mixing varying amounts of concentrated glutaraldehyde to a chitosan solution (1 w/w %). The relative stiffness was determined by forcing a cylindrical probe (15.64 mm diameter) into the gels at a constant velocity of 0.1 mm/s. After correcting for the buoyancy, the measured force could be used to calculate an “apparent Young’s modulus” which is a relative measure of gel stiffness (46).

RESULTS AND DISCUSSION

Purified laccase (33 units) from *C. gallica* was conjugated to chitosan using carbodiimide chemistry. Conjugation was performed at a pH of 4.0 and after conjugation, the solution pH was raised to 8 resulting in the formation of a white precipitate. The precipitate and supernatant were separated, and after adding phosphate buffer (pH 4) to the precipitate it was observed to redissolve in about 10 min. A sample of the redissolved precipitate was tested and observed to have 9.3 units of laccase activity. The unprecipitated, supernatant fraction was observed to have 16.3 units of laccase activity. This sample (designated cycle 1 in Figure 2) shows that 28% of the initial laccase activity was conjugated and the laccase–chitosan conjugate possess pH-responsive behavior.

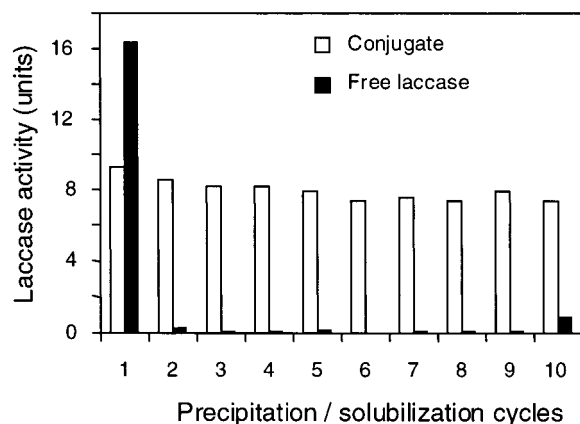


Figure 2. pH-responsive solubility of the laccase–chitosan conjugate. Laccase–chitosan conjugate was subjected to sequential cycles of precipitation at pH 8 and solubilization at pH 4. The activity of the precipitated (conjugate) and soluble (free laccase) fractions were measured under standard conditions (pH 4.5) after allowing about 10 min for the conjugate to redissolve. Cycle 1 represents the conjugation step and the soluble laccase from cycle 1 includes the activity of nonconjugated laccase. Precipitation can completely recover laccase activity of the conjugate.

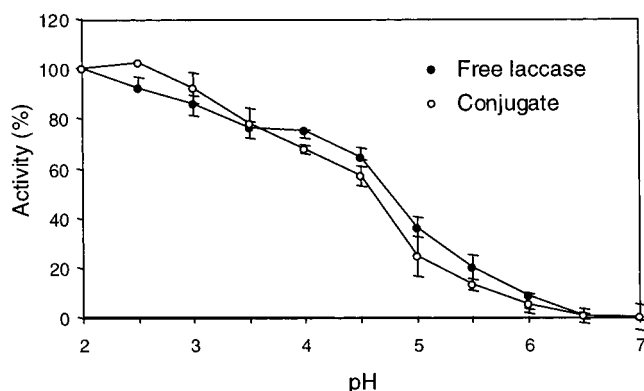


Figure 3. pH-activity profile for free laccase and the laccase–chitosan conjugate. Activity was measured colorimetrically using ABTS as substrate. Conjugation does not affect laccase's pH-activity profile.

To test the reversibility and completeness of precipitation, we subjected the laccase–chitosan conjugate to several cycles of precipitation (pH 8) and redissolution (pH 4). In each cycle, the precipitate was separated from the supernatant and redissolved in fresh buffer. Figure 2 shows that over the course of 10 cycles only small amounts of laccase activity were lost in the supernatants. More importantly, Figure 2 shows that the activity of the laccase–chitosan conjugate was almost quantitatively recovered by precipitation. Further, these results indicate that precipitation does not inactivate laccase.

We measured the activity of free and conjugated laccases at various pHs to determine if conjugation affects the pH-activity profile. Figure 3 shows that laccase is active at pH 2 and the activity decreases monotonically to zero at neutral pHs. This pH-activity profile is similar to that reported by Xu (36) for the ABTS substrate. Figure 3 shows that conjugation to chitosan does not alter the pH-activity profile of laccase. Similar results were reported by D'Annibale et al. (44) for laccases that had been immobilized onto insoluble chitosan supports.

The effect of conjugation on enzyme stability was compared by exposing free and conjugated enzyme to pH extremes. At various times, samples from extreme pH solutions were assayed for laccase activity under stan-

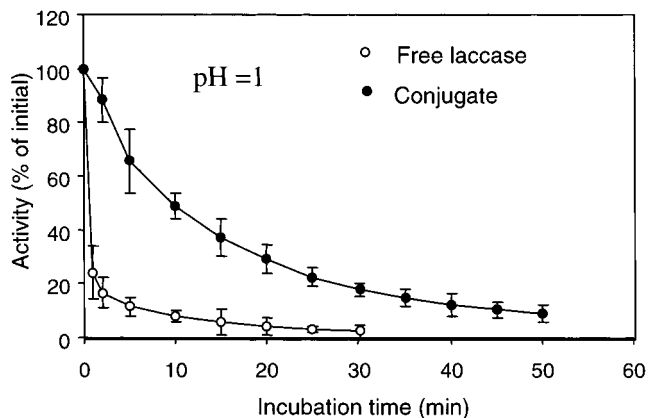


Figure 4. Stabilization of laccase to extreme acid conditions. Enzyme was incubated for varying times at pH 1 and activities were measured under standard conditions (pH 4.5). Conjugation resulted in a 20-fold reduction in the inactivation rate constant (k_{in} was 85 h^{-1} for free laccase and 3.6 h^{-1} for the laccase–chitosan conjugate).

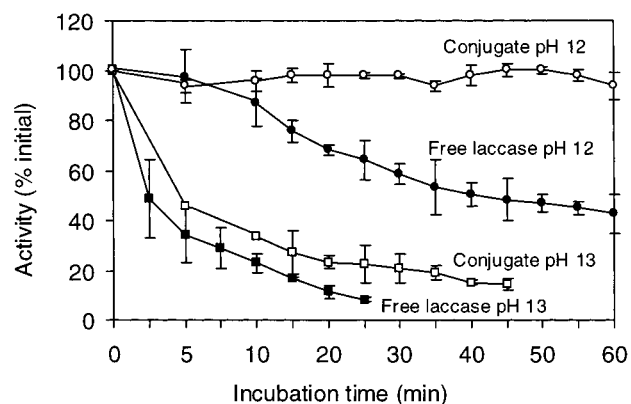


Figure 5. Stabilization of laccase to extreme base conditions. Enzyme was incubated for varying times at pH 12 or 13, and activities were measured under standard conditions (pH 4.5). The laccase–chitosan conjugate was fully stable at pH 12. Conjugation resulted in nearly a 3-fold reduction in the inactivation rate constant at pH 13 (k_{in} was 4.5 h^{-1} for free laccase and 1.6 h^{-1} for the laccase–chitosan conjugate).

dard conditions (pH 4.5). Figure 4 shows that exposure to pH 1 leads to a rapid loss of activity for free laccase, while the soluble laccase–chitosan conjugate loses activity more slowly. The first-order inactivation rate constants (k_{in}) for this extreme acid condition were estimated to be 85 and 3.6 h^{-1} for free and conjugated laccases, respectively. Figure 5 shows results for the alkaline extremes. At pH 12, the free enzyme was gradually inactivated ($k_{in} = 0.89 \text{ h}^{-1}$), while no inactivation could be detected for the insoluble laccase–chitosan conjugate. The protective effect of chitosan persisted even when the laccase–chitosan conjugate was incubated at pH 12 and 3 M NaCl (data not shown). At the extreme pH of 13, Figure 5 shows that the free laccase was inactivated more rapidly ($k_{in} = 4.5 \text{ h}^{-1}$) than the laccase–chitosan conjugate ($k_{in} = 1.6 \text{ h}^{-1}$).

Fungal laccases have relatively good pH stabilities (47) and the results in Figures 4 and 5 show that conjugation to chitosan improves stabilities under acidic and basic pH extremes. This stabilization was observed with both soluble (low pH) and insoluble (high pH) conjugate. D'Annibale et al. (44) also observed that laccases that had been immobilized onto insoluble chitosan supports were more stable to acidic conditions. The nature of this stabilization is not known although various investigators

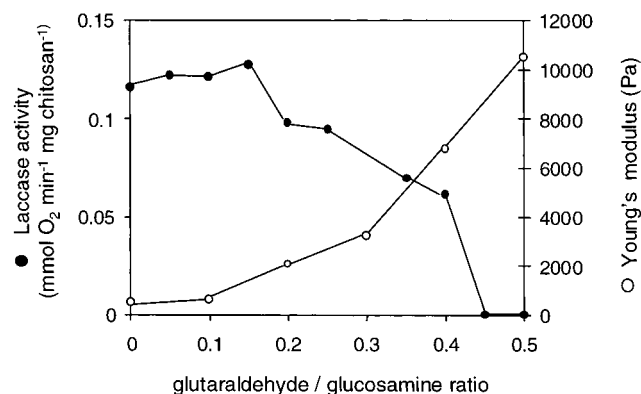


Figure 6. Biocatalytic hydrogels created by glutaraldehyde cross-linking. Laccase-chitosan conjugate was mixed with non-conjugated chitosan (final laccase activity 0.5 units/mL and final chitosan concentration 1 w/w %) and cross-linked with varying levels of glutaraldehyde (a molar ratio of glutaraldehyde to glucosamine monomer of 0.5 corresponds to the use of 32 mM glutaraldehyde). The apparent Young's modulus was measured by a penetration method (46) while laccase activity was measured from dissolved oxygen consumption with catechol as the substrate.

have observed that sugars and sugar-based polymers can stabilize enzymes during lyophilization (23) and in non-aqueous environments (19, 20). Also, stabilization has been reported when enzymes are bound through multiple points (21, 22). Conceivably, the laccase may be covalently conjugated to chitosan at more than one "point". Also, since laccases have a large number of surface carboxylates (48), it is possible that electrostatic interactions with chitosan's amino groups could lead to multi-point, stabilizing interactions. Interestingly, when we "conjugated" laccase to glucosamine (the monomeric unit of chitosan), we observed no stabilization of laccase (data not shown).

Laccase-containing chitosan hydrogels were prepared by glutaraldehyde cross-linking. To prepare these hydrogels, laccase-chitosan conjugate was diluted with nonconjugated chitosan (final polymer concentration 1 w/w % and final laccase activity 0.5 units/mL), and varying levels of glutaraldehyde were added to these solutions (pH 4). Over the course of several hours, these solutions were observed to form gels. As expected, Figure 6 shows that the stiffness, or apparent Young's modulus, of the cross-linked chitosan gels increased with increasing glutaraldehyde levels. The enzymatic activities of glutaraldehyde-cross-linked chitosan hydrogels were measured by adding 100 μ L of gel to substrate solution (1 mL total volume) and measuring consumption of dissolved oxygen. Figure 6 shows that laccase activity was unaffected by cross-linking when less than 9 mM glutaraldehyde was used (<0.15 glutaraldehyde/glucosamine monomer molar ratio). At intermediate glutaraldehyde levels of 12–25 mM (0.2–0.4 molar ratio), Figure 6 shows that laccase activity decreased with increasing glutaraldehyde. At glutaraldehyde levels above 28 mM (>0.45 molar ratios) laccase activity was completely lost. The results in Figure 6 indicate that glutaraldehyde can be used to create laccase-containing chitosan hydrogels and the cross-linker level must be selected to balance the need to retain enzymatic activity with the desire to achieve a given gel strength. It should be noted that this compromise is not limited to conditions examined in Figure 6, as alternative chitosan concentrations and different cross-linkers could also be considered.

One additional characteristic of chitosan that must be considered, is that the amino groups may react with

electrophilic substrates or products. Chitosan's reactivity may limit its use in some applications, but it also provides additional opportunities. For instance, tyrosinase-containing chitosan gels can convert phenols into electrophilic quinones that react with the chitosan matrix (49). Because the substrate specificity of tyrosinase confers selectivity to the conversion and the quinone-chitosan reaction results in strong binding, tyrosinase and chitosan can be used to selectively remove phenolic contaminants from intermediate chemical process streams for industrial waste minimization (50–53).

In conclusion, chitosan is a versatile biopolymer that is obtained from renewable resources and is readily biodegraded in the environment. We report three useful features that result from conjugating enzymes to chitosan. First, the laccase-chitosan conjugate has pH responsive behavior such that the conjugate is soluble at pHs of maximal laccase activity, yet insoluble at neutral pHs. This pH-responsive behavior endows the conjugate with the mass transfer advantages of soluble enzymes, while allowing the enzyme to be readily recoverable, the characteristic benefit of immobilized enzymes. Second, conjugation to chitosan stabilized laccase to extreme pHs. This stabilization occurred with soluble (low pH) and precipitated (high pH) conjugate. Third, the enzyme-chitosan conjugate can be cross-linked under mild conditions to yield biocatalytic hydrogels. This result suggests the potential for cross-linking biocatalytic hydrogels in various shapes and sizes including beads, membranes, fibers, and monolith coatings.

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Synthesis of Fluorogenic Substrates for Continuous Assay of Phosphatidylinositol-Specific Phospholipase C

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An improved synthesis of fluorogenic substrate analogues for phosphatidylinositol-specific phospholipase C (PI-PLC) is described. The water-soluble substrates, which are derived from fluorescein, are not fluorescent until cleaved by the enzyme, and provide a convenient means to continuously monitor PI-PLC activity. The improvement in the synthesis lies in the method used to protect the hydroxyl groups of the inositol portion of the substrate molecule and allows a milder deprotection procedure to be used. The result is a much more reproducible synthesis of the substrate. The improved procedure has been employed to synthesize a series of fluorogenic substrates, which differ in the length of the aliphatic tail attached to the fluorescein portion of the molecule. The length of the tail was found to have a significant effect on the rate of cleavage of these substrates.

INTRODUCTION

The phosphatidylinositol specific phospholipase C (PI-PLC) family of enzymes plays an important role in cellular signaling (1–4). These enzymes cleave phosphatidylinositol and its phosphorylated analogues, forming inositol phosphates and diacylglycerol, which can act as intracellular second messengers in eukaryotes, ultimately leading to a variety of cellular responses. Bacterial PI-PLCs are of interest as model systems for the mammalian signal transduction isozymes, as possible virulence factors, and for their ability to cleave glycosylphosphatidylinositol (GPI)-anchored proteins (5). PI-PLC activity is most often measured in a discontinuous assay using radiolabeled phospholipids as substrates, with the assays requiring the separation of product from uncleaved substrate before enzyme activities can be determined (6). The discontinuous nature of these assays has led to a search for water-soluble substrates for PI-PLC which would also allow continuous monitoring of PI-PLC activity (7). Being able to monitor assays continuously is especially advantageous when carrying out studies of enzyme kinetics (8).

We previously reported the synthesis of the first fluorescein-containing substrate for the continuous fluorescence assay of PI-PLC (9). This compound **1** (Scheme 1) is the only reported fluorogenic substrate that is cleaved by both the bacterial and mammalian forms of PI-PLC (10). Three specific properties make this compound especially attractive as a fluorogenic probe: (a) the high fluorescence quantum yield of fluorescein compounds, (b) the nonfluorescence of the substrate **1** itself, and (c) the relatively low pK_a (~4.8) of the fluorescent product **3**, ensuring that a high percentage of the product is deprotonated, and therefore fluorescent, at physiological pH.

The useful properties of probe **1** prompted us to investigate its closest analogues by varying the aliphatic tail attached to the fluorescein part of the molecule. The different substituents on the aromatic ring can have a significant impact on certain physicochemical characteristics of the substrate, e.g., lipophilicity and water solubility. The availability of a set of such probes would provide a better optimization of the particular substrate to a specific assay experiment. However, as indicated below, problems with reproducibility were encountered with the synthetic scheme previously described for compound **1**. In this paper, we describe a general procedure that overcomes these difficulties and permits the reproducible preparation of high quality fluorogenic substrates for assay of PI-PLC activity.

EXPERIMENTAL PROCEDURES

Reagents and General Procedures. Reagent-grade solvents were used without further purification unless otherwise noted. All reagents were purchased from Aldrich Chemical Co. and were used as received. ¹H NMR spectra were recorded on a Varian INOVA spectrometer at 300 MHz. Chemical shifts are in δ units (ppm) referenced to residual proton signals of the deuterated solvents. Coupling constants (J) are reported in hertz (Hz). NMR splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad. Column chromatography was carried out on Davisil silica gel (60–200 mesh). Analytical TLC was performed on Kieselgel 60 F₂₅₄, and spots were rendered visible by exposing the plate to UV light. Radial chromatography (RC) was performed on a Chromatotron 7924T using 1, 2, and 4 mm disks covered with silica gel 60PF₂₅₄ containing gypsum. High-resolution FAB mass spectrometry was performed by Dr. Brian Arbogast (mass spectroscopy facility, Oregon State University) on a Kratos MS-50TC mass spectrometer (Manchester, England). Elemental analyses were obtained from Robertson Microlit Laboratories, Madison, NJ.

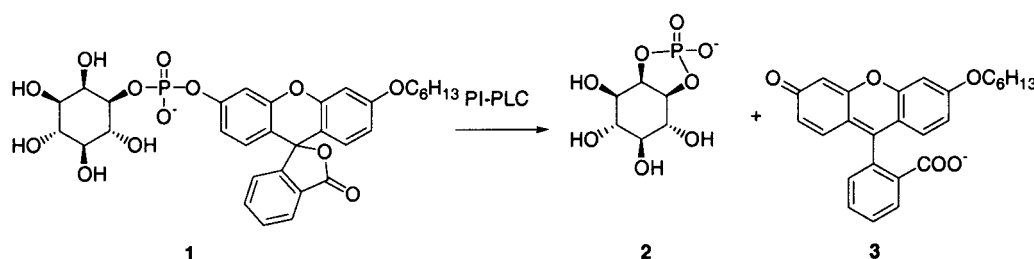
1. Alkyl-Substituted Fluoresceins, 14a–d. General Procedure. To a solution of NaOH (2.00 g, 50.0 mmol) in

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Scheme 1



methanol (200 mL) was added fluorescein **11** (8.30 g, 25.0 mmol). The mixture was stirred for 30 min or until all of the fluorescein dissolved. The resulting solution was evaporated and the residue was dried in a vacuum to give the disodium salt of fluorescein (9.40 g, quantitative). It was powdered and used for the alkylation step. In a typical experiment, the disodium salt **12** (9.40 g, 25.0 mmol) was suspended in DMF (200 mL) containing the alkyl bromide or iodide (200 mmol). The reaction mixture was stirred at RT (for methyl iodide) or at a higher temperature that did not exceed the boiling temperature of the alkyl bromide used. After stirring for 48–60 h the mixture was diluted with 5% NaHCO_3 (200 mL) and extracted with ether (2×100 mL). The combined extracts were washed with brine (50 mL), dried over Na_2SO_4 , and evaporated to give the corresponding ester-ether **13** as a brown oil. This substance was dissolved in methanol (150 mL) and mixed with a 2 M aqueous solution of NaOH (50 mL). The resulting mixture was stirred for 2 h at RT, concentrated under vacuum and diluted with water (80 mL). Impurities were extracted with ether (2×50 mL), and then the aqueous phase was acidified with concentrated HCl to pH about 2. The mixture was extracted with ether (3×80 mL), and the combined extracts were washed with brine (50 mL), dried over Na_2SO_4 , and evaporated to give the crude alkylation product **14** which was finally purified by chromatography on silica gel using a gradient of ether in hexanes (from 1:1 to 3:1). Evaporation of appropriate fractions gave the alkylation product **14** (50–60% yield) which was pure by TLC and NMR.

14a, ^1H NMR (CDCl_3) δ 3.83 (s, 3H), 6.54–6.79 (m, 6H), 7.16 (d, $J = 7.2$, 1H), 7.59–7.69 (m, 2H), 8.01 (d, $J = 7.5$, 1H). **14b**, ^1H NMR (CDCl_3) δ 1.05 (t, $J = 7.5$, 3H), 1.84 (sextet, $J = 6.9$, 2H), 3.98 (t, $J = 6.6$, 2H), 6.60–6.95 (m, 6H), 7.17 (d, $J = 6.6$, 1H), 7.60–7.70 (m, 2H), 8.07 (d, $J = 7.2$, 1H). **14c**, ^1H NMR (CDCl_3) δ 0.98 (t, $J = 7.2$, 3H), 1.46 (sextet, $J = 7.2$, 2H), 1.78 (m, 2H), 3.99 (t, $J = 6.3$, 2H), 6.50–6.70 (m, 5H), 6.79 (dd, $J = 2.4$, 11.7, 1H), 7.16 (d, $J = 6.9$, 1H), 7.25–7.70 (m, 2H), 8.03 (d, $J = 6.6$, 1H). **14d**, ^1H NMR (CDCl_3) δ 0.91 (t, $J = 6.6$, 3H), 1.32 (m, 6H), 1.77 (m, 2H), 3.96 (t, $J = 6.7$, 2H), 6.68–6.72 (m, 2H), 6.76–6.72 (m, 2H), 7.16 (d, $J = 7.2$, 1H), 7.66 (m, 2H), 8.02 (d, $J = 6.9$, 1H).

2. Phosphate Triesters, 17a–d. General Procedure. To a suspension of alkylated fluorescein **14** (1.75 mol) in methylene chloride (10 mL) was added diisopropylethylamine (2.60 mmol) followed by addition of *N,N*-diisopropylmethylphosphonamidic chloride (2.60 mmol). The reaction mixture was stirred for 20 min at RT, diluted with ether (50 mL), washed with 5% NaHCO_3 (10 mL), brine (10 mL), dried over Na_2SO_4 , and evaporated to dryness. The residue was chromatographed on a short silica gel column with 50:50:1 ether-hexanes- Et_3N mixture. The appropriate fractions were combined and evaporated, and the residue was reevaporated twice from toluene to give the corresponding phosphorimidite **15** as a clear oil. This was dissolved in methylene chloride (20

mL) and racemic pentaprotected inositol **16** [0.300 g, 0.930 mmol, prepared as described (11)] was added to the above solution followed by the addition of tetrazole (0.190 g, 2.70 mmol) dissolved in acetonitrile (5 mL, needs warming to dissolve). The reaction mixture was stirred for 3 h at RT, then a 3 M solution of *tert*-butyl hydroperoxide in isooctane (0.7 mL, 2.1 mmol) was added to the solution, and the mixture was stirred for 1 h. Then it was diluted with methylene chloride (25 mL) and washed with 5% NaHCO_3 (15 mL). The solution was dried over anhydrous MgSO_4 and evaporated to give the crude phosphate **17** which was purified on silica gel twice; first with hexanes–ether mixture (from 4:1 to pure ether) then with a methylene chloride–ethyl acetate mixture (from 50:1 to 50:4). The corresponding phosphate **17** was a yellow foam, which was pure by TLC and NMR.

17a, 60%, ^1H NMR (CDCl_3 –py- d_5 50:1) δ 1.10–1.60 (m, 18H), 3.22–3.40 (m, 1H), 3.42–3.60 (m, 1H), 3.60–3.78 (m, 1H), 3.75–3.90 (m, 6H), 4.00–4.18 (m, 1H), 4.40–5.05 (m, 5H), 6.45–6.80 (m, 4H), 6.83–7.00 (m, 1H), 7.10–7.20 (m, 1H), 7.58–7.70 (m, 2H), 7.99 (m, 1H). **17b**, 56%, ^1H NMR (CDCl_3 –py- d_5 50:1) δ 0.80–1.45 (m, 21H), 1.70 (sextet, $J = 7.5$, 2H), 3.20–4.00 (m, 11H), 4.37 (brs, 1H), 4.72 (brs, 1H), 4.87 (m, 1H), 6.30–6.60 (m, 5H), 6.79 (brs, 1H), 6.98 (brs, 1H), 7.45–7.60 (m, 2H), 7.90 (d, $J = 6.9$, 1H). **17c**, 45%, ^1H NMR (CDCl_3 –py- d_5 50:1) δ 0.96 (t, $J = 7.5$, 3H), 1.15–1.60 (m, 20H), 1.70–1.82 (m, 2H), 3.29–3.40 (m, 1H), 3.50–3.60 (m, 1H), 3.65–3.75 (m, 1H), 3.80–4.15 (m, 8H), 4.45–4.65 (m, 1H), 4.75–4.85 (m, 1H), 4.95–5.05 (m, 1H), 6.50–6.75 (m, 4H), 6.85–6.98 (m, 1H), 7.10–7.15 (m, 1H), 7.25–7.36 (m, 1H), 7.55–7.70 (m, 2H), 8.01 (d, $J = 6.6$, 1H). **17d**, 69%, ^1H NMR (CDCl_3 –py- d_5 50:1) δ 0.88 (t, $J = 6.9$, 3H), 1.10–1.60 (m, 24H), 1.76 (quintet, $J = 8.4$, 2H), 3.29–3.40 (m, 1H), 3.45–3.60 (m, 1H), 3.65–3.75 (m, 1H), 3.85–3.98 (m, 5H), 4.00–4.13 (m, 3H), 4.44–4.62 (m, 1H), 4.50–4.82 (m, 1H), 4.94–5.04 (m, 1H), 6.56–6.74 (m, 4H), 6.85–6.96 (m, 1H), 7.08–7.14 (m, 1H), 7.30–7.35 (m, 1H), 7.56–7.68 (m, 2H), 8.00 (d, $J = 6.6$, 1H).

3. Phosphate Diesters, 18a–d. General Procedure. To a solution of phosphate **17** (0.260 mmol) in acetone (5 mL) was added LiI (0.035 g, 0.26 mmol). The resulting mixture was stirred at reflux for 1 h. After that, the acetone was evaporated and the residue was chromatographed using a Chromatotron (2 mm silica gel disk). The product was eluted with a methylene chloride–methanol mixture (from 50:1 to 50:10). The eluate was evaporated to give the appropriate phosphate diester **18** as a viscous oil, which was pure by TLC and NMR.

18a, 89%, ^1H NMR (CDCl_3 –py- d_5 50:1) δ 0.80–1.60 (m, 18H), 3.20–4.30 (m, 9H), 4.49 (brs, 1H), 4.81 (brs, 1H), 4.92 (brs, 1H), 6.30–6.65 (m, 3H), 6.87 (brs, 1H), 7.03 (brs, 1H), 7.26 (brs, 2H), 7.50–7.70 (m, 2H), 7.96 (d, $J = 6.0$, 1H). **18b**, 85%, ^1H NMR (CDCl_3 –py- d_5 50:1) δ 0.93 (t, $J = 7.5$, 3H), 1.00–1.50 (m, 18H), 1.71 (m, 2H), 3.30 (brs, 1H), 3.50 (m, 1H), 3.62 (t, $J = 7.8$, 1H), 3.77 (m, 1H), 3.91 (m, 4H), 4.40 (brs, 1H), 4.75 (brs, 1H), 4.91 (m,

1H), 6.40–6.65 (m, 5H), 6.87 (brs, 1H), 7.02 (brs, 1H), 7.56 (m, 2H), 7.94 (d, $J = 6.3$, 1H). **18c**, 96%, ^1H NMR (CDCl_3 -py- d_5 50:1) δ 0.94 (t, $J = 7.3$, 3H), 1.00–1.60 (m, 20H), 1.75 (m, 2H), 3.20–4.00 (m, 8H), 4.30 (brs, 1H), 4.75 (brs, 1H), 4.95 (brs, 1H), 6.40–6.60 (m, 4H), 6.88 (brs, 1H), 7.02 (brs, 1H), 7.22 (m, 1H), 7.56 (m, 2H), 7.94 (d, $J = 6.3$, 1H). **18d**, 98%, ^1H NMR (CDCl_3 -py- d_5 50:1) δ 0.88 (t, $J = 6.3$, 3H), 0.90–1.50 (m, 24 H), 1.74 (quintet, $J = 7.5$, 2H), 3.30–4.00 (m, 8H), 4.45 (brs, 1H), 4.80 (brs, 1H), 4.93 (brs, 1H), 6.30–6.70 (m, 4H), 6.85 (brs, 1H), 7.02 (brs, 1H), 7.20 (brs, 1H), 7.57 (brs, 1H), 7.95 (brs, 1H).

4. *Deprotected Phosphate Diesters, 19a–c and 1. General Procedure.* Phosphate **18** (268 mmol) was suspended in the mixture of 8 mL of water and 2 mL of acetic acid. The reaction mixture was stirred for 16 h at RT and then extracted with ether (3×20 mL). The aqueous solution was diluted with ethanol and evaporated to dryness (bath temperature did not exceed 40 °C). The residue was re-evaporated from ethanol to remove acetic acid and dissolved in water (about 1 mL). Then ethanol (30 mL) was added to the solution and the resulting solid was filtered. The solid was washed subsequently with ethanol and ether and then it was dried in a vacuum to give the desired phosphate as a white powder, pure by NMR.

19a, 60%, ^1H NMR ($\text{CD}_3\text{OD}-\text{D}_2\text{O}$ 10:1) δ 3.28 (t, $J = 9.3$, 1H), 3.46 (dd, $J = 2.7$, 9.0, 1H), 3.65 (t, $J = 9.3$, 1H), 3.80 (t, $J = 9.9$, 1H), 4.08 (dt, $J = 3.0$, 8.4, 1H), 4.23 (t, $J = 2.7$, 1H), 6.60–6.75 (m, 3H), 6.92 (d, $J = 2.1$, 1H), 7.01 (d, $J = 8.7$, 1H), 7.19 (d, $J = 7.6$, 1H), 7.30 (s, 1H), 7.70–7.90 (m, 2H), 8.05 (d, $J = 7.2$, 1H). Anal. calcd for $\text{C}_{27}\text{H}_{24}\text{LiO}_{13}\text{P}\cdot\text{H}_2\text{O}$: C, 52.95; H, 4.28. Found: C, 53.21; H, 4.05. HRMS (FAB) calcd for $\text{C}_{27}\text{H}_{26}\text{O}_{13}\text{P}$: 589.10996. Found: 589.11026 ($\text{M} + \text{H}$) $^+$. **19b**, 68%, ^1H NMR ($\text{CD}_3\text{OD}-\text{D}_2\text{O}$ 10:1) δ 1.07 (t, $J = 7.2$, 3H), 1.83 (sextet, $J = 6.9$, 2H), 3.28 (t, $J = 9.3$, 1H), 3.44 (dd, $J = 2.4$, 9.9, 1H), 3.55–3.75 (m, 1H), 3.85 (t, $J = 9.6$, 1H), 4.02 (t, $J = 6.3$, 2H), 4.10 (brt, $J = 9.6$, 1H), 4.24 (brs, 1H), 6.65–6.75 (m, 3H), 6.88 (d, $J = 2.1$, 1H), 7.05 (d, $J = 8.7$, 1H), 7.22 (d, $J = 7.5$, 1H), 7.34 (s, 1H), 7.73–7.90 (m, 2H), 8.06 (d, $J = 7.2$, 1H). Anal. calcd for $\text{C}_{29}\text{H}_{28}\text{LiO}_{13}\text{P}\cdot\text{H}_2\text{O}$: C, 54.38; H, 4.72. Found: C, 53.98; H, 4.44. HRMS (FAB) calcd for $\text{C}_{29}\text{H}_{30}\text{O}_{13}\text{P}$: 617.14240. Found: 617.14351 ($\text{M} + \text{H}$) $^+$. **19c**, 49%, ^1H NMR ($\text{CD}_3\text{OD}-\text{D}_2\text{O}$ 10:1) δ 0.96 (t, $J = 7.5$, 3H), 1.48 (sextet, $J = 7.5$, 2H), 1.76 (quintet, $J = 7.5$, 2H), 3.27 (t, $J = 9.3$, 1H), 3.45 (dd, $J = 2.4$, 9.9, 1H), 3.65 (t, $J = 9.6$, 1H), 3.81 (t, $J = 9.3$, 1H), 4.04 (t, $J = 6.3$, 2H), 4.02–4.11 (m, 1H), 4.28 (t, $J = 2.4$, 1H), 6.62–6.72 (m, 3H), 6.87 (d, $J = 1.8$, 1H), 7.02 (d, $J = 8.7$, 1H), 7.19 (d, $J = 7.2$, 1H), 7.30 (t, $J = 1.8$, 1H), 7.72–7.86 (m, 2H), 8.04 (d, $J = 7.2$, 1H). Anal. calcd for $\text{C}_{30}\text{H}_{30}\text{LiO}_{13}\text{P}\cdot\text{H}_2\text{O}$: C, 55.05; H, 4.93. Found: C, 53.39; H, 4.57. HRMS (FAB) calcd for $\text{C}_{30}\text{H}_{32}\text{O}_{13}\text{P}$: 631.15805. Found: 631.15794 ($\text{M} + \text{H}$) $^+$. **1**, 53%, ^1H NMR ($\text{CD}_3\text{OD}-\text{D}_2\text{O}$ 10:1) δ 0.90 (t, $J = 6.9$, 3H), 1.20–1.50 (m, 6H), 1.77 (quintet, $J = 7.8$, 2H), 3.26 (t, $J = 9.3$, 1H), 3.44 (dd, $J = 2.7$, 9.9, 1H), 3.65 (t, $J = 9.3$, 1H), 3.81 (t, $J = 9.6$, 1H), 4.03 (t, $J = 6.3$, 2H), 4.00–4.10 (m, 1H), 4.23 (t, $J = 2.4$, 1H), 6.62–6.74 (m, 3H), 6.87 (d, $J = 1.8$, 1H), 7.02 (d, $J = 8.7$, 1H), 7.19 (d, $J = 7.8$, 1H), 7.31 (s, 1H), 7.70–7.88 (m, 2H), 8.04 (d, $J = 7.2$, 1H). Anal. calcd for $\text{C}_{32}\text{H}_{34}\text{LiO}_{13}\text{P}\cdot\text{H}_2\text{O}$: C, 56.31; H, 5.32. Found: C, 56.58; H, 5.44. HRMS (FAB) calcd for $\text{C}_{32}\text{H}_{34}\text{O}_{13}\text{P}$, 657.17370. Found: 657.17368 ($\text{M} - \text{H}$) $^-$.

5. *Acid, 9.* Phosphate diester **5** [10 mg, prepared by the earlier method (9)] was dissolved in 1 mL of water and the resulting solution was mixed with 875 mL of 0.0286 M aqueous NaOH. The solution was kept for 2 h

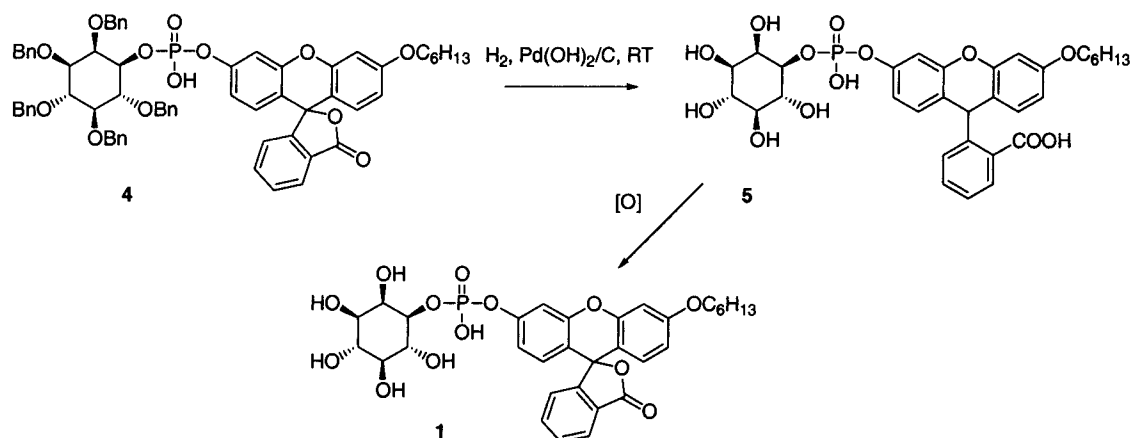
at RT and then acidified with 2 M HCl to pH about 2. The mixture was extracted with ether (2×50 mL), the combined extracts were washed with brine (20 mL), dried over Na_2SO_4 and evaporated. The residue was purified using a Chromatotron (1 mm silica gel disk). The product was eluted with 1:1 hexanes–ether mixture. Evaporation of the eluate gave 2 mg of acid **9** as clear viscous oil, pure by TLC and NMR. ^1H NMR (CDCl_3) δ 0.90 (s, 3H), 1.20–1.50 (m, 6H), 1.76 (quintet, $J = 7.2$, 2H), 3.92 (t, $J = 7.2$, 2H), 6.36 (s, 1H), 6.44 (dd, $J = 2.7$, 8.7, 1H), 6.51 (dd, $J = 2.4$, 8.4, 1H), 6.60–6.63 (m, 2H), 6.89 (d, $J = 8.4$, 1H), 6.94 (d, $J = 9.0$, 1H), 7.11 (d, $J = 7.5$, 1H), 7.18 (t, $J = 6.9$, 1H), 7.32 (t, $J = 8.1$, 1H), 7.92 (dd, $J = 1.2$, 7.8, 1H). HRMS (EI) calcd for $\text{C}_{26}\text{H}_{26}\text{O}_5$: 418.17802. Found: 418.17795 (M^+). Enzymatic hydrolysis of **5** was accomplished as described below for PI-PLC assay. The aqueous phase after hydrolysis was extracted with ether (2×10 mL) and the combined extract was washed with brine (10 mL), dried over Na_2SO_4 and concentrated in a vacuum. The extracted product was identical by TLC with the product obtained after hydrolysis of **5** with sodium hydroxide.

PI-PLC Assays. Stock solutions of substrate (~ 2 mg/mL) were prepared in deionized water, and accurate concentrations were determined by phosphate assay (12). A stock solution containing 0.5 nM PI-PLC (13) from *Bacillus cereus* was prepared in 6 mM HEPES, 0.06 mM EDTA, pH 7.0 [containing 0.1% poly(ethylene glycol) (PEG, average molecular weight 8000)]. Enzyme assays were carried out in 100 mM HEPES, 1 mM EDTA, pH 7.0 (containing 0.008% PEG) in a total volume of 0.8 mL in polymethacrylate cuvettes at 26 °C. Fluorescence measurements were made using a Hitachi F-4500 fluorescence spectrometer equipped with a yellow filter to reduce scatter from the excitation peak. Excitation was at 465 nm, emission was measured at 520 nm, and excitation and emission slits were set at 10 nm. After addition of enzyme, the fluorescence emission was recorded for 3 min in time scan mode. The change in fluorescence over the 3 min period was then converted to activity units ($\mu\text{mol product/min/mg enzyme}$) using a calibration curve of fluorescence vs concentration of product (produced by NaOH-induced cleavage of the substrate) and the concentration of enzyme used in the assay.

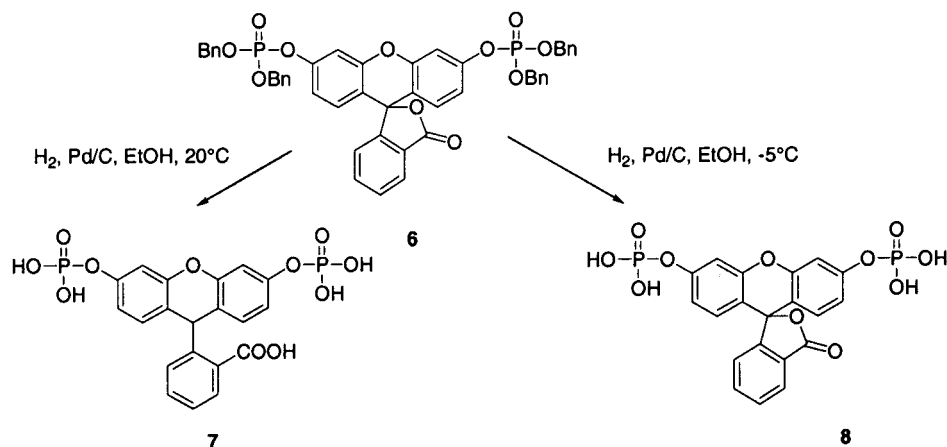
RESULTS

The search for an alternative synthetic route for compound **1** was stimulated by the fact that some of the subsequent batches prepared by the published procedure exhibited 2–5-fold lower rates of production of fluorescence by PI-PLC. To investigate this phenomenon, a larger (100 mg) batch of substrate was prepared using the published protocol. Surprisingly, this larger batch did not show any rise of fluorescence characteristic of substrate **1** after exposure to PI-PLC under standard conditions. The NMR spectrum of the desired compound **1** differs from the NMR spectra of this freshly made batch of product in the aromatic region, and indicates that concomitant hydrogenolysis of the lactone ring took place during the hydrogenolysis reaction used to remove the benzyl groups in **4** (Scheme 2). The HRMS (FAB) spectra of the resulting reduced compound shows a peak at m/e 659.18979 ($[\text{M} - \text{H}]^-$, calcd for $\text{C}_{32}\text{H}_{36}\text{O}_{13}\text{P}$, 659.18936) which corresponds to the opened ring form **5**. By comparison, the presumptive oxidized compound gives a peak 657.17368 ($[\text{M} - \text{H}]^-$, calcd for $\text{C}_{32}\text{H}_{34}\text{O}_{13}\text{P}$, 657.17370) corresponding to the lactone form **1**.

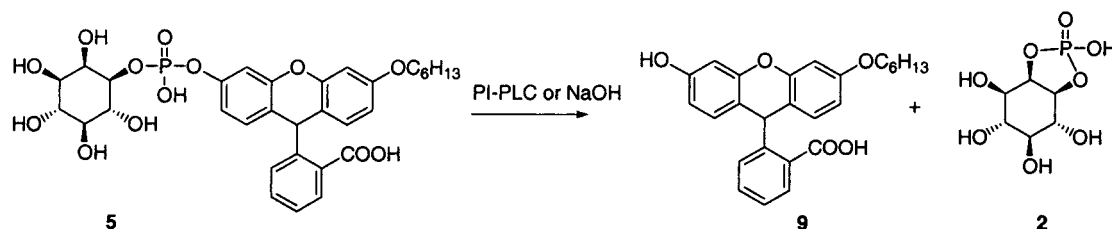
Scheme 2



Scheme 3



Scheme 4

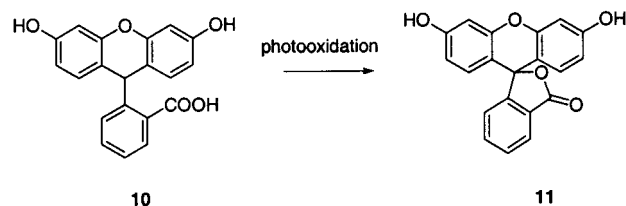


A search of the literature revealed that hydrogenolysis of fluorescein 3,6-bis(dibenzyl phosphate) **6** can produce either ring-opened product **7** or fluorescein biphosphate **8** by using different hydrogenation conditions (Scheme 3). Hydrogenolysis at -5°C at atmosphere pressure in the presence of palladium on carbon gives mostly fluorescein biphosphate **8**. At temperatures higher than 5°C the spiro lactone ring underwent hydrogenolysis to give the reduced acid **7** (14).

Further experiments showed that keeping the diluted solution of compound **5** at room temperature in the presence of room light resulted in spontaneous conversion to substrate **1** (Scheme 2), which did produce a fluorescent product **3** when exposed to *B. cereus* PI-PLC (Scheme 1). Cleavage of **5** with either PI-PLC or sodium hydroxide produced the nonfluorescent reduced fluorescein derivative **9** (Scheme 4).

We did not explore the mechanism of oxidation of nonfluorogenic compound **5** to fluorogenic substrate **1**. It is possible that a photooxidation reaction is responsible for the conversion of **5** to **1**. An example of a similar transformation is the known process of photooxidation

Scheme 5

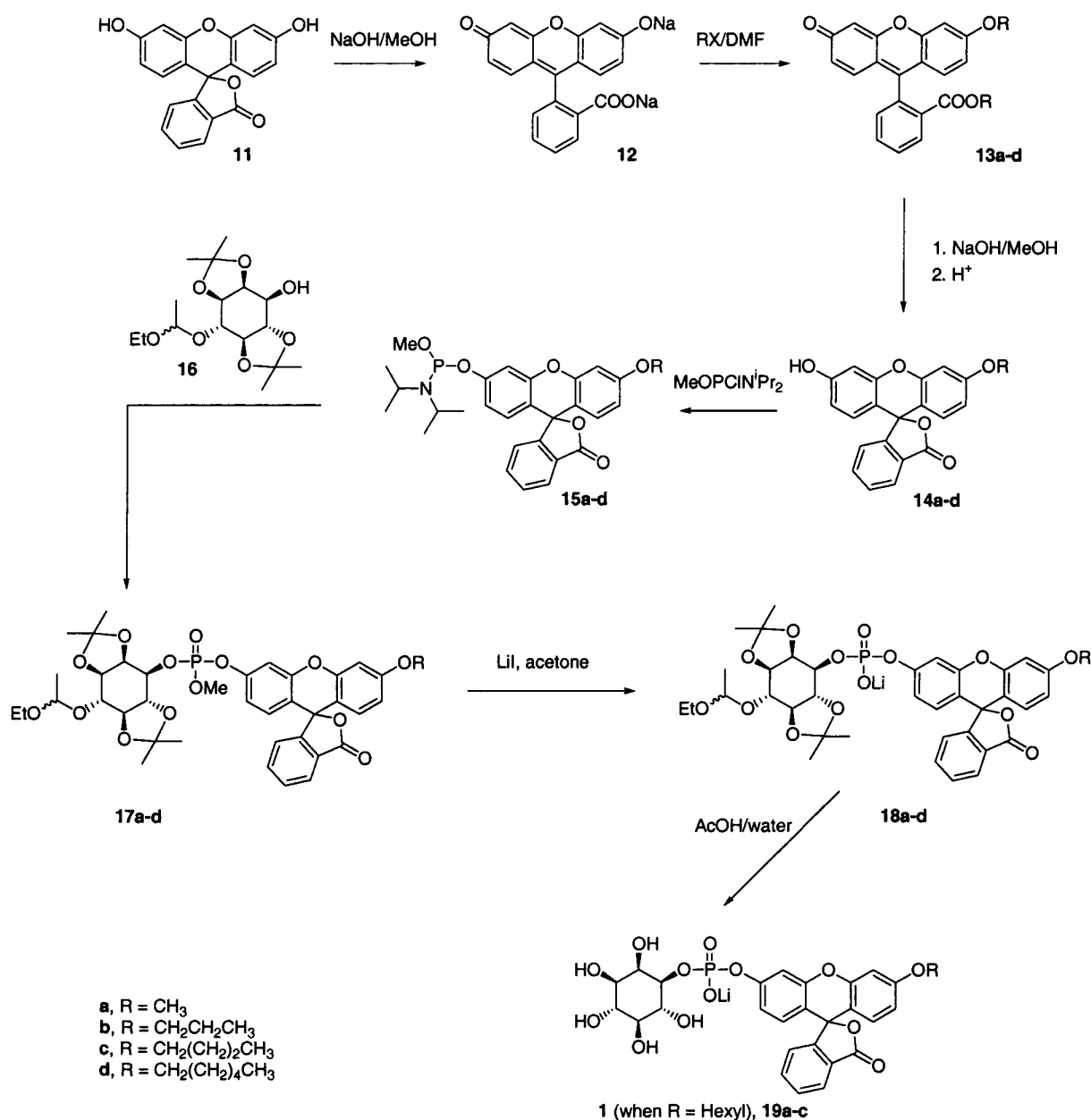


of leuco-fluorescein **10** which gives fluorescein **11**, as shown in the Scheme 5 (15).

To avoid the problematic hydrogenolysis step, we developed an improved synthesis of fluorogenic substrates **1** and **19a–c** (Scheme 6).

This scheme makes use of pentaprotected inositol derivative **16** which was previously utilized for the improved synthesis of the chromogenic substrate 4-nitrophenyl *myo*-inositol-1-phosphate (NPIP) (**11**). Easily hydrolyzable protective groups in **16** facilitate the final acid-mediated deprotection. The fluorescein-derived phosphorimidites **15a–d** were prepared from fluorescein **11**

Scheme 6



as described earlier (9). Different substituents on the aromatic ring were introduced by varying the alkylating agent reacting with fluorescein disodium salt **12**. The resulting ester-ether intermediates **13a-d** were hydrolyzed to provide alkyl substituted fluoresceins **14a-d**. Reaction of the alkylated fluorescein intermediates with MeOPCINⁱPr₂ gave **15a-d**. Protected inositol intermediate **16** was coupled with fluorescein-derived phosphorimidites **15a-d** to form, after oxidation, protected phosphates **17a-d**. Demethylation of the phosphate triester was accomplished using lithium iodide in acetone in place of trimethylsilyl bromide, which was used in our earlier synthesis (9). This change resulted in an increase in the yield of demethylated compound **18** from ~65 to ~95%. Finally, all inositol-protecting groups were removed at once using aqueous acetic acid. The resulting fluorogenic substrates were purified by precipitation with ethanol from aqueous solution followed by washing of the precipitates with ether and drying under vacuum.

The final products **1** and **19a-c** are likely mixtures of two racemic diastereomers because of the racemic nature

of the inositol moiety and the chirality center in the fluorescein moiety. The latter is not expected to have a significant effect on the enzyme-catalyzed hydrolysis reaction given that a wide variety of hydrophobic residues have been attached to D-*myo*-inositol and shown to be enzyme substrates (7). In the present case, the fluorescein moiety and the attached alkyl groups serve as the hydrophobic segment of the substrate. In previous studies of chromogenic substrates (i.e., NPIP) for PI-PLC, we found that racemic phosphoinositols posed no difficulties. One enantiomer (D-NPIP) was a substrate while the other enantiomer (L-NPIP) was neither a substrate nor an inhibitor (16). Other studies demonstrate that PI-PLC cleaves only the D-enantiomer of *myo*-inositol phosphate esters (17, 18). Thus, **1** and **19a-c** should be adequate for most enzymatic and cell biological studies.

Fluorogenic substrates **19a-c** readily dissolved in water. Substrate **1** dissolves in water with some difficulty, due to the longer hydrophobic tail. All of these solutions exhibited only very low levels of fluorescence, which was due to the small quantity of fluorescent

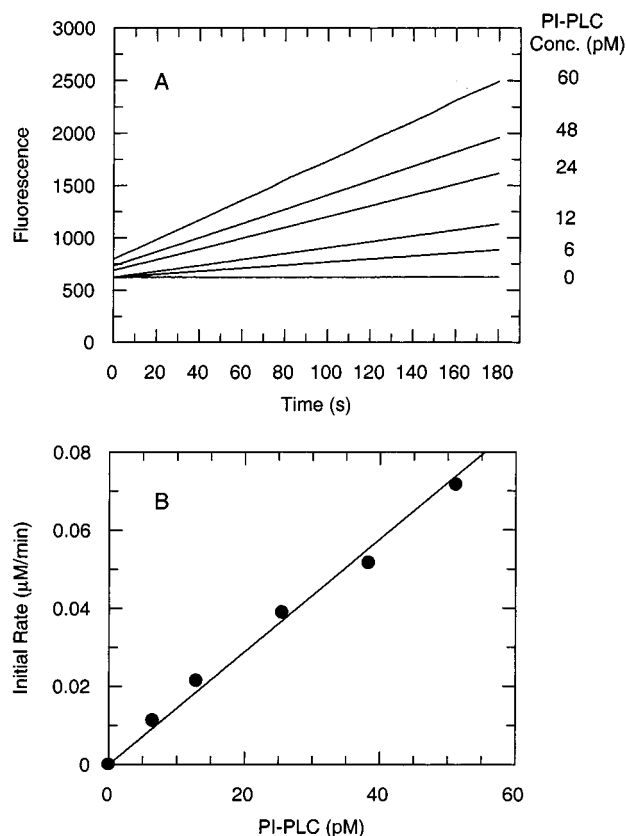


Figure 1. (A) Change in fluorescence emission intensity for the cleavage of 50 μM substrate **1** as a function of time. The numbers on the right indicate the amount of PI-PLC added. (B) Correlation between the initial rates and enzyme concentration. The initial rates were obtained from the slopes of the curves in Figure 1A and a calibration plot of fluorescence vs concentration of product. Assays were carried out at 26 $^{\circ}\text{C}$ in 100 mM HEPES, 1 mM EDTA, pH 7.0 [containing 0.008% poly(ethylene glycol) 8000]. Excitation was at 465 nm and emission (in arbitrary units) was measured at 520 nm.

product (e.g., **3**; <0.1% of the total substrate) present in each preparation of substrate. At ambient temperature and pH 7, this background fluorescence exhibited essentially no change over the course of the 3-min period in which activity data were recorded. On addition of *B. cereus* PI-PLC to solutions of each of the fluorogenic substrates, a rapid increase in fluorescence was observed, as shown in Figure 1 for substrate **1**. The slopes of the fluorescence vs time curves (Figure 1A) were dependent upon both the concentration of the enzyme and that of the substrate. The activity was linear with enzyme concentration (Figure 1B). The increase in fluorescence was also dependent upon the length of the aliphatic tail attached to the fluorescein part of the molecule with the substrate molecules having the shortest tails being cleaved less readily than those with longer tails. The specific activities for the cleavage of the fluorogenic substrates **1** and **19a–c** determined at an enzyme concentration of 6 pM and substrate concentrations of 500 μM are shown in Table 1. The data in Table 1 were obtained using duplicate samples, i.e., one set of data was taken with the four substrates on each of two different days. Both runs used the same batches of the various substrates and a fresh stock of diluted enzyme. Larger differences (10–20%) were observed when different batches of the same substrate were compared. Part of the variation is due to the difficulty in reproducibly pipetting the very small amounts (2 μL) of enzyme from the 50% glycerol solution in which it was stored (at -20

Table 1. Cleavage of Fluorogenic Substrates by *B. cereus* PI-PLC^a

fluorogenic substrate	acronym	no. of carbon atoms in side chain	specific activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)
19a	methyl FLIP	1	27 $\pm$ 3
19b	propyl FLIP	3	86 $\pm$ 2
19c	butyl FLIP	4	122 $\pm$ 4
1	hexyl FLIP	6	110 $\pm$ 4

^a Determined using 500 μM substrate at 26 $^{\circ}\text{C}$ in 100 mM HEPES, 1 mM EDTA, pH 7.0 (containing 0.008% PEG 8000).

$^{\circ}\text{C}$ to preserve activity). In the biochemical literature, it is common practice to use acronyms for chromogenic and fluorogenic substrates. For this purpose, we suggest the name FLIP (fluorescein *myo*-inositol phosphate) for this series of substrates by analogy with NPIP for the chromogenic 4-nitrophenyl *myo*-inositol-1-phosphate. For example, compound **1** is hexyl FLIP.

DISCUSSION

Because of the high sensitivity potentially obtainable using fluorescence (approaching that of radiometry), a number of earlier attempts have been made to prepare fluorogenic substrates for assaying PI-PLC activity. A pyrene derivative, 4-(1-pyrenyl)butylphosphoryl-1-*myo*-inositol, which is cleaved by bacterial PI-PLC, was reported in 1991 (19). However, since the fluorescent properties of both this substrate and its product are similar, it was necessary to separate them by either HPLC or TLC before any analysis could be carried out, resulting in a discontinuous assay (20). In contrast, a continuous assay can be carried out much more rapidly and has the added advantage that the time course of the reaction can be observed directly. Any deviation from linearity can therefore be detected immediately. The first reported fluorogenic substrate for continuous assay of bacterial PI-PLC activity was 2-naphthyl *myo*-inositol-1-phosphate, which is cleaved by the enzyme to form 2-naphthol, which is fluorescent, and inositol phosphates (21). In this case, the fluorescent properties of the substrate and product were sufficiently different that no separation step was required. However, the pH at which maximal fluorescence of 2-naphthol occurs is 10.5, making it necessary to carry out the assay at a pH which is not optimal for the activity of the enzyme (maximal activity occurs around pH 6.5). The result was a very low turnover of substrate by the enzyme in the pH range suitable for fluorescence measurement of product (21). A coumarin-derived fluorogenic substrate for bacterial PI-PLC, 4-methylumbelliferyl-*myo*-inositol-1-phosphate, was recently reported to be useful for detection of bacterial PI-PLC activity (22). This substrate has been only partially characterized, although it should not be fluorescent until hydrolysis occurs. However, like 2-naphthol, phenolic dyes such as β -methylumbelliferone (the product formed by the cleavage of the fluorogenic substrate) have high pK_a values (~ 7.8) and are not fully deprotonated and thus not fully fluorescent if the pH is below 10 (23). Because of this, methylumbelliferyl-based substrates have not been widely used for enzyme assays which are carried out under physiological conditions (23). In contrast, due to the lower pK_a (~ 4.8), the new series of FLIP substrates **1** and **19a–c** provide significantly greater sensitivity in PI-PLC assays carried out under physiological conditions than those derived from β -naphthol and coumarin, and at the present time offer the best prospects for continuous fluorescence assays of PI-PLC activity. The present investigation identified the source

of the high variability in apparent activity as being due to the presence of the nonfluorescent reduced derivative **5** with the desired substrate **1**. The improved synthetic procedure described here solves this problem, yielding pure fluorogenic substrates. A series of substrates prepared with the new procedure show that the specific activity is strongly affected by the length of the hydrocarbon tail, with butyl FLIP and hexyl FLIP exhibiting substantially greater activities than the shorter methyl and propyl derivatives.

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Mechanistic Investigation of Smart Polymer–Protein Conjugates

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Many affinity separation and diagnostic applications rely upon both capture and release steps. There is thus a need for methods to enhance the reversibility of biomolecular interactions. We have previously demonstrated that stimuli-responsive polymers can be used to gate biomolecular reactions when conjugated near the active site of proteins. Here we have used a new smart polymer, *N,N*-dimethyl acrylamide-*co*-4-phenylazophenylacrylate that has allowed a mechanistic investigation of the smart polymer switches. This polymer was conjugated via a vinyl sulfone terminus to cysteine residues of genetically engineered streptavidin mutant E116C, where the polymer is conjugated close to the biotin-binding site, and streptavidin mutant S139C, where the conjugation site is distant. The biotin binding switching activity was strongly dependent on conjugation position, as the E116C conjugate displayed a large thermal response while the S139C conjugate displayed only small effects. Kinetic measurements of biotin release demonstrated that the off-rate of biotin was unperturbed and that the thermally triggered release of biotin with the E116C conjugate was due to the blocking the reassociation of biotin. The addition of free polymer to purified E116C conjugates was also shown to increase the blocking and release properties of the switch. This effect was site dependent, suggesting that the conjugated polymers were directing a physical aggregation near the binding site that effectively enhanced the switching activity. These investigations provide mechanistic insight that can be utilized to design better molecular switches for a variety of stimuli-responsive polymer–protein conjugates.

INTRODUCTION

Capture and release steps are involved in many affinity separation and diagnostic applications, and the reversibility of affinity interactions is thus an important underlying aspect of these technologies. Reversibility is intrinsically problematic, however, because the capture step typically requires high binding energy and specificity. This often makes it necessary to use relatively harsh conditions for release that can damage delicate biological components or targets. A striking example of these conflicting needs is found with streptavidin, one of the most utilized affinity reagents. Streptavidin (SA)¹ displays a remarkably high affinity for the ligand biotin, and this interaction is utilized in a wide variety of biological, clinical, and diagnostic applications (1, 2). The high affinity is also a major limitation, however, for applications where the recovery of the biotinylated molecules are desirable.

Many strategies for reversibly manipulating biomolecular recognition have been devised, but there is still a clear need for improvement. We have been developing a new approach that utilizes site-specific conjugation of

stimuli-responsive polymers near the protein-binding site. These stimuli-responsive polymers undergo a large change in size and physical properties in response to external stimuli such as temperature, pH, or specific wavelengths of light. We have previously demonstrated that the change in physical properties can be used as a temperature- or pH-dependent activity switch to control ligand capture and release (3–5). We report here an investigation into the mechanism of these smart polymer–protein conjugates that utilizes the new end-reactive polymer *N,N*-dimethyl acrylamide-*co*-4-phenylazophenylacrylate (DMAA). The use of this polymer and a control streptavidin mutant has allowed a determination of the role of conjugation site and free polymer, along with the mechanism of biotin release.

EXPERIMENTAL PROCEDURES

Materials. 4-Hydroxyazobenzene (Aldrich, Milwaukee, WI) was recrystallized from ethanol and water, and dried in vacuo. *N,N*-Dimethyl acrylamide (DMA) (Fluka, Milwaukee, WI), diethyl ether, acryloyl chloride, triethylamine, and 2-mercaptoethanol (MEO) (Aldrich, Milwaukee, WI) were purified by distillation under reduced pressure. 2,2'-azoisobutyronitrile (AIBN) (J. T. Baker, Phillipsburg, NJ) was recrystallized from methanol. Ethanol, dimethylformamide (DMF), tetrahydrofuran (THF), methylene chloride, potassium *tert*-butoxide, divinyl sulfone (DVS), 5,5'-thiobis (2-nitrobenzoic acid) (DTNB), ethylenediamine tetraacetic acid (EDTA) (Aldrich, Milwaukee, WI), tris(2-carboxy ethyl)-phosphine hydrochloride (TCEP-HCl) (Pierce, Rockford, IL), iminobiotin beads, cysteine hydrochloride (Pierce, Rockford, IL), Microcon-30, Centrprep-10 (Millipore, Bedford, MA), and [³H]biotin (d-[8,9-³H(N)]biotin, Dupont, Wilmington, DE) were used as received. All other reagents were of

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¹ Abbreviations: AIBN, 2,2'-azobisisobutyronitrile; AZAA, 4-phenylazophenylacrylate; DMA, *N,N*-dimethylacrylamide; DMAA, *N,N*-dimethylacrylamide-*co*-4-phenylazophenylacrylate copolymer; DMAA-VS, vinyl sulfone terminated DMAA; DMF, dimethylformamide; DTNB, 5,5'-thiobis (2-nitrobenzoic acid); DVS, divinyl sulfone; EDTA, ethylenediamine tetraacetic acid; LCST, lower critical solution temperature; MEO, mercaptoethanol; MW, molecular weight; SA, streptavidin; TCEP-HCl, tris(2-carboxyethyl)-phosphine hydrochloride; THF, tetrahydrofuran; VS, vinyl sulfone.

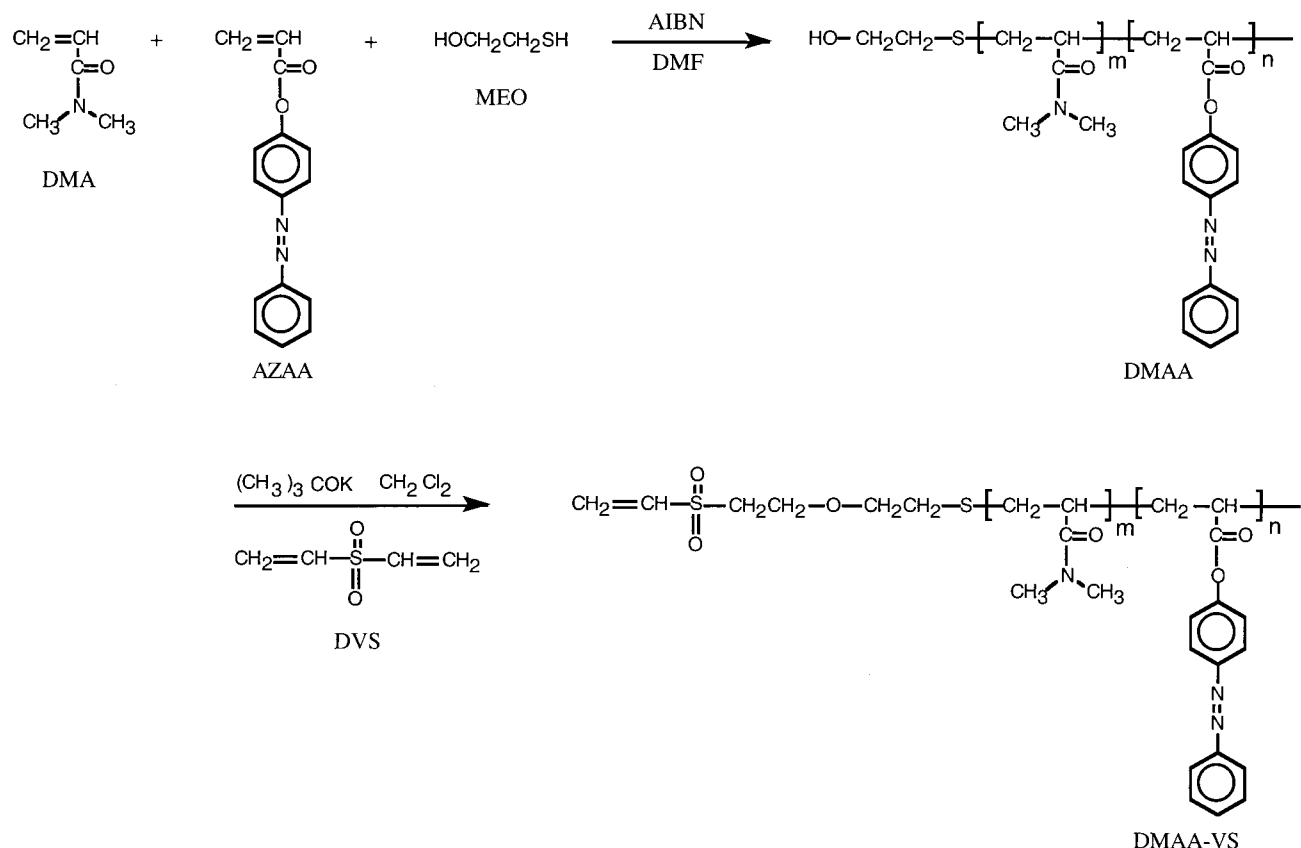


Figure 1. Synthetic scheme for DMAA and DMAA-VS.

analytical grade. Restriction enzymes (*MscI*, *MluI*, and *HindIII*) and T4 DNA Ligase were purchased from New England Biolabs (Beverly, MA). Qiaex II kit for extracting DNA was purchased from Qiagen (Santa Clarita, CA). pET21a vector was obtained from Novagen (Madison, WI).

Azobenzene Monomer Synthesis. We synthesized an azobenzene-containing monomer, 4-phenylazobenzyl acrylate (AZAA). 4-Hydroxyazobenzene (0.2 mol) and triethylamine (0.26 mol) were dissolved in diethyl ether (200 mL). Acryloyl chloride (0.24 mol) dissolved in 80 mL of diethyl ether was added dropwise at 0 °C under nitrogen atmosphere with stirring. The reaction mixture was allowed to come to room temperature and stirred for 4 h. The reaction solution was filtered to remove triethylammonium chloride, washed with water and evaporated. The product was recrystallized from ethanol–water mixture and dried in vacuo. The structures were confirmed by ^1H NMR (d-DMSO). AZAAm: δ 5.7–6.7 (m, 3H), 7.4–7.8 (m, 5H), 7.8–8.2 (m, 4H). AZAA: δ 6.1–6.7 (m, 3H), 7.3–7.7 (m, 5H), 7.8–8.2 (m, 4H).

Temperature Responsive Polymer Preparation. We synthesized a temperature responsive polymer with hydroxyl termini, DMAA (DMA-*co*-AZAA copolymer), by free-radical polymerization in DMF at 60 °C for 20 h, using MEO as a chain transfer reagent and AIBN as an initiator (monomer concentration = 2 mol/L, monomer/MEO/AIBN = 100/1/0.2 molar ratio) (Figure 1). The feed ratios of AZAA was 4.0 mol %. The product was purified by precipitation into diethyl ether three times and dried in vacuo.

The content of AZAA incorporated in the copolymers was determined by ^1H NMR (Spectrospin and Bruker, dpx200), comparing the ratio of aromatic and aliphatic hydrogens. The molecular weight (MW) of the polymer was determined by gel permeation chromatography

(GPC, Water, Styragel HR3 and 4) in THF, using polystyrene as standards.

The hydroxyl terminus of DMAA was converted to a vinyl sulfone (VS) group for conjugation to the protein SH sites (Figure 1). DMAA was dissolved in 20 mL of methylenechloride with 0.03 g of potassium *tert*-butoxide, and 100 μL of divinyl sulfone (DVS) (DVS/OH = 10/1 molar ratio). The solution was stirred for 12 h at room temperature under nitrogen atmosphere. The polymer was precipitated and then washed in diethyl ether and dried in vacuo.

The amount of active VS in VS-terminated DMAA (DMAA-VS) was determined by the reaction with cysteine. Since cysteine was detected colorimetrically by the reaction with 5,5'-thiobis (2-nitrobenzoic acid) (DTNB), the amount of VS was calculated by the consumed cysteine in the solution. Briefly, 0.2 mL of polymer solution (10 mg/mL in 0.1 M PB, pH 8.0) was mixed with 2 mL of cysteine solution (1.5 mM cysteine hydrochloride in 0.1 M PB, pH 8.0, with 10 mM EDTA), and reacted for 4 h. A total of 0.2 mL of the solution was mixed with DTNB solution (0.2 mg of DTNB in 2 mL of 0.1 M PB, pH 8.0) and incubated for 15 min. Then, the absorbance at 412 nm was measured to calculate the amount of cysteine reacted with DTNB.

The LCST of the polymer was determined as the temperature at 10% of the maximum absorbance at 600 nm. The polymer concentration was 2 mg/mL in double-distilled water or 100 mM phosphate buffer (PB) pH 7.4.

Genetic Engineering of Streptavidin. The streptavidin mutant E116C and S139C were constructed by site-directed, cassette mutagenesis, using a synthetic "core" streptavidin gene previously designed and constructed for protein expression in *Escherichia coli* (5). The construction and characterization of the E116C (5) and S139A (6) have been previously described. Analysis of the

streptavidin mutants to confirm the correct production included SDS-PAGE, mass spectrometry, and biotin binding studies.

Conjugation of Polymer to Protein. Conjugation between streptavidin (E116C, S139C) and the polymer (DMAA-VS) was carried out in 100 mM sodium tetraborate buffer, pH 8.0, containing 50 mM sodium chloride and 5 mM EDTA. Cysteine residues in E116C or S139C form disulfide bonds during storage. To reduce disulfide bonds to sulfhydryl groups, TCEP at 50-fold molar excess to the mutants was added into the solution, which was then rotated at room temperature for 20 min. Then, DMAA-VS was added in 50-fold molar excess to the mutants to obtain high conjugation efficiency, and reacted for 1 h at room temperature, followed by the reaction for 24 h at 4 °C. The conjugates were separated from the unconjugated mutants by thermally induced precipitation, i.e., centrifuging the mixture at 15 000 rpm (31000*g*) for 15 min at 50 °C with 4 wt % ammonium sulfate to enhance the precipitation. The unconjugated mutants were retained in the supernatant. The thermal-induced precipitation was repeated three times.

Further purification was carried out by using an iminobiotin column to investigate the biotin-binding properties of the isolated conjugates from free polymers. Iminobiotin has been used for purifying streptavidin and avidin widely owing to its reversible binding to these proteins at different pHs. The conjugates were bound to iminobiotin on the beads in the binding buffer (50 mM sodium carbonate, 500 mM sodium chloride, pH 11) at room temperature, and the free polymers were washed off by the same buffer. Then, the bound conjugates were eluted in the elution buffer (100 mM acetic acid, pH 2.6) at room temperature. The purified conjugates were condensed by a Centriprep-10 (MW cut off: 10 000) to 30–300 µg/mL.

Biotin-Binding Assay. [³H]Biotin was employed to quantitate biotin binding. A total of 10 µg of the conjugate was suspended in 0.9 mL of 100 mM of sodium phosphate buffer, pH 7.4, containing 50 mM of sodium chloride and 5 mM of EDTA. A mixture of [³H]biotin and unlabeled biotin (1% of [³H]biotin) was added to the conjugate solution (total volume was 1 mL) after incubation at 52 °C for 1 h. The ratio of total biotin/biotin binding sites was 1.5 (molar ratio). The solution was incubated at a certain temperature, and a 100 µL aliquot was taken at the end of each incubation. The unbound biotin was separated from the bound biotin by ultrafiltration of the aliquot using a Microcon-30 (MW cutoff: 30 000) at the incubation temperature of the solution. A 30 µL aliquot of the filtrate that contained unbound biotin was counted in a β-counter (LS7000 Liquid scintillation system, Beckman Instruments, Inc. Fullerton, CA), to determine the unbound biotin. The biotin binding could be quantitated by the decrease of free biotin in the solution.

RESULTS AND DISCUSSION

Polymer Characterization and Protein Conjugation. The DMAA copolymer terminated with a hydroxyl group was synthesized by free radical copolymerization of DMA and AZAA in DMF using MEO as a chain transfer reagent (Figure 1). GPC and ¹H NMR analyses showed that the DMAA had a weight average molecular weight (MW) of 18 kDa and an AZAA content of 5.9 mol %. The LCST of DMAA was measured by the absorbance change at 600 nm and determined to be 37 °C. The hydroxyl terminus of DMAA was reacted with DVS to

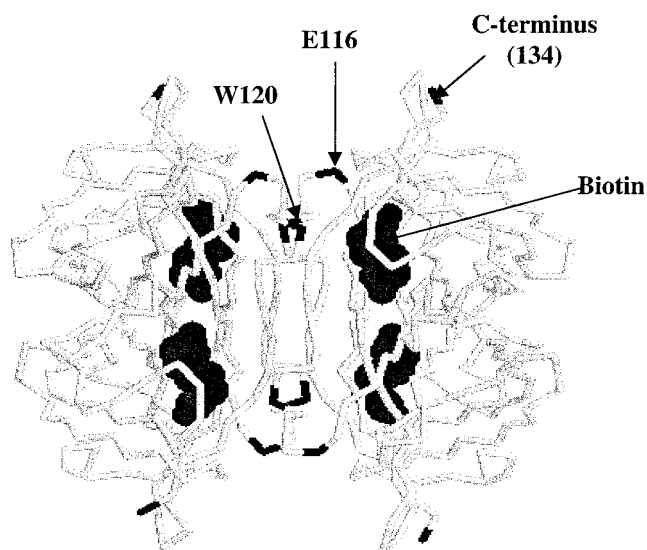


Figure 2. Three-dimensional model of the streptavidin displaying the relative locations of position 116 and the C-terminus. The X-ray crystal structure is defined to the highlighted position 134, with the 139 position three residues away. The biotin-binding site and Trp 120 are also shown for orientation.

make DMAA-VS (Figure 1). DMAA-VS contained 97.5 molar percentage of reactive VS as determined with the colorimetric DTNB assay.

The polymer was conjugated to SA site specifically by the reaction of the vinyl sulfone group of the polymer with the sulfhydryl groups of the protein. The conjugate was separated from the unreacted SA by thermally induced precipitation of the polymer. The polymer–protein conjugates could be subsequently purified from free polymer by affinity chromatography. The number of polymer molecules conjugated to a SA was calculated from spectral analysis of the protein and polymer absorption spectra as 2.03 for the E116C–DMAA conjugate and 2.11 for the S139C–DMAA conjugate. This suggests that one polymer chain is conjugated to each of the two streptavidin binding faces, with steric repulsion likely preventing the conjugation of a second polymer at each face.

Conjugation Site Dependence of Activity Switch. Each subunit of E116C has a unique cysteine residue, which replaces the glutamic acid at position 116 (Figure 2). This conjugation site was previously chosen to be as close as possible to Trp120, one of the energetically most important biotin binding contacts (7). To test the site-dependence of conjugation for the activity switch, we have characterized the S139C mutant, which has a unique cysteine, engineered at the C-terminus of streptavidin (Figure 2). The streptavidin structure is crystallographically determined to residue 134, where the distance to the biotin binding site is approximately 2.4 nm.

The relative change in biotin binding capacity for the E116C conjugate is shown in Figure 3. The thermal response for the purified S139C conjugate was also determined, and compared both to protein alone and physical mixtures of SA and hydroxyl terminated polymers as controls. The E116C conjugate displayed a striking thermally dependent biotin binding capacity that translated into biotin blocking and releasing activities. Moving the conjugation site away from the biotin binding site had a large effect on the switching ability, as the S139C conjugate did not display a thermal dependence above the controls of protein alone and protein/polymer physical mixtures. These results demonstrate that the switching activity is strongly dependent on the location

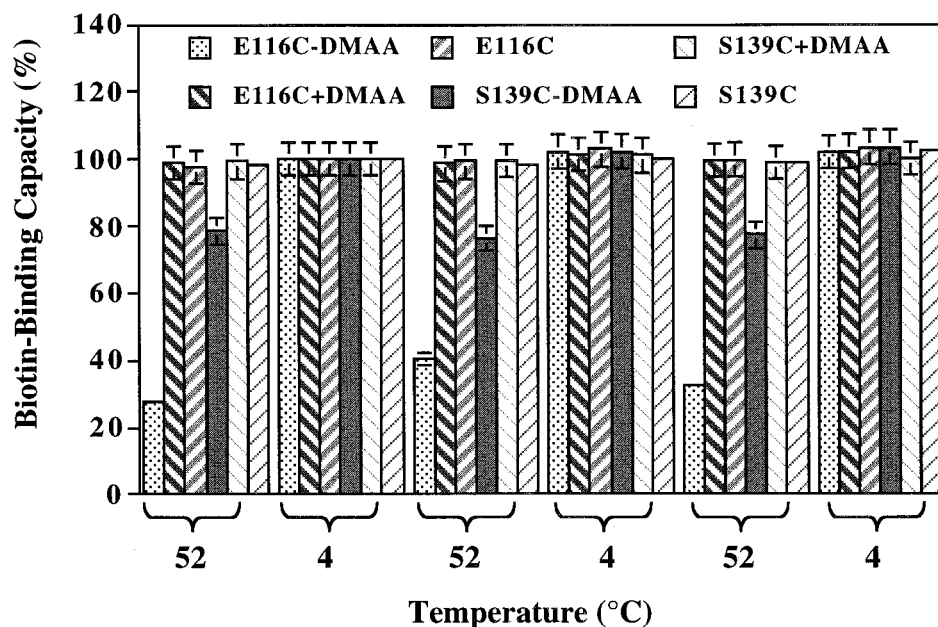


Figure 3. The effect of thermal cycling on the biotin-binding capacity for the E116C–DMAA conjugate, the S139C–DMAA conjugate, physical mixtures of E116C or S139C and DMAA, and E116C and S139C alone in 100 mM sodium phosphate buffer at pH 7.4. The biotin-binding capacity was determined assuming 100% binding at 4 °C after 1 h incubation. For the conjugates, the biotin-binding capacity was plotted as the percentage of conjugated sites.

of the conjugation site, consistent with the need for the polymer to be near the binding site.

Mechanism of Biotin Releasing Activity. To investigate whether the off-rate of biotin was increased when the polymer was collapsed, the time dependence of biotin release was characterized (Figure 4). The unconjugated E116C exhibits the same biotin off-rate as wild-type streptavidin, and the increase in off-rate at 52 °C for unconjugated E116C is not large enough to significantly reduce the amount of bound biotin at equilibrium. This can be observed in Figure 4B, where there is no significant change in the percentage of bound biotin for the control E116C as the temperature is shifted from 4 to 52 °C. When biotin was first added to the DMAA–E116C conjugate at 52 °C, binding was largely blocked by the collapsed polymer. The temperature was then cycled to 4 °C where the polymer was hydrated, and this opened the blocked binding sites.

After recycling back to 52 °C, the time course of biotin release was quantitated. From these data, the first-order dissociation rate constant for biotin was determined to be $1.6 \times 10^{-4} \text{ s}^{-1}$ for the E116C–DMAA conjugate. The off-rate is thus very close to the $3.0 \times 10^{-4} \text{ s}^{-1}$ constant of wild-type SA at 52 °C. This finding suggests that the collapse of DMAA is not inducing an intrinsic increase in the biotin off-rate, but is blocking the reassociation of biotin. It is interesting that the biotin can exit through the collapsed polymer from the binding pocket, but not re-enter from bulk solution.

Role of Free Polymer in Switching Activity. Iminobiotin affinity chromatography was used to purify the E116C–DMAA and S139C–DMAA streptavidin conjugates. This purification allowed the study of the role of free polymer in the blocking and release switching activity. The thermal cycling response for binding and release of purified conjugates is compared in Figure 5 to conjugates where free polymer was also present. Although the purified conjugates showed reversible thermal switching for biotin binding, they exhibited smaller responses than when free polymer was added. The responses increased in magnitude as more of the free polymer was added.

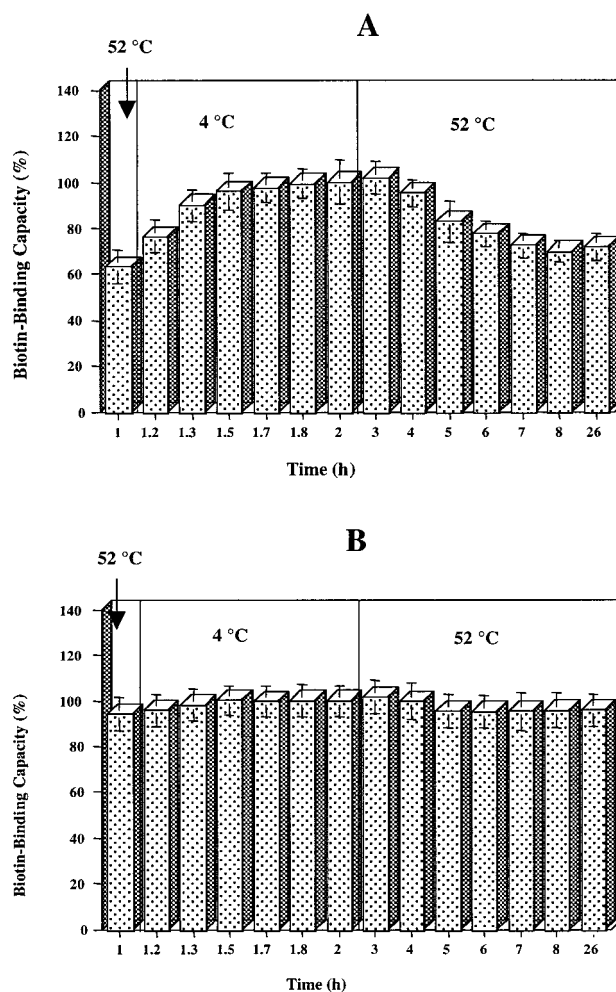


Figure 4. Kinetics of biotin release after cycling to 52 °C from 4 °C. (A) E116C–DMAA conjugate and (B) E116C control. Assays were performed in 100 mM sodium phosphate buffer, pH 7.4. The biotin-binding capacity is determined assuming 100% binding at 4 °C after 1 h incubation.

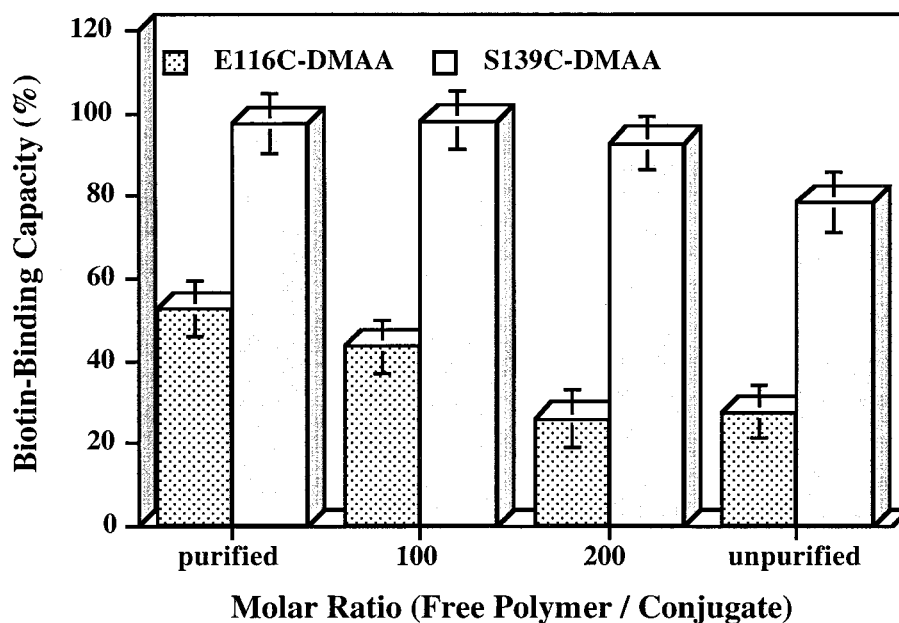


Figure 5. Effect of adding free polymer on the biotin-binding capacity at 52 °C for E116C-DMAA and S139C-DMAA in 100 mM sodium phosphate buffer, pH 7.4. The unpurified samples were those isolated by thermal precipitation from the conjugation reaction. The biotin-binding capacity was determined assuming 100% binding at 4 °C after 1 h incubation. For the conjugates, the biotin-binding capacity was plotted as the percentage of conjugated sites. The molar ratios of free polymer/conjugate in the unpurified states were calculated as 1430 for E116C-DMAA and 1150 for S139C-DMAA conjugates by use of the conjugation conversions.

This effect was clarified by characterizing the S139C-DMAA streptavidin conjugate. The purified S139C-DMAA conjugate did not display any switching capability, and addition of free polymer led to only a small increase in activity. Taken together with the E116C-DMAA results, this suggests that the free polymer is likely aggregating with the conjugated polymer in the collapsed state. When directed to a site near the binding pocket, as with the E116C mutant, this DMAA aggregate more effectively blocks biotin association. But the aggregation effect is insignificant if the free polymer is directed toward a conjugation site that is too distant from the binding site. These findings confirm that the MW and type of smart polymer will likely provide important avenues to engineer the molecular switch activity. For example, larger and/or grafted polymers could provide better barriers near the binding site.

CONCLUSIONS

We have utilized a new thermally responsive polymer to investigate the mechanism of the smart polymer activity switches. The use of the DMAA polymer allowed an accurate determination of the conjugation ratio. The site-selectivity of the switch was first investigated with the S139C streptavidin mutant that contains a unique cysteine at the C-terminus. The purified S139C conjugate, where the DMAA is distant from the binding site, did not display switching activity. The previously characterized E116C streptavidin mutant did display switching activity when the DMAA was conjugated at this cysteine near the biotin-binding site. Because the release of biotin was shown to proceed with the same kinetics as the biotin off-rate for wild-type streptavidin, the mechanism for release was shown to be the blocking of biotin reassociation by the collapsed DMAA. The magnitude of the switching activity could be significantly increased by the addition of free polymer, but only if the conjugated polymer was near the binding site. This suggests that the conjugated polymer is directing a

physical aggregation of the free polymer at the defined position near the biotin-binding site. Taken together, these studies elucidate the importance of conjugation site and suggest that the molecular weight and type of stimuli-responsive polymer will be essential to optimize the switching activity.

ACKNOWLEDGMENT

We would like to thank Dr. J. Milton Harris of Shearwater Polymers for his advice on the addition of the polymer end hydroxyl groups to vinyl sulfone groups of DVS. We also wish to thank C. Lackey, C. J. Long, and Dr. R. To for the construction and expression of the SA mutants. The NIH (Grant 53771), UW Office of Technology Transfer, the Washington Research Foundation, and the Washington Technology Center are gratefully acknowledged for their support of this project.

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An Improved Method for Conjugating Monoclonal Antibodies with *N*-Hydroxysulfosuccinimidyl DOTA

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A simple, water-soluble procedure for conjugation of monoclonal antibodies to 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA) has been improved by optimizing pH, buffer, and temperature conditions for the preparation of *N*-hydroxysulfosuccinimidyl DOTA and its conjugation to the human/murine chimeric anti-carcinoembryonic antigen antibody cT84.66. This improved method results in a 6-fold increase in conjugation efficiency, a 3–7-fold decrease in antibody cross-linking, a more homogeneous population of conjugate species, and a 5-fold decrease in the quantities of reagents needed for conjugation. The cT84.66–DOTA conjugate was labeled to high specific activity with ¹¹¹In, ⁹⁰Y, ⁸⁸Y, ⁶⁴Cu, and ⁶⁷Cu, affording near-quantitative incorporation of the majority of these radiometals. This improved conjugation procedure facilitates large-scale production and radiometal labeling of cT84.66–DOTA for clinical radioimmunotherapy trials.

INTRODUCTION

Cancerous tumors can be targeted specifically by radiolabeled monoclonal antibodies (mAbs)¹ used in clinical imaging and therapy (1, 2), and a wide variety of medically useful radiometals are available for these applications. Considerable effort has been devoted to the development of bifunctional chelating agents (BCAs) for covalent attachment of radiometal ion complexes to mAbs (3–5). An important property of the BCA is that it chelates radiometals with high in vivo stability, resulting in minimal deposition of free radiometal in normal tissues (6). The macrocyclic chelating agent 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA) is an attractive candidate for conjugation of radiometals to antibodies. DOTA binds a large number of metal ions with extremely high thermodynamic (7, 8) and kinetic (9, 10) stability, and mAb–DOTA conjugates have shown minimal loss of chelated radiometal in vivo (11–14).

Recently, we developed a simple, water-soluble chemical method for conjugation of DOTA to proteins using commercially available reagents (15). This method involved the preferential activation of one carboxyl group of DOTA with *N*-hydroxysulfosuccinimide (sulfo-NHS)

and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and reacting the resulting DOTA active ester with proteins at pH 8.5–9.0. The systematic development of elevated temperature, optimum pH, and appropriate buffer conditions allowed rapid, efficient, and kinetically stable labeling of immunoconjugates with ¹¹¹In(III) and ⁹⁰Y(III) at high specific activities. In this report, we describe improvements to this method, which make practical large-scale antibody conjugation for clinical radioimmunotherapy trials and result in near-quantitative labeling yields with radioisotopes of In, Y, and Cu.

EXPERIMENTAL PROCEDURES

Reagents. The antibody cT84.66, a human/murine chimeric anti-carcinoembryonic antigen (anti-CEA) mAb, isotype IgG₁, κ , was prepared as described previously (16). DOTA internal salt was purchased from Parish Chemical Co. (Orem, UT). Sulfo-NHS and EDC were obtained from Pierce (Rockford, IL). Cobalt powder (99.995%) was obtained from Aldrich (Milwaukee, WI). Chelex 100 (Biotechnology Grade, 100–200 mesh, sodium form) was purchased from Bio-Rad (Hercules, CA), and buffers used for radiolabeling reactions were passed over a Chelex 100 column (1 × 15 cm). ⁵⁷CoCl₂, ¹¹¹InCl₃, ⁹⁰YCl₃, ⁸⁸YCl₃, ⁶⁴CuCl₂, and ⁶⁷CuCl₂ were obtained from ICN (Costa Mesa, CA), Amersham (Arlington Heights, IL), NEN (Boston, MA), Los Alamos National Laboratory (Los Alamos, NM), Washington University (St. Louis, MO), and Brookhaven National Laboratory (Upton, NY), respectively. Ultrapure water (18 M Ω cm) was used for all procedures, and all other reagents were of the highest purity obtainable.

Thin-Layer Chromatography. Thin-layer chromatography was performed on ITLC plates (silica gel 60F-254, 0.5 × 10 cm, EM Science, Gibbstown, NJ), using 10% (w/v) ammonium acetate:methanol (1:1) as the mobile phase. In this system, radiometal-labeled antibody remains at the origin, while radiometal-DTPA complexes migrate to *R_f* = 0.7–0.9. Radiation counting of TLC plates

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¹ Abbreviations: mAb, monoclonal antibody; BCA, bifunctional chelating agent; DOTA, 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid; DOTA–OSSu, *N*-hydroxysulfosuccinimidyl DOTA; sulfo-NHS, *N*-hydroxysulfosuccinimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EDTA, ethylenediaminetetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; HSA, human serum albumin; IEF, isoelectric focusing; CEA, carcinoembryonic antigen; cT84.66, human/murine chimeric anti-CEA mAb; cT84.66–DOTA, cT84.66 conjugated to DOTA using DOTA–OSSu; HACA, human anti-chimeric antibody.

was performed with a Packard Cobra Auto-Gamma model 5003 counter with the appropriate energy window set for ^{57}Co or ^{111}In .

High-Performance Liquid Chromatography. HPLC was performed at room temperature on a Gilson chromatograph (models 307 pump, 506C System Interface Module, 715 System Controller Software, and FC 203 Fraction Collector). The column, solvent system, and flow rate used are described below. UV detection was accomplished at 280 nm using a Gilson 112 detector (10-mm, 11- μL analytical flow cell). Radioactivity detection was accomplished using a NaI detector and a Technical Associates PRS-5 analyzing miniscaler/ratemeter.

Ultraviolet Spectrophotometry. Concentrations of antibody were determined by UV spectrophotometry, measuring the absorbance at 280 nm (A_{280} at 1 mg/mL = 1.42, within 5% of the concentration determined by amino acid analysis). UV measurements were obtained on a Pharmacia LKB Ultrospec III spectrophotometer, using a 1-cm sample cell.

Preparation of N-Hydroxysulfosuccinimidyl DOTA (1). A solution of 16.2 mg of DOTA in 810 μL of H_2O was adjusted to pH 5.45 with 80 μL of 1 M NaOH and cooled to 4 $^\circ\text{C}$. To 239 μL (4.35 mg, 10.7 μmol) of the DOTA solution was added 2.33 mg (10.7 μmol) of sulfo-NHS in 117 μL of H_2O , freshly prepared at 4 $^\circ\text{C}$. Then 8.25 μL (0.207 mg, 1.08 μmol) of EDC, freshly prepared in H_2O (25 mg/mL), was added, and the reaction mixture was stirred at 4 $^\circ\text{C}$ for 30 min. The theoretical concentration of **1** in the reaction mixture was 2.97 mM. Prior to being added to the mAb, the pH of the reaction mixture was adjusted to 7.3 with 40 μL of 0.2 M Na_2HPO_4 , pH 9.2.

Preparation of cT84.66-DOTA Conjugate. An aliquot of 8.64 mg of cT84.66 in 1 mL of PBS, pH 7.4, was dialyzed against 1 L of 0.1 M Na_2HPO_4 , pH 7.5, containing approximately 1.2 g of Chelex 100, for 42 h at 4 $^\circ\text{C}$, with one buffer change. Then, the solution of **1** (1.08 μmol of DOTA-OSSu theoretical) was added to 8.06 mg (53.7 nmol) of cT84.66 in 1 mL of 0.1 M Na_2HPO_4 , pH 7.5. The reaction mixture was incubated at 4 $^\circ\text{C}$ for 24 h with continuous end-over-end mixing, after which it was dialyzed against 1 L of 10 mM Na_2HPO_4 /150 mM NaCl, pH 7.5, containing approximately 1.2 g of Chelex 100, for 24 h at 4 $^\circ\text{C}$. The conjugate was then dialyzed against 1 L of 0.25 M ammonium acetate, pH 7.0, containing approximately 1.2 g of Chelex 100, for 139 h at 4 $^\circ\text{C}$, with five buffer changes.

Determination of the Average Number of Chelates per Antibody Molecule. An aliquot of 10 μL of cT84.66-DOTA, containing approximately 400 pmol of conjugate, was added to 10 μL of 0.25 M ammonium acetate, pH 7.0, followed by 10 μL of a standardized CoCl_2 solution (402 μM , 4.02 nmol, containing >350000 cpm/ μL of $^{57}\text{CoCl}_2$) or InCl_3 solution (428 μM , 4.28 nmol, containing $>4.40 \times 10^6$ cpm/ μL of $^{111}\text{InCl}_3$). The reaction mixture was incubated at 43 $^\circ\text{C}$ for 3 h, after which 3.3 μL (33 nmol) of 10 mM DTPA, pH 6.0, was added. The reaction mixture was incubated at room temperature for 15 min. An aliquot of 1 μL of the reaction mixture was spotted onto an ITLC plate, which was developed until the solvent front had migrated 7 cm from the origin. The plate was cut in 1-cm increments from 1 cm above the origin to 6 cm above the origin, and the resulting seven sections were counted in the gamma counter. The number of chelates per antibody molecule was calculated from the ratio of counts remaining at the origin (0–1 cm, R_f = 0–0.14) to the total number of counts, using the method of Meares et al. (17).

Radiolabeling of cT84.66-DOTA. Representative conditions are given for labeling cT84.66-DOTA with the radiometals ^{111}In (III), $^{88,90}\text{Y}$ (III), and $^{64,67}\text{Cu}$ (II). In the ^{111}In and ^{90}Y labeling reactions, 88.8 μL of 0.25 M ammonium acetate, pH 7.0, was added to 2.0 mCi of $^{111}\text{InCl}_3$ in 111 μL of 0.04 N HCl or 6.0 mCi of $^{90}\text{YCl}_3$ in 4.6 μL of 0.05 N HCl, followed by 0.250 mg of cT84.66-DOTA in 50.4 μL of 0.25 M ammonium acetate, pH 7.0. The reaction mixture was incubated at 43 $^\circ\text{C}$ for 1 h, and then the reaction mixture was made 1 mM in DTPA with the addition of one-ninth of a reaction volume of 10 mM DTPA, pH 6.0. The reaction mixture was allowed to stand at room temperature for 15 min, after which the ^{111}In - or ^{90}Y -labeled antibody was purified by size-exclusion HPLC using a TosoHaas TSKgel G2000 SW column (7.5 $\times$ 300 mm, 10 μm), an isocratic mobile phase of normal saline, and a flow rate of 1.0 mL/min. The mAb peak, eluting at 10.3 min retention time, was collected in 0.5-mL fractions containing 1 drop of 25% (w/v) human serum albumin (HSA) each.

For ^{88}Y labeling, a longer reaction time was needed to achieve optimal labeling efficiency, presumably because of higher concentrations of trace metal contaminants in preparations of this isotope. In a typical ^{88}Y labeling reaction, 190 μL of 0.25 M ammonium acetate, pH 7.0, was added to 0.287 mCi of $^{88}\text{YCl}_3$ in 102 μL of 0.1 N HCl, and then 0.300 mg of cT84.66-DOTA in 69 μL of 0.25 M ammonium acetate, pH 7.0, was added. The reaction mixture was incubated at 43 $^\circ\text{C}$ for 3.5 h, after which 40.1 μL of 10 mM DTPA, pH 6.0, was added. The reaction mixture was incubated at 37 $^\circ\text{C}$ for 15 min, and the ^{88}Y -labeled conjugate was purified by size-exclusion HPLC in the same manner as the ^{111}In -labeled antibody.

In the ^{64}Cu labeling reaction, 120 μL of 0.1 M ammonium citrate, pH 5.5, was added to 1.4 mCi of $^{64}\text{CuCl}_2$ in 3.5 μL of 0.1 N HCl, followed by 0.205 mg of cT84.66-DOTA in 45 μL of 0.25 M ammonium acetate, pH 7.0. For ^{67}Cu labeling, 200 μL of 0.1 M ammonium citrate, pH 5.5, was added to 1.17 mCi of $^{67}\text{CuCl}_2$ in 100 μL of 0.1 N HCl, and then 0.228 mg of cT84.66-DOTA in 50 μL of 0.25 M ammonium acetate, pH 7.0, was added. The reaction mixtures were incubated at 43 $^\circ\text{C}$ for 1 h, after which they were made 1 mM in EDTA with the addition of one-ninth of a reaction volume of 10 mM EDTA, pH 5.5. The reaction mixtures were allowed to stand at room temperature for 15 min, and the ^{64}Cu - or ^{67}Cu -labeled mAb was purified by size exclusion HPLC in the same manner as the ^{111}In -labeled conjugate.

Immunoreactivity Determination. A dilution of the purified radiolabeled cT84.66-DOTA conjugate, containing approximately 200 000 cpm of radioactivity, was mixed with a 20-fold molar excess of purified CEA (18, 19) in 150 μL of 1% HSA/PBS, pH 7.4. A control sample was prepared in an identical manner, except that CEA was not added. The reaction mixtures were incubated at 37 $^\circ\text{C}$ for 15 min with continuous end-over-end mixing, after which aliquots of 100 μL were analyzed by gel filtration HPLC, using two Pharmacia Superose 6 HR 10/30 columns (1 $\times$ 30 cm) in series and an isocratic mobile phase of 0.05 M Na_2SO_4 /0.02 M NaH_2PO_4 /0.05% NaN_3 , pH 6.8, at a flow rate of 0.5 mL/min. Immunoreactivity was calculated as the percentage of the total radioactivity shifted to complexes with apparent molecular weights higher than that of the mAb.

Isoelectric Focusing. Aliquots of 5 μg of cT84.66 and each of three lots of cT84.66-DOTA were analyzed by isoelectric focusing (IEF) on pH 3–10 precast gels (NOVEX, Invitrogen Corp., Carlsbad, CA). IEF gels were run at constant voltage for 1 h at 100 V, 1 h at 200 V,

Scheme 1

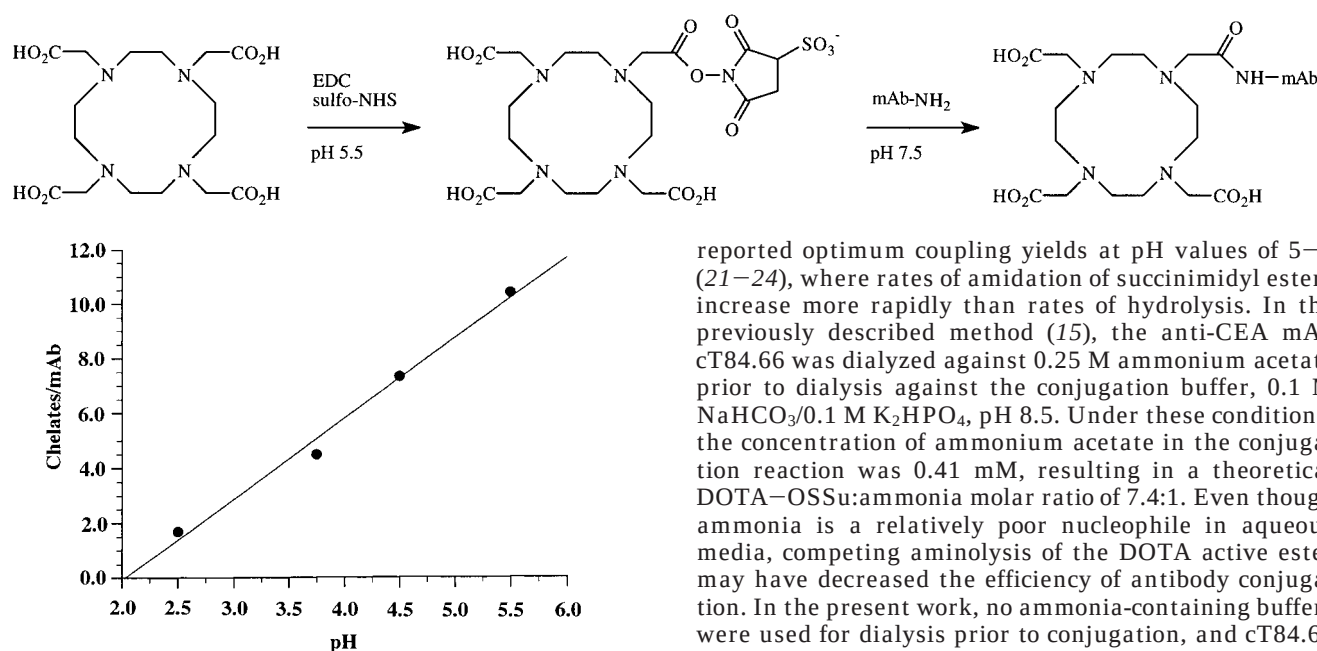


Figure 1. Average number of chelates per molecule of human IgG conjugated with 100 theoretical equiv of DOTA–OSSu, determined by a $^{57}\text{Co}(\text{II})$ binding assay, as a function of the pH at which DOTA–OSSu was prepared.

and then 30 min at 500 V. The gels were fixed in 0.14 M 5-sulfosalicylic acid/0.7 M trichloroacetic acid for 30 min at room temperature, stained with Coomassie Brilliant Blue R-250 for 30 min at room temperature, and destained in H_2O overnight.

RESULTS AND DISCUSSION

The previously reported method for conjugation of monoclonal antibodies with *N*-hydroxysulfosuccinimidyl DOTA (15) was a simple, aqueous phase procedure using only commercially available reagents (Scheme 1). However, large-scale preparation of clinical lots of mAb–DOTA conjugates, using 250 mg of antibody, would consume approximately 675 mg of DOTA. Therefore, it was desirable to improve the efficiency of the conjugation reaction, to reduce the amount of chelating agent required.

Several modifications to the original procedure were made. First, the pH of the active ester synthesis was optimized. In the original method, activation of trisodium DOTA with EDC and sulfo-NHS was performed at ambient pH, approximately 2.5–2.6. However, water-soluble carbodiimides are more stable at higher pH, in the range of 4 to 10 (20, 21). Above pH 7, the hydrolytic stability of sulfo-NHS esters decreases markedly (22), and many active ester-mediated protein coupling reactions have been performed in the range of pH 5–6 (23, 24). Therefore, in the present work, human IgG was used as a model system and conjugated with 100 theoretical equiv of DOTA–OSSu prepared at pH 2.5, 3.75, 4.5, and 5.5. As shown in Figure 1, a linear relationship was found between the pH of activation and the degree of antibody modification. Determination of the average number of chelates per antibody molecule indicated that the efficiency of conjugation increased approximately 6-fold when DOTA–OSSu was synthesized at pH 5.5, compared to ambient pH.

Second, pH and buffer conditions of the antibody conjugation reaction were improved. Several groups have

reported optimum coupling yields at pH values of 5–8 (21–24), where rates of amidation of succinimidyl esters increase more rapidly than rates of hydrolysis. In the previously described method (15), the anti-CEA mAb cT84.66 was dialyzed against 0.25 M ammonium acetate prior to dialysis against the conjugation buffer, 0.1 M NaHCO_3 /0.1 M K_2HPO_4 , pH 8.5. Under these conditions, the concentration of ammonium acetate in the conjugation reaction was 0.41 mM, resulting in a theoretical DOTA–OSSu:ammonia molar ratio of 7.4:1. Even though ammonia is a relatively poor nucleophile in aqueous media, competing aminolysis of the DOTA active ester may have decreased the efficiency of antibody conjugation. In the present work, no ammonia-containing buffers were used for dialysis prior to conjugation, and cT84.66 was reacted with DOTA–OSSu in sodium phosphate buffer at pH 7.5. On the basis of previously published studies, it was anticipated that the lower pH conditions would decrease the rate of hydrolysis of *N*-hydroxysulfosuccinimidyl DOTA by a factor of 2–2.5 (22) while maintaining the reactivity of the active ester with primary amines at neutral to slightly alkaline pH (24, 25).

EDC has been shown to react readily with inorganic phosphate to form an *O*-phosphoisourea, which hydrolyzes rapidly to give a substituted urea and phosphate (20). Thus, the pH of the DOTA–OSSu solution was adjusted to 7.0–7.5 with sodium phosphate before being added to the mAb. This procedure not only minimized the potential for mAb cross-linking by unreacted EDC, but also reduced the acidity of the active ester preparation and the possibility of protein denaturation. When analyzed by gel filtration HPLC, antibody–DOTA conjugates prepared by the previously published procedure were found to contain between 2.35 and 6.00% antibody dimers. Conjugates prepared under the improved pH and buffer conditions contained 0 to 0.862% dimers, as determined by gel filtration HPLC.

Model conjugation studies with cytochrome *c* (15) suggested that the reaction was equally efficient at 4 °C and room temperature. However, cytochrome *c* is a small, hydrophilic, and very lysine-rich protein and, compared to antibodies, may react with active esters at a rate more comparable to that of competing hydrolysis. Hydrolysis of *N*-hydroxysulfosuccinimide esters is an order of magnitude slower at 0 °C than at 31 °C, increasing the half-life of active esters from 0.87 h at 31 °C to 11 h at 0 °C (22). Thus, mAb conjugations with *N*-hydroxysulfosuccinimidyl DOTA were performed at 4 °C to minimize the rate of hydrolysis of the active ester. It was expected that a long incubation time (24 h) at low temperature (4 °C) would maximize both the lifetime of DOTA–OSSu in solution and the efficiency of antibody conjugation.

Using the optimized activation and conjugation conditions, the reaction of *N*-hydroxysulfosuccinimidyl DOTA with antibodies was approximately 6-fold more efficient than when the previously published procedure was used (Figure 1). Therefore, for subsequent conjugations the molar ratio of DOTA–OSSu:cT84.66 was reduced from

Table 1. DOTA Activation and cT84.66 Conjugation Conditions

lot no.	molar ratio DOTA:sulfo-NHS:EDC:mAb	activation pH	conjugation buffer	chelates/mAb
RJ121296	1000:1000:100:1	2.55	0.1 M NaHCO ₃ /0.1 M K ₂ HPO ₄ , pH 8.5	1.7
010297NC	200:200:20:1	5.18	0.1 M Na ₂ HPO ₄ , pH 7.5	3.1
021997	200:200:20:1	5.15	0.1 M Na ₂ HPO ₄ , pH 7.5	3.1
MA071098	200:200:20:1	5.45	0.1 M Na ₂ HPO ₄ , pH 7.5	3.8

Table 2. Radiometal Labeling of cT84.66–DOTA

radiometal	<i>n</i>	labeling ratios (mCi/mg)	labeling efficiency (%)	specific activities (mCi/mg)	immunoreactivity (%)
¹¹¹ In ^b	19	0.723–38.1	98.9 ± 1.0	0.718–37.7	99.9 ± 0.3
⁹⁰ Y ^b	14	0.185–24.2	86.3 ± 9.9	0.181–19.4	98.6 ± 2.9
⁸⁸ Y	2	0.957–1.00	94.1 ± 2.2	0.915–0.925	ND ^a
⁶⁷ Cu	1	5.22	61.0	3.18	ND
⁶⁴ Cu	1	6.83	100.0	6.83	100.0

^a ND = not determined. ^b Reaction of unconjugated, demetalated cT84.66 with ¹¹¹In or ⁹⁰Y resulted in 0% radiometal incorporation.

100:1 to 20:1. Table 1 gives the activation and conjugation conditions, as well as the number of chelates per mAb determined by ⁵⁷Co and ¹¹¹In binding assays, for one lot of cT84.66–DOTA prepared by the original method and three lots of cT84.66–DOTA prepared using the procedure described here. The results of the radiometal binding assays show that conjugates prepared by the two procedures contain similar numbers of functional chelates, yet the quantities of reagents used to conjugate the antibody were reduced by a factor of 5 when the new method was used.

DOTA conjugates of cT84.66, prepared by the original procedure and the improved method, were analyzed by isoelectric focusing (Figure 2). The pI of unmodified cT84.66 (lane 1) was determined to be 6.5–7.3 by IEF. Modification with DOTA resulted in expected decreases in the isoelectric points of the mAb conjugates. The pI of cT84.66–DOTA, conjugated by the previously published method (lot RJ121296, lane 5), was found to be 5.0–6.7. However, a pair of predominant bands at a pI value of 5.3 suggested the presence of a relatively large population of highly modified mAb species. In contrast, cT84.66–DOTA conjugates prepared by the new method (lots MA071098, 010297NC, and 021997; lanes 2, 3, and 4) showed a more uniform distribution of antibody modification over a pI range of 5.2–7.0. Thus, the improved conjugation procedure described here gives a more homogeneous product, with fewer highly modified conjugates. Overmodification of mAbs with BCAs can lead to decreases in immunoreactivity and tumor uptake (26), so it was desirable to reduce the fraction of heavily conjugated species.

The results of ¹¹¹In, ⁹⁰Y, ⁸⁸Y, ⁶⁴Cu, and ⁶⁷Cu labeling reactions of cT84.66–DOTA under no-carrier-added conditions are listed in Table 2. The ¹¹¹In and ⁹⁰Y labeling reactions were performed over a wide range of specific activities, for the purposes of animal biodistribution, patient imaging, patient therapy, and human anti-chimeric antibody (HACA) studies. When unconjugated, demetalated cT84.66 was incubated with ¹¹¹In or ⁹⁰Y, no nonspecific binding of either radiometal to the mAb was observed after DTPA challenge and HPLC purification. Labeling of cT84.66–DOTA proceeded with >85% efficiency with all radiometals except ⁶⁷Cu. The relatively low specific activity of ⁶⁷Cu (185 Ci/mmol, compared to 20 000 Ci/mmol for ⁶⁴Cu) likely contributed to its lower labeling efficiency, because of competition by cold copper for chelation sites on the conjugate. Compared to the previously published work (15), ¹¹¹In labeling was increased by 15%, and ⁹⁰Y labeling was improved by 27%.

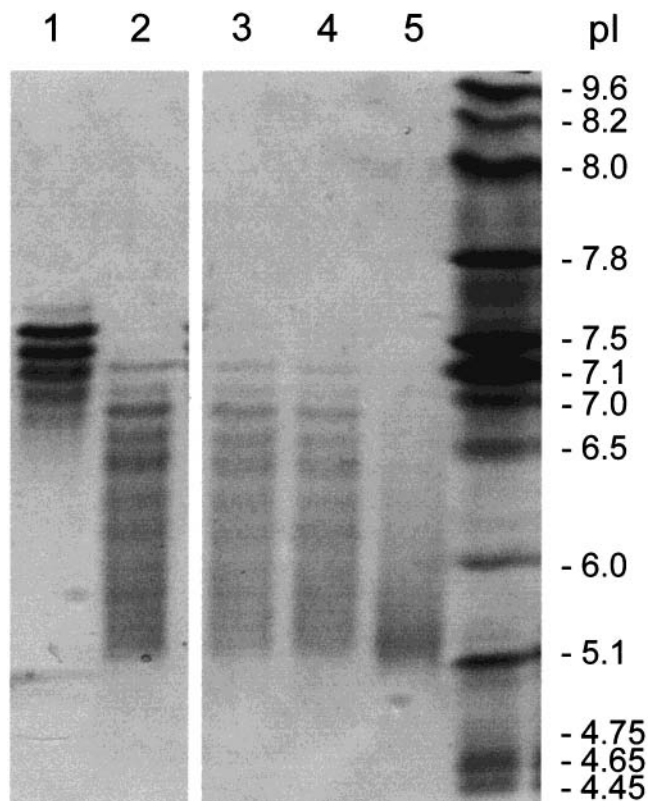


Figure 2. IEF analysis of unconjugated cT84.66 (lane 1) and cT84.66–DOTA conjugates prepared by the method reported herein, Lot MA071098 (lane 2), lot 010297NC (lane 3), lot 021997 (lane 4), and cT84.66–DOTA prepared by the previously published method (15), lot RJ121296 (lane 5). Isoelectric point markers are shown to the right.

However, the standard deviation of ⁹⁰Y labeling efficiency was larger than those of the other radiometals. The variation in ⁹⁰Y labeling did not correlate with specific activity, mAb conjugate lot, or storage of the conjugate over a period of 14 months. Rather, the efficiency of labeling seemed to vary with the lot of ⁹⁰Y used. Table 2 also lists the immunoreactivities of the radiometal-labeled conjugates, as determined by a solution-phase CEA binding assay. For all conjugates analyzed, immunoreactivity was essentially quantitative.

By optimizing pH, buffer, and temperature conditions for both the preparation of *N*-hydroxysulfosuccinimidyl DOTA and its conjugation to the anti-CEA monoclonal antibody cT84.66, several improvements to the previously published procedure were made. These improvements

included a 6-fold increase in conjugation efficiency, a 3–7-fold decrease in antibody dimerization, a more homogeneous population of conjugate species, and a 5-fold reduction in the quantities of DOTA and coupling reagents required for the conjugation. Furthermore, ^{111}In and ^{90}Y labeling of cT84.66–DOTA was increased significantly, and high specific activity labeling with Cu radioisotopes was achieved, while maintaining quantitative immunoreactivity. This procedure has been scaled up to produce two lots of 233 and 215 mg, respectively, of clinical-grade cT84.66–DOTA. We are currently evaluating ^{90}Y -labeled cT84.66–DOTA in a Phase I/II clinical trial for radioimmunotherapy of CEA-positive malignancies.

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COMMUNICATIONS

Synthesis and Biological Evaluation of an Anticancer Vaccine Containing the C-Glycoside Analogue of the Tn Epitope

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The C-saccharide analogue of the GalNAc (Tn epitope) has been covalently linked to the T cell epitope peptide ³²⁸⁻³⁴⁰OVA using a chemoselective convergent synthetic approach. In this way, a non-hydrolyzable synthetic vaccine was obtained composed by a B epitope conjugated to a T cell epitope. This compound was tested in a proliferation assay with spleen cells from DO11.10 mice. The molecule was recognized by transgenic T cells although at a slightly lower efficiency if compared with the reference peptide OVA. An additional experiment with dendritic cells fixed with glutaraldehyde shows that the glycopeptide can bind to extracellular MHC molecules without need of internalization and processing and that the C-glycoside part does not interfere with TCR recognition. These observations constitute an important starting point for the use of this molecule as vaccine against the Tn-expressing TA3-Ha mouse mammary carcinoma.

Tumor immunotherapy is based on the theory that tumor-associated antigens (TAA) become immunogenic if presented to a properly trained immunosystem (1). Many of the tumor antigens are constituted by sugars, because the malignant cells are commonly characterized by incomplete glycosylation or neoglycosylation, and a large number of tumor-associated carbohydrate antigens (TACA) expressed on glycolipids and glycoproteins have been identified (2, 3). In particular, the monosaccharide α -GalNAc, called Tn antigen, has been extensively studied, since it is expressed on mucin-type glycoproteins by the majority of human adenocarcinomas as a consequence of aberrant glycosylation, whereas it is hidden in normal cells (4).

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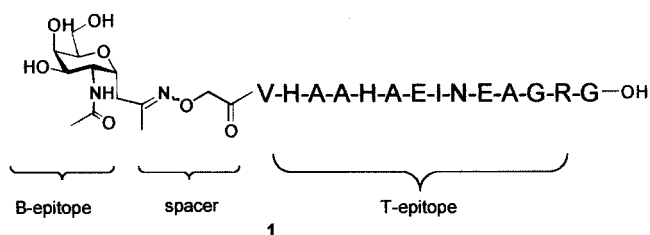


Figure 1. Chemical structure of the synthetic vaccine 1.

An immune response directed against carbohydrate antigens results in the induction of antibodies that could eradicate the micrometastases and the circulating tumor cells in the blood stream, thus providing protection against tumor. However, carbohydrate-based vaccines have so far been unsuccessful in inducing detectable T cell immunity (5). To overcome this limitation, and in

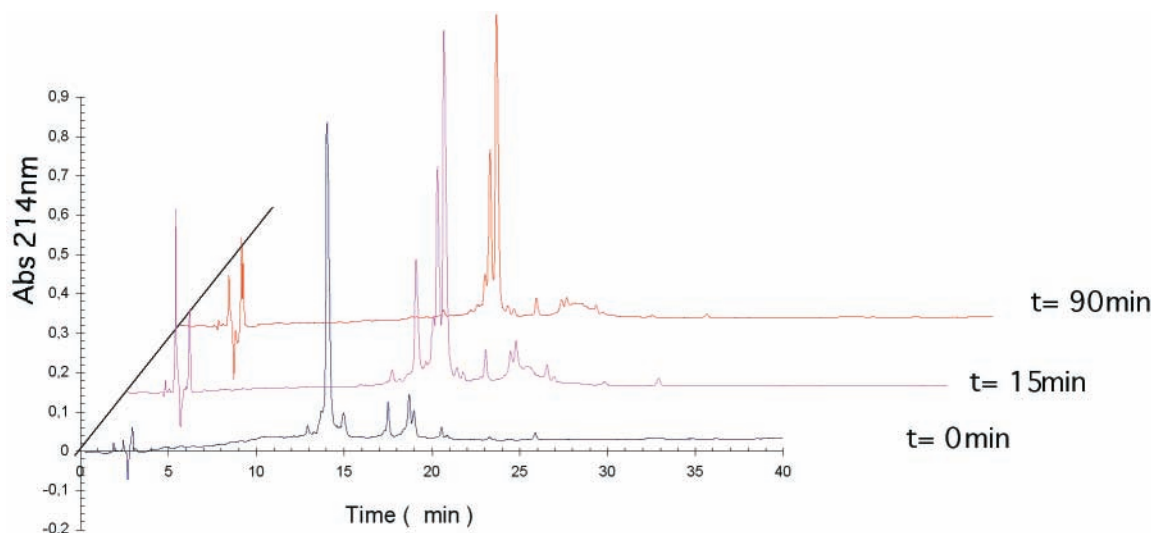


Figure 2. Overlay of RP-HPLC chromatograms of the conjugation reaction. Conditions: A: 0.1% TFA in water, B: 0.1% TFA in CH₃CN; gradient: from 0% to 100% B in 30 min; detection at 214 nm. After 90 min at rt, the HPLC profile of reaction crude shows the complete disappearance of aminoxy-peptide **3** and the appearance of the conjugation product **1** constituted by two peaks (*syn* and *anti* oxime isomers).

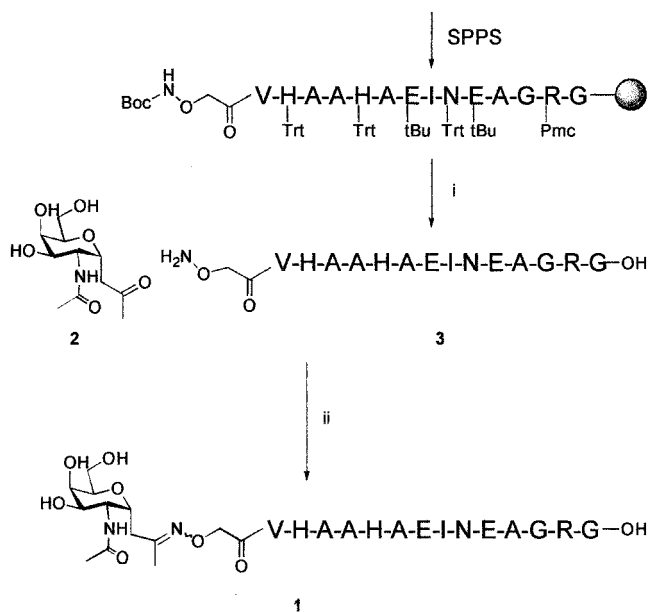
order to induce an immunological memory toward a carbohydrate epitope, a suitable vaccine has to provide an helper T cell response for B cell induction and IgG production. According to this concept, semisynthetic vaccines have been prepared by conjugation of a carbohydrate B-epitope with a protein and it has been shown that these molecules are capable to induce IgM and IgG anti-Tn antibody responses (6). Totally synthetic vaccines composed by a carbohydrate B-epitope covalently linked to the lipopeptide tripalmitoyl-*S*-glycerylcysteinylserine as a combined carrier and adjuvant system have been prepared (7, 8, 9). Vaccines with a more complex molecular structure have been developed by assembling covalently a carbohydrate (B-epitope), a peptide T-epitope and the lipopeptide dipalmitoyloxypropyl-*N*-palmitoyl-cysteine (Pam₃-Cys) (10). However, the outcome of the immune response for this molecule has not been reported.

Following the same concept we designed a totally synthetic vaccine (Figure 1) by linking covalently a sugar B and a peptide T cell epitope through a spacer. In our design, the spacer containing an oxime bond has the function to keep the saccharide and the peptide apart, thus minimizing a possible negative interference of the sugar in the formation of the complex between peptide and MHCII¹ and its recognition by the TCR. We verified our hypothesis *in vitro* by testing the capacity of the glycopeptide to form a complex with MHCII on dendritic cells (DC). However, the main innovation in the design of our vaccine consists of the use of the α -C-glycosidic analogue of the GalNAc instead of the glyco-amino acid GalNAc-Ser/Thr as Tn epitope. The C-glycosidic bond is stable toward acids, bases, and enzymatic hydrolysis; this makes **1** a promising candidate for drug development.

Finally, we tested the ability of dendritic cells to present glycopeptide **1** to TCR.

DC are the only antigen presenting cells capable of priming a T cell response; thus, vaccines targeted to DC are potentially more powerful (11).

Scheme 1^a



^a Reagents and conditions: i, TFA, TIS, H₂O (95: 2.5: 2.5); ii, **2** (1.2 equiv), **3** (1 equiv), acetate buffer 0.1 M, pH 4.5.

The ^{328–340}OVA peptide in **1** corresponds to a part of the epitope for the TCR–OVA transgenic T cells derived from DO11.10 mice (12). The ^{327–339}OVA peptide was chosen as a model antigen to provide T cell help either in DO11.10 mice or in mice that have been previously immunized with soluble OVA in an immunogenic fashion. The latter will mimic a situation of immunized animals and it will be a “proof of principle” for the use of a recall T-helper antigen, such as the tetanus toxoid in humans. In particular, it will be possible to test the effectiveness of the synthetic vaccine **1** in providing protection against challenge with a highly invasive mouse mammary carcinoma, TA3-Ha, which expresses the Tn antigen (13).

The synthesis of the vaccine was accomplished according to a convergent strategy based on the chemoselective coupling (14) of the α -C-glycosyl ketone **2** with peptide **3** bearing an aminoxy group at the N-terminal end (Scheme 1). The preparation of the C-glycosyl analogue

¹ Abbreviations: Boc, *tert*-butoxycarbonyl; DC, dendritic cell(s); DIPEA, diisopropylethylamine; GalNAc, *N*-acetyl-galactosamine; MHCII, major histocompatibility complex II; Fmoc, 9-fluorenylmethoxycarbonyl; TCR, T-cell receptor(s); SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

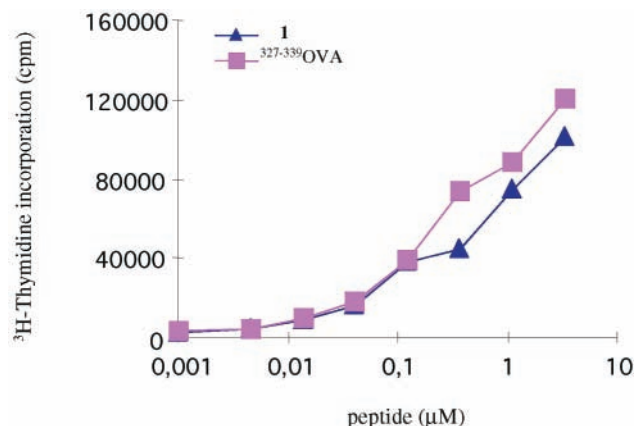


Figure 3. OVA peptide in compound **1** is recognized by TCR–OVA transgenic T cells. Spleen cells from DO11.10 mice were incubated with the indicated concentrations of **1** and of reference $^{327-339}$ OVA peptide. The proliferative response was measured after 2 days as ^3H -thymidine incorporation by TCR–OVA T cells. As shown, compound **1** was recognized although with slightly lower efficiency as compared to the reference peptide.

of GalNAc has been reported by our group (15); the peptide sequence was assembled on Wang resin using the Fmoc/*tert*-butyl solid-phase strategy (16) (SPPS, Scheme

1). The aminoxy functionality was introduced by solid-phase condensation of *N*-hydroxysuccinimide activated Boc-aminoxyacetic acid in the presence of the base DIPEA. Aminoxy antigen **3** was obtained with 40% overall yield after cleavage from resin, complete side chain deprotection, and RP-HPLC purification. The chemoselective oxime bond formation between the Tn-ketone and the aminoxy antigen was carried out in aqueous solution (acetate buffer, pH 4.5) by monitoring the reaction by RP-HPLC (Figure 2); the coupling turned out to be complete after 90 min at rt (35% yield after HPLC product purification). Compound **1** presents two isomeric forms in equilibrium at rt, deriving from the *cis*/*trans* isomerism of the oxime bond, detectable by HPLC analysis and characteristic of oxime-linked glycoconjugates (14). The glycopeptide was tested in a proliferation assay with spleen cells from DO11.10 mice in order to assess if the carbohydrate group interferes with the capacity of TCR–OVA transgenic T cells to recognize the peptide in association with MHC II. As shown in Figure 3, compound **1** was recognized by transgenic T cells although at a slightly lower efficiency if compared with the reference peptide OVA. Next, we analyzed whether the glycopeptide **1** has to be internalized and processed by DC in order to be presented to T cells. We used a well-characterized nonimmortalized DC cell line (D1 cells)–(17) which is the prototype of immature DC able to

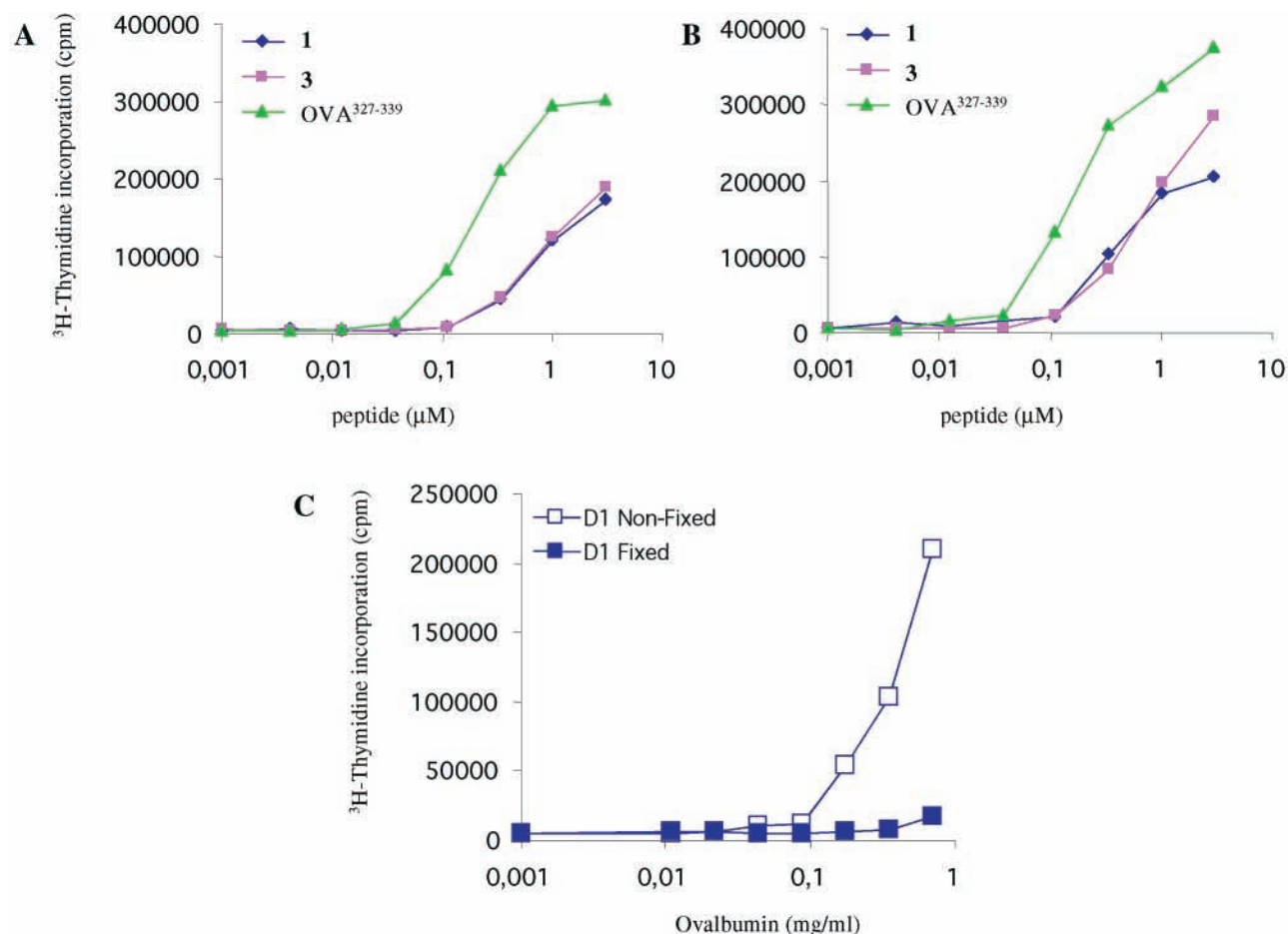


Figure 4. Internalization and processing of compound **1** is not necessary for OVA peptide presentation. D1 cells were either fixed (A) or nonfixed (B) with 0.001% glutaraldehyde for 1 min on ice, loaded with the different peptides and incubated with the costimulation-insensitive hybridoma (BO97.10) which is specific for the $^{327-339}$ OVA peptide. Activation of the hybridoma was tested by measuring the IL-2 produced in culture supernatants as a function of ^3H -thymidine incorporation by a CTL line which is dependent on IL-2 for its growth (CTLL-2). Peptides **1** and **3** were similarly presented to T cells by fixed and nonfixed cells, indicating that processing is not required for efficient antigen presentation. (C) As a control, fixed DC were unable to present whole ovalbumin which requires processing for the generation of the OVA peptide.

present antigens to T cells in vitro. DC were thus fixed with 0.001% glutaraldehyde in phosphate-buffered saline (PBS) before exposure to the peptides to avoid the internalization and processing of the compounds. To overcome the need of costimulation which is essential for T cell priming and cannot be provided by fixed DC, a costimulation-insensitive hybridoma (BO97.10) recognizing the same OVA peptide in association with MHC II molecules was used. As a control we analyzed the capacity of fixed D1 cells to present ovalbumin which has to be internalized and degraded to produce the OVA^{327–339} peptide recognized by the hybridoma. Both the glycopeptide **1** and the aminooxy peptide **3** could be presented by fixed D1 cells (Figure 4A) with similar efficiencies as non fixed cells (Figure 4B) indicating that the peptide can bind to extracellular MHC molecules and that the carbohydrate does not interfere with TCR recognition. The antigen presentation is very similar in both **1** and **3** but less efficient than in the OVA epitope, suggesting that the aminooxy linker interferes with MHC binding or with TCR recognition. As expected, the whole ovalbumin could not be presented by fixed cells (Figure 4C).

Thus, we have validated our vaccine **1** in vitro by showing that it can be presented by DC and that the C-glycosidic analogue of GalNAc, stable toward enzymatic and chemical hydrolysis, does not influence the antigen specificity of the compound. Future perspectives of this work will aim to study the capacity of compound **1** to induce an antibody response to GalNAc in immunized animals in vivo. The requirement of T cell help delivered by the OVA peptide for antibody production will also be evaluated and this will be correlated with the capacity of vaccine **1** to protect mice from lethal challenge with the TA3-Ha mouse mammary carcinoma.

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Design of Acridinium-9-carboxamides and Anti-acridinium Antibodies for Chemiluminescent Signal Enhancement

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A novel system of signal enhancement is presented in which every labeled antibody is capable of generating a signal. Three chemiluminescent acridinium-9-carboxamide haptens (**1**, **2**, and **3**) which incorporated differences in charge and location of the linker were designed and synthesized. Anti-acridinium polyclonal antibodies for each hapten were screened using surface plasmon resonance instrumentation to determine specificity for each hapten. Anti-acridinium **2** antibodies were found to be non-cross-reactive to acridinium **1**. This property was exploited to design secondary antibody conjugates which would bind to primary antibodies labeled with **2** yet could still be labeled with the structurally similar acridinium **1**. Consequently, both layers contributed to the overall chemiluminescent signal. This format is an advance over other signal amplification formats which employ non-signal-generating, labeled antibodies to construct multilayered systems.

A variety of assays used for biological research, clinical diagnostics, and high throughput screening employ a detection reagent labeled with species capable of generating a colorimetric, fluorescent, or chemiluminescent signal (*1*). In turn, the signal generated is used to detect an analyte of interest. Frequently, multiple layers of signal generating conjugates must be applied to a captured analyte to achieve signal enhancement.

In the area of immunoassays, immunoconjugates can be prepared with increasing label-to-antibody ratios until an optimal signal is achieved (*2*). While this approach is straightforward, it does have its practical limitations. Conjugates with high label-to-antibody ratios can result in detection reagents with lowered solubility and compromised binding affinity relative to their unconjugated state (*3, 4*). To overcome such limitations, systems that build multiple layers of detection reagents can be used. In this way, the number of labeled antibodies increases, *not* the number of labels per antibody (*5*). A common method makes use of a biotin label (B) in conjunction with an anti-biotin antibody labeled with a signal generating species (S). In this scheme, the system achieves an enhanced signal relative to a single labeled anti-analyte antibody (Figure 1b). Many analogous systems exist; schemes incorporating labeled avidin are perhaps the best known (*6–8*).

While such systems are effective, they are simultaneously wasteful. In the example detailed in Figure 1a, the biotin "extender" is merely a means to noncovalently bind the signal bearing antibody and itself does not generate a signal. We reasoned that a signal generating extender would increase the overall effectiveness of the system by ensuring the contribution of *every* labeled antibody. In such a proposition, B, the extender, becomes a signal-generating species, S₁, and the signal-generating species on the secondary antibody is designated S₂ (Figure 1c).

The design of a signal generating extender and its corresponding receptor poses certain challenges. First,

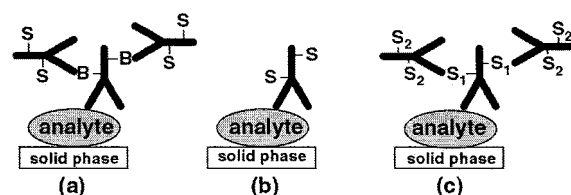


Figure 1. Schematic representation of immunoassay formats.

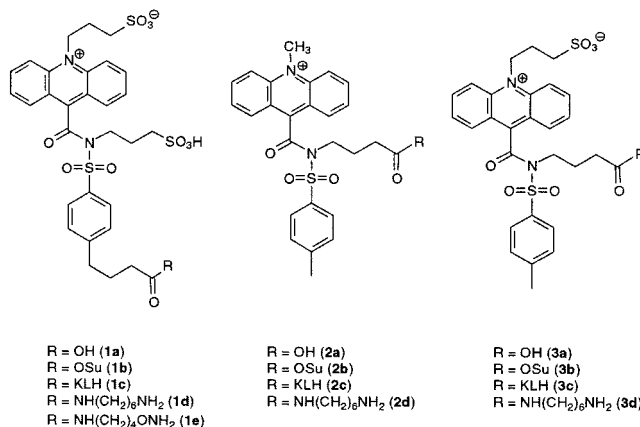


Figure 2. Acridinium-9-carboxamides used in this study.

S₁ and S₂ should be capable of generating a signal using an identical method of induction. Otherwise, multiple triggering events may be needed, thereby increasing the complexity of the system. Finally, because an anti-S₁ antibody would need to be labeled with S₂, cross-reactivity between the secondary label and the secondary antibody must be eliminated. If not, the secondary antibody conjugate would have limited capacity to bind S₁ and would be prone to insolubility caused by immunoprecipitation.

To overcome these challenges, three chemiluminescent acridinium-9-carboxamide haptens (**1a**, **2a**, and **3a**) were designed and synthesized (Figure 2). Acridinium deriva-

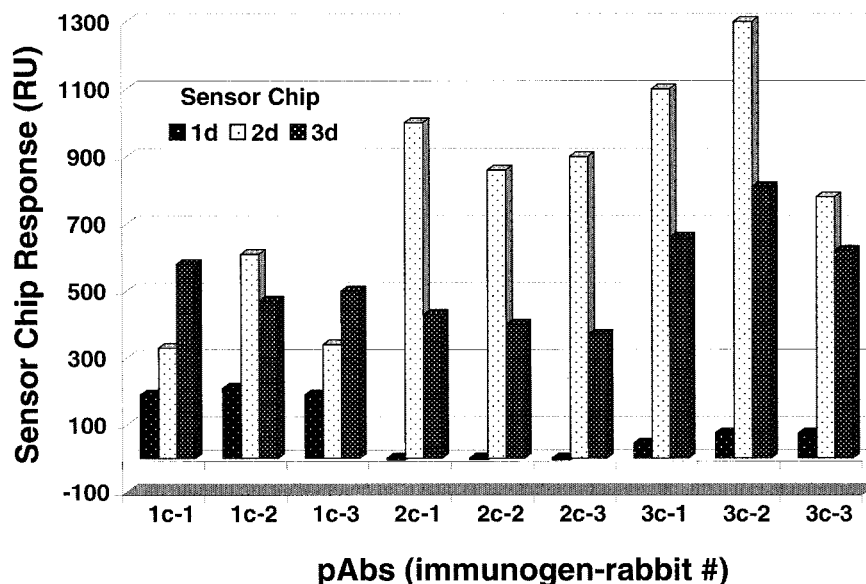


Figure 3. Determination of antibody cross-reactivity using SPR.

tives have proved themselves to be efficient chemiluminescent labels used in a variety of applications (9). In the case of antibodies specific for acridinium-9-carboxamides, the antibodies would need to discriminate between acridinium labels that, by necessity, must be structurally conserved to retain their chemiluminescent properties. Differences between the structures, such as their overall charge and the location of the linker, were incorporated to generate hapten specific antibodies. Conjugation of **1b**, **2b**, and **3b** to keyhole limpet hemocyanin (KLH) yielded the hapten-carrier conjugates **1c**, **2c**, and **3c**, respectively, which were used to immunize New Zealand White rabbits. Three anti-sera for each immunogen were generated. IgG polyclonal antibodies (pAbs) were isolated from rabbit serum using ammonium sulfate precipitation followed by protein A chromatography.

To ascertain specificity, surface plasmon resonance (SPR) was employed to gauge the relative affinities of the polyclonal antibodies for the three different haptens. SPR has previously been shown to be an effective method for characterization of antibody binding phenomena (10). Amine-bearing congeners of the haptens (**1d**, **2d**, and **3d**) were covalently attached to a carboxymethylated dextran sensor chip using NHS and EDAC. The resulting sensor chips were then capped using ethanolamine. Solutions of each of the anti-hapten pAbs were injected over the surface of the sensor chips. The resulting saturated responses were then compared (Figure 3).

Significantly, of the nine possible combinations of pAbs versus hapten screened, one combination exhibited no observable cross-reactivity: Anti-2 pAbs showed no response for an acridinium **1** derivatized chip. This particular combination showed the greatest divergence with respect to charge and position of the linker. Post-conjugation to KLH, **2b**, yielded a hapten with a positive charge in contrast to the negative charge of the conjugated form of **1**. Furthermore, the linker of **2** emanates from the nitrogen of the sulfonamide. By contrast, the linker of **1** is attached to the tosyl ring. In general, antibody specificity was similar among the set of three rabbits immunized with the same immunogen. This trending would support that the observed specificity was the result of a general immunological response to hapten structure as opposed to the isolated response of a single rabbit. Interestingly, the opposite configuration, anti-1

pAbs in combination with a **2d** derivatized sensor chip showed cross-reactivity.

Once a requisite pair of hapten and anti-hapten pAbs was found, immunoconjugates were designed to demonstrate the utility of the combination for construction of a signal generating layer in a model system. An anti-L-T₄ monoclonal antibody (mAb) was used to detect a bovine serum albumin (BSA)-L-T₄ conjugate passively absorbed to a polystyrene microtiter plate (11). The anti-L-T₄ mAb was labeled with the active ester **2b** in sodium borate buffer, pH 8.5. The anti-2 pAbs were oxidized using sodium periodate and purified from excess periodate into sodium acetate buffer, pH 5.1. Coupling of the oxidized pAbs with **1e** resulted in the formation of a stable, oxime-linked immunoconjugate. Both conjugates were purified using a desalting column equilibrated with PBS in order to remove unconjugated label. The resulting conjugates were characterized using UV-visible spectroscopy and were shown to have label-to-antibody ratios of 3.4 and 4.0, respectively. Prior to or after the purification of the labeled anti-2 pAbs, no precipitation of the antibodies was observed, as might be expected if cross-linking and subsequent immunoprecipitation was to be a problem.

To demonstrate the use of **2** as a signal generating extender, the chemiluminescent signals of the labeled anti-L-T₄ mAb complexed to labeled or unlabeled anti-2 pAbs were compared. Specifically, the anti-L-T₄ mAb conjugate was incubated with a BSA-L-T₄ conjugate passively absorbed to a polystyrene plate and unbound antibody then was washed away. This incubation was followed by a second incubation using either labeled or unlabeled anti-2 pAbs (Figure 4a and 4b, respectively). After a second wash, the addition of basic peroxide resulted in a chemiluminescent signal. The observed increase in signal when labeled secondary antibody was used was taken as evidence for the ability of the label **2** to act as a chemiluminescent extender between the primary and secondary antibodies used for detection. To rule out apparent signal enhancement caused by non-specific binding of the labeled antibodies, two controls were performed. Neither replacement of the BSA-L-T₄ conjugate with BSA (Figure 4c) nor the replacement of the anti-L-T₄ mAb conjugate with unlabeled mAb (Figure 4d) in the system yielded any significant signal. Both of these latter experiments indicated that the observed

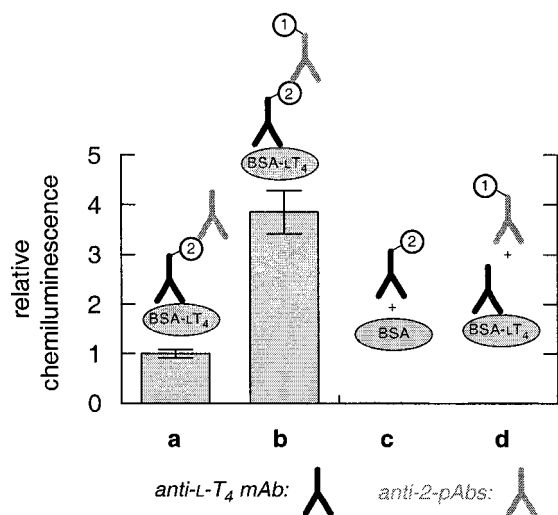


Figure 4. Normalized chemiluminescence microtiter plate results.

signal enhancement in Figure 4b is due to specific binding as opposed to nonspecific absorption of the immunoconjugates.

In conclusion, a novel system of chemiluminescent signal enhancement has been presented in which every antibody layer is capable of generating a signal. A combination of synthetic design and ligand screening was utilized to find the appropriate hapten–antibody pair to enhance signal. Alternative synthetic or enzymatic labels also can be envisioned. Furthermore, the application of this concept to build multilayered systems should provide even higher degrees of signal enhancement.

Supporting Information Available: Synthesis of **1b–e**, **2a–d**, and **3c,d**; preparation and isolation of pAbs; conjugation procedures; experimental procedures for surface plasmon resonance and chemiluminescence analyses. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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A Scanning Near-Field Optical Microscope Approach to Biomolecule Patterning

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In view of future generations of biosensors and advanced biomaterials, photochemistry in the near field using scanning near-field optical microscopy is investigated. The potential of direct near-field-induced photoactivation is demonstrated on standard photoresist. Photoimmobilization of maleimidoaryldiazirine on silicon substrates and bovine serum albumin on glass substrates is achieved, opening the way to a controlled biopatterning of surfaces with submicrometer feature size. The obtained patterns are characterized using atomic force microscopy, time-of-flight secondary ion mass spectrometry (ToF-SIMS), and near-field fluorescence microscopy.

INTRODUCTION

In view of future generations of biosensors and advanced biomaterials, the construction and integration of complex biological systems onto selected materials or transducers is of primary importance. Chemical treatment of a material surface through functionalization is the first step in engineering of biosensors and biomaterials. Immobilization of biomolecules on transducer surfaces can be achieved by different means: physical adsorption (physisorption), receptor-mediated adsorption (bioaffinity), or covalent binding. The first two processes are reversible, while the latter leads to the formation of irreversible chemical bonds (1). An elegant way toward covalent immobilization is the use of photoactivable reagents. Indeed, the interaction of light with the reagents induces reaction intermediates that can lead to covalent chemical bonds to the substrate surface. Among the photoactivable reagents available, aryldiazirine has been successfully used on a wide range of materials (from polymer to metal oxides) (2). Its activation wavelength is in the range of 350 nm, which does not impair biological activity. Meanwhile, topical addressability, which allows multicomponent domain-limited devices, has been achieved with a standard masking technique as it is commonly used in microtechnology. However, this technique is limited in spatial resolution by the diffraction of light. The use of deep-UV wavelengths to improve the resolution is not possible without destroying the biological properties of the attached molecules. Future

needs for photopatterning of biomolecules include high spatial localization of the biomolecules, and thus it involves the development of new techniques going beyond the diffraction limit. Near-field illumination potentially allows to overcome this limit.

Scanning near-field optical microscopy (SNOM) is, in first use, a technique to optically investigate surfaces beyond the diffraction limit. An optical probe is scanned a few nanometers above the object to be investigated where it interacts with the sample via the near electromagnetic field either emitted or scattered by the probe or by the object.

The resolution of SNOM is not limited by diffraction, but rather by the size of the optical probe and the distance between the probe and the sample.

Although already proposed decades ago (3), the principle has been rediscovered and experimentally realized only in 1984 with the demonstration of optical resolution below diffraction limit (4), when technology allowed fabrication of sub-wavelength size probes and control of its distance to the sample with nanometer precision. This technique offers a wide area of applications for the study of biomaterials, allowing exploitation of most of the optical contrast mechanisms (fluorescence, phase, polarization, etc). SNOM has been successfully used in investigation of single molecules (5), cells (6), and in combination with fluorescence energy transfer (7).

This work demonstrates that SNOM can be used not only as an observation tool, but also as a structuring tool, that exploits the high localization of the light in the probe's near field, to very locally induce photochemical effects. Combining SNOM lateral resolution with photochemical immobilization of aryldiazirine on material surfaces, we perform near-field photochemistry to immobilize nanostructures on surfaces obtaining submicrometric domains of recognition. In this paper, we first illustrate the potential of direct near-field induced photoactivation on standard photoresist and show that sub-wavelength patterning is achievable. In a second step, immobilization of maleimidoaryldiazirine (MAD) on a

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silicon substrate is obtained by near-field photoactivation, opening the way to a controlled biopatterning of surfaces. This configuration offers the advantage of a small molecule that forms a thin and homogeneous layer on the sample surface. Finally, the technique is applied to photoimmobilize a larger biomolecule (bovine serum albumin, BSA), obtaining patterns of proteins with submicrometer feature size on a glass substrate.

EXPERIMENTAL SECTION

Near-Field Instrumentation. Near-field photoimmobilization and near-field sample observation are performed with a homemade scanning near-field optical microscope, mounted on a standard inverted optical microscope (Zeiss Axiovert 100). The SNOM head is composed of a near-field probe holder and a compact distance control module. The probe-to-sample distance control is based on interferometric detection of the lateral force induced when the tip is laterally vibrating over the sample. This technique, described in detail in ref 8, allows interaction forces between the probe and the sample to be kept constant. Assuming that the distance-to-sample is directly correlated to the interaction forces, topographical information of the sample is obtained.

The near-field probes are obtained by chemical etching of monomode optical fibers and subsequent metallization with aluminum (9). Before using them for photoimmobilization, the optical probe quality is tested by a set of optical measurements with a characterization of transmission and light distribution at the apex. Scanning of a calibrated test object allows evaluation of the diameter of the optical probe. Tips used in this study show a diameter between 60 and 110 nm with transmission between 6×10^{-4} and 5×10^{-3} .

The samples are scanned under the tip with an x - y - z linear piezo scanner (Physik Instrument). The linearity and the absence of creep are ensured by a closed loop control of the scanner.

Near-field photochemistry is induced by UV light emitted from an argon laser (Spectra Physics 2020) coupled into the optical fiber-based probe. Detection of fluorescein-labeled BSA is performed by near-field fluorescence, excited by 488 nm Argon laser light. Emitted fluorescence light is collected by a microscope objective (Zeiss 40x, N.A.: 0.6) and, after filtration of the excitation light with a notch filter (OD6), it is finally analyzed with an avalanche photodiode (APD, EG&G, SPCMAQ131FC).

Photoresist. Photoresists are radiation-sensitive materials commonly used in semiconductor technology for patterning. The resist is exposed to ultraviolet radiation through a mask containing the desired patterns. In positive photoresists, the exposed regions are made soluble, whereas in negative photoresists they remain insoluble and the nonilluminated resist is dissolved away. A diazoquinone-inhibited novolak positive photoresist S1813 available from Shipley is used for all experiments. The hydrophobic diazoquinone inhibitor strongly absorbs UV light (350–400 nm). The addition of the hydrophobic inhibitor to the hydrophilic novolak resin retards the dissolution of the mixture in alkaline developer. Upon photoexposure, the inhibitor is destroyed and rearranged into hydrophilic acid, which dissolves in the alkaline developer. The coating and the developing processes are made using Rite Track 88 series equipment. Four-inch diameter plain silicon wafers are coated with 1.5 μ m photoresist. MF-319 is used for development of the exposed wafer. The obtained patterns are preliminary

observed with a far-field optical microscope. Precise characterization of patterns is carried out with an atomic force microscope (Autoprobe CP, Thermomicroscopes).

Photoreagents. *N*-(*m*-(3-(Trifluoromethyl)diazirine-3-yl)phenyl)-4-maleimidobutyramide (MAD) is synthesized according to Collioud et al. (10). Silicon wafers with a naturally grown oxide layer are used as substrate. The 5×5 mm² samples are cut from the wafer, washed with an ultrasonic treatment (2×5 min in ethanol Uvasol and then 2×5 min in hexane Uvasol), and dried for 2 h at 10^{-2} mbar. The MAD solution (0.5 mM in ethanol, 10 μ L) is deposited as a droplet released by a micropipette. The samples are dried for 2 h at room temperature under vacuum of 50 mbar before irradiation. Irradiation is performed at 350 nm, keeping a constant irradiance of 3.8 mW/cm² during 5 min. The samples are then washed according to the following process: 2×5 min ethanol Uvasol, 2×5 min hexane Uvasol. Samples are characterized and analyzed with time-of-flight secondary ion mass spectroscopy (ToF-SIMS). The ToF-SIMS system used is a mass spectrometer from PHI-EVANS & Associates (described in detail in (11)) equipped with a pulsed FEI Ga⁺ ion gun operated at 25 kV for high lateral resolution (unbunched beam). Sample surface is biased ± 3 kV with respect to the grounded extraction electrode for positive and negative mode SIMS, respectively. The 400 pA DC ion beam current is pulsed at 5 kHz repetition rate (8 ns pulse width, measured with the unbunched beam). The analyzed area is estimated to 126×126 μ m². The analysis is performed under so-called "static" conditions with an ion dose in the order of 9×10^{11} ions/cm². F[−] is the most characteristic ion of the MAD molecule (12), and images of this ion are displayed to demonstrate the topical addressability with ToF-SIMS imaging.

Bovine serum albumin (BSA) is functionalized with 3-(trifluoromethyl)-3-(*m*-isocyanophenyl)diazirine (TRIMID), a light activable agent that absorbs UV light at 350 nm to obtain T-BSA. The detailed protocol for the preparation of T-BSA is described in (13). After modification, T-BSA carries 7 to 9 mol aryldiazirines per mole of BSA. For subsequent fluorescence detection, BSA labeled with fluorescein (F-BSA) is mixed with T-BSA. Glass substrates are prepared according to the following protocol: overnight treatment in HCl 37%, 3×5 min sonification in Millipore quality water, and drying 2 h under vacuum/3 mbar at ambient temperature. A solution of proteins (4 μ g of F-BSA, 4 μ g of T-BSA, 10 μ L of PBS 1:100 = 0.5 mM sodium phosphate buffer pH 7.4 containing 1.5 mM NaCl) is deposited on the glass substrate as a droplet released from a micropipette and then dried under vacuum at 30 to 40 mbar. After photoimmobilization by UV argon laser light, the sample is washed to eliminate the physically adsorbed molecules according to the following process: 3×10 min in 3 M KSCN, overnight in PBS/0.05% Tween solution, 3×10 min in PBS/0.02% Tween, 2×5 min in PBS 1:100, 10 min in 10 mM HCl, 3×5 min in Millipore quality water. The obtained patterns are observed and characterized using near-field fluorescence optical microscopy and atomic force microscopy in contact and noncontact mode (Autoprobe CP, Thermomicroscopes).

RESULTS

Cruciform patterns have been polymerized into photoresist, scanning the near-field probe above the deposited resist in two successive perpendicular lines. Figure 1 shows an AFM observation (contact mode) of the resulting pattern obtained after resist development. The

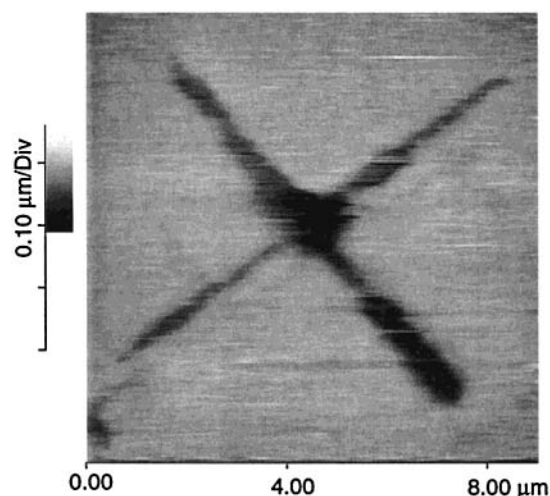


Figure 1. AFM recording of a near-field-induced cruciform pattern in S1813 positive photoresist (AFM image by E. Dupas, IGA-EPFL).

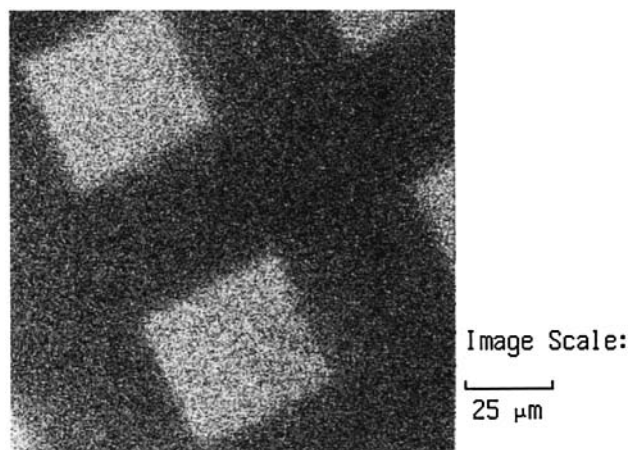


Figure 2. Imaging of ToF-SIMS (registration of F^-) of a MAD-coated surface after mask-assisted photopatterning.

estimated size of the near-field probe used in this experiment is 120 nm (optical aperture), for a transmission of 9.2×10^{-4} . This leads to an estimated illumination dose of 29.8 mJ/cm^2 . The line width measured at a typical line section is 223 nm FWHM, with a depth of 43 nm. This shows that sub-wavelength patterning is possible with the SNOM technique, even with a standard photoresist deposited as a 1.5 μm thick layer.

The same technique applied to polymerize the photoresist is used to photobond a cruciform pattern of biomolecules. Two approaches are considered: In the first one, demonstration of near-field photoimmobilization is performed with a low molecular weight diazirine-based molecule (MAD), that can be used as a “building block” in the functionalization of surfaces. In the second approach, a biologically relevant molecule is directly photoimmobilized onto the surface. This is demonstrated using BSA functionalized with the photoactive diazirine and illuminated in the near-field of the probe.

Figure 2 is a ToF-SIMS image of MAD photobonded onto a Si surface. The photobonding is obtained by illuminating a monolayer of MAD deposited onto a Si wafer, using a mask patterning technique with parallel beam far field illumination with a light irradiance of 8.5 mW/cm^2 . The light dose for optimal photobonding of MAD is measured in the range between 1.1 J/cm^2 to 1.6 J/cm^2 . At lower doses, no immobilization occurs. The square

patterns of $40 \times 40 \text{ μm}^2$ show a transition width of 2 μm from immobilized to nonimmobilized zones. Using near-field immobilization with a comparable light dose, it is possible to immobilize MAD with a finer spatial resolution. However, we have not succeeded in clearly demonstrating sub-wavelength patterns of photoimmobilized MAD. This is related to the difficulty to observe a deposited monolayer with submicrometer lateral feature size. Several analysis techniques have been applied to observe the near-field immobilized MAD pattern, such as ToF-SIMS and scanning microscopies (AFM, SEM, fluorescence SNOM). ToF-SIMS submicrometer imaging is in principle possible. However, it requires a small probed area that implies the use of an increased ion dose. This leads, in the case discussed here, to surface damage, which prohibits the acquisition of images on ultrathin submicrometer MAD structures. Further imaging techniques such as AFM, or SEM of OsO_4 -treated surfaces (OsO_4 reacts with the maleimido group, thus enhancing contrast), do not lead to a sufficient contrast to allow quantitative characterization of the patterns. Even near-field fluorescence microscopy with fluorescence-labeled MAD leads to inconclusive results when performed on immobilized monolayer. This is attributed to quenching of the fluorescence when the fluorescent marker is directly bound to the surface.

In a second approach, biomolecules have been photoimmobilized using the same technique. This is performed onto a glass substrate using BSA, previously functionalized with the photoactivable diazirine and labeled with fluorescein isothiocyanate for subsequent detection. Immobilization in the near-field is obtained with a light dose of 2.1 J/cm^2 . The threshold for immobilization is sharp at the lower end. When the dose is increased up to 5 times above that threshold, no significant difference in the attachment is observed. The thickness of the adsorbed proteins is measured by AFM before immobilization, the average result being $165 \text{ nm} (\pm 30 \text{ nm})$. Far-field fluorescence microscopy is used to localize where immobilization has taken place and to optimize the illumination dose. Working with small structures and low concentration requires an optimal washing process. This process, described in the previous paragraph, is 2.5 times more efficient to eliminate the adsorbed proteins compared to standard procedures without chaotropic agents.

Figure 3 shows the topography (a) and the near-field fluorescence image (b) of the immobilized BSA pattern. Observation and immobilization are performed with the same tip. The cruciform pattern is clearly visible, both in topography and as near-field fluorescence contrast. Figure 4 shows the topographical and light profile of the smallest structure obtained. The width (FWHM) of the topographical profile is 470 nm. The immobilized proteins thickness is $40 \text{ nm} \pm 15 \text{ nm}$ as confirmed by independent AFM observations.

For comparison, the best resolution obtained on BSA by mask-assisted photoimmobilization is 3.8 μm applying mask-contact modes and 1.9 μm with the mask-projection procedure.

DISCUSSION

The potentiality of SNOM for nanostructuring is clearly demonstrated using standard photoresist chemistry. Sub-wavelength feature size has been obtained with standard coating and development processes. Improvement in feature size can be obtained by using an ultrathin photoresist layer ($<100 \text{ nm}$), as recently demonstrated by Alkaisi et al. (14) with evanescent near-field optical lithography.

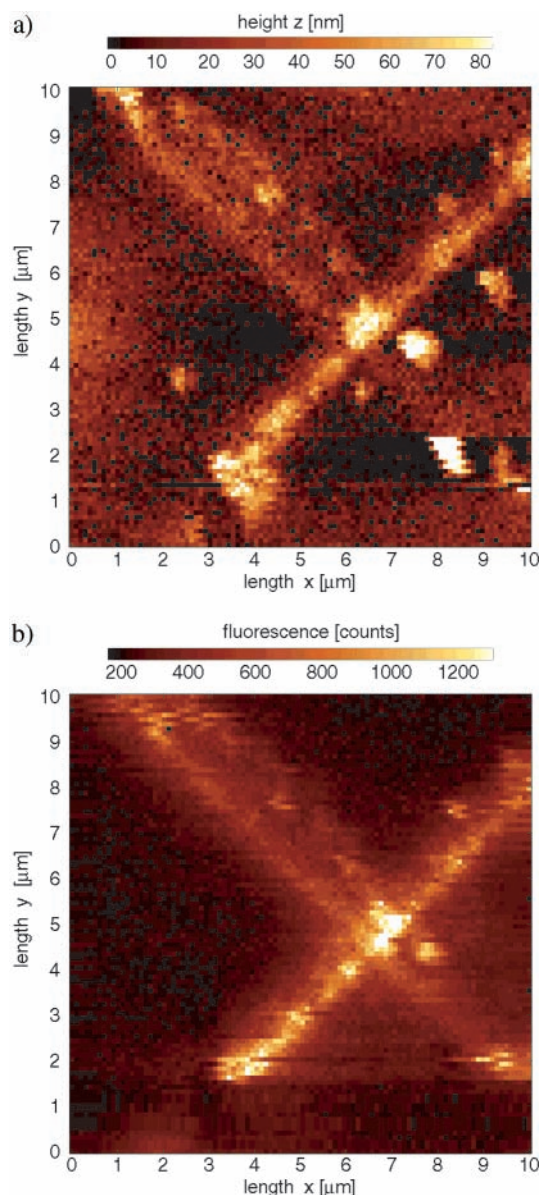


Figure 3. SNOM observation of photobonded BSA. The cruxiform structure is induced by near-field patterning. (a) Topographical image. (b) Fluorescence near-field image.

The aryldiazirine chemistry is appropriate for surface engineering, as shown using mask-assisted illumination. The size of the molecules and their capacity to form ultrathin films is advantageous for near-field structuration at submicrometer size, due to the strong exponential decay of the near-field intensity with the sample to surface distance. Thus, immobilization of the photocrosslinker MAD should be considered as a first step in building more complex structures (15). However, the contrast between derivatized and nonderivatized zones is difficult to monitor except as a chemical contrast (ToF-SIMS), which is limited in lateral resolution in this case due to surface damaging. Topographical and optical contrasts, including derivatization of MAD with a fluorophore (thiolated FITC), did not reveal the surface patterns. Attaching molecules offering functionality to the MAD is, however, a promising route to develop submicrometer scale patterns.

The photoimmobilization of T-BSA shows that near-field photochemistry of more complex structures such as proteins is also achievable using SNOM. The feature sizes obtained in near field are four times smaller than the

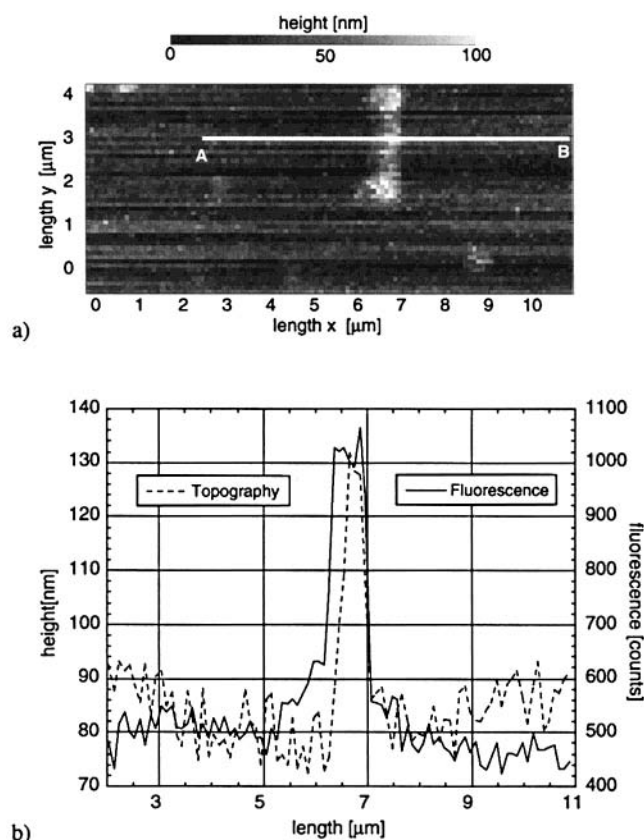


Figure 4. SNOM topographical image of photoimmobilized BSA (a). The curves display the topographical and optical profiles across the BSA structure, along the AB line (b).

structures obtained with mask techniques, showing the advantage of a near-field approach. The present study shows, however, that on the processing side, the key factors for submicrometer scale immobilization are the thickness of the protein layer before photoimmobilization and the efficiency of the washing process. The first factor is important to ensure that photoactivation is performed in the near field, thus exploiting the high spatial localization of the light. The second factor is necessary to maintain a sufficient contrast between photobonded and adsorbed molecules when working at low molecular density. The adsorbed proteins that are not washed away from the surface are of critical importance when the thickness of the proteins layer is reduced. To take full advantage of the near field, it is mandatory to use thin layers, optimally monomolecular layers. For BSA, the decrease of the layer thickness reduces the signal ratio between the immobilized zones and the nonimmobilized zones, as the residual nonspecific adsorption (after washing) does not depend on the thickness of the initially deposited molecule layer.

On the instrumental aspect, the critical point in the achievement of fine immobilized patterns is the quality of the optical probes. This criterion is even more important when SNOM is used as structuring tool, as it is when used as an observation tool. Indeed, tips giving similar results in the test process on calibrated objects can lead to very different pattern size in the immobilization process. The only way so far to determine if a tip is suitable for near-field immobilization is by experimental surface structuration. Reproducibility of the pattern size depends highly on the tips characteristics, and future improvements in tip fabrication will contribute to the development of reliable near-field photochemistry.

CONCLUSION

Investigations presented in this work show that near-field photoimmobilization using the SNOM technique is a yet unexploited way to induce submicrometer biopatterning of surfaces. The potential of using the SNOM as a structuring tool is demonstrated with standard photoresist, showing structures down to 223 nm. This method is also suitable to achieve immobilization of diazirine-based compounds, as it is shown with feature sizes as small as 470 nm.

The two key factors of near-field photoimmobilization are the chemistry process and the optical probes. On one hand, further development and fine-tuning of chemistry aspects are needed to fully exploit the potential of near-field photoimmobilization and reach even smaller structure size. On the other hand, the reproducibility of the method is highly dependent on the quality of the optical probe, and special attention should be paid to the fabrication or the choice of the optical probe. Near field photoimmobilization may thus become a very promising approach for the immobilization of nanometer size domains in future generations of biosensors.

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Water-Soluble Lipopolymer for Gene Delivery

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The use of biocompatible polymeric gene carriers may overcome the current problems associated with viral vectors in safety, immunogenicity, and mutagenesis. Nontoxic water-soluble lipopolymer (WSLP), poly{(ethylenimine)-co-[N-(2-aminoethyl) ethyleneimin]-co-N-(N-cholesteryl oxycarbonyl-(2-aminoethyl)-ethylenimine)} was synthesized using branched poly(ethylenimine) (PEI, mw 1800) and cholesteryl chloroformate. Following synthesis and purification, the structure and molecular weight of WSLP were confirmed by ^1H NMR and MADI-TOF mass spectrometry, respectively. The percentage of cholesterol conjugated to PEI was about 47%, and the average molecular weight of WSLP was approximately 2000 Da. WSLP/pDNA complexes were prepared at different N/P (nitrogen atoms of WSLP/phosphate of plasmid DNA) ratios and characterized in terms of particle size, ζ potential, osmolarity, surface morphology, and cytotoxicity. WSLP condensed plasmid DNA when N/P ratio reached 2.5/1 and no free DNA was detected at N/P ratio of 5/1 and above, as determined by agarose gel electrophoresis. The mean particle size was in the range of 25.9 to 148.5 nm and was dependent on N/P ratios. Atomic force microscopy (AFM) showed complete condensation of plasmid DNA with spherical particles of ~ 50 nm in diameter. WSLP/pDNA complexes or WSLP itself were nontoxic to CT-26 colon adenocarcinoma and 293 T human embryonic kidney transformed cells when formulated at the N/P ratio of 10/1 and below as determined by MTT assay. In contrast, PEI25000/pDNA complexes were toxic to these cells. Erythrocytes aggregated when incubated with PEI25000/pCMV–Luc complexes at high DNA concentrations, but there was little aggregation with WSLP/pCMV–Luc complexes. WSLP/pCMV–Luc complexes demonstrated higher transfection efficiency in both CT-26 and 293 T cells compared to PEI25000- or PEI1800-based formulations. WSLP/pCMV–Luc complexes are nontoxic and showed enhanced in vitro transfection. Thus, WSLP will be a suitable carrier for in vivo gene delivery.

INTRODUCTION

Development of improved gene transfer methods is prerequisite for gene therapy to realize its clinical potential. Although currently less effective than viral vectors, cationic lipids and polymers are increasingly being considered for in vivo gene delivery into the animal models and patients due to their relative stability, greater carrier capacity, and ease of large scale production (1). Current cationic liposomes and polymers from complexes with plasmid DNA via electrostatic interaction and tend to form large aggregates on storage. These complexes are efficient at transfecting cells in culture, yet are poor at diffusing within a tissue or escaping from blood vessels (2–5). Polyethylenimine (PEI) has been reported to be effective for gene delivery, because plasmid DNA can be delivered to the cytoplasm via endosomes due to the proton-sponge effect of PEI (6, 7). However, high molecular weight PEI is toxic to the cells, and PEI/DNA complexes are prone to aggregation (8).

The general structure of a cationic lipid has three parts: (i) a hydrophobic lipid anchor group, which helps in forming micellar structure and can interact with cell membranes; (ii) linker group, such as an ester, amide or

carbamate; and (iii) a positively charged headgroup, which interacts with plasmid DNA, leading to its condensation. The hydrophobic group lipid anchors can be either a cholesterol group or fatty acid chains (9). The linker group is an important component and determines the chemical stability and biodegradability of cationic lipids. These linker groups should be biodegradable yet strong enough to survive in a biological environment. Ester linkage between hydrophobic lipid anchors and cationic headgroups are likely to provide biodegradability to cationic lipids. Cationic lipids with T-shaped headgroups tend to be more effective than linear counterparts (10). However, current cationic lipids are water insoluble, and most of them require the formation of liposomes. Complex formation between plasmid DNA and cationic liposomes usually produces large size complexes, which are poorly extravasated through the capillary endothelia.

In this study, we designed an effective water-soluble lipopolymer (WSLP) by combining the advantages both of cationic liposomes and PEI, which condenses DNA and enhances endosomal release due to its tertiary amines, while the lipid coating on the DNA increases its permeability through cell membranes (11). Monomolecular pDNA collapse has been suggested to occur only for cationic lipid or lipopolymer concentrations below the critical micellar concentrations (CMC) and are used for complex formation with DNA (12). We synthesized WSLP using PEI and cholesteryl chloroformate, and characterized it for its physicochemical properties, cytotoxicity, and in vitro transfection efficiency.

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MATERIALS AND METHODS

Materials. Poly(ethylenimine) (PEI, mw 1800) was purchased from Polysciences, Inc. (Warrington, PA). Cholesteryl chloroformate, PEI (mw 25000), triethylamine, glucose, deuterium oxide (D_2O), and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Aldrich Chemical Co. (Milwaukee, WI). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), terrific broth, ampicillin, Rosewell Park Memorial Institute (RPMI-1640) medium, Dulbecco's modified essential medium (DMEM), and 1,4-dioxane- d_8 were purchased from Sigma Chemical Co. (St. Louis, MO). LipofectAMINE, phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and proteinase K were purchased from Gibco-BRL (Gaithersburg, MD). QIAGEN maxi plasmid purification kit were purchased from QIAGEN (Valencia, CA). Methylene chloride and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Fair Lawn, NJ). RQ1 RNase-free DNase, λ HindIII, ScaI, buffer B, 100bp DNA stepladder, 1kb DNA stepladder, reporter lysis buffer, and bacterial strain DH5 α were purchased from Promega (Madison, WI). Albumin standard and bicinchoninic acid (BCA) protein assay kit were purchased from Pierce Chemical Co. (Rocford, IL). PCMV-Luc was obtained as a kind gift from Dr. Mitsuru Hashida of Kyoto University, Japan.

Synthesis of Water Soluble Lipopolymer. Three grams of PEI (mw 1800) was stirred on ice in a mixture of 10 mL of anhydrous methylene chloride and 100 μ L of triethylamine for 30 min. One gram of cholesteryl chloroformate was dissolved in 5 mL of anhydrous ice-cold methylene chloride and then slowly added to the PEI solution for 30 min. The mixture was stirred for 12 h on ice, and the resulting product was dried on a rotary evaporator. The powder was dissolved in 50 mL of 0.1 N HCl and then filtered through the glass microfiber filter. Cholesteryl chloroformate is well soluble in methylene chloride, diethyl ether, and acetone. Therefore, nonreacted cholesteryl chloroformate was removed from the product by washing three times in these different organic solvents as follows: the aqueous solution was extracted three times with 100 mL of methylene chloride to remove nonreacted cholesterol chloroformate, and then extracted aqueous solution was filtered through a glass microfiber filter. The product was concentrated by solvent evaporation, precipitated with large excess acetone, and dried under vacuum. The powder was dissolved in water and precipitated three times with acetone. After washing with methanol, the product was washed three times with diethyl ether. The white powder product was analyzed using matrix-assisted laser desorption-time-of-flight (MALDI-TOF) mass spectrophotometry (Perspective Voyager-DE STR, PE Applied Biosystem Co., Framingham, MA) with *trans*-4-hydroxy-3-methoxycinnamic acid as matrix and 400-MHz ^1H nuclear magnetic resonance (^1H NMR, Varian, Inc., Palo Alto, CA) and then stored at $-20^\circ C$ until used.

Critical Micellar Concentration. The critical micellar concentration (CMC) of WSLP was determined using the dye solubilization methods as described previously by Jeong et al. (13). Various amounts of WSLP was dissolved in 1 mL of deionized water to adjust the concentrations of WSLP ranging from 9.28 to 76021.76 μ g/mL. To prepare 0.4 mM stock solution of 1,6-diphenyl-1,3,5-hexatriene (DPH), 2.2 mg of DPH was dissolved in 23.54 mL of methanol followed by sonication for 15 min. Ten microliters of 0.4 mM DPH was added to 1 mL of WSLP and stored in dark for 6 h. The reference solution was also prepared by adding 10 μ L of 0.4 mM DPH to 1

mL of water. The absorption spectra of these samples were measured at 200–600 nm using a UV/vis/NIR spectrometer (Perkin-Elmer Lambda 19). The absorbance at 356 nm was plotted against the concentration of WSLP, and the crossing point of the extrapolated two straight lines was defined as the CMC of WSLP.

Amplification and Purification of Plasmid. Plasmid DNA encoding Luciferase driven by CMV promoter (pCMV-Luc) was constructed by inserting the HindIII/XbaI firefly Luciferase cDNA fragment from pGL3-control vector into the HindIII/XbaI site of pcDNA3 (14). This plasmid DNA was transformed in *Escherichia coli* DH5 α and amplified in terrific broth media at $37^\circ C$ overnight with 225 rpm. The amplified plasmid DNA was purified by QIAGEN Maxi plasmid purification kit. Purified plasmid DNA was dissolved in Tris-EDTA (TE) buffer, and its purity and concentration were determined by ultraviolet (UV) absorbance at 260 nm. The optical density ratios at 260–280 nm of these plasmid preparations were in the range of 1.7–1.8. To confirm that there is no rearrangement of the gene during cloning and propagation of the plasmid DNA, restriction enzyme assay was done using ScaI and HindIII followed by 1% agarose gel electrophoresis.

Gel Retardation and DNase Protection Assays. WSLP/pDNA complexes were prepared at various N/P (nitrogen atoms of WSLP/phosphate of plasmid DNA) ratios ranging from 1.25 to 20.0. In phosphate buffered saline, the positively charged polymer/plasmid complexes tend to aggregate on storage. Therefore, WSLP/pLuc complexes were prepared in the presence of 5% glucose to adjust the osmolality at 290–310 mOsm and incubated for 15–20 min at room temperature to allow complex formation between lipopolymer and pDNA. The samples were electrophoresed on agarose gel in $1\times$ Tris-boric acid-EDTA (TBE) buffer at 80 V until the $1\times$ orange blue loading dye ran through 80% of the gel. A 1kb marker was used as a DNA size marker. The gel was stained with 0.5 μ g/mL ethidium bromide for 45 min and analyzed on UV illuminator to show the location of DNA.

For DNase protection assay, WSLP/pDNA (5/1, N/P) complexes were incubated at $37^\circ C$ in the presence of DNase I (45 μ L). At 0, 5, 10, 15, 30, 60, and 120 min postincubation, 50 μ L of the samples was taken into Eppendorf tubes and was mixed with 100 μ L of stop solution (400 mM NaCl, 100 mM EDTA) under mild vortexing. The samples were then mixed with 12 μ L of 10% sodium dodecyl sulfate (SDS) and incubated at $65^\circ C$ overnight. The DNA was then extracted with the mixture of Tris-EDTA saturated phenol: chloroform: isoamyl alcohol (25:24:1, v/v). The extracted DNA was precipitated with 700 μ L of absolute ethanol at 12000 rpm for 30 min and washed with 70% ethanol. The DNA precipitate was dried by air followed by dissolving in 10 μ L Tris-EDTA buffer, and the plasmid DNA integrity was assessed using 1% agarose gel electrophoresis.

Atomic Force Microscopy. Morphology of WSLP/pDNA complexes was analyzed using atomic force microscopy (AFM) as described by Maheshwari et al. (15). Briefly, red mica was freshly cleaved as a thin wafer and then soaked in 33 mM MgAc $_2$ overnight to favor the replacement of potassium ions by divalent magnesium ions for stronger DNA binding. Mica was then sonicated for 30 min in distilled water, and its surface was subjected to glow discharge for 15 s in a vacuum between 100 and 200 mTorr. As soon as the mica surface was exposed to air, 20 μ L of 0.1 mg/mL of WSLP/pDNA complexes was placed on mica surface and allowed to

stick for 2 min. The mica surface was then rinsed gently with deionized water and blow-dried with nitrogen gas. Nanoscope II SFM (Digital Instruments, Santa Barbara, CA) was used for imaging at room temperature in the attractive force regime and under 30–60% relative humidity. The microscope was operated using cantilever oscillation frequencies between 12 and 24 kHz. Force minimization was maintained by reducing the set point voltage to minimize sample damage. To remove the high frequency noise in the slow scan direction, only minimal filtering was applied to the image.

Particle Size and ζ Potential Measurement. ζ potential and particle size of WSLP/pDNA complexes were measured as described by Mahato et al. (16). Briefly, polymer/pDNA complexes were prepared as discussed above and were diluted four times in the cuvette, and the electrophoretic mobility was determined with Zeta-PALS (Brookhaven Instruments Corp, Holtsville, NY). All experiments were performed at 25 °C, pH 7.0 and 677 nm wavelength at a constant angle of 15°. The ζ potential was automatically calculated from the electrophoretic mobility based on Smoluchowski's formula. Following the determination of electrophoretic mobility, the samples were subjected to mean particle size measurement by the same equipment using the same light source and wavelength. The particle size was reported as effective mean diameter.

Cell Lines. 293T human embryonic kidney transformed cells were obtained from ATCC (Rockville, MD) and were grown and maintained in DMEM medium containing 10% FBS, 100 units/mL penicillin and 2.2 mg/mL sodium bicarbonate at 37 °C humidified 5% CO₂.

CT-26 colon adenocarcinoma cell lines was a kind gift from Dr. Charles Tannenbaum of Cleveland Clinic Foundation, Cleveland, OH (17). Tumor cells were grown and maintained in RPMI 1640 medium which was supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 2.2 mg/mL sodium bicarbonate at 37 °C and humidified 5% CO₂.

Cytotoxicity Assay. Cytotoxicity of WSLP/pDNA complexes prepared at various charge ratios were assessed as described by Fisher et al. (18). The fraction of nitrogen atoms that are protonated at neutral pH is unknown. Therefore, the PEI/DNA ratios are usually calculated on the basis of PEI nitrogen per DNA phosphate (i.e., N/P ratio) (19). The optimal N/P ratio of PEI/pLuc complexes was determined, according to several publications, to be around 5/1 (6, 20–23). However, Boussif et al. mentioned that the transfection efficiency of PEI (800 and 50 kDa) was highest at 13/1 N/P ratio (19). In their studies, the transfection efficiency was reported in terms of relative light units (RLU). Transfection efficiency/cytotoxicity balance is known to increase with increase in the polymer molecular weight and charge ($\pm$) or N/P ratios. Although PEI (mw 800 or 50 kDa)/pDNA complexes showed high transfection at 13/1 N/P ratio, many cells might have died due to high toxicity. To normalize the cytotoxic effect of the polymer, we reported our transfection results in terms of RLU/mg of total proteins. CT-26 cells and 293T cells were seeded in 96 well plates at 4000 cells/well and incubated at 37 °C for 24 h. After checking the cell confluency, which was over 70%, gene carriers were added to the cells. Following 48 h of incubation at 37 °C, 25 μ L of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) stock solution in phosphate-buffered saline (5 mg/mL) was poured into each well until it reached a final concentration of 0.5 mg/mL MTT. The plate was then incubated at 37 °C in 5% CO₂ for 5 h. The medium

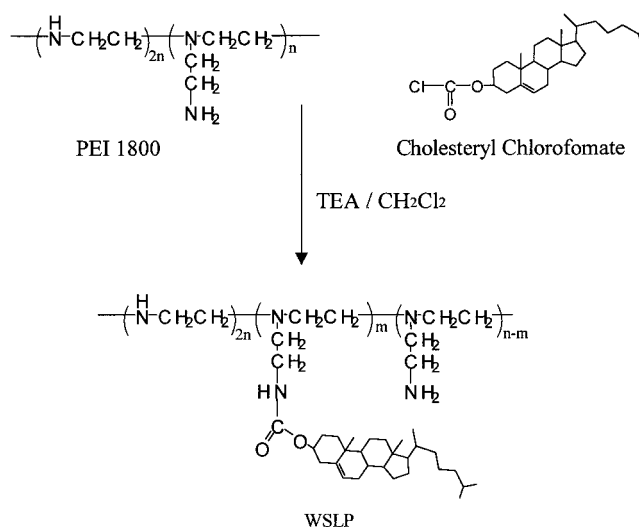


Figure 1. Synthesis scheme of poly{(ethylenimine)-co-[N-2-(aminoethyl)ethylenimine]-co-[N-(N-cholesteryloxycarbonyl-(2-aminoethyl))ethylenimine]}.

was removed, and 150 μ L of DMSO was added to dissolve the formazan crystals. The plate was read spectrophotometrically at 570 nm using an ELISA plate reader. The relative cell viability was calculated as $[\text{Abs}]_{\text{sample}}/[\text{Abs}]_{\text{control}} \times 100$.

Erythrocyte Aggregation Assay. Erythrocyte aggregation assay was done as described by Ogris et al. (24). Briefly, fresh blood was collected from a BALB/c female mouse and immediately mixed with sodium citrate to a final concentration of 25 mM. Erythrocytes were washed on ice three times in Ringer's solution. Five and ten microliters of erythrocytes/well of six well plate were mixed with WSLP/pCMV–Luc (10/1, N/P) complexes at a dose of 20 μ g DNA/well. PEI 25000/pCMV–Luc (5/1, N/P) complexes were used as a control. The cells containing erythrocytes were incubated for 1 h at 37 °C.

In Vitro Transfection. CT-26 cells and 293 T cells were seeded separately in six well tissue culture plates at 3×10^5 cells per well in 10% FBS containing RPMI 1640 or DMEM media. Cells achieved 70% confluency within 24 h after which they were transfected with WSLP/pDNA complexes prepared at different charge (N/P) ratios ranging from 5/1 to 20/1. The total amount of plasmid DNA loaded was maintained constant at 2.5 μ g/well and transfection was carried out in absence of serum. The cells were allowed to incubate at 37 °C in the presence of complexes for 6 h in CO₂ incubator followed by replacement of 2 mL of RPMI 1640 or DMEM containing 10% FBS. Thereafter the cells were incubated 37 °C for additional 36 h. Cells were lysed using 1X lysis buffer after washing with cold PBS. Total protein assays were carried out using BCA protein assay kit. Luciferase activity was measured in terms of relative light units (RLU) using 96 well plate Luminometer (Dynex Technologies Inc, Chantilly, VA). The Luciferase activity was monitored and integrated over a period of 30 s. The final values of Luciferase were reported in terms of RLU/mg total protein. In all the above experiments, both naked DNA as well as untreated cultures were used as positive and negative controls, respectively.

RESULTS

Synthesis of Water-Soluble Lipopolymer. Water-soluble lipopolymer (WSLP) was synthesized using branched PEI of 1800 Da and cholesteryl chloroformate

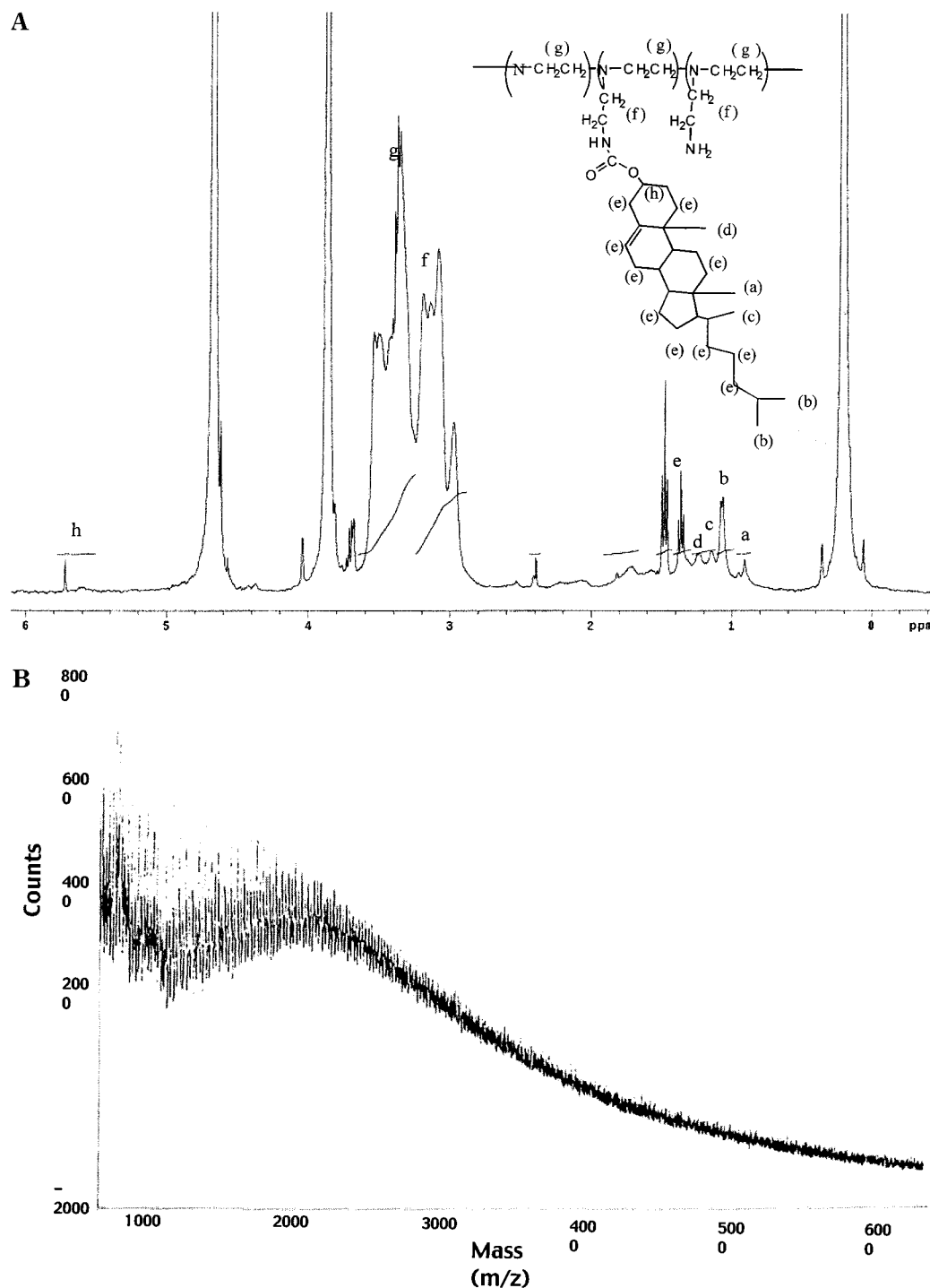


Figure 2. Determination of chemical structure and molecular weight of water-soluble lipopolymer (WSLP) by ¹H NMR spectra (A) and MALDI-TOF mass spectra (B).

(Figure 1). Following synthesis and purification, the structure and molecular weight of WSLP were determined using 400-MHz ¹H NMR and MALDI-TOF mass spectrometry. The NMR results are as follows (Figure 2 A): ¹H NMR (400 MHz, D₂O + 1,4-dioxane-*d*₆) δ 0.93 (s, 4.28 H of CH₃ from cholesterol (a)); δ 1.08 (d, 17.89 H of (CH₃)₂ from cholesterol (b)); δ 1.14 (d, 8.9 H of CH₃ from cholesterol (c)); δ 1.24 (d, 9.0 H of CH₃ from cholesterol (d)); δ 1.35 and δ 1.48 (t, 16.68 and 17.53 H from CH₂-CH₂ and CHCH₂ from cholesterol (e)); δ 3.1 (59.6 H of NHCH₂CH₂ from the side chain of PEI (f)); δ 3.4 (80.8 H of =NCH₂CH₂NH₂ from backbone of PEI (g)); δ 5.65 (1 H of =C=CHC from cholesterol (h)). The peak appearing at δ 0.8–1.9 was from cholesterol. The amount of

cholesterol conjugated to PEI was determined to be about 47%. MALDI-TOF mass spectrophotometric analysis of WSLP showed its molecular weight to be approximately 2000 (Figure 2B). Many intensity peaks appeared from 1000 to 3500 (*m/z*); however, the majority peaks were at around 2000 (*m/z*). Expected position is 2414: 1800 (mw of PEI) + 449–35 (mw of cholesteryl chloroformate after removal of one chloride). The data suggest that the majority of WSLP synthesized were of 1/1 molar ratio of cholesterol and PEI of 1800 Da, although some were either not conjugated or conjugated at the molar ratio of 2/1 (cholesterol/PEI). The molecular weight of WSLP could not be measured using MALDI-TOF mass spectrophotometry at various concentrations and matrices.

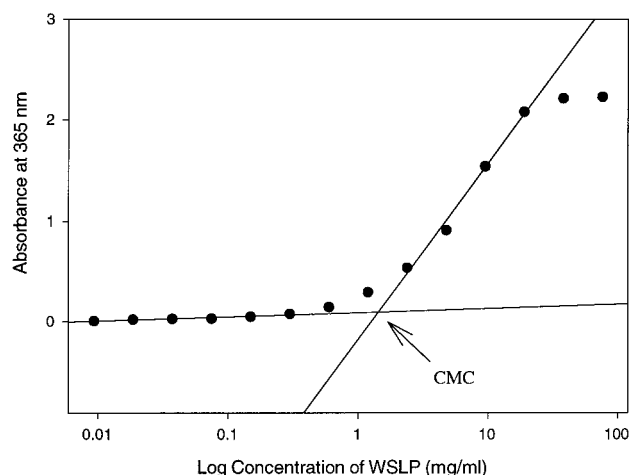


Figure 3. Determination of critical micellar concentration of water-soluble lipopolymer (WSLP) by dye solubilization methods.

Critical Micellar Concentration. WSLP is amphiphilic in nature because water is a poor solvent for hydrophobic cholesterol while it is a good solvent for hydrophilic PEI. With increasing its concentration, WSLP may form multimolecular micelles or micellar aggregates. The solubility of WSLP in water greatly depends on the molar ratio of PEI and cholesterol, and thus it is important to maintain the hydrophobic–hydrophilic balance. DPH is a hydrophobic dye, which has a significantly lower absorptivity at 356 nm in an aqueous environment compared with that in the hydrophobic environment. With the formation of micelles, DPH is preferentially partitioned into the hydrophobic core of micelles, resulting in the increase in the absorbance of DPH (13). The abrupt increase in absorbance reflects the micelle formation. As shown in Figure 3, CMC of WSLP was determined to be 1.43 mg/mL which was the cross-point of extrapolating the absorbance at low and high concentration regions.

Band Retardation and DNase Protection Assays. DNA condensing ability of WSLP was determined by gel retardation assay. The positively charged headgroup of WSLP makes strong complexes with the negatively charged phosphate ions on the base backbone on plasmid DNA. When the value of N/P reached 5/1, free DNA could not be detected on agarose gel electrophoresis (data not shown). WSLP could protect pDNA from digestion by DNase at least for 2 h at 37 °C, whereas naked DNA was completely digested by DNase within 5–10 min of incubation at 37 °C (data not shown).

Surface Morphology. DNA condensation, its reduction in overall size and the resulting surface morphology were captured by AFM (Figure 4). Plasmid DNA when complexed with WSLP at the N/P ratio of 10 was completely condensed and formed spherical particles of ~50 nm. The AFM image revealed that the naked pDNA was condensed from its natural extended state in water to about one-tenth of its size in the complex. The small-sized complexes might be of special significance, as it could facilitate enhanced mobility of the complex through the plasma membrane as well as trafficking across the cytoplasm.

Particle Size and ζ Potential. The compaction of plasmids into particulates of defined colloidal and surface

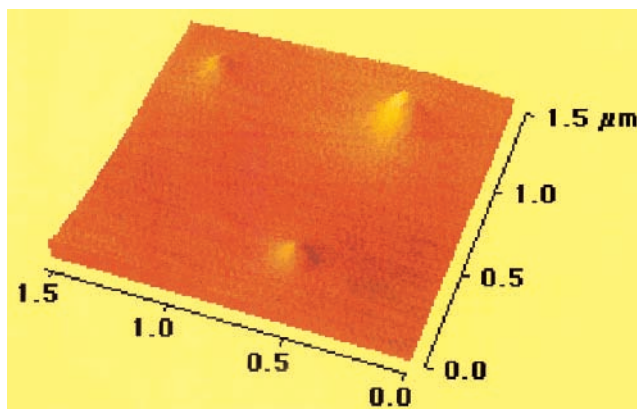


Figure 4. Atomic force microscopy (AFM) image of WSLP/pCMV–Luc complexes (10/1, N/P). Condensed plasmid DNA forms an ellipsoidal structure as seen in z-direction.

properties plays a major role in controlling stability, biodistribution, and intracellular fate of formulated plasmids. Interaction with the tissue components and transport in the interstitial space and uptake characteristics by the cells. Low aggregate formulation and a small-sized complex would enhance the diffusivity and cellular uptake of plasmid DNA in the tissues. The particle size distribution of WSLP/pDNA complexes was determined by dynamic light scattering. In contrast to the large size of naked DNA (25), complex formation between WSLP and plasmid DNA produced small size particles. The mean particle size of the complexes was in the range of 25.9 to 148.5 nm and was dependent on N/P ratios (Table 1). At the N/P ratio of 1.25, plasmid DNA was not completely neutralized, resulting in the formation of heterogeneous particles with the mean diameter of 148.5 nm. In contrast, the mean particle size of WSLP/pDNA complexes 40 nm or below, when the complexes were formed at the N/P ratios of 2.5 and 10. However, at 20/1 (N/P) ratio these complexes had narrow particle size distribution with a mean diameter of 61.8 nm.

ζ potential of WSLP/pDNA complexes prepared at different charge ratios was greatly dependent on the charge ratio and linearly increased with the increase in the charge ratios. At the N/P ratio of 2.5/1, it was -41.35 mV, but increased to 8.23 mV when formulated at N/P ratio of 5/1. The gel retardation data confirm the above finding, as there was no DNA movement seen at the N/P ratio of 2.5/1 or above.

Cytotoxicity Assay. The cytotoxicity of WSLP and WSLP/pCMV–Luc complexes after 24 h of incubation at 37 °C was determined using MTT assay on CT-26 colon adenocarcinoma (Figure 5 A) and 293 T human embryonic kidney transformed cells (Figure 5 B). The cytotoxicity of WSLP and WSLP/pCMV–Luc complexes was compared with commercially available cationic liposomes (LipofectAMINE/pCMV–Luc, 5/1 w/w) and branched PEI of 25000 Da (PEI25000/pCMV–Luc, 5/1 N/P) complexes. LipofectAMINE reagent is 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleoyloxy-*N*-[2(sperminecarboxyamido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA, mw 867) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE, mw 744) in membrane-filtered water. Based on its chemical structure, LipofectAMINE has two primary amines, two

Table 1. Mean Particle Size and ζ Potential of WSLP/pDNA Complexes

N/P ratios of WSLP/pDNA	1.25/1	2.5/1	5.0/1	10.0/1	20.0/1
mean particle size (nm)	148.5 $\pm$ 0.18	25.9 $\pm$ 0.02	41.6 $\pm$ 0.05	42.1 $\pm$ 0.53	61.8 $\pm$ 0.07
mean ζ potential (mV)	-59.12 ± 2.79	-41.35 ± 2.18	8.23 $\pm$ 0.25	37.40 $\pm$ 0.46	61.67 $\pm$ 0.06

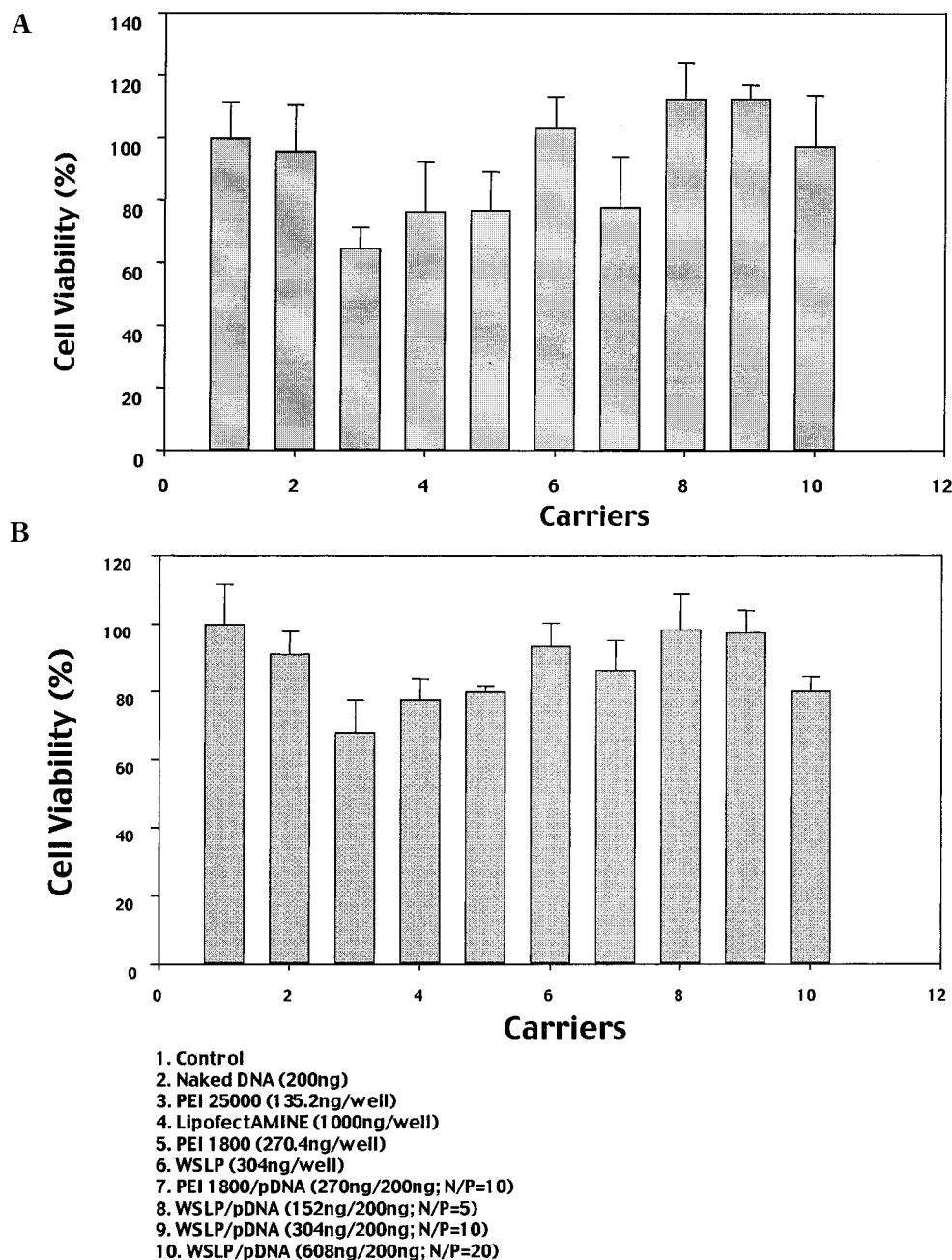


Figure 5. Cell viability assay of CT-26 colon adenocarcinoma (A) and 293 T human embryonic kidney transformed (B) cell lines after transfection with WSLP/pCMV-Luc complexes prepared at different N/P ratios in 5% (w/v) glucose. Naked pCMV-Luc, PEI 25000 (25 Kda) and LipofectAMINE were used for comparison. Relative cell viability was at least 85% for all WSLP/pCMV-Luc complexes. In contrast, PEI 25000/pCMV-Luc (5/1, N/P) complexe-based transfection resulted in less than 60% cell viability.

secondary amines, and one quaternary amine, total five positive charges per molecule (26). Subsequent calculations show that 5/1 (w/w) corresponds to 6.82/1 ($\pm$) for LipofectAMINE/pDNA complexes. Following normalization by ($\pm$) charge ratios, we thus confirmed that WSLP/pDNA complexes or WSLP itself were indeed nontoxic to the cell when formulated at N/P ratio of 10 and below (Figure 5). In contrast, LipofectAMINE/pDNA and PEI 25000/pDNA complexes were toxic to the cells. Compared to 293T cells, these complexes were more toxic to CT-26 cells, which granulated and the cell population decreased under the same or lower concentration as WSLP. Similar to WSLP, PEI of 1800 Da was less toxic to both CT-26 and 293T cells compared to PEI 25000 or LipofectAMINE (Figure 5).

Interaction with Erythrocytes. To investigate the interaction of WSLP/pDNA complexes with erythrocytes,

an erythrocyte aggregation assay was performed (Figure 6). Mouse erythrocytes were incubated with either WSLP/pCMV-Luc (10/1, N/P) or PEI25000/pCMV-Luc (5/1, N/P) complexes at a dose of 20 μ g DNA/well. After 2 h of incubation at 37 °C, several erythrocytes were aggregated, when the cells were incubated with PEI25000/pCMV-Luc complexes. In case of WSLP/pCMV-Luc complexes, the extent of erythrocyte aggregation was minimal (Figure 6). Although we did not check the effect of N/P ratios on erythrocyte aggregation, we expect significant increase in erythrocyte aggregation with the increase in N/P ratios of WSLP/pCMV-Luc complexes.

In Vitro Transfection. WSLP/pCMV-Luc complexes formulated at different N/P ratios in 5% (w/v) glucose were evaluated for their transfection efficiency in CT-26 colon carcinoma and 293 T human embryonic kidney transformed cells. Following transfection, cells were

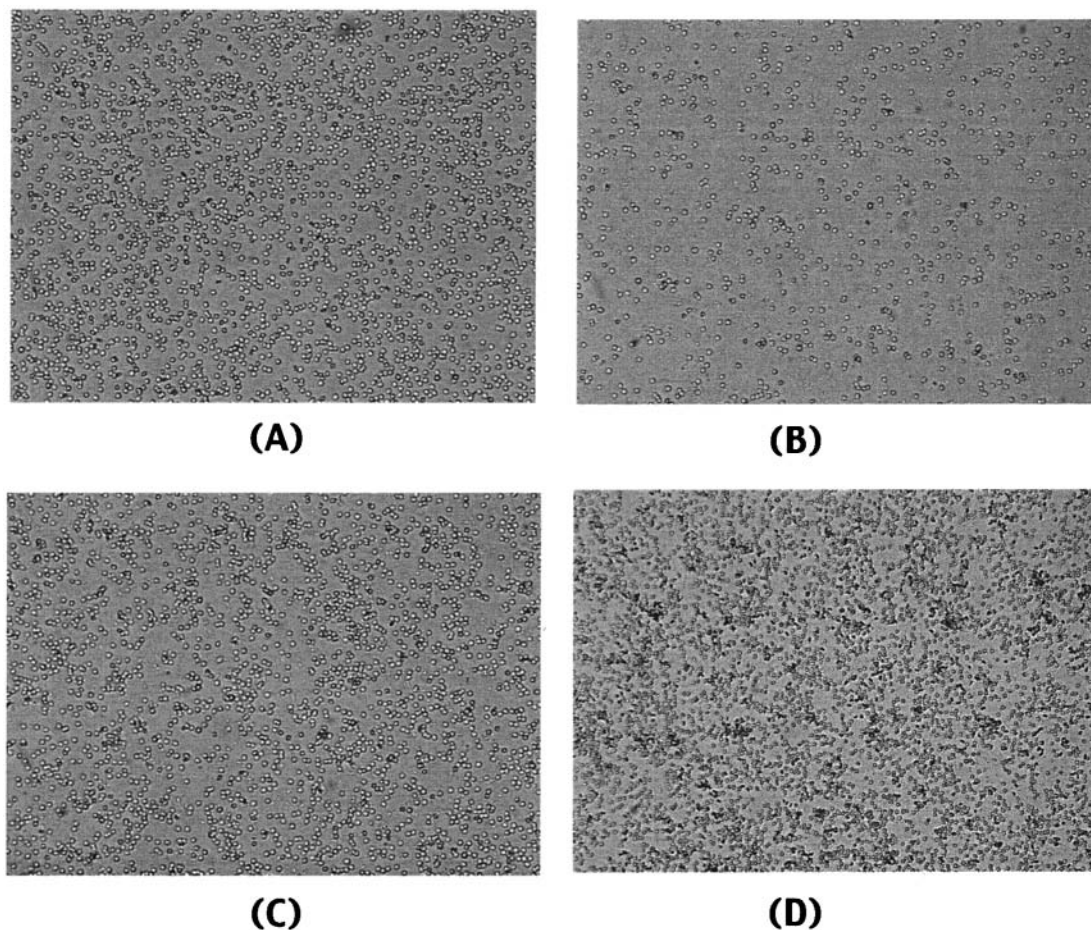


Figure 6. Interaction of WSLP/pCMV-Luc complexes with erythrocytes. Freshly collected erythrocytes were washed thrice with Ringer's solution and incubated with WSLP/pCMV-Luc (10/1, N/P) containing 20 μ g DNA/well of six well plates. Untreated erythrocytes were used as control. PEI25000/pCMV-Luc (5/1, N/P) complexes treated erythrocytes were used for comparison. (A) Nontreated erythrocytes, (B) erythrocytes treated with 5 μ L of WSLP/pCMV-Luc (10/1, N/P), (C) erythrocytes treated with 10 μ L of WSLP/pCMV-Luc (10/1, N/P), and (D) erythrocytes treated with 10 μ L of PEI25000/pCMV-Luc (5/1, N/P) complexes.

lysed, and RLU and total protein concentration were measured. Transfection efficiency increased with the increase in N/P ratios. Among the N/P ratios tested, highest Luciferase activity (RLU/ μ g total protein) was observed at N/P ratio of 20/1 for both CT-26 and 293T cells (Figure 7). The RLU values for WSLP/pCMV-Luc complexes gave several orders of magnitude higher Luciferase levels compared to naked pCMV-Luc. The Luciferase activity of WSLP/pCMV-Luc complexes was also higher than PEI25000/pCMV-Luc (5/1, N/P) complexes when formulated at the N/P ratio of 20/1, but similar when formulated the N/P ratio of 10/1.

DISCUSSION

Current cationic lipid and polymer-based gene carriers are not satisfactory due to the low transfection efficiency, high toxicity, and poor storage stability. In an attempt to combining these two modalities, namely cationic liposomes and polycations, Huang and associates (27) synthesized lipopolylysine for gene transfer. However, treatment for endosomal disruption, such as chloroquine treatment or liposome preparation with colipid DOPE was essential for enhanced gene transfer by lipopolylysine (28). Behr (29) synthesized lipopolyamine, which mediates transfection by itself and does not require any phospholipid to form liposomes. In other words, a micellar complex of lipopolyamine and DNA was sufficient for transfection. Pitard et al. (30) attempted to determine the conditions under which DNA molecules can collapse

into oligomolecular complexes rather than forming large multimolecular aggregates when mixed with lipopolyamine micelles. As lipopoly(L-lysine) requires the addition of chloroquine or the formation of liposomes with colipid DOPE for efficient transfection, Yamazaki et al. (11) grafted cetyl groups as hydrophobic lipid anchors on to PEI with molecular weights of 1800 and 25000 Da and prepared polycation liposomes for gene transfer. However, there is the growing need to avoid the preparation of cationic liposomes and the variability associated with it, especially because the large DNA particles are not good for many in vivo applications, due to their poor extravasation through the capillary endothelia and dispersibility inside the tumor tissues.

Several cholesterol-based cationic lipids have been proven been used for gene transfer. Gao and Huang (2) were the first to synthesize a cholesterol-based cationic lipid 3 β [N, (N',N'-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol) using cholesteryl chloroformate and N,N-dimethylethylenediamine and mixed with colipid DOPE to form cationic liposomes that efficiently transfect mammalian cells. DC-Chol liposomes have already been used in gene therapy applications in the clinical settings (31, 32). These early successes spurred recent interest in the development of novel cholesterol-based cationic lipids. Spermine cholesteryl carbamate and spermidine cholesteryl carbamate have been shown to be more active than DC-Chol, which has only a single protonatable amine. Furthermore, spermine cholesteryl carbamate,

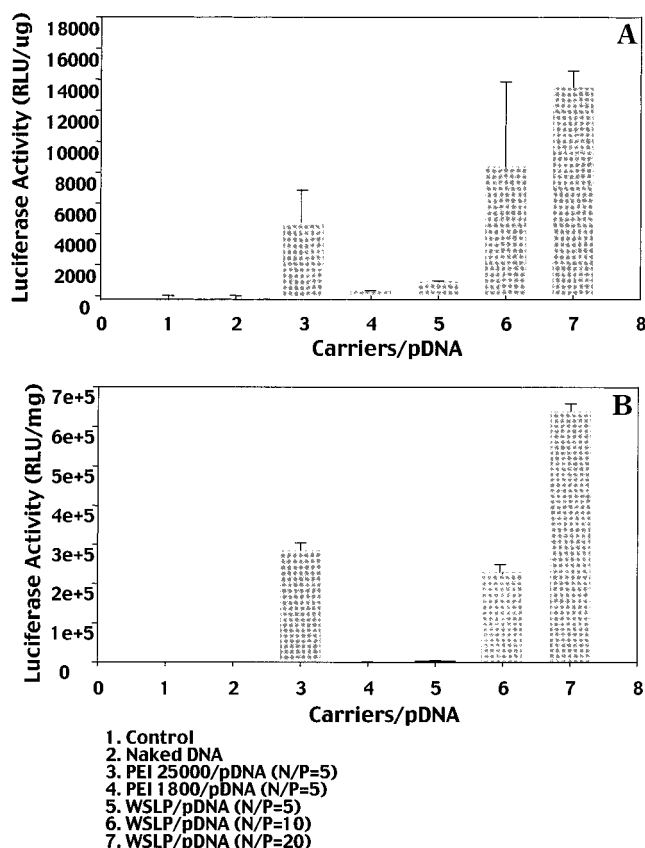


Figure 7. Luciferase activity in cultured CT-26 colon carcinoma (A) and 293 T human embryonic kidney transformed (B) cells after transfection with WSLP/pCMV–Luc complexes prepared at different (N/P) charge ratios in 5% (w/v) glucose. Naked pCMV–Luc, PEI 1800/pCMV–Luc and PEI 25000/pCMV–Luc complexes were used for comparison. Nontransfected cells (in vitro) were used as negative controls. Luciferase activity is expressed as RLU/mg of total protein.

which contains three protonatable amines, was more effective compared to two protonatable spermidine cholesteryl carbamate (10).

In this study, we conjugated cholesterol as a hydrophobic lipid anchor to PEI, a cationic polymer known to have high transfection activity. PEI is a cationic polymer composed 25% primary amines, 50% secondary amines, and 25% tertiary amines. Massive vesicular ATPase-driven proton accumulation followed by passive chloride influx into endosomes buffered with PEI should cause osmotic swelling and subsequent endosome disruption (19). For gene transfer, PEI of high molecular weight, namely 25000 Da or above, is commonly used, which is highly toxic to the cells even at low N/P ratios, and PEI25000/pDNA complexes are prone to aggregation (33). Since micellization may concentrate positive charge on the lipid surface, polycations with high molecular weight might not be required. Furthermore, anchoring of polymers with lipids may strongly be observed with polymers of low molecular weight (11). Thus, in the present study, we used PEI of 1800 Da, which has about 41 monomer units of aziridine and around 13 units of primary amines in one molecule. WSLP can destabilize the endosomal membrane by protonation of PEI itself without the requirement of nonbilayer lipids. PEI has many protonation sites, which may cause influx of chloride ions into the endosomes, and as a result of this proton-sponge effect, endosomes are broken to release their internal contents (1). Thus, this water-soluble lipopolymer (WSLP) should deliver plasmid DNA without the requirement of

any colipid as a liposomal compartment. The percentage of cholesterol conjugated to PEI was about 47%, and the average molecular weight of WSLP was about 2000 Da (Figure 2). WSLP forms micelles in water with a critical micellar concentration value of 1.43 mg/mL (Figure 3). Since WSLP was found to form micellar solutions, we investigated its potential usefulness as transfection reagent by direct mixing its solution with plasmid containing solution. The particle size of WSLP/pDNA complexes was in the range of 25.9 to 148.5 nm and was dependent on N/P ratios. Atomic force micrographs suggest that these complexes are spherical particles of ~50 nm (Figure 4).

WSLP and WSLP/pDNA complexes were not toxic to CT-26 colon carcinoma and 293T human embryonic kidney transformed cells even at N/P charge ratios, whereas both PEI25000 and LipofectAMINE-based formulations were fairly toxic to these cells (Figure 5). WSLP/pDNA complexes did not cause any aggregation of freshly prepared murine erythrocytes, whereas PEI25000/pDNA complexes aggregated the erythrocytes (Figure 6). This result suggests that cholesterol of WSLP may provide some shielding effect to WSLP/pDNA complexes and thus preventing erythrocytes aggregation.

WSLP/pDNA complexes efficiently transfected CT-26 colon carcinoma and 293T human embryonic kidney transformed cells (Figure 7). The transfection efficiency of WSLP was dependent on the N/P ratios, and the highest Luciferase activity was observed at the N/P ratio of 20/1 (Figure 7). Our findings on the usefulness of water soluble lipopolymers are in good agreement with Vigneron et al. (34), who reported the synthesis of guanidinium cholesterol lipids and their use for the efficient transfection of various mammalian cell lines as water-soluble lipopolymer as well as in the form of cationic liposomes.

In summary, we designed a water soluble nontoxic lipopolymer, which efficiently condenses and protects plasmid DNA from degradation by nucleases, and promotes enhanced transfection into mammalian cells.

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Peptide and Small Molecule Microarray for High Throughput Cell Adhesion and Functional Assays

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A novel class of chemical microchips consisting of glass microscope slides was prepared for the covalent attachment of small molecule ligands and peptides through site-specific oxime bond or thiazolidine ring ligation reaction. Commercially available microscope slides were thoroughly cleaned and derivatized with (3-aminopropyl)triethoxysilane (APTES). The amino slides were then converted to glyoxylyl derivatives via two different routes: (1) coupling of Fmoc-Ser followed by deprotection and oxidation, or (2) coupling with protected glyoxylic acid and final deprotection with HCl. Biotin or peptide ligands derivatized at the carboxyl terminus with a 4,7,10-trioxa-1,13-tridecanediamine succinimic acid linker and an amino-oxy group or a 1,2-amino-thiol group (e.g., cysteine with a free N^α-amino group) were printed onto these slides using a DNA microarray spotter. After chemical ligation, the microarray of immobilized ligands was analyzed with three different biological assays: (1) protein-binding assay with fluorescence detection, (2) functional phosphorylation assay using [γ -³²P]-ATP and specific protein kinase to label peptide substrate spots, and (3) adhesion assay with intact cells. In the cell adhesion assay, not only can we determine the binding specificity of the peptide against different cell lines, we can also determine functional cell signaling of attached cells using immunofluorescence techniques in situ on the microchip. This chemical microchip system enables us to rapidly analyze the functional properties of numerous ligands that we have identified from the “one-bead one-compound” combinatorial library method.

INTRODUCTION

Ever since the introduction of solid-phase peptide synthesis by Professor Bruce Merrifield in 1963, there has been a revolutionary advancement in peptide and organic synthesis (1). This technique was rapidly improved and modified for the synthesis of long peptides or proteins, peptide libraries and even small organic molecule libraries (2–5). A decade ago, by adopting the solid-phase peptide synthesis method, we reported the synthesis and screening of a “one-bead one-compound” combinatorial peptide library (6, 7). In this method, using a split synthesis approach (6, 8, 9), millions of peptide-beads were generated such that each bead displays only one chemical entity (6, 7). Because each compound-bead is spatially separable, the “one-bead one-compound” combinatorial library can be viewed as a huge microarray platform that is not addressable. Using various screening techniques (7), we can identify the compound-beads with a specific biological, chemical, or physical property and isolate them for structural determination. Over the last 10 years, we have successfully used this technology to identify ligands for various protein targets (6, 10–13) or small molecule dyes (14), and peptide substrates for various protein kinases (15–17). In addition, using intact cell lines as a probe, we were able to identify ligands that bind to cell surface receptors (18). Many ligands for various molecular targets can be identified with this combinatorial approach. There is an urgent need for the development of a highly minaturized and high-throughput technique to characterize the binding specificity of

these ligands. The DNA microarray technique has been developed in recent years as a powerful tool for genomic analysis. In this method, thousands of genes (cDNA) are immobilized as individual tiny spots on microscope slides, and fluorescent-labeled cDNA derived from mRNA of whole cell lysate is then used to probe the gene microarray (19, 20). This elegant technique enables one to determine expression levels of multiple genes in one single experiment. This method has inspired us to develop a peptide and small molecule microarray based technique to achieve our goal of rapidly analyzing the binding and functional properties of the leads identified from our combinatorial libraries. We reported the chemical microarray work at the American Peptide Symposium in June of 1999 (21). Independently, a similar microarray method has also been reported by Schreiber's group (22). More recently, MacBeath and Schreiber modified their method to protein microarrays (23).

Spot synthesis on cellulose sheets and light-directed parallel synthesis on special glass slides have already been reported for the generation of peptides in a microarray format (24, 25). Photogenerated acids have been introduced to make the light-directed synthesis simpler on a glass slide for microarray preparation (26). Proteins and enzymes have been immobilized on either acrylamide-gel pads or glass slides with special Teflon cover in a microarray format for high throughput screening (27, 28). In these cases, proteins or enzymes are bound to the slides through covalent nonspecific site of attachment, or noncovalently by hydrophobic or electrostatic interaction. Several types of chemistries have been applied in a DNA microarray system using glass surface modified with amino, epoxide, carboxylic acid, aldehyde, or sulfhydryl groups (29, 30). Schreiber's group has reported

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the Michael addition of thiol-containing compounds onto maleimide-derivatized glass slides for the microarray printing of small molecules (22). In the last five years, chemoselective ligation methods have been developed to chemically link unprotected peptide fragments together to form larger peptides or proteins (31–34). These coupling reactions are efficient and highly specific. We have reported the use of these ligation methods to iodinate peptide (35) and to derivatize the N-terminal cysteine of a single chain monoclonal antibody molecule via thiazolidine ring formation (36). In this report we applied this chemoselective ligation reaction to covalently attach peptides or small molecules onto the glyoxylyl-derivatized glass slide via oxime bond or thiazolidine ring formation. These chemical microarrays were then subjected to a number of biological assays, including protein binding, substrate phosphorylation, and cell adhesion.

EXPERIMENTAL PROCEDURES

Materials. Avidin-Cy5 and Streptavidin-Cy3 were obtained from Pharmacia, Amersham; anti-phosphotyrosine antibody-FITC and anti-Mouse IgG-Cy5 conjugates from Sigma, St. Louis, MO; Calcein AM or Cell-Tracker Orange from Molecular Probes, Eugene, OR; anti- β -endorphin monoclonal antibody (clone 3-E7) was obtained from Boehringer Mannheim, Indianapolis, IN; *O*-(hydrazinocarbonyl methyl) *O*-methyl-poly(ethylene glycol)-5000 (PEG₅₀₀₀-NHNH₂) from Fluka, Milwaukee, WI; stearic acid, glyoxylic acid, all other fatty acids and other chemicals used in this study were obtained from Aldrich Chem Co., Milwaukee, WI. All Fmoc-protected amino acids and resins were purchased from Nova-Bachem, Switzerland or Advanced ChemTech, Louisville, KY; human p60^{csrc} protein tyrosine kinase was purchased from Upstate Biotechnology Inc., Lake Placid, NY. Thin-wall polycarbonate 96 well plates were obtained from Corning Inc. New York, NY. Ordinary microscope slides were obtained from Corning or Fisher, and amino-functionalized slides from CEL Associates, Houston TX. The anti-human insulin antibody was purified from ascites derived from HB125 murine hybridoma cell line (ATCC) using a protein G column (Pharmacia).

Preparation of Glyoxylyl Modified Glass Slides. Ordinary glass microscope slides (75 mm $\times$ 25 mm; 1.06 mm thickness) were cleaned extensively by dipping in 1% NaOH followed by 3% HCl both at 90 °C for 10 min. The slides were then placed in boiling 35% HNO₃ in a reaction vessel fitted with a water condenser and the outlet passed through cold 1% NaOH solution for 1 h. The slides were thoroughly washed six times with distilled water, followed by methanol, and dried under vacuum overnight. The dry slides were dipped in 2% (3-aminopropyl)triethoxysilane (APTES) in dry toluene containing anhydrous Na₂SO₄ or 4 Å molecular sieves and stirred at room temperature for 3 h. The amino slides were thoroughly washed with toluene followed by dichloromethane (DCM) and N₂ dried. The slides were then cured overnight at 190 °C. The amino slides were placed in a Fmoc-Ser-OH solution (50 mM) in DMF along with DIC and HOBt (50 mM each) for 1.5 h and washed five times with DMF. The Fmoc group was removed by 20% (v/v) piperidine in DMF, and the deprotected Ser was oxidized by a NaIO₄ solution (100 mM) for 2 h. This procedure results in surface derivatization of both sides of the slides by a glyoxylyl group.

Alternatively, we have also used protected glyoxylic acid to derivatize the amino slide. To a solution of glyoxylic acid (1 M) in DMF was added HCl to give a

concentration of 0.1 mM, followed by addition of ethylene glycol to give a final concentration of 5 M. The solution was allowed to react in a boiling water bath for 30 min to obtain protected glyoxylic acid. The product was directly used for DIC–HOBt coupling reaction. The amino slides were placed in a solution of freshly prepared protected glyoxylic acid (10 mM), stearic acid (40 mM), DIC (50 mM), and HOBt (50 mM) dissolved in DMF and stirred at room temp for 2 h. The slides were washed with DMF (four times) and DCM (three times) and air-dried. The acetal protecting group was removed by placing the slides in 0.01 M HCl solution with stirring at room temp for 2 h. Finally the glyoxylyl slides were washed four times with distilled water and three times with methanol and dried under vacuum overnight. In a similar manner, different fatty acids (in place of stearic acid) along with different proportions of glyoxylic acid were used for the slide preparation.

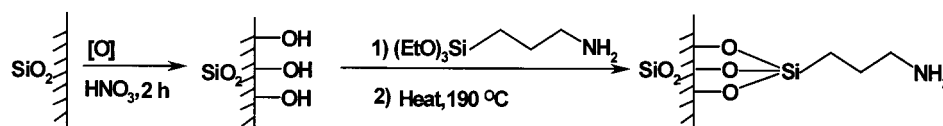
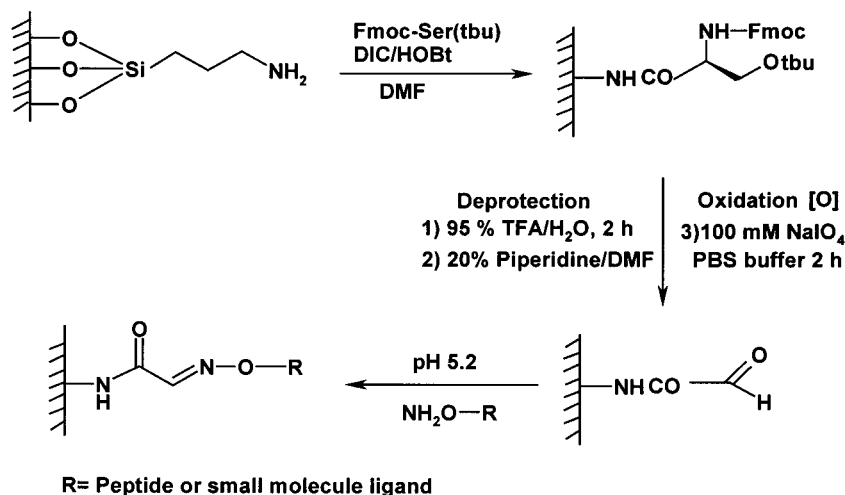
Peptide Synthesis. Peptides were synthesized according to standard Fmoc-chemistry on Rink resin. After TFA cleavage, the peptides were purified on a C-18 reverse phase column (Vydac, Hesperia, CA) using a Beckman HPLC system and characterized by ESMS. The hydrophilic linker, Fmoc-4,7,10-trioxa-1,13-tridecanediamine succinimic acid (Fmoc-Ttds-OH), was synthesized according to our previous report (36), and its purity was confirmed by HPLC and ES MS.

Microarray Spotting. Peptides or biotin containing a Ttds-linker and an amino-oxycetyl group or Lys (Cys) at their carboxyl termini were used for microarray spotting. The compounds were dissolved in 300 mM NaCl and 250 mM NaOAc (pH 5.2) at a concentration of 1–3 mM and distributed into a thin-wall polycarbonate 96 well plate (15 μ L per well). Both linear and array mode of spotting were carried out using the Affymatrix 417 arrayer (Santa Clara, CA). A spacing of 500 μ m or 300 μ m was employed between the spots with one to three repetitions for each spot. Soon after spotting, the slides were transferred to a plastic rack inside a plastic box containing saturated NaCl and incubated at room temperature overnight. The slides were thoroughly washed with distilled water, air-dried, and stored under N₂.

Fluorescent Binding Assay. The microarray slides were blocked for 3 h with 1% (w/v) BSA solution in PBS (1 mL per slide), rinsed with water (six times), and air-dried. Protein labeled with fluorescent dye Cy3 or Cy5 (1–2 μ g/mL) was added to the slides and kept in dark for 45 min. In the case of secondary antibody detection technique, the slides were first incubated with the primary antibody (anti-insulin antibody or anti- β -endorphin antibody) for 1.5 h and washed with distilled water, followed by incubation with fluorescent dye conjugated secondary antibody for 45 min. The slides were washed with deionized water, air-dried, and scanned on both channels of the General Scanning (ScanArray 3000) confocal microscope fluorescence scanner.

Protein Kinase Functional Assay. The microarray slides were blocked with 1% BSA solution in PBS as mentioned above. Human p60^{csrc} protein tyrosine kinase (30 units) and [γ -³³P]-ATP (3000 Ci/mmol) were mixed in 1 mL of MES buffer (50 mM, pH 6.8) and placed over the slides and allowed to react for 5 h. The slides were washed thoroughly with deionized water and placed in boiling HCl (1 M) for 5 min. The slide was rinsed with water, air-dried, and exposed to low energy X-ray film (Kodak Biomax MR film) for 5 h, after which the film was developed.

Cell Adhesion Assay. To block nonspecific binding of the cells, each slide was covered with 1 mL of

Scheme 1. Preparation of Amino Slides**Scheme 2. Preparation of Glass Slides for Microarray Spotting by the Fmoc-Ser Method**

O-(hydrazinocarbonyl methyl)-O-methyl-poly(ethylene glycol)-5000 (PEG₅₀₀₀-NHNH₂, 2 mM) in 0.2 mM NaOAc buffer at pH 5.2. After 4 h, the slides were rinsed with deionized H₂O and dried in a desiccator. The slides were then covered with a suspension of intact cancer cell line (2×10^6 cells in 1 mL of medium) and incubated at room temperature for 30 min. After gentle washing with PBS, if the slides were to be studied under fluorescent microscope or array scanner, the cells were then stained with Calcein AM or Cell-Tracker Orange, respectively. After 30 min of incubation, the slides were washed gently with PBS before analysis. In some experiments, the bound cells were fixed with acetone and stained with Giemsa stain.

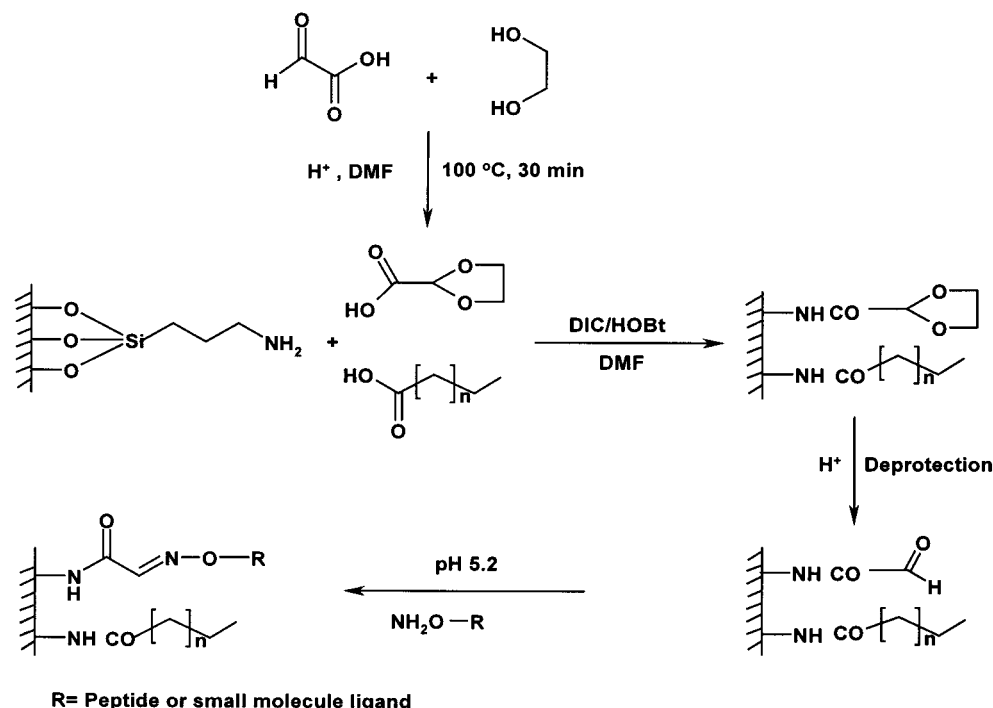
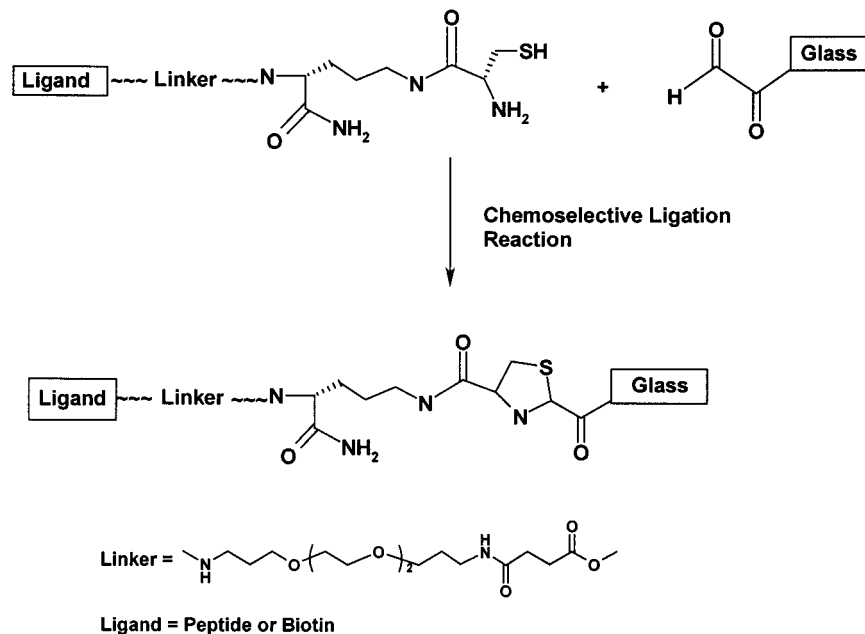
Immunofluorescence Microscopy. Immunofluorescence microscopy was performed in continuation of cell adhesion assay. After cell binding, the slides were covered with 1 mL of 3.7% paraformaldehyde to fix the cells. After 15 min, paraformaldehyde solution was aspirated, and the slides were washed with 0.5% Triton X-100 in TBS buffer for 5 min and TBS for another 5 min. To detect the effect of cell adhesion on intracellular tyrosine phosphorylation of proteins, 45 μ L of FITC conjugated anti-phosphotyrosine antibody (1/40 dilution) was added. The slides were incubated for 1 h at room temperature and washed in TBS for 5 min. The slides were then mounted for observation under fluorescent microscope. For the negative control slide (without peptide), cell attachment to the glass slide was accomplished with cytospin. The cells were then fixed, processed, and examined under the fluorescent microscope as above.

RESULTS AND DISCUSSION

Slide Preparation. We have tried various commercially available microscope slides for this microarray technique. Corning and Fisher slides were found to be the best for our purpose. First the slides were treated with NaOH and HCl, followed by boiling with HNO₃ to remove all organic matter and free up more surface Si-OH groups for the derivatization with APTES. Optimal conditions for the amino modification was to incubate 2% APTES solution in dry toluene for 3 h (Scheme 1). Strict

anhydrous conditions were maintained during this step to avoid white precipitate formation on the surface of the slides (probably a polymer of the silane). We have also tried prederivatized amino slides from CEL Associates with satisfactory results. The amino slides reacted with Fmoc-Ser in the presence of DIC, HOBt, followed by removal of the Fmoc group. Final oxidation with NaIO₄ will generate slides with a uniform coating of glyoxylyl groups (Scheme 2). Alternatively acetal protected glyoxylic acid was directly coupled to the amino slides followed by deprotection with dilute HCl (Scheme 3). We have found that this alternative method is more convenient, efficient, and consistent. To maximize the signal-to-noise ratio, we need to create a glass surface that has minimal nonspecific binding during biological assays. In addition, the printed chemical spots need to be uniform, precise, and cannot spread to the adjacent areas of the microarray. We believe that these requirements can be achieved through (1) the creation of an optimal hydrophobic-hydrophilic balance on the glass surface, and (2) the use of appropriate blocking agents prior to the biological assays. To achieve these goals, we added various fatty acids such as butyric, valeric, hexanoic, heptanoic, octanoic, decanoic, lauric, myristic, palmetic, and stearic acids separately along with protected glyoxylic acid during the coupling step. Stearic acid was found to be best in this regard. We also tried various ratios of glyoxylic acid to stearic acid and found that a 1:4 ratio was optimal for the least nonspecific binding and reproducible well-defined spots. These slides are designated as (10:40) slides. Slides without the addition of fatty acids tend to generate larger spots that often spread to the adjacent areas. This is probably due to the hydrophilic character of the modified glass surface that leads to greatly reduced surface tension between the aqueous spot and the surface of the slide. Spots generated from the (10:40) slides are smaller and usually do not have the problem with spreading.

Peptides were synthesized by Fmoc-chemistry using DIC/HOBt as coupling reagents. The Ttds-linker was used to connect the carboxyl terminus of the peptide to the glass slide as shown in Scheme 4. The linker is

Scheme 3. Preparation of Glass Slides for Microarray Spotting by the Glyoxylic Acid Method**Scheme 4. Covalent Attachment of Peptides or Small Molecules to a Glass Slide via a Thiazolidine Ring**

critical in some biological assays (see below). The glyoxylyl group exhibits desirable reactivity in that it will react specifically with oxy-amine to form oximes, with 1,2-amino-thiols (e.g., N-terminal cysteine) to form thiazolidine rings, or with hydrazines to form hydrazones at acidic pH. We have tried various additives for this ligation reaction. These include glycerol, ethylene glycol, PEG-1000, PEG-4000, and PEG-6000. High molecular weight PEG caused more spreading of the spots, and hence they were avoided. We have tried various blocking agents such as *O*-allyl-hydroxylamine, *O*-ethyl-hydroxylamine, trimethylsilyl hydroxylamine, *tert*-butyldimethylsilyl-hydroxylamine, *O*-*tert*-butyl-hydroxylamine, and PEG₅₀₀₀NHNH₂ to improve the background of slides. PEG₅₀₀₀NHNH₂ was found to be the best in the case of cell adhesion assay. For protein binding assay, we found

that chemical blocking of the glyoxylyl group is not needed. Blocking with 1% BSA prior to biological assay is sufficient.

Fluorescent Binding Assay. Previously we have identified peptide sequences that bind specifically to avidin (e.g., HPYPP) and streptavidin (e.g., HPQ) using the "one-bead one-compound" combinatorial library approach (6, 12). Biotin and peptides (WSHPQFEK and HPYPP) containing Ttds-linker with Dpr (Aoa) at the carboxyl end were printed onto the glass slide and then probed with a mixture of streptavidin-Cy3 and avidin-Cy5 conjugates. As expected, biotin bound to both streptavidin and avidin. Peptide WSHPQFEK only bound to streptavidin, whereas peptide HPYPP bound to avidin only (Figure 1). This result validates that the microarray methodology could be applied to both peptides and small

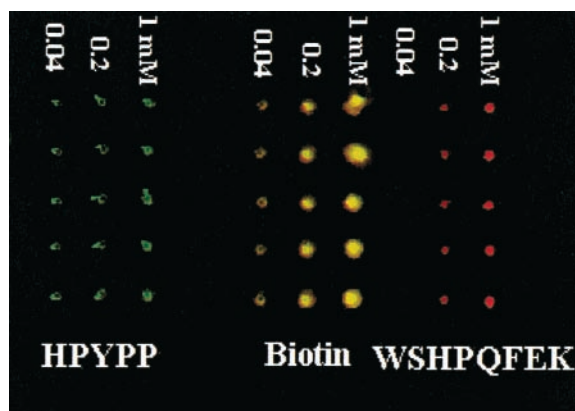


Figure 1. Binding of streptavidin-Cy3 (red) and avidin-Cy5 (green) conjugates to a microarray of biotin, and peptides HPYPP and WSHQPFEK. As expected, avidin bound to HPYPP, streptavidin bound to WSHQPFEK, and both avidin and streptavidin bound to biotin.

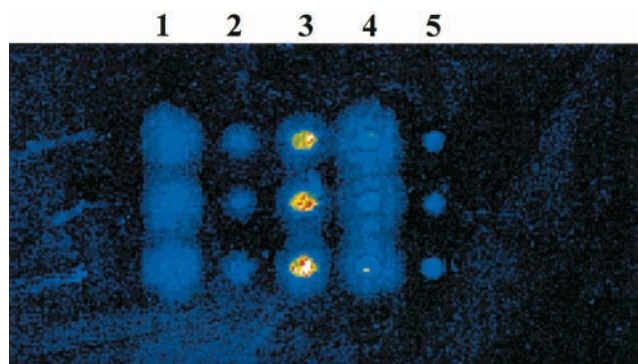


Figure 2. Binding of anti-human insulin monoclonal antibody to the immobilized mimotope peptide pqrGstG in a microarray format. The microarray was first treated with the primary anti-human insulin antibody, followed by secondary anti-mouse IgG-Cy5 conjugate. The peptides were spotted in triplets. Column 1: YGGFL, 2: wGeyidvk, 3: pqrGstG, 4: WSHQPFEK, and 5: biotin. As expected, anti-human insulin antibody bound to pqrGstG peptide only.

molecules, such as biotin. In another experiment, several peptides (YGGFL, wGeyidvk, pqrGstG, WSHQPFEK) and biotin were spotted in a microarray format, and their binding specificity to an anti-insulin MoAb (HB125) was evaluated. From our previous epitope mapping effort, pqrGstG was a mimotope peptide that we had discovered for the anti-insulin MoAb (37). As expected, after incubating with the primary HB125 MoAb followed by anti-mouse IgG-Cy3, only peptide pqrGstG was positive (Figure 2, column 3). To confirm the applicability of this technique in a microarray format, we spotted the pqrGstG peptide on five identical (10:40) slides along with biotin in an alternate pattern. Four of the five slides were incubated with the primary anti-insulin MoAb (HB125) for 1.5 h, followed by one of the following secondary reagents: streptavidin-Cy3, avidin-Cy5, anti-mouse IgG-Cy3, or a mixture of avidin-Cy5 and anti-mouse IgG-Cy3. The remaining slide was treated with a mixture of avidin-Cy5 and anti-mouse IgG-Cy3. As expected, no staining was observed in the microarray that had been incubated with the secondary antibody anti-mouse IgG-Cy3 alone. For the slide that had been incubated with HB125 MoAb followed by a mixture of avidin-Cy5 and anti-mouse IgG-Cy3, the biotin spot was labeled with Cy5 and the pqrGstG spot labeled with Cy3 (Figure 3). On the same slide, the reference spot that had both biotin and pqrGstG was labeled with both Cy3 and Cy5 (see arrow in Figure

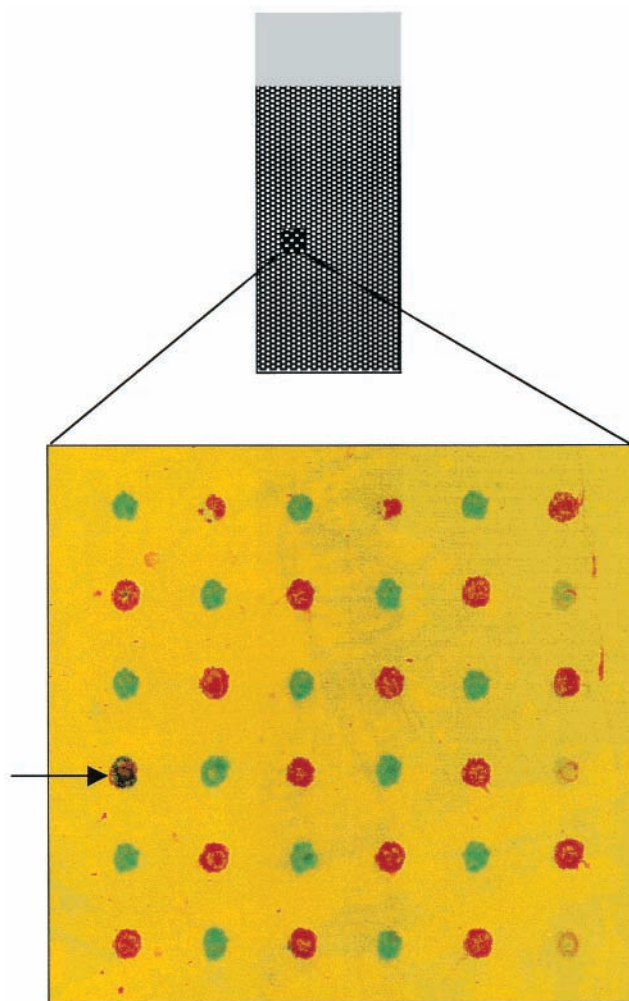


Figure 3. Biotin-linker-Dpr (Aoa) and pqrGstG-linker-Dpr(Aoa) peptide were spotted alternatively on this slide. The amino slide was functionalized with 10:40 glyoxylic acid:stearic acid. The green spots are due to the avidin-Cy5 binding to biotin and red spots due to the anti-insulin monoclonal antibody binding to pqrGstG peptide. The slides were first treated with anti-insulin antibody (1 $\mu\text{g}/\text{mL}$) followed by a mixture of avidin-Cy5 and anti-mouse IgG-Cy3 (1:1000 each). The slide was scanned under red and green channels. The color shown is not original. The final image was obtained by overlaying the two pictures that were scanned separately. The distance between the spots is 500 μm . The arrow marks the spot that both pqrGstG peptide and biotin were printed onto the glass. As expected, this spot bound to both avidin and anti-insulin antibody.

3). For the slides that had been incubated with HB125 MoAb followed by streptavidin-Cy3 or avidin-Cy5, only the biotin spots were labeled (data not shown). For the slide that had been incubated with HB125 MoAb followed by anti-mouse IgG-Cy3, only the pqrGstG spots were labeled (data not shown).

Protein Kinase Functional Assay. To demonstrate the versatility of the chemical microarray approach for functional assays, we carried out a solid-phase phosphorylation assay that is very similar to the condition we used to screen a combinatorial bead library for peptide substrates with specific protein kinases (15–17). A known peptide substrate for p60^{c-src} protein tyrosine kinase EEIYGEFF (38, 39) was used in this study. Figure 4 shows the autoradiogram of the peptide microarray that has been phosphorylated by p60^{c-src} using [$\gamma^{32}\text{P}$]ATP, instead of [$\gamma^{32}\text{P}$]ATP as the phosphoryl donor. ^{32}P was used because it gives a much better resolution of the radiolabeled microarray. In another experiment, we

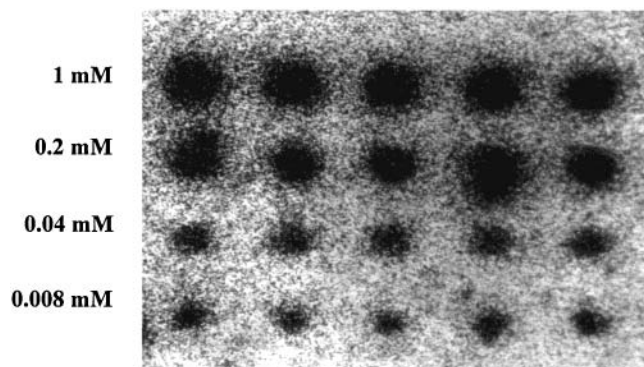


Figure 4. Autoradiogram of [^{32} P]-labeled EEIYGEFF peptide microarray (spotted at various concentrations) that had been phosphorylated by p60^{c-src} protein tyrosine kinase.

evaluated the importance of the Ttds linker in the phosphorylation assay. The peptide substrate EEIYGEFF was ligated directly to the glass slide without the use of the Ttds linker. No phosphorylation was observed on this peptide microarray (data not shown), indicating the importance of a long flexible hydrophilic linker in this assay.

Cell Adhesion Assay. We have reported the use of a whole cell binding assay to screen a “one-bead one-compound” combinatorial library peptide library for the identification of cell surface binding ligands (18). In addition, we have previously reported the use of an enzyme-linked colorimetric screening method, with purified idiotype derived from WEHI-231 murine lymphoma cell line, to screen an all-D-amino acid 8-mer peptide library. We identified wGeyidvk as a peptide that bound specifically to the surface idiotype of the WEHI-231 cells (40). Furthermore, this peptide (in a tetrameric form) was able to induce tyrosine phosphorylation of intact WEHI231 but not WEHI279 cells. We have decided to use this peptide as a model system for the development of a cell adhesion assay for the microarray. Peptide wGeyidvk was synthesized with a Ttds linker and Lys(Cys) at the carboxyl terminus (Scheme 4) and spotted onto the glyoxylyl-functionalized (10:40) slides. After reaction overnight in a moisture chamber, the slide was treated with PEG₅₀₀₀NH₂. Each of the areas of the microarray were then covered separately with various cell lines which includes WEHI-231, Jurkat, OC1LY8, K562, MOLT-4, and HL-60. The slides were then washed and stained with Giemsa stain and observed under a light microscope. Only the WEHI 231 cell was found to bind to the spotted area of the slide with minimal background (Figure 5). The slides that were incubated with other cell lines had no binding (data not shown). To determine whether the cell binding could be quantified, different concentrations of wGeyidvk peptide were spotted on the slide. The slide was then treated with Cell-Tracker Orange stained WEHI 231 cells and scanned by the microarray scanner. The scanned picture was analyzed and quantitated by the ImageQuant 5.0 program. With this quantitative analysis, we were able to determine that maximal cell binding to the microarray spots could be reached with 30 min of incubation (data not shown). As indicated above, wGeyidvk peptide was known to induce tyrosine phosphorylation in WEHI 231 cell line (40). To confirm this finding by the microarray method, cells were fixed after the cell adhesion assay. After permeation with nonionic detergent, FITC-conjugated anti-phosphotyrosine antibody was added. After incubation and washing, the cells were examined under a fluorescent microscope. Cells bound to wGeyidvk peptide fluoresced brightly

(Figure 6a), whereas the negative control where WEHI 231 cells were bound to the slide by cytospin was completely dark (data not shown). To make sure we still had bound cells in the negative control slide, we turned the background light on and showed that the cells were present but did not fluoresce (Figure 6b). To avoid nonspecific binding of cell onto the slide, PEG₅₀₀₀NH₂ blocking was essential.

Comparison with Other Microarray Techniques.

One of the earlier reports on peptide or chemical microarray was the light-directed photolithographic synthetic microchip system first reported by Fodor et al. (25). They synthesized 1042 peptides on microscope slides and used a fluorescent-labeled antibody to probe the peptide microarray. The method, while elegant, is inefficient as the synthetic method requires new synthesis on each slide and requires expensive instrumentation that is not readily available. The spotting of peptides or small molecules onto the slide (the method described in this report and that of Schreiber's (22), on the other hand, is much more efficient. Similar to the DNA microarray method, once the peptides or chemical compounds are synthesized, they can be spotted repeatedly onto hundreds of slides. The main difference between the Schreiber's approach and our approach is that Schreiber used the Michael addition chemistry to couple free thiol group of the ligand to the maleimide-functionalized slide. This free radical addition reaction, while efficient, is not site-specific as the ligation chemistries described in this report and cannot be used for ligands with a sulfhydryl group that may be essential for binding. Furthermore, the chemoselective ligation method employed in this study can also be used to generate a protein microarray by ligating specifically to proteins with a free N-terminal cysteine (36).

Potential Applications. In this report, we describe three distinct biological assays to analyze the chemical microarrays: the fluorescent-protein binding assay, the functional phosphorylation assay, and the whole cell adhesion assay. All these assays have been used in the screening of the “one-bead one-compound” combinatorial libraries (7). As indicated earlier, each compound-bead is spatially separable and the bead-library can be viewed as a huge microarray platform (10^7 compounds) that is nonaddressable. The identity of the compound-bead is known only after one picks up the positive bead and determines the chemical structure. On the other hand, the chemical microarray described in this report can be viewed as a mini-microarray (up to 10 000 compounds per slide) that is addressable. That is, the chemical identity of each spot is known prior to the biological assays. The two methods, although quite different, are highly complementary. Many ligands can be identified from bead-library screening. Once the chemical structure of these positive ligands are identified, they can be resynthesized, spotted as multiple sets of ligands onto the microscope slide, and characterized under several conditions. For instance, one may use the bead-library method to identify hundreds to thousands of specific peptide substrates for a large number of different protein kinases. These peptide substrate sets can then be used to profile protein kinase activities of cell extracts derived from patient specimens. This profile may be able to direct us to use the appropriate kinase inhibitor for cancer therapy. Similarly, the whole cell binding assay in conjunction with the peptide microarray method may enable us to select the most appropriate peptide or peptide cocktails for targeted-therapy of cancer (41). The in situ functional assay after cell adhesion on the

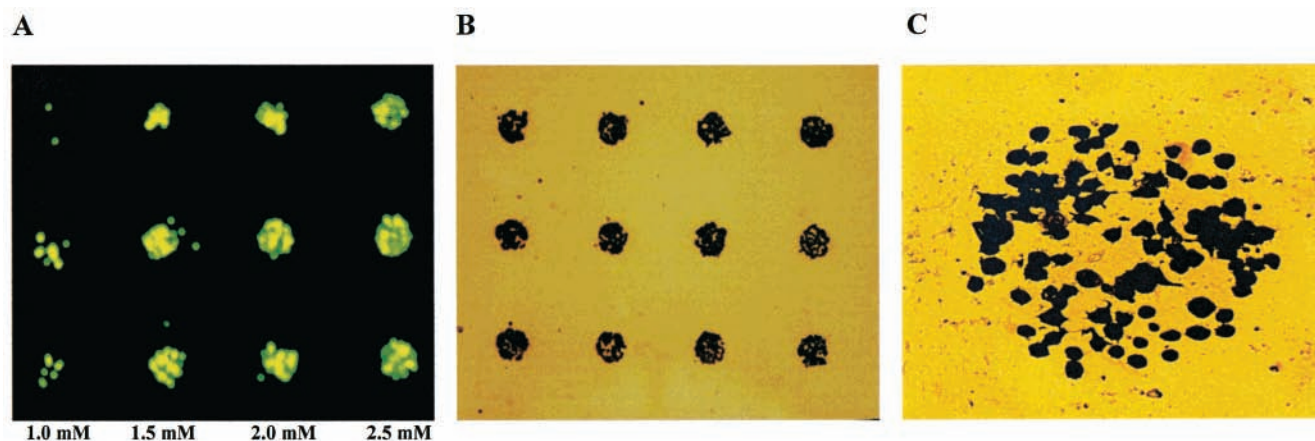


Figure 5. Cell adhesion assay of intact WEHI 231 murine lymphoma cells to wGeyidvk peptide microarray. (A) Fluorescent micrograph of calcein AM-labeled cells bound to peptide microarray that was spotted at different peptide concentrations. (B) Giemsa staining of cells bound to the peptide microarray (low power). (C) Giemsa staining of cells bound to the peptide microarray (higher power).

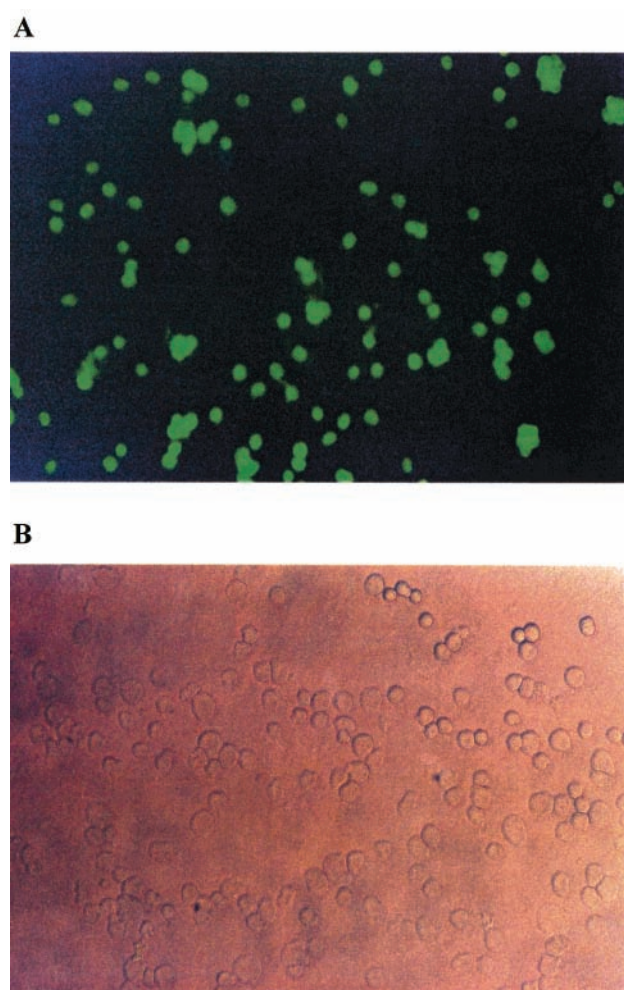


Figure 6. (A) Fluorescent micrograph of WEHI 231 lymphoma cells bound to immobilized wGeyidvk peptide. The bound cells were first fixed with paraformaldehyde and then treated with FITC-labeled anti-phosphotyrosine antibody. Strong fluorescence indicates that cell binding to the immobilized peptide triggers cell signaling. (B) Control slide with WEHI 231 cells bound to the glass by cytospin, fixed with paraformaldehyde, and treated with FITC-labeled anti-phosphotyrosine antibody as above. No fluorescence was observed (data not shown), but the cells could still be seen with dim background light.

microarray is particularly powerful because one can easily envision that hundreds to thousands of interactions between different cell lines and many ligands can

be examined concurrently with respect to binding as well as the different functional responses after binding. Using the appropriate fluorescent-labeled antibodies against a large number of signaling proteins, one can perform numerous experiments and answer many questions on one single microarray slide, using only a minute amount of cell suspension. The method is highly miniaturized and could potentially be fully automatic, from peptide synthesis, to peptide spotting, to cell adhesion, to immunofluorescence staining, to final image analysis with confocal microscopy.

CONCLUSION

We have demonstrated the preparation and optimization of a novel class of microchip, which consists of glyoxylyl-modified microscope glass slides and their application for high-throughput analysis of biomolecules and even whole cells. Peptides or small molecules were spotted and covalently attached to slides by site-specific chemical ligation procedure via thiazolidine ring or oxime bond formation. Slides with optimal hydrophobic–hydrophilic balance were obtained by coupling-protected glyoxylic acid and stearic acid in a ratio of 1:4. The peptide and biotin spots on this microchip bound specifically with their receptor protein conjugates (with dye Cy3 or Cy5) and could be easily visualized under a confocal array scanner. We were able to carry out functional phosphorylation assay as well as whole cell binding assay on this microchip. In the cell adhesion assay, nonspecific binding could be eliminated by modifying the glass surface with PEG₅₀₀₀NH₂. One major advantage of this type of slide comes from the site-specific attachment of C-terminal modified peptides or small molecules with a Ttds linker molecule to the solid-support, thereby facilitating the binding or functional assays on these chemical microarrays.

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Synthesis, Characterization, and Labeling with $^{99m}\text{Tc}/^{188}\text{Re}$ of Peptide Conjugates Containing a Dithia-bisphosphine Chelating Agent

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Radiolabeling of small receptor-avid peptides at specific predetermined chelation sites with radioactive metals has been an effective approach for production of target-specific radiopharmaceuticals for diagnosis and therapy of diseases. Among various electron-donating groups found on chelator frameworks, phosphines are unique because they display versatile coordination chemistry with a wide range of transition metals. We have recently reported the utility of a dithia-bis(hydroxymethyl)-phosphine-based (P_2S_2) bifunctional chelating agent (BFCA) containing air-stable primary phosphine groups to form ^{99m}Tc -labeled receptor-avid peptides by the preconjugation approach. Here we report a novel strategy for labeling small peptides with both ^{99m}Tc and ^{188}Re using the P_2S_2 -COOH (6,8-bis-[3-(bis(hydroxymethyl)phosphanyl)propylsulfanyl]octanoic acid) BFCA by a postconjugation radio-labeling approach. The first step in this approach involves the coupling of the corresponding $(\text{PH}_2)_2\text{S}_2$ -COOH intermediate to the N-terminus of the peptide(s). Formylation of P–H bonds with aqueous formaldehyde in the presence of HCl in ethanol affords the corresponding (hydroxymethyl)phosphine- P_2S_2 -peptide conjugates in the form of an oxidatively stable phosphonium salt. The P_2S_2 -peptide conjugates are generated (where the PH_2 groups are converted to $\text{P}(\text{CH}_2\text{OH})_2$ groups) by treatment of the P_2S_2 -peptide phosphonium salt(s) with 1 M sodium bicarbonate solution at pH 8.5. Complexation of BFCA conjugates with ^{99m}Tc is achieved by direct reduction with Sn(II) tartarate to yield the ^{99m}Tc - P_2S_2 -peptide conjugate in near quantitative yields. Complexation of the BFCA conjugates with ^{188}Re is achieved by transchelation with ^{188}Re citrate in yields of $\geq 90\%$. In this study, $(\text{PH}_2)_2\text{S}_2$ -COOH BFCA was conjugated to model peptides. The glycylglycine ethyl ester (GlyGlyOEt)- $(\text{PH}_2)_2\text{S}_2$ -COOH BFCA conjugate was converted to the hydroxymethylene phosphine form and complexed with ^{99m}Tc to produce the $^{99m}\text{TcO}_2$ - P_2S_2 - GlyGlyOEt conjugate **8** in RCPs of $\geq 95\%$. This singular ^{99m}Tc product is stable over 24 h in aqueous solution as confirmed by HPLC. Identical retention times of the $^{99m}\text{TcO}_2$ - P_2S_2 - GlyGlyOEt complex and its cold rhenium analogue (ReO_2 - P_2S_2 - GlyGlyOEt) on HPLC indicates similarity in structures at the macroscopic and the tracer levels. The utility of this postconjugation strategy was further demonstrated by synthesizing a P_2S_2 -D-Lys⁶-LHRH conjugate and producing its corresponding ^{99m}Tc complex in RCPs of $\geq 88\%$. Finally, the P_2S_2 -5-Ava-BBN[7–14] NH_2 bombesin (BBN) analogue was synthesized, the PH_2 groups converted to $\text{P}(\text{CH}_2\text{OH})_2$ groups and subsequently labeled with ^{188}Re to yield a ^{188}Re -labeled bombesin analogue with a RCP of $\geq 90\%$. The biological integrity of this conjugate was demonstrated in both in vitro and in vivo. The results of this investigation demonstrate that the $(\text{PH}_2)_2\text{S}_2$ -COOH BFCA can be conveniently used as a precursor for labeling small receptor-avid peptides with diagnostic (^{99m}Tc) and therapeutic (^{188}Re) radionuclides via the postconjugation approach in high yields.

INTRODUCTION

The labeling of small peptides with diagnostic and/or therapeutically useful radiometals has attracted a great degree of attention in recent years due to wide applicabil-

ity into the development of target-specific radiopharmaceuticals.^{1–10} ^{99m}Tc continues to be the most commonly used radiometal for SPECT imaging due to its favorable physical properties and availability (i.e., $t_{1/2} = 6$ h).^{11,12} The suitable nuclear emission properties, the physical half-life ($t_{1/2} = 17$ h), the decay characteristics ($E_\beta = 1.7$ MeV), and its availability as a no-carrier-added (NCA) reagent makes ^{188}Re one of the most attractive radionuclides for potential use in therapeutic applications.^{12,13} However, the number of research and development studies with ^{188}Re -labeled biomolecules has been limited.² The reason is due, in part, to the more complex labeling chemistry required for ^{188}Re .^{7,14–20} For example, a large excess of reducing agent [e.g., Sn(II)] is required to convert the rhenium from an oxidation state of VII in the $^{188}\text{ReO}_4^-$, to a lower oxidation state(s) (e.g., +5, +3,

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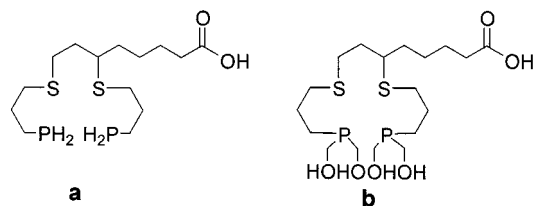


Figure 1. (a) $(\text{PH}_2)_2\text{S}_2\text{-COOH}$. (b) $\text{P}_2\text{S}_2\text{-COOH BFCA}$.

or +1) to facilitate ^{188}Re complexation to coordinating ligand frameworks.^{14–20} In context of $^{99\text{m}}\text{Tc}$, Re(VII) is more difficult to reduce than Tc(VII) , and the presence of strong reducing agents can alter the metalation reactions and/or the integrity of the biomolecular conjugate.^{2,12} Overcoming these and other related issues (e.g., Re is often more readily oxidized and less stable at low oxidation states than Tc) to produce well-defined ^{188}Re -labeled compounds in high yields while also maintaining their ability for high specific in vivo targeting of cancers is not trivial.^{2,12}

The use of phosphines in nuclear medicine has been well established. $^{99\text{m}}\text{Tc}$ –Tetrafosmin and $^{99\text{m}}\text{Tc}$ –Technecard, which utilize the phosphine ligands 1,2-bis(bis(ethoxyethyl)phosphino)ethane and tris(3-methoxy-1-propyl)phosphine are currently being clinically used as myocardial perfusion agents for imaging the heart.^{21,22} Furthermore, Deutsch et al. have demonstrated the stabilization of technetium in the oxidation states (V), (III), and (I) using the bidentate dimethylphosphinoethane (DMPE).^{22–28} Katti et al. have reported the utility of hydroxymethylphosphines (HMPs) as chelating ligands for $^{99\text{m}}\text{Tc}$ in (V) and (I) oxidation states.^{29–35} Alkyl phosphines are excellent ligands for binding the transition metals, as they form stable and well-defined complexes with a variety of transition-metals.³⁶ However, the high affinity of alkyl phosphines toward oxidation limits their use for the radiometal labeling of biomolecules.

Development of hydrophilic chelating moieties to facilitate efficient clearance of the $^{99\text{m}}\text{Tc}/^{188}\text{Re}$ activity from the blood pool through the kidneys and into the urine, with low retention of the radioactivity in the liver and the kidney is an important endeavor in development of effective radiopharmaceuticals.^{1,37–40} With this aim, we previously reported development of a $\text{P}_2\text{S}_2\text{-COOH BFCA}$ based on the dithia-bis(hydroxymethyl)phosphine framework (Figure 1) that can be linked to peptides or other biomolecules to produce $^{99\text{m}}\text{Tc}$ -labeled conjugates.^{33,34} The complexes of $\text{Tc-}^{99\text{m}}$ with the new chelating framework demonstrate excellent in vitro and in vivo stability and desirable clearance and excretion properties (from the blood stream).^{29,32,34,35} This study describes an effective approach for synthesizing P_2S_2 conjugated peptides to produce products in which the two hydroxymethylene phosphine (HMP) groups are not oxidized by convenient routes (e.g., automated SPPS is exceptionally challenging). For example, the HMP groups are oxidized when P_2S_2 conjugated peptides synthesized by SPPS are cleaved from the resin by TFA. This study demonstrates the utility of an effective approach for synthesis of P_2S_2 bioconjugates, using $(\text{PH}_2)_2\text{S}_2$ -precursors or intermediates, that are exceptionally stable to most harsh synthetic conditions. The phosphorus hydride groups (PH_2) present in the $(\text{PH}_2)_2\text{S}_2$ -ligand framework will not be oxidized or chemically altered by a variety of reaction conditions (including exposure to high TFA concentrations at elevated temperatures), yet they can be readily converted to HMP groups for chelating with $^{99\text{m}}\text{Tc}$ and ^{188}Re via the postconjugation approach.

EXPERIMENTAL SECTION

All solvents were either ACS certified or HPLC grade solvents were obtained from Fischer Scientific and used as received. Fmoc-amino acids, coupling agents, and resins were purchased from Calbiochem-Novabiochem Corp., CA. All other reagents were purchased either from Aldrich Chemical Co., Fischer Scientific, or Acros Chemicals. $^{99\text{m}}\text{TcO}_4^-$ and $^{188}\text{ReO}_4^-$ were eluted from either a $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ generator (Mallinckrodt Medical, Inc.) or a $^{188}\text{W}/^{188}\text{Re}$ generator (Oakridge National Laboratories), respectively, using sterile 0.9% saline. $(\text{PH}_2)_2\text{S}_2\text{-COOH}$, $\text{ReO}_2\text{-P}_2\text{S}_2\text{-COOH}$, and $\text{ReO}_2(\text{py})_4\text{Cl}$ were prepared according to previously reported procedures.^{33,34,41} Nuclear magnetic resonance spectra were recorded on a Bruker ARX-300 spectrometer using D_2O , CDCl_3 , or CD_3OD as solvents. The ^1H and ^{13}C chemical shifts are reported in ppm, downfield from external standard tetramethylsilane. The ^{31}P NMR (121.5 MHz) spectra were recorded with 85% phosphoric acid as an external standard, and positive chemical shifts lie downfield of the standard. Infrared spectra were recorded on a Mattson FT-IR Galaxy 3000 spectrometer using KBr pellet for solid samples and NaCl crystals for liquid samples. FAB mass spectral analyses were performed by the Washington University Resource for Biomedical and Bio Organic Mass Spectrometry, St. Louis, MO. Electrospray mass spectral analyses were performed by Synpep Corporation, Dublin, CA.

Solid-Phase Peptide Synthesis (SPPS). Peptide synthesis was carried out on a Perkin-Elmer – Applied Biosystems Model 432 automated peptide synthesizer employing traditional fmoc chemistry with HBTU activation of carboxyl groups on the reactant with the N-terminal amino group on the growing peptide anchored via the C-terminus to the resin. Rink Amide MBHA resin (25 μmol) and fmoc-protected amino acid with appropriate side-chain protections (75 μmol) were used for the synthesis. The final products were cleaved by a standard procedure using a cocktail containing thioanisole, water, ethanedithiol, and trifluoroacetic acid in a ratio of 2:1:1:36 and precipitated into methyl *tert*-butyl ether. Crude peptides were purified by HPLC, and the solvents were removed on a SpeedVac concentrator.

High Performance Liquid Chromatography (HPLC). HPLC analyses of all peptide conjugates and their metal complexes were performed on a Waters 600E system equipped with a Waters 486 tunable absorption detector, a sodium iodide crystal containing a radiometric detector, and a Waters 746 Data Module integrating recorder. HPLC solvents consisted of H_2O containing 0.1% trifluoroacetic acid (Solvent A) and acetonitrile containing 0.1% trifluoroacetic acid (Solvent B). Conditions: Either a C-18 Hamilton PRP-1 column (10 μm , 4.1 $\times$ 150 mm) or a Phenomenex Jupiter C-18 (5 μm , 4.1 $\times$ 250 mm) column was used with a flow rate of 1.5 mL/min. This HPLC gradient system begins with a solvent composition of 95% A and 5% B from 0 to 2 min, followed by a linear gradient 95% A:5% B to 50% A:50% B from 2 to 17 min. The gradient remains at 50% A:50% B for 8 min, after which the column is reequilibrated.

Synthesis of $(\text{PH}_2)_2\text{S}_2\text{-GlyGlyOEt}$ (1a). To a solution of $(\text{PH}_2)_2\text{S}_2\text{-COOH}$ (712 mg, 2.0 mmol) in acetonitrile (25 mL) were added GlyGly ethyl ester hydrochloride (395 mg, 2.0 mmol), triethylamine (606 mg, 6.0 mmol), and HBTU (770 mg, 2.05 mmol). The reaction mixture was stirred for 30 min at room temperature (25 $^\circ\text{C}$) under nitrogen. The solvent was removed under reduced pressure, and the crude product was chromatographed on a silica gel column using a hexane/ethyl acetate solvent

system. The pure conjugate **1a** was obtained (857 mg, 86%) as a colorless viscous oil. ^1H NMR (300 MHz, CDCl_3): δ = 6.71 (bs, 1H), 6.39 (bs, 1H), 4.17 (q, J = 7.8 Hz, 2H), 4.05–3.91 (m, 4H), 3.041–2.94 (m, 2H) 2.71–2.44 (m, 8H), 2.37–2.29 (m, 2H), 2.21 (t, J = 7.5 Hz, 2H), 1.85–1.68 (m, 6H), 1.67–1.36 (m, 8H), 1.23 (t, J = 8.1 Hz, 3H); ^{13}C NMR (75 MHz, CDCl_3): δ = 173.58 (s), 169.54 (s), 169.22 (s), 61.65 (s), 44.69 (s), 43.01 (s), 41.31 (s), 36.15 (s), 34.74 (s), 32.96 (s), 32.73 (s), 30.89 (s), 29.35 (s), 26.43 (s), 25.38 (s), 14.12 (s), 13.05 (bs); ^{31}P NMR (121 MHz, CDCl_3): δ = –136.5 (s), –136.56 (s). HRMS (FAB) calcd m/z for $[\text{M} + \text{H}]^+$: 499.1998; found: 499.1970.

Synthesis of $(\text{PH}_2)_2\text{S}_2$ –5-Ava-BBN[7–14] NH_2 (1b**).** A solution of 5-Ava-BBN[7–14] NH_2 (10 mg, 9.63 μmol) in DMF (0.5 mL) was added to a reaction mixture containing $(\text{PH}_2)_2\text{S}_2$ –COOH (6.0 mg, 16.85 μmol), HBTU (7.0 mg, 18.47 μmol), and triethylamine (10 μL) in DMF (0.5 mL). The reaction mixture was stirred for 30 min at room temperature. The resultant $(\text{PH}_2)_2\text{S}_2$ –5-Ava-BBN[7–14] NH_2 peptide conjugate was purified by reversed-phase HPLC and analyzed by ^{31}P NMR spectroscopy and electrospray mass spectrometry. The pure product (9.3 mg, 70%) was obtained as a white powder. ^{31}P NMR (121 MHz, D_2O): δ = –137.91 (s). ^{31}P NMR (121 MHz, proton coupled, D_2O): δ = –137.90 (t, $J_{\text{P-H}}$ = 190.2 Hz). Electrospray MS calcd m/z for $(\text{M} + 1)^+$: 1377; found: 1377.

Synthesis of $(\text{PH}_2)_2\text{S}_2$ –D-Lys⁶–LHRH (1c**).** The same synthetic procedure described to produce **1b** was used. The pure product was obtained in a yield of 68% as a white powder. ^{31}P NMR (121 MHz, D_2O): δ = –137.91 (s). ^{31}P NMR (121 MHz, proton coupled, D_2O): δ = –137.90 (t, $J_{\text{P-H}}$ = 190.2 Hz). Electrospray MS calcd m/z for $(\text{M} + \text{H})^+$: 1591.9; found: 1592.0.

Synthesis of P_2S_2 –GlyGlyOEt (2a**).** To a solution of conjugate **1a** (500 mg, 1.0 mmol) in degassed ethanol (5 mL) was added 37% aqueous formaldehyde solution (650 μL , 8.0 mmol) in the presence of 5 N hydrochloric acid (0.5 mL) to produce the phosphonium salt. The reaction mixture was stirred at room temperature (25 °C) for 15 min under nitrogen. Removal of the solvent under reduced pressure afforded conjugate **2a** (1.2 g, 97%) as a colorless viscous oil. ^1H NMR (300 MHz, D_2O): δ = 4.67 (bs, 12H), 4.06 (q, J = 5.5 Hz, 2H), 3.88 (s, 2H), 3.82 (s, 2H), 2.76–2.67 (m, 1H), 2.64–2.48 (m, 6H), 2.42–2.27 (m, 4H), 2.26–2.15 (m, 2H), 1.96–1.78 (m, 4H), 1.75–1.55 (m, 2H), 1.54–1.39 (m, 4H), 1.36–1.22 (m, 2H), 1.12 (t, J = 7.1 Hz, 3H); ^{13}C NMR (75 MHz, D_2O): δ = 177.59 (s), 172.35 (s), 171.81 (s), 62.88 (s), 50.44 (d, J = 53.9 Hz), 44.43 (s), 42.71 (s), 41.73 (s), 35.77 (s), 34.13 (s), 32.08 (d, J = 15.6 Hz), 30.53 (d, J = 15.5 Hz), 28.66 (s), 25.99 (s), 25.42 (s), 21.77 (s), 21.29 (s), 13.82 (s), 13.32 (d, J = 10.8 Hz), 12.78 (d, J = 10.8 Hz); ^{31}P NMR (75 MHz, D_2O): δ = 30.14 (s); HRMS (FAB) calcd m/z for $[\text{M} - \text{Cl}]^+$: 715.2383; found: 715.2376.

Synthesis of P_2S_2 –5-Ava-BBN[7–14] NH_2 (2b**).** To a solution of **1b** (5.0 mg, 3.63 μmol) in ethanol (200 μL) and DMF (100 μL) were added 5 N hydrochloric acid (25 μL) and 37% aqueous formaldehyde (50 μL). The reaction mixture was stirred at room temperature (25 °C) for 5 min. The formation of the P_2S_2 –5-Ava-BBN[7–14] NH_2 conjugate phosphonium salt was confirmed by ^{31}P NMR spectroscopy. The reversed-phase HPLC purification yielded the pure P_2S_2 –5-Ava-BBN[7–14] NH_2 conjugate phosphonium salt (4.6 mg, 81%) as a white powder. ^{31}P NMR (121 MHz, D_2O): δ = 30.1 (s). Electrospray MS calcd m/z for $(\text{M} + \text{H})^+$: 1376; found: 1376.

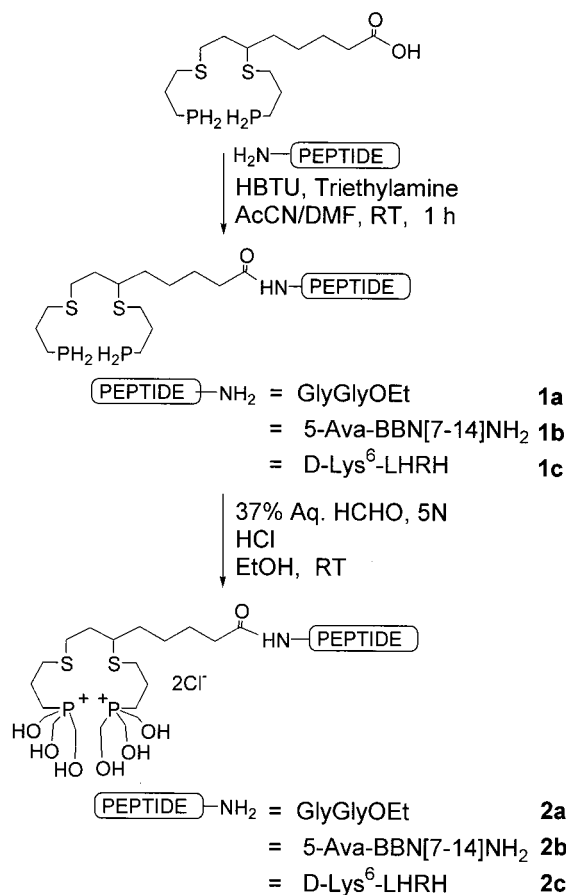
Formylation on solid phase was accomplished by first synthesizing $(\text{PH}_2)_2\text{S}_2$ –5-Ava-BBN[7–14] NH_2 on the resin by SPPS using standard fmoc chemistry. The presence

of PH_2 groups on $(\text{PH}_2)_2\text{S}_2$ –5-Ava-BBN[7–14] NH_2 -resin was confirmed by FTIR spectroscopy indicated by characteristic singlet band at 2284 cm^{-1} due to P–H stretching. To 90 mg of $(\text{PH}_2)_2\text{S}_2$ –5-Ava-BBN[7–14] NH_2 -resin in ethanol (300 μL) was added a solution of 5 N HCl (50 μL) and 37% aqueous formaldehyde (100 μL). The reaction mixture was stirred for 1 h at room temperature. The resultant P_2S_2 –5-Ava-BBN[7–14] NH_2 -resin was washed with water (10 mL) and dried. P_2S_2 –5-Ava-BBN[7–14] NH_2 conjugate phosphonium salt was cleaved from the resin by a standard procedure using a cocktail consisting of thioanisole, water, ethanedithiol, and trifluoroacetic acid in a ratio of 2:1:1:36. The new peptide conjugate was precipitated into methyl *tert*-butyl ether. The crude peptide conjugate was purified by reversed-phase HPLC, and the solvents were removed on a SpeedVac concentrator. ^{31}P NMR spectroscopy and electrospray mass spectrometry confirmed product formation. The analytical data was consistent with the product prepared by the solution phase formylation method. The P_2S_2 –5-Ava-BBN[7–14] NH_2 conjugate phosphonium salt was obtained as a white powder in 31% yield.

Synthesis of P_2S_2 –D-Lys⁶–LHRH (2c**).** To a solution of **1c** (0.5 mg) in ethanol (400 μL) and DMF (100 μL) were added 0.1 N HCl (25 μL) and 37% aqueous formaldehyde (25 μL). The solution was stirred at room temperature (25 °C) for 5 min. The formation of the P_2S_2 –D-Lys⁶–LHRH hydroxymethyl phosphonium chloride was confirmed by ^{31}P NMR spectroscopy (δ = 31.4 ppm). The P_2S_2 –D-Lys⁶–LHRH hydroxymethyl phosphonium chloride was converted to corresponding hydroxymethyl phosphine by the addition of 1 M aqueous sodium bicarbonate (30 μL) to increase pH to 8.5. The conjugate was formed in near quantitative yield as monitored by ^{31}P NMR spectroscopy (δ = –24.2 ppm).

Synthesis of ReO_2 – P_2S_2 –GlyGlyOEt (3a**).** To a solution of compound **2a** (500 mg, 0.81 mmol) in water (25 mL) was added a solution of $[\text{ReO}_2(\text{py})_4]\text{Cl}$ (432 mg, 0.8 mmol) also in water (75 mL). The reaction mixture (pH = 5) was refluxed for 1 h. The reaction mixture was concentrated under reduced pressure and purified on a C-18 Sep-Pak reverse phase column with a water/methanol solvent system. The pure rhenium complex **3a** was obtained (610 mg, 70%) as a purple viscous oil. ^1H NMR (300 MHz, D_2O): δ = 4.47–4.11 (m, 8H), 4.00 (q, J = 7.2 Hz, 2H), 3.81 (s, 2H), 3.76 (s, 2H), 3.41 (bs, 4H), 3.24 (bs, 1H), 3.04–2.86 (m, 2H), 2.55–2.45 (m, 6H), 3.33–3.08 (m, 6H), 1.92–1.18 (bm, 6H), 1.06 (t, J = 5.5 Hz, 3H); ^{13}C NMR (75 MHz, D_2O): δ = 176.86 (s), 171.93 (s), 171.49 (s), 62.51 (s), 59.24–57.82 (m), 42.45 (s), 41.43 (s), 38.37 (s), 38.19 (s), 37.05 (s), 35.41 (s), 33.14 (s), 30.10 (s), 25.22 (s), 25.22 (s), 20.87 (s), 17.83 (s), 17.53 (s), 13.56 (s); ^{31}P NMR (121 MHz, D_2O): δ = –6.26 (d, J = 10.1 Hz), –7.14 (d, J = 9.9 Hz); IR (NaCl): ν = 3385 (bs, $\nu_{\text{O-H}}$), 1730 (s, $\nu_{\text{C=O}}$), 1647 (s, $\nu_{\text{C=O}}$), 793, 912 (s, $\nu_{\text{O=Re=O}}$), (s, $\nu_{\text{as O=Re=O}}$) cm^{-1} ; HRMS (FAB) calcd m/z for $[\text{M} - \text{HCl}]$: 836.1705; found: 836.1699.

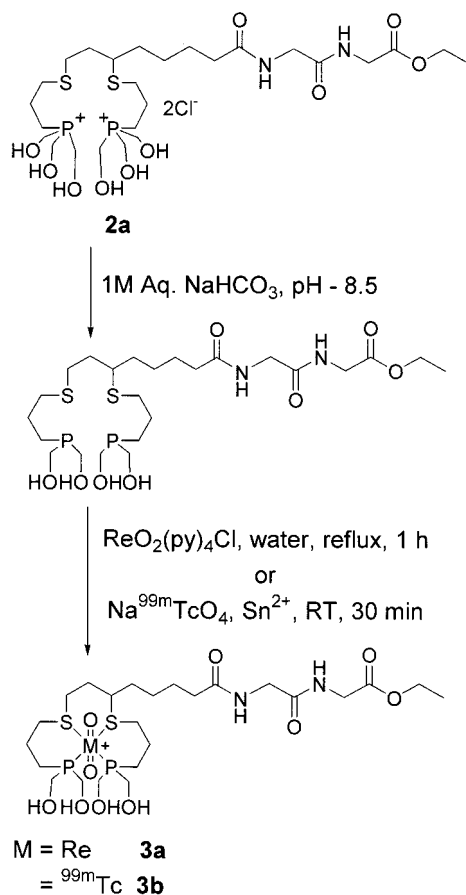
Synthesis of ReO_2 – P_2S_2 –5-Ava-BBN[7–14] NH_2 (4a**).** The ReO_2 – P_2S_2 –5-Ava-BBN[7–14] NH_2 **4a** was synthesized by solid-phase peptide synthesis using traditional fmoc chemistry. The precomplexed ReO_2 – P_2S_2 –COOH was introduced onto the bioconjugate as the last residue in the synthetic sequence.³⁴ Synthesizing the “cold” Re product in this manner allows incorporation of the metal as the ReO_2 – P_2S_2 -moiety at the N-terminal of the peptide. The ReO_2 – P_2S_2 –5-Ava-BBN[7–14] NH_2 **4a** was cleaved from the resin by a standard procedure using cocktail consisting of thioanisole, water, ethanedithiol, and trifluoroacetic acid in a ratio of 2:1:1:36. The new bio-

Scheme 1. Synthesis of $(\text{PH}_2)_2\text{S}_2$ -Peptide and P_2S_2 -Peptide Conjugates

conjugate was precipitated into methyl *tert*-butyl ether. The crude peptide conjugate was purified by reversed-phase HPLC, and the solvents were removed on a SpeedVac concentrator. The $\text{ReO}_2\text{-P}_2\text{S}_2\text{-5-Ava-BBN}[7\text{-}14]\text{NH}_2$ **4a** was obtained as a pale-colored powder in 41% yield. ^{31}P NMR (121 MHz, D_2O): $\delta = -6.44$ (s), -7.41 (s); electrospray MS calcd m/z for $[\text{M} + \text{OH}]$: 1732.9; found: 1732.9.

$^{99\text{m}}\text{Tc}$ Labeling of $\text{P}_2\text{S}_2\text{-GlyGlyOEt}$ (3b**).** *Postconjugation Labeling.* A ligand stock solution of $\text{P}_2\text{S}_2\text{-GlyGlyOEt}$ **2a** was prepared by dissolving 5 mg of the conjugate in 1 mL of water, and the pH was raised to 8.5 with 1 M sodium bicarbonate buffer. A 30 μL (0.37 μmol , concentration in final 550 μL solution = 6.6×10^{-4} M) amount of the stock solution was added to a solution of $\text{Na}^{99\text{m}}\text{TcO}_4$ (500 μL , 8 mCi). An aliquot of saturated aqueous stannous tartarate (10 μL) was added to the solution. The solution (pH = 6) was allowed to incubate at room temperature for 30 min. $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-GlyGlyOEt}$ complex **3b** was formed in a yields of $\geq 98\%$, as a single species. The retention time of the new complex was 10.6 min using those conditions previously described. The tracer level complex is identical to that of the Re complex **3a**, monitored at $\lambda = 300$ nm. HPLC conditions were the same for each of the $^{99\text{m}}\text{Tc}$ and Re complexes.

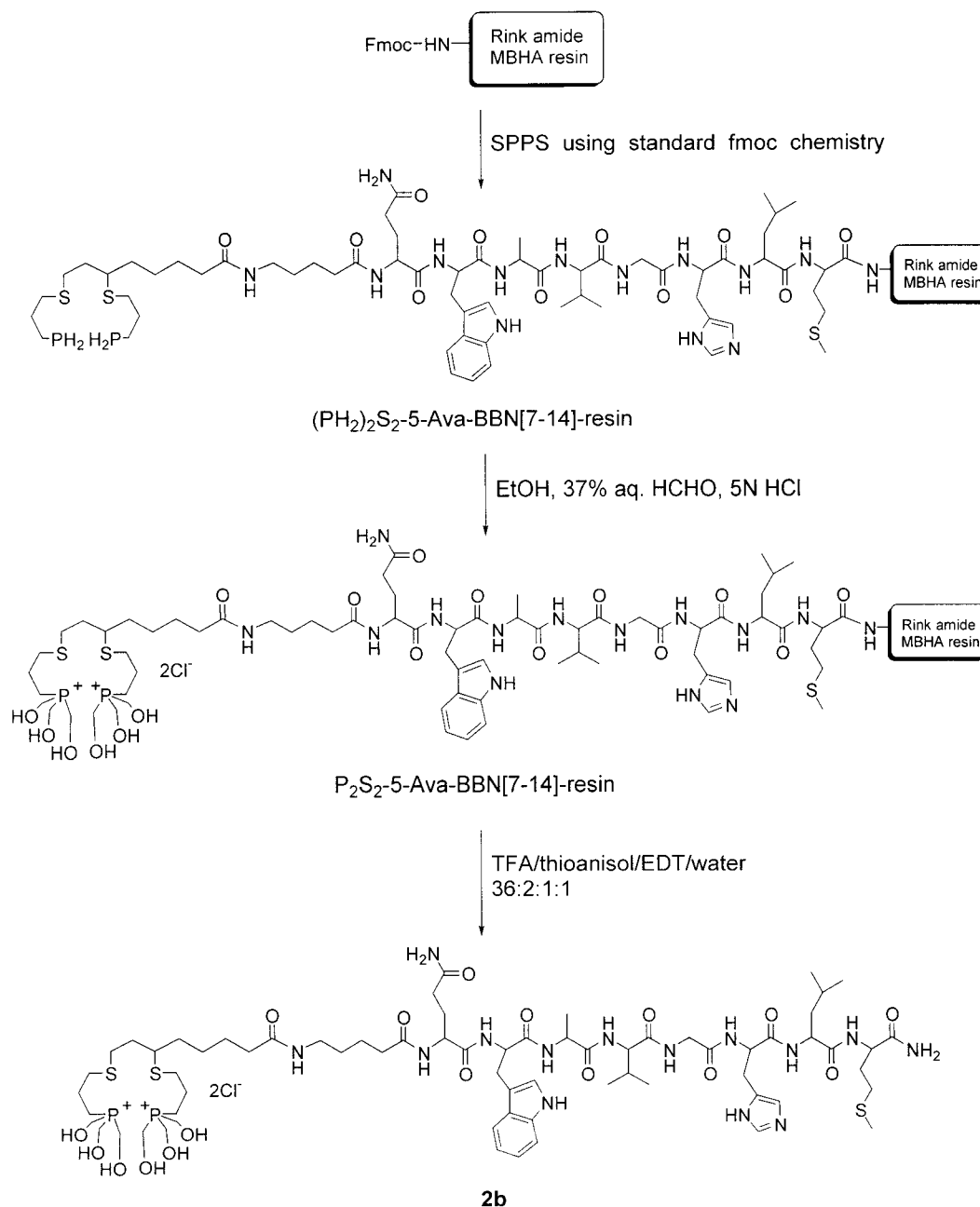
Preconjugation Labeling. The $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-COOH}$ complex was produced by the method described above. Upon confirmation of the formation of the $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-COOH}$ complex by HPLC, the PFP activated ester was prepared by reacting the $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-COOH}$ complex (400 μL , 185–740 MBq) with an excess of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (10 mg) and pentafluorophenol (PFP) (7 mg) in acetonitrile (100 μL).

Scheme 2. Complexation of $\text{P}_2\text{S}_2\text{-GlyGlyOEt}$ **2a with Re(V) and $^{99\text{m}}\text{Tc}$** 

The resultant solution was incubated at room temperature for 1 h. HPLC confirmed the formation of a single species at 17.1 min. An aliquot of the HPLC purified $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-PFP}$ (500 μL) solution was added to GlyGlyOEt (0.5 mg), and the pH was adjusted to 8.5 using 1 M sodium bicarbonate. The reaction mixture was incubated at room temperature for 30 min. HPLC confirmed the formation of the $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-GlyGlyOEt}$ complex **3b** as a single species at 10.6 min, with a yield of $\sim 95\%$.

$^{99\text{m}}\text{Tc}$ Labeling of $\text{P}_2\text{S}_2\text{-D-Lys}^6\text{-LHRH}$ (5**).** To a solution of $(\text{PH}_2)_2\text{S}_2\text{-D-Lys}^6\text{-LHRH}$ **2c** (575 μg , 0.36 μmol) in ethanol (400 μL) and DMF (100 μL) were added 0.1 N HCl (25 μL) and 37% aqueous formaldehyde (25 μL). The (hydroxymethyl)phosphine derivative of $\text{P}_2\text{S}_2\text{-D-Lys}^6\text{-LHRH}$ conjugate was obtained by adjusting the pH of the ligand solution to 8.5 using 1 M NaHCO_3 (25 μL). The concentration of the ligand solution was 1 $\mu\text{g}/\mu\text{L}$. Finally, 50 μL (50 μg , concentration in final 560 μL solution = 5.2×10^{-5} M) of ligand stock solution was added to a solution of $\text{Na}^{99\text{m}}\text{TcO}_4$ (500 μL , 185–370 MBq) in 0.9% saline. A 10 μL aliquot of saturated aqueous stannous tartarate was added to the solution. The solution (pH = 6) was incubated at room temperature for 30 min. The product $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-D-Lys}^6\text{-LHRH}$ **5**, was formed in yields of $\sim 88\%$. A retention time of 12.7 min indicated the formation of a singular species using HPLC conditions previously described.

^{188}Re Labeling of $\text{P}_2\text{S}_2\text{-5-Ava-BBN}[7\text{-}14]\text{NH}_2$ (4b**).** The free (hydroxymethyl)phosphine derivative ligand stock solution of the $\text{P}_2\text{S}_2\text{-5-Ava-BBN}[7\text{-}14]\text{NH}_2$ conjugate **2b** was prepared by treating **2b** (1.0 mg, 0.6 μmol) in water (200 μL) with 1 M aqueous sodium bicarbonate

Scheme 3. Solid-Phase Synthesis of P_2S_2 -5-Ava-BBN[7-14]NH₂ 2b

(20 μ L) (pH = 7.5). ^{188}Re labeling was achieved via transmetalation from ^{188}Re citrate. An aliquot of the P_2S_2 -5-Ava-BBN[7-14]NH₂ ligand stock solution (30 μ L, 150 μ g) was added to ^{188}Re citrate (700 μ L, 37–74 MBq) and heated at 90 $^\circ\text{C}$ for 30 min. Gentisic acid was added to the ^{188}Re citrate solution for quenching of the free radicals produced by high energy β^- radiation. ^{188}Re citrate was prepared by adding $^{188}\text{ReO}_4^-$ (500 μ L, 37–74 MBq) in 0.9% saline to a solution of stannous chloride (15 mg) in 1 M sodium citrate (200 μ L). The reaction mixture (pH = 5.5) was heated at 90 $^\circ\text{C}$ for 30 min. The product, $^{188}\text{Re}-P_2S_2$ -5-Ava-BBN[7-14]NH₂ conjugate **4b**, was formed in yields of $\geq 95\%$, as a single species as confirmed by reversed-phase HPLC.

In Vitro Binding Assay of $\text{Re}-P_2S_2$ -5-Ava-BBN[7-14]NH₂ Conjugate (4a). In vitro gastrin releasing peptide (GRP) receptor binding capabilities of the labeled peptide was assessed utilizing displacement binding assays conducted at 37 $^\circ\text{C}$ in Swiss 3T3 fibroblast cells, which exhibit high GRP receptor expression, by a method

similar to that designed by Mahmoud et al.⁴² The IC₅₀ value was determined for the nonradiolabeled $\text{Re}-P_2S_2$ -5-Ava-BBN[7-14]NH₂ analogue by analyzing displacement of bound ^{125}I -Tyr⁴-bombesin.

In Vivo Biodistribution of $^{188}\text{Re}-P_2S_2$ -5-Ava-BBN[7-14]NH₂ Conjugate (4b) in PC-3 Tumor-Bearing SCID Mice. The biodistribution and uptake of $^{188}\text{Re}-P_2S_2$ -5-Ava-BBN[7-14]NH₂ conjugate (**4b**) with PC-3 cell derived xenografts in SCID mice was studied. The SCID mice (average weight, 25 g) were injected in each flank of the limb leg with 5×10^6 human prostate cancer PC-3 cells (obtained from ATCC). These cells exhibit an overexpression of GRP receptors on their cell surface. When the tumor reached a weight of between 0.5 and 0.7 g (0.64 ± 0.03 g for these studies) at approximately six weeks after inoculation, aliquots (50–100 μ L) of the labeled peptide solution (55–75 kBq) were injected in each animal via the tail vein. Tissues, organs, and tumors were excised from the animals sacrificed at 1 h postinjection. The activity was counted in a NaI counter and

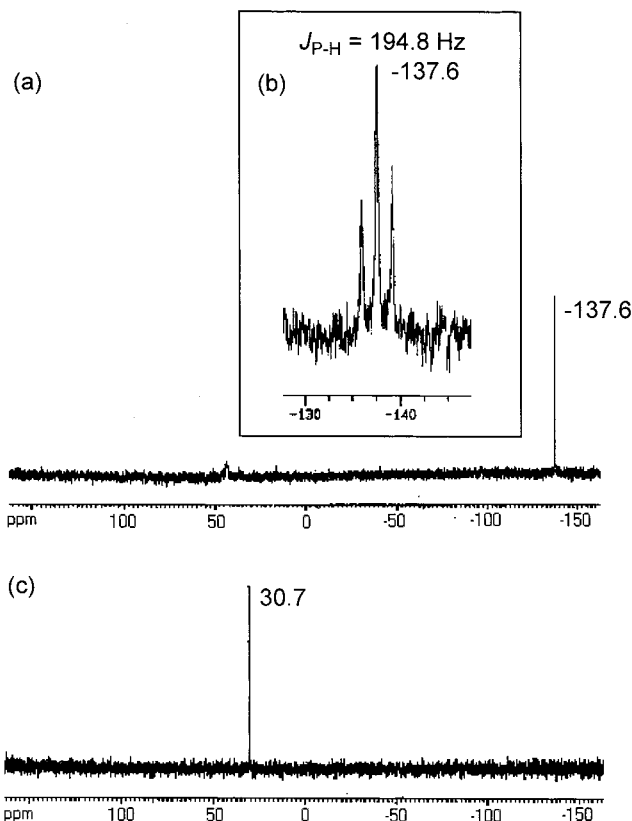


Figure 2. (a) ^{31}P and (b) Proton-coupled ^{31}P NMR spectra of $(\text{PH}_2)_2\text{S}_2$ -5-Ava-BBN[7-14] NH_2 **1b** and (c) ^{31}P NMR spectrum of P_2S_2 -5-Ava-BBN[7-14] NH_2 **2b**.

the mean percent injected dose per gram calculated. The percent injected dose (% ID) in the blood was estimated assuming a blood volume equal to 6.5% of the total body weight.

RESULTS AND DISCUSSION

To initial evaluate the feasibility of using the $(\text{PH}_2)_2\text{S}_2$ -COOH BFCA as an intermediate for labeling biologically active peptides via the postconjugation method with both Tc-99m or Re-188, a model peptide, glycylglycine ethyl ester (GlyGlyOEt) was selected. The $(\text{PH}_2)_2\text{S}_2$ -GlyGlyOEt conjugate **1a** was synthesized according to Scheme 1. The PH_2 groups in the peptide conjugate conjugate **1a** were formylated using aqueous formaldehyde in the presence of acid to yield $^+\text{P}(\text{CH}_2\text{OH})_3$ groups

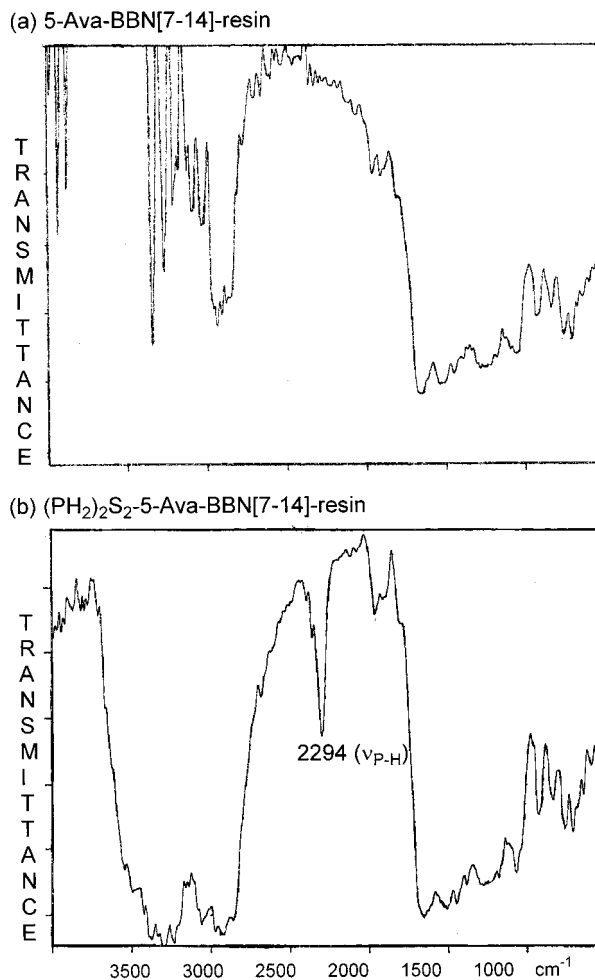
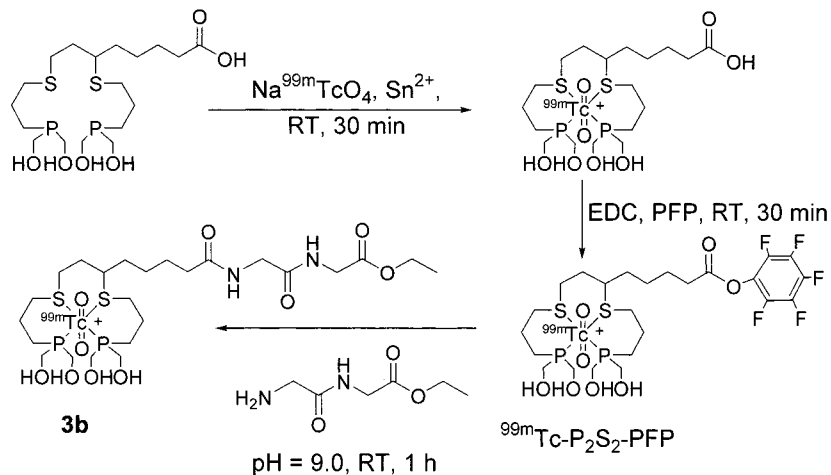
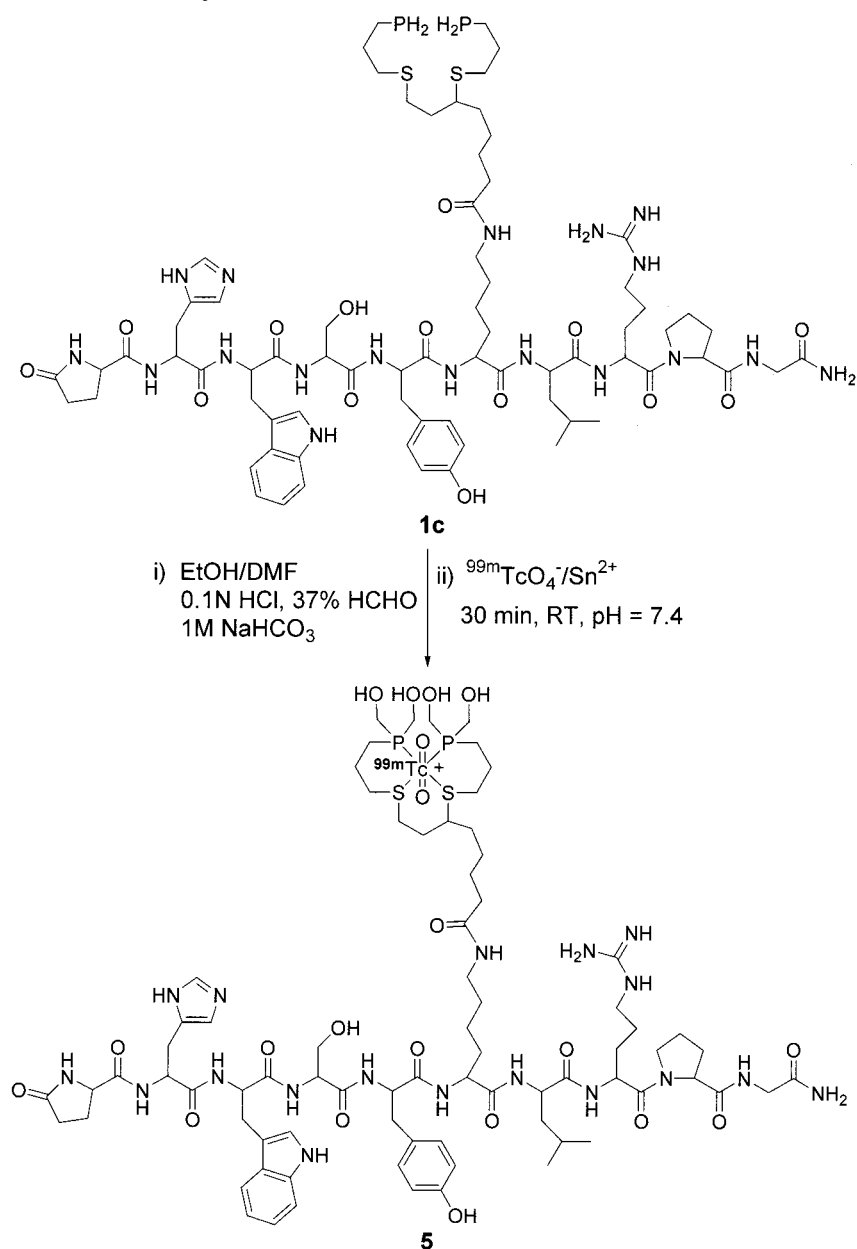


Figure 3. IR spectra of (a) 5-Ava-BBN[7-14]-resin and (b) $(\text{PH}_2)_2\text{S}_2$ -5-Ava-BBN[7-14]-resin.

(phosphonium salt) (Scheme 1). The formation of the phosphonium salt protects the P^{III} centers in the form of $\text{P}(\text{CH}_2\text{OH})_2$ ((hydroxymethyl)phosphine) groups against oxidation.^{43,44} Since the peptide conjugate in the form of the phosphonium salt can be stored for long periods of time under aerobic conditions, this provides an important advantage for formulating stable kit formation of radiopharmaceuticals.⁴⁵ We have previously shown that $\text{RP}(\text{CH}_2\text{OH})_2$ groups are stable, even under high levels of

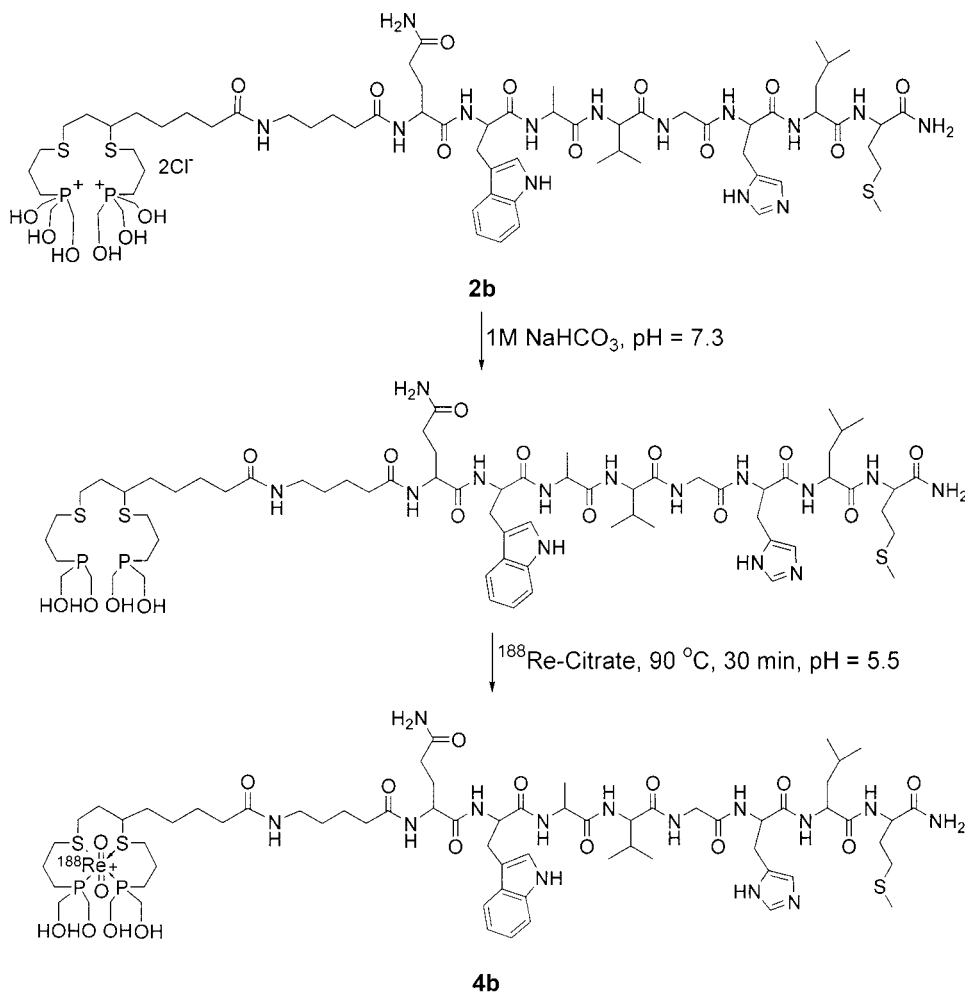
Scheme 4. Synthesis of $^{99\text{m}}\text{Tc-P}_2\text{S}_2$ -GlyGlyOEt **3b** Using Postconjugation Labeling Strategy



Scheme 5. Complexation of P_2S_2 -D-Lys⁶-LHRH **2c with ^{99m}Tc** 

dilution, in aqueous media for several days.^{29–35} The $^+P(CH_2OH)_3$ groups in the peptide conjugates, **2**, can be readily converted to $P(CH_2OH)_2$ groups by treatment with a base (bicarbonate buffer) prior to complexation with a metal center.^{33–35} The high σ -donating nature of the $R_xP(CH_2OH)_{3-x}$ groups allows these peptide conjugates the high affinity for coordination with transition metals.^{46–50} The P_2S_2 -GlyGlyOEt conjugate containing $P(CH_2OH)_2$ groups was reacted with a Re(V) precursor in water to yield the corresponding rhenium complex, ReO_2 - P_2S_2 -GlyGlyOEt **3a** (Scheme 2). The peptide conjugates **1a**, **2a**, and **3a** were characterized by using ^1H , ^{13}C , and ^{31}P NMR and IR spectroscopy and high-resolution FAB mass spectrometry. The spectral data is consistent with our previously reported ReO_2 - P_2S_2 -COOH complex, suggesting that the geometry around the Re(V) center in ReO_2 - P_2S_2 -GlyGlyOEt complex **3a** is identical to that in the ReO_2 - P_2S_2 -COOH complex.^{34,35} The conjugation chemistry of the $(PH_2)_2S_2$ -COOH precursor was extended to produce receptor-avid peptide conjugates P_2S_2 -5-Ava-BBN[7–14]NH₂ **2b** and P_2S_2 -D-Lys⁶-LHRH **2c** (Scheme 1).^{5–8,51–54} The peptide conjugate

2b was also synthesized in an alternative method using a solid-phase peptide synthesizer employing traditional fmoc chemistry (Scheme 3). The $(PH_2)_2S_2$ -COOH BFCA was introduced as the last residue in the sequence. Figure 3 shows the FTIR spectra of the resin containing 5-Ava-BBN[7–14]NH₂ before and after the coupling of the $(PH_2)_2S_2$ -COOH BFCA. The characteristic singlet band at 2294 cm^{-1} , attributed to P–H stretching in the IR spectrum of the $(PH_2)_2S_2$ -5-Ava-BBN[7–14]NH₂-resin, confirms the presence of the PH_2 functionality.³⁶ Treatment of the $(PH_2)_2S_2$ -5-Ava-BBN[7–14]NH₂-resin with aqueous formaldehyde in the presence of dilute hydrochloric acid, followed by cleavage from the resin, yielded the P_2S_2 -5-Ava-BBN[7–14]NH₂ **2b**. The ReO_2 - P_2S_2 -5-Ava-BBN[7–14]NH₂ conjugate **4a** was also synthesized using solid-phase peptide synthetic methods. All new peptide conjugates were characterized by ^{31}P NMR spectroscopy and electrospray mass spectrometry, and the purity was confirmed by HPLC. The presence of phosphorus atoms in these P_2S_2 -peptide conjugates provides an excellent opportunity for using ^{31}P NMR spectroscopy for monitoring the reactions and product for-

Scheme 6. Complexation of P_2S_2 -5-Ava-BBN[7-14]NH₂ **2b with ^{188}Re** 

mation as well as purity of the products in addition to HPLC. The metalated peptide $\text{ReO}_2\text{-P}_2\text{S}_2\text{-5-Ava-BBN-[7-14]NH}_2$ conjugate **4a** exhibited an IC_{50} value of 0.8 ± 0.4 nM for the GRP receptors expressed on Swiss 3T3 fibroblast cells, relative to ^{125}I -Tyr⁴-bombesin, which is similar to that observed with native bombesin.⁴²

Radiolabeling of the P_2S_2 -GlyGlyOEt **2a** with $^{99\text{m}}\text{Tc}$ was carried out using pre- and postconjugation labeling strategies as described in Schemes 2 and 4. In the preconjugation labeling approach (Scheme 4), the $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-COOH}$ complex was first prepared according to our previously reported procedure.^{34,35} The formation of the $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-COOH}$ complex was confirmed by HPLC. The carboxylic functionality was activated by formation of its pentafluorophenol (PFP) ester. The $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-PFP}$ activated ester was produced by a similar procedure reported in the literature.^{18,34,45} The HPLC chromatographic profile indicated the formation of the $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-PFP}$ complex as a single species at 17.1 min. The $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-PFP}$ complex was purified by HPLC and incubated with GlyGlyOEt at room temperature for 30 min at a pH of 8.5. The HPLC profile showed the formation of the $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-GlyGlyOEt}$ complex **3b** as a single species at 10.6 min with a yield of $\geq 95\%$. The retention time of the $^{99\text{m}}\text{Tc}$ -labeled species matched the corresponding $\text{ReO}_2\text{-P}_2\text{S}_2\text{-GlyGlyOEt}$ complex **3a**.

In the postconjugation labeling strategy (Scheme 2), the P_2S_2 -GlyGlyOEt conjugate phosphonium salt **2a** was treated with 1 M aqueous sodium bicarbonate (pH = 8.5) solution prior to complexation. A direct labeling procedure with $^{99\text{m}}\text{TcO}_4^-$ in the presence of stannous tartarate

was used. High labeling yields were obtained at a 0.1 mM P_2S_2 -GlyGlyOEt concentration at room temperature. The product, $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-GlyGlyOEt}$ complex **3b** was formed in a yield $\geq 98\%$, as a single species with a retention time of 10.6 min as determined by reversed-phase HPLC. Radiolabeling using both pre- and postconjugation labeling strategies produced same HPLC chromatograms demonstrating the formation of identical species. The utility of using this postconjugation labeling strategy with larger peptides was further tested by using the $(\text{PH}_2)_2\text{S}_2\text{-D-Lys}^6\text{-LHRH}$ conjugate **1c**. In this case, the $P_2S_2\text{-D-Lys}^6\text{-LHRH}$ conjugate phosphonium salt was produced in situ by formylation of **1c**, which was then treated with 1 M bicarbonate buffer to generate the required (hydroxymethyl)phosphine containing $P_2S_2\text{-D-Lys}^6\text{-LHRH}$ conjugate. The formation of the $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-D-Lys}^6\text{-LHRH}$ conjugate **5** was achieved by the postconjugation labeling reaction conditions described earlier. The product, $^{99\text{m}}\text{Tc-P}_2\text{S}_2\text{-D-Lys}^6\text{-LHRH}$ conjugate **5**, was formed in $\sim 88\%$ yield as a single species as confirmed by reversed-phase HPLC.

This postconjugation labeling strategy was also used for labeling of the P_2S_2 -peptide conjugate **2b** with ^{188}Re (Scheme 6). The $P_2S_2\text{-5-Ava-BBN[7-14]NH}_2$ conjugate **2b** was treated with 1 M aqueous sodium bicarbonate and adjusted to a pH of 7.5. ^{188}Re labeling was achieved via transmetalation from ^{188}Re citrate by heating at 90 °C for 30 min. Gentisic acid was added to the ^{188}Re citrate solution for quenching of the free radicals produced by high-energy β^- particle emissions. ^{188}Re citrate was prepared by addition of $^{188}\text{ReO}_4^-$ to a solution of stannous

chloride in 1 M sodium citrate. The reaction mixture (pH = 5.5) was heated at 90 °C for 30 min. The product, $^{188}\text{Re}-\text{P}_2\text{S}_2-5\text{-Ava-BBN}[7-14]\text{NH}_2$ conjugate **4b**, was formed in yields of $\geq 95\%$ yield, as a single species as confirmed by reversed-phase HPLC. The retention time of the ^{188}Re -labeled matched the corresponding $\text{ReO}_2-\text{P}_2\text{S}_2-5\text{-Ava-BBN}[7-14]\text{NH}_2$ conjugate **4a**, monitored at $\lambda = 280$ nm, and was found to be identical to the corresponding $^{99\text{m}}\text{Tc}-\text{P}_2\text{S}_2-5\text{-Ava-BBN}[7-14]\text{NH}_2$ conjugate previously reported.³⁴ The HPLC purified $^{188}\text{Re}-\text{P}_2\text{S}_2-5\text{-Ava-BBN}[7-14]\text{NH}_2$ conjugate was found to be stable over a period of 18 h with $\leq 5\%$ decomposition. This indicates that the $^{188}\text{Re}-\text{P}_2\text{S}_2-5\text{-Ava-BBN}[7-14]\text{NH}_2$ conjugate **4b** has an identical structure to that of the corresponding $^{99\text{m}}\text{Tc}$ -analogue. Furthermore, the new Re-188 conjugate is stable, even in the absence of excess tin and citrate. For example, the Re-188 conjugate was HPLC purified and found to be stable in vitro for time points in excess of 24 h. Preliminary in vivo studies in SCID mice bearing human prostate cancer xenografts derived from PC-3 cells show that $4.7 \pm 0.8\%$ ID/g of the $^{188}\text{Re}-\text{P}_2\text{S}_2-5\text{-Ava-BBN}[7-14]\text{NH}_2$ conjugate **4b** uptake in these tumors at 1 h postinjection. These data, compiled with the results that an average of 2.3% ID (1.8% ID/g) and average of 0.9% ID (1.0% ID/g) were found in the blood and stomach, respectively, show that this ^{188}Re conjugate retains high in vivo targeting specificity for GRP-receptor-expressing cells and undergoes minimal in vivo decomposition to $^{188}\text{ReO}_4^-$. The postconjugate labeling approach using the $(\text{PH}_2)_2\text{S}_2$ precursor as described herein provides a viable strategy to formulate freeze-dried kit preparations of P_2S_2 -peptide conjugates or other bioactive compounds that can be subsequently labeled in high yields with $^{99\text{m}}\text{Tc}$ or ^{188}Re .

CONCLUSION

The synthetic procedures described here successfully demonstrated the conjugation of the $(\text{PH}_2)_2\text{S}_2\text{-COOH}$ BFCA on peptide backbones that contain a free primary amine group. The peptide conjugates are used as precursors containing phosphine hydrides can be readily formylated to produce the corresponding hydrophilic, (hydroxymethyl)phosphine functionalized, peptide conjugates for potential applications in radiopharmaceutical formulations. The synthesis of the well-defined $^{99\text{m}}\text{Tc}-\text{P}_2\text{S}_2\text{-GlyGlyOEt}$ and radiolabeled receptor-avid peptides, $^{99\text{m}}\text{Tc}-\text{P}_2\text{S}_2\text{-D-Lys}^6\text{-LHRH}$ and $^{188}\text{Re}-\text{P}_2\text{S}_2-5\text{-Ava-BBN}[7-14]\text{NH}_2$, in high yields via postconjugation labeling techniques was demonstrated.

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Nanostructured DNA–Protein Aggregates Consisting of Covalent Oligonucleotide–Streptavidin Conjugates

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Covalent conjugates consisting of streptavidin and a 24-mer single-stranded DNA oligonucleotide have been oligomerized by cross-linking with a 5',5'-bis-biotinylated 169-base-pair double-stranded DNA (dsDNA) fragment. The oligomeric conjugates formed have been analyzed by nondenaturing gel electrophoresis and scanning-force microscopy (SFM). The comparison of analogous oligomers, prepared from native STV and the bis-biotinylated dsDNA fragment, revealed that the covalent STV–oligonucleotide hybrid conjugates self-assemble to generate oligomeric aggregates of significant smaller size, containing on average only about 2.5 times less dsDNA fragments per aggregate. Likely, this is a consequence of electrostatic or steric repulsion between the dsDNA and the single-stranded oligomer covalently attached to the hybrid, as indicated from control experiments. Nevertheless, the single-stranded oligonucleotide moiety within the oligomeric conjugates can be used as a selective molecular handle for further functionalization and manipulation. For instance, it was used for specific DNA-directed immobilization at a surface, previously functionalized with complementary capture oligonucleotides. Moreover, we demonstrate that macromolecules, such as STV and antibody molecules, which are tagged with the complementary oligonucleotide, specifically bind to the supramolecular DNA–STV oligomeric conjugates. This leads to a novel class of functional DNA–protein conjugates, suitable, for instance, as reagents in immuno-PCR or as building blocks in molecular nanotechnology.

INTRODUCTION

Molecular nanotechnology concerns the fabrication of well-defined nanoscaled structural and functional devices, using the “bottom-up” approach, in which small molecular building blocks self-assemble to form larger elements (1). Current advances in this area suggest that biological macromolecules are suitable components in the biomimetic synthesis of nanostructured elements and materials. For this purpose, DNA is a particularly powerful construction material (2–4). Due to its unique recognition capabilities, physicochemical stability, mechanical rigidity, and high precision processibility, DNA has extensively been used to fabricate nanostructured scaffolds (3) as well as for the selective positioning of proteins (5, 6), and metal and semiconductor nanoclusters (7–9). In addition, other biomimetic approaches to assemble inorganic nanoparticles have been reported which are based on the use of protein recognition systems, such as the bacterial surface layer proteins (10, 11) and the streptavidin–biotin system (6, 12).

We have recently reported on the self-assembly of bis-biotinylated DNA **1** and the biotin-binding protein streptavidin (STV) **2** which leads to the formation of oligomeric DNA–protein networks **3** (Figure 1) (13). The nanostructured networks had been characterized using scanning-force microscopy (SFM). These studies revealed that the STV molecules predominantly function as a bivalent or trivalent linker between adjacent double-stranded DNA (dsDNA) molecules, despite the tetravalent binding capacity of STV for biotinylated ligands. As a conse-

quence, the supramolecular DNA–STV networks **3** have a high residual biotin-binding capacity, and thus they can be employed as powerful reagents in Immuno-PCR (IPCR) (14), a method for the ultrasensitive detection of proteins and other antigens. In addition, the oligomeric networks **3** have also great potential for applications in biomolecular nanotechnology. For instance, the networks **3** can be used as a starting material for the synthesis of well-defined nanostructures, supramolecular DNA cycles consisting of a single STV and one dsDNA molecule (15). Moreover, the dsDNA–STV oligomers **3** are suitable model systems for basic studies on self-assembled nanoparticle networks and might even be used for the fabrication of ion-switchable nanoarchitecture (16).

We here report on the incorporation of covalent oligonucleotide–STV conjugates **4** as distinct building blocks within oligomeric dsDNA–STV networks **5** (Figure 1). Since the coupling of biotinylated macromolecules occurs preferentially at terminal STV molecules of the oligomeric chains **3**, and also, since some biotinylated compounds degrade the networks (13), we anticipated that the covalently attached oligonucleotide moiety in **4** might be a suitable molecular handle for the selective modification of oligomeric nanostructures **5**. For instance, the coupling of other macromolecules tagged with a complementary oligonucleotide should be attainable. Thus, oligomeric aggregates **5** containing the covalent hybrids were assembled and characterized using native gel electrophoresis and SFM. It was observed that the oligonucleotide-containing oligomeric aggregates **5** are significantly smaller in size than comparable aggregates **3** assembled from native STV. Nevertheless, the oligomers were found to be functional with respect to the hybridization of complementary nucleic acids. This allowed, for instance, the selective immobilization of **5** to capture oligonucleotide-modified surfaces, and the use of

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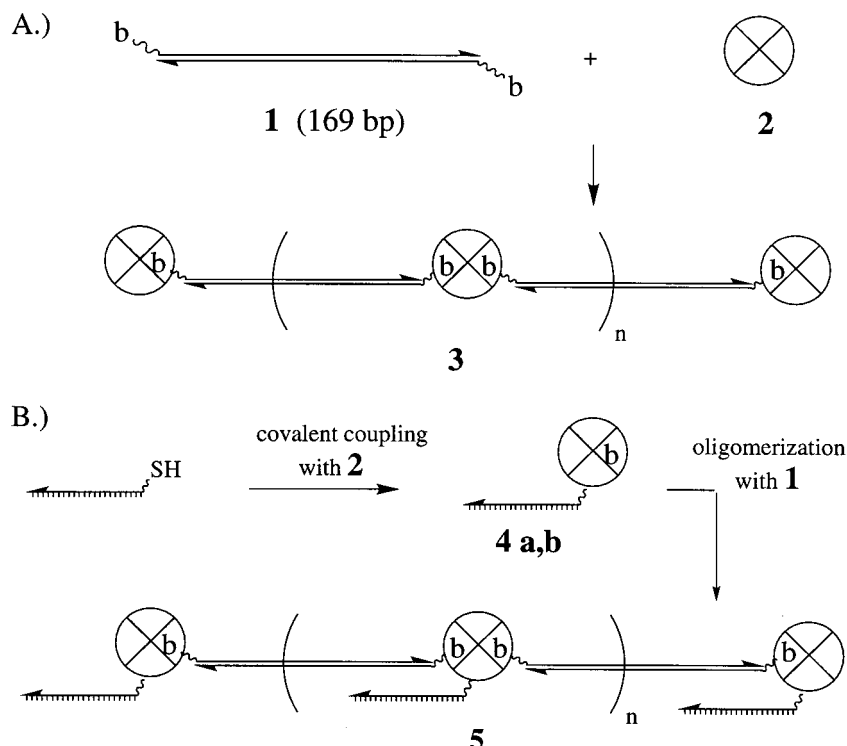


Figure 1. Synthesis of oligomeric streptavidin networks using 5',5'-bis-biotinylated DNA spacers. (A) Formation of supramolecular networks consisting of a 169 bp dsDNA fragment **1** and native streptavidin (STV) **2**. Note that the schematic drawing of the oligomers **3** is simplified, since branch-points occur due to the presence of tri- and tetra- valently conjugated STV (see Figure 3). (B) Assembly of networks from the dsDNA **1** and covalent oligonucleotide-STV conjugates **4**. The latter hybrid molecules were synthesized by covalent coupling of 5'-thiol-modified oligonucleotides and STV using a heterobispecific cross-linker (**5**). The assembly of **2** and **4** leads to the formation of oligomers **5** containing oligonucleotide moieties which can be used for functionalization of **5** by hybridization with complementary oligonucleotide-tagged macromolecules (see Figure 5). For simplification, complementary DNA strands are drawn as parallel lines. The 3'-ends are indicated by the arrowheads.

5 as a novel class of supramolecular reagents in IPCR applications.

MATERIALS AND METHODS

Synthesis and purification of covalent DNA-STV hybrids, **4a** and **4b**, were carried out from the corresponding thiolated oligonucleotides, 5'-thiol-AGC GGA TAA CAA TTT CAC ACA GGA-3' (**4a**), 5'-thiol-TCC GTG GTG AAA TTG TTA TCC GCT-3' (**4b**), respectively, and recombinant streptavidin **2** (IBA, Göttingen), similar as previously described (**5**). In brief, STV (10 nmol) was derivatized with maleimido groups using a heterobispecific cross-linker (sulfo-SMCC, Pierce), reacted with a 5'-thiolated oligonucleotide (10 nmol) and subsequently the unreacted maleimido groups were quenched with an excess of mercaptoethanol. The crude product was purified by anion-exchange chromatography, and the one-to-one molar ratio of the oligonucleotide and STV moiety within the hybrids was verified by gel-electrophoretic and photometric analysis (**5**). For control purposes, STV **2a** was activated with the cross-linker but quenched with an excess of mercaptoethanol instead of the reaction with the thiolated oligonucleotide.

The 169 base pair bis-biotinylated dsDNA fragment **1** was prepared from M13mp18 template (Promega) by preparative polymerase chain reaction (PCR), using two biotinylated primers: 5'-biotin-AGC GGA TAA CAA TTT CAC ACA GGA-3' (bcA) and 5'-biotin-AAG GCG ATT AAG TTG GG-3' (bG) as previously described (**13**). A monobiotinylated 169 bp dsDNA fragment **1a** was synthesized for the preparation of model conjugates. An additional bisbiotinylated 256 bp dsDNA fragment was prepared from pUC19 template, similar as described in

reference (**13**). The dsDNA products were purified by gel-filtration chromatography, quantified by photometry and characterized by nondenaturing agarose gel electrophoresis in the presence of a DNA molecular weight standard. Oligomeric conjugates **3** and **5** were prepared from recombinant STV **2** or the DNA-STV hybrids **4**, respectively. As an example, 1 μ L of the dsDNA, 3 μ M in 10 mM Tris buffer, pH 7.3 containing 5 mM EDTA (buffer A), was mixed with 14 μ L of STV or hybrids **4** (0.2 μ M in Buffer A), and the mixture was incubated typically for out about 24 h at 4 $^{\circ}$ C. The low ionic strength buffer A was chosen to avoid the condensation of the DNA fragments during immobilization on mica for SFM inspection (**16**). For gel electrophoretic analysis, the molar ratio of dsDNA to STV ranged from 4:1, 2:1, 1:1, 1:2, 1:4, to 1:10, respectively, as indicated in Figure 2.

Samples for SFM imaging were prepared by placing a 6 μ L drop of a solution containing 4 μ M MgCl_2 and about 500 nM DNA-STV conjugates on a Parafilm sheet. The droplet was adsorbed onto freshly cleaved mica and left to fix for 1 min. The drop was then quickly washed three times with 50 mL of deionized water and immediately blown dry for 5 min with nitrogen gas. The SFM inspection was carried out with commercial instruments (Digital Instrument, Dimension 3000 TM and Multimode III TM) using Si cantilevers purchased from Nanosensor TM. The SFM images were taken with instruments operating in high-amplitude dynamic mode with a home-made active feedback circuit (**17**), preventing the onset of intermitted contact (tapping). With this circuit, SFM can be run stable in the attractive interaction regime in air so that the interaction between the scanning tip and the sample is minimized.

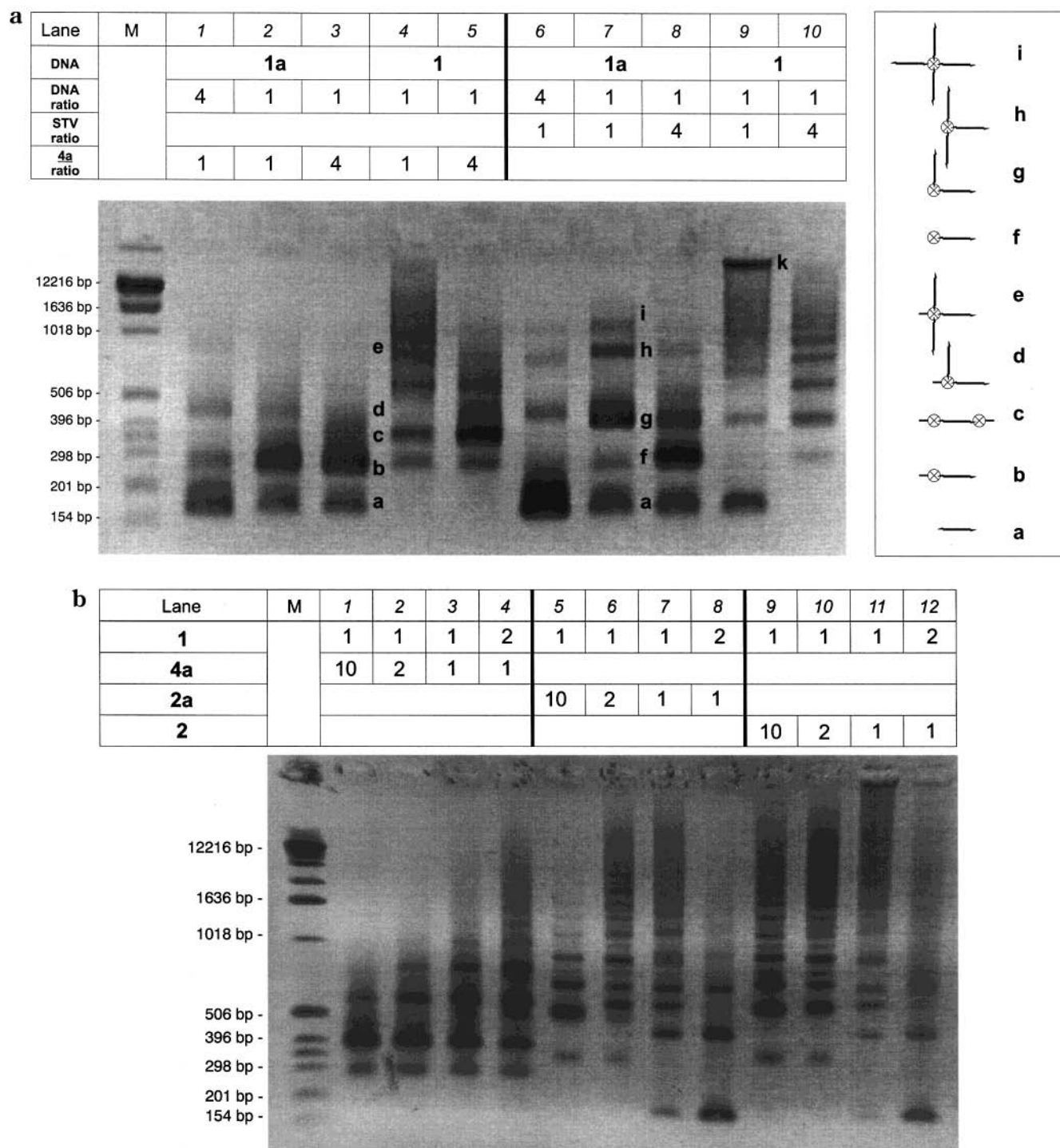


Figure 2. Gel electrophoretic analysis of the self-assembly of biotinylated dsDNA and streptavidin. (A) Formation of adducts from native streptavidin (STV) **2** and DNA–STV hybrid **4** with biotinylated dsDNA fragments. This is a nondenaturing 2% agarose gel stained with ethidium bromide. Adducts were formed by mixing bisbiotinylated dsDNA **1** with 1 or 4 mol equiv of native STV (lanes 9 and 10, respectively). The coupling of equimolar amounts of **1** and **2** leads to the formation of an immobile band (position k in lane 9), while in the case of the coupling of **1** and STV-hybrid **4a** (lanes 4, 5), individual bands of lower molar weight are observed. As model compounds, adducts obtained from STV and varying amounts of monobiotinylated dsDNA **1a** are analyzed in lanes 6–8. The analogous adducts obtained from hybrid **4a** are shown in lanes 1–3. The small letters at the right side indicate the mobilities observed for free dsDNA **1** (a) and the adducts STV–DNA₁ (b, f), STV₂–DNA₁ (c), STV–DNA₂ (d, g), STV–DNA₃ (e, h), and STV–DNA₄ (i). Lane M contains a DNA molecular weight marker, the lengths are indicated as base pairs. The free conjugate **4a** comigrates with the 396bp fragment of the molecular weight marker (not shown). (B) Comparison of native STV **2**, chemically activated STV **2a**, and DNA–STV hybrid **4a** in the aggregation with bisbiotinylated dsDNA **1**. This is a nondenaturing 1.5% agarose gel stained with ethidium bromide. A fingerprint-like band pattern of adducts is formed by mixture either **2**, **2a**, or **4a** with different ratios of **1**, ranging from 0.1 to 2 mol equiv of the DNA. Note the similarity of the patterns observed for **2** (lanes 9–12) and **2a** (lanes 5–8), compared to the smaller products obtained from **4a** (lanes 1–4). Some differences between **2a** and **2** are visible in lanes 7 and 11. While native STV and an equimolar amount of **1** form an immobile product band (lane 11, see also position k in Figure 2a), this band is missing for the STV **2a** (lane 7), and also smaller aggregates are obtained in the case of an excess of the STV components **2** and **2a** in lanes 9 and 5, respectively. In contrast, a completely different band pattern is obtained from **4a**, indicating the formation of a dumbbell-shaped conjugate STV₂–DNA₁ as the major product (position c in Figure 2a).

Solid-phase hybridization of the conjugates **5** were carried out on STV-coated microplates, functionalized with a biotinylated capture oligonucleotide, complementary to **4a**, similar as described earlier (18). A 30 μ L volume of a 3.3 fM solution of **5** in buffer A was incubated in the wells of this microplate for 30 min at room temperature, and after washing, the immobilized DNA was amplified by PCR. The low ionic strength buffer A was chosen to allow for comparison with previous experiments. Quantification of the PCR products was carried out by microtiter-plate based enzyme-linked immunosorbent assay (19).

For Immuno-PCR analysis, serial dilutions of rabbit IgG model antigen (Sigma, St. Louis, MO) immobilized in TopYield modules (Nunc) were used as a model antigen as previously reported (13, 19). For preparation of conjugates **6** and **7**, 5 μ L of a 500 nM solution of biotinylated goat anti-rabbit antibody (Coulter Immuno-tech, Hamburg, Germany) was either coupled with 15 μ L of a 166 nM solution of **3**, or else, similar amounts of the antibody were coupled with 10 μ L of a 250 nM of **4b**. In the latter case, the antibody-conjugated DNA-STV hybrid **4b** was quenched with excess D-biotin and subsequently mixed with equimolar amounts of the D-biotin saturated oligomer **5**. The mixture was incubated for 3 h at room temperature and then used in IPCR assay, as described (13).

RESULTS AND DISCUSSION

Self-Assembly of the DNA-Streptavidin Complexes 3 and 5. A 169 bp bis-biotinylated dsDNA **1** was synthesized by preparative PCR using two 5'-biotin derivatized primer oligonucleotides, and, for control purposes, the analogous mono-biotinylated dsDNA fragment **1a** was similarly prepared from one 5'-biotin derivatized and one unlabeled primer. Covalent hybrids of DNA and streptavidin, **4a** and **4b**, were synthesized from 5'-thiolated oligonucleotides and recombinant streptavidin by chemical cross-linking as previously described (5). The chief products, hybrids containing a single oligonucleotide moiety per STV, were purified by chromatography.

Figure 2a shows the electrophoretic comparison of the adducts of monobiotinylated **1a** and the STV hybrid **4a** (lanes 1–3), or else, with native STV (lanes 6–8), respectively. Lanes 2 and 7 compare the conjugates obtained from the coupling of monobiotinylated dsDNA **1a** with equimolar amounts of **4a** or STV **2**, respectively. The covalently linked oligonucleotide domain in **4a** influences the electrophoretic mobility of the monoadducts, STV–DNA₁ and **4a**–DNA₁, respectively (position b, f in Figure 2a). Moreover, it can be seen that the relative ratio of the mono- and bis-adducts is about 1:4 in the case of native STV, while in the case of the STV hybrid **4a**, this ratio is only about 5:1. This suggests that the affinity of **4a** for the biotinylated dsDNA **1a** is decreased compared to the native STV. The comparison of lanes 2 and 7 in Figure 2a supports this assumption. Native STV forms adducts of a relative stoichiometry of STV–DNA_{3,4} to a small but significant extent. The preferential formation of bis-adducts has previously been observed (13). However, in the case of the hybrid **4a** (lane 2), the analogous complex, **4a**–DNA₄ is entirely missing. The reduced affinity of **4a** for the biotinylated dsDNA can also be deduced from the comparison of lanes 4 and 9 (Figure 2a), in which the bis-biotinylated dsDNA **1** was mixed with equimolar amounts of STV **2** or **4a**, respectively. In the case of native STV, this coupling leads to

the formation of a single immobile band (position k), indicating the formation of large oligomeric networks **3** in lane 9. In contrast, the coupling of **4a** leads to the formation of individual bands, representing the presence of small supramolecular aggregates **5** (lane 4).

The reduced affinity of **4a** for the biotinylated dsDNA might be due to an increased steric or electrostatic repulsion of the dsDNA and the covalently bound oligonucleotide, or else, due to a decreased biotin-binding affinity itself, possibly caused by a conformational change induced by the chemical treatment of the STV's surface. The latter might be possible despite the fact that we had previously established the complete tetravalent binding capacity of the covalent hybrids **4** for the binding of low molecular weight D-biotin (**5**). To further elucidate why only small aggregates are formed from the hybrids **4**, controls were carried out with STV **2a**, activated with the cross-linker but not exposed to the thiolated oligonucleotide (Figure 2b). The coupling of various molar equivalents of the bis-biotinylated dsDNA **1** revealed an almost unchanged capability of the control STV **2a** for binding the biotinylated dsDNA, as compared to either the native STV **2** or the DNA-STV hybrid **4a**. These results suggest that the decrease in oligomer formation of **4a** is mainly due to steric and/or electrostatic repulsion of the dsDNA and the single-stranded oligomer. It seems likely that an increase in the ionic strength of the buffer would compensate for electrostatic repulsion. However, we have used a low salt 10 mM Tris buffer throughout this initial study for two reasons. First, these conditions are equal to that of a previous study, thereby allowing for a direct comparison of the results (13). Second, the use of higher salt concentration, in particular the presence of magnesium, leads to the formation of anomalous aggregate structures (16). In particular, a condensation of the DNA fragments occurs which makes the aggregates difficult to analyze by SFM. Nevertheless, we anticipate that the detailed optimization of coupling conditions, such as a systematic variation of polyvalent cations and ionic strengths, will lead to novel means of affecting the aggregate's supramolecular structure.

To study the structures of the oligomeric DNA-STV complexes, samples of **3** and **5** were imaged using dynamic SFM working in attractive regime mode. Representative images are shown in Figure 3, and statistical analysis of the complexes observed are given in Table 1. As expected from the gel electrophoretic characterization (Figure 2), the average size of **3** is more than 2-fold greater than that of **5**. On average, the oligomers **5** contain only about 2.0 dsDNA fragments per supramolecular conjugate. Even more significant, however, is the complete lack of highly oligomerized networks, containing more than 10 DNA molecules per conjugate (Table 1). Moreover, the comparison of the streptavidin's valency reveals that in the case of the oligomers **5**, on average, only 1.46 dsDNA fragments are coupled per protein particle. In contrast, an average STV valency of 2.4 is observed in the oligomers **3**. Experiments carried out with DNA-STV hybrids containing other sequences of the single-stranded oligomer, such as **4b**, led to similar aggregation patterns, as judged from gel-electrophoretic analysis (data not shown). These observations again indicate the decreased affinity of **4** for the binding of biotinylated dsDNA, as compared to native STV **2**.

Functionality of the Oligonucleotide Domain of Oligomers 5. As an initial test of the functionality of the oligonucleotide domain of **5**, the supramolecular aggregates **5** were hybridized to complementary capture oligonucleotides, previously immobilized in STV-coated

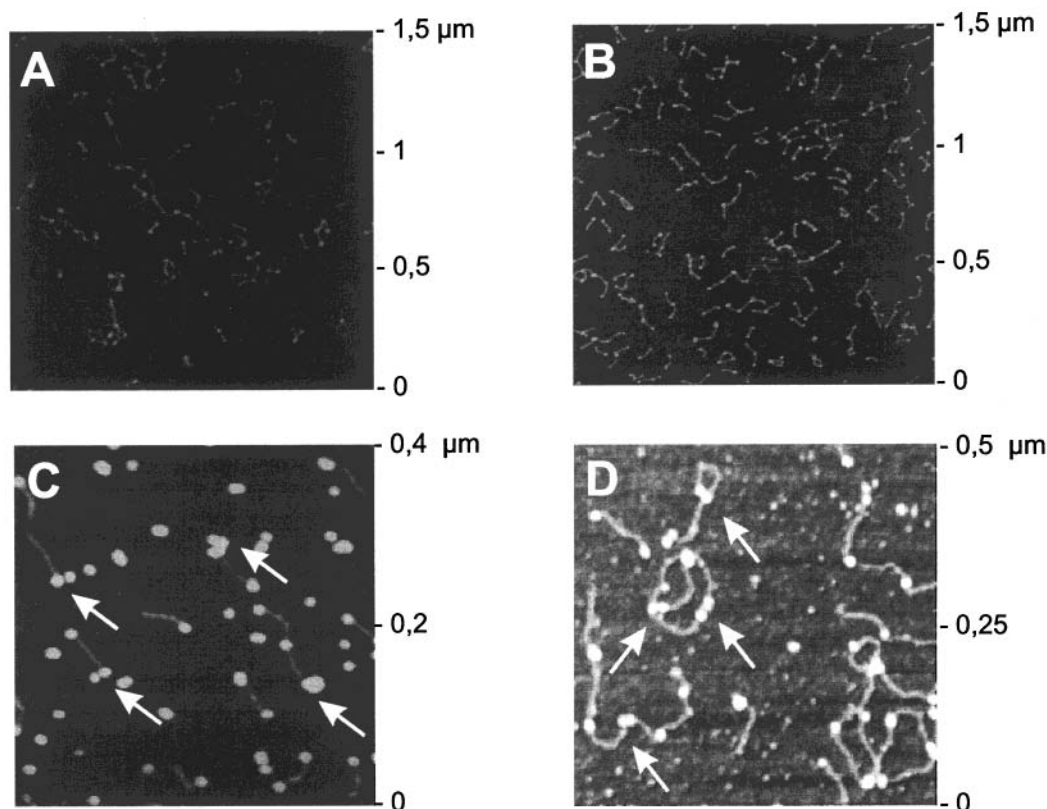


Figure 3. Scanning force microscopy images of oligomeric dsDNA-STV conjugates. (A) DNA-STV networks **3** obtained from native STV. (B) Oligomers **5** obtained from dsDNA **1** and covalent oligonucleotide-STV conjugates **4**. (C) A preformed oligomer **5** was coupled with the complementary DNA-STV hybrid **4b**. The arrows indicate the occurrence of **4a-4b** dimers, formed by hybridization of the two complementary oligonucleotide moieties. (D) Interconnected networks obtained from the self-assembly of two complementary STV hybrids **4a** and **4b**, subsequently oligomerized using a bis-biotinylated 256 bp dsDNA. Note that these nanoparticle networks contain two different dsDNA spacers, the ca. 87 nm dsDNA and the about 7 nm dsDNA formed by the hybridization of **4a** and **4b** (arrows). The latter dsDNA spacer fragment, however, cannot directly be observed by SFM imaging.

Table 1. Statistical Analysis of SFM Images of Oligomeric Complexes **3 and **5** Obtained from dsDNA **1** and Native STV **2** or STV Hybrid **4a****

	native conjugates dsDNA· 2	hybrid conjugates dsDNA· 4a
size distribution [%] ^b		
no. of DNA/complex		
1	26	49
2	36	29
3	19	13
4	7	3
5–10	9	5
>10	2	<0.5
size _{max} [DNA/complex] ^c	30	13
size _{avg} [DNA/complex] ^d	5	2
valency of STV [%] ^e		
monovalent STV	12	52
divalent STV	44	45
trivalent STV	37	3
tetravalent STV	7	<0.2
avg valency [dsDNA/STV]	2.40	1.46

^a The values are not affected by treatment of the oligomers **3** and **5** with an excess of D-biotin. The total number of molecules counted was greater than 2000. ^b The numbers are average values obtained from **4a** and **4b**, with a relative deviation between the two hybrids of less than 5%. ^c Percentage fraction of oligomeric conjugates, containing the number of dsDNA fragments specified below. ^d Maximum size of the supramolecular complexes. ^e Average size of the supramolecular complexes. ^f The valency depicts the number of biotinylated dsDNA fragments that are bound to individual STV molecules within the oligomers **3** and **5**.

microtiter plates (Figure 4). Successful binding of **5** was measured using PCR-amplification of the immobilized material and subsequent quantification by PCR-ELISA analysis (19). For comparison, similar amounts of **5** were

also incubated in wells, previously modified with a noncomplementary capture oligonucleotide. In addition, similar amounts of **5** were directly amplified by PCR to allow for estimation of the immobilization efficiency. As indicated in Figure 4, the immobilization efficiency of **5** was about 40%, and thus the assay provided evidence for the functionality of the oligonucleotide domain within the oligomers **5**. Since a single solid-phase binding contact leads to the adsorption of multiple DNA fragments; however, this efficiency is not necessarily identical with the hybridization efficiency of the oligonucleotide moiety in **5**. To estimate the latter, oligomers **5** were mixed with equimolar amounts of DNA-STV hybrid **4b**, containing a complementary oligonucleotide sequence, and the resulting conjugates were analyzed by SFM after hybridization (Figure 3C). In the resulting bioconjugates, STV-STV dimers were clearly distinguishable by SFM inspection. Statistical analysis revealed that about 20% of the STV hybrids **4a** have hybridized with the complementary hybrid **4b**.

As a further test of the hybridization capabilities, the two complementary STV hybrids **4a** and **4b** were mixed, and after hybridization, the dimers were subjected to oligomerization using bis-biotinylated dsDNA. A sample of the products was characterized by SFM (Figure 3D). The image obtained showed interconnected networks **5** in which the dimer of the two complementary conjugates **4** were clearly distinguishable. The use of the two complementary hybrids leads to the formation of “true” tetravalent connectors, the **4a-4b** dimers, and thus the size of the oligomers **5** is significantly increased. A maximum of up to 40 and, on average, about 4.4 dsDNA

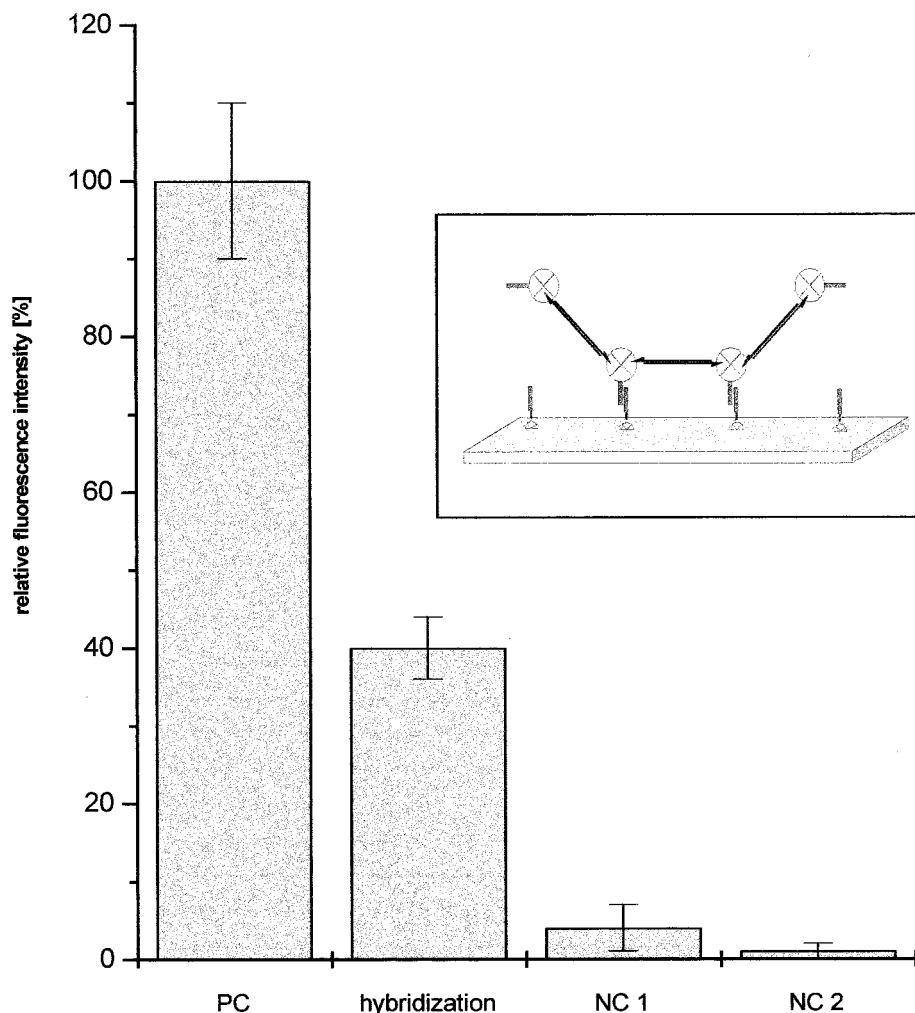


Figure 4. Site-specific immobilization of oligonucleotide-containing networks **5** by hybridization to complementary surface-bound capture oligonucleotides. The schematic drawing of the immobilized oligomers **5** is shown in the boxed inlay. The histograms represent the signal intensities obtained from PCR amplification of surface-bound oligomers **5**. As a positive control (PC), similar amounts of **5** were directly amplified by PCR. Note that the comparison of signals obtained from surface hybridization of **5** and PC indicates an immobilization efficiency of about 40%. As negative controls, similar amounts of **5** were incubated in wells, modified with noncomplementary capture oligonucleotides (NC1), or else no conjugate **5** was applied to the wells (NC2).

fragments were present in the individual oligomers. This is more than twice as much as observed in the regular oligomers **5**, and the size of the networks even exceeds those obtained from native STV **2** (Table 1).

The Use of Oligomers **5 As Reagents in Immuno-PCR.** We had previously demonstrated that immunoglobulin (IgG)-functionalized oligomers **3** are powerful reagents in immuno-PCR (IPCR) (13), in particular, when preformed and purified IgG-containing aggregates are used (see **6** in Figure 5). The employment of purified **6** leads to an about 100-fold enhancement of IPCR sensitivity, compared to the standard IPCR protocol using successive coupling strategies. Likely, this is not only a consequence of the oligomeric nature of **6**, leading the binding of multiple DNA marker fragments per antigen contact. In addition, the oligovalency of **6** should allow for cooperative avidity effects due to a polyvalent interaction with the surface-immobilized target structures to be detected (13). To accomplish an efficient IgG-coupling with the oligomeric dsDNA-STV conjugates, we reasoned that the linkage with biotinylated IgG might be attained by hybridization of the oligomers **5** with a preconjugate, obtained from complementary STV hybrid **4b** and a biotinylated antibody (Figure 5). To investigate this hypothesis, an oligomeric conjugate **7** was prepared from equimolar amounts of **5** and a conjugate of **4b** and

biotinylated anti-mouse IgG. As a control, a similar mixture was prepared from **5** and an antibody-STV hybrid conjugate that is noncomplementary to the oligonucleotide moieties within **5**. The resulting bioconjugates were analyzed by nondenaturing gel electrophoresis (data not shown). While the control sample only showed the typical band pattern of oligomer **5**, the presence of an immobile band in the case of **7** suggested the formation of high molecular weight species, likely the supramolecular oligomer proposed in Figure 5.

To further evaluate the formation of **7** the functionality of the putative bioconjugate was tested in the IPCR detection of a rabbit IgG model antigen. For comparison, the negative control, consisting of the noncomplementary STV hybrid **4b**-IgG conjugate described above, and also the conventional supramolecular oligomer **5** were used as reagents. The IPCR signals obtained in this assay are shown in Figure 6. While in the case of the negative control no significant signal intensity was observed, both supramolecular reagents, **6** and **7**, revealed distinct IPCR performance. Both preparations were used as crude assembly products without any purification, and thus the reagent **6** was of lower performance than the conventional IPCR, using successive incubation of the biotinylated antibody, STV, and the biotinylated dsDNA marker (19). Strikingly, the supramolecular oligomer **7** showed

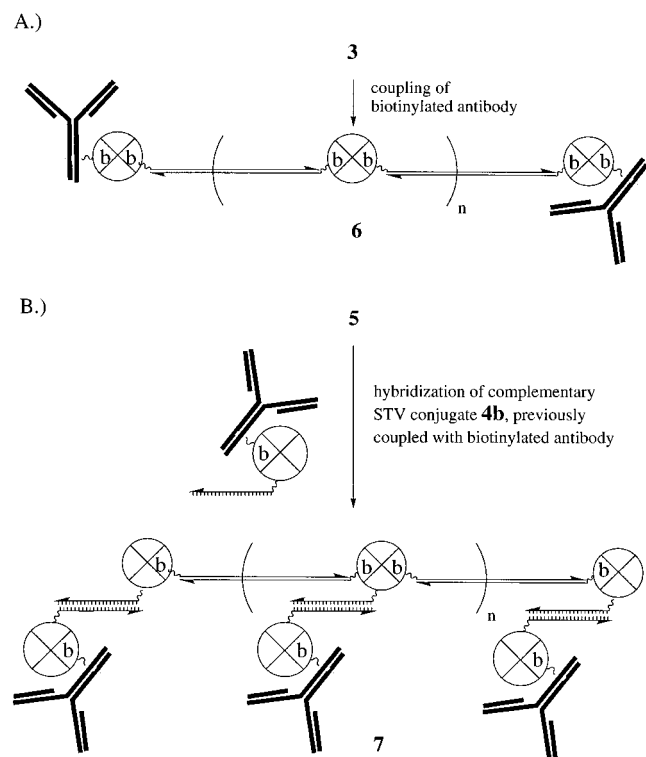


Figure 5. Functionalization of oligomeric DNA–STV networks with biotinylated antibodies. (A) Direct coupling of oligomers **3** with a biotinylated antibody. The conjugation predominantly occurs at the terminal STV molecules of the oligomers (**13**). (B) Functionalization of the oligonucleotide-containing oligomers **5**, using a conjugate previously assembled from a complementary STV hybrid **4b** and a biotinylated antibody.

greater signals, suggesting the efficient incorporation of the biotinylated antibody. We anticipate that the performance of this novel class of reagents might further be optimized, for instance, by variations in the coupling stoichiometry, ionic strength of reaction buffers, and also the purification of the supramolecular oligomers after their assembly.

CONCLUSIONS

In this report, oligomeric aggregates consisting of bis-biotinylated dsDNA fragments and the biotin-binding protein STV have been functionalized by incorporating semisynthetic building blocks, covalent conjugates of STV, and a single-stranded oligonucleotide moiety. The incorporation of the latter hybrid molecules led to significant decrease of the size of the dsDNA–linked oligomeric aggregates, compared to those obtained from native STV which form large oligomeric networks. This indicates a decreased affinity of the covalent STV hybrids for binding biotinylated DNA fragments, likely due to steric or electrostatic repulsion of the DNA moieties. Nevertheless, the covalently attached oligonucleotide of the STV hybrids can be used as a selective molecular handle for convenient modification of the oligomeric DNA–STV aggregates. For instance, the oligonucleotide moiety was used to specifically immobilize the oligomeric bioconjugates to capture oligomer-modified surfaces or else for generating interconnected networks consisting of two different lengths of dsDNA linkers. Moreover, the coupling of immunoglobulins, tagged with complementary oligonucleotides, was attained by nucleic acid hybridization. This leads to a novel class of functional supramolecular bioconjugates, suitable, for instance, as reagents

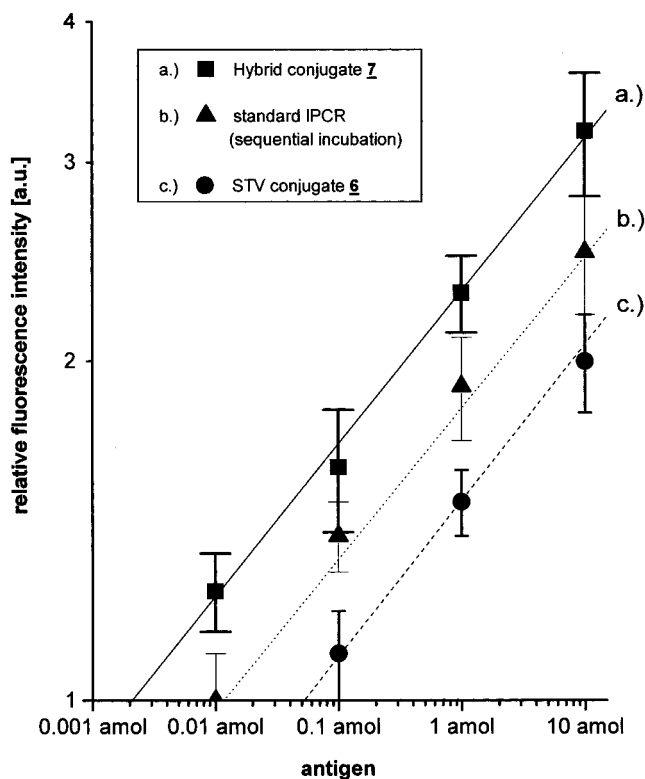


Figure 6. Use of the supramolecular bioconjugates **6** and **7** shown in Figure 5 as reagents in Immuno-PCR. The signals were obtained in the Immuno-PCR detection of rabbit immunoglobulin as a model antigen, using either the antibody-functionalized oligomer **6**, or else the antibody-functionalized supramolecular bioconjugate **7** as a reagent. Signal intensities are given as normalized values, obtained from fluorescence intensities of the samples divided by the intensity of the negative controls in which no antigen was present. Both supramolecular bioconjugates were used as crude assembly products without further purification. The signal intensities were obtained by PCR-ELISA quantification of the IPCR products.

in immuno-PCR. Further studies of this approach will focus on the development of optimized coupling conditions, in particular the systematic variation of cations and ionic strengths, to attain an efficient hybridization of oligonucleotide-containing supramolecular networks and complementary oligonucleotide-tagged macromolecules.

This manuscript describes initial steps of the systematic development of self-assembled DNA–protein conjugates. The novel aggregates described here almost exclusively form chainlike structures and thus are much more regular than aggregates obtained from native STV displaying randomly branched networks. We anticipate that the extensive study of such systems will eventually lead to novel approaches toward the use of such components as building blocks in biomolecular nanotechnology. DNA–STV aggregates might serve as molecular framework for the bottom-up construction of devices, decorated by attachment and selective positioning of biotinylated functional molecular components, such as proteins (20), inorganic metal nanoclusters (6), or low molecular weight peptides and fluorophores (21). Further exploration of nucleic acid- and streptavidin-based recognition systems will open the door to the fabrication of novel biomaterials and thus will contribute to the current development of the novel discipline, descriptively termed as “nanobiotechnology”. Applications might include the fabrication of supramolecular hybrid materials, useful as nanosensors, catalytic and light-harvesting devices, ultrafast

molecular switches and transistors, supramolecular mediators between electrical and living systems, and other bio- and optoelectronic elements.

ACKNOWLEDGMENT

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Quantitative Assays of the Amount of Diethylenetriaminepentaacetic Acid Conjugated to Water-Soluble Polymers Using Isothermal Titration Calorimetry and Colorimetry

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The level of conjugation of diethylenetriaminepentaacetic acid (DTPA) to the polysaccharide sodium hyaluronan (HA) has been measured by a colorimetric assay, isothermal titration calorimetry (ITC), and ^1H NMR spectroscopy. The colorimetric assay is based on the red shift, upon complexation with gadolinium ion (Gd^{3+}), of the wavelength of maximum absorption of the dye arsenazo III. It can be performed in a few minutes using as little as $10\ \mu\text{g}$ of polymer with a detection limit of $\sim 0.03\ \text{mmol}$ of DTPA ($\text{gram of polymer}^{-1}$). The ITC measurements yield values of the amount of DTPA linked to HA identical to those obtained by colorimetry. The levels of DTPA conjugation calculated by integration of signals at 3.1–3.2 ppm (DTPA protons) and at 2.0 ppm (HA acetamide protons) in the ^1H NMR spectrum of HA–DTPA are consistently overestimated by a factor of ~ 2 , compared to the data obtained by ITC and colorimetry. The longer relaxation times of protons of the polymer backbone, compared to those of protons attached to the freely moving DTPA side-chains may account for the discrepancy.

INTRODUCTION

Magnetic resonance imaging (MRI) contrast agents are intended to improve diagnosis of disease by changing tissue signal intensity and, consequently, to enhance detection and characterization of lesions. The more specific the accumulation of contrast agent within a target tissue, the better the resulting lesion–tissue contrast. An effective approach toward precise targeting of the imaging agent consists of its conjugation to a biopolymer, the “carrier” (1), with inherent affinity for a particular lesion. Typical carrier systems include antibodies, proteins, peptides, polysaccharides, such as dextrans (2, 3), sodium hyaluronate (4), and other natural polymers (5–9).

The contrast agents most widely used in MRI are complexes of the gadolinium ion (Gd^{3+}) and polyaminocarboxylate ligands, such as diethyltri-aminepentaacetic acid (DTPA) (2, 5, 10–13). Indeed, Magnevist, the contrast agent currently used clinically is an aqueous solution of DTPA–Gd. To date, no polymer derivative of DTPA–Gd is available for routine clinical applications. We developed recently several syntheses of DTPA–Gd conjugates of hyaluronan (HA), an alternating copolymer of D-glucuronic acid and 2-acetamido-2-deoxy- β -D-glucopyranose (Figure 1) (4). As the project progressed, it became imperative to be able to determine precisely the amount of DTPA–Gd linked to the polymer. At first, we relied on high-field ^1H NMR spectroscopy, since in spectra of the DTPA–HA conjugates, signals attributed to protons linked to the polymer backbone and signals due to the resonance of the chelate protons were distinct enough to allow precise integration. However, the ratios chelate: polymer chain determined by ^1H NMR turned out to be

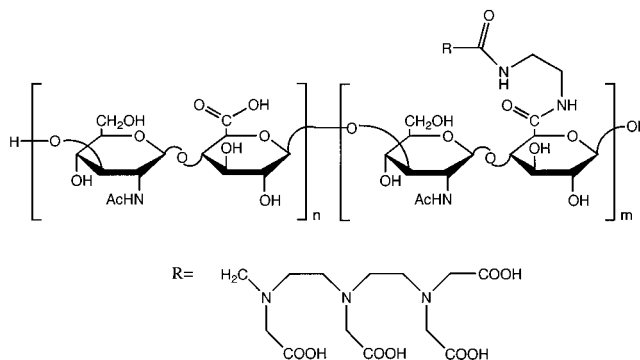


Figure 1. Chemical structure of HA–DTPA.

artificially high, since this analysis does not take into account the fact that relaxation times of protons linked to the freely rotating chelate moieties are usually much shorter than the relaxation times of protons on the polymer backbone (14). Several quantitative analytical methods have been reported for the determination of DTPA/polymer coupling efficiency. They include elemental analysis (5), EDTA titration (2), and acid–base titration (15). We evaluated all the published methods to determine the efficiency of DTPA/HA conjugation and observed that none of the standard protocols yielded sufficiently accurate results to allow reliable comparisons of polymer characteristics and performance as contrast agents *in vivo*. This situation led us to design two new analytical methods: a colorimetric assay with the dye arsenazo III and a Gd binding assay by isothermal titration calorimetry (ITC).

We report here the detailed protocols of the colorimetric and ITC assays using two samples of HA–DTPA. We compare the results of the newly developed assays with data obtained from elemental analysis and ^1H NMR spectroscopy performed on the same polymers. The methods are assessed in terms of ease of performance,

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accuracy, repeatability, and sensitivity. Although the assays have been tested only on hyaluronan–DTPA conjugates, they should be applicable also to the analysis of other DTPA-conjugates of water-soluble polymers and can be modified easily to the quantification of chelators other than DTPA.

EXPERIMENTAL SECTION

Materials. Sodium hyaluronate (HA, ~500 kDa) was a gift of Hyal Pharmaceutical Corporation (Mississauga, ON, Canada). Gadolinium chloride hexahydrate ($\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$), europium chloride (EuCl_3), and arsenazo III (2,2'-[1,8-dihydroxy-3,6-disulfonaphthylene-2,7-bisazo]-bisbenzene arsonic acid) were purchased from Aldrich Chemical Corporation and used as received. The following buffers were prepared using water ($R = 18 \text{ M}\Omega \text{ cm}$) deionized by a Millipore water purification system: pH 5.25: acetic acid–sodium acetate (0.1 M); pH 2.0: hydrochloric acid–sodium chloride (0.1 M); pH 6.5: PBS (4.400 g of NaCl, 7.576 g of Na_2HPO_4 , 1.600 g of NaH_2PO_4 in 1 L, adjusted with HCl 1 N) or MES (2.13 g of morpholineethanesulfonic acid in 1 L, adjusted with HCl 1 N). HA–DTPA (see Figure 1) was prepared following methods described elsewhere (4, 16). Briefly, HA was reacted first with ethylenediamine in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide as the coupling agent in a 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer solution (pH 4.5). The ethylenediamine-modified HA was purified by dialysis. It was reacted with DTPA activated separately with a dicyclohexylcarbodiimide/*N*-hydroxysulfosuccinimide mixture in dimethyl sulfoxide (DMSO) prior to the addition. The resulting HA–DTPA was purified by three consecutive ultrafiltrations using membranes with a molecular cutoff of 3500 Da.

The following test was performed in order to confirm the absence of free DTPA in purified HA–DTPA. A solution of HA and DTPA (0.15 mol DTPA/1 mol Glucuronic acid) in water (pH 9.0) was subjected to three consecutive ultrafiltrations with water. The combined filtrates were lyophilized yielding a solid residue identified as DTPA. The amount of DTPA recovered was determined by the arsenazo test (see below) and by gravimetry. Both techniques agreed, indicating a recovery of $97\% \pm 1\%$.

^1H NMR Experiments. ^1H NMR spectra were run with a 400 MHz NMR spectrometer (Bruker). Samples (polymer concentration: 1% w/w) were prepared in deuterated water (Cambridge Isotope Inc). Typically, 96 scans were recorded with interscan delays of 1 or 5 s and water was used as an internal standard. The intensity of the signal at 3.1 ppm (2 protons of the DTPA amide methylene) was determined and compared with the intensity of the methyl amide group of the glucuronic residue (3 protons). The level of DTPA incorporation was calculated from the ratio of the signals at 3.1 and 2.0 ppm assuming a level of incorporation of 100% for a ratio of 0.66.

Isothermal Titration Calorimetry. Binding isotherms were obtained using a Microcal VP–ITC ultrasensitive titration calorimeter (MicroCal Inc., Northampton, MA). The sample cell had a volume of 1.43 mL. It was filled with an aqueous buffered solution of either DTPA (0.25 mM) or HA–DTPA (0.60 mM Glu). An aqueous buffered solution of Gd^{3+} (6.5 mM) was placed in a 300- μL continuously stirred (300 rpm) syringe and injected into the cell in 15 to 25 5- μL aliquots, delivered over 10 s in intervals of 6–8 min. Data points were

collected every 2 s. The measurements were performed at 25 °C. All titrations were carried out three times to ensure consistency of the data and stability of the solutions. This was particularly important in the case of measurements conducted at pH 2. Even under such acidic conditions, data was reproducible, confirming the stability of HA–DTPA during analysis. Data analysis was carried out using the Microcal ORIGIN software. The principles and basic thermodynamic conventions of ITC are discussed in a recent publication (17).

Colorimetric Assays. Absorbance spectra were measured with a Hewlett-Packard 8542A photodiode array spectrometer. A calibration curve was obtained by measuring the absorbance at 660 nm of a series of standard solutions of the arsenazo III/ Gd^{3+} complex prepared by mixing stock solutions of GdCl_3 (0.2 mM) and arsenazo III (0.2 mM) in pure water.

Method I. Solutions for analysis were prepared by adding to a solution of HA–DTPA (10 mg L^{-1} , 1.0 mL) an excess of Gd^{3+} in aqueous solution (~0.6 to 1.0 mL), followed by an arsenazo III stock solution (1.0 mL), the final solution being made up exactly to 10 mL. The absorbance of the solution at 660 nm was measured. The concentration of Gd^{3+} that is not bound to HA–DTPA was determined using the calibration curve fitted with a 3rd order polynomial function. The concentration of polymer-bound DTPA was obtained by subtracting the excess Gd^{3+} determined colorimetrically from the total amount of Gd^{3+} added to the sample.

Method II. Excess $\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$ (usually about 4–7 mg depending on the expected level of DTPA conjugation) was added to a solution of HA–DTPA (20 mg in 13 mL water). The solution was subjected to ultrafiltration using a Diaflo system fitted with YM-10 membranes (Amicon, Beverly, MA, molecular weight cutoff: 10 kDa). The polymer solution was washed with 10-mL portions of water, and four samples of filtrate (10.0 mL each) were collected during ultrafiltration. The concentration of Gd^{3+} in the filtrate was analyzed as follows: An aliquot of the filtrate (500 μL) was added to the arsenazo III stock solution (1.0 mL). The solution was made up to exactly 10 mL. The absorbance of the solution was measured at 660 nm. The arsenazo III/ Gd^{3+} concentration of the sample was determined using the calibration curve. This value corresponds to the excess Gd^{3+} added to the HA–DTPA solution analyzed. The concentration of polymer-bound DTPA was obtained by subtracting the excess Gd^{3+} determined by colorimetry from the total amount of Gd^{3+} added to the sample.

RESULTS AND DISCUSSION

Isothermal Titration Calorimetry. ITC is commonly used to determine the thermodynamics of substrate/ligand interactions, since it provides in a single measurement the enthalpy of binding ($\Delta H_{\text{binding}}$), the equilibrium constant K , and the stoichiometry of binding (17). The quantitative determination of the number of DTPA groups linked to a polymer backbone relies on the precise measurement of the number of binding sites offered to Gd^{3+} by the conjugated polymer. Such measurement has to be performed at concentrations above K_D of the complex. The binding constant of the DTPA–Gd complex was measured previously by potentiometry [18], but there are no reports of its determination by ITC. To validate our protocol, we set about to carry out a titration of the

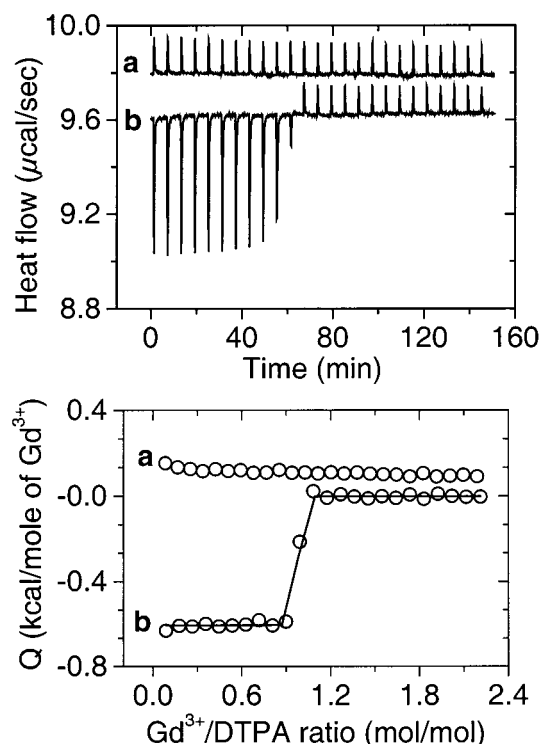


Figure 2. Titration of Gd^{3+} (0.2 mM) into water (a) and aqueous DTPA (0.25 mM) (b) at 25 °C and pH = 5.25. Above: calorimetric traces (heat flow against time). Below: reaction enthalpy (obtained by integrating the peaks in curves above) vs the $\text{Gd}^{3+}/\text{DTPA}$ ratio (mol/mol).

DTPA–Gd system, before undertaking the analysis of polymer-bound DTPA. Experimental titration curves of a concentrated Gd^{3+} solution in buffered water (curve a) and in a buffered aqueous solution of DTPA (curve b) are represented in Figure 2 (top). In the simple dilution experiment (curve a), each injection produces a small endothermic signal corresponding to the heat of dilution of Gd^{3+} in the buffer. In the binding experiment (curve b), the first injections produce strong exothermic signals associated with the formation of the DTPA–Gd complex. Then, as the Gd^{3+} concentration reaches the value corresponding to a DTPA/ Gd^{3+} molar ratio of 1, the signal decreases rapidly and at even higher $[\text{Gd}^{3+}]$ the signal takes a constant endothermic value, corresponding to ΔH_{dil} . The dilution enthalpy and the binding enthalpy were determined by integration of each peak in the respective experimental curves (Figure 2, bottom). We note that ΔH_{dil} decreases slightly with increasing Gd^{3+} concentration. This effect is taken into account in the data treatment by subtracting the heat of dilution from the raw binding data. The curve corresponding to the titration shows a sharp transition for DTPA/Gd = 1. From the enthalpy of the transition one can calculate the binding constant. It turns out that the DTPA–Gd binding constant at pH = 5.25 is too high to be determined accurately by ITC (17). The suggested upper limit of detection is $\sim 10^8 \text{ L mol}^{-1}$. The reported value at this pH is more than 5 orders of magnitude higher. To circumvent this difficulty, we determined a conditional binding constant K_{cond} at a lower pH (2.0). The thermodynamic stability constant, K_{therm} , was calculated using the eq 1 (acidity constants are reported elsewhere (18)) where K_1 is the acidity constant of H_5DTPA , K_2 is the acidity

constant of H_4DTPA^- , K_3 is the acidity constant of $\text{H}_3\text{DTPA}^{2-}$ and so on. The value of pK (23.02) thus determined compares satisfactorily with the reported value 22.46 (18). The binding constant of HA-conjugated DTPA was determined at pH 2.0, following the same protocol. A thermodynamic pK value of 23.3 was calculated from eq 1, using the acidity constant of free DTPA as an approximation. This value turns out to be identical, within experimental error, to the pK of free DTPA, in contrast to previous reports indicating that the DTPA–Gd binding constant for protein conjugated DTPA is lower than that of free DTPA (19). For example, pK values of 18.80 and 18.87 were determined for BSA–DTPA–Gd and IgG–DTPA–Gd, respectively. Further experiments are in progress to assess the generality of this observation.

It is worth noting that choosing the adequate buffer for the ITC experiments is a critical task. The buffer has to satisfy three criteria: (a) it must dissolve a Gd^{3+} salt in sufficiently high concentration; (b) it must maintain a pH close to neutral (~ 5 to 7) to prevent undesirable degradation of the polymer; (c) the enthalpy of dilution (ΔH_{dil}) of the Gd^{3+} solution in the buffer should be small, compared to the enthalpy of binding. In practice ΔH_{dil} varies greatly with the chemical composition of the buffer. For example, in PBS and MES buffers, the ΔH_{dil} is very large, compared to the enthalpy of binding ($\Delta H_{\text{binding}}$). Since the ΔH_{dil} needs to be subtracted from the data collected in a binding study, using these buffers led to large uncertainties in the titration data. In an acetate buffer (pH 5.25), the ΔH_{dil} , although still endothermic, is small relative to $\Delta H_{\text{binding}}$. Since this buffer is also a good solvent for the gadolinium salt, we selected it for all the titrations.

Next, we carried out titrations of Gd^{3+} in solutions of various HA–DTPA samples, as well as in a solution of unmodified HA. Two sets of data are presented in Figure 3: the experimental titration curves for Gd^{3+} in buffer (a), in a buffered solution of HA (b), and in a buffered solution of HA–DTPA (c); and the corresponding binding curves obtained by integration of each peak of the HA–DTPA curve in the top part of Figure 3. We observe that the dilution of Gd^{3+} into the buffer is not affected by the presence of HA (compare traces a and b), confirming that Gd^{3+} does not bind to HA under the conditions of the titration. On the other hand, trace c in Figure 3 undergoes a sharp transition for a $\text{Gd}^{3+}/\text{polymer}$ ratio of 0.321 mmol g^{-1} or 0.150 mol DTPA/mol Glu. If we assume that exactly 1 gadolinium ion binds to 1 DTPA unit, the titration indicates that 15.0 mol % of the carboxylic acid groups of HA bear a DTPA unit in the sample analyzed. Since DTPA forms stable complexes with several transition metals (18), it is not necessary to carry out the titration of HA–DTPA with Gd^{3+} . For instance, the europium ion Eu^{3+} forms a complex with DTPA with a pK value of 22.39 at 25 °C (18). A titration of the same HA–DTPA sample using Eu^{3+} , instead of Gd^{3+} , yielded the same degree of conjugation: 0.149 ± 0.001 mol DTPA/mol Glu, or 0.320 ± 0.003 mol DTPA (g polymer) $^{-1}$.

Colorimetric Studies. The colorimetric assay of the level of DTPA incorporation in HA–DTPA is based on the large bathochromic shift of the absorbance of arsenazo III in the presence of transition metals, such as

$$K_{\text{therm}} = K_{\text{cond}} \frac{K_1 K_2 K_3 K_4 K_5}{[\text{H}]^5 + [\text{H}]^4 K_1 + [\text{H}]^3 K_1 K_2 + [\text{H}]^2 K_1 K_2 K_3 + [\text{H}] K_1 K_2 K_3 K_4 + K_1 K_2 K_3 K_4 K_5} \quad (1)$$

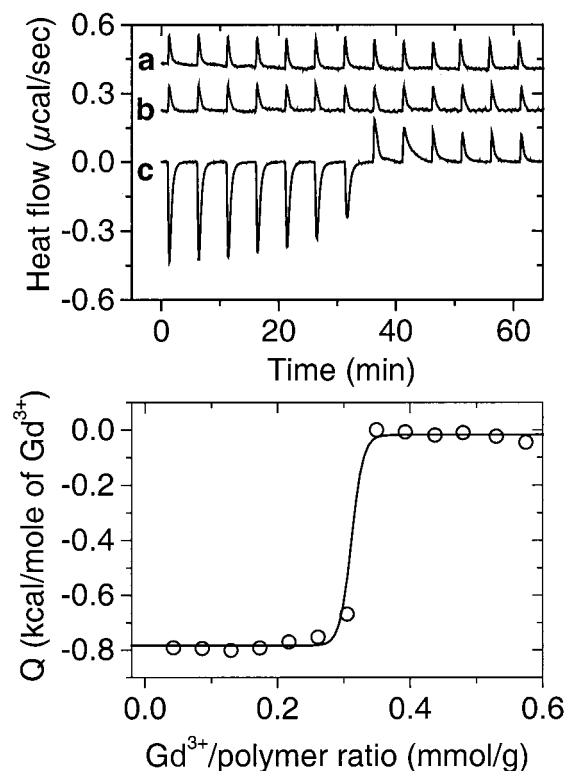


Figure 3. Titration curves of Gd^{3+} into aqueous polymer solutions at 25 °C and pH = 5.25. Above: calorimetric traces (heat flow against time) for (a) the blank, (b) HA, and (c) HA-DTPA1. The curves were shifted vertically for clarity. Below: reaction enthalpy (obtained by integrating the peaks in curves above) vs the Gd^{3+} /polymer ratio (mol/g).

Gd^{3+} , due to the formation of a stable complex (20). The pK of the Gd^{3+} –arsenazo III complex is 15.85 (18), a value approximately 7 orders of magnitude lower than the binding constant of gadolinium to HA-DTPA (vide supra). The free dye has a strong visible absorption at 548 nm. It absorbs at 660 nm when complexed with heavy metals (Figure 4). The colorimetric assay provides the degree of conjugation of DTPA on HA-DTPA via a protocol based on (1) the addition of an excess Gd^{3+} to a solution of HA-DTPA, and (2) the quantitative analysis of the excess of gadolinium detected as its complex with arsenazo III ($\lambda_{\text{max}} = 660$ nm) (see method I, Experimental Section). The changes in the absorption spectrum of arsenazo III as a function of added Gd^{3+} are illustrated in Figure 4, where we display spectra of arsenazo III in the absence of Gd^{3+} ($\lambda_{\text{max}} = 548$ nm), and in the presence of increasing amounts of Gd^{3+} . The intensity of the band with a maximum absorbance at 548 nm decreases, while the band characteristic of the complexed dye ($\lambda_{\text{max}} = 660$ nm) increases in intensity with increasing amounts of Gd^{3+} . We note the occurrence of two isosbestic points at $\lambda = 460$ and 582 nm.

In the titration described, we assume that the DTPA–Gd complex does not dissociate in the presence of arsenazo III, as expected from the large difference in the

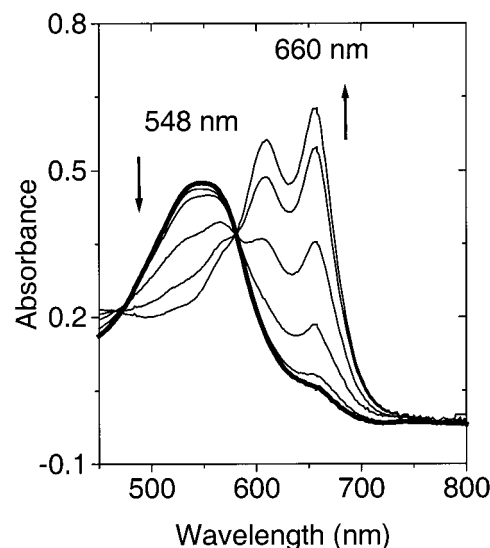


Figure 4. Absorption spectra of arsenazo III as a function of the concentration of Gd^{3+} ; [arsenazo III] = 20 mmol L^{-1} ; $[\text{Gd}^{3+}] = 0$ –18 mmol L^{-1} . The thick curve represents the spectrum of arsenazo III in the absence of Gd^{3+} .

binding constants of the two Gd^{3+} complex. To confirm the validity of this working hypothesis, we performed an analysis of the excess gadolinium added to a HA-DTPA solution, not in the presence of HA-DTPA–Gd as described previously, but after its separation from the polymer via ultrafiltration (Method II, Experimental Section). The two colorimetric methods gave identical results, within experimental error (see Table 1). Therefore, the time-consuming separation of unreacted Gd^{3+} from the polymer solution is not necessary for accurate colorimetric quantitation of the degree of DTPA conjugation. Method I is fast, accurate, and extremely economical in terms of polymer required (minimum amount: ~ 10 μg). The detection limit of this method is about 150 μg $\text{Gd}^{3+} \text{L}^{-1}$. Levels of DTPA incorporation as low as 0.03 mmol (gram of polymer) $^{-1}$ can be quantified accurately.

NMR Studies. All HA-DTPA samples were characterized by NMR spectroscopy. The ^1H NMR spectra, while useful to ascertain linking of DTPA to the polymer backbone, cannot be relied upon for a quantitative determination of the level of DTPA incorporation on the polymer, at least in the case of high molecular HA. Signals due exclusively to either the resonance of DTPA protons or HA protons were identified, as illustrated in Figure 5, which presents the relevant section of the ^1H NMR spectrum of HA-DTPA. The singlet at 2.0 ppm was attributed to the resonance of the acetamide protons linked to HA. The broad signal centered at 3.1 ppm was assigned to the resonance of the two methylene protons of the DTPA moiety, indicating conjugation of DTPA to the polymer backbone. The ratio of the integrated areas of these two signals, weighed for the respective numbers of protons, should yield the degree of DTPA substitution.

Table 1. Level of DTPA Incorporation As Determined by NMR, ITC, and Colorimetry for Two Different HA-DTPA Polymers

method	HA-DTPA1		HA-DTPA2	
	mol DTPA/mol Glu	mmol DTPA/g polymer	mol DTPA/ mol Glu	mmol DTPA/g polymer
NMR	0.27 ± 0.02	0.68 ± 0.05	0.73 ± 0.01	1.03 ± 0.03
ITC	0.155 ± 0.001	0.321 ± 0.003	0.415 ± 0.001	0.720 ± 0.003
UV(I)	0.16 ± 0.01	0.32 ± 0.02	0.44 ± 0.01	0.74 ± 0.02
UV(II)	0.17	0.384	0.40	0.71

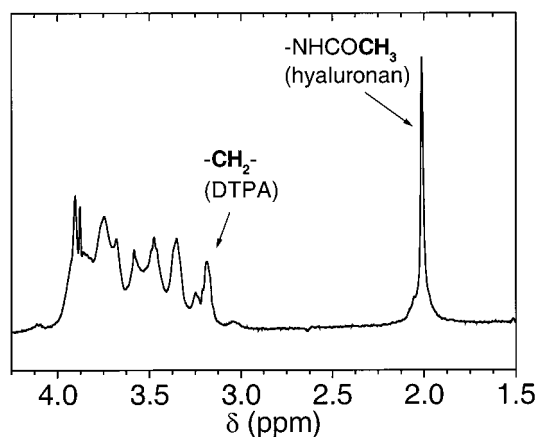


Figure 5. ^1H NMR spectrum of HA-DTPA (solvent: D_2O).

The value extracted from the spectra (Table 1) is significantly higher than the amount determined by either colorimetry or ITC. The enhancement of the NMR-derived value may be attributed to the fact that the relaxation times of the DTPA methylene protons are shorter than those of the methyl protons of the 2-acetamido-2-deoxy- β -D-glycopyranose moieties (16). A ^1H NMR spectrum was obtained using a longer interscan delay (5 s vs 1 s) for the same HA-DTPA sample, to determine if an increased delay would allow enough time for all the spins to get back to equilibrium. A slightly lower ratio HA/DTPA (22%) was calculated from this spectrum. The value remains significantly higher than the true value.

It should be pointed out that the methods described are intended to detect DTPA in a polymer solution, but they do not distinguish between bound DTPA and free DTPA. Therefore, it is important to ascertain the absence of free DTPA. To rule out the possibility of strong ionic interactions between free DTPA and HA, we conducted tests to ensure the effectiveness of the HA-DTPA purification protocol (see Experimental Section). Qualitative indication of covalent binding between DTPA and HA is given by obtained the ^1H NMR spectra (Figure 5).

CONCLUSION

We have compared three analytical techniques for the quantitative determination of the level of DTPA conjugation onto water-soluble polymers. Our study indicates that high-field ^1H NMR spectroscopy grossly overestimates the amount of DTPA incorporation. In contrast, titrations carried out by colorimetry and ITC are more accurate. They yield identical results. The colorimetric test is fast, requires only standard laboratory instrumentation, and can be performed using as little as 200 μg of polymer. The minimal level of DTPA detectable by colorimetry is about 25 nmol and the best detection window is between 50 and 500 nmol. Isothermal titration calorimetry is more demanding in terms of analysis time (a few hours) and of polymer sample (~ 2 mg). The advantage of ITC, however, is that a single experiment yields the level of DTPA conjugation and the thermodynamic parameters of the complexation. It is not limited to the DTPA-Gd complex. Other chelating agents/transition metal complexes can be analyzed by the identical protocol. While the present study was limited to two samples of HA-DTPA, either the colorimetry assay or the ITC measurement can easily be applied to other modified water-soluble polymers.

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C-Terminal and N-Terminal Fusions of Aequorin with Small Peptides in Immunoassay Development

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Aequorin fusion proteins have been used extensively in intracellular Ca^{2+} measurements and in the development of binding assays. Gene fusions to aequorin for production of fusion proteins have been so far limited to its N-terminus, as previous studies have indicated that aequorin loses its activity upon modification of its C-terminus. To further investigate this, two model peptides, an octapeptide (DTLDDDDL), and leu-enkephalin (TGGFL), an opioid peptide, were fused to the C-terminus of a cysteine-free mutant of aequorin through genetic engineering. The octapeptide was also fused to the N-terminus of the aequorin–leu-enkephalin fusion protein, which enables its affinity purification. Contrary to reports of earlier studies, we found that aequorin retains its bioluminescence activity after modification of the C-terminus. The half-life of light emission and the calibration curves obtained with the fusion proteins were comparable to those of the cysteine-free mutant of aequorin. Dose–response curves for the octapeptide were generated using two aequorin–octapeptide fusion proteins with the octapeptide fused to the N-terminus in one case, and to the C-terminus in the other. Similar detection limits for the octapeptide were obtained using both fusion proteins. The C-terminal fusion system has advantages in cases where antibodies recognize only the C-terminus of the peptide, as well as in cases where the functionality of the peptide lies in its C-terminus. The purification is also simplified as the affinity tag can be engineered at one terminus and the peptide of interest at the other.

INTRODUCTION

Aequorin, a bioluminescent photoprotein was originally isolated from *Aequorea victoria*, a hydromedusan jellyfish (1). Aequorin is composed of an apoprotein (apoequorin, 189 amino acid residues), a chromophore (coelenterazine, an imidazopyrazine compound), and molecular oxygen. Aequorin has three calcium binding sites, namely EF-hand structures, that are characteristic of the family of Ca^{2+} binding proteins. Upon binding to Ca^{2+} , aequorin undergoes a conformational change that results in the oxidation of coelenterazine to coelenteramide. This reaction produces CO_2 and emission of a flash of light at 469 nm (2, 3).

The sequencing and subsequent cloning of the cDNA of apoequorin has enabled researchers to expand the potential applications of aequorin (4, 5). This photoprotein has been widely employed in the measurement of Ca^{2+} in various cellular compartments and to monitor gene expression (6, 7). It has also been used as a highly sensitive label in binding assays (8–12) and in the detection of proteolytic bond cleavage (13). Aequorin can be detected in the femtomole to attomole range, which has allowed its employment as an alternative label to fluorescent probes in the calibration of micropipets (14). This photoprotein offers several advantages such as no photobleaching and the production of signal with virtually no background.

Aequorin fusion proteins have been widely employed in the development of immunoassays and in targeting aequorin to specific cellular locations for Ca^{2+} measure-

ments (6–8). Since the modification of the N-terminus of aequorin does not have any adverse effect on its luminescence activity, the gene fusions prepared thus far had been made only through its N-terminus (7, 8, 15). Literature reports had shown that modifications on the C-terminus of aequorin caused a significant decrease in the bioluminescence. Moreover when the C-terminal proline 189 was deleted or its position was changed, the result was complete loss of aequorin bioluminescence (16). In another study, a glycine was added to proline 189, resulting also in loss of the luminescent activity of aequorin (15). Thus, these studies indicated that the C-terminal proline was essential for the activity of aequorin and that its C-terminus was not amenable to modifications, which restricted its use in C-terminal gene fusions. In that respect, the goal of this study was to explore the possibility of preparing gene fusions of aequorin through its C-terminus without deleting or changing the position of proline 189 and to determine whether this modification has any effect on the luminescence activity of the protein. The crystal structure of aequorin which has recently been published shows that its C-terminal region forms a flexible loop structure and extends itself into the solvent, implying the feasibility of gene fusion through its C-terminus (17). These observations led us to believe that gene fusions can be done through the C-terminus of aequorin.

To prepare these fusions, a cysteine-free mutant of aequorin constructed in our laboratory was selected because we had found that this mutant is more stable than native aequorin. We genetically conjugated the N-terminus of a model octapeptide to the C-terminus of a cysteine-free mutant of aequorin to extend the C-terminal region of aequorin. The octapeptide employed

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has an amino acid sequence of Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys and is often used as an affinity tag for protein purification. Since the octapeptide is a highly polar peptide, another study was performed by fusing the N-terminus of a relatively weakly polar peptide to the C-terminus of mutant aequorin to determine whether the polarity of the peptide has any effect on the activity of aequorin. For that purpose, the N-terminus of leu-enkephalin, a mammalian opioid peptide, which has the amino acid sequence of Tyr-Gly-Gly-Phe-Leu was fused genetically to the C-terminus of aequorin. Leu-enkephalin has morphine-like pharmacological properties, and it acts as an agonist on opiate receptor sites (18, 19). The C-terminus of the same octapeptide used in the earlier study described above was also fused genetically to the N-terminus of the aequorin-leu-enkephalin fusion protein, allowing for the affinity purification of the fusion protein. Immunoassays were then developed for the octapeptide using both of these fusion proteins; in one, an octapeptide was fused to the N-terminus, and in another, it was fused to the C-terminus. The results were compared to see whether the sensitivity and the detection limit for the peptide would differ based on the different site of attachment to aequorin.

EXPERIMENTAL SECTION

Materials. Tris(hydroxymethyl)aminomethane (Tris) free base, ethylenediaminetetraacetic acid (EDTA) sodium salt, agar, glucose, sodium dodecyl sulfate (SDS), isopropyl β -thiogalactopyranoside (IPTG), sodium phosphate, glycine, 3-chloro-4-naphthol, hydrogen peroxide, nitrocellulose membrane, and all other reagents were purchased from Sigma (St. Louis, MO). Microlite 2 polystyrene microtiter plates were from Dynex (Chantilly, VA). Reactibind 96-well neutravidin-coated white polystyrene microtiter plates and biotin-free bovine serum albumin (BSA) were obtained from Pierce (Rockford, IL). A Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Coelenterazine was from Biosynth International (Naperville, IL). Biotinylated Anti-FLAG M2 antibody, unlabeled octapeptide and Anti-octapeptide M2 agarose affinity column were from Sigma (St. Louis, MO). Rabbit anti-leu-enkephalin antibody and horseradish peroxidase conjugated affinity purified anti-rabbit secondary antibody was purchased from Chemicon (Temecula, CA). Luria Bertani (LB) broth, *Eco*R I, *Hind* III and *Sal* I restriction enzymes, *Taq* and *Pfu* polymerases, and all other molecular biology reagents were obtained from Gibco-BRL (Gaithersburg, MD). All the primers used for the polymerase chain reaction (PCR) were purchased from Operon Technologies (Alameda, CA). All chemicals were reagent grade or better, and solutions were prepared using deionized (Milli-Q Water Purification System, Millipore, Bedford, MA) distilled water.

Apparatus. PCR was performed on a Perkin-Elmer Gene Amp PCR System 2400 (Norwalk, CT). Cells were sonicated using 550 Sonic Dismembrator from Fisher Scientific (Pittsburgh, PA). Fractions containing the aequorin fusion protein were lyophilized using a VirTis Bench Top 3 freeze-dryer (Gardiner, NY). The purity of the fusion protein was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a Phast System (Pharmacia Biotech, Uppsala, Sweden). The protein was transferred to the nitrocellulose membrane using the Phast transfer system from Pharmacia Biotech (Uppsala, Sweden). Bioluminescence measurements were made on a MLX microtiter plate luminometer from Dynex (Chantilly, VA) using a 100 μ L-fixed volume

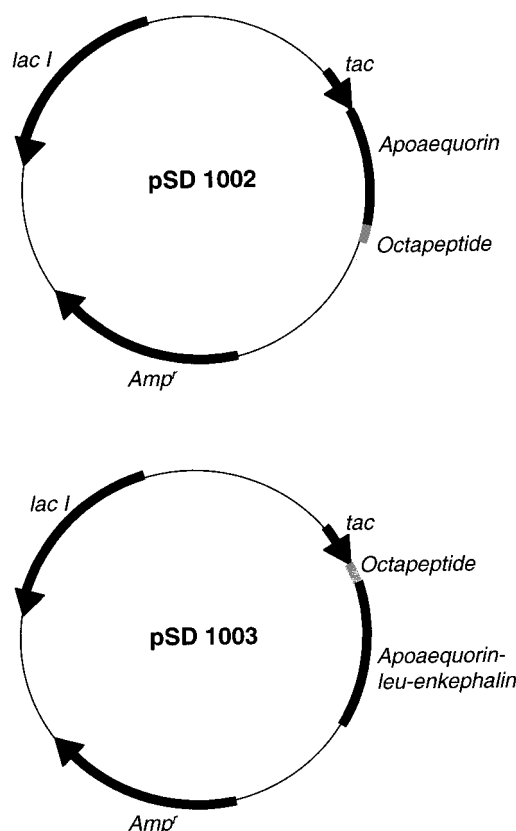


Figure 1. Schematic representation of plasmid pSD1002 containing the oligonucleotide sequences of the apoaequorin-octapeptide fusion protein and plasmid pSD1003 containing the gene sequence encoding for the octapeptide-apoaequorin-leu-enkephalin fusion protein.

injector. All luminescence intensities reported are the average of a minimum of three replicates and have been corrected for the contribution of the blank. All molecular biology procedures were performed using standard protocols (20).

METHODS

Construction of Plasmid pSD1002. The gene of a mutant apoaequorin in which all three cysteines of the native aequorin were replaced by serines was used as a template for the PCR (21). The following two primers were used to introduce the restriction endonuclease site for *Hind* III and *Sal* I restriction enzymes on mutant apoaequorin gene:

(1) TCTTCTTCTTCTTCTAAGCTTGTCAAGCTTACATCAGACTTCGACCCAAGA; (2) AGAAGAAGAAGAGTCGACCAGATCTGGTACCCGGAATT.

The DNA obtained by PCR and the vector pFLAG-CTC which consists of the gene sequence for the octapeptide downstream of the multiple cloning site (MCS) were digested with the restriction enzymes *Hind* III and *Sal* I. Next, the digested DNA fragment was ligated into the plasmid pFLAG-CTC, to yield the pSD1002 vector (see Figure 1). DNA sequencing was performed at the Macromolecular Center (University of Kentucky) to confirm the presence of the gene encoding for the apoaequorin-octapeptide fusion protein.

Construction of Plasmid pSD1003. The gene of a mutant apoaequorin was used as a template for the PCR (18). The following three primers were designed to obtain the gene construct, which codes for the apoaequorin-leu-enkephalin fusion protein:

(1) TCTTCTTCTTCTTCTAAGCTTGTCAAGCTTACATCAGACTTCGACCCAAGA; (2) GTACAGATCTGGTACCCGGAATTGGGGACAGCTCCACCGGAGCTT; (3) AGAAGAATTCTATTACAGGAAACCACCGTACAGATCTGGTACCCGGGAA.

Primer 1 was used to introduce the restriction site for the endonuclease *Hind* III. Primers 2 and 3 were used to introduce the seven amino acid spacer, sequence for leu-enkephalin, and restriction site for the enzyme *Eco*R I. The DNA obtained by PCR was digested with the restriction enzymes *Hind* III and *Eco*R I. The vector pFLAG-MAC, which consists of the sequence for the octapeptide upstream of the MCS, was also digested with the restriction enzymes *Hind* III and *Eco*R I. Next, the digested DNA fragment was ligated into the plasmid pFLAG-MAC, to yield the pSD1003 vector (see Figure 1). DNA sequencing was performed at the Macromolecular Center (University of Kentucky) to confirm the presence of the gene encoding for the octapeptide-apoaequorin-leu-enkephalin fusion protein.

Expression and Isolation of Apoaequorin-Peptide Fusion Proteins. Bacteria (*E. coli*, strain JM109) were transformed with the pSD1002 or pSD1003 vector and then cultured to express the fusion protein. Specifically, bacteria were grown in 2 mL of LB broth containing ampicillin (100 μ g/mL) for 16–18 h. The culture was then transferred to 250 mL of LB broth containing ampicillin (100 μ g/mL) and grown to an absorbance value of 0.6 measured at 600 nm. Next, IPTG was added to the culture, adjusting its final concentration to 1 mM, and the culture was grown for an additional of 3 h. The culture was then centrifuged at 5930g at 4 °C for 30 min to collect the cells. The bacterial pellet obtained was resuspended in 30 mM Tris-HCl, pH 7.0 containing 2 mM EDTA, followed by sonication for 3 min. The mixture was then centrifuged at 5930g at 4 °C for 30 min, and the supernatant containing the fusion protein was obtained.

Purification of the Apoaequorin-Peptide Fusion Proteins. The protein was purified by using an anti-octapeptide M2 agarose affinity column. The column was equilibrated with 50mM Tris-HCl, pH 7.4 containing 150 mM NaCl and 2 mM EDTA (TBS buffer). The solution containing protein was loaded on the column, and the column was washed three times with 15 mL of TBS buffer. The protein was eluted using 1 mL aliquots of 0.1 M glycine, pH 3.0, into tubes containing TBS buffer. The purity of the fractions containing the protein was determined by SDS-PAGE using 12.5% polyacrylamide gels, which were developed by silver staining (Pharmacia Biotech, Uppsala, Sweden). Fractions containing the fusion protein were pooled together, and glucose was added to a final concentration of 30 mM followed by lyophilization. The lyophilized protein was resuspended in deionized distilled water. Excess salt was removed through dialysis using TBS buffer. The protein concentration was determined by using the Bradford protein assay, with BSA as the standard. A 3-fold molar excess of coelenterazine was added to the apoaequorin fusion protein, and this mixture was stirred for 15 h at 4 °C. Excess of the coelenterazine was removed from the protein by dialyzing twice against 1.0 L of TBS buffer.

Bioluminescence Emission Study. A volume of 50 μ L of the two fusion proteins at a concentration of 1.0×10^{-10} M was added to a Microlite 2 polystyrene microtiter plate, and the bioluminescence was measured by injecting 100 μ L of luminescence triggering buffer (100 mM Tris-HCl, 100 mM CaCl₂, pH 7.5). The bioluminescence signal was collected at 0.1-s intervals over a 3-s time period.

Calibration Curves for Aequorin-Peptide Fusion Proteins. Stock solutions of the fusion proteins were serially diluted in assay buffer (30 mM Tris-HCl, pH 7.5 containing 2 mM EDTA, 150 mM NaCl, and 0.1% BSA), and the bioluminescence intensity of 50 μ L of each dilution was measured after injecting 100 μ L of luminescence triggering buffer.

Binder-Dilution Studies. Biotinylated anti-FLAG M2 antibody was serially diluted in the dilution buffer (50 mM sodium phosphate, pH 7.4 containing 100 mM NaCl and 0.1% biotin-free BSA). A volume of 50 μ L of differing concentration of antibody was added to the wells of neutravidin-coated plate and incubated with shaking for 2 h at room temperature. After that, the plate was washed three times with the wash buffer (30 mM Tris-HCl, pH 7.5 containing 2 mM EDTA and 150 mM NaCl). Next, 50 μ L of 1×10^{-10} M of the aequorin-peptide fusion proteins in assay buffer were added to the wells containing immobilized antibody. The plate was incubated for an additional 2 h with shaking at 4 °C. Then, it was washed with the washing buffer three times, and the bioluminescence intensity was measured by injecting 100 μ L of the luminescence triggering buffer.

Association Plots of Aequorin-Peptide Fusion Proteins. Biotinylated anti-FLAG M2 antibody (50 μ L, 8 μ g/mL) was immobilized on the neutravidin-coated wells of the microtiter plate as described previously. A volume of 50 μ L of a 1×10^{-10} M solution of the fusion proteins was added to the plate, and the plate was incubated with shaking for various time periods. At the end of each time period, the plate was washed three times with washing buffer, and the luminescence intensity was measured as described previously.

Dose-Response Curves for the Octapeptide. Dose-response curves were generated in either a competitive or a sequential binding mode. For the competitive binding mode, 50 μ L of unlabeled octapeptide of differing concentrations and 50 μ L of the fusion proteins of a 1×10^{-10} M concentration were added simultaneously to the wells of the plate containing immobilized biotinylated anti-FLAG M2 antibody (50 μ L, 8 μ g/mL). The plate was incubated for 1 h with shaking. After that, the plate was washed with washing buffer three times, and the luminescence intensity was measured by injecting 100 μ L of the luminescence triggering buffer. A dose-response curve was also generated in a sequential binding mode. In this case, the unlabeled peptide of differing concentrations was incubated with the immobilized antibody for 1 h. The plate was then washed three times, and the fusion proteins (50 μ L, 1×10^{-10} M) were added to the plate. The plate was incubated for an additional 1 h with shaking followed by a washing step. The luminescence intensity was measured by injecting 100 μ L of the luminescence triggering buffer.

Western Blotting for Octapeptide-Aequorin-Leu-Enkephalin Fusion Protein. A Western blott was performed using rabbit anti-leu-enkephalin antibody and horseradish peroxidase-conjugated affinity purified anti-rabbit secondary antibody by following a standard protocol (24).

RESULTS AND DISCUSSION

Fusion proteins have been produced for a variety of applications such as affinity purification, receptor-protein interaction studies, and development of binding assays. Gene fusion to a protein is produced through either the N-terminus or the C-terminus of the protein. These fusions can, sometimes, result in the improper

folding of the protein causing loss of its activity (22, 23). Various proteins and peptides have their active site at their N- or C-terminal region (24, 25). Therefore, in such cases, the flexibility of the label with regard to conjugation to either of its termini is of paramount importance. For example, the sequence of the different enkephalin peptides only differs at their C-terminus whereas the sequence of angiotensin II and III differs at their N-terminus. Thus, to develop immunoassays for these peptides, the label should be conjugated either at the C- or N-terminus such that the peptide can be selectively recognized by the antibody. Aequorin is a photoprotein that has been used as a sensitive label in a variety of immunoassays. It has been reported in the literature that the C-terminal proline of aequorin is essential for its bioluminescence activity, and that any modifications at this terminus result in the loss of its activity. Hence, to date, gene fusions of aequorin have been limited to those involving its N-terminus (16). In this study, we decided to investigate the feasibility of generating gene fusions through the C-terminus of aequorin, keeping the C-terminus proline intact. For that, two fusion proteins were constructed with different peptides fused to the C-terminus of aequorin. The N-terminus of an octapeptide and an opioid peptide, leu-enkephalin, were fused genetically to the C-terminus of aequorin.

To produce the fusion protein between aequorin and the octapeptide, the gene encoding for apoequorin was ligated into the multiple cloning site (MCS) of the expression vector pFLAG-CTC, yielding plasmid pSD1002 (see Figure 1). The vector pFLAG-CTC consists of the gene sequence for the octapeptide downstream from the MCS. The fusion protein thus produced has the sequence, apoequorin-Asn-Ser-Arg-Val-Pro-Asp-Leu-octapeptide. The seven amino acids between apoequorin and the octapeptide are encoded in the MCS of the pFLAG-CTC vector between the recognition sequence of the restriction enzyme *EcoR* I and the gene sequence for the octapeptide. These amino acids can act as a spacer between aequorin and the octapeptide, thus allowing for the proper folding of the protein. To produce the fusion protein between aequorin and leu-enkephalin, the DNA encoding for apoequorin-leu-enkephalin was constructed using PCR. This gene was then ligated into the MCS of the expression vector pFLAG-MAC, which also has a sequence for the octapeptide upstream of the MCS, to yield plasmid pSD1003 (see Figure 1). The resulting fusion protein has the sequence octapeptide-apoequorin-Asn-Ser-Arg-Val-Pro-Asp-Leu-leu-enkephalin. This fusion protein has the same spacer as used in the case of the apoequorin-octapeptide fusion protein. *E. coli* cells were then transformed either with pSD1002 or with pSD1003 plasmid. The expressed proteins were purified by affinity chromatography using anti-octapeptide M2 antibody covalently linked to agarose beads. Crude extracts were loaded onto preequilibrated columns followed by a washing step to remove nonspecifically bound proteins. The proteins of interest were eluted using glycine buffer, pH 3.0. The purity and concentration of the protein was determined using SDS-PAGE and the Bradford assay, respectively. The yields of the purified proteins were 2 mg/L and 1.8 mg/L for the apoequorin-octapeptide and the octapeptide-apoequorin-leu-enkephalin fusion protein, respectively. The apoequorin-peptide fusion proteins obtained were stirred with a 3 molar excess of the coelenterazine chromophore to regenerate the aequorin-peptide fusion proteins. Unbound coelenterazine was subsequently removed by dialysis.

As mentioned earlier, previous studies reported that aequorin became inactive upon modification of its C-terminus. Thus, the first step in our study was to determine whether the protein prepared through a C-terminal fusion had any effect on the luminescence activity of aequorin. Bioluminescence emission studies were then performed to characterize the fusion proteins in terms of their luminescence properties. The fusion proteins demonstrated the same flash-type emission characteristics as that of the native protein with 95% of the total light being emitted during a 3-s time period. Thus, a 3-s light integration time was chosen for all remaining experiments. The half-life of the bioluminescence decay of the fusion proteins was determined to be 0.97 s for both, the aequorin-octapeptide and the octapeptide-aequorin-leu-enkephalin fusion protein. These half-life values are comparable to that of the unconjugated cysteine-free mutant aequorin (1.0 s) (18). The luminescence activities of the aequorin-octapeptide and octapeptide-aequorin-leu-enkephalin fusion proteins were found to be 100% and 97%, respectively, when compared to the activity of the aequorin cysteine-free mutant (100%). This virtual no loss of bioluminescence activity after the C-terminal modification of aequorin is contrary to previous observations reported in the literature. It should be pointed out, however, that there are some differences between the literature work (15, 16) and our study. Previously, C-terminal modification studies were carried out using native aequorin, whereas we have employed a cysteine-free mutant of aequorin. This mutant has higher bioluminescence activity than native aequorin (21). Also, in the previous studies either the C-terminal proline, believed to be necessary for aequorin activity, was deleted or its position was changed, but in our study we have maintained this proline in the 189 position (15). The spacer between apoequorin and the peptides may play an important role in the proper folding of aequorin, thus maintaining the luminescence activity of aequorin. Further studies to elucidate the effect of different spacers located at the C-terminus on the aequorin activity are underway in our laboratory. Next, a study was conducted to determine whether the fusion of the octapeptide to either the C- or N-terminus of aequorin would alter the bioluminescence assay performance when aequorin is used as the label. For that, immunoassays were developed for this peptide using the two different fusion proteins.

To select the optimum amount of fusion proteins to develop the assays for the peptides, a calibration study was performed for both fusion proteins. A working range extending over 4 orders of magnitude was obtained for both proteins as shown in Figure 2. A concentration of 1×10^{-10} M of both fusion proteins was selected for the remaining studies, as it generates a signal well above the background one.

The next step in our study was to determine the optimum amounts of antibodies to be used in the assay development. To determine this, binder-dilution curves were generated using biotinylated anti-FLAG M2 antibody. The biotinylated antibody was immobilized on neutravidin coated plates by taking advantage of strong binding between biotin and neutravidin. The antibody was immobilized by shaking the plate at room temperature for 2 h followed by a washing step. Next, the fusion proteins were added to the plate and incubated for an additional 2 h. The luminescence intensity was measured after washing the plate. The binder-dilution curves for both fusion proteins are shown in Figure 3. As it can be seen from the figure, the binder-dilution curves for both

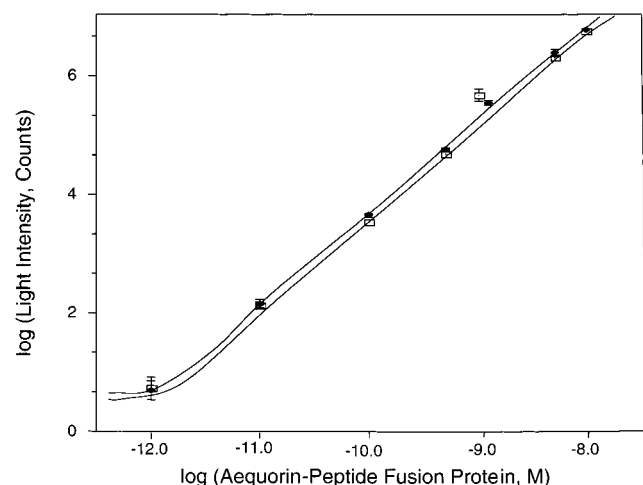


Figure 2. Calibration curves for the aequorin-octapeptide (□) and octapeptide-aequorin-leu-enkephalin (◆) fusion proteins. Data are the average $\pm$ one standard deviation ($n = 3$). All error bars are less than 10% and are represented as obtained.

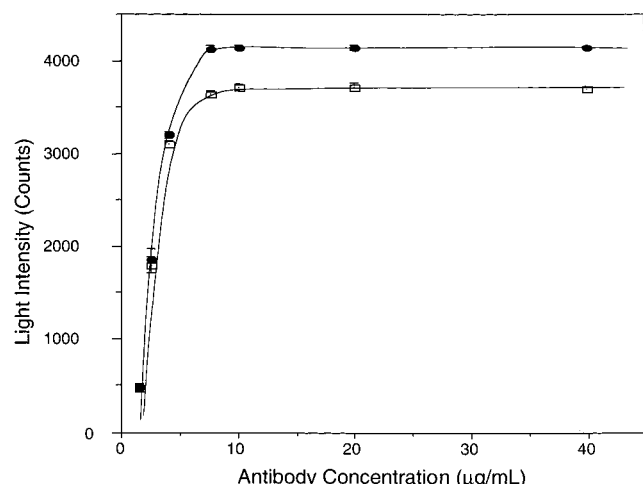


Figure 3. Binder-dilution curves for the aequorin-octapeptide (□) and the octapeptide-aequorin-leu-enkephalin (●) fusion proteins. Data are the average $\pm$ one standard deviation ($n = 3$). All error bars are less than 10% and are represented as obtained.

proteins follow a similar path. This indicates that neither the C- nor the N-terminal fusion of the peptide to aequorin affects the binding of the fusion protein to the antibody. The optimum concentration of antibody to be used for the subsequent steps was determined using these binder-dilution curves. A concentration of antibody of 8 $\mu\text{g/mL}$ was selected for both fusion proteins to be used in the development of the assays for the peptide. This concentration was selected since it was the highest concentration of antibody that was within the linear portion of the curve; at higher antibody concentrations the binding sites on the plate are saturated and no further increase in the signal was observed.

Another important parameter to be determined in assay development is the optimum time required for binding of the fusion protein to the antibody, which allows reduction of the total assay time. An association study was performed in the same manner as the binder-dilution study, except that the incubation time for binding the fusion protein to the antibody was varied. The association curves are shown in Figure 4. Maximum signal was observed at an association time of 1 h, and it was selected for the rest of the experiments.

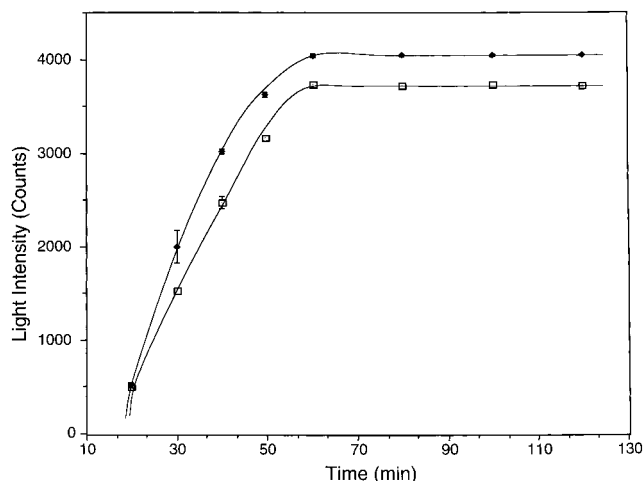


Figure 4. Association curves for the aequorin-octapeptide (□) and the octapeptide-aequorin-leu-enkephalin (◆) fusion proteins. Data are the average $\pm$ one standard deviation ($n = 3$). All error bars are less than 10% and are represented as obtained.

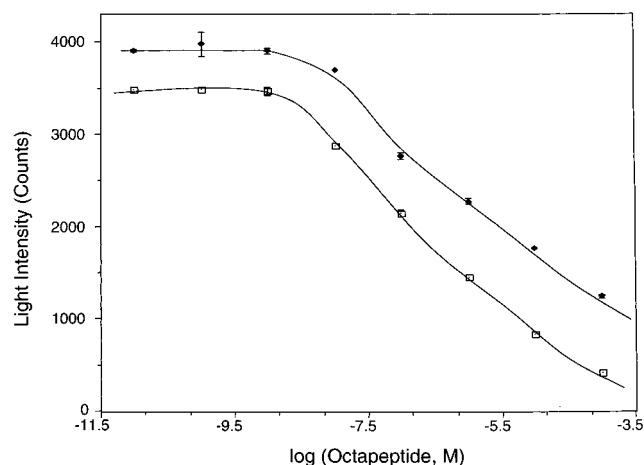


Figure 5. Dose-response curves for the octapeptide generated in a competitive binding mode by employing the aequorin-octapeptide (□) and the octapeptide-aequorin-leu-enkephalin (◆) fusion proteins. Data are the average $\pm$ one standard deviation ($n = 3$). All error bars are less than 10% and are represented as obtained.

Once the optimum amounts of fusion proteins, antibody, and the association time were determined, the final step in the assay development was to generate dose-response curves for the peptides. These curves were generated in two binding modes, namely, a competitive mode and a sequential binding mode. In the competitive binding mode the unlabeled peptide and the fusion proteins were incubated along with the immobilized antibody. This type of assay mode offers the advantage of having fewer steps, thus decreasing the overall assay time. The dose-response curves generated using competitive binding are shown in Figure 5. To generate a dose-response curve in a sequential binding mode, the unlabeled peptide was first incubated with the immobilized antibody followed by a washing step. Next, the fusion proteins were added, and an incubation step was performed. The luminescence intensity was measured after a washing step. In this case, the addition of labeled peptide is delayed; thus, this binding mode offers the advantage of producing lower detection limits (26). The dose-response curves generated in the sequential binding mode are shown in Figure 6. The dose-response

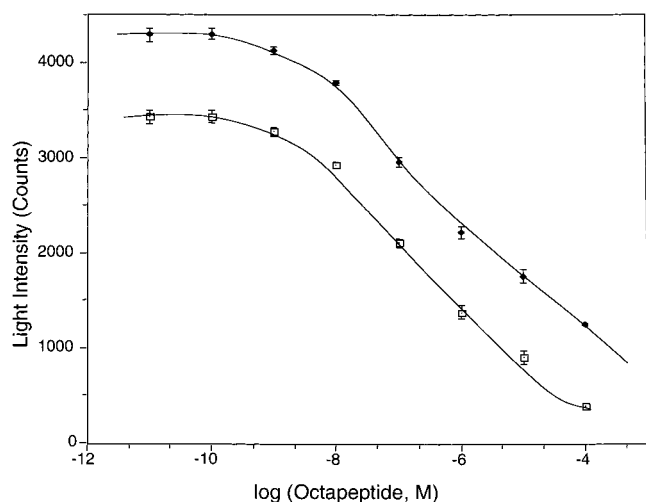


Figure 6. Dose-response curves for the octapeptide generated in a sequential binding mode by employing the aequorin-octapeptide (□) and the octapeptide-aequorin-leu-enkephalin (◆) fusion proteins. Data are the average $\pm$ one standard deviation ($n = 3$). All error bars are less than 10% and are represented as obtained.

curves for both fusion proteins showed a decrease in luminescence intensity with increasing amounts of unlabeled peptide, as expected. The working range of the curve spans over 3 orders of magnitude. The detection limits obtained in the case of the competitive binding mode are higher in comparison to the sequential binding mode. However, the detection limits obtained with the N-terminal and C-terminal aequorin-octapeptide fusion proteins are the same for the same binding mode. This demonstrates that hydrophilic peptides can be fused to either of the aequorin termini without affecting the performance of the assay. In the competitive binding assay mode a detection limit of 4×10^{-9} M for the octapeptide was obtained, whereas in the case of the sequential binding assay mode the detection limit was 1

$\times 10^{-9}$ M. The detection limits obtained compared well with the detection limit obtained when the same octapeptide was fused to the N-terminus of aequorin as reported in the literature (7).

In this study, we have also demonstrated that no loss in activity of aequorin was observed when the N-terminus of another peptide, leu-enkephalin, was genetically fused to the C-terminus of aequorin. A Western blot was performed using anti-leu-enkephalin antibody to determine whether the antibody can bind to the octapeptide-aequorin-leu-enkephalin fusion protein. The Western blot is shown in Figure 7. As it can be seen from the figure, the antibody binds selectively to the fusion protein. Therefore, an immunoassay can be developed for the leu-enkephalin peptide using this system. Currently, work is underway in our laboratory toward achieving this goal. This study also indicates that we can detect two peptides using the same fusion protein reagent by immobilizing the respective antibodies in separate wells. This potentially offers an advantage of reduced reagent preparation cost.

In conclusion, we have demonstrated that the C-terminus of a cysteine-free mutant of aequorin can be modified without any loss of its activity. To our knowledge, this is the first time that the C-terminus of aequorin is being modified without any loss of its luminescence activity. Immunoassays for the octapeptide were developed using aequorin-octapeptide fusion proteins. In one case, the peptide was fused to the N-terminus, whereas in the other case it was fused to the C-terminus of aequorin. No significant difference in performance was observed between the two systems as evident by the similar detection limits for the peptide obtained. This demonstrated that peptides or proteins can be fused through either of the terminus of aequorin, thus increasing the flexibility of aequorin in terms of peptide/protein conjugation. An additional advantage of this system is that dual functionalities can be attached to the aequorin molecule. For example, at one terminus of aequorin an

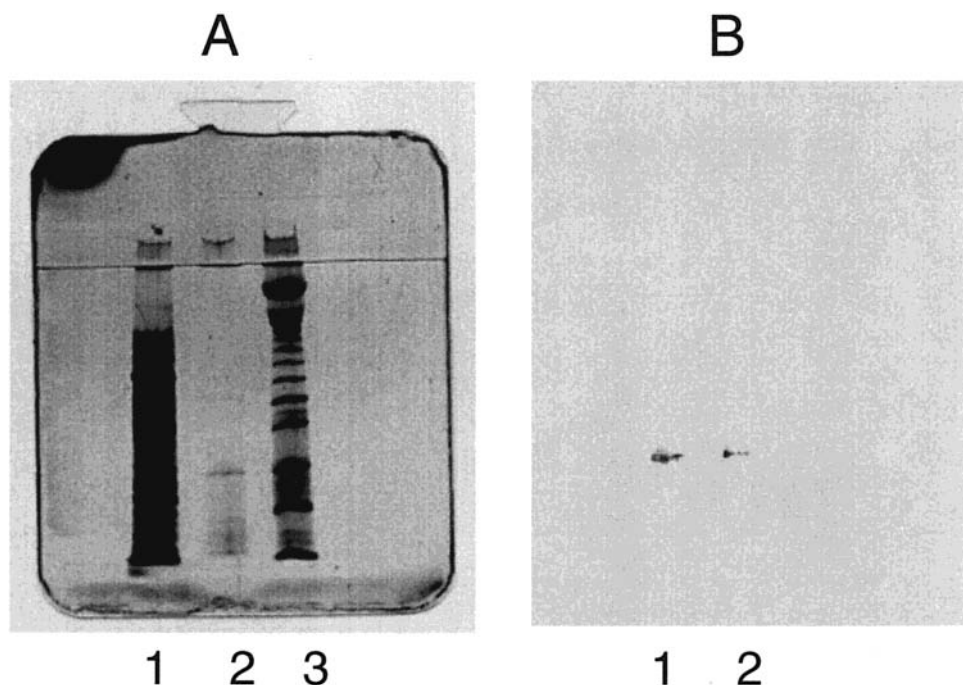


Figure 7. A. SDS-PAGE analysis of the octapeptide-aequorin-leu-enkephalin fusion protein. Lane 1: crude protein, lane 2: purified fusion protein, lane 3: protein molecular weight marker. B. Western Blot performed using rabbit anti-leu-enkephalin antibody and horseradish peroxidase conjugated affinity purified anti-rabbit secondary antibody. Lane 1: crude protein, lane 2: purified fusion protein.

epitope tag for antibody recognition can be attached, while at the other terminus an affinity tag can be attached simplifying the purification of the fusion protein as demonstrated in this study. In summary, given the flexibility that C- and N-terminal fusions afford to aequorin in terms of analyte conjugation, it is envisioned that the range of potential applications of aequorin in assay development will be increased.

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Enzymes by Design: Chemogenetic Assembly of Transamination Active Sites Containing Lysine Residues for Covalent Catalysis

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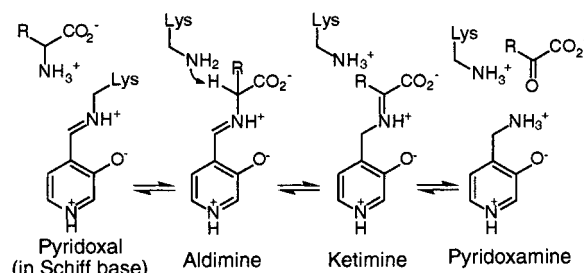
Artificial enzymes can be created by covalent conjugation of a catalytic active group to a protein scaffold. Here, two transamination catalysts were designed via computer modeling and assembled by chemically conjugating a pyridoxamine moiety within the large cavity of intestinal fatty acid binding protein. Each catalyst included a lysine residue, introduced via site-directed mutagenesis, that promotes catalysis by covalent interactions with the pyridoxamine group. Evidence for such interactions include the formation of a Schiff base with the pyridoxal form of the catalyst and a rate versus pH dependence that is bell shaped; both of these features are manifested in natural transaminases. The resulting constructs operate with high enantioselectivity (83–94% ee) and increase the rate of reaction as much as 4200-fold over the rate in the absence of the protein; this is a modest (12-fold) increase in catalytic efficiency (k_{cat}/K_M) compared to the conjugate lacking the lysine residue. Most importantly, these artificial aminotransferases are the first examples of designed bioconjugates capable of covalent catalysis, highlighting the potential of this chemogenetic approach.

INTRODUCTION

Artificial enzymes can be designed by covalent conjugation of a catalytic active group to a protein scaffold (Kaiser and Lawrence, 1984; Distefano et al., 1998). This concept has been successfully applied to the construction of a variety of so-called semisynthetic enzymes, such as thiazol-papain (Suckling and Zhu, 1993), flavo-papain (Kaiser and Lawrence, 1984), flavo-hemoglobin (Kokubo et al., 1987), oligonucleotide-nuclease (Corey and Schultz, 1987), or seleno-subtilisin (Wu and Hilvert, 1989; Häring and Schreier, 1999). The protein scaffold enhances the binding of substrates and provides a chiral environment, thus mimicking an enzymatic active site. However, another important feature of native enzymes has not yet been incorporated in such conjugates: the interaction of functional groups from the protein framework with the cofactor or substrates for covalent catalysis.

Pyridoxamine is a versatile catalytic engine for a diverse array of reactions including transamination, decarboxylation, racemization, and aldol condensation. To achieve highly efficient catalysis, interactions with neighboring functional groups are critical. For example, in all known pyridoxamine phosphate utilizing enzymes, the aldehyde form of the cofactor is bound covalently to the ϵ -amino group of a lysine residue via a Schiff base linkage. Upon nucleophilic attack of this internal imine by the amino group of an incoming substrate, the lysine residue is released and an internal ketimine complex between the substrate and the aldehyde is formed (Scheme 1). Moreover, this same lysine residue also serves as a general base in the subsequent conversion of the ketimine intermediate to the internal aldimine. Deletion of this residue via mutagenesis results in an enzyme exhibiting a 10^6 fold decrease in transamination activity (Malashkevich et al., 1995a).

Scheme 1. Simplified Transamination Half Reaction^a



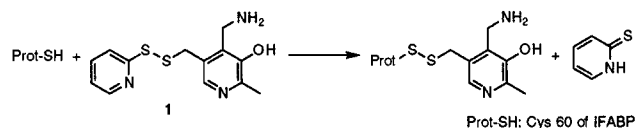
^a For detailed information about the reaction mechanism and the role of lysine cf. literature (Hayashi et al., 1998; Goldberg and Kirsch, 1996).

To correctly position functional groups in a three-dimensional fashion that can modulate catalyst reactivity and selectivity, a sphere-shaped scaffold is useful for the design of an artificial enzyme. Fatty acid binding proteins are a well-characterized family of intracellular transport proteins involved in lipid metabolism (Banaszak et al., 1994; Veerkamp and Maatman, 1995). These proteins bind their cognate ligands in a cavity consisting of a β -barrel and a α -helical lid. Intestinal fatty acid binding protein from rats (IFABP¹) is a cysteine-free 15 kDa protein with an internal cavity volume of 600 Å³. Its established structure (Scapin et al., 1992; Hodson et al., 1996) as well as the convenient overexpression of mutants in *Escherichia coli* make IFABP a conceptually simple framework for the design and assembly of an active site.

¹ Abbreviations: AT, aspartate aminotransferase; DTNB, 2,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; IFABP, intestinal fatty acid binding protein; MES, 2-(*N*-morpholino)ethanesulfonic acid; PMP, pyridoxamine monophosphate; PX, pyridoxamine; TRIS, tris(hydroxymethyl)aminomethane.

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Recently, we developed an approach for catalyst design that combines elements of chemical modification with genetic engineering. For example, a pyridoxamine derivative was covalently linked inside the cavity of IFABP via a disulfide bond to a cysteine residue, which has been introduced by site-directed mutagenesis (Kuang and Distefano, 1998). These experiments demonstrated that the catalytic efficiency of pyridoxamine could be increased by up to 360-fold by introducing the cofactor in the cavity at a specific site.



Here we have used this chemogenetic approach to position functional groups for participation in catalysis in this artificial enzyme. Our goal was to construct a transaminase active site that would contain a lysine residue capable of Schiff base formation and general acid/base catalysis. An approach based on geometric constraints for residue introduction into an existing catalytic system was developed. Two lysine mutants were prepared and conjugated with pyridoxamine. Spectroscopic evidence for covalent bond formation between the new lysine residues and the cofactor was obtained. The rate versus pH profile for both mutants was measured and shown to be bell shaped, similar to natural transaminases. Finally, kinetic investigation showed that the new lysine mutants exhibited an increased catalytic efficiency relative to the free cofactor and the protein without the lysine residues.

MATERIALS AND METHODS

Materials. Pyridoxamine dihydrochloride was converted into 5-(2-pyridyldithio)pyridoxamine **1** as described previously (Kuang et al., 1996). The mutagenic primers were obtained from the Microchemical Facility of the Institute of Human Genetics (University of Minnesota). The site-directed mutagenesis kit ("Quick-Change") was obtained from Stratagene. The expression vector pMON-IFABP was used previously for the preparation of IFABP-V60C (Kuang and Distefano, 1998). The protein purification was carried out at 4 °C unless otherwise noted.

Modeling. Molecular modeling experiments were performed using Insight II 97.0/Discover 3.00 (Molecular Simulations). The potential energy was calculated on basis of a consistent valence force field and minimized sequentially by steepest descents and conjugate gradients. The distances and angles of various native amino-transferases used in this study, listed in Table 1, were averaged from literature data (Peisach et al., 1998; Suigo et al., 1995; Miyahara et al., 1994; Malashkevich et al., 1995b; Okamoto et al., 1998).

Site-Directed Mutagenesis. Site-directed mutations were introduced in the expression vector of pMON-IFABP recombinant plasmid with the QuickChange mutagenesis kit. The sequence for the mutagenic oligomers was 5'-CATGACAACCTGAAAAAGACGATCACACAGGAAG-3' for L38K and 5'-AAATAAATTCACAGTCAAAAAATCAAGCAACTTC-3' for E51K. The proteins were overexpressed in *E. coli* strains JM105, which were grown under shaking at 37 °C in LB media (6 × 1 L) and induced with nalidixic acid.

Protein Purification. IFABP-V60C/E51K was purified according to a published procedures (Jiang and

Frieden, 1993). IFABP-V60C/L38K was expressed as inclusion bodies. They were repeatedly pelleted (3000 g) and resuspended in 0.5 M urea, 50 mM TRIS pH 7.5, 10 mM EDTA, 5 mM dithiothreitol (DTT), and 1% Triton X-100. After a final resuspension in the above buffer without urea, the protein was denatured in 3 M guanidine in buffer A (20 mM HEPES pH 7.5, 10 mM EDTA and 10 mM DTT). Refolding was achieved by slowly dropping the protein solution in a 25-fold volume of 250 mM sodium chloride in buffer A. The IFABP mutant was finally isolated after gel filtration chromatography (Sephacryl S-100).

Conjugation to Pyridoxamine Cofactor. Prior to conjugation, the IFABP-mutants were desalted by gel filtration, and the concentration of free thiol was determined by titration with DTNB. The conjugation reagent 5-(2-pyridyldithio)pyridoxamine **1** was added in 10-fold excess, and the reaction was allowed to proceed for 18 h at 20 °C in 20 mM HEPES (pH 7.5). The conjugated protein was purified by gel filtration and the concentration of pyridoxamine determined by its UV absorbance ($\epsilon_{326} = 6140 \text{ M}^{-1}\text{cm}^{-1}$ in 6 M guanidine, 20 mM HEPES, pH 7.5). To convert the pyridoxamine to the pyridoxal form, the conjugates were incubated with 50 mM α -ketoglutarate for 24 h at 37 °C. The excess of keto acid was removed with a desalting column (Sephadex G25).

Kinetic Analysis. Reactions were performed with 50 μM catalyst, 5 mM phenylalanine (in the case of PMP 5 mM tyrosine was used) and varying concentrations of α -ketoglutarate in 0.2 M HEPES (pH 7.5) at 37 °C. The formation of glutamate was monitored by reversed-phase HPLC (Kuang et al., 1996; Buck and Krummen, 1987).

Electrospray Mass Spectrometry. For mass spectral experiments, protein samples were desalted (NAP-5 column, elution with 0.5% formic acid), lyophilized, and analyzed by direct infusion (5 $\mu\text{L}/\text{min}$) electrospray ionization mass spectroscopy in water/acetonitrile (1:1) containing 0.5% formic acid at a protein concentration of 50 μM .

pH-Dependence. The reactions were performed with 50 μM IFABP-PxK38, 5 mM phenylalanine, and 50 mM α -ketoglutarate acid in "MHP-buffer" (Gloss and Kirsch, 1995) with constant ionic strength $I = 0.2$ (25 mM HEPES, 25 mM MES, 50 mM 4-hydroxy-*N*-methylpiperidine, 0.1 M KCl). The formation of the glutamate-enantiomers was monitored by HPLC (Buck and Krummen, 1987), and the data points of the initial rate were fitted to a standard bell curve.

RESULTS AND DISCUSSION

Our goal in this work was to design and construct a transaminase active site containing a lysine residue capable of Schiff base formation and general acid/base catalysis. Such a site would be prepared by covalently tethering a pyridoxamine moiety to a cysteine residue (introduced via mutagenesis) within the cavity. On the basis of examination of the crystal structure of IFABP, a V60C mutant form of IFABP was chosen, since it is in a region proximal to the proposed site of entry for fatty acids (and hence putative substrates). Furthermore, attachment of the pyridoxamine to Cys60 positions the cofactor close to the cationic residue Arg126 which could facilitate the binding of carboxylate-bearing substrates via electrostatic interactions (Figure 1). The conformation of the pyridoxamine derivative tethered by a disulfide bond to Cys60 was optimized by energy minimization to provide a starting point for exploring possible sites for Lys introduction.

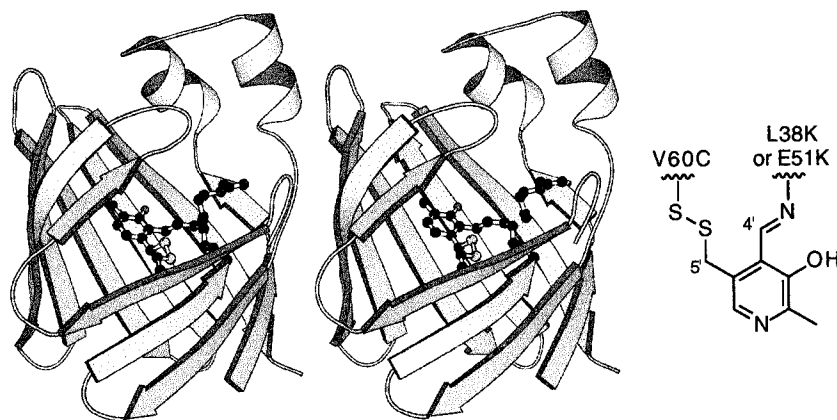


Figure 1. Stereoview of a computational model of IFABP-PxK51 developed from the crystal structure of IFABP (Scapin et al., 1992). The pyridoxal is bound via a disulfide bond to Cys60, and a Schiff base linkage to Lys51; a cationic residue (Arg126) is close to the catalytic center. The figure was generated using MOLSCRIPT (Kraulis, 1991).

Table 1. Critical Geometric Parameters for the Placement of a Lysine Residue near Pyridoxamine in a Transamination Catalyst

	$C_{\alpha}-C_{4'}$ (Å)	$C_{\alpha}-X$ (Å)	$C_{\alpha}-C_{5'}$ (Å)	α (deg)	β (deg)
native AT ^a	6.5–8.7	7.3–8.9	8.1–9.5	70–145	80–135
IFABP-PxK38 ^b	7.1–8.8	8.0–8.8	7.4–9.6	134–153	33–37
IFABP-PxK51 ^b	6.9–7.6	5.6–6.4	6.8–8.1	130–140	22–35

^a The data for native aminotransferases (AT) were averaged from crystal structures of D- and L-aspartate AT as well as aromatic amino acid AT in their pyridoxal, Schiff base, and glutamate ketimine form. ^b The data for the IFABP conjugates were determined by computational modeling (C_{α} of lysine; X: $O-PO_3^{2-}$ for native AT or S-Cys60 for IFABP-PX conjugates; α : angle $N_{Ar}-C_{4'}-C_{\alpha}$; β : dihedral angle $C_{5'}-N_{Ar}-C_{4'}-C_{\alpha}$).

As guidance for the positioning of a Lys residue capable of Schiff base formation with pyridoxal, we used geometric parameters from the active site of native aminotransferase (AT). Table 1 summarizes critical distances and angles between the cofactor and the C_{α} -atom of lysine in various AT structures. The neighborhood of the IFABP-bound pyridoxal was searched with these parameters. This resulted in the identification of 10 possible C_{α} -positions where lysine mutations could be introduced. Four of these mutations were ruled out for reasons of obvious steric hindrance or because they are highly conserved throughout this protein family. Optimized models of the pyridoxal form, the Schiff base to the lysine and the substrate (α -ketoglutarate) ketimine complex were calculated for the other six lysine mutations (L38K, F47K, E51K, Q115K, Y117K, R126K). The geometric parameters of the two most promising mutations, L38K and E51K are summarized in Table 1; a model for one of these constructs is presented in Figure 1.

The structural parameters for the L38K and E51K mutants are closely related to those of the native amino transferases that are also included in Table 1 for comparison. It should be noted that although wild-type IFABP has 15 lysine residues, modeling experiments suggest that none of these are oriented appropriately for Schiff base formation with the pyridoxal linked to Cys 60.

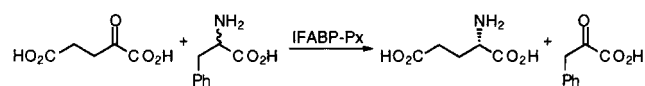
Based on the modeling results described above, two possible catalysts were assembled by a chemogenetic approach. The desired mutations V60C/L38K and V60C/E51K of IFABP were introduced by site-directed mutagenesis and the proteins purified to homogeneity. Using the activated pyridoxamine (Px) derivative 5-(2-pyridylthio)pyridoxamine, the cofactor was chemically linked to Cys60 of both mutants on a 50–100 mg scale with over 90% conjugation efficiency based on DTNB titration and UV/vis spectroscopy of the denatured protein constructs. The resulting assemblies IFABP-PxK38 and IFABP-PxK51 (the number indicates the position of the lysine

Table 2. Masses of Various IFABP-Px Conjugates in Their Pyridoxamine Form (amine) and after Reduction with $NaBH_4$ (red. imine)

conjugate	calcd mass (Da)	found (Da)
IFABP-Px (amine)	15179.3	15178.0
IFABP-Px (red. imine)	15180.3	15179.0
IFABP-PxK38 (amine)	15194.3	15193.0
IFABP-PxK38 (red. imine)	15177.3	15188.0
IFABP-PxK51 (amine)	15178.3	15177.0
IFABP-PxK51 (red. imine)	15161.3	15160.0

mutation) show the UV absorption of both the protein (280 nm) and the pyridoxamine (326 nm). Analysis of the proteins via electrospray mass spectrometry clearly indicates the attachment of the cofactor; all of the conjugates are within 2 Da of their expected calculated masses (the pyridoxamine forms) as noted in Table 2.

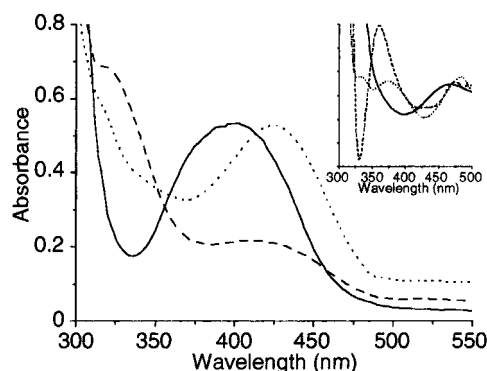
The conjugates IFABP-PxK38 and IFABP-PxK51 were next examined for their ability to catalyze the transamination of α -ketoglutarate and phenylalanine to glutamate and 3-phenylpyruvate.



Kinetic parameters, determined by measuring the initial rate at varying concentrations of α -ketoglutarate, were obtained for IFABP-PxK38 and IFABP-PxK51. The rate data, summarized in Table 3, were compared to the unliganded cofactor as well as to IFABP-Px; this latter construct lacks either of the new lysine residues. Both IFABP-PxK38 and IFABP-PxK51 show improved values for k_{cat}' and K_m' . The overall catalytic efficiency (k_{cat}'/K_m') of IFABP-PxK51 is 4200-fold increased compared to the unliganded pyridoxamine phosphate and 12-fold greater compared to IFABP-Px. The predominant kinetic effect of the lysine residues is on K_m' . IFABP-PxK38 has a K_m' value 2.2-fold lower than IFABP-Px

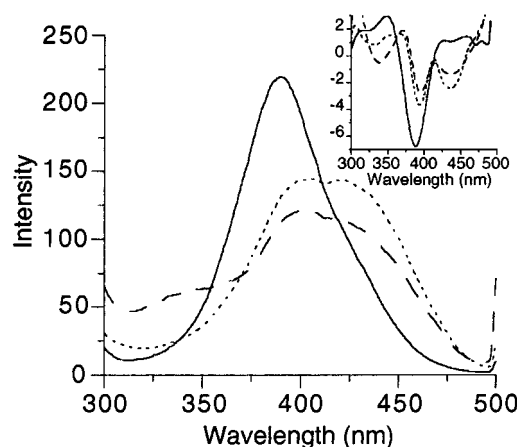
Table 3. Kinetic Parameters for Transaminations Reactions Catalyzed Unbound Pyridoxamine Phosphosphate (PMP) and IFABP-Px Conjugates

	PMP	IFABP-Px	IFABP-PxK38	IFABP-PxK51
K_m' (mM)	73 ± 7	1.8 ± 0.5	0.81 ± 0.08	0.24 ± 0.02
k_{cat} (h^{-1})	$3.2 \times 10^{-2} \pm 0.2 \times 10^{-2}$	0.29 ± 0.02	0.44 ± 0.01	0.44 ± 0.004
k_{cat}/K_m' ($\text{h}^{-1} \text{mM}^{-1}$)	$4.4 \times 10^{-4} \pm 0.7 \times 10^{-4}$	0.16 ± 0.06	0.54 ± 0.07	1.83 ± 0.17
$(k_{cat}/K_m')_{rel}$	1	360	1200	4200

**Figure 2.** UV absorption spectra of IFABP conjugates (IFABP-PxK38 dotted line; IFABP-PxK51 dashed; IFABP-Px solid; all $100 \mu\text{M}$ in 20 mM HEPES, pH 7.5). The insert shows the second derivatives of the spectra.

while IFABP-PxK51 manifests a larger, 7.5-fold decrease. Both conjugates display a small increase in k_{cat} (1.5-fold). Molecular modeling suggests that the decreases in K_m' values for the new conjugates are most likely due to the formation of specific interactions between the substrate-derived α -carboxylate group and the ϵ -amino groups of the new lysine residues. Finally, it should be noted that the catalytic transamination reactions promoted by IFABP-PxK38 and IFABP-PxK51 are enantioselective, yielding L-glutamate with high enantiomeric purities ranging from 83 to 94% ee depending on the amino acid substrate employed. These levels of enantioselectivity are comparable to those previously obtained with the conjugate IFABP-Px that lacks the engineered lysines. In particular, using α -ketoglutarate and phenylalanine as substrates, glutamate was obtained from IFABP-Px, IFABP-PxK38, and IFABP-PxK51 in 93% ee, 92% ee, and 87% ee, respectively. Using α -ketoglutarate and tyrosine as substrates, glutamate was obtained from the same conjugates in 91% ee, 92% ee, and 92% ee, respectively. Thus, the above kinetic results clearly indicate that the new lysine residues are participating in catalysis and are having a positive influence on catalytic transamination without eroding the enantioselectivity observed with the parent conjugate, IFABP-Px.

To gain insight into how the introduced lysine residues were interacting with the catalytic center, mechanistic studies were undertaken. IFABP-PxK38 and IFABP-PxK51 (both in their pyridoxamine forms) were incubated with excess α -ketoglutarate. Due to the absence of an amino acid substrate, only half of the catalytic cycle can be accomplished, resulting in the conversion of the pyridoxamine cofactor to the corresponding aldehyde. The UV absorbance spectra of these catalyst forms, displayed in Figure 2, showed a band at 422–428 nm, which is characteristic of a pyridoxal–Schiff base complex (Shaltiel and Cortijo, 1970; Metzler et al., 1980). The fluorescence excitation spectra (excitation at 300–500 nm; emission at 510 nm) shown in Figure 3 revealed a corresponding band at 421 nm (Shaltiel and Cortijo, 1970; Metzler et al., 1980). After reduction of the Schiff base to a secondary amine with sodium borohydride, the absorbance and

**Figure 3.** Fluorescence excitation spectra of IFABP conjugates (ex at 300–500 nm; em at 510 nm; IFABP-PxK38 dotted line; IFABP-PxK51 dashed; IFABP-Px solid; all $10 \mu\text{M}$ in 20 mM HEPES, pH 7.5). The insert shows the second derivatives of the spectra.

fluorescence bands disappeared. As a control, samples of the IFABP-Px conjugate without the lysine mutation were subjected to the same series of experiments. After reaction with α -ketoglutarate, this conjugate had a maximum in the UV absorption spectrum at 398 nm (Figure 2) and in the fluorescence excitation spectrum at 391 nm (Figure 3), both corresponding to the pyridoxal form (Kallen et al., 1985). Thus, while there are 15 lysine residues present in IFABP-Px, none of these are able to form a Schiff base with the covalently tethered pyridoxal. Presumably, as predicted from modeling experiments, none of these residues have a geometry suitable for Schiff base formation. Only in the case of the lysines introduced by design does Schiff base formation occur.

Additional evidence for Schiff base formation between the engineered lysine residues and the pyridoxal cofactor was obtained via electrospray ionization mass spectroscopy; data for these experiments is summarized in Table 2. Analysis of samples of IFABP-PxK38 and IFABP-PxK51 following treatment with excess α -ketoglutarate and borohydride revealed a mass loss of 17 m/z compared to the pyridoxamine forms of these conjugates. This is consistent with the loss of ammonia (NH_3) that would occur upon intramolecular Schiff base formation with the active site lysine residue. Treatment of IFABP-Px (without a lysine mutation) under similar conditions followed by mass spectrometric analysis resulted in a loss of 1 m/z compared to the amine form, consistent with the simple reduction of the aldehyde to the corresponding alcohol.

Finally, in addition to playing a role in Schiff base formation, the engineered lysine residues also appear to participate in catalysis as possible proton donors or acceptors. In Figure 4 the pH dependence of the initial rate of transamination and the enantiomeric ratio of IFABP-PxK38 are shown; similar bell-shaped curves were found in the case of IFABP-PxK51. In contrast, the pH dependence of IFABP-Px (lacking a lysine mutation) catalyzed transamination, measured under the

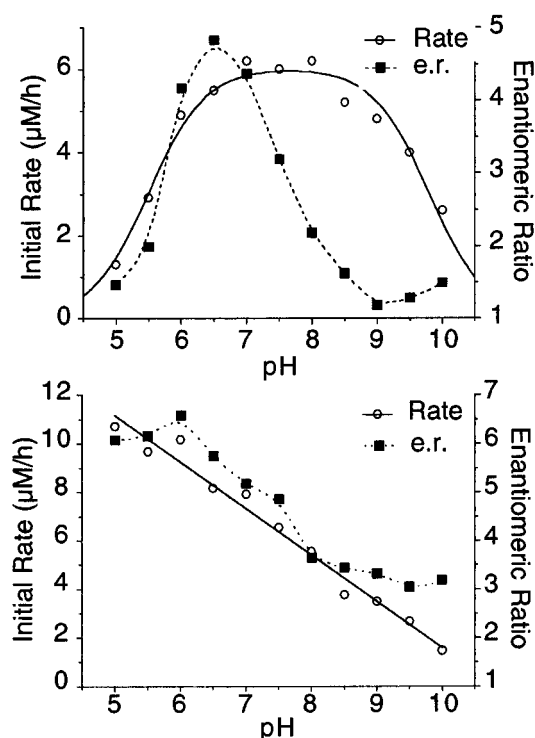


Figure 4. Top: The initial rate and enantiomeric ratio of the IFABP-PxK38 catalyzed transamination depend on the pH value ($pK_{S1} = 5.5$, $pK_{S2} = 9.8$). IFABP-PxK51 revealed a similar curve with $pK_{S1} = 7.2$ and $pK_{S2} = 9.8$. For comparison, native aspartate aminotransferase shows values of $pK_{S1} = 6.7$ – 7.2 and $pK_{S2} = 9.4$ – 10.3 for the pH dependence of k_{cat} in the transamination of α -ketoglutarate and L-aspartate or L-cysteinesulfinate (Gloss and Kirsch, 1995). Bottom: The initial rate and enantiomeric ratio of the IFABP-Px (without a lysine mutation) catalyzed transamination reaction.

same reaction conditions, was linear for both rate and selectivity with a negative slope toward higher pH values. Interestingly, the bell-shaped pH dependence manifested by IFABP-PxK38 and IFABP-PxK51 is similar to that of natural transaminases; clearly the lysine residues introduced into these protein conjugates are influencing catalysis. The plots of the enantiomeric ratio vs pH of IFABP-PxK38 and -PxK51 show a sharp maximum in contrast to that observed with IFABP-Px. Such results suggest that the engineered lysine residues may be directly involved in the protonation of the aldimine intermediate and in determining the enantioselectivity of the reaction.

It is helpful to place the results obtained here with the lysine-containing conjugates into context with earlier work using fatty acid binding proteins. In our initial work, a fatty acid binding protein was derivatized with the pyridoxamine using the same reagent as employed here (Kuang et al., 1996). The resulting construct promoted the reductive amination of various α -ketoacids with significant enantioselectivity but at a rate that was actually slower than that mediated by the free cofactor. Subsequent work in which the pyridoxamine was positioned at several sites within the cavity showed that the cofactor was highly sensitive to its location within the protein host (Kuang et al., 1997). IFABP-PX60 was estimated to be at least 9.4 times faster than the original conjugate in a single turnover reaction in which α -ketoglutarate was reductively aminated to glutamic acid and examined after 24 h. These results prompted us to perform a kinetic analysis of the single turnover reaction promoted by IFABP-PX60 as well as to examine the

potential for this construct to undergo catalytic turnover in which the net transformation would be the transamination reaction shown in eq 1 (Kuang et al., 1998). Interestingly, it was found that the single turnover reaction was accelerated 62-fold relative to free pyridoxamine when measured at a single substrate concentration (50 mM α -ketoglutarate). Further analysis of the concentration dependence under catalytic conditions enabled values for k_{cat}' and K_M' to be determined. Comparison of these values with those for the simple pyridoxamine-catalyzed reaction showed a significant decrease in K_M' (41-fold) and a modest increase in k_{cat}' (9-fold) leading to an overall increase in catalytic efficiency, k_{cat}'/K_M' , of 360-fold. It should be noted that this rate enhancement in the catalytic reaction occurred in concert with significant enantioselectivity (>90% ee). While the increased catalytic efficiency obtained with IFABP-PX60 was exciting, it is important to emphasize that the role of the protein in this conjugate is limited to serving as a chiral, shape-selective recognition element. In particular, residues that participate in the acid/base chemistry and in modulating the reactivity of cofactor-bound intermediates that would exist in a typical enzyme active site are not present in IFABP-PX60. It was with this in mind that we sought to introduce into the IFABP-PX60 scaffold, lysine residues that could participate in covalent catalysis.

The results presented here highlight the successful design and assembly of two catalytic systems based on a protein scaffold that function via covalent catalysis. The interaction of the protein scaffold with the catalytic group was demonstrated by several spectroscopic methods. The introduced lysine residues change the pH profile and the kinetic parameters. The catalytic efficiency increased as much as 4200-fold over that of the free cofactor which is a rate acceleration comparable to that achieved with early examples of catalytic antibodies (Tramontano et al., 1986; Pollack et al., 1986). These semisynthetic aminotransferases are the first examples of bioconjugates designed for covalent catalysis. While the increase in catalytic efficiency due to the lysine residues reported here is only modest, this is not surprising given the absence of additional residues normally present in transaminases that are thought to modulate cofactor/substrate reactivity. Nevertheless, the ability to rationally design catalytic systems that mimic features of enzymatic processes (e.g., covalent catalysis, pH dependence) is a significant achievement. Thus, this approach illustrates the utility of mechanistic information and structural analysis for catalyst design as well as the enormous flexibility that can be harnessed using a combination of chemical and genetic (chemogenetic) methods.

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Surface-Linked Liposomal Antigen Induces IgE-Selective Unresponsiveness Regardless of the Lipid Components of Liposomes

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We have previously reported that antigen coupled with liposomes induced antigen-specific and IgE-selective unresponsiveness in mice. This antigen preparation was investigated for application in a novel vaccine protocol to induce minimal IgE synthesis. In this study, ovalbumin (OVA)–liposome conjugates were made using liposomes of four different lipid components, including unsaturated carrier lipid and three different saturated carrier lipids, after which the induction of anti-OVA antibody production was investigated in mice. All of the OVA–liposome conjugates induced IgE-selective unresponsiveness. The membrane fluidity of liposomes, as measured by detecting changes in the fluorescence polarization of a 1,6-diphenyl-1,3,5-hexatriene (DPH) probe located in the bilayers, was significantly higher in liposomes consisting of unsaturated carrier lipids than those of the other liposomes consisting of saturated carrier lipids. The highest titer of anti-OVA IgG was observed in mice immunized with OVA–liposomes made using liposomes consisting of unsaturated carrier lipids. In addition, among these OVA–liposomes, the one possessing the longest carbon chain induced the lowest IgG antibody production. These results suggest that the membrane fluidity of liposomes might affect the adjuvant effect of liposomes but not the induction of IgE-selective unresponsiveness in immunizations with surface-linked liposomal antigens.

INTRODUCTION

Adjuvants play a key role in the development of novel vaccines, particularly for vaccine antigens with weak immunogenicity. Aluminum-containing compounds such as hydroxides and phosphates (alum) are the only adjuvants currently approved for clinical use. However, alum is known to stimulate only humoral responses (1) and is known to induce IgE antibody production, which elicits an allergic response in the vaccinee (2). Therefore, the availability of improved adjuvants suitable for clinical use is highly desirable. Among the candidates for adjuvants for novel vaccines, liposomes are garnering attention as carriers (vehicles) of antigens because they are known to act as powerful adjuvants when physically associated with a protein antigen (3–6). Most of the liposomal vaccines proposed have been made by antigen entrapment within the aqueous lumen of liposomes (7). However, it is known that differential immune responses are induced by encapsulated and surface-linked liposomal antigens in both humoral- (8) and cell-mediated (9) immunity. We have previously reported that surface-linked liposomal antigens induce antigen-specific IgG

antibody production but not antigen-specific IgE antibody production (10). Thus, surface-linked liposomal antigens are expected to serve as a novel protocol for the development of vaccines to induce minimal allergic response.

In this study, ovalbumin–liposome conjugates were made using liposomes of four different lipid components, and the induction of antibody production by these conjugates was investigated in mice. The effect of liposomal composition on adjuvanticity has been investigated in many different studies (11–14). However, the more specific effect of this composition on IgE unresponsiveness has been more rarely addressed. This study was aimed at correlating liposomal composition and the induction of IgE-unresponsiveness by surface-coupled liposomal antigens.

MATERIALS AND METHODS

Experimental Animals. BALB/c mice (8 weeks of age, female) were purchased from Charles River (Yokohama, Kanagawa, Japan).

Chemicals. All phospholipids were obtained from NOF Co., Tokyo, Japan. Reagent grades of cholesterol and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Wako Pure Chemicals Co., Osaka, Japan.

Liposomal Antigen. Ovalbumin (OVA, Grade VII) was purchased from Sigma (St. Louis, MO).

Liposomes. The liposomes consisting the four different components used in this study are listed in Table 1. The crude liposome solution was passed through a membrane filter (Nucleopore polycarbonate filter, Coster, Cambridge, MA) with a pore size of 0.2 μ m. The diameter of liposomes was analyzed using a NICOMP 370 Submicron Particle Sizer (Pacific Scientific, Silver Spring, MD).

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Table 1. Liposomes Used in This Study

name	liposome formulation	molar ratio
myristoyl	dimiristoyl phosphatidyl choline (DMPC)	4
	dimiristoyl phosphatidyl ethanolamine (DMPE)	3
	dimiristoyl phosphatidyl glycerol (DMPG)	2
	cholesterol	7
palmitoyl	dimiristoyl phosphatidyl choline (DMPC)	4
	dimiristoyl phosphatidyl ethanolamine (DMPE)	3
	dimiristoyl phosphatidyl glycerol (DMPG)	2
	cholesterol	7
stearoyl	dimiristoyl phosphatidyl choline (DMPC)	4
	dimiristoyl phosphatidyl ethanolamine (DMPE)	3
	dimiristoyl phosphatidyl glycerol (DMPG)	2
	cholesterol	7
oleoyl	dimiristoyl phosphatidyl choline (DMPC)	4
	dimiristoyl phosphatidyl ethanolamine (DMPE)	3
	dimiristoyl phosphatidyl glycerol (DMPG)	2
	cholesterol	7

Preparation of Fluorescence-Labeled Liposomes.

Fluorescence-labeled liposomes containing phospholipids consisting of an oleoyl group acting as an acyl chain were prepared as follows: 76.9 mg (97.88 μM) of DOPC, 54.6 mg (73.41 μM) of DOPE, 39.0 mg (48.94 μM) of DOPG, and 66.2 mg (171.29 μM) of cholesterol were dissolved in 2.37 mL of a solvent mixture (chloroform/methanol/water = 65/25/4 by volume), and then 0.2635 mL of 4.3 M DPH in chloroform solution was added. After a vigorous mixing, solvents were removed completely under reduced pressure, followed by the addition of 5 mL of 1 mM phosphate buffer (pH 6.0) containing 8.5% of sucrose to make a lipid vesicle suspension. The suspension was then extruded through a polycarbonate filter (0.2 μm) to adjust the liposome size. The lipid concentration of the resulting liposomes was 4.7 wt % (78.3 mM). Fluorescence-labeled liposomes containing phospholipids that consisted of a myristoyl group, palmitoyl group, or stearoyl group acting as the acyl chain were prepared by the same described above.

Fluorescence Measurement. Fluorescence depolarization of DPH was measured according to the method described by Lents et al. (15) using a Hitachi F-2000 spectrophotometer equipped with a temperature-controlled cuvette holder. DPH was excited at 360 nm, and its fluorescence was measured at 428 nm using an UV-39 instrument filter UV-39 to cutoff wavelengths below 390 nm. The degree of fluorescence polarization was calculated from the formula

$$P = (I_{VV} - I_{VH}G)/(I_{VV} + I_{VH}G)$$

where I_{VV} and I_{VH} are the observed fluorescence intensities measured with polarizers parallel and perpendicular to the vertically polarized exciting beam, respectively, and G is a correction factor for the transmission efficiency of vertically and horizontally polarized light. A 3-mL portion of 1 mM phosphate buffer (pH 6.0) containing 8.5% sucrose was mixed well with 0.3 mL of the liposome suspension prepared above. The mixture was then allowed to stand at 37 °C for 5 min before measurement. Three samples were prepared from each liposome suspension.

Coupling of OVA to Liposomes. Liposomal conjugates with OVA (OVA-liposome) via glutaraldehyde were prepared as described previously (10). Briefly, to a mixture of 90 mg of liposomes and 6 mg of OVA in 2.5 mL of phosphate buffer (pH 7.2) was added 0.5 mL of 2.5% glutaraldehyde solution in drops. The mixture was stirred gently for 1 h at 37 °C, and then 0.5 mL of 3 M glycine-NaOH (pH 7.2) was added to block excess

aldehyde groups. The mixture was then incubated overnight at 4 °C. The liposome-coupled OVA and uncoupled OVA in the resulting solution were separated using CL-4B column chromatography (Pharmacia Fine Chemical Co., Upsala, Sweden). The amount of lipid in the liposomal fraction was measured using a phospholipid content assay kit (Phospholipid-Test-Wako: Wako Pure Chemical Industries, Osaka, Japan). The OVA-liposome solution was adjusted to 10 mg lipid/mL and kept at 4 °C until use. For the measurement of OVA coupled to liposome, radiolabeled OVA ([methyl- ^{14}C]: purchased from New England Nuclear, Boston, MA) was mixed with cold OVA and used for coupling with liposome and for determining the calibration curve. The radioactivity of the resulting OVA-liposome solution was counted by using a calibration curve.

Immunization. Mice were immunized intraperitoneally (i.p.) with OVA-liposome at a dose of 2 mg lipid/200 μL /mouse. Control mice received i.p. injection with plain OVA solution in phosphate-buffered saline (PBS, pH. 7.2) at a dose of 140 μg OVA/200 μL /mouse. Four weeks after primary immunization, mice were boosted in the same manner as that used for primary immunization. For the measurement of serum antibodies, mice were bled from the tail vein.

Measurement of Serum Antibodies. Six weeks after primary immunization, serum anti-OVA IgG and IgG isotypes were determined by ELISA using HRP-labeled goat anti-mouse IgG (H+L), IgG1, IgG2a, IgG2b, and IgG3 (Zymed lab., San Francisco, CA). OVA-specific IgE was determined by monoclonal antibody-captured ELISA using monoclonal antibody against murine IgE produced by clone B1E3.

Spleen Cell Culture. Six weeks after primary immunization, spleen cells (SC) were taken from mice and suspended in RPMI-1640 containing 10% FCS at a cell density of $5 \times 10^6/\text{mL}$. In the preliminary examination, OVA at a final concentration of 1 mg/mL and a 24-h culture period was found to be optimal for the production of IFN- γ and IL-2. The cell suspension was plated at 100 μL per well onto a 96-well culture plate (No. 3072, Becton Dickinson Labware, Franklin Lakes, NJ), and 100 μL of 2 mg/mL OVA solution in the same media was added to each well. After incubation for 24 h in a CO_2 incubator, the culture supernatants were assayed for IFN- γ and IL-2.

Cytokine ELISA. Concentrations of IFN- γ and IL-2 in the culture supernatants were measured using a Biotrak mouse ELISA system (Amersham International, Buckinghamshire, UK). All test samples were assayed in duplicate, and the standard error (SE) in each test was less than 5% of the mean value.

Statistic Analysis. The Student's t-test was employed for the statistical analysis.

RESULTS

Liposome Diameter, Amount of OVA Coupled to Liposomes, and Polarization of DPH. There were no significant difference in diameter and in the amounts of OVA coupled to liposomes in OVA-liposome conjugates between the four liposome formulation (Table 2). Figure 1 shows fluorescence polarization degree of each liposome preparation determined at 37 °C. The degree of fluorescence polarization in "Oleoyl" liposomes, consisting of unsaturated fatty acids, was significantly lower than that in the other three liposomes, consisting of saturated fatty acids. In addition, a significant difference in the degree of fluorescence polarization was also observed among three liposomes consisting of saturated fatty acids.

Table 2. Liposome Diameter and Amount of OVA Coupled to Liposome^{a,b}

liposome formulation	diameter, nm (mean $\pm$ SD)	OVA, mg/10 liposome (mean $\pm$ SD)
myristoyl	241.2 $\pm$ 65.7	0.68 $\pm$ 0.04
palmitoyl	249.5 $\pm$ 50.2	0.70 $\pm$ 0.08
stearoyl	237.3 $\pm$ 54.6	0.59 $\pm$ 0.06
oleoyl	231.3 $\pm$ 28.9	0.62 $\pm$ 0.02

^a The diameter of liposomes was analyzed using a NICOMP 370 Submicron Particle Sizer. Data represent mean and SD of three independent measurements. ^b The amount of OVA coupled to liposome was measured using radiolabeled OVA as described in Materials and Methods. Data represent mean and SD of three independent measurements.

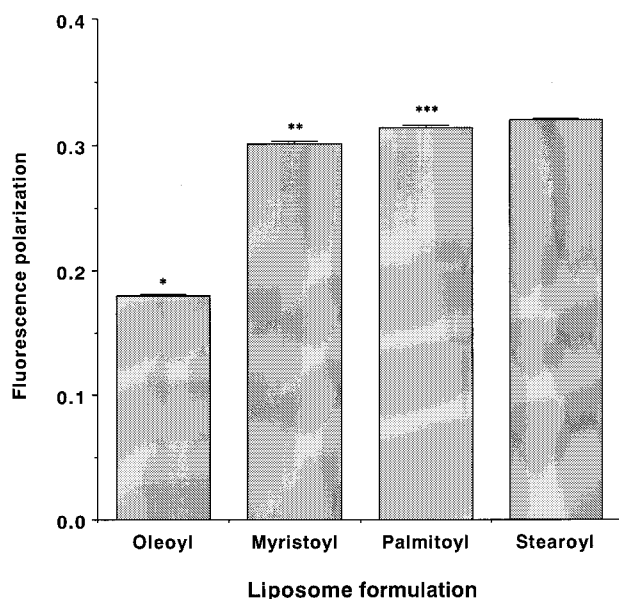


Figure 1. Fluorescence polarization degree in the liposomes with four different lipid formulations. The degree of fluorescence polarization at 37 °C was calculated as described in Materials and Methods. Data represent mean and SE of the triplicate measurements. Asterisk, $P < 0.000001$ compared with "Myristoyl" group; two asterisks, $P < 0.001$ compared with "Palmitoyl" group; three asterisks, $P < 0.01$ compared with "Stearoyl" group.

Anti-OVA Antibody Production in Mice Immunized with OVA-Liposome. Mice were immunized with OVA-liposome conjugates made using four different liposome formulations, and anti-OVA antibody production was investigated. Anti-OVA IgE antibody production was not observed in any of the OVA-liposome-immune groups (data not shown). As shown in Figure 2a, conjugates made using "Oleoyl" liposomes induced a significantly higher level of anti-OVA IgG antibody production as compared with those induced by the other three conjugates. Among the other three conjugates, those made using "Stearoyl" liposomes induced the lowest level of anti-OVA IgG antibody although the levels were higher than those of the control group, which received injections with plain OVA solution. The proportion of anti-OVA IgG isotype antibodies also varied among the immunization groups (Figure 2b). In mice immunized with OVA-liposome conjugates made using "Myristoyl", "Palmitoyl", and "Stearoyl" liposomes, the anti-OVA IgG response was dominated by IgG1 production; 80.8%, 75.9%, and 80.0% of the total anti-OVA IgG, respectively. In contrast, in mice immunized with OVA-liposome conjugates made using "Oleoyl" liposomes, IgG1 was 54.5% of the total anti-OVA IgG, while the rest were IgG2a (20.2%), IgG2b (21.2%), and IgG3 (4.0%).

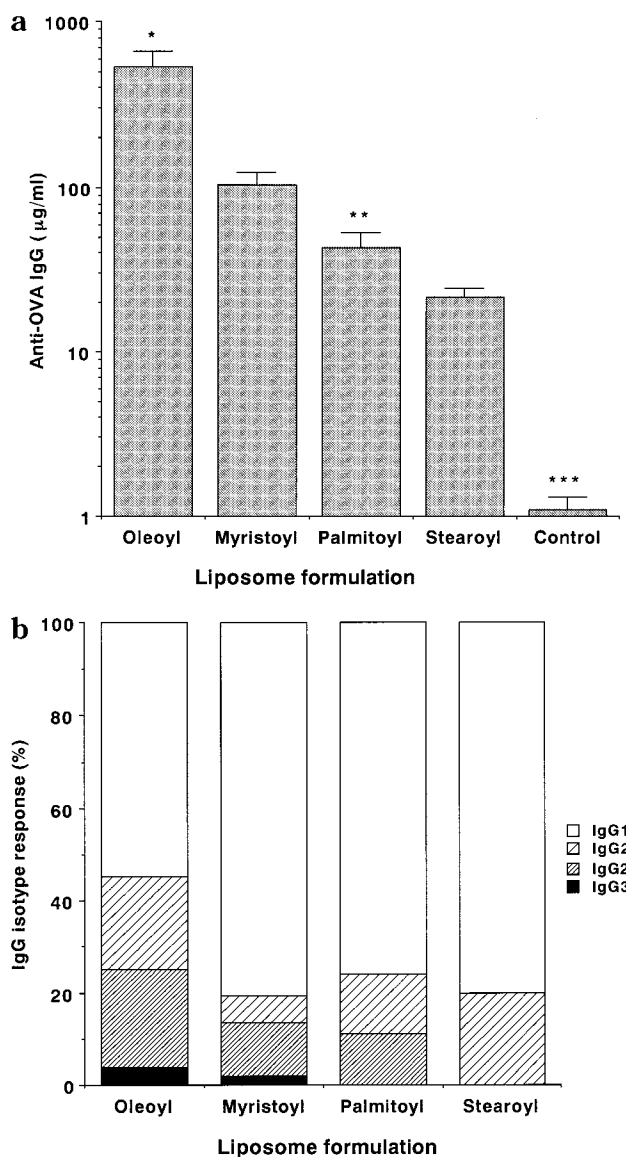


Figure 2. Anti-OVA antibody production in mice. Six weeks after primary immunization, mice were bled from the tail vein and serum anti-OVA antibodies were measured. **a**, Titers of serum anti-OVA IgG. Data represent mean and SE of five mice per group. Asterisk, $P < 0.005$ compared with "Myristoyl" group; two asterisks, $P < 0.05$ compared with "Myristoyl" group and "Stearoyl" group; three asterisks, $P < 0.001$ compared with OVA-liposome-immune groups. **b**, Percentage anti-OVA IgG isotype response. The same serum samples in Figure 2a were assayed for the anti-OVA IgG isotype. Data represent percentage of the total anti-OVA IgG.

Cytokine Production by Spleen Cells of Mice Immunized with OVA-Liposome. Six weeks after primary immunization, cytokine production by SC of mice after *in vitro* restimulation with OVA was investigated. Correlating well with the results of anti-OVA IgG antibody production *in vivo* (Figure 2a), the level of IFN- γ produced by the SC of mice immunized with OVA-liposome made using "Oleoyl" liposomes was significantly higher than that in the other three groups (Figure 3). There were no significant differences in IL-2 production among the four immunization groups.

DISCUSSION

In the present study, liposomes with four different formulations were used to prepare OVA-liposome conjugates. All of the conjugates induced IgE-selective

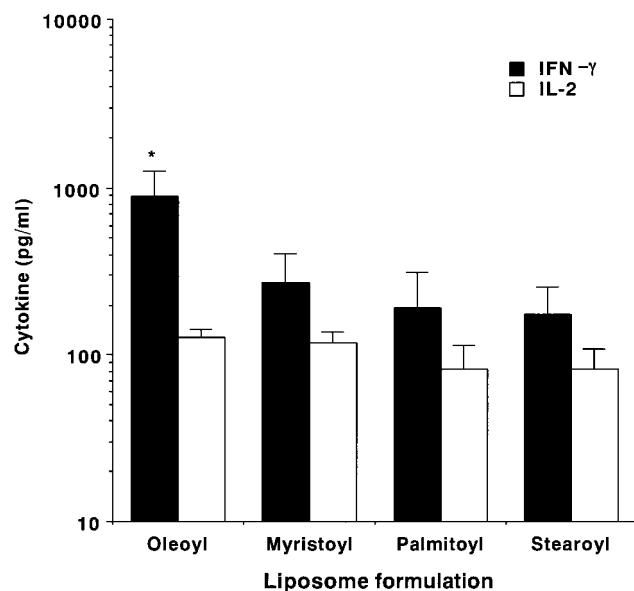


Figure 3. Cytokine production by SC of mice immunized with OVA. Six weeks after primary immunization, SC of mice were cultured in vitro with OVA, and IFN- γ and IL-2 in the supernatants were measured thereafter. Data represent mean and SE of five mice per group. Asterisk, $P < 0.005$ compared with "Myristoyl", "Palmitoyl", or "Stearoyl" group. ■, IFN- γ . □, IL-2.

unresponsiveness. Among liposomes with the four different formulations, the fluorescence polarization values of the Oleoyl liposomes were significantly lower than those of the other three liposome preparations (Figure 1). The decrease in fluorescence polarization values can be interpreted as being due to an increase in mobility of the hydrophobic region of phospholipid bilayers in the membranes. OVA-liposome conjugates made using Oleoyl liposomes induced a significantly higher level of anti-OVA IgG antibody production (Figure 2a). In contrast, OVA-liposome made using Stearoyl liposome induced the lowest level of anti-OVA IgG antibody production. Among the four liposome preparations, only the Oleoyl liposomes consisted of unsaturated fatty acids. Membrane mobility would be higher in liposomes prepared using unsaturated fatty acid than in those liposomes prepared using saturated fatty acids. The chain length of the three saturated fatty acids used in the present study, Myristoyl, Palmitoyl, and Stearoyl, are 14, 16, and 18, respectively. In general, the shorter the acyl chain length, the higher the membrane mobility. Taken together, the membrane mobility of liposomes may correlate with liposomal adjuvanticity in immunizations with surface-coupled antigens of liposomes.

The results obtained in the present study differ from those mentioned in the review by Gregoriadis (3) in two points: (1) the adjuvanticity of liposomes does not depend on any specific vesicle characteristic (e.g., composition, size, surface charge, etc.). However, in this study, the adjuvant effects of liposomes differed strikingly among the different liposome formulations; (2) liposomal adjuvanticity occurs during primary immunization and is observed with most IgG subclasses with no apparent shift observed in subclass responses as compared with responses obtained with the free antigen. However in the present study, an increased production of IgG2a and IgG2b was observed in mice immunized with OVA-liposome made using Oleoyl liposomes (Figure 2a). These difference may be due to Gregoriadis and co-workers having used liposome-encapsulated antigen. In fact,

Shahum and Therien (8) have claimed that liposomal antigens differently influence the activation of the immune system depending on the mode of association of the antigen with the liposomal vehicle, whether it is by encapsulation or surface linkage. The present results further demonstrates that, even among surface-linked antigens of liposomes, the adjuvanticity of liposomes differs among specific vesicle characteristics (e.g., composition).

There are two types of processes for the mechanism of liposome uptake into cultured cells: phagocytosis and fusion (15). The differential fluidity of liposomes may affect the phagocytosis by antigen-presenting cells. In the group of mice immunized with OVA-liposome made using Oleoyl liposomes, a significantly higher level of IFN- γ production was observed as compared with that in the other groups (Figure 3). In the course of macrophage-T cell interactions, IFN- γ secreted from T cells may activate macrophages to increase phagocytic activity and antigen processing, which may result in the increased antigen recognition by T cells and an enhancement of antibody production.

Thus, in the present study, liposomes with different formulations were used to prepare OVA-liposome conjugates, and the induction of anti-OVA antibody production was investigated in mice. The liposomal adjuvanticity was strikingly different among different formulations of liposomes, although all of the OVA-liposome conjugates induced IgE-selective unresponsiveness. These results suggest that liposomal adjuvanticity, membrane mobility, or the lipid formulation of the liposomes did not affect the effect of the surface-coupled liposomal antigens on the induction of IgE-selective unresponsiveness. Efforts to clarify the mechanism of the induction of IgE-selective unresponsiveness with surface-coupled antigens of liposomes are in progress.

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Synthesis of the First Ferrocene-Labeled Dideoxynucleotide and Its Use for 3'-Redox End-Labeling of 5'-Modified Single-Stranded Oligonucleotides

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The target ferrocene-labeled dideoxynucleotide compound 5-[*N*-(β -ferrocenyl-propanoyl)3-amino-propyn-1-yl]-2',3'-dideoxyuridine 5'-triphosphate, Fc-ddUTP, was synthesized and tested with terminal deoxynucleotidyl transferase for enzymatic 3'-redox-active end-labeling of 5'-phosphorylated single-stranded oligodeoxynucleotides. Starting from readily available 5-iodouridine and 3-ferrocenylpropanoic acid, the synthetic strategy elaborated here follows a mild multistep route. Each step involves reliable methods, and all ferrocene intermediates can be easily purified. Enzymatic 3'-ferrocene end-labeling of 5'-phosphorylated oligonucleotides is remarkably efficient, and 3'-ferrocene-labeled oligonucleotides can thus be prepared in sufficient amounts for further use in surface modifications.

Redox-active labeling of DNA has received recent substantial attention (1–14). In this regard, ferrocene-ended single stranded DNA oligomers have emerged as important versatile tools for the investigation of nucleic acids. In particular, ferrocene–oligodeoxynucleotide (ODN) conjugates have been recognized as viable alternatives to the use of radioactive isotopes in traditional DNA sequencing techniques, and their usefulness as hybridization redox probes for electrochemical sensing of target DNA/RNA sequences has been demonstrated in a number of modern applications (6–11). More specifically, ferrocene-labeled ODNs are routinely finding utility in the development and miniaturization of bioelectronic gene-sensing systems (12, 13).

A great, yet nonexploited, potential lies in the development of ferrocene-ended DNA probes bearing at the other terminus a function able to create a stable attachment to solid supports, surfaces and biomolecules. To our knowledge, only one group has been interested to address this promising research area. In this context, Letsinger et al. can be acknowledged for the pioneering synthesis of a 5'-ferrocene-nucleotide 3'-thiol-ended adsorbate for chemisorption onto gold surfaces and characterization of a redox-active nucleotide monolayer (14). However, despite the potential interest recognized by the authors in preparing redox-active oligonucleotide molecular assemblies on well-defined surfaces, it appears that the proposed chemical synthetic strategy for introducing a ferrocene label and a chemical functionality within an individual nucleotide was not expanded any further to oligonucleotides. Their strategy which focuses primarily on the construction of a ferrocene-labeled phosphoramidite for subsequent coupling to a nucleotide by standard automated DNA solid-phase synthesis suffers from a serious drawback: the ferrocene subunit does not suitably withstand the chemical oxidation step conditions required (iodine in basic THF/H₂O) in the coupling cycle. As noted in ref 14, the bound-ferrocene is readily converted into its corresponding ferrocenium form. However,

in the absence of an appropriate reductant, ferrocenium cations are well-known to rapidly hydrolyze in neutral/basic media with only partial regeneration of the parent ferrocene (15–17). Therefore, failure sequences lacking the ferrocene function inevitably are formed as byproducts, thus affecting product yield and reproducibility. While the phosphoramidite method generally offers the versatility of incorporating a metal complex at any site along the synthetic ODN (3, 18), postmodifications of a functionalized oligonucleotide after standard solid-phase synthesis are more appropriate approaches for incorporation of the sensitive ferrocene functionality (6, 10).

As part of an ongoing program on the investigation of the dynamic properties of biopolymer chains end-tethered to surfaces (19, 20), our interest was focused on developing redox-active ferrocene end-labeled DNA oligomers having a transformable monophosphate group at the 5'-end. The phosphate at this terminus is of importance since it can be used directly for ready attachment to amino-bearing surfaces through phosphoramidate linkages while preserving the integrity of the DNA single-strand sequence. Moreover, this 5'-end group can be profitably exploited for diverse bioconjugations and extension modifications (21, 22). Our approach to these complex labeled oligonucleotides combines key features that make it well-suited for preparation of 3'-ferrocene-5'-phosphorylated oligonucleotides that vary considerably in nature and length, from short ODNs accessible by chemical synthesis to enzymatically synthesized long nucleic acid sequences. Specifically, the route involves enzymatic 3'-end-labeling of presynthesized 5'-phosphorylated oligonucleotides that leaves the 5'-end intact and makes use of terminal deoxynucleotidyl transferase (terminal transferase, TDT). Terminal transferase catalyzes a template-independent addition of deoxyribonucleoside triphosphates to the 3'-OH ends of nucleic acids (23, 24). The enzyme accepts also a number of deoxynucleoside triphosphates containing modified base and/or sugar moieties. In this regard, C-5 labeled uridine 2',3'-dideoxynucleotides (ddUTPs) comprise a unique class of monomer substrates that are compatible with polymerase enzymes, and due to lack of the 3'-hydroxyl

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functionality on the 2'-deoxyribose, offer a unique way to add a single label group such as biotin and digoxigenin (25, 26) or fluorophore groups (27, 28), to a DNA fragment. These labeled nucleotides are to date critical compounds in DNA sequencing techniques and antiviral chemotherapy (29, 30), as well as in molecular biology and biochemistry studies. Thus it seemed to us quite reasonable to expect a tolerance of terminal transferase to modification of the uracyl base moiety with a ferrocene unit.

As noted recently in a comprehensive review, no general and high-yielding route for preparing 2',3'-dideoxynucleosides 5'-triphosphates is currently available, but a renewed interest in the synthesis of these important compounds is highly expected (31). We felt that application of modern synthetic organic methodologies utilized in 2,3'-dideoxyuridines (ddUs) chemistry (29, 30) coupled with recent methods of analysis for the detection and isolation of nucleoside triphosphates would lead to a useful synthetic approach to ferrocene-labeled 2',3'-dideoxynucleoside triphosphates. In this paper, we describe the complete chemical synthesis of a first representative of the series, 5-[*N*-(β -ferrocenyl-propanoyl) 3-amino-propyn-1-yl]-2',3'-dideoxyuridine 5'-triphosphate, Fc-ddUTP, from inexpensive 5-iodo-2'-deoxyuridine. All reactions utilize readily available reagents and standard laboratory conditions. We further demonstrate that Fc-ddUTP is an excellent substrate for terminal deoxynucleotidyl transferase, a commercially available enzyme, and can be employed for a routine preparation of redox active 3'-end-labeled DNA probes.

EXPERIMENTAL SECTION

Ferrocenylmethyltrimethylammonium iodide was purchased from Strem Chemicals and from Aldrich Chemical Co. 5-Iodo-2'-deoxyuridine and propargylamine were purchased from Aldrich Chemical Co. Tetra-*n*-butylammonium dihydrogenophosphate (NBu₄KH₂PO₄) (99+%) was purchased from Fluka. Tri-*n*-butylammonium pyrophosphate ((HNBu₃)₂H₂P₂O₇) was obtained from Sigma Chemical Co. Other chemicals were obtained from commercial sources and were reagent grade or better quality and used as received. All solvents were anhydrous unless otherwise noted.

Terminal deoxynucleotidyl transferase (EC 2.7.7.31), TDT, and tailing reaction buffer were purchased from Roche Molecular Biochemicals (Boehringer, Mannheim). Synthetic 5'-phosphorylated oligonucleotides (sodium salts) were purchased from ESGS Cybergene (Evry, France) and were HPLC purified grade products. Centricon-30 YM cellulose membranes were purchased from Amicon.

Unless otherwise specified, all reactions were carried out in oven-dried glassware under argon atmosphere. All the ferrocene compounds prepared in this work were stored in vacuo (over P₂O₅) at ambient temperature, protected from light, and dried for at least 12 h before first use.

Chromatography. RP high-performance liquid chromatography (HPLC) was performed on a system equipped with two Gilson HPLC pumps (Model 305 and 306), a Gilson 119 UV-vis detector operating at 260 or 270 nm. Pump control and data processing used a Rainin Dynamax chromatographic software package (Macintosh).

For nonionic ferrocenes, analytical HPLC was carried out on a Waters (Milford, MA) Novapak C₁₈ (4 μ m, 3.9 mm i.d. $\times$ 15 cm) column with a linear gradient elution from 35% to 75% acetonitrile in water in 30 min (system

I). For other analytical runs, HPLC solutions were as follows: solvent A, 10% acetonitrile in aqueous KH₂PO₄ (20 mM) and NBu₄KH₂PO₄ (5 mM), pH adjusted to 7.5 with KOH; solvent B, acetonitrile. HPLC on a Novapak C₁₈ was performed isocratically with 25% B (system II) or following a linear gradient elution from 10 to 40% B in 60 min (system III). Purification of ferrocene-end-labeled oligonucleotides was carried out by HPLC on a Nucleosil 120C₁₈ (5 μ m, 4.6 mm i.d. $\times$ 15 cm) column (20 μ L loop) following a linear gradient elution from 25% to 35% B in 20 min (system IV). For other experiments performed on a Nucleosil 120C₁₈, the following conditions were used: solvent A, 10% acetonitrile in aqueous triethylammonium acetate, Et₃NH⁺, AcO⁻ (TEAA, 0.1 M) pH adjusted to 7.0 with acetic acid; solvent B, acetonitrile, (100 μ L loop). Elutions were done with a linear gradient from 2.5% to 25% B over 30 min (system V). For all HPLC runs the flow rate was 0.8 mL/min.

Thin-layer chromatography (TLC) analyses were performed on 0.25 mm silica gel Polygram SIL G/UV₂₅₄ plates. Preparative column chromatography was carried over Magerey-Nagel silica gel (0.063–0.2 mm), or over Sigma Chemical Co. ion-exchange DEAE cellulose or DOWEX 50WX4–100 (H⁺ form, strongly acidic) resins.

A 1 M solution of triethylammonium hydrogenocarbonate Et₃NH⁺, HCO₃⁻ (TEAC) was prepared by bubbling CO₂ through an aqueous mixture of triethylamine until the organic layer disappeared and adjusted to pH 7.5 with Et₃N.

Spectrometries. ¹H and proton-decoupled ¹³C NMR spectra were recorded on a Bruker AM-200 spectrometer operating at 200 and 50.3 MHz, respectively, in CDCl₃, CD₃OD, and/or DMSO-*d*₆. Chemical shifts are reported in parts per million relative to the solvents' residual protons CDCl₃ δ 7.27 ppm, CD₃OD, 3.31 ppm, DMSO-*d*₆ 2.50 ppm for ¹H experiments and the center line of CDCl₃ (77.00 ppm) CD₃OD (49.00) for ¹³C NMR experiments. ³¹P NMR spectra were recorded on a Bruker AM-400 spectrometer operating at 161.9 MHz in D₂O and referenced to 85% H₃PO₄.

Fast atom bombardment (FAB) mass spectra were obtained in the negative ion mode from the Ecole Normale Supérieure, Département de Chimie (Paris, France) using "magic bullet" (MB) as matrix. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a PerSeptive Biosystems Inc. Voyager Elite instrument, operating in the negative mode using 3-hydroxypicolinic acid in diammonium citrate as the matrix and 355-nm pulsed light (49). External calibration was carried out using HPLC de-salted 5'-p-(dT)₁₀ (system V) in the above matrix.

UV-visible spectra and optical density (OD) were measured on a Hewlett-Packard HP 8452 diode array spectrophotometer. The quantity and yield of the ferrocene-nucleotides was determined by measuring the OD₂₉₀ of aqueous solutions of ferrocene-nucleotides and taking the value of ϵ_{290} for Fc-ddU **7** (ϵ_{290} = 9500 M⁻¹ cm⁻¹) as a standard value for **8–11**.

3-Ferrocenylpropanoic Acid (1). 3-Ferrocenylpropanoic acid **1** was prepared from ferrocenylmethyltrimethylammonium iodide following an adaptation of the three-step procedure reported in the past (50). The main modification is that ferrocenylmethylmalonic diethyl ester obtained in the first step was rigorously purified by fractional distillation before use. Without that additional purification step, we have found that the ferrocene monocarboxylic **1** could not be obtained as a clean sample.

A solution of diethyl malonate (8.8 g, 55 mmol) in 25

mL of absolute ethanol was added dropwise to a well-stirred solution containing freshly cut sodium (1.26 g, 55 mmol) in 50 mL of absolute ethanol. After complete addition, ferrocenylmethyltrimethylammonium iodide (19.3 g, 50 mmol) was added. The solution was heated under reflux for 72 h. After cooling to ambient temperature, the reaction mixture was diluted with water (200 mL), neutralized with 1 N HCl, and then extracted with two 100-mL portions of diethyl ether. The combined extracts were washed with saturated aqueous sodium hydrogenocarbonate, dried over magnesium sulfate, filtered, and evaporated to give an oily residue that was subjected to fractional distillation under reduced pressure. Discarding the first colorless fraction corresponding to residual diethyl malonate, the desired ferrocenylmethylmalonic diethyl ester was distilled as an orange oil (3 mmHg, 139–141 °C) which slowly solidified on standing (12.1 g, 68%). ¹H NMR (CDCl₃) δ 4.19 (q, 4H, OCH₂), 4.13 (s, 5 H, CpH), 4.08 (m, 4 H, CpH), 3.46 (t, *J* = 7.45 Hz, 1H, CH), 2.97 (d, *J* = 7.45 Hz, 2H, FcCH₂), 1.25 (t, *J* = 7.13 Hz, 6H, CH₃). ¹³C NMR (CDCl₃) δ 168.87, 84.55, 68.52, 67.57, 61.24, 54.15, 29.15, 13.96.

Ferrocenylmethylmalonic ester (10.7 g, 30 mmol) was saponified in 25 mL of 6 N potassium hydroxide in 90% aqueous ethanol under reflux for 24 h. The reaction mixture was diluted with water (150 mL) and extracted with two 100-mL portions of diethyl ether. The aqueous phase was neutralized with 6 N HCl. After addition of diethyl ether (150 mL), the mixture was cautiously acidified with 6 N HCl under stirring until extraction with ether. The organic extract was washed with water, dried over magnesium sulfate, and evaporated to obtain 2-ferrocenylmethyl malonic acid (8.2 g, 90%). ¹H NMR (DMSO-*d*₆) δ 12.73 (br s, 2H, COOH), 4.2–4.0 (m, 9H, CpH), 3.32 (t, *J* = 7.33 Hz, 1H, CH), 2.79 (d, *J* = 7.33 Hz, 2H, FcCH₂).

A sample of 2-ferrocenylmethyl malonic acid (7.6 g, 35 mmol) was heated at 150–160 °C for 0.5 h. The resulting residue was then purified by column chromatography on silica gel in chloroform/methanol, CHCl₃/MeOH (95:5) to afford analytically pure 3-ferrocenylpropanoic acid **1** (6.0 g, 93%). mp 121–122 °C (116–118 °C (50), 119–120 °C (51)). ¹H NMR (CDCl₃) δ 4.2–4.05 (m, 9H, CpH), 2.75–2.55 (m, 4H, FcCH₂CH₂). ¹³C NMR (DMSO-*d*₆) δ 174.08, 87.73, 68.30, 67.67, 66.94, 34.98, 24.31.

5-*N*-(β-Ferrocenyl-propanoyl)-3-amino-1-propyne (2). To a stirred solution of ferrocene **1** (5.16 g, 20 mmol) in 200 mL of dichloromethane were added 1-hydroxybenzotriazole (HOBT) (270 mg, 2 mmol) and propargylamine (2.06 mL, 40 mmol). After addition of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, EDC (4.22 g, 22 mmol), the mixture was allowed to react under stirring for 24 h at ambient temperature. The solution was successively washed with three 100-mL portions of 0.5 M H₂SO₄, water (100 mL), and saturated aqueous sodium hydrogenocarbonate. The organic phase was dried over magnesium sulfate, and the solvent was removed under reduced pressure. The solid residue was chromatographed on silica gel in CHCl₃/MeOH (95:5), yielding analytically pure ferrocene conjugate **2** (5.54 g, 94%). mp 78–79 °C. ¹H NMR (CHCl₃) δ 5.56 (br s, 1H, CONH), 4.3–3.9 (m, 11H, CpH + NHCH₂), 2.70 (m, 2H, FcCH₂), 2.38 (m, 2H, CH₂CO), 2.23 (t, *J* = 2.55 Hz, 1H, CH). ¹³C NMR (CD₃OD) δ 175.14, 88.79, 80.60, 72.12, 69.48, 68.96, 68.30, 38.27, 29.39, 26.63.

5-[*N*-(β-Ferrocenyl-propanoyl)-3-amino-propyn-1-yl]-2'-deoxyuridine (3) (Fc-dU). To a stirred solution of 5-iodo-2'-deoxyuridine (3.54 g, 10 mmol) in 50 mL of degassed DMF were added cuprous iodide (CuI) (38 mg,

0.2 mmol) and ferrocene **2** in excess (7.38 g, 25 mmol). The solution was stirred for 30 min at ambient temperature at which time triethylamine (Et₃N) (2.78 mL, 20 mmol) and tetrakis(triphenylphosphine)palladium(0) (PhP₃)₄Pd (116 mg, 0.1 mmol) were added. After stirring of the solution for 48 h, CuI (0.2 mmol) and (PhP₃)₄Pd (0.1 mmol) were added anew followed by addition of Et₃N (20 mmol). The reaction mixture was allowed to stir for another 24 h. At this point, TLC (CHCl₃/MeOH (85:15)) indicated quantitative conversion of the nucleoside reagent (*R*_f 0.42) to give the desired nucleoside–ferrocene product **3** (*R*_f 0.51). The solution was concentrated under vacuum at a temperature maintained at ≤35 °C, and the liquid residue was separated on silica gel. A first yellow fraction containing remaining starting ferrocene was eluted with CHCl₃/MeOH (95:5). A second yellow fraction was eluted with CHCl₃/MeOH (85:15), affording the desired product contaminated with a small amount of triethylammonium iodide. Crystallization from acetonitrile yielded analytically pure **3** (3.76 g, 72%). mp 196–198 °C (with some decomposition). ¹H NMR (CD₃OD) δ 8.30 (s, 1H, H-6), 6.24 (app t, *J* = 6.6 Hz, 1H, H-1'), 4.40 (m, 1H, H-3'), 4.2–4.0 (m, 11H, CpH + NHCH₂), 3.94 (m, 1H, H-4'), 3.85–3.70 (m, 2H, H-5'), 2.65 (m, 2H, FcCH₂), 2.42 (m, 2H, CH₂CO), 2.4–2.1 (m, 2H, H-2'). ¹³C NMR (CD₃OD) δ 175.16, 164.52, 151.11, 145.38, 99.94, 90.07, 89.15, 88.80, 87.03, 75.18, 72.07, 69.50, 68.97, 68.34, 62.62, 41.71, 38.36, 30.43, 26.63. HPLC (system I), retention time, *t*_r = 5.88 min.

A second chromatography using chloroform was performed on crude collected ferrocene **2** and allowed recovery of 2.95 g (67% from starting excess) of an essentially pure sample which could be used without further purification for a next preparation of **3**.

5'-O-(tert-Butyldimethylsilyl)-5-[*N*-(β-ferrocenyl-propanoyl)-3-amino-propyn-1-yl]-2'-deoxyuridine (4) (Fc-dU-5'TBDMS). Ferrocene–nucleoside **3** (1.56 g, 3 mmol) and imidazole (0.51 g, 7.5 mmol) were dissolved in 30 mL of DMF under stirring. To the solution was added *tert*-butyldimethylsilyl chloride (0.57 g, 3.75 mmol). The reaction mixture was stirred with the exclusion of moisture at ambient temperature for 1 h. The solvent was removed under vacuum at a temperature maintained at ≤35 °C, and the yellow residue was purified by column chromatography on silica gel in ethyl acetate/cyclohexane (95:5). Isolation of the major yellow component afforded the desired 5'-monosilylated ferrocene–nucleoside **4** as an orange solid (1.62 g, 85%). mp 165–166 °C. ¹H NMR (CD₃OD) δ 8.12 (s, 1H, H-6), 6.24 (dd, *J* = 7.45, 6.00 Hz, 1H, H-1'), 4.46 (m, 1H, H-3'), 4.2–4.0 (m, 12H, CpH + NHCH₂ + H-4'), 3.94 (dd, *J* = 11.50, 2.33 Hz, 1 H, H-5'), 3.84 (dd, *J* = 11.50, 2.59 Hz, 1H, H-5'), 2.66 (m, 2H, FcCH₂), 2.40 (m, 2H, CH₂CO), 2.4–2.1 (m, 2H, H-2'), 0.97 (s, 9H, *t*-BuSi), 0.19 (m, 6H, CH₃Si). ¹³C NMR (10% DMSO-*d*₆ in CD₃OD) 174.28, 163.85, 150.83, 144.35, 99.89, 90.67, 89.22, 89.02, 87.00, 75.60, 72.54, 69.56, 68.95, 68.33, 64.57, 42.22, 38.11, 30.27, 26.74, 26.44, 16.33, –4.90, –5.02. HPLC (system I) *t*_r = 19.49 min.

5'-O-(tert-Butyldimethylsilyl)-3'-O-(phenoxythiocarbonyl)-5-[*N*-(β-ferrocenyl-propanoyl)-3-amino-propyn-1-yl]-2'-deoxyuridine (5). (Fc-dU-5'TBDMS-3'PTC). To a solution of **4** (1.50 g, 2.36 mmol) in 3 mL of dichloromethane containing 1.4 mL of pyridine (17.3 mmol), cooled to 0 °C, was added dropwise a solution of phenoxythiocarbonyl chloride (0.94 mL, 6.84 mmol) in 3 mL of dichloromethane over a period of 30 min. After complete addition, the reaction mixture was stirred at ambient temperature for 2 h. At this point, TLC (ethyl acetate/cyclohexane (80:20)) showed the complete absence

of the starting nucleoside **4** (R_f 0.27) and its replacement by the thioacylated nucleoside **5** (R_f 0.63) and a byproduct (R_f 0.83). The solution was diluted with 100 mL of dichloromethane, washed successively with three 100-mL portions of 0.5 M H_2SO_4 , water, and brine. After drying over magnesium sulfate, the solvent was removed under vacuum to give a solid residue which was chromatographed on silica gel. Elution with ethyl acetate/cyclohexane (95:5) afforded the desired product **5** (1.10 g, 60%) as an orange amorphous solid. ^1H NMR (CD_3OD) δ 8.13 (s, 1H, H-6), 7.5–7.0 (m, 5H, ArH), 6.32 (dd, J = 8.7, 5.5 Hz, 1H, H-1'), 5.79 (app d, J = 5.6 Hz, 1H, H-3'), 4.46 (m, 1H, H-4'), 4.15–4.0 (m, 13H, CpH + $NHCH_2$ + H-5'), 2.65 (m, 2H, $FcCH_2$), 2.41 (m, 2H, CH_2CO), 2.9–2.2 (m, 2H, H-2'), 0.97 (s, 9H, t -BuSi), 0.19 (s, 3H, CH_3Si), 0.17 (s, 3H, CH_3Si). HPLC (system I) t_r = 34.22 min.

5'-O-(tert-Butyldimethylsilyl)-5-[N-(β -ferrocenylpropanoyl)-3-amino-propyn-1-yl]-2',3'-dideoxyuridine (6) (Fc-5'TBDMs-ddU). A well-stirred mixture of **5** (1.10 g, 1.42 mmol) and tributyltin hydride (0.42 mL, 1.56 mmol) in 70 mL of benzene was degassed for 30 min. After addition of 2,2'-azobis(2-methylpropionitrile) (AIBN) (23 mg, 0.14 mmol), the stirring reaction mixture was heated to reflux for 90 min. Solvent removal under reduced pressure gave a residue which was chromatographed on silica gel in ethyl acetate/cyclohexane (80:20), yielding the expected deoxygenated product **6** (0.46 g, 52%) as an orange amorphous solid. ^1H NMR (CD_3OD) δ 8.19 (s, 1H, H-6), 5.99 (m, 1H, H-1'), 4.2–4.0 (m, 13H, CpH + $NHCH_2$ + H-4' + H-5'), 3.77 (dd, J = 11.7, 2.81 Hz, 1H, H-5'), 2.65 (m, 2H, $FcCH_2$), 2.41 (m, 3H, CH_2CO + H-2'), 2.05–1.95 (m, 3H, H-2' + H-3'), 0.97 (s, 9H, t -BuSi), 0.17 (s, 6H, CH_3Si). HPLC (system I) t_r = 25.89 min.

5-[N-(β -Ferrocenylpropanoyl)-3-amino-propyn-1-yl]-2',3'-dideoxyuridine (7) (Fc-ddU). Product **6** (0.80 g, 1.29 mmol) was reacted with tetrabutylammonium fluoride in THF (1 M solution, 6.4 mL, 6.4 mmol) for 30 min. At this point, TLC (ethyl acetate–cyclohexane (80:20)) indicated complete disappearance of the starting nucleoside **6** (R_f 0.45) into the desired deprotected product **7** (R_f 0.07). After solvent removal, the residue was chromatographed on silica gel in $CHCl_3/MeOH$ (90:10). Fractions containing **7** were combined and concentrated under vacuum to give a viscous liquid which was taken up in 100 mL of acetonitrile. After washing with six 100 mL portions of cyclohexane which removed silylated byproducts, the acetonitrile phase was evaporated under vacuum yielding **7** (0.60 g, 92%) as an amorphous orange solid. ^1H NMR (CD_3OD) δ 8.44 (s, 1H, H-6), 6.01 (dd, J = 6.6, 3.0 Hz, 1H, H-1'), 4.2–4.0 (m, 12H, CpH + $NHCH_2$ + H-4'), 3.91 (dd, J = 12.2, 2.9 Hz, 1H, H-5'), 3.69 (dd, J = 12.2, 3.5 Hz, 1H, H-5'), 2.65 (m, 2H, $FcCH_2$), 2.41 (m, 3H, CH_2CO + H-2'), 2.2–1.9 (m, 3H, H-2' + H-3'). ^{13}C NMR (CD_3OD) δ 175.03, 164.65, 151.09, 145.56, 99.11, 89.81, 88.81, 88.20, 83.32, 75.43, 69.50, 68.94, 68.32, 63.26, 38.30, 33.85, 30.50, 26.56, 25.41. HPLC (system I) t_r = 7.81 min.

5-[N-(β -Ferrocenylpropanoyl)-3-amino-propyn-1-yl]-2',3'-dideoxyuridine 5'-monophosphate (9) (Fc-ddUP) as Morpholinium Salt. A 0.4 M stock solution of β -cyanoethyl phosphate pyridinium salt in pyridine was prepared by the procedure of Tener (46).

Nucleoside-ferrocene **7** (300 mg, 0.6 mmol) was dissolved in 3 mL of degassed anhydrous pyridine containing β -cyanoethyl phosphate in excess (3 mL of the stock solution, 1.2 mmol). To this solution was added dicyclohexylcarbodiimide (DCC) (495 mg, 2.4 mmol). The reac-

tion mixture was stirred at ambient temperature for 48 h. At this point, HPLC (system II) showed the quantitative conversion of the starting nucleoside (t_r = 7.1 min) into the less hydrophobic β -cyanoethyl phosphate derivative **8** (t_r = 6.5 min). To the solution was added 12 mL of water, and the mixture was stirred at ambient temperature for 1 h. The precipitate of N,N' -dicyclohexylurea was removed by filtration and washed well with water. The filtrate and washings were combined and concentrated under vacuum. The resulting residue was purified from excess undecomposed phosphorylating reagent by chromatography on DEAE cellulose using 0.1 M triethylammonium hydrogenocarbonate (TEAC) as the eluant. The yellow eluate was collected and lyophilized to afford intermediate **8** as a chromatographically pure solid: ^{31}P (D_2O) δ 0.96 (s, 1P, P_α).

The solid was dissolved in 30 mL of 9 N aqueous ammonia, and the solution was heated at 55 °C for 1 h. At this point, HPLC (system II) indicated conversion of the intermediate **8** into the expected monophosphate product **9** Fc-ddUP (t_r = 4.6 min) contaminated as shown by ^{31}P NMR spectroscopy with what appeared to be the corresponding diphosphate derivative. The solution was concentrated under vacuum and the residue was passed onto a DEAE (HCO_3^- form). The column was eluted with a linear gradient of TEAC (0.0 to 0.4 M, pH 7.5). Appropriate fractions were dried by lyophilization, and excess salt was removed by repeated lyophilization with deionized water to yield the triethylammonium salt of Fc-ddUP. An aqueous solution of Fc-ddUP was then applied to a Dowex 50 WX4 cation-exchange column (morpholinium form) and eluted with H_2O . The solution was lyophilized to afford morpholinium ferrocene-2',3'-dideoxyuridine phosphate **9**. The isolated yield (58%, based on starting **7**) was determined by optical density measurement. Attempts to separate the product from a phosphorus impurity gave the monophosphate compound, still not completely pure. The content of "morpholine" was determined to be 1.1 equiv by ^1H NMR. ^1H NMR (CD_3OD) δ 8.32 (s, 1H, H-6), 6.01 (m, 1H, H-1'), 4.27 (m, 1H, H-4'), 4.20–4.05 (m, 11H, CpH + $NHCH_2$), 4.03 (m, 2H, H-5'), 3.79 (br s, 1.1 $\times$ 8H, CH_2 morpholinium), 2.65 (m, 2H, $FcCH_2$), 2.55–2.35 (m, 3H, CH_2CO + H-2'), 2.25–1.95 (m, 3H, H-2' + H-3'). ^{13}C NMR (CD_3OD) δ 175.17, 164.71, 151.29, 145.39, 99.63, 90.08, 89.95, 88.10, 82.26 (d, J = 8.8 Hz, 1H, C-4'), 75.65, 69.51, 69.02, 68.29, 66.85 (d, $J_{C,P}$ = 4.8 Hz, 1H, C-5'), 65.98, 45.04 (morpholinium), 38.27, 33.18, 30.65, 26.62, 26.37. ^{31}P NMR (D_2O) δ 2.07 (s, 1P, P_α). HPLC (system II) t_r = 4.6 min. FAB-MS (–) data: m/z ($M - H$) $^-$, 584.0, $[C_{25}H_{27}N_3O_8PFe]H_3$ requires 584.1.

^{31}P NMR signals from what appears to be the diphosphate are as follows: δ –8.7 (d, 1P, J = 21 Hz, P_β), –13.2 (d, 1P, J = 21 Hz, P_α).

5-[N-(β -Ferrocenylpropanoyl)-3-amino-propyn-1-yl]-2',3'-dideoxyuridine 5'-triphosphate (9) (Fc-ddUTP) as Triethylammonium Salt. A solution of the monophosphate **9** (168 mg, 0.25 mmol) in water (2.5 mL) was mixed with *tert*-butyl alcohol (2.5 mL) and morpholine (85 μ L, 1 mmol). To the stirred refluxing solution was added a solution of N,N' -dicyclohexylcarbodiimide (206 mg, 1 mol) in *tert*-butyl alcohol dropwise over a period of 1.5 h. As shown by HPLC analysis, the quantitative formation of the phosphoromorpholidate **10** (system II, t_r = 6.2 min) was obtained after an additional 1 h. The reaction mixture was then cooled and evaporated under reduced pressure. The residue was dissolved in water (20 mL) and filtered; the solid was washed with an additional portion of water (5 mL). The combined

aqueous portions were extracted with diethyl ether (3×20 mL) and then coevaporated under reduced pressure with pyridine and finally benzene.

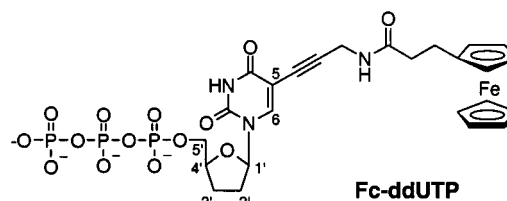
A solution of 47 mg of the above solid in dry DMSO (1.4 mL) was added slowly to a solution of tri-*n*-butylammonium pyrophosphate (101 mg, 0.28 mmol) in dry DMSO (0.4 mL), and the mixture was stirred under an inert atmosphere at ambient temperature for 3 days. The resulting solution was applied to a DEAE cellulose column (HCO_3^- form). The column was washed with water until the absorbance of the effluent fell to zero and then eluted with a linear gradient of triethylammonium hydrogenocarbonate (0.1 to 0.4 M). Appropriate fractions (0.2–0.3 M) monitored by HPLC for phosphorus content were pooled and lyophilized to yield Fc-ddUTP which was finally purified by HPLC on Nucleosil C₁₈ (system V). The isolated yield was determined by optical density measurement (0.12 mmol, 48%). The content of "triethylamine" was determined to be 3.2 equiv by ^1H NMR. ^1H NMR (CD_3OD) δ 8.24 (s, 1H, H-6), 6.01 (m, 1H, H-1'), 4.28 (m, 1H, H-4'), 4.20–4.05 (m, 11H, + CpH + NHCH_2), 4.04 (m, 2H, H-5'), 2.87 (m, 3.2×6 H, CH_2 triethylammonium), 2.63 and 2.48 (A_2B_2 system, 4H, $\text{FcCH}_2\text{CH}_2\text{CO}$), 2.38 (m, 1H, + H-2'), 2.20–2.00 (m, 3H, H-2' + H-3'), 1.18 (m, 3.2×3 H, CH_3 triethylammonium). ^{31}P NMR (D_2O) δ -9.80 (d, 1P, $J = 20$ Hz, P_γ), -10.1 (d, 1P, $J = 20$ Hz, P_α), -22.1 (t, 1P, $J = 20$ Hz, P_β). HPLC (system II), $t_r = 5.5$ min. FAB-MS (–) data: m/z ($\text{M} - \text{H}$) $^-$, 744.0, [$\text{C}_{25}\text{H}_{27}\text{N}_3\text{O}_{14}\text{P}_3\text{Fe}$] H_3 requires 744.0; ($\text{M} - 2\text{H} + \text{Na}$) $^-$, 766.0, [$\text{C}_{25}\text{H}_{27}\text{N}_3\text{O}_{14}\text{P}_3\text{Fe}$] H_2Na requires 766.0; ($\text{M} - 3\text{H} + 2\text{Na}$) $^-$, 788.0, [$\text{C}_{25}\text{H}_{27}\text{N}_3\text{O}_{14}\text{P}_3\text{Fe}$] HNa_2 requires 788.0.

5'-Phosphorylated Oligonucleotides Containing a 3'-Terminal ddU-Fc (5'-p-(dT)₁₀-ddU-Fc-3'). In a typical experiment, approximately 20 nmol of 5'-phosphorylated ODN 5'-p-(dT)₁₀ (23 μM) and 200 nmol of Fc-ddUTP (0.23 mM) in tailing buffer (850 μL) containing 25 mM Tris-HCl and 100 mM potassium cacodylate, pH 7.2, 1 mM CoCl_2 and 0.2 mM dithiothreitol were mixed with 500 units of terminal deoxynucleotidyl transferase (20 μL). The reaction was incubated for 30 min at 37 °C. The enzyme was then removed by filtration through a Centricon 30 YM cellulose membrane (30000 MW cutoff). For complete recovery of the ferrocene-labeled oligonucleotide, the membrane unit was washed by successive dilutions with water and filtrations (5×500 μL). The filtrates were concentrated by lyophilization and purified by HPLC using a linear gradient of sodium phosphate buffer (system IV) to remove reaction buffer components and excess Fc-ddUTP ($t_r = 7.7$ min). Appropriate fractions ($t_r = 13.8$ min) were collected in sterile Eppendorf tubes and were lyophilized to afford the oligonucleotide extended from the 3'-end with a ddU-Fc label (5'-p-(dT)₁₀-ddU-Fc-3') in a ca. 75–80% yield. Quantitation was done from the HPLC peak integral at 270 nm, the molar extinction coefficient being the sum of the estimated molar extinction coefficient for 5'-p-(dT)₁₀ ($\epsilon_{270} = 97800 \text{ M}^{-1} \text{ cm}^{-1}$) (9) and of the molar extinction coefficient of the Fc-ddU moiety ($\epsilon_{270} = 6640 \text{ M}^{-1} \text{ cm}^{-1}$) which was determined separately. Finally, the purified ferrocene-labeled DNA probe was desalted using a linear gradient of TEAA in acetonitrile buffer (system V, $t_r = 22.5$ min) prior to MALDI-TOF analysis. MS (MALDI-TOF) (–) data: m/z ($\text{M} - \text{H}$) $^-$, 3623.8, $\text{C}_{125}\text{H}_{157}\text{N}_{23}\text{O}_{78}\text{P}_{11}\text{Fe}$ requires 3624.5.

RESULTS AND DISCUSSION

Synthesis and Characterization of C-5 Ferrocene Nucleosides and Nucleotides. Of major concern for

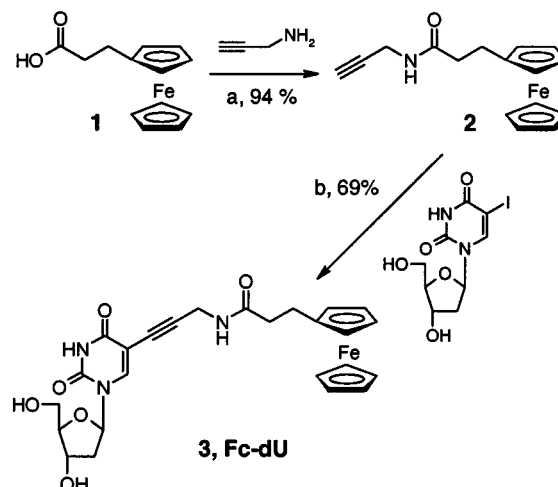
Scheme 1



successful 3'-end-labeling of single-stranded DNAs is the appropriate choice of the linkage between the label and the uridine base in the C-5 labeled nucleotide substrate. For the Fc-ddUTP species we chose to synthesize (Scheme 1), the precise positioning of the ferrocene label is ensured through a triple-bonded cross-linking to the base. This structural feature originally designed by Prober et al. (32) is actually found in a variety of commercially available C-5 modified chain terminators. While the rigid triple bond is expected to reduce the interaction between the label and the nucleotide, the alkynyl $\text{C}=\text{CH}-\text{CH}_2-\text{NH}-\text{C}(\text{O})-(\text{CH}_2)_2$ arm we selected for Fc-ddUTP generally minimizes interference with enzyme activity. Choice of this linker arm would also offer an interesting redox-labeled nucleotide endowed with the low redox potential of an alkyl ferrocene derivative. Finally, because the C-5 positions of uridines are not involved in the Watson–Crick base pairing, the ferrocene label is expected not to interfere with probe hybridization, a criteria of major importance for development of valuable ferrocene-labeled DNA probes.

Our synthetic approach to the target compound Fc-ddUTP follows a chemical route which consists of three main stages: (a) synthesis of a C-5 ferrocene-labeled 2'-deoxyuridine precursor Fc-dU (Scheme 2); (b) specific 3'-deoxyoxygenation to Fc-ddU (Scheme 3); (c) triphosphorylation of the 5'-end via the monophosphate form Fc-ddUP (Scheme 4). This strategy capitalizes on well-established and mild methodologies in uridine chemistry that we anticipated to be compatible with the ferrocene unit. As a second main advantage, the route starts from inexpensive and readily available materials. In particular, 5-iodo-2'-deoxyuridine was selected for modification because of its commercial availability and current use at the gram level. Additionally, incorporation of the ferrocene label facilitates the purification work because of its hydrophobicity and coloration, therefore providing a quick entry to a variety of new ferrocene-nucleosides.

Scheme 2^a



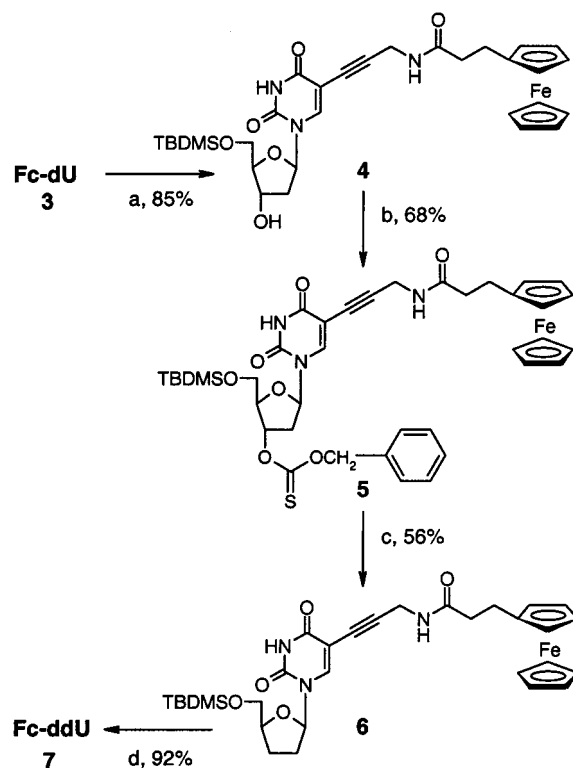
^a (a) EDC, HOBT, CH_2Cl_2 ; (b) $(\text{PPh}_3)_4\text{Pd}$, CuI, Et_3N , DMF.

As outlined in Scheme 2, the synthetic stage to Fc-dU **3** relies on the classical palladium-mediated coupling methodology of terminal alkynes with 5-iodo-nucleosides. The starting 5-*N*-(β -ferrocenyl-propanoyl)-3-amino-1-propyne linker **2** was readily prepared by carbodiimide EDC-mediated condensation reaction of 3-ferrocenylpropanoic acid **1** with propargylamine in dichloromethane. The presence of 1-hydroxybenzotriazole (HOBT) as an additive was necessary for efficient reaction. This afforded **2** in an excellent isolated yield (94%) and purity. An improved synthesis of **1** is given in the Experimental Section.

Coupling of alkyne **2** with 5-iodo-2'-deoxyuridine was carried out in DMF following the Pd(0)-catalyzed methodology reported by Hobbs (33). It seemed from literature observations that formation of the cyclized furano pyrimidin-2-one byproduct which generally occurs under the Sonogashira Pd(II)-catalyzed coupling reactions (33–35) would be prevented. Indeed, at ambient temperature and using the (PhP₃)₄Pd/CuI catalytic system, the coupling reaction proceeded cleanly to give FcdU as the sole ferrocene-containing nucleoside. Compound Fc-dU was readily isolated by silica gel chromatography followed by crystallization in 72% yield. Its structure and purity were confirmed by ¹H NMR and ¹³C NMR spectra and reversed-phase (RP) HPLC.

Our route for specific chemical 3'-deoxygenation of ferrocene-2'-deoxyuridine Fc-dU to the corresponding ferrocene-2',3'-dideoxyuridine Fc-ddU involves the classical and mild four-step sequence: selective-protection of the 5'-hydroxyl function, thioacylation followed by radical-induced reductive cleavage, and deprotection (36) (Scheme 3). We chose to protect the 5'-hydroxy of Fc-dU with the *tert*-butyl-dimethyl silyl TBDMS group to avoid acid conditions during deprotection since 2',3' dideoxyuridines are acid labile (37, 38). Thus, Fc-dU was treated with *tert*-butylchlorodimethylsilane (TBDMSCl) in the presence of imidazole in DMF to give the 5'-O-TBDMS derivative **4** which was obtained after chromatographic separation from minor amounts (<5%) of the 3',5'-bis-silylated product in 85% yield. The removal of the 3'-hydroxy group of ferrocene-nucleoside **4** was basically achieved by adaptation of the Barton–McCombie deoxygenation procedure of alcohols (39) as modified by Robins et al. for nucleosides (36). Accordingly, **4** was converted in a first step into its 3'-(phenyl thionocarbonate) ester **5** using phenoxythiocarbonyl chloride (PTC-Cl).

However, it appeared that the acetonitrile/4-dimethylaminopyridine catalytic system classically used for thioacylation of hindered alcohols (36) was unsuitable for large-scale preparation of **5**. In addition to the limited solubility of **4** in acetonitrile, thioacylation of **4** was complicated by formation of a ferrocene-nucleoside byproduct in an approximate 50% amount. This product is believed to be the corresponding O²,3'-anhydronucleoside derivative of **4** (Figure 1), judging from the marked downfield position of the H-3' (δ 5.75 ppm) signal, slightly displaced 0.06 ppm upfield from the corresponding signal in the ester **5**, and the absence of the aromatic phenoxy signals in the NMR spectrum of a byproduct chromatography fraction. Similar O²,3'-anhydronucleosides have been reported in the literature and can be obtained by direct treatment with various reagents of the appropriate 2'-deoxyuridine but also by base treatment of the corresponding 3'-O-thiocarbonyl-nucleoside precursor (40–42). The low yield and the difficulty of purification of the desired **5** led us to use alternate conditions for the 3'-thioacylation step of **4**. In dichloromethane and in the presence of ~7 mol equiv of pyridine (43), O²,3'-cyclo-

Scheme 3^a

^a (a) *tert*-Butyldimethylsilyl chloride, DMF; (b) phenoxythiocarbonyl chloride, CH₂Cl₂, pyridine; (c) tributyltin hydride, AIBN, benzene; (d) TBAF, THF.

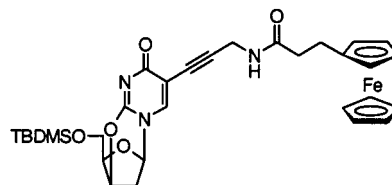
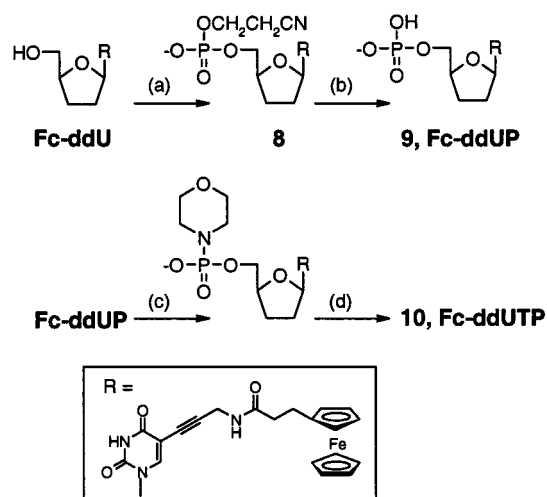


Figure 1. Ferrocene-cyclonucleoside byproduct formed during thioacylation of **4**.

nucleoside formation could be reduced to less than 10%, possibly due to the shorter reaction times (<2 h). 3'-O-(Phenoxythiocarbonyl)ferrocene-nucleoside **5** was obtained as the major product and was isolated in this work in 62% (pure) as an amorphous solid.

Compound **5** was then smoothly deoxygenated with commercial tri-*n*-butyltin hydride (Bu₃SnH) and a catalytic amount of 2,2'-azobisisobutyronitrile (AIBN). The best chemical yield in the desired 2',3'-dideoxyuridine product **6** was obtained after a short heating at benzene reflux under argon. Under such conditions, no dethiation or alcohol byproducts were detected. Interestingly, the plausible unwanted hydrostannylation of the yne function (44) was also fully prevented. After purification by silica gel chromatography, the deoxygenated **6** whose structure was confirmed by ¹H and ¹³C NMR spectra was obtained in a 56% (pure) yield.

Removal of the silyl protecting group from **6** was then easily achieved by treatment with ~5 mol equiv of tetrabutylammonium fluoride (TBAF) in tetrahydrofuran for 30 min at ambient temperature. This afforded the desired deprotected ferrocene-2',3'-dideoxyuridine Fc-ddU in excellent yield. Full characterization of the compound is described in the Experimental Section. All physical data confirm the structure.

Scheme 4^a

^a (a) β -Cyanoethyl phosphate, DCC, pyridine; (b) aq NH_3 (c) DCC, morpholine, *t*-BuOH/ H_2O ; (d) $(\text{HNBu}_3)_2\text{H}_2\text{P}_2\text{O}_7$, DMSO.

5'-Phosphorylated Ferrocene-2',3'-dideoxyuridines. Several methods have been described for the chemical preparation of nucleoside triphosphates in the literature (31). Most of them involve the coupling of a pyrophosphate with an activated nucleoside monophosphate. Of the commonly used methods a priori compatible with the ferrocene subunit, we judged that the Moffatt phosphoromorpholidate coupling approach (45) would offer the mildest and most practical route to ferrocene–nucleoside triphosphate Fc-ddUTP **11** from **7** via the monophosphate derivative **9** (Scheme 4).

All phosphorylation reactions (**7** to **11**) were monitored by RP-HPLC, and chemical modifications were characterized by ^{31}P NMR spectroscopy of the products in crude and/or isolated pure forms. The free 5'-hydroxyl group of Fc-ddU was first phosphorylated by condensation with β -cyanoethyl phosphate (46) in the presence of *N,N*-dicyclohexylcarbodiimide (DCC) in anhydrous pyridine to afford the 5'-(β -cyanoethyl phosphate) sodium salt intermediate **8** in essentially quantitative yield. The ^{31}P NMR spectrum of **8** obtained after purification on DEAE cellulose anion-exchange chromatography showed one signal at 0.96 ppm corresponding to α -phosphorus. The β -cyanoethyl group of the intermediate **8** was removed in concentrated ammonia at 55 °C for 1 h to afford crude ferrocene–nucleoside 5'-phosphate **9**. The presence of two doublets centered at -8.7 ppm and -13.2 ppm in the ^{31}P NMR spectrum of the reaction mixture suggested the formation of the ferrocene–nucleoside diphosphate as a minor byproduct (<5%). Purification of the product on DEAE cellulose followed by chromatography on DOWEX 50 WX4 cation-exchange resin yielded the morpholinium salt of **9** (58% yield, based on starting **7**, UV–visible determination) which was used without further purification. The presence of the 5'-monophosphate group was proved by the singlet found at 2.1 ppm in the ^{31}P NMR spectrum of **9**. The structure of compound **9** was verified by ^1H NMR spectroscopy and confirmed by its negative-ion FAB spectrum which displayed the $[\text{M} - \text{H}]^-$ ion at m/z 504 as the base peak.

Monophosphate **9** was then converted into its activated 5'-phosphomorpholidate derivative **10** in quantitative yield by refluxing with morpholine in the presence of DCC in aqueous *tert*-butyl alcohol (45). In agreement with the proposed intermediate **10** (Scheme 4) was the observation of a new singlet at 4.9 ppm in the ^{31}P NMR spectrum of the crude product obtained after an aqueous

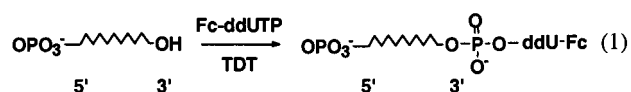
workup procedure (45, 47). The solid was rigorously dried and treated with a 5 equiv of commercial tri-*n*-butylammonium pyrophosphate $(\text{HNBu}_3)_2\text{H}_2\text{P}_2\text{O}_7$ in dry DMSO for 3 days. This yielded a crude product that was first purified by anion-exchange chromatography to yield a crude sample of ferrocene nucleoside 5'-triphosphate, Fc-ddUTP, **11**. Compound **11** could be further purified and desalted by HPLC using the volatile triethylammonium acetate (TEAA) buffer as eluant.

Chromatography thus provided spectroscopically pure **11** as a triethylammonium salt in 48% yield. In particular, the ^{31}P NMR spectrum only showed the three characteristic phosphorus atom signals due to the triphosphate chain with the expected P–P geminal coupling. The structure of compound **11** was also confirmed by its negative-ion FAB-MS.

Synthesis of 5'-Phosphorylated Oligonucleotides Containing a 3'-Terminal Ferrocene Label. To obtain uniformly successful results in 3'-end-tailing experiments, an important practical problem we studied preliminarily was the quality of the starting single-stranded 5'-phosphate-labeled oligonucleotide products supplied by ODN manufacturers. As it is known, the purity of crude unlabeled synthetic oligonucleotides depends on the length of the sequence to synthesize. The main impurities that are difficult to separate from (*n*)-mer (*n* nucleotides in length) are the *n* – 1 and *n* – 2 congener species (called runts of synthesis). In addition to these contaminants, chemical phosphorylation, which is achieved during solid-phase oligonucleotide syntheses using phosphoramidites, is often nonquantitative. Runts and unphosphorylated 5'-OH-ODN sequences cannot be eliminated through a simple desalting step and should be removed using purification methods. After reversed phase HPLC or ion-exchange HPLC, the 5'-modified oligonucleotide with the correct length and label is usually provided from the manufacturer as a HPLC-grade product.

Therefore, we developed a fast and informative C_{18} RP-HPLC analytical method for routine quality control of these 5'-modified synthetic products (up to 20 bases). HPLC traces A and B in Figure 2 clearly illustrate the high degree of variability in quality encountered among two HPLC-grade 5'-phosphorylated ODNs differing only in their commercial origin. As evidenced from HPLC profiles, some products regarded by their manufacturers as "gold standard" are actually unsuitable samples for most experiments due to high contamination with the parent 5'-hydroxylated sequence (slower-eluting species, trace A) and not well-resolved secondary structures. Nevertheless we have found that HPLC-grade 5'-phosphorylated products from ESGS Cybergene were practically free of impurities, their purity being >85% (Figure 3, trace B) in complete agreement with the analytical certificate of the provider.

An optimized 3'-ferrocene end-labeling procedure using terminal deoxynucleotidyl transferase TDT was set up at the 20 nmol scale of a HPLC-purified 5'-phosphorylated oligonucleotide of defined length (10-mer 5'-p-(dT)₁₀ sequence, ~ 2 OD₂₆₀ units) by adaptation of the standard protocol reported by Roychoudhury and Wu (48). The enzymatic incorporation reaction of the redox-labeled dideoxynucleotide Fc-ddUTP at the 3'-terminus of the oligonucleotide (eq 1) was analyzed by C_{18} RP-HPLC



using a linear gradient of acetonitrile in phosphate buffer,

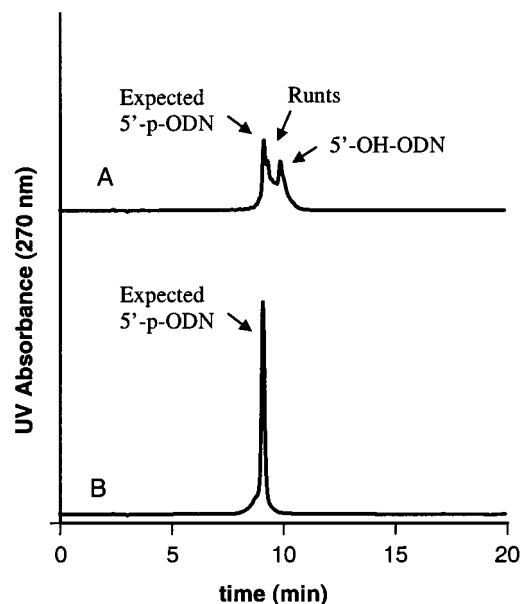


Figure 2. RP-HPLC Profiles of Commercial HPLC-Purified Grade 5'-Phosphorylated Oligonucleotides. 5'-p-(dT)₂₀ sample provided (A) from Genset (B) from ESGS Cybergene. Column: Nucleosil C₁₈, 120 Å; eluant A: 10% acetonitrile in 0.1 M aqueous triethylammonium acetate, pH 7.0; solvent B, acetonitrile, linear gradient 2.5–25% B in 30 min, flow-rate 0.8 mL/min.

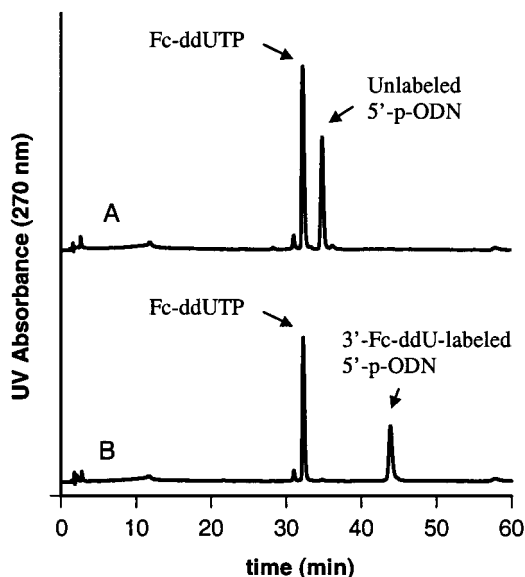


Figure 3. Analytical HPLC Control of 3'-End-labeling of 5'-Phosphorylated Oligonucleotide with Fc-ddUTP. Reaction mixture 200 nmol Fc-ddUTP + 20 nmol 5'-p-(dT)₁₀ in enzymatic buffer at 37 °C: (A) before TDT addition; (B) 30 min after TDT addition and after first Centricon-30 membrane ultrafiltration. Column: Novapak C₁₈, 60 Å; solvent A, 10% acetonitrile in aqueous KH₂PO₄ (20 mM) and NBu₄KH₂PO₄ (5 mM), pH 7.5; solvent B, acetonitrile, linear gradient 10–40% B in 60 min, flow-rate 0.8 mL/min.

at different times, after removal of the TDT enzyme by ultrafiltration on 30000 molecular weight cutoff membrane. As demonstrated in Figure 3, a rapid and complete ferrocene-labeling of the 10-mer sequence (trace A, retention time 34.4 min) could be achieved when using approximately 10-fold molar excess of Fc-ddUTP (traces A and B, retention time 32.0 min). Under our analytical reversed-phase HPLC conditions, the oligonucleotide extended at its 3'-end by a single hydrophobic ferrocene-nucleoside label Fc-ddU (trace B, retention time 43.9 min)

was a quite slower-eluting species than Fc-ddUTP. This interesting chromatographic separation feature was also obtained at the preparative scale level using a similar appropriate chromatographic system (see Experimental Section for details), and thus made purification of the ferrocenylated-oligonucleotide from excess Fc-ddUTP and other fastest eluting reaction contaminants a facile and efficient step. The final yield in ferrocenylated-oligonucleotide 5'-p-(dT)₁₀-ddU-Fc-3' product was about 75–80% (based on starting oligonucleotide) as determined from HPLC peak integral, and purity was confirmed by HPLC quality control method. Finally, the ferrocenylated-oligonucleotide sample was desalted from the chromatographic phosphate salts by RP-HPLC with volatile TEAA buffer and lyophilized. The identity of the Fc-ddU-terminated oligonucleotide product was confirmed by matrix-assisted laser desorption/ionization/time-of-flight (MALDI-TOF) mass spectrometry in the negative mode, using the carboxylic acid 3-hydroxypicolinic acid (3-HPA)/diammonium citrate as a useful two component matrix for detection of the molecular ion peak, (M – H)[–] of labeled 5'-p-(dT)₁₀ (49).

In conclusion, by starting from the readily available 5-iodouridine and ferrocene-alkyne **2** we developed an efficient synthetic approach for preparing the first ferrocene-labeled dideoxynucleotide Fc-ddUTP **11**. Although the overall yield of Fc-ddUTP cannot be regarded as outstanding (<10%), the methods presented here are reliable and all ferrocene intermediates can be easily purified. It is worth emphasizing that the ferrocene-dideoxynucleoside intermediate Fc-ddU **7** can be prepared in gram batches and that the target compound Fc-ddUTP can be obtained pure in practical quantities for incorporation into single-stranded DNA. Our results also clearly show that enzymatic 3'-ferrocene end-labeling of small 5'-phosphorylated synthetic oligonucleotides with TDT using Fc-ddUTP as a substrate is a highly efficient reaction which can be extended with great promise to larger and more complex 5'-modified oligomers. Finally, by proving that significant quantities of 3'-ferrocene-labeled oligonucleotides can be prepared, this work sets the stage for our studies of various redox-labeled DNA probes required for attaching monolayers of interesting redox-active oligonucleotide on surfaces.

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Chemical and Biochemical Issues Related to X-ray Crystallography of the Ligand-Binding Domain of Estrogen Receptor α

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Careful attention to technical issues preceded successful crystallography of the ligand-binding domain of estrogen receptor α (ER α) complexed with CP-336156, a nonsteroidal estrogen agonist/antagonist. An affinity column based on immobilized estradiol was prepared according to the scheme of Greene et al. (Greene, G. L., Nolan, C., Engler, J. P., and Jensen, E. V. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 5115–5119). It was shown by X-ray crystallography that the major and less polar isomer of the affinity column precursor was 17 α -((S)-2',3'-epoxyprop-1'-yl)estra-1,3,5(10)-triene-3,17 β -diol. This diastereomer was coupled to Thiopropyl Sepharose, with coupling monitored by observing loss of the phenolic absorption band of estradiol from the reaction supernatant, and gave an affinity matrix containing about 9 μ mol of estradiol per milliliter of wet gel. Recombinant ER α ligand binding domain was selectively removed from *E. coli* cell lysate by binding to the column and was partly S-carboxymethylated by treatment with iodoacetic acid while bound to the column as described by previous workers. After being eluted from the column as a complex with drug, the receptor fragment was shown by mass spectrometry to be a mixture of differently modified forms. It was further S-carboxymethylated in solution, after which anion-exchange chromatography was used to isolate protein in which two of the four cysteine residues were S-carboxymethylated. This material, which afforded diffraction-quality crystals, was subjected to digestion with trypsin and peptide mapping analysis by HPLC coupled with mass spectrometry. For this experiment, the two previously unmodified cysteines were alkylated with 4-vinylpyridine to allow definitive identification. It was shown that Cys-417 and Cys-530 were S-carboxymethylated in the crystallized protein, while Cys-381 and Cys-447 remained unmodified. Close attention to such technical issues may be important in structural studies of other nuclear receptors, a very important class of potential drug targets.

INTRODUCTION

ER¹ belong to the nuclear receptor superfamily and are composed of discrete ligand-binding and DNA-binding domains. There are two principal isoforms, ER α and ER β . In the presence of 17 β -estradiol or another agonist, the receptors associate with accessory proteins known as coactivators to mediate the effects on gene expression that are the end-point of estrogen action in many cells or tissues. Details of these interactions have only recently begun to be elucidated (1–3).

Crystallographic structural biology is one of the approaches being used to scrutinize this complex machinery. Structures have been reported for the ligand-binding domains of ER α (4–6) and ER β (7) complexed separately with agonist ligands (17 β -estradiol and diethylstilbestrol) and drugs of the class called selective estrogen receptor modulators (SERMs) (tamoxifen and raloxifene; for reviews of this drug class, see refs 8–10). The results illustrate brilliantly how internal details of a protein–ligand interaction can modulate the protein's external conformation, as the packing site of the C-terminal structural element helix 12 is different in the agonist and SERM complexes. They complement other demonstra-

tions that the nature of the ligand governs the types of interactions with coactivators available to the receptor and that this underlies the differences in action between agonists and SERMs. The results with ER form part of a more general picture that is emerging of how ligand–protein interactions modulate the function of nuclear receptors (11).

Isolation of ER from natural or recombinant sources is necessary in many studies and an essential precursor to crystal growth. Affinity chromatography has been an important technical asset, as the high affinity of ER for immobilized derivatives of estradiol allows single-step isolations (12). The same high affinity, however, has also been a detriment, as conditions that are probably at least partly denaturing have been needed to elute bound receptor from columns. In all four publications to date describing crystallography of ER–ligand complexes (4–7), a single specific affinity matrix (13) and generally similar procedures have been used.

In most of these published procedures, the receptor fragment was partly S-carboxymethylated while it was immobilized on the affinity column. When this was not done, the resulting structure contained an artifactual intermolecular disulfide (5). The exact outcome of these treatments has not been described in every case, and it has largely been left unstated but assumed that the modifications have no bearing on the substance of the results. We present peptide mapping analysis of a homogeneous protein preparation from which crystals were grown, data that clearly show the extent and

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¹ Abbreviations: ER, estrogen receptor(s); ER α , estrogen receptor α ; mCPBA, *m*-chloroperoxybenzoic acid; DTT, dithiothreitol, reduced form; RP-HPLC, reversed-phase HPLC.

location of the modifications in the protein that we crystallized.

Our experience with these various technical issues came as we prepared recombinant ER α ligand-binding domain complexed with CP-336156 (lasofoxifene) (14, 15) for crystallography. The structural results (J. Pandit et al., unpublished) will appear elsewhere. Some technical aspects of the task of obtaining crystallographic-quality material were unusually subtle and challenging, and included elements not fully described in existing literature. Among these were crystallographic determination of the absolute conformation of the estradiol derivative needed to make the affinity column, details of its immobilization to Thiopropyl Sepharose, and the isolation of incompletely but uniformly S-carboxymethylated ER α protein. This note describes our experience with these key steps preceding successful crystal growth and structure analysis.

EXPERIMENTAL PROCEDURES

Synthesis of 17 α -Epoxypropylestradiol. 17 α -(2'-Propen-1'-yl)estra-1,3,5(10)-triene-3,17 β -diol (1). To 100 mL of a 1 M allylmagnesium chloride solution in ether (100 mmol) was added a solution of estrone (4.05 g, 15.0 mmol) and THF (100 mL) over 20 min. The reaction was heated to reflux for 3 h and then allowed to cool to room temperature overnight. After pouring into an aqueous solution of ammonium chloride (250 mL) and extracting with ether (2 $\times$ 150 mL), the combined organic fraction was washed sequentially with water (100 mL), 5% NaHCO₃ (50 mL), and brine (100 mL) and then dried over MgSO₄ and concentrated to give the title compound (4.48 g, 96%). Recrystallization from ethyl acetate/cyclohexane afforded a single diastereomer, mp 106–107 °C (dec). ¹H NMR (CDCl₃) δ 0.90 (s, 3H), 1.2–1.7 (multiplets, 9H), 1.86 (ddt, J = 2.7, 2.7, 12.5 Hz, 1H), 1.97 (m, 1H), 2.11 (m, 1H), 2.24 (dd, J = 7.1, 13.5 Hz, 1H), 2.29 (m, 1H), 2.35 (dd, J = 7.1, 13.5 Hz, 1H), 2.79 (d, J = 5.0 Hz, 2H), 5.18 (m, 2H), 5.98 (ddt, J = 7.3, 10, 17 Hz, 1H), 6.53 (d, J = 2.7 Hz, 1H), 6.60 (dd, J = 2.7, 8.4 Hz, 1H), 7.12 (d, J = 8.4 Hz, 1H). MS (APCI) 295 (M-17, 100), 253 (18). ¹³C NMR (CDCl₃) δ 14.6, 23.7, 26.5, 27.7, 29.9, 32.0, 35.1, 39.8, 42.0, 44.0, 46.7, 49.8, 82.8, 112.9, 115.5, 119.5, 126.7, 132.8, 135.0, 138.5, 153.6. Anal. Calcd for C₂₁H₂₈O₂·¹/₃EtOAc: C, 79.00; H, 9.04. Found: C, 78.78; H, 8.87.

3-(tert-Butyldimethylsilyloxy)-17 α -(2'-Propen-1'-yl)estra-1,3,5(10)-triene-3,17 β -ol (2). To a solution of tert-butyldimethylsilyl chloride (1.68 g, 11.1 mmol), imidazole (1.58 g, 23.2 mmol), and DMF (7 mL) was added **1** (9.01 mmol), and the reaction was heated to 35 °C for 3 h. An additional portion of silyl chloride (0.80 g, 11.6 mmol) was added, and the reaction was heated an additional 2 h at 35 °C. After cooling to room temperature, the reaction was poured into 2% aqueous NaHCO₃ (200 mL) and extracted with ethyl acetate (2 $\times$ 100 mL). The combined organic layer was washed with water (100 mL) and brine (100 mL), dried (MgSO₄), and concentrated to give an oil. This was chromatographed on silica gel with 5% ethyl acetate/hexane to give the title compound (3.61 g, 94%) isolated as an oil. ¹H NMR (CDCl₃) δ 0.16 (s, 6H), 0.89 (s, 3H), 0.95 (s, 9H), 1.2–1.7 (multiplets, 9H), 1.86 (ddt, J = 2.7, 2.7, 12.5 Hz, 1H), 1.97 (m, 1H), 2.11 (m, 1H), 2.24 (dd, J = 7.1, 13.5 Hz, 1H), 2.29 (m, 1H), 2.35 (dd, J = 7.1, 13.5 Hz, 1H), 2.79 (d, J = 5.0 Hz, 2H), 5.18 (m, 2H), 5.98 (ddt, J = 7.3, 10, 17 Hz, 1H), 6.53 (d, J = 2.5 Hz, 1H), 6.57 (dd, J = 2.5, 8.3 Hz, 1H), 7.10 (d, J = 8.3 Hz, 1H).

17 α -((R,S)-2',3'-Epoxyprop-1'-yl)estra-1,3,5(10)-triene-3,17 β -diol (3). To a solution of **2** (3.61 g, 8.47 mmol) and

methylene chloride (85 mL) was added mCPBA (2.93 g, 17.0 mmol). After stirring overnight, the reaction was diluted with an additional 100 mL of methylene chloride and then washed sequentially with 2% KI (aqueous, 100 mL) and 10% aqueous sodium bisulfite (100 mL). The remaining solution was dried (MgSO₄) and concentrated to give an oil which was chromatographed on silica gel (40% ethyl/hexane) to give two compounds (only clean fractions were collected): less polar diastereomer, 1.38 g; more polar diastereomer, 0.47 g.

These compounds were not further characterized, but rather a solution of the less polar isomer (1.38 g, 3.12 mmol) and THF (10 mL) was treated with tetrabutylammonium fluoride (4.6 mL, 1 M in THF) at 0 °C. After 5 min the ice bath was removed and the reaction allowed to warm to room temperature. It was then diluted with ethyl acetate and washed with 5% NaHCO₃ (2 $\times$ 50 mL) and brine (50 mL). This was dried over MgSO₄ and concentrated to give a solid. Purification on silica gel with 60% ether/hexane gave the title compound as a white solid; mp 202–203 °C. ¹H NMR (CDCl₃) δ 0.90 (s, 3H), 1.2–1.7 (m, 9H), 1.85 (m, 1H), 2.10 (m, 2H), 2.27 (m, 1H), 2.36 (br s, 1H), 2.53 (dd, J = 2.8, 4.9 Hz, 1H), 2.80 (m, 2H), 2.85 (dd, J = 4.2, 4.9 Hz, 1H), 3.26 (m, 1H), 4.72 (br s, 1H), 6.53 (d, J = 2.7 Hz, 1H), 6.59 (dd, J = 2.7, 8.3 Hz, 1H), 7.11 (d, J = 8.3 Hz, 1H). ¹³C NMR (CDCl₃) δ 14.0, 23.6, 26.4, 27.6, 29.8, 31.7, 35.0, 39.3, 39.7, 44.0, 46.8, 47.7, 49.7, 50.3, 83.7, 112.9, 115.4, 126.7, 132.8, 138.5, 153.5. Anal. Calcd for C₂₁H₂₈O₃: C, 76.79; H, 8.59. Found: C, 76.48; H, 8.65.

Crystallography of the Resolved Less Polar Isomer of 17 α -Epoxypropylestradiol. Light yellow crystals of the purified less polar isomer were grown by slow evaporation from acetonitrile. A representative crystal (0.24 $\times$ 0.26 $\times$ 0.44 mm) was surveyed and a 1 Å data set collected on a Siemens R4/sealed tube diffractometer using copper radiation (λ = 1.54178 Å) at room temperature. All crystallographic calculations were facilitated by the SHELXTL system. The crystal belonged to the monoclinic space group $P2_1$ with the following cell dimensions: a = 6.824(1), b = 26.662(2), c = 9.839 Å, β = 100.67(1)°, V = 1759.2(2) Å³. There were two molecules in the asymmetric unit (Z = 4). The structure was phased using direct methods and refined routinely to a R -index of 4.96%. Hydrogen positions were calculated wherever possible. The methyl hydrogens and hydrogens on oxygen were located by difference Fourier techniques. A final difference map revealed no missing or misplaced electron density (0.22, -0.28 eÅ⁻³).

Preparation of the Affinity Matrix. With minor variations, the coupling procedure was based on the method used by Salman et al. (16) to immobilize 17 α -(6'-iodohex-1'-ynyl)estr-4-en-17 β -ol-3-one on Thiopropyl Sepharose. Solutions and buffers were not bubbled with N₂ before use in the present work, and coupling was monitored by observing the phenolic 280 nm absorption band of the intended ligand rather than by chemical assay of the beads.

To generate the free-thiol form of Thiopropyl Sepharose 6B (Amersham Pharmacia Biotech), 9 g of the dry resin was slurried in 50 mL of 0.3 M NaHCO₃, 1.1 mM EDTA, pH 8.4 containing 1.1 g of DTT, and the resulting mixture in a screw-capped polypropylene tube was inverted slowly and continuously for 1 h at 22 °C. After this, the resin was washed on a coarse glass sinter with (i) an aqueous solution made to 3 L containing 18 mL of acetic acid, 87.7 g of sodium chloride, and 1.1 g of disodium EDTA, and (ii) 300 mL of 0.3 M NaHCO₃, 1.1 mM EDTA, pH 9.2.

The reduced, filtered, and washed Thiopropyl Sepharose

beads (now containing 0.48–0.84 mmol of reactive thiol, according to the manufacturer's specifications) were then added to a 50 mL polypropylene screw-cap tube containing 81 mg (0.25 mmol) of **3** dissolved in 18 mL of DMF. Some spontaneous warming of the mixture was noticed. This addition was followed by 30 mL of 0.3 M NaHCO₃, 1.1 mM EDTA, pH 9.2, after which a small portion of the mixture (about 0.05 mL) was quickly withdrawn, and the full tube was capped. The sample withdrawn was centrifuged to pellet the suspended beads, and the absorption spectrum of the supernatant (0.02 mL diluted by addition to 1 mL of aqueous sodium phosphate buffer, pH 7.4) was recorded. The local maximum near 280 nm due to the phenol absorption band had an absorbance of 0.12 in a 1 cm cuvette.

The reaction mixture was inverted slowly and continuously overnight at 22 °C, after which a check of the absorption spectrum of the supernatant from a small sample (done as before) showed no trace of the phenol band. This indicated complete attachment of the estradiol derivative to the resin. The beads were filtered on a coarse sinter and washed with 300 mL of 0.3 M NaHCO₃, 1.1 mM EDTA, pH 9.2, after which they were added to a solution of 0.16 g of iodoacetamide in 45 mL of 0.3 M NaHCO₃, 1.1 mM EDTA, pH 8.4, in a screw-capped polypropylene tube. The resulting slurry was inverted continuously and slowly for 1 h at 22 °C to alkylate remaining free thiol groups on the Thiopropyl Sepharose and then washed in turn with 0.3 L of each of the following: (i) 0.3 M NaHCO₃, 1.1 mM EDTA, pH 8.4, also containing 1.5 g of DTT; (ii) the same solution without DTT; (iii) 0.05 M Tris HCl, pH 7.4; (iv) water; (v) 25% methanol in water; (vi) 50% methanol in water; (vii) 70% methanol in water. The beads were then stored at 4 °C as a slurry in 70% methanol in water.

The absorption spectrum of a slurry of a small portion of the derivatized beads in sodium phosphate buffer, pH 7.4, clearly showed the presence of the phenol absorption band of the immobilized estradiol derivative. Based on complete coupling of the epoxide to Thiopropyl Sepharose, the gel was calculated to contain 9 μ mol of estradiol per milliliter.

Estradiol Affinity Chromatography. This step was performed at 22 °C using the method of Hegy et al. (17) for the most part, but with important modifications after ER α was eluted from the affinity column. A 11.4 g amount of *Escherichia coli* cells harboring the expression construct for ER α was resuspended in 50 mL of 0.1 M Tris HCl, 0.1 M KCl, 1 mM EDTA, 4 mM DTT, pH 8.5, containing leupeptin (25 μ g/mL) and aprotinin (5 μ g/mL), and lysed by sonication on ice (4 $\times$ 45 s treatment at power setting 4 with a 40% duty cycle using a Branson Sonifier). The lysate was centrifuged at 18 000g for 30 min, the pellet was discarded, and the supernatant was recovered and centrifuged again in the same way. The resulting clarified 50 mL sample was loaded (0.5 mL/min) onto a 5 mL column of the estradiol-Sepharose affinity medium previously equilibrated with lysis buffer. After loading, the column was washed sequentially at 1 mL/min with 25 mL of lysis buffer, 25 mL of 0.05 M Tris HCl, 0.7 M KCl, 1 mM EDTA, 1 mM DTT, pH 8.5, and 25 mL of 0.01 M Tris HCl, pH 8.1. After this, the column was treated (0.5 mL/min) with 18 mL of 5 mM iodoacetic acid in 0.01 M Tris HCl, pH 8.1, made by 1:200 dilution of a 1 M solution of iodoacetic acid in 1 M Tris base with 0.01 M Tris HCl, pH 8.1. Flow was then stopped, and the column (protected from light) was allowed to stand at 22 °C for 18 h. It was then washed (1 mL/min in each case) with 25 mL of 0.01 M Tris HCl, pH 8.1, and with

25 mL of 0.05 M Tris HCl, 0.25 M NaSCN, 1 mM EDTA, 1 mM DTT, 10% DMF, pH 8.5. Finally, bound ER α was eluted (0.5 mL/min) as a broad peak using 50 μ M CP-336156 in the same buffer, and a 60 mL product pool was recovered.

Mass spectrometry (see Results) showed that the eluted protein was unevenly carboxymethylated. Following dialysis against 0.02 M Tris HCl, 5 mM DTT, pH 8.2, the product (32 mg of ER α in 42 mL) was further treated with 10 mM iodoacetic acid for 2 h at 22 °C under argon in the dark. The reaction was followed by mass spectrometry with the objective of maximizing the yield of twice-carboxymethylated ER α and was stopped by adding DTT to 20 mM. The product was then dialyzed against 0.02 M Tris HCl, 5 mM DTT, pH 8.2, and fractionated by anion-exchange chromatography on a MonoQ HR 10/10 column (Amersham Pharmacia Biotech) using a NaCl gradient in 0.02 M Tris HCl, pH 8.2. The main peak consisting of twice-carboxymethylated ER α was isolated and used for crystal growth trials. Digestion and peptide mapping to locate the sites of S-carboxymethylation were performed as described below.

Mass Spectrometry. Mass spectra of intact proteins were obtained using an open-access system (Dr. J. G. Stroh) consisting of a PE Sciex API 100 single-quadrupole spectrometer with electrospray sample interface. Multistage MS of tryptic digests was obtained using a Finnigan LCQ ion-trap spectrometer interfaced with a Hewlett-Packard Model 1090 HPLC (see below).

Tryptic Digestion. Purified complex of ER α with CP-336156 (0.6 mg in 1.2 mL) was desalted by RP-HPLC on a Poros R2/H 2.1/30 column as described (18), and the protein peak (now lacking drug) in two equal aliquots was dried in a centrifugal concentrator (SpeedVac, Savant). To the dried residue of one portion in a microfuge tube was added 0.05 mL of 0.25 M Tris HCl, 6 M GnHCl, 1 mM EDTA, 5 mM DTT, pH 8.8. The sample was flushed with argon, closed, incubated at 37 °C for 2 h, and then allowed to come to 22 °C and treated with 1 μ L of a 10% solution of 4-vinylpyridine in ethanol, giving a final concentration of 4-vinylpyridine of about 0.02 M. The tube was purged with argon and stored in the dark at 22 °C for 30 min, after which the sample was again desalted by RP-HPLC and dried in the SpeedVac. The dried sample was redissolved in 0.05 mL of 0.4 M NH₄HCO₃, 8 M urea, pH 8.5 and incubated for 1 h at 37 °C. It was then diluted to 0.2 mL with water, giving final concentrations of 0.1 M NH₄HCO₃ and 2 M urea. Trypsin (Promega chemically modified) was added to give a 1:50 ratio of trypsin to ER α , the sample was incubated for 2 h at 37 °C, and a further addition of trypsin was made to give a 1:25 ratio of trypsin to ER α . Digestion was then allowed to continue for 18 h at 37 °C. Digests were then analyzed by LC-MS using a Vydac C18 narrowbore column (type 218TP52) and the Finnigan LCQ. Solvents were A: 0.1% TFA, and B: 0.085% TFA in acetonitrile.

RESULTS

Synthesis and Crystallography. 17 α -Epoxypropyl-estradiol was prepared as described by el Garrouj et al. (19) (Scheme 1). Briefly, estrone was treated with excess allyl Grignard to give a single diastereomeric tertiary alcohol (**1**) after recrystallization. The phenolic hydroxy was then protected as the *tert*-butyldimethyl silyl ether (**2**), the terminal olefin was oxidized with mCPBA, and the intermediate silyloxy epoxides were separated chromatographically into pure fractions. These products were not fully characterized, but the major diastereomer was subjected to desilylation with fluoride ion to give **3**,

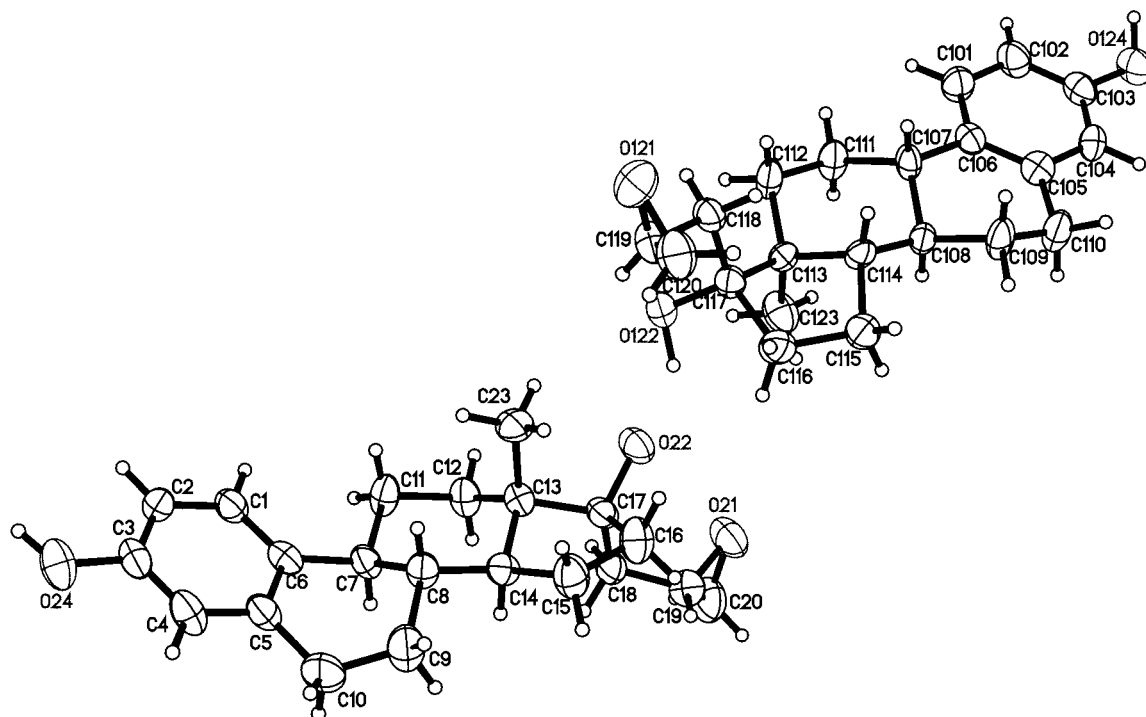
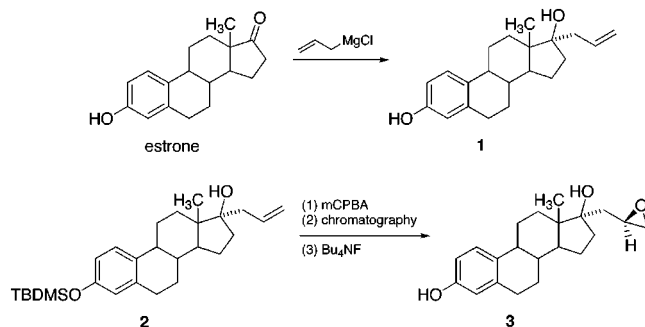


Figure 1. Crystallographically defined structure of 17 α -((*S*)-2',3'-epoxyprop-1'-yl)estra-1,3,5(10)-triene-3,17 β -diol. Two views of the molecule are shown. In the upper molecule, the A ring with the 3-hydroxyl group is to the right and the epoxide is to the extreme left. In the lower molecule, the A ring is at the lower left and the epoxide is to the extreme right.

Scheme 1



crystallized, and identified as *S* at the epoxide (Figure 1) by crystallography (see Experimental Procedures). This compound was then coupled to the solid support.

Coupling. Complete disappearance of the absorption band corresponding to estradiol from the supernatant of the coupling reaction indicated quantitative attachment of the steroid epoxide to Thiopropyl Sepharose (see Experimental Section). The product was stored as a slurry in 70% methanol at 4 °C and performed well after several months of storage.

Affinity Chromatography and Modification of Cysteines. A 29 kDa protein fragment consisting of methionine followed by residues 301–553 of ER α (SWISS-PROT accession P03372; see Figure 2 for the amino acid sequence) was expressed as a soluble intracellular protein in *E. coli* and purified by affinity chromatography on immobilized estradiol (Figure 3). ER α was treated with iodoacetic acid while bound to the column, as described by other groups reporting successful crystallization of an ER–ligand complex (4, 6, 7). After elution of the ER α with CP-336156, anion-exchange chromatography on MonoQ resolved the complex into three main peaks (Figure 4A), and ES-MS (Figure 4B) showed that about 5% of the polypeptide was unmodified, 60% was singly

310	320	330	340	350	360
SKKNSLALS	TADQMVSA	LDAEPPILY	SEAYDPTRP	FSEASMMGL	LTLNLA
370	380	390	400	410	420
AKRVPGFV	DLTLHDQV	HLLECAWLE	ILMIGLVW	RSMEHPG	KLLFAPN
430	440	450	460	470	480
MVEIFDML	LLATSSRF	RMNMLQ	GEFVCLKS	IILLNSGV	TYTFLSST
490	500	510	520	530	540
KITDTLIH	LMAKAGLT	LQQQHQL	RAQLLLI	LSHIRHMS	NKGMERLY
550					
LEMLDAHR	HLHAPT				

Figure 2. The amino acid sequence of recombinant ER α ligand-binding domain numbered according to the full-length sequence of ER α . Theoretical masses: unmodified protein 28 852 Da; once-carboxymethylated 28 910 Da; twice-carboxymethylated 28 968 Da; three times carboxymethylated 29 026 Da. An initiator methionine preceded this sequence, but was absent from the purified product.

carboxymethylated, and 35% was doubly carboxymethylated. Size-exclusion chromatography on a Superdex 75 HR 10/30 column showed that the protein was a dimer of the 29 kDa subunits (data not shown), and ES-MS of the MonoQ peaks (not shown) showed that these were (in order of elution) homodimer of singly carboxymethylated ER α , heterodimer of once- and twice-modified ER α , and homodimer of twice-carboxymethylated ER α .

In an effort to make the protein more homogeneous, it was treated in solution with iodoacetic acid (see Experimental Section). ES-MS was used to follow pilot reactions, and it was found that double carboxymethylation was readily accessible, while a third *S*-carboxymethylation would require more forcing conditions to be completed. A 33 mg sample in 43 mL was therefore treated with 0.02 M iodoacetic acid (2 h, 22 °C) until twice-carboxymethylated product was predominant (mass of 28 970 in Figure 4C). The reaction was quenched with DTT, and anion-exchange chromatography on MonoQ

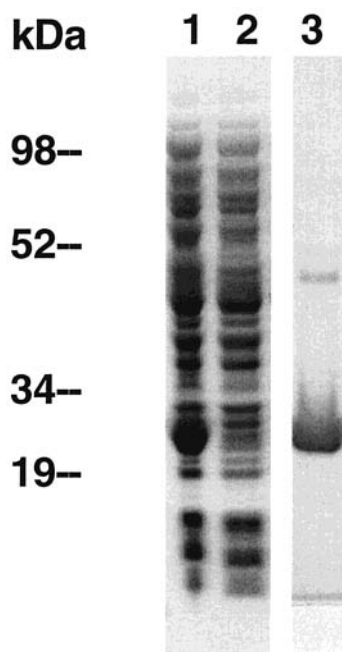


Figure 3. SDS-polyacrylamide gel electrophoresis showing affinity chromatography of ER α ligand binding domain on a column packed with the Thiopropyl Sepharose conjugate of **3**. The procedure used was essentially that described in reference (17).

(Figure 4D) resolved the product into a minor peak (heterodimer of once- and twice-carboxymethylated ER α) and a major peak which was the homodimer of twice-carboxymethylated ER α (mass observed 28 971 Da) (Figure 4D, inset). The purified twice-alkylated protein was then used successfully for crystal growth.

Identification of S-Carboxymethylation Sites. To allow definitive assignment of the modification sites, the two remaining cysteines were S-pyridylethylated using 4-vinylpyridine before the protein was digested with trypsin and subjected to LC-MS peptide mapping. Having each of the four cysteines in one of two stable, alkylated forms created the possibility of showing conclusively which were carboxymethylated and which were unmodified in the crystallized ER α .

LC-coupled mass analysis was conducted using a Finnigan LCQ ion-trap spectrometer. The instrument was programmed to enable data-dependent multistage analysis of any mass peak exceeding a threshold, including a narrow-range scan that allowed confident assignment of its mass and subsequent analysis of a sequence-related pattern of fragment ions created by collision-induced dissociation in the ion trap (20). Postrun analysis of the data using the SEQUEST algorithm (21) frequently allows a peptide corresponding to the experimental ion to be identified even in a large database, with the best success tending to come for peptides of about 8–20 amino acids. For known protein sequences, if used with due care, this analysis is as reliable as chemical peptide sequencing.

Using a combination of automated and manual interpretation, peptides accounting for all but a few small fragments of ER α were accounted for in the peptide map (Figure 5). The SEQUEST program was run more than once to search for Cys-containing peptides modified by S-carboxymethylation (+58 Da) and for Cys-containing peptides modified by S-pyridylethylation (+103 Da), and UV and mass peaks unaccounted for after this process were interpreted manually by reference to the sequence.

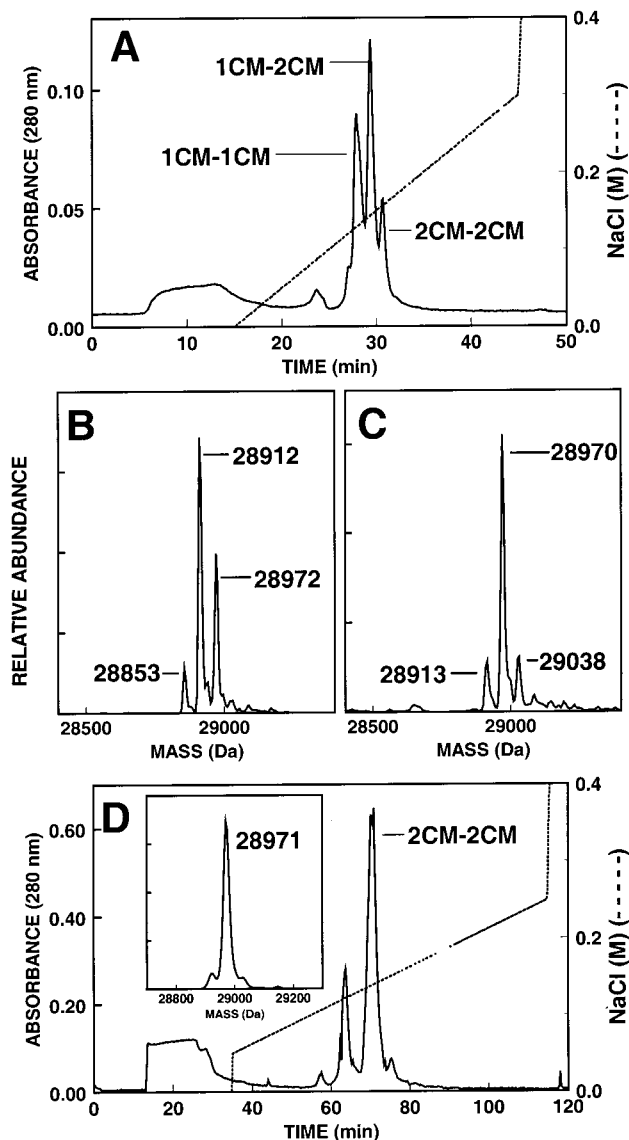


Figure 4. Use of anion-exchange chromatography and mass spectrometry to analyze the extent of S-carboxymethylation in ER α and to isolate twice S-carboxymethylated ER α . (A) Analytical fractionation on MonoQ HR 5/5 of ER α after elution from estradiol-Sepharose by CP-336156. Peak labels indicate indications from mass spectrometry; e.g., 1CM-2CM indicates a heterodimer of once- and twice-carboxymethylated ER α . (B) ES-MS of the protein sample applied to MonoQ in panel A. (C) ES-MS of ER α subjected to further treatment with iodoacetic acid in solution, but before the anion-exchange step in the next panel. (D) Preparative anion-exchange chromatography on MonoQ HR 10/10 of the ER α after the secondary treatment with iodoacetic acid. Inset: ES-MS of the material purified in the major peak, showing that twice-carboxymethylated ER α (mass theor. 28 968 Da) is the major component.

Cys-417 and Cys-530 were detected only in the S-carboxymethylated form, showing that these were the two cysteines that were alkylated in the crystallized ER α . Cys-381 and Cys-447 were detected only in S-pyridylethylated form, showing that they were unmodified in the crystallized protein.

DISCUSSION

The preparation of protein–drug complexes for crystallography is often quite straightforward. Drug is added to soluble protein, and a concentrated solution of the complex is combined with a range of solutions that often induce protein crystallization. Conditions that yield

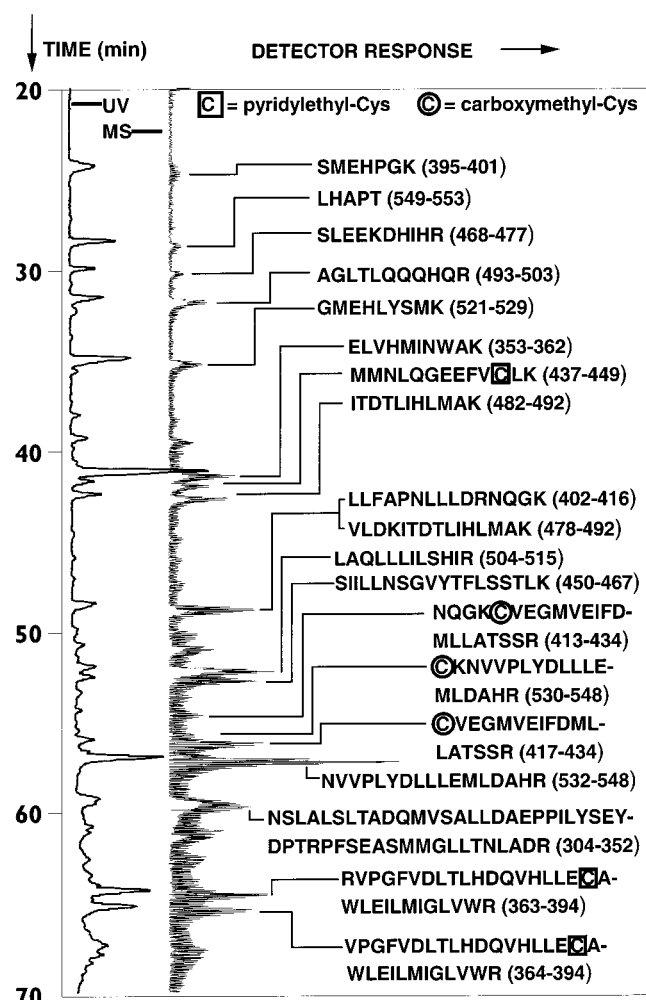


Figure 5. LC-MS peptide mapping of a tryptic digest of ER α ligand binding domain, S-alkylated with iodoacetic acid first and subsequently with 4-vinylpyridene. Cysteine-containing peptides were detected with Cys in either S-carboxymethyl or S-pyridylethyl form, as indicated on the figure.

acceptable crystals are ultimately discovered by a combination of strategy and chance.

This method does not appear to have worked so far with ER, as no published reports describe success with it, and we were also unsuccessful with it. Instead (4–7), ER ligand-binding domain has been allowed to bind to the affinity matrix introduced by Greene et al. (13), treated while bound to the affinity medium with iodoacetic acid to alkylate some of the four sulfhydryl groups in each polypeptide and then eluted from the column with soluble ligand in a buffer containing 0.25 M NaSCN, a chaotrope and potential denaturant, as well as (in one case) 10% DMF. Why this method works when the conventional one does not is open to speculation. An important possibility is that the ER binding site must be fully occupied with ligand, and that this is not achieved by adding ligand to protein in solution. It is possible, for example, that the column method monomerizes the ER fragment, forcing each polypeptide to be separately displaced from the column by soluble hormone or drug and so giving full occupancy.

The Greene affinity column is not the first affinity matrix applied to ER isolation. Vonderhaar and Mueller (22) prepared 17 α -n-propylthiol estradiol which was immobilized on polyvinyl-(N-phenylenemalimide) at a level of 0.08 mmol/g of dried resin (presumably about 0.03

mmol/mL of drained wet gel, or 3% of the ligand density of material made according to Greene). The same authors made another affinity medium in which estradiol was immobilized by diazotization at the 2 or 4 position, although this was unstable on storage. Both their products removed estrogen receptor from a homogenate of rat uterus, but the high affinity exhibited by the receptor for these media caused difficulty.

Estradiol bound to ER α is wholly engulfed by the protein (4), and it is difficult to see how estradiol immobilized through an A ring atom can be an effective affinity ligand if immobilized ligand is bound in the same orientation as soluble ligand. Sica et al. (23) tried other linkages of estradiol to agarose through A ring substituents, but the resulting media were ineffective. Among the many options that they examined, better success came from coupling 17 β -estradiol hemisuccinate to amino groups on an immobilized amino acid copolymer. Bound receptor was eluted by warming the medium to 30 °C in the presence of soluble estradiol. Efforts to produce media based on immobilized estradiol have continued; for example, a detailed discussion of a medium prepared using 17 β -estradiol 17-hemisuccinate linked to bovine serum albumin and immobilized on agarose appeared in reference (24).

Greene et al. (13, 25) described the synthetic plan (Scheme 1) for preparing the affinity medium used by all workers so far reporting crystal structures and showed that bound receptor could be eluted by soluble ligand in a buffer containing 10% DMF and 0.25–0.50 M sodium thiocyanate. We followed this general track to obtain the crystal structure of ER α complexed with CP-336156 (not shown here), but made departures from and additions to previously described methods.

17 α -Epoxypropylestradiol is the key reagent required to prepare the affinity column. Its diastereomers at the epoxide center have previously been used in attempts to prepare compounds that bind irreversibly to ER (19, 26) and were shown to differ not only in their chromatographic properties but also in their affinities for ER. To the best of our knowledge, the stereochemical identities of the two isomers have not previously been reported. The diastereomers were prepared and resolved by chromatography as described (19), and crystals suitable for X-ray diffraction were prepared by slowly evaporating a solution of **3** in acetonitrile. Analysis of the diffraction pattern identified **3** as the (S) epoxide (Figure 1), and this compound was used to prepare the affinity matrix by coupling it to Thiopropyl Sepharose.

Proteins with free cysteines are problematic for crystal growth, because long incubations of droplets create the risk of disulfide formation that destroys molecular homogeneity. For example, when cysteines were left unmodified in ER α , crystals were still obtained but an artifactual intermolecular disulfide was present in the final structure (5). This problem was avoided by the two groups who treated the column-bound protein with iodoacetic acid (4, 6). In addressing these problems, we encountered important issues that have not been discussed in detail elsewhere. The first was that ER α eluted from the affinity column with CP-336156 was inhomogeneous with respect to charge (Figure 4A) and mass (Figure 4B). As homogeneity is so highly valued in efforts to grow protein crystals, we “topped up” the chemical modification of cysteines by treating the protein–drug complex in solution with iodoacetic acid until doubly S-carboxymethylated ER α was the major species (Figure

4C). Anion-exchange chromatography was then used to purify the doubly S-alkylated form (Figure 4D), and this was the protein used to grow diffraction-quality crystals.

The main question is how many and which cysteines need to be carboxymethylated in order to allow crystal growth. Peptide mapping of a tryptic digest was used to determine which cysteines had been modified in our ER α cocrystallized with CP-336156. Cys-417 and Cys-530 were found to be S-carboxymethylated, and Cys-381 and Cys-447 were found not to be (Figure 5; also, see Figure 2 for the amino acid sequence of the recombinant ER α).

This result indicated a difference between our results and those of Brzozowski et al. (4). Their paper indicated only that Cys-381 was uniformly modified and Cys-447 was not modified. Their protein–ligand complexes were prepared as described in Hegy et al. (17), which states that triply carboxymethylated ER α with Cys-447 unmodified was the main product in their protein separately cocrystallized with estradiol and raloxifene. The crystal structures show that Cys-447 is sufficiently buried to be highly unlikely to react with iodoacetic acid in the folded protein. Thus it would appear that crystallizable forms of ER α described so far are those in which Cys-447 is unmodified, Cys-417 and Cys-530 are modified, and Cys-381 either is or is not modified. In the protein as used by Shiau et al. (6) (Protein Data Bank accession 3ERD), carboxymethylation was modeled into the X-ray structure on the basis of electron density at Cys-417 and Cys-530, but the detailed results of protein chemical analyses were not reported in the paper (although ES-MS was stated to have been done on the modified protein).

All of the above represents discussion in detail of material that some will consider purely technical and even marginal. To this it can only be replied that nuclear receptors represent a very large family of molecules of enormous biological significance, and that analysis of their structures is a process now in its infancy. The gains represented by work on a small number of nuclear receptors now need to be followed by success with more family members. The sharing of detailed experimental information can often be helpful and occasionally essential in accelerating such progress.

ACKNOWLEDGMENT

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Synthesis and Preclinical Characterization of a Paclitaxel Prodrug with Improved Antitumor Activity and Water Solubility

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The development of novel chemotherapy strategies based on prodrugs remains a major challenge for effective treatment of malignancies. We tested the hypothesis that this can be achieved by a prodrug of paclitaxel where one biologically active center, represented by the C7 hydroxyl group, was blocked by a dihydroxypropyl side chain which can be hydrolytically cleaved by a pH-dependent, slow-release mechanism. The prodrug was synthesized by condensation of solketal chloroformate with the C7 hydroxyl group of paclitaxel followed by a ring-opening reaction to the dihydroxyl derivative. The cytotoxicity of the prodrug was similar to paclitaxel, when tested in vitro against a variety of human tumor cell lines. In vitro cell cycle analysis indicated that concentrations within the micromolar range of both drug and prodrug are required to induce sufficient G2M arrest. The hydrophilic paclitaxel prodrug proved to be more than 50-fold more water soluble than the parental drug and effectively converted to paclitaxel by pH dependent hydrolysis. Importantly, the prodrug could be used at a 3-fold higher maximum tolerated dose (MTD) and revealed a markedly improved antitumor activity in mice compared to paclitaxel. Taken together, our results demonstrate, that a hydrolytically activated paclitaxel prodrug exhibits greater water solubility and superior antitumor activity than the parental drug.

INTRODUCTION

Paclitaxel is an anticancer agent with established value for clinical application (1) including breast, lung, and ovarian cancer (2–8). However, limited response rates, significant side effects, and low solubility of this drug remain as major obstacles for its optimally effective use in cancer therapy.

The chemical structure of paclitaxel, a natural product obtained from the bark of the pacific yew tree (*Taxus brevifolia*), was first described by Wani et al. in 1971 (9). Like taxotere and unlike other anti-microtubule drugs, such as vinca alkaloids, colchicine, or podophyllotoxin, paclitaxel promotes the irreversible polymerization of tubulin (10–15). The stable and thus dysfunctional microtubules disrupt cell division by cell cycle arrest in the premitotic G2 and the mitotic phases, the so-called G2M-arrest, which is the primary cytotoxic mechanism of this drug (16). A second cytotoxic mechanism of paclitaxel is to assist the induction of tumor necrosis factor α , an event unrelated to the polymerization of microtubules (17). The scope of paclitaxel's therapeutic use, especially in combination with other drugs, has been extensively reviewed (36, 37). Importantly, systemic use of paclitaxel in large doses is limited primarily by hematologic toxicity and neurotoxicity. Life-threatening hypersensitivity reactions are reported in 1–3% of patients despite appropriate premedication including dexamethasone, histamine H2 antagonists, and diphenhydramine (18, 19, 35). A second limitation of paclitaxel chemotherapy is neutropenia, starting usually 10 days

after induction of treatment with neutrophil counts below 500/ μ L in the majority of cases. Severe neutropenia can usually be avoided with doses of less than 200 mg/m² (1, 19). Neutropenia seems to be more severe with longer infusion times (4, 20). The third common side effect of paclitaxel is dose dependent neurotoxicity, characterized by peripheral symmetric distal loss of sensation, pain, and paresthesia. More severe adverse effects occur at dosages above 250 mg/m² such as motor and autonomic dysfunction as well as myopathy (26, 27). These symptoms are often judged to be the most discomforting side effects experienced by the patient. Thus, neurotoxicity and neutropenia are considered as principle dose-limiting factors of paclitaxel (19, 21, 22).

In view of these limitations of paclitaxel chemotherapy, novel prodrug design maintaining antitumor activity combined with fewer side effects is imperative to allow the administration of larger doses of paclitaxel or to diminish side effects. Indeed, the chemical modification of the paclitaxel molecule to achieve better tolerance and efficacy has been the goal of numerous projects (23, 33). One such approach is to induce molecular modifications to release paclitaxel by enzymatic or other physicochemical mechanisms. Thus a strategy was developed based on antibody-directed enzymes for prodrug activation, commonly referred to as ADEPT (23). However, in spite of promising initial results, this strategy is severely limited by antigenic heterogeneity and immunogenicity of the antibody–enzyme constructs used for tumor targeting.

An alternative strategy for tumor specific prodrug activation is provided by acidic conditions in the tumor microenvironment, a common characteristic of many solid tumors (24–29). Here, we describe a novel chemotherapeutic strategy using a hydrolytically activated prodrug with dramatically improved water solubility, allowing the

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delivery of higher doses of active drug to the tumor with less toxicity. We demonstrate for the first time that a modified paclitaxel prodrug with slow and pH-dependent release of parental drug exhibits clearly superior antitumor activity in preclinical models. This method of conjugating a natural compound using a multifunctional linker can also be used to couple targeting molecules such as monoclonal antibodies to generate other types of bioconjugates.

MATERIAL AND METHODS

The paclitaxel prodrug was synthesized in two steps:

1. Utilization of the 2' and 7 hydroxyl groups via a condensation with 2,2-dimethyl-1,3-dioxolane-4-methanol chloroformate (solketal chloroformate) to form the bi-functional carbonate intermediate.

2. This intermediate was then hydrolyzed to remove the 2' carbonate and open the acetal ring to form the final dihydroxypropyl carboxy paclitaxel. The following procedure describes the detailed preparation.

Synthesis of the Paclitaxel Prodrug. 2',7-[Bis-(2'',3''-isopropylidene-glycerol carboxy)]paclitaxel was synthesized in dry glassware, under a nitrogen atmosphere: paclitaxel (5.0 mg, 5.86×10^{-3} mmol) was dissolved in CH_2Cl_2 (250 μL) and cooled to minus 70 °C. Solketal chloroformate (22.8 mg, 0.117 mmol) was added to the solution, followed by addition of dry pyridine (7.1 mL, 87.8×10^{-3} mmol). The reaction was completed after 4.5 h at room temperature. The reaction mixture was diluted with CH_2Cl_2 (1.0 mL) and brine (1 $\times$ 2.0 mL). The organic layer was dried over MgSO_4 , filtered, and then reduced to 100 mL volume under a flow of N_2 . The resulting solution was purified by preparative HPLC to yield 5.2 mg of 2',7-[bis(2'',3''-isopropylidene-glycerol carboxy)]paclitaxel (76.5%). Purity by HPLC was greater than 98%.

Proof of structure by ^1H NMR (500 MHz, CDCl_3): δ 8.13 (d, 2H, *o*-C(O) *pH*), 7.75 (d, 2H, *o*-NHC(O) *pH*), 7.62 (t, 1H, *p*-OC(O) *pH*), 7.53–7.49 (band, 3H), 7.43–7.35 (band, 7H), 6.91 (t, 1H, NH), 6.35 (s, 1H, C(10)-H), 6.27 (t, 1H, C(13)-H), 5.99 (t, 1H, C(3')-H), 5.96 (d, 1H, C(2)-H), 5.51 (dd, 1H, C(7)-H), 5.44 (dd, 1H, C(2')-H), 4.39–4.25 (band, 3H), 4.24–4.10 (band, 7H), 3.96 (d, 1H), 3.85 (dd, 1H), 3.75 (m, 1H), 2.61 (p, 1H, C(6)-H), 2.46 (d, 3H, C(4)-OAc), 2.41 (m, 1H), 2.23 (m, 1H), 2.14 (d, 3H, C(10)-OAc), 2.01 (s, 3H, C(12)-CH₃), 1.97 (m, 1H), 1.81 (s, 3H, C(8)-CH₃), 1.41 (d, 3H), 1.38 (s, 3H), 1.35 (d, 6H), 1.21 (s, 3H, C(15)-CH₃), 1.16 (s, 3H, C(15)-CH₃).

7-(2'',3''-Dihydroxypropyl carboxy)paclitaxel: To a solution of 2',7-[bis(2'',3''-isopropylidene-glycerol carboxy)]paclitaxel (5.0 mg, 5.86×10^{-3} mmol) in tetrahydrofuran (THF) (100 μL) was added 1 N HCl (100 μL) at room temperature. The reaction was stirred at 35 °C for 3 h to give complete conversion to 2',7-[bis-2'',3''-dihydroxypropyl carboxy)]paclitaxel. At this point the reaction was stopped and the product isolated by preparative HPLC following an aqueous workup. After allowing the reaction to reach ambient temperature, it was diluted with THF (1.0 mL) and then washed with brine (3 $\times$ 2.0 mL). The organic layer was dried over MgSO_4 and filtered and the volume reduced to about 100 mL volume under a flow of N_2 (g) followed by the addition of 0.1 M KH_2PO_4 (100 μL , pH = 6.0) at room temperature. The reaction was stirred at ambient temperature for 24 h, diluted with THF (1.0 mL), and washed with brine (3 $\times$ 2.0 mL). The organic layer was dried over MgSO_4 , filtered, and reduced in volume to approximately 100 mL. The resulting solution was purified by preparative HPLC

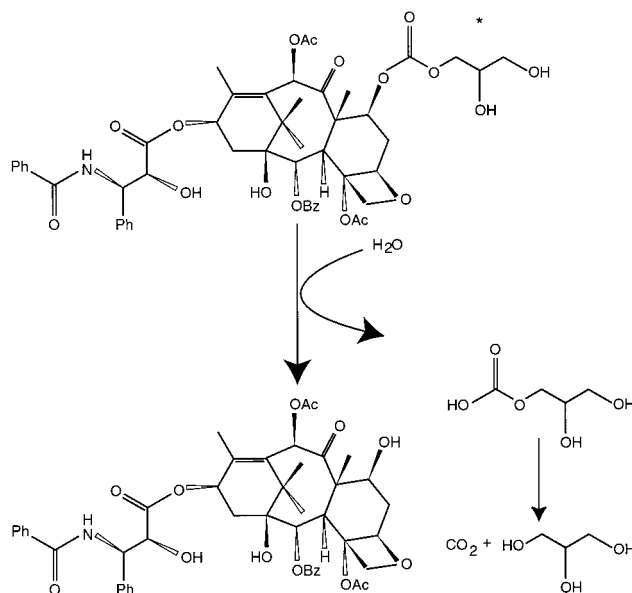


Figure 1. Schematic depicting structure and hydrolytic activation of paclitaxel prodrug. The hydroxyl group in position 7 of paclitaxel was masked by a hydrophilic side chain (*). The resulting 7-(2'',3''-dihydroxypropyl carboxy)paclitaxel is activated, pH-dependently, by hydrolytic cleavage of the carbonate moiety, resulting in active paclitaxel, dihydroxypropanol, and CO_2 .

to yield 4.7 mg of 7-(2'',3''-dihydroxypropyl carboxy)paclitaxel (82.5%). Purity by HPLC was greater than 99%.

Proof of structure by ^1H NMR (500 MHz, CDCl_3): δ 8.11 (d, 2H, *o*-C(O) *pH*), 7.76 (d, 2H, *o*-NHC(O) *pH*), 7.62 (t, 1H, *p*-OC(O) *pH*), 7.52–7.48 (band, 5H), 7.43–7.34 (band, 5H), 7.03 (d, 1H, NH), 6.26 (s, 1H, C(10)-H), 6.19 (t, 1H, C(13)-H), 5.80 (dd, 1H, C(3')-H), 5.67 (d, 1H, C(2)-H), 5.42 (dd, 1H, C(7)-H), 4.95 (d, 1H, C(5)-H), 4.80 (d, 1H, C(2')-H), 4.58–4.39 (dd, 1H, C(2'')-H), 3.72–3.59 (band, 5H), 2.67 (m, 1H, C(6)-H), 2.39 (s, 3H, C(4)-OAc), 2.36–2.29 (band, 3H), 2.22 (d, 4H), 1.98–1.93 (band, 2H), 1.83 (s, 3H, C(4)-OAc), 1.82 (s, 3H, C(11), CH₃), 1.23 (s, 3H, C(15)-CH₃), 1.16 (s, 3H, C(15)-CH₃).

^{13}C NMR (500 MHz, CDCl_3): 201.28, 201.18, 172.85, 171.58, 171.44, 170.70, 167.48, 166.95, 154.46, 154.34, 141.41, 141.34, 138.18, 134.00, 133.84, 132.73, 132.20, 130.37, 129.27, 129.21, 128.93, 128.54, 127.26, 84.02, 81.15, 78.62, 77.48, 77.23, 76.97, 76.57, 76.24, 76.19, 76.12, 74.40, 73.31, 72.39, 70.02, 69.06, 62.96, 62.77, 56.19, 56.16, 55.21, 47.25, 43.47, 35.76, 33.42, 26.80, 22.72, 21.18, 21.12, 21.07, 14.82, 10.92; FAB/MS (NBA/CsI) *m/e* 1104, $\text{M} + \text{Cs}^+$ calculated for $\text{C}_{51}\text{H}_{57}\text{NO}_{18}$ 1104.30; UV_{max} 230 nm (ϵ 27891), min 274 nm (ϵ 1489).

The structure of the prodrug and one mechanism of activation is depicted in Figure 1.

Solubility and Partition Coefficients of Paclitaxel and Paclitaxel Prodrug in Octanol/Water. Solubility was determined by sonicating drug and prodrug for 15 min in water at room temperature, followed by centrifugation at 10000g for 2 min. The supernatants were then analyzed by HPLC.

The partition coefficients (octanol/water) were determined by vortexing the mixtures for 10 min. The concentrations in each layer were then determined by HPLC. Coefficients were calculated according to $[C]_{\text{octanol}}/[C]_{\text{water}}$.

Cytotoxicity. Antiproliferative activities of the prodrug and paclitaxel were determined using the XTT cytotoxicity assay. This assay has been described in detail elsewhere (30). Briefly, 1×10^4 cells/well were added to

96 well microtiter plates and incubated for 24 h before addition of drug at concentrations ranging from 1×10^{-4} to 1×10^{-12} mol/L by direct addition from stock solutions prepared in DMSO. After 72 h, XTT (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) was added to a final concentration of 280 $\mu\text{g/mL}$. The plates were then incubated for 4 h, and the inhibition of cell proliferation was quantified by determining the absorbance at 450 nm with a microplate reader (Molecular Devices, San Diego, CA). The drug concentration which inhibited growth by 50% (IC_{50}) was calculated from duplicate runs.

Stem cell toxicity was determined by a standard Salmon assay, which has been described in detail before (31, 32). Briefly, CD34 positive stem cells from cord blood were cultured in soft agar plates, followed by an incubation of various concentrations of prodrug and paclitaxel for a period of two weeks (37 °C, 5% CO_2). Stem cell toxicity was evaluated by colony counting.

Cell Cycle Analysis by Flow Cytometry. The human neuroblastoma cell line SK-N-SH was used as a model to determine the percentage of cells in G2/M phase as a function of prodrug concentration and time. Tumor cells were fixed and permeabilized in the presence of 70% ethanol. Cells were centrifuged (1000g, 5 min) and resuspended in phosphate-buffered saline (PBS, pH 7.4). To reduce background staining, RNase A (0.3 mg/mL) was added, followed by specific DNA staining using propidium iodide (50 $\mu\text{g/mL}$). The DNA histograms were determined using a Beckton-Dickenson FACS scan.

Animals and Experimental Tumor Xenograft Nude Mouse Models. Male athymic Ncr-nu (nu/nu) mice were obtained from Harlan Sprague Dawley (Indianapolis, IN) at 6–8 weeks of age. Animals were housed in the pathogen-free mouse colony at our institution in groups of four mice each. Mice were fed ad libitum on standard mouse laboratory chow. Animal experiments were performed according to the NIH Guide for The Care and Use of Laboratory animals.

Evaluation of Maximum Tolerated Dose (MTD). For this study, MTD was defined as the treatment dose at which all animals survived without significant weight loss, i.e., less than 15% as compared to the control. The MTD for protaxoid and paclitaxel was determined in Balb/c mice, which were injected iv with increasing concentrations of either of these drugs. Doses used included 10, 20, 40, 60 mg/kg in case of the protaxoid and 8, 10, 12, 14, 16, and 18 mg/kg in case of paclitaxel. A total of 80 animals were used, and each group consisted of eight mice. Body weight was monitored and the MTD defined as a >15% loss in body weight compared to controls treated with PBS.

Treatment of Human Tumor Xenografts. Three groups of eight mice each were selected randomly for each treatment regimen (PBS, paclitaxel, and prodrug) with each tumor cell line. MT-39, MDA-MB-468, OVCAR-3, and PC-3 tumor cell lines were harvested, and 2×10^6 cells were injected subcutaneously in 200 μL Hanks medium in the right front flank.

Mice were examined daily, and the tumors were measured in two dimensions with a microcaliper. Established tumors with mean areas of about 20–50 mm^2 were treated with a vehicle control containing DMSO, chremophore, ethanol, and PBS (8%, 6%, 6%, 80%), paclitaxel prodrug (40 mg/kg), and paclitaxel (16 mg/kg), four times on alternative days. Primary tumor size was determined by microcaliper measurements three times weekly and calculated according to width $\times$ length $\times$ 2/3.

Statistics. Differences between experimental findings were analyzed by the students t-test. Differences were considered statistically significant with p values of <0.05 .

RESULTS

Design of a Prodrug Based on Paclitaxel Activated by a pH-Dependent Mechanism. Carbonates of paclitaxel with hydrophilic group substitutions were shown to be more soluble in water and inert to tubulin (33). Based on this observation, a paclitaxel prodrug was designed and synthesized with a pH-dependent cleavable dihydroxypropyl carbonoxyl group ($-\text{O}-\text{CO}-\text{O}-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}_2\text{OH}$) attached to the C7 position by a carbonate bond. This group is hydrolytically cleaved (Figure 1). The structure was designed based on its superior physiochemical characteristics including slow drug release, substantially increased aqueous solubility, nontoxic metabolites, and a route of synthesis with a high yield. The solubility of this compound was increased more than 50-fold over that of paclitaxel (<0.004 mg/mL) (data not shown). Furthermore, this modification was demonstrated to abrogate paclitaxel-specific activities including the polymerization of tubulin to microtubules in the presence of GTP and inhibition of the depolymerization caused by CaCl_2 in vitro (data not shown).

Solubility and Partition Coefficients of Paclitaxel and Paclitaxel Prodrug in Octanol/Water. The solubility determined for the paclitaxel prodrug was 8.7×10^{-4} M, while paclitaxel revealed a solubility of $<1.5 \times 10^{-5}$ M, making the prodrug 50-fold more water soluble than its parental compound. The partition coefficients in octanol/water was determined as 120 for the prodrug and >10000 for paclitaxel, respectively.

Analysis of Paclitaxel Prodrug Stability and Storage Life. The characterizations of this novel paclitaxel prodrug involved the assessment of stability and storage life in its formulation for intravenous infusion. Importantly, the prodrug was found to be stable to hydrolysis at ambient temperature in its formulation for intravenous infusion for at least 24 h with less than 5% conversion after 48 h.

Evaluation of Cytotoxic Potential and Mechanism against human Cancer and Nonmalignant Cell Lines. To assess cytotoxicity and selectivity for malignant cells over normal cells, paclitaxel prodrug and paclitaxel were tested against a range of malignant and nonmalignant cell lines in vitro (Figure 2). The IC_{50} values observed reveal similar potencies and selectivities for prodrug and drug. The selectivity as defined by an increased cytotoxic potential against malignant cells over normal cells is maintained for the paclitaxel prodrug, which is consistent with an activity based on paclitaxel release (33).

Based on the side effects observed with paclitaxel chemotherapy including agranulocytosis and thrombocytopenia, the toxicity of paclitaxel and the prodrug against hematopoietic stem cells was determined (Figure 3). Two separate sets of experiments demonstrated a 1.0 to 1.5 log decrease in stem cell toxicity of the paclitaxel prodrug in contrast to paclitaxel. No colonies grew in the presence of 1×10^{-5} M concentration of both drugs. Cell colonies were counted starting in the presence of 1×10^{-7} M paclitaxel and at 5×10^{-6} M concentration of prodrug. The number of colonies counted at a prodrug concentration of 1×10^{-6} was equivalent to the colonies counted at a paclitaxel concentration of 5×10^{-8} .

Cell Cycle Analysis by Flow Cytometry. Cell cycle analysis of SK-N-SH tumor cells following incubation

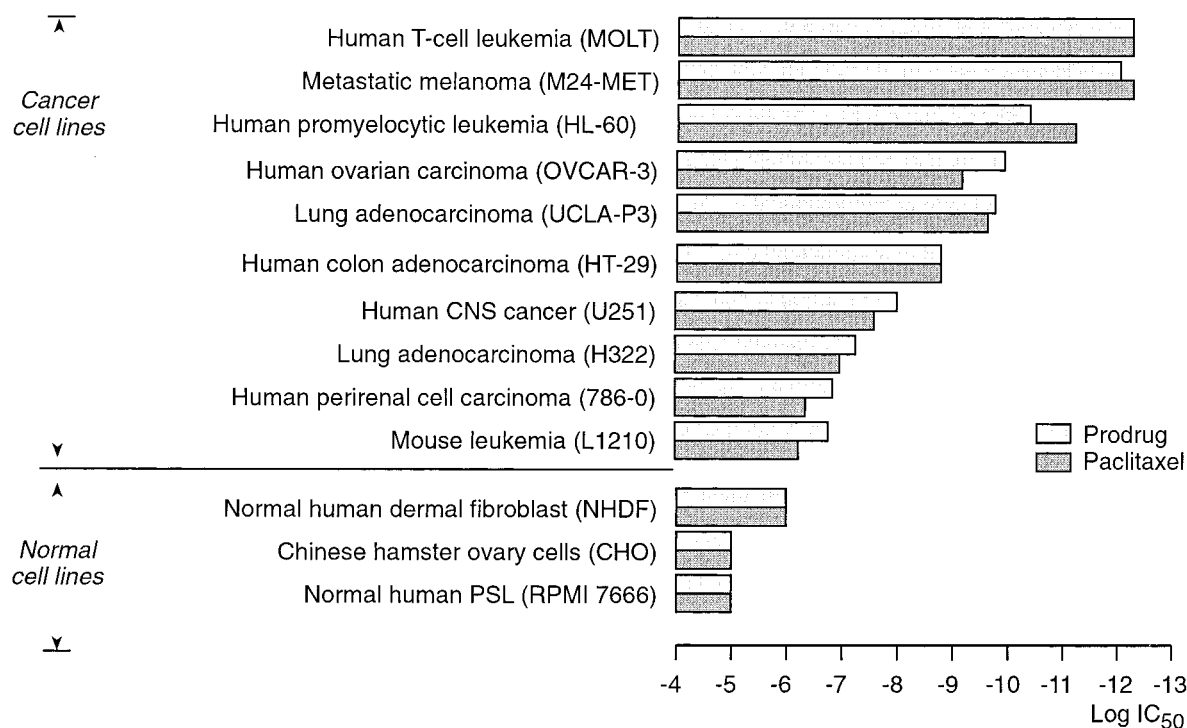


Figure 2. Cytotoxicity of paclitaxel prodrug and paclitaxel. The cytotoxic potential of paclitaxel prodrug and paclitaxel was tested against a variety of malignant and nonmalignant human cell lines. IC₅₀ values were determined as the molarity at which 50% of tumor cell viability was observed after 72 h exposure under standard tissue culture conditions.

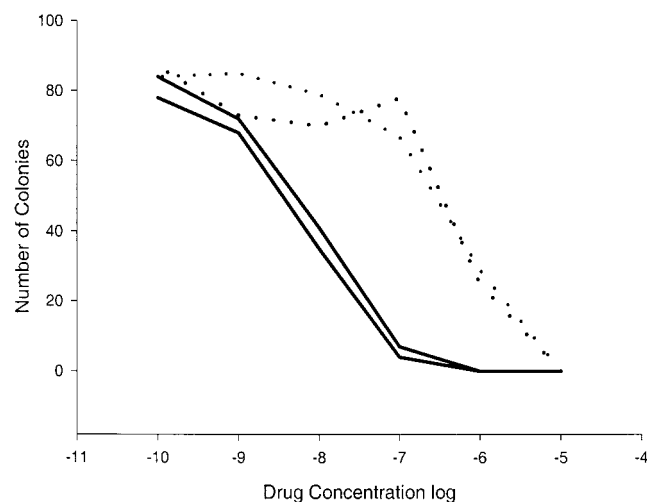


Figure 3. Effect of paclitaxel prodrug and paclitaxel on hematopoietic stem cells. This assay was performed to test the in vitro toxicity of the prodrug and the parental compound on human stem cells. Colonies were counted in duplicates after two weeks of exposure (solid line, paclitaxel; dashed line, paclitaxel prodrug).

with paclitaxel prodrug revealed the prodrug to induce G2M arrest, a mechanism described for paclitaxel which is a prerequisite for synergisms in combination therapies. Importantly, a drug concentration within the micromolar range is required over a period of 24h in vitro to induce G2M-arrest of 40% (Figure 4). These results are similar to those obtained under the same conditions for paclitaxel (data not shown). These findings strongly emphasize that high concentration levels of paclitaxel or paclitaxel prodrug are required over an extended period of time to elicit a strong antitumor effect.

Evaluation of MTD and Antitumor Efficacy in Mice. The preclinical evaluation of paclitaxel prodrug involved the determination of antitumor activity in

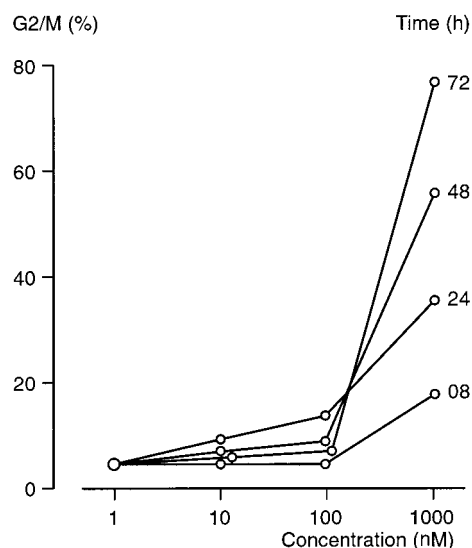


Figure 4. Effect of paclitaxel prodrug on the cell cycle of malignant cells. The percentage of human neuroblastoma (SK-N-SH) cells in G2M phase was determined at varying concentrations of paclitaxel prodrug over time. G2M phase was determined by flow cytometry after DNA staining with propidium iodide. Data points are mean values of triplicate flow cytometry analysis of each sample. The deviations of each scan were within 5%.

human tumor xenograft models and the maximum tolerated dose (MTD) as defined by a loss in body weight of <20%. Typically, Balb/c mice were injected with increasing amounts of paclitaxel prodrug, and the body weight of individual animals was monitored over time. These experiments resulted in a MTD for paclitaxel prodrug of 40 mg/kg, contrasting with a MTD of 16 mg/kg for paclitaxel and consistent with a 2.5-fold decrease in toxicity in mice achieved by our prodrug design. At these dosages all animals survived for more than eight weeks.

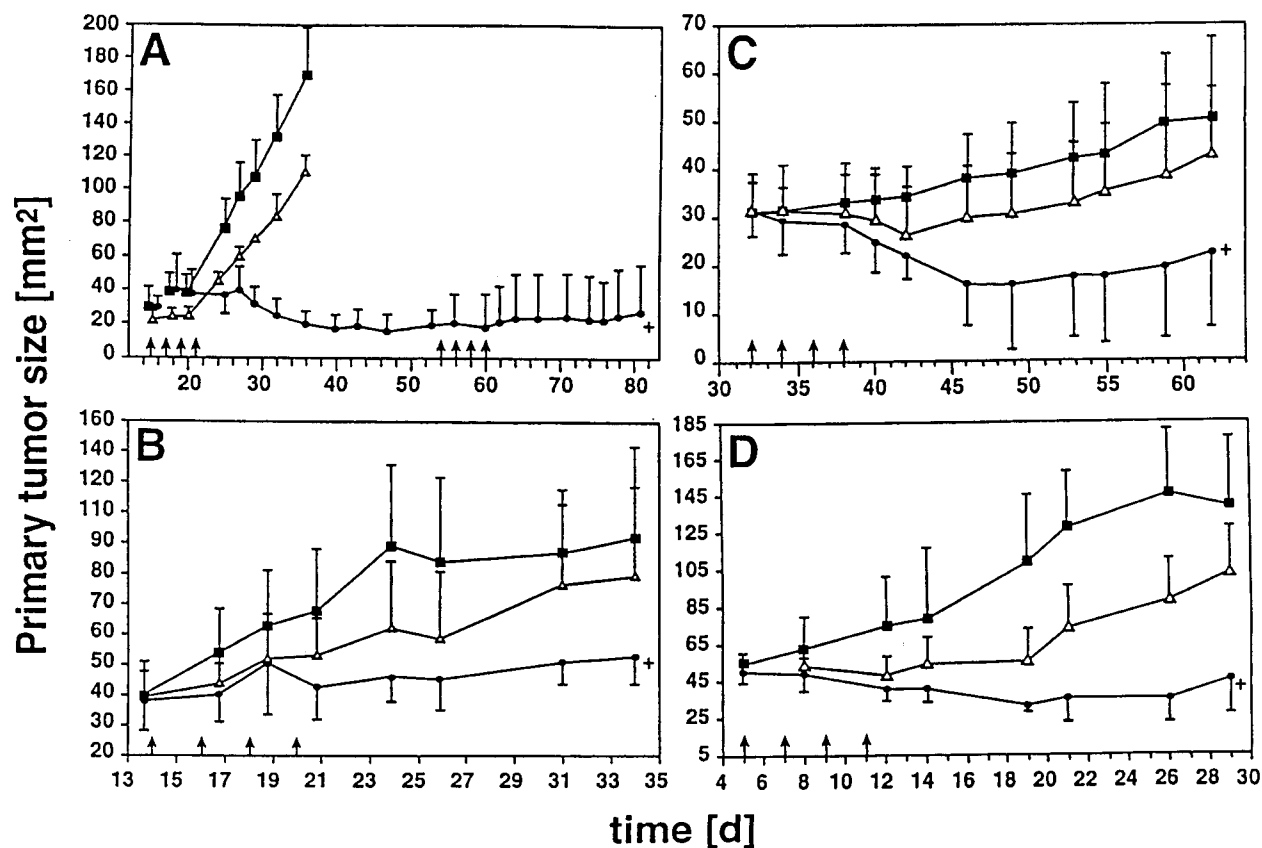


Figure 5. Regression and suppression of growth of established primary tumors by treatment with paclitaxel prodrug. Ncr-nu (nu/nu) mice ($n = 8$) with established subcutaneous human primary tumors were treated with intraperitoneal injections of paclitaxel prodrug (40 mg/kg) (closed circle) and compared to controls treated with paclitaxel (16 mg/kg) (open triangle) or vehicle control (closed square). Primary tumor growth was monitored by microcaliper measurements over time and tumor size calculated according to width $\times$ length $\times$ 2/3. Figures represent mean values and standard deviations for each experimental group of mice for human prostate carcinoma PC-3 (A), human ovarian carcinoma OVCAR-3 (B), human mammary carcinoma MDA MB 469 (C), and human colon carcinoma MT-39 (D). The differences between experimental groups treated with paclitaxel prodrug and controls treated with paclitaxel (open triangle) or vehicle control (closed square) was statistically significant ($+ = p < 0.05$).

Subsequently, the antitumor activity of the prodrug compared to paclitaxel was determined when administered at the MTD in four different human tumor xenograft models using prostate, colon, breast, and ovarian cancer cell lines in nude mice (Figure 5A–D). The paclitaxel prodrug administered four times every other day at the MTD induced tumor regression or significant reduction in tumor growth in all four tumor models. This was in contrast to paclitaxel and the vehicle control, which were ineffective in this regard. For example, the growth of the PC-3 human prostate carcinoma cell line regressed until day 47 to an average tumor surface area of 18 mm² with a standard deviation of 9.5 mm². In contrast, treatment with paclitaxel failed to inhibit extensive tumor growth, and the mice had to be sacrificed 35 days after treatment initiation. Average tumor surface area at that time was 111 mm² as compared to 170 mm² for the vehicle control with a standard deviation of 9 mm² (Figure 5A). The ovarian carcinoma cell line OVCAR-3 revealed an average tumor surface area of 48 mm² (SD 8 mm²) on day 34, as compared to 78 mm² when treated with paclitaxel (Figure 5B). The treatment of the mammary cancer cell line MDA-MB-468 with the prodrug led to an average regression in tumor size from 30 mm² at initiation of treatment to 22 mm² at day 63. In contrast, the treatment with paclitaxel showed no significant difference compared to the vehicle control (Figure 5C). The treatment of the human colon carcinoma cell line MT-39 led to a tumor surface area of 45 mm² on day

29 versus 47 mm² at initiation of treatment and compared to 105 mm² following treatment with paclitaxel (Figure 5D).

DISCUSSION

The addition of paclitaxel to the repertoire of chemotherapeutic drugs offers a significant improvement in outcome and quality of life for patients suffering from a number of solid tumors, including breast, prostate, and ovarian cancer. However, the usefulness of paclitaxel is limited by its toxicity and the development of drug resistance. Here, we describe a novel prodrug designed to deliver paclitaxel via a sustained in vivo release mechanism with the intent to use larger doses or similar doses with lower toxicity. This design included modifications to increase water solubility. We demonstrated in four preclinical human tumor xenograft models an increase of the maximum-tolerated (MTD) dose for the paclitaxel prodrug by a factor of 2.5 over that of active paclitaxel, facilitating the use of higher dosages of paclitaxel prodrug in mice. This reduction in toxicity is also reflected by a 100 times lower stem cell toxicity of the prodrug as compared to the parental drug. This is important, since one of the dose limiting toxicities of paclitaxel is the induction of neutropenia. In addition, the prodrug was at least equally effective as paclitaxel against tumor cell lines in vitro. This finding also translated into improved antitumor effects of the paclitaxel prodrug over paclitaxel in four different human

tumor xenograft models. Three possible mechanisms may be involved in the superior effect of paclitaxel prodrug over parental paclitaxel.

First, higher doses of the prodrug lead to an increase in the maximum plasma levels of both the prodrug and subsequently released drug which may ensure a favorable tissue distribution, resulting in higher concentrations of the prodrug in the tumor microenvironment. Sufficient concentration levels of paclitaxel prodrug are most important for achieving G2M arrest, the primary effect of paclitaxel on malignant cells (15). This was clearly demonstrated in vitro as the paclitaxel prodrug elicited a significant G2M arrest at a concentration of 1 μ M after 24 h of incubation (Figure 4). Therefore, the exposure of tumor tissue to high prodrug concentration over a long period of time appears to be a most critical parameter for effective therapy with such protaxoids. This finding is of particular interest for combination chemotherapy strategies using paclitaxel prodrug with improved efficacy in the induction of G2M arrest, thereby increasing the susceptibility of cancer cells to subsequent attacks by other cytotoxic compounds or irradiation, all of which induce DNA damage. Such combination strategies should clearly enhance synergistic effects, which were already described for paclitaxel (16, 34).

Second, the 50-fold increase in water solubility achieved by the unique prodrug design involving the addition of a hydrophilic side chain decreases protein binding and increases the amount of free drug, thereby further improving tissue penetration. This increase in solubility may allow infusion of the prodrug requiring dramatically lower amounts of additives such as ethanol and Cremaphore EL, the later being associated with infusion related hypersensitivity reactions (35). Also, the total infusion volume to deliver a therapeutic dose of prodrug can be decreased and therefore volume associated problems can be avoided particularly in patients with renal insufficiency.

Third, it is known that favorable activation of the protaxoid in tissue is influenced by high or low pH. Therefore, preferential activation of the prodrug might occur pH dependent in malignant tumors (25, 27–29).

In summary, we described the synthesis and preclinical characterization of a novel prodrug of paclitaxel with increased water solubility and a pH-dependent hydrolytic mechanism of activation. Preclinical evaluation of the protaxoid clearly indicated decreased toxicity in vivo as demonstrated by a substantial increase in MTD over the parental drug. Furthermore, treatment of established subcutaneous xenografts of four different human malignancies resulted in marked suppression of tumor growth in athymic mice treated at the MTD. These findings provide a basis for clinical evaluation of hydrolytically activated paclitaxel prodrug in Phase I/II trials.

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A Robust Method for the Preparation and Purification of Antibody/Streptavidin Conjugates

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The many uses of antibody–protein conjugates, especially antibody–streptavidin conjugates, give rise to the need for a reliable conjugation method offering reasonable yields and reproducible quality. We describe a method for preparing antibody–streptavidin conjugates that has consistently produced conjugates of quality and in sufficient quantity to be used in the clinical development and evaluation of the Pretarget delivery system. In this method antibody disulfides are reduced to generate reactive thiols, and maleimides are linked to streptavidin with the heterobifunctional cross-linking agent, SMCC. The two activated proteins are then mixed briefly before the conjugation is terminated with an oxidizing agent that reforms disulfides from unreacted thiols. The preponderance of the conjugate produced is 1:1 and 1:2 Ab:SA conjugate. This fraction is isolated from unconjugated proteins and high molecular weight byproduct by iminobiotin affinity and ion-exchange chromatography. The resulting conjugate is at least 90% 1:1 + 1:2 Ab:SA conjugate, contains no SA or Ab, and is produced reproducibly in 37% yield.

INTRODUCTION

Protein conjugates of monoclonal and polyclonal antibodies are used in many in vitro and in vivo applications. Researchers at NeoRx Corporation have investigated an approach to the treatment of solid and hematologic malignancies, “Pretarget” technology, which makes use of antibody–streptavidin conjugates to localize therapeutic radionuclides to tumors (1). The antibody causes the conjugate to localize at antigen bearing disease sites; the streptavidin captures subsequently administered biotinylated radionuclide chelates. This strategy has been shown (1–12) to circumvent the dose-limiting toxicity associated with covalent delivery of radionuclides by labeled antibodies (13–18).

Recent literature (9, 11, 12, 19, Pierce Catalog) offers a plethora of methods for the covalent conjugation of proteins. These methods produce conjugates with a wide range of yields and purity and are often incompletely characterized with respect to reproducibility and scalability. The conjugation method described in this report was used throughout the development and evaluation of the Pretarget delivery system and has been compared with a number of other conjugation methods either in the laboratory or in the literature. It meets the key criteria for the development of Pretarget: it produces a high yield (relative to other available methods) of 1:1 + 1:2 Ab:SA conjugate; it succeeds on scales from a few milligrams to tens of grams; and it succeeds, with some optimization, for many different antibodies.

The ideal targeting vehicles for the Pretarget method and many other applications are fusion proteins, which are homogeneous, and which, given a suitable expression system, may be prepared in large quantities at minimal cost. However, synthetic conjugates may be preferable during the investigation of a combined-function protein

because development of a suitable expression system for a fusion protein can be a lengthy and uncertain process. Furthermore, synthetic conjugation methods offer a relatively straightforward process for generating conjugates with a range of properties such as specificity, molecular weight, and binding valency to find out which are most suitable, and to aid the design of an optimal fusion protein. Hence, antibody–protein conjugation methods, and especially antibody–streptavidin conjugation methods can be helpful in the evaluation of new therapeutic modalities.

Streptavidin is a 52 kD tetrameric protein produced by *Streptomyces avidinii* that is commercially available in high purity and shows good in vivo stability (20, 21). The strong interaction and rapid association between streptavidin and biotin ($K_d = 10^{-15}$ M) are ideal for capture of the biotinylated radionuclide chelate (^{90}Y DOTA biotin) from circulation at the low concentrations reaching the disease site following systemic administration (20). Streptavidin was chosen in preference to avidin as a receptor because it is not glycosylated and has a reported lower retention in normal tissue (22).

The murine NR-LU-10 antibody recognizes a noninternalizing 40 kD glycoprotein antigen abundantly expressed on carcinomas of the lung, colon, breast, prostate, and ovary (23, 24). This antibody has been studied both in animals and in human clinical trials (25, 26).

The conjugation method takes advantage of thiols generated by reducing disulfides in IgG's. These thiols react with maleimides placed on the surface of streptavidin at an appropriate loading by reaction with SMCC. The reduced IgG and the maleimide-functionalized streptavidin are combined at an equimolar ratio, and the progress of the reaction is monitored by size exclusion chromatography. At an optimal reaction time, when the modified proteins are maximally consumed, but before too much high molecular weight byproduct has formed, the reaction is terminated by addition of the oxidizing

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agent sodium tetrathionate, which reforms disulfides from unreacted thiols. Thus, the proteins are conjugated through thioether and amide linkages.

While the conditions described represent an optimum incorporation of reactants into usable conjugate, the reactants are not entirely consumed; some streptavidin remains underivatized with SMCC and, hence, unreactive. Consequently, the final conjugation reaction mixture contains, in addition to usable conjugate, unreacted streptavidin, unreacted antibody, and cross-linked byproduct. The purification procedure separates the three impurities in two steps. Unreacted antibody is removed by iminobiotin affinity chromatography. Unreacted streptavidin is removed by ion-exchange chromatography under conditions at which conjugate binds to the matrix but streptavidin does not. Cross-linked byproduct is separated by careful elution of the desired 1:1 + 1:2 conjugate from the ion-exchange matrix (after the streptavidin has flowed through) at a salt concentration at which the cross-linked byproduct remains immobilized on the matrix.

Conjugates produced using this process were evaluated in LS-180 and SW1222 nude mouse xenograft models. The conjugates show favorable pharmacokinetic properties and biodistribution.

MATERIALS AND METHODS

DL-Dithiothreitol (DTT), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), 2-iminobiotin *N*-hydroxysuccinimide, and sodium tetrathionate dihydrate were purchased from Sigma (St. Louis, MO). Succinimidyl 4-(*N*-maleimido-methyl)cyclohexane 1-carboxylate (SMCC) was purchased from Pierce (Rockford, IL). Recombinant core streptavidin (SA) was purchased from Boehringer Mannheim (Mannheim, Germany). The monoclonal antibody, NR-LU-10, was prepared by Boehringer Ingelheim for exclusive use by NeoRx Corporation. Coarse G-25 Sephadex chromatography matrix was purchased from Pharmacia LKB (Piscataway, NJ). Epoxy-activated Marcoprep chromatography support 45–90 μ m was purchased from Bio-Rad (Hercules, Ca) and supplied as a dry powder. *N*-(3-Aminopropyl)-1,3-propanediamine was purchased from Aldrich (Milwaukee, WI). Fractogel EMD TMAE-650 (M) was purchased from EM Separations (Wakefield, RI). Calcium- and magnesium-free phosphate-buffered saline (PBS) was from BioWhittaker (Walkersville, MD). Preparative chromatography was done using XK series columns on a Pharmacia FPLC system. LS-180 cells were obtained from ATCC (Rockville, MD).

Size Exclusion Chromatography. Analytical size exclusion chromatography (SEC) was performed on a Beckman 114M HPLC system fitted with a 9.4 mm $\times$ 250 mm Zorbax GF-250 column (MacMod Analytical, Inc., Chadds Ford, PA) using Borwin data processing software. The mobile phase consisted of 20 mM phosphate, pH 6.8 plus 0.5M NaCl at a flow rate of 1.0 mL/min. The effluent was monitored at 254 nm.

Immunoreactivity. Immunoreactivity of the conjugate was determined using a competitive ELISA (27). Briefly, a solution of crude LS-180 cell extract was allowed to dry onto 96 well microtiter plates as the target. Peroxidase-labeled NR-LU-10 was used as the competitor and reporting agent at a concentration that gave OD values of 0.5 at 490 nm. NR-LU-10 and NR-LU-10/SA were serially diluted into aliquots of optimized NR-LU-10/HRP conjugate and reacted with the antigen-coated microtiter plate for 1 h at room temperature. The microtiter plates were washed, and a substrate solution

of ABTS was added, incubated for 30 min, and then read at 490 nm on a Molecular Devices Spectra Max Plus spectrophotometer.

Polyacrylamide Gel Electrophoresis. PAGE analyses were done using the Mini-PROTEAN II system from Bio-Rad Laboratories (Hercules, CA). All electrophoresis was done using the procedure reported by Laemmli (28) using 5% acrylamide with sodium dodecyl sulfate (SDS). Samples were not boiled prior to electrophoresis, to maintain the tetrameric form of streptavidin. IEF gels were run on a PHAST system from Pharmacia. Precast IEF gels resolving proteins with isoelectric points between pH 3 and 9 were used in all evaluations, under the running conditions recommended by Pharmacia.

Molecular Weight Determination. Molecular weights of NR-LU-10/SA conjugates were determined using a Zorbax GF-250 column connected in series with a Varian Star 9040 refractive index detector and a MiniDawn light scattering instrument (Wyatt Technologies, Santa Barbara, CA). This method involves no assumptions regarding protein shape. Molecular weights were determined as described by Wyatt (29) using a dn/dc value of 0.185 for a protein in an aqueous buffer solution.

Iminobiotin Affinity Matrix Preparation. Immobilized iminobiotin affinity matrix was prepared by suspending a known amount of the dry Marcoprep epoxy-activated matrix in dry methanol. *N*-(3-Aminopropyl)-1,3-propanediamine was added to the slurry at 10 mmol/g of dry matrix. An excess of triethylamine was added, and the slurry was continuously rotated on a Buchi RE 111 rotary evaporator. After 16 h, an equal volume of 1.0 M Tris pH 10.6 buffer was added, and the slurry was rotated for an additional 2 h. The slurry was filtered, washed with several volumes of H₂O, resuspended in 0.1 M H₂SO₄, and rotated for an additional 16 h to hydrolyze any residual epoxide residues. After filtration and H₂O wash, the matrix was resuspended in 0.5 M sodium borate, pH 8.0 buffer, and 50 μ mol of 2-iminobiotin *N*-hydroxysuccinimide hydrobromide was added per gram of dry gel. The reaction mixture was rotated overnight, filtered, and washed with alternate cycles of 1.0 M acetic acid and 1.0 M NaOH. The affinity matrix was capable of binding 3.0 mg of SA/mL of packed bed volume. Iminobiotin resin, available from Pierce, is suitable for this purification, but its expense precluded use on large scale.

Affinity Purification of Streptavidin. Recombinant streptavidin (SA), $pI = 7.3$; $A_{280}^{1\%} = 34$, was supplied as a lyophilized powder. Removal of excipients, endotoxin, and residual proteases was accomplished by affinity purification using immobilized iminobiotin. Lyophilized SA (500 mg) was reconstituted in 50 mM glycine buffer pH 9.0, 0.5 M NaCl, and applied to a sanitized 5.0 cm $\times$ 10 cm (196 mL) iminobiotin affinity column which was preequilibrated in the SA reconstitution buffer. After the 280 nm absorbance returned to baseline, the column was washed with five additional column volumes of buffer. SA was eluted with 0.1 M acetate buffer, pH 3.0, 0.1 M NaCl, buffer-exchanged into PBS, and concentrated using an Amicon 1 ft² 10 kDa molecular weight cut off CH2 spiral ultrafiltration system. Following concentration, 370 mg of purified SA was recovered at 23.0 mg/mL.

Preparation of SMCC-Derivatized Streptavidin. Streptavidin, 368 mg (7.08 μ mol), was diluted to 20 mg/mL in PBS, and 1.84 mL (10% v/v) sodium borate buffer, 0.5 M pH 8.0, was added to increase the pH to 8.0. SMCC (7.10 mg, 21.24 μ mol, or 3 $\times$ over SA mol/mol) in 1.17 mL of DMSO was added to the gently stirring SA solution and reacted for 30–60 min at room temperature. The

solution was then loaded onto a 2.6 cm $\times$ 42 cm Sephadex G-25 size exclusion column equilibrated with PBS at 8.0 mL/min, and the effluent was monitored at 254 nm. SMCC-derivatized SA (368 mg) was collected at the void volume in 71 mL of PBS. The extent of maleimide derivatization (MSR) was determined using cysteine followed by DTNB (30). Briefly, a known quantity of SMCC-SA was reacted with excess cysteine for 15 min. The amount of cysteine consumed by maleimide was determined by treating the solution with DTNB and subtracting the sample OD₄₁₂ at pH 8.0 from that of a cysteine control.

DTT-Reduced NR-LU-10. NR-LU-10 at 23.4 mg/mL (819 mg), $pI = 6.2$; $A_{280}^{1\%} = 14$, was reduced by treatment with 25 mM DTT. After incubation for 30 min, residual DTT was removed by gel filtration as described above using a 2.6 $\times$ 42 cm G-25 column with helium-sparged PBS, containing 1 mM DTPA at 8.0 mL/min. A quantitative yield of reduced NR-LU-10 was recovered from the column in 795 mL. The thiol content of the antibody was measured using DTNB at pH 8.0 ($\epsilon = 13\,600\text{ cm}^{-1}\text{M}^{-1}$).

NR-LU-10/SA Conjugate. Equimolar amounts of DTT-reduced NR-LU-10 (824 mg) and SMCC-SA (286 mg) were diluted to 110 mL each (24 μM) with He-sparged PBS. The two components were combined simultaneously over a 2 min period into a sterile 250 mL container using a P-1 peristaltic pump (Pharmacia LKB) to obtain a final protein concentration of 5.0 mg/mL. The stirred protein solution was monitored by HPLC SEC to evaluate the extent of the conjugation reaction. After 45 min, the reaction was quenched, and the remaining reduced disulfides were reoxidized, by the addition of solid sodium tetrathionate dihydrate to a final concentration of 5 mM.

NR-LU-10/SA Conjugate Purification. For conjugate purification, glycine was added to the crude reaction mixture to obtain a concentration of 50 mM, NaCl was added to bring the total NaCl concentration to about 500 mM, and the pH was adjusted to 9.0 by addition of 1.0 M NaOH while stirring. The extinction coefficient for a 0.1% solution of an equimolar mixture of NR-LU-10 and SA is 2.0, which also corresponds to the extinction coefficient of a 1:1 NR-LU-10/SA conjugate. For simplicity, this value was used in all purification steps to estimate process yield.

A 5.0 cm $\times$ 7.5 cm (147 mL) iminobiotin affinity column was prepared and equilibrated in 50 mM glycine buffer pH 9.0 containing 0.5 M NaCl. Crude conjugate solution (255 mL), pH 9.0, with a conductance of 34 mS/cm was loaded onto the iminobiotin column at 8.0 mL/min. Conjugate products and residual streptavidin bound to the matrix while unreacted NR-LU-10 and tetrathionate were collected in the flow-through. After the column was washed with five additional column volumes of equilibration buffer, 715 mg of bound material was eluted in 550 mL at 1.3 mg/mL with 0.2 M sodium acetate buffer, pH 4.0 containing 0.4 M NaCl. The pH of the eluted material was adjusted to 5.0 with 0.5 M borate buffer, pH 8.0 for storage.

Following iminobiotin affinity purification, the product was buffer exchanged into 50 mM NaPi buffer pH 9.0 using a CH2 spiral diafiltration cartridge with a 30 kDa cutoff yielding 609 mg in 420 mL of buffer. The material (<3 mS/cm) was loaded onto a preequilibrated TMAE column (2.6 $\times$ 5.6 cm), monitored at 280 nm, and washed until the eluate OD₂₈₀ returned to baseline to remove all residual SA. Purified conjugate (413 mg) was recovered from the matrix by elution with 130 mL of 50 mM NaPi buffer containing 90 mM NaCl (15.6 mS/cm) resulting

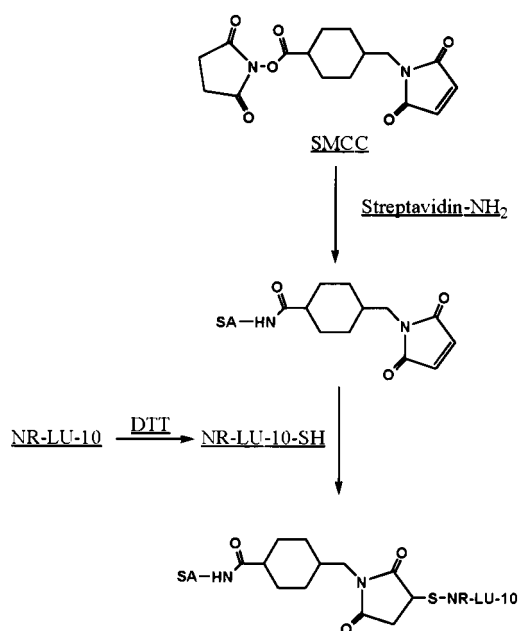


Figure 1. Formation of NR-LU-10/SA conjugate using SMCC.

in an overall product yield of 37%. After recovery of desired conjugate, higher molecular weight cross-linked conjugates could be removed from the column by washing with 50 mM NaPi buffer containing 250 mM NaCl.

RESULTS

The conjugation scheme utilizes the heterobifunctional cross-linker, succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), to form a stable amide/thioether linkage between antibody and streptavidin (32) (Figure 1). SMCC was chosen as the cross-linker since it reacts rapidly and quantitatively and is commercially available, and the molar substitution ratio (maleimides per SA) could be readily determined after SA derivatization.

SMCC Derivatization of SA. Treatment of SA with a 3-fold molar excess of SMCC yielded a molar substitution ratio (MSR) of 1.5–1.8 maleimides per SA. SA and SMCC-SA both eluted from the SEC column at 10 min, but SMCC-SA showed an additional minor peak at 9 min (<10%) corresponding to dimerized SA (Figure 2). On SDS PAGE, underivatized SA migrated mainly as a single band midway down the resolving portion of the gel, while SMCC-SA migrated as multiple bands (Figure 3, lanes 6, 7). This “ladder” effect reflects the alteration of the charge of the SA rather than a change in molecular weight. Because SA was not boiled in SDS, PAGE migration was not a direct reflection of molecular weight. Maleimides on SA were stable in PBS over an 8 h period at 25 °C. However, measurable hydrolysis occurred over 20 h. SMCC-functionalized SA was stored frozen indefinitely at $-70\text{ }^{\circ}\text{C}$.

Reduction of NR-LU-10. Reduction of NR-LU-10 with 25 mM DTT (33) resulted in the formation of 9–12 reactive thiols as determined by DTNB. Use of N₂-sparged buffer and DTPA greatly reduced the thiol reoxidation rate so that the reduced NR-LU-10 could be stored at 25 °C for up to 8 h prior to conjugation without significant loss of reactive thiols. No difference was observed between the reduced and nonreduced forms of NR-LU-10 by SEC (Figure 4) or SDS PAGE (Figure 3, lanes 4 and 5). Noncovalent interactions maintain the

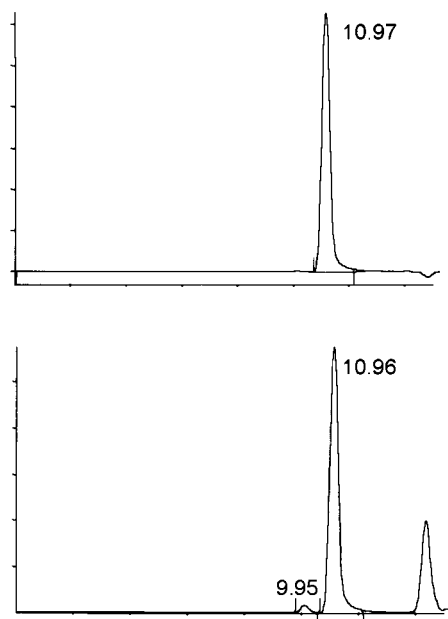


Figure 2. Size exclusion HPLC profile of SA (top) and SMCC-SA (bottom).

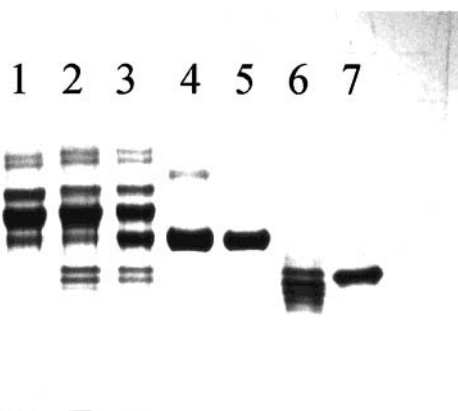


Figure 3. 5% SDS PAGE (nonboiled) of final purified NR-LU-10/SA conjugate (lane 1), conjugate after iminobiotin purification (lane 2), crude conjugate (lane 3), DTT-reduced NR-LU-10 (lane 4), NR-LU-10 (lane 5), SMCC-SA (lane 6), and SA (lane 7).

heavy-heavy and heavy-light chain association while the disulfides are reduced, unless the samples are boiled in SDS.

Conjugation of NR-LU-10 to SMCC-SA. The extent of the conjugation reaction was observed in the SEC profiles of the reaction mixture at 2 and 40 min (Figure 5). Reaction progress is indicated by consumption of SMCC-SA in the reaction mixture. In this reaction 50% of the 10.0 min peak, SMCC-SA, was consumed after 40 min. The consumption of NR-LU-10 is more difficult to quantitate by SEC because the free antibody peak elutes as an unresolved shoulder on the 8.2 min conjugate peak. Higher consumption of SA in the reaction mixture leads to the accumulation of a high molecular weight cross-linked species that appears before the desired monosubstituted conjugate peak at 7.5 min in the SEC trace.

The effect of changing the SMCC offering can be seen in an SDS PAGE analysis of the reaction mixture comparing conjugations performed with SA-SMCC prepared at different offering levels (Figure 6). The maximum yield of desired monosubstituted conjugate occurred at a 3:1 molar offering of SMCC to SA. A lower offering of SMCC to SA gave lower overall conjugate yields due

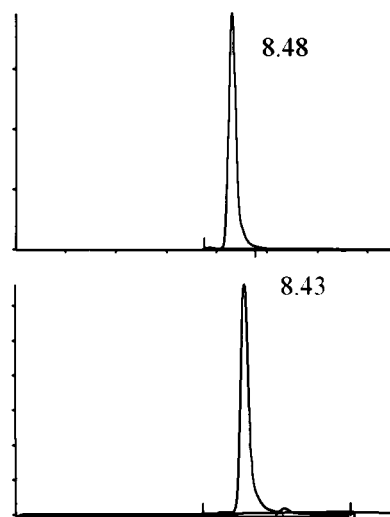


Figure 4. Size exclusion HPLC profile of NR-LU-10 (top) and DTT-reduced NR-LU-10 (bottom).

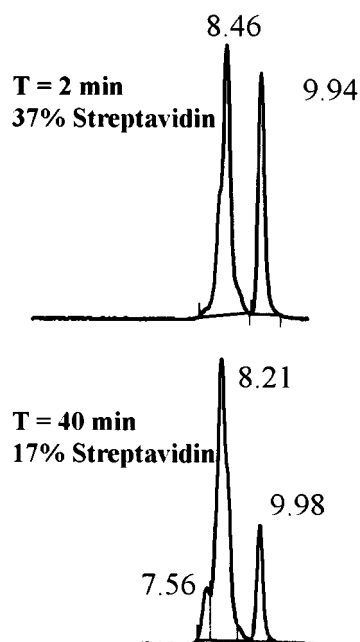


Figure 5. Size exclusion HPLC profile of crude conjugate at 2 and 40 min reaction time.



Figure 6. 5% SDS PAGE comparison of crude NR-LU-10/SA conjugates prepared using different molar offerings of SMCC.

to a decreased consumption of SA. A higher SMCC derivatization resulted in increased consumption of

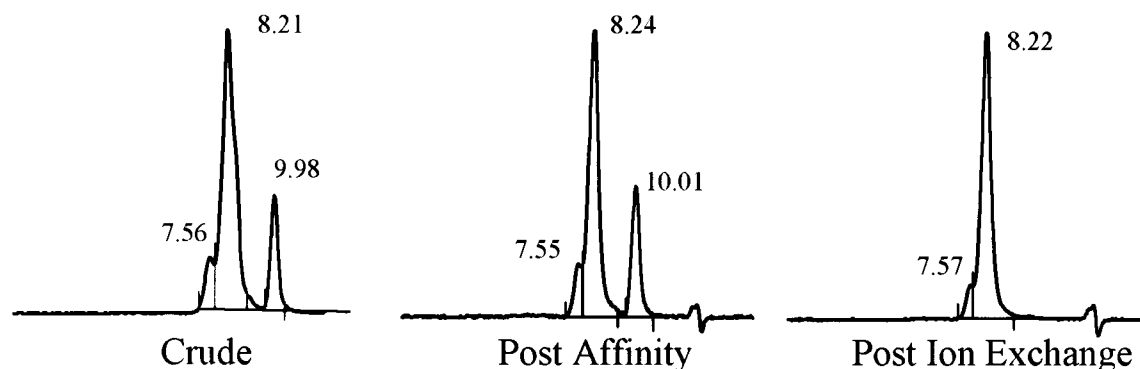


Figure 7. Size exclusion HPLC profiles of crude NR-LU-10/SA conjugate (left), conjugate after iminobiotin purification (middle), and after ion exchange purification (right).

Table 1. SEC Retention Times of Conjugation Reaction Mixture Components

component	ret time (min)
streptavidin	10.0
NR-LU-10	8.4
1:1 conjugate	8.2
1:2 conjugate	7.6
high molecular weight byproduct	<7.6

SMCC-SA yet lower desired product yield due to formation of higher molecular weight cross-linked species. Addition of 3 equiv of SMCC to SA provided a good balance between conjugation efficiency and percent desired conjugates. All reactions in this study were done at room temperature for 1 h at a total protein concentration of 5.0 mg/mL. If the conjugation reaction was allowed to proceed for longer times a steady increase in the percentage of cross-linked products was observed until these species dominated the reaction mixture.

Conjugate Purification. Removal of residual NR-LU-10 from the crude reaction mixture was accomplished by employing the pH dependent binding of iminobiotin (IB) to SA (34). Anion-exchange chromatography (Fractogel) was used to remove SA and enrich product purity by removal of most HMW species. Using the initial phosphate buffer conditions described, the anion-exchange matrix retained conjugate products while unconjugated SA was collected in the flow-through. Desired conjugate was eluted by increasing the NaCl concentration in the eluting buffer in a single step to 90 mM. Fractionation and analysis of the collected eluate (not shown) indicates that the fraction of high molecular weight byproduct increases as the elution progresses, so it is possible to obtain conjugate with reduced levels of this contaminant by judicious pooling. Eluate was pooled so that the total quantity of high molecular weight material was no more than 10% of the total conjugate mixture, to maximize yield without adversely affecting in vivo performance of the conjugate. Residual high molecular weight byproduct was washed from the column with 250 mM NaCl. The purity of the conjugate was determined at each step by SEC (Figure 7). Byproducts were also analyzed to ensure that loss of desired conjugate was minimized. The product contained no detectable streptavidin or NR-LU 10 and about 7% high molecular weight byproduct.

In Vitro Characterization. HPLC Analysis/SDS PAGE. SEC retention times of each of the components present in the conjugation mixture are given in Table 1.

Conjugate and SA were analyzed by SDS PAGE under nonboiled conditions to avoid dissociation into monomeric species (Figure 3). SDS-PAGE analysis of crude conjugate showed bands corresponding to unreacted SA, NR-LU-10, and conjugate products. After iminobiotin puri-

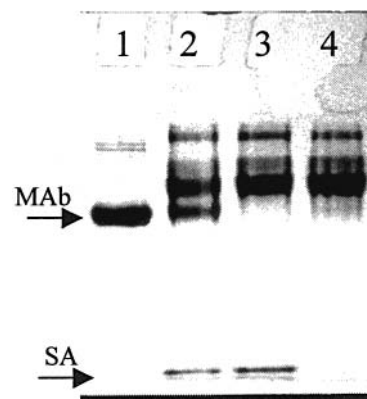


Figure 8. Native 5% PAGE of NR-LU-10 (lane 1), crude NR-LU-10/SA conjugate (lane 2), conjugate after iminobiotin purification (lane 3), conjugate after ion exchange purification (lane 4).

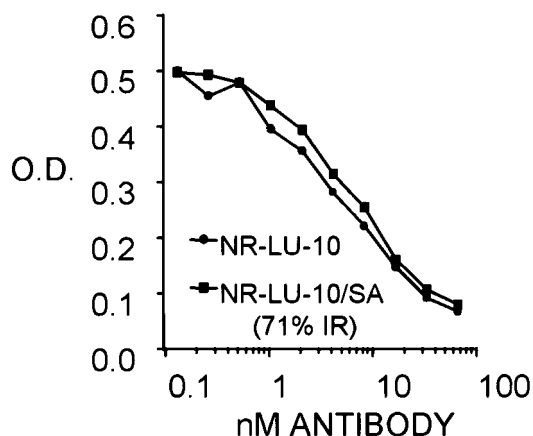


Figure 9. Competitive ELISA of NR-LU-10 vs. NR-LU-10/SA on LS-180 cell extracts.

fication, the IgG band was absent; after anion exchange purification, the SA band was absent leaving desired conjugate and a small percentage of cross-linked conjugates. The band migrating like NR-LU-10 in the final product was produced by the SDS treatment since the corresponding native gel shows only product bands (Figure 8).

Immunoreactivity/Biotin Binding/IEF. Competitive ELISA indicated that NR-LU-10/SA conjugate was 71% as immunoreactive as unmodified NR-LU-10 (Figure 9). Biodistribution studies in tumor-bearing nude mouse xenografts demonstrated the antigen-mediated localization of this conjugate (5, 6). The observed minimal disruption of the antibody's antigen binding capability

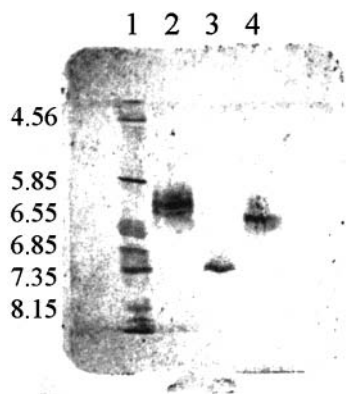


Figure 10. IEF gel (pI 3–9) of markers (lane 1), NR-LU-10 (lane 2), SA (lane 3), and NR-LU-10/SA (lane 4).

indicated that NR-LU-10 was not impaired by derivatization through thiol moieties. This has been the case with most antibodies we have tested. The conjugate bound 4.5 biotins per conjugate molecule as determined using a HABA displacement assay (31), reflecting the retention of biotin binding function of the conjugate, and the portion of conjugate that contains >1 streptavidin moiety per conjugate molecule. Conjugate and starting materials were also analyzed by IEF on a pH 3–9 gel (Figure 10).

Laser Light Scattering Analysis. The molecular weight of purified conjugate eluting at 8.22 min by SEC HPLC was determined using laser light scattering analysis. The 210 kDa value obtained corresponds closely with the theoretical value of 202 kDa. The peak eluting as a front shoulder at 7.57 min had an average molecular weight of 260 kDa which corresponds closely to the theoretical 254 kDa value for the disubstituted conjugate.

DISCUSSION

The conjugation method described here has been guided by the pharmacologic behavior of radiolabeled conjugate and by the Pretarget delivery. Although the degree of heterogeneity with respect to attachment site is not characterized, the molecular weight heterogeneity is inferred from the molecular weight profile determined by static light scattering measurements. Biodistribution studies indicate that the presence of greater than 10% cross-linked material has a measurable effect on pharmacokinetics, while complete elimination of cross-linked material, and even 1:2 conjugate, (at substantial sacrifice in yield), does not improve the observed biodistribution. Hence, the method has been developed to produce the highest possible yield of experimentally suitable conjugate.

Cross-linkers other than SMCC have also been used successfully. The SA-maleimide reacts rapidly with antibody thiols. This conjugation rate can vary with different antibodies, as well as with concentration. For antibodies that react more rapidly, it may be impossible to follow the progress of the conjugation because the time required for two consecutive SEC analyses may be longer than the optimum reaction time, leading to over-reaction and excess cross-linking. In these cases less reactive thiol-selective cross-linkers such as α -halo-carbonyls may be preferable to SMCC. While instability of maleimide-derived thioethers has been reported by other researchers, conjugates prepared in our laboratory have remained apparently unchanged for extended periods at 4 °C and neutral pH.

One factor that directly affects the yield of conjugate is the loading level of maleimide on streptavidin as

described in Results. Lower yields of 1:1 + 1:2 conjugate are also obtained in either of two modified methods. If the antibody is conjugated through thiols synthetically attached by the use of thiolating agents such as iminothiolane or SPDP, the conjugation reaction mixture is more heterogeneous, and the preponderance of 1:1 and 1:2 conjugate is much smaller. Likewise, if the antibody is insufficiently reduced so that less than ~ 8 thiols/Ab are generated, the conjugation reaction proceeds more slowly, and the products are substantially more heterogeneous. These observations suggest that a single thiol on the antibody is substantially more reactive than the others and that this reactive thiol is released from its disulfide more slowly or at greater reducing agent concentration than the others. Hence, if the antibody is only partially reduced, the "reactive" thiol is not available, and the conjugation is a competition between a number of less reactive thiols.

Another key aspect of this conjugation method rests with the ion-exchange purification. Despite repeated attempts in our laboratory, purification by preparative SEC HPLC uniformly results in a dramatic decrease in yield. Determination of suitable ion-exchange conditions for conjugation of a new antibody involves three basic steps. First, pH and ionic strength conditions must be determined at which the conjugate binds to the matrix while streptavidin does not. If such conditions cannot be found, an additional purification step must be incorporated, namely affinity chromatography with Protein A or Protein G, which retains IgG containing species, but not streptavidin. Second, conjugate is bound to the matrix and then eluted with a linear NaCl gradient to identify the NaCl concentration at which conjugate is released from the matrix. Third, a series of step gradient elutions is performed. Both the eluate and the fraction that remains bound (subsequently eluted with 250 mM NaCl) are analyzed by SEC to identify a step gradient that will elute the maximum quantity of 1:1 + 1:2 conjugate with an acceptable level of high molecular weight component.

This conjugation method appears to be adaptable to the conjugation of enzymes and other proteins to antibodies (35). The purification of conjugates by size exclusion chromatography as reported is useful for preparation of small quantities of high quality conjugates. However, optimized ion-exchange procedures combined with affinity chromatography is more applicable to preparation of large quantities of conjugates. The two primary limitations in applying the conjugation method to proteins other than streptavidin are that the protein contain no reactive thiols and that the protein be insensitive (function and structural integrity) to lysine modification.

A number of reports describe methods for preparation of antibody/streptavidin conjugates (9, 11, 12, 19). We find that the method described in this paper is superior because of its reliability, yield, scalability, the quality of conjugate produced, and its adaptability to different antibodies and proteins. The conjugation method described in this paper enabled the preclinical development and optimization of the Pretarget delivery system. It was also used for preparation of conjugate employed in the clinical investigation of the therapeutic modality in hundreds of patients. While the method may be suboptimal for production of a pharmaceutical product because of its expense and the heterogeneity of the conjugate, it has been nonetheless invaluable in evaluating the utility of the Pretarget delivery system and in optimizing it. Less efficient conjugation methods would have been incapable of producing the required quantity and quality of material to fulfill this function.

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A Polar Substituent-Containing Acylation Agent for the Radioiodination of Internalizing Monoclonal Antibodies: *N*-Succinimidyl 4-Guanidinomethyl-3-[¹³¹I]iodobenzoate ([¹³¹I]SGMIB)

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The objective of this study was to develop an acylation agent for the radioiodination of monoclonal antibodies that would maximize retention of the label in tumor cells following receptor- or antigen-mediated internalization. The strategy taken was to add a polar substituent to the labeled aromatic ring to impede transport of labeled catabolites across lysosomal and cell membranes after antibody degradation. Preparation of unlabeled *N*-succinimidyl 4-guanidinomethyl-3-iodobenzoate (SGMIB) was achieved in six steps from 3-iodo-4-methylbenzoic acid. Preparation of 4-guanidinomethyl-3-[¹³¹I]-iodobenzoic acid from the silicon precursor, 4-(*N*¹,*N*²-bis-*tert*-butyloxycarbonyl)guanidinomethyl-3-trimethylsilylbenzoic acid proceeded in less than 5% radiochemical yield. A more successful approach was to prepare [¹³¹I]SGMIB directly from the tin precursor, *N*-succinimidyl 4-(*N*¹,*N*²-bis-*tert*-butyloxycarbonyl)guanidinomethyl-3-trimethylstannylbenzoate, which was achieved in 60–65% radiochemical yield. A rapidly internalizing anti-epidermal growth factor receptor variant III antibody L8A4 was labeled using [¹³¹I]SGMIB in 65% conjugation efficiency and with preservation of immunoreactivity. Paired-label in vitro internalization assays demonstrated that the amount of radioactivity retained in cells after internalization for L8A4 labeled with [¹³¹I]SGMIB was 3–4-fold higher than that for L8A4 labeled with ¹²⁵I using either Iodogen or [¹²⁵I]SIPC. Catabolite assays documented that the increased retention of radioiodine in tumor cells for antibody labeled using [¹³¹I]-SGMIB was due to positively charged, low molecular weight species. These results suggest that [¹³¹I]-SGMIB warrants further evaluation as a reagent for labeling internalizing antibodies.

INTRODUCTION

Monoclonal antibodies (mAb¹) that bind to cell membrane associated molecules are often internalized into the cell, thereby exposing the mAb to intracellular catabolic processes including lysosomal proteolysis. For radiolabeled mAbs, intracellular processing can result in loss of label from the target cell, with the magnitude of this effect dependent on the nature of the radionuclide and the labeling method (Govindan et al., 1999; Kobayashi et al., 2000; Reist et al., 1999). After lysosomal catabolism, radioactivity from radiometalated mAbs is generally trapped in the cell in contrast to activity from directly

radioiodinated mAbs which escapes from the cell in the form of monoiodotyrosine (Press et al., 1996).

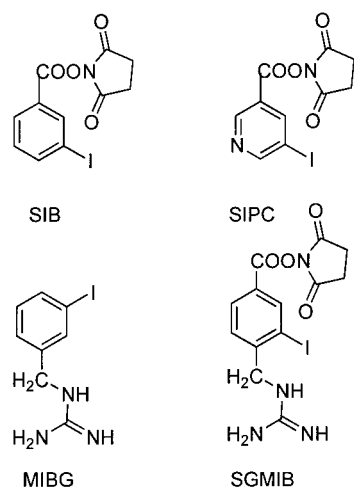
To surmount this problem, residualizing labeling methods have been pursued for protein radioiodination that involve labeled templates that are inert to lysosomal digestion and not transported across lysosomal or cell membranes. The first generation of such labels contained nonmetabolizable carbohydrate residues as their main feature, for example, tyramine–cellobiose (TCB) and inulin–tyramine (Ali et al., 1990; Maxwell et al., 1988; Pittman et al., 1983; Thorpe et al., 1993). Although these reagents enhanced cellular retention of radioiodinated proteins after internalization, this approach also results in considerable protein cross-linking, diminished immunoreactivity, and in many cases, poor protein conjugation efficiencies (Ali et al., 1988; Moerlein et al., 1988; Schilling et al., 1992; Reist et al., 1995; Stein et al., 1995). Because mAb–DTPA–radiometal conjugates exhibit good retention of the radiometal in tumor cells (Kobayashi et al., 2000), Govindan et al. (1999) developed an acylation agent consisting of DTPA coupled to a radioiodinated, proteolytically inert, D-amino acid peptide. An increase of up to 3-fold in cellular retention of radioiodine in vitro was achieved for two internalizing mAbs labeled using this method in comparison to those labeled using Chloramine-T.

Another strategy for residualization of activity within tumor cells has attempted to exploit the fact that exocytosis of catabolites by passive transport across the membrane can be minimized if they are charged species.

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¹ Abbreviations: Boc, *tert*-butyloxycarbonyl; DTPA, diethylenetriamine pentaacetic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; EGFRvIII, epidermal growth factor receptor variant III; FIAU, 5-[¹³¹I]iodo-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)uracil; GMIBA, 4-guanidinomethyl-3-iodobenzoic acid; IgG, immunoglobulin; INA, 5-iodonicotinic acid; IUdR, 5-iodo-2'-deoxyuridine; mAb, monoclonal antibody; MES, 2-(*N*-morpholino)ethanesulfonic acid; MIBG, *m*-iodobenzylguanidine; PMSF, phenylmethylsulfonylfluoride; SGMIB, *N*-succinimidyl 4-guanidinomethyl-3-iodobenzoate; SIB, *N*-succinimidyl 3-iodobenzoate; SIPC, *N*-succinimidyl 5-iodopyridine-3-carboxylate; TCA, trichloroacetic acid; TCB, tyramine cellobiose; TFA, trifluoroacetic acid; TMSE, 2-(trimethylsilyl)ethyl.

Chart 1



Lysosomal pH is ~ 5 , and molecules containing basic functionalities would be protonated under these conditions and thus should be trapped within the cells. The feasibility of increasing the tumor retention of mAbs by labeling them with positively charged templates has been demonstrated (Reist et al., 1996; 1997). The acylation agent, *N*-succinimidyl 5-[¹³¹I]iodopyridine-3-carboxylate ([¹³¹I]SIPC; Chart 1; Garg et al., 1991; 1993), contains a basic nitrogen in its structure and was shown to be protonated at lysosomal pH. Indeed, the intracellular retention of a mAb labeled with SIPC increased up to 65% in vitro, compared with the same mAb radioiodinated using Iodogen or *N*-succinimidyl 3-[¹³¹I]iodobenzoate; however, the advantage was only observed at time points less than 24 h ([¹³¹I]SIB, Reist et al., 1996). Similar differences were seen in vivo, but the enhanced tumor retention associated with SIPC labeling was again a transient effect.

Guanidines have a pK_a of about 13 and thus a substantially higher proportion of guanidine-substituted molecules should be protonated than those with most other basic moieties such as the pyridine nitrogen present in SIPC. On this basis, we hypothesized that higher and more prolonged tumor retention might be achievable if the radioactive catabolites of an internalizing mAb contained a guanidine function. Herein we describe the synthesis of *N*-succinimidyl 4-guanidinomethyl-3-[¹³¹I]iodobenzoate ([¹³¹I]SGMIB), derived from the attachment of a guanidinomethyl group to SIB. Its use for labeling mAb L8A4, which rapidly internalizes after binding to the mutant epidermal growth factor receptor variant III (EGFRvIII; Wikstrand et al., 1995), is also described.

MATERIALS AND METHODS

General. All chemicals were purchased from Aldrich unless otherwise noted. *N*-Succinimidyl 5-iodopyridine-3-carboxylate and 5-iodonicotinic acid were prepared as reported before (Garg et al. 1991; Vaidyanathan et al., 2000). Sodium [¹²⁵I]iodide and sodium [¹³¹I]iodide with specific activities of 2200 Ci/mmol and 1200 Ci/mmol, respectively, were obtained from DuPont-New England Nuclear. Radioiodinated SIPC was prepared according to previously published procedures (Garg et al. 1991).

Melting points were determined on a Haake Buchler apparatus and were uncorrected. High-pressure liquid chromatography was performed using one of two systems: (1) a Beckman System Gold HPLC equipped with a Model 126 programable solvent module, a Model 168 diode array detector, a Model 170 radioisotope detector,

and a Model 406 analog interface module; or (2) a Perkin-Elmer Series 4 Liquid Chromatograph connected to a Perkin-Elmer LC-95 UV/visible spectrophotometer detector and a Perkin-Elmer LCI-100 Laboratory Computing Integrator. Methods were programmed using a Perkin-Elmer 6312 display terminal. For reversed-phase chromatography, a Waters μ Bondapak C18 (10 μ m, 3.9 $\times$ 300 mm) column or a 3.9 $\times$ 300 mm Bondclone 10 (10 μ m) C18 column (Phenomenex, Torrance, CA) was used. Normal-phase HPLC was done on a 4.6 $\times$ 250 mm Partisil (10 μ m) silica column (Alltech, Deerfield, IL). Size-exclusion HPLC was performed using a 7.5 mm $\times$ 60 cm Tosohaas TSKgel G3000 SW (10 μ m) gel filtration column (Montgomeryville, PA) eluted with PBS at 1 mL/min. Aluminum-backed sheets (Silica gel 60 F₂₅₄) were used for analytical TLC, and normal-phase column chromatography was performed using silica gel 60, both obtained from EM Science (Gibbstown, NJ). Column chromatographic fractions were collected using a Gilson model 203 micro fraction collector (Middleton, WI) or an ISCO Foxy 200 fraction collector (Lincoln, NE), and the products were identified by TLC. In some cases, an ISCO UA-6 UV-vis detector was placed between the column outlet and the fraction collector to identify products. Preparative thick layer chromatography was performed using 20 $\times$ 20 cm, 1000 μ m plates (Whatman, Clifton, NJ). Before applying the sample, the plates were run in ethyl acetate to remove any adsorbed impurities. Radio-TLC was initially analyzed using a System 200 Imaging Scanner (BioScan, Washington, DC); sheets were then cut into strips and counted using an automated gamma counter (LKB 1282, Wallac, Finland). Proton NMR spectra (300 MHz) were obtained on a Varian Mercury 300 spectrometer or a General Electric Midfield GN-300 spectrometer. Chemical shifts are reported in δ units; solvent peaks were referenced appropriately. Mass spectra were obtained on a Hewlett-Packard GC/MS/DS Model HP-5988A instrument or on a JEOL SX-102 high-resolution mass spectrometer. Elemental analyses were provided by Galbraith Laboratories (Knoxville, TN).

Cells, mAb, and Culture Conditions. Murine IgG₁ L8A4 is a mAb which reacts specifically with the 145 kDa deletion mutant EGFRvIII molecule found on multiple human malignancies including gliomas and breast carcinomas (Wikstrand et al., 1995). The EGFRvIII-expressing cell line used for these studies was U87 MG/EGFR which was established from U87 MG cells by transfection with EGFRvIII cDNA (Nishikawa et al., 1994). In cell culture, this line expresses an average of 4–13 $\times$ 10⁵ EGFRvIII molecules per cell (Reist et al., 1997). Cells were grown in zinc option media (Life Technologies, Inc., Grand Island, NY) containing 10% fetal calf serum and Geneticin sulfate (600 mg/mL).

2-(Trimethylsilyl)ethyl (3-Iodo-4-methyl)benzoate (1). To a solution of 3-iodo-4-methylbenzoic acid (1.17 g, 4.5 mmol) in dry ethyl acetate (20 mL) at room temperature were added DMAP (34 mg, 0.3 mmol), 2-trimethylsilyl ethanol (990 mg; 8.4 mmol), and DCC (1.65 g; 8.2 mmol) in that order. The mixture was stirred at room temperature overnight, adsorbed on silica gel, and chromatographed (0–5% EtOAc/hexane) to yield 1.22 g (74%) of an oil: ¹H NMR (CDCl₃): δ 0.08 (s, 9H), 1.13 (m, 2H), 2.48 (s, 3H), 4.40 (m, 2H), 7.29 (d, 1H), 7.95 (d, 1H), 8.46 (s, 1H). MS (FAB⁺), m/z : 362 (M⁺).

2-(Trimethylsilyl)ethyl (3-Iodo-4-bromomethyl)benzoate (2). A mixture of **1** (970 mg, 2.7 mmol), NBS (477 mg; 2.7 mmol), and benzoyl peroxide (5–6 mg) in dry CCl₄ (15 mL) was refluxed for 3–4 h. The mixture was cooled to room temperature, the precipitate was

filtered off, and CCl_4 was evaporated from the filtrate. Column chromatography using hexane followed by 1% EtOAc in hexane afforded 400 mg (34%) of an oil. ^1H NMR (CDCl_3): δ 0.08 (s, 9H), 1.13 (m, 2H), 4.41 (m, 2H), 4.60 (s, 2H), 7.52 (d, 1H), 7.96 (d, 1H), 8.50 (s, 1H). MS (CI), m/z : 458 and 460 ($\text{M} + \text{NH}_4^+$), 413 and 415, 378, 362. This compound was used in the next step without extensive purification.

2-(Trimethylsilyl)ethyl 4-[N^1,N^2 -Bis(*tert*-butyloxy-carbonyl)guanidinomethyl]-3-iodobenzoate (3). The title compound was prepared from **2** as reported before (Vaidyanathan and Zalutsky, 1997). ^1H NMR (CDCl_3) δ 0.08 (s, 9H), 1.14 (t, 2H), 1.25 (s, 9H), 1.47 (s, 9H), 4.41 (t, 2H), 5.22 (s, 2H), 7.05 (d, 1H), 7.95 (d, 1H), 8.45 (s, 1H), 9.45 (br s, 2H). HRMS (FAB $^+$) Calcd for $\text{C}_{24}\text{H}_{39}\text{IN}_3\text{O}_6\text{Si}$ (MH^+): 620.1653. Found: 620.1649. Anal. Calcd for $\text{C}_{24}\text{H}_{38}\text{IN}_3\text{O}_6\text{Si}$: C, 46.53; H, 6.18; N, 6.78. Found: C, 46.66; H, 6.25; N, 6.76.

4-[N^1,N^2 -Bis(*tert*-butyloxycarbonyl)guanidinomethyl]-3-iodobenzoic Acid (4). To a solution of **3** (160 mg; 0.26 mmol) in dry DMF (5 mL, Pierce) was added TBAF (1 M in THF, 0.36 mL), and the solution was stirred at room-temperature overnight. The reaction mixture was partitioned between EtOAc and water, and the crude product was purified by preparative TLC using 50% EtOAc/hexane to afford 26 mg (20%) of an off-white solid: mp 133–135 °C. ^1H NMR (CDCl_3) δ 1.24 (s, 9H), 1.50 (s, 9H), 5.16 (s, 2H), 6.93 (d, 1H), 7.76 (d, 1H), 8.22 (s, 1H), 9.5 (br d, 2H). MS (FAB $^+$), m/z : 520 (MH^+), 464, 406.

4-Guanidinomethyl-3-iodobenzoic Acid (5). To 14.1 mg (27 μmol) of **4** in 1 mL of EtOAc at room temperature was added 45–50 mg of SnCl_4 (Miel and Rault, 1997), and the solution was stirred for 3–4 h. Ethyl acetate was removed under aspirator vacuum using a rotary evaporator, and the residue was further subjected to rotary evaporation under high vacuum. The residue was triturated with ether to get an oil in almost quantitative yield: ^1H NMR (CD_3OD) δ 4.48 (s, 2H), 7.45 (d, 1H), 8.20 (dd, 1H), 8.51 (d, 1H). MS (ESI), m/z : 359 ($\text{MH} + \text{K}^+$), 320 (MH^+), 261. HRMS (FAB $^+$) Calcd for $\text{C}_9\text{H}_{11}\text{IN}_3\text{O}_2$ (MH^+): 319.9896. Found: 319.9895 $\pm$ 0.0014 ($n = 2$).

***N*-Succinimidyl 4-[N^1,N^2 -Bis(*tert*-butyloxycarbonyl)guanidinomethyl]-3-iodobenzoate (6).** To a mixture of **4** (117 mg, 0.23 mmol) and *N*-hydroxysuccinimide (34 mg, 0.3 mmol) in dry THF (5 mL) was added DCC (47 mg; 0.23 mmol), and the mixture was stirred at room-temperature overnight. The precipitated DCU was filtered, and the filtrate concentrated. The residue was subjected to preparative TLC using 25% EtOAc/hexane to yield 75 mg (54%) of a solid: mp 225–230 °C (decomp). ^1H NMR (CD_3OD) δ 1.30 (s, 9H), 1.47 (s, 9H), 2.92 (s, 4H), 5.25 (s, 2H), 7.09 (d, 1H), 8.05 (d, 1H), 8.59 (s, 1H), 9.44 (br d, 2H). MS (ES), m/z : 617 (MH^+), 561, 505. HRMS (FAB $^+$) Calcd for $\text{C}_{23}\text{H}_{30}\text{IN}_4\text{O}_8$ (MH^+): 617.1108. Found: 617.1100 $\pm$ 0.0025 ($n = 2$).

***N*-Succinimidyl 4-Guanidinomethyl-3-iodobenzoate (7).** A sample of this compound was prepared for use as an HPLC standard by the treatment of **6** (about 5 mg) with TFA (0.1 mL) at room temperature for 5–10 min. TFA was evaporated, and the residue was triturated with ethyl acetate: ^1H NMR (CD_3CN) δ 2.86 (s, 4H), 4.40 (d, 2H), 7.48 (d, 1H), 8.19 (d, 1H), 8.82 (s, 1H). MS (ES), m/z : 417 (MH^+).

3-Methyl-4-(trimethylsilyl)benzoic Acid (8). To a solution of 3-bromo-4-methylbenzoic acid (1.26 g, 5.9 mmol) in dry THF (30 mL) at -78 °C was added dropwise a solution of *n*-BuLi in hexanes (6.9 mL, of 1.6 M, 11 mmol), and the mixture was stirred for 15 min at -78

°C. Chlorotrimethylsilane (1.79 g, 16.6 mmol) was added gradually to the above mixture. The reaction mixture was gradually warmed and was stirred overnight at room temperature. The THF was evaporated, and the residue was partitioned between EtOAc and water. The crude product was chromatographed using a stepwise gradient of 10–50% EtOAc/hexane to yield 340 mg of a crystalline solid: mp 134–136 °C. ^1H NMR (CDCl_3) δ 0.37 (s, 9H), 2.53 (s, 3H), 7.25 (d, 1H), 7.98 (d, 1H), 8.20 (s, 1H). MS (CI), m/z : 226 ($\text{M} + \text{NH}_4^+$), 209 (MH^+). Anal. Calcd for $\text{C}_{11}\text{H}_{16}\text{O}_2\text{Si}$: C, 63.42; H, 7.74. Found: C, 64.08; H, 7.75.

2-(Trimethylsilyl)ethyl (4-Methyl-3-trimethylsilyl)benzoate (9). The title compound was prepared from **8** (433 mg, 2 mmol) by a procedure similar to that used for the preparation of **1**, affording 335 mg (52%) of an oil: ^1H NMR (CDCl_3) δ 0.09 (s, 9H), 0.35 (s, 9H), 1.13 (m, 2H), 2.50 (s, 3H), 4.41 (m, 2H), 7.21 (d, 1H), 7.92 (d, 1H), 8.12 (s, 1H). This compound was used in the next step without extensive purification.

2-(Trimethylsilyl)ethyl (4-Bromomethyl-3-trimethylsilyl)benzoate (10). Compound **10** was prepared from **9** (319 mg, ~ 1 mmol) following a procedure similar to that used for the preparation of **2** yielding 195 mg (48%) of an oil: ^1H NMR (CDCl_3) δ 0.09 (s, 9H), 0.35 (s, 9H), 1.13 (m, 2H), 4.42 (m, 2H), 4.63 (s, 2H), 7.52 (d, 1H), 8.02 (d, 1H), 8.18 (s, 1H). MS (CI), m/z : 404 and 406 ($\text{M} + \text{NH}_4^+$), 386, 388 (MH^+), 376 and 378, 361 and 363. Anal. Calcd for $\text{C}_{16}\text{H}_{27}\text{BrO}_2\text{Si}_2$: C, 49.60; H, 7.02. Found: C, 49.00; H, 6.94.

2-(Trimethylsilyl)ethyl 4-[N^1,N^2 -Bis(*tert*-butyloxy-carbonyl)guanidinomethyl]-3-(trimethylsilyl)benzoate (11). This compound was prepared from **10** as reported before (Vaidyanathan and Zalutsky, 1997). ^1H NMR (CDCl_3) δ 0.08 (s, 9H), 0.40 (s, 9H), 1.14 (t, 2H), 1.26 (s, 9H), 1.41 (s, 9H), 4.41 (t, 2H), 5.37 (s, 2H), 7.05 (d, 1H), 7.95 (d, 1H), 8.18 (s, 1H), 9.45 (br s, 2H). HRMS (FAB $^+$) Calcd for $\text{C}_{27}\text{H}_{49}\text{N}_3\text{O}_6\text{Si}_2$ (MH^+): 566.3082. Found: 566.3069. Anal. Calcd for $\text{C}_{27}\text{H}_{47}\text{N}_3\text{O}_6\text{Si}_2$: C, 57.31; H, 8.37; N, 7.42. Found: C, 57.23; H, 8.33; N, 7.28.

4-[N^1,N^2 -Bis(*tert*-butyloxycarbonyl)guanidinomethyl]-3-(trimethylsilyl)benzoic Acid (12). THF was evaporated from a solution of TBAF (0.7 mL of 1 mM), and the residue was dried under vacuum. A solution of **11** (367 mg, 0.65 mmol) in DMF (10 mL) was added to the above, and the resultant solution was stirred overnight at room temperature. The reaction mixture was partitioned between water and EtOAc, and the pooled EtOAc layer was washed, dried, and concentrated. Chromatography of the crude product using a stepwise gradient of 30% EtOAc/hexane to 100% EtOAc yielded 170 mg (56%) of a solid: mp 223–228 °C (decomp). ^1H NMR (CDCl_3) δ 0.41 (s, 9H), 1.27 (s, 9H), 1.42 (s, 9H), 5.38 (s, 2H), 7.05 (d, 1H), 7.95 (d, 1H), 8.17 (s, 1H), 9.45 (br s, 2H). MS (FAB $^+$), m/z : 466 (MH^+). HRMS (FAB $^+$) Calcd for $\text{C}_{22}\text{H}_{36}\text{N}_3\text{O}_6\text{Si}$ (MH^+): 466.2373. Found: 466.2363.

2-(Trimethylsilyl)ethyl 4-[N^1,N^2 -Bis(*tert*-butyloxy-carbonyl)guanidinomethyl]-3-(trimethylstannyl)benzoate (13). A mixture of **3** (787 mg, 1.3 mmol), hexamethylditin (2.1 g, 6.4 mmol), and $(\text{Ph}_3\text{P})_2\text{PdCl}_2$ (425 mg, 0.6 mmol) in 35 mL of dry dioxane was heated under reflux for 1 h. The dark slurry was filtered through a bed of Celite, and the filtrate was concentrated. The residue was initially subjected to column chromatography using 0–10% EtOAc/hexane to obtain 211 mg of an oil. This was further purified by preparative TLC using 8% EtOAc/hexane to obtain 83 mg (10%) of a clear oil: ^1H NMR (CDCl_3) δ 0.07 (s, 9H), 0.40 (s, 9H [$^{119}\text{Sn}-\text{H}$, d]), 1.16 (m, 2H), 1.25 (s, 9H), 1.45 (s, 9H), 4.42 (m, 2H), 5.24 (s, 2H), 7.02 (d, 1H), 7.94 (d, 1H), 8.10 (s, 1H), 9.46 (br s,

2H). MS (FAB⁺), *m/z*: cluster peaks at 658 (MH⁺) and several others.

4-[N¹,N²-Bis(*tert*-butyloxycarbonyl)guanidinomethyl]-3-(trimethylstannyl)benzoic Acid (14**).** The solvent was evaporated from 0.3 mL of a 1 M solution of TBAF in THF, and the residue was dried using a vacuum pump for several hours. The TBAF residue was redissolved in 0.3 mL of dry DMSO, and the resultant solution was added to a vial containing 78 mg (0.12 mmol) of **13**. The reaction mixture was stirred overnight at room temperature. Aqueous workup and preparative TLC of the crude product using EtOAc gave 30 mg (45%) of a solid: mp 162–165 °C (decomp). ¹H NMR (CDCl₃) δ 0.42 (s, 9H [¹¹⁹Sn–H, d]), 1.26 (s, 9H), 1.44 (s, 9H), 5.28 (s, 2H), 7.06 (d, 1H), 7.95 (d, 1H), 8.11 (s, 1H), 9.42 (br s, 2H). MS (FAB⁺), *m/z*: cluster peaks at 558 (MH⁺), 502, 356. HRMS (FAB⁺) Calcd for C₂₂H₃₆N₃O₆¹¹⁸Sn (MH⁺): 556.1620. Found: 556.1643.

N-Succinimidyl 4-[N¹,N²-Bis(*tert*-butyloxycarbonyl)guanidinomethyl]-3-(trimethylstannyl)benzoate (15**).** A mixture of **14** (22 mg, 39 μmol), *N*-hydroxy-succinimide (12 mg, 0.1 mmol), and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (39 mg, 0.1 mmol) in 0.5 mL of dry THF was stirred at room temperature for 5–6 h. The reaction mixture was filtered, and the crude mixture obtained from concentrating the filtrate was chromatographed (preparative TLC) using 35% EtOAc/hexane to yield 23 mg (90%) of a colorless oil: ¹H NMR (CDCl₃) δ 0.40 (s, 9H [¹¹⁹Sn–H, d]), 1.29 (s, 9H), 1.42 (s, 9H), 2.90 (s, 4H), 5.28 (s, 2H), 7.08 (d, 1H), 8.03 (d, 1H), 8.17 (s, 1H), 9.42 (br d, 2H). MS (FAB⁺), *m/z*: cluster peaks at 655 (MH⁺), 455. HRMS (FAB⁺) Calcd for C₂₆H₃₉N₄O₈¹²⁰Sn (MH⁺): 655.1790. Found: 655.1787 ± 0.0019 (*n* = 4).

Unlabeled Glycine and Lysine Conjugates of Potential Acid Metabolites. These conjugates were prepared on a small scale for use as HPLC standards. The products were isolated by semipreparative reversed-phase HPLC and were characterized by mass spectrometry. For HPLC, a C18 column (Bondclone) was eluted with the following gradient consisting of 0.2% (v/v) HOAc in water (A) and 0.2% (v/v) HOAc in methanol (B) at 1 mL/min: 5% B for 15 min, 70% B for 10 min, and then held at 70% B for 5 min. Radioiodinated forms of these potential catabolites were also prepared similarly from the corresponding labeled esters and had the same retention times as the unlabeled compounds.

Lysine Conjugate of 4-Guanidinomethyl-3-iodobenzoic Acid. To a solution of **6** (3.3 mg, 5.4 μmol) in 60 μL of THF was added a solution of *N*-α-Boc-lysine in 0.1 M borate buffer, pH 8.5 (2 mg in 5 μL, 8.1 μmol). The mixture was stirred overnight at room temperature. THF was evaporated, and the residue was treated at room temperature for 10 min with 100 μL of TFA. TFA was evaporated, and the residue was triturated with methanol. Finally, portions of a solution of the residual mixture were injected onto HPLC and the product (*t*_R = 14 min) was isolated. MS (FAB⁺), *m/z*: 448 (MH⁺), 390, 330.

Glycine Conjugate of 4-Guanidinomethyl-3-iodobenzoic Acid. To 1.8 mg (4.4 μmol) of **7** was added 25 μL of THF followed by a solution of glycine in 0.1 M borate, pH 8.5 (0.6 mg, 8 μmol). The hazy reaction mixture was stirred overnight at room temperature, and the product was isolated by reversed-phase HPLC (*t*_R = 22 min). MS (FAB⁺), *m/z*: 379 (MH⁺).

Lysine Conjugate of 5-Iodonitrocinic Acid. A solution of SIPC (5.6 mg, 16 μmol) in 90 μL of THF was treated at room temperature with 10 μL of a solution of *N*-α-Boc-lysine in 0.1 M borate buffer, pH 8.5 (4 mg, 8.1

μmol), and the mixture was stirred overnight at room temperature. Deprotection with TFA and HPLC purification were accomplished as described above (*t*_R = 27.6 min). MS (FAB⁺), *m/z*: 378 (MH⁺).

Glycine Conjugate of 5-Iodonitrocinic Acid. Glycine in borate buffer, pH 8.5 (2.5 mg in 50 μL, 33 μmol) was added to a solution of SIPC (5 mg, 14.4 μmol) in 90 μL of THF, and the mixture was stirred at room temperature overnight. The product was isolated by semipreparative HPLC as above (*t*_R = 26.3 min). MS (FAB⁺), *m/z*: 307 (MH⁺).

4-Guanidinomethyl-3-[¹³¹I]iodobenzoic Acid ([¹³¹I]5**).** (A) From **12**. The radioiodination of **12** to give [¹³¹I]**5** was attempted following conditions used for the preparation of [¹³¹I]MIBG from its silicon precursor (Vaidyanathan and Zalutsky, 1993a). To 100–200 μCi of ¹³¹I in 1–2 μL of 0.1 N NaOH in a Reacti vial was added a solution of NCS in TFA (0.4 mg in 10 μL) followed by 0.2 mg of **12** in 5 μL of TFA. The vial was vortexed, and the reaction was allowed to proceed for 5 min at room temperature. The mixture was injected onto a C18 column (Bondapak) and eluted with 90:10 water/acetonitrile each containing 0.1% TFA (w/v) at a flow rate of 1 mL/min. Less than 5% of the injected activity eluted with a retention time (14–15 min) corresponding to that of **5**.

(B) From **14**. A solution of **14** in CHCl₃ (~0.3 mg in 10 μL) was added to a 1/2-dram vial, and the CHCl₃ was evaporated using argon. To this was added about 0.5 mCi of ¹³¹I in 1 μL of 0.1 N NaOH followed by a solution of NCS in HOAc (0.4 mg in 20 μL). The mixture was heated in an oil bath at 70 °C for 10 min, 5 μL of TFA was added, and the mixture was heated for an additional 5 min. The contents of the vial were injected onto a C18 HPLC column (Bondapak) and eluted with a gradient of 0.1% TFA (w/v) in water (A) and acetonitrile (B) at 1 mL/min. The gradient had an initial composition of 5% B for 5 min, and the percentage of B was increased linearly to 90% over 15 min and maintained at that value for another 5 min. More than 95% of the injected radioactivity was associated with the only radioactive peak observed, which had a retention time (15.5 min) corresponding to that of **5**. The retention times of **4** and **14** under these HPLC conditions were 22.4 and 20.7 min, respectively.

N-Succinimidyl 4-[N¹,N²-Bis(*tert*-butyloxycarbonyl)guanidinomethyl]-3-[¹³¹I]iodobenzoate ([¹³¹I]6**).** To a Reacti vial containing 1–2 mCi of ¹³¹I in 1–3 μL of 0.1 N NaOH was added a solution of 3% (v/v) HOAc in CHCl₃ (3 μL), 30% (w/v) *tert*-butyl hydroperoxide in CHCl₃ (5 μL, dried with a few crystals of Na₂SO₄), and a solution of **15** in CHCl₃ (0.2 mg in 15 μL). The reaction mixture was stirred at room temperature for 30 min. The product [¹³¹I]**6** (*t*_R = 16 min) was isolated in 55–60% radiochemical yield by normal-phase HPLC using a Partisil column eluted with 30/70 (v/v) EtOAc/hexane containing 0.12% (v/v) HOAc at 1 mL/min.

N-Succinimidyl 4-Guanidinomethyl-3-[¹³¹I]iodobenzoate ([¹³¹I]7**).** The solvents from HPLC fractions containing [¹³¹I]**6** were evaporated, and the residual activity was transferred to a 1/2-dram vial and further concentrated to dryness. TFA (50–100 μL) was added to the above vial, and the reaction was allowed to proceed for 5 min at room temperature. TFA was evaporated, and the residue was triturated 3 times with EtOAc. This activity was used as such for mAb labeling.

Radiolabeling of L8A4 mAb. (A) *Iodogen Method.* This was performed as described previously (Reist et al., 1996). To a 1/2-dram vial coated with 10 μg of Iodogen was added a solution of L8A4 in phosphate buffer, pH

7.14 (100 μ L, $\sim$ 2 mg/mL), and 1–2 μ L of ^{131} I (0.2–0.5 mCi) in 0.1 N NaOH. The mixture was incubated at room temperature for 20 min and the labeled mAb was isolated by gel filtration over a PD10 column (Pharmacia) eluted with PBS.

(B) *Conjugation of 4-Guanidinomethyl-3-[^{131} I]iodobenzoic Acid Using EDC.* A 200 mM solution of EDC in 100 mM MES buffered saline pH 4.7 (Pierce) was prepared. To 0.5–1.0 mCi of [^{131} I]**5** in a $1/2$ -dram vial was added 5 μ L of the EDC solution followed by 5 μ L of sulfo *N*-hydroxysuccinimide (20 mM in MES). The vial was vortexed, and the reaction was allowed to proceed for 5 min at room temperature. Unreacted EDC was quenched by adding 5 μ L of 750 mM sodium acetate, and 50 μ L of L8A4 (375 mg) in pH 8.5, 0.1 M borate buffer was added. The mixture was incubated for 30 min at room temperature, and the labeled mAb was isolated by gel filtration chromatography as described above.

(C) *Acylation with N-Succinimidyl 5-[^{125} I]iodo-pyridine-3-carboxylate, N-Succinimidyl 4-[N^1,N^2 -Bis(*tert*-butoxy-carbonyl)guanidinomethyl]-3-[^{131} I]iodobenzoate or N-Succinimidyl 4-Guanidinomethyl-3-[^{131} I]iodobenzoate.* A solution of L8A4 in 0.1 M borate buffer pH 8.5 (50 μ L, 3 mg/mL) was added to about 1 mCi of [^{125} I]SIPC, [^{131} I]**6**, or [^{131} I]**7**, respectively, and the mixture was incubated for 20 min at room temperature. Labeled proteins were isolated using a PD10 column.

Evaluation of Immunoreactivity. The immunoreactivity of radiolabeled L8A4 was evaluated using a magnetic bead assay using beads coated with the extracellular domain of EGFRvIII or to control for nonspecific binding, BSA, as described (Foulon et al., 2000). Assays were done in paired-label format using two mAb preparations, one labeled with ^{125} I (SIPC or Iodogen) and the other with ^{131} I (GMIBA-EDC, SGMIB–Boc, or SGMIB). To increasing volumes (12.5, 25, and 50 μ L) of both positive- and negative-control beads in triplicate were added 5 ng (10 μ L) each of the two labeled mAbs. The immunoreactive fractions were calculated by plotting the reciprocals of the percent specific binding against the reciprocals of the bead volumes (Lindmo et al., 1984).

Internalization Assays. These assays were performed in either a single- or paired-label format using U87 MGDEGFR plated at a density of 5×10^5 cells per well in 3 mL. After an overnight incubation at 37 °C, the cells were allowed to equilibrate at 4 °C for 30 min. The medium was removed and fresh medium containing about 1 μ Ci (0.5–1.0 μ g, in mAb excess) of each labeled L8A4 preparation in 2 mL was added, and the cells were incubated for 1 h at 4 °C. Cells were washed with cold medium to remove unbound activity, and fresh zinc option medium supplemented with 10% fetal calf serum was added. Cells were brought to 37 °C and processed in triplicate at 0, 1, 2, 4, 8, 16, and 24 h. Cells were centrifuged, and the supernatants were removed. The surface-bound activity was removed by washing the cells twice with pH 2 zinc option medium. Finally, the cells were harvested and solubilized in 0.5 mL of 0.5 N NaOH. The supernatants, acid washes, and the cell pellets were all counted in an automated gamma counter. The number of counts in each compartment (released, cell surface, internalized) were expressed as the percentage of the activity initially bound to the cells immediately after the 1 h incubation at 4 °C.

Catabolism of L8A4 Labeled with [^{125} I]SIPC and [^{131} I]SGMIB by U87 MGDEGFR Cells. To determine the nature of the labeled catabolites present inside the cell and in the cell culture supernatant, an internalization assay was performed as above using 3–5 μ Ci of each

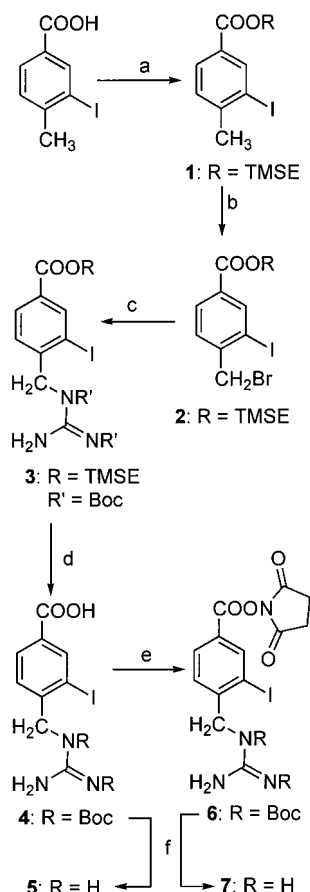
labeled mAb. After removing the surface-bound activity with acid washing, the cell pellets were lysed with 200 μ L of 0.5% NP40 in PBS containing aprotinin (0.02 mg/mL), leupeptin (0.05 mg/mL), PMSF (4 mM), and pepstatin (0.07 mg/mL) for 10 min at room temperature. The membranes were pelleted by centrifugation, and the lysates were isolated.

High molecular weight components in lysates and cell culture supernatants were analyzed by size-exclusion HPLC using a TSK column eluted with 0.1 M sodium phosphate, 0.05% sodium azide, pH 6.8 at 1 mL/min. Fractions of 0.25 mL were collected and counted for ^{125} I and ^{131} I in an automated gamma counter, and the activity was plotted versus time. For comparison, gel filtration standards (BioRad) were also run under identical conditions. To determine the low molecular weight catabolites, cell lysates and supernatants at each time point were filtered through Centricon-10 tubes (Amicon; 10,000 MW cut off). The filtrates and the cartridges were counted for radioactivity, prior to HPLC analyses of the filtrates, to determine the proportion of low and high molecular weight component at each time point. Filtrates were analyzed by reversed-phase chromatography using a C18 column (Bondclone) eluted with a mobile phase gradient consisting of 0.2% HOAc each in water (solvent A) and methanol (solvent B) at a flow rate of 1 mL/min. The mobile phase was maintained initially at 5% B for 15 min and then increased linearly to 70% B over 10 min, and then kept at that composition for another 5 min. Fractions of 0.25 mL were collected and counted for radioactivity. Under these HPLC conditions, the retention times of INA, INA-glycine, INA-lysine, GMIBA, GMIBA-glycine, GMIBA-lysine were 29.9, 27.5, 26.5, 23.0, 22.0, and 14.0 min, respectively.

Octanol–Water Partition Coefficient. [^{125} I]INA was prepared by incubating [^{125} I]SIPC (50–100 μ Ci) with 1 mL of 1 N NaOH at room temperature for 1 h. This mixture was extracted twice with 1 mL of EtOAc to remove any unreacted ester. The pH of the aqueous layer was adjusted to about 6 with 1 N HCl, and [^{125} I]INA was extracted with EtOAc. The partition coefficient was determined at both lysosomal and physiological pH using PBS at pH 4.75 and 7.4, respectively. The buffer and octanol were each saturated with the other before use. A few thousand counts each of [^{125} I]INA and [^{131} I]GMIBA in 5–10 μ L PBS were added in triplicate to a mixture of 1 mL each of octanol and buffer, and the contents were thoroughly mixed by vortexing for a few minutes. The layers were separated by centrifugation. A 50 μ L aliquot of each layer in duplicate was withdrawn from each tube and counted for radioactivity, and the ratios of activities in octanol and buffer were calculated.

RESULTS AND DISCUSSION

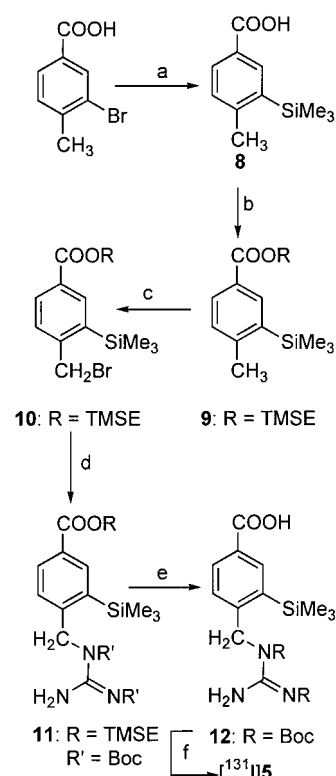
Perhaps the most widely known radiopharmaceutical bearing a guanidinoaryl substituent is *m*-iodobenzylguanidine (Chart 1) which has been utilized for cardiac applications as well as the diagnosis and treatment of neuroendocrine tumors such as neuroblastoma (Tepmongkol and Heyman, 1999). This compound has shown excellent stability in vivo and rapid clearance from normal tissues. Thus, in designing a guanidino-substituted protein acylation agent, it might be ideal to select a compound incorporating the structural features of both SIB and MIBG and with a 1,3,5-substitution pattern. However, we embarked on the preparation of a 1,3,4-system due to the ready availability of 3-iodo-4-methylbenzoic acid. The synthetic strategy utilized for the

Scheme 1^a

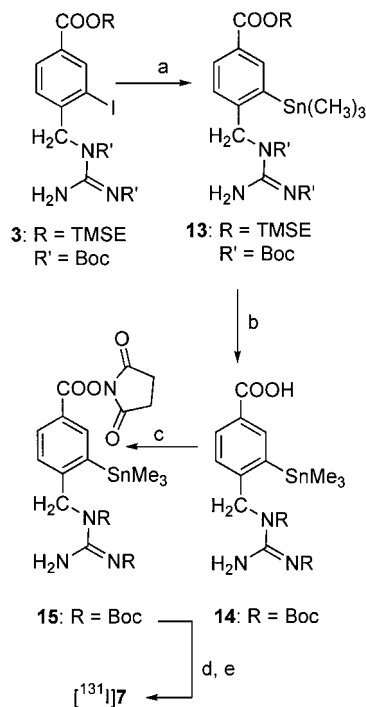
^a (a) TMS-ethanol, DCC, DMAP, THF; (b) NBS, benzoyl peroxide, CCl₄, incandescent light; (c) *N,N'*-bis-Boc-guanidine, NaH, DMF; (d) TBAF, THF; (e) *N*-hydroxysuccinimide, DCC, THF; (f) TFA.

preparation of unlabeled SGMIB is presented in Scheme 1. Attempts to prepare 4-(*N*¹,*N*²-bis-*tert*-butoxycarbonyl)guanidinomethyl-3-iodobenzoic acid directly from 4-bromomethyl-3-iodobenzoic acid based on a previously described approach (Vaidyanathan and Zalutsky, 1997) were unsuccessful. For this reason, the acid moiety was first protected as a 2-(trimethylsilyl)ethyl (TMSE) ester. Benzylic bromination of **1** and subsequent guanidinylation yielded **3**. Removal of the TMSE group from **3**, and the reesterification of the resultant acid with *N*-hydroxysuccinimide afforded the active ester **6**. Both **4** and **6** were converted to the respective free guanidines by treatment with TFA, or SnCl₄ (Miel and Rault, 1997).

Because NCA [¹³¹I]MIBG has been prepared in excellent yields from its silicon precursor (Vaidyanathan and Zalutsky, 1993a), initial efforts were directed toward the preparation of a silicon derivative from which 4-guanidinomethyl-3-iodobenzoic acid (GMIBA, **5**) or its active ester could be obtained. Scheme 2 shows the synthesis of 4-(*N*¹,*N*²-bis-*tert*-butoxycarbonyl)guanidinomethyl-3-(trimethylsilyl)benzoic acid (**12**). Because the radioiodination of **12** to give [¹³¹I]**5** was not successful (vide infra), no attempts were made to prepare the *N*-hydroxysuccinimide ester. Compound **12** was prepared essentially following the approach taken to prepare the iodo counterpart. The starting material **8** was synthesized from 3-iodo-4-methylbenzoic acid via lithium/iodide exchange and subsequent silylation. In the case of the tin precursor **15**, the intermediate **3** was first stannylated using a modified Stille coupling protocol (Wigerinck et al., 1993;

Scheme 2^a

^a (a) *n*-BuLi, Me₃SiCl, THF; (b) TMS-ethanol, DCC, DMAP, THF; (c) NBS, benzoyl peroxide, CCl₄, incandescent light; (d) *N,N'*-bis-Boc-guanidine, NaH, DMF; (e) TBAF, THF; (f) [¹³¹I]**5**, NCS, TFA.

Scheme 3^a

^a (a) (Ph₃P)₂PdCl₂, (Me₃Sn)₂, dioxane; (b) TBAF, THF; (c) *N*-hydroxysuccinimide, DCC, THF; (d) [¹³¹I], HOAc, TBHP CHCl₃; (e) TFA.

Scheme 3). The resultant compound **13** was deprotected to **14** and converted to **15** following the methods used for the preparation of iodo derivatives.

Radioiodination of **12** under conditions used for the preparation of [^{131}I]MIBG from its silicon precursor (Vaidyanathan and Zalutsky, 1993a) gave [^{131}I]**5** in less than 5% radiochemical yield. Difficulty in the synthesis of radioiodinated 3-guanidinomethyl-5-iodopyridine from its silicon precursor was encountered earlier (Vaidyanathan et al., 1998a). While electronic factors might have been the reason for failure in the above instance, steric hindrance from the *o*-guanidinomethyl group may have contributed to the lower yields in the present situation. However, it was possible to convert **14** to [^{131}I]**5** in a one-pot reaction, by sequential radioiodination and deprotection, in an overall radiochemical yield of more than 90%. This radioiodination was performed at 70 °C in HOAc and lead to removal of a significant proportion of the Boc group; however, the mixture was further treated with TFA to ensure complete deprotection.

Preparation of [^{131}I]**7** from **15** in a similar fashion was not attempted because the active *N*-hydroxysuccinimidyl ester functionality is not expected to survive the reversed-phase HPLC and/or the subsequent concentration of the HPLC fraction. To avoid this, milder conditions for the radioiodination of **15** were sought. Radioiodinated IUdR and its fluorinated analogue FIAU have been prepared by a iododestannylation route under mild, but high yielding, conditions (Vaidyanathan and Zalutsky, 1998b). However, only modest yields were obtained when attempts were made to convert **15** to [^{131}I]**6** under these conditions. On the other hand, a radiochemical yield of 55–60% was obtained for the reaction when conditions used for the preparation of [^{131}I]SIB (Zalutsky and Narula, 1987) were adapted. Compound [^{131}I]**6** was isolated by normal-phase HPLC, and after deprotection, investigated for labeling mAbs.

Compound [^{131}I]**5** was initially pursued for mAb labeling because of the questionable stability of the *N*-succinimidyl function to TFA, the reagent used to convert [^{131}I]**6** to [^{131}I]**7**. Coupling of [^{131}I]**5** to mAb L8A4 under conditions reported for the EDC-mediated conjugation of carboxylic acids to protein (Davis and Preston, 1981; Sehgal and Vijay, 1994) was not successful. Therefore, conditions for EDC-mediated coupling were first optimized using [^{131}I]iodobenzoic acid (Vaidyanathan and Zalutsky, 1993b) and then applied for labeling L8A4 with [^{131}I]**5**. Consistent conjugation efficiencies of 35–40% were obtained with specific activities of 0.5–2.0 $\mu\text{Ci}/\mu\text{g}$. The immunoreactive fraction for L8A4 labeled by this method was similar to that labeled using Iodogen and was in the range of 75–80% and size-exclusion HPLC revealed less than 5% of aggregates (Figure 1). Nonetheless, this approach was not pursued because of the greater simplicity and higher yields anticipated with the *N*-succinimidyl ester acylation agents.

Initially, we attempted to label mAbs directly with [^{131}I]**6**, the *N*-succinimidyl ester with a protected guanidine functionality. It was anticipated that the protecting groups would be cleaved within the lysosomal compartment due to the susceptibility of the Boc group to acidic environments; carbamate groups have been used in the design of prodrugs (Hansen et al., 1991). Although it is an active ester, [^{131}I]**6** could be coupled to L8A4 in only 10–15% yield, presumably due to its high lipophilicity from the presence of Boc protecting groups, resulting in poor solubility in aqueous buffers. While this method yielded labeled mAb with immunoreactive fractions of about 80% and no obvious protein cross-linking, only low specific activities were obtained due to poor coupling yields. Thus, it was necessary to convert [^{131}I]**6** to [^{131}I]**7**. A pilot study was undertaken to test the stability of the

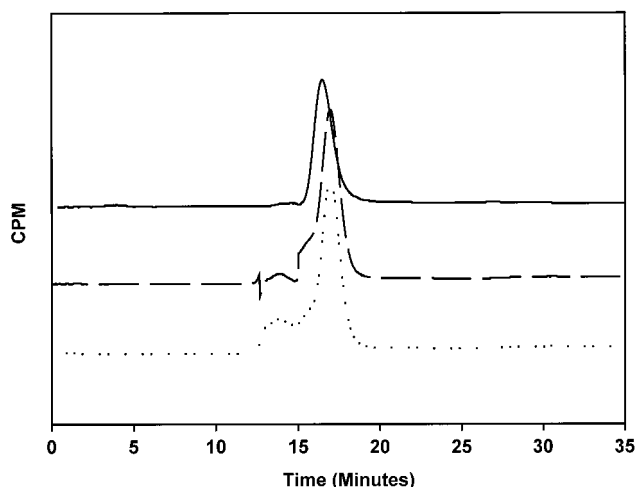


Figure 1. Size-exclusion HPLC radiochromatogram of L8A4 mAb labeled with ^{131}I using Iodogen (dotted line), [^{131}I]SGMIB (solid line), and [^{131}I]GMIBA using EDC-mediated coupling (dashed line).

N-hydroxysuccinimidyl ester to TFA using SIB as a model compound. No significant degradation of SIB in the presence of TFA was seen for up to 30 min at room temperature by TLC. Subsequently, it was demonstrated that [^{131}I]**6** could also be converted to [^{131}I]**7** without any adverse effects. Labeling of L8A4 with [^{131}I]**7** (SGMIB) resulted in 60–65% coupling yields with specific activities of 0.5–2.0 $\mu\text{Ci}/\mu\text{g}$. This method did not produce any observable cross-linking, and the immunoreactive fraction of ^{131}I -labeled L8A4 was more than 80%.

To test the hypothesis that the presence of a highly polar guanidine function in the structure of radioiodinated acylation agents will enhance the intracellular retention of activity for mAbs labeled with these molecules, internalization assays were performed with mAb L8A4 and the EGFRvIII-expressing U87 MGDEGFR cell line. In the first study, L8A4 labeled with the Boc-protected SGMIB analogue and Iodogen were compared in paired-label format. As shown in Figure 2, the percentage of activity retained inside the cell for L8A4 labeled using [^{131}I]**6** was higher than that for L8A4 labeled via Iodogen at all time points. At 16 h, the value for the former ($25.9 \pm 1.3\%$) was 3.5-fold higher than that for the latter ($7.5 \pm 0.4\%$). The activity pattern in the supernatant over this period was complementary to that observed for internalized activity. We speculate that the higher intracellular retention of activity for L8A4 labeled via [^{131}I]**6** reflects the removal of Boc groups from the prosthetic group within the lysosomal compartment generating the cationic guanidine moiety.

The results from a second paired-label study comparing the internalization of L8A4 labeled with [^{131}I]**7** versus that labeled using Iodogen is depicted in Figure 3. Again, L8A4 labeled with [^{131}I]**7** demonstrated significantly higher amounts of internalized activity and lower supernatant counts compared with the Iodogen-labeled mAb, with 3 to 4 times higher intracellular activity observed with SGMIB labeling at later time points. These results are in marked contrast to those obtained when this mAb was labeled using SIPC (Reist et al., 1996). In that study, SIPC offered no advantage compared with Iodogen in terms of intracellular counts after 8 h; however, it should be noted that a different EGFRvIII-expressing cell line (HC2 20 d2) was used in that study. A final experiment was performed in a paired-label format to directly compare the intracellular retention of radioiodine activity

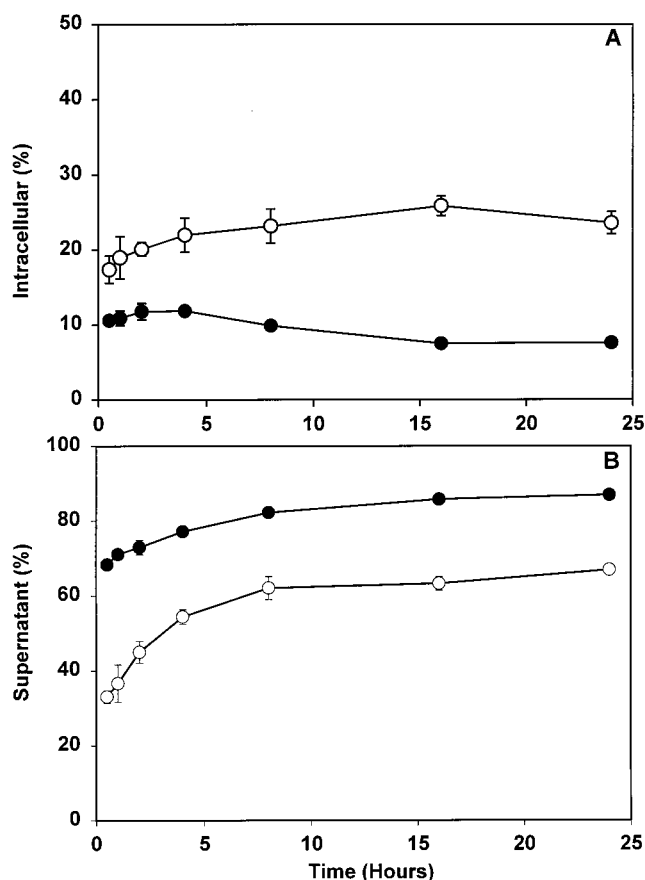


Figure 2. Paired-label in vitro internalization of L8A4 by U87 MGDEGFR cells. L8A4 radioiodinated with ¹²⁵I via Iodogen method (●) and with ¹³¹I using Boc-SGMIB (○). (A) Percentage of radioactivity initially bound to cells retained intracellularly. (B) Percentage of radioactivity initially bound to the cells recovered from the cell culture supernatant.

in U87 MGDEGFR cells incubated with L8A4 labeled with ¹³¹I using SGMIB and ¹²⁵I using SIPC. At all time points, intracellularly trapped activity from L8A4 labeled with SGMIB was significantly higher than that from mAb labeled with SIPC. Use of the guanidino-substituted acylation reagent resulted in about 3 times higher internalized counts at the 16 and 24 h time points (Figure 4).

We next investigated whether the increased intracellular retention of iodine activity indeed was related to the nature of the catabolites generated during the processing of mAbs labeled using SGMIB. As a first step, the percentage of low molecular weight catabolites associated with the cell lysates was determined by passage through a 10 kDa filter for L8A4 labeled via SGMIB and SIPC. As summarized in Table 1, a considerably higher percentage of the low molecular weight catabolites that were generated from SGMIB-labeled mAb were recovered from the cell lysates. By 8 h, a nearly 20 times higher fraction of low molecular weight catabolites were found in the cell lysates with SGMIB labeling with the difference increasing to a factor of about 75 by 24 h. The trends observed for L8A4 labeled with SIPC were similar to those observed previously with a different EGFRvIII-expressing cell line (Reist et al., 1996). These results demonstrate that SGMIB labeling yields catabolites that remain inside the cell to a considerably greater extent than seen with mAb labeled via SIPC.

The percentage of the total radioiodine activity that was present in the cell culture supernatant and cell

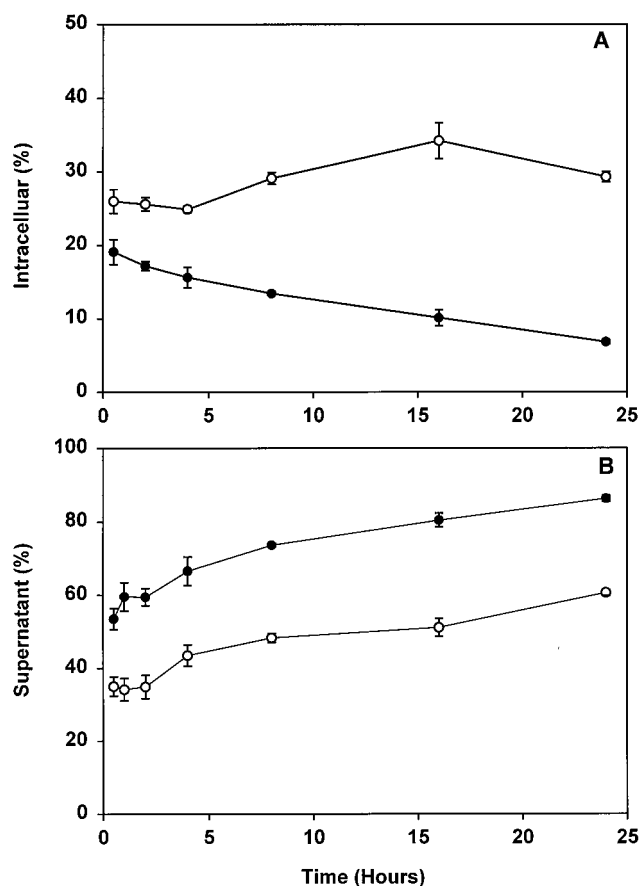


Figure 3. Paired-label in vitro internalization of L8A4 by U87 MGDEGFR cells. L8A4 radioiodinated with ¹²⁵I via Iodogen method (●) and with ¹³¹I using [¹³¹I]SGMIB (○). (A) Percentage of radioactivity initially bound to cells retained intracellularly. (B) Percentage of radioactivity initially bound to the cells recovered from the cell culture supernatant.

lysates in the form of low molecular weight catabolites also was calculated, and the results are summarized in Table 2. At early time points, low molecular weight species accounted for only a small fraction of the total activity, and in the cell culture supernatant, the contribution of low molecular weight species was similar for mAb labeled by reaction with SIPC and SGMIB. However, clear differences were observed between the two labeling methods in the cell lysate. With SIPC, low molecular weight species accounted for less than 6% of the radioiodine activity recovered from the cells. In contrast, 45 to 64% of the radioactivity present in the cells after 8–24 h incubation at 37 °C was in the form of low molecular weight molecules. These results demonstrate that the increased intracellular retention observed for mAb L8A4 labeled via SGMIB in the internalization assays is due to low molecular weight degradation products.

Previous studies have shown that for mAbs labeled using SIB and similar reagents, the primary low molecular weight catabolites are the free acid as well as its glycine and lysine conjugates (Garg et al., 1995). The higher intracellular retention of low molecular weight species from mAbs labeled with SGMIB compared to those labeled with SIPC is consistent with the relative polarity of their expected catabolites. Retention times on reversed-phase HPLC (vide supra) indicate that GMIBA, GMIBA–glycine, and GMIBA–lysine are all more polar than INA and its corresponding derivatives. The higher polarity of GMIBA compared with INA was confirmed

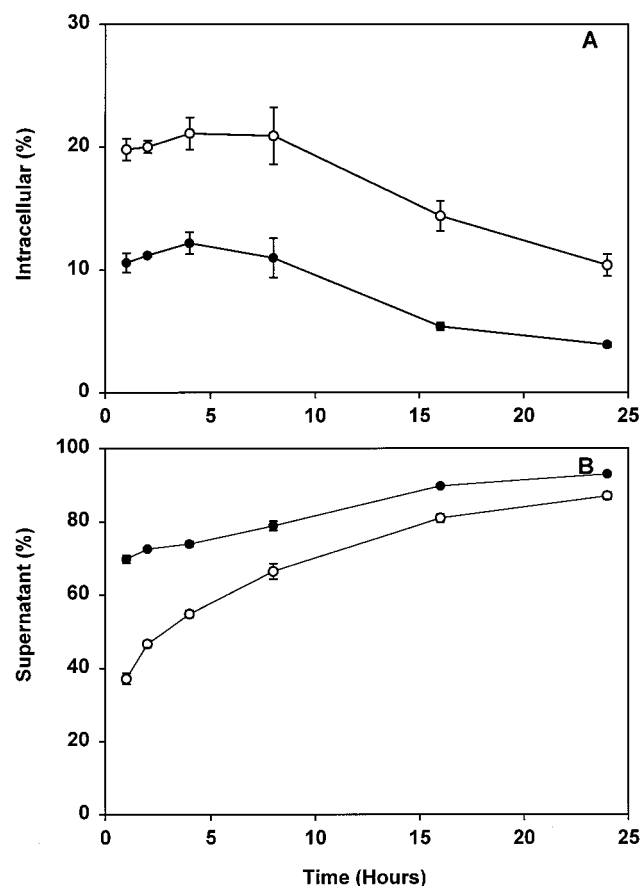


Figure 4. Paired-label in vitro internalization of L8A4 by U87 MG/EGFR cells. L8A4 radioiodinated with ^{125}I using [^{125}I]SIPC (●) and with ^{131}I using [^{131}I]SGMIB (○). (A) Percentage of radioactivity initially bound to cells retained intracellularly. (B) Percentage of radioactivity initially bound to the cells recovered from the cell culture supernatant.

Table 1. Low Molecular Weight Catabolites Retained by H898 Cells Following Paired-Label Intracellular Processing of L8A4 Labeled Using [^{131}I]SGMIB and with [^{125}I]SIPC

time (h)	low molecular weight catabolites retained in the cell (percent of total)	
	SGMIB	SIPC
1	12.6	6.14
2	40.0	9.72
4	56.9	7.65
8	58.9	3.01
16	29.3	0.63
24	14.9	0.20

by measuring their octanol/saline partition coefficients at physiologic and lysosomal pH (pH 4.75). At a pH of 7.4, GMIBA had a partition coefficient (0.02 ± 0.00), three times smaller than that for INA (0.06 ± 0.00). The difference was even more pronounced at lysosomal pH (GMIBA 0.06 ± 0.00 ; INA 1.63 ± 0.01). Thus, INA probably exists predominantly as an uncharged molecule at lysosomal pH, whereas it may exist as a zwitter ion at physiologic pH. On the other hand, the guanidine function of GMIBA is likely to exist in its protonated form at both pH values.

The catabolism of mAb L8A4 labeled with [^{131}I]SGMIB and [^{125}I]SIPC following exposure to U87 MG/EGFR cells in vitro was evaluated by HPLC. Size exclusion HPLC failed to detect any aggregates in the mAb preparations used in these studies and less than 1% of the activity was associated with low molecular weight

Table 2. Percent of Total Activity Associated with Low Molecular Weight Catabolites in Cell Lysate and Cell Culture Supernatant

time (h)	supernatant ^a (%)		cell lysate ^a (%)	
	I-131	I-125	I-131	I-125
1	1.13	0.71	3.48	1.12
2	1.41	1.20	11.4	2.33
4	1.84	3.15	24.5	4.19
8	5.12	12.1	45.4	5.30
16	17.4	26.7	64.2	5.23
24	26.4	33.1	61.8	2.98

^a (Activity in the low mw fraction $\times$ 100)/(activity in the low mw fraction + activity in the high mw fraction).

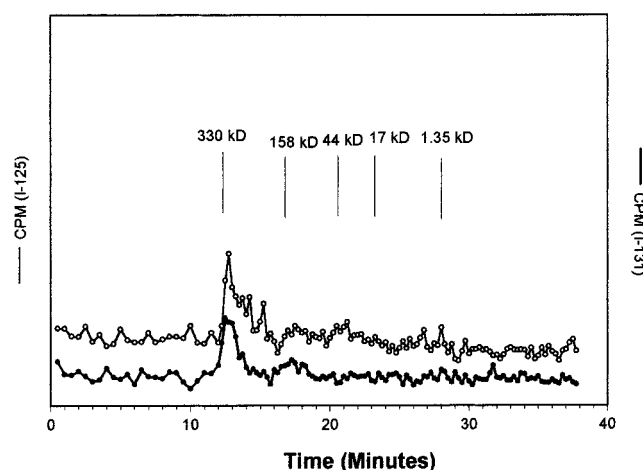


Figure 5. Size-exclusion HPLC of radioiodine activity recovered from cell lysates after 1 h incubation with L8A4 mAb labeled with ^{125}I using SIPC (●) and labeled with ^{131}I using SGMIB (○). Retention times of molecular weight standards are indicated.

species. Size-exclusion HPLC of the cell lysate at 1 h showed that both radionuclides were primarily associated with a peak with a retention time of about 300 kDa (Figure 5) and a similar pattern was observed in cell lysate samples obtained at latter time points. Because of the lack of mAb dimers in the preparations used in this experiment, it is likely that the 300 kDa species represents a monomeric complex between mAb L8A4 and the 145 kDa EGFRvIII.

Analysis of reversed-phase HPLC radiochromatograms indicated that the predominant low molecular weight catabolites present in the cell culture supernatant at 2 h from the SGMIB labeled mAb were the free acid, GMIBA, and a peak eluting at the void volume. We speculate that this peak could be either free iodide, or more likely, the glucuronide conjugate (Laznick and Laznickova, 1999). At 4 h, a peak corresponding to GMIBA-glycine became apparent, and it was the predominant peak present in the supernatant at 24 h. The major peak at 2 h from the SIPC labeled mAb eluted in the void volume, and there were also two minor peaks corresponding to INA and INA-lysine. The intensity of the peak corresponding to INA-lysine increased with time, and by 24 h, it was the only major peak present in the cell culture supernatant.

Up until the 24 h time period, GMIBA-glycine was the only predominant peak that could be detected in the cell lysates for mAb labeled using SGMIB (Figure 6); however, the only peak seen at 24 h eluted in the void volume. From SIPC-labeled mAb, the major peak at 2 h has a retention time corresponding to that of INA while

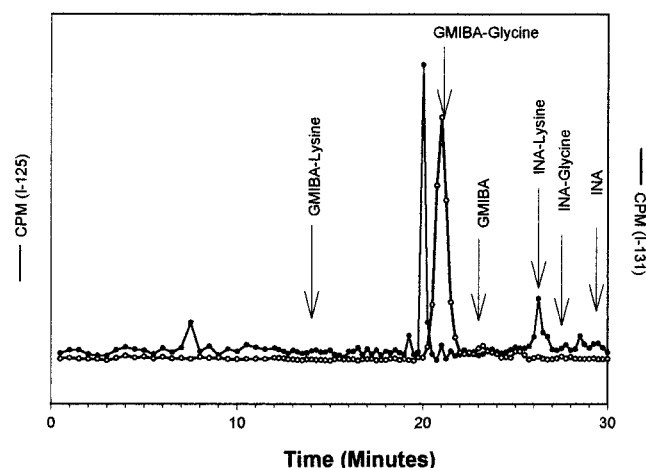


Figure 6. Reversed-phase HPLC of low molecular weight species recovered from cell lysates 8 h after incubation with L8A4 mAb labeled with ¹²⁵I using SIPC (●) and that labeled with ¹³¹I using SGMIB (○). Retention times of potential catabolites indicated are those of authentic standards.

at 4 h, two peaks were seen, one corresponding to INA-lysine and the other, an unidentified more polar peak ($t_R = 20$ min). This peak was the significant component at 8 h in addition to a lower level of INA-lysine (Figure 6). At 24 h, there were two peaks near the void volume, one coeluting with the only SGMIB-labeled mAb catabolite, and the other unidentified peak eluted slightly earlier.

Although it is difficult to fully account for these catabolic patterns, it is clear that no lysine conjugate of GMIBA was detected. In contrast, INA-lysine was one of the major metabolites from SIPC labeled mAb, and similar lysine conjugates have been observed in the degradation of mAbs labeled on lysine ϵ -amino groups with other molecules (Duncan and Welch, 1993; Arano et al., 1995; Rogers et al., 1995). It appears that when mAbs labeled using SGMIB are degraded, free GMIBA is released from the protein itself and then further converted to the glycine conjugate, a normal pathway for the metabolism of aromatic carboxylic acids seen earlier with SIB-labeled mAbs (Garg et al., 1995). This suggests that the amide bond between GMIBA and the lysine on the mAb (or its lower molecular weight protein degradation product) is more susceptible to cleavage within the cell than the bond created when the mAbs were labeled using SIPC. Nonetheless, the retention of the GMIBA-glycine conjugate inside the cell after proteolysis of the mAb is consistent with the highly basic nature of its guanidino substituent.

In summary, a method has been developed for the preparation of SGMIB, an acylation agent containing a polar guanidine function, via a tin precursor. The anti-EGFRvIII mAb L8A4 could be radioiodinated efficiently using SGMIB with preservation of immunoreactivity and without any protein cross-linking. Internalization assays demonstrated that radioiodination of this mAb using SGMIB significantly enhanced the retention of radioiodine activity in EGFRvIII tumor cells after mAb internalization. Catabolism studies showed that this behavior was likely related to the generation of highly polar catabolites which were trapped within the cell. Experiments are in progress in mice with EGFRvIII-expressing tumors to determine whether SGMIB will be a useful reagent for labeling internalizing mAbs such as L8A4.

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Fluorine-Substituted Ligands for the Peroxisome Proliferator-Activated Receptor Gamma (PPAR γ): Potential Imaging Agents for Metastatic Tumors

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The peroxisome proliferator-activated receptor gamma (PPAR γ), a primary regulator of lipid metabolism, is present in many tumor cell lines and animal tumor systems and, in some cases, can mediate effective antitumor therapy with potent synthetic ligands. In an approach to image tumors with positron-emission tomography (PET) based on their content of PPAR γ , we have synthesized two fluorine-substituted analogues of a high affinity ligand from the phenylpropanoic acid class. The analogue having the highest affinity for PPAR γ was labeled with the positron-emitting radionuclide fluorine-18. In tissue distribution studies in normal rats and in SCID mice bearing human breast tumor xenografts, this compound did not show evidence of receptor-mediated uptake. The prospects for using PPAR γ as a target for imaging tumors may be limited by the low receptor concentrations in tumors and by the pharmacokinetic behavior of this class of ligands, which appears to be more favorable for therapy than for imaging.

The peroxisome proliferator-activated receptor gamma (PPAR γ), formerly considered together with its paralogs PPAR α and PPAR δ to be an orphan receptor, is now known to be a primary regulator of lipid metabolism, capable of inducing differentiation and lipid storage in preadipocytes (1, 2). More recently, endogenous ligands (prostaglandin-J's and unsaturated fatty acids) (3, 4) as well as potent synthetic ligands for PPAR γ have been identified (5).

PPAR γ appears to play an intriguing role in cancer, because PPAR γ ligands can induce certain cancer cell lines (namely, liposarcomas) to stop proliferating, lose their malignant character, and undergo terminal differentiation to adipocytes (6–9). Thus, these ligands might provide a novel route to therapy. PPAR γ levels, assayed by immunohistochemistry, are also elevated in other tumor cells, including several breast cancer cell lines (7), and in animal tumor models for breast cancer, receptor levels were especially high in metastases. When tested in some of the breast cancer cell lines, PPAR γ ligands again induced tumor cell differentiation, but not all cell lines that were PPAR γ positive responded. The key to responsiveness may be the state of PPAR γ phosphorylation in the tumor cells; the receptor phosphorylated on S112 is much less responsive to ligand than the nonphosphorylated receptor (10) and has lower affinity for ligands (11), whereas both are detected equally well by the antibody used in the PPAR γ immunoassay. Thus, it appears that PPAR γ might be a novel target for tumor therapy, but one in which responsiveness is determined

not only by the presence of the receptor, but its presence in a dephosphorylated form capable of responding to ligand.

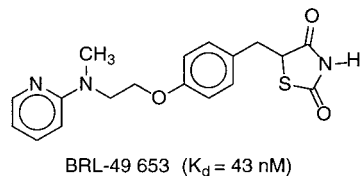
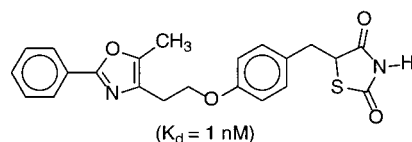
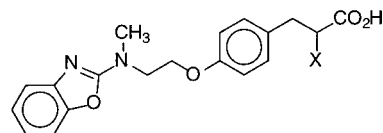
In such a situation, functional imaging could play a critical role: The selective uptake of appropriate PPAR γ ligands, developed as *in vivo* radiotracers, could be used to identify those tumors containing receptors that have retained their ability to bind PPAR γ ligands. These might be the tumors more likely to show a favorable response to therapy with PPAR γ ligands. Thus, as has proved to be the case with estrogen receptor-based imaging of breast tumors (12–14), functional imaging of tumors based on their content of ligand-binding PPAR γ might enable hormonal therapy to be directed to those patients most likely to benefit. In this manner, time would not be lost in providing alternative therapies with higher morbidity to those who are unlikely to respond.

The affinity of endogenous ligands for PPAR γ , unsaturated fatty acids or prostaglandin J's (3,4), is only in the micromolar range, too low to be useful for imaging. However, a number of synthetic ligands, exemplified by the 5-substituted-thiazolidine-2,4-diones (15, 16) and the 2-aryloxy-3-phenylpropanoic acids (17, 18) have been identified as potent, high affinity PPAR γ ligands (Scheme 1). As targets in this first study, we selected compounds **9** and **22** as fluorine-substituted analogues of one of the most potent and selective PPAR γ ligand from the 3-phenylpropanoic acid class (SB 213 068, Scheme 1). The sites of fluorine substitution were selected to make these compounds reasonable analogues of the parent ligand and to allow for the introduction of a fluorine-18 label by a fluoride ion displacement reaction late in the synthetic sequence. In compound **9**, fluorine substitution is made by replacing the *N*-methyl group with a 2-fluoroethyl substituent, and in compound **22**, fluorine substitution is on C(2) of the α -ethoxy substituent of the parent ligand. The former, *N*-(2-fluoroethyl) for *N*-methyl substitution, has been used frequently in the design of

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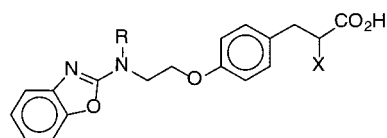
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Scheme 1. Two Classes of High Affinity Ligands for PPAR γ and Synthetic Targets in This Study**Thiazolidinediones:****Phenylpropanoic Acids:**

SB 213 068

X	K_i
-OEt ^a	2.5 nM
-OPh	0.4 nM

^a most potent *in vivo***Synthetic Targets:**

	R	X
9	-CH ₂ CH ₂ F	-O-Ph
22	-CH ₃	-OCH ₂ CH ₂ F

ligands for imaging neuroreceptors by PET (19, 20). It is of note that the parent compound, SB 213 068, also has good potency and relatively rapid blood clearance rates. The latter, in particular, is an important consideration for achieving the good contrast between target and nontarget areas needed for imaging.

We have synthesized these compounds, determined their binding affinity toward receptors in the PPAR family, prepared the highest affinity analogue labeled with the positron-emitting radionuclide fluorine-18, and conducted tissue distribution studies on this analogue in two animal model systems. Despite the high binding affinity of the best analogue (**22**), its tissue distribution does not show evidence of receptor-mediated uptake. This and the pharmacokinetic factors that characterize most high affinity/high potency ligands from these and other classes suggest that PPAR γ is likely to be a challenging target for tumor imaging by PET.

EXPERIMENTAL SECTION

Materials and Methods. All reactions using water- and air-sensitive reagents were carried out under N₂ atmosphere with dry solvents. Solvents were distilled under N₂ as follows: pyridine from KOH, tetrahydrofuran (THF) from sodium benzophenone ketyl, dimethylformamide (DMF) from MgSO₄. All other reagents were purchased from commercial suppliers and used without further purification. Reaction were monitored using thin-layer chromatography (TLC), performed on 0.255 mm silica gel plates containing F-254 indicator, with visualization under UV light (254 nm). Flash chromatography was performed using Woelm 32–63 μ m silica gel packing unless otherwise noted. Methods for the preparation of methanesulfonyloxyethanol (21), *tert*-butyldimethylsilyloxyethanol (22), methyl 2-phenoxydiethylphosphonoacetate (23), and methyl 2-diazodiethylphosphonoacetate (23) have been described in the cited literatures.

¹H NMR and ¹³C NMR spectra were recorded on Varian U400 and U500 instruments. NMR spectra chemical shifts (δ) are reported in parts per million downfield from TMS and referenced with either TMS internal standard for CDCl₃, MeOH-*d*₄, acetone-*d*₆, or

DMSO-*d*₆. NMR coupling constants are reported in Hertz. Low-resolution electron impact mass spectra and fast-atom bombardment mass spectra were obtained on Finnigan MAT CH-5 and ZAB-SE spectrometers, respectively. High-resolution mass spectra were obtained on a VG 70-SE-4E spectrometer.

2-[Di(2-hydroxyethyl)amino]benzoxazole (1). To the solution of 2-chlorobenzoxazole (1.22 g, 7.95 mmol) in the mixture of THF (15 mL) and DMF (5 mL) at room temperature was added diethanolamine (1.8 mL, 18.8 mmol). The reaction mixture was stirred for 3 h at the same temperature and then poured into ethyl acetate (50 mL) and water (50 mL). The organic layer was separated, extracted with ethyl acetate (50 mL $\times$ 3), dried over MgSO₄, filtered, and evaporated to give compound **1** (1.2 g, 68%) as a white solid: mp 109–110 °C; ¹H NMR (methanol-*d*₄, 500 MHz) δ 3.75 (t, $J = 5.5$ Hz, 4H), 3.84 (t, $J = 5.5$ Hz, 4H), 7.04 (t, $J = 7.5$ Hz, 1H), 7.15 (t, $J = 7.5$ Hz, 1H); MS (FAB) (%) m/z 223 ($M^+ + 1$, 100), 155 (28), 119.

2-[(2-(Methanesulfonyloxy)ethyl-2-hydroxyethyl)-amino]benzoxazole (2). To the solution of compound **1** (1.30 g, 5.86 mmol) in pyridine (5 mL) was added dropwise methanesulfonyl chloride (340 mg, 2.97 mmol) at 0–5 °C with a small syringe. After stirring continuously for 30 min, the same amount of MsCl was once more added to the reaction mixture. After an additional 30 min, the reaction mixture was added to 20 mL of water and extracted with ethyl acetate (30 mL $\times$ 3). The ethyl acetate solution was washed with brine, dried over MgSO₄, and concentrated in vacuo. The column chromatography on silica gel with ethyl acetate as eluent gave the desired compound **2** (850 mg, 48%) as a liquid together with unreacted starting material **1** (309 mg, 23%) and 2-[bis(2-methanesulfonyloxyethyl)amino]benzoxazole (580 mg, 25%). Spectra for compound **2**: ¹H NMR (CDCl₃, 500 MHz) δ 2.99 (s, 3H), 3.78 (t, $J = 5.5$ Hz, 2H, CH₂OH), 3.92–3.95 (m, 4H, CH₂NCH₂), 4.52 (t, $J = 5.5$ Hz, 2H, MsOCH₂), 7.08 (t, $J = 7.5$ Hz, 1H), 7.17 (t, $J = 7.5$ Hz, 1H), 7.27 (d, $J = 8.0$ Hz, 1H), 7.34 (d, $J = 8.0$ Hz, 1H); ¹³C NMR (CDCl₃, 125 MHz) δ 37.51, 49.56, 53.22, 61.40, 67.06, 109.02, 116.39, 121.08, 124.30,

142.50, 148.81, 162.19; MS (FAB) (%) m/z 301 ($M^+ + 1$, 100), 223 (100), 205 (50), 119 (33).

4-[2-[Benzoxazol-2-yl-(2-hydroxyethyl)amino]ethoxy]benzaldehyde (3). (A) To the suspension of NaH (58 mg, 1.44 mmol, 60%) in DMF (5 mL) was added portionwise *p*-hydroxybenzaldehyde (168 mg, 1.38 mmol) at 0 °C. To this solution was added compound **2** (375 mg, 1.25 mmol) at room temperature, and the reaction was heated at 80 °C for 5 h. The solution was poured into water (10 mL), and the solid precipitate was collected by filtration and chromatographed on silica gel with the mixture of CH_2Cl_2 and EtOAc (1:2) to afford a trace of 2-morpholinobenzoxazole and title compound **3** (350 mg, 86%) as a colorless liquid: ^1H NMR ($CDCl_3$, 500 MHz) δ 3.84 (t, $J = 5.5$ Hz, 2H, NCH_2CH_2OH), 3.95 (t, $J = 5.5$ Hz, 2H, NCH_2CH_2OH), 4.01 (t, $J = 5.5$ Hz, 2H, NCH_2CH_2OAr), 4.36 (t, $J = 5.5$ Hz, 2H, NCH_2CH_2OAr), 6.97 (d, $J = 8.5$ Hz, 2H), 7.08 (t, $J = 7.5$ Hz, 1H), 7.15 (t, $J = 8.0$ Hz, 1H), 7.24 (d, $J = 8.0$ Hz, 1H), 7.33 (d, $J = 8.0$ Hz, 1H), 7.79 (d, $J = 8.5$ Hz, 2H), 9.84 (s, 1H, CHO); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 49.45, 53.31, 61.17, 66.38, 108.92, 114.73, 116.14, 120.84, 124.19, 130.24, 132.03, 142.67, 148.76, 163.29, 190.89; MS (EI) (%) m/z 327 (M^+ , 100), 224 (20), 205 (24), 161 (15).

(B) The mixture of **12** (924 mg, 2.28 mmol), K_2CO_3 (400 mg), and ethylene carbonate (300 mg, 3.41 mmol) in DMF (2 mL) was heated at 110 °C for 4 h. When compound **12** was no longer present upon TLC analysis, water (15 mL) was added to the reaction mixture, and the products were extracted with ethyl acetate (50 mL $\times$ 3), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was chromatographed on SiO_2 column with EtOAc as an eluent to give title compound **3** (520 mg, 70%).

4-[2-[Benzoxazol-2-yl-(2-trimethylsilyloxyethyl)amino]ethoxy]benzaldehyde (4). A solution of compound **3** (287 mg, 0.88 mmol), $TMSCl$ (115 mg, 1.06 mmol), and triethylamine (107 mg, 1.06 mmol) in THF at room temperature was stirred for 4 h and then filtered and evaporated under vacuum to give title compound **4** (350 mg, 100%) as a colorless liquid. ^1H NMR ($CDCl_3$, 500 MHz) δ 0.09 (s, 9H), 3.81 (t, $J = 5.5$ Hz, 2H, NCH_2CH_2OTMS), 3.89 (t, $J = 5.5$ Hz, 2H, NCH_2CH_2OTMS), 4.07 (t, $J = 5.5$ Hz, 2H, NCH_2CH_2OAr), 4.38 (t, $J = 5.5$ Hz, 2H, NCH_2CH_2OAr), 7.00–7.04 (m, 3H), 7.17 (t, $J = 7.5$ Hz, 1H), 7.26 (d, $J = 7.5$ Hz, 1H), 7.37 (d, $J = 7.5$ Hz, 1H), 7.82 (d, $J = 9.0$ Hz, 2H), 9.80 (s, 1H, CHO); ^{13}C NMR ($CDCl_3$, 125 MHz) δ -0.64, 49.54, 52.47, 60.75, 66.31, 108.82, 114.73, 114.76, 116.18, 120.65, 121.31, 124.13, 130.22, 132.05, 148.19, 163.54, 190.80.

Methyl 3-[4-[2-[Benzoxazol-2-yl-(2-hydroxyethyl)amino]ethoxy]phenyl]-2-phenoxyacrylate (5). To the solution of methyl 2-phenoxy-2-diethylphosphonoacetate (261 mg, 0.86 mmol) in THF (15 mL) was added portionwise NaH (38 mg, 0.95 mmol, 60% mineral oil dispersion) at 0 °C. When gas evolution ceased, a solution of compound **4** (345 mg, 0.87 mmol) in THF (5 mL) was added dropwise. After stirring for 5 h, water (50 mL) was added to the reaction mixture, and the pH of the aqueous solution was adjusted to 2 with 0.1 M HCl. An EtOAc extract (30 mL $\times$ 3) was washed with brine, dried ($MgSO_4$), and evaporated to give title compound **5** ($E:Z = 1:1$) (309 mg, 75%) as a colorless liquid. ^1H NMR ($CDCl_3$, 400 MHz) [most peaks are doubled because of the geometric isomers] δ 3.65, 3.76 (s, combined, 3H, CH_3O), 3.82, 3.85 (t, $J = 5.5$ Hz, combined, 2H, CH_2OH), 3.90–4.02 (m, 4H, CH_2NCH_2), 4.26, 4.30 (t, $J = 5.5$ Hz, 2H, NCH_2CH_2OAr), 6.70 (s, 0.5H, *E*-olefinic H), 6.80–7.64 (m, 13.5H, *Z*-olefinic H and ArH); MS (EI) (%) m/z

474 (M^+ , 10), 430 (15), 326 (18), 296 (44), 205 (60), 161 (100), 147 (77).

Methyl 3-[4-[2-[Benzoxazol-2-yl-(2-hydroxyethyl)amino]ethoxy]phenyl]-2-phenoxypropanoate (6). The mixture of compound **5** (309 mg, 0.65 mmol) and Pd/C (10%, 3 mg) in MeOH (20 mL) was placed under 30 psi of H_2 in a Parr shaker and agitated at room temperature for 20 h. Filtration and solvent evaporation afforded the title compound **6** (306 mg, 99%) as a colorless liquid. ^1H NMR ($CDCl_3$, 400 MHz) δ 3.15 (dd, $J = 17.0$, 5.2 Hz, 1H, $ArCH_aH_bCH$), 3.19 (dd, $J = 17.0$, 7.6 Hz, 1H, $ArCH_aH_bCH$), 3.67 (s, 3H), 3.90 (t, $J = 5.2$ Hz, 2H, NCH_2CH_2OH), 3.96 (t, $J = 5.2$ Hz, 2H, NCH_2CH_2OH), 4.00 (t, $J = 5.2$ Hz, 2H, NCH_2CH_2OAr), 4.28 (t, $J = 5.2$ Hz, 2H, NCH_2CH_2OAr), 4.74 (dd, $J = 7.6$, 5.2 Hz, 1H, $ArCH_2CH$), 6.79–6.83 (m, 4H), 6.92–7.07 (m, 2H), 7.17–7.41 (m, 7H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 38.08, 49.75, 52.18, 53.67, 61.37, 65.97, 77.68, 108.89, 114.22, 115.04, 115.83, 120.98, 121.61, 124.22, 129.00, 129.41, 130.48, 141.62, 148.29, 156.94, 157.49, 161.99, 171.56; MS (EI) (%) m/z 476 (M^+ , 18), 432 (22), 205 (56), 161 (100), 135 (68).

Methyl 3-[4-[2-[Benzoxazol-2-yl-(2-methanesulfonyloxyethyl)amino]ethoxy]phenyl]-2-phenoxypropanoate (7). To the solution of compound **6** (255 mg, 0.54 mmol) in pyridine (5 mL) was added methanesulfonyl chloride (41.0 μ L, 0.54 mmol) with a microsyringe at 0–5 °C. When starting material was no longer detected by TLC, the reaction mixture poured into water (30 mL), extracted with EtOAc (20 mL $\times$ 3), washed with 1 N HCl solution, brine, and water, and dried over $MgSO_4$. Evaporation of solvent under pressure gave the title compound **7** (210 mg, 71%) as a colorless liquid. ^1H NMR ($CDCl_3$, 500 MHz) δ 2.93 (s, 3H), 3.13–3.21 (m, 2H, $ArCH_2CH$), 3.70 (s, 3H), 3.98–4.10 (m, 4H, CH_2NCH_2), 4.24 (t, $J = 5.5$ Hz, 2H, NCH_2CH_2OAr), 4.55 (t, $J = 5.5$ Hz, 2H, NCH_2CH_2OMs), 4.74 (dd, $J = 7.5$, 5.5 Hz, 1H, $ArCH_2CH$), 6.80–6.83 (m, 4H), 6.94 (t, $J = 7.5$ Hz, 1H), 7.04 (t, $J = 7.5$ Hz, 1H), 7.16–7.28 (m, 6H), 7.36 (d, $J = 7.5$ Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 37.42, 38.22, 49.57, 49.80, 52.28, 66.24, 66.96, 77.89, 108.99, 114.36, 115.21, 116.49, 120.98, 121.76, 124.23, 129.01, 129.57, 130.63, 142.88, 148.91, 157.32, 157.70, 161.44, 171.71; MS (EI) (%) m/z 554 (M^+ , 1.2), 476 (32), 275 (26), 205 (100), 161 (42).

Methyl 3-[4-[2-[Benzoxazol-2-yl-(2-fluoroethyl)amino]ethoxy]phenyl]-2-phenoxypropanoate (8). To a solution of *n*-Bu₄NF (62 μ L, 0.1 M CH_2Cl_2 solution) in DMSO (0.2 mL), preheated to 90 °C, was added compound **7** (28 mg, 0.05 mmol) dissolved in DMSO (0.2 mL) through a microsyringe, and heating was continued for 10 min. A lot of solid deposited upon addition of water (5 mL). After the solvent was decanted, the residue was chromatographed on silica gel (1 $\times$ 3 cm), with an ether eluent. 4-[2-[(benzoxazol-2-yl)amino]ethoxy]benzaldehyde (4 mg, 18%) eluted in the first 20 mL, and the desired fluorinated title compound **8** (16 mg, 67%) in the next 25 mL, as a colorless oil. A small amount of unreacted starting material (4 mg, 14%) was eluted with ethyl acetate. ^1H NMR ($CDCl_3$, 400 MHz) δ 3.14 (dd, $J = 18.0$, 5.2 Hz, 1H, $ArCH_aH_b$), 3.19 (dd, $J = 18.0$, 7.6 Hz, 1H, $ArCH_aH_b$), 3.67 (s, 3H), 4.00 (tt, $J = 25.6$, 4.8 Hz, 2H, FCH_2CH_2N), 4.02 (t, $J = 5.6$ Hz, 2H, $ArOCH_2CH_2N$), 4.25 (t, $J = 5.6$ Hz, 2H, $ArOCH_2CH_2N$), 4.75 (tt, $J = 47.6$, 4.8 Hz, 2H, FCH_2CH_2N), 4.74 (dd, $J = 7.6$, 5.2 Hz, 1H, $ArCH_2CH$), 6.80–6.83 (m, 4H), 6.94 (t, $J = 7.6$ Hz, 1H), 7.02 (t, $J = 7.6$ Hz, 1H), 7.15–7.27 (m, 6H), 7.37 (d, $J = 7.6$ Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 38.24, 49.51, 50.48 (d, $J = 20$ Hz), 52.27, 66.13, 77.96, 82.12 (d, $J = 168.5$ Hz, FCH_2), 108.88, 114.39, 115.24, 116.31, 120.73,

121.76, 124.13, 128.86, 129.56, 130.57, 136.10, 142.81, 148.70, 157.46, 157.72, 171.92; MS (FAB) (%) m/z 479 ($M^+ + 1$, 100), 155 (46), 135 (40), 119 (93).

3-[4-[2-(Benzoxazol-2-yl)-(2-fluoroethyl)amino]ethoxy]phenyl-2-phenoxypropionic Acid (9). The mixture of compound **8** (6 mg, 0.011 mmol), 2 N NaOH (0.2 mL), and MeOH (0.4 mL) was stirred for 2.5 h at room temperature and then diluted with water (0.5 mL). White solid was deposited by adjusting the pH to 2–3 with 1 N HCl. Isolation of this material by filtration gave the title compound **9** (5 mg) as an off-white solid: mp 73–75 °C; ^1H NMR (acetone- d_6 , 500 MHz) δ 2.81 (brs, 1H, OH and H_2O), 3.15 (dd, $J = 14.5$, 8.0 Hz, 1H, ArCH_aH_b), 3.21 (dd, $J = 14.5$, 5.0 Hz, 1H, ArCH_aH_b), 3.99–4.06 (m, 4H, CH_2NCH_2 -), 4.30 (t, $J = 6.0$ Hz, 2H, $\text{ArOCH}_2\text{CH}_2\text{N}$), 4.77 (tt, $J = 47.5$, 5.5 Hz, 2H, $\text{FCH}_2\text{-CH}_2\text{N}$), 4.86 (dd, $J = 8.0$, 5.0 Hz, 1H, ArCH_2CH), 6.86–6.91 (m, 5H), 7.00 (t, $J = 7.5$ Hz, 1H), 7.14 (t, $J = 7.5$ Hz, 1H), 7.21–7.31 (m, 6H); ^{13}C NMR (CDCl_3 , 125 MHz) δ 37.77, 48.79, 49.87 (d, $J = 22$ Hz), 65.53, 77.26, 82.66 (d, $J = 165.0$ Hz, FCH_2), 108.70, 114.24, 115.10, 116.03, 120.35, 121.16, 123.84, 129.34, 130.58, 143.66, 149.08, 157.64; FABMS m/z (%) 465 ($M^+ + 1$, 98), 207 (35), 119 (100), 115 (45); FABHRMS Calcd/Found for $\text{C}_{26}\text{H}_{26}\text{FN}_2\text{O}_5$ ($M + \text{H}^+$) 465.18256/465.18257.

2-(2-Hydroxyamino)benzoxazole (10). To a solution of 2-chlorobenzoxazole (3.30 g, 21.5 mmol) in THF (30 mL) was added 2-hydroxyethylamine (2.70 g, 45.0 mmol) at room temperature. The mixture was stirred for 2 h, poured into water (200 mL), and extracted with EtOAc (50 mL $\times$ 3). The extract was dried over MgSO_4 and evaporated under reduced pressure to give title compound **10** (3.53 g, 92%) as a white solid: mp 156–157 °C; ^1H NMR (methanol- d_4 , 500 MHz) δ 3.45 (t, $J = 6.0$ Hz, 2H), 3.74 (t, $J = 6.0$ Hz, 2H), 6.99–7.25 (m, 4H, ArH); MS (EI) m/z (%) 178 (M^+ , 57), 147 (97), 134 (100).

2-(2-Methanesulfonyloxyamino)benzoxazole (11). To a solution of compound **10** (2.0 g, 11.2 mmol) in pyridine (5 mL) was added methanesulfonyl chloride (1.3 g, 11.3 mmol) through a microsyringe at room temperature. After stirring for 2 h, the mixture was poured into water (30 mL) and extracted with EtOAc (20 mL $\times$ 3). The extract was washed with 0.1 N HCl solution and brine, dried (MgSO_4), and concentrated under reduced pressure. The residue was chromatographed on SiO_2 with ether to give title compound **11** (2.15 g, 86%) as a colorless solid; mp 94–95 °C; ^1H NMR (CDCl_3 , 400 MHz) δ 3.01 (s, 3H), 3.84 (t, $J = 4.8$ Hz, 2H), 4.82 (t, $J = 4.8$ Hz, 2H, $\text{CH}_2\text{-OMs}$), 7.05 (t, $J = 8.0$ Hz, 1H), 7.18 (t, $J = 8.0$ Hz, 1H), 7.26 (d, $J = 8.0$ Hz, 1H), 7.36 (d, $J = 8.0$ Hz, 1H); MS (EI) m/z (%) 256 (M^+ , 34), 177 (32), 160 (47), 147 (100).

4-[2-[(Benzoxazol-2-yl)amino]ethoxy]benzaldehyde (12). To the suspension of NaH (95 mg, 2.38 mmol, 60%) in DMF (15 mL) was added portionwise *p*-hydroxybenzaldehyde (280 mg, 2.28 mmol) at 0 °C. To this solution was added compound **11** (580 mg, 2.27 mmol) at room temperature, and the mixture was heated at 80 °C for 5 h. The solution was poured into water (40 mL), and the solid that deposited was collected by filtration to give the title compound **12** (510 mg, 80%) as a colorless solid; mp 115–116 °C; ^1H NMR (acetone- d_6 , 500 MHz) δ 3.90 (t, $J = 5.5$ Hz, 2H, NHCH_2), 4.41 (t, $J = 5.5$ Hz, 2H, CH_2OAr), 7.00 (t, $J = 7.5$ Hz, 1H), 7.11–7.28 (m, 6H, NH and ArH), 7.86 (d, $J = 9.0$ Hz, 2H), 9.89 (s, 1H, CHO); MS (FAB) m/z (%) 283 ($M^+ + 1$, 100), 118 (77).

Methyl 2-(2-Methanesulfonyloxy)ethoxy-2-diethylphosphonoacetate (13). The mixture of methyl 2-diazo-2-diethylphosphonoacetate (0.5 g, 2.12 mmol), 2-methanesulfonyloxyethyl alcohol (0.59 g, 4.24 mmol), and

rhodium(II) acetate (10 mg, 2.26×10^{-5} mol, 1 mol %) in the solvent mixture of benzene (15 mL) and chloroform (7 mL) was refluxed for 8 h. Evaporation of the solvent, followed by chromatography of the residue on SiO_2 with EtOAc gave the title compound **13** (580 mg, 79%) as a colorless liquid. ^1H NMR (CDCl_3 , 500 MHz) δ 1.30–1.35 (m, 6H, $2 \times (\text{OCH}_2\text{CH}_3)$), 3.10 (s, 3H), 3.82 (s, 3H), 3.83–3.87 (m, 1H, $\text{MsOCH}_2\text{CH}_a\text{H}_b$), 3.94–3.98 (m, 1H, $\text{MsOCH}_2\text{-CH}_a\text{H}_b$), 4.12–4.24 (m, 4H, $2 \times (\text{OCH}_2\text{CH}_3)$), 4.33 (d, $J = 18.5$ Hz, 1H, POCH), 4.42 (t, $J = 4.5$ Hz, 2H, MsOCH_2); ^{13}C NMR (CDCl_3 , 125 MHz) δ 16.35, 16.39 (2C, $2 \times (\text{OCH}_2\text{CH}_3)$), 37.78, 52.82 (1C, OMe), 63.85 (d, 1C, $J = 6.5$ Hz, P(O) (OCH_2CH_3)), 63.95 (d, 1C, $J = 6.5$ Hz, P(O) (OCH_2CH_3)), 68.76, 70.33, 76.44 (d, 1C, $J = 156.5$, POCH), 167.34; MS (FAB) m/z (%) 349 ($M^+ + 1$, 100); FABHRMS Calcd/Found for $\text{C}_{10}\text{H}_{21}\text{PSO}_9$ ($M + \text{H}^+$) 349.0723/349.0722.

Methyl 2-(2-Fluoroethoxy)-2-diethylphosphonoacetate (14). The method described for the preparation of compound **13**, using 2-fluoroethanol in place of 2-mesyloxyethanol, furnished the desired product **14** as a colorless liquid (yield 91%); ^1H NMR (CDCl_3 , 500 MHz) δ 1.30–1.36 (m, 6H, $2 \times (\text{OCH}_2\text{CH}_3)$), 3.83 (s, 3H), 3.79–3.91 (m, 1H, $\text{FCH}_2\text{CH}_a\text{H}_b$), 3.92–4.01 (m, 1H, $\text{FCH}_2\text{-CH}_a\text{H}_b$), 4.19–4.27 (m, 4H, $2 \times (\text{OCH}_2\text{CH}_3)$), 4.45 (d, $J = 19$ Hz, 1H, POCH), 4.51–4.60 (m, 1H, $\text{FCH}_a\text{CH}_b\text{CH}_2$), 4.60–4.70 (m, 1H, $\text{FCH}_a\text{CH}_b\text{CH}_2$); ^{13}C NMR (CDCl_3 , 125 MHz) δ 16.32, 16.37 (2C, $2 \times (\text{OCH}_2\text{CH}_3)$), 52.74 (1C, OMe), 63.90 (d, 1C, $J = 6.5$ Hz, FCH_2CH_2), 71.57 (d, 1C, $J = 12$ Hz, P(O) (OCH_2CH_3)), 71.72 (d, 1C, $J = 12$ Hz, P(O) (OCH_2CH_3)), 77.18 (d, 1C, $J = 156.6$, POCH), 82.82 (d, 1C, $J = 169.4$ Hz, FCH_2), 167.65; MS (FAB) m/z (%) 273 ($M^+ + 1$, 100); FABHRMS Calcd/Found for $\text{C}_9\text{H}_{18}\text{-PO}_6$ ($M + \text{H}^+$) 273.0903/273.0903.

Methyl 2-(2-*tert*-Butyldimethylsilyloxy)ethoxy-2-diethylphosphonoacetate (15). The method described for the preparation of compound **13**, using 2-(*tert*-butyldimethylsilyloxy)ethanol in place of 2-mesyloxyethanol, furnished the desired product **15** as a colorless oil (yield 66%); ^1H NMR (CDCl_3 , 500 MHz) δ 0.05 (s, 6H), 0.89 (s, 9H), 1.32–1.36 (m, 6H, $2 \times (\text{OCH}_2\text{CH}_3)$), 3.61–3.67 (m, 1H, $\text{SiOCH}_a\text{H}_b\text{CH}_2$), 3.72–3.83 (m, 6H, OCH_3 , $\text{SiOCH}_a\text{-H}_b\text{CH}_2$), 4.20–4.24 (m, 4H, $2 \times (\text{OCH}_2\text{CH}_3)$), 4.56 (d, $J = 19$ Hz, 1H, POCH); ^{13}C NMR (CDCl_3 , 125 MHz) δ 16.32, 16.37 (2C, $2 \times (\text{OCH}_2\text{CH}_3)$), 52.74 (1C, OMe), 63.90 (d, 1C, $J = 6.5$ Hz, FCH_2CH_2), 71.57 (d, 1C, $J = 12$ Hz, P(O) (OCH_2CH_3)), 71.72 (d, 1C, $J = 12$ Hz, P(O) (OCH_2CH_3)), 77.18 (d, 1C, $J = 156.6$, POCH), 82.82 (d, 1C, $J = 169.4$ Hz, FCH_2), 167.65; MS (FAB) m/z (%) 273 ($M^+ + 1$, 100); ^{13}C NMR (CDCl_3 , 125 MHz) δ -5.49, -5.42, 16.35, 16.40 (2C, $2 \times (\text{OCH}_2\text{CH}_3)$), 18.24, 25.82, 52.74 (1C, OMe), 62.87 (SiOCH_2), 63.67, 63.73 (2C, P(O) (OCH_2CH_3)), 73.75, 73.84, 77.28 (d, 1C, $J = 155.6$, POCH), 168.06; MS (FAB) m/z (%) 385 ($M^+ + 1$, 100).

2-(2-Benzoxazolylmethylamino)ethanol (16). Reaction of 2-chlorobenzoxazole with *N*-methylethanolamine, according to the method described for the preparation of **10**, furnished the desired product **16** as a colorless solid after recrystallization from EtOAc; mp 61–62 °C (lit. (24) 62–63 °C); yield 86%; ^1H NMR (CDCl_3 , 400 MHz) δ 3.22 (s, 3H, NCH_3), 3.67 (t, $J = 5.5$ Hz, 2H), 3.90 (t, $J = 5.5$ Hz, 2H), 4.25 (brs, 1H, OH), 6.96 (t, $J = 7.5$ Hz, 1H), 7.00 (t, $J = 7.5$ Hz, 1H), 7.23 (d, $J = 7.5$ Hz, 1H), 7.30 (d, $J = 7.5$ Hz, 1H); ^{13}C NMR (CDCl_3 , 125 MHz) δ 36.64, 53.20, 60.55, 108.61, 115.71, 120.35, 123.86, 142.66, 148.65, 163.05; MS (FAB) 193 ($M^+ + 1$, 100) 161 (10).

2-[(*N*-Methyl-2-methanesulfonyloxyethyl)amino]benzoxazole (17). Mesylation, according to the method

described for the preparation of **11**, furnished the desired product as a colorless solid; mp 95–96 °C (lit. (24) 97–98 °C); yield 71%; ^1H NMR (CDCl_3 , 400 MHz) δ 3.00 (s, 3H, NCH_3), 3.31 (s, 3H), 3.91 (t, $J = 5.5$ Hz, 2H), 4.52 (t, $J = 5.5$ Hz, 2H), 7.02 (t, $J = 7.5$ Hz, 1H), 7.18 (t, $J = 7.5$ Hz, 1H), 7.28 (d, $J = 7.5$ Hz, 1H), 7.35 (d, $J = 7.5$ Hz, 1H); MS (FAB) 271 ($\text{M}^+ + 1$, 100) 161 (10), 119 (6).

4-[2-(2-Benzoxazolylmethylamino)ethoxy]-benzaldehyde (18). Reaction of mesylate **17** with *p*-hydroxybenzaldehyde, according to the method described for the preparation of compound **12**, furnished the desired product **18** as a colorless solid (yield 96%); mp 90–91 °C (lit. (24) 96–98 °C); ^1H NMR (CDCl_3 , 500 MHz) δ 3.36 (s, 3H, NCH_3), 3.99 (t, $J = 5.5$ Hz, 2H, NHCH_2), 4.36 (t, $J = 5.5$ Hz, 2H, CH_2OAr), 7.00 (d, $J = 8.5$ Hz, 2H), 7.03 (t, $J = 7.5$ Hz, 1H), 7.16 (t, $J = 7.5$ Hz, 1H), 7.26 (d, $J = 7.5$ Hz, 1H), 7.36 (d, $J = 7.5$ Hz, 1H), 7.83 (d, $J = 8.5$ Hz, 2H), 9.88 (s, 1H, CHO); ^{13}C NMR (CDCl_3 , 125 MHz) δ 37.48, 49.94, 66.47, 108.78, 114.75, 116.20, 120.57, 124.09, 130.31, 132.05, 149.01, 158.20, 162.28, 163.40, 190.76; MS (FAB) m/z (%) 297 ($\text{M}^+ + 1$, 100), 175 (24), 119 (28).

Methyl 3-{4-[2-[Benzoxazol-2-yl-methylamino]ethoxy]phenyl}-2-(2-fluoroethoxy)acrylate (19). (A) The compound **19** was prepared from the reaction of aldehyde **18** and phosphonate **14**, as described in the preparation of compound **5**. The geometric isomers were separated by silica gel column chromatography with a mixture of EtOAc and hexane (1:1.2) as eluent. *Z*-isomer: colorless liquid; yield 58%; ^1H NMR (CDCl_3 , 400 MHz) δ 3.35 (s, 3H), 3.83 (s, 3H), 3.96 (t, $J = 5.2$ Hz, 2H, CH_2N), 4.18 (two pseudotriplets, $J = 30$ Hz, 2H, $\text{FCH}_2\text{CH}_2\text{O}$), 4.29 (t, $J = 5.2$ Hz, 2H, $\text{ArOCH}_2\text{CH}_2\text{N}$), 4.67 (two pseudotriplets, $J = 47.6$ Hz, 2H, $\text{FCH}_2\text{CH}_2\text{O}$), 6.88 (d, $J = 8.8$ Hz, 2H), 7.00 (s, 1H, vinyl H), 7.01 (t, $J = 8.0$ Hz, 1H), 7.14 (t, $J = 8.0$ Hz, 1H), 7.26 (d, $J = 8.0$ Hz, 1H), 7.36 (d, $J = 8.0$ Hz, 1H), 7.75 (d, $J = 8.8$ Hz, 2H); ^{13}C NMR (CDCl_3 , 100 MHz) δ 37.33, 49.87, 52.01, 66.04, 70.42 (d, $J = 19$ Hz, 1C, FCH_2CH_2), 82.41 (d, $J = 169.0$ Hz, FCH_2), 108.60, 114.34, 115.96, 120.32, 123.87, 124.79, 126.08, 132.02, 141.84, 148.79, 158.98, 164.75; FABMS m/z (%) 415 ($\text{M}^+ + 1$, 100), 175 (64); FABHRMS Calcd/Found for $\text{C}_{22}\text{H}_{24}\text{FN}_2\text{O}_5$ ($\text{M} + \text{H}^+$) 414.1671/414.1669.

E-isomer: colorless liquid; yield 34%; ^1H NMR (CDCl_3 , 400 MHz) δ 3.35 (s, 3H), 3.69 (s, 3H), 3.95 (t, $J = 5.6$ Hz, 2H, CH_2N), 4.08 (two pseudotriplets, $J = 27.6$ Hz, 2H, $\text{FCH}_2\text{CH}_2\text{O}$), 4.26 (t, $J = 5.6$ Hz, 2H, $\text{ArOCH}_2\text{CH}_2\text{N}$), 4.72 (two pseudotriplets, $J = 47.2$ Hz, 2H, $\text{FCH}_2\text{CH}_2\text{O}$), 6.21 (s, 1H, vinyl H), 6.82 (d, $J = 8.8$ Hz, 2H), 7.01 (t, $J = 8.0$ Hz, 1H), 7.15 (d, $J = 8.8$ Hz, 2H), 7.16 (t, $J = 8.0$ Hz, 1H), 7.26 (d, $J = 8.0$ Hz, 1H), 7.35 (d, $J = 8.0$ Hz, 1H); FABMS m/z (%) 415 ($\text{M}^+ + 1$, 50), 175 (25), 154 (100); FABHRMS Calcd/Found for $\text{C}_{22}\text{H}_{24}\text{FN}_2\text{O}_5$ ($\text{M} + \text{H}^+$) 414.1671/414.1669.

(B) The title compound **19** was also prepared by the reaction of compound **25** (30 mg, 0.06 mmol) and tetrabutylammonium fluoride (0.07 mL of a 1 M solution in THF) in DMSO (0.5 mL) at 80 °C for 10 min as described in the preparation of compound **9**. The *Z*- and *E*-isomers were obtained in the ratio of 33% and 21%, using the same purification procedure stated in preparation A, above.

3-{4-[2-[Benzoxazol-2-yl-methylamino]ethoxy]phenyl}-2-(2-fluoroethoxy)acrylic Acid (20; 20(*E*) and 20(*Z*)). Hydrolysis of the separated *E* and *Z* isomers of ester **19**, according to the method described for the preparation of compound **9**, with a reaction time of 1 h, gave the *E* and *Z* isomers of acid **20**. *Z*-isomer: mp 154–

155 °C; yield 92%; ^1H NMR (methanol- d_4 , 500 MHz) δ 3.30 (s, 3H), 3.96 (t, $J = 5.0$ Hz, 2H, CH_2N), 4.14 (tt, $J = 30.5$ Hz, 2H, $\text{FCH}_2\text{CH}_2\text{O}$), 4.30 (t, $J = 5.0$ Hz, 2H, $\text{ArOCH}_2\text{CH}_2\text{N}$), 4.63 (tt, $J = 47.6$ Hz, 2H, $\text{FCH}_2\text{CH}_2\text{O}$), 6.90 (d, $J = 9.0$ Hz, 2H), 7.00 (s, 1H, vinyl H), 7.03 (t, $J = 8.0$ Hz, 1H), 7.16 (t, $J = 8.0$ Hz, 1H), 7.27 (d, $J = 8.0$ Hz, 1H), 7.30 (d, $J = 8.0$ Hz, 1H), 7.75 (d, $J = 9.0$ Hz, 2H); ^{13}C NMR (methanol- d_4 , 500 MHz) δ 35.74, 49.37, 65.30, 70.31 (d, $J = 19$ Hz, FCH_2CH_2), 82.39 (d, $J = 168.5$ Hz, FCH_2), 108.53, 114.14, 115.07, 120.57, 123.87, 124.00, 126.41, 131.80, 142.38, 142.48, 148.65, 159.22, 166.17, 166.28; FABMS m/z (%) 401 ($\text{M}^+ + 1$, 100), 175 (50); FABHRMS Calcd/Found for $\text{C}_{21}\text{H}_{22}\text{FN}_2\text{O}_5$ ($\text{M} + \text{H}^+$) 401.1514/401.1513.

E-isomer: mp 128–129 °C; yield 90%; ^1H NMR (methanol- d_4 , 500 MHz) δ 3.29 (s, 3H), 3.94 (t, $J = 5.5$ Hz, 2H, CH_2N), 4.07 (tt, $J = 21.0$ Hz, 2H, $\text{FCH}_2\text{CH}_2\text{O}$), 4.25 (t, $J = 5.5$ Hz, 2H, $\text{ArOCH}_2\text{CH}_2\text{N}$), 4.67 (tt, $J = 47.5$ Hz, 2H, $\text{FCH}_2\text{CH}_2\text{O}$), 6.18 (s, 1H, vinyl H), 6.82 (d, $J = 8.5$ Hz, 2H), 7.03 (t, $J = 8.0$ Hz, 1H), 7.16 (t, $J = 8.0$ Hz, 1H), 7.20 (d, $J = 8.5$ Hz, 1H), 7.27 (d, $J = 8.0$ Hz, 1H), 7.30 (d, $J = 8.0$ Hz, 1H); ^{13}C NMR (methanol- d_4 , 500 MHz) δ 35.74, 49.44, 65.30, 68.22 (d, $J = 20$ Hz, FCH_2CH_2), 81.35 (d, $J = 168.5$ Hz, FCH_2), 108.56, 110.54, 113.75, 115.03, 120.59, 123.89, 127.26, 129.65, 142.29, 146.20, 148.62, 157.70, 162.69, 166.18; FABMS m/z (%) 401 ($\text{M}^+ + 1$, 100), 175 (23), 155 (38), 119 (69); FABHRMS Calcd/Found for $\text{C}_{21}\text{H}_{22}\text{FN}_2\text{O}_5$ ($\text{M} + \text{H}^+$) 401.1514/401.1513.

Methyl 3-{4-[2-[Benzoxazol-2-yl-methylamino]ethoxy]phenyl}-2-(2-fluoroethoxy)propanoate (21). (A) The title compound **21** was prepared by hydrogenation of the isomeric unsaturated esters **19**, as described in the preparation of compound **6**: Colorless liquid; yield ~ 100%; ^1H NMR (CDCl_3 , 500 MHz) δ 2.96 (dd, $J = 14.5$, 8.0 Hz, 1H, ArCH_aH_b), 3.01 (dd, $J = 14.5$, 5.0 Hz, 1H, ArCH_aH_b), 3.35 (s, 3H), 3.58 (double multiplet, $J = 27.5$ Hz, 1H, $\text{FCH}_2\text{CH}_a\text{H}_b$), 3.70 (s, 3H), 3.79 (double multiplet, $J = 27.5$ Hz, 1H, $\text{FCH}_2\text{CH}_a\text{H}_b$), 3.95 (t, $J = 5.0$ Hz, 2H, $\text{ArOCH}_2\text{CH}_2\text{N}$), 4.06 (dd, $J = 8.0$, 5.0 Hz, 1H, ArCH_2CH), 4.24 (t, $J = 5.0$ Hz, 2H, $\text{ArOCH}_2\text{CH}_2\text{N}$), 4.49 (double multiplet, $J = 53$ Hz, 2H, $\text{FCH}_2\text{CH}_2\text{N}$), 6.80 (d, $J = 8.0$ Hz, 2H), 7.01 (t, $J = 7.5$ Hz, 1H), 7.13–7.18 (m, 3H), 7.26 (d, $J = 7.5$ Hz, 1H), 7.36 (d, $J = 7.5$ Hz, 1H); ^{13}C NMR (CDCl_3 , 125 MHz) δ 37.46, 38.33, 50.12, 51.96, 66.17, 69.87 (d, $J = 20$ Hz, FCH_2CH_2), 80.86, 82.93 (d, $J = 169.5$ Hz, FCH_2CH_2), 108.76, 114.28, 116.07, 116.54, 120.47, 124.04, 129.39, 130.49, 144.61, 148.90, 157.32, 172.36; MS (FAB) (%) m/z 417 ($\text{M}^+ + 1$, 45), 154 (100), 136 (62); FABHRMS Calcd/Found for $\text{C}_{22}\text{H}_{26}\text{FN}_2\text{O}_5$ ($\text{M} + \text{H}^+$) 417.1825/417.1825.

(B) This compound was also prepared by the reaction of compound **26** (30 mg, 0.06 mmol) and TBAF (0.07 mL, 1 M THF solution) in DMSO (0.5 mL) at 80 °C for 10 min, followed by chromatography on SiO_2 with ether as eluent, as described in the preparation of compound **5** (12 mg, 48%).

3-{4-[2-[Benzoxazol-2-yl-methylamino]ethoxy]phenyl}-2-(2-fluoroethoxy)propionic Acid (22). Hydrolysis of ester **21**, according to the method described for the preparation of compound **9**, with a reaction time of 30 min, gave the acid **22**: colorless solid; mp 106–107 °C; yield 91%; ^1H NMR (methanol- d_4 , 500 MHz) δ 2.89 (dd, $J = 14.0$, 8.0 Hz, 1H, ArCH_aH_b), 2.99 (dd, $J = 14.0$, 4.5 Hz, 1H, ArCH_aH_b), 3.28 (s, 3H), 3.57 (double multiplet, $J = 28.5$ Hz, 1H, $\text{FCH}_2\text{CH}_a\text{H}_b$), 3.76 (double multiplet, $J = 28.5$ Hz, 1H, $\text{FCH}_2\text{CH}_a\text{H}_b$), 3.93 (t, $J = 5.0$ Hz, 2H, $\text{ArOCH}_2\text{CH}_2\text{N}$), 4.07 (dd, $J = 8.0$, 4.5 Hz, 1H, ArCH_2CH), 4.23 (t, $J = 5.0$ Hz, 2H, $\text{ArOCH}_2\text{CH}_2\text{N}$), 4.43 (double multiplet, $J = 48$ Hz, 2H, $\text{FCH}_2\text{CH}_2\text{N}$), 6.80 (d, $J = 8.0$

Hz, 2H), 7.03 (t, $J = 7.5$ Hz, 1H), 7.13–7.18 (m, 3H), 7.27 (d, $J = 7.5$ Hz, 1H), 7.30 (d, $J = 7.5$ Hz, 1H); ^{13}C NMR (CDCl_3 , 125 MHz) δ 35.74, 37.75, 49.50, 65.29, 69.56 (d, $J = 20$ Hz, FCH_2CH_2), 80.26, 82.55 (d, $J = 167.6$ Hz, $\text{F-CH}_2\text{CH}_2$), 108.55, 113.92, 115.01, 120.58, 123.88, 129.67, 130.21, 142.25, 148.61, 157.37, 166.28, 174.11; MS (FAB) (%) m/z 403 ($\text{M}^+ + 1$, 100), 175 (32) FABHRMS Calcd/Found for $\text{C}_{21}\text{H}_{23}\text{FN}_2\text{O}_5$ ($\text{M} + \text{H}^+$) 403.1669/403.1669.

Methyl 3-{4-[2-[Benzoxazol-2-yl-methylamino]-ethoxy]phenyl}-2-(2-dimethyl-*tert*-butylsilyloxy-ethoxy)propanoate (23). Condensation of aldehyde **18** with phosphonate **15**, according to the method described for the preparation of compound **5**, furnished the desired product **23** as a colorless liquid (yield 90%). ^1H NMR (CDCl_3 , 500 MHz) [Peaks are doubled because of geometric isomerism] δ 0.05, 0.09 (s, combined, 6H), 0.89, 0.90 (s, combined, 9H), 3.34, 3.35 (s, combined, 3H, NCH_3), 3.67, 3.81 (s, combined, 3H), 3.88–4.00 (m, 6H, NCH_2 , $\text{CH}_2\text{CH}_2\text{OSi}$), 4.25, 4.29 (t, $J = 5.0$ Hz, combined, 2H, ArOCH_2), 6.14 (s, 0.4H, *E*-vinyl H), 6.93 (s, 0.6H, *Z*-vinyl H), 6.80–7.78 (m, 8H); MS (FAB) (%) m/z 527 ($\text{M}^+ + 1$, 100), 175 (60), 148 (47).

Methyl 3-{4-[2-[Benzoxazol-2-yl-methylamino]-ethoxy]phenyl}-2-(2-hydroxyethoxy)propanoate (24). The mixture of TBS ether **23** (158 mg, 0.3 mmol) and acetic acid (1.4 mL) in THF–water (2 mL, 2:1, v/v) was stirred for 5 h at 45 °C. CH_2Cl_2 (20 mL) was added to the reaction mixture, which was then washed with sat. NaHCO_3 solution, brine, and water. After drying the extract over MgSO_4 , evaporation gave the title compound **24** (103 mg, 88%) as a colorless liquid. ^1H NMR (CDCl_3 , 500 MHz) [Peaks are doubled because of geometric isomerism] δ 3.34 (s, 3H, NCH_3), 3.67, 3.83 (s, combined, 3H), 3.80–3.83 (m, 1.2H, *Z*-isomer, CH_2OH), 3.90–4.00 (m, 4.8H, NCH_2 , OCH_2 of *E* and *Z*, and CH_2OH of *E*), 4.25, 4.29 (t, $J = 6.5$ Hz, combined, 2H, ArOCH_2), 6.22 (s, 0.4H, *E*-vinyl H), 6.80–7.78 (m, 8.4H, *Z*-vinyl H and ArH); MS (FAB) (%) m/z 413 ($\text{M}^+ + 1$, 100), 175 (59).

Methyl 3-{4-[2-[Benzoxazol-2-yl-methylamino]-ethoxy]phenyl}-2-(2-methanesulfonyloxyethoxy)acrylate (25). (A) Reaction of aldehyde **18** with phosphonate **13**, according to the method described for the preparation of compound **5**, furnished the desired product **25** as a mixture of geometric isomers that was separated by SiO_2 column chromatography with EtOAc as eluent, giving 51% (*Z*-isomer) and 27% (*E*-isomer). *E*-isomer: colorless liquid; ^1H NMR (CDCl_3 , 500 MHz) δ 3.12 (s, 3H), 3.35 (s, 3H, NCH_3), 3.67 (s, 3H, OCH_3), 3.95 (t, $J = 5.5$ Hz, 2H, NCH_2), 4.10–4.12 (m, 2H, $\text{OCH}_2\text{CH}_2\text{OMs}$), 4.26 (t, $J = 5.5$ Hz, 2H), 4.54–4.56 (m, 2H, $\text{OCH}_2\text{CH}_2\text{OMs}$), 6.23 (s, 1H, *E*-vinyl H), 6.82 (d, $J = 8.0$ Hz, 2H), 7.00 (t, $J = 7.5$ Hz, 1H), 7.15 (d, $J = 8.0$ Hz, 2H), 7.16 (t, $J = 7.5$ Hz, 1H), 7.26 (d, $J = 7.5$ Hz, 1H), 7.36 (d, $J = 7.5$ Hz, 1H); ^{13}C NMR (CDCl_3 , 100 MHz) δ 37.45, 37.83, 50.67, 52.67, 52.09, 66.19, 67.08, 67.73, 108.75, 113.92, 114.15, 114.62, 116.11, 120.47, 124.03, 126.50, 130.02, 132.15, 144.82, 148.98, 157.94, 164.21; MS (FAB) (%) m/z 491 ($\text{M}^+ + 1$, 50), 381 (28), 175 (100).

Z-isomer: colorless liquid; ^1H NMR (CDCl_3 , 500 MHz) δ 2.98 (s, 3H), 3.35 (s, 3H, NCH_3), 3.83 (s, 3H, OCH_3), 3.96 (t, $J = 5.5$ Hz, 2H, NCH_2), 4.21–4.22 (m, 2H, $\text{OCH}_2\text{CH}_2\text{OMs}$), 4.30 (t, $J = 5.5$ Hz, 2H, ArOCH_2), 4.48–4.50 (m, 2H, $\text{OCH}_2\text{CH}_2\text{OMs}$), 6.89 (d, $J = 8.5$ Hz, 2H), 7.01 (t, $J = 7.5$ Hz, 1H), 7.01 (s, 1H, *Z*-vinyl H, overlap with ArH of benzoxazole), 7.16 (t, $J = 7.5$ Hz, 2H), 7.26 (d, $J = 7.5$ Hz, 1H), 7.36 (d, $J = 7.5$ Hz, 1H), 7.72 (d, $J = 8.5$ Hz, 1H); ^{13}C NMR (CDCl_3 , 100 MHz) δ 37.45, 37.59, 49.99, 52.23, 66.23, 68.62, 68.95, 108.77, 114.45, 114.62, 116.14, 116.63, 120.48, 124.03, 125.21, 126.00, 132.16, 141.73,

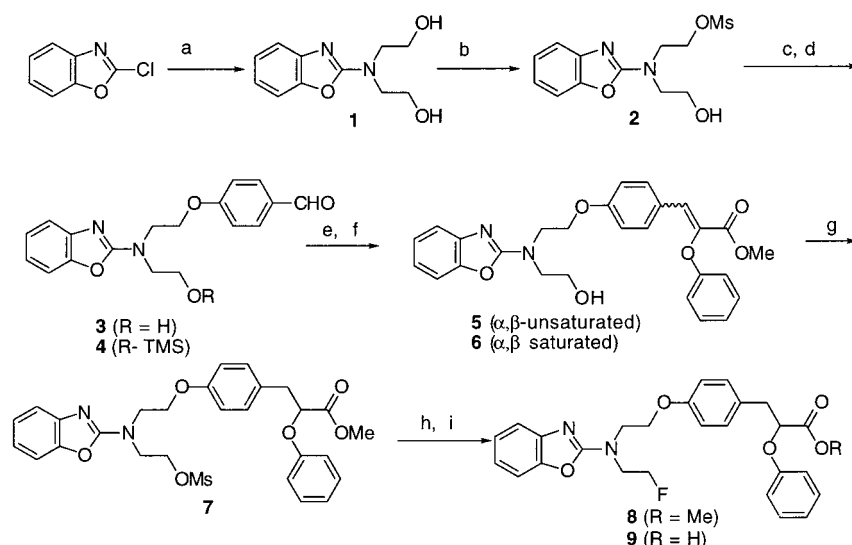
149.00, 159.34, 164.73; MS (FAB) (%) m/z 491 ($\text{M}^+ + 1$, 50), 381 (28), 175 (100).

(B) The title compound was also prepared by the reaction of the isomeric mixture of alcohols **24** (100 mg, 0.26 mmol) and methanesulfonyl chloride (30 mg, 0.26 mmol) in pyridine (2 mL) at room temperature, as described in the preparation of **11** (yield 87%).

Methyl 3-{4-[2-[Benzoxazol-2-yl-methylamino]-ethoxy]phenyl}-2-(2-methanesulfonyloxy-ethoxy)propanoate (26). Hydrogenation of the unsaturated ester **25**, according to the method described for the preparation of compound **6**, furnished the desired product **26** as a colorless liquid (yield ~ 100%); ^1H NMR (CDCl_3 , 500 MHz) δ 2.91 (s, 3H, OSO_2CH_3), 2.91 (dd, 1H, $J = 8.5$, 14.5 Hz, ArCH_aHCH), 3.01 (dd, 1H, $J = 4.5$, 14.5 Hz, ArCHH_bCH), 3.33 (s, 3H, NCH_3), 3.53–3.57 (m, 1H, $\text{OCH}_a\text{HCH}_2\text{OMs}$), 3.70 (s, 3H), 3.80–3.84 (m, 1H, $\text{OCHH}_b\text{CH}_2\text{OMs}$), 3.93 (t, $J = 5.0$ Hz, 2H CH_2N), 4.06 (dd, $J = 4.5$, 8.5 Hz, 1H, ArCH_2CH), 4.22 (t, $J = 5.0$ Hz, 2H, $\text{NCH}_2\text{CH}_2\text{OAr}$), 4.28–4.30 (m, 2H, CH_2OMs), 6.83 (d, $J = 9.0$ Hz, 2H), 7.00 (t, $J = 7.5$ Hz, 1H), 7.10 (d, $J = 9.0$ Hz, 2H), 7.15 (t, $J = 7.5$ Hz, 1H), 7.24 (d, $J = 7.5$ Hz, 1H), 7.34 (d, $J = 7.5$ Hz, 1H); ^{13}C NMR (CDCl_3 , 125 MHz) δ 37.40, 37.52, 38.22, 50.06, 52.03, 66.19, 68.47, 68.96, 80.63, 108.73, 114.36, 116.09, 120.39, 123.98, 129.07, 130.44, 143.38, 149.00, 157.43, 162.44, 172.04; MS (FAB) (%) m/z 493 ($\text{M}^+ + 1$, 100), 175 (99), 148 (47).

Radiochemical Synthesis of Compound [^{18}F]-22.

Proton bombardment of $\text{H}_2[^{18}\text{O}]\text{O}$ yielded no-carrier added [^{18}F]fluoride. Tetrabutylammonium [^{18}F]fluoride ($\text{n-Bu}_4\text{N}[^{18}\text{F}]\text{F}$) was obtained by the addition of [^{18}F]fluoride in $\text{H}_2[^{18}\text{O}]\text{O}$ to 2.6 μL of $\text{n-tetrabutylammonium hydroxide}$ (1 M in H_2O). The water was removed by azeotropic distillation upon three successive additions of acetonitrile while heating at 110 °C under N_2 . With 200 μL of THF, $\text{n-Bu}_4\text{N}[^{18}\text{F}]\text{F}$ was transferred to a reactivial containing 1.5 mg of compound **20**. Typically 80–90% of the activity was transferred. The reactivial was capped and immersed in an 80 °C oil bath for 30 min to effect incorporation. After cooling for 3 min, the reaction mixture was loaded directly onto a Florisil “plug” (Pasteur pipet filled with ca. 100 mg of Florisil above a Kimwipe plug) and eluted (45:55 hexane, 1:19 2-propanol/ CH_2Cl_2 with 0.1% TFA added) into a flask. Activity in the flask, Florisil “plug” and reactivial was counted to determine the yield of fluoride incorporation (typically 65–90%). The [^{18}F]fluorine-substituted methyl ester [^{18}F]-**21** was purified by normal phase semipreparative HPLC (45:55 hexane, 1:19 2-propanol/ CH_2Cl_2 with 0.1% TFA added) on a silica column (Mag 9, 0.9 cm $\times$ 50 cm, Whatman) under isocratic conditions of 5 mL/min. The eluent was monitored by UV (254 nm) and radioactive (NaI/Tl) detection. The filtrate was loaded into the injection loop through a 0.45 μm filter. The filter was washed with an additional 1.0 mL of eluent (stated above) prior to injection. The fractions that corresponded to the fluorine-substituted methyl ester [^{18}F]-**21** were combined, counted, and concentrated under reduced pressure. To this sample of [^{18}F]-**21** was added 100 μL of MeOH and 25 μL of 10% NaOH solution. After heating for 15 min at 80 °C, the solution was acidified with 100 μL of 1 N HCl, extracted with 2 mL of 1:19 hexane/ CH_2Cl_2 (v/v), and passed directly through a MgSO_4 plug (Pasteur pipet with ca. 100 mg MgSO_4 above a Kimwipe plug). The plug was washed with an additional 1 mL of HPLC eluent (35:65 hexane, 1:19 2-propanol/ CH_2Cl_2 with 0.1% TFA added). The filtrate, MgSO_4 plug, and reaction flask were then counted to determine the deprotection yield (typically 80–90%). The filtrate was loaded onto a semipreparative

Scheme 2^a

^a Reagents and conditions: (a) diethanolamine, THF-DMF (3:1), 3 h, rt, 68%; (b) MsCl, pyr, 30 min, 0–5 °C, 48%; (c) NaH, DMF, *p*-hydroxybenzaldehyde, 8 °C, 86%; (d) TMSCl, TEA, THF, rt, 4 h, ~100%; (e) methyl 2-phenoxy-2-diethylphosphonoacetate, NaH, THF, 5 °C, 75%; (f) 10% Pd/C, H₂, MeOH, 99%; (g) MsCl, pyr, 0–5 °C, 71%; (h) TBAF, DMSO, 90 °C, 10 min, 67%; (i) 2 N NaOH, MeOH, rt, 2.5 h, 98%.

HPLC column (as specified above) through a 0.45 μm filter. An additional 1.0 mL of HPLC eluent was washed through the filter prior to injection onto the column. The fractions containing the desired fluoroacid [**¹⁸F]-**22** were combined, counted, concentrated under reduced pressure, and counted again. Typically, the overall radiochemical yield (two reactions and two purifications) was 20–30% decay corrected from n-Bu₄N[**¹⁸F]F. The entire synthesis and purification processes, starting from H₂[**¹⁸O]O, typically required 2.5–3.5 h.******

Tissue Distribution Studies of Compound [¹⁸F]-**22** in Rats and Mice.** All animal experiments were conducted in compliance with the Guidelines for the Care and Use of Research Animals established by Washington University's Animal Studies Committee. Animal doses were prepared by removing HPLC solvent under reduced pressure from collected fractions containing purified [**¹⁸F]-**22**. The radiolabeled compound was then dissolved in absolute EtOH and diluted with isotonic saline to give a 10% EtOH:90% saline solution.****

Anesthetized mature female Sprague-Dawley rats (200–225 g) were injected via the tail vein with either 30 μCi (normal dose) or 5 μCi (low dose) in a volume of 100 μL. The animals receiving a blocking dose were coinjected with 34 μg of unlabeled compound **22** (dissolved in 15% EtOH:85% saline) along with [**¹⁸F]-**22** giving a total injection volume of ca. 200 μL. Animals were sacrificed by decapitation at 1 h after receiving either a normal dose, low dose, or a normal dose with block, while animals sacrificed at 3 h received a low dose with or without block. Blood and tissues were removed, weighed, and counted in a Beckman Gamma 8000 counter. The upper and lower small intestine were voided of their contents, after being removed from the animal, by applying gentle pressure along the length of the tissue with a blunt instrument. Brown fat was removed from between the shoulder blades, while white fat was sampled from alongside the kidneys. Brown fat differs from white fat in appearance having a slightly darker color and a more solid and lumpy consistency.**

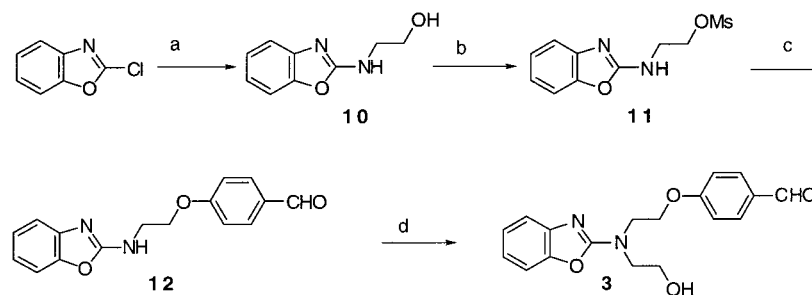
In female SCID mice (ca. 4 months, 20 g), 1 × 10⁷ MCF-7 cells (in 100 μL) were implanted bilaterally by subcutaneous injection in the left and right flanks. By

this method, two tumors were grown in each animal. These cells are a human line that is estrogen receptor-positive and require estradiol for growth; therefore, a 1.7 mg/60 day release pellet of estradiol (Innovative Research of America, Sarasota, FL) was inserted into the side of the neck of each animal. After 4 weeks of tumor growth, the biodistribution study of [**¹⁸F]-**22** in the tumor bearing animals was performed. Anesthetized SCID mice were injected via the tail vein with either 32 μCi (normal dose) or 5 μCi (low dose) in a volume of 100 μL. The animals receiving a blocking dose were coinjected with 29 μg of unlabeled compound **22** along with [**¹⁸F]-**22** giving a total injection volume of ca. 200 μL. Animals were sacrificed by decapitation at 1 h after receiving either a normal dose, low dose, or a normal dose with block, while animals sacrificed at 3 h received a low dose. A 3 h block was not included in the study due to a fewer number of tumor bearing animals. Tissue sampling and handling were as stated for the rats, except the gallbladder and tumors were harvested from the mice.****

RESULTS

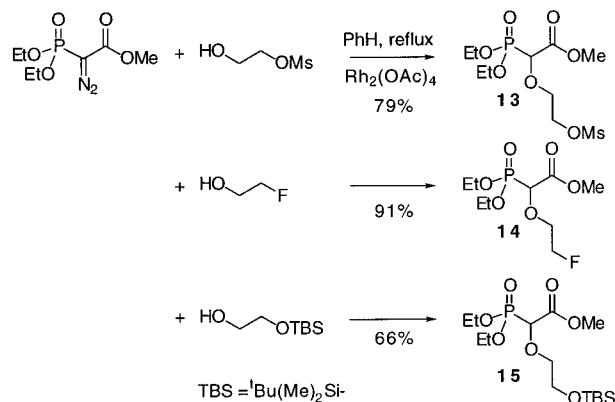
Chemical Synthesis. Ultimately, labeling of the PPARγ ligands with the positron emitter fluorine-18 is to be done by fluoride ion displacement of a suitable leaving group on a precursor molecule. Therefore, we designed a synthetic route to both of the fluorine-substituted ligands that furnished such a reactive intermediate (**7** and **26**).

Synthesis of 3-[4-[2-[Benzoxazol-2-yl-(2-fluoroethyl)amino]ethoxy]phenyl]-2-phenoxypropionic Acid (9**).** As shown in Scheme 2, compound **7**, the precursor needed for preparation of the ultimate fluorine-substituted product **9**, was synthesized in seven steps from 2-chlorobenzoxazole, using a modification of known methods (17, 24). The reaction of diethanolamine with 2-chlorobenzoxazole gave the tertiary amine diol **1**. The monomethanesulfonate **2** (mesylate) could be prepared quite selectively by two sequential additions of 0.5 equiv of methanesulfonyl chloride. Sulfonate displacement with *p*-hydroxybenzaldehyde, followed by protection of the free hydroxyl group in intermediate **3** as the trimethylsilyl ether, gave the protected aldehyde **4**. A somewhat more

Scheme 3^a

^a Reagents and conditions: (a) ethanolamine, THF, rt, 92%; (b) MsCl, pyr, rt, 2 h, 86%; (c) NaH, DMF, *p*-hydroxybenzaldehyde, 80 °C, 80%; (d) ethylene carbonate, K₂CO₃, DMF, 110 °C, 70%.

Scheme 4

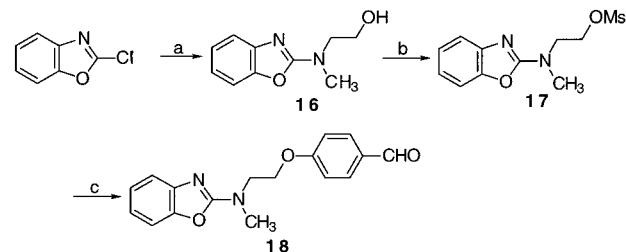


controlled and efficient route to the hydroxyaldehyde **3** is shown in Scheme 3. Continuing in Scheme 2, Horner–Emmons addition of methyl 2-phenoxycarbonylphosphonate to the aldehyde **4** (**23**) and subsequent hydrogenation of enoate **5**, followed by mesylation of hydroxyester **6**, provided the fluorination precursor **7**. This mesylate is relatively unstable and needs to be used soon after its preparation.

The crucial fluoride ion substitution reaction was examined using reagents (tetrabutylammonium fluoride (TBAF) or potassium 2.2.2 Kryptofix) and conditions (90 °C in DMSO or THF) suitable for ¹⁸F labeling (25). The best conditions proved to be TBAF in DMSO at 90 °C, and under these conditions, the displacement reaction, product isolation, and purification steps could be accomplished in ca. 2 h. The product of the fluorine substitution step consisted of the desired fluorinated methyl ester (**8**, 67%), together with small amounts of an undesired byproduct (18%) and unreacted starting material (**7**) (14%). Hydrolysis of methyl ester **8** with methanolic NaOH solution proceeded under mild conditions to give the desired fluorine-substituted phenylpropanoic acid **9** in 98% yield.

Synthesis of 3-{4-[2-[Benzoxazol-2-yl-methylamino]ethoxy]phenyl}-2-(2-fluoroethoxy)propionic Acid (22**).** To synthesize compound **22**, the fluorination precursor to the second PPAR_γ ligand, aldehyde **18** and the matched Horner–Emmons reagents **13**–**15** were prepared, as shown in Schemes 4 and 5, according to literature methods (21–23).

We first obtained an authentic sample of the final product (**22**) by the condensation of aldehyde **18** with the fluoroethylphosphonate **14**, followed by hydrogenation of the enoate **19** and hydrolysis of ester **21** (Scheme 6, right side). However, this approach is not practical for synthesis of the labeled material, because of the short half-life of fluorine-18.

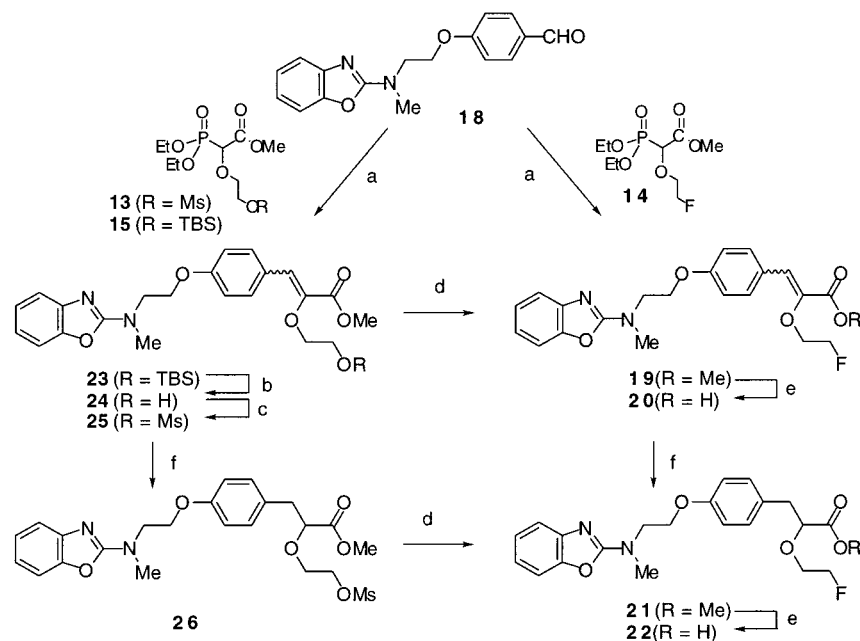
Scheme 5^a

^a Reagents and conditions: (a) *N*-methylhydroxyethylamine, THF, rt, 86%; (b) MsCl, pyr, rt, 71%; (c) NaH, *p*-hydroxybenzaldehyde, DMF, 96%.

The number of steps following the introduction of fluorine could be reduced by using the methanesulfonyloxyethyl analogue **13** in place of the fluoroethylphosphonate **14**, to give the enoate–methanesulfonate **25** (Scheme 6, left side). Alternatively, this compound can be prepared from aldehyde **18** and the TBS-protected phosphonate **15**, through compounds **23** and **24**. All of these unsaturated esters (**19**, **23**–**25**) were mixtures of *E*- and *Z*-isomers that could be separated by chromatography. Hydrogenation of enoate **25** (30 psi) over Pd/C afforded the saturated ester **26**. Fluoride substitution on either the unsaturated methanesulfonates **25** (as separated *E*- and *Z*-isomers) or the saturated methanesulfonate **26** with TBAF in DMSO at 80 °C for 10 min furnished the corresponding fluorine-substituted products **19** and **21** in around ca. 50% yields. The final acids, **20(E)**, **20(Z)**, and **22**, were obtained after mild alkaline hydrolysis.

Binding Affinity Determinations. The binding affinity of four fluorine-substituted ligands (**9**, **20(E)**, **20(Z)**, and **22**) for PPAR_γ, PPAR_α, and PPAR_δ was determined in a competitive radiometric scintillation proximity assay, as previously described (26,27), using receptor preparations consisting of the ligand binding domains of the three PPARs, expressed in *E. coli*. The results are shown in Table 1.

The two alkenes, **20(E)** and **20(Z)**, have low affinity, especially the *Z*-isomer. Of the remaining two, the *O*-fluoroethyl compound **22** is the better, having a *K_i* value of 7 nM for PPAR_γ, 25 nM for PPAR_α, and much lower affinity for PPAR_δ. Because the concentration of receptors used in this assay (5 nM) is rather high with respect to the *K_i* value (7 nM), the *K_d* value is, in fact, considerably lower, possibly as low as 5 nM. By comparison with the *K_d* value of the parent compound SB 213 068 (Scheme 1), one can see that substitution of fluorine on the α-ethoxy group (as in compound **22**) has only a small effect on the binding, which is consistent with established structural and functional tolerance at the α position (17, 18). By contrast, substitution of the *N*-

Scheme 6^a

^a Reagents and conditions: (a) THF, NaH, 0 °C 5 h 80–90%; (b) AcOH, THF–H₂O, 45 °C, 5 h 88%; (c) MsCl, pyr, 87%; (d) TBAF, DMSO, 80 °C, 10 min, 45%; (e) 2 N NaOH, rt, 1 h, 90%; (f) 10%Pd/C, H₂, MeOH, 100%.

Table 1. Binding Affinity of Fluorine-Substituted Ligands to PPARs

compd no.	binding affinity, K _i (nM)		
	PPAR γ	PPAR α	PPAR δ
9	17	350	>10000
22	7	25	2800
20(Z)	>10000	6200	>10000
20(E)	210	830	>10000

^a Determined by competitive binding, as described in refs 26, 27. Values represent the mean of replicate experiments.

methyl group with an *N*-(2-fluoroethyl) group reduces affinity substantially. The effect of alterations in *N*-substitution has not been widely investigated in this series (16, 17), and our result suggests that this site might not be very tolerant of structural changes.

It is of note that compounds **9** and **22** are racemates. On the basis of results in related series (28–30), it is likely that the *S* enantiomers of both compounds are responsible for most of the binding of their racemates to PPAR γ .

Synthesis of PPAR γ Ligand 22 in Fluorine-18 Labeled Form ([¹⁸F]-22). Because of its higher affinity for PPAR γ and its reasonable selectivity with respect to binding to the other two PPAR subtypes, we selected ligand **22** for preparation in fluorine-18 labeled form. The labeling reaction followed the same procedure as did the synthesis of the unlabeled compound, simply with substitution of fluorine-18 at the tracer level for fluorine-19 at the macroscopic level.

Fluorine-18 was generated from an oxygen-18 water target in a ¹⁸O(p,n)¹⁸F reaction and converted to tetrabutylammonium [¹⁸F]fluoride, using methodology described by us previously (25). At the tracer level, fluoride ion substitution on the methanesulfonate precursor **26** proceeded well, with more than 65% incorporation of radioactivity occurring within 30 min at 80 °C. The methyl ester intermediate **21** was purified by HPLC to remove unreacted starting material, OH-substituted byproduct, and a small amount of ester hydrolysis product, and the purified ester (**21**) was then saponified and the product

acid **22** repurified by HPLC. These normal-phase chromatographic purifications were facilitated by the good separation of the desired product ([¹⁸F]-**22**) from the precursors and byproducts. The overall radiochemical yield was 20–30% decay corrected, and the entire synthesis and purification, starting from H₂[¹⁸O]O, took 2.5–3.5 h.

Tissue Distribution Studies on [¹⁸F]-22. We evaluated the tissue distribution of compound [¹⁸F]-**22** in two animal model systems, adult male rats and SCID mice containing human MCF-7 tumor xenografts (Tables 2 and 3). Animals were injected IV with the indicated dose of compound [¹⁸F]-**22** alone, or, in the case of blocking studies, together with an excess of unlabeled compound **22** to block receptor-mediated uptake. In some cases, a lower dose of compound [¹⁸F]-**22** was used to ascertain that uptake was not being limited by receptor saturation. Tissue distribution was determined at the indicated times (1 or 3 h postinjection). On the basis of mRNA expression, the normal tissues having the highest PPAR γ levels are brown and white adipose tissue; significant levels are also found in the spleen and the mucosa of the duodenum (2, 31, 32). MCF-7 cells have significant levels of PPAR γ , so one would expect these receptors to be present in these xenografts (7). If the tissue distribution of this ligand were to be guided by its binding to PPAR γ , one would expect to see selective uptake in these target tissues (or tumor) that is displaceable by coinjection of an excess of the unlabeled ligand.

The distribution of compound [¹⁸F]-**22** in mature male rats (Table 2) shows no evidence of target tissue selective uptake. Very high levels are found in liver and kidney. In the presumed target tissues that we could evaluate, namely white and brown fat and spleen, there is some evidence of blockable uptake, but it is not statistically significant nor different from that of some other tissues (e.g., muscle, heart). Overall uptake at these target sites is also low, with uptake in spleen and white fat being the same as muscle, uptake in brown fat being somewhat higher, but only comparable to that of heart. There is no clear evidence that any of the tissue uptake in the

Table 2. Tissue Distribution of [¹⁸F]-22 in Mature Female Rats (% ID/g)

tissue	1 h ^a	1 h (low) ^a	1 h (blocked) ^a	3 h (low) ^a	3 h (low, blocked) ^a
blood	0.193 ± 0.018	0.205 ± 0.037	0.172 ± 0.045	0.0509 ± 0.0117	0.565 ± 0.0097
liver	4.04 ± 1.16	3.64 ± 0.475	3.41 ± 1.16	1.10 ± 0.33	1.22 ± 0.45
kidney	0.833 ± 0.239	0.870 ± 0.114	0.620 ± 0.106	0.181 ± 0.045	0.248 ± 0.061
lung	0.133 ± 0.028	0.131 ± 0.029	0.105 ± 0.025	0.312 ± 0.0108	0.0367 ± 0.0065
heart	0.0665 ± 0.0111	0.0748 ± 0.0161	0.0559 ± 0.0127	0.0221 ± 0.0089	0.0239 ± 0.0084
muscle	0.297 ± 0.0042	0.0335 ± 0.0070	0.0275 ± 0.0082	0.0098 ± 0.0022	0.0115 ± 0.0018
bone	0.0386 ± 0.0135	0.0329 ± 0.0078	0.0252 ± 0.0103	0.0260 ± 0.0104	0.0243 ± 0.0094
brain	0.0097 ± 0.0033	0.0089 ± 0.0032	0.0080 ± 0.0025	0.0032 ± 0.0014	0.0067 ± 0.0041
white fat	0.0228 ± 0.0049	0.0308 ± 0.0075	0.0159 ± 0.0052	0.0073 ± 0.0038	0.0101 ± 0.0024
brown fat	0.0619 ± 0.0112	0.0726 ± 0.0205	0.0523 ± 0.0152	0.0235 ± 0.0197	0.0213 ± 0.0026
spleen	0.0455 ± 0.0082	0.0460 ± 0.0114	0.0367 ± 0.0085	0.0108 ± 0.0071	0.0166 ± 0.0046
upper sm int	1.52 ± 0.50	1.45 ± 0.34	1.51 ± 0.07	0.147 ± 0.134	0.454 ± 0.147
lower sm int	1.87 ± 0.65	1.96 ± 0.70	2.46 ± 1.79	2.13 ± 0.64	1.67 ± 0.10

^a Normal dose is 30 μ Ci/animal, low dose is 5 μ Ci; blocked dose includes 34 μ g unlabeled compound **22**. Values represent the mean $\pm$ SD for four animals.

Table 3. Tissue Distribution of [¹⁸F]-22 in Female SCID Mice with MCF-7 Tumors (% ID/g)

tissue	1 h ^{a,b} (n = 4)	1 h (low) ^{a,b} (n = 5)	1 h (blocked) ^{a,b} (n = 5)	3 h (low) ^{a,b} (n = 4)
blood	3.95 ± 0.67	4.16 ± 1.00	6.00 ± 1.10	2.65 ± 0.47
gall bladder	252 ± 114	362 ± 164	461 ± 112	165 ± 70
liver	11.2 ± 1.1	13.6 ± 1.4	13.9 ± 2.7	6.98 ± 1.12
kidney	2.59 ± 0.29	3.24 ± 0.73	3.29 ± 1.02	1.96 ± 0.22
lung	2.12 ± 0.27	2.10 ± 0.46	3.20 ± 0.88	1.44 ± 0.24
heart	1.09 ± 0.14	1.18 ± 0.19	1.54 ± 0.37	0.712 ± 0.093
muscle	1.68 ± 0.65	1.03 ± 0.27	1.73 ± 0.41	1.64 ± 0.45
bone	0.408 ± 0.224	0.440 ± 0.146	0.672 ± 0.235	0.880 ± 0.119
brain	0.254 ± 0.028	0.252 ± 0.021	0.316 ± 0.030	0.236 ± 0.032
white fat	0.586 ± 0.231 ^d	0.650 ± 0.129 ^c	1.13 ± 0.20 ^c	0.458 ± 0.088 ^d
brown fat	0.968 ± 0.209	1.05 ± 0.36	1.17 ± 0.30	0.703 ± 0.120
spleen	0.687 ± 0.071	0.763 ± 0.129	1.00 ± 0.19	0.565 ± 0.061
combined tumors	1.50 ± 0.63	1.23 ± 0.217	1.61 ± 0.45	0.837 ± 0.122
upper small intestine ^e	20.8 ± 11.0	20.3 ± 1.7	19.7 ± 1.7	6.37 ± 1.46
lower small intestine ^e	13.6 ± 2.8	18.1 ± 10.5	11.6 ± 5.3	10.2 ± 5.9

^a Normal dose is 32 μ Ci/animal, low dose is 5 μ Ci; blocked dose includes 29 μ g unlabeled compound **22**. ^b Number of animals given at head of each column, except where otherwise noted. ^c n = 3. ^d n = 4. ^e The contents of the small intestine were voided.

unblocked experiments is limited by receptor saturation, because none of the uptake values in the 1 h low dose experiment is significantly greater than the 1 h normal dose experiment. We were unable to evaluate the uptake into intestinal mucosa because of the very high levels of radioactivity in the intestinal contents, presumed to be due to hepatobiliary excretion of metabolites derived from compound [¹⁸F]-22.

Tissue distribution studies of compound [¹⁸F]-22 in SCID mice bearing MCF-7 xenografts is given in Table 3. Because mice are smaller animals, all of the uptake values, expressed as % ID/g tissue, are greater in mice than they are in rats. Again, high uptake is seen in liver and kidney, and, as well, in lung. The very high uptake seen in the gall bladder is consistent with the hepatobiliary excretion route of this compound. (Note: because rats do not have a gall bladder, uptake at this site could not be evaluated.) Uptake in normal target tissues (white and brown fat, and spleen) is also low in the SCID mice, again being similar to that in muscle and heart. There is no evidence that the uptake can be blocked with excess unlabeled ligand. Uptake in the tumors is no different than in the target tissues and is neither selective nor blockable.

DISCUSSION

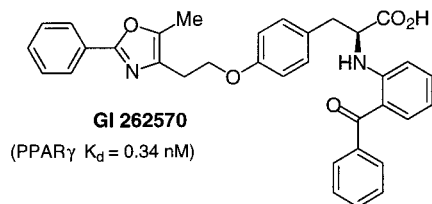
The presence of PPAR γ receptors in certain cancer cell lines and experimental tumor systems, and the utility of PPAR γ ligands in treating some cancers, offers the tantalizing prospect that this receptor might be useful as a target for in vivo tumor imaging using PET with PPAR γ ligands labeled with positron-emitting radionuclides. If successful, the results of such imaging might

be useful in selecting those patients most likely to benefit from PPAR γ -based hormone therapy.

In this initial study, we have synthesized two fluorine-substituted analogues from the phenylpropionic acid class as high affinity ligands for PPAR γ . The particular parent ligand (SB 213068) that we selected for fluorine substitution, compound **22**, appeared to have a good combination of high binding affinity, good in vivo potency, and an adequate rate of clearance to permit the development of good target to nontarget tissue contrast during the lifetime of the radionuclide fluorine-18 ($t_{1/2}$ = 110 min). Both of the fluorine-substituted analogues **9** and **22** retain good affinity for PPAR γ , with the fluoroethoxy analogue **22** having somewhat higher affinity than the *N*-fluoroethyl analogue **9**. It was on the basis of this higher affinity that we chose to prepare compound **22** in fluorine-18 labeled form. The radiolabeling reaction proceeded in a reasonable manner and furnished sufficient material for tissue distribution studies in female rats and in female SCID mice bearing human breast tumor (MCF-7) xenografts. The distribution of activity, however, was not consistent with selective uptake or retention by PPAR γ , by either normal target tissue or tumor, suggesting that higher performing radiopharmaceuticals will be needed to achieve the type of selective uptake required for imaging applications.

PPAR γ ligands with higher affinity than the parent ligand used in this study have been described recently (5, 30, 33, 34). In particular, members of the novel tyrosine-benzophenone class (such as GI 262570) have significantly higher affinity, and they show high potency in vivo. In this respect, these newer ligands would appear to hold new promise as parent systems for the develop-

ment of fluorine-18 labeled PPAR γ -based imaging agents. The use of the high affinity enantiomer should also increase receptor binding affinity (28–30), which might also improve target tissue uptake selectivity. Nevertheless, both the low PPAR γ titer in tumors and the pharmacokinetic behavior of PPAR γ ligands are likely to prove to be limiting in the development of imaging agents for this receptor.



In general, there is a parallelism between the in vivo potency of PPAR γ ligands and their extent of plasma protein binding, which correlates in an inverse fashion with their clearance rates. Members of the highest affinity tyrosine–benzophenone class, in particular, have serum half-lives of several hours, which is thought to contribute to their high in vivo potency. By contrast, long serum half-lives are generally considered unfavorable for the development of good target to nontarget contrast during the lifetime of the radionuclide. For these reasons, we believe that it will be a significant challenge to develop effective radiopharmaceuticals to image PPAR γ .

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Incorporation of an Aldehyde Function in Oligonucleotides

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A nucleotide-like phosphoramidite building block that has the nucleic base replaced by the *tert*-butyldimethylsilyl-protected styrene glycol was synthesized. After the automatic synthesis of an oligonucleotide incorporating this synthon, the benzaldehyde function was generated by fluoride deprotection and oxidation by sodium periodate. In a similar manner, an oligonucleotide where a nucleic base was replaced by the $(\text{CH}_2)_8\text{CH}=\text{O}$ chain was synthesized and conjugated with biotin derivatives.

INTRODUCTION

The aldehyde function is often used to couple biopolymers to other molecules by the process of reductive amination. In a typical reaction, this function is condensed with an amino group to form an imine bond, reduced in situ by sodium cyanoborohydride, under slightly acidic conditions (Lane, 1975 and recent examples: Westfall et al., 1998; Maruyama et al., 1998; Bentley et al., 1998; Zhao et al., 1999). A fully irreversible linkage is so obtained. The imine bond itself is quite labile. More stable links are obtained using hydroxylamines, hydrazines, and semicarbazides as nucleophiles. Stable oximes and semicarbazones functions are indeed present in well-established drugs such as cefuroxime and nitrofurantoin. The chemical properties of the aldehyde adducts are such that a tuning of the reversibility of the cross-link is thus possible. This is of prime importance if the in vivo targeting and delivery of a molecule of interest is envisioned.

Modification of oligonucleotides (ODNs) through incorporation of an appropriate molecule such as intercalating agent, nonnatural analogue of nucleic bases, metal chelating agent, fluorescent dye, etc., has been widely used to obtain functionalized ODNs as tools for biological and biophysical studies (Shinozuka et al., 2000 and references therein). The coupling of ODN's to cellular penetration enhancing polymers or peptides is also an important topic in the antisense ODN field (Lemaitre et al., 1987; Bongartz et al., 1994; Soukchareun et al., 1998; Peyrottes et al., 1998; Aubert et al., 2000).

Aldehyde functions were rarely introduced in ODNs. It is surprising, as reductive amination is largely used in protein chemistry and does not interfere with the functions present in DNA. The reason is probably that suitable aldehyde building blocks for automatic ODN synthesis are not currently available (Dechamps and Sonveaux, 1998). Aldehyde functions are randomly generated on DNA by depurination: these are the apurinic sites or, more broadly, the abasic sites. Abasic sites condense with hydroxylamine and methoxyamine to form rather stable adducts (Coombs and Livingston, 1969; Talpaert-Borlé and Liuzzi, 1983; Liuzzi and Talpaert-

Borlé, 1985; Liuzzi et al., 1987; Vasseur et al., 1986). When a ribose residue is introduced at the 3' or 5'-end of an ODN (by a 3'-5' or a 5'-5' linkage, respectively), the sodium periodate oxidation of the glycol function gives a dialdehyde that can be coupled with, for example, polylysine (Agrawal et al., 1986; Lemaitre et al., 1987). The intermediate dialdehyde is, however, prone to β -elimination in basic media (Nadeau et al., 1984). Postsynthetic 3', 5', or internal functionalizations of ODNs by aldehyde functions have also been reported (Kremsky et al., 1987; Urata and Akagi, 1993; Trévisiol et al., 1997). It should, however, be more practical to have at one's disposal aldehyde phosphoramidite building blocks mimicking nucleotides, especially if ODNs bearing multiple aldehyde functions are required. This could allow the polylabeling of ODNs (e.g., Teigelkamp et al., 1993). More specifically, a series of aldehyde functions situated at the end of an antisense ODN could in principle allow attachment of a bouquet of fusogenic peptides to mimic the viral proteins responsible for the cellular penetration of viruses. The influenza virus, for example, needs the concerted action of numerous fusogenic peptides to induce membrane destabilization and fusion (Chernomordik et al., 1998). Devising a method to replace a nucleic acid base by a benzaldehyde motif also opens perspectives in hybridization-triggered cross-linking. Formaldehyde (Chaw and al., 1980; Huang and Hopkins, 1993), acetaldehyde (Matsuda et al., 1998), and glucose (Bucala et al., 1984) react with DNA. Inside a double helix, an aldehyde function placed in the right position on one strand could form an adduct with the exocyclic amino function of cytosine, adenine, or guanine on the other strand (Gao and Orgel, 1999) and could, if the reaction is exoergonic, enhance the stability of the duplex. The testing of these ideas required easy access to aldehyde-oligonucleotides. This paper describes the synthesis of two phosphoramidite building blocks tailored for the construction of such ODNs.

EXPERIMENTAL PROCEDURES

The proportions in solvent and eluent mixtures are expressed as volumic fractions. The NMR spectra were recorded on a 200 MHz instrument, except when otherwise stated. Chemical shifts are given in ppm relative to TMS (^1H , ^{13}C) or phosphoric acid (^{31}P).

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3,5-Di-O-*p*-chlorobenzoyl-1,2-dideoxy-1-(4-vinylphenyl)- β (and α)-D-ribofuranose (3a). Commercial 4-bromostyrene is stabilized by sterically hindered catechols. Such contaminants have to be removed in order to allow the formation of the Grignard reagent. 4-Bromostyrene (25 mL) was thus diluted by dichloromethane (75 mL), this solution was washed with 1 M NaOH (3 $\times$), dried (MgSO₄), filtered, and concentrated, and the residue was distilled under vacuum (bp 88 °C, 0.2 mmHg). The so-purified 4-bromostyrene was used immediately.

A solution of 4-bromostyrene (2.56 g, 14 mmol) in dry ether (10 mL) was added dropwise to a suspension of magnesium (0.374 g, 15.4 mmol) in the same solvent (40 mL) (the reaction was started with iodine and a few drops of the halide solution). After 2 h stirring, cadmium chloride (1.608 g, 8.75 mmol, dried at 100 °C for 1 h) was added portionwise. The mixture was further stirred for 1 h at RT. Solid 3,5-di-O-*p*-chlorobenzoyl-1,2-dideoxy-1-chloro-D-ribofuranose **1** was then added (1.5 g, 3.5 mmol, synthesized according to Fox et al., 1961. See also Ness et al., 1961 for the first step of this synthesis). After 40 h, cold water was added, the organic phase was collected, and the aqueous phase was washed with toluene (2 $\times$ 100 mL). The gathered organic extracts were washed with NaHCO₃ (5%) and water (2 $\times$), dried on MgSO₄, filtered, and evaporated under vacuum. The isomers were isolated by flash chromatography on silica (eluent: hexane/ethyl acetate 19:1), giving an amount of pure β epimer (10%), a mixture of β and α (18%) epimers, and an amount of pure, slower eluting α epimer (22%). A major side-product was *p*-chlorobenzoyl furfuryl alcohol, obtained from **1** by two successive β -eliminations.

Anal. β epimer (C₂₇H₂₂Cl₂O₅) C, H.

¹H β epimer (ppm, CDCl₃): 2.2 (1H, H_{2'}, ddd, $J_{gem} = 13.5$ Hz, $J_{2'-1} = 10.8$ Hz, $J_{2'-3} = 6.3$ Hz), 2.55 (1H, H_{2''}, m, $J_{gem} = 13.5$ Hz, $J_{2''-1} = 5.1$ Hz), 4.5 (1H, H₄, m), 4.65 (2H, H₅, m), 5.2 (2H, H₁ and one of the olefinic CH₂'s, m), 5.6 (1H, H₃, m, $J_{3-2'} = 6.3$ Hz), 5.75 (1H, the second olefinic CH₂, d, $J_{vic} = 17.7$ Hz), 6.7 (1H, isolated olefinic CH, dd, $J_{vic1} = 17.7$ Hz, $J_{vic2} = 10.8$ Hz), 7.2–7.5 (8H, phenyl and *p*-chlorobenzoyl, m), 7.95 and 8.0 (4H, *p*-chlorobenzoyl, 2 $\times$ d, $J = 8.5$ Hz).

¹³C β epimer (ppm, CDCl₃): 41.90 (C₂), 65.54 (C₅), 78.12 (C₃), 81.06 (C₁), 83.29 (C₄), 114.54 (olefinic CH₂), 126.50 to 131.56 and 136.89 to 140.59 (11 resolved signals, aromatics and olefinic CH), 165.63 and 165.84 (2 $\times$ C=O).

¹H α epimer (ppm, CDCl₃, 300 MHz NMR): 2.34 (1H, H_{2''}, ddd, $J_{gem} = 14.0$ Hz, $J_{2''-1} = 5.5$ Hz, $J_{2''-3} = 3.5$ Hz), 2.89 (1H, H_{2'}, m, $J_{gem} = 14.0$ Hz, $J_{2'-1} = 7.2$ Hz, $J_{2'-3} = 6.5$ Hz), 4.58 (2H, H₅, m), 4.65 (1H, H₄, m), 5.27 (1H, one of the olefinic CH₂'s, d, $J_{vic} = 10.7$ Hz), 5.38 (1H, H₁, pseudo-t, $J_{1-2'} = 7.2$ Hz, $J_{1-2''} = 5.5$ Hz), 5.58 (1H, H₃, m, $J_{3-4} = 3.1$ Hz, $J_{3-2'} = 6.5$ Hz, $J_{3-2''} = 3.5$ Hz), 5.76 (1H, the second olefinic CH₂'s, d, $J = 17.6$ Hz), 6.73 (1H, isolated olefinic CH, q, $J_{vic1} = 17.6$ Hz, $J_{vic2} = 10.7$ Hz), 7.30–7.45 (8H, phenyl and *p*-chlorobenzoyl, m), 7.64 and 8.01 (4H, *p*-chlorobenzoyl, 2 $\times$ d, $J = 8.5$ Hz).

¹³C α epimer (ppm, CDCl₃): 40.11 (C₂), 64.75 (C₅), 76.69 (C₃), 79.94 (C₁), 81.99 (C₄), 113.99 (olefinic CH₂), 125.66 to 131.11 and 136.40 to 141.85 (13 signals, aromatics and olefinic CH), 165.13 and 165.45 (2 $\times$ C=O).

(2R,3S,5S)-1,3-O-Bis(*p*-chlorobenzoyl)-1,2,3,5-tetrahydroxy-2,5-anhydropentadec-14-ene (3b). This stereoisomer (β epimer) was obtained along the same lines. Yield, 30%. Anal. (C₂₉H₃₄Cl₂O₅) C, H.

Reference Compounds: 3,5-O-Bis(*p*-chlorobenzoyl)-1,2-dideoxy-1-(phenyl)- β (and α)-D-ribofuranose (3c). These compounds were also obtained by the

same procedure, except that the sugar derivative was condensed to the cadmium compound in a hot (40 °C) THF/benzene solution (1:1), distilling ether out of the reaction mixture (1 h). The crude mixture was chromatographed on silica (eluent: ethyl acetate/petroleum ether 1:20), giving a crop of pure α anomer as fast-migrating product, followed by the α/β mixture. The two constituents of this last fraction were separated by thin-layer chromatography, eluting three times consecutively with ethyl acetate/petroleum ether 1:15 ($R_f \alpha = 0.23$; $R_f \beta = 0.20$). Total yield 50% (*O*-*p*-chlorobenzoylfurfuryl alcohol was a major side-product). Yield of the α anomer (mp 232–233 °C): 37%. Yield of the β anomer (mp 227–228 °C): 13%.

The β anomer was also synthesized by a stereospecific method, starting from β -1-phenyl-1-deoxy-D-ribofuranose (Klein et al., 1971), by deoxygenation at position 2 followed by acylation (Robins et al., 1983). The properties of the compound obtained by this stereospecific synthesis were identical to those of the slow-eluting isomer.

500 MHz NMR data:

¹H β epimer (ppm, CDCl₃): 2.23 (1H, H_{2'}, ddd, $J_{gem} = 13.7$ Hz, $J_{2'-1} = 10.9$ Hz, $J_{2'-3} = 6.1$ Hz), 2.55 (1H, H_{2''}, m, $J_{gem} = 13.7$ Hz, $J_{2''-1} = 5.1$ Hz, $J_{2''-3} = 1.0$ Hz) (lit., 100 MHz: Millican et al., 1984: H_{2'} and H_{2''}: 2.05–2.70), 4.53 (1H, H₄, m), 4.66 (2H, H₅, m) (lit., ibidem: H₄ and H₅: 4.50–4.80), 5.26 (1H, H₁, dd, $J_{1-2'} = 10.9$ Hz, $J_{1-2''} = 5.1$ Hz) (lit., ibidem: H₁: 5.51–5.30, $J_{1-2'} = 9$ Hz, $J_{1-2''} = 5$ Hz), 5.60 (1H, H₃, m, $J_{3-4} = 1.5$ Hz, $J_{3-2'} = 6.1$ Hz, $J_{3-2''} = 1.0$ Hz) (lit., ibidem: H₃: 5.5–5.7), 7.24–7.46 (9H, phenyl and *p*-chlorobenzoyl, m), 7.95 and 8.02 (4H, *p*-chlorobenzoyl, 2 $\times$ d, $J = 8.5$ Hz).

¹³C β epimer (ppm, CDCl₃) (DEPT): 42.22 (C₂, t) (lit., ibidem: C₂, 41.6), 65.80 (C₅, t) (lit., ibidem: C₅, 64.9), 78.32 (C₃, d) (lit., ibidem: C₃, 82.8), 81.55 (C₁, d) (lit., ibidem: C₁, 80.8), 83.51 (C₄, d) (lit., ibidem: C₄, 77.4), 126.53 to 131.84 and 140.41 to 141.13 (12 signals, aromatics), 166.01 and 166.26 (C=O, s).

¹H α epimer (ppm, CDCl₃): 2.35 (1H, H_{2''}, ddd, $J_{gem} = 13.7$ Hz, $J_{2''-1} = 5.5$ Hz, $J_{2''-3} = 3.7$ Hz), 2.90 (1H, H_{2'}, m, $J_{gem} = 13.7$ Hz, $J_{2'-1} = 7.0$ Hz, $J_{2'-3} = 6.9$ Hz), 4.58 (2H, H₅, m), 4.67 (1H, H₄, m), 5.38 (1H, H₁, pseudo-t, $J_{1-2'} = 7.0$ Hz, $J_{1-2''} = 5.5$ Hz), 5.58 (1H, H₃, m, $J_{3-4} = 3.2$ Hz, $J_{3-2'} = 6.9$ Hz, $J_{3-2''} = 3.7$ Hz), 7.25–7.41 (9H, phenyl and *p*-chlorobenzoyl, m), 7.64 and 8.01 (4H, *p*-chlorobenzoyl, 2 $\times$ d, $J = 8.5$ Hz).

¹³C α epimer (ppm, CDCl₃) (DEPT): 40.68 (C₂, t), 65.49 (C₅, t), 78.05 (C₃, d), 80.83 (C₁, d), 82.70 (C₄, d), 126.17 to 131.72 and 140.40 to 142.95 (12 signals, aromatics), 165.85 and 166.18 (C=O, s).

3,5-O-Bis(*p*-chlorobenzoyl)-1,2-dideoxy-1-[4-(1,2-dihydroxyethyl)phenyl]- β -D-ribofuranose (4a). Compound **3a β** (1.8 mmol) was dissolved in an dichloromethane/acetone/water mixture (2:9:1, 35 mL) kept at 0 °C. *N*-methylmorpholin *N*-oxide (3 mmol) was added, followed by osmium tetroxide (0.16 mmol, i.e., 2 mL of a 2.5 (weight %) solution in *tert*-butyl alcohol). The mixture was magnetically stirred and the progress of the reaction was followed by TLC. (CH₂Cl₂/CH₃OH, 95:5; **3a**, $R_f = 0.8$, **4**, $R_f = 0.1$). The mixture was evaporated after 8 h and partitioned between water and CH₂Cl₂. The organic phase was dried (MgSO₄), filtered, and evaporated. The compound was purified by column chromatography (eluent, CH₂Cl₂). Yield: 94%. Anal. (C₂₇H₂₄Cl₂O₇) C, H.

¹H NMR (ppm, CDCl₃): 2.2 (1H, H_{2'}, m), 2.5 (1H, H_{2''}, dd, $J_{gem} = 13.5$ Hz, $J_{2''-1} = 5$ Hz), 3.25 (2H, broad band, 2 $\times$ OH), 3.55 (1H, one of the two diastereotopic CH₂-OH's, pseudo-t, $J_{gem} = 10.5$ Hz, $J_{vic} = 7.5$ Hz), 3.7 (1H,

the second of the two diastereotopic CH_2OH 's, dd, $J_{\text{gem}} = 10.5$ Hz, $J_{\text{vic}} = 3$ Hz), 4.5 (1H, H_4 , m), 4.6 (2H, H_5 , m), 4.75 (1H, CHOH , dd, $J_{\text{vic1}} = 7.5$ Hz, $J_{\text{vic2}} = 3$ Hz), 5.2 (1H, H_1 , dd, $J_{1-2'} = 11$ Hz, $J_{1-2''} = 5$ Hz), 5.6 (1H, H_3 , m, $J_{3-2'} = 5.5$ Hz), 7.25–7.5 (8H, phenyl and *p*-chlorobenzoyl, m), 7.95 and 8.0 (4H, *p*-chlorobenzoyl, $2 \times \text{d}$, $J = 9$ Hz).

As a further proof of structure, **4a** (0.18 g, 0.34 mmol) was oxidized by NaIO_4 (2.13 g, 10 mmol, 1.5 h, RT) in THF/phosphate buffer pH 7 (125 mL, 1:4) to give the corresponding aldehyde in quantitative yield. The ^1H NMR of the aldehyde (CDCl_3) featured the lack of the $\text{CH}(\text{OH})\text{CH}_2(\text{OH})$ signals, the appearance of the $\text{CH}=\text{O}$ signal at 10 ppm integrating for 1 proton, and the modification of the aromatic CH absorptions, appearing now as two sets of three doublets (12H, $J = \text{ca. } 9$ Hz) in the 7.3–7.7 and the 7.8–8.1 ppm ranges, respectively.

(2R,3S,5S)-1,3-O-Bis(*p*-chlorobenzoyl)-1,2,3,5,14,15-hexahydroxy-2,5-anhydropentadecane (4b). The oxidation was performed as for **4a**. Yield, 90%. Anal. ($\text{C}_{29}\text{H}_{36}\text{Cl}_2\text{O}_7$) C, H.

5-O-[(4,4'-Dimethoxy)triphenylmethyl]-1,2-dideoxy-1-{4-[1,2-bis(*tert*-butyldimethylsilyloxy)ethyl]phenyl}- β -D-ribofuranose (7a). The reaction was performed under argon under magnetic stirring. The diol **4a** (0.313 g, 0.6 mmol) was dissolved in dry DMF (5 mL), and dried imidazole (0.401 g, 6 mmol) was added, followed by *tert*-butyldimethylsilyl chloride (0.355 g, 2.4 mmol). The progress of the reaction was followed by TLC (eluent CHCl_3 , R_f **4a** = 0.1, R_f **5a** = 0.6). After 48 h, the mixture was partitioned between water (15 mL) and CH_2Cl_2 (15 mL). The organic phase was washed ($3 \times$) with water, dried (MgSO_4), filtered, and evaporated to give the silylated derivative **5a**.

^1H NMR (ppm, CDCl_3): 0.0–0.2 (12H, $\text{Si}(\text{CH}_3)_2$), 0.9–0.95 (18H, $\text{SiC}(\text{CH}_3)_3$), 2.3 (1H, $\text{H}_{2'}$, ddd, $J_{\text{gem}} = 14$ Hz, $J_{2'-1} = 11$ Hz, $J_{2'-3} = 6$ Hz), 2.6 (1H, $\text{H}_{2''}$, dd, $J_{\text{gem}} = 14$ Hz, $J_{2''-1} = 5$ Hz), 3.55 (1H, one of the two diastereotopic CH_2OSi 's, dd, $J_{\text{gem}} = 10$ Hz, $J_{\text{vic}} = 5$ Hz), 3.7 (1H, the second of the two diastereotopic CH_2OSi 's, dd, $J_{\text{gem}} = 10$ Hz, $J_{\text{vic}} = 7$ Hz), 4.6 (1H, H_4 , m), 4.7 (3H, H_5 and CHOSi , m), 5.3 (1H, H_1 , dd, $J_{1-2'} = 11$ Hz, $J_{1-2''} = 5$ Hz), 5.6 (1H, H_3 , m, $J_{3-2'} = 6$ Hz), 7.3–7.6 (8H, phenyl and *p*-chlorobenzoyl, m), 8.0 and 8.1 (4H, *p*-chlorobenzoyl, $2 \times \text{d}$, $J = 9$ Hz).

Compound **5a** (0.405 g) was deacylated by stirring 10 h in a mixture of dichloromethane (5 mL) and hydrazine hydrate (1 mL). The reaction mixture was evaporated, the residue dissolved in ether, and the solution washed with water (3×20 mL). The organic phase was dried (MgSO_4), filtered, and evaporated to leave **6a**.

^1H NMR (ppm, CDCl_3): –0.06, –0.05, –0.04, and 0.05 (12H, diastereotopic $\text{Si}(\text{CH}_3)_2$), 0.85 and 0.87 (18H, $\text{SiC}(\text{CH}_3)_3$), 2.0 (1H, $\text{H}_{2'}$, ddd, $J_{\text{gem}} = 13$ Hz, $J_{2'-1} = 10$ Hz, $J_{2'-3} = 6$ Hz), 2.2 (1H, $\text{H}_{2''}$, ddd, $J_{\text{gem}} = 13$ Hz, $J_{2''-1} = 6$ Hz, $J_{2''-3} = \text{ca. } 2$ Hz), 2.5 (2H, OH, broad signal), 3.4–3.9 (4H, diastereotopic CH_2OSi 's and H_5 , m), 4.0 (1H, H_4 , m), 4.4 (1H, H_3 , m, $J_{3-2'} = 6$ Hz, $J_{3-2''} = J_{3-4} = \text{ca. } 2$ Hz), 4.7 (1H, CHOSi , dd, $J_{\text{vic1}} = 5$ Hz, $J_{\text{vic2}} = 7$ Hz), 5.15 (1H, H_1 , dd, $J_{1-2'} = 10$ Hz, $J_{1-2''} = 6$ Hz), 7.15–7.35 (4H, aromatics, $2 \times \text{d}$, $J_{\text{vic}} = 8.5$ Hz).

Compound **6a** (0.204 g, 0.43 mmol) was coevaporated three times with dry pyridine and then stirred for 3 h with dimethoxytrityl chloride (0.194 g, 0.57 mmol) in pyridine (15 mL). Methanol (15 mL) was added to quench the residual chloride (during 10 min), followed by dichloromethane (15 mL), and the mixture was extracted three times with NaHCO_3 (5%), to eliminate the pyridinium hydrochloride. The organic phase was dried (MgSO_4),

filtered, and evaporated. The residue was chromatographed on silica (eluent $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ 98:2 + a few drops of triethylamine). Overall yield: 0.255 g, 0.33 mmol, 55%). Anal. ($\text{C}_{46}\text{H}_{64}\text{O}_7\text{Si}_2$) C, H.

^1H NMR (ppm, CDCl_3): –0.1 to 0.1 (12H, diastereotopic $\text{Si}(\text{CH}_3)_2$), 0.8–0.9 (18H, $\text{SiC}(\text{CH}_3)_3$), 1.9–2.1 (1H, $\text{H}_{2'}$ and OH, m), 2.2 (1H, $\text{H}_{2''}$, ddd, $J_{\text{gem}} = 12.3$ Hz, $J_{2''-1} = 5.4$ Hz, $J_{2''-3} = \text{ca. } 2$ Hz), 3.3 (1H, H_5 , dd, $J_{\text{gem}} = 10$ Hz, $J_{\text{vic}} = 5.4$ Hz), 3.4 (1H, $\text{H}_{5'}$, dd, $J_{\text{gem}} = 10$ Hz, $J_{\text{vic}} = 4.3$ Hz), 3.5 (1H, one of the diastereotopic CH_2OSi , dd, $J_{\text{gem}} = 10$ Hz, $J_{\text{vic}} = 4.8$ Hz), 3.6 (1H, the second diastereotopic CH_2OSi , dd, $J_{\text{gem}} = 10$ Hz, $J_{\text{vic}} = 7$ Hz), 3.8 (6H, OCH_3), 4.0 (1H, H_4 , m), 4.4 (1H, H_3 , m), 4.7 (1H, CHOSi , dd, $J_{\text{vic1}} = 4.8$ Hz, $J_{\text{vic2}} = 7$ Hz), 5.15 (1H, H_1 , dd, $J_{1-2'} = 10$ Hz, $J_{1-2''} = 5.4$ Hz), 6.8 (4H, DMTr, d, $J = 8.5$ Hz), 7.15–7.55 (13H, aromatics).

(2R,3S,5S)-1-O-(4,4'-dimethoxytrityl)-14,15-O-bis-(*tert*-butyldimethylsilyl)-1,2,3,5,14,15-hexahydroxy-2,5-anhydropentadecane (7b). This compound was obtained by the same procedure. Yield, 61%. Anal. ($\text{C}_{48}\text{H}_{76}\text{O}_7\text{Si}_2$) C, H.

3-{5-O-[(4,4'-Dimethoxy)triphenylmethyl]-1,2-dideoxy-1-{4-[1,2-bis(*tert*-butyldimethylsilyloxy)ethyl]phenyl}- β -D-ribofuranosyl}-2-methoxy}(N,N'-diisopropylamino)phosphine (8a). The reaction was performed under argon. Compound **7a** (0.178 g, 0.23 mmol) was coevaporated three times with acetonitrile, dried under high vacuum (2 h), and dissolved in dry CH_2Cl_2 (10 mL) containing diisopropylammonium tetrazolide (0.022 g, 0.13 mmol). Methyl tetraisopropylphosphorodiamidite (0.064 g, 0.24 mmol) was added. The mixture was magnetically stirred for 2 h [control by TLC, hexane/ $\text{N}(\text{CH}_2\text{CH}_3)_3$, 6:1]. Sodium bicarbonate (5%, 10 mL) was added. The organic phase was washed with a phosphate buffer (pH 7, 10 mL) and then with brine (sat., 10 mL), dried (MgSO_4), filtered, and evaporated. The residue was chromatographed on silica [eluent: hexane/ $\text{N}(\text{CH}_2\text{CH}_3)_3$, 6:1, R_f of **8a** = 0.7]. Yield, 60%. ^{31}P NMR, $\delta = 148.536$ and 148.366 ppm. Anal. ($\text{C}_{53}\text{H}_{80}\text{NO}_8\text{PSi}_2$) C, H, N.

^1H NMR (ppm, CDCl_3): –0.1 to 0.1 (12H, diastereotopic $\text{Si}(\text{CH}_3)_2$), 0.8–0.9 (18H, $\text{SiC}(\text{CH}_3)_3$), 1.0–1.3 (12H, $\text{NCH}(\text{CH}_3)_2$, m), 1.9–2.5 (2H, $\text{H}_{2'}$ and $\text{H}_{2''}$, m), 3.2 (2H, H_5 , m), 3.3 and 3.4 (3H, OCH_3 of the two diastereoisomers, $2 \times \text{d}$, $J_{\text{H-P}} = 13$ Hz), 3.6 (4H, CH_2OSi and $\text{NCH}(\text{CH}_3)_2$), 3.8 (6H, OCH_3), 4.2 (1H, H_4 , m), 4.5 (1H, H_3 , m), 4.7 (1H, CHOSi , m), 5.2 (1H, H_1 , m), 6.8 (4H, DMTr, d, $J = 8.5$ Hz), 7.15–7.55 (13H, aromatics).

3-[2R,3S,5S)-1-O-(4,4'-dimethoxytrityl)-14,15-O-bis-(*tert*-butyldimethylsilyl)-1,2,5,14,15-pentahydroxy-2,5-anhydro]pentadecoxy}-2(cyanoethoxy), (N,N'-diisopropylamino)phosphine (8b). The same protocol was used, engaging 0.06 mmol of diisopropylammonium tetrazolide and 0.12 mmol of bis(diisopropylamino)(β -cyanoethoxy)phosphine. Yield, 70%. ^{31}P NMR, $\delta = 148.811$ and 148.641 ppm. Anal. ($\text{C}_{57}\text{H}_{93}\text{N}_2\text{O}_8\text{PSi}_2$) C, H, N.

Synthesis and Purification of Oligonucleotides 9a,b. Building blocks **8a,b** were introduced at the X position in the sequence d(GTCGTGAC-X-GGGAAAAC) by automatic synthesis. The trityl-on oligomer was deprotected by the classical sequence of thiophenol (only for **9a**), concentrated ammonia (50 °C), and tetrabutylammonium fluoride (THF, 0.1 M). The oligomer was then desalted (Pharmacia NAP-10), adsorbed on a C-18 cartridge (Pharmacia Oligo-Pak), and eluted by detritylation (2% aqueous trifluoroacetic acid). A final purification, when needed, was performed by anion-exchange chromatography (Pharmacia Mono Q column), followed by desalting (NAP-10).

Oxidation of 9a,b to 10a,b and Titration by NaBT₄.

The aldehyde function was generated from the vicinal diol according to the protocol of Bayard et al. (1984).

Oligonucleotide **9a** (2.8 nmoles) was oxidized by NaIO₄ (2.5 μ L of 1.6 mM solution in 0.02 M sodium acetate buffer, pH 4.75) at 4 °C for 5 h in the dark. Ethylene glycol was added to destroy the excess NaIO₄. The aldehyde function was then reduced in situ by NaBT₄ (+ 2.5 μ L of 10 mM solution in 0.01 M sodium borate buffer, pH 9). The excess hydride was decomposed by the addition of acetate buffer. The yield of tritium incorporation was determined by passing the sample through two successive NAP-10 cartridges, isolating the high molecular weight fraction and determining both the A₂₆₀ (2.5 nmol of DNA recovered) and the immobilized tritium content (2.37 nmol, by scintillation counting). The tritium incorporation yield was thus 95%. The yield of **10b**, obtained by the same procedure, was quantitative.

Synthesis of 11 and 12. A solution of NaIO₄ (120 mM) in phosphate buffer (20 mM, pH 7) was made. It was degassed by helium (to avoid overoxidation of the aldehyde to be obtained). This solution (1 mL) was added to the glycol-oligonucleotide **9b** (100 OD in 25 μ L). The reaction was stopped after 30 min in the dark at room temperature by desalting on NAP-10. The success of the reaction was checked by reversed phase HPLC [BIORAD Bio-Sil C₁₈ HL 150 $\times$ 4.6 mm column. Linear gradient of eluent B (triethylammonium acetate 0.1 M in CH₃CN: H₂O, 60:40) in eluent A (triethylammonium acetate 0.1 M in CH₃CN:H₂O, 5:95), from 0 to 30% in 25 min. Flow, 1 mL/min]. The retention times were 15.4 and 16.3 min for **9b** and **10b**, respectively. The solution of the aldehyde-oligomer was not kept, but immediately used for the next step.

A solution of biotin hydrazide (Sigma, 10 μ L, 15 mM) and acetate buffer (5 μ L, 500 mM, pH 5) and a solution of sodium cyanoborohydride (5 μ L, 100 mM in acetate buffer) were added to the aldehyde-oligonucleotide **10b** dissolved in water (1.5 mmol in 500 μ L). The mixture was desalted on NAP-10 after 2 h at room temperature. The conjugate **11** was isolated by semipreparative HPLC, under the same conditions as indicated above.

Similarly, a solution of D-biotinyl-1,8-diamino-3,6-dioxaoctane (Boehringer Mannheim, 10 μ L, 15 mM) and phosphate buffer (100 μ L, 20 mM, pH 6.8) and a solution of sodium cyanoborohydride (10 μ L, 5 mM in water) were added to the aldehyde-oligonucleotide **10b** dissolved in water (1.5 nmol in 200 μ L). After 4 h at room temperature, the mixture was diluted by water to 500 μ L and desalted on NAP-5. The conjugate **12** was also isolated by semipreparative HPLC.

A titration of conjugate **12** by avidin was performed. Aliquots of a solution of avidin (Sigma, 5.2 nmol/mL of sites of complexation) were added to solutions of the biotin-oligonucleotide conjugate (66 pmol of oligonucleotide in 200 μ L). Each solution was ultrafiltrated (ULTRAFREE-MC from Millipore, membrane UFC-3-LTK, cutoff 30.000), and the filtrate was checked by HPLC. Oligomer **9b** was used as a not-retained internal standard. The addition of 65 pmol of avidin sites of complexation was necessary to completely retain the biotinylated oligomer on the filter.

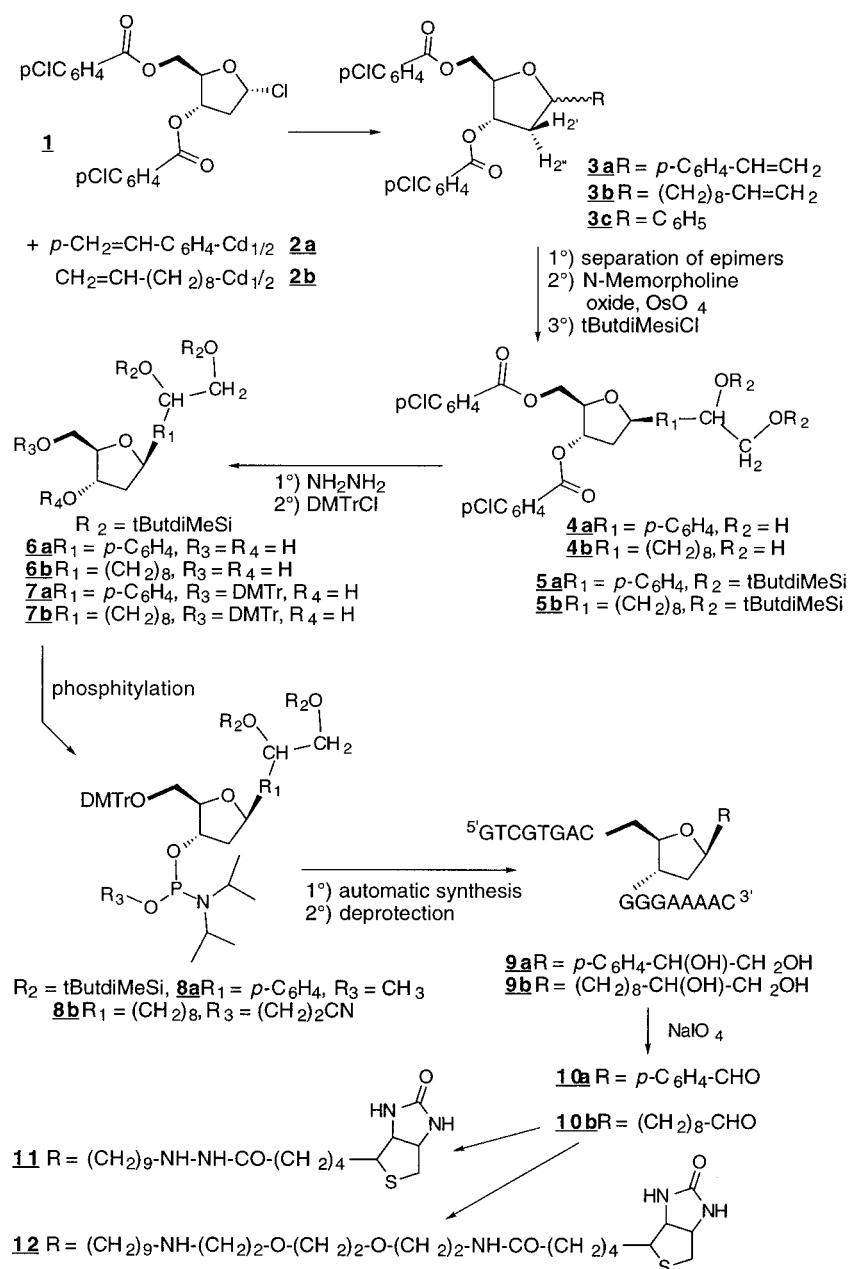
RESULTS AND DISCUSSION

Proper Protection of the Aldehyde Function. The conditions of automatic DNA synthesis should arm an

unprotected aldehyde (nucleophilic attack of the P(III) derivatives on the carbonyl, oxidation of the aldehyde by iodine, and possible Cannizzaro reaction in the strongly basic conditions used for full deprotection). A classical acetal protection is not convenient, as it would be cleaved during the detritylation steps. Our strategy was to use a bis-silylated glycol as a precursor of aldehyde. ODNs can be treated with aqueous sodium periodate without degradation, as reported in the Introduction. This reagent selectively oxidizes glycols. A glycol is of course not compatible with the conditions of automatic synthesis. The OHs have to be properly protected. *tert*-Butyldimethylsilyl protection is used to cap the 2'-OH in solid-supported RNA synthesis (Wu and Ogilvie, 1990). A reasonable strategy leading to aldehyde-ODNs was thus to use a phosphoramidite synthon incorporating a *O,O'*-bis(*tert*-butyldimethylsilyl)glycol function.

Synthesis. The synthesis started with the coupling of organocadmium compounds **2a,b** with the highly electrophilic anomeric carbon of the deoxyribofuranosyl chloride **1** (Scheme 1). This type of coupling was pioneered in the hexose field (Hurd and Holysz, 1950) and expanded later in the ribose series (Klein et al., 1971). The yields were, however, low, ca. 20%. We and others have successfully used this method for the synthesis of various C-nucleosides in the deoxyribofuranose series, with, in our hands, yields higher than 80% (François et al., 1994; Chaudhuri and Kool, 1995; Ren et al., 1996; Dechamps and Sonveaux, 1998; Strässler et al., 1999). The coupling is not stereoselective, a mixture of α and β isomers being obtained. The formation of the *p*-styrenylmagnesium bromide, the obligate precursor of the corresponding organocadmium compound **2a**, proved to be impossible if *p*-bromostyrene was not freed from its radical trap stabilizers. The determination of the configuration of **3a,b** needed a detailed study, the Karplus equation being difficult to use (Chaudhuri and Kool, 1995) because of the flexibility of the deoxyribofuranose ring (François et al., 1990). *O,O'*-Dibenzoyl α and β 1-phenyl-1,2-dideoxy-D-ribofuranoses (e.g., **3c**) are known compounds, but their NMR spectra were not reported in sufficient detail (Millican et al., 1984; Gunning et al., 1985). For the sake of recording reference NMR spectra, we thus also obtained by our method the mixture of α -**3c** and β -**3c** and separated them by chromatography, and we synthesized independently β -**3c** by a reported stereospecific procedure (the route used the stereospecific synthesis of β -1-phenyl-1-deoxy-D-ribofuranose, followed by deoxygenation at position 2 and benzylation) (Klein et al., 1971; Robins et al., 1983). The stereoisomers α -**3a,b** and β -**3a,b**, separated by chromatography, were then easily identified by comparison. The synthesis was pursued with pure stereoisomers in order to simplify the analysis of the spectral data. On a practical point of view, if the method is used for labeling purpose only, the synthesis could be pursued on the mixture of epimers. We chose the less abundant β epimer because it corresponds to the configuration of natural nucleotides, a point of importance if hybridization-triggered cross-linking is envisioned. Osmium tetroxide oxidation (Jacobsen et al., 1988), followed by silyl capping, gave the fully protected nucleoside analogues **5a,b**. Selective aminolysis of the ester functions of **5a,b** led to **6a,b**, that were dimethoxytritylated on the primary alcohol function and then phosphitylated. Compounds **8a,b** are building blocks tailored for the automatic phosphoramidite-type DNA synthesis.

Scheme 1



We incorporated **8a,b** at the **X** position in the sequence GTCGTGAC-**X**-GGGAAAAC. The trityl-on oligomer was cleaved from the glass beads by concentrated ammonia, fully deprotected, and purified to homogeneity by anion-exchange chromatography. The oxidation of the glycol by sodium periodate, followed by the titration of the generated aldehyde function by NaBT₄, proved that the yields of these reactions were practically quantitative.

We also synthesized the biotine conjugates **11** and **12**, to demonstrate that the aldehyde function of the oligonucleotides can be coupled with amino compounds by reductive amination. The biotinylated oligomers were quantitatively captured by avidin.

CONCLUSION

We thus described a practical method to introduce an aldehyde function and perform a reductive amination at any position in an ODN. Both 3' and 5' ends of the ODN remain free for further functionalization or ³²P labeling.

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Supporting Information Available: Elemental (C, H) analysis data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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COMMUNICATIONS

Synthesis of Peptide–Oligonucleotide Conjugates for Chromium Coordination

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The synthesis of the first peptide–oligonucleotide conjugate designed to coordinate chromium(III) is reported. The overall goal of this work is to synthesize di-deoxynucleotides tethered with chromium(III)-coordinating appendages to model chromium–DNA–protein cross-links, which are a type of DNA lesion that may be involved in chromium-induced cancers. The conjugate dGp(NHCH₂CH₂S-Ac-Gly-Ser-Gly-OH)G was made by coupling the peptide, ClAc-Gly-Ser-Gly-OH, and dinucleotide, dGp(NHCH₂CH₂SH)G, through a thioether moiety. The conjugate was characterized by HPLC and mass spectrometry. Previously reported methods for small-scale solid-phase synthesis of peptides and dinucleotide were unsuitable; therefore, gram-scale solution-phase methods were developed. We also report the gram-scale syntheses of two other serine-containing peptides, ClAc-βAla-Ser-Gly-OH and ClAc-Ser-Gly-OH, and three histidine-containing peptides, ClAc-Gly-His-Gly-OH, ClAc-βAla-His-Gly-OH, and ClAc-His-Gly-OH. The synthesis and characterization of chromium-containing peptide–oligonucleotide conjugates will ultimately help us to understand chromium–DNA interactions at a molecular level, which is necessary before we can determine how chromium causes cancer.

INTRODUCTION

Peptide oligonucleotide conjugates have been synthesized for a variety of applications, providing control of cellular uptake and intracellular delivery of oligonucleotides, improvement of DNA or RNA binding, creation and modification of artificial nucleases, and enhancement of nuclease resistance of oligonucleotides (1). We are interested in synthesizing peptide oligonucleotide conjugates to ultimately model chromium–DNA–protein cross-links, which are a type of DNA lesion that may be involved in chromium-induced cancers.

Chromium is a carcinogenic metal that may produce DNA damage by two different pathways: oxidative DNA

damage by high valent chromium intermediates and free radicals, and adduct formation by low valent chromium(III). We are investigating the covalent interaction between Cr(III) and DNA. Amino acid–Cr(III)–DNA adducts and Cr(III)-containing DNA–protein cross-links (Cr-DPC) have been reported in cells (2, 3), animals (4, 5), and humans (6) exposed to Cr(VI) compounds. These Cr-containing DNA lesions are mutagenic (7) and are likely to be involved in Cr-induced cancers because they are more slowly repaired than strand breaks (2).

Although Cr(III)–DNA adducts have been detected and quantitated in bulk DNA from cells and animals, there is still very little information available about their structures. Chromium(III) will associate electrostatically with the phosphate backbone. This noncovalent interaction can be disrupted by EDTA or high NaCl (8). There

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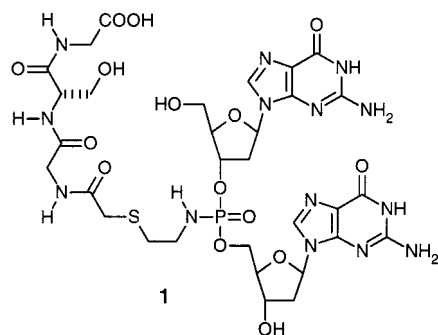


Figure 1. Peptide–dinucleotide conjugate.

is growing evidence that Cr(III) also associates with guanine bases *in vitro* (9–15), which is consistent with the preference for guanine coordination shown by platinum (16), cobalt and barium (17), and nickel (18).

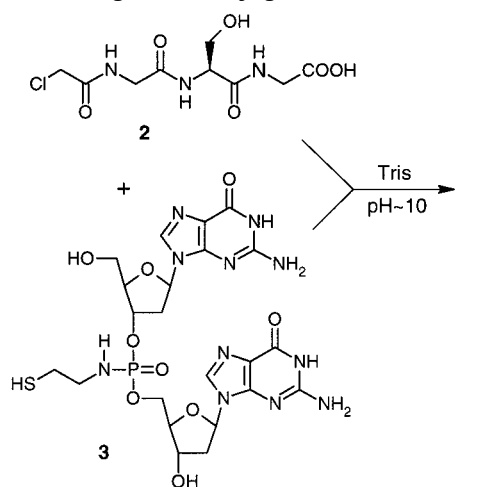
The overall goal of this work is to synthesize di-deoxynucleotides tethered with Cr(III)-coordinating appendages to model Cr-DPCs. A tethered Cr(III) will optimize metal DNA interactions by localizing the metal in the coordination environment of the nucleotide and will produce a more stable complex for crystallization and structural characterization. The synthesis of a series of Cr–DNA–peptide conjugates will help us to explain the difference in stabilities between Cr(III) coordination to the nucleotide versus the phosphate backbone. It will also help us to understand the stereochemical and electronic effects of different amino acids when they complete the coordination environment of Cr(III). Characterization of these conjugates will elucidate the mechanisms of Cr(III)-induced mutations. An understanding of chromium–DNA interactions at the molecular level is necessary before we can ultimately determine how chromium causes cancer.

The design of our initial targets is based on current knowledge of Cr–DNA–protein cross-links. The DNA portion contains guanine dinucleotides because of their known ability to bind metals described above. A series of peptide tethers were made, differing in length, with either serine-glycine or histidine-glycine sequences for the Cr(III)-binding region because these were among the amino acids found in Cr-DPC in cultured cells (3, 19, 20). In these first series of conjugates the tether has been attached to the phosphate bridge of the dinucleotide so that the phosphate oxygen will be blocked from association with Cr(III), thus favoring the coordination of Cr(III) to the guanine bases. Future conjugates will contain the tether attached to the 5'-OH group (21) or the base residue such as the N4-position of cytosine (22).

RESULTS AND DISCUSSION

Herein we describe the synthesis of our first peptide–dinucleotide conjugate, dGp(NHCH₂CH₂S-Ac-Gly-Ser-Gly)G (**1**), specifically designed to bind chromium(III) (Figure 1). We anticipate chromium to bind strongly to the C-terminal peptide sequence, -Ser-Gly-OH. This will serve as a tether holding chromium in proximity to the dinucleotide to allow for the isolation and characterization of the complex. Conjugate **1** was made by the Fragment Conjugation method (1), in that the peptide and dinucleotide were synthesized separately and then coupled through the thioether moiety (Scheme 1). While both the functionalized peptide ClAc-Gly-Ser-Gly-OH (**2**) and dinucleotide dGp(NHCH₂CH₂SH)G (**3**) can be synthesized by solid-phase methods, we found this approach to be unsuitable for our needs. To crystallize and char-

Scheme 1. Fragment Conjugation

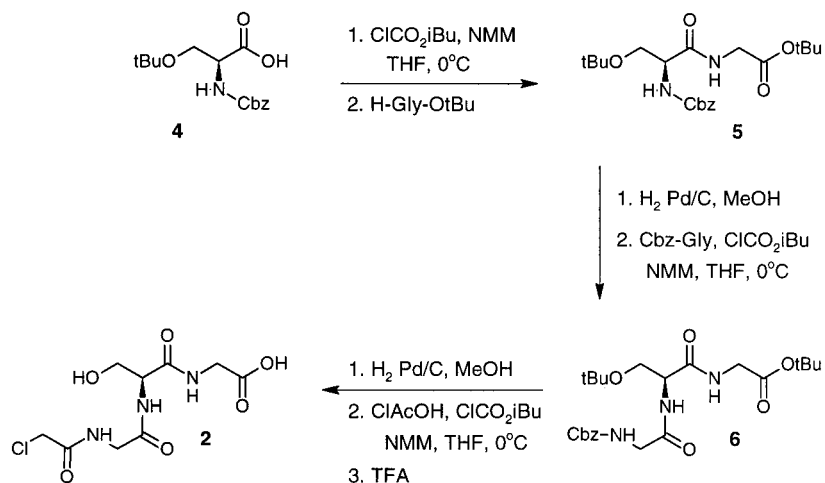
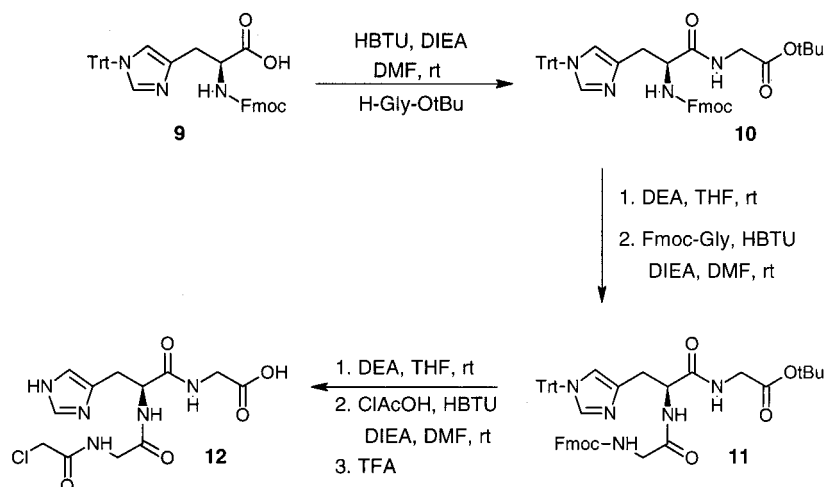


acterize the chromium complex, we envision that gram quantities of **1** will be necessary, and the high cost and small scale of solid-phase methods made this approach uneconomical. Therefore, our first goal was to develop gram-scale solution-phase methods for the synthesis of the peptide and dinucleotide fragments.

The synthesis of peptide **2** is outlined in Scheme 2. Diisopropylcarbodiimide (DIC) and *N*-hydroxysuccinimide (HOSu) were originally used for the coupling of Cbz-Ser(OtBu)-OH (**4**) to H-Gly-OtBu. However, at multigram scales, the diisopropylurea (DIU) byproduct became problematic. The large amounts of DIU were removed by precipitation from cold CH₂Cl₂ followed by normal-phase column chromatography. However, these methods proved inefficient, and often repeated chromatography was necessary which made this procedure impractical. A superior procedure was found in the mixed anhydride approach. Using isobutyl chloroformate to activate the carboxylic acid moiety of **4**, the reaction with H-Gly-OtBu was clean and efficient. The byproducts, isobutanol and *N*-methylmorpholine (NMM), were easily removed in the workup. Column chromatography was not necessary, and near quantitative yields of dipeptide **5** were obtained. Removal of the benzyloxycarbonyl (Cbz) protecting group by catalytic reduction was straightforward and could be done quantitatively on a multigram scale also without chromatographic purification. These steps were repeated for the subsequent coupling of glycine and chloroacetic acid. The protecting groups were then removed by treatment with TFA, and the final peptide was purified by preparative reversed-phase HPLC.¹ Tetrapeptide **2** was isolated in over 90% yield on a 3-g scale. Two other peptides were made by this method, ClAc-βAla-Ser-Gly-OH (**7**) and ClAc-Ser-Gly-OH (**8**), representing a longer and shorter version of peptide **2**, respectively. These peptides were designed to investigate the ideal tether length between the peptide and dinucleotide binding regions.

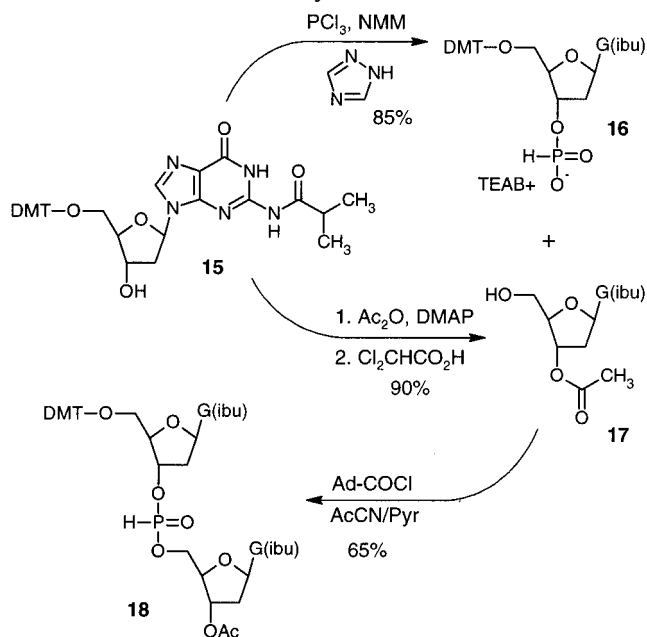
The three corresponding histidine peptides, ClAc-Gly-His-Gly-OH (**12**), ClAc-βAla-His-Gly-OH (**13**), and ClAc-His-Gly-OH (**14**), were made by the method outlined in Scheme 3. Due to known problems with imidazole protecting groups (23), we chose to adapt standard solid-

¹ Reversed-phase HPLC: Varian Dynamax C-18 column, 41.4 × 250 mm, 8 μm, 100 Å; solvent A: 0.1% trifluoroacetic acid (TFA) in water; solvent B: 0.1% TFA in 90/10 acetonitrile/water; gradient: 0–30% solvent B over 30 min at 50 mL/min; UV detection at 220 nm.

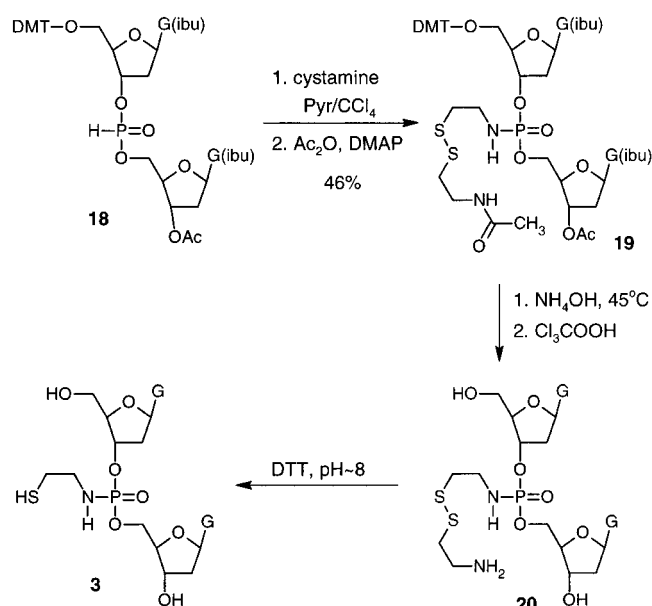
Scheme 2. Synthesis of ClAc-GlySerGly-OH (2)**Scheme 3. Synthesis of ClAc-GlyHisGly-OH (12)**

phase Fmoc methods for the synthesis of these peptides. Peptide couplings were achieved with the commercially available HBTU reagent (2-(1*H*-benzotriazol-1-yl 1,1,3,3-tetramethyluronium hexafluorophosphate), and the protected peptide intermediates were purified by normal-phase column chromatography. The Fmoc protecting group was removed by treatment with diethylamine (DEA) in THF. The free-amine peptides were separated from the dibenzofulvene byproduct by precipitation from cold hexanes. After the chloroacetylation step, the peptides were deprotected with TFA and purified by prep-HPLC.¹ All three histidine peptides were produced on a 3- to 5-g scale in overall yields ranging from 85 to 92%. In due course, all six peptides will be conjugated to the functionalized dinucleotide **3** to produce a series of peptide–dinucleotide conjugates. These conjugates will then be incubated with chromium(III) to study chromium–DNA binding.

Our synthetic approach to the functionalized dinucleotide fragment **3** was inspired by the solid-phase synthesis reported by Fidanza and McLaughlin (24). In our solution-phase synthesis, both guanine nucleoside pieces **16** and **17** were synthesized from DMT-ibu-deoxyguanosine (**15**) which is commercially available and relatively inexpensive (Scheme 4). Treatment of **15** with PCl_3 and triazole afforded the corresponding H-phosphonate **16**, which was isolated and purified as the triethylammonium bicarbonate salt (25, 26). H-Phosphonate **16** could be made on a 3-g scale and purified by normal-phase column chromatography using a gradient of 0–10%

Scheme 4. Dinucleotide Synthesis

MeOH in CHCl_3 in an overall yield of 85%. Nucleoside **17** was made from **15** by acetylating the 3'-hydroxy group followed by the removal of the 5'-DMT protecting group. Nucleoside **17** was also purified by normal-phase chromatography (0–3% MeOH in CHCl_3) and could be

Scheme 5. Dinucleotide Functionalization

prepared on a gram scale in over 90% yield from **15**. The coupling of **16** and **17** was accomplished with adamantanecarbonyl chloride (AdCOCl) in a mixture of acetonitrile and pyridine. The timing for this reaction is critical due to the reactivity of the acylated phosphonate intermediate. H-Phosphonate **16** is treated with AdCOCl and pyridine under anhydrous conditions for 25 s and is then immediately quenched with an anhydrous solution of **17** in acetonitrile. The bis-acyl phosphite (**27**) and trimetaphosphite (**28**) byproducts from this reaction continue to make the scale-up difficult. Currently, we have been able to produce dinucleotide **18** in 65% yield on a 500 mg scale. This dinucleotide was characterized by mass-spec² and 2D NMR (COSY).

The functionalization of the H-phosphonate moiety in **18** was accomplished through oxidation with CCl₄ in the presence of cystamine (Scheme 5). The terminal amine was subsequently acetylated to facilitate its isolation and purification by normal-phase chromatography (0–3% MeOH in CHCl₃). Compound **19** was isolated in 46% yield on a 500 mg scale, and characterized by mass-spec³ and

² Compound **18** mass-spec: monoisotopic mass $M = 1064.4$, calculated for C₅₁H₅₇N₁₂O₁₄P₂; Found MH⁺ at 1065, MNa⁺ at 1087, and [M – H][–] at 1063.

³ Compound **19** mass-spec: monoisotopic mass $M = 1256.4$, calculated for C₅₇H₆₉N₁₂O₁₅PS₂; Found MH⁺ at 1257, MNa⁺ at 1279, and [M – H][–] at 1255.

⁴ Reversed-phase HPLC: Varian Dynamax C-18 column, 41.4 × 250 mm, 8 μm, 100 Å; solvent A: 50 mM HCO₂NH₄ in water (pH ~ 6); solvent B: 90/10 acetonitrile/solvent A; gradient: 0–20% solvent B over 20 min at 50 mL/min; UV detection at 220 nm.

⁵ Compound **20** mass-spec: monoisotopic mass $M = 730.2$, calculated for C₂₄H₃₅N₁₂O₉PS₂; Found MH⁺ at 731, MNa⁺ at 753, and [M – H][–] at 729.

⁶ Compound **3** mass-spec: monoisotopic mass $M = 655.2$, calculated for C₂₂H₃₀N₁₁O₉PS; Found MH⁺ at 656 and [M – H][–] at 654.

⁷ Reversed-phase HPLC: Varian Dynamax C-18 column, 41.4 × 250 mm, 8 μm, 100 Å; solvent A: 50 mM HCO₂NH₄ in water (pH ~ 6); solvent B: 90/10 acetonitrile/solvent A; gradient: 5–15% solvent B over 40 min at 50 mL/min; UV detection at 220 nm.

⁸ Compound **1** mass-spec: monoisotopic mass $M = 914.2$, calculated for C₃₁H₄₃N₁₄O₁₅PS; Found MNa⁺ at 937 and [M – H][–] at 913.

2D NMR (COSY and ROESY). Removal of the isobutryl and acetyl protecting groups was accomplished on a small scale by heating with concentrated NH₄OH at 45 °C for 2 h. The crude product was then treated with 2.5% Cl₃COOH/CH₂Cl₂ to remove the DMT group and then purified by reverse-phase HPLC⁴ to afford approximately 23 mg of compound **20**.⁵ The disulfide moiety was reduced with dithiothreitol (DTT), and the crude product **3**⁶ was then treated with an excess of peptide **2** at pH ~ 10. Approximately 3 mg of peptide–dinucleotide conjugate **1** was isolated by reverse-phase HPLC⁷ and characterized by mass-spec.⁸

CONCLUSIONS

We have synthesized the first of a series of peptide–dinucleotide conjugates that will be used to study chromium(III)–DNA binding. The isolation and characterization of conjugate **1** validates our synthetic approach. Our continuing efforts are aimed at the gram-scale synthesis of **3** and its corresponding conjugates with peptides **2**, **7**, **8**, **12**, **13**, and **14**. With sufficient quantities of our peptide–dinucleotide conjugates in hand, we will then begin to explore their interaction with Cr(III).

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ARTICLES

Syntheses of Curcumin Bioconjugates and Study of Their Antibacterial Activities against β -Lactamase-Producing Microorganisms

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In the present study curcumin bioconjugates, viz. di-*O*-glycinoylcurcumin (**I**), di-*O*-glycinoyl-*C*⁴-glycylcurcumin (**II**), 5'-deoxy-5'-curcuminylylthymidine (5'-cur-T) (**IV**), and 2'-deoxy-2'-curcuminylyluridine (2'-cur-U) (**V**) have been synthesized and characterized by elemental analysis and ¹H NMR. The turmeric peptide (Tp) was isolated from the aqueous turmeric extract of the turmeric rhizome. The antibacterial activity of these four bioconjugates and also of the turmeric peptide and sodium salt of curcumin (**III**) have been tested particularly for β -lactamase-producing microorganisms.

INTRODUCTION

Most novel drug-delivery systems have stemmed from work on new polymers, lipid vesicles, cyclodextrins, prodrugs, and viral vectors. The rationale behind systemic drug delivery is to achieve greater efficacy with lower toxicity. Enhancing the specificity of therapeutic drugs and thereby improving their site-specific delivery is the primary goal of today's pharmaceutical industries. The past decade has seen extensive use of liposomes and lipid-based delivery systems to improve the pharmacological properties of a variety of drugs (1–7). The principal benefits afforded by therapeutic agents through their liposomal encapsulation were enhanced plasma circulation lifetimes, increased delivery to specific sites, and changes in tissue distribution, which can result in reduced toxic effects.

Lipid–oligonucleotide complexes can provide levels of encapsulation, but their size and charge severely impair their ability to distribute systemically after parenteral administration and subsequently extravasate to the disease site. As a result, this type of liposomal carrier has a limited utility for the treatment of systemic diseases (8).

Keeping this rationale in mind, a bioconjugate can be synthesized composed of a nucleoside and any suitable molecule which should itself be active against inflammatory disorders, and it must be devoid of any charge. Nucleosides and amino acids rapidly cross the plasma membrane of the cells by a facilitated transport mechanism (9), thus gaining rapid entry into the cells. The curcumin molecule, meeting such requirements, was chosen for this dual purpose. It is the main coloring component of turmeric, the rhizome of the plant *Curcuma*

longa linn. Curcumin (1,7-bis(4-hydroxy-5-methoxyphenyl)-1,6-heptadiene-3,5-dione/diferuloyl methane) has been used for centuries as a traditional medicine for external/internal wounds, liver diseases (particularly jaundice), blood purification, and inflamed joints (rheumatoid arthritis) (10–13). It has been reported to have antioxidant properties (14, 15). Turmeric and curcumin have been shown to inhibit carcinogen-induced mutation in the Ames assay and the formation of tumors in several experimental systems, suggesting antiinitiating and/or antipromoting activity against several chemical carcinogens (16–20). Recently, it has been reported to decrease total cholesterol and LDL cholesterol levels in serum and also to increase the beneficial HDL cholesterol level. Curcumin is reported to inhibit the proliferation of HUVEC (human umbilical vein endothelial cells); therefore, it could turn out to be a useful compound for the development of novel anticancer therapy (21). It has also been claimed that curcumin inhibits HIV-I integrase protein, thus potentially preventing HIV-I from infecting CD-4 and CD-8 cells (22). The other attractive features of curcumin to explore as a vulnerary agent is that, despite being eaten daily for centuries in Asian countries, curcumin has not been reported to be toxic (11).

Curcumin has an interesting structure with two phenolic groups and one active methylene function, which are potential sites for attaching biomolecules. There is not much scope for structural variation in the curcumin molecule in order to enhance its activity, since it is reported that blocking the phenolic groups decreases its antioxidant activity; therefore, phenolic groups appear to be the sites involved in enzymatic activity at receptor sites. The double bonds are essential for proper conformational flexibility of the molecule.

However, its meager absorption through the intestinal wall on oral intake needs to be improved in order to achieve significant concentration inside the cells for appropriate activity. One of the most easily accessible approaches is to make bioconjugates of this molecule by

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covalent attachment with ligands to promote entry into the pathogen cell. Since glycine and uridine are essential components of bacterial cell wall, when linked covalently to curcumin they may act as carrier molecules and thereby increase the intracellular delivery of curcumin.

Henceforth, the nucleoside–curcumin bioconjugates and amino acid–curcumin bioconjugates can serve a dual purpose of systemic delivery as well as a therapeutic agent against viral diseases. The size of these molecules as compared to liposomes ($>1\text{--}2\text{ }\mu\text{m}$) is small enough to get thorough passage in pulmonary capillaries, and the approach for improving the uptake involves such a design that would allow enzyme-mediated transformation of the bioconjugate within the target organ. The conjugate bonds reported herein in the case of amino acid–curcumin are exclusively enzyme sensitive to cause a positive systemic delivery. During the present work, a number of such conjugates have been synthesized, e.g., di-*O*-glycinoylcurcumin (**I**), di-*O*-glycinoyl-*C*⁴-glycylcurcumin (**II**), 5'-deoxy-5'-curcuminythymidine (5'-cur-T) (**IV**), and 2'-deoxy-2'-curcuminyluridine (2'-cur-U) (**V**), and tested against a number of Gram-positive and Gram-negative bacteria isolated from clinical specimens.

MATERIALS AND METHODS

All solvents used were thoroughly dried and distilled prior to use. Curcumin, glycine, allyl bromide, and triphenyl phosphite were purchased from Merck-Schuchardt, Germany. Nucleosides were purchased from Sigma Chemical Company, St. Louis, MO. Mueller–Hinton broth, agar, and sterile disks were purchased from Hi Media Laboratory Ltd, Mumbai, India. The water-soluble turmeric peptide (Tp) was purified from aqueous turmeric extract, lyophilized, and stored at $-30\text{ }^{\circ}\text{C}$.

Mueller–Hinton broth and agar have been selected for testing aerobic and facultative anaerobic bacterial isolates for fastidious organisms such as *Streptococci* and *Peptococci*. The agar was supplemented with 5% defibrinated blood. The microsusceptibility tests were standardized at pH 7.4, and agar and broth were incubated in an ambient air incubator at $37\text{ }^{\circ}\text{C}$.

The inoculum was prepared from broth culture that had been incubated for 4–6 h, when growth was considered in the logarithmic phase. Amoxyclave, taken as standard drug, is a combination of amoxicillin and clavulanate.

Experimental Guidelines To Determine Zone of Inhibition by Kirby–Baur's Method (24). 100 mg each of **I**, **II**, **III**, **IV**, **V**, curcumin, and peptide were weighed and dissolved in 10 mL of acetone. From this stock solution, serial dilutions were performed to get 30, 15, 7.5, 3.75, 1.88, and $0.94\text{ }\mu\text{mol/mL}$ with acetone in sterile test tubes. Sterilized filter disks were dipped in these solutions and subsequently dried to remove acetone. Nutrient agar medium plates were prepared using Mueller–Hinton agar and allowed to solidify. Seven different bacterias were selected, viz. *Enterobacter cloacae*, *Peptococcus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Micrococcus cocci*, *Staphylococcus aureus*, and *Streptococcus pyogenes* and 1 mL each of the bacteria's culture broth was added to the plates and spread with the help of a sterile spreader. The filter paper disks soaked in bacterial strain were placed aseptically over the inoculated plates using sterile forceps. The plates were incubated at $37\text{ }^{\circ}\text{C}$ for 18 h in upright position. The zone of inhibition was measured using scale.

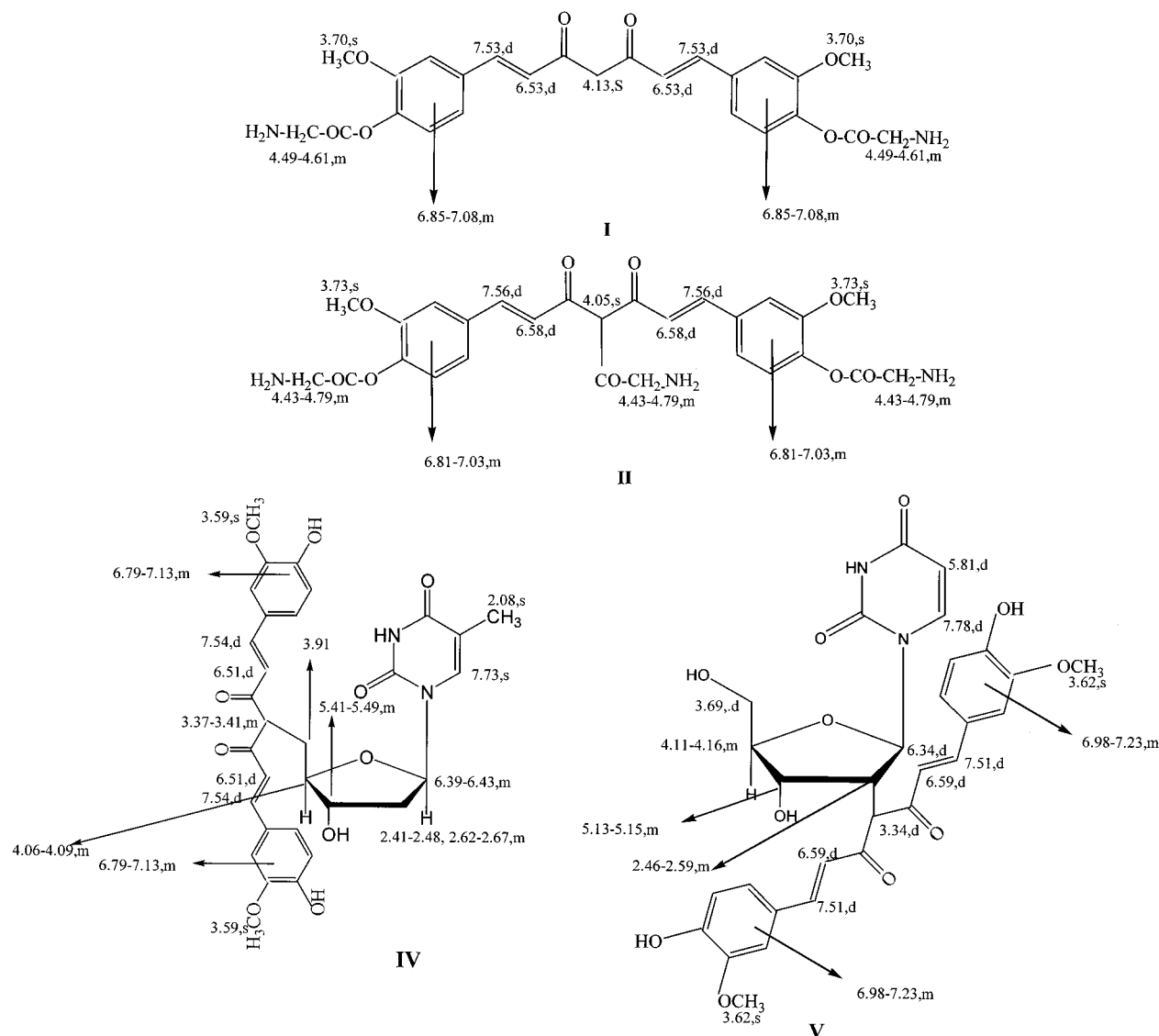
Experimental Guidelines To Determine Mic by the Micro Dilution Broth Susceptibility Test. Dif-

ferent concentrations 30, 15, 7.5, 3.75, 1.88, and $0.94\text{ }\mu\text{mol/mL}$ of **I**, **II**, **III**, **IV**, **V**, curcumin, and turmeric peptide were prepared in sterile dry test tubes. Nutrient broth was prepared using Mueller–Hinton broth (M391), and 4.9 mL of it was added to each test tube and then sterilized after plugging. After cooling, 0.1 mL of each of the dilutions was added to the test tubes, and final volume was brought to 5.0 mL. To each of the test tubes was added 0.1 mL of bacterial culture broth. The test tubes were shaken to uniformly mix the inoculum with the broth. The tubes were incubated at $37\text{ }^{\circ}\text{C}$ for 18 h. Appearance of any turbidity shows that the compound is not able to inhibit the growth of bacteria, while no turbidity indicates the inhibition of microorganism by the sample.

1,7-Bis(4-*O*-glycinoyl-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (I**).** Curcumin (368 mg; 1 mmol) was dissolved in dry pyridine, mixed with *N*-phthaloylglycinoyl chloride (536 mg; 2.4 mmol), and stirred at room temperature for 6 h. After the completion of reaction, the reaction mixture was poured into crushed ice and thoroughly extracted with EtOAc. The organic layer was concentrated and treated with ammonia: pyridine (9:1 v/v) for 1 min at room temperature. The organic layer was concentrated and purified by silica gel column chromatography using a DCM:methanol gradient, yield 40% (192 mg). The pure product was characterized by elemental data and ^1H NMR. Anal. Found: C, 62.05; H, 5.56; N, 5.61%. Calcd for $\text{C}_{25}\text{H}_{26}\text{O}_8\text{N}_2$: C, 62.17; H, 5.39; N, 5.80%. ^1H NMR (CDCl_3) δ = 3.70 (s, 6H, OCH_3), 4.13 (s, 2H, $\text{C}_4\text{-H}$), 4.49–4.61 (m, 4H, CH_2NH_2), 6.53 (d, 2H, $\text{C}_2\text{-H}$ and $\text{C}_6\text{-H}$), 6.85–7.08 (m, 6H, Ar-H), 7.53 (d, 2H, $\text{C}_1\text{-H}$ and $\text{C}_7\text{-H}$).

1,7-Bis(4-*O*-glycinoyl-3-methoxyphenyl)-1,6-heptadiene-*C*⁴-glycinoyl-3,5-dione (II**).** Curcumin (368 mg; 1 mmol) was dissolved in EtOH, NaOEt (containing 83 mg of metallic sodium; 3.6 mmol) was added dropwise for 10 min, and the reaction mixture was stirred at room temperature for 30 min. The resulting sodium salt was concentrated under vacuo, thoroughly washed with EtOH, dissolved in dry pyridine, mixed with *N*-phthaloylglycinoyl chloride (804 mg; 3.6 mmol), added to the reaction mixture, and stirred at room temperature for 6 h. After completion of reaction, the reaction mixture was poured into crushed ice and thoroughly extracted with EtOAc. The organic layer was concentrated and treated with ammonia:pyridine (9:1 v/v) for 1 min at room temperature. The reaction mixture was poured into crushed ice and extracted again with EtOAc. The organic layer was concentrated and purified by silica gel column chromatography using a DCM:methanol gradient, yield 38% (205 mg). The pure product was characterized by elemental data and ^1H NMR. Anal. Found: C, 59.88; H, 5.60; N, 7.51%. Calcd for $\text{C}_{27}\text{H}_{29}\text{O}_9\text{N}_3$: C, 60.04; H, 5.48; N, 7.78%. ^1H NMR (CDCl_3) δ = 3.73 (s, 6H, OCH_3), 4.05 (s, 1H, $\text{C}_4\text{-H}$), 4.43–4.79 (m, 6H, CH_2NH_2), 6.58 (d, 2H, $\text{C}_2\text{-H}$ and $\text{C}_6\text{-H}$), 6.81–7.03 (m, 6H, Ar-H), 7.56 (d, 2H, $\text{C}_1\text{-H}$ and $\text{C}_7\text{-H}$).

5'-Deoxy-5'-curcuminythymidine (IV**).** 5'-Deoxy-5'-bromothymidine (305 mg, 1 mmol), synthesized by a procedure developed in our laboratory (23), was dissolved in dry pyridine and mixed with **III** (627 mg, 1.3 mmol). The reaction mixture was stirred at room temperature for 8 h. The product was treated with 2 N alcoholic NaOH at room temperature for 45 min to afford 5'-deoxy-5'-curcuminythymidine by the usual workup procedure as described above, yield 40% (237 mg). The pure product was characterized by elemental data and ^1H NMR. Anal. Found: C, 62.59; H, 5.56; N, 4.54%.

Chart 1. ^1H NMR Assignments for **I**, **II**, **IV**, and **V**

Calcd for $\text{C}_{31}\text{H}_{32}\text{O}_{10}\text{N}_2$: C, 62.77; H, 5.40; N, 4.72%. ^1H NMR (CDCl_3) δ = 2.08 (s, 3H, C_5-CH_3 of **T**); 2.41–2.48, 2.62–2.67 (m, 2H, 2'-H); 3.37–3.41 (m, 1H, C_4-H of **Cur**); 3.59 (s, 6H, OCH_3); 3.91 (m, 2H, 5'-H); 4.06–4.09 (m, 1H, 4'-H); 5.41–5.49 (m, 1H, 3'-H); 6.39–6.43 (m, 1H, 1'-H); 6.51 (d, 2H, C_2-H and C_6-H of **Cur**); 6.79–7.13 (m, 6H, Ar-H); 7.54 (d, 2H, C_1-H and C_7-H); 7.73 (s, 1H, C_6-H of **T**).

2'-Deoxy-2'-curcuminylluridine (V). 2'-Deoxy-2'-chlorouridine (263 mg, 1 mmol), synthesized by the known procedure (22), was dissolved in dry pyridine and mixed with **Ia** (627 mg, 1.3 mmol). The reaction mixture was stirred at room temperature for 8 h. The product was treated with 2 N alic NaOH to get 2'-deoxy-2'-curcuminylluridine by the usual workup procedure as describe above, yield 40% (237 mg). The pure product was characterized by elemental data and ^1H NMR. Anal. Found: C, 60.37; H, 5.19; N, 4.66%. Calcd for $\text{C}_{30}\text{H}_{30}\text{O}_{11}\text{N}_2$: C, 60.55; H, 5.05; N, 4.71%. ^1H NMR (CDCl_3) δ = 2.46–2.59 (m, 1H, 2'-H); 3.34 (d, 1H, C_4-H of **Cur**); 3.62 (s, 6H, OCH_3); 3.69 (d, 2H, 5'-H); 4.11–4.16 (m, 1H, 4'-H); 5.13–5.15 (m, 1H, 3'-H); 5.81 (d, 1H, C_5-H of uridine); 6.34 (d, 1H, 1'-H); 6.59 (d, 2H, C_2-H and C_6-H of **Cur**); 6.98–7.23 (m, 6H, Ar-H); 7.51 (d, 2H, C_1-H and C_7-H of **Cur**); 7.78 (d, 1H, C_6-H of uridine).

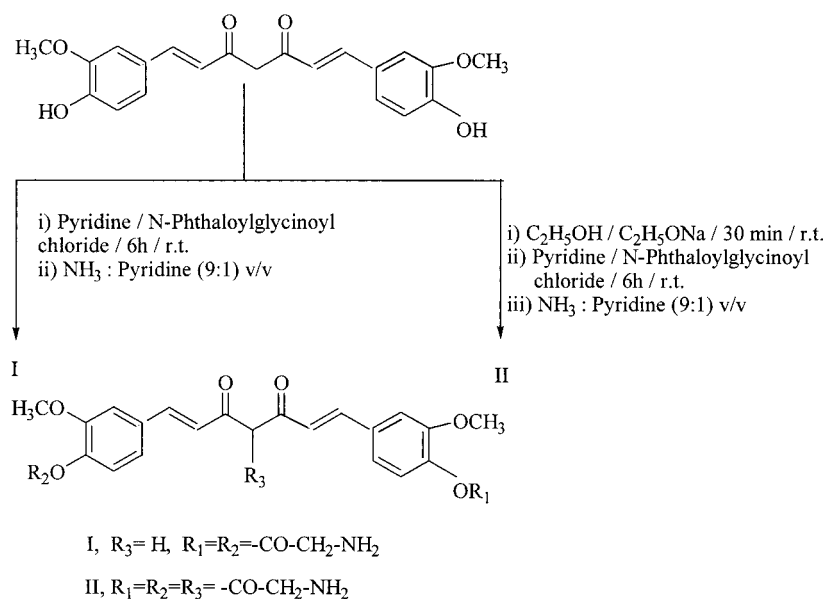
RESULTS AND DISCUSSION

Schemes 1 and 2 show the experimental steps involved in the preparation of **I** and **II**, and **IV** and **V**, respectively. In the case of **II**, a strong base, NaOEt, was used whereas in the case of **I**, curcumin itself was treated with *N*-phthaloylglycinoyl chloride (Scheme 1).

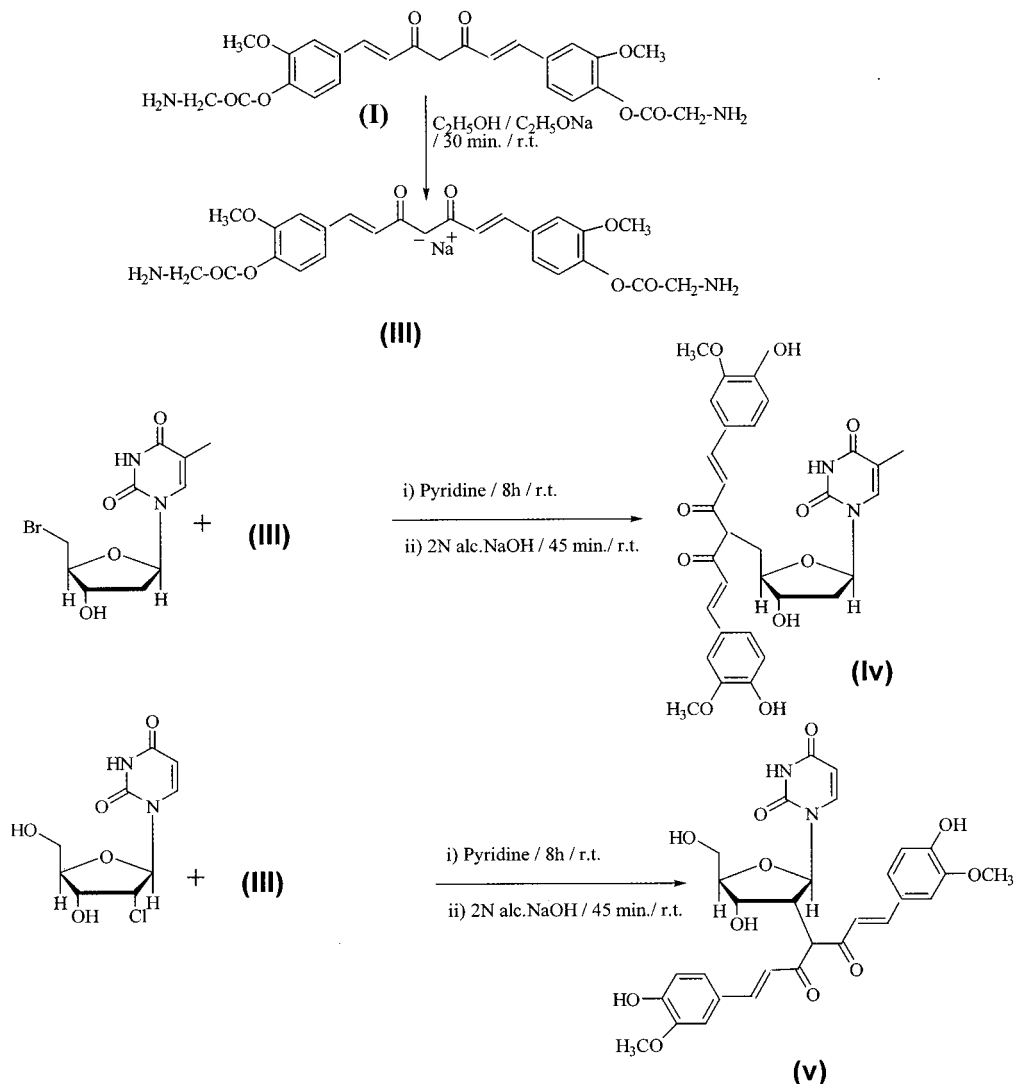
5'-Deoxy-5'-curcuminylluridine (5'-cur-T) (**IV**) and 2'-deoxy-2'-curcuminylluridine (2'-cur-U) (**V**) were synthesized by adding 5'-deoxy-5'-bromothymidine and 2'-deoxy-2'-chlorouridine (23) to pyridine mixed with **III** as shown in (Scheme 2).

The antibacterial activity of bioconjugates **I**, **II**, **III**, **IV**, **V**, and **TP** were compared with curcumin from a known microdilution broth susceptibility test method. These were serially diluted (30, 15, 7.50, 3.75, 1.88, 0.94 $\mu\text{mol/mL}$) and added to Mueller–Hinton broth, after which a standardized bacterial suspension was added. One of the disks was kept free of antibiotic and served as growth control, and the other disks were inoculated with a calibrated suspension of microorganism to be tested and incubated at 37 °C for 18 h. The lowest concentration of curcumin bioconjugates in $\mu\text{mol/mL}$ that prevents the in vitro growth of microorganism is represented as MIC (minimum inhibitory concentration) (Table 1) and correlated with the zone of inhibition (Table 2).

Scheme 1



Scheme 2



Each test was performed in triplicate, and the MICs reported represent the result of at least two repetitions. Out of four, three bioconjugates (**I**, **II**, and **V**) show a positive result on multiresistant microorganisms. The

most encouraging result was found against *Streptococcus pyogenes*, with **I** having an MIC of $1.88 \mu\text{mol/mL}$, compared with one of the most widely marketed antibiotics, viz. Amoxycylav, that has an MIC of $7 \mu\text{mol/mL}$ (6

Table 1. MIC Correlation Diagram (in $\mu\text{mol/mL}$)^{a,b}

no.	name of bacteria	I	II	V	Cs	Cur	T
1	<i>E. cloacae</i>	3.75	7.50	15.00	30	R ^c	R
2	<i>Peptococcus</i>	3.75	7.50	30.00	R	R	3.75
3	<i>Staphylococcus epidermidis</i>	15.00	15.00	7.50	7.50	R	R
4	<i>Staphylococcus saprophyticus</i>	3.75	30.00	15.00	7.50	R	R
5	<i>Micrococcus cocci</i>	3.75	15.00	3.75	7.50	R	R
6	<i>Staphylococcus aureus</i>	15.00	30.00	7.50	15.00	R	R
7	<i>Streptococcus pyogenes</i>	1.88 ^d	3.75	15.00	R	15.00	R

^a **I**: Di-O-glycinoylcurcumin, **II**: di-O-glycinoyl-C⁴-glycylcurcumin, **V**: 2'-deoxy-2'-curcuminylluridine (2'-cur-U), **Cs**: sodium salt of curcumin, **Cur**: curcumin, **T**: turmeric. ^b Results with 5'-deoxy-5'-curcuminyllthymidine (5'-cur-T) are not included. ^c R: resistant (below 10 mm). ^d **I** shows the best result against *Streptococcus pyogenes* (1.88 $\mu\text{mol/mL}$), and it was compared with Amoxycylav (7 $\mu\text{mol/mL}$).

Table 2. Antibacterial Activity of Curcumin Bioconjugates (zone of inhibition in mm)^{a,b}

no.	name of bacteria	I	II	V	Cs	Cur	T
1	<i>E. cloacae</i>	24, 22, 20, 16	20, 18, 16	14, 12	13	R ^c	R
2	<i>Peptococcus</i>	18, 16, 14, 12	16, 12, 11	13	R	R	22, 20, 18, 12
3	<i>Staphylococcus epidermidis</i>	14, 12	15, 13	16, 15, 14	15, 12, 11	R	R
4	<i>Staphylococcus saprophyticus</i>	18, 16, 14, 12	12	13, 12	20, 16, 14	R	R
5	<i>Micrococcus cocci</i>	16, 15, 13, 11	15, 12	18, 16, 14, 12	16, 13, 11	R	R
6	<i>Staphylococcus aureus</i>	14, 12	12	13, 12, 11	14, 12	R	R
7	<i>Streptococcus pyogenes</i>	28, 24, 20, 18, 16	22, 24, 18, 16	14, 12	R	20, 16	R

^a **I**: Di-O-glycinoylcurcumin, **II**: di-O-glycinoyl-C⁴-glycylcurcumin, **V**: 2'-deoxy-2'-curcuminylluridine (2'-cur-U), **Cs**: sodium salt of curcumin, **Cur**: curcumin, **T**: turmeric. ^b Zone of inhibition is reported with respect to serial dilution (30, 15, 7.5, 3.75, 1.88, 0.94 $\mu\text{mol/mL}$). ^c R: resistant (below 10 mm).

$\mu\text{mol/mL}$ reported). This shows that **I** is 3.7 times more effective than Amoxycylav.

The result of zone of inhibition is also encouraging, as the disk containing 30 μg of Amoxycylav was purchased and same amount of **I** was loaded on a separate disk. The zone of inhibition of Amoxycylav was found to be 20 mm while **I** showed 28 mm zone of inhibition. The MICs of **II**, **V**, and **III** were found between 4 and 8 $\mu\text{mol/mL}$ in most cases as compared to available antibiotics on the market having an MIC between 6 and 10 $\mu\text{mol/mL}$. **Ip** has a significant result against *Peptococcus*, MIC 3.75 $\mu\text{mol/mL}$. In comparison to the above results, curcumin and turmeric were found to be resistant in most cases except against *Streptococcus pyogenes* where curcumin shows MIC 15 $\mu\text{mol/mL}$. The results with 5'-deoxy-5'-curcuminyllthymidine (**IV**) were far from satisfactory against the selected bacterial strains, which may be due to the fact that thymidine is not a natural component of bacterial genome and hence unable to internalize in the bacterial cell wall (data not shown). The results suggest that amino acids glycine and D-alanine are natural components of the bacterial cell wall, thus the positive results of **I** and **II**, whereas the positive result of **V** is due to uridine, which is an essential component of the bacterial cell wall and thus well recognized by bacteria. However, the amino acid bioconjugates have been found to be bacteriologically more active (approximately two times) than nucleoside bioconjugates, since the former, in addition to being internalized like the latter, are also more water soluble. The hydrophilic nature of these bioconjugates may also help in active transport across cellular membrane. It is therefore anticipated that bioconjugates composed of D-alanine would give the best results. This work is in progress and will be published elsewhere.

CONCLUSION

Conventionally, antibiotics are used to kill bacteria or to halt their division with the intention of preventing replication of the bacterial genome, but overuse of antibiotics in humans and livestock has led to the rapid evolution of bacteria that are resistant to multiple drugs (25), and recently there has been a call for worldwide use

of antibiotic rotation schemes to conquer the problems of resistance (26). β -Lactamase present in bacterial cellular fluid hydrolyzes the amide bond of the β -lactam ring of penicillins and cephalosporins, producing acidic derivatives which have no antibacterial properties, thereby making the bacteria resistant to that particular antibiotic drug.

To develop a new armory of agents active against antibiotic-resistant bacteria, nonconventional antibiotics could be designed that can internalize themselves in the bacterial cell wall easily. The structures should be devoid of the β -lactam ring for better efficacy.

Curcumin, the main molecule studied in this paper, is reported to be capable of expanding the cell membrane in in vivo conditions in eukaryotic cells, inducing echinocytosis (27), and changes in cell shape are accompanied by transient exposure to phosphatidyl serine. Membrane asymmetry was recovered in the presence of aminophospholipid traslocase, which remains active in the presence of curcumin, thereby suggesting that curcumin is able to be transported if covalently attached to a molecule which can easily cross the cell wall both in eukaryotic and in prokaryotic cells. This fact is supported by the studies reported here, by curcumin bioconjugates, where curcumin is conjugated with a biomolecule which is the active part of either the bacterial genome, i.e., uridine (**V**), or of the bacterial cell wall, i.e., amino acids (**I** and **II**) gave excellent results, whereas **IV**, i.e., curcumin conjugated with thymidine, failed to show positive results since thymine is not a natural component of the bacterial genome, thus unable to internalize in the bacterial cell wall.

More such bioconjugates may be designed by keeping one of the components common to either the bacterial cell wall or bacterial genome conjugated with curcumin, thereby improving the efficiency of curcumin comprehensively at low doses, and may prove to be better systemic drugs by concentrating mainly on and around malignant and ill-fated cells by recognizing the bacterial cells. This study proves once again the efficacy of curcumin and its bioconjugates and their antibacterial properties.

The present work suggests that the need for nonantibiotic drugs (28) which may overcome antibiotic resis-

tance may be fulfilled in the near future. A new series of inexpensive and highly bioactive drugs with no toxicity may be in the offing.

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Studies on Protein–Liposome Coupling Using Novel Thiol-Reactive Coupling Lipids: Influence of Spacer Length and Polarity

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To optimize the preparation of immunoliposomes, we investigated the coupling of thiolated IgG and BSA to liposomes using a novel group of coupling lipids. All lipids consist of cholesterol as membrane anchor and a thiol-reactive maleimide headgroup, linked by a spacer that differs in length and polarity (ethylene glycol, tetraethylene glycol, PEG 400, PEG 1000, dodecyl). In addition, lipids differ in the electrophilicity of the maleimide group (*p*- or *m*-maleimidobenzoic ester). In the case of BSA, coupling efficiency strongly depended on the electrophilicity of the maleimide group as well as on the spacer polarity: The less electrophilic meta constitution seems to be an advantage over the *p*-maleimidobenzoic ester, resulting in higher coupling efficiency. Polar spacers (tetraethylene glycol, 46%) achieved a higher coupling efficiency than a nonpolar spacer with approximately the same length (dodecyl, 15%). When liposomes containing coupling lipids with the spacers tetraethylene glycol, PEG 400, and PEG 1000 were linked to BSA, coupling efficiencies were in a medium range and similar (41–46%) but were lower for the short ethylene glycol spacer (30%). In contrast, for IgG coupling efficiencies correlated with increasing spacer length. Best results were obtained using coupling lipids with a long polar spacer (PEG 1000) (65%), whereas a coupling lipid bearing a short spacer (ethylene glycol) resulted in a low coupling efficiency of 12%.

INTRODUCTION

Encapsulation of chemotherapeutic drugs into liposomes has been shown to be an effective strategy to increase the pharmacological index, i.e., to increase the antitumor activity and to decrease adverse side effects (1–3). When liposomes are used as drug carriers, not only the drugs can be prevented from their rapid degradation but also passive and active targeting can be achieved.

Passive targeting is an accumulation of liposomes in tumors or in inflamed tissues, based on the effect that blood vessels in such tissues are very often leaky, so that liposomes can extravasate (4–6). These particular morphological changes in the vasculature of tumors and inflamed tissues are most probably caused by angiogenesis (7, 8). Passive targeting of liposomes can be enhanced by increasing their half-life in blood, because extravasation is more probable with prolonged circulation in the blood stream. The lifetime of liposomes in the blood stream can be extended by preventing their interactions with serum components (e.g., antibodies or complement factors). The adsorption of serum proteins (opsonization) results in an increased uptake of liposomes by cells of the mononuclear phagocyte system (MPS) (9). This process can be prevented by coating liposomes with long poly(ethylene glycol) (PEG) chains (10–12) or defined glycolipids (13, 14), e.g., monosialo gangliosids (sterical stabilization). Clinical studies with nontargeted sterically stabilized liposomes containing doxorubicin have been reported (15, 16). Meanwhile, liposomal doxorubicin and daunorubicin have been admitted to clinical approach.

In contrast to passive targeting, active targeting of liposomes is based on specific interactions between liposome-bound structures (mostly antibodies or Fab-

fragments, immunoliposomes) and their defined antigens on the cell surfaces, for example, immunoliposomes bearing antibodies against defined structures on human ovarian carcinoma cells, human breast cancer cells, human B-cell lymphoma, and many other targets (17–21). The interaction between immunoliposomes and their target structures depends on liposome half-life. Hence, investigations on sterical stabilization of immunoliposomes have been reported (22–25).

An important prerequisite for the preparation of immunoliposomes for clinical use is a highly reproducible and effective method for liposome–protein coupling, which can easily be performed. Different methods based on noncovalent and covalent protein coupling have been described (23, 26–29). A widespread strategy is the use of coupling lipids containing maleimide groups. This coupling procedure is based on the rapid reaction of proteins bearing thiol groups with the thiol-reactive maleimide moiety, linked to the liposome surface by a lipid anchor.

Maleimide derivatives have been shown to be immunogenic (30, 31). Nevertheless, coupling lipids con-

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¹ Abbreviations used: BSA, bovine serum albumin; PEG, poly(ethylene glycol); MPB-PE, *N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine; *m*-MCS-1, *O*-(3-cholesterylloxycarbonyl)propionyl-*O'*-*m*-maleimidobenzoyl ethylene glycol; *m*-MCS-4, *O*-(3-cholesterylloxycarbonyl)propionyl-*O'*-*m*-maleimidobenzoyl tetraethylene glycol; *p*-MCS-4, *O*-(3-cholesterylloxycarbonyl)propionyl-*O'*-*p*-maleimidobenzoyl tetraethylene glycol; *m*-MCS-9, *O*-(3-cholesterylloxycarbonyl)propionyl-*O'*-*m*-maleimidobenzoyl poly(ethylene glycol) 400; *m*-MCS-22, *O*-(3-cholesterylloxycarbonyl)propionyl-*O'*-*m*-maleimidobenzoyl poly(ethylene glycol) 1000; *m*-MCS-Do, *O*-(3-cholesterylloxycarbonyl)propionyl-*O'*-*m*-maleimidobenzoyl 1,12-dodecanediol; EPC, egg phosphatidylcholine, SATA, *N*-succinimidyl *S*-acetylthioacetate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid), HPTLC, high-performance thin-layer chromatography, BCA, bicinchoninic acid; DPPC, dipalmitoyl phosphatidylcholine.

taining maleimide groups are widely used for immuno-liposome preparation (17, 19, 25, 32). A common method is the use of *N*-[4-(*p*-maleimidophenyl)butyl]phosphatidylethanolamine (MPB-PE), first described by Martin and Papahadjopoulos in 1982 (32). MPB-PE is a coupling lipid consisting of phosphatidylethanolamine as membrane anchor that is linked to a thiol-reactive maleimide group via a short butyryl spacer. Unfortunately, no systematic studies on the influence of variations of the spacer moiety and the thiol-reactive head-group of MPB-PE on coupling efficiency are available.

Recently, we synthesized a group of thiol-reactive coupling lipids based on cholesterol as lipid anchor and bearing different spacer moieties. These lipid derivatives vary in length and polarity of the spacer unit and in electrophilicity of the thiol-reactive maleimide group (33, 34). The aim of this study was to investigate the influence of these structural variations on the coupling efficiency of IgG and BSA to the respective liposomes.

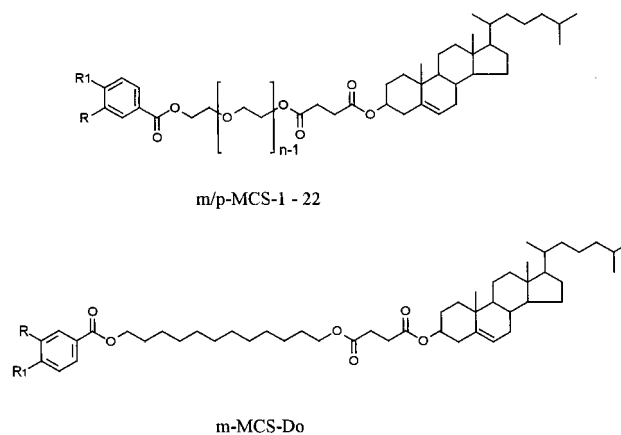
MATERIALS AND METHODS

Materials. Egg-phosphatidylcholine (EPC) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (isolated from lanolin and recrystallized from ethanol), dimethylformamide, and *N*-ethylmaleimide were from Fluka (Buchs, Switzerland). Bovine albumin fraction V (BSA), bovine IgG (reagent grade), 2-iminothiolane HCl (Traut's reagent), *N*-succinimidyl *S*-acetylthioacetate (SATA), 5,5'-dithiobis(2-nitrobenzoic acid) (Ellmans reagent), and hydroxylamine HCl grade I were purchased from Sigma (Deisenhofen, Germany). Chloroform, methanol, diisopropyl ether, 2-propanol, ethyl acetate, phosphoric acid (85%), cupric sulfate, HPTLC plates, and HEPES were obtained from Merck (Darmstadt, Germany). Sephadex G 25 superfine was purchased from Pharmacia (Uppsala, Sweden). The bicinchoninic acid protein assay kit (used with bovine serum albumin and bovine IgG, respectively, as standard) was obtained from Pierce (Rockford, IL).

Thiol-Reacting Coupling Lipids. The thiol-reactive lipids used in this study were prepared as described previously (33): In brief, lipids were synthesized by a linear reaction sequence starting from cholesterol hemisuccinate, obtained from cholesterol and succinic anhydride. Cholesterol hemisuccinate was converted into the respective acid chloride and esterified with a 10-fold excess of the respective α,ω -diol spacer to achieve monoacylation. After separation of the diester byproduct, the desired monoester product was esterified with the *m*- or *p*-maleimidobenzoic acid chloride. Structures and abbreviations of all lipids are shown in Figure 1.

Preparation of Liposomes. Liposomes were composed of EPC/cholesterol/coupling lipid at a molar ratio of 58.8:39.2:2. A mixture of the appropriate amounts of lipids (typically: 120 μ mol of total lipid) was dissolved in chloroform/2-propanol 2:1 (v/v) and evaporated to dryness by rotary evaporation at 40 °C. Afterward, the lipid film was rehydrated in 4 mL of HEPES buffer (20 mM HEPES, 135 mM NaCl, pH 6). The resulting lipid dispersion was extruded sequentially 15 times through polycarbonate membrane filters with a pore diameter of 400 nm and subsequently 15 times through filters with a pore diameter of 100 nm using a Lipofast Extruder (Avestin, Canada). The temperature during extrusion was 40 °C. The liposome dispersion was diluted with HEPES buffer (20 mM HEPES, 135 mM NaCl, pH 6) to a final lipid concentration of 10 mM.

Determination of Liposome Composition by HPTLC. Prior to chromatography, the liposome disper-



name	spacer	n	R	R ¹
m-MCS-1	ethylene glycol	1	maleimido	H
m-MCS-4	tetraethylene glycol	4	maleimido	H
p-MCS-4	tetraethylene glycol	4	H	maleimido
m-MCS-9	PEG 400	9 (8-10)	maleimido	H
m-MCS-22	PEG 1000	22 (20-25)	maleimido	H
m-MCS-Do	dodecyl	-	maleimido	H

Figure 1. Structures of the coupling lipids used in this study.

sion was diluted with 2-propanol at a ratio of 1:3 (v/v). Standard solutions of the respective coupling lipid dissolved in ethyl acetate were prepared from 0.25 to 1 mM. The HPTLC plates were prerun with a mobile phase of diisopropyl ether/ethyl acetate/methanol/acetic acid (20:10:1:0.2, v/v/v/v). Samples and standard solutions were applied to the HPTLC plates using a Linomat IV (Camag, Berlin). Then the plates were developed in the mobile phase mentioned above. After the plate was dried at 110 °C for 15 min on a heating plate, lipid spots were visualized by staining with cupric sulfate solution (10%, w/v) in phosphoric acid (85%). The HPTLC plate was dipped into the dye solution for 15 s and then dried on the heating plate (5 min at 110 °C and subsequently 10 min at 170 °C). Finally, the lipid spots were quantified using a densitometer (Camag, Berlin) at a wavelength of 420 nm.

Thiolation of Proteins with 2-Iminoethiolane. A 0.05 mM solution of BSA in phosphate buffer (0.1 M NaH₂PO₄, pH 8) was incubated with 2-iminoethiolane at a molar ratio of iminoethiolane/BSA of 15:1 for 60 min at room temperature. For the thiolation of IgG, a 20-fold excess of 2-iminoethiolane was added to a 0.04 mM solution of IgG in phosphate buffer. Unbound 2-iminoethiolane was separated from the protein by gel filtration using a 1 × 10 cm Sephadex G-25 superfine column (Pharmacia, Uppsala, Sweden). The products were eluted with sterile 0.1 M phosphate buffer (pH 8). The thiolated BSA or IgG were immediately used for covalent coupling.

The thiolation grade was measured using 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) as described by Benzinger et al. (34). A 20-fold excess of 2-iminoethiolane resulted in two to three thiol groups per IgG. In the case of BSA, the same thiol density was achieved by using a 15-fold excess of 2-iminoethiolane.

Thiolation of IgG with SATA. Just before use, 4 mg of *N*-succinimidyl *S*-acetylthioacetate (SATA) (35) was dissolved in 1 mL of dimethylformamide and added to 1

mL of a 0.04 mM IgG solution in 0.1 M phosphate buffer (pH8) at a SATA/IgG molar ratio of 7:1. A 7-fold excess of SATA was necessary to achieve the desired density of two to three thiol groups per protein. After 20 min of incubation at room temperature, IgG was separated from unreacted SATA by gel filtration with a Sephadex G-25 superfine column in 0.1 M phosphate buffer (pH 8).

Coupling of Proteins to Liposomes. For the covalent attachment of proteins to liposomes, 5 nmol of thiolated BSA or IgG, respectively, was added to 1 mL of freshly prepared 10 mM MCS-liposomes at a maleimide/protein molar ratio of 40:1. The pH was adjusted to 7.6, adding 0.01 N NaOH to the incubation mixture. The coupling reaction was carried out for 12 h at 4 °C under constant and gentle shaking and protection from light. Afterward, the liposomes were separated from unconjugated IgG or BSA, respectively, by ultracentrifugation at 30000g (40 min, 4 °C). The resulting pellet was redispersed in 10 mL of HEPES buffer (20 mM HEPES, 135 mM NaCl, pH 7.4) and ultracentrifuged for a second time (40 min, 30000g, 4 °C). Finally, the pellet was redispersed in 1 mL of HEPES buffer. Liposome dispersions were stored at 4 °C.

If SATA was used as thiolating agent, the IgG bearing the protected thiol groups were added to liposomes containing 2 mol % *m*-MCS-22. Hydroxylamine HCl (0.5 M hydroxylamine HCl, 25 mM EDTA, pH 8, 100 μ L/mL protein solution to be deacetylated) was added, the pH was adjusted to 7.6, and the mixture was incubated for 12 h at 4 °C under constant and gentle shaking. Afterward, the liposomes were separated from unconjugated IgG as described above.

Kinetics of the Coupling Reaction. SATA-thiolated IgG was added to liposomes containing 2 mol % *m*-MCS-22. The procedure was performed as described in the Experimental Section. Afterward, the liposome/protein mixture was divided into 18 samples and incubated at 4 °C. After 0.5, 1, 2, 4, 6, and 12 h, the coupling reaction was stopped by adding a 100-fold excess of *N*-ethylmaleimide to the incubation mixture. The samples were ultracentrifuged in order to separate liposomes from unconjugated protein (see above).

Characterization of Liposomes. The mean liposome size was analyzed by using a Nicomp 370 photon correlation spectroscopy system (Nicomp, St. Barbara, CA). Lipid phosphate was determined by the method of Eibl and Lands (36). For the quantification of protein (IgG or BSA, respectively), coupled to the liposomes, a commercially available BCA-assay (37) was used. Prior to the protein assay, lipids were separated from proteins according to the method of Wessel and Flügge (37). All results are given as coupling efficiency, i.e., the percentage of the added protein linked to liposomes.

RESULTS AND DISCUSSION

Preparation of Immunoliposomes. Just after the extrusion process, the size of the resulting liposomes containing the different coupling lipids were examined by photon correlation spectroscopy. The diameters of the resulting liposomes (as well as the liposomes containing no coupling lipid) were similar (85.8 ± 22.7 nm; all Gaussian distribution). Thus, the incorporation of coupling lipid neither influenced the liposome size considerably nor induced spontaneous aggregation.

Since knowledge of the stability of coupling lipids incorporated into liposomal bilayers is important, we analyzed the liposomal content of *m*-MCS-4 and *p*-MCS-4 by HPTLC after different time points and temperatures

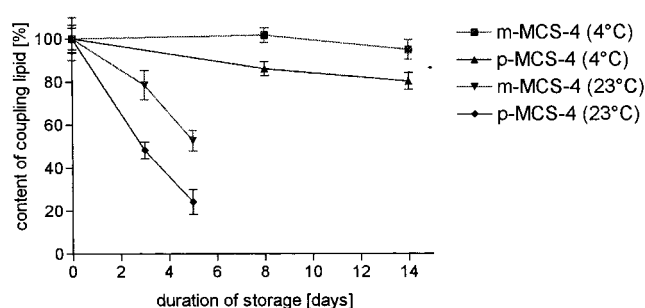


Figure 2. Stability of *m*-MCS-4 and *p*-MCS-4, incorporated into liposomes, at 23 and 4 °C.

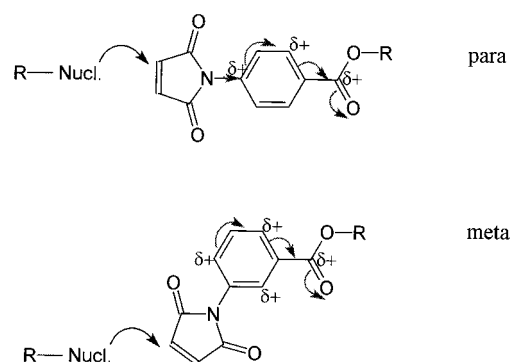


Figure 3. Influence of the mesomeric effect on the electrophilicity of *p*- and *m*-maleimido benzoic acid esters.

(4 and 23 °C). Lipid analysis immediately after preparation process proved that the liposomes regularly contained the full concentration of coupling lipid and no destruction of coupling lipid occurred. In contrast, coupling lipids were unstable during storage of the liposomes, especially at room temperature. At 23 °C, 5 days after liposome preparation only 53% of the initial content of *m*-MCS-4 and 24% of the content of *p*-MCS-4 were detected (Figure 2). At 4 °C, the stability of the coupling lipids is much higher: 14 days after liposome preparation nearly the entire content of *m*-MCS-4 (95%) and a high amount of *p*-MCS-4 (80%) were detected (see Figure 2). Thus, to achieve high and reproducible coupling results, the following protein-liposome coupling experiments were performed immediately after liposome preparation (for discussion of the different stability of *p*-MCS-4 and *m*-MCS-4, see the following paragraph).

Influence of the Electrophilicity of the Maleimide Group on Coupling Efficiency. The formation of a thioether linkage between a thiol group on a protein and a thiol-reactive maleimide group of a coupling lipid was expected to be faster and probably more efficient with increasing electrophilicity of the maleimide group. Thus, we compared the coupling efficiencies of two coupling lipids that contain the same spacer (tetraethylene glycol), but differ in the constitution of the maleimido benzoic esters (*m*- or *p*-maleimido benzoic acid esters, *m*-MCS-4 and *p*-MCS-4). The difference in structure of the maleimido benzoic acid esters influences their electrophilicity: *p*-Maleimido benzoic acid esters are more electrophilic than *m*-maleimido benzoic acid esters, caused by a stronger $-M$ (mesomeric) effect of the benzoic ester group on the maleimide group (Figure 3). Interestingly, we found that the less electrophilic (less reactive) coupling lipid *m*-MCS-4 resulted in a higher coupling efficiency ($46 \pm 7\%$) than the more electrophilic *p*-MCS-4 ($30 \pm 5\%$). This might be explained by the increasing susceptibility to (competing) nucleophiles (water) of the maleimide group with higher electrophilicity. This result

corresponds well with the lower stability of *p*-MCS-4 in aqueous environment (see Preparation of Immunoliposomes, above). In the following experiments, we only used the *m*-maleimido benzoic acid ester group as the thiol-reactive moiety because of its higher coupling efficiency.

Influence of Spacer Length and Polarity on Coupling Efficiency. Coupling of thiolated proteins using the commercially available lipid *N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine (MPB-PE) is one of the most common methods to prepare immunoliposomes (32). The coupling lipid MPB-PE is based on phosphatidylethanolamine as lipid anchor, bearing a short nonpolar butyryl spacer. Previous experiments, coupling thiolated IgG with liposomes that consisted of DPPC, cholesterol, and MPB-PE or *m*-MCS-4, respectively, showed better results for *m*-MCS-4 (25%) than for MPB-PE (16%) (data not shown). We assume that a short nonpolar spacer might be disadvantageous for the coupling of thiolated proteins because it is not long enough to present the maleimide group at a sufficient distance from the liposome surface. Furthermore, a nonpolar spacer could interact with the hydrophobic part of the liposome bilayers, thereby reducing the effective distance between maleimide group and liposome surface. Both effects could reduce the accessibility of the thiol-reactive maleimide groups, especially to sterically hindered thiol groups. Considering the structure of the liposomal bilayer, a long and polar spacer (e.g., PEG) might be more suitable to present the thiol-reactive maleimide group at the liposome surface.

Polarity. We studied the influence of the spacer polarity on coupling efficiency by comparing *m*-MCS-4 and *m*-MCS-Do. *m*-MCS-Do (see Figure 1) is an isosteric analogue of *m*-MCS-4 that contains a nonpolar hydrocarbon chain with approximately the same length (C-12) as the tetraethylene glycol spacer of *m*-MCS-4. The use of *m*-MCS-4, containing a polar PEG spacer, resulted in a 3-fold higher coupling efficiency compared to *m*-MCS-Do ($46 \pm 7\%$ vs $15 \pm 5\%$). This striking difference is probably caused by the better accessibility of the thiol-reactive moiety, presented via the polar tetraethylene glycol spacer at the liposome surface. A polar spacer is more likely to penetrate the outer hydrophilic part of the bilayer and to project from the membrane. This result partly explains the different coupling efficiencies using *m*-MCS-4 and MPB-PE (see above).

Spacer Lengths. To evaluate the influence of different spacer lengths, we compared the coupling efficiencies of four lipids with systematic variations in their polar PEG spacer length (*m*-MCS-1, *m*-MCS-4, *m*-MCS-9, *m*-MCS-22).

The coupling of BSA to liposomes containing *m*-MCS-4, *m*-MCS-9, and *m*-MCS-22 resulted in medium and statistically similar coupling efficiencies (41–46%). As expected, the use of *m*-MCS-1 bearing a spacer of only one ethylene glycol unit resulted in a statistically significant lower coupling efficiency (30%) (Figure 4).

Using BSA, we achieved coupling efficiencies of no more than 46%. That means that a high percentage of thiolated BSA molecules are not available for the maleimide groups, although a 40-fold excess of coupling lipid was present in the liposomes. Probably many thiol groups on BSA were not accessible for the following reasons.

(i) Some of the thiol groups might be located in unfavorable positions (e.g., in folds and inaccessible regions of the protein).

(ii) Formation of disulfide bonds may reduce the coupling efficiency: Thiol groups either of the same or different BSA molecules can react with each other,

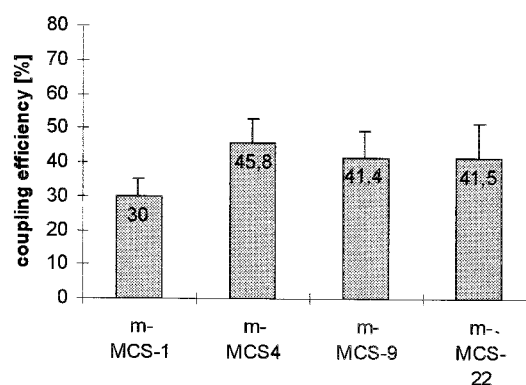


Figure 4. Influence of spacer length on coupling efficiencies of thiolated BSA to liposomes, containing *m*-MCS-1, *m*-MCS-4, *m*-MCS-9, or *m*-MCS-22.

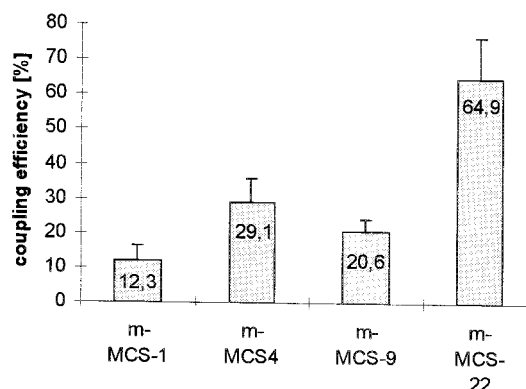


Figure 5. Influence of spacer length of coupling efficiencies of thiolated IgG to liposomes, containing *m*-MCS-1, *m*-MCS-4, *m*-MCS-9, or *m*-MCS-22.

forming disulfide bonds and thereby reducing the number of free thiol groups.

(iii) There may be still some BSA-proteins that bear just one thiol group (although the mean thiol group density was adjusted “statistically” to two to three thiol groups per protein).

All these factors may reduce the number of free thiol groups presented by the BSA surface and therefore result in lower coupling efficiencies. Our results show that a minimal spacer length of about four ethylene glycol units seems to be decisive for the successful interaction with albumin.

In contrast to BSA, the coupling of IgG to liposomes containing *m*-MCS-1, *m*-MCS-4, *m*-MCS-9, or *m*-MCS-22 is more dependent on spacer length: Experiments using *m*-MCS-1 resulted in a low coupling efficiency (12%). Using the longer coupling lipids *m*-MCS-4 and *m*-MCS-9, we achieved coupling efficiencies in a medium range (*m*-MCS-4: 29%, *m*-MCS-9: 21%). The best results could be achieved by using liposomes containing *m*-MCS-22 (65%) (Figure 5).

The different profiles of BSA and IgG coupling can be explained by the different shapes of albumin and IgG. IgG are Y-shaped proteins, consisting of two heavy chains and two light chains, forming a complicated bulky three-dimensional structure. Probably many thiol groups, introduced to IgG, are hardly available for coupling because they are located in inaccessible regions of the antibody. Therefore, the long distances between the thiol group and the liposome surface that are fixed by the shape and the surface structure of IgG result in lower coupling efficiencies of lipids with short spacers. Only coupling lipids bearing long polar spacers such as PEG

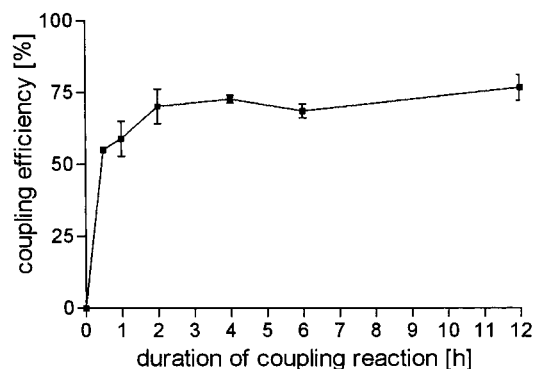


Figure 6. Kinetics of the coupling reaction of SATA-thiolated IgG and liposomes, containing *m*-MCS-22.

1000 (*m*-MCS-22) are able to reach these hidden thiol groups.

The coupling efficiency of *m*-MCS-1 was higher for albumin than for IgG (12% vs 30%), whereas coupling lipids with spacers longer than one ethylene glycol unit resulted in similar coupling efficiencies (41–46%). This may be due to the fact that BSA, unlike IgG, is a more globular protein. Hence, thiol groups on the protein surface are easier to access and also short polar spacers may be able to reach these thiol groups.

In the case of *m*-MCS-22, coupling efficiency of albumin was lower than IgG (BSA 42% vs IgG 65%). This seems to be contradictory to the fact mentioned above but might be caused by the formation of disulfide bonds by BSA. This may result in the formation of dimers because thiol groups at the surface of BSA are well accessible. This effect is less probable for IgG because of the bulky Y-shaped structure of antibodies.

Thiolation Using SATA. The thiolating agent, so far used for all previous experiments, was 2-iminothiolane (Traut's reagent). Iminothiolane introduces thiol groups by reaction with the amino group of lysine, present in the proteins. The use of 2-iminothiolane is a cheap and easy method for protein thiolation; unfortunately, the thiol group of iminothiolane is unprotected, and therefore, the thiolated protein has to be used immediately for coupling.

To improve the coupling process, we used *N*-succinimidyl *S*-acetylthioacetate (SATA) as thiolating agent. SATA introduces thiol groups into proteins by a two-step reaction. Like iminothiolane, SATA reacts with the amino group of lysine and introduces acetylated thiol groups into the protein. In a second step prior to protein coupling the thiol groups have to be deacetylated under mild conditions by adding hydroxylamine. Under appropriate conditions, the intermediate acetylated thio-proteins can be stored for at least 1 month without loss of thiol groups (35).

We prepared immunoliposomes by coupling SATA-thiolated IgG with liposomes, containing 2% *m*-MCS-22. Apart from the method of thiolation, all steps were identical with the previous experiments. The coupling of IgG to *m*-MCS-22 liposomes using SATA resulted in a high coupling efficiency ($72 \pm 13\%$), which is statistically identical with the results achieved by 2-iminothiolane ($65 \pm 12\%$).

Kinetics of the Coupling Reaction. The thiol-reactive lipid bearing the longest PEG spacer (*m*-MCS-22) proved to be the best agent for IgG coupling. To optimize the protocol of immunoliposome preparation, the kinetic parameters of the coupling reaction of IgG to *m*-MCS-22 liposomes were determined.

The coupling of IgG to liposomes containing *m*-MCS-22 was characterized by a rapid increase of liposome bound proteins within the first 2 h, reaching a plateau between 2 and 4 h. There was no further change of coupling efficiency within the next 8 h (Figure 6). The kinetics show that the reaction of IgG with liposomes, containing *m*-MCS-22, is almost completed after 4 h; a longer incubation time does not enhance coupling efficiency. Hence, the duration of the coupling reaction can be drastically reduced from 12 h (standard protocol) to approximately 4 h, which is more convenient.

CONCLUSIONS

The coupling of a sufficient number of antibodies to liposomes in an effective and reproducible way is an important prerequisite for immunoliposome preparation. Therefore, we recently synthesized novel group of thiol-reactive lipids (33, 34) in order to optimize the preparation of immunoliposomes. These coupling lipids differ in length and polarity of the spacer moiety and in electrophilicity of the thiol-reactive maleimide group. The new coupling lipids can be used for effective coupling of IgG and BSA to the liposome surface. Spacer polarity, spacer length, and electrophilicity of the maleimide group are important determinants for the coupling process: Polar spacers achieved a higher coupling efficiency than non-polar spacers. The less electrophilic meta constitution of the maleimide group (*m*-MCS-4) seems to be in advantage over the more electrophilic para conformation (*p*-MCS-4), resulting in increased stability as well as higher coupling efficiencies. Interestingly the optimal PEG-spacer length for a high coupling efficiency depends on the proteins which are coupled to the liposomes. The best coupling efficiency for IgG was achieved by using *m*-MCS-22 (65%), a lipid bearing a long hydrophilic spacer moiety (PEG 1000). Moreover, the use of long polar spacers might be essential with regard to the preparation of sterically stabilized immunoliposomes (22–25). Studies on optimization of the coupling process demonstrated, that the thiolation of proteins using SATA or iminothiolane resulted in identical coupling efficiencies and that the reaction was completed after 2–4 h.

The novel coupling lipids used for these experiments were based on cholesterol as lipid anchor, but the findings might also be applied to future coupling lipids: Such lipids might be based on phosphatidylethanolamine or diacylglycerol as lipid anchor or may contain a less immunogenic thiol-reactive moiety, e.g., succinimidyl 6-(*N*-maleimido)-*n*-hexanoate (30). These novel thiol-reactive lipids may further help to optimize the design of immunoliposomes and improve tumor and tissue targeting.

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Enhancement of Gene Expression by Polyamidoamine Dendrimer Conjugates with α -, β -, and γ -Cyclodextrins

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To improve the transfection efficiency of nonviral vector, we synthesized the starburst polyamidoamine dendrimer conjugates with α -, β -, and γ -cyclodextrins (CDE conjugates), expecting the synergistic effect of dendrimer and cyclodextrins (CyDs). The ^1H NMR spectroscopic data indicated that α -, β -, and γ -CyDs are covalently bound to dendrimer in a molar ratio of 1:1. The agarose gel electrophoretic studies revealed that CDE conjugates formed the complexes with plasmid DNA (pDNA) and protected the degradation of pDNA by DNase I in the same manner as dendrimer. CDE conjugates showed a potent luciferase gene expression, especially in the dendrimer conjugate with α -CyD (α -CDE conjugate) which provided the greatest transfection activity (approximately 100 times higher than those of dendrimer alone and of the physical mixture of dendrimer and α -CyD) in NIH3T3 and RAW264.7 cells. In addition, the gene transfer activity of α -CDE conjugate was superior to that of Lipofectin. The enhancing gene transfer effect of α -CDE conjugate may be attributable to not only increasing the cellular association, but also changing the intracellular trafficking of pDNA. These findings suggest that α -CDE conjugate could be a new preferable nonviral vector of pDNA.

INTRODUCTION

There are two categories of gene therapy vectors, i.e., viral vectors and nonviral vectors. Viral vectors take advantage of being a higher gene expression. However, they have a failing in which the virus replicates and the inflammatory reaction occurs. On the other hand, the nonviral vectors have many advantages over viral vectors, such as easy to manufacture, safety, low immunogenicity, and molecular attachment of targeting ligand (1, 2). However, the problem is that the efficiency of nonviral vector-mediated gene transfer to cells is markedly low, compared to the viral vectors. This is because of the lack of receptor recognition, endosome escape, and nuclear pore targeting (3). To date, several strategies to enhance the gene expression of nonviral vectors are being developed, e.g., the application of helper lipids, pH-sensitive lipids, cationic polymers, endosome-disruptive peptides, nuclear proteins, and nuclear localization signal, etc. (3–5). Starburst polyamidoamine dendrimer is a spherical, highly ordered, dendritic polymer with positively charged primary amino groups on the surface at physiological pH (6). Recently, their usefulness as nonviral vectors has been reported (7, 8). However, dendrimers with lower generations (generation 1–3) do not have a good gene transfer activity, whereas dendrimers with higher generations may exhibit cytotoxicity (9).

Cyclodextrins (CyDs) are cyclic (α -1,4)-linked oligosaccharides of α -D-glucopyranose containing a hydrophobic central cavity and hydrophilic outer surface (10–12). The most common CyDs are α -, β -, and γ -CyDs, which consist

of six, seven, and eight D-glucopyranose units, respectively. CyDs are known to form inclusion complexes with a variety of guest molecules in solution and in a solid state. The solubilization of lipophilic compounds by CyDs has many uses in pharmaceutical fields (13, 14), while CyDs at higher concentration induce hemolysis and decrease the integrity of the nasal and intestinal epithelial cells, leading to an increase in the permeability of impermeable drugs through the membrane (15–17). The dissolution of lipids by CyDs from biological membranes may be useful in the study of cellular biology, e.g., caveolae, rafts, and cholesterol transport (18–20). Recently, CyDs have also been applied to the gene transfer and the oligonucleotide delivery (21–24): (1) The cationic β -CyDs such as tertiary amino- β -CyD acted as a viral dispersant, which resulted in an increase in adenoviral transduction in human colon adenocarcinoma Caco-2 cells by enhancing both viral binding and internalization (21). (2) β -CyD increased the gene transfer in the lungs of rats (22). In addition, 2-hydroxypropyl- β -CyD (HP- β -CyD) and 2-hydroxyethyl- β -CyD (HE- β -CyD) increased the uptake of the antisense phosphorothioate oligodeoxynucleotides in human T cell leukemia H9 cell line, without cytotoxicity (23). 6-Deoxy-6-S- β -D-galactopyranosyl-6-thio-CyD improved the antiviral activity of the antisense phosphodiester oligonucleotide in human adenocarcinoma cells (24). Unfortunately, these enhancing effects of CyDs were not great.

The potential use of the conjugation with CyDs has been studied in the pharmaceutical field, e.g., the sulfonylurea LY237868 conjugate with α -CyD (25) and the epoxysuccinyl peptide conjugate with β -CyD (26) as antitumor agents and the bioactive peptide conjugates with CyDs as protease inhibitors (27). In our laboratory, the potential uses of CyD conjugates with biphenyl acetic acid and prednisolone for colon-specific drug delivery have been reported. (28, 29). To our knowledge, however, there is only one paper reporting the beneficial

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¹ Abbreviation: CyDs, cyclodextrins; CDE conjugate, cyclodextrin–dendrimer conjugate; pDNA, plasmid DNA; dendrimer, Starburst polyamidoamine dendrimer; [^{32}P]pDNA, [^{32}P]-labeled plasmid DNA; FITC-pDNA, Fluorescein isothiocyanate labeled pDNA.

use of CyD conjugates for gene transfer; a new class of β -CyD-containing polymers was successful in the gene transfer in vitro (30). In the present study, we synthesized the potent new dendrimer conjugates with α -, β -, and γ -CyDs (CDE conjugates), in anticipation of the following synergic effect; i.e., (1) dendrimer has the abilities to complex with plasmid DNA (pDNA) and to enhance the cellular uptake of pDNA and (2) CyDs have a disrupting effect on biological membranes by the complexation with membrane constituents such as phospholipids and cholesterol. Thus, we investigated the effects of CDE conjugates on the gene transfer efficiency in the cells, and the enhancing effects were compared with commercial transfection reagents, Lipofectin and TransFast.

EXPERIMENTAL SECTION

Materials. α -, β -, and γ -CyDs were donated by Nihon Shokuhin Kako (Tokyo, Japan) and recrystallized from water. Starburst PAMAM dendrimer (ethylenediamine core, generation 2, the terminal amino groups = 16, molecular weight = 3256) was purchased from Aldrich Chemical (Tokyo, Japan). *p*-Toluenesulfonyl chloride and *p*-naphthalenesulfonyl chloride were purchased from Nakarai Tesque (Kyoto, Japan). Fetal calf serum (FCS) was purchased from Nichirei (Tokyo, Japan). Dulbecco's modified Eagle's medium and RPMI1640 media were obtained from Nissui Pharmaceuticals (Tokyo, Japan). TransFast and Lipofectin transfection reagents were obtained from Promega (Tokyo, Japan) and Life Technologies (Rockville, MD), respectively. The plasmid pGL3 control vector (luciferase reporter vector, pDNA) and DNase I were obtained from Promega (Tokyo, Japan). The purification of pDNA amplified in bacteria was carried out using QIAGEN EndoFree plasmid maxi kit (<0.1 EU/ μ g endotoxin). [α - ^{32} P]CTP was obtained from Amersham Pharmacia Biotech (Tokyo, Japan). Other chemicals and solvents were of analytical reagent grade.

Preparation of CDE Conjugates. The CDE conjugates were prepared by the method of Suh et al. (31). Monotosyl- α -CyD, monotosyl- β -CyD, and mononaphthalenesulfonyl- γ -CyD were prepared according to the methods of Brown et al. (32) and Melton et al. (33), respectively. The methanol solution containing dendrimer (0.39 mL, 2.6×10^{-5} mol) was added to a flask and evaporated under reduced pressure. Dimethyl sulfoxide (DMSO, 0.5 mL) was added into the flask and then the monotosyl- α -CyD (25 mg, 2.6×10^{-5} mol), monotosyl- β -CyD (30 mg, 2.6×10^{-5} mol), or mononaphthalenesulfonyl- γ -CyD (34 mg, 2.6×10^{-5} mol) was added, respectively. Under a stream of nitrogen gas, the mixture was stirred at 60 °C for 24 h. The α -, β -, and γ -CDE conjugates were purified by gel-filtration (TOSOH TSKGel HW-40S, Tokyo, Japan) and then precipitated with methanol. The gel filtration was carried out to remove the free and monotosylated or mononaphthalenesulfonylated CyDs. The methanol precipitation was conducted to remove the free dendrimer, because it is highly soluble in methanol whereas the CDE conjugates are less soluble in the solvent. The CDE conjugates gave ^1H NMR spectra consisting of protons of both CyDs and the dendrimer. The ratios of peak areas of the anomeric proton of CyDs and ethylene protons of the dendrimer were approximately 0.95–1.11, indicating that α -, β -, and γ -CyDs covalently bind to the dendrimer in a molar ratio of 1:1, as shown in Figure 1. α -CDE conjugate: yield: 18%; ^1H NMR (500 MHz, D_2O) δ (from TMS) 5.29 and 4.96 (H1, α -CyD), 3.94–3.61 (H3, H5, H6, α -CyD), 3.60–3.33 (H2, H4, α -CyD), 3.33–3.08 (dendrimer methylene), 3.08–2.94 (dendrimer methylene),

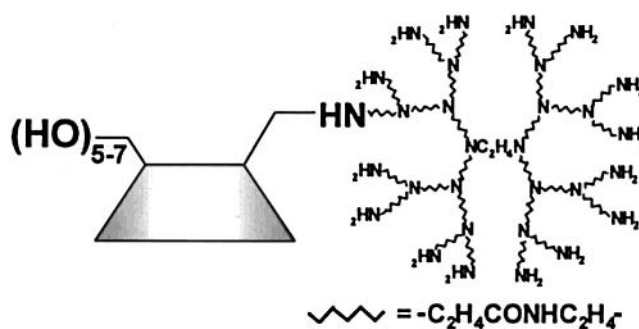


Figure 1. Chemical structure of CDE conjugates.

2.94–2.60 (dendrimer methylene), 2.60–2.43 (dendrimer methylene), 2.43–2.15 (dendrimer methylene). β -CDE conjugate: yield: 21%; ^1H NMR (500 MHz, D_2O) δ (from TMS), 4.96 (H1, β -CyD), 3.93–3.67 (H3, H5, H6, β -CyD), 3.59–3.40 (H2, H4, β -CyD), 3.34–3.08 (dendrimer methylene), 3.08–2.96 (dendrimer methylene), 2.96–2.62 (dendrimer methylene), 2.62–2.44 (dendrimer methylene), 2.44–2.22 (dendrimer methylene). γ -CDE conjugate: yield: 29%; ^1H NMR (500 MHz, D_2O) δ (from TMS) 5.29 and 5.00 (H1, γ -CyD), 3.91–3.60 (H3, H5, H6, γ -CyD), 3.60–3.39 (H2, H4, γ -CyD), 3.39–3.05 (dendrimer methylene), 3.05–2.97 (dendrimer methylene), 2.97–2.57 (dendrimer methylene), 2.57–2.39 (dendrimer methylene).

Gel Electrophoresis. Electrophoretic mobility of the pDNA complex with dendrimer or CDE conjugates was measured using a gel electrophoresis system. Varying amounts of dendrimer or CDE conjugates were mixed with 0.2 μ g of pDNA in Hank's balanced salt solution (HBSS, pH 7.4). Gel electrophoresis was carried out at room temperature in TBE (45 mM Tris-Borate, 1 mM EDTA) buffer (pH 8.0) in 1% (w/v) agarose gel (include 0.1 μ g/mL ethidium bromide) using Mupid system (Cosmo Bio, Tokyo, Japan) at 100 V for 50 min. pDNA bands were visualized by an UV illuminator.

Measurement of Particle Size. Dendrimer and α -CDE conjugate solutions (0.1 mL in HBSS) at various charge ratios were added to pDNA solution (20 μ g/1.5 mL in HBSS) and incubated for 15 min. Particle sizes of the pDNA complexes with dendrimer and α -CDE conjugate were determined by quasi-elastic (dynamic) light scattering using a Beckman Coulter N4 Plus machine (CA). The wavelength of the He–Ne laser used was 632.8 nm. The measurement was carried out at a fixed angle of 62.6°, and the measuring time was 102 s at 25 °C. The measurements were carried out at least in triplicates.

DNase I Protection Assay. The complexes of pDNA (0.2 μ g) with dendrimer or CDE conjugates were prepared as described above and then incubated for 2 h at 37 °C in the reaction buffer (pH 7.4, 10 mM Tris-HCl, 10 mM NaCl, 10 mM CaCl_2 , 6 mM MgCl_2 and 2 mM DTT, 0.2 μ g/mL bovine serum albumin) containing 0.1 unit/ μ L DNase I. After incubation, DNase I was inactivated by heating the solution at 70 °C for 10 min, and 1 μ L of EDTA (0.5 M) and 10 μ L of SDS (8% w/v) were added to each sample and incubated at 37 °C for 60 min to dissociate pDNA from the complexes. After the incubation, gel electrophoresis was carried out in the same manner as described in gel electrophoresis section.

Cell Culture. NIH3T3 cells, a mouse fibroblast cell line, and RAW264.7 cells, a mouse macrophage-like cell line, were obtained from the Japan Health Sciences Foundation (Osaka, Japan) and the Riken cell bank (Tsukuba, Japan), respectively. Both cell lines were grown in Dulbecco's modified Eagle's medium (include 1

$\times 10^5$ mU/mL penicillin, 0.1 mg/mL streptomycin) and RPMI1640 medium (include 4×10^5 mU/mL penicillin, 0.2 mg/mL streptomycin) supplemented with 10% (v/v) FCS at 37 °C in a humidified 5% CO₂ and 95% air atmosphere, respectively.

Transfection. Transfection of the complexes of pDNA with dendrimer, CDE conjugates, or the commercial cationic lipid transfection reagents (Lipofectin and TransFast) was performed and analyzed using the expression of pDNA containing firefly luciferase gene under the control of Rous sarcoma virus enhancer/promoters. One to two micrograms of purified pDNA (per dish of transfected cells) was mixed with dendrimer or conjugates at a variety of charge ratios from 1/0 to 1/200 (pDNA/the vectors). The pDNA/dendrimer or CDE conjugates were then allowed to stand for 15 min at room temperature. The cells (2×10^5 per 35 mm culture dish) were seeded 18 h before transfection and then washed two times with serum-free medium. Eight hundred microliters of serum-free medium containing pDNA or the complexes with the various vectors (equivalent to 2 μ g of pDNA) were added to each well and then incubated for 1 h at 37 °C. The culture medium (1.5 mL) supplemented with 10% (v/v) FCS was added to each well. The cells remained in culture for 6, 12, and 24 h before being harvested for analysis of the expression of the transfected pDNA. Control transfection was performed with TransFast and Lipofectin in the same manner as described above. The charge ratios of pDNA and TransFast and pDNA and Lipofectin were 1:1, at which the optimal transfection activity was observed under the present experimental conditions.

Measurement of Luciferase Activity. After transfection for 25 h, firefly luciferase content of the cell lysate was quantified, using the Promega luciferase assay reagent and a luminometer (Lumat LB9507, EG&G Berthold Japan, Tokyo, Japan). Absolute light emission generated from the luciferase enzyme reaction was determined. It was confirmed that CyDs and CDE conjugates have no influence on the luciferase assay under the experimental conditions. Total protein contents of the supernatant were determined by BCA protein assay kit (Pierce, Rockford, IL).

Cellular Association. pDNA was labeled with [α -³²P]-CTP using a nick translation kit II (Nippon Gene, Toyama, Japan) according to manufacturer's protocols. [³²P] labeled pDNA ([³²P]pDNA) was purified using QIAquick PCR purification Kit (Qiagen, Tokyo, Japan). NIH3T3 and RAW264.7 cells were cultured in 35-mm culture dish. The cells were washed two times with 1 mL of medium, and [³²P]pDNA (1 μ g/mL, 5.0×10^4 cpm/ μ L) with or without dendrimer and CDE conjugates was added. After incubation at 37 °C for the indicated time, the cells were washed two times with 0.5 mL of ice-cold phosphate-buffered saline (PBS) and then solubilized with 0.5 mL of 1 N NaOH and incubated for 2 h. One-half milliliter of the total solutions was added to Hionic-Flour (Packard Japan, Tokyo, Japan) and then taken for the determination of ³²P radioactivity using a liquid scintillation counter (Aloka LSC-5100, Tokyo, Japan). Nonspecific binding of labeled pDNA was defined as the binding to the dishes rather than the cells and then that count subtracted from the total binding to calculate the specific binding.

Confocal Microscopic Study. Fluorescein isothiocyanate (FITC) labeled pDNA (FITC-pDNA) was prepared using Mirus label ITTM fluorescein labeling kit (Madison, WI). FITC-pDNA/dendrimer or CDE conjugates were then allowed to stand for 15 min at room temperature.

The transfection was performed as described above. After 25 h incubation, the cells were rinsed two times with 2 mL of PBS (pH 7.4) and fixed with 3.7% (v/v) formaldehyde in PBS for 40 min. Then, the cells were washed two times with 2 mL of PBS, and the lipids were removed by washing with 2 mL of methanol (−20 °C) for 10 min and then followed by washing two times with 2 mL of PBS. The cells were scanned with a confocal fluorescence microscopic system (Olympus FV300-BX, Tokyo, Japan) with an argon ion laser (excitation wavelength, 488 nm).

Data Analysis. Data are given as means $\pm$ SEM. Statistical significance of mean coefficients was performed by analysis of variance followed by Student's-t test. *p*-Values for significance were set at 0.05.

RESULTS

Interaction between pDNA and CDE conjugates.

We first studied the complexation between pDNA and dendrimer or CDE conjugates using agarose electrophoresis. As shown in Figure 2A, the intensity of the band derived from pDNA decreased, with increasing the charge ratio of pDNA/dendrimer, and at the charge ratio of 1/1 the band disappeared. The similar patterns were observed in the α -, β -, and γ -CDE conjugate systems (Figures 2B,C,D). CyDs did not change the electrophoretic band pattern of pDNA (data not shown). These results suggested that CDE conjugates form the complex with pDNA in a manner similar to dendrimer.

DNase I Protection Assay. Condensation of pDNA by cationic polymers has been shown to protect supercoiled pDNA from degradation by DNase I. We examined the stabilizing effect of dendrimer or CDE conjugates on the degradation of pDNA via the endonuclease activity of DNase I. In the present study, the concentration of DNase I was 0.1 unit/ μ L, because the activity in human serum is 2×10^{-4} – 8.2×10^{-2} unit/ μ L (34). As shown in Figure 3, when pDNA alone was treated with DNase I, pDNA degraded into smaller size fractions. On the other hand, the addition of dendrimer or CDE conjugates increased the band intensity of pDNA with the increase in the charge ratio up to about 1/10, although the intensity decreased at higher ratios ($> 1/20$). Since CyDs alone possessed no protecting ability against the DNase I-catalyzed degradation of pDNA (data not shown), these results suggest that both dendrimer and CDE conjugates protect pDNA, and the optimal charge ratio exists in their protecting effects. The protecting mechanism may be same between dendrimer and CDE conjugates.

Transfection. The gene transfer efficiency of dendrimer and CDE conjugates was compared with that of commercial transfection reagents, TransFast and Lipofectin, which were employed as positive controls. Figures 4A and 4B show the transfection efficiency of CDE conjugates in NIH3T3 and RAW264.7 cells, respectively. When pDNA alone with or without CyDs was transfected in both cells, no luciferase activity was observed (data not shown). In NIH3T3 cells, the combination of pDNA/dendrimer (1/2) gave the luciferase activity of about 10^4 RLU/ μ g proteins. However, this activity was only slightly enhanced even when the charge ratio increased to 1/200. On the other hand, the combination of pDNA/CDE conjugates significantly increased the luciferase activity with increasing the charge ratio; especially, α -CDE conjugate enhanced the activity by approximately 100 times that of pDNA/dendrimer. At the charge ratios of 1/100 and 1/200, the luciferase activity of the α -CDE conjugate system was higher than that of the Lipofectin system, although it was lower than that of the TransFast

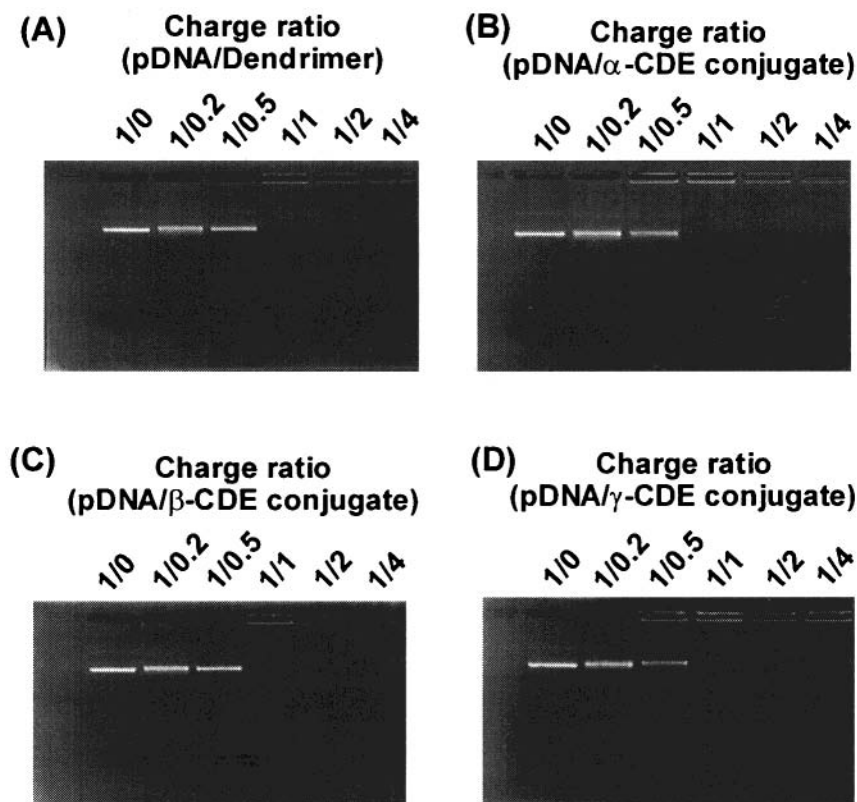


Figure 2. Agarose gel electrophoretic analysis of pDNA/dendrimer or pDNA/CDE conjugates using TBE buffer (pH 8.0). The solutions containing pDNA and dendrimer or CDE conjugates were incubated for 15 min at room temperature after slight agitation. The electrophoresis was performed at 100 V for about 50 min. A, dendrimer; B, α -CDE conjugate; C, β -CDE conjugate; D, γ -CDE conjugate.

system. In RAW264.7 cells, the same effect of CDE conjugates was observed, as shown in Figure 4B. Since the luciferase activity was saturated at the charge ratio of 1/100–1/200 (pDNA/CDE conjugate), the complex with the 1/200 charge ratio was employed in the following experiment. Figures 5A and 5B show the time-courses of the gene transfer ability of CDE conjugates in NIH3T3 and RAW264.7 cells, respectively. Of CDE conjugates, the great transfection activities of α -CDE were observed at 7, 13, 25 h after transfection in NIH3T3 cells and at 13 and 25 h in RAW264.7 cells, although their activities were inferior to those of TransFast. These results indicate that α -CDE conjugate has the superior transfection ability than β - and γ -CDE conjugates.

Next, we compared the transfection ability of CDE conjugates with that of the simple mixtures of dendrimer and CyDs. As shown in Figure 6, the luciferase activities of the physical mixtures were almost the same as those of dendrimer alone in both NIH3T3 and RAW264.7 cells. These results indicate that the covalent conjugation between dendrimer and CyDs is necessary for the induction of the efficient transfection activity.

Cellular Association of pDNA. The enhanced transfection effects of CDE conjugates may be attributable to the increasing cellular association and/or to changing the intracellular trafficking of pDNA. To clarify the mechanism for the higher transfection efficiency of the CDE conjugates, we examined the effects of dendrimer and CDE conjugates on the cellular association of pDNA to NIH3T3 and RAW264.7 cells, using [^{32} P]pDNA. As shown in Figure 7, the association of pDNA to the cells increased as the charge ratio increased up to about 1/2–1/5, and there was a good correlation between the cellular association and the luciferase activity. This suggests that

the cellular entry is a rate-limiting step for gene transfer in lower molar ratios. However, the association was saturated above the charge ratio of about 1/2–1/5 and was almost the same between the dendrimer and the CDE conjugates, which was inconsistent with the results of luciferase activity (Figures 4 and 5). CyDs showed no effects of the cellular association of pDNA under the experimental conditions, implying a lack of perturbing effect of CyDs on the barrier function of plasma membrane (data not shown). These results suggest that CDE conjugates, especially α -CDE conjugate, may change the trafficking of pDNA in the cells.

Intracellular Distribution of FITC-Labeled pDNA.

To examine the effects of α -CDE conjugate on the distribution of pDNA in the cells, we observed the cells under a confocal laser microscope after transfection of FITC-pDNA for 25 h in NIH3T3 cells. The results are shown in Figure 8. The transfection of pDNA alone gave no fluorescence in the cells (Figure 8A), reflecting a lower cellular uptake of pDNA. The pDNA/dendrimer system gave a moderate fluorescence in cytoplasm (Figure 8B). On the other hand, more intense fluorescence was observed in cytoplasm when α -CDE conjugate was used as a vector (Figure 8C). Separately, we revealed that dendrimer and CDE conjugates entered into the cells in terms of the endocytosis mechanism (data not shown). These results suggest that α -CDE conjugate may change the distribution of pDNA in the cells, possibly in terms of the increase in the release of pDNA from the endosome into cytoplasm after endocytosis. Furthermore, α -CDE conjugate gave the fluorescence similar to that of the dendrimer alone at the low charge ratio (1/5) of the complexes, whereas it gave the stronger fluorescence than that of the dendrimer alone at the higher charge

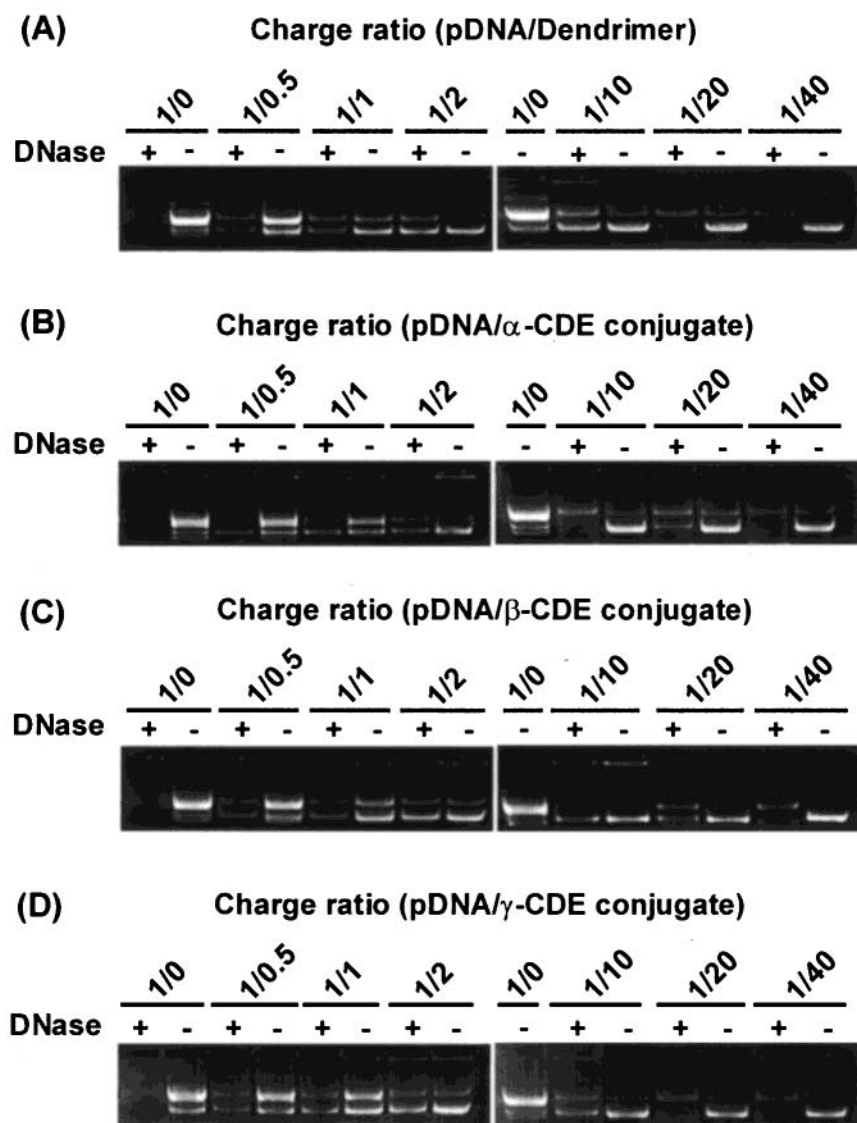


Figure 3. Effects of dendrimer and CDE conjugates on electrophoretic mobility of pDNA treated with DNase I. The complexes of pDNA/dendrimer or pDNA/CDE conjugates were incubated with 0.1 unit/ μ L DNase I for 2 h. After DNase I was inactivated at 70 °C for 10 min, 1 μ L of EDTA (0.5 M) and SDS (8% w/v) were added to each sample and incubated at 37 °C for 1 h to dissociate pDNA from the complexes. After incubation, gel electrophoresis was carried out at room temperature in TBE (45 mM Tris-Borate and 1 mM EDTA) buffer (pH 8.0) in 1% (w/v) agarose gel (include 0.1 μ g/mL ethidium bromide) using Mupid system at 100 V for 50 min. A, dendrimer; B, α -CDE conjugate; C, β -CDE conjugate; D, γ -CDE conjugate.

ratio (1/200), as shown in Figures 8D and 8E. These results support the preceding idea that α -CDE conjugate changes the distribution of pDNA in the cells.

DISCUSSION

We prepared three CDE conjugates in which α -, β -, and γ -CyDs were covalently bound to the dendrimer (generation 2) in a molar ratio of 1:1. These CDE conjugates may be unique as gene transfer vectors, because the only conjugates with β -CyD have been published (30). We revealed here that of three CDE conjugates, α -CDE conjugate possessed the greatest transfection activity. This activity was approximately more than 100-fold than that of dendrimer under an optimal condition.

There are three major barriers to cationic polymers-mediated transfection: (1) interactions between polyplexes and cell surfaces, (2) the endosomal release of polyplexes, and 3) entry of pDNA into the nucleus (35). None of the CyDs alone had the protecting ability of pDNA against DNase I, or the transfection activity at various concentrations, or the enhancing effects of the

cellular association of pDNA (data not shown). In addition, neither dendrimer alone nor the physical mixture of the dendrimer with CyDs showed the significant enhancing effect on the transfection activity (Figures 4–6). On the other hand, the complexes of pDNA with CDE conjugates, especially α -CDE conjugate, possessed a superior transfection activity over the dendrimer and the physical mixtures. The effects of the CDE conjugates on these barriers were investigated to gain an insight into the enhancing mechanism of α -CDE conjugate.

From the results of gel mobility shift assay, dendrimer formed the complexes with pDNA (Figure 2). This may be attributed to electrostatic interaction, because terminal amines of dendrimer are only partially protonated because of their low pK_a 6.9 (36), thereby interacting with negatively charged pDNA. However, this result was inconsistent with that of Fukowska-Latallio et al. (9), in which the pDNA migration is not retarded in the presence of dendrimers with the generation of 1 to 3. This discrepancy in the complexation of dendrimer with pDNA is not clear; possibly the pH value and components of

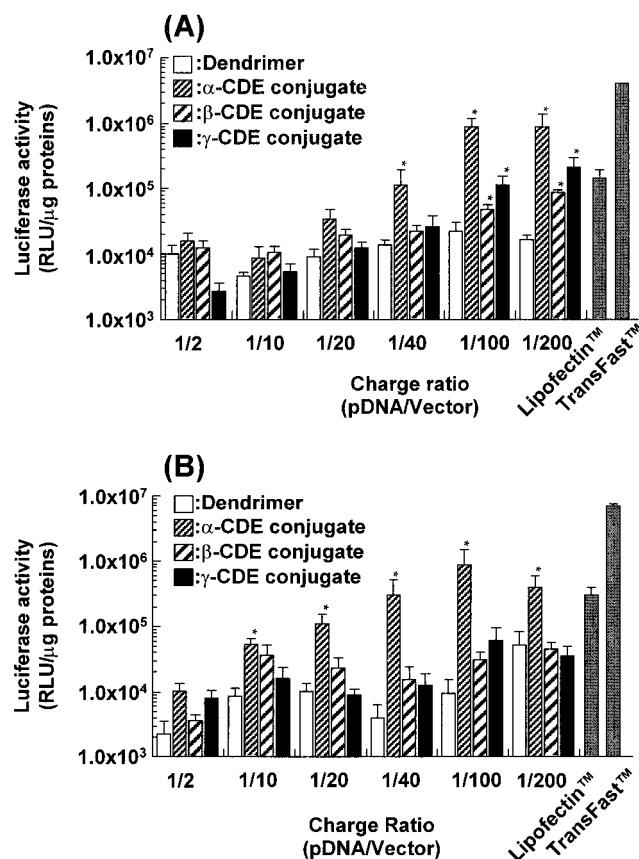


Figure 4. Transfection efficiency of the complexes of pDNA/dendrimer or pDNA/CDE conjugates complexes at various charge ratios in NIH3T3 cells (A) and RAW264.7 cells (B). The luciferase activity in cell lysates was determined 25 h after incubation with pDNA alone or various complexes. The charge ratios of pDNA and TransFast and pDNA and Lipofectin were 1:1; open column, dendrimer alone; dark hatched column, α-CDE conjugate; light hatched column, β-CDE conjugate; closed column, γ-CDE conjugate; shade column, TransFast and Lipofectin. Each value represents the mean $\pm$ SEM of 5–11. * p < 0.05, compared with dendrimer alone.

buffers might be different. CDE conjugates formed the complex with pDNA in a similar manner as dendrimer (Figure 2), suggesting that the CyD moiety is not involved in the complexation, because the 15 terminal amino groups remain on surface of CDE conjugates. Therefore, it is apparent that the complexation ability of CDE conjugates with pDNA is not necessarily a crucial factor for the enhanced transfection activity of CDE conjugates, although the complexation is needed for the induction of transfection activity. Interestingly, the band driven from pDNA disappeared at the charge ratio of 1/1 (pDNA/CDE conjugates), at which the molar ratio of complexes of pDNA with CDE conjugates was about 1/700 (Figure 2). This suggests that the relatively larger and complicated complexes may be formed at more than 1/1 charge ratios. At the charge ratios of 1/100 and 1/200 (pDNA:CDE conjugates), the conjugates are largely in a free form, although their accurate fractions were unknown. The α-CDE conjugate in a free form may assist in the transfection by increasing the release of pDNA from endosome, due to its endosome-membrane perturbing effect, as shown in Figure 8. To examine the average size of the complex of pDNA with dendrimer or α-CDE conjugate, the diameter of the complex was measured by quasi-elastic (dynamic) light scattering (see Experimental Section). The mean diameters of the complexes of pDNA with dendrimer at the charge ratios of 1/0.5, 1/1, 1/2, 1/10,

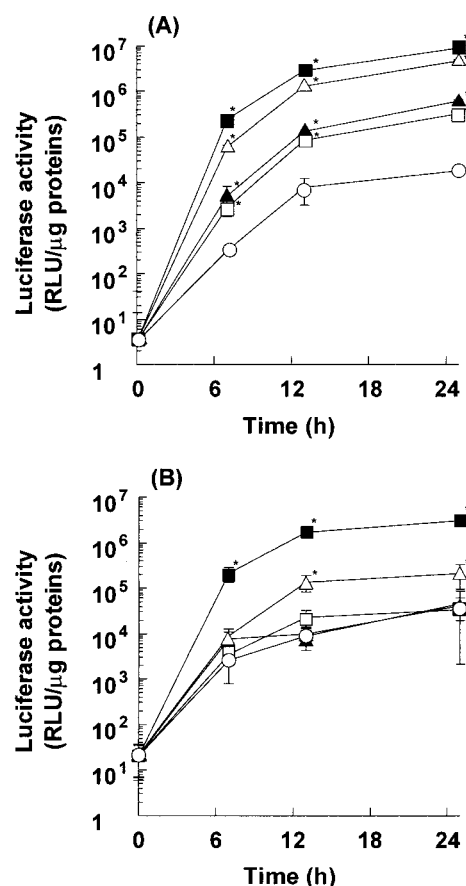


Figure 5. Time course of transfection efficiency of the complexes of pDNA/dendrimer or pDNA/CDE conjugates complexes at the charge ratio of 1/200 in NIH3T3 cells (A) and RAW264.7 cells (B). The charge ratio of pDNA and TransFast was 1:1. ○, dendrimer alone; △, α-CDE conjugate; □, β-CDE conjugate; ▲, γ-CDE conjugate; ■, TransFast. Each point represents the mean $\pm$ SEM of 4. * p < 0.05, compared with dendrimer alone.

1/20, 1/100, and 1/200 (pDNA/vectors) were 794, 594, 737, 731, 804, 717, and 743 nm, respectively, and those with α-CDE conjugate were 781, 753, 718, 735, 761, 747, and 720 nm, respectively. Therefore, the diameter of the complex of pDNA with dendrimer or α-CDE conjugate was not significantly affected by the charge ratios, and there was an insignificant difference in the diameter between the complexes with dendrimer and the conjugate. These results suggest that morphology of the complexes of pDNA with dendrimer or α-CDE conjugate might be independent of the charge ratio, and both dendrimer and α-CDE conjugate may exist as a free form in the solution containing the complexes with the higher molar ratios.

The DNase I protection assay indicated that the pDNA/dendrimer and pDNA/CDE conjugates resist the nuclease-catalyzed degradation (Figure 3). These results were in agreement with the previous reports in which the nuclease-catalyzed degradation is inhibited by the complexation with poly-L-lysine, DC-cholesterol, and Lipofectin (37, 38). However, the optimum charge ratio for the stabilizing effect of CDE conjugates existed, suggesting that factors other than the electrical charge may be associated with the stabilizing effect, e.g., the mode of the complexes and the existence of free fraction of CDE conjugate. Furthermore, there was an insignificant difference in the stabilizing ability between dendrimer and CDE conjugates, indicating that the greater transfection activity of α-CDE conjugate cannot be ex-

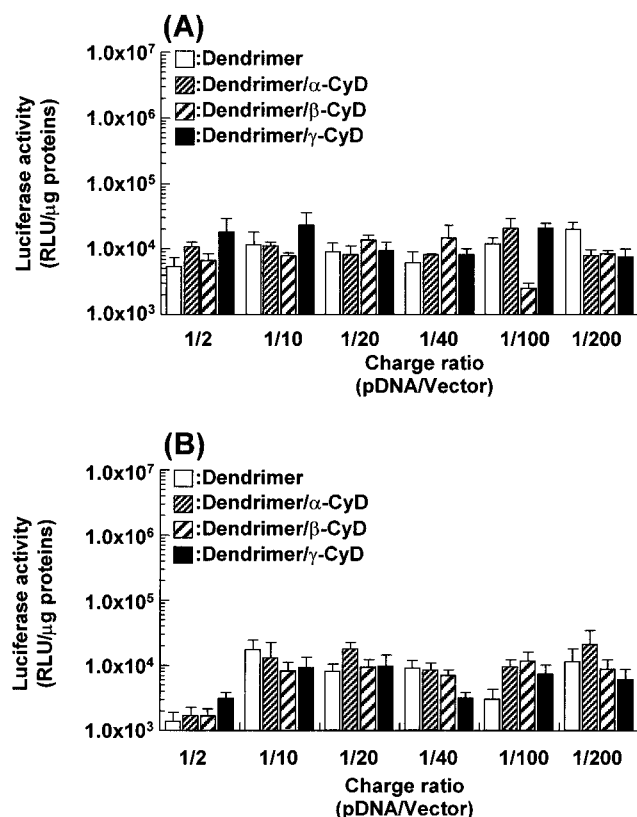


Figure 6. Transfection efficiency of physical mixtures of pDNA and dendrimer or pDNA and CDE conjugates in NIH3T3 cells (A) and RAW264.7 cells (B). Open column, dendrimer alone; dark hatched column, α -CDE conjugate; light hatched column, β -CDE conjugate; closed column, γ -CDE conjugate. Each value represents the mean $\pm$ SEM of 5–11. * $p < 0.05$, compared with dendrimer alone.

plained simply by the inhibition of the DNase I-catalyzed degradation of pDNA.

The entry of the complexes of pDNA with cationic polymers into the cell is the first step of transfection. In the present study, we confirmed a good correlation between cellular association and luciferase activity in lower charge ratios (1/0.2 to 1/2–1/5) of pDNA/CDE conjugates (Figure 7). This suggests that the cellular entry is rate-limiting step for gene transfer. However, no difference in the cellular association of pDNA between dendrimer and CDE conjugates was observed (Figure 7), suggesting that the conjugation of α -CyD with dendrimer, particularly in higher molar ratios, affects intracellular trafficking of pDNA. The fluorescence in cytoplasm was stronger when α -CDE conjugate was used, compared with dendrimer alone (Figure 8), supporting that α -CDE conjugate changes the distribution of pDNA in the cells, possibly in terms of the increase in the release of pDNA from the endosomes into cytoplasm. This hypothesis is based on facts that CyDs induce hemolysis and collapse of liposomes via the release of biological components, such as phospholipids and cholesterol from the membranes (15, 41, 42). It is well recognized that CyDs disrupt the liposomes composed of egg phosphatidylcholine or dipalmitoylphosphatidylcholine in the order of $\alpha > \beta > \gamma$ -CyD, whereas the incorporation of cholesterol into the liposomes changes the membrane-disrupting ability of CyDs in the order of $\beta > \alpha > \gamma$ -CyD (41). In addition, CyDs cause hemolysis of human erythrocytes in the order of $\beta > \alpha > \gamma$ -CyD (15). This difference can be explained by the fact that the species and amounts of released membrane components were dependent upon the cavity

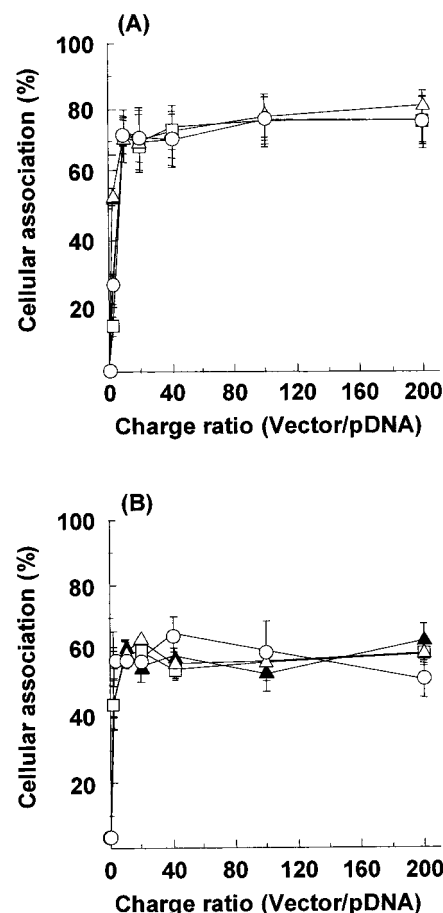
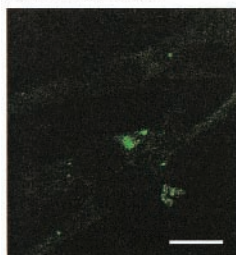
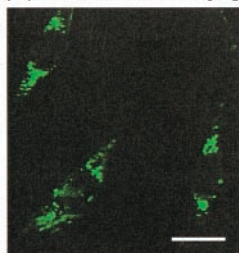
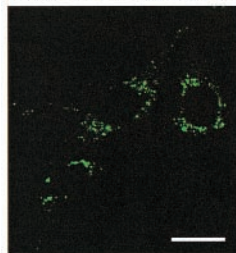


Figure 7. Cellular associations of [α -³²P] labeled pDNA at various charge ratios in NIH3T3 cells (A) and RAW264.7 cells (B). $\circ$, dendrimer alone; Δ , α -CDE conjugate; $\square$, β -CDE conjugate; $\blacktriangle$, γ -CDE conjugate. Each point represents the mean $\pm$ SEM of three experiments.

size of CyDs, i.e., α -CyD extracted mainly phospholipids, β -CyD cholesterol, and γ -CyD both components in smaller amounts (43). However, since endosomal membranes have a relatively higher cholesterol content, the enhanced transfection activity of α -CDE conjugates could not simply be related to the membrane-disrupting effects of CyDs. Furthermore, α -CyD concentration (12 mM) to induce 50% hemolysis (43) is higher than that to enhance the transfection efficiency (for example, about 50 μ M in the charge ratio of 1/100). In addition, CDE conjugates at less than micromolar concentrations could not increase the calcein release from the egg phosphatidylcholine liposomes (data not shown). Therefore, the exact mechanism for the enhanced transfection activity of α -CDE conjugate is currently unclear, but it is apparent that the conjugate affects the intracellular trafficking of pDNA. Further studies are necessary to elucidate the enhancing mechanism of CDE conjugates, e.g., whether β -CDE and γ -CDE conjugates change the intracellular trafficking of pDNA, how the complexes of CDE conjugates with pDNA dissociate, and how pDNA enters into the nucleus, compared with those of dendrimer alone.

Under the present experimental conditions, no cytotoxicity of CDE conjugates and their complexes with pDNA was observed in NIH3T3 and RAW264.7 cells in WST-1 assay, up to the charge ratio of at least 1/800 (data not shown). Therefore, CDE conjugates, particularly α -CDE conjugate, have great advantages as nonviral vectors, i.e., an easy preparation, a superior transfection efficiency, and less cytotoxicity. In a preliminary study,

(A) pDNA alone

(B) With Dendrimer (1/200) (C) With α -CDE conjugate (1/200)

(D) With Dendrimer (1/5)

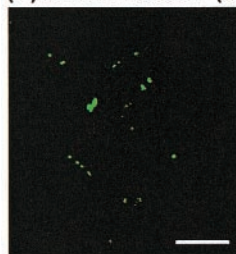
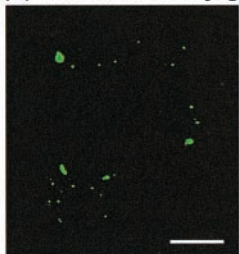
(E) With α -CDE conjugate (1/5)Scale bars = 25 μ m

Figure 8. Confocal laser microscope images for the distribution of FITC-labeled pDNA in NIH3T3 cells after transfection of pDNA alone (A), the complex of pDNA/dendrimer at charge ratio of 1/200 (B), the complex of pDNA/ α -CDE conjugate complex at charge ratio of 1/200 (C), the complex of pDNA/dendrimer at charge ratio of 1/5 (D), and the complex of pDNA/ α -CDE conjugate complex at charge ratio of 1/5 (E). The cells were incubated with pDNA alone or the complexes for 25 h at 37 °C. Scale bars represent 25 μ m. The images shown are typical among similar ones in the several visual fields.

we prepared the α -CDE conjugates containing more than one CyD molecules (average α -CyD/dendrimer molar ratios of 1.8 and 3.2) and then evaluated their transfection activities. The luciferase activities after the transfection of the 1.0, 1.8, and 3.2 α -CDE conjugates in NIH3T3 cells were $3.69 \times 10^5 \pm 0.22 \times 10^5$, $4.82 \times 10^5 \pm 0.27 \times 10^5$, and $6.72 \times 10^5 \pm 0.31 \times 10^5$, respectively, at the charge ratio of 1/200. These results indicated that the transfection activity of pDNA/ α -CDE conjugate complex increased with the increasing of molar ratio of α -CyD/dendrimer.

In conclusion, the present findings suggest that α -CDE conjugate could be a preferable nonviral vector of plasmid DNA among CDE conjugates. This strategy is applicable to other cationic lipids and polymers such as poly-L-lysine and polyethylenimine, which are now under investigation.

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Significance of Substrate Hydrophobicity for Recognition by an Oligopeptide Transporter (PEPT1)

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Our previous paper [(1999) *Bioconjugate Chem.* 10, 24–31] pointed out that hydrophobicity of substrates/inhibitors plays an important role in the recognition by an oligopeptide transporter (PEPT1) expressed in the human intestinal epithelial cell line Caco-2. To determine the significance of that hydrophobicity, we have now synthesized dipeptide analogues conjugating the ϵ -amino group of Lys in Val-Lys with aliphatic carboxylic acids: acetic acid (C2), propanoic acid (C3), pentanoic acid (C5), hexanoic acid (C6), and decanoic acid (C10). The affinities of these conjugates were estimated by their inhibition of the accumulation rate of Gly-Sar, a well-established substrate for PEPT1. With the increase in length of the hydrocarbon chain of the conjugates, i.e., in the hydrophobicity of the conjugates, the inhibition strengthened. Dixon–Webb plot analysis of the inhibition by the C10-conjugated dipeptide showed competitive inhibition. The trans-stimulation effect of Val-Lys conjugated to C10 or C5 on the uptake of Ceftibuten was observed using rat brush border membrane vesicles. This findings showed that these conjugates are transportable substrates. These results confirmed that the hydrophobicity of substrates/inhibitor is one of the factors in the recognition by PEPT1.

INTRODUCTION

Dipeptide transporters in the small intestine play an important role in the efficient absorption of small peptides following the digestion of dietary proteins (1). In some cases, this transporter serves as a carrier of pharmacological-active peptide-like compound such as oral β -lactam antibiotics (2–4), an antineoplastic agent bestatin (5), and an angiotensin-converting enzyme (ACE) inhibitor (6, 7). Because of the structural diversity of the substrates, this oligopeptide transporter (PEPT1) is believed to have very broad specificity. It can therefore be used as a channel to increase the intestinal absorption of poorly absorbable drugs when these drugs are conjugated with a dipeptide or tripeptide core, thus making them recognizable to the transporter (8). On the basis of this idea, we synthesized dipeptide analogues conjugated at ϵ -amino group of Lys in Val-Lys or Lys-Sar with the fluorescent dyes, fluorescein isothiocyanate (FITC, abbreviated as Flu) or coumarin-3-carboxylic acid (Coum¹) (9). These dyes were employed as model drugs which are poorly absorbed.

Unfortunately, the fluorescent dipeptide analogues synthesized were not transported by the oligopeptide

transporter, PEPT1, in Caco-2 cells. Quite interestingly, however, they were recognized by PEPT1 and their affinity was very high; Val-Lys(Flu) inhibited competitively the Gly-Sar uptake by PEPT1 with the inhibition constant being as low as 5 μ M. Here, Val-Lys(Flu) stands for the fluorescent analogue where Flu is conjugated with the free NH_2 of Lys in the dipeptide, Val-Lys. Since the fluorescent dyes, Flu and Coum, are hydrophobic, the increase in the affinity to PEPT1 led us to hypothesize that the hydrophobicity is one of the important factors for the substrate recognition of PEPT1.

To check this hypothesis, we have synthesized dipeptide analogues conjugated with various linear aliphatic acids, by which we systematically changed the degree of the hydrophobicity only while leaving the peptide core unchanged; aliphatic acids were acetic acid (C2), propanoic acid (C3), pentanoic acid (C5), hexanoic acid (C6), and decanoic acid (C10). In the presence of these conjugates, Gly-Sar uptake by Caco-2 cells was measured. With an increase in the chain length, i.e., hydrophobicity, the conjugates more strongly inhibited the uptake. In addition, the conjugates preloaded inside the vesicles increased the uptake rate of Ceftibuten, a substrate, meaning that the conjugate can be transported by the transporter. We thus concluded that the hydrophobicity is one of factors in the substrate recognition by PEPT1.

MATERIALS AND METHODS

Materials. ¹⁴C-Glycylsarcosine ([¹⁴C]-Gly-Sar)(1.78 GBq/mmol) was purchased from NEN Life Science Products (Boston, MA). The culture medium (Dulbecco's modified Eagle's medium, nonessential amino acids) and fetal bovine serum (FBS) were purchased from GIBCO BRL Life Technologies (Grand Island, NY). Dehydrated THF was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). DMF was distilled from CaH_2 at reduced

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¹ Abbreviations: Coum, coumarin-3-carboxylic acid; FITC, fluorescein isothiocyanate; Flu, fluorescein; Gly-Sar, glycylsarcosine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; *n*, number of carbons of the acyl group of Val-Lys-linear aliphatic acid synthesized; Tris, Tris(hydroxymethyl)-aminomethane; WSCD, water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide).

pressure. Toluene and Et₂O were distilled from sodium metal/benzophenone ketyl. Unless otherwise stated, starting materials were obtained from commercial suppliers and were used without further purification. All amino acids were L-isomers. Boc-Val were purchased from Peptide Institute, Inc. (Osaka, Japan). Lys(Cbz)-O-*t*-Bu was purchased from Kokusan Chem. Co., Inc. (Tokyo, Japan). FITC was purchased from Molecular Probes, Inc. (Eugene, OR). Other chemicals used were of the highest purity available.

Synthesis. General Procedures. ¹H NMR spectra were obtained on a JEOL EX270 or Bruker ARX500, and were presented in ppm (δ) relative to either tetramethylsilane or the solvent signal as internal reference (CHCl₃, 7.27 ppm; DMSO, 2.49 ppm). Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Analytical thin-layer chromatography was carried out on silica gel 60 F254 plates (Merck). Preparative column chromatography was performed on silica gel 60 (Merck, 70–230 mesh or 230–400 mesh). All reactions were performed under a nitrogen atmosphere, unless stated otherwise (9).

Boc-Val-Lys(Cbz)-O-*t*-Bu (1) and Boc-Val-Lys-O-*t*-Bu·HCl (2). The preparation and the characterization were described previously (9).

Boc-Val-Lys(C2)-O-*t*-Bu (3a). Acetic anhydride (101 μL, 1.07 mmol) was added to a solution of **2** (304 mg, 0.717 mmol) in DMF (15 mL), followed by Et₃N (100 mL, 0.717 mmol) at 0 °C, and the mixture was stirred at room temperature for 3 h. It was diluted with AcOEt (60 mL) and washed successively with 0.6 M citric acid (40 mL), H₂O (30 mL), aqueous saturated NaHCO₃ (30 mL), H₂O (30 mL), and brine (20 mL) and then dried (Na₂SO₄) and concentrated. The resulting residue was purified by column chromatography (SiO₂, 25:1 CHCl₃–MeOH) to give **3a** (248 mg, 81% as white amorphous): ¹H NMR (CD₃OD, 270 MHz) δ 0.94(3 H, d, *J* = 6.6 Hz, Val-CH₃), 0.99(3 H, d, *J* = 6.6 Hz, Val-CH₃), 1.16–1.89(24 H, overlapping m, 2 × *t*-Bu, Lys β, γ, δ-CH₂), 1.99 (3 H, s, Acetyl), 2.07–2.23 (1 H, m, Val β-CH), 3.11–3.33 (2 H, m, Lys ε-CH₂), 4.00 (1 H, dd, *J* = 5.9, 7.9 Hz, Val α-CH), 4.39 (1 H, dt, *J* = 4.6, 7.9 Hz, Lys α-CH), 5.14 (1 H, d, *J* = 7.9 Hz, Val α-NH), 6.16 (1 H, br s, Lys ε-NH), 6.70 (1 H, d, *J* = 7.3 Hz, Lys α-NH); FAB–HRMS calcd for [C₂₂H₄₁N₃O₆ + H]⁺ 444.3073, found 444.3080.

Boc-Val-Lys(C3)-O-*t*-Bu (3b). The product from **2** (302 mg, 0.712 mmol) and propanoic anhydride (137 μL, 1.04 mmol) was prepared using the procedure for **3a**. The crude product was purified by column chromatography (SiO₂, 40:1 CHCl₃–MeOH) to give **3b** (246 mg, 78% as white amorphous): ¹H NMR (CD₃OD, 270 MHz) δ 0.93–(3 H, d, *J* = 6.6 Hz, Val-CH₃), 0.98 (3 H, d, *J* = 6.6 Hz, Val-CH₃), 1.16 (3 H, t, *J* = 7.3 Hz, propanoyl-CH₃), 1.26–1.90 (24 H, overlapping m, 2 × *t*-Bu, Lys β, γ, δ-CH₂), 2.08 (1 H, m, Val β-CH), 2.21 (2 H, q, *J* = 7.3 Hz, propanoyl-CH₂), 3.28 (2 H, m, Lys ε-CH₂), 3.97 (1 H, dd, *J* = 5.9, 7.9 Hz, Val α-CH), 4.41 (1 H, dt, *J* = 4.6, 7.3 Hz, Lys α-CH), 5.13 (1 H, d, *J* = 7.9 Hz, Val α-NH), 5.90 (1 H, br s, Lys ε-NH), 6.62 (1 H, d, *J* = 7.3 Hz, Lys α-NH); FAB–HRMS calcd for [C₂₃H₄₃N₃O₆ + H]⁺ 458.3230, found 458.3225.

Boc-Val-Lys(C5)-O-*t*-Bu (3c). A solution of **2** (313 mg, 0.738 mmol), *n*-pentanoic acid (88 μL, 0.812 mmol), HOBt (304 mg, 1.47 mmol), and WSCD·HCl (162 mg, 0.848 mmol) in DMF (15 mL) was stirred at 0 °C for 15 min and then Et₃N (103 μL, 0.738 mmol) was added. After 30 min, the reaction mixture was warmed to room temperature and stirred for a further 2 h. It was diluted with EtOAc (60 mL), washed successively with 0.6 M

citric acid (50 mL), H₂O (30 mL), aqueous saturated NaHCO₃ (40 mL), H₂O (30 mL), and brine (30 mL), and then dried (Na₂SO₄) and concentrated. The resulting residue was purified by column chromatography (SiO₂, 35:1 CHCl₃–MeOH) to give **3c** (270 mg, 75% as a white solid): ¹H NMR (CDCl₃, 270 MHz) δ 0.91 (3 H, t, valeryl-CH₃), 0.93 (3 H, d, *J* = 7.3 Hz, Val-CH₃), 0.98 (3 H, d, *J* = 7.3 Hz, Val-CH₃), 1.17–1.89 (28 H, overlapping m, 2 × *t*-Bu, Lys β, γ, δ-CH₂, 2 × valeryl-CH₂), 2.11 (1 H, m, Val β-CH), 2.18 (3 H, t, *J* = 7.3 Hz, valeryl-CH₂), 3.21 (2 H, m, Lys ε-CH₂), 3.95 (1 H, dd, *J* = 2.6, 8.6 Hz, Val α-CH), 4.41 (1 H, dt, *J* = 4.6, 7.9 Hz, Lys α-CH), 5.10 (1 H, d, *J* = 8.6 Hz, Val α-CH), 5.83 (1 H, br s, Lys ε-NH), 6.56 (1 H, d, *J* = 7.3 Hz, Lys α-NH).

Boc-Val-Lys(C6)-O-*t*-Bu (3d). The product from **2** (289 mg, 0.682 mmol) and *n*-hexanoic acid (94 μL, 0.750 mmol) was prepared using the procedure described for **3c**. The crude product was purified by column chromatography (SiO₂, 60:1 CHCl₃–MeOH) to give **3d** (221 mg, 68% as white solid): ¹H NMR (CDCl₃, 270 MHz) δ 0.89(3 H, t, *J* = 6.9 Hz, hexanoyl-CH₃), 0.93 (3 H, d, *J* = 7.3 Hz, Val-CH₃), 0.98 (3 H, d, *J* = 6.9 Hz, Val-CH₃), 1.21–1.85 (30 H, overlapping m, 2 × *t*-Bu, Lys β, γ, δ-CH₂, 3 × hexanoyl-CH₂), 2.11 (1 H, m, Val β-CH), 2.17 (2 H, t, *J* = 7.3 Hz, hexanoyl-CH₂), 3.21 (2 H, m, Lys ε-CH₂), 3.96 (1 H, dd, *J* = 5.6, 8.3 Hz, Val α-CH), 4.42 (1 H, dt, *J* = 4.6, 7.9 Hz, Lys α-CH), 5.11 (1 H, d, *J* = 8.6 Hz, Val α-NH), 5.87 (1 H, br s, Lys ε-NH), 6.58 (1 H, d, *J* = 7.3 Hz, Lys α-NH); FAB–HRMS calcd for [C₂₆H₄₉N₃O₆ + H]⁺ 500.3700, found 500.3697.

Boc-Val-Lys(C10)-O-*t*-Bu (3e). This product was prepared using the procedure for **3c** with starting materials being **2** (252 mg, 0.594 mmol) and *n*-decanoic acid (126 μL, 0.653 mmol). The crude product was purified by column chromatography (SiO₂, 40:1 CHCl₃–MeOH) to give **3e** (252 mg, 76% as white solid): ¹H NMR (CDCl₃, 270 MHz) δ 0.87(3 H, t, *J* = 6.6 Hz, caproyl-CH₃), 0.93 (3 H, d, *J* = 6.8 Hz, Val-CH₃), 0.98 (3 H, d, *J* = 6.8 Hz, Val-CH₃), 1.26–1.89 (38 H, overlapping m, 2 × *t*-Bu, Lys β, γ, δ-CH₂, 7 × caproyl-CH₂), 2.17 (3 H, overlapping m, Val β-CH, caproyl-CH₂), 3.23 (2 H, m, Lys ε-CH₂), 3.95 (1 H, dd, *J* = 5.9 Hz, Val α-CH), 4.41 (1 H, dt, *J* = 5.0, 7.9 Hz, Lys α-CH), 5.11 (1 H, d, *J* = 8.6 Hz, Val α-NH), 5.87 (1 H, br s, Lys ε-NH), 6.58 (1 H, d, *J* = 7.3 Hz, Lys α-NH); FAB–HRMS calcd for [C₃₀H₅₇N₃O₆ + H]⁺ 556.4325, found 556.4337.

Val-Lys(C2)·HCl (4a). A solution of 4 M HCl/dioxane solution (10 mL) was added to a solution of **3a** (225 mg, 0.521 mmol) in THF (1 mL). After 30 min, the reaction mixture was evaporated under reduced pressure. The resulting residue was triturated with Et₂O and dried in vacuo to give **4a** (168 mg, 100% as white powder): ¹H NMR (DMSO, 270 MHz) δ 0.97 (6 H, d, *J* = 6.6 Hz, Val-CH₃), 1.26–1.85 (9 H, overlapping m, Lys β, γ, δ-CH₂, acetyl-C), 2.11 (1 H, m, Val β-CH), 3.01 (2 H, m, Lys ε-CH₂), 4.09 (1 H, t, *J* = 5.9 Hz, Val α-CH), 4.20 (1 H, m, Lys α-CH), 7.96 (1 H, m, Lys ε-NH), 8.22 (3 H, br, Val α-NH₃), 8.67–8.86 (1 H, m, Lys α-NH); FAB–HRMS calcd for [C₁₃H₂₅N₃O₄ + H]⁺ 288.1923, found 288.1923.

Val-Lys(C3)·HCl (4b). The desired product **4b** (173 mg, 100% as a white powder) was obtained from **3b** (227 mg, 0.512 mmol) in a similar manner as described for **4a**: ¹H NMR (DMSO, 270 MHz) δ 0.89–1.04 (9 H, overlapping m, Val-CH₃, propanoyl-CH₃), 1.19–1.93 (6 H, overlapping m, Lys β, γ, δ-CH₂), 2.07 (2 H, q, *J* = 7.3 Hz, propanoyl-CH₂), 2.15 (1 H, m, Val β-CH), 3.02 (2 H, m, Lys ε-CH₂), 4.09 (1 H, t, *J* = 5.9 Hz, Val α-CH), 4.24 (1 H, m, Lys α-CH), 7.86 (1 H, m, Lys ε-NH), 8.22 (3 H, br, Val α-NH₃),

8.56–8.99 (1 H, m, Lys α -NH); FAB–HRMS calcd for $[\text{C}_{14}\text{H}_{27}\text{N}_3\text{O}_4 + \text{H}]^+$ 302.2080, found 302.2070.

Val-Lys(C5)·HCl (4c). The desired product **4c** (195 mg, 100% as white powder) was obtained from **3c** (260 mg, 0.535 mmol) in a similar manner as **4a**: ^1H NMR (DMSO, 270 MHz) δ 0.86 (3 H, t, $J = 7.3$ Hz, valeryl-CH₃), 0.95 (6 H, d, $J = 6.6$ Hz, Val-CH₃), 1.04–1.91 (10 H, overlapping m Lys β , γ , δ -CH₂, 2 $\times$ valeryl-CH₂), 2.06 (2 H, t, $J = 7.3$ Hz, valeryl-CH₂), 2.15 (1 H, m, Val β -CH), 3.02 (2 H, m, Lys ϵ -CH₂), 3.68 (1 H, m, Val- α -CH), 4.18 (1 H, m, Lys α -CH), 7.90 (1 H, m, Lys ϵ -NH), 8.23 (3 H, br, Val α -NH₃), 8.71 (1 H, d, $J = 7.3$ Hz, Lys α -NH); FAB–HRMS calcd for $[\text{C}_{16}\text{H}_{31}\text{N}_3\text{O}_4 + \text{H}]^+$ 330.2393, found 330.2401.

Val-Lys(C6)·HCl (4d). The desired product **4d** (153 mg, 95% as white powder) was obtained from **3d** (206 mg, 0.424 mmol) in a similar manner as described for **4a**: ^1H NMR (DMSO, 270 MHz) δ 0.85 (3 H, t, $J = 6.6$ Hz, Hexanoyl-CH₃), 0.97 (6 H, d, $J = 6.6$ Hz, Val-CH₃), 1.13–1.84 (12 H, overlapping m, Lys β , γ , δ -CH₂, 3 $\times$ hexanoyl-CH₂), 2.05 (2 H, t, $J = 7.9$ Hz, Hexanoyl-CH₂), 2.13 (1 H, m, Val β -CH), 3.01 (2 H, m, Lys ϵ -CH₂), 4.11 (1 H, m, Val α -CH), 4.22 (1 H, m, Lys α -CH), 7.89 (1 H, m, Lys ϵ -NH), 8.21 (3 H, br, Val α -NH₃), 8.62–8.93 (1 H, m, Lys α -NH); FAB–HRMS calcd for $[\text{C}_{17}\text{H}_{33}\text{N}_3\text{O}_4 + \text{H}]^+$ 344.2549, found 344.2549.

Val-Lys(C10)·HCl (4e). The desired product **4e** (183 mg, 97% as a white powder) was obtained from **3e** (239 mg, 0.430 mmol) in a similar manner as **4a**: ^1H NMR (DMSO, 270 MHz) δ 0.86 (3 H, t, $J = 5.9$ Hz, caproyl-CH₃), 0.97 (6 H, d, $J = 6.6$ Hz, Val-CH₃), 1.23–1.76 (20 H, overlapping m, Lys β , γ , δ -CH₂, 7 $\times$ caproyl-CH₂), 2.04 (2 H, t, $J = 7.3$ Hz, caproyl-CH₂), 2.10 (1 H, m, Val β -CH), 3.01 (2 H, m, Lys ϵ -CH₂), 4.09 (1 H, m, Val α -CH), 4.23 (1 H, m, Lys α -CH), 7.86 (1 H, m, Lys ϵ -NH), 8.20 (3 H, br, Val α -NH₃), 8.66–8.85 (1 H, m, Lys α -NH); FAB–HRMS calcd for $[\text{C}_{21}\text{H}_{41}\text{N}_3\text{O}_4 + \text{H}]^+$ 400.3175, found 400.3172.

Cell Culture. Caco-2 cells (ATCC HTB-37) at passage 28 were purchased from the American Type Culture Collection. Although a detailed method was described previously (9), the followings are brief description. Cells were passaged in 75 cm² culture dish in a culture medium consisting of Dulbecco's modified Eagle's medium supplemented with nonessential amino acids (1%), fetal bovine serum (15%), HEPES (5 mM) and NaHCO₃ (2 g/L) without antibiotics. The cells were maintained at 37 °C in an atmosphere of 5% CO₂. Cells between the 30th and 40th passage were used in this study. At 80–90% confluence, cells were seeded using 0.02% EDTA and 0.05% trypsin at a density of 53000 cells/cm² on 60 mm plastic culture dishes. The cells were fed fresh medium every 3 days and were used between the 14th and 16th day for the transport studies.

Uptake Experiments in Monolayer Caco-2. The uptake of [¹⁴C]-Gly-Sar was essentially measured using the manner described elsewhere (9). Unless otherwise indicated, values were corrected for the protein content which was also the same as described elsewhere (9). Initial uptake rates were determined using a linear regression of the linear portion in Gly-Sar uptake vs time (measured at 5, 10, and 15 min).

Kinetic Analysis of Gly-Sar Uptake. The analysis was performed using a Michaelis–Menten type equation with the nonsaturable component described earlier (see eq 1) by an iterative nonlinear least-squares method using a MULTI program (10).

$$v = v_{\max}[\text{S}]/(K_m + [\text{S}]) + K_d[\text{S}] \quad (1)$$

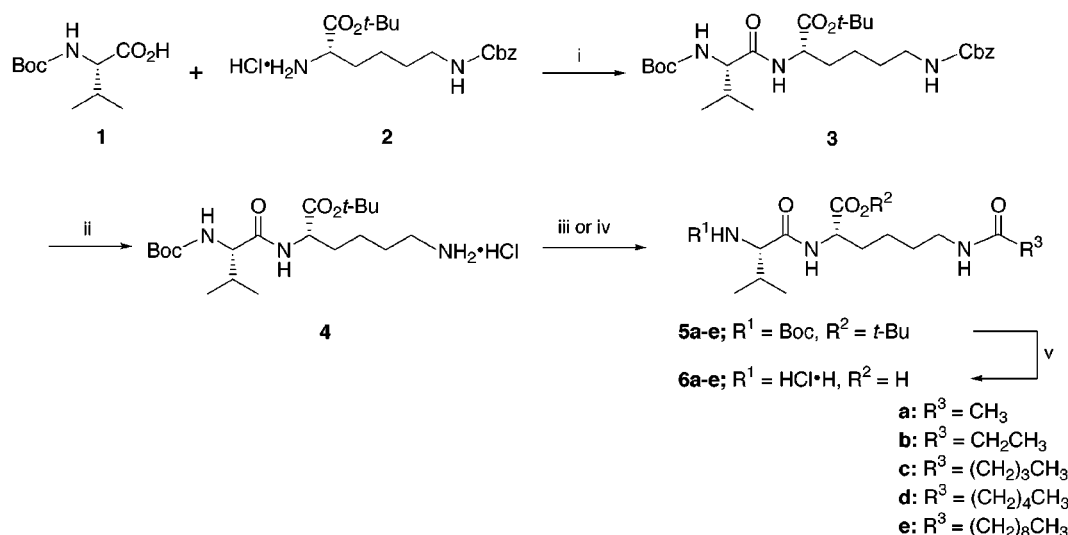
where v represents the initial uptake rate of Gly-Sar; $v_{\max}$, the maximum uptake velocity; $[\text{S}]$, the concentration of Gly-Sar; K_m , Michaelis constant; K_d , the coefficient of the passive diffusion.

Counter-Transport Experiments. These were done using microvilli vesicles from rat intestine. The increase in the uptake rate of Ceftibuten, a substrate of PEPT1, was measured by preloading Val-Lys (C10 or C5) inside vesicles. The entire small intestine of male Wistar rats (250–300 g) was exised under anaesthesia with diethyl ether, and brush border membrane vesicles were isolated according to calcium chloride precipitation method of Kessler et al. (11) which was described previously in some length (12, 13). The purity of the vesicles was assessed by measuring the specific activity of alkaline phosphatase, and the enrichment was more than 12-fold. Membrane vesicles were suspended in an experimental buffer containing 20 mM MES/Tris (pH 5.5), 100 mM D-mannitol, and 100 mM KCl. Twenty microliters of the membrane vesicle suspension (10–15 mg protein/mL) was mixed with 20 μL of the same buffer containing 1 or 10 mM of the test chemical, followed by incubation for 1 h at 30 °C, to load the chemical inside the vesicle. Ceftibuten (400 μL of 1.1 mM solution of the buffer) was added to start the uptake. After designated time (usually 30 s), the uptake was terminated by diluting the mixture with 5 mL of ice-cold washing buffer (150 mM NaCl, 20 mM HEPES/Tris (pH 7.5)) followed by filtration through a Millipore filter (0.45 μm , 2.5 cm diameter; HAWP). The filter was immediately washed once with 10 mL of the ice-cold washing buffer. Ceftibuten trapped on the filter was extracted with 300 μL of mili-Q water using a bath-type sonicator. Nonspecific adsorption of Ceftibuten to the filter was estimated using the experimental buffer without vesicles. The binding was corrected and did not exceed 10% of the total uptake. The concentrations of Ceftibuten were determined by HPLC (L-6200, Hitachi Co. Tokyo) equipped with an UV detector L-4200 (Hitachi Co.). The HPLC conditions were: column, PR-18 (5 μm , 4.2 mm i.d. $\times$ 250 mm, Kanto Chemical Co., Tokyo); a mobile phase, acetonitrile/5 mM citric acid/100 mM KCl (pH 2.5); flow rate 0.7 mL/min; injection volume, 20 μL ; room temperature; detection wavelength, 262 nm.

RESULTS

Synthesis of Hydrophobic Dipeptide Analogues. Our preceding paper (9) suggested the following two points with regard to the substrate recognition by PEPT1: (1) the hydrophobic nature is important and (2) α -carboxyl group is more essential than α -amino group. To confirm the importance of the hydrophobicity, we synthesized hydrophobic dipeptide conjugates: ϵ -Amino group of Lys in Val-Lys was conjugated with aliphatic acids of varying hydrocarbon chain lengths such as acetic acid (C2), propanoic acid (C3), pentanoic acid (C5), hexanoic acid (C6), and decanoic acid (C10). We thus obtained peptide–conjugate homologues with varying hydrophobicity but with the dipeptide backbone kept constant. Val-Lys was selected as a core dipeptide because it is highly resistant to the hydrolysis by peptidase owing to the bulkiness of Val (14) and to the existence of the free amino group. We designated the Val-Lys conjugated to an aliphatic acid whose carbon number is n as Val-Lys(Cn).

Compound **6a** was synthesized from commercially available protected amino acids **1** and **2** (Scheme 1). To remove the Cbz-protecting group of **3**, we chose acetic acid as a reaction solvent since **4** was unstable and an

Scheme 1. Synthesis of Conjugates of Val-Lys with Linear Aliphatic Acids, Val-Lys(Cn)^a

^a Reagents: (i) WSCD·HCl, HOBT, DMAP, DMF; (ii) 10% Pd/C, AcOH, then ion-exchange resin; (iii) Ac₂O or (CH₃CH₂CO)₂O, Et₃N, DMF; (iv) CH₃(CH₂)_nCO₂H ($n = 3$ or 4 or 8), WSCD·HCl, Et₃N, DMF; (v) 4 M HCl/dioxane.

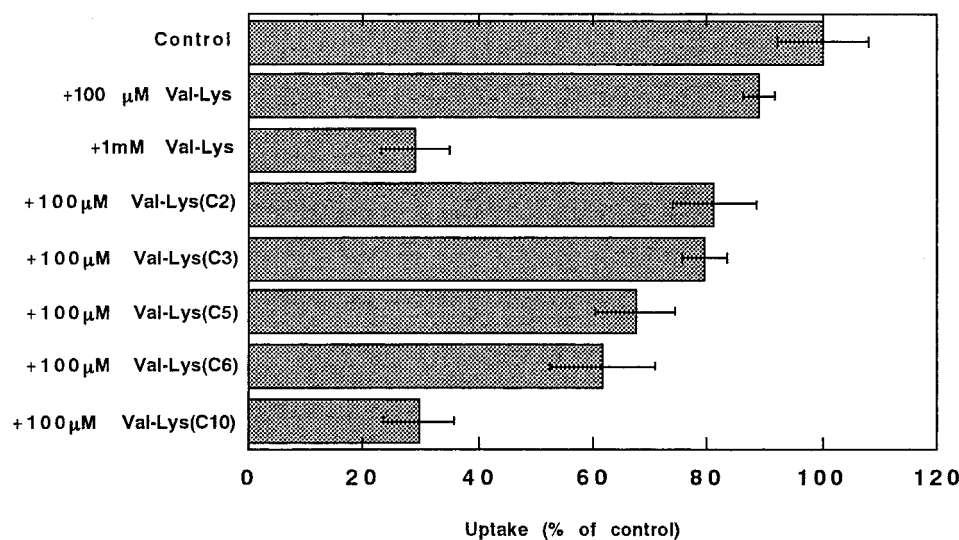


Figure 1. Inhibitory effects of Val-Lys conjugated with linear aliphatic acids, Val-Lys(Cn) on the uptake of 100 μM Gly-Sar, a good substrate for PEPT1. Here, n in Val-Lys(Cn) stands for the hydrocarbon numbers of conjugated linear aliphatic acids. Initial uptake rates of Gly-Sar were measured in the presence of 100 μM or 1 mM of Val-Lys (second and third bar, respectively), or 100 μM Val-Lys(Cn). Control means the uptake rate of 100 μM Gly-Sar.

undesired isomerization occurred in a neutral solution. After removal of Cbz-protecting group, the product was applied to a weakly acidic ion-exchange resin column, and **4** was obtained as a hydrochloride salt. Acylation of **4** at the ϵ -amino group gave **5a**. The desired compound **6a** was easily obtained from **5a** by removal of Boc and *tert*-butyl ester protecting group with 4 M HCl/dioxane. Similarly, other compounds **4b**, **4c**, **4d**, and **4e** were obtained.

The Importance of Hydrophobicity on Recognition by PEPT1. Gly-Sar is a good substrate of PEPT1 (15). Gly-Sar uptake kinetics by Caco-2 cells satisfactorily followed eq 1. The values of K_m and $v_{\max}$ were 0.84 mM and 0.54 nmol/min/mg protein, respectively. A reference (15) described that the rate obtained at 10 μM Gly-Sar was about 0.13 nmol/10 min/mg protein. Our kinetic data give 0.07 nmol/10 min/mg protein at this condition, which is consistent with those reported by previous authors (15).

The uptake rate of 100 μM Gly-Sar was measured in the presence of Val-Lys and various Val-Lys(Cn). The uptake rate of Gly-Sar was inhibited to be 30% of the control level by 1 mM Val-Lys, whereas 100 μM Val-Lys

slightly influenced the uptake rate of Gly-Sar (Figure 1). These are consistent with the reported affinity of Val-Lys for PEPT1. As n increases, Val-Lys(Cn) more potently inhibits the Gly-Sar uptake. The conjugates synthesized here are hydrophobic and thought to interact with membrane lipid bilayer, so there might be possibility that Val-Lys(Cn) inhibit the transport *nonspecifically*. The effect of glucose transport was, hence, examined using Val-Lys(C10) as a most potent inhibitor among the Val-Lys(Cn) synthesized. Uptake rate of 100 μM glucose was 0.23 nmol/mg protein/min as a control, while those in the presence of 50 or 100 μM of Val-Lys(C10) were 0.23 or 0.24 nmol/mg protein/min, respectively, suggesting that the inhibition of the conjugates may be specific to PEPT1.

To evaluate the kinetic type of inhibition by Val-Lys-(C10) which is the strongest, we performed Dixon-Webb plot analysis (16, 17). Results are detailed in Figure 2 where the three lines cross at one point, and the slopes of these lines in this Dixon plot were replotted against the reciprocals of the Gly-Sar concentrations. The slopes

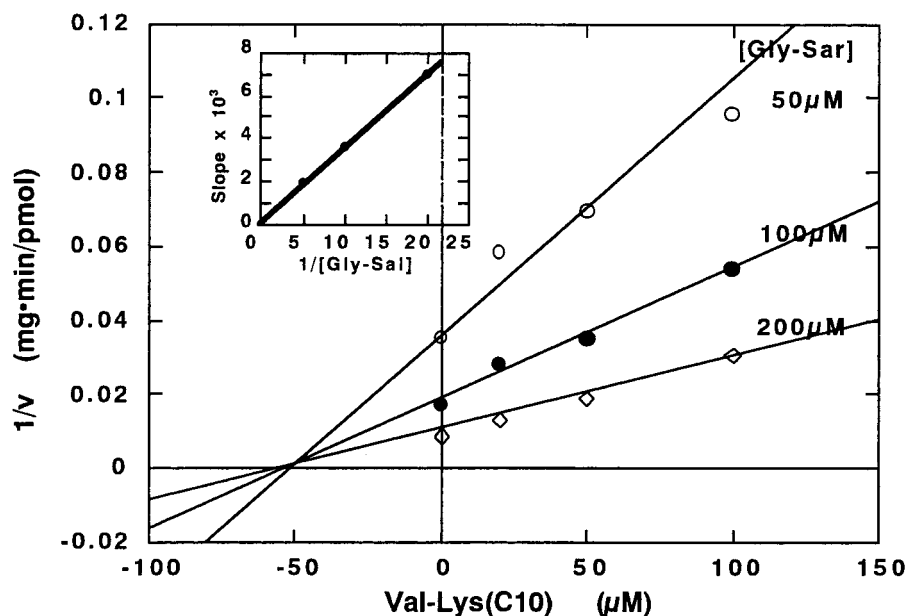


Figure 2. Val-Lys(C10) is a competitive inhibitor of Gly-Sar uptake by PEPT1 as analyzed by Dixon–Webb plot. Gly-Sar uptakes were measured in the presence of varying concentrations of Val-Lys(C10) plotted in abscissa. Open circles, closed circles, and diamonds represent data obtained in the presence of 50, 100, and 200 μM Gly-Sar, respectively. It is noted that the three lines nearly cross at one point. The abscissa and ordinate value of this cross point means the inhibition constant (K_i) and the reciprocal of V_{max} for Gly-Sar uptake, respectively. From this cross point, K_i and V_{max} were estimated to be about 50 μM and 0.65 $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$. This V_{max} value agrees well with 0.54 $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ that was obtained from eq 1 (see text). Inset shows the plot of the slope of this Dixon plot against the reciprocal concentration of Gly-Sar, indicating the proportional relationship. As described in the text, these data clearly show competitive inhibition.

were proportional to the reciprocals as shown in an inset. This shows the competitive inhibition of Gly-Sar by Val-Lys(C10). The inhibition constant (K_i) was calculated to be 50 μM . In a previous paper (9), we found that the K_i value of Val-Lys(Flu) is as small as 5 μM . Despite this, the present Val-Lys(C10) may be a strong inhibitor because the values of K_i or K_m reported for various peptide transporter systems are in the range of 1–10 mM (18–20).

Dipeptide conjugates (Val-Lys(Cn)) are, thus, verified to be recognized by PEPT1. We further examined the trans-stimulation effect of the conjugate on the substrate transport to determine directly whether the conjugate is a transportable substrate. This trans-stimulation experiment was employed by previous authors (21) to examine whether a test compound is a transport substrate. Cefutibuten was chosen as a PEPT1 transport substrate. As depicted in Figure 3, the initial uptake of cefutibuten was markedly enhanced in the vesicles preloaded with Gly-Sar or β -lactam antibiotics cephalixin, whereas not with valine. These mean that Cefutibuten is a good substrate for PEPT1, and show good control results. Cefutibuten uptake was enhanced about 2-fold by the preloading of 1 mM Val-Lys(C10) and more than 2-fold by 1 mM Val-Lys(C5), revealing that Val-Lys(Cn) are transport substrates of PEPT1. The reason C5 has larger effect than C10 is not clear; it might be caused by the difference of the intravesicle concentrations after the loading or by the cis-inhibition of C10 due to its higher affinity.

DISCUSSION

Figure 1 implies that more hydrophobic Val-Lys(Cn) inhibits the uptake of Gly-Sar more potently. Figure 2 shows that Val-Lys(C10) is a competitive inhibitor of Gly-Sar, meaning that Val-Lys(C10) interacts directly with the substrate binding site. Figure 3 implies that Val-Lys (C5 and C10) are substrates. These findings suggest the

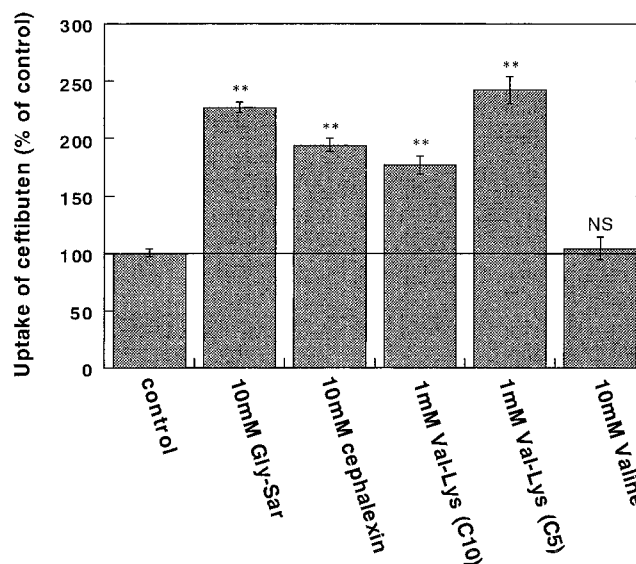


Figure 3. Trans-stimulation effect of Val-Lys(C5) and Val-Lys(C10) on the uptake of Cefutibuten. The conjugate was preloaded into rat brush border membrane vesicles, followed by the Cefutibuten uptake experiments. In this figure, control means the uptake rate of 100 μM Cefutibuten, and its absolute value was 526 $\text{pmol}/30 \text{ s}/\text{mg}$ protein. Bars with 10 mM Gly-Sar and with 10 mM cephalixin show the stimulation of Cefutibuten uptake after Gly-Sar or cephalixin, well-established PEPT1 substrates, were preloaded. 10 mM valine, which is not a substrate, did not induce the stimulation. These are results of control experiments for the counter-transport experiments. ** indicates $P < 0.05$, significant differences from the control using Student t-test.

importance of hydrophobicity of the substrate recognition of PEPT1. The same suggestion was recognized previously (9) using fluorescent dipeptide analogues.

Here a question might arise that the significance of hydrophobic properties is found only in artificial substrates, and this might not be valid for physiological

Table 1. Correlation between Inhibition of Gly-Sar Uptake by Various Dipeptides and Their Hydrophobicity Indexes

dipeptide	hydrophobicity index ^a (kcal/mol)	uptake ^b (% of control)
Gly-Sar	—	100
Gly-Phe	4.7	20.6
Gly-Leu	3.8	19.4
Gly-Pro	0.8	30.1
Gly-Glu	-7.2	64.1
Ala-His	-1.4	79.0
Val-Lys	-6.2	36.1

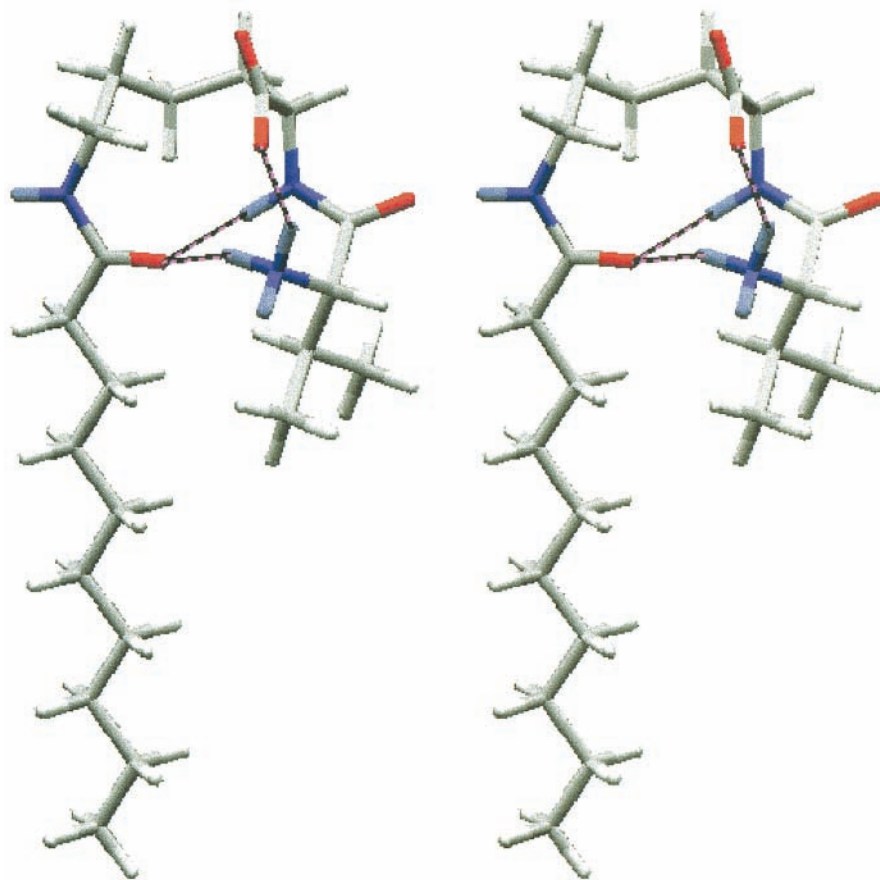
^a Sum of hydrophobicity indexes of two amino acids composing the dipeptide. The hydrophobicity indexes are taken from ref 22.

^b Data were taken from ref 9. Initial uptake rate of 100 μ M Gly-Sar by Caco-2 cells was measured in the absence or presence of 1 mM of various dipeptides.

substrates. We measured the inhibition of Gly-X dipeptide on the Gly-Sar uptake (see Figure 2 in ref 9). The order of inhibition by X was Phe > Leu > Pro > Glu, which is consistent with the order of hydrophobicity of X. One way to express the degree of the hydrophobicity of amino acids is to evaluate the free energy changes from a hydrophobic medium of low dielectric constant to water, as was done by Engelman et al. (22). Table 1 lists the percent uptake of 100 μ M Gly-Sar inhibited by various dipeptides and the hydrophobicity indexes (sum of the free energy change of two amino acids composed of dipeptide). Dipeptides used are Gly-X, Val-Lys, and Ala-His. For those of Gly-X dipeptide, the inhibition shows fairly good linearity against the hydrophobicity indexes, the free energy change (plot not shown). The data points of Val-Lys and Ala-His, however, do not follow the line of Gly-X, suggesting that total hydrophobicity of dipeptide

is not important but that the hydrophobicity of the X residue of Y-X dipeptide might determine the affinity when Y is fixed. Similar results were obtained by Brandsch et al. (23) who investigated the decisive structural factors relevant for dipeptide-PEPT1 interaction by measuring the ability of Pro-X dipeptide derivatives to inhibit Gly-Sar transport in Caco-2 cells: They demonstrated that the affinity of dipeptide to PEPT1 is determined by the hydrophobicity of C-terminal amino acid. They further concluded that structural parameters which play an important role in Pro-X recognition is a rigidity of the structure, since the affinity of a dipeptide with much high rigidity, Pro-Pro is 8-fold higher than Pro-Ala. To confirm these aspects of importance of the hydrophobicity, however, would require testing many dipeptides other than those.

The most probable conformation of Val-Lys(C10) (in vacuo, 25 °C) was calculated by 20,000-step Monte Carlo conformational search using AMBER*, and the obtained conformation is shown in Figure 4. It is assumed that the dielectric constant within the membrane is small, so our calculation in vacuo was done. Three hydrogen bonds are formed: one is between NH₃ of Val and C=O of Lys, the second is between N of the peptide-bonding and C=O of the caproyl group, and the third is between NH₃ of Val and C=O of the caproyl. These hydrogen bonds give rise to the bend structure of Val-Lys(C10) shown in the figure. It is worthwhile to note that the inhibition becomes stronger with increase in *n*, and that the inhibition is still observed at the C10 conjugate (see Figure 1), suggesting that the hydrophobic moiety near the substrate-binding site is large enough to accommodate the C10 hydrocarbon chain. This observation and the simulated structure of Val-Lys(C10) led us to assume

**Figure 4.** Stereoview of the simulated structure of Val-Lys(C10) in vacuo. The red broken lines represent the hydrogen bonds.

that the hydrophobic moiety might be membrane phospholipids or a helix whose surface toward the inner pore of the PEPT1 is hydrophobic. The latter may be less probable because a series of hydrophobic amino residues might not be present at the surface toward the inner pore of the transporter.

Döring et al. (24) showed that ω -amino fatty acid was transported by PEPT1, indicating that the presence of a peptide bond in a substrate is not a structural requirement. In addition, removal of either the amino group or the carboxyl group in ω -amino fatty acid maintained the affinity of the compound for interaction with the transporter but abolished the capability for the transport. EC₅₀ values of hexylamine and octylamine were 6.19 and 0.37 mM, respectively. The values for hexanoic acid and octanoic acid were 2.75 and 1.36 mM. These facts also suggest the importance of hydrophobicity for the recognition by PEPT1.

Miyamoto et al. (25) reported the inhibition by various dipeptides or tripeptides on the Gly-Sar uptake into rabbit renal vesicles. Analysis focusing on the hydrophobicity showed a similar tendency although the data points are scattered (plot not shown). Moore et al. (26) found that hydrophobic oligonucleotide conjugates inhibit the Gly-Pro uptake by Caco-2 cells. Otto et al. (27) also showed the preference of hydrophobic dipeptides by primary culture cells from rat anterior pituitaries, although property of this dipeptide transporter is somewhat different from the PEPT system because of there being no recognition of captopril, thiorphan, and benzylpenicillin. The physiological meaning that peptides having hydrophobic amino acids have high affinity is not known at present. On the other hand, there is an opposite report of the oligopeptide transport in human fibrosarcoma cell line HT1080 when we analyze the data of Nakanishi et al. (28). We do not know the reason, but should take the difference of the transporter into consideration.

The present data suggest the existence of a hydrophobic moiety in PEPT1, and this moiety is composed of the substrate binding site because Val-Lys(C10), Val-Lys(C5), and probably other Val-Lys(Cn) are substrates. Bailey et al. (29) also suggested the presence of hydrophobic part of the substrate recognition. Identification of this moiety on a molecular basis will be further investigated, and its physiological meaning should be clarified. It has been shown that ω -amino fatty acids are transportable substrates under the condition that two charged groups are separated within a certain distance (24). This and the finding presented here may be an interesting clue to develop the transportable carrier conjugated with a poorly absorbable drug.

ACKNOWLEDGMENT

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Energy-Transfer Cassette Labeling for Capillary Array Electrophoresis Short Tandem Repeat DNA Fragment Sizing

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Energy-transfer (ET) dye-labeled primers significantly improve fluorescent DNA detection because they permit excitation at a single common wavelength and they produce well separated and intense acceptor dye emission. Recently, a new ET cassette technology was developed [Berti, L. et al. (2001) *Anal. Biochem.* 292, 188–197] that can be used to label any PCR, sequencing, or other primer of interest. In this report we examine the utility of this ET cassette technology by labeling seven different short tandem repeat (STR) specific primers with each of the four ET cassettes and analyzing the PCR products generated on a MegaBACE-1000 capillary array electrophoresis system. More than 60 amplicons were generated and successfully analyzed with the ET cassette-labeled primers. Both forward and reverse primers were labeled for multiplex PCR amplification and analysis. Single base pair resolution was achieved with all four ET cassettes. This ET cassette-primer labeling procedure is ideally suited for creating four-color fluorescent ET primers for STR and other DNA assays where large numbers of different loci are analyzed including sequencing, genetic identification, gene mapping, loss of heterozygosity testing, and linkage analysis.

INTRODUCTION

Microsatellite DNA or short tandem repeat (STR) sequences have proven invaluable in many aspects of genomic analysis. The power of these microsatellite loci is derived from their high degree of polymorphism and genome-wide distribution (Edwards et al., 1991). There are more than 5200 mapped microsatellite loci available for human genomic studies (Dib et al., 1996). Microsatellite loci are important for gene discovery and genome mapping, genome wide searches for disease markers and susceptibility genes, parentage testing and forensic identification, clinical diagnosis, evolutionary studies, loss of heterozygosity testing associated with cancer diagnosis, and linkage analysis (March, 1999; Reed et al., 1994; Cornelis et al., 1998; Ober et al., 1998; Davies et al., 1994; Edwards et al., 1991; Wiegand et al., 2000; Chakraborty et al., 1999; Caskey et al., 1992; Medintz et al., 2000a).

The intrinsic nature of short tandem repeat DNA sequences dictates that their analysis is heavily reliant on electrophoretic sizing separations. STR analysis usually consists of a locus-specific PCR amplification followed by electrophoretic separation of the products (Mansfield et al., 1996; Cornelis et al., 1998; Medintz et al., 2000a). In order for STR-based genetic assays to be highly informative, large numbers of microsatellite loci have to be screened in thousands of individuals (Cornelis et al., 1998; Ober et al., 1998; Davies et al., 1994; March, 1999), and each study will require multicolor fluorescently labeled locus-specific primers. A steady progression of improved slab gel and capillary array electrophoresis (CAE) instruments have been developed that can increase the amount of information gathered from these loci (Kheterpal and Mathies, 1999; Meldrum 2000) including the MegaBACE-1000 CAE system and the ABI 3700 (Fox,

1999; Meldrum, 2000). Analyses on these instruments have relied, at least in part, on energy-transfer (ET) dye-labeling technology.

In comparison to single dye-labeled primers, ET labeling offers the advantages of excitation at a single common wavelength with distinctive and intense acceptor dye emission and matched electrophoretic mobilities of the labeled targets (Hung et al., 1997; Ju et al., 1995; 1996a). ET labeling is particularly well suited to multicolor fluorescence assays such as STR analysis requiring coanalysis of analyte and standard (Mansfield et al., 1998).

Our original ET primer constructs introduced the donor and acceptor dyes onto a modified primer, or spacer, after automated oligonucleotide synthesis, resulting in a lengthy synthetic procedure (Ju et al., 1995; Ju et al., 1996a). An ET cassette synthetic scheme allowing the attachment of an ET label to any primer has been developed (Ju et al., 1996b). However, this early procedure required synthesis of the cassette on the 5' end of the primer during automated synthesis and postsynthetic dye conjugation. A convenient and simple synthetic method for ET labeling any primer of interest would be valuable.

Recently, an approach was developed (Berti et al., 2001) for easily tagging any primer sequence or other target of interest with a reactive fluorescent ET cassette label. A schematic representation of such an ET cassette-labeled primer is presented in Figure 1A. In our construct, the ET cassette-labeled primer consists of three parts: the ET cassette itself, the primer, and the disulfide linker joining the two. The chemical structure of the reactive ET cassette is presented in Figure 1B. The cassette consists of a sugar–phosphate spacer with a 6-FAM (6-carboxyfluorescein) donor at the 3'-end, an acceptor dye linked to a modified T-base at the 5'-end of the spacer, and a mixed disulfide group for coupling to the 5'-end of a thiol-modified primer. The coupling reaction is a two-step process (see Figure 1C) consisting

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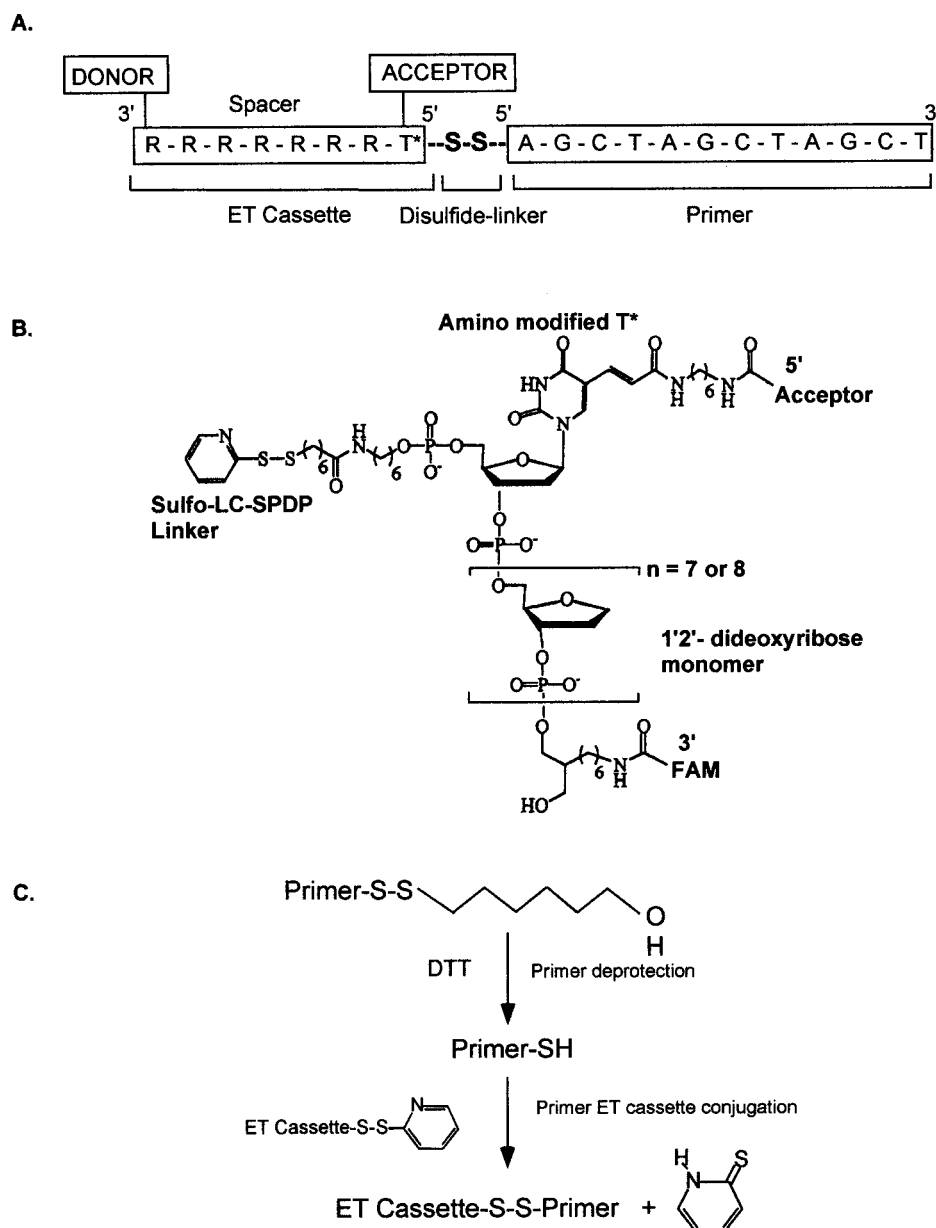


Figure 1. A. Schematic representation of an ET-cassette labeled primer. R = 1',2'-dideoxyribose monomer. B. Chemical structure of an ET cassette. C. Schematic of the two-step ET cassette primer conjugation.

of primer deprotection followed by conjugation with the ET cassette. By quantitative coupling to a thiol-activated M13-target primer through disulfide exchange, a four-color set of ET cassette-labeled M13-primers have been constructed and used for both PCR and DNA sequencing analysis. The acceptor dye emission intensities of primers produced in this manner are comparable to commercially available ET primers (Berti et al., 2001).

In this report we labeled seven STR specific primers in each of four colors with our ET cassettes and analyzed the amplicons generated from these primers on a MegaBACE-1000 CAE system. Amplicons from a primer set where either the forward or reverse primer was labeled were generated as well as amplicons from multiplex amplification mixtures. Single basepair (bp) resolution is easily achieved with amplicons generated from four-color ET cassette labeled STR primers. This study illustrates that this ET-cassette labeling technology is particularly well suited to microsatellite analysis and other fluorescent DNA assays where large numbers of custom-labeled ET primers are required.

MATERIALS AND METHODS

DNA. Purified K562 cell line DNA was purchased from Promega Corp. (Madison, WI). Universal CEPH (Centre d'Etude du Polymorphisme Humain) Donor DNA, catalog number NA10859, was purchased from the Coriell Cell Repository (Camden, NJ). A genomic DNA sample containing the previously characterized THO1 9.3/10 alleles was generously provided by G. Sensabaugh, School of Public Health, U. C. Berkeley (Wang et al., 1995; 1996).

ET Cassette Synthesis. Briefly, starting with a 6-FAM-labeled controlled-pore glass (CPG) column on an Applied Biosystems (Foster City, CA) 392 DNA Synthesizer, the 1',2'-dideoxyribose monomers, amino modified T-base, and a monomethoxytrityl (MMT)-protected 5'-amino-modifier group were added sequentially through standard phosphoramidite chemistry. Cassettes with both seven and eight 1',2'-dideoxyribose monomer spacings were synthesized for this study. The 6-FAM-labeled cassette precursor was cleaved from the CPG support by NH_4OH treatment. The trifluoroacetamide protecting

Table 1. Primer Sequences^a

primer name	chromosomal location	locus definition	repeat sequence	labeled sequence 5'-3'	PCR reverse primer sequence 5'-3'
CSFIPO-forward	5q33.3-q34	human c-fms proto-oncogene for CSF-1 receptor gene	AGAT	AACCTGAGTCTGCCAAGGACTAGC	TTCCACACACCACTGGCCATCTTC
CSFIPO-reverse	5q33.3-q34	human c-fms proto-oncogene for CSF-1 receptor gene	AGAT	TTCCACACACCACTGGCCATCTTC	AACCTGAGTCTGCCAAGGACTAGC
D7S820	7q11.21-q22	NA ^b	AGAT	TGTCATAGTTTAGAACGAACCTAACG	CTGAGGTATCAAAAACTCAGAGG
D13S317	13q22-q31	NA	AGAT	ACAGAAGTATGGGATGTGGA	GCCCCAAAAGACAGACAGAA
THO1	11p15.5	human tyrosine hydroxylase gene	AATG	GTGGGCTGAAAAGCTCCCATTAT	ATTCAAAGGGTATCTGGGCTCTGG
TPOX	2p25.1-pter	human thyroid peroxidase gene	AATG	ACTGGCACAGAACAGGCACCTTAGG	GGAGGAACTGGGAACACACAGGT
vWA-reverse	12p12-pter	human von Willebrand factor gene	AGAT	GGACAGATGATAAATACATAGGATG GATGG	GAAAGCCCTAGTGGATGATAAGAATAAT

^a Ober et al., 1998; www.cstl.nist.gov. ^b NA: Not available.

group on the amino-modified T-base was also removed by this NH_4OH treatment. Succinimidyl ester derivatives of the acceptor dyes were then conjugated with the amino modified T-base. The acceptor dyes 6-carboxyrhodamine-110 (R110, emission maximum at ~ 525 nm), 6-carboxyrhodamine-6G (R6G, emission maximum at ~ 555 nm), carboxytetramethylrhodamine (TAMRA, emission maximum at ~ 572 nm), and 6-carboxy-X-rhodamine (ROX, emission maximum at ~ 620 nm) were obtained from Molecular Probes (Eugene, OR). The donor 6-FAM dye has an emission maximum at ~ 497 nm. Unreacted dyes were removed by size exclusion chromatography on PD-10 cartridges (Amersham/Pharmacia Biotech, Piscataway, NJ), and then the MMT-5'-aminomodifier group was deprotected with aqueous acetic acid treatment. A sulfosuccinimidyl 6-[3'-(2-pyridyldithio)propionamido]-hexanoate (Sulfo-LC-SPDP) linker (Pierce, Rockford, IL) was then coupled to the amino-functionalized ET cassette, and the activated cassette was purified by reverse phase HPLC. A particular ET cassette is described by the abbreviation D-S_n-A, where D is the donor, A is the acceptor, and S_n indicates the number of sugar phosphate monomers constituting the spacer (Ju et al., 1995). The ET cassettes with R110 and TAMRA as the acceptor dyes utilized a spacer with eight sugar phosphates (S-8) while cassettes with R6G and ROX as the acceptors utilized seven sugar phosphate spacers (S-7). In this cassette format the modified T to which the acceptor dye is attached will function as part of the spacer. Therefore, the spacings for the cassettes used in this study are functionally nine and eight units, respectively. The donor-acceptor dye pairings and the sugar phosphate spacers in our current cassettes are similar to those found in commercially available M13 ET labeled primers. The donor-acceptor dye pairs and spacings were selected to maximize the acceptor emission intensity while minimizing the relative donor emission.

ET Cassette-Primer Conjugation. Primer sequences are presented in Table 1. Modified primers for ET cassette conjugation were purchased from Operon Technologies (Alameda, CA) with a 5'-protected thiol (C6 S-S modification) and the trityl protection groups already removed. Additional primers were obtained from GIBCO BRL (Gaithersburg, MD). The thiol primer, protected as a disulfide, was deprotected by incubating in a 0.17 M phosphate buffer pH 8.0 solution containing 0.04 M dithiothreitol (DTT) for 16 h at 37 °C in a MJ Research PTC-100 Programmable Thermal Cycler (Watertown, MA). The thiol-deprotected primers can be stored for prolonged periods of time in this same solution at -20 °C. Just prior to coupling, the thiol-deprotected primers were purified using a PD-10 cartridge. A typical primer-ET cassette coupling reaction was performed as

follows: equimolar amounts (~ 1 nmol) of activated ET cassette and purified thiol-deprotected primer are mixed together in a 0.5 μL Eppendorf tube, dried down in a vacuum centrifuge, and reconstituted in 20 μL of 20 mM phosphate buffer containing 1 mM EDTA pH 7.5 and incubated at 45 °C overnight in the thermal cycler. The ET cassette-conjugated primers were then purified by reverse phase HPLC and quantitated by UV spectroscopy. ET cassette-labeled primers were stored in 1X TE at -20 °C.

PCR Amplification. Fifty nanograms of genomic DNA was utilized for each PCR reaction along with 15 pmol of both the ET cassette label and appropriate reverse primer. Reactions utilized Qiagen PCR Master Mix (Valencia, CA). Reaction conditions used were as described for specific STR loci (Promega, 1998) on the MJ Research Thermal Cycler. Successful PCR was verified by agarose gel electrophoresis.

Sample Preparation and Sizing Standards. PCR amplicons were desalted using the Qiagen PCR Purification Kit. Samples were resolubilized in 50 μL of 0.5X TE buffer. Approximately 1 μL of sample was then mixed with 5 μL of 75% deionized formamide containing dye-labeled sizing markers at a concentration of 10 fM. The TET (tetrachlorofluorescein, emission maximum at ~ 538 nm) Mapmarker Sizing Standard consisting of 20 fragments (70, 80, 90, 100, 120, 140, 160, 180, 190, 200, 220, 240, 260, 280, 300, 320, 340, 360, 380, and 400 bp) was obtained from Bio Ventures Inc. (Murfreesboro, TN). The ET-400-R sizing standard obtained from Amersham/Pharmacia consists of 20 fragments (60, 90, 100, 120, 150, 160, 170, 190, 200, 220, 250, 270, 290, 300, 310, 330, 350, 360, 380, and 400 bp) labeled in an energy transfer format with FAM as the donor dye and ROX as the acceptor dye. Samples were placed in a 96-well microtiter injection plate and centrifuged for 5 s to remove air bubbles. Less than 10 min before injection, the samples were denatured at 95 °C for at least 5 min and immediately placed on ice.

Capillary Array Electrophoresis. A MegaBACE-1000 capillary array electrophoresis system (Molecular Dynamics, Sunnyvale, CA) was utilized for sizing separations (Fox, 1999; Medintz et al., 2000a; Meldrum, 2000). This CAE system contains 96 capillaries, each with a 40 cm length (Bashkin et al., 1996a,b). Long Read linear polyacrylamide matrix, LPA, containing 7 M urea in a Tris/Taps buffer, was obtained from Amersham/Pharmacia Biotech. The matrix was introduced into the capillaries at 400 psi for 200 s followed by pre-electrophoresis for 5 min at 10 kilovolts (kV). Denatured samples were introduced into the capillaries by electrokinetic injection for 45 s at 3 kV and then electrophoresed at 10 kV for 75 min. Fresh matrix was introduced into

Table 2. Comparison of Predicted and Observed Allele Sizes Using ET Cassette-Labeled STR Primers

locus	alleles	predicted ^c size (bp)	determined size (bp) ^a				avg of determined/ predicted size (bp) ^b	std deviation of four- color sizing values
			FAM-8-R110	FAM-7-R6G	FAM-8-TAMRA	FAM-7-ROX		
CSFIPO (forward)	9/10	303/307	316.8/321.1	315.6/321.2	316.5/320.5	317.5/321.5	13.6/14.1	0.79/0.42
CSFIPO (reverse)	9/10	303/307	318.1/321.3	317.4/321.5	318.3/322.4	317.3/321.4	14.8/14.7	0.50/0.50
D7S820	9/11	210/218	223.5/231.6	222.7/231.6	224.6/232.8	222.9/231.1	13.4/13.8	0.85/0.72
D13S317	8/8	169	188.4	188.5	187.8	187.6	18.1	0.44
THO1	9.3/9.3	198	210.3	209.7	210.3	209.7	12.0	0.35
TPOX	8/9	232/236	244.8/248.7	243.8/247.9	244.9/248.9	244.1/248.1	12.4/12.4	0.53/0.48
vWA	16/16	151	163.5	163.1	163.8	163.4	12.5	0.29

^a Size of each allele in each color averaged from two capillaries. ^b Average size of all four colors. ^c www.cstl.nist.gov; Ober et al., 1998.

the capillaries between each run. The instrument was set up to run in sequencing mode with the 488 nm laser only used for fluorescent excitation. Four-channel fluorescent detection was performed with the following emission wavelength detection ranges: Channel 1, 555 ± 10 nm; Channel 2, 520 ± 10 nm; Channel 3, ≥ 610 nm; and Channel 4, 580 ± 10 nm.

Data Analysis. Data were analyzed with Genetic Profiler Software Version 1.1 (Molecular Dynamics, Sunnyvale, CA). The raw signal data files were imported into the Genetic Profiler program where they underwent background subtraction using a spectral separation or cross-talk matrix. Unknown samples were sized against the DNA sizing ladders included in each capillary using a third-order local algorithm (Medintz et al., 2000a; Wedemayer et al., 2001).

RESULTS

Evaluation of ET Cassette-Labeled STR Amplicons. All the primers utilized in this study were successfully labeled in parallel with the four different ET cassettes. Amplicons were generated from the K562 DNA and the CEPH control DNA and were genotyped on the MegaBACE-1000 CAE instrument. Figure 2 presents the electropherograms generated by genotyping all the FAM-7-ROX-primer derived alleles amplified from the CEPH control DNA. As can be seen, each of the amplicons is clearly identified and genotyped even though the amount of PCR product utilized for these separations represents only 1/50th of the total amount of purified PCR amplicon. Furthermore, these strong signals are obtained in the ROX channel, which has the reddest absorption (~595 nm) and emission (~620 nm); without using ET-labeling the ROX emission would be more than 10-fold weaker (Medintz et al., 2000b; 2001). The spacings between determined allele sizes corresponds to 4-bp increments as predicted for the TPOX, THO1, D7S820, and vWA loci. The difference between labeling either the forward or reverse primer for the CSF1PO locus is only 0.2 bp. This very slight (0.06%) difference is achieved even though the DNA strands that are labeled and separated are distinct in sequence.

Since ET cassette-primer labeling results in the addition of the ET cassette to a modified primer, a slight difference in predicted versus determined fragment size is expected. The approximate sizes of the cassettes were predicted by taking the molecular weight of the cassette and dividing by the average molecular weight of a nucleotide (330 g/mol). Using this method, the predicted size of an S-8 cassette should range from 9.5 bp for the FAM-8-TAM cassette to 9.8 bp for the FAM-8-ROX primer. Use of an S-7 cassette will drop these predicted sizes into the 8.5 to 8.8 bp range. To determine the actual effect of ET cassette conjugation on fragment sizes,

amplicons were generated from the K562 genomic DNA. The predicted sizes of the alleles generated from K562 DNA with the corresponding unlabeled primers have been characterized (Promega Corp, 1998; www.cstl.nist.gov). Table 2 presents a comparison of the predicted K562 allele sizes at the seven loci with those determined using all 4 ET cassette-labeled primers. On average, the ET labeled alleles were increased in size by 12.0 to 18.1 bp for the THO1 9.3 allele and the D13S317 8 allele, respectively. The standard deviations of the four-color sizing values for each allele ranged from 0.29 for the vWA 16 allele to 0.85 for the 9 allele at the D7S820 locus.

Multiplex Amplification. To explore the utility of ET cassette-labeled primers for multiplex coamplification and analysis, three separate amplicons were generated in a single reaction. Using K562 DNA, amplicons were generated with the THO1 FAM-8-TAM, TPOX FAM-7-R110, and CSF1PO reverse FAM-8-R6G labeled primers. Figure 3 presents the separation and genotyping of this triplex analysis against the ET-400-R sizing ladder. All the expected amplicons were generated, and their sizing did not differ from values presented in Table 2.

Single Base-Pair Resolution. The original characterization of these disulfide ET cassettes showed through four-color sequencing of M13 DNA that single base-pair resolution is easily achieved (Berti et al., 2001). A similar test in the context of STR analysis was performed using a THO1 9.3/10 allele-containing sample that was genotyped with all four ET labels. The THO1 9.3 allele represents a common variant that differs from the 10 allele by only a single base, instead of the usual 4 bp repeat (Promega 1998; Wang et al., 1995; 1996). Figure 4 presents the genotyping of this sample with all ET cassette labeled THO1 primers. Single bp resolution between the two alleles is achieved for all four colors. The interallele size difference in every case is no more than 1/10th of a bp, or 0.04%.

Effects of S-7 and S-8 Sugar Phosphate Spacers. To determine the effects of cassettes containing different number of sugar phosphate spacers on electrophoretic separations, the CEPH sample was genotyped at the THO1 locus with FAM-7-TAM and FAM-8-TAM ET cassette labeled primers. The expected size differences for these two cassettes should be around 1 bp. Figure 5 presents the separation of the alleles generated in this experiment. The 4 bp difference between alleles is still-maintained while the difference caused by the change in spacer number is less than 1 bp. Similar results were obtained with K562 DNA for the same primers as well as for the D7S8 primer labeled with FAM-7-R110 and FAM-8-R110 cassettes (data not shown).

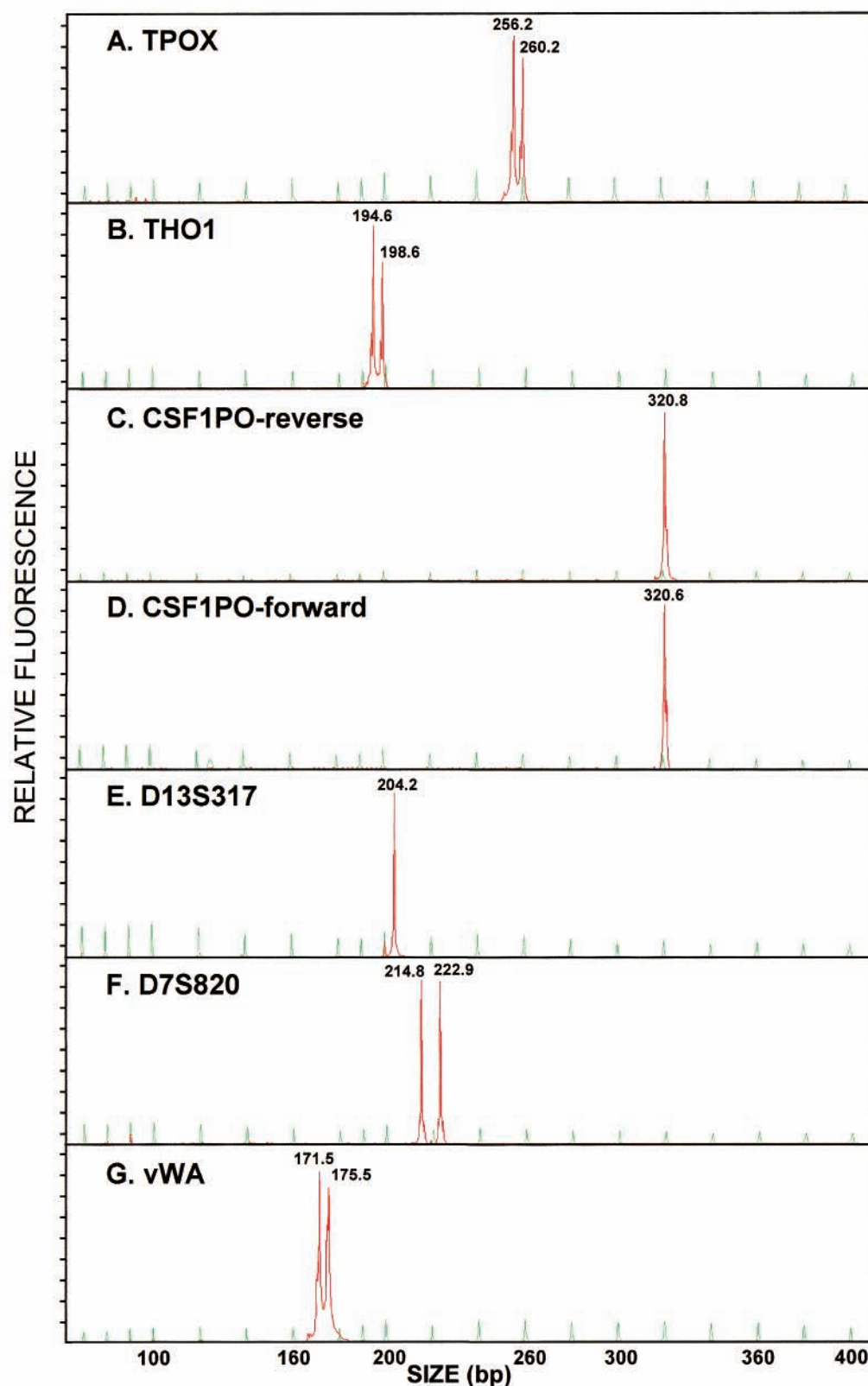


Figure 2. Panels A–G present electropherograms generated by genotyping all the FAM-7-ROX primer derived alleles amplified from the CEPH control DNA. Samples (red traces) were separated against the TET Mapmarker Sizing Standard (green traces) on the MegaBACE-1000 CAE system, and resulting data were analyzed using Genetic Profiler Version 1.1 software. The allele sizes as determined are displayed above the peaks. Peak signal strength range is 600–10000 fluorescence units.

DISCUSSION

This study was performed to explore the feasibility of using ET cassette labeling to tag any primer of interest in a multicolor fluorescent format. To accomplish this we

labeled seven different STR specific primers, each with four different ET cassette labels, and genotyped amplicons generated from these primers. Our ET cassette labeling methodology has numerous advantages. One can tag any primer of interest with any of the multicolor ET

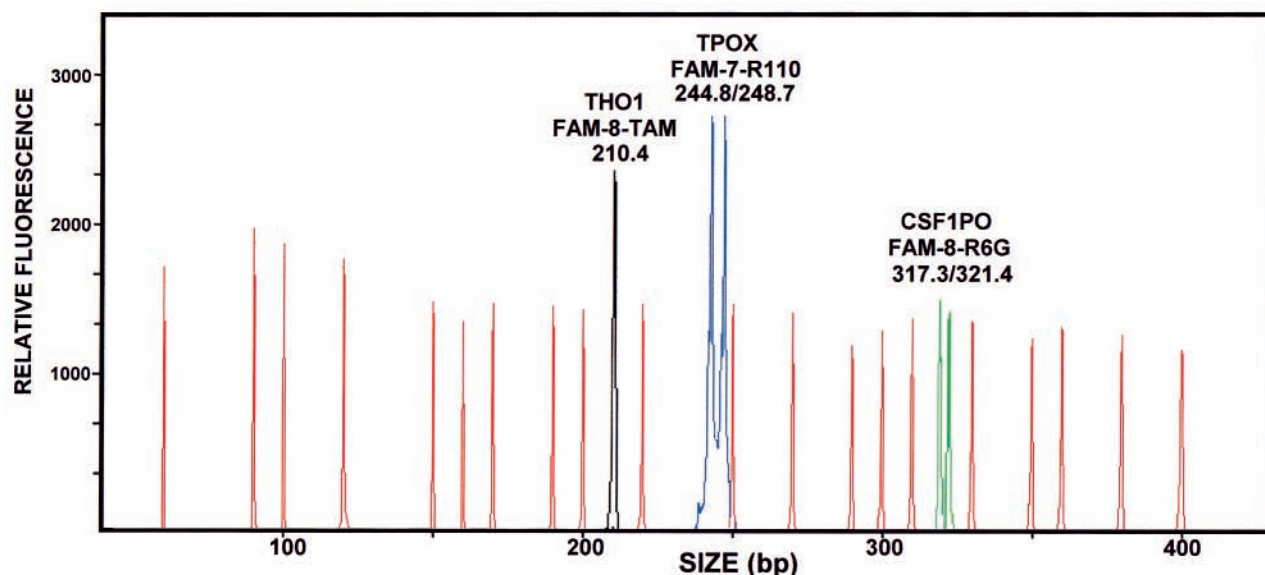


Figure 3. Four-color electropherogram demonstrating multiplex coamplification and analysis. Three separate amplicons were generated in a single reaction using K562 DNA and the THO1 FAM-8-TAM, TPOX FAM-7-R110, and CSF1PO reverse FAM-8-R6G labeled primers. The THO1 (black), TPOX (blue), and CSF1PO (green) alleles were separated and sized against the ET-400-R Sizing Standard (red). Allele sizes are indicated above the peaks.

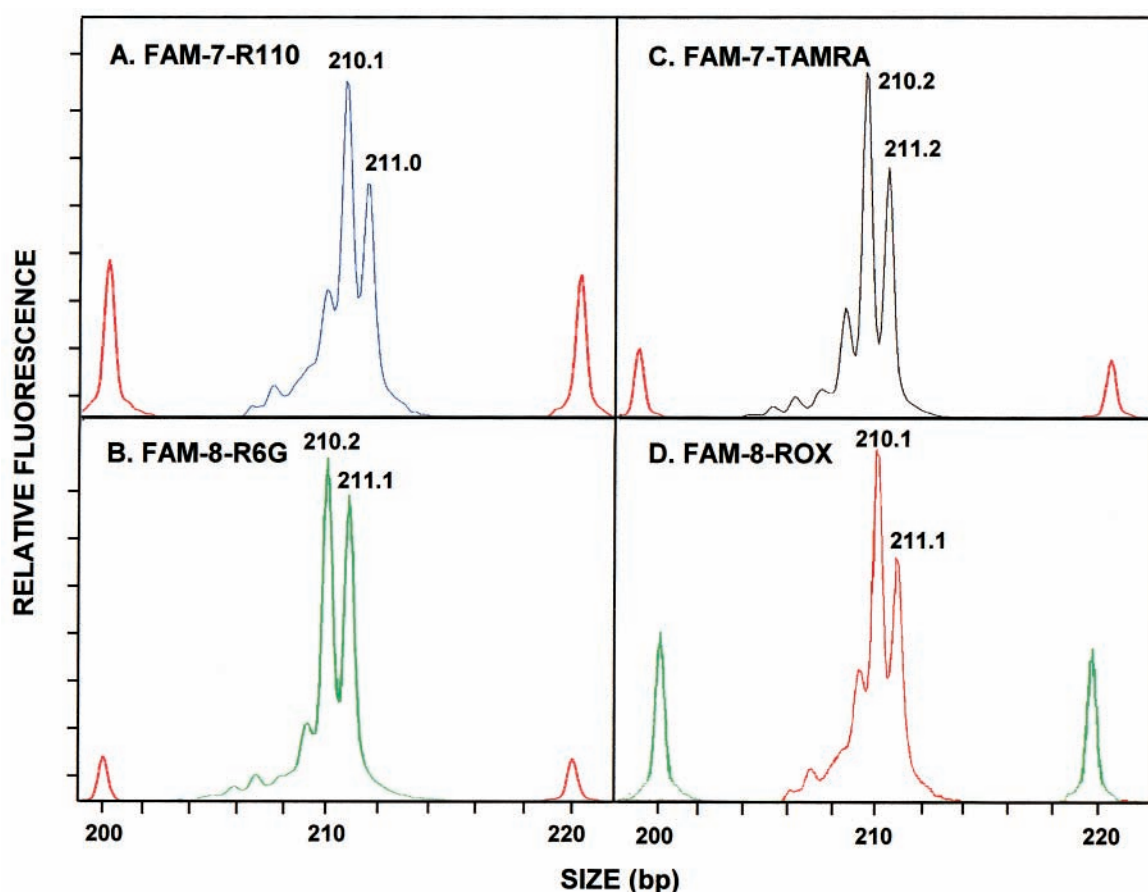


Figure 4. Genotyping of the 9.3/10 allele containing sample with all 4 ET cassette-labeled THO1 primers. The small peak just to the left of the first allele represents a PCR stutter artifact common in STR DNA typing of these loci (Medintz et al., 2000a; Promega, 1998). The red (ET-400-R) or green (TET Mapmarker) peaks at 200 and 220 bp are generated from the sizing standard. Cassettes used for labeling are indicated and the determined sizes are presented above the peaks.

cassette labels. Primers destined for ET cassette labeling can be obtained commercially with a 5' protected thiol modifier; the primer can be deprotected in one step and stored for subsequent tagging. This ET cassette labeling technology is easily adapted to 96 well parallel robotic preparation formats, and both the primer deprotection

and ET cassette labeling reactions can be performed with a PCR thermal cycler. Due to the high sensitivity obtained when using ET cassette labels, only small amounts of labeled amplicons are needed for actual analysis. This means that primer concentrations and reaction volumes can be substantially reduced.

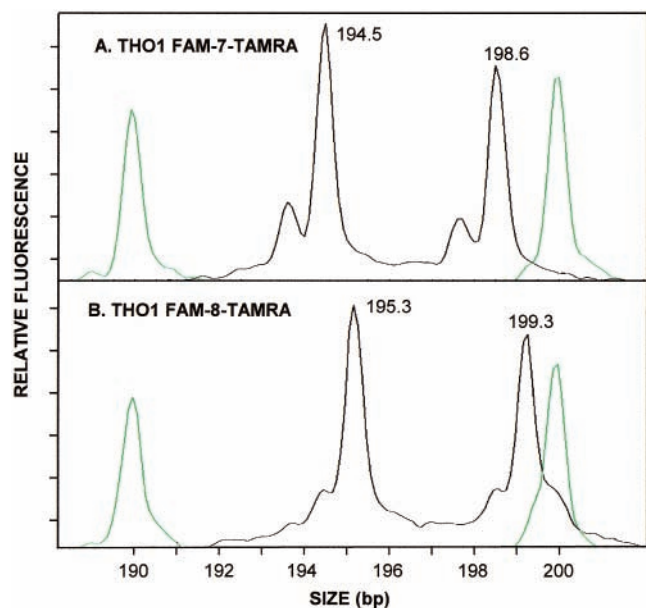


Figure 5. Effect of S-7 and S-8 sugar phosphate spacers on the mobility shift. The CEPH sample was genotyped at the THO1 locus with primers that had been labeled with either the FAM-7-TAM (A) or the FAM-8-TAM (B) cassettes. Alleles appear in black against the TET sizing markers in green at 190 and 200 bp.

To demonstrate multiplex analysis we evaluated the amplicons generated from ET cassette-labeled primers on a MegaBACE-1000 CAE system. All primers were labeled in all four colors and alleles from K562 and the CEPH control DNA were successfully generated by PCR and analyzed for all seven primers. The addition of the ET cassettes to these primers was expected to increase the sizes of amplicons by approximately 8–10 bp. The results presented in Table 2 show that the actual average fragment size for each of the seven loci increases by 12.0 to 18.1 bp. However, the standard deviation between each of the four cassette labels for each primer is very small (0.29 to 0.85 bp). This indicates that the addition of any one of the ET cassettes to a primer will result in a very small sizing difference as compared to another ET cassette for that same primer. The effect of the ET cassette on predicted versus determined fragment sizes can be ascribed to the increased mass and bulk of the cassette, the difference in charge, as well as conformational influences. The fact that the TET Mapmarker has a single fluorescent label directly attached to each DNA strand as well as the noncassette structure of the ET-400-R ladder also have to be taken into account. These structural differences will influence electrophoretic migration rates and cause relative mobility differences. Additionally, the sizing markers are all present as single-stranded DNA fragments while the amplicon sample consists of both the labeled and unlabeled complementary DNA strand. Although the samples are denatured prior to analysis, this may still influence electrophoretic migration. However, these mobility shift effects can be easily corrected by labeling all the fragments in the separation with ET cassettes or through the use of mobility shift correction software.

The 4 bp incremental size difference between STR alleles is strictly maintained in this ET format (see Table 2 and Figures 2–5). Furthermore labeling either the forward or reverse primer does not alter sizing differences as evidenced by the 0.2 bp difference in amplicon size at the CSF1PO locus (Figure 2). This small difference is achieved even though the analyzed strands are distinct

in sequence. Finally, primers labeled in this manner are functional for multiplex coamplification and analysis. This is demonstrated through a triplex amplification and four-color genotyping analysis (Figure 3). Moreover, single bp resolution is easily achievable when using these primers (Figure 4).

An added benefit of this labeling format is the flexibility to choose ET cassettes with different numbers of dideoxyribose monomer spacers. This becomes important when analysis requires differentiating amplicons that migrate very close to one another (Hung et al., 1997). By utilizing cassettes with varying spacers to label different primers, coeluting amplicons can be forced to migrate separately. Adjustment of cassette monomer spacing will allow adjustment in the differences between predicted vs electrophoretic size of fragments. Additionally, varying the number of spacers in an ET pair can substantially increase or decrease the relative fluorescent emission of the acceptor dye (Hung et al., 1998). For instance, cassettes can be constructed that have very high acceptor dye emissions, ideal for sequencing, or very low donor spectral cross-talk, ideal for multiplex fragment sizing/genotyping analysis.

ET cassette primer labeling is not limited to STR analysis. This labeling format can be used in any other assay where fluorescent tagging of analyte is required. This includes microarray hybridization formats, allele specific amplification, as well as protein and cell labeling (Meldrum, 2000; Medintz et al., 2000b, 2001). Additionally, the ability to label any sequencing primer in a four-color ET cassette format will facilitate custom sequencing methods and plasmid vector formats beyond those containing the M13 sequence.

In summary, these experiments have successfully demonstrated the wide applicability of our ET cassette labeling method and the ease with which the ET cassette labeling technology can be used for fluorescent tagging of any DNA oligonucleotide target. This ET cassette labeling technology will increase, expand, and diversify fluorescent DNA analysis.

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Triple Helix-Forming Oligonucleotides Conjugated to Indolocarbazole Poisons Direct Topoisomerase I-Mediated DNA Cleavage to a Specific Site

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Topoisomerase I is an ubiquitous DNA-cleaving enzyme and an important therapeutic target in cancer chemotherapy for camptothecins as well as for indolocarbazole antibiotics such as rebeccamycin. To achieve a sequence-specific cleavage of DNA by topoisomerase I, a triple helix-forming oligonucleotide was covalently linked to indolocarbazole-type topoisomerase I poisons. The three indolocarbazole-oligonucleotide conjugates investigated were able to direct topoisomerase I cleavage at a specific site based upon sequence recognition by triplex formation. The efficacy of topoisomerase I-mediated DNA cleavage depends markedly on the intrinsic potency of the drug. We show that DNA cleavage depends also upon the length of the linker arm between the triplex-forming oligonucleotide and the drug. Based on a known structure of the DNA-topoisomerase I complex, a molecular model of the oligonucleotide conjugates bound to the DNA-topoisomerase I complex was elaborated to facilitate the design of a potent topoisomerase I inhibitor-oligonucleotide conjugate with an optimized linker between the two moieties. The resulting oligonucleotide-indolocarbazole conjugate at 10 nM induced cleavage at the triple helix site 2-fold more efficiently than 5 μ M of free indolocarbazole, while the other drug-sensitive sites were not cleaved. The rational design of drug-oligonucleotide conjugates carrying a DNA topoisomerase poison may be exploited to improve the efficacy and selectivity of chemotherapeutic cancer treatments by targeting specific genes and reducing drug toxicity.

INTRODUCTION

Sequence-specific modulation of gene expression can be achieved by oligonucleotides that bind to double-helical DNA via triplex formation (1–5). These triple-helical DNA structures offer new prospects to modulate the interactions between proteins and DNA. Cleaving or cross-linking agents have been tethered to triple helix-forming oligonucleotides [TFOs] in order to target chemical reactions to specific sequences on DNA (1, 2, 6–12). An attractive option for biological applications would be the recruitment of a cellular enzyme whose action on DNA would be directed by triplex formation to perform a sequence-specific DNA cleavage. One such enzyme is topoisomerase I, which is an ubiquitous nuclear enzyme involved in the control of DNA topology (13).

The reaction of topoisomerase I with double-stranded DNA produces a covalent 3'-phosphotyrosyl adduct, usually referred to as the cleavage complex. Under physiological conditions, the covalent intermediate is barely detectable, because a fast religation step occurs after relaxation of DNA constraints. A number of drugs, such as the antitumor alkaloid camptothecin [CPT], can convert topoisomerase I into a cell poison by blocking the

relication step, thereby enhancing the formation of persistent DNA breaks responsible for cell death (13, 14). Apart from the camptothecins, several classes of antineoplastic drugs have been shown to stabilize DNA-topoisomerase I complexes. One of the most promising series of antitumor agents targeting topoisomerase I is represented by indolocarbazole analogues of the antibiotic rebeccamycin [RBC], which strongly stimulate topoisomerase I-mediated DNA cleavage (15–17). In contrast to the camptothecins, RBC and analogues intercalate in DNA even in the absence of topoisomerase I. Structure-activity relationship studies have provided key information concerning the stabilization of DNA-topoisomerase I covalent complexes by indolocarbazoles (15, 18–21).

These considerations prompted us to attach a rebeccamycin derivative to a TFO in order to recruit topoisomerase I and direct its cleavage to specific DNA sequences. Upon intercalation into DNA, the attached indolocarbazole chromophore can stabilize the triple-helical structure and, in addition, can direct the topoisomerase I enzymatic reaction to particular sites at proximity to the triple helix binding site (22, 23). In a previous study, Matteucci and co-workers (24) have shown that a triplex-forming oligonucleotide conjugated to camptothecin is able to direct topoisomerase I-mediated DNA cleavage to a specific site. In this study, three indolocarbazole derivatives, R6, R0, and R95 (see Figure 1 for chemical structures) were covalently attached to the 3'-end of a 16-nt triplex-forming oligonucleotide and their potencies for topoisomerase I recruitment compared. The

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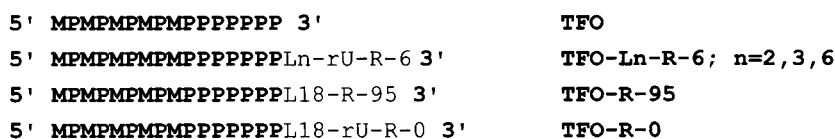
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A

Target duplex:

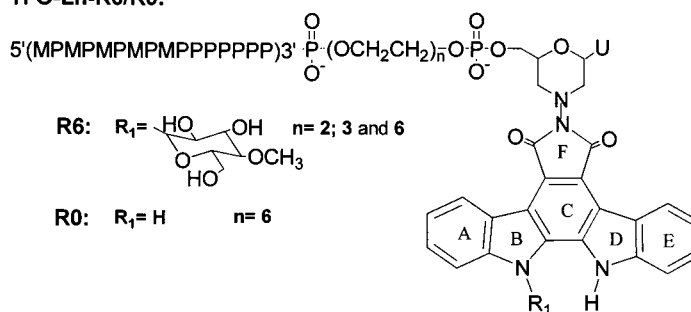


Oligonucleotides:



B

TFO-Ln-R6/R0:



TFO-R95:

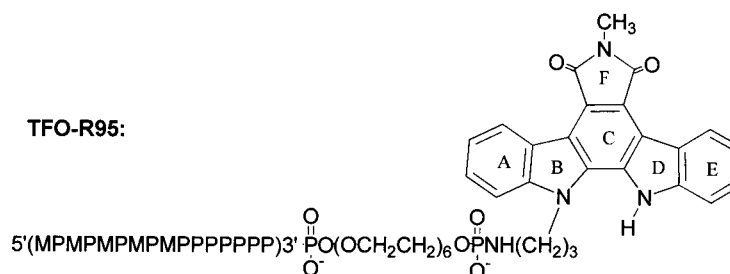


Figure 1. (A) Sequence of the TFOs and the 77-bp target used in this study. The TFO binds in the major groove to the oligopurine strand of the duplex and parallel to it. The target site is in bold and underlined. The 77-bp duplex target sequence was inserted between the *Bam*H I and *Eco*R I sites of pBSK(±). M = 5-methyl-2'-deoxycytidine, P = 5-propynyl-2'-deoxyuridine, rU = uridine, L6 = hexaethylene glycol, L3 = triethylene glycol, L2 = diethylene glycol. (B) Structure of the indolocarbazole-TFO derivatives used in this study. U = uracil. The TFO-R6/TFO-R0 was synthesized with the spacer linked at one end to the 3'-phosphate of the TFO and at the other end to a uridylic acid. The glycol function of the ribose was oxidized by NaIO_4 and reacted with the NH_2 group linked to the maleimide function of R6 or R0. For the synthesis of the TFO-R95 conjugate, the hexaethylene glycol linker at the 3'-end of the TFO was phosphorylated and after activation by *N*-methylimidazole, dipyridyl disulfide, and triphenylphosphine, it was reacted with the NH_2 -terminal group of the 1-aminopropyl side chain of the B cycle of R95 (see reference (22) for details).

compounds differ by the nature of their functional groups, which allowed us to use different types of linkage to tether them to the TFO.¹ These different conjugates were used to further investigate the parameters involved in the interaction between topoisomerase I and the triple-helical structure in order to rationally design topoisomerase I poison-oligonucleotide conjugates that efficiently cleave DNA at specific sites.

¹ Abbreviations: TFO = triplex-forming oligonucleotide, bp = base pair, nt = nucleotide, • = Watson-Crick base pairing, x = Hoogsteen or reverse Hoogsteen base pairing, ssDNA = single-stranded DNA, dsDNA = double-stranded DNA, PAGE = polyacrylamide gel electrophoresis, RBC = rebeccamycin, CPT = camptothecin, DNase I = deoxyribonuclease I, K_d = dissociation constant defined as: $K_d = ([\text{Duplex}][\text{Oligonucleotide}])/[\text{Triplex}]$, M = 5-methyl-2'-deoxycytidine, P = 5-propynyl-2'-deoxyuridine, rU = uridine, L2 = diethylene glycol, L3 = triethylene glycol, L6 = hexaethylene glycol.

EXPERIMENTAL PROCEDURES

Drugs, Oligonucleotides, and DNA Fragment. The synthesis of the indolocarbazoles R-6, R-0, and R-95 have been previously described (18, 22, 25). All the drugs were dissolved in dimethyl sulfoxide at 3 mg/mL and then diluted further with water. The final dimethyl sulfoxide concentration never exceeded 0.3% (v/v) in all assays. They were attached to the 3'-end of the TFO as described in Figure 1.

Oligonucleotides were purchased from Eurogentec and purified using quick spin columns and sephadex G-25 fine (Boehringer, Mannheim). Concentrations were determined spectrophotometrically at 25 °C using molar extinction coefficients at 260 nm calculated from a nearest-neighbor model (26).

The rebeccamycin derivatives-oligonucleotide conjugates were synthesized and purified as reported in (22) and their structure is shown in Figure 1. The NH_2 group

of the aliphatic chain of R-95 was covalently linked to the phosphorylated hexaethylene glycol linker arm at the 3' end of TFO (Figure 1B) [22]. The 3' phosphorylated oligonucleotide (150 μ g) was first precipitated as a hexadecyltrimethylammonium salt and the oligonucleotide salt was then dissolved in 50 μ L of dry DMSO. A 5 μ L sample of *N*-methylimidazole and 25 μ L each of dipyridyl disulfide and triphenylphosphine solutions [1.2 M DMSO] were added. After 15 min incubation at room temperature, 5 μ L of triethylamine was added followed by the drug solution [20 μ L, 30 mM in DMSO]. After 20 min, the oligonucleotide was precipitated with LiClO₄. Reverse phase HPLC using a linear acetonitrile gradient (5–80% CH₃CN in 0.2 M (NH₄)OAc) was used to separate the product from the initial oligonucleotide. A Lichrosorb C-18 column (4.6 $\times$ 25 cm) and Shimadzu CR4-A Chromopack instrument were used (average yield 60%). The product was eluted as a single peak absorbing both at 310 nm (oligonucleotide) and 410 nm (rebeccamycin) with a retention time of 11.4 min compared to 10.4 min for initial TFO.

To covalently link R6 or R0 to the 3'-end of the oligonucleotide, we used the 16-mer TFO bearing at the 3'-end an uridine linked through an poly(ethylene glycol) linker arm [22]. 300 nmoles of oligonucleotide (ethanol precipitated), and 6.5 mg of sodium periodate (NaIO₄) were dissolved in 100 μ L of 200 mM sodium acetate, pH 5.2. After 20 min incubation at room temperature and in the dark, 100 μ L of 2 M potassium chloride was added in order to precipitate the excess of periodate. The pellet was washed several times with 50 μ L of H₂O. All water phases were collected, and the oligonucleotide was precipitated with ethanol. It was then dissolved in 50 μ L of 200 mM NaOAc, pH 5.2 and added to 1 mg of the indolocarbazole compound R6 or R0 dissolved in 50 μ L of DMF. The solution was incubated for 12 h at 4 $^{\circ}$ C in the dark. After ethanol precipitation, the oligonucleotide was dissolved in water and treated with 0.5 mg of sodium borohydrate and again precipitated with ethanol. The conjugates were recovered by reverse phase HPLC with a Lichrosorb C-18 column (4.6 $\times$ 25 cm) using a linear acetonitrile gradient (5–40% CH₃CN in 0.2 M (NH₄)OAc) and a Shimadzu CR4-A Chromopack instrument (average yield 40%). The product was eluted as a single peak absorbing both at 310 nm (oligonucleotide) and 410 nm (rebeccamycin). Retention times: TFO-L2-R6, 13.1 min vs 11.1 min; TFO-L3-R6, 13.0 min vs 11.2 min; TFO-L6-R6, 13.6 min vs 11.7 min.; TFO-L6-R0, 13.0 min vs 11.7 min for the drug-free oligonucleotide.

All conjugates were analyzed by UV spectroscopy and gel electrophoresis under denaturing conditions. In each case, the ratio of $A_{310\text{ nm}}/A_{410\text{ nm}}$ from the UV-vis absorbance spectrum corresponded to a 1:1 oligonucleotide:rebeccamycin complex. On a 20% denaturing gel electrophoresis only one band migrating slower than the one of the initial TFO was observed. The band revealed blue fluorescence as the free rebeccamycin derivative.

The plasmid pBSK($\pm$) was bought from Promega, and the 77-bp target duplex was inserted between the *Bam* HI and *Eco* RI sites. The digestion of the plasmid by *Pvu*II and *Eco* RI yielded a 324-mer fragment suitable for 3'-end labeling by the Klenow polymerase and α [³²P]-ddATP (Amersham). The detailed procedures for isolation, purification and labeling of this duplex DNA fragment have been described previously (27).

Topoisomerase I Cleavage Assays. The radiolabeled 324-bp target duplex (50 nM) was incubated for 1 h at 30 $^{\circ}$ C, in 50 mM Tris-HCl, pH 7.5, 60 mM KCl, 10 mM

MgCl₂, 0.5 mM DTT, 0.1 mM EDTA, and 30 μ g of BSA, in the presence of the TFO (at the indicated concentration) in order to form the triplex (total reaction volume 20 μ L). To analyze the topoisomerase I DNA cleavage products, 10 units of enzyme (Life Sciences) were added to the duplex, preincubated as described above with either the TFO or/and the drugs, followed by incubation for 30 min at 30 $^{\circ}$ C. DNA-topoisomerase I cleavage complexes were dissociated by addition of SDS (final concentration 0.25%) and of proteinase K (Sigma) to 250 μ g/mL, followed by incubation for 30 min at 55 $^{\circ}$ C. After ethanol precipitation, all samples were resuspended in 6 μ L of formamide, heated at 90 $^{\circ}$ C for 4 min, and then chilled on ice for 4 min, before being loaded onto a denaturing 8% polyacrylamide gel [19:1 acrylamide:bisacrylamide] containing 7.5 M urea in 1xTBE buffer (50 mM Tris base, 55 mM boric acid, 1 mM EDTA). To quantitate the extent of cleavage, the gels were scanned with a Molecular Dynamics 445SI Phosphorimager. For the determination of cleavage levels, a normalization relative to total loading was performed.

RESULTS AND DISCUSSION

Prior to investigating the effects of triplex formation with oligonucleotides conjugated to topoisomerase I poisons on DNA cleavage by topoisomerase I, the ability of the conjugates to form the triple helix was determined.

Triple Helix Formation by TFO-RBC Conjugates. As previously described (22), modified 16-nt TFO, containing 5-propynyl-2'-deoxyuracils [P] and 5-methyl-2'-deoxycytosines [M] (Figure 1), can form a stable triple helix on the target sequence (Figure 1, bold and underlined), at 37 $^{\circ}$ C and at pH 7.2 in a buffer containing 50 mM KCl, 10 mM MgCl₂. We covalently attached the 3'-end of the oligonucleotide to the indolocarbazole derivatives R6, R0, and R95 as described in Figure 1B. Triplex formation involves major groove binding of the conjugates via recognition of the oligopurine strand of the duplex (28). The binding should allow the planar indolocarbazole ring to intercalate at the duplex/triplex junction as previously described for other intercalating agents (29, 30).

Initially the RBC derivatives were attached to the TFO by a hexaethylene glycol linker (L6). The choice for such a long linker chain was based on previously reported data suggesting that topoisomerase I protected both strands of DNA over a 15–19-bp region in which the cleavage site was centrally located (31). This suggested that the enzyme might need an accessible 7–9-bp double helical region around the cleavage site, and therefore we reasoned that a relatively long linker (L6) should be used to allow the drug to get access to the catalytic site of topoisomerase I and to prevent steric hindrance between the TFO and the enzyme. But as shown below, this linker can be further shortened to improve topoisomerase I-mediated cleavage on the basis of the crystal structure of topoisomerase I. The indolocarbazole drugs, R6, R0, and R95, bear different functional groups used to tether the indolocarbazole chromophore to the TFO. R0 and its glycosylated analogue R6 were attached through reaction of the NH₂ group on the nitrogen of the maleimide function with the ribose of an uridine attached at the 3'-end of the oligonucleotide through a linker arm (Figure 1). In contrast, the R95 derivative was linked to a phosphate attached to the hexaethyleneglycol linked to the 3'-end of TFO via the NH₂ group of the 1-aminopropyl side chain attached to the B cycle of the drug.

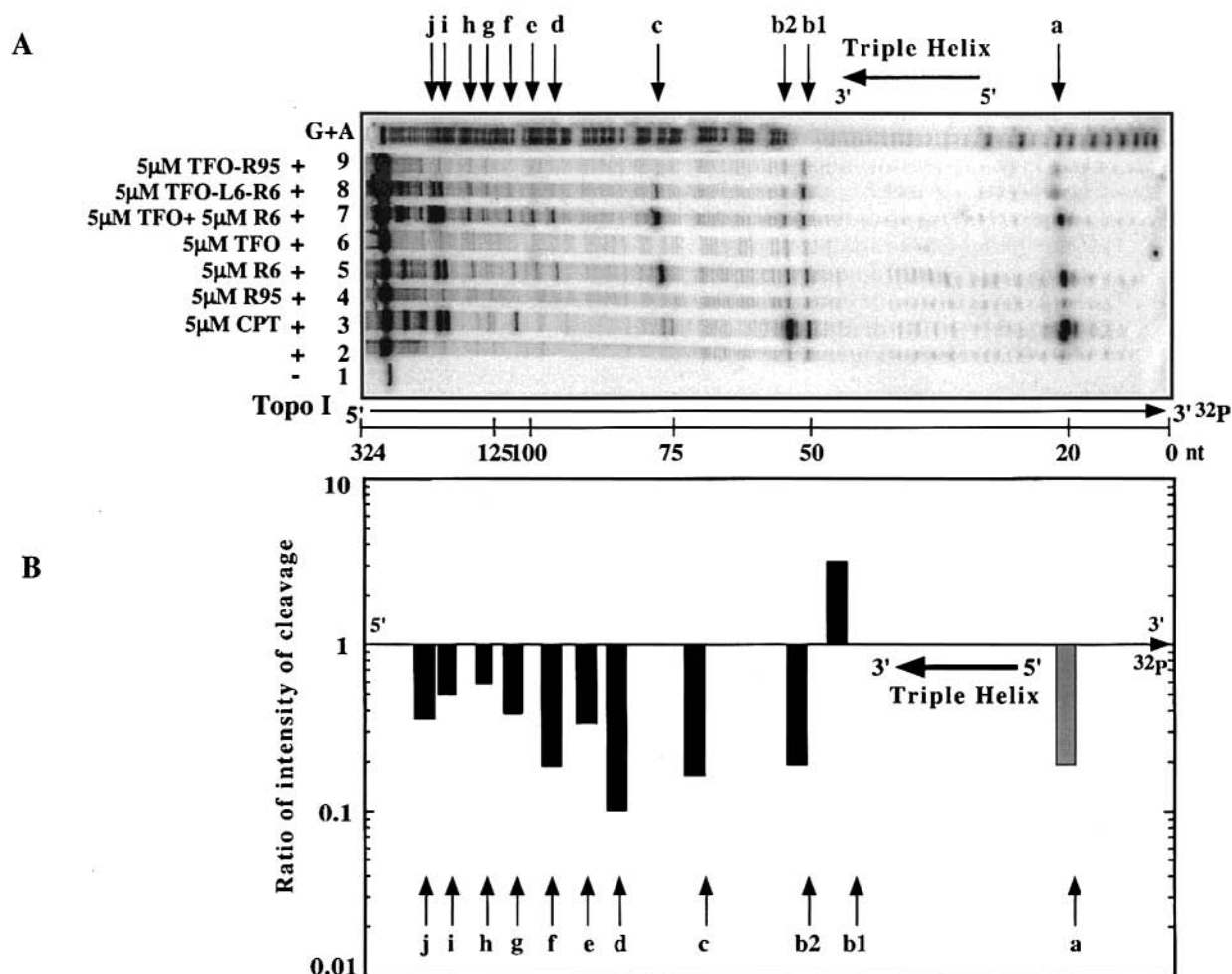


Figure 2. (A) Sequence analysis of the topoisomerase I-mediated cleavage products on the 324-bp target duplex (50 nM) 3'-end radiolabeled on the oligopyrimidine-containing strand. Adenine/guanine-specific Maxam–Gilbert chemical cleavage reactions were used as markers. The positions of the cleavage sites are indicated (sites *a* to *j*). Lane 1, target duplex; duplex incubated with topoisomerase I (10 units) in the absence (lane 2) or in the presence of 5 μ M camptothecin (lane 3), 5 μ M R95 (lane 4), 5 μ M R6 (lane 5), 5 μ M TFO (lane 6), 5 μ M TFO and R6 (lane 7), 5 μ M TFO-L6-R6 (lane 8), and 5 μ M TFO-R95 (lane 9). The triple helix region and the nucleotide positions along the radiolabeled oligopyrimidine strand are indicated. (B) The ratio between the intensity of cleavage in the presence of TFO-L6-R6 and the intensity in the presence of the untethered R6 is represented on a logarithmic scale at different sites along the DNA fragment used as a substrate for topoisomerase I cleavage (data from lanes 8 and 5 of Figure 2A).

The binding of the conjugated third strands to the duplex was analyzed by gel retardation experiments, and the affinity of the TFOs for the duplex was measured. The following dissociation constants (K_d) were determined (as described in (22)): TFO: $5.3 \pm 0.5 \mu\text{M}$; TFO-R6: $0.20 \pm 0.06 \mu\text{M}$; TFO-R0: $0.50 \pm 0.1 \mu\text{M}$; TFO-R95: $0.85 \pm 0.07 \mu\text{M}$. Only the RBC-conjugates were studied, because as intercalating agents the RBC derivatives could greatly modify the stability of the triplex. Indeed, all conjugates bind more strongly to the duplex than the corresponding drug-free oligonucleotide. These results showed that the TFO–drug conjugates formed stable triple helices in the absence of topoisomerase I. Of the three conjugates, only TFO–R6 and TFO–R0 are comparable, because they share the same type of attachment point to the TFO. A 2-fold higher binding affinity was observed with the R6 conjugate, presumably due to the presence of the β -glucose residue attached to the indole nitrogen on ring B, which has been previously shown to stabilize drug–DNA complexes in the case of the free drugs (19).

Topoisomerase I-Mediated DNA Cleavage in the Presence of the Triple Helix. The 3'-end-radiolabeled restriction fragment (324-bp) bearing the 16-bp triple helix target site was incubated in the presence of TFOs and topoisomerase I. After protease treatment, DNA

cleavage products were analyzed by denaturing polyacrylamide gel electrophoresis. Figure 2 shows the results obtained when using the duplex target labeled at the 3'-end of the oligopyrimidine-containing strand. We compared the different topoisomerase I poisons, R6, R95, and R0, and their TFO conjugates.

Lane 2 shows the cleavage pattern of topoisomerase I alone in the absence of TFO and topoisomerase I poisons. Two sites are present near the 3' triplex/duplex junction (sites *b*) and one near the 5' junction (site *a*). There are also several other sites at some distance from the triplex binding site. The indolocarbazoles R95 (lane 4) and R0 (data not shown) are weak topoisomerase I poisons and enhance only slightly topoisomerase I-mediated cleavage (15, 19). In the presence of R6 (lane 5), sites *b1* and *b2*, located at 4-bp and 7-bp from the 3' triplex end, respectively, are strongly enhanced, like that of site *a* on the 5'-side of the triplex at 8-bp from the 5' triplex end. Additional sites were also detected in the oligopyrimidine–oligopurine region and in the regions marked *c*, *d*, *e*, *f*, *g*, *h*, *i*, and *j* in Figure 2. Sequence analysis showed that most of these cleavage sites correspond to T $\downarrow$ G sites, in agreement with previous findings showing that RBC derivatives stimulate DNA cleavage by topoisomerase I

preferentially at sites having a T and a G on the 3'- and 5'-sides of the cleaved bond, respectively (15).

The topoisomerase I cleavage profile changed only slightly in the presence of the triplex formed with the untethered oligonucleotide TFO (lane 6 compared to lane 2). The formation of the triple helix protected the duplex against enzymatic cleavage in this region. Site *a* at 8-bp from the 5' duplex/triplex junction and sites *b* at the 3'-triplex/duplex junction were still present. The addition of R6 to the triplex (lane 7) restored cleavage at sites *a* and *b*. Again there were no cleavage sites in the triple helix region. It is interesting to observe that the presence of the triplex enhanced the cleavage at the site situated at 4-bp from the 3' triplex end (site *b1*) compared to cleavage at the *b2* site. This could be due to an increase of local drug concentration at this site as a consequence of its intercalation at the triplex/duplex junction. The very strong site characteristic of R6 at 33-bp from the 3'-end of the triplex was still present (region *c*), as well as the other sites *d* to *j*.

Triplex formation with the TFO-L6-R6 conjugate (lane 8) diminished the cleavage at sites located far away from the triple helix (*c*, *d*, *e*, *f*, *g*, *h*, *i*, *k*) and at site *a* on the 5'-side of the TFO. Only site *b1*, at the 3'-end of the triple helix, was maintained and enhanced. This is consistent with a sequence-specific recruitment of topoisomerase I by triplex formation with TFO conjugates bearing topoisomerase I poisons at the 3' end. The TFO bearing the drug R6 at the 3'-end favors DNA cleavage by the enzyme only on the 3'-side of the triplex and decreases the other characteristic sites of the drug: this is clearly due to the targeting of the drug by triplex formation specifically to the *b1* site at the 3'-end of the triple helix. In fact, the triple helix formed by the uncoupled oligonucleotide stimulates cleavage equally at the three sites adjacent to the triplex (lane 6, sites *a*, *b1*, and *b2*) and upon addition of the free drug, all the characteristic cleavage sites of the drug are present (lane 7, sites *a*, *b*, *c*, *d*, *e*, *f*, *g*, *h*, *i*, and *j*). In Figure 2B is shown the ratio of the intensities measured with the conjugate TFO-L6-R6 and the free drug (R6) at 5 μ M. Only site *b1* shows an enhanced cleavage whereas all other sites reveal a decrease in cleavage efficiency, demonstrating that the drug-TFO conjugate is able to target the topoisomerase I poison specifically near the triplex site and to increase the efficiency of cleavage of the agent at this site. The enhancement observed at site *b1* was 3-fold for the TFO-L6-R6 as compared to free R6. It was only 2.6-fold when compared to the TFO+R6 mixture at 5 μ M concentration. The decrease at site *b2* was 5- and 2-fold, respectively.

The TFO-R95 conjugate was much less potent at inhibiting topoisomerase I than its congener TFO-L6-R6 (compare lanes 9 and 8, Figure 2A). Nevertheless, cleavage at the expected site *b1* remained visible and enhanced. The cleavage at site *a* on the 5' end of the triple helix and at site *b2* located 7-bp away from the 3' end of the triplex was less efficient compared to the free R95 (lane 4). Again this is consistent with a triple helix-induced effect. As for the TFO-R95 conjugate, in the presence of the triplex formed by the TFO-R0 conjugate, cleavage was maintained only at site *b1* (data not shown).

Therefore, TFOs bearing topoisomerase I poisons at the 3'-end favor DNA cleavage by the enzyme only on the 3'-side of the triplex and decreases the other characteristic sites of the free drug. This is clearly due to the targeting of the poison by triplex formation specifically to site *b1* at 4-bp from the 3'-end of the triple helix.

The lower efficacy of TFO-R95 and TFO-R0 compared to TFO-R6 is entirely consistent with the data presented

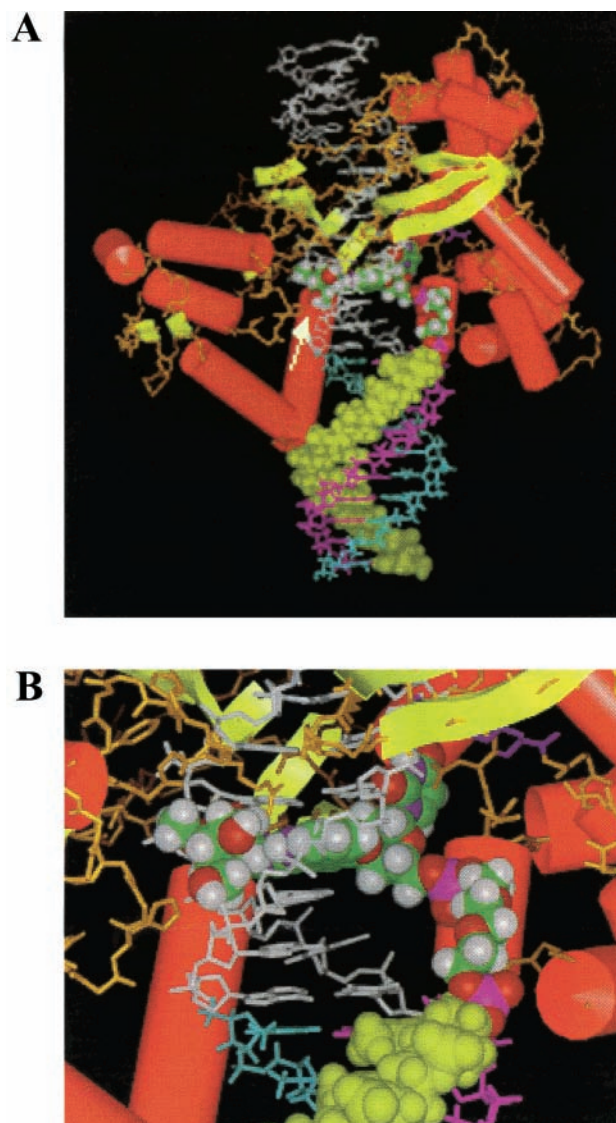


Figure 3. Molecular model for the complex formed between the TFO-L3-R6 conjugate and the topoisomerase I bound to DNA. The atomic coordinates of the topoisomerase I/DNA complex published by Rebinbo et al. (32) were used to construct this model. The α -helix, β -sheets, and the backbone are colored in red, yellow, and brown, respectively. The duplex region of the DNA is represented in white; the double helix site involved in triple helix formation is represented in pink (oligopurine strand) and in light blue (oligopyrimidine strand). The third strand moiety of the TFO-L3-R6 conjugate is in yellow. The extended linker chain (L3) positions the drug moiety at 4-bp from the triplex/duplex junction with the indolocarbazole chromophore intercalated in the double helix at the cleavage site. The glucose moiety of the drug is located in the minor groove, in a suitable position to interact with the Arg364 residue of topoisomerase I. Closer view of the model showing the drug binding pocket around the topoisomerase cleavage site.

above indicating that R95 and R0 are much less efficient than R6 both in terms of triplex stabilization and stimulation of topoisomerase I cleavage. As mentioned above, TFO-R95 not only lacks the essential glucose residue but the F-ring on the indolocarbazole chromophore is oriented toward the minor groove, which is an unfavorable orientation for optimal topoisomerase I inhibition. The F ring is presumed to interfere with the DNA-enzyme complex from the major groove (19). This might explain why the cleavage efficiency of the R95 conjugate is poor. The cleavage profile obtained with R0 was similar to that found with R95. The absence of the sugar moiety is presumably the most important element

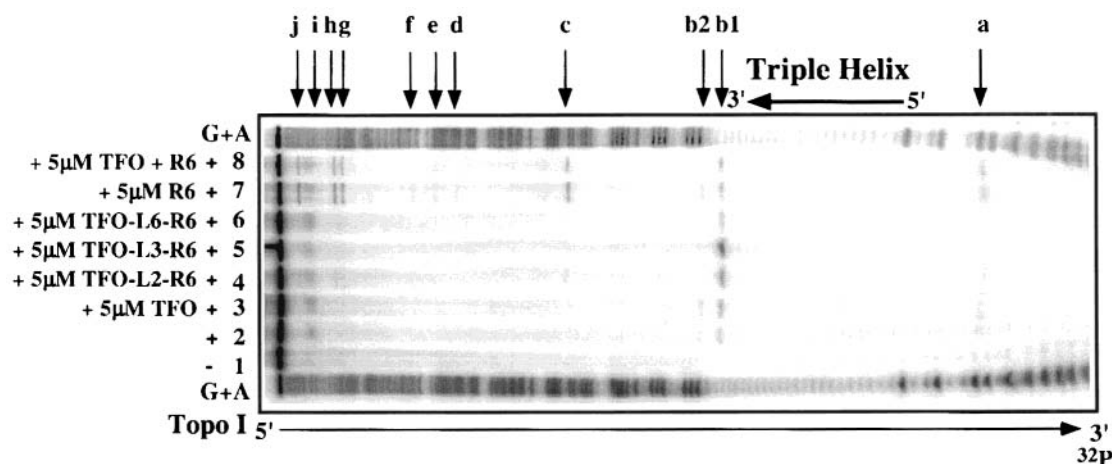


Figure 4. Sequence analysis of topoisomerase I-mediated cleavage products with R6 conjugates containing linker arms of different length. Adenine/guanine-specific Maxam–Gilbert chemical cleavage reactions were used as markers. The positions of the cleavage sites are indicated (sites *a* to *j*). Lane 1, target duplex; duplex incubated with topoisomerase I (10 units) in the absence (lane 2) or in the presence of 5 μ M TFO (lane 3), 5 μ M TFO-L2-R6 (lane 4), 5 μ M TFO-L3-R6 (lane 5), 5 μ M TFO-L6-R6 (lane 6), 5 μ M R6 (lane 7), and 5 μ M TFO and 5 μ M R6 (lane 8). The triple helix region and the nucleotide positions along the radiolabeled oligopyrimidine strand are indicated. The band with an asterisk corresponds to a labeling artifact and was used as loading control.

responsible for the weak effect on topoisomerase I because the results obtained with the conjugate TFO–R0 were comparable to those obtained with TFO–R95. This corroborates the findings that the sugar moiety is important for both binding to DNA and poisoning of topoisomerase I (15, 19).

At this stage, the results suggest that topoisomerase I recruitment by a TFO coupled to a potent poison can be achieved with RBC derivatives, with efficiencies corresponding to the potency of the free drug. Furthermore, we have shown that the attachment point of the poison to the TFO orients the drug in the drug/topoisomerase I/DNA complex and thus changes the intensity of DNA cleavage. The conjugates could thus be used to study the orientation of the drug in the ternary complex, by measuring the efficiency of cleavage as a function of the attachment point of the drug to the TFO.

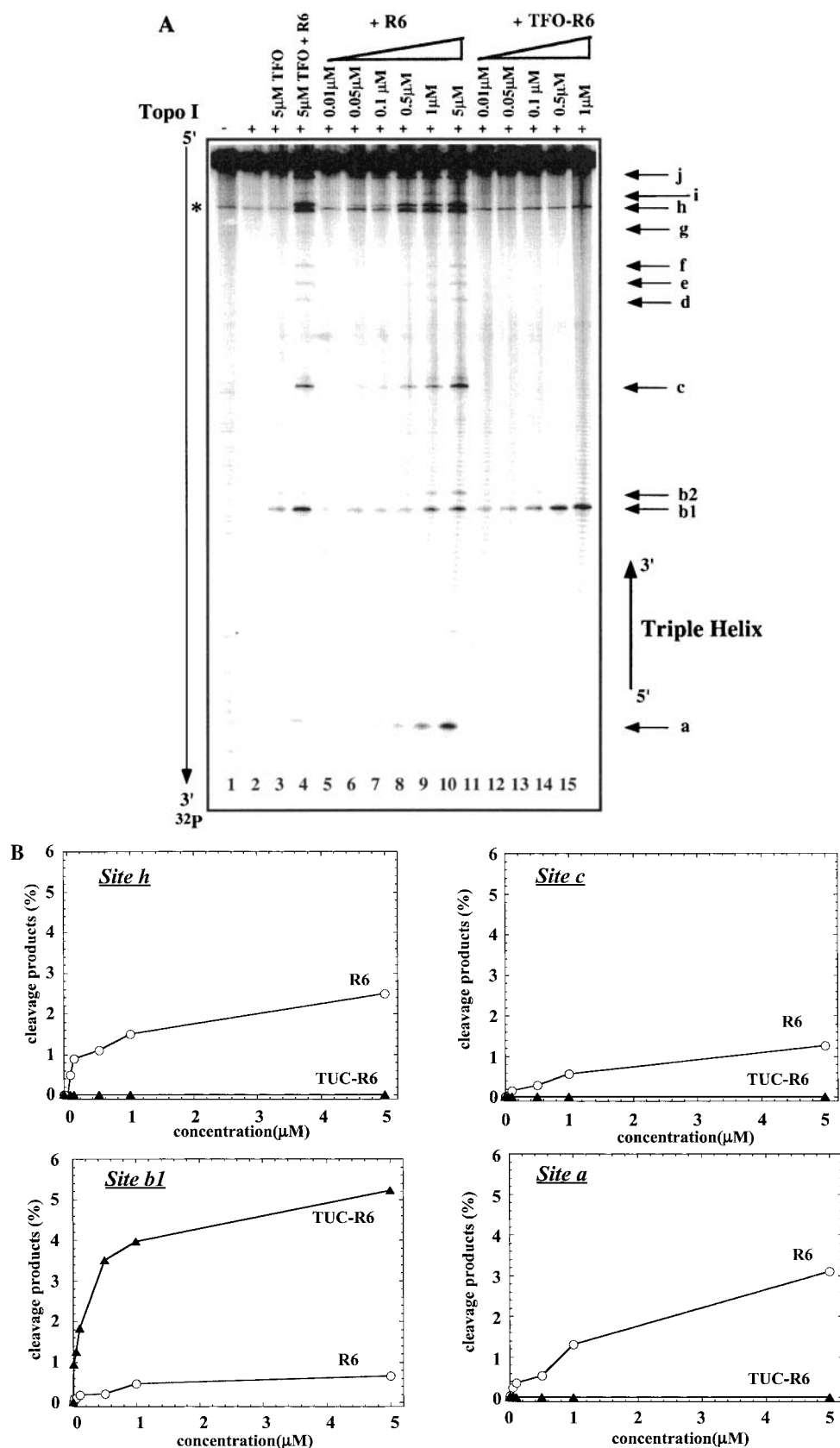
Design of an Optimized Linker for Oligonucleotide–Rebeccamycin Conjugates. The reported crystal structure of the complex formed between a 22-bp DNA duplex and a large fragment of human topoisomerase I (32) allowed us to build a molecular model where the drug moiety of the TFO–R6 conjugate was positioned in the catalytic site of the enzyme. According to this model, a triplex could be formed without steric clash with topoisomerase I at the 3′-side of the cleavage site, while the 5′-side of the cleavage site was not accessible (Figure 3). A pyrimidine motif triple helix was docked into the crystal structure at 4-bp from the cleavage site, called *b1*, identified in Figure 2A. The indolocarbazole derivative was attached through the linker arm to the 3′-end of the TFO and placed in the cleavage site. The model in Figure 3 shows the intercalated indolocarbazole derivative with its methoxy-glucose moiety attached to the B-ring of the chromophore. The sugar lies in the minor groove, in a suitable position to interact with amino acid Arg364, and to a lesser extent with the Asp533 residue of topoisomerase I. This model was used to investigate further the influence of the linker chain between the TFO and the drug. The modeling suggested that a triethylene glycol linker (L3) would be more appropriate than the hexaethylene glycol linker (L6) to position the drug at the *b1* cleavage site on the 3′ side of the triplex. Moreover, this shorter linker should permit major groove binding of the TFO 4-bp away from the cleavage site without steric interference with topoisomerase I.

To test this prediction, we synthesized the conjugates TFO-L3-R6 and the analogue TFO-L2-R6 bearing a diethylene glycol linker (Figure 1B). Both conjugates were tested in parallel with the TFO-L6-R6 conjugate initially constructed.

The 324-bp restriction fragment bearing the 16-bp triple helix target site was incubated in the presence of the conjugates and topoisomerase I. After protease treatment, DNA cleavage products were analyzed by denaturing polyacrylamide gel electrophoresis (Figure 4A). Lane 2 shows the cleavage pattern of topoisomerase I alone in the absence of TFO and drugs. As above-described, the presence of the triple helix formed by the untethered oligonucleotide (lane 3) has only a slight effect on the topoisomerase I cleavage pattern. In the presence of the R6 compound alone (lane 7), the two sites *b1* and *b2* were detected on the 3′ side of the triplex site, as well as site *a* on the opposite side of the triplex. Additional sites were observed in the regions marked *c* to *j*. The addition of the TFO (lane 8) did not affect the cleavage profile seen with the free drug. The TFO–[L2, L3, L6]–R6 conjugates (lanes 4–6) enhanced cleavage specifically at site *b1*. Cleavage at sites *a* at the 5′ end of the triple helix and *b2* located 7-bp away from the 3′ end of the triplex are strongly reduced compared to the free R6 compound (lane 7), as the other sites *c* to *j*. Again this is consistent with a triple helix-induced effect. In the R6 series, the 9 atoms linker (L3) produced a 4- and 2.6-fold stronger topoisomerase I-mediated DNA cleavage than TFO–L2–R6 and TFO–L6–R6, respectively, and 8-fold stronger than R6 alone. The TFO–R6 conjugate with the shorter linker arm enhanced cleavage at site *b1* about 8 times as compared to free R6 and 3 times as compared to TFO+R6 at 5 μ M concentration.

The experimental data fully agrees with the modeling predictions: the triethylene glycol is the most appropriate linker to allow positioning of the R6 compound at the same site in the complex. Collectively, the data demonstrate that the elaboration of a conjugate molecule requires an optimization of the two linked components but also a precise design of the linker chain.

Concentration Effect. Next we studied the efficacy of the TFO–drug conjugate compared to that of the free drug. We compared topoisomerase I-mediated DNA cleavage in the presence of increasing concentrations of R6 (from 0.01 μ M to 5 μ M, Figure 5A, lanes 5 to 10) and of



TFO-L3-R6 (from 0.01 μ M to 1 μ M, lanes 11 to 15). The conjugate induces efficient cleavage even at a concentration as low as 0.01 μ M (lane 11) compared to R6 alone (lane 5). Figure 5B compares the percentage of cleavage in the presence of R6 (red circles) and in the presence of TFO-L3-R6 (blue triangles) at sites *h*, *c*, *b1*, and *a* as a function of the concentration. The R6 conjugate not only strongly enhanced topoisomerase I cleavage at the triplex site *b1* but it is 10-fold more efficient at 10 nM than the poison alone at the same concentration. In contrast, at sites other than *b1* cleavage was abolished.

CONCLUSION

The designed TFO-indolocarbazole conjugates are able to direct the action of the topoisomerase I poison to a specific site in vitro. They induce sequence-specific DNA cleavage by topoisomerase I. Upon binding of the TFO-drug conjugate to its specific target, DNA cleavage is strongly enhanced at the triplex/duplex junction where the poison is positioned and strongly decreased at other sites. Three novel aspects were studied. First, the combined computational and experimental approach helped to identify the determinants, predominantly drug inhibitory potency and length of the linker arm, which shape the TFO-directed DNA cleavage mediated by topoisomerase I. Second, the comparison between the R0 and R6 conjugates provides important information on the role of the sugar residue attached to the indolocarbazole chromophore. Third, the positioning of the drug by triplex formation increases its local concentration at this site, and thus the conjugates induce cleavage in the nanomolar concentration range as compared to micromolar for the free drug.

In the future, triple helix-directed targeting of antitumor-active topoisomerase I poisons, such as camptothecin and indolocarbazole analogues, may be exploited further to improve the efficacy of chemotherapeutic cancer treatments by reducing drug toxicity and targeting topoisomerase I-induced cleavage to appropriately chosen genes. In addition to their utilization as therapeutic tools, TFO conjugates could be used as research tools: these conjugates provide a new approach for understanding the interactions involved in the topoisomerase I/drug/DNA ternary complex. In fact, with this approach, by varying the attachment sites and the functional groups as well as the nature and length of the linker, the drug can be located in different and controlled orientations with respect to topoisomerase and DNA.

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Paramagnetic Water-Soluble Metallofullerenes Having the Highest Relaxivity for MRI Contrast Agents

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Water-soluble gadolinium (Gd) endohedral metallofullerenes have been synthesized as polyhydroxyl forms ($\text{Gd@C}_{82}(\text{OH})_n$, Gd-fullerenols) and their paramagnetic properties were evaluated by in vivo as well as in vitro for the novel magnetic resonance imaging (MRI) contrast agents for next generation. The in vitro water proton relaxivity, R_1 (the effect on $1/T_1$), of Gd-fullerenols is significantly higher (20-folds) than that of the commercial MRI contrast agent, Magnevist (gadolinium–diethylenetriaminepentaacetic acid, Gd-DTPA) at 1.0 T close to the common field of clinical MRI. This unusually high proton relaxivity of Gd-fullerenols leads to the highest signal enhancement at extremely lower Gd concentration in MRI studies. The strong signal was confirmed in vivo MRI at lung, liver, spleen, and kidney of CDF1 mice after i.v. administration of Gd-fullerenols at a dose of 5 μmol Gd/kg, which was 1/20 of the typical clinical dose (100 μmol Gd/kg) of Gd-DTPA.

INTRODUCTION

With the advent of gadolinium-chelate complex for a magnetic resonance imaging (MRI)¹ contrast agent, Magnevist (Gd-DTPA) (1, 2), where the contrast agent can induce extra proton spin relaxation due to the paramagnetic property, many efforts have been given for the design and development of new compounds, such as Eovist (Gd-3,6,9-triaza-3,6,9-tris(carboxymethyl)-4-(4-ethoxybenzyl)undecandicarboxylic acid, Gd-EOB-DTPA), ProHance (Gd-1,4,7-tris(carboxymethyl)-10-(2'-hydroxypropyl)-1,4,7,10-tetraazacyclododecane, Gd-HP-DOTA) or Omnican (Gd-diethylenetriaminepentaacetate-bis(methylamide), Gd-DTPA-BMA) (3–5). These agents have been used as a routine clinical tool to reveal anatomical details in vivo and to detect lesions for diagnosis. For the future real-time in vivo imaging of molecular/cellular diagnosis, many efforts have been given by using the fluorescent (6, 7) and nuclear imaging techniques (8, 9) as well as MRI technique (10–13). Although fluorescent technique can have excellent temporal resolution, a common shortcoming is limited depth penetration. The nuclear techniques are free from this limitation but suffer from the inherently lower spatial resolution. In contrast, the MRI technique has much less

limitation on the observation depth and spatial resolution. As compared to fluorescent and nuclear imaging techniques, however, molecular probe detection by magnetic resonance is several orders of magnitude less sensitive (14, 15). It is necessary, therefore, to accumulate more targeting probes or enforce its potential enhancing ability for molecular/cellular diagnosis by MRI. Thus, novel materials having strong proton relaxivity and a higher capacity for MR signal enhancement at a significantly lower concentration are required for the next generation.

Here we have investigated the possibility of application of novel materials, water-soluble Gd metallofullerenes ($\text{Gd@C}_{82}(\text{OH})_n$, Gd-fullerenols) (16–20), for MRI contrast agents, and have evaluated them in vivo as well as in vitro. Extremely high water proton relaxivity, R_1 (the effect on $1/T_1$), was obtained with $\text{Gd@C}_{82}(\text{OH})_{40}$, 81 ($\text{mM}^{-1} \text{s}^{-1}$) at 1.0 T and 25 °C which is more than 20 times as high as that of the commercial Gd-DTPA. This unusually high proton relaxivity of $\text{Gd@C}_{82}(\text{OH})_{40}$ led to the highest signal enhancement at significantly lower Gd concentration by in vitro and in vivo MRI studies so far reported.

MATERIALS AND METHODS

Gd@C₈₂. Soot containing Gd endohedral metallofullerenes such as Gd@C_{82} was produced by the direct current arc evaporation method (350 A, 21 V) of Gd/graphite composite rods (0.8 wt % Gd atom, Toyo Tanso Co.) under a 17 l/min flow of helium at 50 Torr, followed by Soxhlet extraction with carbon disulfide for 12 h (21, 22). The purification and isolation of Gd@C_{82} was achieved by the two-stage high performance liquid chromatography (HPLC) (23) with a Buckyprep column (20 mm dia $\times$ 300 mm, Nacalai Tesque). The purity of Gd@C_{82} (<99%) was analyzed by both HPLC and matrix-assisted laser desorption/ionization (MALDI) TOF mass spectrometry (Shimadzu Kompact MALDI V).

Gd@C₈₂(OH)_n. The water-soluble Gd metallofullerene ($\text{Gd@C}_{82}(\text{OH})_n$, Gd-fullerenols) was synthesized as poly-

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¹ Abbreviations: MRI, magnetic resonance imaging; Gd, gadolinium; DTPA, diethylenetriaminepentaacetic acid; EOB-DTPA, 3,6,9-triaza-3,6,9-tris(carboxymethyl)-4-(4-ethoxybenzyl)undecandicarboxylic acid; HP-DOTA, 1,4,7-tris(carboxymethyl)-10-(2'-hydroxypropyl)-1,4,7,10-tetraazacyclododecane; DTPA-BMA, diethylenetriaminepentaacetate-bis(methylamide); TBAH, tetrabutylammonium hydroxide; ICP-AES, inductively coupled plasma atomic emission spectroscopy; RES, reticular-endothelial system.

hydroxyl forms by vigorously stirring a mixture of a toluene solution of isolated Gd@C_{82} (<99%, 0.1 g) and a 50 wt % aqueous NaOH solution containing a few drops of tetrabutylammonium hydroxide (40% in water, TBAH) or 15-crown-5 as catalyst at room temperature under ambient conditions (24, 25). Within a few minutes, a brown sludge precipitated. After removal of toluene by decantation, the sludge and remaining aqueous solution was stirred with additional water for 1 night. The brown solution was added with MeOH to produce brown aqueous precipitates. The precipitation was rinsed with MeOH to ensure the removal of the catalyst and NaOH and was dissolved in water again to produce a brown solution which was put into cellulose dialyze tube (cut off MW = 500) in water for 1 week for complete removal of residual catalyst and NaOH. After freeze-drying, fine aqueous solution gave brown solid product Gd-fullerenols, $\text{Gd@C}_{82}(\text{OH})_n$.

On the basis of the elemental analysis and water contents, the average number of hydroxyl groups of $\text{Gd@C}_{82}(\text{OH})_n$ was obtained. The Gd concentration of the Gd-fullerenols solution was determined by using inductively coupled plasma atomic emission spectroscopy (ICP-AES, Seiko Electro Ind.) (26, 27). The sample for ICP-AES was prepared by the dilution with water. This preparation method was validated in comparison with the following two sample preparation methods. First, sample was digested in concentrated HNO_3 at 120 °C. Secondly, sample was digested in a mixture of concentrated H_2SO_4 and 30 % aqueous H_2O_2 (4:1 by volume) at 70 °C. Gd standards were made from a ICP standard solution (Wako Pure Chem.). The standards and samples were evaluated for intensity of their Gd emission line at 342.247 nm.

Proton Relaxivity. The proton relaxivities, R_1 and R_2 (the effect on $1/T_2$ as R_1 for T_1) of $\text{Gd@C}_{82}(\text{OH})_{40}$ in aqueous solution were determined by 0.47 T (Minispec, Bruker), 1.0 T (MAGNETOM Harmony, Siemens), and 4.7 T (Unity INOVA, Varian) at 25 ± 1 °C.

In Vitro MRI. The MRI phantom images of Gd-DTPA as well as $\text{Gd@C}_{82}(\text{OH})_{40}$ solutions at concentrations of 0.05, 0.1, and 0.2 mmol Gd/L were obtained at 4.7 T (Unity INOVA, Varian) by conventional spin-echo with pulse repetition time/echo time (TR/TE) = 300 ms/9 ms, field of view (FOV) = 10×10 cm², at 256×64 data, points zero-filled to 1024×1024 at 24 °C.

In Vivo MRI. In vivo MRI studies were performed after i.v. administration of $\text{Gd@C}_{82}(\text{OH})_{40}$ to CDF1 mice (ca. 20 g, female, 8 weeks age anesthetized with halothane (0.5–1%) and treated according to the Institutional Animal Care and Use Guidelines) at a dose of 5 μmol Gd/kg, which was 1/20 of the typical clinical dose (100 μmol Gd/kg) of Gd-DTPA ($n = 3$). The biodistribution studies at lung, liver, spleen, and kidney after i.v. administration of $\text{Gd@C}_{82}(\text{OH})_{40}$ ($n = 3$) was performed by Gd assay according to ICP-AES.

RESULTS AND DISCUSSIONS

Synthesis of Gd@C_{82} and $\text{Gd@C}_{82}(\text{OH})_n$. For the biomedical application of the metallofullerenes, modification to the water-soluble forms is essential. In this study, we employed the polyhydroxyl form for two reasons; one, for the simplicity of the synthesis, i.e., one-step procedure, and two, for the possibility of further molecular modifications such as conjugation of the bioreactive ligands. As the starting material of water-soluble metallofullerenes, highly purified Gd@C_{82} (<99%), which was achieved by the two-stage HPLC with a Buckyprep column (20 mm

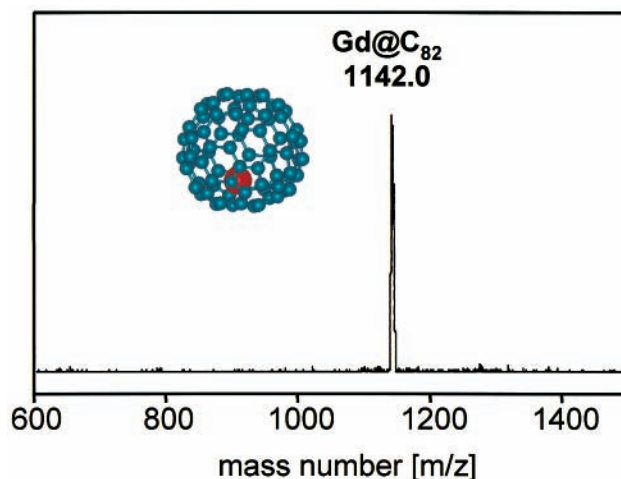


Figure 1. MALDI-TOF mass spectrum of highly purified Gd@C_{82} (negative ion). The purity of Gd@C_{82} (<99%) was quantified by matrix-assisted laser desorption/ionization (MALDI) TOF mass spectrometry (Shimadzu Compact MALD IV).

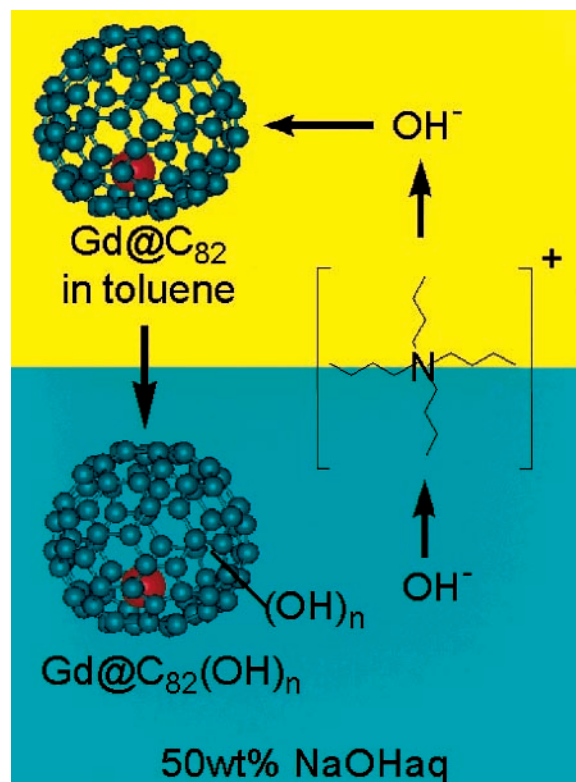


Figure 2. Scheme of the synthesis of the water-soluble polyhydroxylated Gd@C_{82} , $\text{Gd@C}_{82}(\text{OH})_n$ (Gd-fullerenols) by the tetrabutylammonium hydroxide (40% in water, TBAH) phase-transfer reaction.

dia $\times$ 300 mm, nacalai tesque), was used. The purity was determined by HPLC and by matrix-assisted laser desorption/ionization (MALDI) TOF mass spectrometry (Figure 1). The polyhydroxylation of Gd@C_{82} to $\text{Gd@C}_{82}(\text{OH})_n$ (Gd-fullerenols) was performed by the tetrabutylammonium hydroxide (40% in water, TBAH) phase-transfer reaction as Figure 2. On the basis of the elemental analysis and water contents, the average number of hydroxyl groups of $\text{Gd@C}_{82}(\text{OH})_n$ was calculated to be approximately 40. The obtained Gd-fullerenols solution, whose Gd concentration was determined by the ICP-AES at 342.247 nm, was used for the further in vitro and in vivo MRI studies.

Table 1. Proton Relaxivities, R_1 and R_2 of $\text{Gd@C}_{82}(\text{OH})_{40}$ and Gd-DTPA at 0.47, 1.0, and 4.7 T, 25 °C and pH 7.5

[mM ⁻¹ s ⁻¹]	Gd@C ₈₂ (OH) ₄₀ ^a			Gd-DTPA ^b		
	0.47 T	1.0 T	4.7 T	0.47 T	1.0 T	4.7 T
R_1	67	81	31	3.8	3.9	3.8
R_2	79	108	131	4.2	6.2	5.9

^a The proton relaxivities R_1 and R_2 (the effect on $1/T_1$ and $1/T_2$) of $\text{Gd@C}_{82}(\text{OH})_{40}$ were determined at 0.47 T (Minispec, Bruker), 1.0 T (MAGNETOM Harmony, Siemens), and 4.7 T (Unity INOVA, Varian) at 25 °C. ^b The values of the conventional MRI contrast agent, Gd-DTPA (Magnevist), were also determined for comparison.

In Vitro MRI Study. The ability of proton relaxation enhancement of a paramagnetic compounds is commonly expressed by the term relaxivity, R_i , which is denoted as eq 1;

$$(1/T_i)_{\text{obsd}} = (1/T_i)_d + R_i \times [M] \quad i = 1, 2 \quad (1)$$

where $1/T_1$ and $1/T_2$, are the longitudinal and transverse relaxation rates of the solvent, $(1/T_i)_{\text{obsd}}$ is the observed value in the presence of the paramagnetic species, $(1/T_i)_d$ is the one in the absence of the paramagnetic species, and $[M]$ is the concentration of the paramagnetic species. The proton relaxivities, R_1 and R_2 of $\text{Gd@C}_{82}(\text{OH})_{40}$ determined at 0.47 T (Minispec, Bruker), 1.0 T (MAGNETOM Harmony, Siemens), and 4.7 T (Unity INOVA, Varian) at 25 ± 1 °C are summarized in Table 1 with the values of Gd-DTPA for comparison. R_1 of $\text{Gd@C}_{82}(\text{OH})_{40}$ is significantly higher than that of Gd-DTPA especially at 1.0 T which is the field strength of the common clinical MRI. Another important result is that R_2/R_1 is comparable to that of Gd-DTPA at lower field, which is one of the most crucial features for the T_1 -contrast agent. A Gd^{3+} ion with seven unpaired electrons in half filled 4f orbital has been known to exhibit a strong relaxation capability of proton nuclear spin of nearby water molecules. Earlier reports on R_1 measurements at 0.47 T gave lower values [20 mM⁻¹s⁻¹ (17) or 47 mM⁻¹s⁻¹ (18)] than that of our results (67 mM⁻¹s⁻¹). The factors causing the difference may be the number of the hydroxyl groups on the surface of fullerene cage and/or the differences in the aqueous environments, such as pH and ionic strength. Our preliminary results indicated the R_1 s of the Gd-fullerenols, obtained at 4.7 T at 37 °C, were significantly influenced by the pH and ionic strength. For example, a bell shape relation of R_1 vs pH was observed where maximal R_1 s values were from pH ca. 3 to pH ca. 8. R_1 values were lowered from 67 to 61 mM⁻¹s⁻¹ at 0.47 T when the NaCl concentration of Gd-fullerenol solution was changed from 0 to 1.0 M.

The conventional MRI contrast agents consisting of the Gd^{3+} -chelate complexes have one direct coordination window to a water molecule that rapidly exchanges with the bulk water molecules surrounding the Gd^{3+} ion, which leads to an efficient nuclear spin relaxation of solvent water molecules (3–5). In contrast, in $\text{Gd@C}_{82}(\text{OH})_n$ there are no water molecules which can form a direct coordination to the encaged Gd^{3+} ion. Although the details of the electronic and magnetic properties of the current Gd-fullerenols is not known at present, it is highly probable that $\text{Gd@C}_{82}(\text{OH})_n$ also has the paramagnetic property, as in the intact Gd@C_{82} fullerene (28), due to three electron transfer from Gd to the C_{82} fullerene cage [$\text{Gd}^{3+}@\text{C}_{82}(\text{OH})_n^{3-}$] with seven unpaired electrons in the Gd-4f orbitals inside the C_{82} cage and one on the cage (25). The encapsulated Gd^{3+}

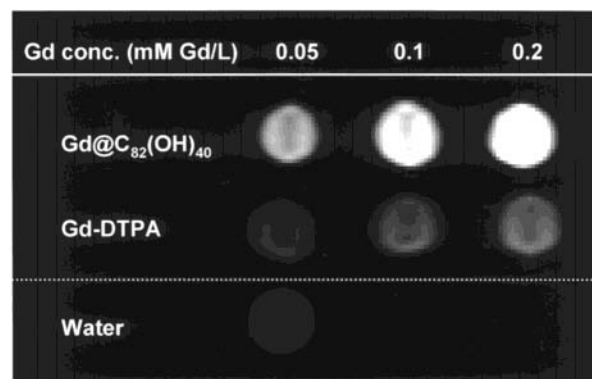


Figure 3. T_1 -weighted MRI of $\text{Gd@C}_{82}(\text{OH})_{40}$ and Gd-DTPA phantom at the concentration of 0.05, 0.1, and 0.2 mmol Gd/L, respectively, by 4.7 T Unity INOVA (Varian, by conventional spin-echo with TR/TE = 300 ms/9 ms, FOV = 10×10 cm², 256×64 , points zero-filled to 1024×1024 , 24 °C) Extremely strong signals of $\text{Gd@C}_{82}(\text{OH})_{40}$ were demonstrated at the Gd concentration of 0.05 mM whereas Gd-DTPA of the same concentration showed only a slight enhancement of MRI signals as compared with pure water.

ion is unable to interact with the surface water directly. Therefore, the most plausible interpretation of the observed efficient relaxation of water protons is the dipolar interaction between these unpaired electrons and many water molecules surrounding the large spherical surface area of $\text{Gd}^{3+}@\text{C}_{82}(\text{OH})_n^{3-}$ with H side heading toward the negatively charged surface of the cage.

The present high values of relaxivity, R_1 , prompted us to carry out in vitro MRI phantom study of $\text{Gd@C}_{82}(\text{OH})_{40}$ with the expectation that the sufficient MRI signal enhancement should be obtained at lower Gd concentration than that required for Gd-DTPA. The measurement was performed by the T_1 -weighted spin-echo method and was evaluated according to the following relation (29):

$$\text{signal intensity} \propto \rho (1 - e^{-\text{TR}/T_1})e^{-\text{TE}/T_2} \quad (2)$$

where the symbols of ρ is proton density.

An extremely strong MRI signal enhancement was observed for $\text{Gd@C}_{82}(\text{OH})_n$ at the Gd concentration of 0.05 mM whereas Gd-DTPA at the same concentration showed only a slight enhancement as compared with pure water (Figure 3). These MRI data were consistent with the high R_1 and R_2 values in Table 1.

In Vivo MRI Study. The biodistribution studies after in vivo MRI were performed by Gd assay according to ICP-AES after i.v. administration of $\text{Gd@C}_{82}(\text{OH})_{40}$ to CDF1 mice (ca. 20 g, female, 8 weeks age). As shown in Figure 4 a, stronger signal in the T_1 -weighted image was obtained at lung, liver, spleen, and kidney after i.v. administration of $\text{Gd@C}_{82}(\text{OH})_{40}$ at a dose of 5 $\mu\text{mol Gd/kg}$ ($n = 3$), which was 1/20 of the typical clinical dose of Gd-DTPA (100 $\mu\text{mol Gd/kg}$). The signal intensity had good correspondence with the concentration of $\text{Gd@C}_{82}(\text{OH})_{40}$ found in these organs (Figure 4b and Figure 5). The biodistribution indicated that the $\text{Gd@C}_{82}(\text{OH})_{40}$ tends to be entrapped in the reticular-endothelial system (RES) supposedly by forming large particles by aggregation or by the interaction with the plasma components such as albumin (5, 15). This result of biodistribution suggested the potential of $\text{Gd@C}_{82}(\text{OH})_{40}$ as a RES specific MRI contrast agent. It was reported that the biodistribution of the other metallofullerenols, e.g., $\text{Ho@C}_{82}(\text{OH})_n$, showed an accumulation greater than 10% within bone tissues (19, 20). If it holds for the present system, $\text{Gd@C}_{82}(\text{OH})_n$ may accumulate in bone tissues, which

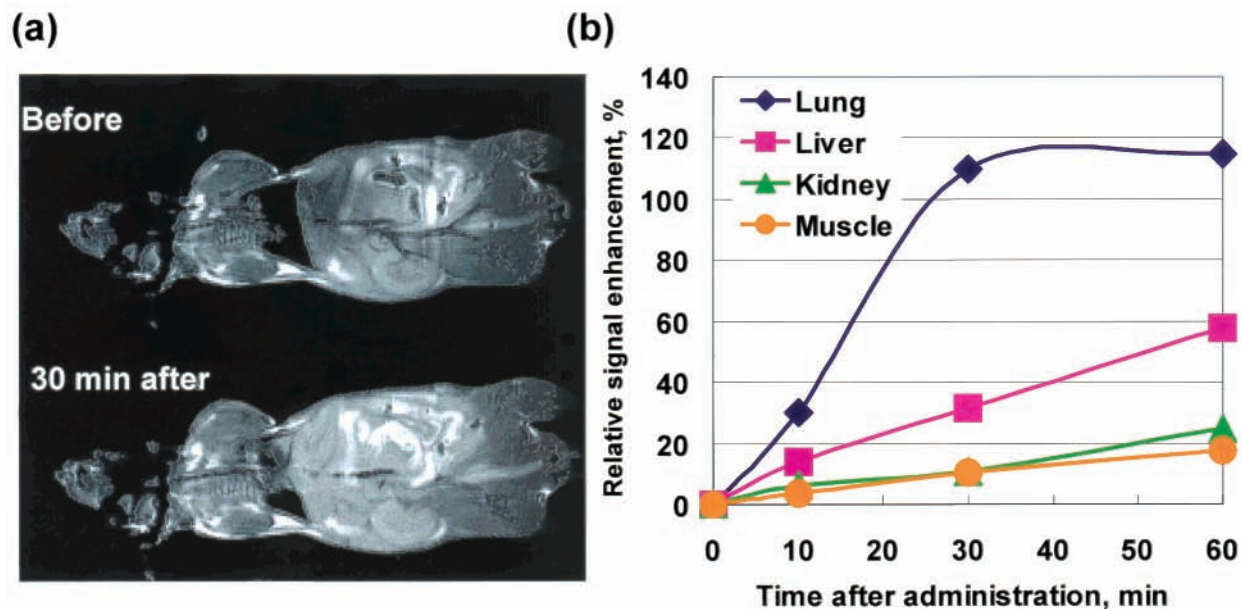


Figure 4. (a) T_1 -weighted MRI of CDF1 mice before and 30 min after i.v. administration of $\text{Gd@C}_{82}(\text{OH})_{40}$ via tail vein as the dose of $5 \mu\text{mol Gd/kg}$ [which was 1/20 of a typical clinical dose of Gd-DTPA ($100 \mu\text{mol Gd/kg}$)] ($n = 3$), and (b) its time dependent signal intensity change in various organs. MRI conditions: 4.7 T Unity INOVA (Varian), at $\text{TR/TE} = 300 \text{ ms}/11 \text{ ms}$. At 30 min. after i.v. administration of $\text{Gd@C}_{82}(\text{OH})_{40}$, enormous signal enhancement was observed at lung, liver, spleen, and kidney (Figure 4(b)).

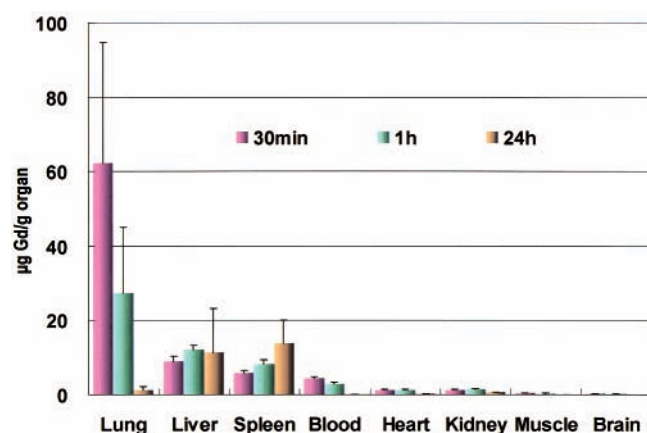


Figure 5. Biodistribution of Gd in CDF1 mice at 30 min, 1 h, and 24 h after i.v. administration of $\text{Gd@C}_{82}(\text{OH})_{40}$. Assay of Gd ions was carried out by ICP. The signal enhancement shown in Figure 3 had good correspondence with the concentration of $\text{Gd@C}_{82}(\text{OH})_{40}$ found in these organs (Figure 4a and Figure 4b). The biodistribution indicated that the $\text{Gd@C}_{82}(\text{OH})_{40}$ tends to be entrapped in the reticular-endothelial system (RES), such as lung, liver, and spleen.

might not be well-tolerable. To avoid bone uptake and to target specific tissues, further surface modification may be required.

In the utilization of Gd-chelate complexes for the RES agents, one must be apprehensive of the possible in vivo Gd^{3+} dissociation. In contrast, $\text{Gd@C}_{82}(\text{OH})_{40}$ has much less potency of toxicity because of its solid carbon cage structure. The unique fullerene structure of $\text{Gd@C}_{82}(\text{OH})_{40}$ protects the central metal atom from the outer environments, hence scarce release of Gd^{3+} from the entrapped fullerene cage. Furthermore, our preliminary 3-D MR contrast-enhanced angiography clearly demonstrated rat blood vessels within a few minutes after i.v. administration of Gd-fullerenols at the dose of $10 \mu\text{mol Gd/kg}$. It is very likely that this angiogram can be further improved by simple modification on the fullerene surface for the prolongation of the blood circulation period and the avoidance of RES uptake.

The present paramagnetic water-soluble metallofullerenes (Gd-fullerenols), which have extremely strong MR signal enhancement ability, will be applied as novel MRI contrast agents for the present anatomical and functional imaging. Furthermore, this will be a promising novel core material of MRI probes for high-quality molecular/cellular and future genetic imaging for next generation.

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Immunological Properties of Uricase Conjugated to Neutral Soluble Polymers

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For a comparative study of immunological properties of protein–polymer conjugates, uricase was modified with (a) poly(*N*-vinylpyrrolidone) 6000 Da, (b) poly(*N*-acriloylmorpholine) 6000 Da, (c) branched monomethoxypoly(ethylene glycol) 10000 Da, and (d) linear monomethoxypoly(ethylene glycol) 5000 Da. Spectroscopic studies performed by UV, fluorescence, and circular dichroism did not show any relevant difference in protein conformation among the native and the conjugates. Immunological studies showed that both uricase antigenicity and immunogenicity were altered by polymer conjugation to an extent that depended upon the polymer composition; in particular, monomethoxypoly(ethylene glycol) 10000 Da remarkably reduced the protein antigenicity, while unexpectedly, the poly(*N*-vinylpyrrolidone) derivative presented higher antigenicity than the native protein. In Balb/c mice, the native protein elicited a rapid and intense immunoresponse whereas all the conjugates induced a lower production of anti-native uricase antibodies. The rank order of immunogenicity was native uricase > uricase–poly(*N*-vinylpyrrolidone) ≥ uricase–poly(*N*-acriloylmorpholine) > uricase–monomethoxypoly(ethylene glycol) 5000 Da > uricase–monomethoxypoly(ethylene glycol) 10000 Da. The four conjugates also induced anti polymer immunoresponse. Anti poly(*N*-vinylpyrrolidone) and anti poly(*N*-acriloylmorpholine) antibodies were generated from the first immunization while low levels of anti polymer antibodies were found with both poly(ethylene glycol) conjugates only after the second immunization.

INTRODUCTION

Polymer conjugation has so far been successfully used to enhance the therapeutic potential of many pharmacologically active proteins and peptides since it allows for the alteration of their physicochemical and biological properties improving permanence in circulation, stability, and solubility and reducing immunogenicity (1–3).

Immunological properties of conjugates are of primary importance for their therapeutic application, but a number of items should be taken into consideration regarding both the conjugate components: polymer and protein (4).

Although absence of immunogenicity is a general prerequisite for the polymers used in drug modification, some of them were shown to present inherent immunogenic character because of their repetitive structure or high molecular weight. Examples are polyamino acids, polyacryloyl peptides, and polysaccharides (5, 6). Furthermore, in some cases, conjugation can provoke a dramatic change of polymer immunological properties. Low or nonimmunogenic macromolecules can in fact acquire haptogenic character by conjugation with immunogenic compounds. Poly(hydroxyethylstarch), poly(lysine), poly(ornithine), and dextrans, for example, were found to gain relevant antigenicity and immunogenicity after coupling with proteins and peptides (7–9). Also, poly(hydroxyethylacrylate), a polymer largely investigated for drug conjugation, was found to acquire unexpected immunogenicity after binding to oligopeptides, and anti poly(ethylene glycol) antibodies were generated using PEG-

superoxide dismutase and PEG-ovoalbumin although this polymer is nonimmunogenic when administered alone (10, 11).

As far as the protein side is concerned, reduction of immunogenicity often represents the target of conjugation when medical application of proteins entails the risk of severe immunoresponse. However, it is worth noting that although protein immunogenicity can be improved by modification with several macromolecules this result cannot be considered a general consequence of polymer coupling. Dextrans, for example, have been found to ameliorate the immunogenic behavior of many proteins, but paradoxically, in some cases they increase the protein immunogenicity as reported with bovine serum albumin or chicken serum albumin. (12, 13). Increased immunogenicity was also observed when polymer coupling induces partial modification of protein structure or dissociation into subunits that can be accompanied by introduction of neo-antigens (2).

Another interesting immunological aspect of polymer binding is the possibility to obtain derivatives that modulate the immunoresponse toward allergens. In this regard, several studies demonstrated that polymer conjugation can switch immunogenic proteins into tolerogenic compounds, providing new therapeutic perspectives (14).

These features indicate that conjugation can reflect in immunological properties of the derivatives, which can be quite different from those of the starting protein and polymer. These are often unpredictable since, as already reported in the literature, they depend on a number of parameters.

In the framework of this research field, we carried out an immunological characterization of synthetic polymers

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recently developed in our laboratory for protein conjugation: monofunctional poly(*N*-vinylpyrrolidone) (PVP) (15), poly(*N*-acryloilmorpholine) (PACM) (16), and a branched form of poly(ethylene glycol) (PEG2) (17). These polymers were selected because they possess physicochemical properties similar to the most used linear PEG 5000 Da, namely solubility, chemical stability, monofunctionality in reaction, and absence of charge. However, these polymers were found to confer to proteins different physicochemical and biopharmaceutical properties (18).

For the present comparative investigation, derivatives of uricase, an immunogenic protein of outstanding therapeutic value, bearing a close number of PVP, PACM, PEG2, and PEG were prepared and spectroscopic studies and antigenic and immunogenic characterizations were carried out on the native enzyme and the four conjugates.

EXPERIMENTAL SECTION

Uricase from *Candida utilis* was obtained from Toyobo (Osaka, Japan). [^3H]Succinimidyl propionate was from Amersham International (Amersham, U.K.) and Instagel for radioactivity determinations from Canberra Packard (Groningen, The Netherlands). The gel filtration Superose columns and the FPLC system were supplied by Pharmacia Biotech (Uppsala, Sweden); the ultrafiltration system was from Amicon Inc. (Beverly, MA). The alkaline phosphatase conjugated goat anti mouse IgG, IgM, and [IgG + IgM] were from Sigma (St. Louis, MO). All other reagents, of analytical grade, were purchased by Fluka (Buchs, Switzerland). Male Balb/c mice, weighing 24 ± 2 g and fed ad libitum, obtained from Charles River (Calco, Italy), were used for in vivo experiments. Animal treatments were performed according to institutional European guidelines.

Polymer Preparation and Activation. Succinimidyl ester activated linear monomethoxypoly(ethylene glycol) MW 5000 Da (PEG 5000) and branched monomethoxypoly(ethylene glycol) MW 10000 Da (PEG 10000) were prepared as previously reported (17, 19). Carboxy-terminating poly(*N*-vinylpyrrolidone) MW 6000 Da (PVP) and poly(*N*-acryloilmorpholine) MW 6000 Da (PACM) were synthesized, fractionated, and activated according to methods previously described (15, 16).

Preparation of Protein-Polymer Conjugates. Uric acid (77 μmol) was dissolved in 1 mL of 0.2 M borate buffer, pH 8.0 containing 10 mg of uricase. Proper amounts of activated polymer (77 mg of PEG 5000, 270 mg of PEG 10000, 115 mg of PVP, or 140 mg of PACM) were added to the protein solution under vigorous stirring. The reaction mixture was gently stirred for 1 h at room temperature, and 9 mL of 0.1 M borate buffer pH 8.0 was added. The solution was ultrafiltered with an Amicon system using a 10000 Da cut off membrane to reach a volume of 1 mL and the solution diluted with 5 mL of the same buffer and further ultrafiltered. The ultrafiltration procedure was repeated three times. The solution was finally loaded on a gel filtration Superose 12 preparative column that was eluted with 10 mM phosphate buffer, 0.15 M NaCl, pH 7.2 (PBS). The eluate was analyzed by OD at 280 nm for protein content, by iodine assay for polymer determination (20), and by enzyme activity (21). The protein-polymer conjugate peak was concentrated by ultrafiltration, and the protein concentration was determined by biuret assay (22). The degree of modification was estimated by a trinitrobenzenesulfonic acid based colorimetric method (23).

Preparation of Tritium-Labeled Derivatives. [^3H]Succinimidylpropionate (10 μg , 519 mCi/mg) was added

under stirring to 0.5 mL of 0.2 M borate buffer, pH 8.0, containing 5 mg of native uricase or equimolar amounts of its conjugates. The solution was gently stirred for 1 h at room temperature and then loaded on an analytical Superose 6 column eluted with PBS. The eluted fractions were analyzed for protein content by OD at 280 nm, enzyme activity, polymer content, and radioactivity using the Instagel cocktail. The radioactive peak corresponding to the [^3H]-labeled uricase or its conjugates was collected and protein concentration and radioactivity were assessed.

Spectroscopic Evaluations. The spectroscopic characterizations were carried out using solutions of 0.38 μM of native or polymer conjugated uricase in PBS. The circular dichroism spectra were recorded in the range of 200–250 nm while fluorescence spectra were determined in the range of 300–440 nm by excitation at 276 nm.

ELISA Determinations. ELISA microplates were incubated overnight at 4 °C with 100 μL /well of coating solution (0.1 M NaHCO_3 , pH 9.5) containing 1 $\mu\text{g}/\text{mL}$ of antigen. The wells were washed three times with 200 μL of PBS, 0.05% Tween, pH 7.2 (PBS-T) and then incubated for 2 h at room temperature with 200 μL of 0.1 M NaHCO_3 , pH 9.5, containing 50 $\mu\text{g}/\text{mL}$ of bovine serum albumin. The wells, washed as described above, were incubated for 2 h at room temperature with 100 μL of serum samples serially diluted in PBS-T and, after further washing, for 2 h with 100 μL of alkaline phosphatase conjugated goat anti mouse antibodies (anti IgG or anti IgM or anti [IgG + IgM]) properly diluted in PBS-T. The wells were washed as above and 100 μL of 1 M diethanolamine, MgCl_2 , pH 9.8, containing 1 mg/mL of *p*-nitrophenol phosphate (Sigma¹⁰⁴). The enzymatic reaction was stopped after 1 h by addition of 50 μL of 3 N NaOH and the o.d. at 405 nm was determined.

Antigen Coating Estimation. The amount of adsorbed protein on the well surface was estimated as follows: 200 μL of coating solutions containing serial dilutions (0.05–5 $\mu\text{g}/\text{mL}$) of tritium-labeled native protein or equimolar concentrations of its tritium-labeled conjugates in 0.1 M NaHCO_3 , pH 9.5, were incubated overnight at 4 °C in the ELISA wells. The solutions were completely removed and conserved, and the wells were washed three times with 200 μL of PBS-T. The coating and the washing solutions were pooled, lyophilized, and examined for radioactivity content.

Antigenicity Determinations. Native uricase (10 μg) in 200 μL of PBS/complete Freund's adjuvant (50/50) were intraperitoneously injected into seven male Balb/c mice on day 0. The animals were intraperitoneously boosted on days 7, 14, 21, and 28 with 10 μg of native uricase dissolved in 200 μL of PBS/incomplete Freund's adjuvant (50/50). The blood was taken on day 35, pooled, and centrifuged, and the serum serially diluted in PBS-T was used for antigenicity determinations by ELISA. The assay was performed as described above using, for well coating, 0.2 $\mu\text{g}/\text{mL}$ of native uricase or equimolar dilutions of uricase-PEG, uricase-PEG2, uricase-PVP, or uricase-PACM.

Immunogenicity Evaluations. Fifty male Balb/c mice were divided into five groups of 10 animals and immunized on days 0, 4, and 11 with 10 μg of native uricase (group 1) or equimolar amounts of uricase-PEG (group 2), uricase-PEG2 (group 3), uricase-PVP (group 4), and uricase-PACM (group 5). The immunizing solutions were prepared by dissolution of the immunogen in 100 μL of PBS and 100 μL of Freund's adjuvant (complete adjuvant was used in the first immunization, incomplete adjuvant was used for boosting). The solutions were

Table 1. Reaction Conditions, Composition and Molecular Mass of Native Uricase, Uricase-PVP, Uricase-PACM, Uricase-PEG, and Uricase-PEG2

	polymer/protein molar ratio	mean no. of polymer molecules per protein molecule ($\pm$ SD ^a)	molecular mass (KDa)
native uricase			130
uricase-PVP	250/1	47 $\pm$ 2	400
uricase-PACM	300/1	43 $\pm$ 4	380
uricase-PEG	300/1	44 $\pm$ 3	350
uricase-PEG2	350/1	40 $\pm$ 4	530

^a $\pm$ SD: the standard deviation was calculated on the basis of five experiments carried out to point out the conjugation reaction conditions.

intraperitoneously injected, and blood samples were taken by retrobulbar bleeding at scheduled times. The blood samples were centrifuged for 2 min at 3500 rpm, and the serum was properly diluted in PBS for ELISA.

Anti conjugate (whole construct) antibodies were assessed by ELISA using the corresponding uricase derivative for well coating. For the determination of anti native uricase antibodies the well coating was performed with native enzyme while for determination of anti-polymer antibodies, ribonuclease modified with PVP, PACM, PEG2, and PEG was used.

RESULTS AND DISCUSSION

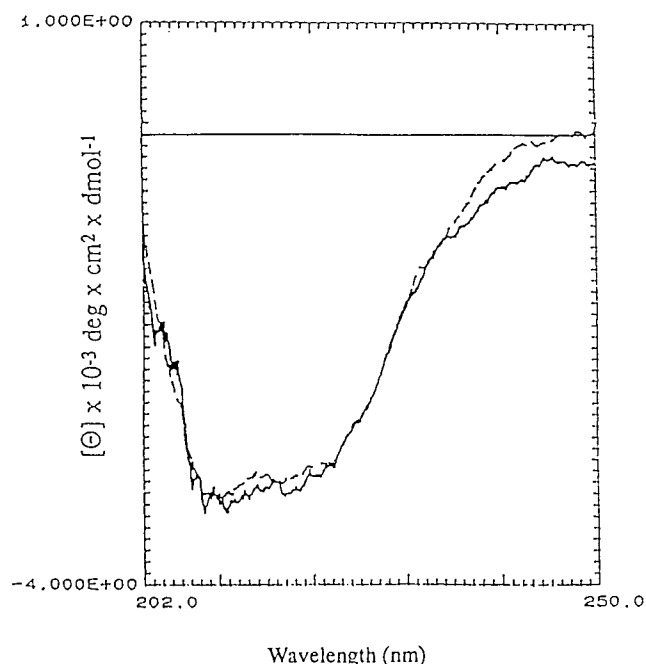
To provide alternatives to monomethoxypoly(ethylene glycol) MW 5000 Da (PEG) for protein modification, we recently developed new soluble polymers: monofunctional poly(*N*-vinylpyrrolidone) MW 6000 (PVP), poly(*N*-acryloilmorpholine) MW 6000 Da (PACM), and a branched form of monomethoxypoly(ethylene glycol) MW 10000 Da (PEG2).

Previous studies showed that these macromolecules possess the prerequisites for medical application, and encouraging results were obtained in the conjugation of few protein models. Conjugation of these polymers endowed in fact proteins with improved biopharmaceutical and immunological properties (17, 18, 24, 25).

Aiming to investigate closely the immunological aspects of PVP, PACM, and PEG2 conjugation, we carried out a comparative study using uricase as protein model. Uricase conjugated to the most known linear PEG 5000 was used as a comparison model. PVP and PACM with MW 6000 Da were selected because they have a molecular weight similar to PEG 5000, the polymer of choice for protein modification. PEG2 was considered in order to investigate the influence of the polymer molecular weight and shape on the immunological properties of the conjugate. Uricase was chosen because, despite its therapeutic value in treatment of uric acid overproduction, it presents a poor immunogenic character that greatly limits its medical use. For this reason, dextran and PEG derivatives of uricase with improved immunogenic and biopharmaceutical properties were already prepared (26, 27).

Polymer Conjugation. Because of the comparative nature of this investigation, conjugates at a similar level of modification were prepared. To overcome the differences in reactivity of the polymer, which depend on both polymer activation degree and accessibility to the protein surface, different polymer/protein molar ratios were used in the conjugation reaction.

Table 1 reports the reaction conditions, degree of modification, and molecular mass of the various uricase species. In all of the derivatives, an average of 44 protein

**Figure 1.** Circular dichroism spectra of native uricase (—) and uricase-PVP (---).

amino groups were modified by polymer. This yielded an higher molecular mass in the case of the PEG2 conjugate with respect to the other ones because of the higher molecular weight of this polymer that is about twice that of PVP, PACM, and PEG. It is important to note that since PEG2 is a branched form of the linear PEG, the number of polymer chains bound to the protein surface corresponds to a double number with respect to the other single chain polymer conjugates.

In all preparations, the addition of uric acid in the reaction mixture allowed for partial maintenance of the enzyme activity (20–80% of the unmodified uricase) probably because the substrate prevents the conjugation at the active site region (28).

Structural Studies. Spectroscopic studies were carried out to investigate whether the polymer conjugation induced protein denaturation that would be reflected in increased protein antigenicity and immunogenicity.

Native uricase and the four polymer conjugates displayed identical UV-vis and fluorescence spectra indicating that no macroscopic changes in protein structure occurred by polymer attachment. Also, no differences were found among the CD spectra of the various uricase species demonstrating that the secondary structure of the protein was not affected to a detectable degree by polymer conjugation. As an example, the CD spectra of native uricase and uricase-PVP are reported in Figure 1. The maintenance of the protein structure after polymer modification seems to indicate that the generation of new protein determinants, which could convey poor immunogenic properties to the conjugates, is unlikely.

The spectroscopic data suggest also that the low enzyme activity of conjugates is not due to partial denaturation of uricase but most probably to masking of the active site by the polymer or to chemical modification of protein amino groups involved in the catalytic activity that takes place despite the use of uric acid.

Antigenic Properties. The antigenicity of native and conjugated uricase was estimated by ELISA using mouse anti serum enriched with anti native uricase antibodies.

In a preliminary study, the coating levels of the various enzyme forms on the ELISA microplate wells were

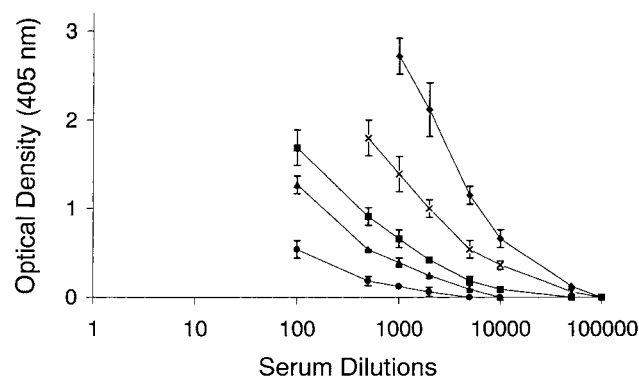


Figure 2. Antigenic profiles of native uricase (X), uricase-PVP (♦), uricase-PACM (■), uricase-PEG (◆), and uricase-PEG2 (●). $\pm$ SD was calculated on the basis of the values obtained with five experiments.

estimated using the following: [^{3} H]uricase, [^{3} H]uricase-PVP, [^{3} H]uricase-PACM, [^{3} H]uricase-PEG, and [^{3} H]uricase-PEG2. This was essential for a correct comparison of the results obtained with the native and the polymer conjugated protein. Comparable protein adhesion was obtained with the various uricase species provided that the protein concentration of the coating solutions was 0.2 μ g/mL.

The antigenic profiles of native and polymer modified uricase are depicted in Figure 2. The different behavior obtained with the conjugates underlines the influence of the structural and physicochemical properties of the polymers on the protein antigenic character. PACM, PEG, and PEG2 were found to reduce remarkably the uricase antigenicity. The reduction of uricase antigenicity obtained with both poly(ethylene glycol) derivatives confirms the known efficiency of these polymers in enhancing the antigenic character of this protein (29). PACM also was found to reduce the protein antigenicity although at a lower extent with respect to the poly(ethylene glycol)s.

Most probably, the reduction of protein antigenicity is due on one side to direct polymer masking of the protein epitopes and, on the other, to the steric hindrance of the polymer around the protein that prevents the antibody approach. Both of these events are strictly related to the physicochemical nature of the polymers that can be arranged on the protein surface in an extended or coiled conformation providing a different degree of hiding of the protein epitopes. Furthermore, these polymers possess a different capability to coordinate water molecules, which is reflected in different hydrodynamic volume of the conjugates. Therefore, the protein located in the hydrated polymeric core of the construct is differently accessible to the antibody interaction. To observe that the high efficiency of PEG2 in decreasing the uricase antigenicity with respect to the linear polymer is in agreement with the results obtained with other proteins (30). This can be ascribed to the higher molecular mass of polymer on the protein surface that efficiently enables it to mask the protein antigenic sites and reduces the approach of the antibodies to the protein structure as well as to prevent the proteolytic digestion (30).

Unexpected results were instead obtained with the PVP derivative. In this case, the protein antigenicity is about 2-folds higher than the native enzyme one. This intriguing result is in contrast with the data obtained in our laboratory with superoxide dismutase and ribonuclease where PVP conjugation lowered the protein antigenicity. The different behavior observed with uricase with respect to ribonuclease and superoxide dismutase demonstrates that the antigenic performance of the

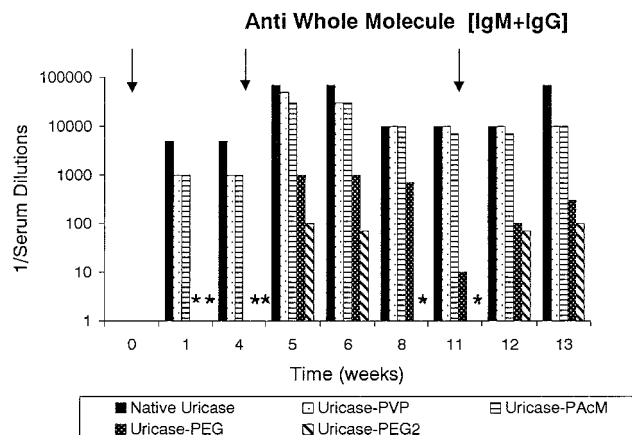


Figure 3. Anti whole molecule immunogenic profiles. IgM + IgG levels toward native uricase, uricase-PVP, uricase-PACM, uricase-PEG, and uricase-PEG2. $\downarrow$: immunization time. *: undetectable values.

conjugate is strictly related to the protein properties. In this regard we must remember that studies reported in the literature indicate that also PVP-conjugated D-N-acetyl hexosamidase A can strongly interact with the anti native protein antibodies (4).

Conjugate Immunogenicity. The generation of antibodies against the native protein or the whole constructs was determined by ELISA using the various uricase forms for well coating and mouse serum obtained from the animals immunized with the corresponding conjugates.

The immunological profiles depicted in Figure 3 indicate that the derivatives present a lower immunogenicity with respect to the native uricase, though at a different degree from one another. Uricase-PVP and uricase-PACM are the most immunogenic conjugates since they stimulate a high production of antibodies from the first immunization, and similar to the native enzyme, high antibody levels were maintained for the length of the experiment. On the other hand, the PEG and PEG2 derivatives present a remarkably lower immunogenicity with respect to native uricase. The very low amount of antibodies elicited by the PEG2 conjugate is worthy of note.

Protein Immunogenicity. The anti native protein antibody levels obtained by animals immunized with the four conjugates were estimated and compared with the ones obtained with native uricase.

The anti native protein [IgG + IgM] time courses depicted in Figure 4 A point out the high immunogenicity of native uricase. Anti uricase antibodies were found from the first immunization, and the maximal immunoreponse was obtained after animal boosting. The separate evaluation of IgM and IgG (Figure 4B,C) indicated that native uricase could elicit a rapid and intense IgM production since after the first immunization, and high IgM levels were maintained for 10 weeks while high amounts of anti native uricase IgG were elicited after the second immunization and maintained throughout the experiment.

All the conjugates were found to stimulate a lower anti uricase immunoreponse with respect to the native protein, although a different behavior was obtained with the four derivatives. PVP was the least effective in reducing the protein immunogenicity; anti uricase IgM were generated from the first immunization and IgG from the second one. PACM was more effective than PVP in suppressing uricase immunogenicity. By treatment with

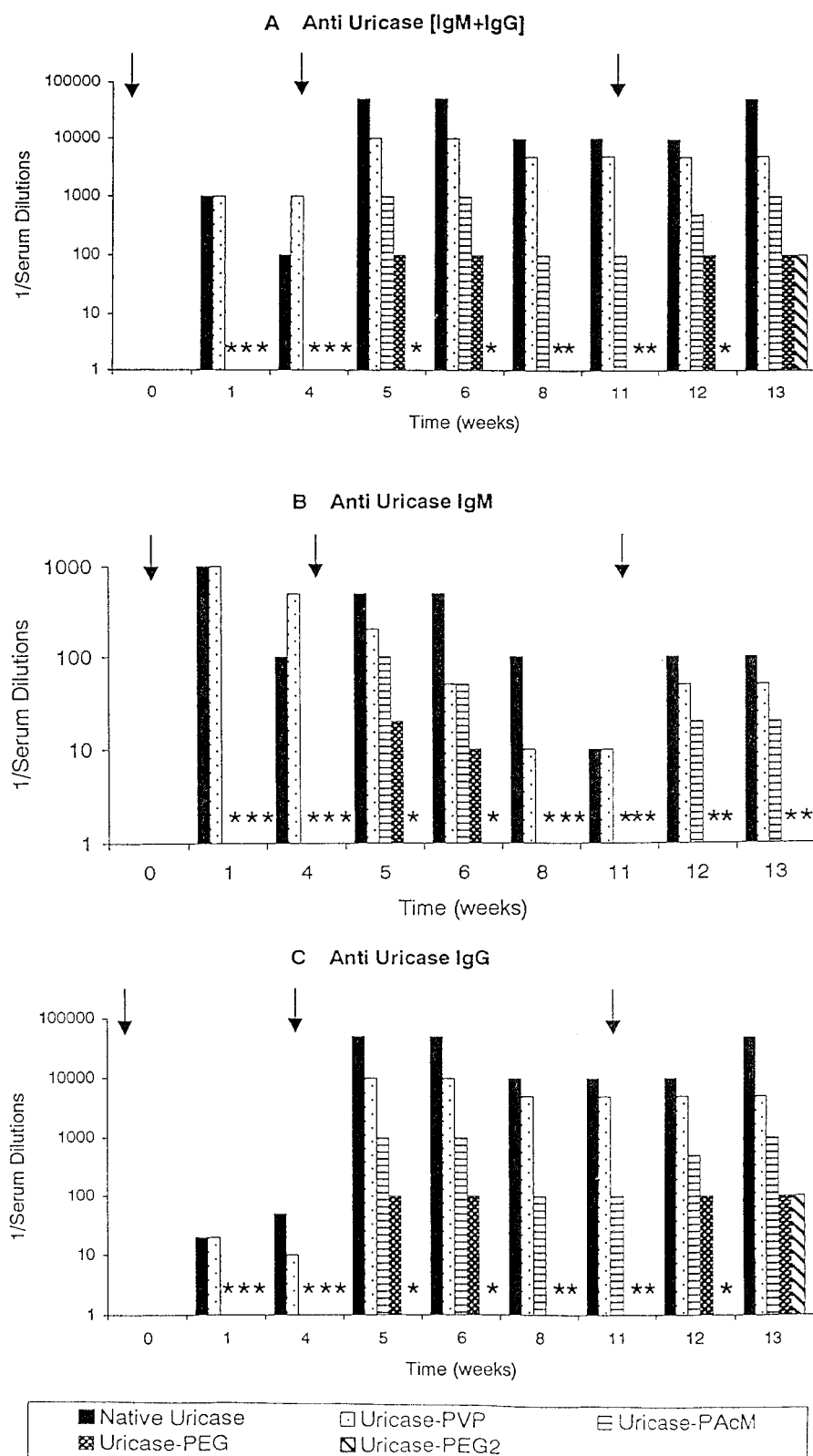


Figure 4. Anti native uricase immunogenic profiles: IgM + IgG(A), IgM (B), and IgG (C) immunoresponse after immunization with native uricase, uricase-PVP, uricase-PACM, uricase-PEG, and uricase-PEG2. ↓: immunization time. *: undetectable values.

PACM-uricase, anti protein IgM and IgG were detected only after the second immunization, and the reduction in immunoresponse was reduced to 2% of the native enzyme one. Even higher reduction in uricase immunogenicity was found with the linear PEG conjugate (below 1% of the native protein one). In this case, detectable amounts of anti native uricase IgM were determined only after the second immunization to disappear rapidly while

low amounts of IgG were generated after the third treatment. With the PEG2 derivative anti native protein IgM were not found, and negligible amounts of IgG were detected after the third immunization only.

Similar to what reported for the conjugate immunogenicity, the different immunogenic profiles depicted in Figure 4 can be explained with the different ability of the polymer to mask the protein epitopes and to prevent

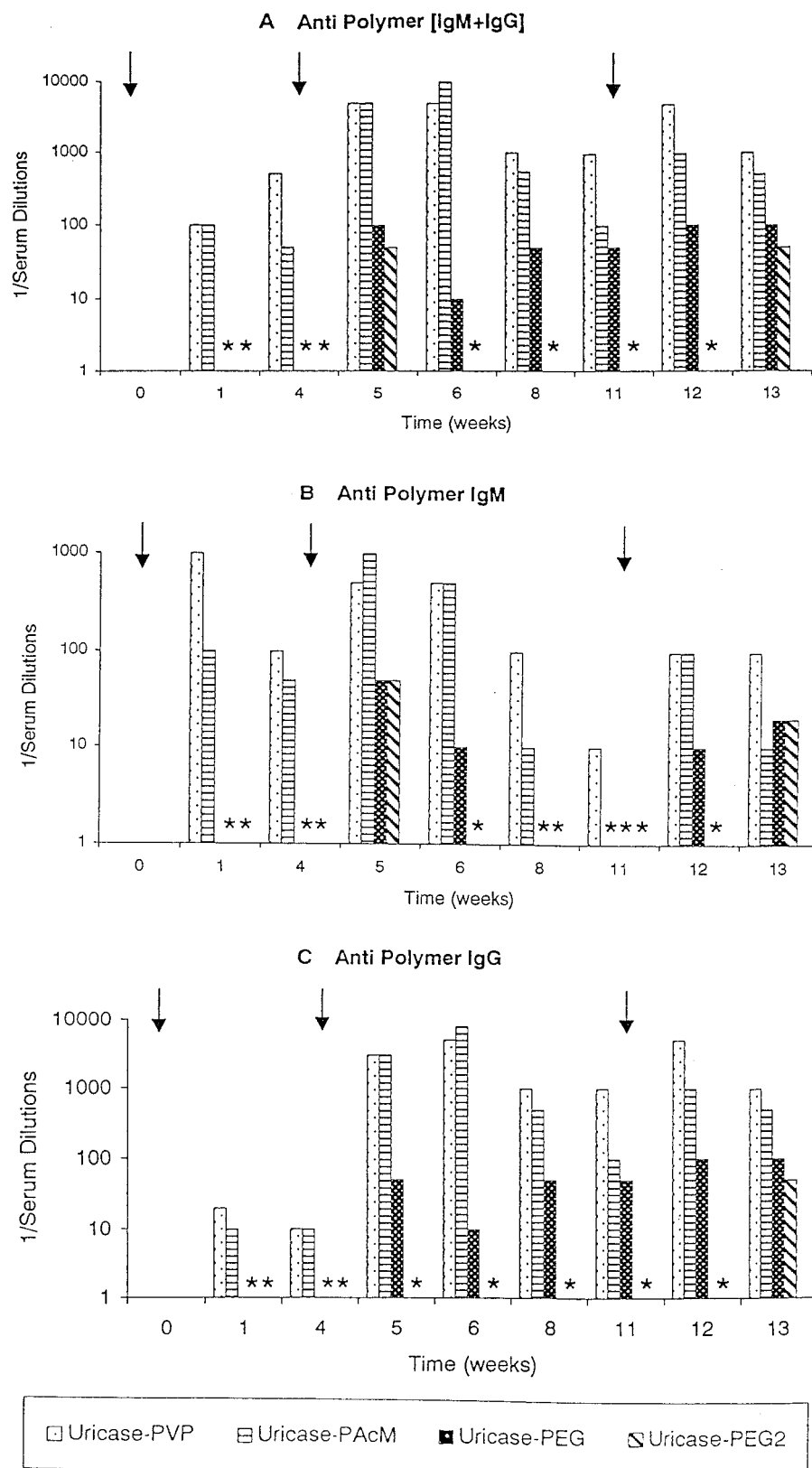


Figure 5. Anti polymer immunogenic profiles: IgM + IgG(A), IgM (B), and IgG (C) immunoresponse after immunization with uricase-PVP, uricase-PAcM, uricase-PEG, and uricase-PEG2. ↓: immunization time. *: undetectable values.

the approach of the immunocompetent cells to the conjugate. Probably the unlike arrangement of the four polymers on the protein surface as well as their degree of hydration affect in a different way the uricase immunogenicity.

Also in this case, the immunogenic behavior of uricase-PVP is in contrast with the results previously obtained

in our laboratory with other proteins. Superoxide dismutase-PVP, for example, was found to be less immunogenic than the PEG derivative (24). However, the low effectiveness of PVP in reducing uricase immunogenicity could be related to the high immunogenic character of this protein as well as to a possible low in vivo stability of the protein induced by PVP attachment that could

induce the molecule dissociation into high immunogenic subunits.

The rapid and intense production of anti native uricase IgM obtained with uricase-PVP indicates that a T cell independent mechanism may be involved in antibody response while with the other conjugates the immunoresponse appears to take place by a T cell dependent mechanism only. On the basis of such a hypothesis, we can think that PAcM, linear PEG, and PEG2 can convey to the conjugates a different capability to interact with macrophages and different stability toward the cell proteolytic processing.

As far as the two poly(ethylene glycol) derivatives are concerned, the PEG2 conjugate presents an immunogenic character that is more convenient for therapeutic application as already observed in our laboratory (30).

Polymer Immunogenicity. Interesting results were obtained regarding the ability of the conjugates to induce anti polymer immunoresponse. For this study, ELISA microplate wells were coated with ribonuclease (RNase) extensively modified with the four polymers. To avoid misinterpretation of the results, the absence of antibody cross reactivity with native RNase was previously verified.

Figure 5 reports the anti-polymer immunogenic profiles obtained with the four uricase-polymer conjugates. Unexpectedly, all the conjugates were found to induce anti polymer immunoresponses although at different levels.

The PVP and PAcM conjugates were found to induce a relevant production of IgM soon after the first immunization, suggesting a T cell independent mechanism antibody response (Figure 5B), while high levels of anti polymer IgG were estimated after the second treatment (Figure 5C). On the contrary, PEG and PEG2 derivatives gave rise to slight and delayed anti polymer antibody production. In particular, the immunogenic profiles reported in parts B and C of Figure 5 show that low levels of anti PEG IgM and IgG were found by immunization with uricase-PEG after the second and the third boosting, respectively, while the PEG2 derivative stimulated only negligible amounts of anti polymer IgM and IgG.

These results indicate that despite the absence of inherent immunogenic character of the polymers used for the present investigation (7, 31, 32) their immunogenicity can significantly increase by linkage to proteins. In this regard, we must remember that conjugation to proteins generally induces a deep modification of polymer physicochemical properties that can be reflected in different immunological properties. Covalent binding of polymers to the protein surface achieves high molecular weight constructs that may result in partial loss of polymer flexibility and prolonged exposure to the biological environment due to their long permanence in circulation. These events could be the reason immunocompetent cells are stimulated at a greater extent to produce anti polymer antibodies.

With regard to the molecular weight, it is worth noting that this parameter is of primary importance in dictating polymer immunogenicity. Immunogenic character of many polymers has been, in fact, demonstrated to increase as their molecular mass increases as, for instance, in the case of poly(*N*-vinylpyrrolidone)s of high molecular weights that are definitely immunogenic (32, 33). In the case of conjugates, the protein in the core of the construct behaves as an anchoring point for the polymer so that the conjugate could virtually acquire the properties of a high mol wt polymer. This may contribute

to increase immunogenic character of the polymer after protein conjugation. However, this relationship between polymer immunogenicity and molecular weight is not a general rule since data reported in the literature indicate that poly(ethylene glycol) immunogenicity decreases as the molecular weight increases. This is in agreement with the lower anti polymer immunoresponse obtained with the PEG2 derivative relative to the PEG one (7).

CONCLUSIONS

The data reported in this paper demonstrate that the four polymers investigated here, PVP, PAcM linear and branched PEG, can improve the immunogenic character of uricase. However, despite their apparently similar physicochemical properties, they convey different immunological properties to the conjugates.

In particular, our data demonstrate that the immunogenic properties of the conjugates are strictly related to the protein structure. This is in agreement with that reported in the literature and explains the different results obtained in our laboratory with other protein models modified with the same polymers. In the case of uricase, the high protein immunogenicity is probably the reason for the low efficiency of PVP and PAcM in preventing the anti native protein immunoresponse. Furthermore, this protein can confer to the polymers haptogenic character allowing for stimulation of anti polymer antibody production.

On the other hand, our results underline the relevance of the polymer choice in fabrication of conjugates with suitable immunogenic properties. Polymer flexibility, extended or coiled conformation, hydrophilic/hydrophobic balance, water coordination, and arrangement on the protein surface, together with molecular weight and chemical structure, are, in fact, all parameters that dictate the immunological properties of the conjugate.

In conclusion, from the investigation reported here we can say that, although neutral amphiphilic polymers seem to possess suitable immunological properties on their own for protein modification, the immunogenic performance of conjugates is not easily predictable because of the complexity of the immunoresponse process. Accurate immunological investigations are therefore required in the development of any new derivative, and both protein and polymer immunogenicity before and after conjugation must be thoroughly investigated.

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New TFO Conjugates Containing a Carminomycinone-Derived Chromophore

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Conjugates obtained by linking the anthracycline intercalating chromophore to triple helix forming oligonucleotides (TFOs) have been used in a physicochemical study of the stability of triple helices with DNA sequences of pharmacological relevance. The intercalating moiety is represented by carminomycinone derivatives obtained upon O-demethylation and hydrolysis of the glycosidic linkage of daunomycin followed by the introduction of an alkylating residue at two different positions. Results of experiments with a polypurinic region present in the multidrug resistance (MDR) gene indicate that the stability of the triple helix is significantly enhanced by replacement of C's with ⁵-MeC's in the TFO sequences tested. The stability is not changed when a 3'-TpT is present in place of a 3'-CpG at the presumed intercalation site of the anthraquinone chromophore. The same carminomycinone derivatives were used for the preparation of conjugates able to form triple helices with the polypurine tract (PPT) present in the human integrated genome of HIV-1 infected cells. Three different TFOs (T₄^{Me}CT₄^{Me}CC, **C2**; T₄^{Me}CT₄^{Me}CC^{Me}CC^{Me}CCT, **C6**; and T₄^{Me}CT₄G₆, **G6**) were designed and linked to the anthraquinone moiety. These conjugates showed a significantly enhanced ability to bind the PPT region of HIV with respect to the nonconjugated TFOs.

INTRODUCTION

One promising new technique used to inhibit the expression of genes consists of the formation of a local triple helix able to interfere with the transcription machinery. Local triple helices can be formed by an exogenous oligonucleotide that binds to a DNA in its natural duplex form, providing it has the right recognition pattern for Hoogsteen's type of hydrogen bonds, i.e., a polypurine tract. Since the pioneering studies of Hélène and co-workers, this approach has been known as anti-gene methodology (1). Even if polypurine tracts of suitable length for selective recognition (14–20 bases) are relatively rare within genomes, they are often present in the control region of genes (indicating, perhaps, a possible natural mechanism of regulation). In general, triple helices have a rather low thermodynamic stability under physiological conditions of pH and temperature. However, stability may be enhanced, inter alia, by tagging an intercalating agent at one or both termini of the triplex-forming oligonucleotide (TFO). Using different derivatives of daunorubicin as intercalating moieties at pH values between 5.5 and 6.8, we have already increased from 16 to 20 °C the melting temperature of a triplex formed by a pyrimidinic T/C oligonucleotide with a DNA duplex featuring a polypurine region present in the *hMDR1* gene (2).

The first part of the present work studied the effect of replacing cytidines with 5-methyl-cytidines on the stabil-

ity at pH 7.0 of the same triplex used with the daunorubicin-derived TFO conjugates (2). Using the same model system, we also addressed the question of the possible dependence of the triplex stabilizing effect of this type of intercalator upon the base pair sequence of their putative intercalation site. The second part investigated the use of the same type of conjugation for increasing the thermal stability at pH 7.0 of triplexes formed with the polypurine tract (PPT) region of HIV-1 proviral DNA. This region of the proviral DNA is accessible to TFOs within cell nuclei (3, 4), but native oligonucleotides do not lead to stable triplexes under physiological conditions. Hence, some extra binding strength is needed to obtain a significant inhibition of transcription.

MATERIALS AND METHODS

General Methods. All chemicals were of commercial analytical grade and used as received.

The following abbreviations were used throughout the text: AcOH, acetic acid; DMF, *N,N*-dimethylformamide; DTT, 1,4-dithio-D,L-threitol (or 1,4-dithioerythritol); TEAA, triethylammonium acetate; TEAB triethylammonium bicarbonate; Tris, tris(hydroxymethyl)aminomethane.

HPLC Analysis and Purifications. HPLC analysis and purifications were performed on a Waters 600E Millipore system control equipped with a Waters 484 tuneable absorbance detector as previously described (2).

UV-Melting Experiments. Ultraviolet measurements and melting profiles were recorded on a Perkin-Elmer 554 spectrophotometer equipped with a MGW Lauda RC5 thermostat and a MGW Lauda R40/2 digital thermometer; heating was controlled by an electronic device generating a linear gradient of temperature of 0.5 °C/min. Samples were prepared by dissolving equimolar amounts of the duplex strand in 0.1 M Tris–AcOH buffer, 0.05 M NaCl, 0.01 M MgCl₂ at pH 7.0 at a concentration

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of 1 or 2 μM per strand. The UV cuvettes were heated to 80 °C for 20–30 min and then slowly cooled (at least in 2–3 h) to room temperature to favor annealing; the conjugates were then added, and the cuvettes were again heated to 60 °C to favor the annealing of the third strand without destruction of the duplex. The cuvettes were then gently cooled to 4 °C. Condensation of moisture on the cell walls, in the range 4–20 °C, was prevented by flushing nitrogen.

Fluorescence Experiments. Fluorescence quenching experiments were monitored on a SPEX 3000 spectrofluorometer at room temperature with 1.25 mm slits, 455 and 495 nm filters, $\lambda_{\text{exc}} = 470$ nm; the emission was recorded in the interval 520–700 nm. Interference of water was electronically subtracted from the recorded spectra.

Electrophoretic Mobility Shift Assay. Electrophoresis was run using a 20% polyacrylamide gel in a thermostatic slab gel unit at 10 V/cm. The samples were prepared by mixing 1 μL of a 0.1 mM buffered solution of duplex with 0.8–1.5 μL of a 0.1 mM buffered solution of conjugate–TFO. The samples were then diluted with buffer to 5 μL and allowed to anneal at 15 or 36 °C overnight; 5 μL of a 50% sucrose solution was added, and the 10 μL samples were applied on the gel. A 0.1 M Tris–AcOH buffer, 0.05 M NaCl, 0.01 M MgCl_2 at pH 7.0 was used both for the preparation of samples and for the run.

Gel staining was obtained by soaking the gel with 0.01% stains-all dye in 1/1 (v/v) water/formamide. The gels were then directly digitalized on a PC scanner.

Synthesis and Purification of the Oligonucleotides. The oligodeoxynucleotides were prepared on a Pharmacia gene assembler II-plus using the manufacturers' protocols with commercially available amidites. The oligonucleotides were deblocked with 30% aqueous ammonia (16 h at 50 °C) and then purified by ion exchange on a Sephacel column (Pharmacia), eluting with a linear gradient from 0.05 to 1.5 M TEAB. The fractions were analyzed by HPLC, and those with purity greater than 90% were pooled together, evaporated several times with water, and then converted to the sodium salt by ion exchange with Dowex WX-8 resin in the sodium form.

Synthesis and Purification of Conjugates (Series A and B). Oligonucleotides containing the 5'-PS linkage were prepared using a modified (home-tested) protocol on a Pharmacia gene assembler II-plus employing dicyanoethyl-(*N,N*-diisopropyl)-phosphoramidite. After deblocking and night-standing on DTT (5 mg/mL), the crude compound was precipitated with 1-butanol (10 volumes); the oligonucleotide was then dried, converted to the sodium salt (as above), and lyophilized. The sodium salt of a 5'-PS-oligonucleotide (10 ODU about 75% pure by HPLC) was dissolved in 125 μL of DMF, 20 μL of water, 11 μL of 15-crown-5, 1 mg of anthracycline iodo derivatives (2), and 0.2 mg of DTT. The reaction mixture was kept in a sealed vial for 16 h in a thermostat at 45 °C. The crude mixture was purified from the excess of iodoalkylcarminomycinone by diluting the mixture with 150 μL of water and extracting the unreacted iodo derivative with a 85/15 v/v solution of dichloromethane/methanol. The pure conjugate was then recovered by purification of the aqueous solution on a reverse-phase silica gel column using a gradient of acetonitrile (0–30%) in a 0.1 M TEAA aqueous buffer. Usually 5–6 ODU of pure conjugate was obtained.

RESULTS AND DISCUSSION

Part 1: hMDR1 Target. The sequences of the TFOs and the target duplexes used in part 1 of this study are

Table 1. Oligonucleotides (ODNs) Synthesized and Used in the MDR Work (Boldface C indicates 5-Me₂dC)^a

ODN	entry code
5' TTT CTT CTT CTT 3'	MDR-1
5' TTT CTT CTT CTT 3'	MDR-2
5' AGG AGC AAA GAA GAA CTT T 3'	DNA-1
3' TCC TCG TTT CTT CTT CTT GAA A 5'	
5' AGG ATT AAA GAA GAA GAA CTT T 3'	DNA-2
3' TCC TAA TTT CTT CTT CTT GAA A 5'	

^a The evidenced regions encompassing the intercalation site are common to both **DNA-2** and PPT duplex.

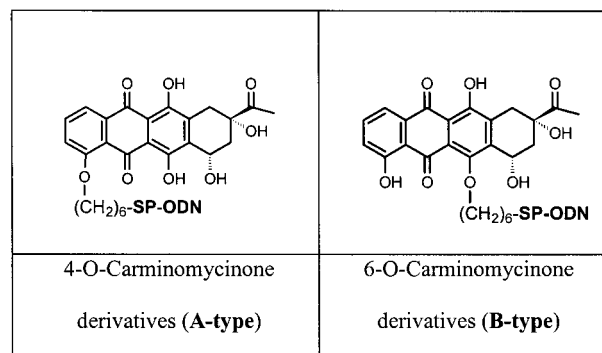


Figure 1. The two types of conjugates employed. The hexamethylene spacer is attached to the 5'-base of the ODN through a phosphorothioate (PS) linkage (2).

shown in Table 1, and the structures of the conjugates are presented in Figure 1. Our previous work (2) demonstrated that derivatization of **MDR-1** as in the compounds of the type shown in Figure 1 enhances the stability of the triplex with the **DNA-1** target (Table 1). In particular, we observed that, while the triplex formed at pH 6.8 by unconjugated **MDR-1** exhibited a T_m of 11 °C, the 4-O- and the 6-O-carminomycinone derivatives of the same TFO (**MDR-1-A** and **MDR-1-B**) melted at 27 and 31 °C, respectively. The increased stability of the triplex is related to a specific structure including both recognition of the PPT region by the complementary Hoogsteen strand and intercalation of the anthracycline chromophore. This was deduced from observation of the biphasic melting transition and gel mobility shift in electrophoretic experiments, together with the fluorescence quenching typical of the intercalation process. Fluorescence quenching was observed only as a consequence of the triple helix formation. First, to explore the possibility of further enhancing the stability of the triple helix by substituting the cytidines with the 5-methylcytidine's sequence, **MDR-2** was synthesized and linked to the appropriate carminomycinone derivatives, according to the procedure already described (2), to give **MDR-2-A** and **MDR-2-B**. Second, although the precise position of the intercalation of the tagged molecule in the triple-helical complex remains to be established, the length of the linker allows it to occur either at the interface between the duplex and the triplex region or, at most, one base pair away from it. As **DNA-1** exhibits a 3'-CpG next to the said interface, we evaluated the effect of the substitution of this structural element with, for example, a 3'-TpT as in **DNA-2**.

The stability of the triplexes formed by the TFOs and by the conjugates with **DNA-1** and **DNA-2** was studied by UV-melting experiments, and the results are summarized in Table 2. As expected, replacing the C's with 5-MeC's (**MDR-2** versus **MDR-1**) enhanced the stability of the triple helix by more than 12 °C at pH 7.0. The melting data also show that, at least for the conjugates of type **A**

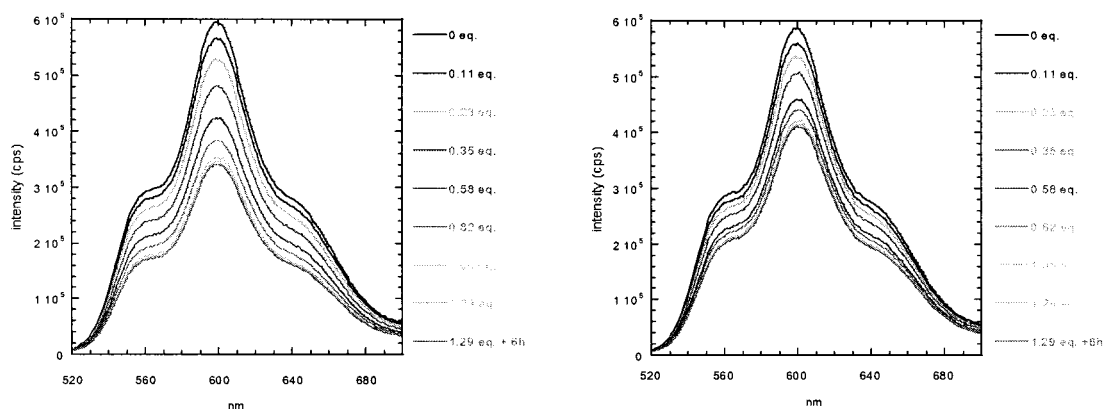


Figure 2. Fluorescence quenching of **MDR-2-A** with increasing equivalents of **DNA-1** duplex (left panel) or **DNA-2** duplex (right panel). Measurements were made at pH 7.0 and 22 °C.

Table 2. Results of the Melting Experiments with TFO MDR-1 and MDR-2 and Conjugates of the Latter Targeting DNA-1 and DNA-2 at pH 7.0 in 100 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 2 μ M per Strand

	<i>T_m</i> triplex (°C)	
	DNA-1^a	DNA-2^b
MDR-1	<11 at pH 6.8	<11 at pH 6.8
MDR-2	23	24
MDR-2-A	53	53
MDR-2-B	53	53

^a *T_m* of **DNA-1** duplex is 65 °C. ^b *T_m* of **DNA-2** duplex is 60 °C.

or **B**, the identity of the base pairs present in the duplex at the duplex/triplex interface (i.e., at the putative intercalation site) played no role in the stabilization of the resulting triplex, as there is no appreciable difference in the effect on the *T_m* between targeting **DNA-1** or **DNA-2**. This finding is important in that it allows the extension of the applicability of this class of conjugates to the targeting of polypurinic regions devoid of a flanking 3'-

CpG motif (such as the PPT region of the HIV). The CpG sequence is considered to be the preferred intercalation site of the anthracyclines (5).

As described in our previous work (2), the formation of a triplex with daunorubicin-derived conjugates quenches the fluorescence signal of the anthraquinone moiety, as shown for the present sequences in Figure 2. This figure illustrates the increasing fluorescence quenching of **MDR-2-A** (2.0 μ M) at neutral pH, following the progressive addition of **DNA-1** (left panel) or **DNA-2** (right panel). The signals were recorded 5 min after each addition of a small volume of a concentrated duplex solution. Since the measurements were done at 22 °C, it is assumed that the triplex was the only stable species, the *T_m* of the complex being about 53 °C (Table 2). In both cases, the signal decreases regularly up to the addition of 1.05 molar equiv of the duplex, with a further negligible change upon the addition of a further 0.14 equiv. This last measurement was repeated, and found unchanged, after 6 h. Since we know from the value of

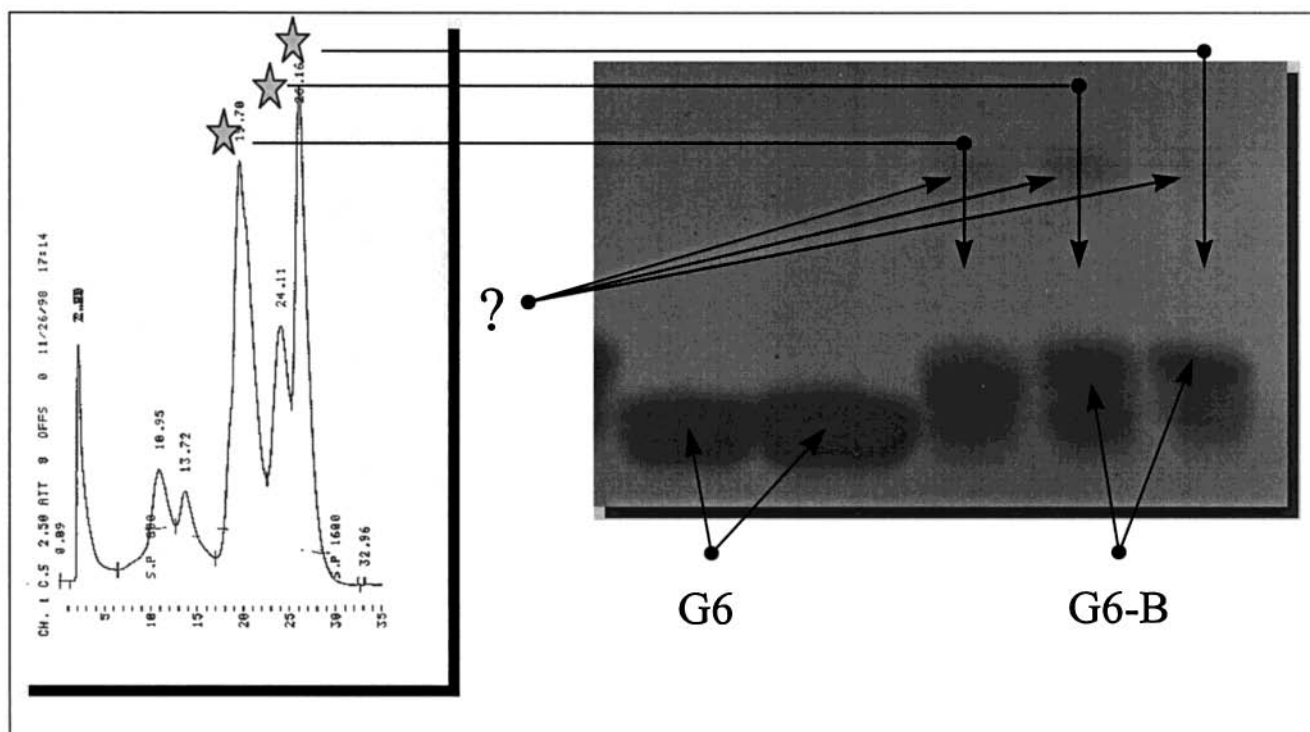


Figure 3. Reverse-phase (C-18) HPLC analysis of the crude reaction mixture at the end of the synthesis of **G6-B** (left panel). The three main peaks were separately collected and analyzed under denaturing conditions (6 M urea) by PAGE (right panel). They resulted mainly in the formation of various aggregates between the original **G6** and the desired **G6-B**.

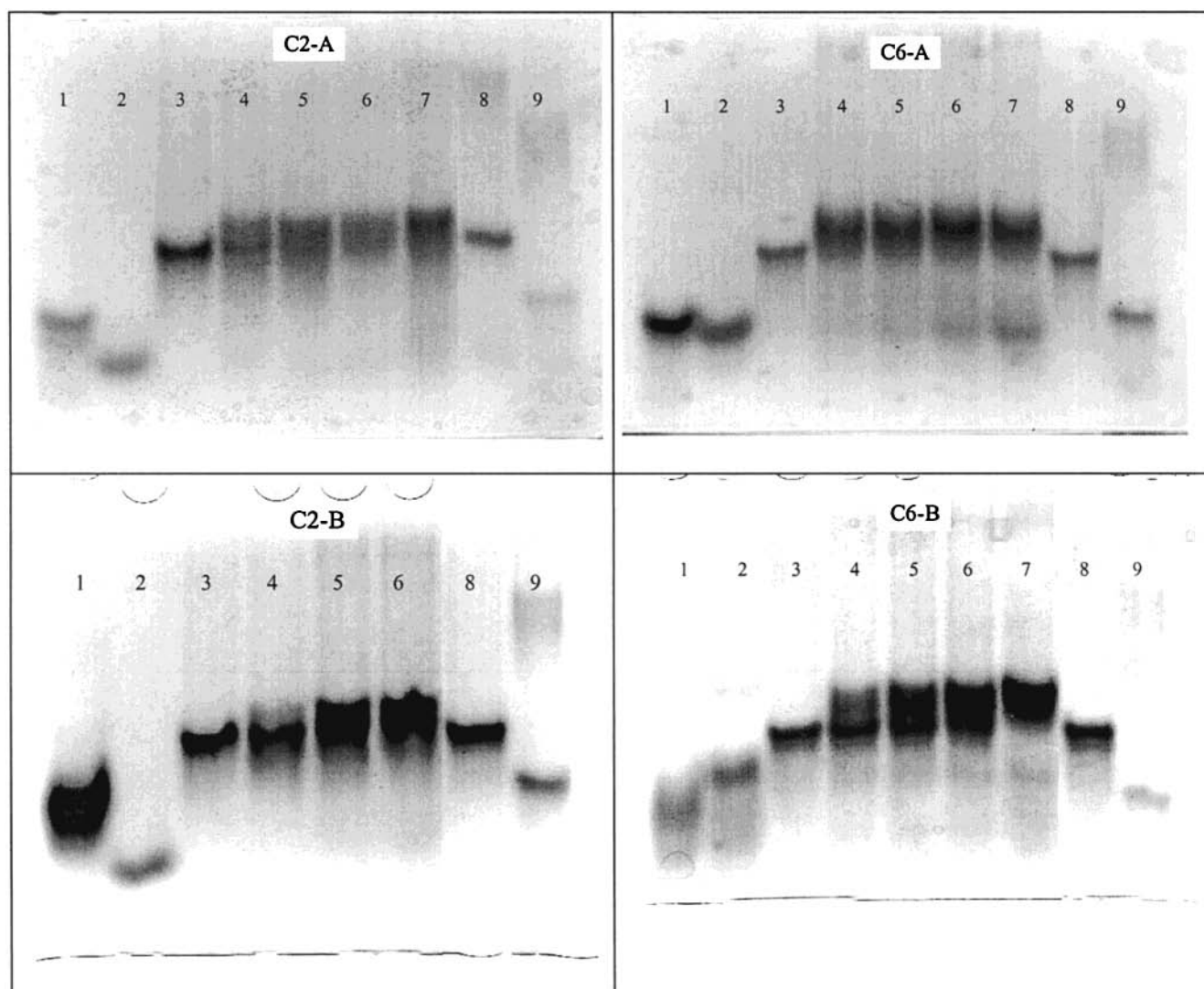


Figure 4. Gel mobility shift experiments of the triplex formed by **C2-A**, **C2-B**, **C6-A**, and **C6-B** at pH 7.0 and 15 °C. Lane 1, 200 pmol of single-stranded **PPT-Py**. Lane 2, 200 pmol of the indicated conjugate-TFO. Lanes 3 and 8, 100 pmol of duplex **PPT**. Lane 4, 100 pmol of **dx-PPT** + 80 pmol of conjugate-TFO. Lane 5, duplex **PPT** + conjugate-TFO, both 100 pmol. Lane 6, 100 pmol of **dx-PPT** + 120 pmol of conjugate-TFO. Lane 7, 100 pmol of **dx-PPT** + 150 pmol of conjugate-TFO. Lane 9, 200 pmol of single-stranded **PPT-Pu**. Total applied volumes, 10 μ L per lane.

$T_m = 53$ °C and from the melting profile (not shown) that the triplex was the largely predominant species at the equilibrium at 22 °C, we may assume that the fluorescence signals recorded at the end of the two experiments are those of the intercalated chromophore. The quenching due to intercalation was 40% with **DNA-1** and 29% with **DNA-2**; this behavior reflects the known property of the daunomycin chromophore to modulate its fluorescence yield according to the structure of the intercalating site (5).

In summary, the above experimental findings demonstrate that, in the case of the *hMDR1* target, the conjugates between suitable derivatives containing the daunorubicin chromophore and a polypyrimidinic oligonucleotide containing methylated cytidines form triple helices which are stable at physiological pH; the high increase in thermal stability (+30 °C) with respect to the underivatized TFO is due to intercalation of the tagged aglycon moiety.

Part 2: The HIV-1 PPT Target. Since we demonstrated that daunomycinone conjugates were able to stabilize a triplex formed with a duplex featuring a TpT step at the 5' side of the purinic *hMDR1* region, we turned to the targeting of the PPT region of HIV-1. We

Table 3. Oligonucleotides (ODNs) Synthesized and Used in the HIV Work (Boldface C indicates 5-Me dC)

ODN	entry code
5' TTT TCT TTT CC 3'	C2
5' TTT TCT TTT CCC CCC T 3'	C6
5' TTT TCT TTT GGG GGG T 3'	G6
5' TT TTT AAA AGA AAA GGG GGG ACT GG 3'	PPT-Py
3' AA AAA TTT TCT TTT CCC CCC TGA CC 5'	PPT-Pu

used as TFOs oligomers **C2**, **C6**, and **G6**, whose sequence is reported in Table 3 together with that of the target duplex. According to previous literature data (3), acridine-oligonucleotide conjugates with very similar structures selectively blocked RNA polymerase at this target HIV-PPT region in a transcription elongation experiment.

The base sequence of **C2** and **C6**, alternating normal and methylated cytidines, was designed to avoid the destabilization due to the presence of a row of protonated, self-repelling, 5-methylcytidines, particularly in the case of **C6** (6).

The attempt to select the best candidate for triplex formation at neutral pH, among the three unconjugated TFOs, did not lead to any conclusion, as gel electrophore-

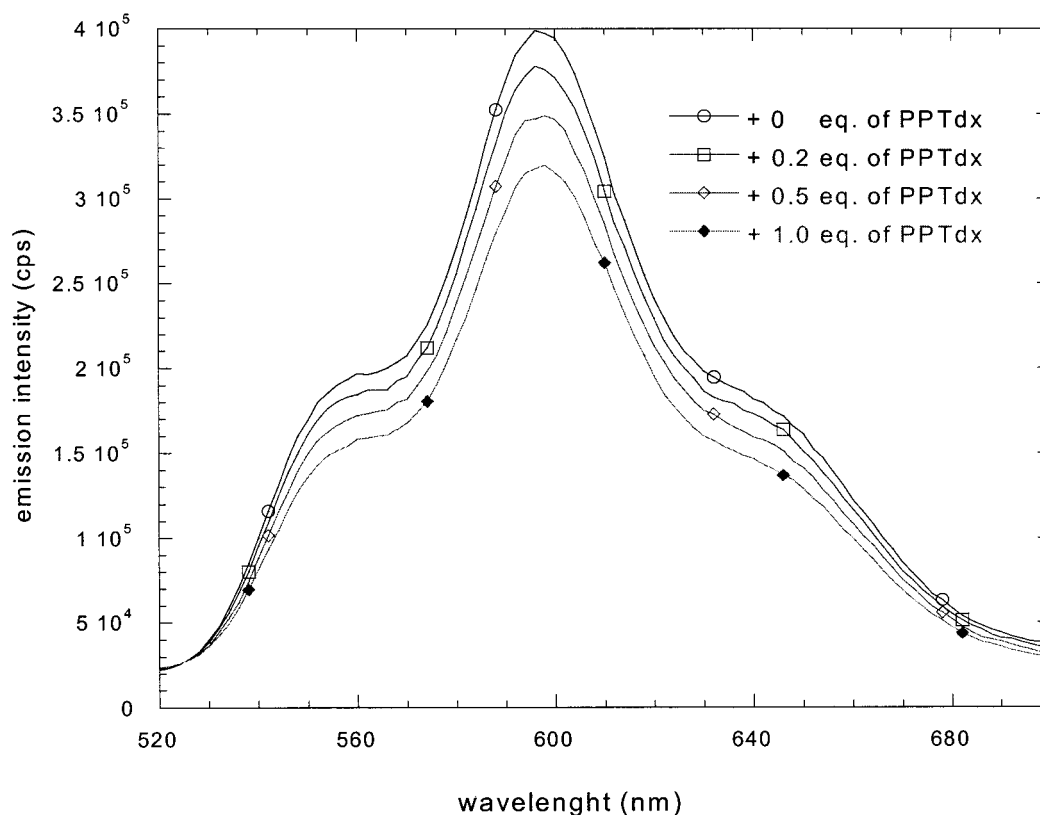


Figure 5. Quenching of the fluorescence of conjugate **C2-A** upon addition of PPT duplex. Measurements were recorded in a buffer containing 100 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, and 2 μ M conjugates.

sis at low temperature failed to disclose the formation of a retarded species between these ODNs and the target PPT-duplex (**PPT-dx**). In the case of **G6**, the experiment was also hampered by the self-aggregation of this oligomer at the concentration used to load the gel (10 μ M). [This same sequence had been reported to form a triple helix with $T_m = 34$ °C at pH 6.2 and at lower concentration (3)]. Hence, all three oligonucleotides were used for the preparation of conjugates of series **A** and **B**, following the already reported procedure with minor modifications (2).

During HPLC purification of the final products, we observed, by RP-18 HPLC analysis of concentrated solutions at neutral pH, the formation of easily reversible aggregates with both **C6-A** and **C6-B**, possibly due to the presence of a "i-motif" (7, 8). These aggregates spontaneously disappeared at lower concentrations or at basic pH, and did not affect the binding experiments. On the contrary, during the purification of crude mixtures of **G6-A** and **G6-B**, we faced severe problems of conjugate aggregation with themselves and with the starting **G6**, making it practically impossible to isolate the desired product with the needed quality (Figure 3).

The formation of triple helices between the **C2** and **C6** conjugates of general structure **A** and **B** with the **PPT-dx** was investigated at 10 μ M concentrations of each partner by electrophoretic retardation experiments at 15 °C and pH 7.0. The results are shown in Figure 4. All four gels show that, upon increasing the amount of conjugate added, the duplex spot is less and less visible and a retarded product is formed in increasing amounts. With the **C2** conjugates, the retarded spot, corresponding to the triplex, is very close to that of the duplex, reflecting the small mass difference between the 50 base duplex and the 61 base triplex. Accordingly, the retardation is slightly improved with the **C6** conjugates. Staining was

used to detect the species present in the gel; thus, only a qualitative estimate of the binding phenomenon can be made, with the indication that, at pH 7, at 15 °C and 10 μ M concentrations, at least 50% of the duplex is converted into the triplex upon addition of 1.5 equiv of the conjugates. In all four cases, UV-melting experiments from 5 to 80 °C failed to reveal the biphasic profile that allows the assessment of the thermal stability of the triple-helical complex. Hence, the gel electrophoresis experiments were repeated at 36 °C, but again failed to show any evidence of retarded species with any of the conjugates. To prove that the retarded species detected at 15 °C were, indeed, the triplexes formed by the conjugates with the target duplex, we used fluorescence spectroscopy. In all cases, the intensity of the fluorescence signal of the conjugate decreased following the addition of increasing amounts of the duplex, as expected because of the intercalation of the chromophore moiety of the conjugate within the triple-helical complex. The results obtained with conjugate **C2-A** are shown in Figure 5. Furthermore, the initial fluorescence values were restored when, at the end of the "titrations" with the duplex, the pH of the solution was brought to 8.2, i.e., a condition that is incompatible with the existence of triplexes formed by cytidine-containing TFOs.

In summary, the experimental results for the HIV-1 PPT target indicated that conjugation of the anthracycline aglycons with (T,C)-containing TFOs (**C2** and **C6**) significantly enhances their binding affinity for the target, even though further stabilization will be required to obtain triple helices sufficiently stable under physiological conditions. Conjugation of (T,G)-containing TFOs with anthracycline derivatives strongly promotes self-association, at least with the **G6** conjugates, thus preventing their recognition of the PPT region.

ACKNOWLEDGMENT

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Different Strategies for Formation of PEGylated EGF-Conjugated PEI/DNA Complexes for Targeted Gene Delivery

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With the aim of generating gene delivery systems for tumor targeting, we have synthesized a conjugate consisting of polyethylenimine (PEI) covalently modified with epidermal growth factor (EGF) peptides. Transfection efficiency of the conjugate was evaluated and compared to native PEI in three tumor cell lines: KB epidermoid carcinoma cells, CMT-93 rectum carcinoma cells, and Renca-EGFR renal carcinoma cells. Depending on the tumor cell line, incorporation of EGF resulted in an up to 300-fold increased transfection efficiency. This ligand-mediated enhancement and competition with free EGF strongly suggested uptake of the complexes through the EGF receptor-mediated endocytosis pathway. Shielded particles being crucial for systemic gene delivery, we studied the effect of covalent surface modification of EGF–PEI/DNA complexes with a poly(ethylene glycol) (PEG) derivative. An alternative way for the formation of PEGylated EGF-containing complexes was also evaluated where EGF was projected away from PEI/DNA core complexes through a PEG linker. Both strategies led to shielded particles still able to efficiently transfect tumor cells in a receptor-dependent fashion. These PEGylated EGF-containing complexes were 10- to 100-fold more efficient than PEGylated complexes without EGF.

INTRODUCTION

Cancer gene therapy by systemic gene delivery would be a most attractive way for treatment of cancer. Approaching the tumor via blood circulation should allow several advantages over the local intratumoral injection such as reaching multiple distant metastases. Unfortunately, the required tumor-targeted gene delivery system does not exist yet. An ideal gene delivery system has to fulfill several criteria to access tumor tissue: (1) ligands able to mediate cell specific recognition and internalization into target cells must be incorporated; (2) the gene delivery system must be inert against nonspecific interactions with the biological environment such as blood components and nontarget cells.

Synthetic gene delivery systems have recently attracted much attention particularly those based on polyethylenimine (PEI) polymers. Native PEI polymers of various molecular weights and topological isomers have been shown to be efficient for transfection of eukaryotic cells (1–4), and receptor-mediated endocytosis was achieved by chemical modification of PEI backbone with ligands such as sugars (5–8), peptides (9), and proteins (10). PEI polymers have also been used for gene delivery in vivo (1, 11–17) and particularly for systemic delivery to the lung (18, 19). However, unspecific interactions of the DNA complexes with blood components and nontarget cells strongly reduce the applicability of complexes. Recently, our group reported tumor-targeted gene delivery based on transferrin–PEI conjugate (20, 21).

Surface modification of transferrin-containing complexes with hydrophilic poly(ethylene glycol) (PEG) polymers strongly reduced interactions with blood components and prolonged circulation in blood after systemic delivery. Moreover, PEGylated transferrin-containing complexes mediated gene expression in distant tumor and showed reduced tropism for the lung compared to nonmodified PEI/DNA complexes.

Epithelial growth factor (EGF) receptor is an attractive therapeutic target for tumor targeting because it is overexpressed in a high percentage of human carcinomas including glioblastoma and lung, liver, breast, head, neck, and bladder cancers (22). The natural EGF ligand is a 53-residue polypeptide that binds specifically and with high affinity (23) to the EGF receptor. The binding of EGF triggers the dimerization of the receptor and the clustering of EGF–EGF receptor into coated pits that are internalized.

With the aim of specific gene delivery to EGF receptor-bearing tumor cells we synthesized a EGF–PEI conjugate and tested its ability to deliver DNA in three carcinoma cell lines. Keeping in mind future systemic gene delivery, the effect of PEGylation of preformed EGF-containing complexes was studied. We also describe an alternative novel way to generate PEGylated ligand-containing complexes where ligands are projected away from core complexes through a PEG linker.

EXPERIMENTAL PROCEDURES

Chemicals. Branched polyethylenimine with an average molecular weight of 25 kDa (PEI 25K) was obtained from Aldrich (Vienna, Austria). For complex preparation, PEI 25K was diluted with water, neutralized with HCl solution, and used as a 0.43 mg/mL working solution (10 mM of amine functions, assuming a MW of 43 Da for the repeating unit). Linear polyethylenimine

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(PEI 22K), kindly provided by Jean-Paul Behr (Faculté de Pharmacie, Illkirch, France) and commercially available from Euromedex (Exgen 500, Euromedex, Souffelweyersheim, France), was also used as a neutral 10 mM working solution. Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was purchased from Fluka (Buchs, Switzerland); chloroquine, 1,4-dithio-L-threitol (DTT), bovine serum albumin (BSA) from Sigma (St. Louis, MO); α -vinyl sulfone- ω -N-hydroxy succinimide ester poly(ethylene glycol) (VS-PEG-NHS, MW 3400), α -maleimide- ω -N-hydroxysuccinimide ester poly(ethylene glycol) (MAL-PEG-NHS, MW 3400) from Shearwater Polymers (Huntsville, AL); mouse epidermal growth factor (mEGF) from Serotec (Oxford, England); cell culture media, antibiotics, foetal calf serum (FCS), Geneticin (G418-sulfate) from Life Technologies (Gaithersburg, MD). Plasmid pCMVLuc (Photinus pyralis luciferase under control of the CMV enhancer/promoter) described in Plank et al. (24) was purified with the EndoFree Plasmid Kit from Qiagen (Hilden, Germany).

Synthesis of Thiol-Functionalized PEI 25K. PEI 25K was diluted in water, neutralized with 32% aqueous hydrochloric and subjected to gel filtration as described previously (10). To 50 mg of PEI 25K (1.16 mmol of amine functions, quantified by ninhydrine assay at 570 nm (25)) in 758 μ L was added 394 μ L of a 20 mM ethanolic solution of SPDP (2.5 mg, 7.9 μ mol). The reaction was carried out for 1 h at room temperature. Low molecular weight products were removed by gel filtration on a Sephadex G25 superfine (Pharmacia, Uppsala, Sweden; 300 $\times$ 10 mm column) in 0.25 M NaCl, 20 mM Hepes, pH 7.3. After dialysis and concentration under vacuum, a 720 μ L solution containing 0.97 mmol of PEI monomer (41.5 mg) with 4.2 μ mol of pyridyldithiopropionate linker (PDP; quantified spectrophotometrically at 343 nm by release of thiopyridone after reduction of an aliquot with excess DTT (26)) was obtained (i.e., ca. 2.5 PDP groups per PEI molecule). Half of this solution (20.7 mg PEI, 0.48 mmol of amine functions) was mixed under argon with an excess of DTT (73 μ mol) and was left to react for 1 h at room temperature. For removal of the low molecular weight products an additional gel filtration on a Sephadex G25 superfine (0.25 M NaCl, 20 mM Hepes pH 7.3; 100 $\times$ 10 mm) was performed yielding 1.5 mL of thiol-functionalized PEI 25K that contained 242 μ mol of PEI monomer (10.4 mg) modified with 1.25 μ mol of thiol functions (quantified by Ellman assay at 412 nm (27)); i.e., ca. three thiol groups per PEI molecule.

Synthesis of PDP- and Thiol-Functionalized mEGF. A solution of 4 mg mEGF (0.66 μ mol) in 1 mL of 16 mM Hepes buffer pH 7.9 was mixed with 3.1 mg of SPDP (10 μ mol) in 0.5 mL of ethanol. The reaction and the purification steps (see below) were carried out in the presence of ethanol (20–50%) to avoid precipitation of the PDP-functionalized product. The reaction was left to react for 3 h at room temperature. The functionalized peptide was purified by either dialysis (MWCO 1000; Spectra Por, Spectrum, Houston, TX) against 50% aqueous ethanol or gel filtration on a Sephadex G10 (Pharmacia, Uppsala, Sweden; 100 $\times$ 10 mm column) in 20 mM Hepes pH 7.3/20% ethanol.

The purified conjugate contained about 0.9 PDP group per mEGF peptide ($\pm$ 10%, depending on the preparation).

Thiol-functionalized mEGF (mEGF-SH) was obtained by reduction of an aliquot of mEGF-PDP (at a concentration of about 50 μ M) with DTT (1 mM) for 1 h under argon. Low molecular weight products were removed by gel filtration on a Sephadex G25 PD-10 column (Pharmacia, Uppsala, Sweden) in 20 mM Hepes pH 7.3/30%

ethanol. Quantification of thiol functions with Ellman assay indicated that the conjugate contained about one thiol group per mEGF peptide ($\pm$ 15%).

Synthesis of mEGF-PEI 25K Conjugate. Two solutions of PDP-functionalized mEGF were pooled, and 2 mL of the resulting solution that contained 4.2 mg EGF (0.69 μ mol) was modified with 0.56 μ mol of pyridyldithiopropionate linker was mixed under argon with 7.5 mg of thiol-functionalized PEI 25K (174 μ mol of amine functions, 0.9 μ mol thiol functions) in 1.08 mL of 0.25 M NaCl, 20 mM Hepes, pH 7.9. After 1 day at room temperature, the reaction mixture was loaded on a cation-exchange column (Macro-Prep High S, BioRad, München, Germany; 100 $\times$ 10 mm column) and was fractionated with a salt gradient from 0.5 to 3.0 M sodium chloride with a constant content of 20 mM Hepes, pH 7.3. The major amount of conjugate eluted between 2.7 and 3.0 M salt and was pooled. After dialysis against HBS (MWCO 6000–8000; Gibco BRL, Gaithersburg, MD) and concentration under vacuum, 6.5 mL of mEGF-PEI solution was obtained that contained 148 μ mol PEI of monomer (6.4 mg) modified with 0.32 μ mol of mEGF peptide (2.0 mg); i.e., approximately 1.3 mEGF peptides per PEI molecule. Before use, EGF conjugate samples were diluted to 10 mM PEI monomer.

Formation of PEI/DNA, mEGF-PEI/DNA, and PEGylated mEGF-PEI/DNA Complexes. DNA complexes used for the transfection of KB and Renca-EGFR cells were prepared as described by Boussif et al. (1) with some modifications. Briefly, 4 μ g of pCMVLuc plasmid (12 nmol of phosphate) and 7.2 μ L of a 10 mM PEI or EGF-PEI (72 nmol of amine functions) were each diluted in 100 μ L of the indicated buffer: HBS (Hepes buffered saline: 145 mM NaCl/20 mM Hepes pH 7.3), HBS 1/2 (HBS twice diluted with water) or 5 mM Hepes pH 7.3, and mixed together. After 15 min incubation an aliquot of the complex solution was added to the cells (100 μ L/well for example corresponding to 2 μ g DNA/well).

Complexes that were subsequently PEGylated were prepared in advance. Fifteen minutes after mixing DNA and polymer solutions, 0.6 μ L of a 6 mM VS-PEG-NHS (or MAL-PEG-NHS) stock solution in DMSO (3.6 nmol) was added and left to react overnight.

For titration of different N/P ratios on CMT-93 cells mEGF-PEI/DNA or PEI/DNA complexes were prepared by mixing 5 μ g of pCMVLuc plasmid, prediluted in 250 μ L of HBS, with different amounts of mEGF-PEI 25K or PEI 25K prediluted in 250 μ L of HBS (2.25 μ g of PEI corresponding for example at N/P 3.6).

Sequential Addition of Bifunctional PEG and mEGF-SH to PEI/DNA Complexes. pCMVLuc plasmid (20 μ g, 60 nmol of phosphate) and PEI (360 nmol of amine) were each diluted in 0.5 mL of the indicated buffer previously saturated with argon. The two solutions were gathered and mixed with argon stream. The mixture was kept under argon until the end of the protocol. After 15 min, 3 μ L of a 6 mM VS-PEG-NHS (or MAL-PEG-NHS) stock solution in DMSO (18 nmol) were added. Two hours later, various amounts of EGF-SH (between 7.2 and 144 pmol; as indicated in the Figure legend) were added to 200 μ L aliquots of the complex solution and left to react overnight.

Cells and Cell Culture. KB human epidermoid carcinoma cells (ATCC CCL-17) and CMT-93 murine rectum carcinoma cells (ATCC CCL-223) were cultured in DMEM medium supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/mL streptomycin. Renca mouse renal carcinoma cells stably cotransfected with the plasmids pLTR-EGFR and pSV2neo (28)

(kindly provided by Winfried Wels, Georg-Speyer-Haus, Frankfurt am Main, Germany), named Renca-EGFR, were grown in RPMI-1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.5 mg/mL Geneticin. All cell lines were maintained at 37 °C in a 5% CO₂ humidified atmosphere.

Transfection. One day before transfection, KB and Renca-EGFR cells were seeded at 4×10^4 cells per well in 24-well dishes. Prior to transfection, medium was removed and replaced with 1 mL (or as indicated in Figure legend) of fresh culture medium complemented with 10% FCS. Transfection complexes were added to the cells, and after 4 h incubation, transfection medium was removed and replaced by 1 mL fresh culture medium.

Murine CMT-93 cells were seeded at 5×10^5 cells per 25 cm² tissue culture flask 1 day before transfection. Prior to transfection, medium was removed and replaced by 2 mL of fresh culture medium complemented with 10% FCS. Transfection complexes (in 0.5 mL of HBS) were added to the cells. After 4 h incubation, transfection medium was removed and replaced by fresh culture medium.

The cells were grown for additional 20 h and then washed in PBS and lysed (0.25 M Tris·HCl pH 7.4, 0.1% Triton X-100). Luciferase activity and protein concentration were determined from aliquot of the lysate using a luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany) and Bio-Rad protein assay (Bio-Rad, Hercules, CA), respectively. Values are given as light units integrated over 10 s per mg of protein.

RESULTS

Synthesis of mEGF-PEI 25K Conjugate. mEGF was covalently coupled to the PEI backbone through a disulfide bridge as described in Experimental Procedures. Murine EGF can bind both the murine and human EGF receptor (29) and was chosen because it lacks lysine residues in its primary sequence and can react with SPDP-activated ester only through the N-terminus. It has been shown that the derivatization of this free amino group had little effect on EGF receptor binding compared to native EGF (30). Modification of this amino group with the heterobifunctional cross-linker SPDP (26) rendered the peptide poorly soluble, and ethanol had to be present in the reaction and purification steps. The reaction was carried out with an excess of SPDP (15 molar equiv excess) and led to a mEGF-PDP conjugate containing almost one linker per peptide. PEI with an average molecular weight of 25 kDa was also modified with SPDP and then treated with an excess of DTT. A thiol-functionalized PEI conjugate that contained approximately three thiol groups per PEI molecule was obtained. Reaction between thiol-functionalized PEI and mEGF-PDP followed by purification by cation-exchange chromatography resulted in a mEGF-PEI conjugate that contained 1.3 mEGF peptides per PEI molecule.

EGF Receptor-Mediated Gene Transfer in Various Carcinoma Cell Lines. Transfection efficiency of mEGF-PEI 25K-containing DNA complexes was compared with EGF-free PEI 25K/DNA complexes in three carcinoma cell lines (Figure 1). Formulation of complexes in three different Hepes buffers containing different concentrations of NaCl (Hepes: 0 mM, HBS 1/2: 75 mM and HBS: 150 mM) at a PEI-nitrogen to DNA-phosphate ratio of 6 (N/P = 6) resulted in complexes of various sizes (Table 1) which were tested in transfection experiments with Renca-EGFR cells (Figure 1A) and KB cells (Figure

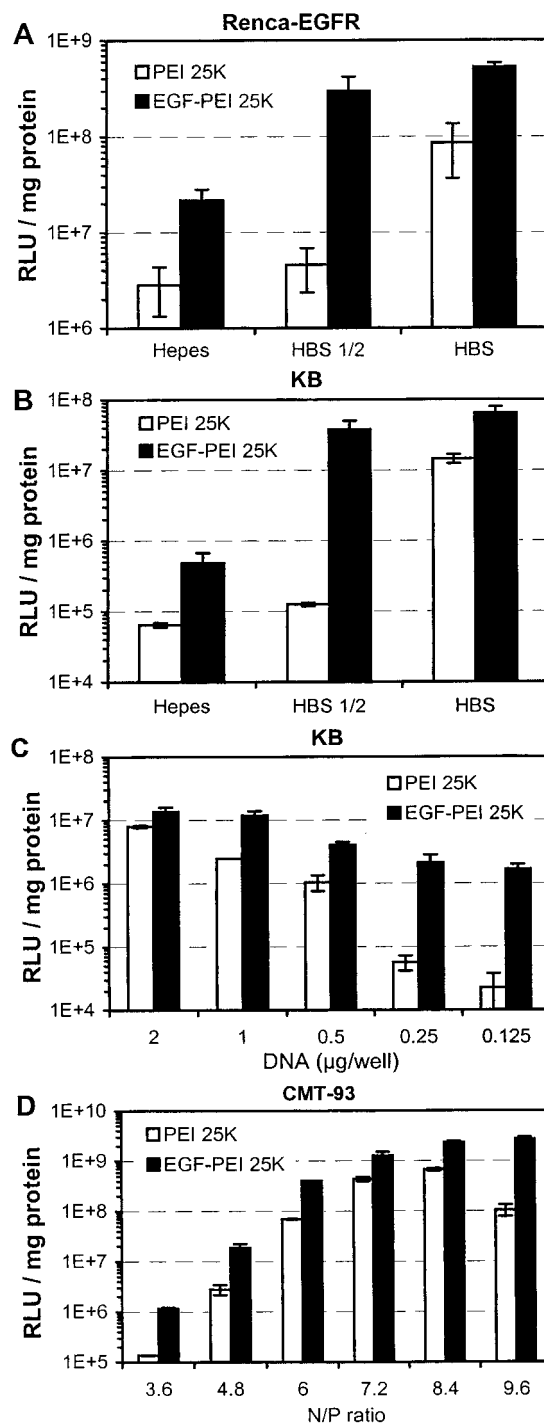


Figure 1. EGF-mediated gene transfer to Renca-EGFR mouse renal, KB human epidermoid and CMT-93 murine rectum carcinoma cells. Renca-EGFR (A) and KB cells (B) were transfected with pCMVLuc plasmid (2 μ g/well) complexed with PEI 25K or mEGF-PEI 25K/PEI 25K (1:1) at a PEI (nitrogen)/DNA (phosphate) ratio of 6 (N/P = 6) in either 5 mM Hepes pH 7.3, HBS 1/2 or HBS. After 4 h, transfection medium was replaced with fresh culture medium and cells were grown for additional 20 h. Luciferase activity was measured as described in Experimental Procedures. Values are the mean $\pm$ SD of two independent experiments made in duplicate. (C) KB cells were transfected with various amounts of pCMVLuc plasmid (2 to 0.125 μ g per well) complexed with PEI 25K or mEGF-PEI 25K/PEI 25K (1:1) at N/P = 6 in HBS. Luciferase activity is the mean $\pm$ SD of two independent experiments made in duplicate. (D) CMT-93 cells were transfected with pCMVLuc plasmid (5 μ g/flask) complexed with various amounts of PEI 25K or EGF-PEI 25K (at indicated N/P ratios) in HBS. Luciferase activity is shown as mean $\pm$ SD of duplicate.

Table 1. Biophysical Characteristics of DNA Complexes and Their PEGylated Derivatives^a

	size (nm)			zeta (mV) Hepes
	Hepes	HBS 1/2	HBS	
PEI 25K	57 ± 14	116 ± 36	478 ± 111	+20.0 ± 1.7
PEI 25K + MAL-PEG-NHS	47 ± 11	96 ± 30	446 ± 118	-0.7 ± 1.1
PEI 25K + MAL-PEG-NHS + EGF-SH	49 ± 15	81 ± 23	403 ± 118	-0.6 ± 1.4
EGF-PEI 25K/PEI 25K	53 ± 12	447 ± 82	590 ± 132	+21.5 ± 2.3
EGF-PEI 25K/PEI 25K + MAL-PEG-NHS	48 ± 14	553 ± 128	> 1 μm	-0.6 ± 1.0
PEI 25K/PEI 22K	43 ± 11	~ 1 μm	~ 1 μm	+28.0 ± 2.5
PEI 25K/PEI 22K + MAL-PEG-NHS	42 ± 10	> 1 μm	> 1 μm	-0.1 ± 1.4

^a Complexes were prepared as described in Experimental Procedures in 1 mL total volume of the indicated buffer containing 20 μg DNA (for zeta potential measurement of PEI 25K/PEI 22K/DNA complexes DNA concentration was 50 μg/mL). Size and zeta potential of non-PEGylated and PEGylated complexes were measured 20 min and 18 h after their formation respectively, using a Zetasizer 3000HS (Malvern Instruments, Worcestershire, England). Three measurements were carried out per sample.

1B). EGF-containing DNA complexes showed a 5- to 300-fold increase in gene delivery efficiency compared to unmodified PEI/DNA complexes. This ligand-mediated enhancement was found for all formulations. Large complexes formulated in physiological salt concentration (HBS) were more efficient than small complexes prepared in salt-free medium (5 mM Hepes pH 7.3), similar as observed previously with other cell lines using transferrin-modified PEI/DNA complexes (31). Chemical ligation of mEGF to the PEI 25K backbone did not change the biophysical properties (size, zeta potential) of resulting complexes generated either in Hepes or HBS buffer (Table 1). This strongly suggests the expected biological involvement of EGF in the increase of gene expression. In contrast, in HBS 1/2, the difference of size between both type of complexes might also contribute to the improved gene expression.

Comparative transfection was also investigated with sub-micromolar amounts of DNA on KB cells (Figure 1C). Luciferase expression of PEI complexes generated in HBS dropped rapidly with the decrease of DNA amount while expression of EGF-PEI complexes remained relatively high.

Luciferase gene expression was examined as a function of the PEI-nitrogen to DNA-phosphate ratio used to generate the complexes. Gene expression in CMT-93 murine rectum carcinoma cells was strongly dependent on the N/P ratio (Figure 1D). Increasing the N/P ratio from 3.6 to 8.4 increased the efficiency of both EGF-containing and EGF-free DNA complexes by more than three orders of magnitude. There was a 3- to 30-fold ligand-mediated enhancement at all N/P ratios tested. With ligand-free complexes a drop in transfection efficacy was found at high N/P ratio correlating with some toxicity.

Small particles generated in salt-free medium led to low gene expression in comparison with large particles generated in HBS (Figures 1A and 1B). We tested whether the addition of an endosomolytic compound during the transfection could enhance the efficiency of small complexes. Therefore, the lysomotropic drug chloroquine was added during transfection in cell culture medium of Renca-EGFR cells at a concentration of 100 and 200 μM (Figure 2A). Addition of chloroquine resulted in a 100-fold increase of gene expression with both EGF-containing DNA complexes and EGF-free DNA complexes while the ligand-mediated enhancement remained approximately constant.

To test whether the EGF receptor was involved in EGF-PEI-mediated gene transfer, a competition assay was performed using small complexes formulated in salt-free medium. Addition of exogenous mEGF in cell culture medium during transfection of Renca-EGFR did not interfere with PEI/DNA receptor-independent gene trans-

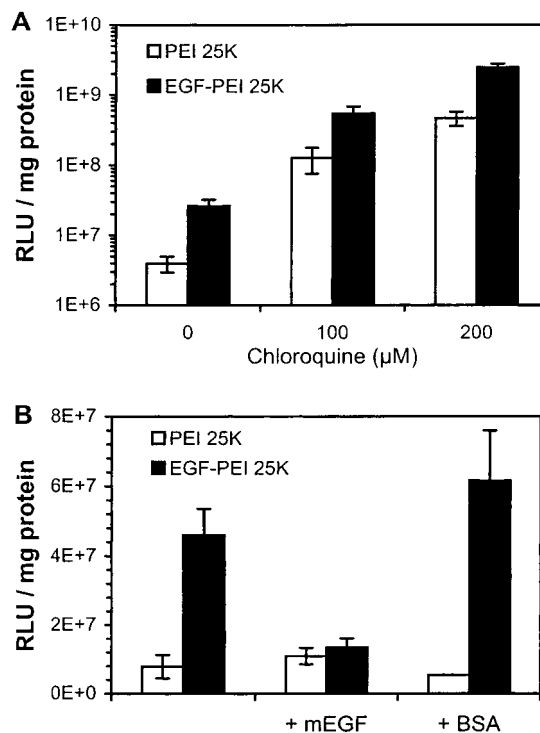


Figure 2. (A) Chloroquine strongly enhances transfection efficiency of small particles generated in salt free medium on Renca-EGFR cells. Renca-EGFR were transfected with pCMVLuc plasmid (2 μg/well) complexed with PEI 25K or mEGF-PEI 25K/PEI 25K (1:1) at N/P = 6 in Hepes. Transfection was carried out in the presence of 0, 100, or 200 μM chloroquine. After 4 h, transfection medium was replaced with fresh culture medium and cells were grown for additional 20 h. Luciferase activity values are the mean ± SD of two independent experiments made in duplicate. (B) Competitive inhibition of EGF receptor-mediated gene transfer on Renca-EGFR cells. Renca-EGFR cells incubated in 0.5 mL of fresh culture medium containing 200 μM chloroquine were transfected with pCMVLuc plasmid (1 μg/well) complexed with PEI 25K or mEGF-PEI 25K/PEI 25K (1:1) at N/P = 6 in Hepes. For the inhibition and the control experiments transfection was carried out in the presence of free mEGF (12 μg/well) and BSA (12 μg/well), respectively. After 4 h, transfection medium was replaced with fresh medium and cells were grown for additional 20 h. Luciferase activity is shown as mean ± SD of duplicate.

fer (Figure 2B). However, competition of EGF-containing DNA complexes with free mEGF (100 molar equiv excess) decreased gene transfer efficacy down to nearly the values found for the receptor-independent transfection. On the other hand, addition of BSA, a protein that does not interact with EGFR, did not significantly affected gene transfer efficiency of both type of complexes. Taken together, competitive and comparative transfection ex-

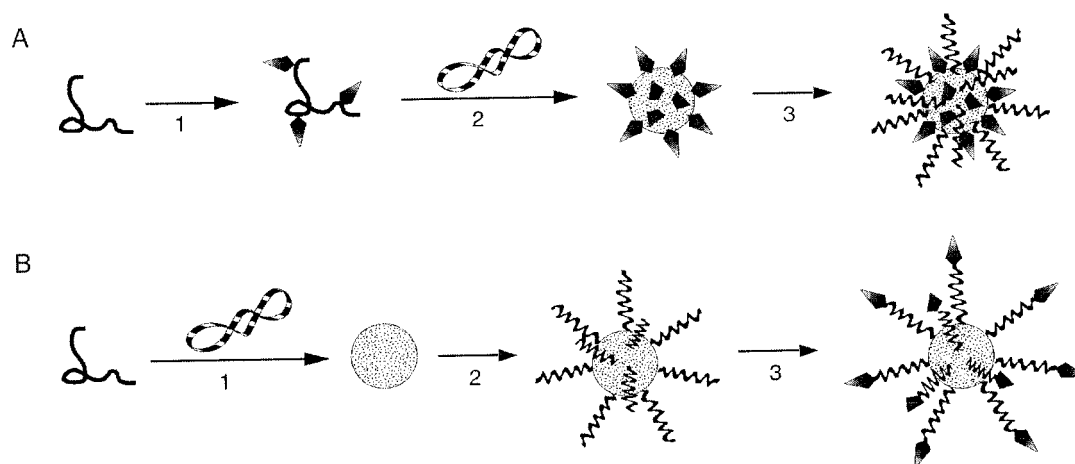


Figure 3. Schematic presentation of alternatives for the formation of PEGylated ligand-containing DNA complexes. Strategy A: A ligand is covalently conjugated to a polycation (step 1). Condensation of DNA with this conjugate leads to ligand-containing DNA complexes (step 2) which are subsequently modified with a PEG derivative reacting with free amino groups of the polycation (step 3). Strategy B: DNA is condensed with a polycation (step 1), and the resulting complexes are modified by a heterobifunctional PEG which first reacts with amino groups of the polycation (step 2). Subsequently ligands are incorporated into the complexes by covalent conjugation with the distal end of the PEG (step 3). The latter strategy should allow a better accessibility of the grafted ligand for its receptor.

periments strongly suggested involvement of EGF receptor in gene delivery with EGF-PEI-based systems.

Transfection Efficiency of PEGylated EGF-Containing DNA Complexes. Targeted transfection particles for systemic gene delivery should have uncharged surface to avoid nonspecific electrostatic interaction with blood components and nontarget cells. On the other hand, DNA is usually efficiently condensed using an excess of polymer, giving rise to cationic particles. One way to overcome this dilemma is to covalently modify the surface of ligand-containing DNA complexes with an electro-neutral polymer such PEG (20) (Figure 3, strategy A). Coating the DNA particle with PEG shields its cationic surface and prevents interaction with macromolecules by steric hindrance. On the other hand PEG potentially might also shield small ligands bound to the polymer and thus preventing to some extent the binding to the receptor on the target cell. As described in strategy B (Figure 3), incorporation of the ligand at the distal end of PEGylated PEI/DNA complexes should allow better accessibility of the ligand for its receptor and thus should increase cellular internalization of DNA complexes.

These two strategies using EGF as ligand were evaluated with Renca-EGFR cells (Renca mouse renal carcinoma cells stably transfected with pLTR-EGFR plasmid carrying human EGF receptor cDNA (28)) and KB cells (human epidermoid carcinoma cells that endogenously express human EGF receptor). Transfection of Renca-EGFR was performed in the presence of chloroquine with small complexes formulated in salt free medium. It was previously observed that addition of chloroquine on Renca-EGFR cells increases considerably PEI-mediated gene transfection (Figure 2A), in contrast to KB cells where no beneficial effect is observed. PEGylation of PEI/DNA complexes dramatically decreased the zeta potential (which reflects the surface charge) close to the neutrality (Table 1) and was accompanied by a reduction of gene expression (Figure 4A). Addition of increasing amounts of thiol-functionalized mEGF to PEGylated complexes without changing their biophysical properties (size, zeta potential) increased gene transfection by two-orders of magnitude. In contrast, addition of the functionalized ligand to PEI/DNA complexes without reactive PEG linker had no effect. This finding strongly suggested that

EGF binding at the distal end of the PEG allows the delivery of PEGylated complexes in a ligand-dependent manner. We compared this strategy with the one previously developed in our group (20) (Figure 3, strategy A). As previously observed, EGF-PEI conjugate-containing DNA complexes were more efficient than those prepared with native PEI 25K. PEGylation of EGF-containing DNA complexes decreased the zeta potential close to the neutrality (Table 1) and also gene expression. This PEGylated complexes were still about 5-fold more effective than EGF-free PEI/DNA complexes but about one order less efficient than PEGylated complexes obtained according to strategy B (Figure 3).

The two strategies were also used for the delivery of PEGylated EGF-containing DNA complexes in KB cells in the absence of chloroquine (Figure 4B). Subsequent addition of PEG and thiol-functionalized EGF to PEI/DNA complexes generated in HBS 1/2 increased their efficiency by more than one order of magnitude. Complexes resulting from the PEGylation of EGF-PEI conjugate-containing DNA complexes showed approximately the same level of gene expression.

Transfection with Different PEI Polymers Complexes and Their PEGylated Derivatives. Among all available PEI, which differ by their average molecular weight and topology, linear PEI 22K had shown the most promising properties to deliver DNA (4, 18). We were interested to know whether the combination of PEI 22K with mEGF-PEI 25K could enhance the ligand-mediated gene delivery in our in vitro cell models. KB cells were transfected with various amount of DNA complexed with PEI 25K, EGF-PEI 25K/PEI 25K (1:1), PEI 25K/PEI 22K (1:1), or EGF-PEI 25K/PEI 22K (1:1) in HBS 1/2 at N/P = 6 (Figure 5A). Incorporation of PEI 22K in the preparation of ligand and ligand-free PEI 25K/DNA complexes strongly increased gene expression especially at low DNA amounts. At 60 ng of DNA, PEI 22K-containing complexes remained efficient (10^7 – 10^8 RLU/mg) whereas PEI 22K-free complexes showed only low activity. Interestingly, a ligand-dependent enhancement was observed with PEI 22K-containing complexes on KB cells but also on Renca-EGFR cells independently of the medium in which the complexes were prepared (Figure 5B).

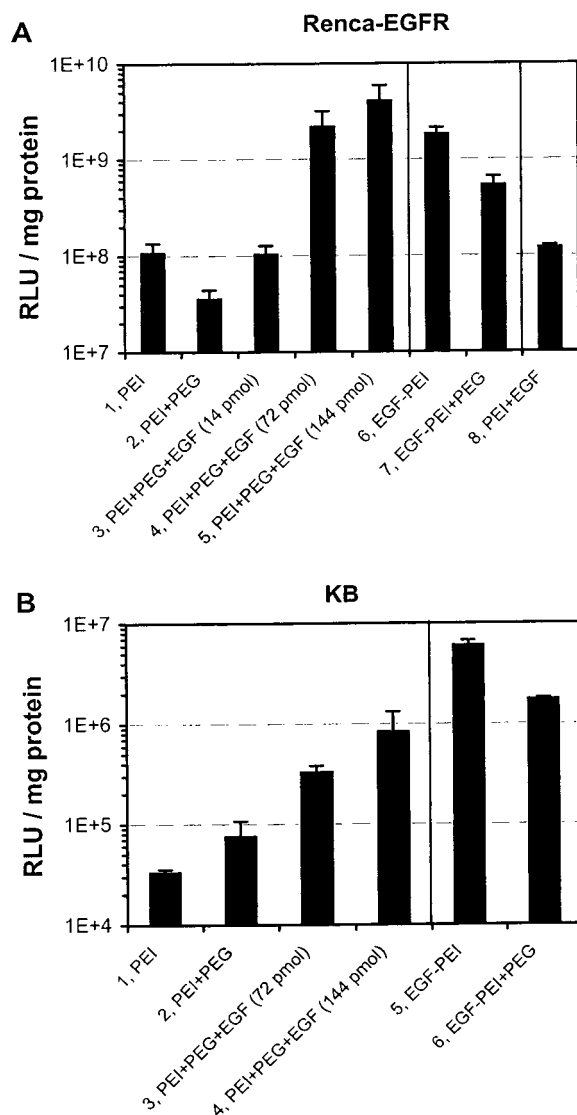


Figure 4. Transfection efficiency of PEGylated EGF-containing DNA complexes on Renca-EGFR and KB cells. (A) Renca-EGFR cells incubated in fresh culture medium containing 200 μ M chloroquine were transfected with the following complexes generated as described in Experimental Procedures in HEPES buffer at a N/P ratio of 6 (2 μ g DNA/well). Column 1: PEI 25K/DNA; 2: PEI 25K/DNA modified with VS-PEG-NHS; 3, 4, and 5: as in 2 and subsequently modified with 14, 72, and 144 pmol of thiol-functionalized mEGF, respectively; 6: mEGF-PEI 25K/PEI 25K (1:1)/DNA; 7: mEGF-PEI 25K/PEI 25K (1:1)/DNA modified with VS-PEG-NHS; 8: as in 1 in the presence of 144 pmol mEGF in culture medium during the transfection. Luciferase activity values are the mean $\pm$ SD of two independent experiments made in duplicate. (B) KB cells incubated in fresh culture medium were transfected with the following complexes prepared in HBS 1/2 at a N/P ratio of 6 (2 μ g DNA/well). Column 1: PEI 25K/DNA; 2: PEI 25K/DNA modified with MAL-PEG-NHS; 3 and 4: as in 2 and subsequently modified with 72 pmol and 144 pmol of thiol-functionalized mEGF, respectively; 5: EGF-PEI 25K/PEI 25K (1:1)/DNA; 6: EGF-PEI 25K/PEI 25K (1:1)/DNA modified with MAL-PEG-NHS. Luciferase activity is shown as mean $\pm$ SD of duplicate.

Transfection efficiency of PEGylated EGF-containing PEI 25K/PEI 22K complexes was studied in KB cells (Figure 6). As observed previously with PEI 22K-free complexes (Figure 4B), subsequent addition of PEG and thiol-functionalized EGF to PEI/DNA complexes strongly increased transfection efficiency to a level comparable to the one obtained by PEGylation of EGF-containing DNA complexes.

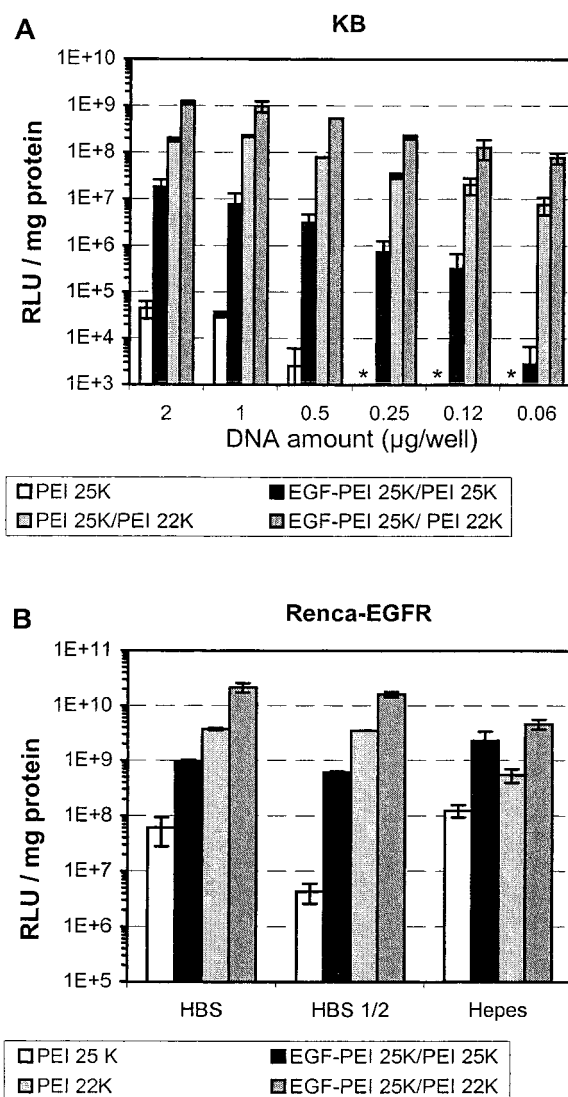


Figure 5. Effect of different PEI polymers (linear PEI 22K versus branched PEI 25K) on transfection efficiency. Complexes were generated from a mixture of mEGF-PEI 25K and different PEIs and tested on KB and Renca-EGFR cells. (A) KB cells were transfected with various amounts of pCMVLuc plasmid (2 to 0.06 μ g per well) complexed with PEI 25K, mEGF-PEI 25K/PEI 25K (1:1), PEI 25K/PEI 22K (1:1) or mEGF-PEI 25K/PEI 22K (1:1) at N/P=6 in HBS 1/2. Luciferase activity is shown as mean $\pm$ s.d. of duplicate (* is $<10^3$ RLU/mg of protein). (B) Renca-EGFR cells were transfected with pCMVLuc plasmid (2 μ g/well) complexed with PEI 25K, mEGF-PEI 25K/PEI 25K (1:1), PEI 22K or mEGF-PEI 25K/PEI 22K (1:1) at N/P = 6 in either Hepes buffer (in the presence of 200 μ M chloroquine in cell culture medium during the transfection), HBS 1/2 or HBS. Luciferase activity is shown as mean $\pm$ SD of duplicate.

DISCUSSION

Our aim is to develop synthetic gene delivery systems that can be administered by intravenous application. One important aspect for the construction of such systems is the use of a convenient ligand able to mediate cell specific recognition and internalization into target cells. EGF receptor is overexpressed in many human tumors of epithelial origin and may therefore serve as a useful cell surface target (22). Gene delivery into EGF receptor-expressing cells was previously demonstrated using polylysine conjugates where receptor ligand (EGF or anti-EGF receptor antibody) and polylysine were covalently coupled (32, 33) or bound through a biotin/streptavidin linkage (34–36). Also other synthetic systems were

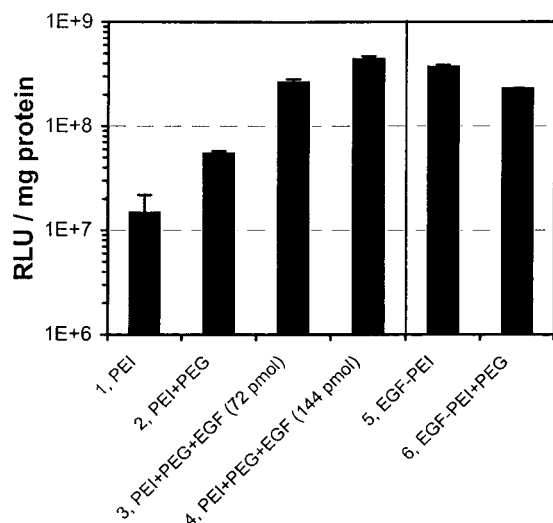


Figure 6. Transfection efficiency of PEGylated EGF-containing PEI 25K/PEI 22K complexes on KB cells. Complexes were prepared as described in Figure 4B with PEI 25K/PEI 22K (1:1) instead of PEI 25K (Columns 1–4) and EGF–PEI 25K/PEI 22K instead of EGF–PEI 25K/PEI 25K (Columns 5 and 6). Column 1: PEI 25K/PEI 22K/DNA; 2: PEI 25K/PEI 22K/DNA modified with MAL-PEG-NHS; 3 and 4: as in 2 and subsequently modified with 72 pmol and 144 pmol of thiol-functionalized mEGF respectively; 5: EGF–PEI 25K/PEI 22K/DNA; 6: EGF–PEI 25K/PEI 22K/DNA modified with MAL-PEG-NHS. Luciferase activity is shown as mean $\pm$ SD of duplicate.

described including chimeric TGF- α fusion protein (37) and EGF-containing liposomes (38, 39). In these cases gene delivery in EGF receptor-expressing cells was effective only in the presence of an endosomolytic agent such as chloroquine or inactivated adenovirus. Chloroquine would be difficult to apply *in vivo* at the required dose due to its toxicity. The use of inactivated adenovirus could lead to undesired immune problems inherent of the virus.

Recently some new polycations such as PEI (1) and polyamidoamine dendrimers (40, 41) have been described as particularly efficient gene delivery vectors without the need for additional endosomolytic agents. These cationic polymers share with chloroquine a strong buffering capacity that promotes escape of the complexes out of the endosomal compartment (1, 42). We combined the powerful gene delivery activity of PEI with the specificity of EGF-receptor binding by generating an EGF–PEI conjugate able to deliver DNA into tumor cells with considerably higher efficiency compared to the native PEI complexes. When EGF was incorporated in polyplexes, 10-fold lower amounts of DNA complexes were required for similar efficiency. High transfection ability with low dose of DNA is essential for *in vivo* application. This ligand-mediated enhancement and competition experiments performed with free EGF strongly suggest that EGF-containing DNA complexes are taken up specifically by the EGF-receptor mediated endocytosis pathway. This is also supported by recent data from our group by FACS analysis (Ogris et al., submitted to *AAPS Pharm Sci.*) where enhanced uptake of EGF-containing complexes was demonstrated.

For *in vivo* administration, small DNA particles are required for escape out of normal blood vessels to reach surrounding tissues such as hepatocytes (43) and much effort is applied in this direction (44–46). Preparation of EGF-polyplexes in salt-free buffer generates small particles that, however, are relatively inefficient. These complexes are not limited by the EGF receptor uptake

process, but by ineffective endosomal release, as suggested by the large increase of transfection efficiency in the presence of chloroquine. This finding is in good agreement with previous observations using transferrin-PEI particles (31). For tumor-targeted gene delivery the DNA particle size is less problematic because of the leakiness of the tumor vasculature. Indeed accumulation of large DNA complexes (approximately 500 nm) was observed recently in our group (20, 21). Therefore, we evaluated also EGF–PEI complexes of medium-large size. Interestingly linear PEI 22K but also in mixture with PEI 25K (1:1) was more efficient to deliver DNA than the branched PEI 25K alone, particularly at low DNA doses. Indeed, gene expression obtained with 60 ng of DNA complexed with EGF–PEI 25K/PEI 22K was higher (10^8 RLU/mg protein) even compared to much higher amounts (2 μ g) of EGF–PEI 25K/PEI 25K complexes (Figure 5).

Gene delivery systems for intravenous application must be inert against nonspecific interactions with biological environment such as nontarget cells and blood components in order to maintain their physical integrity and to avoid rapid elimination via activation of the immune system. Covalent surface modification of pre-formed polyplexes with PEG has been shown to strongly reduce nonspecific interactions with erythrocytes and plasma proteins (20) and it also reduces complement system activation (47). Incorporation of PEG in polyplexes was also achieved using block copolymer as condensing agent such polylysine–polysaccharide (48), polylysine–PEG (49–54), and PEI–PEG (55, 56). Major drawback of this latter strategy is the crowding effect of the neutral polymer that can hinder proper DNA condensation in dense particles (49, 55).

We evaluated two post-PEGylation strategies to generate shielded EGF-containing complexes, where EGF was either coupled to PEI (Figure 3A) or to the distal end of a PEG spacer arm (Figure 3B). In the latter strategy the ligand is incorporated at the final step of complex preparation. Optimal amounts of ligand giving best transfection efficiencies can be rapidly found. The later strategy should also allow a better accessibility of the ligand as suggested by studies on liposomes. Indeed, it was shown that incorporation of PEG in immunoliposomes can substantially weaken antigen/antibody interaction (57, 58). In contrast, coupling the ligand to the distal end of the PEG produced stabilized liposomes still allowing efficient binding to the target receptor (59, 60). We showed that both types of shielded EGF-containing complexes were effective to deliver DNA. Efficiencies were approximately 10- to 100-fold higher compared to EGF-free PEGylated complexes. In contrast to the published liposome work, the beneficial effect of grafting the ligand at the distal end of PEG compared to coupling to PEI and PEGylation was less clear with polyplexes. With small complexes this strategy (Figure 3B) resulted in 8-fold higher gene expression than with strategy illustrated in Figure 3A, but with large complexes efficacies were nearly similar. This strongly suggests that the PEG covalently coupled to the surface of EGF-containing PEI/DNA complexes only weakly interferes with the binding to the EGF-receptor. This is quite surprising knowing that a brush of PEG (MW 5000) extending out of the surface of liposomes has been described to have a thickness of 10 nm (61). Nevertheless, this is in line with a recent finding by Ogris et al. (submitted to *AAPS Pharm Sci.*) suggesting that PEGylation does not reduce internalization of EGF-containing complexes.

In summary we have generated EGF-containing polyplexes able to efficiently transfect cells in a receptor-dependent fashion. PEGylated EGF-containing complexes maintained high efficiency and are interesting candidates for in vivo evaluation in tumor models.

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Mixed-Ligand Technetium(III) Complexes with Tetradentate/Monodentate NS₃/Isocyanide Coordination: A New Nonpolar Technetium Chelate System for the Design of Neutral and Lipophilic Complexes Stable in Vivo

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Starting from the tripodal ligand 2,2',2''-nitrilotris(ethanethiol) (NS₃) and isocyanides (CNR) as co-ligands, neutral mixed-ligand technetium(III) complexes of the general formulation [Tc(NS₃)(CNR)] have been synthesized and characterized. The ⁹⁹Tc complexes can be obtained by a two-step reduction/substitution procedure starting from [TcO₄]⁻ via the phosphine-containing precursor complex [Tc(NS₃)(PMe₂Ph)]. As shown by X-ray structural analyses, the complexes adopt a nearly ideal trigonal-bipyramidal geometry with the trigonal plane formed by the three thiolate sulfurs of the tripodal ligand. The central nitrogen atom of the chelate ligand and the monodentate isocyanides occupy the apical positions. The no-carrier-added preparation of the corresponding ^{99m}Tc complexes was performed by a one-step procedure starting from ^{99m}[TcO₄]⁻ with stannous chloride as reducing agent. Biodistribution studies in the rat demonstrated for the nonpolar, lipophilic compounds a significant initial brain uptake. In vitro challenge experiments with glutathione clearly indicated that no transchelation reaction occurs. Furthermore, there were no indications for reoxidation of Tc(III) to Tc(V) species or pertechnetate. We propose this type of complexes as a useful tool in the design of lipophilic ^{99m}Tc or ¹⁸⁶Re/¹⁸⁸Re radiopharmaceuticals.

INTRODUCTION

Complexes of the γ -emitting nuclide technetium-99m find broad application in diagnostic nuclear medicine because of its optimal nuclide properties (1–4). To design new and broadly applicable “bioactive” Tc-99m radiopharmaceuticals able to be involved in metabolic processes or to bind to receptors, the radioactive metal has to be incorporated into biologically active molecules in form of chelates (5, 6). The most frequently used technetium compounds suitable for coupling the metal to biologically active molecules are square-pyramidal complexes of the oxo ion [Tc=O]³⁺ based on tetradentate N₂S₂ ligands (7, 8). In this context, the “3 + 1” mixed-ligand approach is of high interest too (9–11). Properties and thus the in vivo behavior of such complexes are strongly influenced by the presence of the quite polar [Tc=O]³⁺ unit. Whether such a polarity is beneficial or not is not obvious and depends on the requirements for specific radiotracers, e.g., for receptor targeting agents or for metabolic tracers. Another crucial point in tracer design is the stability of the respective Tc chelate toward ligand exchange in vivo. Recently, it was shown that the applicability of the “3 + 1” mixed-ligand Tc(V) chelates is limited because of their reaction with glutathione in vivo (12, 13). Thus, there is a considerable interest in alternative chelate systems that offer lower polarity and enhanced in vivo stability. Such systems are based on

oxo-free cores with the metal at lower oxidation states. Thus, Tc(I) carbonyl complexes resulting from the reaction of the organometallic Tc(I) aqua ion [Tc(OH)₂(CO)₃]⁺ as a precursor with either N-containing ligands, phosphines, or thioether ligands (14–19) are hopeful candidates for stable binding of Tc to peptides and other polar biomolecules.

Here we offer a new type of Tc(III) chelate formed by the tripodal 2,2',2''-nitrilotris(ethanethiol) and a monodentate isocyanide that should fulfill the requirements for a lipophilic, nonpolar building block stable against ligand exchange reaction in vivo. Part of the family of “n + 1” mixed-ligand technetium and rhenium species (20–22), such a “4 + 1” chelate offers the advantage of high versatility in conjugating biomolecules as described for the “3 + 1” series (23).

In the present paper, we report on the synthesis and structural analysis of ⁹⁹Tc complexes with simple isocyanides serving as models for functionalized derivatives. The no-carrier-added preparation of the analogous ^{99m}Tc complexes, studies on stability in aqueous solution and in plasma, as well as biodistribution studies in rats are dealt with.

EXPERIMENTAL PROCEDURES

General Methods. Dimethylphenylphosphine (PMe₂-Ph) was purchased from Aldrich. Isocyanides were obtained from Aldrich (benzyl isocyanide), Merck (cyclohexyl isocyanide), and Fluka (isocyanoacetic acid ethyl ester). All solvents and commercially available substances were of reagent grade and used without further purification. The tripodal ligand 2,2',2''-nitrilotris(ethane-

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Table 1. Crystallographic Data for X-ray Diffraction Studies of Tc Complexes $^{99}\text{Tc1}$, $^{99}\text{Tc2}$, $^{99}\text{Tc3}$, and $^{99}\text{Tc4}$

	$^{99}\text{Tc1}$	$^{99}\text{Tc2}$	$^{99}\text{Tc3}$	$^{99}\text{Tc4}$
formula	$\text{C}_{14}\text{H}_{23}\text{NPS}_3\text{Tc}$	$\text{C}_{14}\text{H}_{19}\text{N}_2\text{S}_3\text{Tc}$	$\text{C}_{13}\text{H}_{23}\text{N}_2\text{S}_3\text{Tc}$	$\text{C}_{11}\text{H}_{19}\text{N}_2\text{O}_2\text{S}_3\text{Tc}$
formula wt	430.48	409.49	401.51	405.46
crystal system	monoclinic	monoclinic	orthorhombic	monoclinic
space group	$P2(1)/c$	$P2(1)/c$	$Pbca$	$P2(1)/c$
a (Å)	13.3502(5)	8.2607(4)	11.0095(4)	9.9088(4)
b (Å)	9.4833(4)	11.1868(5)	12.7034(5)	22.2838(8)
c (Å)	15.3085(6)	17.6186(9)	24.5798(9)	7.5060(3)
α (deg)	90	90	90	90
β (deg)	112.7420(10)	93.8360(10)	90	107.6780(10)
γ (deg)	90	90	90	90
volume (Å ³)	1787.44(12)	1624.50(14)	3437.7(2)	1579.10(11)
Z	4	4	8	4
T (K)	293(2)	293(2)	293(2)	293(2)
ρ (g/cm ³)	1.600	1.674	1.552	1.705
absorption coeff (mm ⁻¹)	1.236	1.263	1.191	1.307
$F(000)$	880	832	1648	824
λ (Mo K_{α}) (Å)	0.71073	0.71073	0.71073	0.71073
crystal size (mm ³)	$0.9 \times 0.324 \times 0.072$	$0.90 \times 0.72 \times 0.36$	$1.8 \times 0.144 \times 0.036$	$0.6 \times 0.36 \times 0.04$
θ -range for data collection	1.65–23.31	2.16–28.98	1.66–23.29	1.83–27.98
index ranges	$-11 \leq h \leq 14$ $-10 \leq k \leq 10$ $-17 \leq l \leq 16$	$-11 \leq h \leq 8$ $-14 \leq k \leq 11$ $-23 \leq l \leq 20$	$-10 \leq h \leq 12$ $-14 \leq k \leq 14$ $-27 \leq l \leq 25$	$-13 \leq h \leq 13$ $-25 \leq k \leq 28$ $-9 \leq l \leq 8$
reflections collected	7757	9450	13310	9262
independent reflections	2561	3900	2467	3579
goodness-of-fit on F^2	1.096	1.206	1.056	1.049
R [$I > 2\sigma(I)$]	$R1 = 0.0218$ $wR2 = 0.0578$	$R1 = 0.0982$ $wR2 = 0.2441$	$R1 = 0.0426$ $wR2 = 0.1036$	$R1 = 0.0411$ $wR2 = 0.1135$
R (all data)	$R1 = 0.0230$ $wR2 = 0.0590$	$R1 = 0.1033$ $wR2 = 0.2618$	$R1 = 0.0476$ $wR2 = 0.1072$	$R1 = 0.0436$ $wR2 = 0.1165$

thiol) (NS_3) was prepared according to standard procedures starting from tris(2-chloroethyl)amine hydrochloride (22). ^{99}Tc as NH_4TcO_4 was obtained from Amersham as a 0.3 M aqueous solution. $^{99m}\text{TcO}_4^-$ was eluted from a commercial $^{99}\text{Mo}/^{99m}\text{Tc}$ generator (Mallinckrodt).

Instrumentation. Elemental analyses were performed on a LECO elemental analyzer CHNS-932. Melting points were obtained on a BOETIUS-Mikroheiztisch and are uncorrected. IR spectra were measured as KBr pellets on a Perkin-Elmer FTIR-spectrometer SPEC-TRUM 2000. UV/vis spectra were measured on a SPECORD S10 spectrometer from Carl Zeiss Jena.

^1H NMR spectra (400 MHz) were recorded on a Varian spectrometer INOVA-400. Samples were dissolved in deuterated chloroform at a concentration of ca. 2%. Chemical shifts are given as δ in ppm.

TLC and HPLC analyses were used to determine the radiochemical purity and stability of the preparations. TLC analyses were performed using silica gel strips (Kieselgel 60) developed with chloroform/acetone (6:1 v/v) as solvents. For HPLC studies, a Perkin-Elmer device consisting of a Turbo LC system with a quaternary pump (Series 200 LC Pump), a programmable absorbance detector model 785A, and a homemade γ -detector (Bohrloch NaI(Tl) crystal) was used. HPLC analyses were carried out with a Hypersil ODS column (250 $\times$ 4 mm) using a premixed eluent of 80% methanol and 20% 0.01 M phosphate buffer of pH 7.4 and a flow rate of 1.0 mL/min. The eluate from the column was monitored by UV absorbance at 254 nm for ^{99}Tc reference complexes or γ detection for the ^{99m}Tc complexes.

X-ray Data Collection and Processing. The X-ray data were collected at room temperature (293 K) on a SMART-CCD diffractometer (SIEMENS), using graphite-monochromatized Mo K_{α} radiation ($\lambda = 0.71073$ Å). A summary of the crystallographic data is given in Table 1. The positions of the non-hydrogen atoms were determined by the heavy atom technique. After anisotropic refinement of the positions of these, the hydrogen positions were calculated according to ideal geometries.

Empirical absorption corrections were made using ψ scans. Most of the calculations were carried out in the SHELXTL system with some local modifications.

Relevant bond lengths and angles are contained in Table 2. Atomic positional and thermal parameters, full lists of bond lengths and angles, and F_o/F_c values have been deposited at the Cambridge Crystallographic Data Centre (24).

Synthesis of [$^{99}\text{Tc}(\text{NS}_3)(\text{PMe}_2\text{Ph})$] – ($^{99}\text{Tc1}$). A 35 mg (0.25 mmol) sample of dimethylphenylphosphine and 20 mg (0.101 mmol) of 2,2',2''-nitritoltris(ethanethiol) were dissolved in 3 mL of ethanol. This solution was added to a solution of 20 mg (0.1 mmol) KTcO_4 in 2 mL of water/ethanol (2:1 v/v). The reaction mixture was acidified with three drops of acetic acid and was stirred at ambient temperature for 5 h. During this time, the color changed to deep-violet. The solvents were stripped in vacuo, and the complex was purified by column chromatography (column 10 $\times$ 250 mm, stationary phase silica gel 0.04–0.063 mm, eluent CHCl_3 /methanol 9:1 v/v). Recrystallization from chloroform/methanol (1:3 v/v) gave violet crystals of $^{99}\text{Tc1}$ suitable for X-ray structure analysis.

Yield: 31 mg (73% relative to KTcO_4). Mp = 221 °C. Anal. ($\text{C}_{14}\text{H}_{23}\text{NPS}_3\text{Tc}$) Calcd: C, 39.0; H, 5.3; N, 3.2; S, 22.3. Found: C, 39.1; H, 5.8; N, 3.3; S, 22.6. IR (KBr) (cm^{-1}): 943 and 901 ($\text{PhP}(\text{CH}_3)_2$). UV/vis (CHCl_3): λ_{max} ($\log \epsilon$) = 273 nm (4.2), 342 (3.8), 544 (2.9). ^1H NMR (400 MHz, CDCl_3): 1.91 (d, $\text{P}(\text{CH}_3)_2\text{Ph}$, 6H), 2.9 and 3.1 (m, $-\text{CH}_2\text{S}$, $-\text{CH}_2\text{N}$, 12H), 7.42 and 7.92 (m, $\text{P}(\text{CH}_3)_2\text{C}_6\text{H}_5$, 5H).

General Synthesis Procedure for the Complexes [$^{99}\text{Tc}(\text{NS}_3)(\text{CNR})$] – ($^{99}\text{Tc2}$ – $^{99}\text{Tc4}$). A 40 mg (0.093 mmol) sample of $^{99}\text{Tc1}$ was dissolved in 5 mL of chloroform. At ambient temperature, 0.232 mmol of the appropriate isocyanide, dissolved in 1 mL of chloroform, was added in one portion. The reaction mixture was refluxed for 1 h. TLC showed the presence of the appropriate products $^{99}\text{Tc2}$ – $^{99}\text{Tc4}$ and of unreacted $^{99}\text{Tc1}$. The isocyanide complexes were isolated by column chromatography (chromatography (column 10 $\times$ 250 mm, sta-

Table 2. Selected Bond Length (Å) and Angles (deg) of Complexes $^{99}\text{Tc1}$, $^{99}\text{Tc2}$, $^{99}\text{Tc3}$ and $^{99}\text{Tc4}$

$^{99}\text{Tc1}$		$^{99}\text{Tc2}$		$^{99}\text{Tc3}$		$^{99}\text{Tc4}$	
Tc(1)–N(1)	2.2048(19)	Tc(1)–C(1)	1.932(10)	Tc(1)–C(1)	1.942(6)	Tc(1)–C(7)	1.945(3)
Tc(1)–S(2)	2.2230(6)	Tc(1)–N(2)	2.216(7)	Tc(1)–N(2)	2.184(4)	Tc(1)–N(2)	2.199(2)
Tc(1)–S(1)	2.2258(6)	Tc(1)–S(3)	2.227(3)	Tc(1)–S(3)	2.214(2)	Tc(1)–S(3)	2.2239(9)
Tc(1)–S(3)	2.2295(7)	Tc(1)–S(2)	2.232(2)	Tc(1)–S(1)	2.2153(14)	Tc(1)–S(2)	2.2292(9)
Tc(1)–P(1)	2.3172(6)	Tc(1)–S(1)	2.242(3)	Tc(1)–S(2)	2.2422(15)	Tc(1)–S(1)	2.2357(9)
N(1)–Tc(1)–S(2)	85.72(6)	C(1)–Tc(1)–S(1)	93.8(3)	C(1)–Tc(1)–N(2)	178.7(2)	C(7)–Tc(1)–S(1)	95.32(10)
N(1)–Tc(1)–S(1)	85.97(5)	N(2)–Tc(1)–S(1)	86.2(2)	C(1)–Tc(1)–S(3)	95.2(2)	N(2)–Tc(1)–S(1)	85.62(7)
S(2)–Tc(1)–S(1)	118.39(3)	S(3)–Tc(1)–S(1)	120.04(11)	N(2)–Tc(1)–S(3)	86.05(13)	S(3)–Tc(1)–S(1)	120.22(4)
N(1)–Tc(1)–S(3)	85.59(6)	S(2)–Tc(1)–S(1)	119.18(10)	C(1)–Tc(1)–S(1)	92.8(2)	S(2)–Tc(1)–S(1)	119.18(4)
S(2)–Tc(1)–S(3)	119.46(3)	C(1)–Tc(1)–N(2)	178.0(3)	N(2)–Tc(1)–S(1)	86.35(12)	C(7)–Tc(1)–N(2)	178.09(12)
S(1)–Tc(1)–S(3)	120.53(3)	C(1)–Tc(1)–S(3)	95.6(3)	S(3)–Tc(1)–S(1)	117.74(6)	C(7)–Tc(1)–S(3)	92.18(10)
N(1)–Tc(1)–P(1)	176.10(5)	N(2)–Tc(1)–S(3)	86.1(2)	C(1)–Tc(1)–S(2)	93.9(2)	N(2)–Tc(1)–S(3)	85.91(7)
S(2)–Tc(1)–P(1)	96.78(2)	C(1)–Tc(1)–S(2)	92.6(3)	N(2)–Tc(1)–S(2)	85.61(12)	C(7)–Tc(1)–S(2)	95.47(10)
S(1)–Tc(1)–P(1)	95.44(2)	N(2)–Tc(1)–S(2)	85.6(2)	S(3)–Tc(1)–S(2)	120.19(6)	N(2)–Tc(1)–S(2)	85.50(7)
S(3)–Tc(1)–P(1)	90.57(2)	N(2)–Tc(1)–S(2)		S(1)–Tc(1)–S(2)	120.62(6)	S(3)–Tc(1)–S(2)	118.91(4)

tionary-phase silica gel 0.04–0.063 mm, eluent $\text{CHCl}_3/\text{acetone}$ 12:1(v/v). Recrystallization from chloroform/methanol (1:3 v/v) gave violet crystals of $^{99}\text{Tc2}$, $^{99}\text{Tc3}$, and $^{99}\text{Tc4}$ suitable for X-ray structure analysis.

$^{99}\text{Tc}(\text{NS}_3)(\text{CNCH}_2\text{C}_6\text{H}_5)] - ^{99}\text{Tc2}$. Yield: 26 mg (67% relative to $^{99}\text{Tc1}$). Mp: = 207 °C. Anal. ($\text{C}_{14}\text{H}_{19}\text{N}_2\text{S}_3\text{Tc}$) Calcd: C, 41.0; H, 4.6; N, 6.8; S, 23.4. Found: C, 40.9; H, 4.3; N, 6.7; S, 22.9. IR (KBr) (cm^{-1}): 2056, 1997 (CN). UV/vis (CHCl_3): $\lambda_{\text{max}}(\log \epsilon) = 278 \text{ nm}$ (4.2), 353 (3.7), 520 (2.9). ^1H NMR (400 MHz, CDCl_3): 2.9 and 3.1 (m, $-\text{CH}_2\text{S}$, $-\text{CH}_2\text{N}$, 12H), 5.2 (s, CNCH_2 , 2H), 7.2 (m, $\text{CH}_2\text{C}_6\text{H}_5$, 5H).

$^{99}\text{Tc}(\text{NS}_3)(\text{CNCH}_2\text{C}_6\text{H}_{11})] - ^{99}\text{Tc3}$. Yield: 23 mg (61% relative to $^{99}\text{Tc1}$). Mp = 239 °C. Anal. ($\text{C}_{13}\text{H}_{23}\text{N}_2\text{S}_3\text{Tc}$) Calcd: C, 38.8; H, 5.7; N, 6.9; S, 23.9. Found: C, 39.2; H, 5.8; N, 6.8; S, 23.6. IR (KBr) (cm^{-1}): 2022 (CN). UV/vis (CHCl_3): $\lambda_{\text{max}}(\log \epsilon) = 277 \text{ nm}$ (4.1), 350 (3.7), 527 (2.8). ^1H NMR (400 MHz, CDCl_3): 1.5 and 1.9 (m, C_6H_{10} , 10H), 2.9 and 3.1 (m, $-\text{CH}_2\text{S}$, $-\text{CH}_2\text{N}$, 12H), 4.1 (s, $\text{CNCH}(\text{CH}_2)_2$, 1H).

$^{99}\text{Tc}(\text{NS}_3)(\text{CNCH}_2\text{C}(\text{O})\text{OC}_2\text{H}_5)] - ^{99}\text{Tc4}$. Yield: 25 mg (65% relative to $^{99}\text{Tc1}$). Mp = 194 °C. Anal. ($\text{C}_{11}\text{H}_{19}\text{N}_2\text{O}_2\text{S}_3\text{Tc}$) Calcd: C, 32.5; H, 4.7; N, 6.9; S, 23.6. Found: C, 32.8; H, 4.9; N, 6.7; S, 23.1. IR (KBr) (cm^{-1}): 2056 (CN), 1746 (CO). UV/vis (CHCl_3): $\lambda_{\text{max}}(\log \epsilon) = 280 \text{ nm}$ (4.1), 355 (3.7), 505 (2.8). ^1H NMR (400 MHz, CDCl_3): 1.32 (t, $-\text{OCH}_2\text{CH}_3$, 3H), 2.8 and 3.2 (m, $-\text{CH}_2\text{S}$, $-\text{CH}_2\text{N}$, 12H), 4.6 (q, OCH_2- , 2H), 4.8 (s, CNCH_2 , 2H).

General Synthesis Procedure for the No-Carrier-Assisted Preparation of the Compounds $^{99}\text{Tc}(\text{NS}_3)(\text{CN-R})] - (^{99}\text{Tc2}-^{99}\text{Tc4})$. A solution of 20 μL of SnCl_2 (1.0–1.5 mg of $\text{SnCl}_2/5.0 \text{ mL}$ of 0.1 N HCl) was added to a mixture of 0.5 mL of pertechnetate solution (10–500 MBq generator eluate), 0.3 mL of propylene glycol, 0.2 mL of acetonitrile, 0.3–0.4 mg of 2,2',2''-nitrilotris-(ethanethiol), dissolved in ethanol, 0.05 mg of the appropriate isocyanide, dissolved in ethanol, and 0.02 mL of 0.1 M NaOH. The vial was closed, and the reaction solution was heated at 50–60 °C for 15 min. The labeling yields for all complexes were between 75% and 80% as established by HPLC analysis. The identity of the species obtained was confirmed by comparison with the HPLC profiles of ^{99}Tc analogues. After HPLC separation the radiochemical purity of the $^{99}\text{Tc}(\text{III})$ “4 + 1” complexes was about 95%.

Determination of Partition Coefficients. Partition coefficients of the ^{99}Tc complexes were determined by shake-flask method by mixing 0.5 mL of the complex solution (0.9% NaCl solution and 20% propylene glycol) and 0.5 mL of octanol and shaking for 5 min. After centrifugation aliquots of both phases were counted and the partition coefficient calculated.

Ligand Exchange (Challenge) Experiments with Glutathione (GSH). ^{99}mTc complexes, dissolved in a mixture of propylene glycol/MeOH (2:1 v/v), were diluted with an equivalent volume of an aqueous solution of 20 mM GSH containing 25% of propylene glycol immediately after HPLC separation, resulting in a final glutathione concentration of 10 mM. The analyses were performed by HPLC as described above.

Stability Studies in Plasma. A 50 μL sample of the appropriate ^{99}mTc complex solution (0.9% NaCl, 12% propylene glycol) was incubated in 200 μL of rat plasma at 37 °C. After an incubation time of 30 min, the samples were analyzed by HPLC with a Supelguard column (20 $\times$ 4.6 mm, 10 μm , flow rate 1.0 mL/min) using a linear gradient 95% A to 40% A in 15 min [(A) 2-propanol/0.1% trifluoroacetic acid (TFA) (10/90), (B) 2-propanol/0.1% TFA (90/10)].

Alternatively, the HPLC analyses were carried out with a PRP-3 column (Hamilton, 150 $\times$ 4 mm, 10 μm , flow rate 1.0 mL/min) using a linear gradient system [t (min)/% B]: (5/0), (10/70), (5/70) of 10 mM phosphate buffer (PBS) of pH 7.4 (A) and acetonitrile (B).

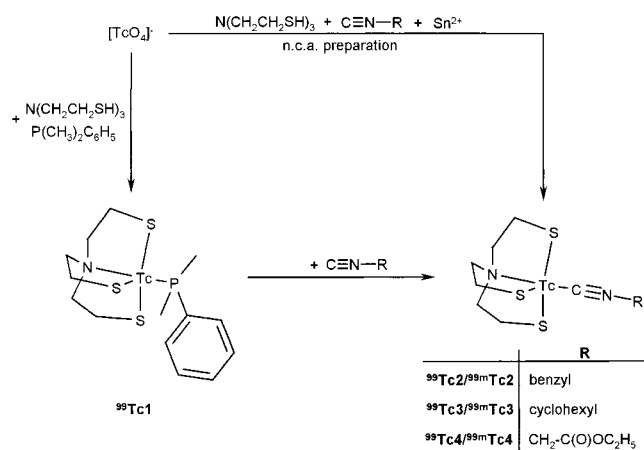
Biodistribution Studies. The animal studies in male Wistar rats (5–6 weeks old) were carried out according to the relevant national regulations. A 500 μL sample of ^{99}mTc complex solution (saline, propylene glycol 25%) was injected into the tail vein of rats. After the injection, the rats were sacrificed by heart puncture under ether anaesthesia 5 and 120 min p.i. Selected organs were isolated for weighing and counting. The accumulated radioactivity in the tissue of organs was calculated in terms of percentage of injected dose per organ as well as percent injected dose per gram blood.

RESULTS AND DISCUSSION

Technetium-99 Chemistry. The ^{99}Tc complexes $^{99}\text{Tc2}-^{99}\text{Tc4}$ were synthesized by a two-step procedure starting from pertechnetate(VII) via the phosphine-containing precursor $^{99}\text{Tc1}$ according to Scheme 1. To a mixture of pertechnetate(VII) and 2,2',2''-nitrilotris-(ethanethiol) (NS_3) was added an excess of dimethylphenylphosphine. The phosphine acts as a reducing agent as well as a coligand to yield $^{99}\text{Tc1}$. The complex can be isolated as an air-stable deep-violet powder. This Tc(III) phosphine derivative undergoes in chloroform solutions facile substitution to the corresponding neutral “4 + 1”-coordinated Tc(III) complexes $^{99}\text{Tc2}-^{99}\text{Tc4}$ of the general formulation $[\text{Tc}(\text{NS}_3)(\text{CN-R})]$ (Scheme 1).

Elemental analyses, as reported in the Experimental Section, are in agreement with the proposed formulation. IR spectra of Tc(III) complexes $^{99}\text{Tc2}-^{99}\text{Tc4}$ exhibit

Scheme 1



strong absorptions characteristic of the Tc–CN– stretching vibrations. No additional bands indicating the presence of the Tc=O core in the region 900–1000 cm⁻¹ are observed. The UV/vis spectra of ⁹⁹Tc2, ⁹⁹Tc3, and ⁹⁹Tc4 (recorded in chloroform) are characterized by intense bands at about 280 and 350 nm and less intense absorptions in the visible region (505–527 nm).

The Tc(III) complexes show sharp proton signals characteristic of diamagnetic compounds, in agreement with low-spin d⁴ t₂g configurations. NMR profiles confirm the solution structures of complexes ⁹⁹Tc1–⁹⁹Tc4 to be identical to solid-state structure found by X-ray analysis.

Five-coordinate Tc(III) complexes ⁹⁹Tc1–⁹⁹Tc4, as prototypic representatives of this novel class of neutral Tc(III) compounds, were studied by X-ray structure analysis. A summary of the crystallographic data is given in Table 1, and selected bond lengths and angles are cumulated in Table 2.

As illustrated in Figures 1–4, the complexes adopt a nearly ideal trigonal-bipyramidal geometry with the trigonal plane formed by the three thiolate sulfurs of the tripodal ligand. The central nitrogen atom of the chelate ligand and the phosphine phosphorus in ⁹⁹Tc1 or the monodendate isocyanide ligand in ⁹⁹Tc2–⁹⁹Tc4 occupy the apical positions.

The Tc–S_{thiolato} distances are restricted in a narrow range (2.21–2.24 Å) and compare well with those previously reported for trigonal bipyramidal [Tc(SR)₃(L)₂]-type complexes (SR = sterically hindered arenethiolates, L = small π-accepting molecules) (25, 26). The Tc–C distances are found in a narrow range between 1.93 and 1.94 Å.

The N2–Tc–C1 axis shows only slight deviation from linearity. Also the other angles of the inner coordination sphere are consistent with the t₂g arrangement.

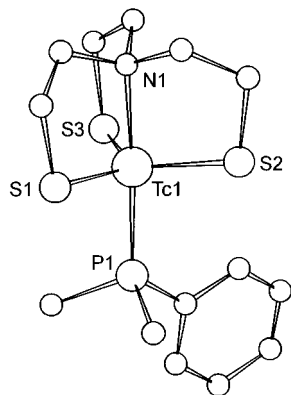


Figure 1. Molecular structure of complex ⁹⁹Tc1.

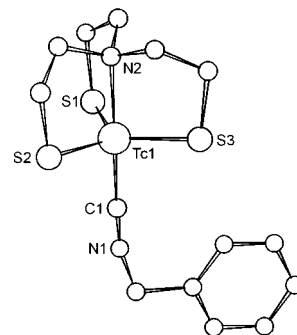


Figure 2. Molecular structure of complex ⁹⁹Tc2.

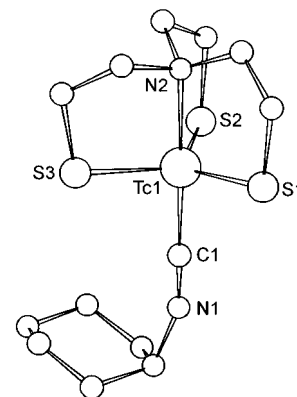


Figure 3. Molecular structure of complex ⁹⁹Tc3.

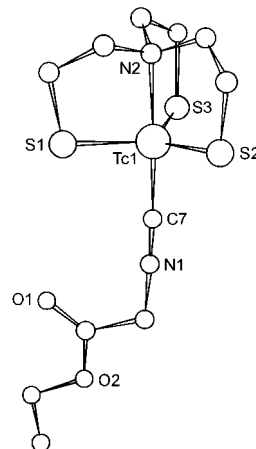


Figure 4. Molecular structure of complex ⁹⁹Tc4.

The compounds constitute the second example of an Tc(III) complex with an “umbrella” ligand, a concept introduced by Davison and co-workers (27, 28). The “umbrella” in Davison’s complexes of the general formula [Tc(PS₃)(CN-R)] consists of a central phosphorus atom and three aromatic thiolate functions (PS₃), while in ^{99m}Tc2–^{99m}Tc4 three aliphatic thiolates and a central nitrogen form the “umbrella” ligand (NS₃).

Technetium-99m Chemistry and Biodistribution Studies. *No-Carrier-Added Preparation of the ^{99m}Tc Complexes.* The no-carrier-added preparation of the complexes ^{99m}Tc2–^{99m}Tc4 was performed by a one-step procedure starting from ^{99m}[TcO₄]⁻ with stannous chloride as reducing agent (Scheme 1). The yields were between 75% and 80% using unoptimized preparation conditions. In the appropriate reaction mixtures, a byproduct occurs that is not eluted from the Hypersil column. Therefore, TLC has to be used for determining the yields of the preparation. After HPLC separation, the

Table 3. HPLC and TLC Data^a of Complex Couples $^{99m}\text{Tc2}/^{99m}\text{Tc2}$, $^{99m}\text{Tc3}/^{99m}\text{Tc3}$, and $^{99m}\text{Tc4}/^{99m}\text{Tc4}$ (Partition Coefficients ($\log P$) of $^{99m}\text{Tc2}$ – $^{99m}\text{Tc4}$ in Comparison to the Data of Corresponding “3 + 1” Mixed-Ligand Oxotechnetium(V) Complexes [$\text{TcO}(\text{SCH}_2\text{CH}_2\text{N}(\text{CH}_3)\text{CH}_2\text{CH}_2\text{S})(\text{SR})$] Bearing the Same Substituent R (A–C))

complex	t_R (min)	$\log P$ (octanol/saline)	R_f
$^{99m}\text{Tc2}$	5.8		0.4
$^{99m}\text{Tc2}$	5.9	1.5	0.4
A		1.0	
$^{99m}\text{Tc3}$	7.1		0.3
$^{99m}\text{Tc3}$	7.3	1.7	0.3
B		0.6	
$^{99m}\text{Tc4}$	7.7		0.1
$^{99m}\text{Tc4}$	7.7	1.6	0.1
C		0.5	

^a Hypersil ODS: MeOH/0.01 M PBS pH 7.4, linear gradient 70–100% A in 5 min, TLC data: TLC aluminum sheets RP-18 (Merck); eluent, methanol/water (80/20).

radiochemical purity of the $^{99m}\text{Tc(III)}$ “4 + 1” complexes was determined by TLC and found to be about 95%.

To establish the structure of the ^{99m}Tc complexes, comparison by HPLC with the well characterized ^{99m}Tc analogues was pursued applying parallel radiometric and photometric detection. Thus, after co-injection of complexes $^{99m}\text{Tc2}$ and $^{99m}\text{Tc2}$, practically identical retention times were observed, while the recovery through the column was quantitative. Similarly, co-injections of the complex couples $^{99m}\text{Tc3}/^{99m}\text{Tc3}$ and $^{99m}\text{Tc4}/^{99m}\text{Tc4}$ led to identical t_R values for the respective compounds, revealing their structural analogy. t_R values of HPLC, R_f values of TLC, and partition coefficients (P) of the ^{99m}Tc complexes in the system octanol/saline are given in Table 3.

Examination of the octanol/water partition coefficients suggests high lipophilicity for the resulting Tc species. A comparison with the values of so-called “3 + 1” mixed-ligand oxotechnetium(V) complexes A–C of the general formula [$\text{TcO}(\text{SCH}_2\text{CH}_2\text{N}(\text{Me})\text{CH}_2\text{CH}_2\text{S})(\text{SR})$] (23) bearing the same substituent R (complex (A) benzyl, (B) cyclohexyl, (C) carbethoxymethylene) indicates considerably higher lipophilicity of the studied “4 + 1” complexes. These observations are consistent with the expectation that absence of an oxo group and better shielding of the metal will enhance the lipophilicity (Table 3).

Glutathione Challenge Experiments. Glutathione (GSH), the most abundant thiol compound in tissues, is present in almost all animal cells in relatively high concentrations (0.5–12 mM) (29, 30). Therefore, it has to be considered as a potential agent for transchelating reactions in vivo. In fact, Neirinckx et al. (31) pointed out that the intracellular reaction of the brain perfusion agent $^{99m}\text{Tc(V)}$ -HMPAO with GSH is responsible for the trapping of the radiopharmaceutical in the brain. GSH is also responsible for the in vivo reactivity of “3 + 1” mixed-ligand $^{99m}\text{Tc(V)}$ complexes that consist of a monodentate thiol ligand and a tridentate dithiol (9–11). Here, a ligand exchange in terms of the replacement of the monodentate ligand by GSH occurs in vivo and in vitro as recently shown (12, 13, 32). In view of these experiences, it would be useful to explore also the behavior of $^{99m}\text{Tc(III)}$ “4 + 1” complexes against GSH ligand-exchange reactions.

Saline-containing preparations of the ^{99m}Tc compounds were diluted with a 10 mM GSH solution immediately after HPLC purification. In all cases, no additional peaks of Tc-GSH mixed ligand species were observed in HPLC studies. These results clearly indicate that glutathione has no negative effect on the stability of ^{99m}Tc complexes in solutions. Obviously, no transchelation reaction by

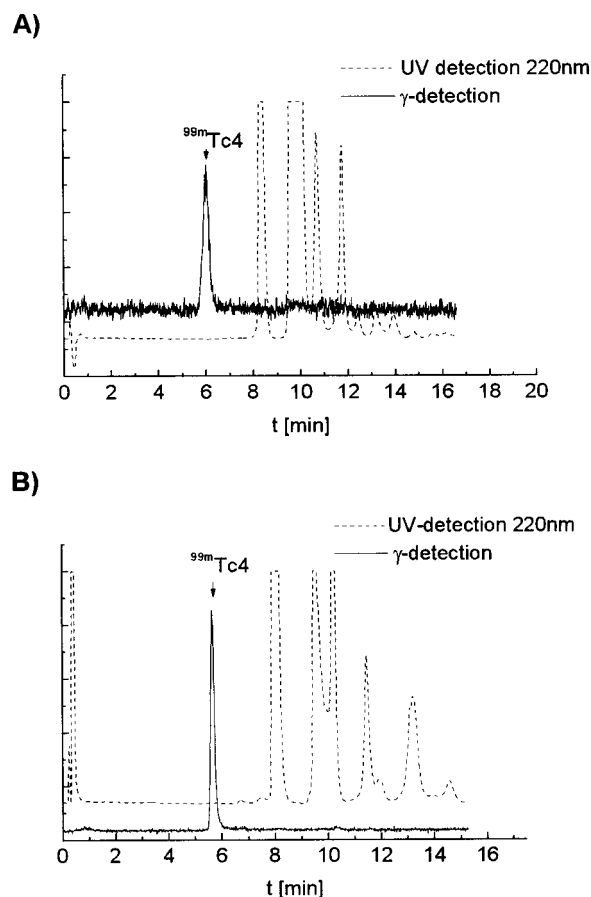


Figure 5. HPLC pattern of the complex $^{99m}\text{Tc4}$; Supelguard column (20 $\times$ 4.6 mm, 10 μm , flow rate 1.0 mL/min) using a linear gradient 95% A to 40% A in 15 min [(A) 2-propanol/0.1% trifluoroacetic acid (TFA) (10/90); (B) 2-propanol/0.1% TFA (90/10)]; (A) 120 min incubation in rat erythrocyte hemolysate; (B) 120 min incubation in rat plasma.

GSH occurs in vitro. This behavior is considered to be an important advantage of the $^{99m}\text{Tc(III)}$ “4 + 1” complexes over mixed-ligand ^{99m}Tc oxocomplexes in the oxidation state +5.

Stability Studies in Plasma and in Rat Blood. For stability studies, the ^{99m}Tc complexes were incubated in rat blood and rat plasma for 120 min. After incubation, the blood was centrifuged and the supernatant plasma was separated from the erythrocytes. The plasma as well as the erythrocyte hemolysate were analyzed using a Supelguard column as described above. For the complexes $^{99m}\text{Tc2}$ – $^{99m}\text{Tc4}$ no additional peaks were observed, indicating some kind of decomposition, modification, or alteration of the complexes. There is also no tendency of these complexes to bind on plasma components.

To confirm these results, the plasma samples as well as the hemolysates were extracted with ethyl acetate. In this way, about 95% of the activity could be extracted. Analyzing the extracts by HPLC using a Hypersil column and by TLC as described in the Experimental Section only the parent compounds were detected. These results indicate that the $^{99m}\text{Tc(III)}$ “4 + 1” complexes behave absolutely stable in plasma and blood of rat for at least 120 min as illustrated for $^{99m}\text{Tc4}$ in Figure 5.

Biodistribution Studies in Rats. Data of the biodistribution studies of ^{99m}Tc complexes $^{99m}\text{Tc2}$ – $^{99m}\text{Tc4}$ are shown in Table 4. All three complexes show low radioactivity in blood at 5 min p.i., which thereafter decreases within 120 min to a similar extent. The neutral, lipophilic

Table 4. Biodistribution Pattern of Complexes $^{99m}\text{Tc2}$ – $^{99m}\text{Tc4}$ in Wistar Rats (% ID/Organ Except for Blood as % ID/g; means $\pm$ SD; $n = 5$)

complex	min p.i.	blood	brain	heart	lungs	kidneys	liver
$^{99m}\text{Tc2}$	5	0.34 $\pm$ 0.09	0.79 $\pm$ 0.11	0.4 $\pm$ 0.1	1.5 $\pm$ 0.5	2.1 $\pm$ 0.2	31.6 $\pm$ 4.0
	120	0.11 $\pm$ 0.04	<0.05	0.1 $\pm$ 0.02	0.3 $\pm$ 0.1	1.7 $\pm$ 0.4	33.2 $\pm$ 5.6
$^{99m}\text{Tc3}$	5	0.23 $\pm$ 0.11	1.12 $\pm$ 0.31	0.5 $\pm$ 0.2	1.2 $\pm$ 0.3	1.8 $\pm$ 0.2	25.2 $\pm$ 2.1
	120	0.13 $\pm$ 0.07	0.05 $\pm$ 0.01	0.1 $\pm$ 0.02	0.4 $\pm$ 0.1	1.5 $\pm$ 0.3	22.5 $\pm$ 3.6
$^{99m}\text{Tc4}$	5	0.13 $\pm$ 0.02	0.44 $\pm$ 0.07	0.6 $\pm$ 0.1	1.1 $\pm$ 0.4	5.2 $\pm$ 0.7	13.8 $\pm$ 1.6
	120	0.07 $\pm$ 0.01	<0.05	0.1 $\pm$ 0.01	0.5 $\pm$ 0.1	3.2 $\pm$ 1.7	13.6 $\pm$ 2.0

complexes can cross the blood–brain barrier. They show a significant initial brain uptake from 0.13 up to 1.1%, obtained for the most lipophilic cyclohexyl-substituted complex $^{99m}\text{Tc3}$ (logP 1.7). The complexes are quickly cleared from the brain. $^{99m}\text{Tc2}$, $^{99m}\text{Tc3}$, and $^{99m}\text{Tc4}$ are distributed predominantly to the liver with slightly increased retention after 120 min p.i. Except for the excretory organs, no prominent uptake and retention was observed. The compounds are stable in whole-blood of rats and reveal no affinity to protein components of rat plasma (Figure 5).

CONCLUSION

The tripodal, tetradentate ligand 2,2',2''-nitritoltris(ethanethiol) and isocyanides as co-ligands form nonpolar, lipophilic technetium complexes that contain sterically well shielded oxo-free Tc(III) ions. The monodentate isocyanide ligand enables easy functionalization in order to fine-tune physicochemical properties of the complexes or to link the chelate unit to biomolecules. ^{99m}Tc complexes can easily be prepared at no-carrier-added level in high radiochemical yield. There is no tendency of these complexes to bind on rat plasma components. In vitro challenge experiments with glutathione clearly indicate that no transchelation reaction occurs. This behavior is considered to be an important advantage over mixed-ligand ^{99m}Tc oxocomplexes in the oxidation state +5. Furthermore, there are no indications for reoxidation of Tc(III) to Tc(V) species or pertechnetate. Therefore, we propose the complexes as a useful tool in designing of lipophilic ^{99m}Tc or $^{186}\text{Re}/^{188}\text{Re}$ radiopharmaceuticals.

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Poly(ethylene glycol)-Conjugated Anti-EGF Receptor Antibody C225 with Radiometal Chelator Attached to the Termini of Polymer Chains

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Several biological barriers, including significant liver uptake, limit the clinical application of radiolabeled antibodies in radioimmunoscintigraphy. Here, a general approach is described for radiolabeling of monoclonal antibodies conjugated with poly(ethylene glycol) (PEG). This strategy is demonstrated with C225, a monoclonal antibody directed against epidermal growth factor (EGF) receptor. We synthesized a heterofunctional PEG with one end attached to a radiometal chelator, diethylenetriaminepentaacetic acid (DTPA), and the other end to a protected thiol group, S-acetylthioacetate. After a deprotection step, the resulting DTPA-PEG-SH was conjugated to maleimide-activated C225 to yield DTPA-PEG-C225 conjugate. Characterization of DTPA-PEG-C225 with immunoprecipitation and Western blot analysis revealed that the conjugate was biologically active in binding to the EGF receptor in A431 cells. Competitive EGF receptor binding assay in MDA-MB-468 cells showed that DTPA-PEG-C225, with up to 60% of the amino groups in C225 substituted, retained 66% of C225's binding affinity. Moreover, DTPA-PEG-C225 with increasing degrees of NH₂ substitution from 20% to 70% retained the activity of C225 to induce apoptosis in DiFi cells. More importantly, DTPA-PEG-C225 demonstrated less nonspecific interaction than DTPA-C225. Pharmacokinetic analysis using ¹¹¹In-labeled compounds revealed narrower steady-state distribution of ¹¹¹In-DTPA-PEG-C225 than ¹¹¹In-DTPA-C225, probably due to reduced nonspecific binding of PEG-modified antibody to tissues. The terminal half-life (*t*_{1/2,γ}) of ¹¹¹In-DTPA-PEG-C225, 21.1 h, was shorter than that of ¹¹¹In-DTPA-C225, 52.9 h. These data suggest that ¹¹¹In-DTPA-PEG-C225 may provide better imaging characteristics than ¹¹¹In-DTPA-C225, and that using PEG as a linker between the monoclonal antibody and DTPA may be a promising strategy in optimizing the imaging characteristics of immunoscintigraphic agents.

INTRODUCTION

Radioimmunodetection for diagnostic purposes has many potential applications in the management of cancer patients. These applications include presurgical staging of extent of disease, postsurgical evaluation of residual disease, confirmation of viable tumors known by other methods, disclosure of occult recurrence, confirmation of tumor targeting of antibody to be used for immunotherapy, and assessment of therapeutic response (1, 2). Although the potential usefulness of radiolabeled monoclonal antibody (mAb)¹ is high, the actual progress concerning their clinical applications has not been satisfactory. Despite nearly 50 years of investigation and refinement, to date only one radiolabeled mAb has been approved by the FDA for use in humans (3). Limitations

of radioimmunodetection have been multifactorial, and include low uptake by tumor confined by biological barriers, unfavorable pharmacokinetics, and poor tumor-to-background ratio, etc. (1).

Among a wide range of isotopes applied to the labeling of antibodies, the majority are transition metals. It is evident that in order to obtain optimal imaging characteristics, it is necessary to achieve a high ratio of radioactivity between the tumor and normal tissues. Unfortunately, although immunoconjugates labeled with radiometals such as yttrium and indium usually have greater retention in cancer than the corresponding iodinated radioimmunoconjugates, they also tend to be retained in normal tissues, particularly the liver (4, 5). To decrease the rate of radiometal detachment from the mAb and reduce the subsequent hepatic uptake of the

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¹ Abbreviations: mAb, monoclonal antibody; DTPA, diethylenetriaminepentaacetic acid; PEG, poly(ethylene glycol); EGF, epidermal growth factor; SATA, S-acetylthioacetate; GMBS, N-γ-maleimidobutyryloxysuccinimide ester; TNBS, 2,4,6-trinitrobenzenesulfonic acid; SDS, sodium dodecyl sulfate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TFA, trifluoroacetic acid; TEA, triethylamine; MWCO, molecular weight cutoff; DMF, dimethylformamide; GPC, gel permeation chromatography; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine albumin; BSA, bovine serum albumin.

radiolabel, modified DTPA molecule (6) and also macrocyclic chelates, such as 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA) (7), have been used to increase the stability of radiometal–chelate complexes. Chelates with labile linkers can also exhibit such favorable characteristics as high tumor accumulation, rapid blood clearance, and low liver uptake in animal experiments (8, 9). For example, a peptide linker, triglycyl-L-phenylthiourea, susceptible to hepatic endopeptidase activity was designed and inserted between DOTA and chimeric mAb L6 (9). The resulting immunoconjugate exhibited reduced liver uptake due to increased hepatocyte cleavage and excretion of the radiochelate (10).

A different conjugation strategy is to manipulate the biodistribution patterns of the antibodies by employing the uncharged, amphiphilic linear polymer poly(ethylene glycol) (PEG) as a linker between the monoclonal antibody and the metal chelators. PEG-modified proteins, antibodies, and liposomes have been shown to exhibit reduced liver uptake and increased blood circulation half-lives, resulting in improved biological activity (11–13). In this report, we present the synthesis of a DTPA-PEG conjugate of an anti-epidermal growth factor (EGF) receptor antibody, C225, its radiolabeling with indium-111, and the biological activities of the resulting immunoconjugate. EGF receptor is a transmembrane glycoprotein with an intracellular tyrosine kinase domain. EGF receptor is overexpressed on the cells of over one-third of all solid tumors, including bladder, breast, colon, ovarian, prostate, renal cell, and squamous cell (nonsmall cell lung and head and neck) carcinomas (14). C225 is a human–mouse chimeric monoclonal antibody directed against human EGF receptor. It specifically binds to the external domain of the receptor with an affinity comparable to the natural ligand. C225 has been demonstrated to inhibit the proliferation of a variety of human cancer cells stimulated by the transforming growth factor- α (TGF- α) and EGF receptor autocrine loop (15).

EXPERIMENTAL PROCEDURES

Materials. C225 was kindly provided by ImClone Systems Inc. (New York, NY). *t*-Boc-NH-PEG-NH₂ (MW 3400) was obtained from Shearwater Polymers, Inc. (Huntsville, AL). *N*-Succinimidyl *S*-acetylthioacetate (SATA), *N*- γ -maleimidobutyryloxysuccinimide ester (GMBS), 2,4,6-trinitrobenzenesulfonic acid (TNBS, 5% w/v aqueous solution), 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's Reagent), sodium dodecyl sulfate (SDS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and PBS (0.01 M phosphate-buffered saline containing 138 mM NaCl, 2.7 mM KCl, pH 7.4) were purchased from Sigma Chemicals (St. Louis, MO). DTPA-dianhydride, trifluoroacetic acid (TFA, anhydrous), ninhydrin, triethylamine (TEA), and all the other solvents and reagents were purchased from Aldrich Chemical Co. (St. Louis, MO). All the chemicals and solvents were at least ACS grade and were used without further purification. Indium-111 radionuclide was obtained from Dupont-NEN (Boston, MA). PD-10 disposable column, Sephadex G-75 gel, and Sephacryl S-200 high-resolution gel were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Spectra/Pro 6 dialysis tubing [molecular weight cutoff (MWCO) 2000] and Centricon-YM-10 centrifugal filter devices (MWCO 10 000) were purchased from Fisher Scientific (Houston, TX). Silica gel 60 TLC plates were obtained from EM Sciences (Gibbstown, NJ).

DTPA-PEG-NH₂. To a stirred suspension of DTPA-dianhydride (143 mg, 0.4 mmol) in 4 mL of chloroform

were added TEA (81 mg, 0.8 mmol) and *t*-Boc-NH-PEG-NH₂ (340 mg, 0.1 mmol). The mixture was allowed to react at room temperature for 2 h. The reaction was followed by silica gel TLC using CHCl₃–MeOH (4:1 v/v) as the mobile phase; the plates were visualized by both iodine vapor and ninhydrin spray (0.1% ninhydrin solution in ethanol). TLC showed complete conversion of NH₂-PEG-NH-*t*-Boc (*R_f* = 0.55, purple in ninhydrin) to DTPA-PEG-NH-*t*-Boc (*R_f* = 0.4 with iodine vapor, negative in ninhydrin). After the reaction, the chloroform and TEA were removed under vacuum. The *t*-Boc protecting group was removed without purification by adding TFA (2 mL) to the resulting residue and stirring the mixture at room temperature for 4 h. The resulting DTPA-PEG-NH₂ was purified by dialysis against PBS and deionized water using dialysis tubing (MWCO, 2000). *R_f*: 0.18 (chloroform–methanol, 4:1 v/v; ninhydrin spray); yield: 360 mg, 95%.

DTPA-PEG-ATA. DTPA-PEG-NH₂ (182 mg, 0.05 mmol) was reacted with SATA (14 mg, 0.06 mmol) in chloroform at room temperature for 1 h, and then purified by dialysis (MWCO, 2000) and by gel filtration on a PD-10 column to afford DTPA-PEG-ATA, *R_f* = 0.27 (chloroform–methanol, 4:1 v/v; iodine vapor). ¹H NMR, 300 MHz, CDCl₃: 2.34 (s, 3H, CH₃COS-), 3.17–3.28 (m, 8H, -CH₂CH₂- in DTPA), 3.50 (s, 308H, -CH₂CH₂- in PEG with 77 repeating units). Anal Calcd for C₁₆₉H₃₃₇N₅O₅S: C, 52.54; H, 8.73; N, 1.81; S, 0.83. Found: C, 51.52; H, 8.90; N, 1.61; S, 0.81. Molecular mass determined by MALDI-TOF mass spectroscopy (BiFlexIII, Bruker Daltonics, Billerica, MA): 3750. No peaks of dimer or oligomers were found in the spectrum (data not shown). Yield: 180 mg, 92%.

DTPA-PEG-C225. Maleimide-activated C225 with different ratios of C225 to maleimide was prepared according to the following general procedure. To an aqueous solution of C225 (2.4 mg/mL; 4.8 mg, 0.032 μ mol) at room temperature were added aliquots of GMBS in dimethylformamide (DMF) (2.8 mg/mL). The mixture was stirred for 1 h, and then purified by gel filtration using a PD-10 column. Prior to conjugation with activated C225, the acetyl protecting group in DTPA-PEG-ATA was removed using hydroxylamine. For this purpose, an aliquot of NH₂OH (50 μ L) in 0.1 M Na₂HPO₄ (0.5 M) was added to a solution of DTPA-PEG-ATA (7.7 mg, 1.92 μ mol) in 0.1 M Na₂HPO₄ (pH 8.5, 0.5 mL), and then incubated at room temperature for 30 min. The resulting DTPA-PEG-SH containing free sulfhydryl group was then mixed with maleimide-activated C225 with a DTPA-PEG-SH-to-maleimide molar ratio of 2:1 and incubated at 4 °C overnight. The final product was separated from unreacted DTPA-PEG by gel filtration on a Sephacryl S-200 HR column (1.5 cm $\times$ 20 cm) with PBS as eluent. The presence of free sulfhydryl group was monitored using Ellman's agent (16).

DTPA-C225. DTPA-C225 was prepared using a previously described method (17). Briefly, DTPA-dianhydride (4.6 mg, 12.8 μ mol) was added to an aqueous solution of C225 (2.4 mg, 0.016 μ mol; 2.4 mg/mL). For reaction efficiency, the pH of the reaction solution was kept at 7–8 by adding 0.1 M Na₂HPO₄. After incubation at room temperature for 1 h, the solution was concentrated to half-volume on a Centricon-YM 10 centrifugal filter and purified from free DTPA by gel filtration on a PD-10 column.

Radiolabeling. Generally, 40 μ g of each antibody conjugate in 100 μ L of PBS was incubated with 350–400 μ Ci of ¹¹¹InCl₃ (in 20 μ L of 1 M sodium acetate buffer, pH 5.5) at room temperature for 15 min. The resulting radioisotopic product was purified from free indium-111

by gel filtration on a PD-10 column using PBS as the eluent. Fractions of 0.5 mL each were collected. The radioactivity of each fraction was measured by a radioisotope calibrator (Capintec Instruments, Ramsey, NJ). The protein content in each fraction was determined using a Bio-Rad protein assay kit according to the manufacturer's instruction (Bio-Rad Laboratories, Hercules, CA). The fractions containing the protein were combined. The radiochemical yield, expressed as a percentage of the radioactivity of the protein fractions to the total loaded radioactivity, was calculated. The radiochemical purity was determined by gel permeation chromatography (GPC).

Determination of Degree of Modification. The degree of substitution of C225 by maleimide was determined by quantifying the free amino groups remaining in the antibody using TNBS assay according to the published protocol (18). Briefly, samples were dissolved in 0.1 M sodium bicarbonate (pH 8.5) at a concentration of 20–200 μ g/mL. To 1 mL of each sample solution was added 0.5 mL of TNBS solution in 0.1 M sodium bicarbonate (0.01%, w/v). After incubation at 37 °C for 2 h, 0.5 mL of 10% SDS and 0.25 mL of 1 N HCl were sequentially added to each sample. The percentage of the reacted amino groups was determined by comparing the UV absorbance (335 nm) of the free amino groups in the modified antibody with that in the intact antibody.

Gel Permeation Chromatography. Analytical GPC was performed with a Waters HPLC system (Waters Corp., Milford, MA) consisting of a 2410 refractive index detector and a 2487 dual λ UV detector applying a TSK-G3000 PW 7.5 mm $\times$ 30 cm gel column (Tosoh Corp., Japan). Samples were eluted with PBS containing 0.1% LiBr at a flow rate of 1 mL/min, and the products were detected by the refractive index and UV absorbance at 254 nm. Radio-GPC was performed using an HPLC unit equipped with LDC pumps (Laboratory Data Control, Rivera Beach, FL), an LUDLUM radiometric detector (Measurement Inc., Sweetwater, TX), and an SP 8450 UV/VIS detector (Spectra-Physics, San Jose, CA). The samples were separated by a Phenomenex Biosep SEC-S3000 7.8 mm $\times$ 30 cm column, eluted with PBS containing 0.1% LiBr at a flow rate of 1 mL/min, and detected by radioactivity and UV absorbance at 254 nm.

Cell Lines. Human breast adenocarcinoma MDA-MB-468, human vulvar squamous carcinoma A431, and human colorectal carcinoma DiFi were maintained in 1:1 (v/v) Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 mixture supplemented with 10% fetal bovine serum (FBS) (Gibco Laboratories, Grand Island, NY) at 37 °C in 5% CO₂/95% air.

Competitive Binding Assay. MDA-MB-468 cells were seeded at 5×10^7 cells/well onto 12-well plates in 10% FBS medium and allowed to attach overnight. The medium was replaced by DMEM/F-12 medium plus 0.2% bovine serum albumin (BSA), and 1 μ g/mL of 1:30 ¹¹¹In-DTPA-PEG-C225 or ¹¹¹In-DTPA-C225 plus native C225 mAb at the indicated concentrations was added to the wells. After incubation at 37 °C for 2 h, the cells were washed 5 times with PBS containing 0.2% BSA. The cells were then trypsinized and transferred to 5 mL disposable culture tubes. The level of radioactivity in each tube was measured with a Cobra Auto-gamma Counter (Packard Instrument Co., Downers Grove, IL).

Immunoprecipitation and Western Blot Analysis. A431 cells were cultured with C225 or DTPA-PEG-C225 conjugates at 37 °C for 30 min, followed by washing the cells twice with cold PBS and lysis of the cells with a buffer containing 50 mM Tris-HCl, pH 7.4, 50 mM NaCl,

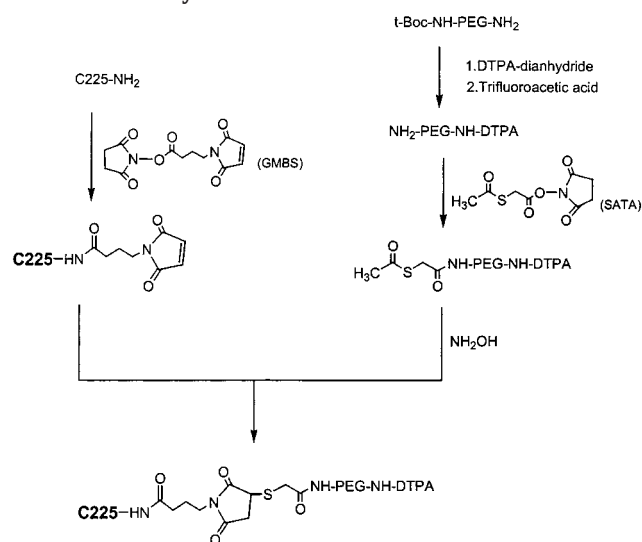
0.5% NP-40, 50 mM NaF, 1 mM Na₃PO₄, 1 mM phenylmethylsulfonyl fluoride, 25 μ g/mL leupeptin, and 25 μ g/mL aprotinin. The lysates were centrifuged at the full speed of a microcentrifuge for 15 min, and the supernatants were collected for protein concentration determination. Immunoprecipitation was performed by incubation of 100 μ g of cell lysate with 40 μ L of Sepharose 4B-conjugated protein A at room temperature for 1 h, followed by washing the immunoprecipitates 3 times with the lysing buffer and separation of immunoprecipitates with 7% polyacrylamide–SDS electrophoresis. Western blot was carried out by electronically transferring the samples into a nitrocellulose membrane and incubation of the membrane for 1 h with an anti-EGF receptor antibody. The EGF receptor signals in the membrane were developed by the ECL chemoluminescence detection kit (Amersham, Arlington Heights, IL).

MTT Assay. DiFi cells were seeded at 5×10^4 cells/well onto 24-well culture plates. Cell viability after 72 h treatment of the cells with C225 or DTPA-PEG-C225 was assayed by adding 50 μ L of 10 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) into 0.5 mL of culture medium and incubating the cells for 3 h at a 37 °C in a CO₂ incubator, followed by cell lysis with 500 μ L of lysis buffer containing 20% SDS in dimethylformamide/H₂O, pH 4.7, at 37 °C for more than 6 h. An optical absorbance of cell lysate was determined by measuring the cell lysate at a wavelength of 595 nm and normalizing the value with the corresponding control of untreated cells.

Pharmacokinetics. Nude mice (Harlan Sprague Dawley, Indianapolis, IN) were divided into two groups of three mice each and administered i.v. with 1:30 ¹¹¹In-DTPA-PEG-C225 at a dose of 1.5 μ g/mouse or with ¹¹¹In-DTPA-C225 at a dose of 5 μ g/mouse. At predetermined intervals, blood samples (30–60 μ L) were taken from the tail vein, and the radioactivity of each sample was measured with a gamma counter. The pharmacokinetic parameters for ¹¹¹In-DTPA-PEG-C225 and ¹¹¹In-DTPA-C225 were calculated from mean blood concentration values observed from the time of initial administration to 96 h after administration using WinNonlin 2.1 software (Scientific Consulting, Inc., Lexington, KY).

RESULTS AND DISCUSSION

Synthesis and Characterization. Previous studies have shown that an ¹¹¹In-labeled DTPA-mAb C225 specifically localizes in human cancer cell xenografts that overexpress EGF receptor (19). Phase I studies demonstrated that ¹¹¹In-DTPA-C225 was able to image squamous cell lung carcinoma expressing high levels of EGF receptor and metastases greater than 1 cm in diameter. However, considerable hepatic radioactivity was seen as a result of nonspecific uptake (20). The significant liver uptake made it difficult to visualize tumors or lesions inside or close to the liver. To overcome this drawback and optimize the imaging properties of C225, we sought to introduce flexible, linear PEG molecules into mAb C225 to reduce nonspecific interaction. In the past, attempts have been made in altering the pharmacological characteristics of mAbs by shielding the antibody with PEG or other synthetic polymers (21–23). Kitamura et al., for instance, reported the attachment of PEG (MW 5000) to murine mAb A7 and its F(ab')₂ fragment (21). These antibodies were radiolabeled with iodine-125 by the chloramine-T method. Both PEG-modified mAb and its fragment exhibited lower tissue-to-blood ratios in all resected organs than the parent antibodies, suggesting

Scheme 1. Synthesis of PEG-Modified C225 Monoclonal Antibody

reduced nonspecific interaction. Unfortunately, the tumor-to-blood ratio was also decreased after PEG modification. A similar study by Pedley et al. with PEG-modified and ¹²⁵I-labeled anti-CEA antibody A5B7 showed that the localization index was inferior to that of the unmodified antibody, and there was little increase in the tumor-to-blood ratio with time (22). A major problem of radioiodinated antibodies is the in vivo instability due to circulating dehalogenases, with the released radioiodine accumulating in the thyroid. We were also concerned with avoiding excessive manipulation of the mAb by both PEG modification and radioiodination procedures. Therefore, we were especially interested in developing a simple, one-step method to introduce both PEG molecules and radiometal chelators to mAbs such as C225.

Our approach was to conjugate the metal chelator DTPA through a PEG linker to C225 instead of attaching DTPA and PEG to the antibodies directly in a stepwise manner. The synthesis of a key intermediate, hetero-functional DTPA-PEG-ATA, with one end attached with a DTPA and the other with S-acetylthioacetate, is shown in Scheme 1. The sequence of reactions, i.e., the reaction with DTPA-dianhydride first, followed by deprotection and coupling with SATA, was necessary to achieve high yield. When t-Boc-NH-PEG-NH₂ was first coupled to SATA, the S-acetyl group was found to be unstable in the presence of TFA in the deprotection step. The prematurely released SH group could be easily destroyed by DTPA-dianhydride in the subsequent step. The 1:1 ratio of DTPA and ATA in DTPA-PEG-ATA was established by ¹H NMR analysis, elemental analysis, and by MALDI-TOF mass spectroscopic characterization. The theoretical ratio of integrals between -CH₂CH₂- in DTPA and CH₃- in ATA is 2.67. The ratio derived from NMR was 2.57, suggesting that the molar ratio of DTPA to ATA in DTPA-PEG-ATA was close to 1. The percentage weight

ratio of N to S in DTPA-PEG-ATA should be 2.18. The ratio derived from elemental analysis was 1.99. These data again confirm the 1:1 ratio of DTPA and ATA in DTPA-PEG-ATA. Finally, the calculated molecular mass of DTPA-PEG-ATA is 3770 Da; the molecular mass of the conjugate determined by MALDI-TOF mass spectroscopy was 3750 Da. No peaks of dimer and oligomers were found in the spectrum. These data support the proposed structure of DTPA-PEG-ATA. DTPA-PEG-ATA is stable for more than 6 months when stored as lyophilized powder at 4 °C.

DTPA-PEG-ATA was conveniently coupled to maleimide-activated C225 following a simple in situ deprotection step to release the free SH group (Scheme 1). Four DTPA-PEG-C225 conjugates with different degrees of C225 modification were synthesized. These conjugates were designated as 1:10, 1:20, 1:30, and 1:40 DTPA-PEG-C225, with the numbers being the molar ratios of antibody to GMBS in the maleimide-activating reaction. The physicochemical properties of the newly synthesized conjugates and some of the ¹¹¹In-labeled molecules are summarized in Table 1. Each C225 molecule contains approximately 50–60 free amino groups as measured by TNBS assay. In the 1:10, 1:20, 1:30, and 1:40 DTPA-PEG-C225 conjugates, approximately 20–25%, 40%, 60%, and 70% of amino groups were substituted, respectively. DTPA-C225 with DTPA directly attached to C225 mAb was also synthesized for the purpose of comparison (Table 1). Because DTPA-anhydride was readily hydrolyzed in aqueous media, coupling of DTPA directly to C225 was an inefficient reaction. Only 10–20% of the amino groups in C225 were substituted by DTPA when the molar ratio of DTPA-dianhydride to C225 reached 800:1.

Two PEG-modified antibody conjugates, 1:10 DTPA-PEG-C225 and 1:30 DTPA-PEG-C225, as well as DTPA-C225 were radiolabeled with indium-111. The radiochemical yields of the two ¹¹¹In-DTPA-PEG-C225 conjugates were over 70%, whereas the yield of ¹¹¹In-DTPA-C225 was only 40%. The lower yield with ¹¹¹In-DTPA-C225 reflects the fact that a large amount of DTPA was introduced into the coupling reaction between DTPA-dianhydride and C225. Although extensive purification procedures including ultracentrifugation and gel filtration on GPC column were used, DTPA-C225 could still be contaminated by trace amounts of DTPA molecules, leading to low labeling efficiency.

GPC was used to monitor the purity of C225 conjugates and ¹¹¹In-labeled C225 conjugates. As shown in Figures 1–3, coupling of PEG to C225 increased the hydrodynamic volume of C225. The retention time of intact C225 (6.0 min) on the TSK-G3000 column was shortened to 4.9 min for 1:30 DTPA-PEG-C225, suggesting that PEG molecules were chemically bound to the mAb (Figure 1). The GPC chromatogram of purified DTPA-PEG-C225 also indicated that gel filtration on the Sephacryl S-200 HR column adequately removed unconjugated C225. However, when 1:30 DTPA-PEG-C225 was labeled with indium-111, it gave two peaks in radio-GPC, with reten-

Table 1. Physicochemical Properties of DTPA-PEG-C225 Conjugates

C225 conjugates	molar ratio of C225 to GMBS	degree of NH ₂ substitution (%)	retention time in GPC (min)	radiochemical yield (%)	radiopurity (%)
1:10 DTPA-PEG-C225	1:10	20–25	6.1	>70	>97
1:20 DTPA-PEG-C225	1:20	40			
1:30 DTPA-PEG-C225	1:30	60	5.7	>70	>99
1:40 DTPA-PEG-C225	1:40	70			
DTPA-C225	1:800 ^a	10–20	6.7	40	>99

^a Molar ratio of C225 to DTPA-dianhydride.

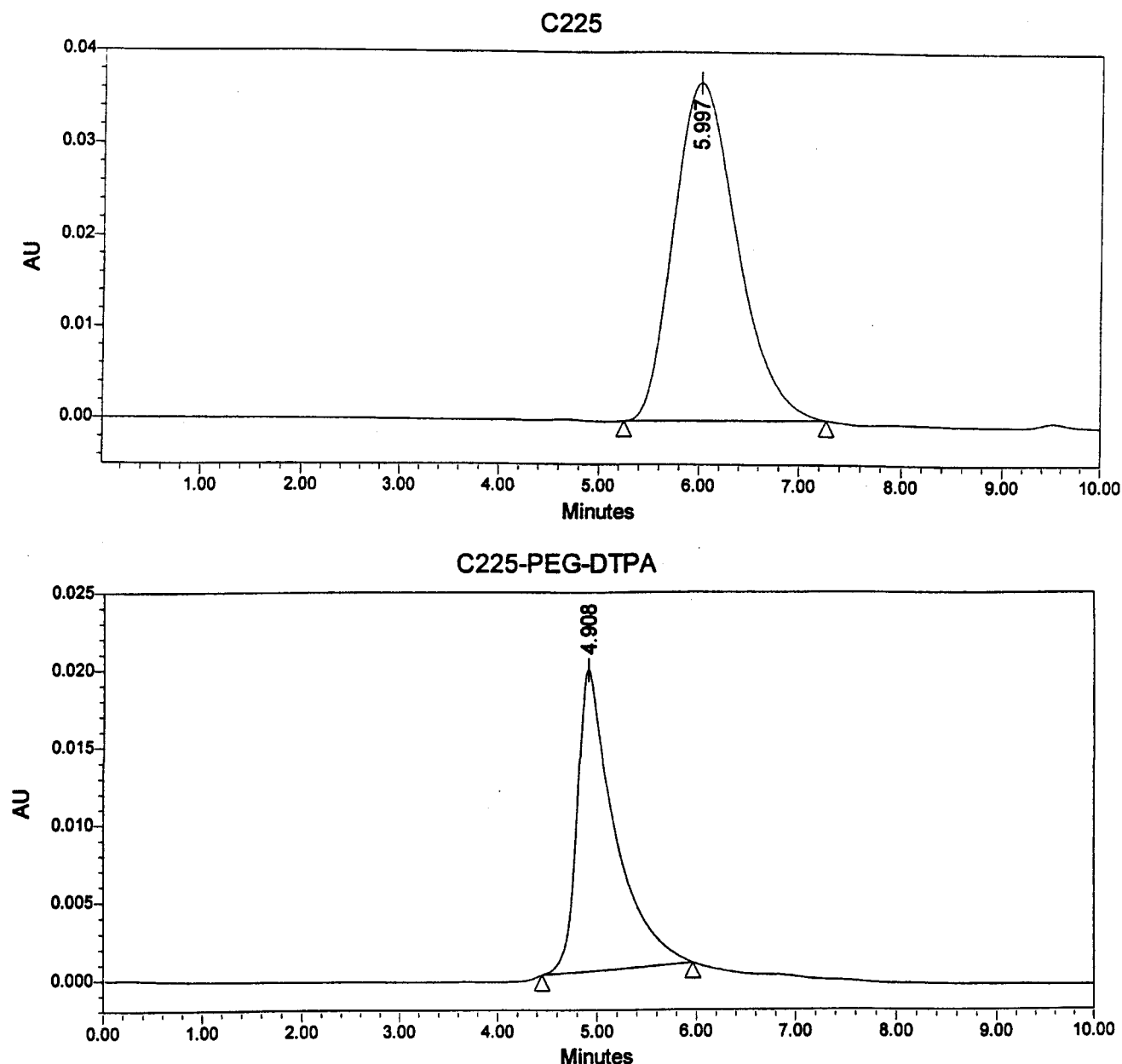


Figure 1. TSK-G3000 size-exclusion HPLC (UV absorbance at 254 nm) of native C225 mAb and 1:30 DTPA-PEG-C225 conjugate after purification on a Sephadex G-75 column. DTPA-PEG-C225 was eluted 1.1 min earlier than the native C225 mAb, revealing the conjugation of PEG molecules to the C225 mAb.

tion times of about 5.9 and 8.5 min, respectively (Figure 2A). The major peak at 5.9 min corresponded to 1:30 ^{111}In -DTPA-PEG-C225 while the minor peak at 8.5 min, which reflects a retention time identical to that of ^{111}In -DTPA-PEG (Figure 2B), was attributed to unconjugated DTPA-PEG. Thus, another gel filtration procedure was necessary to remove ^{111}In -DTPA-PEG. Radio-GPC chromatograms of purified 1:30 ^{111}In -DTPA-PEG-C225, 1:10 ^{111}In -DTPA-PEG-C225, and ^{111}In -DTPA-C225 are presented in Figure 3. The 1:30 and 1:10 conjugates were eluted at 5.7 and 6.1 min, respectively, reflecting shorter retention times than ^{111}In -DTPA-C225 (6.7 min). These results further confirmed that there were more PEG molecules attached to the 1:30 DTPA-PEG-C225 conjugate than to the 1:10 conjugate. The radiopurities of purified 1:30 ^{111}In -DTPA-PEG-C225, 1:10 ^{111}In -DTPA-PEG-C225, and ^{111}In -DTPA-C225 were >99%, >97%, and >99%, respectively (Table 1).

In Vitro Biological Functions. To determine whether PEG-modified C225 retains the binding affinity of the

parent mAb, we evaluated the *in vitro* biological properties of DTPA-PEG-C225 conjugates using three methods: competitive binding to human breast cancer MDA-MB-468 cells, immunoprecipitation of EGF receptor from human vulvar squamous carcinoma A431 cell lysates, and inhibition of growth of human colorectal carcinoma DiFi cells. All three cell lines express high levels of EGF receptor (24–26).

Studies on the cellular uptake of 1:30 ^{111}In -DTPA-PEG-C225 and ^{111}In -DTPA-C225 in MDA-MB-468 cells have shown that cell-associated radioactivity increased with increasing concentrations of radiolabeled C225. A plateau was reached at 2 $\mu\text{g/mL}$ for 1:30 ^{111}In -DTPA-PEG-C225 (data not shown). Therefore, a concentration of 1 $\mu\text{g/mL}$ for the radiolabeled C225 was chosen for the competitive binding assay. The binding of both 1:30 ^{111}In -DTPA-PEG-C225 and ^{111}In -DTPA-C225 to MDA-MB-468 cells was displaced by C225 in a dose-dependent manner, suggesting that the binding is EGF receptor specific (Figure 4). Furthermore, ^{111}In -DTPA-PEG-C225 was almost fully

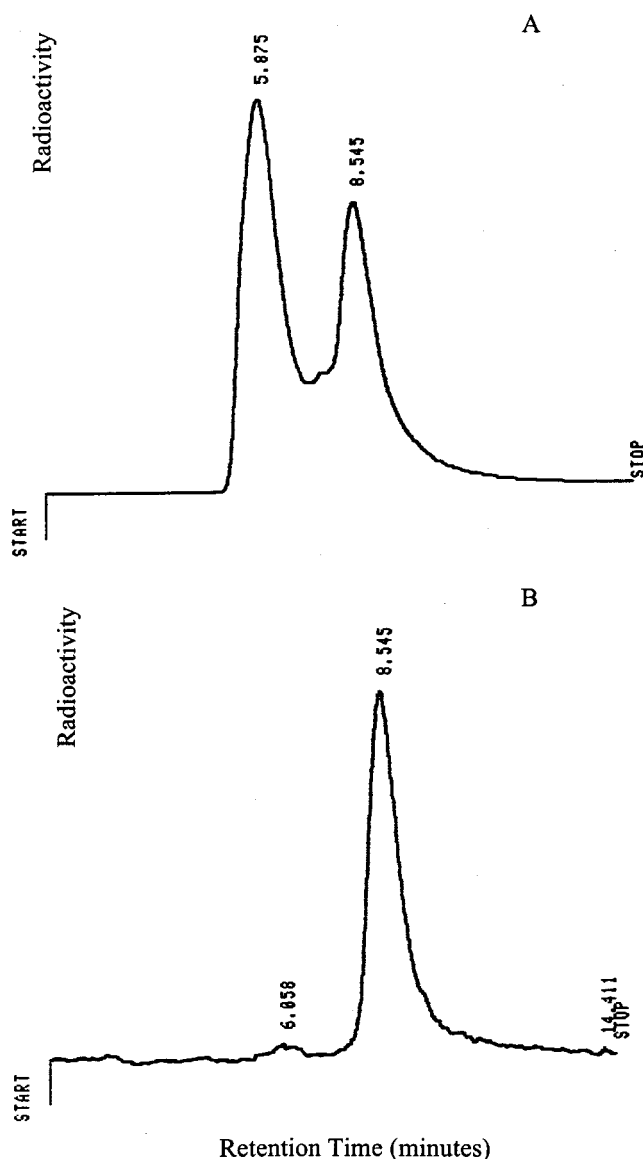


Figure 2. Radio-gel permeation chromatography of (A) ^{111}In -labeled 1:30 DTPA-PEG-C225 prior to purification and (B) ^{111}In -DTPA-PEG. In panel A, the peak at 5.9 min corresponds to the DTPA-PEG-C225 conjugate, and the peak at 8.5 min, which is identical to the peak in panel B, corresponds to unreacted DTPA-PEG.

displaced by a 16-fold excess of C225, whereas ^{111}In -DTPA-C225 was only 80% displaced by a 16-fold excess of C225 and could not be fully displaced even by 40-fold excess of C225. The remaining 20% of cell-associated ^{111}In -DTPA-C225 represent nonspecific binding to the cells. These results suggest that modification of C225 with PEG reduced the nonspecific interaction of the antibody. When the contribution of nonspecific binding is taken into consideration and is deducted from the cell-associated radioactivity, 44% of ^{111}In -DTPA-C225 and 33% of 1:30 ^{111}In -DTPA-PEG-C225 remained bound to the cells when the concentration of native C225 mAb was equal to the concentration of the labeled molecules (1 $\mu\text{g}/\text{mL}$). This means that ^{111}In -DTPA-C225 retained about 88% of the receptor binding affinity of native C225, while 1:30 ^{111}In -DTPA-PEG-C225 retained about 66% of the binding affinity.

To further assess the receptor binding affinity of DTPA-PEG-C225 conjugates, the ability of DTPA-PEG-C225 conjugates to bind to EGF receptor was investigated in A431 cells, which express a very high level of EGF

Table 2. Pharmacokinetic Parameters of 1:30 ^{111}In -DTPA-PEG-C225 and ^{111}In -DTPA-C225

parameters ^a	DTPA-C225	DTPA-PEG-C225 (1:30)
A (% ID/mL)	11.7	11.3
B (% ID/mL)	14.8	37.3
C (% ID/mL)	13.3	7.6
α (h^{-1})	1.74	2.82
β (h^{-1})	0.08	0.10
γ (h^{-1})	0.01	0.03
$t_{1/2,\alpha}$ (h)	0.40	0.24
$t_{1/2,\beta}$ (h)	9.11	6.82
$t_{1/2,\gamma}$ (h)	52.9	21.1
V_1 (mL)	2.52	1.78
V_{ss} (mL)	5.41	2.94
CL (mL/h)	0.08	0.16
k_{10} (h^{-1})	0.03	0.09
k_{12} (h^{-1})	0.48	0.53
k_{21} (h^{-1})	1.24	2.27
k_{13} (h^{-1})	0.03	0.02
k_{31} (h^{-1})	0.04	0.04

^a % ID/mL is the injected dose per milliliter of blood. A, B, C, α , β , and γ are hybrid constants. V_1 and V_{ss} are volume distributions in the central compartment and at steady state (V_{ss}). CL is clearance. k_{10} , k_{12} , k_{21} , k_{13} , and k_{31} are microconstants.

receptors. The cells exposed to C225 or one of the three 1:10, 1:20, and 1:40 DTPA-PEG-C225 conjugates were lysed, followed by immunoprecipitation of the antibody-bound EGF receptor with protein A-Sepharose beads and visualization of the EGF receptors with Western blot analysis. The results shown in Figure 5 indicate that all three DTPA-PEG-C225 conjugates, with 20%, 50%, and up to 70% of the amino groups in C225 substituted, retained their EGF receptor binding activities. However, the amounts of EGF receptor immunoprecipitated by C225 conjugates decreased with increasing degree of substitution, indicating that the binding affinity of the PEG-modified C225 decreased with the increasing number of PEG molecules attached to the mAb.

The activities of DTPA-PEG-C225 conjugates were further assessed by an independent antiproliferation assay. We have previously reported that blocking EGF receptor tyrosine kinase activity with C225 leads to cell cycle arrest and subsequent cell death through apoptosis in DiFi cells (26). As shown in Figure 6, while the linker molecule PEG-DTPA itself had no effect on DiFi cell growth, all three conjugates, 1:10, 1:20, and 1:40 DTPA-PEG-C225, inhibited the tumor cell growth to the same extent as native C225, indicating that all conjugates were capable of inducing apoptosis in the DiFi human colon cancer cells (Figure 6).

Pharmacokinetics. The radioactivities of the blood samples obtained at different time intervals after i.v. injection of 1:30 ^{111}In -DTPA-PEG-C225 and ^{111}In -DTPA-C225 were measured with a gamma counter. Figure 7 plots blood radioactivity expressed as a percentage of injected dose per milliliter of blood (% ID/mL of blood) versus time following i.v. injection of radiolabeled C225. The profiles of both ^{111}In -DTPA-PEG-C225 and ^{111}In -DTPA-C225 fit well into three-compartment models, and can be mathematically described by the triexponential equations: $Ct = 11.3e^{-2.82t} + 37.3e^{-0.10t} + 7.6e^{-0.03t}$ and $Ct = 11.7e^{-1.74t} + 14.8e^{-0.08t} + 13.3e^{-0.01t}$, respectively, where Ct is % ID/mL of blood at any given time t .

The pharmacokinetic parameters of volume distributions in the central compartment (V_1) and at steady state (V_{ss}), clearance (CL), hybrid constants (A, B, C, α , β , and γ), and microconstants (k_{10} , k_{12} , k_{21} , k_{13} , and k_{31}) are summarized in Table 2. The volume distribution at steady state (V_{ss}) of 1:30 ^{111}In -DTPA-PEG-C225, 2.94 mL,

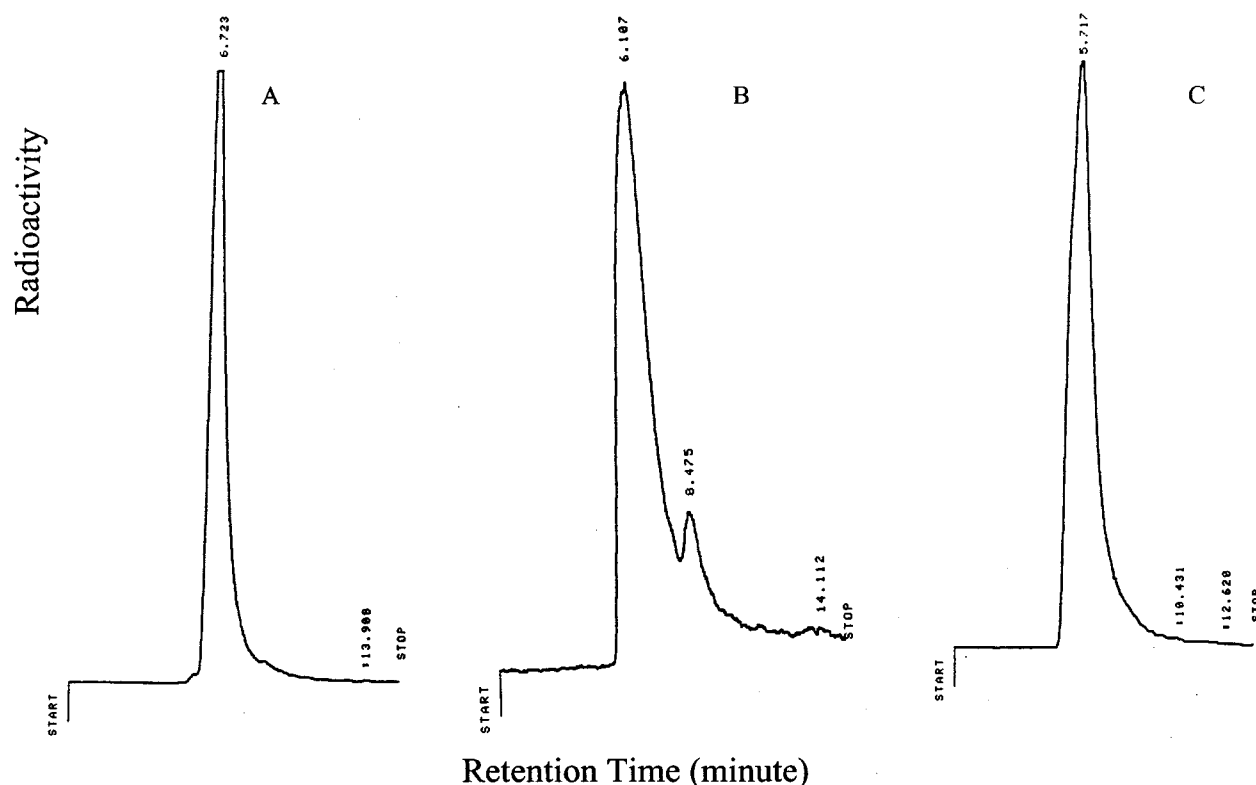


Figure 3. Radio-gel permeation chromatography of purified (A) ^{111}In -DTPA-C225, (B) 1:10 ^{111}In -DTPA-PEG-C225, and (C) 1:30 ^{111}In -DTPA-PEG-C225. The three molecules were eluted through a Phenomenex Bioseep SEC-S3000 column. The radiochemical purity of each conjugate was greater than 97%.

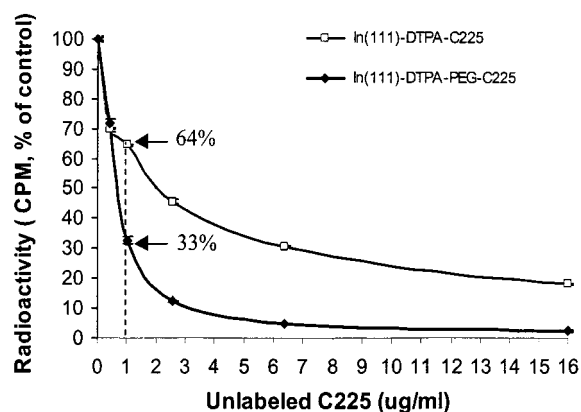


Figure 4. Competitive binding of ^{111}In -DTPA-C225 and 1:30 ^{111}In -DTPA-PEG-C225 with native C225 to MDA-MB-468 cells. The cells were incubated with 1 $\mu\text{g/mL}$ of each ^{111}In -labeled C225 conjugate plus unlabeled C225 at different concentrations. After incubation at 37 $^{\circ}\text{C}$ for 2 h, the cell-associated radioactivity was measured with a gamma counter. The data are expressed as counts per minute (CPM) as a percentage of control and presented as the means of triplicates with standard deviations.

was smaller than that of ^{111}In -DTPA-C225, 5.41 mL. The narrower distribution of PEG-modified molecules might be due to the reduced nonspecific binding of these molecules to tissues and the faster returning rate constant from tissues to the central compartment, reflected by the larger k_{21} . The elimination rate constant from the central compartment (k_{10}) and the clearance (CL) of 1:30 ^{111}In -DTPA-PEG-C225 were 0.09 h^{-1} and 0.16 mL/h, respectively, higher than those of ^{111}In -DTPA-C225, 0.03 h^{-1} and 0.08 mL/h, respectively. The terminal half-life ($t_{1/2,\gamma}$) of ^{111}In -DTPA-PEG-C225, 21.1 h, was shorter than that of ^{111}In -DTPA-C225, 52.9 h, resulting from its smaller volume distribution and faster clearance.

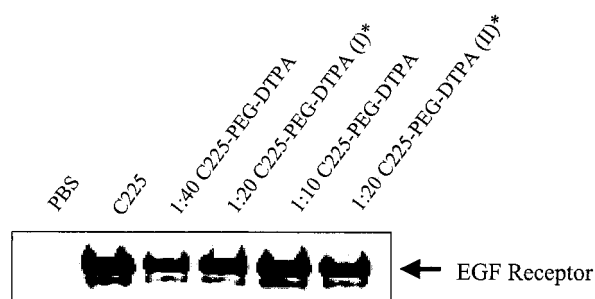


Figure 5. Immunoprecipitation of EGF receptor from A431 cells by C225 and its PEG conjugates. Western blotting with anti-EGF receptor antibody revealed the binding of DTPA-PEG-C225 molecules to EGF receptor in A431 cells. In 1:10, 1:20, and 1:40 DTPA-PEG-C225s, PEG molecules were attached to 25%, 50%, and 70% of the amino groups in C225, respectively. The asterisks represent different batches of DTPA-PEG-C225 with the same degree of substitution (1:20).

Because the molecular weight of C225 is relatively high (150 000), its modification with PEG (34 000) was not expected to have profound effects on its blood circulation time. Nevertheless, our finding that the terminal half-life of 1:30 ^{111}In -DTPA-PEG-C225 was actually shorter than that of ^{111}In -DTPA-C225 is somewhat unexpected. A possible explanation is in vivo cleavage of ^{111}In -DTPA-PEG from ^{111}In -DTPA-PEG-C225. The shortened blood retention of ^{111}In -DTPA-PEG-C225 could be advantageous in obtaining improved images of target organs (8–10).

SUMMARY

The procedures for synthesizing and radiolabeling DTPA-PEG-C225 are efficient and reproducible. DTPA-PEG-C225 retained its binding affinity to EGF receptor with reduced nonspecific interaction with cells, although

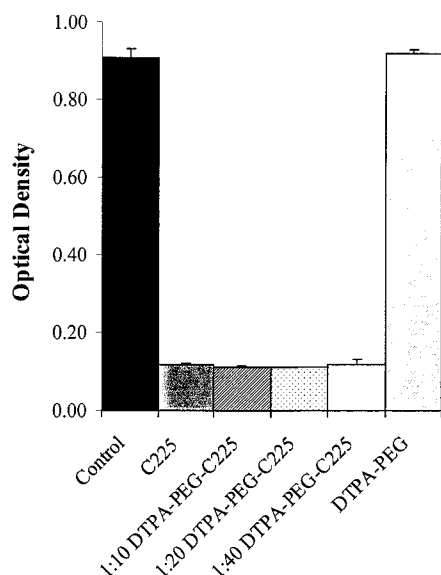


Figure 6. Blockage of DiFi cell growth by C225, DTPA-PEG-C225 conjugates, and free DTPA-PEG. The DiFi cells were exposed to the indicated reagents at concentrations of 20 nM. After 3 days of incubation, the numbers of surviving cells were measured by MTT assay. The results are presented as the means of triplicates with standard deviations.

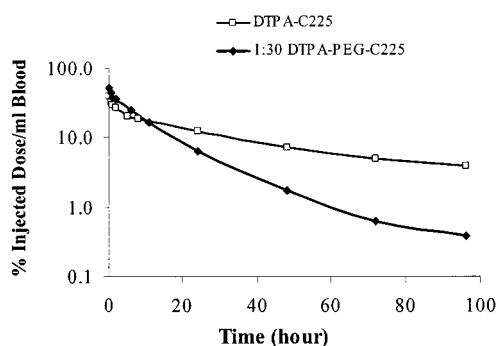


Figure 7. Pharmacokinetics of ^{111}In -DTPA-C225 and 1:30 ^{111}In -DTPA-PEG-C225. Each mouse was given 1.5 μg of a radiotracer i.v. The blood samples were collected at different time intervals, and the radioactivity of each sample was measured. The data are expressed as percentages of injected dose per milliliter of blood (% ID/mL) and presented as the means of triplicates. The standard deviation for each time point is less than 10%. The profiles of both ^{111}In -DTPA-C225 and 1:30 ^{111}In -DTPA-PEG-C225 fit well into the three-compartment model.

a high degree of PEG modification appeared to interfere with the binding activity of the antibody. Modification of C225 with PEG (MW 3400), even at a high degree of substitution, did not appear to increase blood circulation time of the antibody. PEG-modified C225 is less widely distributed to normal tissues than C225 without PEG, suggesting reduced nonspecific binding of PEG-modified C225 to tissues. Taken together, our results suggest that this method, introducing DTPA through a PEG linker, may be a useful strategy in developing radioimmunodetection imaging agents. This approach should be applicable to the labeling of other mAbs as well as their F(ab')_2 , Fab' , and ScFv fragments.

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Stabilization of ^{90}Y -Labeled DOTA-Biomolecule Conjugates Using Gentisic Acid and Ascorbic Acid

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Radiolytic degradation of radiolabeled compounds is a major challenge for the development of new therapeutic radiopharmaceuticals. The goal of this study is to explore the factors influencing the solution stability of a ^{90}Y -labeled DOTA-peptide conjugate (RP697), including the amount of total activity, the activity concentration, the stabilizer concentration, and the storage temperature. In general, the rate of radiolytic decomposition of RP697 is much slower at the lower activity concentration (<4 mCi/mL) than that at the higher concentration (>10 mCi/mL). RP697 remains relatively stable at the 20 mCi level and room temperature while it decomposes rapidly at the 100 mCi level under the same storage conditions. Radical scavengers, such as gentisic acid (GA) and ascorbic acid (AA), were used in combination with the low temperature (-78°C) to prevent the radiolytic decomposition of RP697. It was found that RP697 remains stable for at least 2 half-lives of ^{90}Y when GA or AA (10 mg for 20 mCi of ^{90}Y) is used as a stabilizer when the radiopharmaceutical composition is stored at -78°C . The stabilizer (GA and AA) can be added into the formulation either before or after radiolabeling. The post-labeling approach is particularly useful when the use of a large amount of the stabilizer interferes with the radiolabeling. The radiopharmaceutical composition developed in this study can also apply to other ^{90}Y -labeled DOTA-biomolecule conjugates. The amount of the stabilizer used in the radiopharmaceutical composition and storage temperature should be adjusted according to the sensitivity of the radiolabeled DOTA-biomolecule conjugate toward radiolytic decomposition.

INTRODUCTION

We have been interested in the development of new diagnostic and therapeutic radiopharmaceuticals based on small-molecule receptor ligands (1–18). In our previous contribution (19), we reported the synthesis of a DOTA-conjugated vitronectin receptor antagonist (SU015: 2-(1,4,7,10-tetraaza-4,7,10-tris(carboxymethyl)-1-cyclododecyl)-acetyl-Glu(cyclo{Lys-Arg-Gly-Asp-D-Phe})-cyclo{Lys-Arg-Gly-Asp-D-Phe}) and its ^{90}Y complex (RP697 in Figure 1). Through a series of radiolabeling experiments, we developed a formulation for routine preparations of RP697 in high yield with the radiochemical purity (RCP) of $>95\%$. However, RP697 prepared using this formulation was not stable over time at room temperature, particularly at high activity levels.

^{90}Y is an β -emitter with a 2.27 MeV β -particle and a 2.67 day half-life. Due to the high energy of the β -particle, the radiolabeled cyclic peptide is very susceptible to radiolytic decomposition. Since the tumor uptake of RP697 is largely dependent on the receptor binding of the two cyclic RGD-containing peptide motifs, radiolytic decomposition may lead to the decreased therapeutic efficacy and unwanted radiation toxicity to other normal organs such as liver and bone marrow. The mechanism for radiolytic decomposition is thought to be mediated by the formation of free radicals, such as superoxide ($\text{O}_2^\bullet$) and hydroxyl radicals ($\text{OH}^\bullet$), in the presence of a large amount of high-energy β -particles (20). Radical scavengers such as human serum albumin (HAS), gentisic acid (GA), and ascorbic acid (AA) have been used as stabilizers

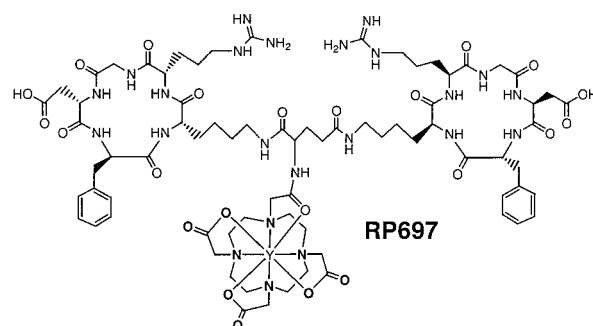


Figure 1. Structure of RP697.

for the radiolabeled antibodies (21–23). It was also found that freezing the antibody after radiolabeling up to the time of administration could considerably improve the immunoreactivity by decreasing the diffusion and interaction of free radicals with the radiolabeled biomolecule (22).

The purpose of this study is to explore the factors influencing the solution stability of RP697, including the activity concentration, the total activity level, the stabilizer concentration, and the storage temperature. We have chosen AA and GA as stabilizers since they have been approved for pharmaceutical or radiopharmaceutical applications. The ultimate goal is to find the optimized radiopharmaceutical composition to maintain the solution stability of RP697. In principle, the radiopharmaceutical composition developed in this study should also apply to other ^{90}Y -labeled DOTA-biomolecule conjugates.

EXPERIMENTAL PROCEDURES

Materials. Acetic acid (ultrapure), ammonium hydroxide (ultrapure), ascorbic acid (sodium salt), diethylene-

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triaminepentaacetic acid (DTPA), and gentisic acid (sodium salt) were purchased from either Aldrich or Sigma Chemical Co., and were used as received. $^{90}\text{YCl}_3$ (in 0.05 N HCl) was purchased from New England Nuclear Life Sciences, North Billerica, MA. The cyclic pentapeptide cyclo(Arg-Gly-Asp-D-Phe-Lys), as its trifluoroacetic acid (TFA) salt, was prepared according to the literature method (24). Synthesis of SU015, 2-(1,4,7,10-tetraaza-4,7,10-tris(carboxymethyl)-1-cyclododecyl)-acetyl-Glu(cyclo-{Lys-Arg-Gly-Asp-D-Phe})-cyclo{Lys-Arg-Gly-Asp-D-Phe}, as its trifluoroacetic acid (TFA) salt will be described in a companion paper (19).

General Procedure for the Synthesis of RP697.

To a shielded clean 5 mL vial containing 100 μg of SU015 and 0–10 mg of sodium gentisate (GA) or sodium ascorbate (AA) dissolved in 0.5 mL of 0.5 M ammonium acetate buffer (pH 8.0) was added 20–40 μL of $^{90}\text{YCl}_3$ stock solution (~ 20 mCi) in 0.05 N HCl. The reaction mixture was heated at 95–100 $^\circ\text{C}$ for 5 min. A sample of the resulting solution was diluted 20-fold with 2 mM DTPA solution (pH 5), and then analyzed by radio-HPLC and ITLC. Each condition was run twice, and the radiochemical purity (RCP) data are presented as an average of two independent measurements.

Analytical Methods. The HPLC method used a HP-1100 HPLC system with a UV/visible detector ($\lambda = 220$ nm), an IN-US radio-detector, and a Zorbax C₁₈ column (4.6 mm $\times$ 250 mm, 80 $\text{\AA}$ pore size). The flow rate was 1 mL/min. The mobile phase was isocratic from 0 to 18 min using 87% solvent A (0.025 M ammonium acetate buffer, pH 6.8) and 13% solvent B (acetonitrile), followed by an isocratic wash using 40% solvent A and 60% solvent B from 19 to 25 min. The retention time of RP697 is 14–16 min. The ITLC method used Gelman Sciences silica gel ITLC paper strips and a 1:1 mixture of acetone and saline as eluant. By this method, RP697 migrates to the solvent front while ^{90}Y colloid and ^{90}Y acetate remain at the origin. The corrected RCP for RP697 was calculated by subtracting the percentage of ^{90}Y colloid and ^{90}Y acetate obtained by ITLC from that obtained by radio-HPLC.

RESULTS

Room Temperature Stability of RP697 at Low Concentration (4 mCi/mL). In the first experiment, we prepared three RP697 vials according to the standard procedure. The first vial contains only 100 μg of SU015, the second vial contains 100 μg of SU015 and 5 mg of GA, and the third vial contains 100 μg of SU015 and 5 mg of sodium ascorbate (AA). After addition of 20 mCi of $^{90}\text{YCl}_3$ in each vial, the reaction mixtures were heated at 100 $^\circ\text{C}$ for 5 min. After radiolabeling, the resulting solution was diluted to a concentration of 4 mCi/mL with saline, and the solution stability of RP697 at room temperature was monitored by radio-HPLC over 6 days. Figure 2 shows a typical radio-HPLC chromatogram for RP697. The peak at 14 min is from RP697. The peak at ~ 3.5 min is probably from a combination of several hydrophilic ^{90}Y -containing species while the peak at 24 min is from more lipophilic ^{90}Y -containing species. Since most of the radioimpurities are less than 1%, no efforts were made for further characterization. Figure 3 shows the RCP change over time for RP697. It is clear that RP697 remains relatively stable (RCP = 96.5% at 0 h and 94% at 144 h post-labeling) in the presence of GA and AA while it decomposes rapidly in the absence of GA and RCP is only 86% at 6 days post-labeling.

GA Concentration and Solution Stability. In this experiment, RP697 was prepared according to the general

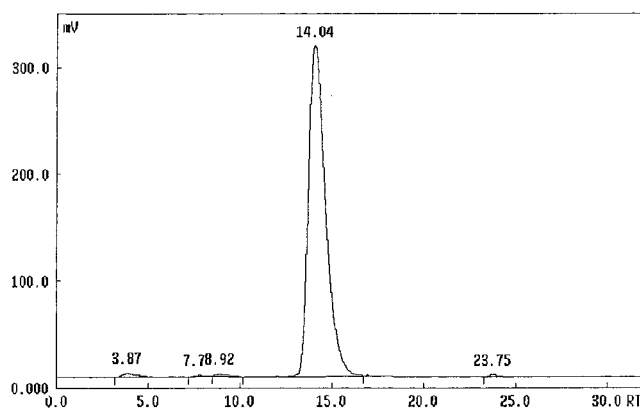


Figure 2. Typical radio-HPLC chromatogram of RP697.

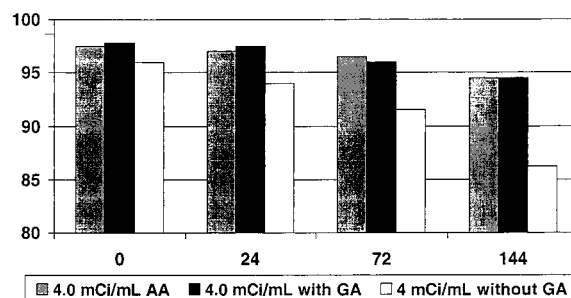


Figure 3. Effect of stabilizers on solution stability of RP697 at room temperature. Each condition was run twice, and the radiochemical purity (RCP) data are presented as an average of two independent measurements.

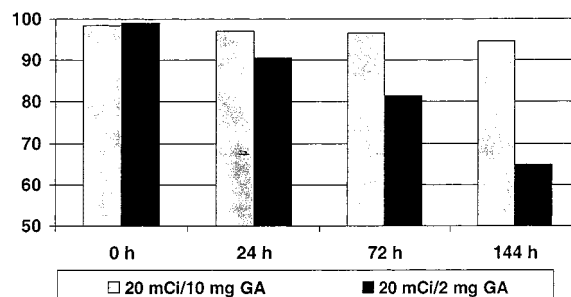


Figure 4. Effect of GA concentration on solution stability of RP697 at room temperature. Each condition was run twice, and the radiochemical purity (RCP) data are presented as an average of two independent measurements.

procedure using two levels (2 and 10 mg) of GA and 100 μg of SU015 for 20 mCi of $^{90}\text{YCl}_3$. The total volume was 0.5 mL, and the activity concentration was 40 mCi/mL. After radiolabeling, the resulting solution was kept at room temperature, and the solution stability of RP697 was monitored by radio-HPLC over 6 days. Figure 4 shows the RCP change over 6 days for RP697. The RCP of RP697 decreased from 96.5% at 0 h to 93% at 144 h post-labeling when 10 mg of GA was used for 20 mCi of activity. The RCP dropped much more rapidly from 97% at 0 h to 65% at 144 h when only 2 mg of GA was used to stabilize 20 mCi of RP697. This suggests that 2 mg of GA is not sufficient to maintain RCP above 90% and 10 mg of GA is needed to stabilize RP697 over 2 half-lives of ^{90}Y . Compared to the results from the previous experiment, it seems that the rate of decomposition of RP697 is also dependent on the activity concentration.

Activity Level and Solution Stability of RP697.

In this experiment, we prepared two RP697 vials using two different levels of ^{90}Y . In one vial, it contained 10 mg of GA, 100 μg of SU015, and 20 mCi of $^{90}\text{YCl}_3$ (total

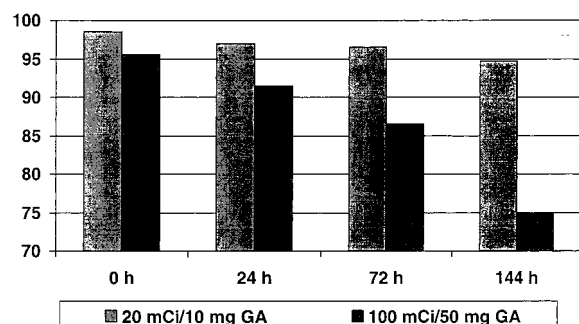


Figure 5. Effect of the activity concentration on solution stability of RP697 at room temperature. Each condition was run twice, and the radiochemical purity (RCP) data are presented as an average of two independent measurements.

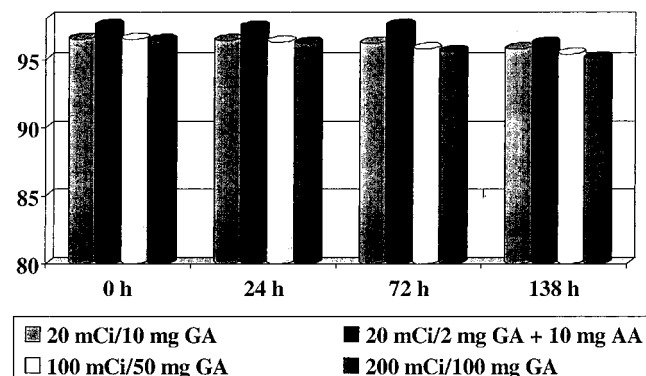


Figure 6. Solution stability of RP697 at -78°C . Each condition was run twice, and the radiochemical purity (RCP) data are presented as an average of two independent measurements.

volume = 0.5 mL). In another vial, RP697 was prepared using 500 μg of SU015, 50 mg of GA, and 100 mCi of $^{90}\text{YCl}_3$ (total volume = 2.5 mL). After radiolabeling, the resulting solution was kept at room temperature, and the solution stability of RP697 was monitored by radio-HPLC over 6 days, as shown in Figure 5.

Solution Stability of RP697 at -78°C . RP697 was prepared using the standard radiolabeling procedure (100 μg of SU015, 10 mg of GA for 20 mCi of $^{90}\text{YCl}_3$, and heating at 100°C for 5 min). For the vials containing 100 and 200 mCi of activity, all the component levels were increased proportionally. For the vial using AA as the stabilizer, AA (200 mg/mL) was dissolved in 0.5 N ammonium acetate buffer (pH 7.4) and added to the reaction mixture (10 mg of AA for 20 mCi of ^{90}Y) after radiolabeling. After addition of AA, all the vials containing RP697 were kept in a dry ice box (-78°C). Samples of the reaction mixture were analyzed by HPLC at 0, 24, 56, and 138 h. Figure 6 shows the RCP change over 6 days for RP697. Apparently, RP697 can be stabilized by the combination use of a stabilizer and low-temperature storage at -78°C .

DISCUSSION

There has been great current interest in radiolabeled small peptides as therapeutic radiopharmaceuticals (25–27). In developing a new receptor-based therapeutic radiopharmaceutical, several factors need to be considered to satisfy the clinical need and to comply with FDA regulations. The therapeutic radiopharmaceutical must demonstrate therapeutic efficacy and favorable pharmacokinetics, including high and fast tumor uptake, high tumor-to-background ratio, long tumor residence time, and fast renal clearance. High tumor uptake and fast

renal clearance are particularly important to improve the tumor-to-background ratio and to reduce the radiation burden to other major organs (kidney, liver, and bone marrow). The new radiopharmaceutical must have high radiochemical purity (RCP $\geq 90\%$). Unlike diagnostic radiopharmaceuticals, therapeutic radiopharmaceuticals have to be manufactured and released under GMP (Good Manufacturing Practice) conditions, and delivered for clinic applications. Therefore, the new therapeutic radiopharmaceutical must retain its chemical and biological integrity during release and transportation.

Therapeutic radiopharmaceuticals comprising β -emitting radionuclides may undergo autoradiolysis during preparation, release, transportation, and storage. During radiolysis, emissions from the radionuclide attack the metal chelate, targeting the biomolecule, and other compounds in proximity, which results in inter- and intramolecular decomposition or destruction of the radiometal chelate or the biomolecule. Radioactivity, which is not linked to the targeting biomolecule, will accumulate in nontargeting tissues and lead to the unwanted radiation toxicity to nontargeting tissues. Thus, it is important that the radionuclide remains linked to the targeting moiety, and the specificity of the targeting biomolecule is preserved. As a matter of fact, a major challenge for the development of a therapeutic radiopharmaceutical is to stabilize the radiolabeled compound during radiolabeling, release, and transportation.

Radiolysis is caused by the formation of free radicals such as hydroxyl and superoxide radicals. Free radicals are very reactive toward organic molecules such as peptides. To prevent radiolysis and stabilize the radiolabeled biomolecule, a radical scavenger or radiolytic stabilizer is often used either during or after the radiolabeling. A stabilizer is often an antioxidant, which readily reacts with hydroxyl and superoxide radicals. In general, the stabilizer for the therapeutic radiopharmaceutical should have the following characteristics: low or no toxicity or immunoreactivity when it is used for human administration, no interference with the receptor binding of the radiolabeled compound to the target cells or tissue(s), and the ability to stabilize the therapeutic radiopharmaceutical for a reasonable period of time (preferably 2 half-lives of ^{90}Y) for preparation, release, storage, and transportation.

There are several commercially available antioxidants. Human serum albumin (HAS) has been used as a stabilizer for the radiolabeled antibodies (21–23). Ascorbic acid is an antioxidant, and an FDA-approved pharmaceutical suitable for human injection. Gentisic acid has been used as a stabilizer in $^{99\text{m}}\text{Tc}$ radiopharmaceutical formulations (28–30). The stabilizer can be added into a therapeutic radiopharmaceutical formulation either before or after the radiolabeling. For pre-labeling addition, the stabilizer is added before addition of $^{90}\text{YCl}_3$ stock solution. During the radiolabeling process, the stabilizer is subjected to a certain degree of thermal decomposition, particularly at elevated temperatures. For the post-labeling addition, the stabilizer is not exposed to the heating process; thereby it may have longer stabilizing effect and higher stabilizing efficiency. The post-labeling approach is particularly useful when the addition of a large amount of stabilizer in the radiopharmaceutical formulation interferes with the radiolabeling.

There are several factors influencing the solution stability of RP697. These include the amount of activity in each vial, the activity concentration, the relative amount of stabilizer, and the storage temperature. For example, the rate of radiolytic decomposition of RP697

is much slower at the activity concentration of 4 mCi/mL than that at a concentration of 40 mCi/mL (Figure 3). It needs 10 mg of GA or AA to maintain the RCP of RP697 (20 mCi) above 90% over 2 half-lives of ⁹⁰Y (Figure 4). RP697 remains relatively stable at room temperature if 10 mg of GA is used for 20 mCi of activity. It decomposes much faster when 50 mg of GA is used for 100 mCi of activity under the same storage conditions, even though the GA/activity ratio remains the same (Figure 5). Therefore, the solution stability data obtained from the low activity level (<20 mCi) cannot be simply extrapolated to high activity levels (>100 mCi).

In addition to the use of an antioxidant in the radiopharmaceutical formulation, storing the reaction mixture at low temperature (−78 °C/dry ice) also has a dramatic impact on the solution stability of RP697. For example, RP697 remains stable for at least 2 half-lives of ⁹⁰Y at −78 °C (Figure 6) while it decomposes rapidly at room temperature. Freezing the reaction mixture containing RP697 offers two advantages: stopping the diffusion of free radicals and slowing down the reaction kinetics between free radicals and radiolabeled cyclic peptide. Thus, it is recommended that the radiopharmaceutical be stored at low temperatures to avoid extensive radiolysis during release and transportation. The amount of antioxidant used in the radiopharmaceutical formulation and the storage temperature during release and transportation should be adjusted according to the sensitivity of a specific radiolabeled receptor ligand toward radiolytic decomposition.

CONCLUSIONS

In this study, we explored the factors influencing the solution stability of RP697, a ⁹⁰Y-labeled DOTA-peptide conjugate, and found that both the amount of total activity and the activity concentration have significant impact on the stability of the ⁹⁰Y-labeled cyclic peptide. The rate of radiolytic decomposition of RP697 is much slower at the lower activity concentration (<4 mCi/mL) than that at the higher concentration (>10 mCi/mL). RP697 remains relatively stable at room temperature when it is prepared at the 20 mCi level while it decomposes rapidly at the 100 mCi level under the same storage conditions. Therefore, the solution stability data obtained from the low activity level (<20 mCi) cannot be simply extrapolated to high activity levels (>100 mCi).

To prevent extensive radiolysis, we used both GA and AA as stabilizers. The stabilizer can be added into the formulation either before or after radiolabeling. For the post-labeling addition, the stabilizer is not exposed to the heating process; thereby it may have a longer stabilizing effect and higher stabilizing efficiency. The post-labeling approach is particularly useful when the addition of a large amount of stabilizer in the radiopharmaceutical formulation interferes with the radiolabeling of the DOTA-biomolecule conjugate.

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^{90}Y and ^{177}Lu Labeling of a DOTA-Conjugated Vitronectin Receptor Antagonist Useful for Tumor Therapy

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The ^{90}Y and ^{177}Lu complexes (RP697 and RP688, respectively) of a DOTA-conjugated vitronectin receptor antagonist (SU015: 2-(1,4,7,10-tetraaza-4,7,10-tris(carboxymethyl)-1-cyclododecyl)acetyl-Glu-(cyclo{Lys-Arg-Gly-Asp-D-Phe})-cyclo{Lys-Arg-Gly-Asp-D-Phe}) were prepared by reacting SU015 with the radiometal chloride in ammonium acetate buffer (pH > 7.2) in the presence of an antioxidant (sodium gentisate, GA). Through a series of radiolabeling experiments, it was found that there are many factors influencing the rate of ^{90}Y chelation and the radiolabeling efficiency of SU015. These include the purity of SU015, the pH, reaction temperature, and heating time, as well as the presence of trace metal contaminants, such as Ca^{2+} , Fe^{3+} , and Zn^{2+} . The chelation of ^{90}Y by SU015 is slow, so that heating at elevated temperatures (50–100 °C) is needed to complete the ^{90}Y -labeling. The rate of ^{90}Y chelation is also dependent on the pH of the reaction mixture. Under optimized radiolabeling conditions (pH 7.2–7.8 and heating at 50–100 °C for 5–10 min), the minimum amount of SU015 required to achieve 95% RCP for RP697 is $\sim 25\text{ }\mu\text{g}$ for 20 mCi of $^{90}\text{YCl}_3$ corresponding to a SU015: ^{90}Y ratio of $\sim 30:1$.

INTRODUCTION

We have been interested in the development of diagnostic radiopharmaceuticals based on small molecule receptor ligands (1–15). We have been using a ternary ligand system for the $^{99\text{m}}\text{Tc}$ labeling of a variety of hydrazinonicotinamide-conjugated biomolecules (HYNIC-BM), including a GPIIb/IIIa receptor antagonist for thrombus imaging (1–8), and chemotactic peptides (9) and LTB_4 receptor antagonists for imaging infection and inflammation (12–14). In a separate communication (15), we report the $^{99\text{m}}\text{Tc}$ labeling of an HYNIC-conjugated vitronectin receptor antagonist (SQ168: 2-sulfonatobenzenesulfonic acid hydrazine of *N*-(6-hydrazinonicotinamido)-Glu(cyclo{Lys-Arg-Gly-Asp-D-Phe})-cyclo{Lys-Arg-Gly-Asp-D-Phe}) and biological properties of the complex [$^{99\text{m}}\text{Tc}(\text{SQ168})(\text{tricine})(\text{TPPTS})$] (RP593: TPPTS = trisodium triphenylphosphine-3,3',3''-trisulfonate). In the c-Neu Oncomouse tumor model (15), RP593 shows high tumor uptake and long tumor residence time (3.4% ID/g at 2 h and 1.5% ID/g at 24 h postinjection) and is excreted predominantly via the renal system. This suggests that the corresponding ^{90}Y and ^{177}Lu complexes have the potential as target-specific radiopharmaceuticals for tumor therapy.

As a continuation of our efforts in developing new peptide-based diagnostic and therapeutic radiopharmaceuticals, a DOTA-conjugated vitronectin receptor antagonist (Figure 1: SU015 = 2-(1,4,7,10-tetraaza-4,7,10-tris(carboxymethyl)-1-cyclododecyl)-acetyl-Glu(cyclo{Lys-Arg-Gly-Asp-D-Phe})-cyclo{Lys-Arg-Gly-Asp-D-Phe}) has been synthesized. In this report, we present the synthesis and characterization of its corresponding ^{90}Y and ^{177}Lu complexes (Figure 1: RP697 and RP688). Through a series of radiolabeling experiments, we developed a for-

mulation for routine preparations of RP697 and RP688. This formulation may be used for the ^{90}Y - and ^{177}Lu -labeling of other DOTA-conjugated biomolecules.

EXPERIMENTAL SECTION

Materials. Acetic acid (ultrapure), ammonium hydroxide (ultrapure), diethylenetriaminepentaacetic acid (DTPA), and sodium gentisate were purchased from either Aldrich or Sigma Chemical Co. and were used as received. $^{90}\text{YCl}_3$ (in 0.05 N HCl) was purchased from NEN Life Sciences, N. Billerica, MA. High specific activity $^{177}\text{LuCl}_3$ was obtained from University of Missouri Research Reactor, Columbia, MO. The cyclic pentapeptide, cyclo(Arg-Gly-Asp-D-Phe-Lys), as its trifluoroacetic acid (TFA) salt was prepared according to the literature method (16).

Synthesis of 2-(1,4,7,10-Tetraaza-4,7,10-tris(*tert*-butoxycarbonylmethyl)-1-cyclododecyl)acetyl-Glu-(cyclo{Lys-Arg-Gly-Asp-D-Phe})-cyclo{Lys-Arg-Gly-Asp-D-Phe}. To a solution of tris(*tert*-butyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (45 mg, 0.078 mmol) in DMF (2 mL) were added HBTU (18 mg, 0.047 mmol) and Hunig's base (20 μL). The mixture was stirred for 15 min. To this were added a solution of Glu-(cyclo{Lys-Arg-Gly-Asp-D-Phe})-cyclo{Lys-Arg-Gly-Asp-D-Phe} (63 mg, 0.038 mmol) and Hunig's base (20 μL) in DMF (1 mL). The reaction mixture was allowed to stir under nitrogen at room-temperature overnight. The solvent was removed in vacuo, and the residue was triturated in ethyl acetate (10 mL), filtered, and washed with ethyl acetate to give the desired product (75.2 mg). Purification of the DOTA conjugate is achieved by preparative HPLC (Vydac reverse phase C_{18} column; solvent A: 0.1% TFA in water; solvent B: 0.1% TFA 90% aq. ACN). Removal of the mobile phase gave the product as a lyophilized solid. ES-MS: $m/z = 1873.9$ for $[\text{M} + \text{H}]^+$ ($\text{C}_{87}\text{H}_{138}\text{N}_{23}\text{O}_{23}$) and 937.2 for $[\text{M} + 2\text{H}]^{+2}$. The analytical HPLC method for the DOTA-conjugate used

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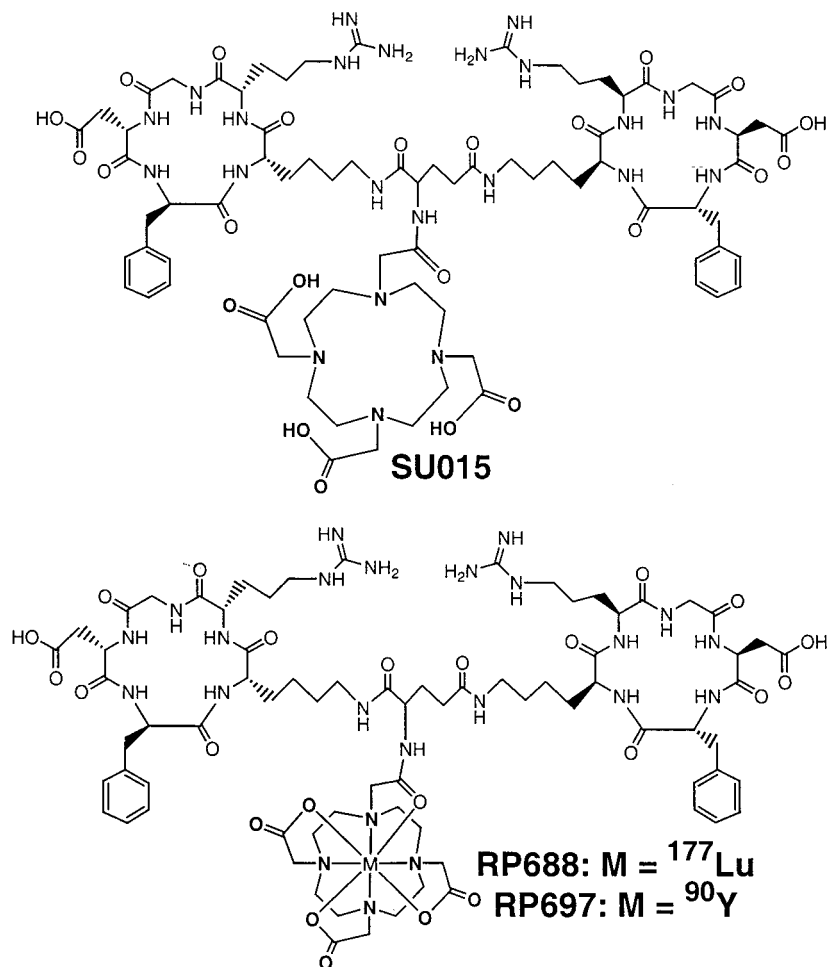


Figure 1. Structures of SU015 and its yttrium and lutetium complexes.

a HP-1050 HPLC system with a Diode array detector ($\lambda = 220$ nm) and a Vydac C₁₈ column (4.6 mm $\times$ 250 mm, 80 Å pore size). The column temperature was 50 °C. The flow rate was 1 mL/min with the mobile phase starting 98% solvent A (0.1% TFA in water) and 2% solvent B (0.1% TFA in 90% acetonitrile) to 63.2% solvent A and 36.8% solvent B at 16 min. The retention time was 20.0 min, and the purity was 99%.

Synthesis of 2-(1,4,7,10-Tetraaza-4,7,10-tris(carboxymethyl)-1-cyclododecyl)-acetyl-Glu(cyclo{Lys-Arg-Gly-Asp-D-Phe})-cyclo{Lys-Arg-Gly-Asp-D-Phe} (SU015). A solution of 2-(1,4,7,10-tetraaza-4,7,10-tris(*tert*-butoxycarbonylmethyl)-1-cyclododecyl)acetyl-Glu(cyclo{Lys-Arg-Gly-Asp-D-Phe})-cyclo{Lys-Arg-Gly-Asp-D-Phe} (4.5 g, 2.4 mmol) in TFA (110 mL) was stirred at room temperature under nitrogen for 2 h. The solution was concentrated in vacuo, and the residue was purified by preparative HPLC (Vydac C₁₈ column; solvent A: 0.1% TFA in water; solvent B: 0.1% TFA 90% aq. ACN). Removal of the mobile phase gave the product as a lyophilized solid (1.5 g, tetra TFA salt). ES-MS: $m/z = 1703.8$ for $[M + H]^+$ (C₇₅H₁₁₄N₂₃O₂₃) and 853.0 for $[M + 2H]^{2+}$. The analytical HPLC method for SU015 used a HP-1050 HPLC system with a Diode array detector ($\lambda = 220$ nm) and a Vydac C₁₈ column (4.6 mm $\times$ 250 mm, 80 Å pore size). The column temperature was 50 °C. The flow rate was 1 mL/min with the mobile phase starting 98% solvent A (0.1% TFA in water) and 2% solvent B (0.1% TFA in 90% acetonitrile) to 63.2% solvent A and 36.8% solvent B at 16 min. The retention time was 13.4 min, and the purity was 97%.

A General Procedure for Synthesis of RP688 and RP697. To a shielded, clean 5 mL vial containing 100 μ g of SU015 and 1–10 mg of sodium gentisate (GA) dissolved in 0.5 mL of 0.5 M ammonium acetate buffer (pH = 7.0, 7.5, or 8.0) was added 10–40 μ L of ⁹⁰YCl₃ or ¹⁷⁷LuCl₃ solution (~ 20 mCi) in 0.05 N HCl. The reaction mixture was allowed to stand at room temperature or was heated at 50–100 °C for 5–30 min. For RP697, a sample of the resulting solution was diluted 20-fold with 4 mM DTPA solution (pH = 5) and then analyzed by HPLC (injection volume = 1–2 μ L) and by ITLC. For RP688, the resulting solution was used for HPLC analysis without dilution (injection volume = 3–5 μ L).

The HPLC method for analysis of RP688 and RP697 used a HP-1100 HPLC system with a UV/visible detector ($\lambda = 220$ nm), an IN-US radio-detector, and a Zorbax C₁₈ column (4.6 mm $\times$ 250 mm, 80 Å pore size). The flow rate was 1 mL/min with an isocratic mobile phase from 0 to 18 min using 87% solvent A (0.025 M ammonium acetate buffer, pH 6.8) and 13% solvent B (acetonitrile), followed by an isocratic wash using 40% of solvent A and 60% solvent B from 19 to 25 min. The ITLC method used Gelman Sciences silica gel ITLC paper strips and a 1:1 mixture of acetone and saline as eluant. By this method, RP697 and RP688 migrate to the solvent front while [⁹⁰Y]/[¹⁷⁷Lu]colloid and [⁹⁰Y]/[¹⁷⁷Lu]acetate remain at the origin.

Synthesis of [¹⁷⁵Lu]RP688 for LC-MS. To a clean 5 mL vial containing 5 mg of SU015, 10 mg of GA, and 10 mg of Lu(NO₃)₃·6H₂O was added 1.0 mL of 0.5 M ammonium acetate buffer (pH = 7.5). The reaction mixture was heated at 100 °C for 20 min. After cooling

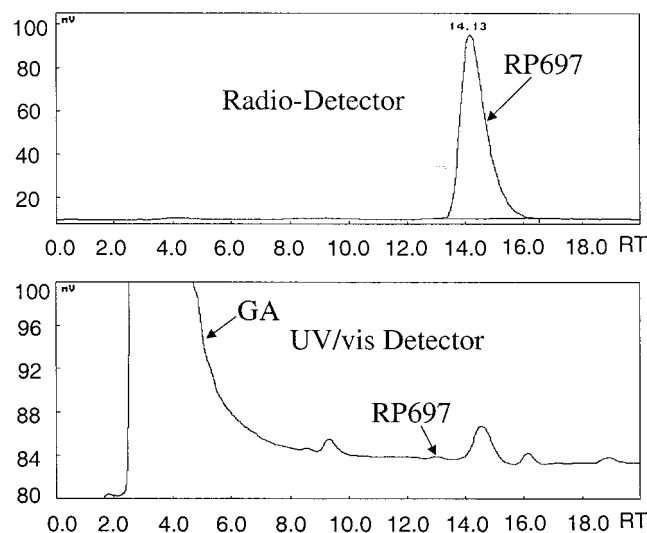


Figure 2. Typical radio (top) and UV/vis (bottom) HPLC chromatograms for the reaction mixture containing RP697. The UV/vis trace was recorded by injecting the reaction mixture without dilution. There is a retention time difference (~ 1.0 min) for the two detectors since they were not synchronized.

to room temperature, a sample of the resulting solution was filtered, and the filtrate was analyzed by LC-MS. No further purification was performed for the LC-MS analysis.

The LC-MS spectra were collected using a HP1100 LC/MSD system with API-electrospray interface. The LC-MS method used a Zorbax C_{18} column (4.6 mm $\times$ 150 mm, 3.5 μm particle size) at a flow rate of 1 mL/min and a gradient mobile phase starting from 92% solvent A (10 mM ammonium acetate buffer, pH 7.0) and 8% solvent B (methanol) to 100% B at 23 min. The MSD parameters are as follows: detection mode: positive mass range: 600–2000; gain: 1.0; fragmentor: 30 V; gas temperature: 350 $^{\circ}\text{C}$; drying gas flow: 13 L/min; nebulizer pressure: 60 psig (max.); V capillary: 4000 V; UV/vis detector: $\lambda = 220$ nm.

RESULTS

Synthesis of RP688 and RP697. RP697 was prepared by reacting SU015 with $^{90}\text{YCl}_3$ in an ammonium acetate buffer (pH = 6.5–8) at 50–100 $^{\circ}\text{C}$. GA was used as a stabilizer to prevent radiolytic degradation of RP697. The total volume was always 0.5 mL, and the $^{90}\text{YCl}_3$ concentration was 40 mCi/mL. We typically used 100 μg of SU015 for 20 mCi of $^{90}\text{YCl}_3$ corresponding to a SU015: ^{90}Y ratio of $\sim 130:1$. The use of excess SU015 is to compensate for the presence of other trace metal contaminants. Under optimized conditions, RP697 can be prepared in high yield with the RCP > 95%. RP688 was prepared in a similar fashion by reacting SU015 with $^{177}\text{LuCl}_3$ (100 μg of SU015, 10 mg of GA for 20 mCi of $^{177}\text{LuCl}_3$, pH > 7.2, heating at 100 $^{\circ}\text{C}$ for 5 min).

HPLC Characterization. Figures 2 and 3 show typical HPLC (radio and UV) chromatograms for RP688 and RP697, both of which show only one radiometric peak. The retention time for RP688 and RP697 is almost identical under the same chromatographic conditions. There are several small peaks due to radioimpurities in the HPLC chromatogram of both RP688 and RP697. Since these radioimpurities are less than 1.0%, no further characterization was performed. Many attempts were made to use LC-MS for the characterization of the

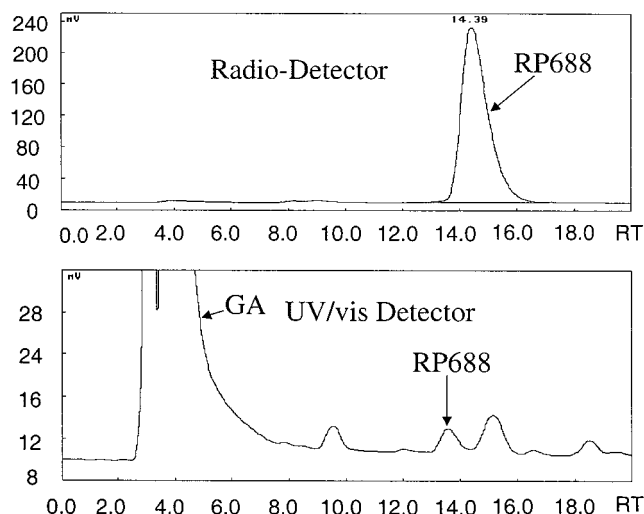


Figure 3. Typical radio (top) and UV/vis (bottom) HPLC chromatograms for the reaction mixture containing RP688. There is a retention time difference (~ 1.0 min) for the two detectors since they were not synchronized.

reaction mixture containing RP697, but the results were often difficult to interpret, due to dilute concentration of RP697.

LC-MS Characterization of RP688. We have used LC-MS to determine the composition of RP688. To identify the peak due to RP688 in the UV profile, we also prepared the corresponding “cold” complex Lu(SU015) by reacting SU015 with excess of lutetium(III) nitrate in a 0.5 M ammonium acetate buffer (pH = 7.5). Figure 4 shows the UV/vis and MSD profiles for RP688 and ^{175}Lu (SU015). Figure 5 shows the LC-MS spectra of RP688 (bottom) and ^{175}Lu (SU015) (top). The LC-MS patterns of both complexes are identical except the difference in isotope. The LC-MS spectral data support the proposed structure (Figure 1). There are several other intense peaks due to complexes of trace metal contaminants in the reaction mixture containing RP688. The molecular weights (Table 1) corresponding to these peaks match those from Ca(SU015) ($t_R = 10.85$ – 10.91 min), Co(SU015) ($t_R = 10.05$ – 10.08 min), Zn(SU015) ($t_R = 10.11$ – 10.21 min), and Fe(SU015) ($t_R = 10.22$ – 10.32 min).

Buffer Concentration. In this experiment, we used 100 μg of SU015 and 10 mg of GA, 20 mCi of $^{90}\text{YCl}_3$, and ammonium acetate buffer (0.1, 0.25, or 0.5 M) to prepare RP697. The pH in the mixture was 7.2 after addition of $^{90}\text{YCl}_3$. The reaction mixture was heated at 100 $^{\circ}\text{C}$ for 10 min. Figure 6 shows that the buffer concentration does not have a significant impact on RCP as long as the pH is well controlled. The 0.5 M ammonium acetate buffer was chosen for the remaining radiolabeling experiments mainly due to its higher buffering capacity.

^{90}Y -Chelation Kinetics at Room Temperature. In this experiment, we explored the possibility of preparing RP697 by reacting 100 μg of SU015 with 20 mCi of $^{90}\text{YCl}_3$ in the presence of GA (10 mg) at room temperature. The pH in the reaction mixture was 6.6, 7.2, or 7.8 after addition of $^{90}\text{YCl}_3$. Samples of the reaction mixture were analyzed by radio-HPLC at 5, 30, 60, and 120 min. The RCP data are summarized in Figure 7. It is obvious that the rate of ^{90}Y chelation depends on the pH of the reaction mixture. At pH 6.6, the RCP for RP697 was only $\sim 60\%$ after 2 h at room temperature. It is possible to prepare RP697 with RCP > 90% at room temperature at pH > 7.0,

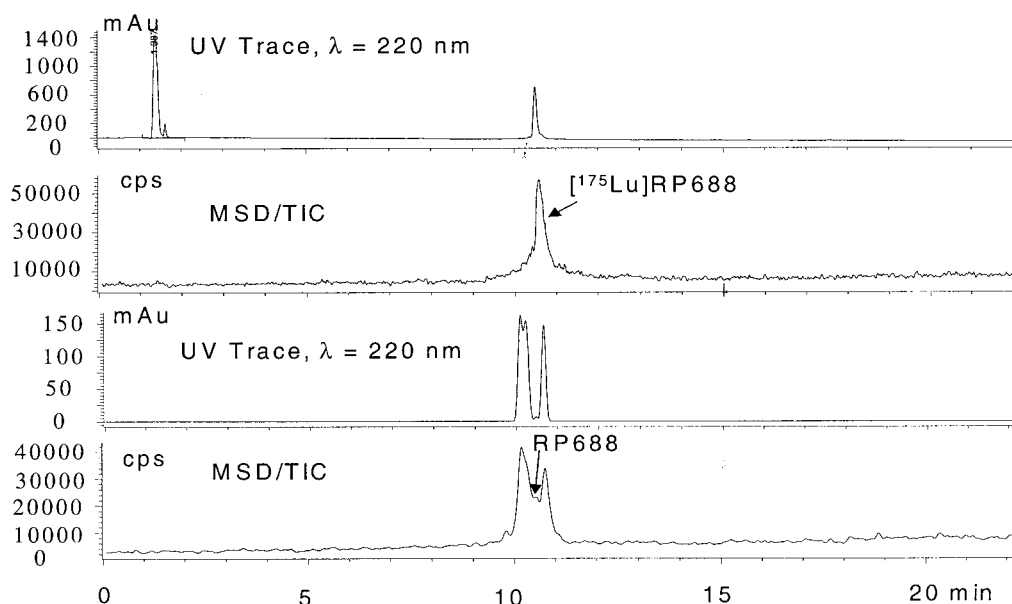


Figure 4. UV/vis ($\lambda = 220$ nm) and MSD profiles of the reaction mixture containing $[^{175}\text{Lu}]\text{RP688}$ (top) and RP688 (bottom).

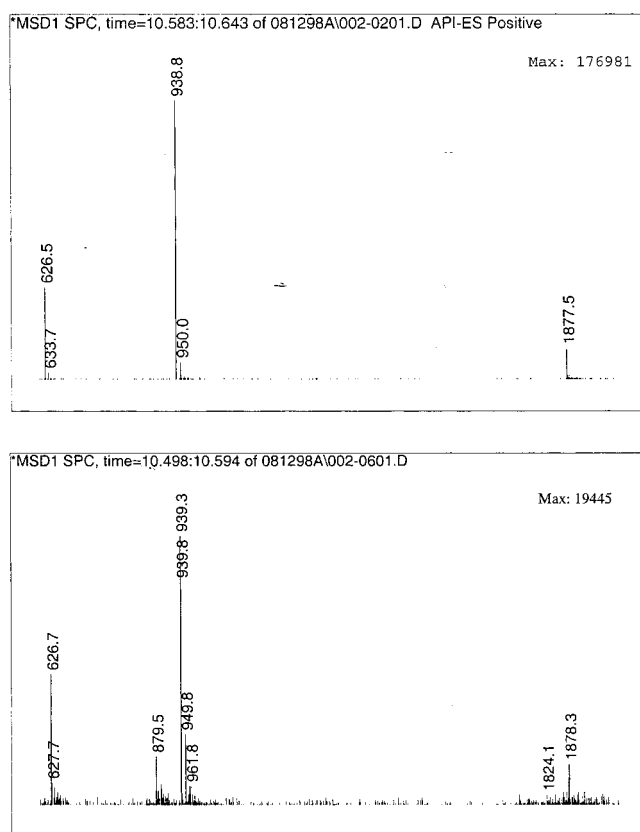


Figure 5. LC-MS spectra for $[^{175}\text{Lu}]\text{RP688}$ (top) and RP688 (bottom). The y-axis represents intensity of the signal. The x-axis represents m/z ratio.

but the RCP of RP697 could not be further improved. The elevated temperatures are needed to complete the ^{90}Y chelation.

Preparation of RP697 at 50°C . In this experiment, we prepared RP697 using $100\ \mu\text{g}$ of SU015 and $10\ \text{mg}$ of GA for $20\ \text{mCi}$ of $^{90}\text{YCl}_3$. The pH in the mixture was 7.2 or 7.8 after addition of $^{90}\text{YCl}_3$. After heating at 50°C for 5, 10, and 30 min, a sample of the reaction mixture was analyzed by radio-HPLC and ITLC. It was found (Figure 8) that at pH 7.8 heating at 50°C for 5 min is sufficient

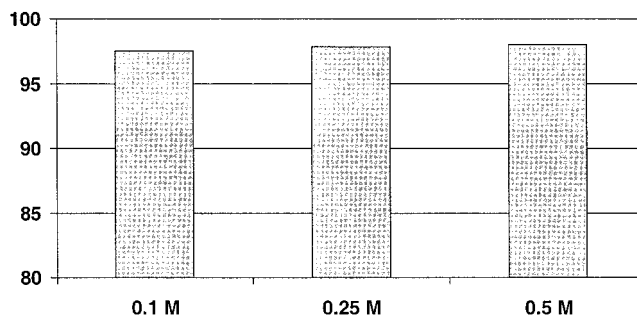


Figure 6. Effect of the buffer concentration on RCP of RP697 .

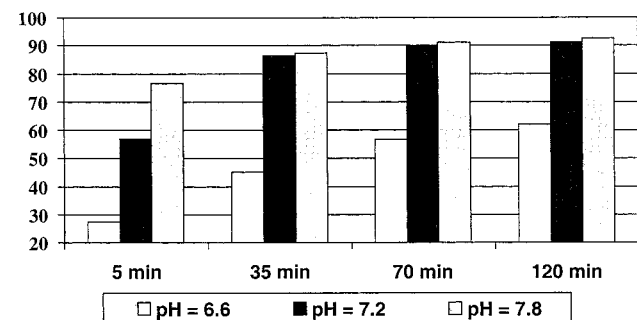


Figure 7. Room temperature ^{90}Y chelation kinetics at pH = 6.6, 7.2, and 7.8.

to achieve 95% RCP for RP697 . The maximum RCP was obtained by heating the reaction mixture at 50°C for 10 min.

Preparation of RP697 at 100°C . In this experiment, we prepared RP697 using $100\ \mu\text{g}$ of SU015 and $10\ \text{mg}$ of GA for $20\ \text{mCi}$ of $^{90}\text{YCl}_3$ at pH 6.6, 7.2 or 7.8. The reaction mixture was heated at 100°C for 5, 10, 15, and 30 min. Figure 9 shows the effect of heating time on the RCP of RP697 at different pH values. It is clear that 5 min heating is sufficient to complete the ^{90}Y chelation at pH 7.8. Prolonged heating is detrimental due to hydrolysis and radiolytic decomposition of the radiolabeled peptide.

^{90}Y -Labeling Efficiency of SU015 . We studied the ^{90}Y -labeling efficiency of SU015 by determining the minimal amount of SU015 required to achieve 95% RCP for RP697 . In this experiment, the heating temperature was 100°C and the heating time was 10 min. Figure 10

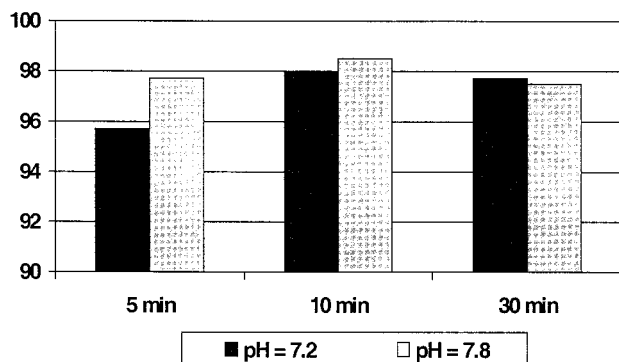


Figure 8. Effect of heating (at 50 °C) time on RCP of RP697 at pH 7.2 and 7.8.

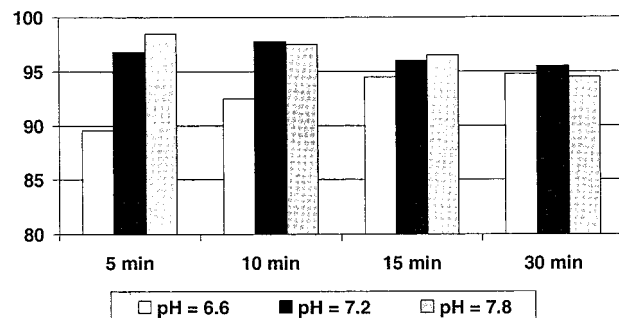


Figure 9. Effect of heating (at 100 °C) time on RCP of RP697 at pH 6.6, 7.2, and 7.8.

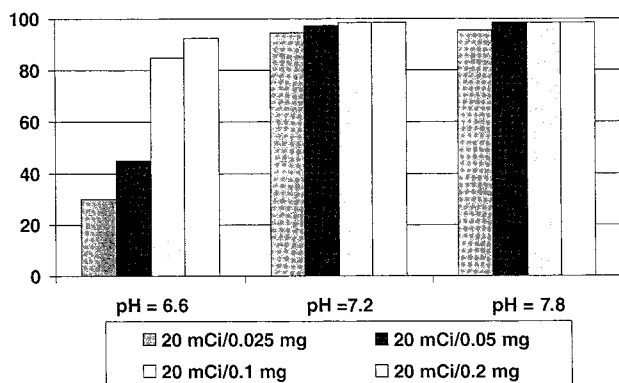


Figure 10. Effect of the pH on radiolabeling efficiency.

shows RCP data for RP697 prepared using 25, 50, or 100 μg of SU015, 10 mg of GA, 20 mCi of $^{90}\text{YCl}_3$, at pH 6.6, 7.2, or 7.8. Obviously, the pH has dramatic impact on the ^{90}Y -labeling efficiency of SU015. At pH 7.2–7.8, the minimum amount of SU015 required to achieve 95% RCP for RP697 is about 25 μg for 20 mCi of $^{90}\text{YCl}_3$ corresponding to a SU015: ^{90}Y ratio of $\sim 30:1$.

DISCUSSION

Therapeutic radiopharmaceuticals are radiolabeled molecules designed to deliver therapeutic doses of ionizing radiation to specific disease sites (17). Most systemically administered therapeutic radiopharmaceuticals are small organic or inorganic compounds with definite composition. They can also be macromolecules such as monoclonal antibodies and antibody fragments labeled with a therapeutic radionuclide. Ideally, a therapeutic radiopharmaceutical should localize at the tumor sites in high concentration and deliver sufficient radiation dose to tumor cells, while clearing rapidly from the blood to minimize radiation damage to normal tissues.

While tumor imaging relies heavily on a high target-to-background ratio for the best contrast, tumor therapy depends largely on a high concentration of radioactivity for a long duration. Thus, the therapeutic radiopharmaceutical must have high and fast tumor uptake, high tumor-to-background ratio, long tumor residence time, and fast renal clearance. High tumor uptake and fast renal clearance are particularly important to a high tumor-to-background ratio and to reduce the radiation burden to other major organs (kidney, liver, and bone marrow). The new radiopharmaceutical must have high radiochemical purity (RCP $\geq 90\%$) and high solution stability. Unlike diagnostic radiopharmaceuticals, which are typically made by radiopharmacists before injection, therapeutic radiopharmaceuticals have to be manufactured and released under GMP (Good Manufacturing Practice) conditions. Therefore, the radiopharmaceutical must retain its chemical and biological integrity during release and transportation.

Choice of Biomolecules. Angiogenesis, the formation of new blood vessels, is a requirement for malignant tumor growth and metastasis (18–20). The angiogenic process depends on vascular endothelial cell migration and invasion, regulated by cell adhesion receptors (21–29). The integrin $\alpha\beta_3$ (vitronectin receptor) is such a cell adhesion receptor and interacts with proteins and peptides containing the RGD tripeptide recognition sequence. Many peptide vitronectin receptor antagonists have very high binding affinity for the vitronectin receptor and have been shown to be able to inhibit neovascularization, tumor-induced angiogenesis, and tumor growth (27–33). Recently, Kessler and co-workers (34, 35) reported two ^{125}I -labeled cyclic pentapeptides: 3- ^{125}I -D-Tyr⁴-cyclo(RGDyV) and 3- ^{125}I -D-Tyr⁴-cyclo(RGDyK(SAA1)) (SAA = sugar amino acid). It was found that 3- ^{125}I -D-Tyr⁴-cyclo(RGDyV) has fast hepatobiliary and renal excretion (34). The tumor/muscle and tumor/blood ratios for melanoma in nude mice were 5.5 and 9.5, respectively, at 60 min postinjection. Substitution of leucine with a SAA-functionalized lysine amino acid residue resulted in improved blood retention time, renal excretion, and better target-to-background ratio. A blocking study using 3 mg/kg of the $\alpha\beta_3$ selective cyclo(RGDfV) demonstrated that the localization of radioactivity in the tumor is due to $\alpha\beta_3$ binding.

In our previous communication (15), we reported a $^{99\text{m}}\text{Tc}$ -labeled vitronectin receptor antagonist (RP593) and its biological properties in two spontaneous tumor models (c-Neu Oncomouse and dog). The high tumor uptake and long tumor residence time (3.4% ID/g at 2 h and 1.5% ID/g at 24 h postinjection in the c-Neu Oncomouse tumor model) led us to prepare the corresponding ^{90}Y and ^{177}Lu complexes, which have the potential for radiotherapy of a wide spectrum of tumors.

Choice of Radionuclides. Identifying an appropriate isotope for radiotherapy is often a difficult task and requires weighing a variety of factors (36, 37). These include tumor uptake, blood clearance, duration of receptor binding, rate of radiation delivery, half-life and specific activity of the radionuclide, and the feasibility of large-scale production of the radionuclide in an economical fashion. The ultimate goal for a therapeutic radiopharmaceutical is to deliver the maximum amount of radiation dose to the tumor cells to achieve a cytotoxic effect without causing unmanageable side-effects.

The physical half-life of the therapeutic radionuclide should match the biological half-life of the radiopharmaceutical at the tumor site. If the half-life of the radionuclide is too short, much of the decay will have occurred

Table 1. LC-MS Data for ^{177}Lu and Nonradioactive Metal Complexes

complex	formula	molecular mass	found (M + H) ⁺	found (M + 2H) ²⁺
RP688	C ₇₅ H ₁₁₀ N ₂₃ O ₂₃ ^{176/177} Lu	1877.8	1878.3	939.3
¹⁷⁵ Lu(SU015)	C ₇₅ H ₁₁₀ N ₂₃ O ₂₃ Lu	1876.8	1877.5	938.8
Ca(SU015)	C ₇₅ H ₁₁₁ N ₂₃ O ₂₃ Ca	1741.8	1742.9	871.9
Co(SU015)	C ₇₅ H ₁₁₁ N ₂₃ O ₂₃ Co	1760.8	1760.8	881.4
Cu(SU015)	C ₇₅ H ₁₁₁ N ₂₃ O ₂₃ Cu	1766.4	1766.3	883.9
Zn(SU015)	C ₇₅ H ₁₁₁ N ₂₃ O ₂₃ Zn	1768.2	1768.6	884.8
Fe(SU015)	C ₇₅ H ₁₁₀ N ₂₃ O ₂₃ Fe	1758.7	1758.5	879.8

Table 2. Trace Metal Contaminants in a Commerical ^{90}Y Stock Solution and the Stability Constants for Their DOTA Complexes

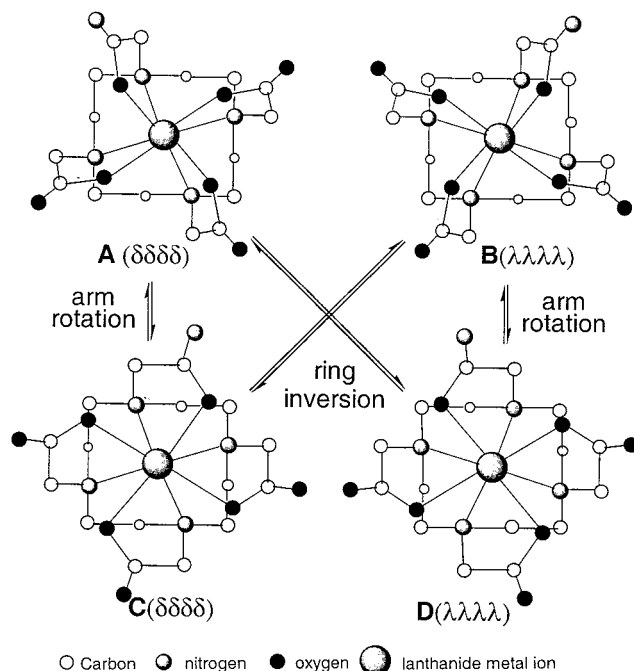
metal ion	max. amount (mg/Ci Y-90)	max. amount (mmol/Ci Y-90)	stability constant for M-DOTA
Y	~2.0	~0.022	24.3 ^a
Al	<30.0	<1.11	
Ca	<100	<2.50	17.2 ^b
Ce	<20	<0.143	23.0 ^c
Co	<20	<0.339	20.2 ^b
Cr	<20	<0.385	
Cu	<30	<0.469	22.5 ^b
Fe	<20	<0.357	29.4 ^d
La	<20	<0.144	21.7 ^d
Ni	<20	<0.339	20.5 ^d
Pb	<20	<0.100	22.7 ^c
Sr	<20	<0.227	15.22 ^d
Zn	<40	<0.615	21.05 ^d
Zr	<20	<0.217	

^a Reference 53. ^bReference 54. ^cReference 55. ^dReferences 56 and 57. ^eSmith, R. M., and Martell, A. E. NIST Critical Selected Stability Constants of Metal Complexes Database. Version 3. Program developed by Motekaitis, R. J., 1997.

before the radiopharmaceutical has reached the maximum target/background ratio. In this situation, attaining a sufficient integrated tumor dose for cytotoxicity (>50 Gy) will prove problematic. On the other hand, too long a half-life would cause unnecessary radiation dose to normal tissues. Ideally, the radionuclide should have a long enough half-life to attain a minimum dose rate (>0.4 Gy/h) and to irradiate all the cells during the most radiation sensitive phases of the cell cycle. In addition, the half-life of the therapeutic radionuclide has to be long enough to allow adequate time for manufacturing, release, and transportation.

We are interested in ^{177}Lu and ^{90}Y for their opposite nuclear properties. ^{90}Y is a high-energy β -emitter ($E_{\text{max}} = 2.23$ MeV) and has a half-life of 2.67 days while ^{177}Lu is a low energy β -emitter ($E_{\text{max}} = 0.5$ MeV) with a half-life of 6.67 days. Yttrium and lutetium share similar coordination chemistry, and chelator technologies have been well established for the radiolabeling of biomolecules with these isotopes. Depending on the tumor size and location, the low energy β -emitter, ^{177}Lu , may be better for smaller metastases while the high-energy β -emitter, ^{90}Y , may be superior for large tumors.

Choice of BFC. Polyazamacrocycles have been widely used as BFCs for the radiolabeling of biomolecules with various radionuclides (36–52). We chose DOTA as the BFC for several reasons. The macrocyclic framework is well organized so that it forms yttrium and lutetium complexes with high thermodynamic stability and kinetic inertness (40, 41). The pK_a values of the carboxylic groups are in the range 2–5. Lower pK_a values result in less competition from proton, high stability of the metal complex, and minimum acid-assisted demetalation, even under acidic ($\text{pH} \leq 2$) conditions. Conjugation of biomolecules can be easily achieved using one of the carboxylic groups. The acetate groups attached to the nitrogen donor atoms have low molecular weight, and are very hydro-

**Figure 11.** Possible structures for reaction intermediates and Y(DOTA-MA).

philic. Therefore, the contribution of the BFC to the overall molecular weight of the DOTA-BM conjugate can be minimized. The high hydrophilicity improves the pharmacokinetics of the radiopharmaceutical.

Characterization of RP688 and RP697. For the past decade, HPLC has become a routine technique for quality control of various radiopharmaceuticals. The advantage of radio-HPLC is its capability to determine the RCP for RP688 and RP697, and to separate different $^{90}\text{Y}/^{177}\text{Lu}$ species in the reaction mixture. It is also a very powerful tool for separation of diastereomers. However, radio-HPLC also has its limitations. For example, it cannot be used for the assessment of the amount of [$^{90}\text{Y}/^{177}\text{Lu}$]colloid in the reaction mixture. Therefore, a TLC method is needed in combination with radio-HPLC to assess the radiochemical purity of the RP688 and RP697. In addition, a 4 mM DTPA solution ($\text{pH} = 5$) is recommended for dilution of the reaction mixture in order to accurately determine unchelated $^{90}\text{Y}/^{177}\text{Lu}$ during the standard quality control by HPLC (51). If DTPA is not used, the unchelated $^{90}\text{Y}/^{177}\text{Lu}$ will be readily bound to the column, thereby giving a false indication of the radiochemical purity of the radiopharmaceutical.

In this study, we used a reverse phase HPLC method to analyze RP688 and RP697. The mobile phase was isocratic at 13% organic solvent (acetonitrile) to maximize the separation of different radiometal-containing species. We also recorded the UV/vis ($\lambda = 220$ nm) profiles in order to assess the extent of trace metal contamination. Figures 2 and 3 show typical HPLC (both radio and UV/vis) chromatograms for RP688 and RP697, respectively. Both RP697 and RP688 show one radiometric peak. We

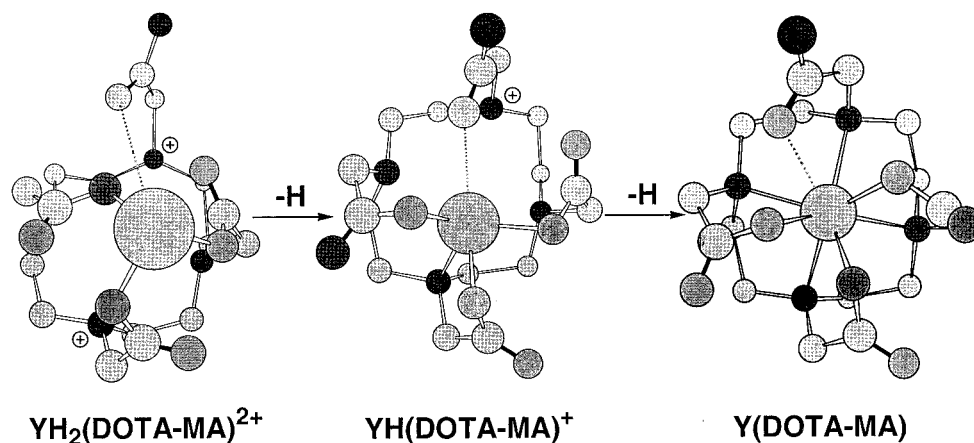


Figure 12. Possible structures of $\text{Y}(\text{DOTA-MA})^{2+}$, $\text{Y}(\text{DOTA-MA})^+$, and $\text{Y}(\text{DOTA-MA})$.

also tried several other reverse phase HPLC methods. In all the cases, RP697 and RP688 show only one radiometric peak, suggesting that they exist in solution as only one *detectable* isomeric form. RP688 and RP697 have almost identical retention time under the same chromatographic conditions, indicating that yttrium and lutetium share the same coordination chemistry in solution.

In the solid state, the complex $\text{Y}(\text{DOTA-MA})$ (MA = monoamide) has a square antiprismatic geometry with four amine-nitrogen, one carbonyl-oxygen, and three carboxylate-oxygen atoms bonding to the yttrium metal (52). In principle, there should be four possible isomers (**A–D** in Figure 11) for the complex $\text{Y}(\text{DOTA-MA})$. Isomers **A** and **B** are enantiomers and indistinguishable by the reverse phase HPLC method. Isomers **C** and **D** represent the other pair of enantiomers. Isomers **A** and **B** are close to square prismatic with the acetate arms in a different layout and the cyclododecane ring conformation being identical to those of **C** and **D**. Conjugation of an optically pure peptide will not produce extra isomers, but it may result in the formation of one predominant isomeric form. These four possible isomers are not enantiomers but diastereomers, which are theoretically expected to be separable using a reverse phase HPLC method, particularly with an isocratic mobile phase. The fact that both RP688 and RP697 showed only one radiometric peak in their radio-HPLC chromatograms suggests that they exist in solution as only one predominant isomer, and is consistent with the observation of only m-isomer in the solid state of the complex $\text{Y}(\text{DOTA-D-Phe-NH}_2)$ (52).

Factors Influencing ^{90}Y Chelation. There are many factors influencing the ^{90}Y chelation and RCP of RP697. These include the purity of SU015, the pH, reaction temperature and heating time, buffer agent and buffer concentration, stabilizing agent, as well as trace metal contaminants, such as Ca^{2+} , Fe^{3+} , and Zn^{2+} . The goal of this study was to explore the factors influencing the RCP and to prepare RP697 in high RCP to support preclinical studies.

Trace Metal Contaminants. DOTA and its derivatives form complexes with most divalent and trivalent metal ions. These metal complexes often have extremely high thermodynamic stability and kinetic inertness. As a matter of fact, one of the most important factors influencing the radiolabeling efficiency of a DOTA-BM conjugate is the presence of trace metal contaminants in the reaction mixture. The trace metal contaminants may come from the buffering agent, glass vials, stoppers,

the radiometal stock solution, and the stabilizing agent. To minimize the amount of contaminants, we always used acid-treated 5 mL glass vials, and Teflon-coated stoppers to prevent leaking of metal ions. If possible, all the reagents should be extra-pure.

Table 1 shows the maximum limits of trace metal contaminants (as of the production date) in the $^{90}\text{YCl}_3$ stock solution from NEN and the stability constants of their DOTA complexes (53–57). Most of the trace metals (Al, Ca, Ce, Co, Cr, Cu, Fe, La, Ni, Pb, Zn, and Zr) in the $^{90}\text{YCl}_3$ stock solution have the potential to influence the ^{90}Y labeling of a DOTA-BM conjugate. The actual metal contaminant concentration may not be as high as specified by the manufacturer, but the UV profile of the reaction mixture containing RP697 (Figure 2) shows that the concentration of total trace metal contaminants is much higher than that of ^{90}Y .

Unlike ^{90}Y , a generator-produced radionuclide, ^{177}Lu is a reactor-produced isotope, made by irradiating ^{176}Lu -enriched lutetium metal. While other trace metal contaminants can be separated, ^{176}Lu remains in the $^{177}\text{LuCl}_3$ stock solution (in 0.05 N HCl) after chromatographic purification. The specific activity for ^{177}Lu is ~ 20 Ci/mg. Although the specific activity of ^{177}Lu is much lower than that of ^{90}Y (~ 500 Ci/mg, carrier free), RP688 can be prepared using identical radiolabeling conditions as RP697 (100 μg SU015 and 10 mg of GA for 20 mCi of $^{177}\text{LuCl}_3$).

Recently, we reported a LC-MS method for the characterization of $^{99\text{m}}\text{Tc}$ -labeled bioconjugates at the tracer level (58). In this study, we used the same MSD parameters to analyze the reaction mixture containing RP688. We found several other metal complexes: including Ca-(SU015) ($t_R = 10.85\text{--}10.91$ min), Zn-(SU015) ($t_R = 10.11\text{--}10.21$ min), and Fe-(SU015) ($t_R = 10.22\text{--}10.32$ min). The concentration of RP688 is much lower than those of Ca-(SU015), Zn-(SU015), and Fe-(SU015). This is especially true when reagent grade ammonium acetate was used as the buffering agent and the Teflon-coated stoppers were not used for the radiolabeling.

^{90}Y Chelation Kinetics. We first explored the possibility of preparing RP697 at room temperature. We found that the ^{90}Y chelation is very much dependent on the pH of the reaction mixture. We also found that the buffer concentration does not have a significant impact on the ^{90}Y chelation as long as the pH is well controlled. While it is possible to prepare RP697 with RCP > 90% at room temperature at pH > 7.0, the RCP of RP697 could not be further improved. Thus, it is necessary to use elevated temperatures to complete the ^{90}Y chelation.

These results are consistent with those reported by Meares and co-workers (48). Higher temperature increases the rate of ^{90}Y chelation, but prolonged heating is detrimental to the RCP of RP697 due to hydrolysis and radiolytic decomposition. For example, heating at 50 °C for 5 min is sufficient to achieve 95% RCP for RP697 if the pH is 7.2–7.8 (Figure 8). However, the RCP can be increased by heating at 50 °C for > 10 min. At 100 °C, only 5 min is needed to complete the ^{90}Y chelation at pH 7.8, while the optimal heating time at pH 7.2 is 10 min. In addition, the pH also shows a dramatic impact on the ^{90}Y -labeling efficiency of SU015. At pH 7.2–7.8, the minimum amount of SU015 required to achieve 95% RCP for RP697 is $\sim 25\ \mu\text{g}$ for 20 mCi of $^{90}\text{YCl}_3$ corresponding to a SU015: ^{90}Y ratio of $\sim 30:1$.

Like DOTA, DOTA-MA (MA = monoamide) also exists in solution as its zwitterion form, and the conformation of the chelator is determined by the degree of protonation of DOTA-MA (59, 60). For example, the predominant form at pH 3.6–5.0 is $\text{H}_3(\text{DOTA-MA})^-$ while DOTA-MA exists primarily as $\text{H}_2(\text{DOTA-MA})^{2-}$ and $\text{H}(\text{DOTA-MA})^{3-}$ at pH > 7.0. The formation of $\text{Y}(\text{DOTA-MA})$ proceeds via two intermediate complexes: $\text{YH}_2(\text{DOTA-MA})^{2+}$ and $\text{YH}(\text{DOTA-MA})^+$. The rate-limiting step is most likely the proton transfer from $\text{YH}(\text{DOTA-MA})^+$ to form the eight-coordinated complex $\text{Y}(\text{DOTA-MA})$ since it requires a significant rearrangement of the macrocyclic ring, including rotation of C–C and C–N bonds and inversion of the N-atom. Once the proton is removed, chelation of the remaining three N-atoms will be fast to give the final product. Therefore, higher pH gives a faster chelation rate.

Different protonated intermediates of DOTA-MA form yttrium complexes with different structures (Figure 12). It is most likely that in the doubly protonated complex $\text{YH}_2(\text{DOTA-MA})^{2+}$, the Y atom is coordinated solely by one carbonyl and three carboxylate oxygens. Upon removal of one proton from the ring nitrogen to form the intermediate, $\text{YH}(\text{DOTA-MA})^+$, the Y atom moves toward the ring and becomes five-coordinated to four oxygen atoms and one ring nitrogen atom. Because of static interaction, the N-atom bonding to the metal is most likely to be the one trans to the protonated N-atom. With the removal of the last proton, the expected eight-coordinated complex $\text{Y}(\text{DOTA-MA})$, with a square antiprismatic coordination geometry, is formed.

CONCLUSIONS

In conclusion, we synthesized ^{90}Y and ^{177}Lu complexes (RP697 and RP688, respectively) of a DOTA-conjugated vitronectin receptor antagonist (SU015). Through a series of radiolabeling experiments, we found that there are many factors influencing the rate of ^{90}Y chelation and the labeling efficiency of SU015. These include the purity of the SU015, the pH, reaction temperature, and heating time, as well as trace metal contaminants, such as Ca^{2+} , Fe^{3+} and Zn^{2+} . Among them, pH is the most dominant factor. Compared to that of the corresponding DTPA analogues (58), the ^{90}Y chelation kinetics are slow and heating at elevated temperatures (50–100 °C) is needed to complete the ^{90}Y labeling. Under optimized radiolabeling conditions (pH 7.2–7.8 and heating at 50–100 °C for 5–10 min), the minimum amount of SU015 required to achieve 95% RCP for RP697 is $\sim 25\ \mu\text{g}$ for 20 mCi of $^{90}\text{YCl}_3$ corresponding to a SU015: ^{90}Y ratio of $\sim 30:1$. The formulation developed in this study may be used for the ^{90}Y and ^{177}Lu labeling of other DOTA-conjugated biomolecules.

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In Vitro Gene Transfer with a Novel Galactosylated Spermine Bolaamphiphile

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We describe the synthesis of a α -galacto- ω -spermine bolaamphiphile (GalSper) and report on the gene transfer mediated with lipoplexes it forms either when used alone or in conjunction with DOPE or with DOGS (Transfectam). Lipofection with GalSper was investigated with human HepG2 or murine BNL-CL2 hepatocytes expressing the asialo-glycoprotein (ASGP) receptor, which displays a high affinity for galactosyl residues, or with A549 cells which do not express ASGP. Although lower luciferase expression levels in BNL-CL2 and in HepG2 cells were obtained with GalSper/DOPE N/P 2.5 lipoplexes as compared with control DOGS/DOPE N/P 2.5 particles or with the more positively charged N/P 5 particles (yet through a different mechanism), specific receptor-mediated endocytosis of DNA can be achieved with this targeted cationic GalSper bolaamphiphile presenting a single galactose residue. The present work suggests that GalSper-based DNA formulations appear as promising synthetic vectors for specific gene delivery to ASGP(+) cells.

INTRODUCTION

Nonviral gene transfer devices hold considerable potential for gene therapy of inherited or acquired diseases. This has led over the past decade to the development of various (poly)cationic lipids and polymers which form with DNA lipoplexes (1–8) and polyplexes (3, 5, 8–15), respectively, through electrostatic interactions. These synthetic systems provide receptor-independent gene transfer into various cell types, as the cationized gene particles bind to the anionic proteoglycans which are expressed on the cell surface (16, 17).

Successful in vivo gene transfer and gene therapy will depend on the efficient delivery of genetic material to specific cells and its effective expression within these cells. Aiming at these goals, various cell-targeting ligands (transferrin (18), mannose, galactose and other oligosaccharides, RGD-peptides and other peptides, antibodies, folate; for a recent review, see ref 19) were conjugated to the DNA complexes in order to trigger receptor-mediated endocytosis. To our knowledge, only a few targetable lipids and targetable quaternary ammonium amphiphiles linked to sugar residues (20–23), for which a selective lipoplex-mediated gene transfer has been demonstrated (20–22), have been described. As part of our contribution into this field, we have performed the synthesis of a α -galacto- ω -spermine bolaamphiphile (GalSper in Scheme 1). The rationale for such a design is that it combines spermine, which is known to spontaneously compact a nucleic acid (lipospermines are among the cationic lipids tested so far the most efficient synthetic gene transfer agents) linked, through a hydrophobic group, to a galactosyl residue which serves as a molecular signal for cell targeting. As a high density of galactose residues (which is important for effective receptor-mediated gene transfer

(21)) can be provided on the lipoplex surface on compacting DNA with such bolaamphiphiles, the sugar moieties are likely to prevent the lipoplexes from aggregation, coating with serum lipophilic proteins and/or binding to the anionic proteoglycans of the extracellular matrix (16, 17).

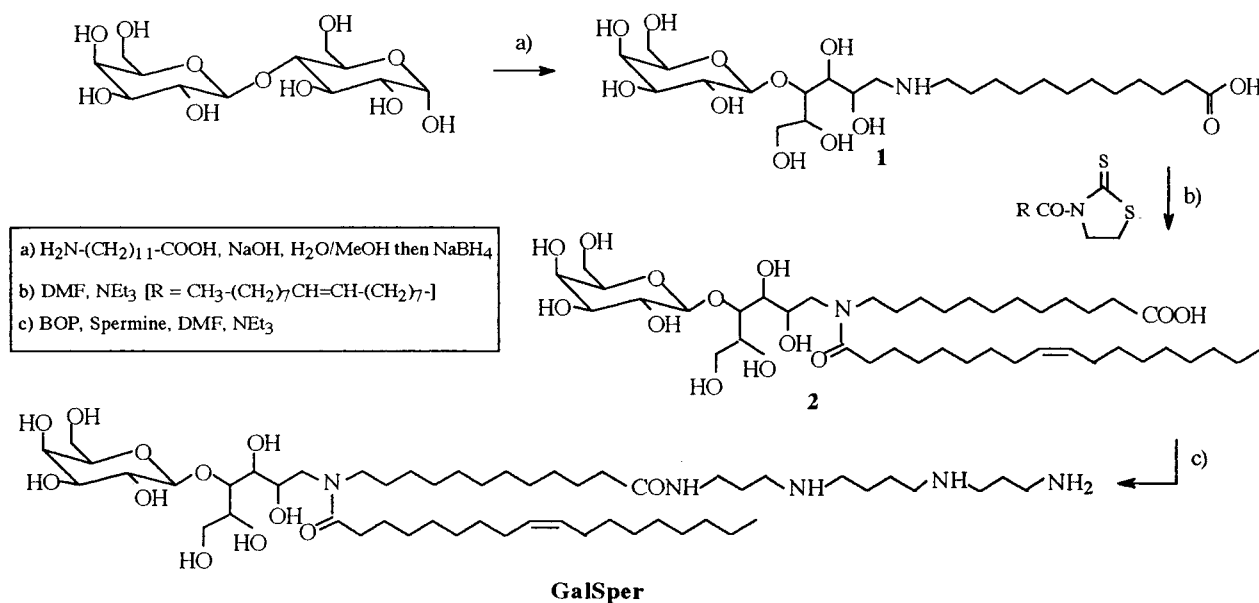
Here we describe the synthesis of the GalSper bolaamphiphile (Scheme 1) and report on the gene transfer mediated with lipoplexes it forms either when used alone or in conjunction with DOPE (which, as colipid, was found in several cases to enhance the transfection activity of cationic lipids (24, 25)) or with DOGS (or Transfectam) (5). Lipofection with GalSper was investigated with various cells (human HepG2 or murine hepatocytes BNL-CL2) expressing the asialo-glycoprotein (ASGP) receptor, which displays a high affinity for galactosyl residues (20–22, 26–29), or not (A549 cells which derive from a human carcinoma). Although the remainder of our initial goal was not fully reached, GalSper constitutes a very promising lead molecule worth studying.

MATERIALS AND METHODS

Analytical Conditions, Chemicals, Expression Vectors, and Cells. Column chromatography purifications were carried out using Merck silica gel 60 (mesh 70–230) and/or Sephadex LH 20 gel (Aldrich). Thin-layer chromatography (TLC) analyses were performed on pre-coated silica gel F₂₅₄ plates (Merck) and visualized by UV light ($\lambda = 254$ nm) and by charring with KMnO₄, 50% MeOH–H₂SO₄, or ninhydrine. ¹H and ¹³C NMR spectra were recorded at 200 and 50.3 MHz, respectively, on a Bruker AC-200. Chemical shifts were measured relative to CHCl₃ (δ 7.27 ppm) or CH₃OD (δ 3.35 ppm) for ¹H, relative to CDCl₃ (δ 76.9 ppm) for ¹³C and expressed in ppm indirectly in relation to TMS. The following abbreviations are used to describe the signal multiplicities: s (singlet), d (doublet), t (triplet), q (quadruplet), and m (multiplet). Chemical shifts are listed as follows: shift in ppm (multiplicity, coupling, integration, and attribution). Electrospray ionization mass spectrometry

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Scheme 1. Synthetic Route to the α -Galacto- ω -spermine Bolaamphiphile, GalSper

(ESI-MS) in positive and negative mode of GalSper was performed on a Finnigan MAT TSQ 7000 equipped with an atmospheric pressure ionization (API) source.

1-(2-Thioxo-thiazolidin-3-yl)-octadec-9-enyl-1-one was obtained from oleic acid chloride and 2-thiazoline-2-thiol according to ref 30. TLC ($\text{CH}_2\text{Cl}_2/\text{hexane}$: 1/1: v/v): R_f 0.46. ^1H NMR (CDCl_3): δ 0.89 (t, $^3J = 6.4$ Hz, 3H, CH_3); 1.18–1.50 (broad s, 20H, $(\text{CH}_2)_6\text{CH}_2\text{CH}=\text{CHCH}_2(\text{CH}_2)_4$); 1.50–1.72 (m, 2H, $\text{CH}_2\text{CH}_2\text{C}(\text{O})$); 1.90–2.12 (m, 4H, $=\text{CHCH}_2$); 3.23 (t, $^3J = 7.4$ Hz, 2H, $\text{CH}_2\text{C}(\text{O})$); 3.28 (t, $^3J = 7.5$ Hz, 2H, CH_2N); 4.57 (t, $^3J = 7.5$ Hz, 2H, CH_2S); 5.24–5.46 (m, 2H, CH). ^{13}C NMR (CDCl_3): δ 14.1 (CH_3); 22.7 (CH_3CH_2); 24.7 ($\text{CH}_2\text{CH}_2\text{C}(\text{O})$); 27.2, 27.3 ($=\text{CHCH}_2$); 28.3 (CH_2S); 29.1, 29.2, 29.4, 29.6, 29.7, 29.8 ($(\text{CH}_2)_4\text{CH}_2\text{CH}=\text{CHCH}_2(\text{CH}_2)_3$); 31.9 ($\text{CH}_3\text{CH}_2\text{CH}_2$); 38.6 ($\text{CH}_2\text{C}(\text{O})$); 56.2 (CH_2N); 129.7, 130.0 (CH), 175.0 ($\text{C}=\text{O}$); 201.6 ($\text{C}=\text{S}$).

The lipoplexes investigated here were prepared either from plasmid pTG11033 (pCMV-intronHMG-luciferase-SV40pA, 9514 bp) or from plasmid pTG11236 (pCMV-SV40-luciferase-SV40pA, 5739 bp). These plasmids contain the luciferase gene under the control of the CMV immediate early 1 promoter. Their endotoxin content (<1 endotoxin unit/mg of plasmid) was checked using a Limulus Amebocyte Lysate kit (Biogenic, France).

For in vitro transfection, epithelial A549 cells (from human pulmonary carcinoma), human hepatoma HepG2 cells or murine embryo BNL-CL2 hepatocytes were grown 24 h before the experiments in Dulbecco-modified Eagle culture medium (DMEM; Gibco-BRL), containing 10% foetal calf serum (FCS; Sigma), in 96-well plates (2×10^4 cells per well), in a wet (37°C) and 5% $\text{CO}_2/95\%$ air atmosphere.

Chemistry. *Synthesis of N-[11-(Sodium oxycarbonyl)-undecyl]-1-amino-1-deoxylactitol (1).* A solution of sodium 12-aminododecanoate (5.0 g, 23 mmol; Aldrich) in 60 mL of MeOH was added to lactose (5.0 g, 14 mmol; Aldrich) in 30 mL of H_2O . The resulting mixture was stirred during 18 h at room temperature and then heated at 55°C for 6 h. Then, sodium borohydride (570 mg, 15 mmol; Aldrich) was added at room temperature, and the resulting mixture was stirred during 12 h. After evaporation, the residue was chromatographed on a silica gel column (200 g, CHCl_3 to $\text{CHCl}_3/\text{MeOH}/\text{NH}_4\text{OH}$: 5/5/1.5: v/v/v) leading to 3.1 g (40%) of **1** as white powder. TLC ($\text{CHCl}_3/\text{MeOH}/\text{NH}_4\text{OH}$: 5/5/1.5: v/v/v): R_f 0.53. ^1H NMR(D_2O): δ

1.12–1.40 (broad s, 14H, $\text{N}(\text{CH}_2)_2(\text{CH}_2)_7$); 1.40–1.57 (m, 2H, $\text{CH}_2\text{CH}_2\text{C}(\text{O})$); 1.57–1.80 (m, 2H, NCH_2CH_2); 2.12 (t, $J = 7.3$ Hz, 2H, $\text{CH}_2\text{C}(\text{O})$); 2.91–3.19 (m, 4H, CH_2N); 3.19–4.29 (m, 12H, CHOH , CHO , CH_2OH); 4.46 (d, $J = 7.5$ Hz, 1H, Gal H1). ^{13}C NMR ($\text{CDCl}_3/\text{CD}_3\text{OD}/\text{NH}_4\text{OH}$): δ 26.1, 26.4, 27.7, 28.7, 28.8, 29.1 ($\text{NCH}_2(\text{CH}_2)_9$); 37.7 ($\text{CH}_2\text{C}(\text{O})$); 51.1 (CH_2N); 61.1 (CH_2OH); 62.0 (Gal C6); 68.8, 70.9, 71.1 (CHOH); 69.3 (Gal C4); 71.0 (Gal C2); 72.9 (Gal C3); 75.3 (Gal C5); 79.6 ($\text{OCH}(\text{CHOH})_2$); 103.1 (Gal C1); 182.0 ($\text{C}=\text{O}$).

Synthesis of N-[11-(Sodium oxycarbonyl)-undecyl]-N-oleoyl-1-amino-1-deoxylactitol (2). A solution of **1** (0.60 g, 1.05 mmol), 1-(2-thioxo-thiazolidin-3-yl)-octadec-9-enyl-1-one (0.76 g, 2.17 mmol), and NEt_3 (0.2 mL; 1.44 mmol) in 100 mL of DMF was stirred during 7 days at 60°C . After evaporation and chromatography on a silica gel column (30 g, CHCl_3 to $\text{CHCl}_3/\text{MeOH}/\text{NH}_4\text{OH}$: 6/4/0.5: v/v/v), 140 mg (15%) of **2** was obtained as a white powder. TLC ($\text{CHCl}_3/\text{MeOH}/\text{NH}_4\text{OH}$: 5/5/1.5: v/v/v): R_f 0.66. ^1H NMR (CD_3OD): δ 0.81 (t, $J = 6.4$ Hz, 3H, CH_3); 1.06–1.41 (broad s, 34H, $\text{N}(\text{CH}_2)_2(\text{CH}_2)_7$, $(\text{CH}_2)_4\text{CH}_2\text{CH}=\text{CHCH}_2(\text{CH}_2)_6$); 1.41–1.72 (m, 6H, $\text{CH}_2\text{CH}_2\text{C}(\text{O})$, NCH_2CH_2); 1.81–2.08 (m, 4H, $=\text{CHCH}_2$); 2.15 (t, $J = 7.3$ Hz, 2H, $\text{CH}_2\text{C}(\text{O})$); 2.24–2.47 (m, 2H, $\text{CH}_2\text{C}(\text{O})\text{N}$); 3.06–4.07 (16H, CHOH , CHO , CH_2OH , $\text{C}(\text{O})\text{NCH}_2$); 4.38 (d, $J = 7.5$ Hz, 1H, Gal H1); 5.16–5.35 (m, 2H, $=\text{CH}$). ^{13}C NMR (CD_3OD): δ 14.5 (CH_3); 23.7 (CH_3CH_2); 26.4 ($\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{N}$); 26.6, 26.8, 27.8, 28.0, 28.5, 29.9, 30.3, 30.4, 30.6, 30.8 ($(\text{CH}_2)_9\text{CH}_2\text{C}(\text{O})$, $(\text{CH}_2)_4\text{CH}_2\text{CH}=\text{CHCH}_2(\text{CH}_2)_4$); 28.1 ($=\text{CHCH}_2$); 33.1 ($\text{CH}_3\text{CH}_2\text{CH}_2$); 34.1, 34.4 ($\text{CH}_2\text{C}(\text{O})\text{N}$); 36.2 ($\text{CH}_2\text{C}(\text{O})\text{O}$); 47.7, 50.5, 50.7, 51.7 (CH_2N); 62.5 (CH_2OH); 63.6 (Gal C6); 70.2, 70.3, 70.8, 71.9, 72.0, (CHOH); 72.7 (Gal C4); 72.9 (Gal C2); 74.9 (Gal C3); 77.2 (Gal C5); 83.6 ($\text{OCH}(\text{CHOH})_2$); 105.7 (Gal C1); 130.9, 131.0 (CH); 176.3, 176.4 ($\text{NC}(\text{O})$); 179.5 ($\text{C}(\text{O})\text{O}$). The doubling of some signals indicates the presence of conformational isomers due to restricted rotation of the amide bound.

Synthesis of GalSper. To a solution of spermine (250 mg, 1.20 mmol; Fluka), **3** (100 mg, 0.12 mmol), and 0.2 mL of NEt_3 (1.4 mmol) in 4 mL of DMF was added 60 mg of BOP (0.13 mmol; Novabiochem). After 1 h at room temperature, evaporation and chromatography on Sephadex LH20 (5 g, MeOH), GalSper (102 mg; 84%) was isolated as a white powder. ^1H NMR (CD_3OD): δ 0.86 (t,

$J = 6.4$ Hz, 3H, CH_3); 1.12–1.43 (broad s, 34H, $\text{N}(\text{CH}_2)_2-(\text{CH}_2)_7$, $(\text{CH}_2)_4\text{CH}_2\text{CH}=\text{CHCH}_2(\text{CH}_2)_6$); 1.43–1.82 (m, 14H, $\text{CH}_2\text{CH}_2\text{C}(=\text{O})$, $\text{N}(\text{H})\text{CH}_2\text{CH}_2$, NCH_2CH_2); 1.89–2.07 (m, 4H, $=\text{CHCH}_2$); 2.14 (t, $J = 7.3$ Hz, 2H, $\text{CH}_2\text{C}(=\text{O})\text{N}(\text{H})$); 2.24–2.51 (m, 2H, $\text{NC}(=\text{O})\text{CH}_2$); 2.51–2.89 (m, 10H, $\text{CH}_2\text{-NH}_2$, $\text{CH}_2\text{N}(\text{H})\text{CH}_2$); 3.19 (t, $J = 7.5$ Hz, $\text{C}(=\text{O})\text{N}(\text{H})\text{CH}_2$); 3.25–4.11 (16H, CHOH , CH_2OH , CHO , $\text{CH}_2\text{NC}(=\text{O})$); 4.44 (d, $J = 7.5$ Hz, 1H, Gal H1); 5.25–5.36 (m, 2H, $=\text{CH}$). ^{13}C NMR (CD_3OD): δ 14.4 (CH_3); 23.6 (CH_3CH_2); 27.0 ($\text{CH}_2\text{CH}_2\text{C}(=\text{O})\text{N}$); 26.5, 26.7, 27.9, 28.3, 30.1, 30.2, 30.3, 30.5, 30.7 ($(\text{CH}_2)_9\text{CH}_2\text{NC}(=\text{O})$, $\text{CH}_2\text{CH}_2\text{N}(\text{H})$, $(\text{CH}_2)_4\text{-CH}_2\text{CH}=\text{CHCH}_2(\text{CH}_2)_4$); 28.0 ($=\text{CHCH}_2$); 32.9 ($\text{CH}_3\text{-CH}_2\text{CH}_2$); 33.9, 34.2 ($\text{NC}(\text{O})\text{CH}_2$); 37.0 ($\text{CH}_2\text{C}(=\text{O})\text{NH}$); 37.8 ($\text{C}(=\text{O})\text{N}(\text{H})\text{CH}_2$); 40.2, 47.4, 47.8, 49.9, 50.0 ($\text{CH}_2\text{-NHCH}_2$, CH_2NH_2); 50.7, 51.8 ($\text{CH}_2\text{NC}(=\text{O})$); 62.4 ($\text{CH}_2\text{-OH}$); 63.6 (Gal C6); 70.0, 70.1, 70.7, 70.7, 71.8, 72.0 (CHOH); 72.7 (Gal C4); 73.0 (Gal C2); 74.7 (Gal C3); 77.0 (Gal C5); 83.6, 83.7 ($\text{OCH}(\text{CHOH})_2$); 105.6, 105.7 (Gal C1); 130.7, 130.8 ($=\text{CH}$); 170.4 ($\text{C}(=\text{O})\text{N}(\text{H})$); 176.1, 176.2 ($\text{NC}(=\text{O})$). The doubling of some signals indicates the presence of conformational isomers due to restricted rotation of the amide bond. ESI-MS: $m/z = 990.8$, in agreement with the calculated mass for $[\text{M}]^+ = \text{C}_{52}\text{H}_{103}\text{-N}_5\text{O}_{12}$.

Lipoplex Preparation and Characterization. The lipoplexes were prepared in 20 mM HEPES pH 7.5 and incubated with the cells at a DNA concentration of 0.1 to 2 $\mu\text{g}/\text{well}$. The GalSper-based DNA complexes are formulated by adding a desired volume of a micellar GalSper preparation at a concentration of 10 mg/mL in HEPES or a liposomal GalSper/DOPE (1:1) or GalSper/DOGS/DOPE (1:1:2) at a concentration of 10 mg/mL in HEPES to the desired volume of the DNA solution to reach a final DNA concentration of 0.1 mg/mL. Thus, for the preparation of the GalSper/DOPE/DNA complex of N/P ratio 5 and 0.1 mg/mL DNA, 50 μL of the GalSper/DOPE solution (10 mg/mL in EtOH) was transferred to a borosilicate glass tube (16 $\times$ 100 mm). The solvent was evaporated in a Rotavap evaporation system (45 $^\circ\text{C}$, 30 pm, 0.2 bar, 40 min). 50 μL of HEPES 20 mM, pH 7.5, was added to the film obtained. The preparation was vortexed for 12 h. Then, 47.9 μL of this preparation was added to 952.1 μL of DNA solution [100 μL of DNA (1 mg/mL) diluted with 852.1 μL of HEPES buffer]. This preparation was used for the following experiments.

The N/P ratio of 5 corresponds to the molar amount of cationic lipid necessary to have a ratio of 5 amino group nitrogens (for 1 mol of cationic lipid) per one phosphate in the DNA (330 Da mean Mw), as described elsewhere (26). The average sizes of the lipoplexes were measured by photon correlation spectroscopy using a Coulter N4Plus particle size analyzer. Each sample (0.5 μg of plasmid) was electrophoresed for about 90 min under 75 V, through a 0.8% agarose gel in TAE 1 x (Tris-acetate-EDTA) buffer and stained by spreading a 1 $\mu\text{L}/\text{mL}$ ethidium bromide (EtBr) solution in TAE buffer. The DNA was visualized in 200 mL of TAE buffer containing 20 μL of ethidium bromide (Sigma, 10 mg/mL) after photographing on a UV transilluminator. The plasmid integrity in each sample (0.25 μg) was confirmed by electrophoresis after decomplexing the lipoplex (10 μL of a plasmid 0.05 mg/mL) with sodium dodecyl sulfate (10 μL , 80 g/L).

In Vitro Transfection. A volume of DNA/lipospermine lipoplex (20, 10, 5, and 1 μL , respectively) was diluted to 100 μL in DMEM in order to obtain various amounts of DNA (2, 1, 0.5, and 0.1 μg , respectively) in the preparation. For the transfection experiments performed in the presence of serum, the lipoplex preparation

was diluted to 100 μL in DMEM supplemented with 10% FCS. The cell culture medium was removed and replaced by 100 μL of lipoplex/DMEM solution. For the transfection experiments performed in the presence of ASF, ASF (1 mg in 50 μL of DMEM) was added to the cells 10 min before the addition of the lipoplex preparation diluted to 50 μL in DMEM. After 4 and 24 h, 50 μL and 100 μL of DMEM supplemented with 30 and 10% FCS, respectively, were added. 48 h after transfection, the culture medium was discarded, and the cells were washed twice with 100 μL of PBS and then lysed with 50 μL of lysis buffer (Promega, Charbonnières, France). The lysates were frozen at -40 $^\circ\text{C}$ awaiting analysis of luciferase activity. This measurement was done for 10 s on 10 μL of the lysis mixture in a LB96P luminometer (Berthold, Evry, France) in dynamic mode, using the "Luciferase" determination system (Promega) in 96-well plates. The total protein concentration per well was determined by the BCA test (Pierce, Montluçon, France). For cells grown in the absence of lipoplexes, a well contains around 30–60 μg of proteins. Luciferase activity was calculated as fg of luciferase/mg of protein. The percentage of cell viability of the lipoplexes was calculated as the ratio of the total protein amount per well of the transfected cells relative to that measured for untreated cells $\times$ 100%. All transfection experiments were performed at least in triplicate and are expressed as the mean $\pm$ SEM. Statistical analyses were performed using a Student *t*-test.

RESULTS AND DISCUSSION

Synthesis. The synthesis of the galacto-lipospermines has been performed using the three-step procedure outlined in Scheme 1. Although the overall yields by which GalSper was obtained are low, this strategy remains nevertheless very advantageous, as it did not require protection/deprotection of the sugar and spermine residues. The first step consisted into the opening of lactose, a low cost disaccharide, by 12-amino dodecanoic acid, followed by the *in situ* NaBH_4 reduction of the imine formed. Acylation of amine **1** thus obtained with the mercaptothiazoline derivative of oleic acid for one week afforded compound **2**. Such mild acylating agents are selective for amino groups and obviate the need for protection of the sugar hydroxyls (30). The low reactivity of this acylating agent and of the starting amine **1**, and the hydrophilicity of compound **3** which complicates its purification, are likely to be responsible for the low yields of this synthesis. Spermine was then condensed onto the free acid function of **3** leading to GalSper in 85% yield. This step was performed in the presence of BOP as coupling agent and with a large excess of spermine in order to avoid the coupling of activated acid onto two amino groups of spermine. GalSper was purified by size-exclusion chromatography on Sephadex LH-20 and characterized by ^1H and ^{13}C NMR spectroscopy, and by electrospray ionization mass spectrometry in positive and negative mode.

Lipoplex Formation and Characterization. The capability of GalSper to condense DNA and to form lipoplexes was analyzed with or without an equimolar amount of DOPE or of DOGS. The sizes of the lipoplexes were analyzed by light scattering spectroscopy. These studies were performed with pTG11033 plasmid, also used for the *in vitro* transfection screening assays (see below). The procedure applied for the lipoplex preparation relies on the dilution from a GalSper micellar solution or from GalSper/DOPE (1:1) or GalSper/DOGS/DOPE (1:1:2) liposomes in buffer, using N/P ratios of 10,

Table 1. Mean Sizes Determined by Light Scattering Spectroscopy of the Lipoplexes Formed between Plasmid PTG11033 and GalSper, GalSper/DOPE (1:1), GalSper/DOGS/DOPE (1:1:2), or DOGS/DOPE (1:1), and Prepared in 20 mM HEPES pH 7.5

N/P ratio ^a	mean size (standard deviation) in nm			
	GalSper	GalSper/DOPE (1:1)	GalSper/DOGS/DOPE (1:1:2)	DOGS/DOPE (1:1)
10	140 (50)	100 (40) 60% ^b 800 (200) 40% ^b	90 (70) 70% ^b 1100 (300) 30% ^b	200 (70)
5	150 (50)	100 (40) 60% ^b 750 (150) 40% ^b	500 (300) 40% ^b 4900 (1300) 60% ^b	300 (120)
2.5	230 (80)	250 (70)	p ^c	p ^c
1.25	p ^c	p ^c	p ^c	p ^c
0.8	280 (100)	290 (70)	400 (140)	300 (140)

^a N = amine equivalents; P = plasmid phosphate equivalents. ^b Percent (%) of the overall population. ^c p = precipitate.

5, 2.5, 1.25, and 0.8 (*N* = number of lipid amines; *P* = number of DNA phosphates). Table 1 collects the mean particle sizes measured for the different lipoplexes. These formulations were also analyzed by gel electrophoresis (data not shown) which indicated the absence of "free" plasmid for *N/P* ratios from 10 to 1.25 and the presence of accessible plasmid to ethidium bromide in the case of the GalSper/DOGS/DOPE *N/P* 1.25 formulation and of all *N/P* 0.8 preparations.

When used alone, GalSper was able to condense DNA into lipoplexes with mean particle sizes in the 140–280 nm range, except for the *N/P* ratio of 1.25 for which a precipitate was observed. When an equimolar amount of DOPE was used for the formulation of the lipoplexes, similar results were obtained, although for *N/P* 10 and 5, larger-sized particles (~800 nm) were also detected. When the lipoplexes were formulated using a combination of GalSper, DOGS, and DOPE, polydispersed preparations of large-sized particles and precipitates were measured.

It is further noticeable that GalSper and GalSper/DOPE form lipoplexes that do not precipitate at a *N/P* ratio of 2.5 while most often precipitates were formed with (poly)cationic lipids for *N/P* 2.5 (7, 31) (e.g., see DOGS/DOPE in Table 1). This result is indeed worth being noted if *in vivo* uses of such formulations are contemplated.

In Vitro Transfection. Our main objective was to demonstrate that targeted lipoplexes formulated with the cationic GalSper bolaamphiphile were able to deliver specifically genes to cells expressing ASGP receptors. However, cationic lipids also provide receptor-independent gene transfer to such cells (see below). It was therefore necessary to determine the conditions under which a specific gene transfer can be obtained *in vitro* with the cationic GalSper. Toward these goals, the transfection potential of the various above-described GalSper pTG11033-lipoplexes was first screened *in vitro* on the human hepatoma HepG2 cells. These assays, using the luciferase-encoding plasmid, were performed in the presence of 10% foetal calf serum for 24 h. The transfection efficiency of the lipoplexes (expressed in fg of luciferase/mg of protein) was evaluated for gradual *N/P* ratios (0.8, 1.25, 2.5, 5, and 10) and various amounts of DNA (2, 1, 0.5, and 0.1 μ g/well) as compared to naked DNA and to the control lipoplexes based on DOGS/DOPE (1/1 mol) which are known to have a very high potential for gene transfection (32). Cells treated with naked DNA under equivalent conditions showed expression levels of 10^{-3} fg luciferase/mg of protein. The cell viability of the lipoplexes was also checked by determining the total protein amount per well of the transfected cells relative to that measured for untreated cells (for which the total protein amount per well is in a 30–50 μ g/well range).

The transfection and cell viability results are illustrated in Figure 1. These results indicate that GalSper, when used alone, is not an efficient gene transfer agent. Transfection was observed only for high *N/P* ratios and for the highest concentrations of plasmid tested (1 and 2 μ g/well). Furthermore, these formulations led to low levels of luciferase expression ($<2 \times 10^5$ fg luciferase/mg protein) and were rather cytotoxic (cell viability below 10%).

However, its transfection potential was substantially improved when the GalSper lipoplexes were coformulated with an equimolar amount of DOPE, highlighting again the unique properties of DOPE as helper lipid (7, 24, 25, 32). Luciferase expression levels in the 10^6 – 10^7 fg/mg protein were indeed measured for the *N/P* 5 and 2.5 formulations and for a DNA amount of 0.5 μ g/well. When compared to control DOGS/DOPE lipoplexes, GalSper/DOPE *N/P* 2.5 formulations were found to be more efficient than the *N/P* 2.5 reference (at least by 2 orders of magnitude, $p < 0.05$) in mediating gene transfer and expression. For *N/P* ratios ≥ 5 or ≤ 1.25 , GalSper/DOPE formulations were found to be statistically as efficient or less efficient than their corresponding DOGS/DOPE lipoplexes, respectively.

Coformulation of DOGS/DOPE lipoplexes (which transfect HepG2 cells through a nonspecific mechanism) with GalSper could also constitute a mean to obtain specific cell interactions and gene transfer. We found that the transfection levels of DOGS/DOPE remained statistically unaffected by replacing half of the molar amount of DOGS by the same molar amount of GalSper. However, this result does not inform on the entry pathway specificity of the vector.

Most importantly, this transfection screening suggests that GalSper/DOPE *N/P* 5 and 2.5 lipoplexes could trigger a receptor-mediated transfection, although such a mechanism of gene transfer is more likely to be observed with lipoplexes having lower electrical surface charge in order to minimize the transfection electrostatic pathway. Unfortunately, the electrically less charged GalSper/DOPE lipoplexes *N/P* 1.25 and 0.8 that should exhibit increased specific binding displayed luciferase expression levels that are too low to allow the detection of a receptor-mediated endocytosis pathway leading to gene expression. It should be noted that substantially higher levels of transfection were measured for GalSper/DOGS/DOPE *N/P* 1.25 and 0.8 as compared to their respective GalSper/DOPE ones. This most likely resulted, consequent to the replacement of GalSper by DOGS, from increased nonspecific binding to the negatively charged proteoglycans which are expressed by these adherent cells.

To demonstrate cell specific transfection with GalSper-based lipoplexes, we performed a series of experiments

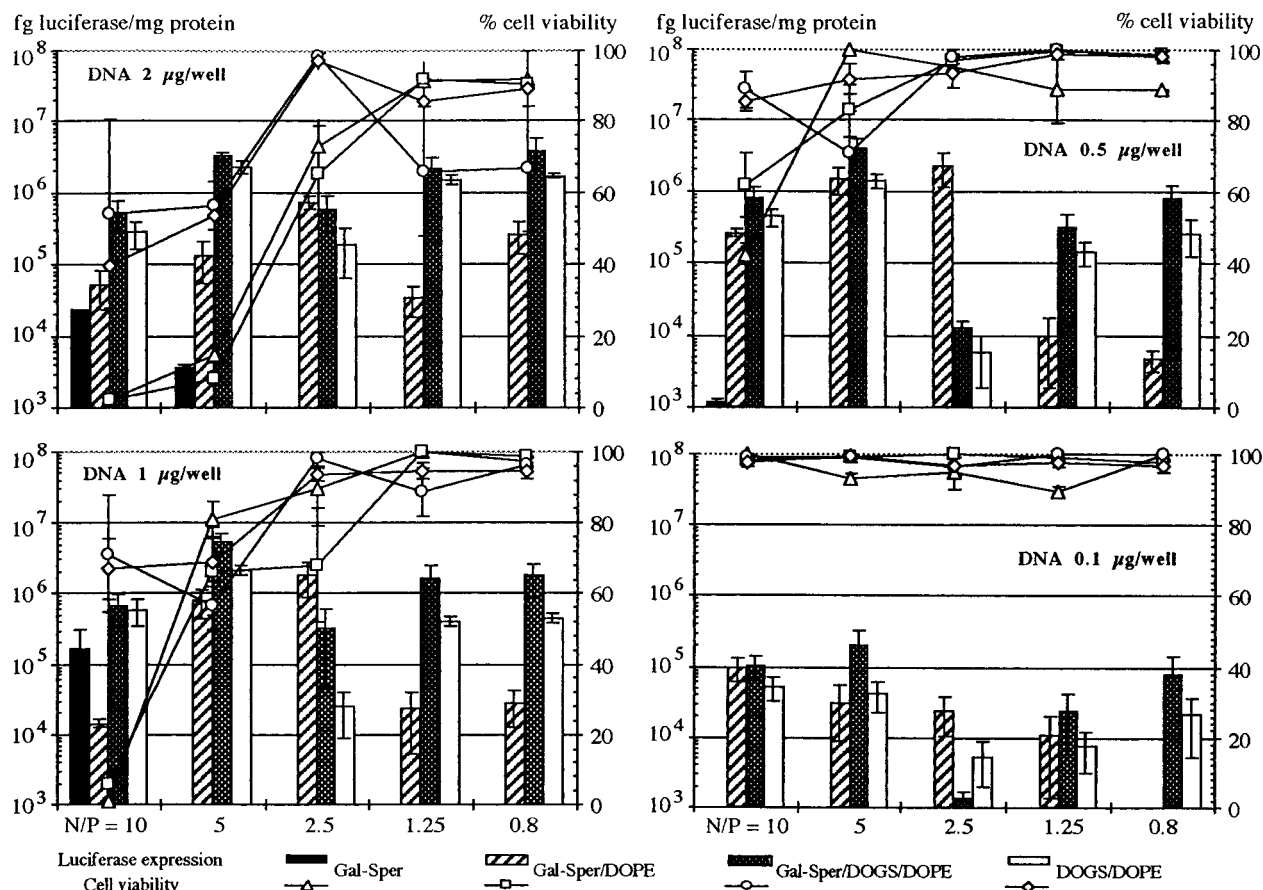


Figure 1. Luciferase expression (bars) and cell viability (points) in HepG2 cells of the lipoplexes prepared in 20 mM HEPES pH 7.5 and made of GalSper, GalSper/DOPE (1:1), or GalSper/DOGS/DOPE (1:1:2) and plasmid pTG11033 (DNA) for various N/P ratios, as compared to control DOGS/DOPE (1:1). The transfection experiments were performed in DMEM supplemented with 10% fetal calf serum. The given means $\pm$ SEM were calculated from four independent experiments.

on ASGP expressing cells (HepG2 and BNL-CL2) in the absence and in the presence of an excess of asialofetuin (ASF, 20- and 10-fold molar excess over galactose for the N/P 2.5 and 5 formulations, respectively) which is a high affinity ligand of ASGP (20, 26). To show the effects of ASF on nonspecific-mediated transfection, we performed also a series of control experiments on A549 cells which do not express ASGP. Control experiments were also realized with DOGS/DOPE lipoplexes as nontargeted galactose lipoplexes. These transfection assays were performed (i) with lipoplexes formulated from the luciferase encoding plasmid pTG11236, (ii) with N/P 5 and 2.5 formulations, for the above-discussed reasons, and (iii) in the absence of serum, to avoid the possible inhibitory effects on ligand–receptor interactions that might be due to the serum constituents. The results are illustrated in Figure 2.

As compared with the first series of experiments on HepG2 cells which were run in the presence of serum, a surprisingly lower luciferase expression level was measured for the GalSper/DOPE N/P 2.5 in the absence of serum while for the other formulations higher or comparable levels were found, as expected. The surface of these lipoplexes most probably displays the highest galactose/cation ratio among the different formulations investigated. This formulation corresponds to lipoplexes having 0.83 equiv of galactose for 2.5 amines and one DNA phosphate while GalSper/DOGS/DOPE (1:1:2) N/P 2.5 lipoplexes for example contain only 0.36 equiv of galactose for 2.5 amines and one DNA phosphate. The interactions of the lipoplexes and the different serum constituents being highly dependent upon the nature and

residual charge of the lipoplex surface, one expect therefore substantial different biological behaviors for the lipoplexes.

Before interpreting unambiguously the transfection results on ASGP(+) cells with GalSper-based lipoplexes, it was essential to estimate the effects of ASF on nonspecific-mediated transfection. Although one expects that ASF has no determinant influence on transfection of (i) the ASGP(–) A549 cells mediated with any type of lipoplexes, or (ii) ASGP(+) cells mediated with the DOGS/DOPE lipoplexes, its interactions with the cell surface and/or with the positively charged lipoplexes are likely to interfere with the transfection process.

Concerning the effects of ASF on nonspecific-mediated transfection, our results did not demonstrate any systematic trend, these effects being rather cell- and lipoplex-type dependent. Indeed, and as expected, the presence of ASF had almost no effect (at most a 5-fold decrease, which is however statistically not significant) on the transfection of A549 and HepG2 cells with DOGS/DOPE lipoplexes (Figure 2). However, ASF induced a substantial transfection increase of the A549 cells (64-fold) mediated with the GalSper/DOPE N/P 5 formulation. In contrast, it decreased the nonspecific transfection (i) of the A549 cells mediated with the GalSper/DOGS/DOPE lipoplexes and (ii) of the BNL-CL2 cells mediated with the DOGS/DOPE lipoplexes, by a factor of 15 in the latter case (ASF was also found to inhibit the transfection of BNL-CL2 cells by PEI polyplexes (26)). Altogether, these data are taken to indicate that, to be unambiguously interpreted as a receptor-mediated transfection process, competition experiments with ASF should inhibit

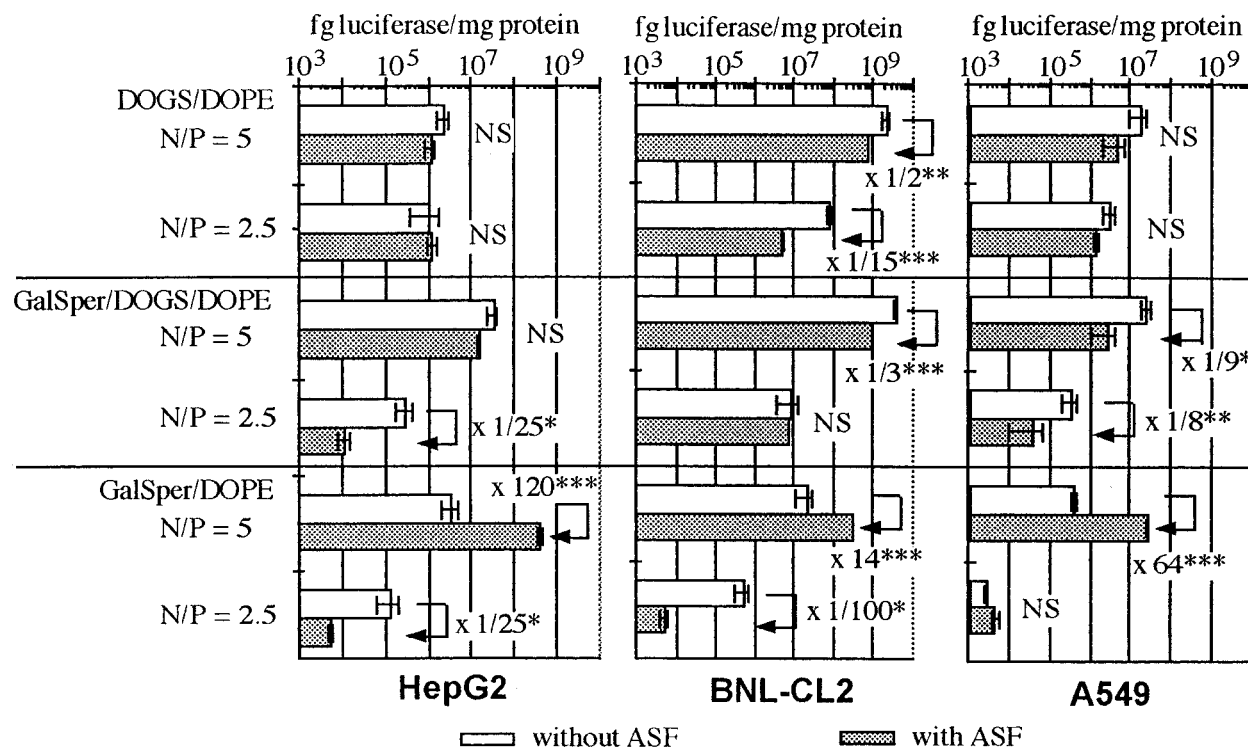


Figure 2. Luciferase expression in ASGP(+) cells (human HepG2 and murine BNL-CL2 hepatocytes) and ASGP(−) cells (A549 cells deriving from a human carcinoma) of the lipoplexes prepared in 20 mM HEPES pH 7.5 in the presence of ASF (grey bars) or not (open bars). The lipoplexes were made of GalSper/DOPE (1:1) or GalSper/DOGS/DOPE (1:1:2) and plasmid pTG11236 (DNA) for N/P 2.5 and 5, as compared to control DOGS/DOPE (1:1). The lipoplexes were incubated at a concentration of 0.5 μ g DNA/well with the cells. The transfection experiments were performed in DMEM supplemented with 1 mg/well of ASF which was added to the cells 10 min before addition of the lipoplexes. The given means $\pm$ SEM were calculated from three independent experiments. * p < 0.05, ** p < 0.01 and *** p < 0.001 were carried out in a pairwise comparison.

transfection of the HepG2 and BNL-CL2 cells with the GalSper-based lipoplexes by a factor significantly larger than 5 for HepG2 and 15 for BNL-CL2 cells.

Concerning the transfection of HepG2 and BNL-CL2 cells with the GalSper/DOPE N/P 2.5 lipoplexes, ASF was found to significantly inhibit this process by a factor 25 and 100 (p < 0.05), respectively. This likely indicates a receptor-mediated endocytosis pathway for the transfection of these two cell types. This is further supported by the very low ability of the GalSper/DOPE N/P 2.5 lipoplexes to transfect the control A549 cells through a receptor-independent process. The galactosyl coat interferes with the binding of the cationic particles to the anionic cell surface, providing a shielding effect that was also observed with other glycosylated-DNA complexes (21, 26, 27). Very surprisingly, ASF induced a transfection increase of the BNL-CL2 (14-fold) and HepG2 cells (120-fold) when performed with the GalSper/DOPE N/P 5 lipoplexes, indicating rather that transfection of these ASGP(+) cells with such highly cationic complexes is mainly dominated by nonspecific electrostatic interactions which involve anionic cell proteoglycans (16, 17).

Concerning the transfection of the HepG2 cells with the GalSper/DOGS/DOPE N/P 2.5 lipoplexes, the 25-fold luciferase expression inhibition detected in the presence of ASF could also be attributed to partial blockade by ASF of the targeted cell entry of the plasmid. On the other hand, no ASF inhibition was detected for these lipoplexes in the case of the BNL-CL2 cells. These results indicate that GalSper/DOGS/DOPE N/P 2.5 lipoplexes can transfect cells through a receptor-dependent and/or a receptor-independent mechanism, and when the former cell entry pathway is blocked, the other one takes over. This is further supported by the larger propensity of the

BNL-CL2 cells, as compared with HepG2 and A549 cell lines, to be transfected by the cationic nontargeted DOGS/DOPE lipoplexes.

Although lower luciferase expression levels in BNL-CL2 and in HepG2 cells were obtained with GalSper/DOPE N/P 2.5 lipoplexes as compared with control DOGS/DOPE N/P 2.5 particles or with the more positively charged N/P 5 particles (yet through a different mechanism), specific receptor-mediated endocytosis of DNA can be achieved with this targeted cationic GalSper bolaamphiphile presenting a single galactose residue. The present work suggests that targetable lipopolyamines appear as promising synthetic vectors for specific gene delivery to ASGP(+) cells. Although encouraging, the transfection efficiency and the selectivity obtained here with this first head compound should however be improved.

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A Convenient Solid-Phase Method for Synthesis of 3'-Conjugates of Oligonucleotides

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We present a new procedure for the preparation of 3'-conjugates of oligonucleotides through solid-phase synthesis. A suitable universal solid support was readily prepared using a series of peptide-like coupling reactions to incorporate first a spacer and then an L-homoserine branching unit. The N- α -position of the homoserine carries an Fmoc protecting group that is removed by treatment with piperidine to liberate an amino group suitable for attachment of the conjugate (e.g., small organic molecule, fluorescent group, cholesterol, biotin, amino acid, etc.) or for assembly of a short peptide. The side-chain hydroxyl group of the homoserine carries a trityl protecting group. After TFA deprotection, the hydroxyl group acts as the site for oligonucleotide assembly. An additional spacer, such as aminohexanoyl, may be incorporated easily between the conjugate molecule and the oligonucleotide. A number of examples of synthesis of 3'-conjugates of oligonucleotides and their analogues are described that involve standard automated oligonucleotide assembly and use of commercially available materials. The linkage between oligonucleotide and 3'-conjugate is chirally pure and is stable to conventional ammonia treatment used for oligonucleotide deprotection and release from the solid support. The homoserine-functionalized solid support system represents a simple and universal route to 3'-conjugates of oligonucleotides and their derivatives.

INTRODUCTION

Oligonucleotides and their analogues have found wide application for the sequence-specific inhibition of gene expression within cells (Crooke and Lebleu, 1993; Chadwick and Cardew, 1997; Stein and Krieg, 1998). One of their main limitations, however, has been their generally poor uptake by cells in culture in the absence of a carrier. This is because the predominant routes of cell entry involve adsorptive or fluid-phase endocytosis, which often result in difficulties in release of the oligonucleotides from endosomes and lysosomes into other cellular compartments (Stein and Narayan, 1996). Uptake may be enhanced by co-administration of certain additives of which cationic liposomes are the most popular (Bennett et al., 1992). In particular, these agents have been found to assist the release of oligonucleotides into the cytosol. Yet use of these reagents is often limited due to their cellular toxicity and their instability in the presence of serum. Further, there are very large variations in effectiveness of these reagents for a particular cell line (Kang et al., 1999).

An alternative approach to enhance cellular uptake is to conjugate the oligonucleotide to another molecule known for its ability to penetrate or interact with cell membranes. Such molecules include lipophilic compounds, e.g., cholesterol (Letsinger et al., 1989; Krieg et al., 1993), lipids (Shea et al., 1990), long-chain hydrocarbons (Rait et al., 2000), and cholic acid and folic acid (Harrison and Balasubramanian, 1997). Conjugates of polycations such as spermine (Kabanov et al., 1995), homopolymers such as poly(ethylene glycol) (Jäschke et al., 1994), and a range of peptides (see recent reviews:

Stetsenko et al., 2000; Tung and Stein, 2000) have also been suggested as carriers. In addition, other types of molecules have been conjugated to oligonucleotides in order to provide specific labels for tracking oligonucleotides diagnostically or within cells (such as biotin or fluorophores (Agrawal et al., 1986)) or to enhance binding to nucleic acids (such as acridine (Asseline and Thuong, 1989)).

Conjugations to oligonucleotides are usually carried out at either the 5' or 3' ends to prevent interference with the ability of the oligonucleotides to hybridize with complementary target sequences. Synthetically, conjugation has been most readily achieved at the 5'-end through attachment of an appropriate amino linker (Agrawal et al., 1986) or by the preparation of a suitable phosphoramidite that can be used during or at the end of oligonucleotide assembly (Nelson et al., 1989b; Misiura et al., 1990). However, 5'-modification precludes the ability of the oligonucleotide to be ^{32}P -labeled by 5'-phosphorylation. By contrast, conjugation at the 3'-end of an oligonucleotide not only permits 5'- ^{32}P -labeling but has the added advantage that 3'-modification usually results in significant protection of the oligonucleotide against attack by 3'-exonucleases, which are the major source of nucleases within serum and cells.

A number of methods for the preparation of 3'-conjugates of oligonucleotides have been published. Early methods have involved a post-assembly conjugation protocol. Since oligonucleotide synthesis is carried out usually by solid-phase assembly from the 3'-end to the 5'-end, most methods have involved the development of special linkages between the solid support and the 3'-end of the first residue. After assembly of the oligonucleotide, it is cleaved from the support to generate a specific functional group to which the desired molecule, label, or peptide is then conjugated. The most common functional

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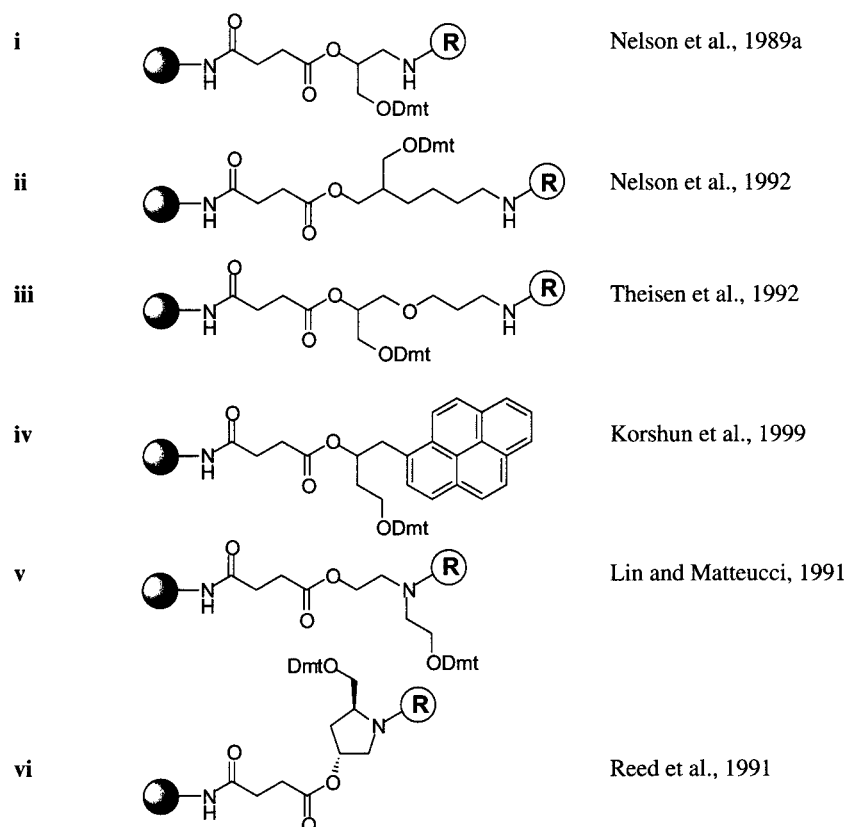


Figure 1. Examples of various solid supports used for preparation of oligonucleotide 3'-conjugates.

groups generated are primary amines (Nelson et al., 1989a; Asseline and Thuong, 1990; Nelson et al., 1992; Petrie et al., 1992) or thiols (Zuckerman et al., 1987; Bonfils and Thuong, 1991; Gupta et al., 1991).

Linear 3'-linkers are only useful for post-assembly conjugation once the oligonucleotide has been cleaved from the solid support. By contrast, a more general 3'-linker incorporates a chain branch where a convenient functional group, usually a protected amino group, is present on a sidearm. In principle, a branched linker should allow one (a) to introduce the conjugation molecule before assembly of the oligonucleotide, (b) to assemble the oligonucleotide first and carry out post-assembly conjugation after release into solution, or (c) to use the functionality to act as an attachment point for solid-phase conjugation or assembly of a peptide or other biomolecule prior to release of the oligonucleotide from the support (total solid-phase conjugate synthesis).

Examples of such branched linkers are shown in Figure 1. The two most commonly used branched 3'-linkers are those based on 3-amino-1,2-propane diol (type i) (Nelson et al., 1989a; Nelson, 1992, 1995) or 3-aminobutyl-1,3-propane diol (type ii) (Nelson et al., 1992; Nelson and Kent, 1995, 1999). In variations of this type of scaffold, a 3-O-aminopropyl glycerol linker has been used for the preparation of 3'-fluorescein conjugates of oligonucleotides (type iii) (Theisen et al., 1992) and a hexadecyl chain has been introduced at the 3'-end via a glyceryl linker (Rait et al., 2000). Similarly, a 3'-pyrene conjugate was prepared via a 1,3-propanediol linker (type iv) (Korshun et al., 1999). All of these types of linkers introduce chirality at the linker branch point. Further, synthesis of solid supports incorporating these linkers generally involves the prior multistep preparation of a specialized linker reagent for attachment to the support, sometimes leading to low linker loading.

Alternative linkers that are achiral have been suggested. Thus, the nonprochiral diethanolamine has been used for the synthesis of 3'-anthraquinone-conjugated oligonucleotides, where the conjugate is attached to the secondary amine (type v) (Lin and Matteucci, 1991; Lin and Matteucci, 1995). This method also requires prior preparation of a specialized conjugate reagent and the route is also not suitable for post-assembly conjugation. Another type of branched linker is based on *trans*-4-hydroxy-L-prolinol (type vi). Linker reagents containing, for example, cholesterol or acridine are attached through the secondary amino moiety of the linker leaving the secondary alcohol for attachment to the support and the primary alcohol as the oligonucleotide growth point (Reed et al., 1991; Gamper et al., 1993; Cheng et al., 1997). Although this linker is chirally pure and avoids the problem of racemates obtained with other linkers, the preparation of conjugates still requires the multistep synthesis of a specialized linker reagent for each conjugate type, and the route is not suitable for post-assembly functionalization.

We now describe a novel method for functionalization of solid supports suitable for synthesis of oligonucleotides conjugated at the 3'-end with a wide variety of labels or other functionalities. The synthetic route involves use of inexpensive, commercially available materials to prepare functionalized supports under standard conditions of solid-phase peptide synthesis and avoids the need for presynthesis of a complex linker reagent for each conjugate type. Examples are presented of solid-phase synthesis of a range of 3'-conjugated oligonucleotides starting from prefunctionalized supports carrying small organic groups 4-pentynoyl or 4-iodophenylacetyl, the fluorescent dye 5(6)-carboxyfluorescein, D-(+)-biotin, the amino acid cysteine, and the tripeptide (Lys)₃.

MATERIALS AND METHODS

Reagents and solvents were obtained from Sigma-Aldrich or Fluka (Poole, U.K.) unless otherwise mentioned. LCAA-CPG¹ 500 was obtained from Pierce. HATU was obtained from Applied Biosystems. 6-*N*-(9-Fluorenylmethoxycarbonyl)aminohehexanol (**1a**) is commercially available from Fluka. TLC plates (Kieselgel 60 F₂₅₄, Merck) were developed in 10% MeOH–CHCl₃. Oligodeoxyribonucleotide synthesis was carried out on a ABI 380B or 394 DNA/RNA synthesizer on a 0.2 or 1 μ mol scale using 2'-deoxynucleoside phosphoramidite reagent materials obtained from Cruachem (Scotland) or 2'-*O*-methyl ribonucleoside phosphoramidites obtained from Glen Research (via Cambio, Cambridge, U.K.) and standard synthetic procedures (Brown and Brown, 1991). Phosphorothiate linkages were incorporated by replacing the iodine oxidation step by sulfurization with the Beaucage reagent (Glen Research). *O*-*trans*-4-(*N*- α -Fmoc-*S*-*tert*-butylsulfenyl-L-cysteinyl)piperidyl *O*-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (Stetsenko and Gait, 2000a,b) was used to prepare the 5'-cysteinyl-3'-carboxy-fluoresceinyl conjugate.

Reversed-phase HPLC of oligonucleotides and conjugates was carried out using a Phenomenex RP-C18 column (4.6 $\times$ 250 mm) and dual-wavelength (218 and 254 nm) UV detection using gradients of acetonitrile (0–2%, 5 min; 2–40%, 20 min; 40–100%, 25 min) in either 0.1 M ammonium acetate buffer, pH 7.0 (a), or 0.1 M triethylammonium acetate buffer, pH 7.0 (b). Ion-exchange HPLC was carried out using a NucleoPac PA-100 (Dionex) (9 $\times$ 250 mm) using gradients of sodium perchlorate (1–400 mM) in a buffer of 20 mM Tris·HCl (pH 6.8), 25% formamide, and at UV detection at 280 nm wavelength. The mass of each oligonucleotide was checked by MALDI-TOF mass spectrometry on a Perseptive Biosystems Voyager DE mass spectrometer in positive-ion mode as previously described (Farrow et al., 1998).

2-(2-*N*-(9-Fluorenylmethoxycarbonyl)aminoethoxy)ethanol (1b). 9-Fluorenylmethyl succinimidyl carbonate (16 g, 47.5 mmol) was dissolved in ethyl acetate (100 mL), and 2-(2-aminoethoxy)ethanol (6 mL, 60 mmol) was added dropwise with stirring during ca. 10 min. A yellow waxy precipitate was formed immediately that gradually dissolved while the reaction proceeded. The mixture was stirred for another 12 h, until TLC (5% MeOH in CHCl₃) showed complete disappearance of the starting carbonate. The solution was transferred to a separation funnel and washed successively with 5% (w/v) citric acid (2 $\times$ 75 mL), water (75 mL), 5% aqueous NaHCO₃ (2 $\times$ 75 mL), and brine and dried over anhydrous sodium sulfate. The solvent was then removed by evaporation in vacuo, and the compound was crystallized from ethyl acetate–hexane (2:1 v/v) to give bunches of white needles. After filtration and washing with cold ethyl acetate–hexane (1:1 v/v), a first portion of product (13.41 g, 86%) was obtained. Evaporation of the mother liquor gave a further 1.68 g of product. Total yield of the title product was 15.09 g (97%). TLC (5% MeOH in CHCl₃): *R*_f 0.35. MALDI-TOF MS (2,5-dihydroxybenzoic acid in MeOH, 10 mg mL⁻¹): [M + H]⁺ 328.35 (obsd), 328.38 (calcd), [M + Na]⁺ 350.19 (obsd), 350.36 (calcd),

[M + K]⁺ 366.13 (obsd), 366.34 (calcd). ¹H NMR (DMSO-*d*₆, δ , ppm): 3.13 (d, 2H, *J* = 4.9 Hz, CH₂), 3.33 (d, 2H, *J* = 5.8 Hz, CH₂), 3.40 (t, 2H, *J* = 5.2 Hz, CH₂), 3.46 (d, 2H, *J* = 5.7 Hz, CH₂), 4.20 (t, 1H, *J* = 6.2 Hz, 9-fluorene CH), 4.29 (d, 2H, *J* = 6.8 Hz, fluorenylCH₂), 4.59 (m, 1H, NH), 7.32 (t, 2H, *J* = 7.2 Hz, Fmoc aromatics), 7.4 (t, 2H, *J* = 7.4 Hz, Fmoc aromatics), 7.68 (d, 2H, *J* = 7.2 Hz, Fmoc aromatics), 7.88 (d, 2H, *J* = 7.5 Hz, Fmoc aromatics).

6-*N*-(9-Fluorenylmethoxycarbonyl)aminohehexyl Succinate (2a). 6-*N*-Fmoc-aminohehexanol (3.73 g, 11 mmol), succinic anhydride (1.49 g, 14.85 mmol), and 4-(dimethylamino)pyridine (134 mg, 1.1 mmol) were suspended in 75 mL of 1,2-dichloroethane, 5 mL of pyridine was added, and the mixture was refluxed for 24 h. The solution was then transferred to a separation funnel, washed successively with 0.5 M NaHSO₄ (3 $\times$ 50 mL), water (50 mL), 5% aqueous NaHCO₃ (3 $\times$ 50 mL), and brine, and dried over anhydrous sodium sulfate. The solvent was removed in vacuo, and the mixture was flash chromatographed on Kieselgel 60 column eluted with 1% MeOH in CHCl₃. The appropriate fractions were pooled and evaporated, and the residue was crystallized from ethyl acetate–hexane (2:1 v/v) mixture to give 4.31 g (89%) of the title compound. TLC (5% MeOH in CHCl₃): *R*_f 0.46. MALDI-TOF MS: [M + H]⁺ 439.85 (obsd), 440.51 (calcd), [M + Na]⁺ 461.84 (obsd), 462.49 (calcd), [M + K]⁺ 477.36 (obsd), 478.60 (calcd). ¹H NMR (DMSO-*d*₆, δ , ppm): 1.26 (m, 4H, CH₂), 1.37 (t, 2H, *J* = 6.1 Hz, CH₂), 1.53 (t, 2H, *J* = 6.4 Hz, CH₂), 2.47 (s, 4H, succinyl CH₂), 2.94 (t, 2H, *J* = 6.9 Hz, CH₂), 3.98 (t, 2H, *J* = 6.4 Hz, CH₂), 4.19 (t, 1H, *J* = 6.9 Hz, 9-fluorene CH), 4.28 (d, 2H, *J* = 6.8 Hz, fluorenyl CH₂), 7.31 (t, 2H, *J* = 7.4 Hz, Fmoc aromatics), 7.40 (t, 2H, *J* = 7.3 Hz, Fmoc aromatics), 7.67 (d, 2H, *J* = 7.3 Hz, Fmoc aromatics), 7.87 (d, 2H, *J* = 7.4 Hz, Fmoc aromatics).

2-(2-*N*-(9-Fluorenylmethoxycarbonyl)aminoethoxy)ethyl Succinate (2b). 2-(2-*N*-Fmoc-aminoethoxy)ethanol (3.93 g, 12 mmol), succinic anhydride (1.50 g, 15 mmol), and 4-(dimethylamino)pyridine (147 mg, 1.2 mmol) were suspended in anhydrous ethyl acetate (75 mL), containing 2 mL of pyridine, and the mixture was stirred at 60 °C for 12 h. After TLC revealed total consumption of the starting alcohol, the mixture was transferred to a separation funnel, washed successively with 0.5 M sodium hydrogen sulfate (3 $\times$ 50 mL), water (50 mL), 5% aqueous NaHCO₃ (3 $\times$ 50 mL), and brine, and dried over anhydrous Na₂SO₄. The solvent was removed in vacuo, and the mixture was flash chromatographed on Kieselgel 60 column eluted with 2% MeOH in CHCl₃. The appropriate fractions were pooled and evaporated, and the residue was crystallized from ethyl acetate–hexane (3:1 v/v) to give 3.9 g (76%) of the title compound. TLC (5% MeOH in CHCl₃): *R*_f 0.40. MALDI-TOF MS (2,5-dihydroxybenzoic acid in MeOH, 10 mg/mL): [M + H]⁺ 427.84 (obsd), 428.17 (calcd), [M + Na]⁺ 449.93 (obsd), 450.15 (calcd), [M + K]⁺ 465.83 (obsd), 466.13 (calcd). ¹H NMR (DMSO-*d*₆, δ , ppm): 1.47 (m, 4H, succinyl CH₂), 2.12 (t, 2H, *J* = 6.7 Hz, CH₂), 2.40 (t, 2H, *J* = 5.7 Hz, CH₂), 2.55 (t, 2H, *J* = 4.4 Hz, CH₂), 3.09 (t, 2H, *J* = 4.4 Hz, CH₂), 3.18 (t, 1H, *J* = 6.7 Hz, 9-fluorene CH), 3.27 (d, 2H, *J* = 6.8 Hz, fluorenylCH₂), 6.30 (t, 2H, *J* = 7.4 Hz, Fmoc aromatics), 6.39 (t, 2H, *J* = 7.3 Hz, Fmoc aromatics), 6.67 (d, 2H, *J* = 7.3 Hz, Fmoc aromatics), 6.86 (d, 2H, *J* = 7.4 Hz, Fmoc aromatics), 11.20 (s, 1H, COOH).

Fmoc-sarcosine-Functionalized LCAA-CPG-500 (S1). Fmoc-sarcosine (148 mg, 0.47 mmol) and HATU

¹ Abbreviations: CPG, controlled pore glass; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; Dmt, dimethoxytrityl; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; LCAA, long-chain alkylamine; MALDI-TOF, matrix-assisted laser desorption ionisation time-of-flight; TFA, trifluoroacetic acid; Trt, trityl.

(162 mg, 0.43 mmol) were dissolved in DMF (4 mL), DIEA (81 μ L, 0.47 mmol) was added, and the mixture was shaken for 5 min. More DIEA (81 μ L, 0.47 mmol) was added, and the yellow solution was then poured onto LCAA-CPG-500 beads (2 g, 0.19 mmol, 94.8 μ mol g⁻¹ amino group loading, for example, obtained from Pierce). The slurry was kept for 1 h with occasional swirling, the solution was filtered, and the CPG beads were washed with DMF (2 $\times$ 5 mL) and acetonitrile (2 $\times$ 5 mL). The CPG was treated on the filter with equal volumes (2 mL of each) of capping solutions (Cap I: 20% acetic anhydride in THF, Cap II: 20% 1-methylimidazole and 40% 2,4,6-trimethylpyridine in THF) for 15 min, washed successively with DMF (5 $\times$ 5 mL), MeOH (5 $\times$ 5 mL), and diethyl ether (5 $\times$ 5 mL), and finally dried in vacuo. A total yield of 1.95 g of Fmoc-sarcosine LCAA-CPG-500 **S1** was obtained, with estimated loading of 92.1 μ mol g⁻¹ (97% substitution based on Fmoc group determination).

6-Fmoc-aminoethyl-succinyl-sarcosine LCAA-CPG-500 (S2a). The above support **S1** (1 g, 0.09 mmol) was treated with piperidine/DMF (2:8 v/v, 3 mL) for 15 min and washed with DMF (10 $\times$ 3 mL). 6-N-Fmoc-aminoethyl succinate (41 mg, 0.09 mmol) and HATU (32 mg, 0.08 mmol) were dissolved in DMF (2 mL), DIEA (16 μ L, 0.09 mmol) was added, and the mixture was shaken for 5 min. More DIEA (61 μ L, 0.09 mmol) was added, and the solution was then poured on the Fmoc-deprotected support **S1**. The slurry was kept for 1 h with occasional swirling, and the solution was filtered. The CPG beads were washed with DMF (2 $\times$ 3 mL) and acetonitrile (2 $\times$ 3 mL) and treated on the filter with equal volumes of capping solutions I and II (1.5 mL of each) for 15 min, washed successively with DMF (5 $\times$ 3 mL), MeOH (5 $\times$ 3 mL), and diethyl ether (5 $\times$ 3 mL), and finally dried in vacuo. A total of 1.01 g of 6-Fmoc-aminoethyl succinyl sarcosine LCAA-CPG-500 **S2a** was obtained with estimated loading of 74.3 μ mol g⁻¹ (90% substitution).

6-(N-Fmoc-O-trityl-homoseryl)aminoethyl-succinyl-sarcosine LCAA-CPG-500 (S3a). The above support **S2a** (1 g, 0.07 mol) was treated with piperidine/DMF (2:8 v/v, 3 mL) for 10 min and washed with DMF (10 $\times$ 3 mL). Fmoc-Hse(Trt)-OH (108 mg, 0.19 mmol, Novabiochem) and HATU (64 mg, 0.17 mmol) were dissolved in DMF (2 mL), DIEA (32 μ L, 0.19 mmol) was added, and the mixture was shaken for 5 min. More DIEA (32 μ L, 0.19 mmol) was added, and the solution was poured onto the Fmoc-deprotected support **S2a**. The slurry was kept for 1 h with occasional swirling, the solution was filtered, and the CPG beads were washed with DMF (2 $\times$ 3 mL) and acetonitrile (2 $\times$ 3 mL). The CPG was treated on the filter with equal volumes of capping solutions I and II (1.5 mL of each) for 15 min, washed successively with DMF (5 $\times$ 3 mL), MeOH (5 $\times$ 3 mL), and diethyl ether (5 $\times$ 3 mL), and finally dried in vacuo. A total of 0.96 g of 6-(N-Fmoc-O-trityl-homoseryl)aminoethyl-succinyl-sarcosine LCAA-CPG-500 **S3a** was obtained with estimated loading of 71.6 μ mol g⁻¹ (93% substitution).

2-(2-Fmoc-aminoethoxy)ethyl-succinyl-sarcosine LCAA-CPG-500 (S2b). A fresh batch of Fmoc-sarcosine LCAA-CPG-500 **S1** (4.74 g, 0.68 mmol, 0.144 mmol g⁻¹ amino groups) was treated with piperidine/DMF (2:8 v/v, 15 mL) for 15 min and washed with DMF (10 $\times$ 15 mL). 2-(2-Fmoc-aminoethoxy)ethyl succinate (146 mg, 0.34 mmol) and HATU (117 mg, 0.31 mmol) were dissolved in DMF (14 mL), DIEA (59 μ L, 0.34 mmol) was added, and the mixture was shaken for 5 min. More DIEA (59 μ L, 0.34 mmol) was added, and the solution was poured onto the Fmoc-deprotected support **S1**. The slurry was kept for 1 h with occasional swirling, the

solution was filtered, and the CPG beads were washed with DMF (2 $\times$ 15 mL) and acetonitrile (2 $\times$ 15 mL). The CPG was treated on the filter with equal volumes of capping solutions I and II (7.5 mL of each) for 15 min, washed successively with DMF (5 $\times$ 15 mL), MeOH (5 $\times$ 15 mL), and diethyl ether (5 $\times$ 15 mL), and finally dried in vacuo. A total of 4.71 g of 2-(2-Fmoc-aminoethoxy)-ethyl succinyl sarcosine LCAA-CPG-500 **S2b** was obtained with estimated loading of 62.2 μ mol g⁻¹ (96% substitution).

2-[2-(N-Fmoc-O-tritylhomoseryl)aminoethoxy]ethyl-succinyl-sarcosine LCAA-CPG-500 (S3b). The above support **S2b** (4.70 g, 0.30 mol) was treated with piperidine/DMF (15 mL) for 10 min and washed with DMF (10 $\times$ 15 mL). Fmoc-Hse(Trt)-OH (172 mg, 0.30 mmol) and HATU (101 mg, 0.27 mmol) were dissolved in DMF (14 mL), DIEA (51 μ L, 0.30 mmol) was added, and the mixture was shaken for 5 min. More DIEA (51 μ L, 0.30 mmol) was added, and the solution was poured onto the Fmoc-deprotected support **S2b**. The slurry was kept for 1 h with occasional swirling, the solution was filtered, and the CPG beads were washed with DMF (2 $\times$ 15 mL) and acetonitrile (2 $\times$ 15 mL). The CPG was treated on the filter with equal volumes of capping solutions I and II (7.5 mL of each) for 15 min, washed successively with DMF (5 $\times$ 15 mL), MeOH (5 $\times$ 15 mL), and diethyl ether (5 $\times$ 15 mL), and finally dried in vacuo. A total of 4.67 g of 2-[2-(N-Fmoc-O-trityl-homoseryl)-aminoethoxy]ethyl-succinyl-sarcosine LCAA-CPG-500 **S3b** was obtained with estimated loading of 47.5 μ mol g⁻¹ (85% substitution).

General Procedure for Synthesis of 3'-Modified LCAA-CPG-500 Supports S4 and S5 from S3a and S3b, Respectively, Exemplified for Preparation of 2-[2-(O,O'-Diacetylfluorescein-6-ylcarbonylhomoseryl)aminoethoxy]ethyl-succinyl-sarcosine LCAA-CPG-500 (S5a). Support **S3b** (0.46 g, 0.02 mmol) was treated with piperidine/DMF (2:8 v/v, 2 mL) for 10 min and washed with DMF (10 $\times$ 2 mL). 6-Carboxyfluorescein diacetate (100 mg, 0.23 mmol) and HATU (74 mg, 0.19 mmol) were dissolved in DMF (1.5 mL), DIEA (37 mL, 0.22 mmol) was added, and the mixture was shaken for 5 min. More DIEA (37 μ L, 0.22 mmol) was added, and the resulting brown solution was poured onto the Fmoc-deprotected support **S3b**. The slurry was kept for 1 h with occasional swirling, the solution was filtered, the CPG beads were washed with DMF (3 $\times$ 2 mL), and the washings were repeated. After 1 h, the CPG was filtered and washed with DMF (2 $\times$ 2 mL) and acetonitrile (2 $\times$ 2 mL). The CPG was treated on the filter with equal volumes of capping solutions I and II (1 mL of each) for 15 min and then washed with DMF (5 $\times$ 2 mL), MeOH (5 $\times$ 2 mL), and CH₂Cl₂ (2 $\times$ 2 mL). A solution of TFA in CH₂Cl₂ (3% v/v, 2 mL) was added, and beads were kept in contact with the acid for 1 min, washed with 3% TFA in CH₂Cl₂ (2 $\times$ 2 mL), MeOH (2 $\times$ 2 mL), CH₂Cl₂ (5 $\times$ 2 mL), and diethyl ether (3 $\times$ 2 mL), and finally dried in vacuo. A total of 0.45 g of 2-[2-(O,O'-diacetylfluorescein-6-ylcarbonylhomoseryl)aminoethoxy]ethyl-succinyl-sarcosine LCAA-CPG-500 **S5a** was obtained as colorless beads.

Other S4 Supports: 6-[N-(4-Pentynoyl)homoseryl]aminoethyl-succinyl-sarcosine LCAA-CPG-500 (S4a). Support **S3a** (0.2 g, 27.5 μ mol/g, 5.5 μ mol) was treated with piperidine/DMF (2:8 v/v, 0.5 mL) for 15 min and washed with DMF (10 $\times$ 0.5 mL). 4-Pentynoic acid (12 mg, 0.11 mmol) and HATU (38 mg, 0.10 mmol) were dissolved in DMF (0.3 mL), DIEA (19 μ L, 0.11 mmol) was added, and the mixture was shaken for 5 min. More

DIEA (19 μ L, 0.11 mmol) was added, and the yellow solution was then poured on Fmoc-deprotected support **S3a**. The CPG beads were washed, capped, treated with TFA in CH_2Cl_2 (3%), washed, and dried in vacuo to yield 0.18 g of 6-[*N*-(4-pentynoyl)homoseryl]aminoethylsuccinyl-sarcosine (**S4a**).

6-(Iodophenylacetylhomoseryl)aminoethylsuccinyl-sarcosine LCAA-CPG-500 (S4b). Support **S3a** (0.14 g, 60.8 μ mol/g, 8.7 μ mol) was treated with piperidine/DMF (2:8 v/v, 0.5 mL) for 15 min and washed with DMF (10 $\times$ 0.5 mL). 4-Iodophenylacetic acid (26 mg, 0.09 mmol) and HATU (30 mg, 0.08 mmol) were dissolved in DMF (0.3 mL), DIEA (15 μ L, 0.09 mmol) was added, and the mixture was shaken for 5 min. More DIEA (15 μ L, 0.09 mmol) was added, and the yellow solution was then poured on Fmoc-deprotected support **S3a**. The CPG beads were washed, capped, treated with TFA in CH_2Cl_2 (3%), washed, and dried in vacuo to yield 0.13 g of 6-[*N*-(4-iodophenylacetyl)homoseryl]aminoethylsuccinyl-sarcosine (**S4b**).

6-(*N*- α -Fmoc-*S*-tert-butylthiocysteinylhomoseryl)aminoethylsuccinyl-sarcosine LCAA-CPG-500 (S4c). Support **S3a** (0.5 g, 60.8 μ mol/g, 30.4 μ mol) was treated with piperidine/DMF (2:8 v/v, 2 mL) for 15 min and washed with DMF (10 $\times$ 2 mL). *N*- α -Fmoc-*S*-tert-butylcysteine pentafluorophenyl ester (179 mg, 0.3 mmol) and HOAt (41 mg, 0.3 mmol) were dissolved in DMF (1.2 mL), DIEA (52 μ L, 0.3 mmol) was added, the mixture was shaken for 5 min, and the yellow solution was then poured on Fmoc-deprotected support **S3a**. The CPG beads were washed, capped, treated with TFA in CH_2Cl_2 (3%), washed, and dried in vacuo to yield 0.48 g of 6-[*N*-(*N*- α -Fmoc-*S*-tert-butylcysteinyl)homoseryl]aminoethylsuccinyl-sarcosine LCAA-CPG-500, and the loading was estimated to be 56.6 μ mol/g (93%).

A Tripeptide S4 Support: 6-(*N*- α -Fmoc-Lys(Tfa)-Lys(Tfa)-Lys(Tfa)-homoseryl)aminoethylsuccinyl-sarcosine LCAA-CPG-500 (S4d). A batch of support **S3a** (0.25 g, 7.7 μ mol, 27.5 μ mol g⁻¹ substitution) was treated with piperidine/DMF (2:8 v/v, 1 mL) for 10 min and washed with DMF (10 $\times$ 1 mL). Fmoc-Lys(Tfa)-OH (36 mg, 0.08 mmol) and HATU (26 mg, 0.07 mmol) were dissolved in DMF (0.5 mL), DIEA (13 μ L, 0.08 mmol) was added, and the mixture was shaken for 5 min. More DIEA (13 μ L, 0.08 mmol) was added, and the solution was poured onto Fmoc-deprotected support **S3a**. The slurry was kept for 1 h with occasional swirling, the CPG beads were filtered off and washed with DMF (3 $\times$ 1 mL), and the procedure was repeated. After 1 h, the CPG beads were filtered off, washed with DMF (2 $\times$ 1 mL) and acetonitrile (2 $\times$ 1 mL), and treated on the filter with equal volumes of capping solutions I and II (0.5 mL of each) for 15 min, washed successively with DMF (5 $\times$ 1 mL), MeOH (5 $\times$ 1 mL), and diethyl ether (5 $\times$ 1 mL), and finally dried in vacuo to obtain 0.23 g of 6-(*N*- α -Fmoc-*N*- ϵ -Tfa-lysyl-*O*-tritylhomoseryl)aminoethylsuccinyl-sarcosine LCAA-CPG-500 with estimated loading 27.1 μ mol g⁻¹ (99% substitution). A portion of the above support (0.2 g, 5.4 μ mol) was treated with Fmoc-deprotected, coupled again with Fmoc-Lys(Tfa)-OH, capped, washed, and dried as before to yield 0.19 g of di-Lys support with an estimated loading 27.2 μ mol g⁻¹ (99% substitution). A third cycle of Fmoc deprotection, coupling, and capping and washing as before was followed by treatment with a solution of TFA in CH_2Cl_2 (3% v/v, 1 mL). The beads were kept in contact with acid for 1 min, washed with 3% TFA in CH_2Cl_2 (2 $\times$ 0.75 mL), MeOH (2 $\times$ 0.75 mL), CH_2Cl_2 (5 $\times$ 0.75 mL), and diethyl ether (3 $\times$ 0.75 mL), and finally dried in vacuo to give 0.18 g of 6-(*N*- α -Fmoc-Lys-

(Tfa)-Lys(Tfa)-Lys(Tfa)-homoseryl)aminoethylsuccinyl-sarcosine LCAA-CPG-500 **S4d** with estimated loading of 26.1 μ mol g⁻¹ (96% substitution).

Other S5 Supports: 2-[(2-Cholesterylloxycarbonylhomoseryl)aminoethoxy]ethylsuccinyl-sarcosine LCAA-CPG-500 (S5b). Support **S3b** (0.25 g, 0.01 mmol) Fmoc-deprotected and washed was treated with 2-cholesterylchloroformate (53 mg, 0.11 mmol) in CH_2Cl_2 (1 mL) and DIEA (40 μ L, 0.22 mmol) for 1 h, washed, capped, washed again, and dried to yield 0.24 g of 2-[(2-cholesterylloxycarbonylhomoseryl)aminoethoxy]ethylsuccinyl sarcosine LCAA-CPG-500 **S5b**.

6-(6-*N*-Fmoc-aminohexanoyl-*O*-tritylhomoseryl)aminoethylsuccinyl-sarcosine LCAA-CPG-500 (S6a). A batch of support **S3a** (0.25 g, 7.7 μ mol, 27.5 μ mol g⁻¹ substitution) was treated with piperidine/DMF (2:8 v/v, 1 mL) for 10 min and washed with DMF (10 $\times$ 1 mL). 6-Fmoc-aminohexanoic acid (27 mg, 0.08 mmol) and HATU (26 mg, 0.07 mmol) were dissolved in DMF (0.5 mL), DIEA (13 μ L, 0.08 mmol) was added, and the mixture was shaken for 5 min. More DIEA (13 μ L, 0.08 mmol) was added, and the solution was poured onto Fmoc-deprotected support **S3a**. The slurry was kept for 1 h with occasional swirling, the CPG beads were filtered off and washed with DMF (3 $\times$ 1 mL), and the procedure was repeated. After 1 h, the CPG beads were filtered off, washed with DMF (2 $\times$ 1 mL) and acetonitrile (2 $\times$ 1 mL), and treated on the filter with equal volumes of capping solutions I and II (0.5 mL of each) for 15 min, washed successively with DMF (5 $\times$ 1 mL), MeOH (5 $\times$ 1 mL), and diethyl ether (5 $\times$ 1 mL), and finally dried in vacuo. A total of 0.24 g of 6-(6-*N*-Fmoc-aminohexanoyl-*O*-tritylhomoseryl)aminoethylsuccinyl-sarcosine LCAA-CPG-500 **S6a** was obtained with estimated loading of 28.2 μ mol g⁻¹ (100% substitution).

2-[2-(6-*N*-Fmoc-aminohexanoyl-*O*-tritylhomoseryl)aminoethoxy]ethylsuccinyl-sarcosine LCAA-CPG-500 (S6b). Support **S3b** (0.50 g, 0.02 mmol) was treated analogously to the synthesis of support **S6a** to yield 0.49 g of 2-[2-(6-*N*-Fmoc-aminohexanoyl-*O*-tritylhomoseryl)aminoethoxy]ethylsuccinyl-sarcosine LCAA-CPG-500 **S6b** with estimated loading of 41.9 μ mol g⁻¹ (88% substitution).

Synthesis of 6-[*N*-(6-(Biotinylamino)hexanoyl)homoseryl]aminoethylsuccinyl-sarcosine LCAA-CPG-500 (S7a). Support **S6a** (0.1 g, 6.0 μ mol) was treated with piperidine/DMF (2:8 v/v, 0.5 mL) for 15 min and washed with DMF (10 $\times$ 0.5 mL). D-(+)-Biotin 4-nitrophenyl ester (44 mg, 0.12 mmol) and HOAt (16 mg, 0.12 mmol) were dissolved in DMF (0.25 mL), DIEA (25 μ L, 0.14 mmol) was added, and the mixture was shaken for 5 min. The yellow solution was then poured onto Fmoc-deprotected support **S6a**. The slurry was kept for 16 h with occasional swirling, and the CPG beads were filtered off, washed with DMF (2 $\times$ 0.5 mL) and acetonitrile (2 $\times$ 0.5 mL), treated on the filter with equal volumes of capping solutions I and II (0.25 mL of each) for 15 min, and washed successively with DMF (5 $\times$ 0.5 mL), MeOH (5 $\times$ 0.5 mL), and CH_2Cl_2 (2 $\times$ 0.5 mL). A solution of TFA in CH_2Cl_2 (3% v/v, 0.5 mL) was added, and the beads were kept in contact with acid for 1 min, washed with 3% TFA in CH_2Cl_2 (2 $\times$ 0.5 mL), MeOH (2 $\times$ 0.5 mL), CH_2Cl_2 (5 $\times$ 0.5 mL), and diethyl ether (3 $\times$ 0.5 mL), and finally dried in vacuo. A total of 0.1 g of 6-[*N*-(6-biotinylamino)hexanoyl]homoseryl]aminoethylsuccinyl-sarcosine LCAA-CPG-500 (**S7a**) was obtained.

2-[2-(*O*,*O'*-Diisobutyl-2,7-dichlorofluorescein-5(6)-ylcarbonylamino)hexanoylhomoseryl]aminoethoxy]ethylsuccinyl-sarcosine LCAA-CPG-500 (S8a).

Support **S6b** (0.25 g, 0.01 mmol) was Fmoc-deprotected and coupled with 2,7-dichloro-5(6)-carboxyfluorescein diacetate (50 mg, 0.1 mmol), washed, capped with a mixture of 10% v/v isobutyric anhydride solution in THF (0.5 mL) and Cap B solution (0.5 mL) for 30 min, and washed again as for the preparation of support **S5a**. A solution of TFA in CH₂Cl₂ (3% v/v, 1 mL) was added, and the beads were kept in contact with the acid for 1 min, washed with 3% TFA in CH₂Cl₂ (2 × 1 mL), MeOH (2 × 1 mL), CH₂Cl₂ (5 × 1 mL), and diethyl ether (3 × 1 mL), and finally dried in vacuo. A total of 0.23 g of 2-[2-(*O,O'*-diisobutryl-2,7-dichlorofluorescein-5(6)-ylcarbonylamino)hexanoylhomoserine]aminoethoxy]ethylsuccinyl-sarcosine LCAA-CPG-500 (**S8a**) was obtained as slightly yellow beads.

RESULTS

Preparation of Solid Supports. We wished to develop a method of functionalization of solid supports that has both flexibility and convenience. First, the chemical reactions should be readily carried out in high yield and with reagents that are either available commercially or simply prepared. Second, the method must be compatible in principle with conjugation either pre- or post-assembly of the oligonucleotide chain. Third, for pre-assembly conjugation, a wide range of conjugate molecules should be able to be introduced at a late stage in support functionalization and without need for a multistep synthesis of the conjugate reagent. Fourth, the linker should not be racemic as this will lead to formation of a mixture of conjugate diastereomers. Finally, the procedure must be flexible enough to allow a range of spacers to be included between oligonucleotide and conjugate for different applications. Such requirements are well met by peptide-type coupling reactions and use of linker molecules containing the 9-fluorenylmethoxycarbonyl group (Fmoc) protecting group that is removable under mild piperidine deprotection conditions, techniques standard in solid-phase peptide synthesis (Chan and White, 2000). Use of the Fmoc group allows for careful monitoring of the loading of the solid support at various stages of its preparation, due to the high UV absorbance of its piperidyl adduct.

In contrast to other strategies of pre-synthesis of complex linker reagents, we have applied the "submonomer" principle (Figliozzi et al., 1996), where small, economically prepared Fmoc-protected synthons are coupled sequentially by means of efficient peptide-like coupling reactions. For the crucial chain-branching agent, we chose the bifunctional amino acid L-homoserine which is chirally pure. Use of a single enantiomer of the amino acid excludes the possibility of multiple stereoisomer formation inevitable in the case of racemic linkers such as 3-amino-1,2-propanediol (Nelson et al., 1989). Further and as reported previously (Beltrán et al., 1997), the spacing of three carbon atoms between the hydroxyl and carbonyl groups in homoserine renders it incapable of β -elimination of a nucleotide substituent that is observed to occur under basic conditions for β -hydroxy amino acids such as serine or threonine. The hydroxyl group of homoserine is used as an anchoring point for growth of the oligonucleotide chain by reaction with the first nucleoside as its phosphoramidite derivative, whereas the α -amino group can be utilized as the site of attachment of the conjugate or other reporter group. Conjugates can be coupled directly to this amino group or after elongation with a spacer, such as 6-aminohexanoic acid.

Homoserine was suggested recently as a branching agent for the stepwise synthesis of 3'-peptide conjugates

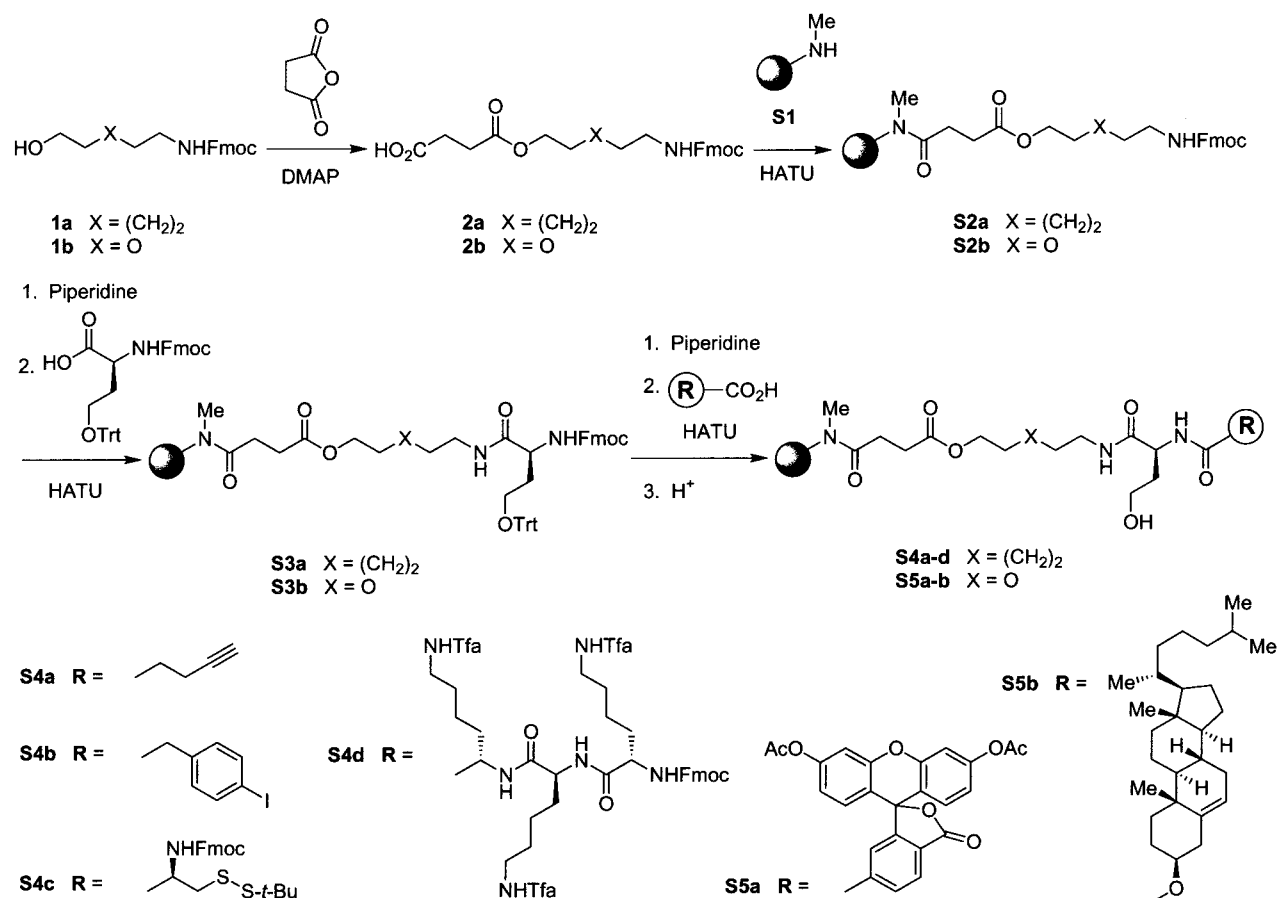
of oligonucleotides but in this case required the four-step pre-synthesis of a glycyl dipeptide to avoid racemization expected to occur upon homoserine esterification of a hydroxybutyrate-substituted solid support (Antopolsky and Azharyev, 1999). By contrast in our route, we have used directly the commercially available, protected homoserine derivative, Fmoc-Hse(Trt)-OH, without further modification (Scheme 1).

Thus, a solid support such as LCAA-CPG is first functionalized to provide both spacing and suitable ammonia lability required for deprotection and cleavage of oligonucleotides from the support. This is carried out by coupling of amino-functionalized LCAA-CPG with a long-chain amino alcohol, such as 6-aminohexanol or 2-(2-aminoethoxy)ethanol esterified to the support via an ammonia-labile succinate linker. Whereas a 6-(4-methoxytrityl)aminohexyl succinate linker has been used previously to attach peptide nucleic acids to a solid support (Will et al., 1995), we decided to use stable, crystalline and easily synthesized compounds 6-Fmoc-aminohexyl and 2-(2-Fmoc-aminoethoxy)ethyl succinates (**2a** and **2b**) as building blocks in linker construction (Scheme 1). We found that amino alcohols containing less than four methylene units gave poor loadings when coupled to the solid support as succinate hemiesters, presumably due to intramolecular O–N migration (D. A. Stetsenko and M. J. Gait, unpublished results). However, both long-chain succinic acid hemiesters **2a** and **2b** were efficiently coupled to the LCAA-CPG-500 using the HATU/DIEA in situ activation protocol commonly used in solid-phase peptide synthesis (Chan and White, 2000) to give the solid supports **S2a** and **S2b**.

Subsequently, Fmoc-Hse(Trt)-OH was attached to the Fmoc-deprotected aminoester support by use of the same coupling method to give the homoserine supports **S3a** and **S3b**. Low reagent consumption and high loading efficiencies (routinely 40–70 $\mu\text{mol g}^{-1}$ based on incorporation of Fmoc) are worth noting in both cases. Since we found that the succinate linkage attached through a primary amino group on the LCAA-CPG support is progressively degraded by repetitive piperidine treatment, we introduced instead a sarcosine (*N*-methylglycine) unit between the succinyl group and solid support (**S1**), with consistently better results. Once again, this is introduced by the same peptide coupling reaction. The *O*-trityl protecting group on homoserine serves the dual purpose of preventing the acylation of the side-chain hydroxyl group during the coupling of the homoserine unit to the solid-phase and in the following capping reactions, as well as excluding the possibility of homoserine lactone formation during carboxyl group activation. The trityl group can be cleaved off prior to oligonucleotide synthesis by dilute trifluoroacetic acid treatment.

The Fmoc-homoserine-derivatized aminoalkyl succinate CPG supports **S3a** and **S3b** are now ready for conjugate synthesis by either pre- or post-assembly of the oligonucleotide chain. Since most types of conjugate are stable to the conditions of oligonucleotide synthesis and deprotection, we have concentrated on more convenient pre-assembly conjugation. Thus, we used the α -amino group of the homoserine for direct attachment of a variety of reporter groups (Scheme 1), once again via a peptide-type coupling reaction with the corresponding carboxylic acid of the reporter group. As examples, we have prepared a number of functionalized supports **S4** starting from support **S3a** that carries the aminohexyl spacer: the small organic groups 4-pentynoyl (**S4a**) or 4-iodophenylacetyl (**S4b**) or the amino acid derivative *N*- α -Fmoc-*S*-tert-butylthio-cysteine (**S4c**). Some other functionalized

Scheme 1



supports **S5** were prepared starting from the 2-aminoethoxyethyl-spaced support **S3b**: the fluorescent group 6-carboxyfluorescein (**S5a**) or the steroid cholesterol (**S5b**). In each case, the conjugation was carried out by piperidine treatment of the support to cleave off the Fmoc group, followed by reaction of the resultant primary amino group with the conjugate either as a carboxylic acid in the presence of HATU/DIEA or in the case of cholesterol, the corresponding chloroformate derivative.

As a monitor of the accessibility of the primary amino group and to demonstrate the feasibility of use of this type of support in the total synthesis of peptide-oligonucleotide conjugates, we assembled the short tripeptide Lys-Lys-Lys on the homoserine support **S4** using a *N*- ϵ -Tfa-protected Lys monomer and standard HATU/DIEA coupling conditions. The three coupling reactions proceeded in average coupling yield of 98.3% per cycle as judged by incorporation of the Fmoc group, to give support **S4d**.

Another option is to introduce a hexanoyl spacer between the homoserine and the reporter group. Thus coupling of Fmoc-amino hexanoic acid to the piperidine-treated support **3a** and **3b** afforded the extended supports **S6a** and **S6b** respectively (Scheme 2). Coupling with the appropriate reporter group or amino acid can now be carried out as before to give the supports **S7** and **S8**, respectively. Examples of these supports are those carrying biotin (**S7a**) or 2,7-dichloro-5(6)-carboxyfluorescein (**S8a**). In the case of biotin, the 4-nitrophenyl ester was used in the coupling reaction. In the case of 2,7-dichloro-5(6)-carboxyfluorescein, the *O,O'*-diacetate was found to be partially unstable under HATU/DIEA-mediated coupling conditions, leading to partial *N*-acetylation of support-bound amino groups. We therefore used iso-

butyric anhydride to cap any liberated phenolic groups on the conjugate.

It should be noted that in principle any reporter molecule that can be activated for reaction with an amino group (e.g., carboxylic, carbonic, isothiocyanate, or sulfonyl derivatives) can be coupled using the same technique. Thus, we have successfully loaded these supports with a large range of other functionalities such as the fluorescent group pyrene-1-ylacetic acid, lipophilic groups such as stearic and adamantane-1-acetic acids, ($\pm$)-lipoic acid, deoxycholic acid, as well as other amino acids such as His and Arg (data not shown).

Synthesis of 3'-Conjugated Oligonucleotides. To demonstrate the utility of these supports in solid-phase synthesis of 3'-conjugates of oligonucleotides we have carried out a number of oligonucleotide assemblies (Table 1). Starting from support **S4a** (4-pentynoyl), an 11-mer oligodeoxyribonucleotide was assembled by standard phosphoramidite synthesis (Dmt on). After ammonia deprotection, reversed-phase HPLC showed a large main product corresponding to the 5'-trityl-3'-pentynoyl oligonucleotide derivative (Figure 2a). MALDI-TOF mass spectrometry showed the expected mass for the desired product (Table 1). Note that the ammonia treatment of the support liberates the oligonucleotide as a 3'-phosphodiester that is linked to the homoserine moiety. The *N*- α -position of the homoserine carries the conjugate moiety (pentynoyl), and the carboxyl is linked as an amide to the detached spacer which remains as the corresponding alcohol (in this case 6-aminohexanol). Similarly, good results were obtained for synthesis of another 11-mer oligodeoxyribonucleotide starting from support **S4b** (4-iodophenylacetyl) and a 15-mer starting

Scheme 2

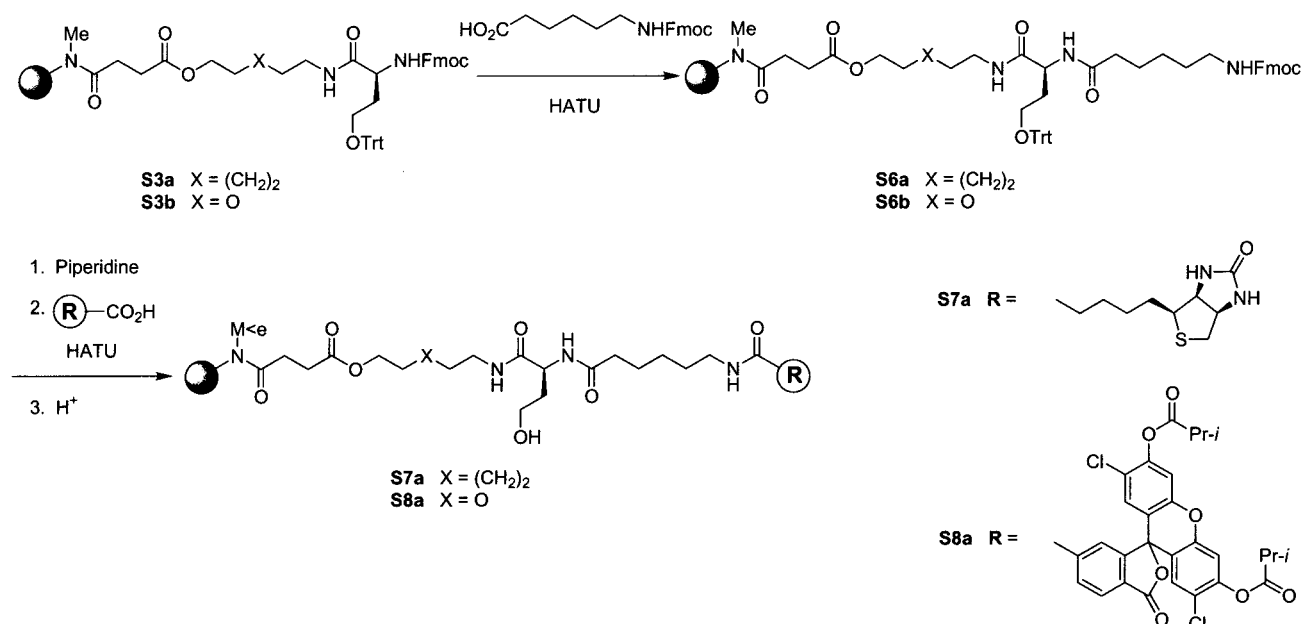


Table 1.

oligonucleotide sequence, type of solid support	3'-labeling group	molecular mass, obsd/calcd	t _R , min	yield, ^d %
5'-Dmt-d(GTTAGCCAGAG), S4a	4-pentynoyl	4049.30/4044.95	20.88 ^a	80.8
5'-Dmt-d(CCCAGGCTCAG), S4b	4-iodophenylacetyl	4137.86/4129.83	21.74 ^a	76.3
5'-Dmt-d(GTCCCAGGCTCAA), S4c	<i>s</i> -tert-butylthiocysteine	5299.99/5296.91	21.05 ^a	62.7
2'-OMe(CUCCCAGGCUCA), S5a	6-carboxyfluorescein	4528.05/4526.11	10.65 ^b	82.3
2'-OMe(CUCCCAGGCUAAA), S5b	cholesterol	5273.41/5266.92	12.59 ^b	85.1
5'- <i>S</i> -tert-butylthiocysteinyl ^c -2'-OMe (CUCCCAGGCUCA), S5a	6-carboxyfluorescein	4880.70/4880.54	11.37 ^b	75.8
2'-OMe(CUCCCAGGCU), S4d	Lys-Lys-Lys	3905.48/3901.94	10.85 ^b	92.9
2'-OMe(CsUsCsCCAGGCUCA), S5a	6-carboxyfluorescein	4567.59/4574.31	15.63–16.88 ^b	63.1
d(AGGCTCAAAT), S7a	D-(+)-biotin	3661.61/3656.75	8.93 ^b	79.9
2'-OMe(CUCCCAGGCUCA), S8a	2,7-dichloro-5(6)-carboxy-fluorescein	4714.19/4708.16	12.75 ^b	47.2

^a Reversed-phase HPLC. ^b Ion-exchange HPLC. ^c See Stetsenko and Gait, 2000. ^d Area under curve as a percentage of the total absorbance in the HPLC at the relevant wavelength.

from support **S4c** (*S*-tert-butylthiocysteinyl) (Figure 2b and Table 1).

Our recent goal has been to investigate antisense effects of the nuclease-stable and tight binding 2'-*O*-methyloligoribonucleotides against the HIV-1 TAR RNA target (Arzumanov and Gait, 1999; Mestre et al., 1999). Thus, starting from support **S5a** (6-carboxyfluorescein) that carries the 2-(2-aminoethoxy)ethyl linkage, a 12-mer anti-TAR 2'-*O*-methyloligoribonucleotide was synthesized. In this case, the trityl group was removed before ammonia deprotection and purification (Dmt off). Ion-exchange HPLC showed a major product of oligonucleotide-6-carboxyfluorescein conjugate (Figure 2c), once again with the correct mass (Table 1). Similarly, a 14-mer anti-TAR 2'-*O*-methyloligoribonucleotide was synthesized on support **S5b** (cholesterol) again with good results (Figure 2d). We also wished to prepare a 5',3'-bis-conjugate of the 2'-*O*-methyl anti-TAR 12-mer where the 3'-end was conjugated to 6-carboxyfluorescein, and the 5'-end was functionalized with a cysteine residue suitable for our recently described procedure for solution-phase conjugation to a peptide via "native ligation" (Stetsenko and Gait, 2000). Thus, starting from support **S5a**, the 12-mer 2'-*O*-methyl oligoribonucleotide was assembled and in a final coupling step the reagent *O*-4-(*N*-α-Fmoc-*S*-tert-butylsulfenyl-L-cysteinyl)piperidyl *O*-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (Stetsenko

and Gait, 2000a,b) was used. After ammonia deprotection and cleavage from the support, HPLC showed again a major 3'-carboxyfluorescein-5'-cysteinyl conjugate product (Figure 2e and Table 1).

To demonstrate the potential for sequential peptide and oligonucleotide synthesis, a 10-mer 2'-*O*-methyl oligoribonucleotide was assembled on the tri-Lys support **S4d**. After ammonia deprotection and cleavage from the support, conditions which also result in removal of *N*-ε-trifluoroacetyl protecting groups from the Lys residues, HPLC showed an excellent yield of peptide-oligonucleotide conjugate (Figure 2f and Table 1).

To show that these methods are also compatible with phosphorothioate linkages in oligonucleotides, we prepared the 12-mer anti-TAR 2'-*O*-methyl oligoribonucleotide where three phosphorothioate linkages were introduced at the 5'-end by a standard sulfurization reaction in the assembly cycle. After ammonia deprotection, ion exchange HPLC showed a series of five closely eluting product peaks (data not shown) representing partial separation of the nine phosphorothioate diastereomers. The combined product showed the correct mass and a good yield (Table 1).

Two further oligonucleotide assemblies were also carried out on the supports containing an additional amino-hexyl spacer between conjugate and the homoserine branching point. Thus, a 10-mer oligodeoxyribonucleotide

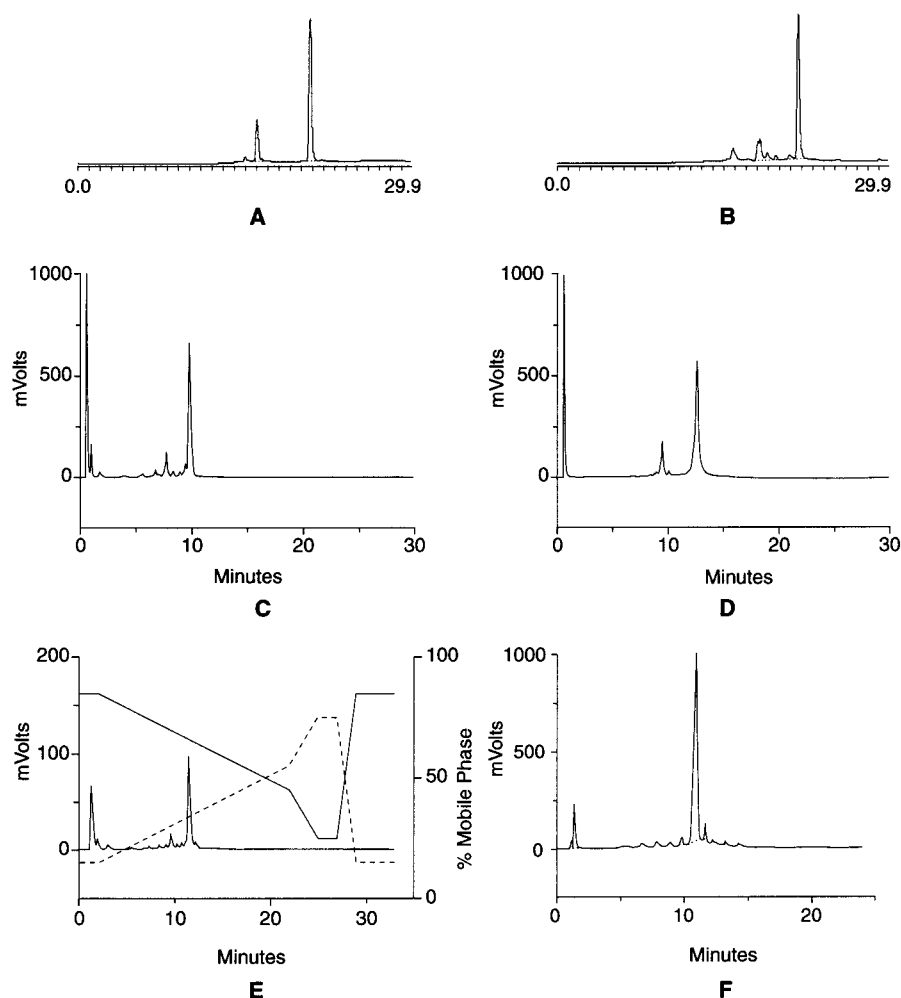


Figure 2. Analytical HPLC of crude oligonucleotide 3'-conjugates synthesized on homoserine supports **S4-S8** (A-B, reversed-phase HPLC, C-F, ion-exchange HPLC, for HPLC conditions see Materials and Methods). (A) 5'-Dmt-d(GTTAGCCAGAG), **S4a** 4-pentynoyl; (B) 5'-Dmt-d(CCCAGGCTCAG), **S4b** 4-iodophenylacetyl; (C) 2'-OMe(CUCCCAGGCUCA), **S5a** 6-carboxyfluorescein; (D) 2'-OMe(CUCCCAGGCUCAAA), **S5b** cholesteryl; (E) 2'-OMe(CUCCCAGGCUCA), 5'-*S*-*tert*-butylthiocysteinyl, **S5a** 6-carboxyfluorescein; (F) 2'-OMe(CUCCCAGGCU), **S4d** lysine tripeptide.

was synthesized on support **S7a** (biotin) and a 12-mer 2'-*O*-methyl oligoribonucleotide on support **S8a** (2,7-dichloro-5(6)-carboxyfluorescein). In the case of the biotin-functionalized 10-mer oligonucleotide synthesis, HPLC of the deprotected product showed a main product peak with high yield (Table 1). In the case of the 2,7-dichloro-5(6)-carboxyfluorescein-functionalized 12-mer oligonucleotide, the main product peak in the HPLC was the desired conjugate that was obtained in 47.2% yield (Table 1). Additionally, we isolated some *N*-acetylated amino-hexanoylhomoserine oligonucleotide as a side product resulting from the dye coupling step (28.7% yield).

DISCUSSION

Previous methods for synthesis of 3'-conjugates of oligonucleotides either require the conjugation reaction to be carried out after oligonucleotide assembly and cleavage from the support (linear linkers) or generally require the multistep synthesis of a specialized linker incorporating the conjugate. Our synthetic route allows the flexibility for both pre- and post-assembly conjugation and without the requirement for a multistep synthesis of a specialized linker reagent. Post-assembly conjugation is achievable, if desired, by standard synthesis of an oligonucleotide starting from supports **S3a** or **S3b** followed by ammonia treatment. The resultant amino group on the homoserine could then be coupled to the conjugate

molecule in solution in an analogous way to the use of C3 or C7 3'-amino-modifier CPG supports available commercially (e.g., Glen Research).

We have concentrated instead on pre-assembly conjugation, since this has been harder to achieve by a general method in the past. We have demonstrated the ready ability to make supports containing a wide range of different functionalities (fluorescent, aromatic, aliphatic, amino acid, etc.). One obvious application would be in the preparation of bis-5',3'-fluorescent oligonucleotide probes for use in FRET or Taqman-type assays as an alternative to the commonly used 3-aminobutyl-1,3-propane diol 3'-linker (Mullah and Andrus, 1997). Similarly, we have shown the synthesis of a bis-conjugated 2'-*O*-methyl 12-mer oligonucleotide containing a 3'-carboxyfluorescein and a 5'-cysteine. The cysteine residue may be used for further conjugation to a thioester peptide by the method of native ligation we recently described (Stetsenko and Gait, 2000a). Another potential application of our technology would be in the parallel preparation of a range of 3'-conjugated oligonucleotides for use in combinatorial screening in diagnostic or therapeutic applications. In addition, the route allows one to vary the length between conjugated molecule(s) and oligonucleotide by choice of appropriate spacer molecule (e.g., amino acid or aminohexanoic acid).

A special case of conjugation is the synthesis of peptides linked through the 3'-end of the oligonucleotide. There have been a number of recent papers in this area aimed at preparing 3'-peptide conjugates or 3', 5'-bis conjugates. Post-assembly conjugation at the 3'-end has been most popular (Kahl et al., 1998; Kahl and Greenberg, 1999; Aubert et al., 2000). However, there is now renewed interest in total solid-phase oligonucleotide-3'-peptide synthesis. For example, a linear 3'-dimethyl-hydroxypropionic acid spacer was used for on column synthesis of 5'-dipeptide, 3'-amino acid conjugates of oligonucleotides (Schwope et al., 1999). A more general route of 3'-peptide conjugate synthesis involves attachment of the oligonucleotide to the support via the exocyclic amino group of a cytidine residue, synthesis of an oligonucleotide toward the 5'-direction and then synthesis of a short peptide (Gly-Gly-His shown) toward the 3'-direction after attachment of a 3'-aminolinker as a phosphoramidite reagent (De Napoli et al., 1999). A less cumbersome method involves attachment of the oligonucleotide to a peptide through a serine residue, but this route requires special allyl-protected nucleotide derivatives to avoid the use of basic conditions during oligonucleotide assembly and deprotection (Sakakura and Hayakawa, 2000). The most encouraging total synthesis route utilizes a 3-amino-1,2-propane diol linker attached to the support via one hydroxyl group and a succinate spacer. Sequential peptide and oligonucleotide assemblies are carried out on the two arms of the linker in turn (Antopolsky and Azhayev, 1999). Our new supports can be used in principle in such a total synthetic route, as demonstrated by our efficient assembly of a tripeptide conjugate Lys-Lys-Lys of an oligonucleotide. In addition to chiral purity and ease of preparation, another advantage of the use of our linker strategy is the ability to vary the length between oligonucleotide and peptide by use of aminohexanoic or other spacer.

In summary, we have presented a convenient and flexible method for the preparation of 3'-conjugates of oligonucleotides that should have wide synthetic, diagnostic, and therapeutic applications.

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Avidin-dendrimer-(1B4M-Gd)₂₅₄: A Tumor-Targeting Therapeutic Agent for Gadolinium Neutron Capture Therapy of Intraperitoneal Disseminated Tumor Which Can Be Monitored by MRI

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Peritoneal carcinomatosis is a late stage in cancer progress, for which no effective therapeutic modality exists. Targeting therapeutic agents to disseminated lesions may be a promising modality for treating peritoneal carcinomatosis. Gadolinium (^{157,155}Gd) is known to generate Auger and internal conversion electrons efficiently by irradiation with a neutron beam. Auger electrons from neutron-activated Gd(III) are strongly cytotoxic, but only when Gd(III) atoms have been internalized into the cells. In the present investigation, we have developed a quickly internalizing tumor-targeting system to deliver large quantities of Gd(III) atoms into tumor cells to generate the Auger emission with an external neutron beam. Simultaneously, one would be able to image its biodistribution by MRI with a shortened T1 relaxation time. Avidin-G6-(1B4M-Gd)₂₅₄ (Av-G6Gd) was synthesized from generation-6 polyamidoamine dendrimer, biotin, avidin, and 2-(*p*-isothiocyanatobenzyl)-6-methyl-diethylenetriaminepentaacetic acid (1B4M). The Av-G6Gd was radiolabeled with Gd(III) doped with ¹⁵³Gd. All of the 1B4M's on the conjugate were fully saturated with Gd(III) atoms. An *in vitro* internalization study showed that Av-G6Gd accumulated and was internalized into SHIN3 cells (a human ovarian cancer) 50- and 3.5-fold greater than Gd-DTPA (Magnevist) and G6-(1B4M-Gd)₂₅₆ (G6Gd). In addition, accumulation of Gd(III) in the cells was detected by the increased signal on T1-weighted MRI. A biodistribution study was performed in nude mice bearing intraperitoneally disseminated SHIN3 tumors. Av-G6Gd showed specific accumulation in the SHIN3 tumor (103% ID/g) 366- and 3.4-fold greater than Gd-DTPA (0.28% ID/g, *p* < 0.001) and G6Gd (30% ID/g, *p* < 0.001) 1 day after *i.p.* injection. Seventy-eight percent of the tumor-related radioactivity of Av-G6Gd in the SHIN3 tumor was located inside the cells. The SHIN3 tumor-to-normal tissue ratio was greater than 17:1 in all organs and increased up to 638:1 at 1 day after *i.p.* injection. In conclusion, a sufficient amount (162 ppm) of Av-G6Gd was accumulated and internalized into the SHIN3 cells both *in vitro* and *in vivo* to kill the cell using ^{157/155}Gd with external irradiation with an appropriate neutron beam while monitoring with MRI. Thus, Av-G6Gd may be a promising agent for Gd neutron capture therapy of peritoneal carcinomatosis. This reagent also has the potential to permit monitoring of its pharmacokinetic progress with MRI.

INTRODUCTION

Neutron capture therapy (NCT) is a biomedical therapy consisting of two steps: (1) uptake/accumulation of a neutron-absorbing and principally nontoxic substance preferentially in the tumor; followed by (2) conversion/activation of the nontoxic agent into a radiotoxic sub-

stance after the neutron capture reaction by selectively irradiating the tumor with a neutron beam (1–3). Two isotopes, ¹⁰B and ¹⁵⁷Gd, are of major interest because of their significant large capture cross sections (CCS). Although the vast majority of NCT research has involved various aspects of boron chemistry for BNCT, ¹⁵⁷Gd has also been suggested from theoretical and experimental studies with limited success (4–9). The ¹⁵⁷Gd nuclide has the following possible advantages over ¹⁰B. First, ¹⁵⁷Gd has a significantly large neutron CCS of 255 000 barns, compared with 3840 barns for ¹⁰B (9). Second, Gd(III) complexes have been employed clinically as MRI contrast agents. The potential to trace the *in vivo* pharmacokinetics of a Gd-NCT agent directly by MRI (10, 11) would be useful for planning the neutron irradiation and to predict the therapeutic effect.

Peritoneal carcinomatosis is a late manifestation of cancer progress. Therapeutic options for treatment of the widely spread tumors in the peritoneal cavity are limited to intravenous or *i.p.* chemotherapy, usually with unsat-

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isfactory results. To treat peritoneal carcinomatosis effectively, targeted delivery of therapeutic agents to disseminated lesions may be a promising tactic. The Auger electrons from Gd-NCT are strongly cytotoxic, but only when the Gd(III) atoms have been internalized into the cells (12). Therefore, targeted delivery with effective internalization of the Gd(III)-containing agent into the cancer cells of disseminated tumors in the peritoneal cavity followed by neutron beam irradiation is a potential schema for therapy of peritoneal carcinomatosis (13).

Avidin is a quickly internalizing molecule into either normal hepatocytes or cancer cells, especially ovarian and colorectal adenocarcinoma cells, which expressed β -D-galactose receptors (14–17). Polyamidoamine dendrimers are a class of highly branched spherical polymers that are highly soluble in aqueous solution and possess a unique surface of primary amino groups (18, 19). The defined structure and large number of available surface terminal primary amino groups of polyamidoamine dendrimers have led to their use as substrates for the attachment of multiple chelating agents to a single antibody molecule (19–22), and for the preparation of novel MRI contrast agents (11, 23, 24). A combination of these components, therefore, appeared possible to provide a reagent suitable for Gd-NCT.

Herein, we report the preliminary results of our ongoing investigation to provide a quickly internalizing tumor-targeting reagent into cancer cells that can be monitored by MRI to deliver large quantities of Gd(III) atoms for Gd-NCT. We describe the preparation and characterization of a macromolecular construct comprised of avidin, polyamidoamine dendrimer, and a bifunctional DTPA derivative for complexing Gd(III), and its in vitro and in vivo targeting and internalization properties.

EXPERIMENTAL PROCEDURES

PAMAM Dendrimers. The generation-6 PAMAM dendrimer (G6) (Aldrich Chemical Co., Milwaukee, WI) with an ethylenediamine core, 256 primary terminal amino groups, and a molecular mass of 57 991 Da was used.

Biotinylation and Conjugation of Chelates to G6 Dendrimers. A 10 mg/mL aliquot of G6 (170 nmol) was dissolved in PBS, pH 7.4, and mixed with 510 nmol of sulfosuccinimidyl-6-(biotinamide) hexanoate (Bt) (NHS-LC-biotin, MW = 340; Pierce Chemical Co., Rockford, IL) at a molar ratio of 1:3, at room temperature for 30 min. The mixture was applied to a diafiltration membrane (Centricon 30, Amicon, Inc., Beverly, MA) to remove unbound Bt as well as to change buffer to 0.1 M phosphate buffer, pH 9. After purification, a small aliquot of biotinylated G6 (Bt-G6) was examined with a modified HABA assay, as described previously in detail (25, 26). In brief, the 2-(4'-hydroxyazobenzene)benzoic acid (HABA; Pierce Chemical Co.) reagent was prepared according to the manufacturer's instructions by adding 10 mg of avidin and 600 μ L of 10 mM HABA to 19.4 mL of PBS. One hundred microliters of serially diluted Bt-G6 solutions was added to 900 μ L of the avidin-HABA solution. Then, the absorbance was measured at 500 nm. Approximately 0.88 biotin was conjugated to each G6 molecule.

The biotinylated G6 was concentrated to $\sim$ 5 mg/mL and reacted with a 256-fold molar excess of 2-(*p*-isothiocyanatobenzyl)-6-methyl-diethylenetriaminepentaacetic acid (1B4M, MW = 555) (44 μ mol) at 40 $^{\circ}$ C. The reaction was maintained at pH 9 with 1 M NaOH for 24 h. Another additional equal amount of 1B4M was added

to the mixture after 24 h as a solid, and the pH was maintained as before for another 24 h. The resulting preparation was purified by diafiltration using a Centricon 30 (Amicon Co.). Approximately $98.5 \pm 1.8\%$ of the amine groups on the surface of the G6 dendrimer reacted with the 1B4M, as determined by ^{153}Gd (NEN DuPont, Boston, MA) labeling of the reacted samples (27). In brief, $\sim$ 500 000 cpm ($\sim$ 0.4 pmol) of ^{153}Gd citrate was added to $\sim$ 40 μ g/10 μ L ($\sim$ 160 pmol) of the reaction mixture before diafiltration and incubated in 0.2 M acetate buffer for 15 min at room temperature. Then, the bound fractions to the conjugates and free 1B4M were separated using a PD-10 column (Pharmacia) and counted, after which the numbers of 1B4M chelates bound to the conjugate were calculated from the count of the radioactivity in the bound fraction to the conjugates. The binding ability to the avidin–Sephacrose gel (Pierce Chemical Co.) of ^{153}Gd -labeled Bt-G6-(1B4M-Gd) $_{254}$ (MW = 240 000) was examined (17). In brief, 3 ng/0.1 μ Ci of Bt-G6-(1B4M-Gd) $_{254}$ was incubated with 0.5 mL of avidin–Sephacrose gel for 15 min at room temperature. Then the gel fraction was washed and separated from the supernatant using a paper filter. The radioactivity of both fractions was counted using a γ -counter (Aloka, Tokyo, Japan). Then 82% of the radioactivity of the ^{153}Gd -labeled Bt-G6-(1B4M-Gd) $_{254}$ sample was bound to the avidin–Sephacrose gel.

For a control drug, G6-(1B4M-Gd) $_{256}$ (G6Gd; MW = 240 000) was prepared without conjugation with biotin as described previously (28).

Preparation of Avidin-G6-(1B4M-Gd) $_{254}$ Agent. Approximately 37 mg (0.16 μ mol) of biotinylated G6-1B4M conjugate (containing 40 μ mol of 1B4M) was mixed with 40 μ mol of nonradioactive Gd(III) citrate in 0.3 M citrate buffer for 2 h at 40 $^{\circ}$ C. The unbound Gd(III) atoms in each preparation were removed by diafiltration using the Centricon 30 (Amicon Co.). The purified samples were mixed with a 2-fold molar excess of avidin (19 mg/0.31 μ mol) (Pierce Chemical Co.) and incubated for 15 min at room temperature.

Radiolabeling of Avidin-G6-(1B4M-Gd) $_{254}$. Carrier-free $^{153}\text{GdCl}_3$ was purchased from NEN DuPont (Boston), and 1 mg (containing 1.3 μ mol of 1B4M) of avidin-G6-(1B4M-Gd) $_{254}$ (Av-G6Gd) (3 nmol; MW = 300 000) conjugate was reacted with 40 μ Ci of ^{153}Gd citrate in 0.3 M citrate buffer at pH 5 for 30 min at room temperature. To remove any nonincorporated free radiometal, 10 μ L of 0.5 M EDTA (Nakarai, Tokyo, Japan) was added, and the product was purified by passage through a PD-10 column (Pharmacia, Uppsala, Sweden) eluted with PBS (pH 7.4).

The radiolabeling yields ranged from 67 to 79%. Eighty-two percent of the conjugated sample bound to biotin–Sephacrose gel (Pierce Chemical Co.).

DTPA (Nakarai, Tokyo, Japan) was also labeled with ^{153}Gd in 0.3 M citrate buffer for 15 min at room temperature.

Cell Lines and Animal Models. The SHIN3 cell line, obtained originally from a human ovarian cancer, was used for performing an in vitro internalization assay and also for generating in vivo intraperitoneal disseminating tumors in this study. The cell lines were grown in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) containing 10% fetal bovine serum (FBS) (GIBCO Laboratories) and 0.03% L-glutamine at 37 $^{\circ}$ C in 5% CO_2 with 1 day doubling time.

The intraperitoneal tumor xenografts were established by i.p. injection of 4×10^6 cells suspended in 350 μ L of PBS into 7-week-old female BALB/c nude mice, which

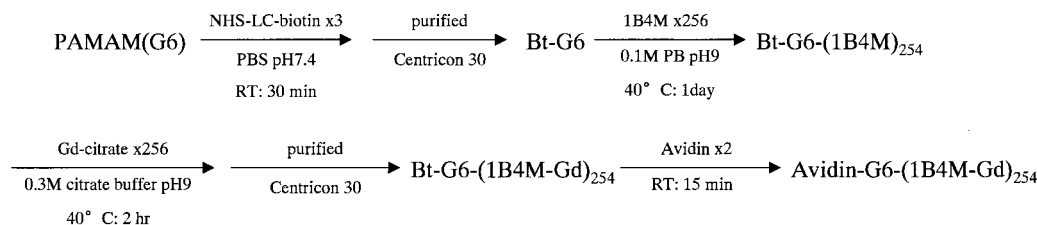


Figure 1. Flowchart of the protocol for preparing Avidin-G6-(1B4M-Gd)₂₅₄.

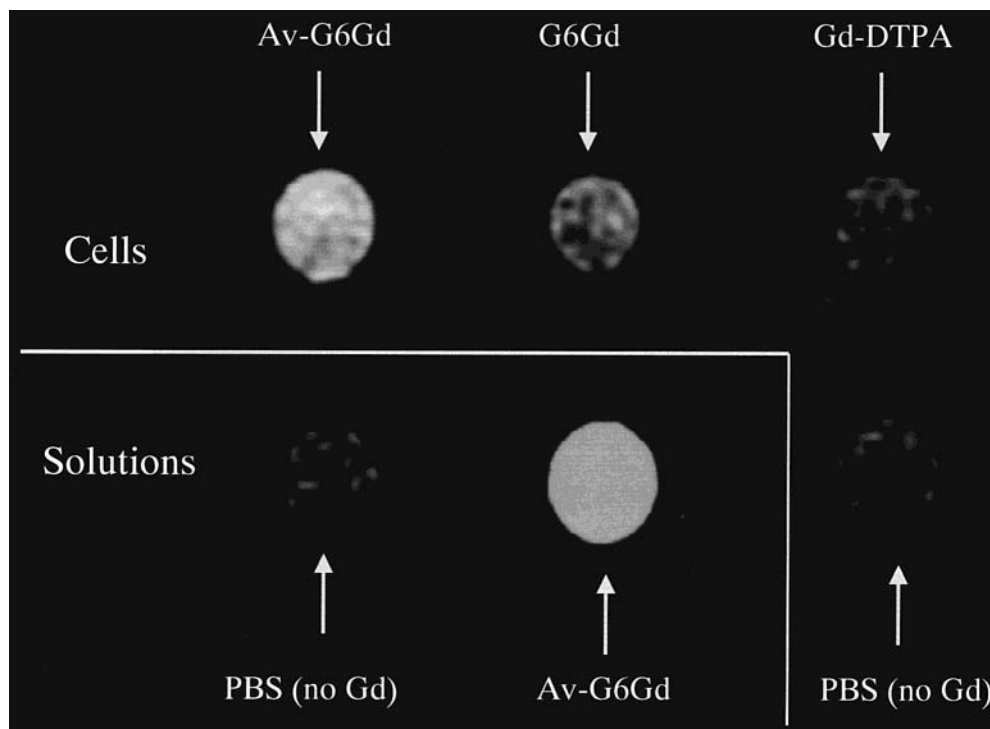


Figure 2. Micro-MR image of SHIN3 cell pellets incubated with 4 μmol of Av-G6Gd, G6Gd, Gd-DTPA, or PBS [no Gd(III)] (upper row), and a solution (lower row) of Av-G6Gd containing the same concentration of Gd(III) as the cell pellet incubated with Av-G6Gd based on radioactivity, and a PBS solution was obtained using the 3D-SPGR method.

were feeding in the clean animal facility. Experiments with tumor-bearing mice were performed at 3–4 weeks after injection when the large numbers of tumors of ~ 2 mm diameter had grown in the peritoneal cavity, especially at the hilum of the spleen, the root of the mesenterium, and around the stomach. All the mice will be died with malignant ascites within 2 months after inoculation of SHIN3 cells.

Internalization Assay and Signal Changes on MRI. SHIN3 cells (5×10^5) were preseeded into 150 cm^2 dishes and cultured overnight in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FBS (GIBCO) and 0.03% L-glutamine, in a 5% CO_2 environment. Subconfluent cells were incubated with 300 000 cpm/4 μmol of Gd of either ^{153}Gd -labeled Av-G6Gd, ^{153}Gd -labeled G6Gd, ^{153}Gd -DTPA, or PBS in 25 mL of RPMI 1640 medium with 10% FBS for 24 h at 37 $^\circ\text{C}$. The medium was removed, and cells were washed with 0.5 M Na_2HPO_4 /0.5 M NaH_2PO_4 , 0.1 M NaCl, pH 7.4, containing 0.53 mM EDTA and 0.05% trypsin (GIBCO). The membrane-bound radioactivity was removed by washing the cells with acidic solution (0.05 M glycine hydrochloride/0.1 M NaCl, pH 2.8). Radioactivity in the acid-resistant (internalized) and acid-sensitive (membrane-bound) fractions was counted using a γ -counter (Aloka, Tokyo, Japan). The percentages of the membrane-bound fraction and internalized fraction of the total added radioactivity were calculated.

Then, MR images of the cell pellets of each preparation incubated with Av-G6Gd, G6Gd, Gd-DTPA, PBS, or an Av-G6Gd solution containing the same concentration of Gd(III) as the cell pellet incubated with Av-G6Gd were obtained using the 3D-fast spoiled gradient echo technique (fSPGR) method. In brief, the fSPGR (TR/TE 150/4.2; flip angle 60 $^\circ$; 4 numbers of excitation) and 3D data acquisitions were used. The coronal images were reconstructed with 0.7-mm section thickness without gaps. The field of view was 8 $\times$ 6 cm, and the size of the matrix was 256 $\times$ 192. The signal intensities of cells in each preparation (quadricate) were measured. The regions of interest were set on each cell pellet, and their average intensities were analyzed (Advantage Windows, General Electric Medical System).

Furthermore, cells were added with the same volume of 20% SDS solution and solubilized at room temperature for 1 week. At that time, MR images of the cell solutions of each preparation were obtained using the same method described above.

Biodistribution and Internalization Study of Gadolinium into Tumor Cells. SHIN3 cancer cells [$(3-4) \times 10^6$] were injected i.p. into 7-week-old female BALB/c athymic mice. Groups of mice bearing i.p. tumors received an i.p. injection of 37 kBq (1 μCi) of ^{153}Gd -labeled Av-G6Gd, ^{153}Gd -labeled G6Gd, or ^{153}Gd -DTPA. The injected samples were added to nonradioactive Av-G6Gd or G6Gd (3.9 nmol), and the total gadolinium dose was

adjusted to approximately 1 $\mu\text{mol}/\text{mouse}$ (0.05 $\mu\text{mol}/\text{g}$). The mice were sacrificed at 4 or 24 h after the injection of radioactivity by ether inhalation ($n = 4-6$). Blood, i.p. disseminated tumors, and organs were removed and weighed, and their radioactivities were determined using a γ -counter. The percentages of injected dose per gram of tissue (% ID/g) were determined, and the results were normalized to a 20-g mouse. The tumor-to-normal tissue uptake ratios of the radioactivity were also calculated. To evaluate the internalized fraction of Gd(III) atoms in the SHIN3 cells *in vivo*, the removed tumors were incubated in 0.5 M $\text{Na}_2\text{HPO}_4/0.5$ M NaH_2PO_4 , 0.1 M NaCl, pH 7.4, containing 0.53 mM EDTA and 0.05% trypsin (GIBCO) for 15 min at 37 $^\circ\text{C}$ immediately after being weighed and γ -counted to obtain the single cell suspension. The membrane-bound radioactivity was removed by washing the cells with an acidic solution (0.05 M glycine hydrochloride/0.1 M NaCl, pH 2.8), after which the tumor cells were completely suspended in the solution. Radioactivity in the acid-resistant (internalized) and acid-sensitive (membrane-bound) fractions was counted using a γ -counter (Aloka). The percentages of the membrane-bound fraction and internalized fraction in the added radioactivity were calculated.

All animal experiments were carried out in accordance with the regulations of Kyoto University animal facility regarding animal care and handling.

Statistical Analysis. Statistical analysis was performed using the Student's *t*-test (Sigmatat, Jandel Scientific, San Rafael, CA).

RESULTS

Internalization Assay and Signal Changes on MRI. The *in vitro* internalization study showed that ^{153}Gd -labeled Av-G6Gd accumulated in the SHIN3 cells (3.2%) 50- and 3.5-fold greater than ^{153}Gd -DTPA (0.064%) and ^{153}Gd -labeled G6Gd (0.91%) 1 day after incubation, respectively. By T1-weighted MRI (Figure 1), the increase in the T1 signal of SHIN3 cells incubated with Av-G6Gd (545 ± 58) was significantly greater than that with Gd-DTPA (120 ± 42 ; $p < 0.01$) and G6Gd (276 ± 75 ; $p < 0.01$), but smaller than that of a stock solution of the Av-G6Gd containing the same concentration of Gd atoms (967 ± 104 ; $p < 0.01$) (Figure 2a). After solubilizing the SHIN3 cells with SDS, the signal intensities of the cell solution containing Av-G6Gd (560 ± 41) were not significantly different from that of the Av-G6Gd solution containing the same amount of Gd atoms (630 ± 107 ; $p = 0.27$) (Figure 2b).

Biodistribution Study. ^{153}Gd -labeled Av-G6Gd showed specific accumulation in the i.p. disseminated SHIN3 tumors (103% ID/g) 366- and 3.4-fold greater than Gd-DTPA (0.28% ID/g, $p < 0.001$) and G6Gd (30% ID/g, $p < 0.001$) 1 day after i.p. injection (Figure 3). The SHIN3 tumor-to-normal tissue ratio was greater than 17 for all organs. The SHIN3 tumor-to-blood ratio increased up to 638 at 1 day after i.p. injection (Figure 4). In addition, 78 and 93% of the tumor-related radioactivity of ^{153}Gd -labeled Av-G6Gd in SHIN3 tumors was located inside the cells at 6 h and 1 day postinjection, respectively (Figure 5). In contrast, 35 and 78% of the tumor-related radioactivity of ^{153}Gd -DTPA in SHIN3 tumors was located inside the cells at 6 h and 1 day postinjection, respectively ($p < 0.01$) (Figure 6).

DISCUSSION

In previous studies, gadolinium atoms chelated with DTPA or macrocyclic compounds were used for Gd-NCT

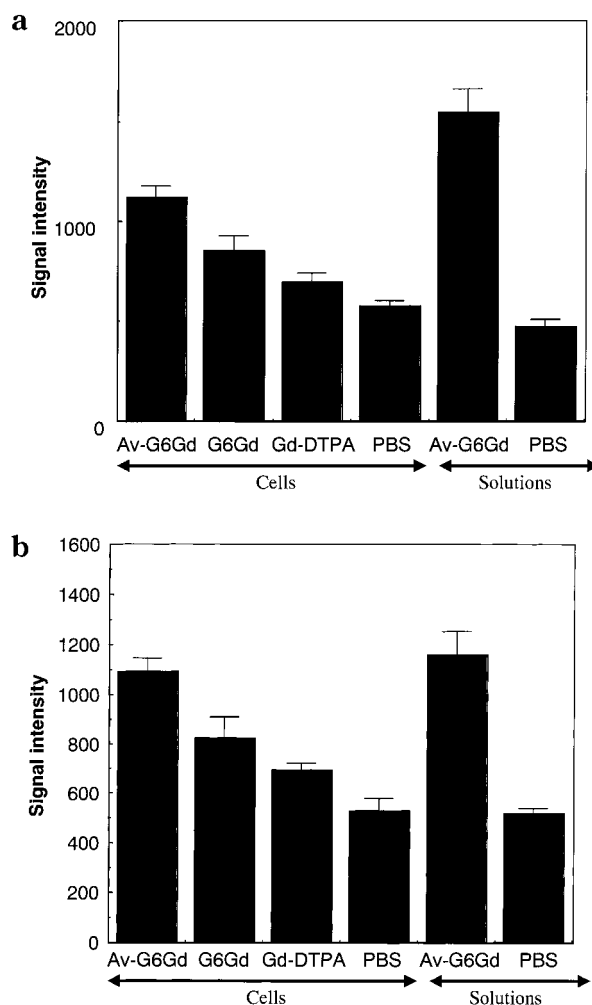


Figure 3. Plots of the signal intensity of cell pellets incubated with 4 μmol of Av-G6Gd, G6Gd, Gd-DTPA, or PBS [no Gd(III)], and a solution of Av-G6Gd containing the same concentration of Gd(III) as the cell pellet incubated with Av-G6Gd based on radioactivity, and a PBS solution was obtained using the 3D-SPGR method shown in Figure 1. The data are expressed as the mean signal intensity of four different sets of samples and standard deviation before (a) and after (b) solubilizing cells by SDS solution.

(5, 6, 8). When those compounds were injected either intravenously or intraperitoneally, they were located in extracellular fluid and rapidly cleared from the body. However, limited but significant therapeutic effects on tumor cells by irradiation with a neutron beam were reported (5, 6, 8, 29). In the present study, differences in pharmacokinetics between Gd-DTPA and Av-G6Gd were clearly demonstrated. Av-G6Gd specifically accumulated in the tumor cells and was internalized at 436-fold greater amounts than Gd-DTPA 1 day after injection. To the best of our knowledge, no successful internalization of massive numbers of Gd(III) atoms into cancer cells for targeted imaging or therapy has been previously reported. The internalized Gd(III) is theoretically more effective than extracellular Gd(III) atoms since the path length of the emitted Auger electrons, a major product of the neutron capture reaction, is so short.

In this study, Gd(III) accumulated at 162 ppm intracellularly, calculated on the basis of the ^{153}Gd radioactivity, in SHIN3 tumors *in vivo* at 1 day after injection, and 93% of Gd(III) was located in the SHIN3 cells. In a recent study, 400–800 ppm of extracellular Gd(III)-DTPA as well as 30–51 ppm of ^{10}B was reported as effective to kill cancer cells with neutron beam irradiation (30).

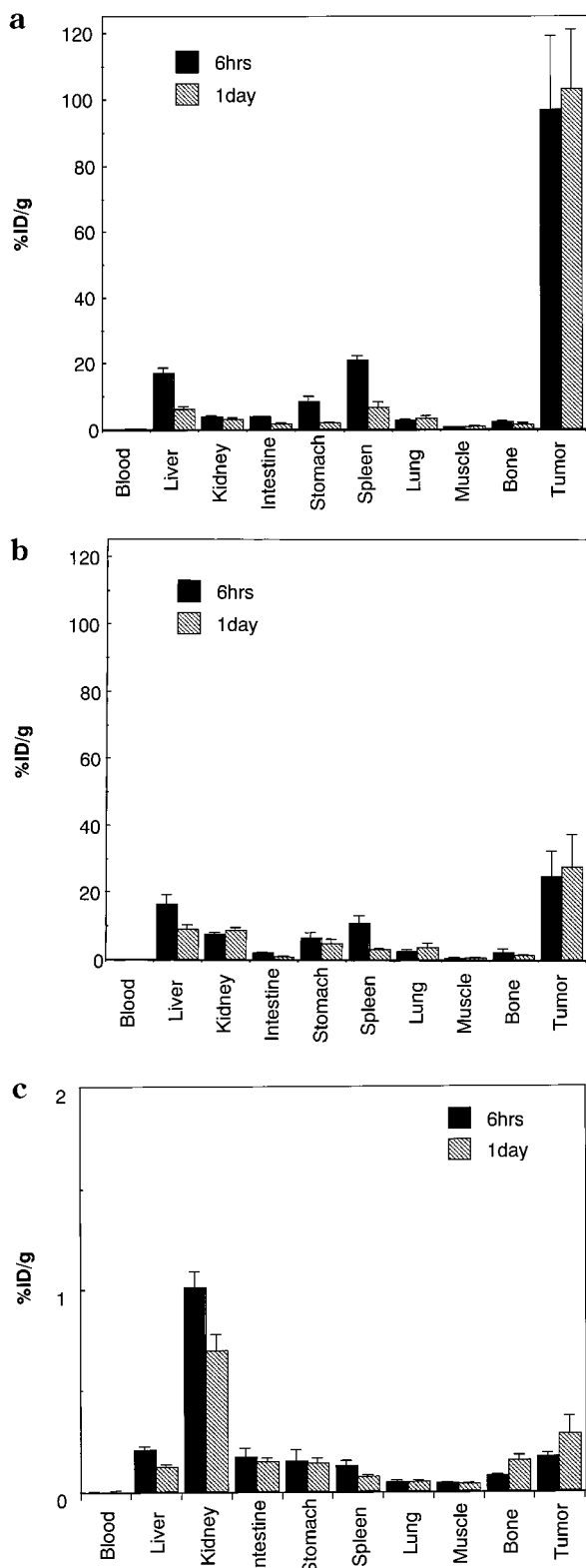


Figure 4. Biodistribution of ^{153}Gd -labeled Av-G6Gd (a), G6Gd (b), and Gd-DTPA (c) at 6 h (black bars) and 1 day (hatched bars) after i.p. injection in nude mice bearing SHIN3 intra-peritoneal disseminated tumors. The data are expressed as the mean percentages of injected dose per gram of normal tissues and standard deviation ($n = 4-6$). All data show significant differences ($p < 0.01$) compared with the appropriate group of data with Gd-DTPA.

Intracellular Gd(III) should be more cytotoxic than the extracellular Gd(III) because of the limited range of the high linear energy transfer Auger electron emission.

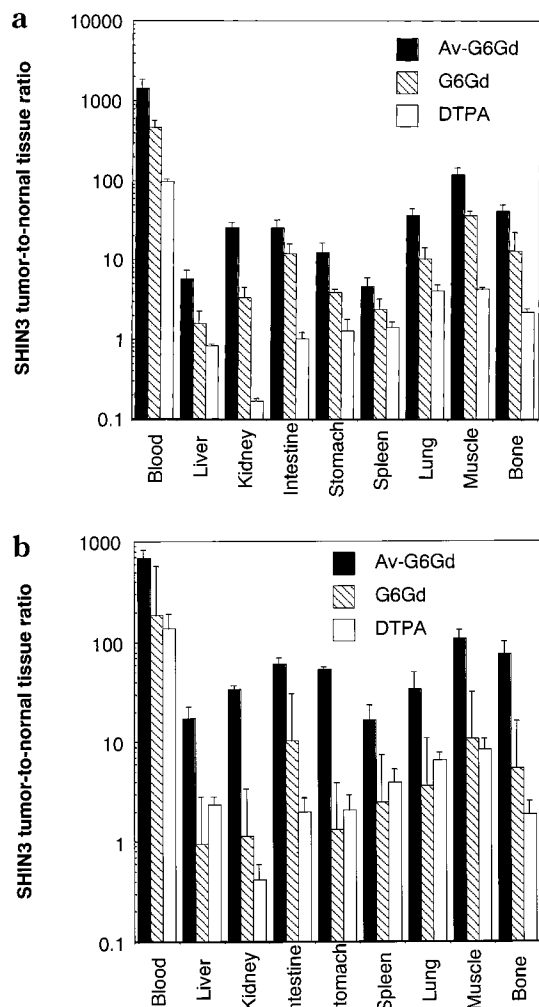


Figure 5. Plots of the serial SHIN3 tumor-to-normal tissue ratio of ^{153}Gd -labeled Av-G6Gd (black bars), G6Gd (hatched bars), and ^{153}Gd -labeled Gd-DTPA (white bars) 6 h (a) and 1 day (b) after i.p. injection. The data are expressed as the mean tumor-to-normal tissue ratio and standard deviation ($n = 4-6$). All data show significant differences between Av-G6Gd and G6Gd or Gd-DTPA ($p < 0.01$).

Therefore, the concentration of Gd(III) with Av-G6Gd in SHIN3 cells should be adequate for Gd-NCT. The use of $^{157/155}\text{Gd}$ enrichment would optimize this potential.

Av-G6Gd accumulation into the SHIN3 cells could also be evaluated by the signal change in the cell pellets by T1-weighted MRI. However, the T1 signal of Av-G6Gd in the SHIN3 cells was lower than that of Av-G6Gd in a PBS solution of the same Gd(III) concentration based on the ^{153}Gd radioactivity. In addition, when the Av-G6Gd-containing cells were solubilized with SDS solution, the T1 signals of the cell solution increased to be insignificantly different from the signal of the Av-G6Gd in PBS solution. Signal changes on the MRI with Gd(III) atoms are directly related to the interaction between Gd(III) atoms and free water molecules. In a recent study, the inconsistent change in T1-relaxation time/signals with Gd-DTPA in a solution containing macromolecular contents was discussed (31). Although a reported theory evaluated the changes of T1-relaxation time in the extracellular solution with macromolecules, when Av-G6Gd is internalized into cells, the Gd(III) atoms were not homogeneously located in the cell and thus did not increase the T1 signals.

Additionally, this observed discrepancy between radioactivity and the signal change on MRI may also be

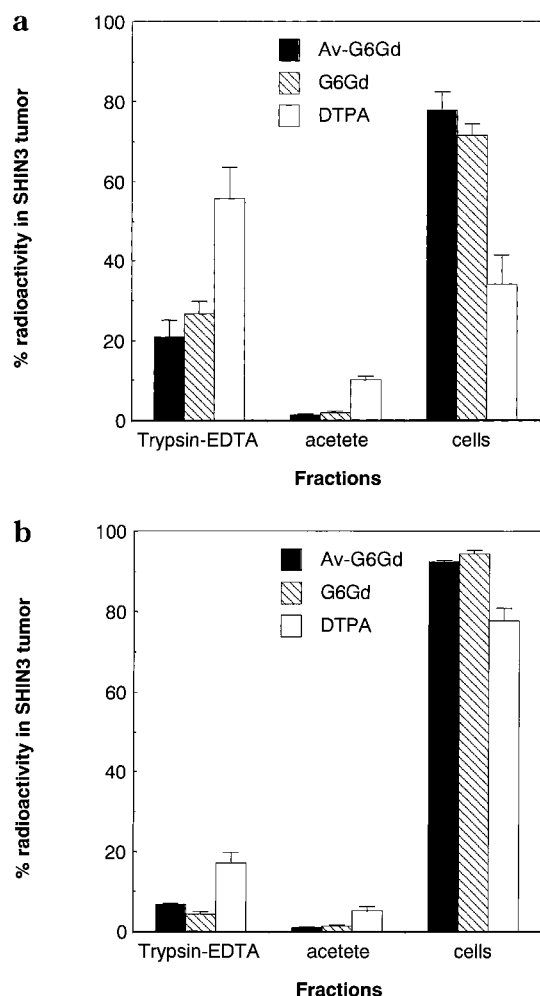


Figure 6. Data of the internalized fraction and the cell-surface fraction of ^{153}Gd -labeled Av-G6Gd (black bars), G6Gd (hatched bars), and ^{153}Gd -labeled Gd-DTPA (white bars) 6 h (a) and 1 day (b) after i.p. injection in the SHIN3 tumors is plotted. The cell-surface radioactivity was determined using a wash with 0.53 mM DTPA and 0.05% trypsin (GIBCO Laboratories) for 15 min at 37 °C followed by an acid wash with 1 mL of 0.05 M glycine hydrochloride/0.1 M NaCl, pH 2.8, at 37 °C for 5 min. Then the cell pellets were separated from the supernatant by centrifugation and counted to determine the internalized fraction. The supernatants of trypsin/EDTA washing and acid washing were separately counted. Each data point was performed in quadruplicate, and the data were calculated as the mean percentage and standard deviation recovered in the supernatant, cell surface or internalized.

evidence that the Gd(III) atoms were internalized. No conventional imaging method has been able to evaluate intra- or extracellular localization of agents containing nonradioactive metal atoms *in vivo*. Detection of the cellular localization of Gd(III) atoms contained in Av-G6Gd was also possible by the discrepancy between MRI and scintigraphy and with the use of ^{111}In as an alternative to Gd(III).

In this study, we used the 3D-SPGR sequence rather than 2D-T1 spin-echo (SE) sequence because the minimal slice thickness of 2D-T1SE was a 2-mm slice with a 0.3-mm slice gap, too thick to evaluate the T1 signal of the cell pellets of approximately 2-mm thickness. In contrast, the 3D-SPGR allows a 0.7-mm slice without gaps and choice of the slice with maximal signal intensity to evaluate the T1 signal.

In conclusion, a massive number of Gd(III) atoms up to 162 ppm were specifically delivered into the intraperitoneally disseminated SHIN3 tumor cells with the

newly synthesized Av-G6Gd molecule for Gd-NCT. In addition, this reagent can be dynamically traced by MRI to optimize Gd-NCT protocols and then to address the associated dosimetry considerations.

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Fluorescent Gramicidin Derivatives for Single-Molecule Fluorescence and Ion Channel Measurements

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Single-molecule spectroscopies in combination with single-channel patch-clamp measurements have the potential for providing new information on ion channel gating processes. Fluorescent gramicidin derivatives could provide a means for calibrating such experiments since the structure of the open channel is known and the mechanism of gating (peptide dimerization) is generally agreed. We describe here the synthesis and characterization of two pairs of gramicidin derivatives that should prove useful for such studies. They contain robust fluorophores, undergo resonance energy transfer (FRET) when they dimerize, and have single-channel properties close to those of the wild-type channel.

INTRODUCTION

Ion channels have been subjects of intensive study ever since the demonstration of their central importance for the function of nerve and muscle tissue (1, 2). The molecular events underlying channel gating (opening and closing) are of particular interest (3, 4). The difficulty of producing large quantities of these proteins and the necessity of maintaining a membrane environment for their proper function have hampered NMR-based and X-ray-based structural studies (5–7).

Single-molecule fluorescence spectroscopy, in combination with site-specific fluorescent-labeling strategies and simultaneous electrical detection of ion flux, offers the potential for obtaining structural information on the gating process under near-native conditions (8, 9). The possibility of obtaining information on structural rearrangements accompanying gating using simultaneous fluorescence and electrical measurements under multichannel conditions has already been demonstrated by Isacoff and colleagues and by Cha and Bezanilla (10–13). Single-molecule optical measurements offer the possibility of more detailed structural analyses. For instance, substates in opening transitions, that might be obscured in a multichannel analysis, could be resolved (14). The potential of combined optical and electrical detection for providing new information on the structures and dynamics of single ion channels has been realized (15), and several reports demonstrating the feasibility of such studies have appeared (16–19). In particular, single-molecule fluorescence resonance energy transfer (FRET)¹ could provide a direct means for probing structural changes underlying channel gating. Several reports of single-molecule fluorescence resonance energy transfer (FRET) have now appeared (20–24).

To fully exploit the power of single-molecule optical methods to extract structural information on complex membrane proteins, it will be useful to have well-characterized model systems with which to calibrate the measurements (16). Changes in apparent FRET ef-

iciency can arise from several sources including reversible transitions to nonemitting states ('blinking') (25) and dye dynamics (26) (e.g., cis–trans isomerizations). Failure to account for different photostates can lead to errors in the interpretation of FRET experiments (27).

The ion channel formed by the peptide gramicidin (HCO-Val-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val-Trp-D-Leu-Trp-D-Leu-Trp-D-Leu-Trp-NHCH₂CH₂OH) meets the requirements of a model system for simultaneous optical and electrical detection of ion channel gating. The structure and function of the gramicidin channel are well-established (28–30). Indeed, simultaneous fluorescence and conductance measurements with gramicidin (in a multichannel configuration) were reported 25 years ago by Veatch and Stryer (31). This classic work helped establish the dimeric model for the gramicidin channel.

Gramicidin is hydrophobic and readily incorporates into bilayers when added to the aqueous phase nearby. Movement of gramicidin monomers in the plane of the membrane is relatively fast [diffusion rates measured using fluorescence recovery after photobleaching techniques are similar to those measured for fluorescently labeled lipids (32)]. The channel is formed upon association of two gramicidin monomers, one from each leaflet of the bilayer membrane, in an N-terminal to N-terminal (head-to-head) fashion (Figure 1) (28, 29, 33). The $\beta^{6.3}$ helical structure of the peptide provides a pore ~ 28 Å long and 4 Å in diameter that traverses the membrane when a dimer forms. The pore is lined by backbone amide groups and permits the transmembrane flux of small monovalent cations at maximum rates of 10^6 – 10^7 ions

¹ Abbreviations: BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; BLM, bilayer lipid membrane; C:M:W, chloroform:methanol:water; Cy5-NHS, 1-[5-(*N*-succinimidyl-oxycarbonyl)-pent-1-yl]-2-[5-(3,3-dimethyl-1-ethyl-5-sulfonyl-indolin-2-ylidene)-1,3-pentadien-1-yl]-3,3-dimethyl-3*H*-indolium-5-sulfonate; DMF, dimethylformamide; EDA, ethylenediamine; FRET, fluorescence resonance energy transfer; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MeOH, methanol; NHS, *N*-hydroxysuccinimide; PyMPO, benzyl-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium; R6G, 5-(and 6)-carboxyrhodamine-6G; ROX, 5-carboxy-X-rhodamine; TEA, triethylamine; TFA, trifluoroacetic acid; TFE, trifluoroethanol; THF, tetrahydrofuran; TLC, thin-layer chromatography.

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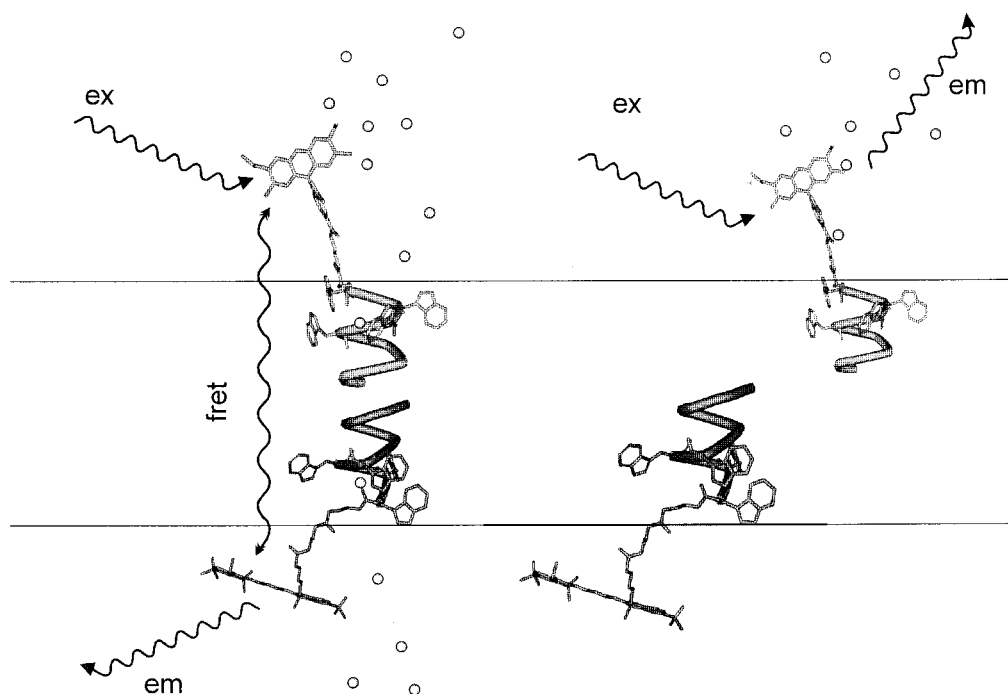


Figure 1. Schematic diagram showing the dimerization of fluorescent gramicidin derivatives in a lipid bilayer. The bilayer is represented as two parallel lines. Dimerization provides a pathway for monovalent cations (small circles) through the membrane. This cation flux can be detected electrically at the single-channel level. Dimerization is also expected to permit efficient fluorescence resonance energy transfer (FRET) between donor (gram-R6G) and acceptor (gram-Cy5) peptides. In principle, this FRET signal could also be detected at the single-channel level.

per second. The channel is stabilized by six hydrogen bonds at the dimer junction. Channel closing is believed to be due to dissociation of the dimer into monomers. At 25 °C, the average lifetime of a dimer is on the order of 1 s (34, 35).

If gramicidin dimerization could be detected optically at the single-molecule level, one would expect the optical change to coincide with a change in ionic current, measured electrically, through the membrane. With fluorescently labeled gramicidin derivatives, optical detection of peptide dimerization should be possible by measuring resonance energy transfer that would occur between dyes with appropriate absorption and emission spectra. Figure 1 shows the process diagrammatically. Peptides bearing a donor dye are added to one side of a lipid bilayer and peptides bearing an acceptor dye to the other. Transmembrane ionic current is measured using conventional patch-clamp or BLM techniques, and dimerization is detected as a step increase in this current. Upon peptide dimerization, the donor and acceptor dyes are 50 Å or less apart, a distance that should lead to efficient resonance energy transfer for many donor–acceptor pairs (36).

We describe here the synthesis and characterization of two pairs of fluorescently labeled gramicidin derivatives that should prove useful for combined optical and electrical single-molecule studies. The synthetic methods are general and could be used to prepare other fluorescent gramicidin derivatives from commercially available amine-reactive dyes. We show that the derivatives incorporate into unilamellar lipid vesicles and into planar bilayers. Single-channel properties of the derivatives measured electrically in planar bilayers were found to be similar to those of the unmodified channel. When donor and acceptor peptides are incorporated into the same lipid vesicles, fluorescence resonance energy transfer was observed under conditions where the channel can be shown to be active using a diffusion potential assay.

Thus, the fluorescent gramicidin derivatives appear to have properties suitable for combined optical and electrical single-molecule experiments. Some further requirements for such experiments are considered.

MATERIALS AND METHODS

1. Synthesis of Gramicidin-EDA (Gram-EDA). The C-terminal end of gramicidin was derivatized as described previously to install a primary amino group (37). Briefly, commercial gramicidin D (38 mg, 20 μmol) was combined with *p*-nitrophenyl chloroformate (200 μmol) in dry tetrahydrofuran (2 mL) (4 °C), and then triethylamine (TEA) (100 μL) was added and the mixture stirred for 1 h at 4 °C. The resulting carbonate ester was filtered through Celite into a 100-fold molar excess of ethylenediamine in 2 mL of dimethylformamide (DMF). The product [gramicidin-ethylenediamine (gram-EDA)] was then separated by gel filtration using Sephadex LH-20 (2.5 × 20 cm gravity column) in methanol. The product was further purified using reverse-phase HPLC [Zorbax-RX-C8 column (4.6 × 250 mm), isocratic conditions, 80% MeOH/20% H₂O, 0.1% TFA adjusted to a pH of 3.0 with TEA, flow rate = 1 mL/min, retention time 6.2 min]. Electrospray mass spectrometry: gram-EDA = C₁₀₂H₁₄₇N₂₂O₁₈ (M+H⁺) calcd = 1969.4, obsd = 1969.5; TLC (C:M:W = 65:25:4): gramicidin *R_f* = 0.70, gram-EDA *R_f* = 0.47.

2. Synthesis of Gramicidin-Rhodamine-6G (Gram-R6G). Gramicidin-EDA (2.0 mg, 1.02 μmol) was dissolved in 1.0 mL of dry DMF in a round-bottom flask under a nitrogen atmosphere. To the stirring solution of gram-EDA was added 100 μL of dry TEA. In a separate reaction vessel, also under nitrogen, 1.0 mg (1.8 μmol) of (5/6)-carboxyrhodamine-6G-NHS ester (Molecular Probes) was dissolved in 1.0 mL of dry DMF. The gram-EDA solution was then added dropwise over a period of 1 h via syringe to the stirring dye solution. After the reaction had stirred

for 24 h, protected from light, the DMF was removed under high vacuum and the product resuspended in methanol and separated from unreacted dye using gel-filtration chromatography (LH-20 in methanol). The product was further purified using reverse-phase HPLC [Zorbax-RX-C8 column (4.6 × 250 mm), isocratic conditions, 80% MeOH/20% H₂O, 0.1% TFA adjusted to a pH of 3.0 with TEA, flow rate = 1 mL/min, retention time 11.6 min]. MALDI mass spectrometry: gram-R6G = C₁₂₉H₁₇₁N₂₄O₂₂ (M+H⁺) calcd = 2409.8, obsd = 2409.7; TLC (C:M:W = 65:25:4): gramicidin *R_f* = 0.70, gram-R6G *R_f* = 0.77.

3. Synthesis of Gramicidin-Cy5 (Gram-Cy5). Gramicidin-Cy5 was prepared in a manner similar to that described for gram-R6G. Gram-EDA (0.4 mg, 0.2 μmol) was mixed with 20 μL of dry TEA in 0.2 mL of dry DMF; then the contents of one vial (nominally 0.2–0.3 mg) of Cy5-NHS-ester (monoreactive)(Amersham Pharmacia Biotech) dissolved in 0.2 mL of dry DMF were added dropwise. This solution was stirred at room temperature overnight protected from light. The solvent was removed under high vacuum, and the residue was dissolved in 1 mL of methanol. The product was then purified by HPLC [Zorbax-C8 column (4.6 × 250 mm), isocratic conditions, 98% solvent A (0.1% TFA in 80% MeOH/20% H₂O)/2% solvent B (0.1% TFA in H₂O), flow rate = 1 mL/min, retention time 5.4 min]. MALDI mass spectrometry (negative ion mode): gram-Cy5 = C₁₃₅H₁₈₃N₂₄O₂₅S₂ calcd = 2606.2, obsd = 2605; TLC (C:M:W = 65:25:4): gramicidin *R_f* = 0.70, gram-Cy5 *R_f* = 0.44.

4. Synthesis of Gramicidin-PyMPO (Gram-PyMPO). PyMPO-NHS [1-(3-(succinimidylloxycarbonyl)-benzyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium iodide] (Molecular Probes) (1.7 mg, 3 μmol) was combined with 0.17 mL of dry DMF, vortexed, and added slowly to 1 mL of dry DMF containing gram-EDA (1.2 mg, 1 μmol). After 1 h, dry TEA (20 μL, 0.14 mmol) was added, and the solution was stirred overnight at room temperature protected from light. The DMF was pumped off, and the product was purified by gel filtration using LH-20 in methanol. The product was further purified using reverse-phase HPLC [Zorbax-RX-C8 column (4.6 × 250 mm), isocratic conditions, 10% MeOH/90% solvent B (80% MeOH/20% H₂O, 0.1% TFA adjusted to a pH of 3.0 with TEA), flow rate = 1.2 mL/min, retention time 6.2 min]. MALDI mass spectrometry: gram-PyMPO = C₁₂₅H₁₆₃N₂₄O₂₁ (M⁺) calcd = 2337.7, obsd = 2336.9; TLC (C:M:W = 65:25:4): gramicidin *R_f* = 0.70, gram-PyMPO *R_f* = 0.76.

5. Synthesis of Gramicidin-ROX (Gram-ROX). ROX-NHS (5-carboxy-X-rhodamine-*N*-hydroxysuccinimidyl ester, Molecular Probes, 1.9 mg, 3 μmol) was reacted with gram-EDA using the same procedure as described for gram-PyMPO. After purification using the LH-20 column, the gram-ROX was further purified with a silica gel column (2 × 20 cm) using C:M:W = 65:25:4 as eluant. The desired product was the first band eluted. The product was then purified using reverse-phase HPLC [Zorbax-RX-C8 column (4.6 × 250 mm), isocratic conditions, 15% MeOH/85% solvent B (80% MeOH/20% H₂O, 0.1% TFA adjusted to a pH of 3.0 with TEA), flow rate = 1.2 mL/min, retention time 10 min]. MALDI mass spectrometry: gram-ROX = C₁₃₅H₁₇₅N₂₄O₂₂ (M+H⁺) calcd = 2485.9, obsd = 2485.5; TLC (C:M:W = 65:25:4): gramicidin *R_f* = 0.70, gram-ROX *R_f* = 0.72.

6. Fluorescence Measurements of Gramicidin Derivatives in Lipid Vesicles. Unilamellar lipid vesicles containing gramicidin derivatives were prepared as follows: An aliquot of dioleoylphosphatidylcholine (DOPC)

(Avanti Polar Lipids) (2 mg, 2.54 μmol) dissolved in methanol was added to a 1.5 mL Eppendorf tube containing either a given number of moles (e.g., 1 nmol) of an individual fluorescent gramicidin derivative or a 1:1 mixture (e.g., 0.5 nmol each) of two fluorescent gramicidin derivatives (total methanol volume = 400 μL). Concentrations of fluorescent gramicidins were determined by UV–Vis absorbance measurements of stock solutions in methanol (extinction coefficients used: gram-R6G = 92 000 at 520 nm, gram-Cy5 = 250 000 at 649 nm, gram-PyMPO = 26 000 at 415 nm, gram-ROX = 78 000 at 574 nm). Methanol was then removed under a gentle stream of dry nitrogen to form a thin lipid film, and the film was pumped under high vacuum for >2 h. The lipid/gramicidin film was resuspended in 400 μL of 400 mM KCl, 6.5 mM phosphate buffer, pH 7.0. Unilamellar vesicles containing fluorescent gramicidins were then formed by repeated extrusion (20 passes) of the lipid suspension through a polycarbonate membrane (Avanti Polar Lipids) with a pore diameter of 100 nm. Fluorescence measurements were made of vesicle solutions prepared from 1:1 mixtures of fluorescent gramicidins and compared to measurements of 1:1 mixtures of vesicle solutions prepared from pure fluorescent gramicidins. Final lipid concentrations and peptide concentrations are given in the figure legends. Steady-state fluorescence measurements were made using a Perkin-Elmer LS50B spectrofluorometer (equipped with a red-sensitive photomultiplier for Cy5 detection) and a 150 μL cuvette with 0.5 cm excitation and emission path lengths. All measurements were made at room temperature (23 ± 2 °C).

7. Fluorescence Detection of Diffusion Potentials Generated by Gramicidin in Lipid Vesicles. The establishment of a diffusion potential across lipid vesicle membranes was assayed using the potential-sensitive fluorescent dye safranin O (38). Unmodified gramicidin (0.1, 1, 3, 5, 50 nmol) was combined with lipid (32 μmol) in methanol. Vesicles were then prepared exactly as described above except that 500 μL of 200 mM KCl, 5 mM phosphate buffer, pH 7.0, was used. After extrusion through 100 nm polycarbonate filters, 20 μL of this vesicle solution was added to 3 mL of a solution of 340 mM sucrose, 5 mM phosphate buffer, pH 7.0, containing 1 μM safranin O (Aldrich). The fluorescence emission of safranin O was monitored at 581 nm (excitation 521 nm, 5 nm slits, 0.5 s response time).

8. Single-Channel Conductance Measurements of Gramicidin Derivatives. The general techniques for making single-channel conductance measurements of gramicidin derivatives have been described previously (39). A solution of 1 M CsCl, 5 mM BES, pH 7.0, was used as the electrolyte. Gramicidin derivatives were HPLC-purified at least twice (using the conditions given above) before use in single-channel measurements to ensure highly uniform conductance events (40). Peptides (~10 nM in methanol) were added to membranes formed from diphytanoylphosphatidylcholine/decane (50 mg/mL) (Avanti Polar Lipids). Membranes were formed by painting the lipid solution across a ~100 μm aperture in a polypropylene pipet tip mounted horizontally in a Teflon well. Silver/silver chloride electrodes were placed, one on either side of the membrane, and were connected to the CV-4B headstage of an Axopatch 1D patch-clamp amplifier (Axon Instruments) controlled by Synapse (Synergetic Research Systems) software that permitted transmembrane voltage to be set and current recorded using the same pair of electrodes. Current records were filtered

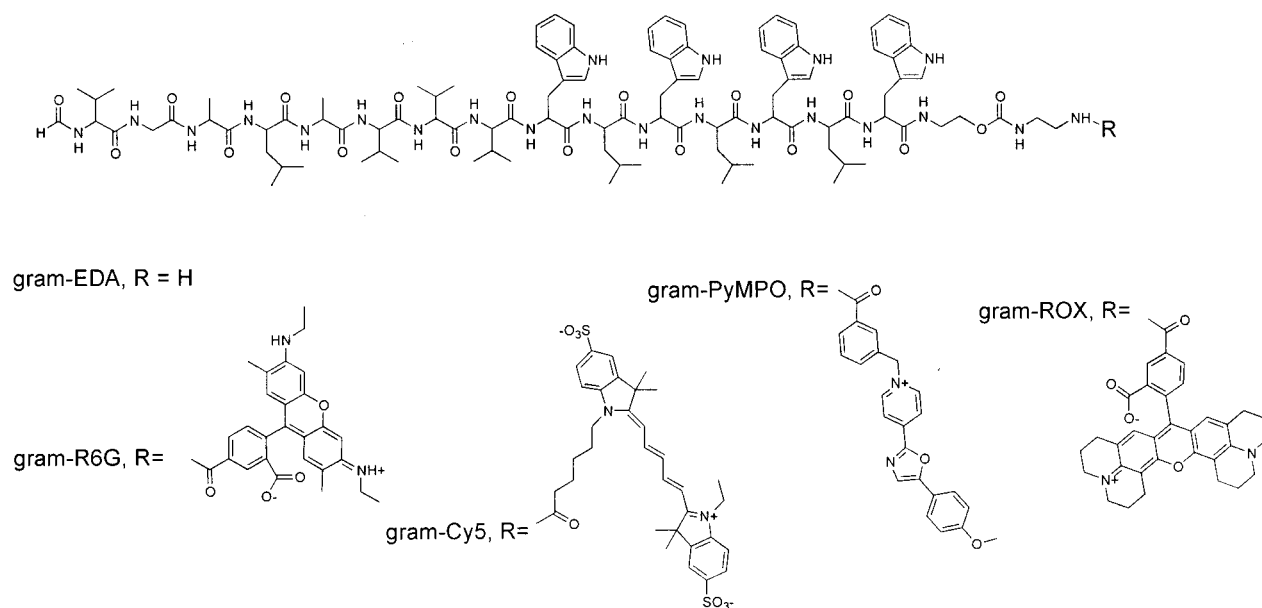


Figure 2. Chemical structures of gramicidin derivatives.

at 50 or 100 Hz, sampled at 1 kHz, stored directly to disk, and analyzed using Synapse and Igor (Wavemetrics Inc.) software.

RESULTS AND DISCUSSION

Synthesis of Fluorescently Labeled Peptides.

Gramicidin isolated from *Bacillus brevis* is a mixture of peptides (denoted gramicidin D) with the general structure formyl-Xxx-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val-Trp-D-Leu-Yyy-D-Leu-Trp-D-Leu-Trp-NHCH₂CH₂OH, where Xxx is Val or Ile and Yyy is Trp (gramicidin A), Phe (gramicidin B), or Tyr (gramicidin C) (Figure 2) (41). Previous work with fluorescent gramicidins (31, 32, 42–44) relied primarily on a dansylated (Tyr¹¹-O-dansyl) gramicidin C derivative. Although this derivative was reported to have channel properties similar to those of native gramicidin, modifications at position 11 in the sequence to install various fluorescent groups might not be generally well tolerated (28). We decided instead to focus on derivatization of the C-terminal end of the peptide since chemical modifications at this end (the membrane/solution interface) do not significantly affect the overall structure of the channel (29).

Chemical modifications were carried out on the mixture (gramicidin D) since it is predominantly (>80%) Val¹-gramicidin A and, in any case, all peptides are HPLC-purified after modification, which removes minor species. Val¹-gramicidin A has no functional groups suitable for reaction with typical fluorescent labeling reagents, so we first derivatized the C-terminal end to provide a convenient site for attachment of commercially available amine-reactive fluorescent labels (37).

To be useful for single-molecule work, fluorescent dyes must be robust (i.e., resistant to photobleaching). We therefore focused on cyanine-based and rhodamine-based dyes that have been used successfully for these purposes (45). In addition, for fluorescence resonance energy transfer (FRET) measurements, dyes with overlapping emission and excitation spectra are required (46). The amine-functionalized gramicidin (gram-EDA, Figure 2) was reacted with four different fluorescent labels (rhodamine-6G, Cy5, PyMPO, ROX) all purchased as *N*-hydroxysuccinimide esters. Each product was purified by gel filtration chromatography in methanol (the pep-

tides are not water soluble), followed by reverse-phase high-performance liquid chromatography (HPLC) to ensure that no unreacted gram-EDA or unreacted dye was present (40). Mass spectrometry [matrix-assisted laser desorption ionization (MALDI)], in addition to absorbance and fluorescence data, confirmed the identity of the products. The chemical structures of the four fluorescently labeled gramicidin derivatives are shown in Figure 2. In addition, uniform single-channel conductance properties (vide infra) are an excellent indication of purity (40).

Channel Formation in Lipid Vesicles. Before performing fluorescence measurements, we wished to ensure that the fluorescent gramicidins were incorporated into a membrane in a functional form. Accordingly, dilute solutions of the peptides were combined with lipid in methanol. Under these conditions, the peptides are expected to be predominantly monomeric (47). As the methanol is evaporated, the peptide forms an intimate mixture with the lipid. When aqueous buffer is then added and the lipid/peptide suspension is extruded through 100 nm polycarbonate filters, unilamellar lipid vesicles are expected to form with peptide incorporated in the single-helix (channel-forming) structure. Although gramicidin peptides are known to form intertwined double helices in organic solvents, these convert to the single-helix form in the presence of lipid (47). Polar organic solutions facilitate this conversion. In particular, trifluoroethanol has been reported to function well in this regard (48). However, we found that traces of TFE remaining after extensive drying led to interference with fluorescence measurements, particularly where membrane potential was assayed using safranin O (see below). Consequently, we used methanol as the solvent for mixing peptide and lipid.

Lipid vesicles of 100 nm diameter may be estimated to contain ~100 000 molecules of lipid taking an average area per lipid molecule of 0.6 nm² (49). If one takes 1 nmol of gramicidin and 2.5 μmol of lipid, an average of 40 gramicidin molecules per vesicle is obtained. This corresponds to a total surface density of gramicidin in the membrane of 2.3×10^{-13} mol/cm².² The fraction of dimeric (active channel) form will depend on the dimerization constant for the particular membrane type.

Dimerization constants have been estimated for the dansyl gramicidin C derivative in dioleoylphosphatidylcholine (DOPC)/decane membranes by Veatch et al. (31) to lie in the range of 10^{13} – 10^{14} cm²/mol. Values between 10^{14} (for 0 mV) and 10^{15} cm²/mol (for 200 mV) have been reported for gramicidin A by Bamberg and Lauser (35) based on current relaxation measurements also in DOPC/decane membranes (the voltage dependence arises from a voltage-induced thinning of the membrane). If we use the same range of values for the present case, 63–95% of the peptide is estimated to be in dimeric form. As the concentration of peptide is lowered, the equilibrium fraction of dimers decreases; however, even with only two gramicidin molecules per vesicle, ion transport should still be possible over time. Transport should not be possible in vesicles with less than two peptide molecules since one molecule cannot form a channel and molecules cannot move between vesicles assuming there is no vesicle fusion (50).

To test the prediction that active channels should be present in vesicles when two or more peptide molecules are present, we investigated whether a diffusion potential could be established across the vesicle membranes. Vesicles were prepared in 200 mM KCl solution and diluted into isotonic sucrose solution. Were a gramicidin channel present, K⁺ ions should diffuse out of the vesicle, but, since no other cations are present and gramicidin is impermeable to anions, a potential should develop, negative inside, that opposes K⁺ efflux. Development of this potential can be followed by observing the redistribution of the cationic fluorescent dye safranin O between aqueous and membrane-bound compartments (38). Figure 3 shows the fluorescence response observed with various concentrations of (unmodified) gramicidin per vesicle. The channel is clearly active. As expected, no fluorescence response is seen if equimolar NaCl is included in the external medium (data not shown), in keeping with the known ability of gramicidin to transport sodium ions.

Fluorescence Spectroscopy. Having established that the present procedure for incorporating gramicidin channels into lipid vesicles led to active channel species, we wished to examine the fluorescence properties of these channels and to test whether the fluorescent tags would serve as functional donor–acceptor pairs for use in fluorescence resonance energy transfer studies. Figure 4 shows normalized fluorescence excitation and emission spectra for each of the four fluorescent gramicidins incorporated (individually) into lipid vesicles. The small size of the lipid vesicles used (100 nm diameter) results in light scattering intensities at these wavelengths that can be readily separated from fluorescence (51).

To test for resonance energy transfer, two different types of sample containing vesicle-bound fluorescent gramicidins were prepared. In case A, a vesicle solution containing only donor peptide and a vesicle solution containing only acceptor peptide were prepared, and then these vesicle solutions were mixed in a 1:1 ratio. In case B, donor and acceptor peptides were mixed directly in a 1:1 ratio and then incorporated together into vesicles. The result is two solutions with equal concentrations of lipid, donor peptide, and acceptor peptide, but with different distributions of the donors and acceptors. Since the aqueous solubility of the peptides is very low, the peptides are not expected to redistribute between vesicles (50). In case A, the following molecular species may be present:

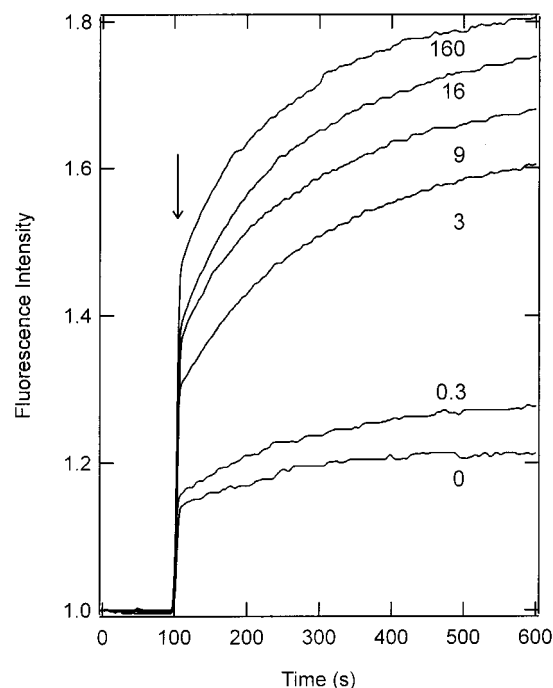


Figure 3. Fluorescence detection of channel formation in vesicles. At the time indicated by the arrow, K⁺-loaded vesicles alone (0) or K⁺-loaded vesicles containing gramicidin (average number of gramicidin molecules per vesicle is indicated) were added to K⁺-free buffer containing isotonic sucrose and the potential-sensitive dye safranin O. Concentrations are given under Materials and Methods. Fluorescence enhancement is observed as the dye associates with vesicle membranes in response to the development of a K⁺ diffusion potential (38).

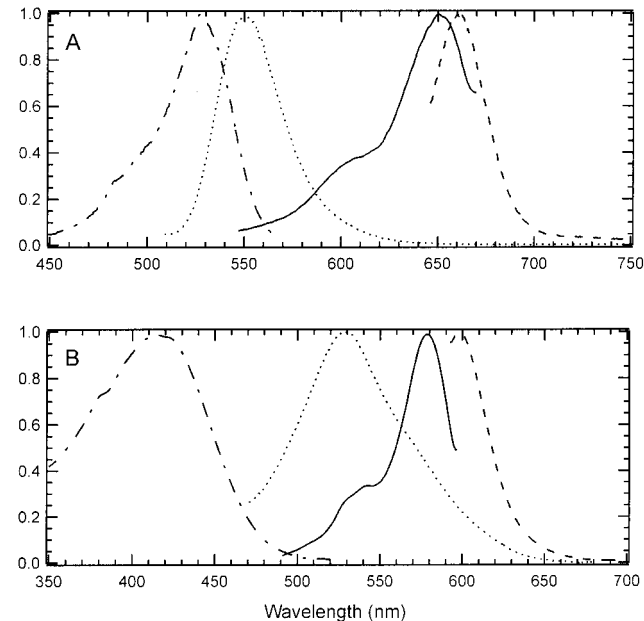


Figure 4. (A) Excitation and emission spectra (normalized) of gram-R6G and gram-Cy5 in lipid vesicles: (— · —) gram-R6G excitation, (····) gram-R6G emission, (—) gram-Cy5 excitation, (— —) gram-Cy5 emission. (B) Excitation and emission spectra (normalized) of gram-PyMPO and gram-ROX in lipid vesicles: (— · —) gram-PyMPO excitation, (····) gram-PyMPO emission, (—) gram-ROX excitation, (— —) gram-ROX emission.

donor monomers, donor dimers, acceptor monomers, and acceptor dimers. In the premixed sample (case B), donor–acceptor dimers (i.e., FRET pairs) will also be present. Since the concentration of vesicles in these experiments is ~64 nM (6.4 mM DOPC present in vesicles of 100 000

² Forty molecules of peptide (6.6×10^{-23} mol) divided by the area of a 100 nm diameter vesicle (2.9×10^{-10} cm²).

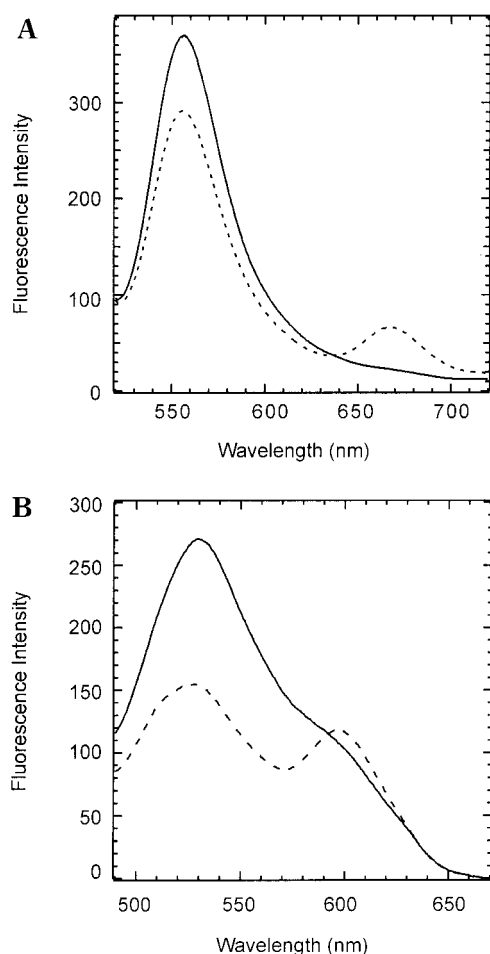


Figure 5. (A) Fluorescence resonance energy transfer between gram-R6G and gram-Cy5: (—) gram-R6G (0.3 μ M) and gram-Cy5 (0.31 μ M) in separate vesicles (6.4 mM DOPC); (---) gram-R6G (0.31 μ M) and gram-Cy5 (0.34 μ M) in the same vesicles (6.4 mM DOPC), excitation at 490 nm, 10 nm slit widths. (B) Fluorescence resonance energy transfer between gram-PyMPO and gram-ROX: (—) gram-PyMPO (1.1 μ M) and gram-ROX (0.9 μ M) in separate vesicles (6.4 mM DOPC); (---) gram-PyMPO (1.3 μ M) and gram-ROX (1 μ M) in the same vesicles (6.4 mM DOPC), excitation at 422 nm, 15 nm slit widths.

molecules each), the average separation between vesicles is ~ 3000 Å. This value is much larger than the expected Förster distance (~ 50 Å) so that FRET should only be possible in case B, where donor and acceptor gramicidins are co-incorporated into the same vesicles.

Figure 5A compares the fluorescence spectra obtained in case A and case B for the gram-R6G/gram-Cy5 pair where the peptide:lipid ratio is $\sim 1:10\,000$. Figure 5B compares the spectra obtained with the gram-PyMPO/gram-ROX pair where the peptide:lipid ratio is $\sim 1:3200$. In each case, significant quenching of donor fluorescence is observed for case B, the premixed samples, in comparison to case A. Additional evidence for FRET is the enhancement of acceptor fluorescence in case B for each dye pair. Note that there is minimal direct excitation of the acceptor in the gram-R6G/gram-Cy5 case as compared to the gram-PyMPO/gram-ROX case. Energy transfer efficiencies were calculated by the method of Clegg et al. (52) using both donor quenching and acceptor enhancement data. Calculated transfer efficiencies were $\sim 23 \pm 5\%$ for the gram-R6G/gram-Cy5 pair and $60 \pm 10\%$ for the gram-PyMPO/gram-ROX pair for the particular conditions described in the legend to Figure 5. The lower transfer efficiency of the gram-R6G/gram-Cy5 pair is

consistent with a lower peptide-to-lipid ratio, and therefore a smaller fraction of dimers, for that case. Increasing the peptide-to-lipid ratio gave increasing FRET efficiencies as expected; however, with acceptor concentrations > 1 μ M, self-quenching of Cy5 was observed (data not shown).

In principle, FRET can also occur between donors and acceptors that are present in the same vesicle but not part of a donor/acceptor dimer. Such lateral energy transfer in lipid bilayers has been analyzed previously by several authors (53–56). While lateral FRET may be observed even when the average donor–acceptor distance exceeds the Förster distance severalfold (53), FRET will nevertheless occur most efficiently between donors and acceptors that are actually associated (55). The total surface density of gramicidin in the membrane in the present FRET experiments ranges from 5.8×10^{-14} to 2.3×10^{-13} mol/cm², which corresponds to average distances between noninteracting peptides in the range of 550–270 Å if they were monomeric and 750–380 Å if they were dimeric. These values are larger than the expected Förster distances for these dye pairs, and substantially larger than the distance between donor and acceptor dyes in a gramicidin dimer (~ 50 Å). Since the fluorescent gramicidin peptides are able to form active dimers as demonstrated by the conductance data in bilayers (vide infra), we expect the observed FRET to be due primarily to the presence of heterodimer (donor–acceptor) gramicidin channels rather than the mere coexistence of the donor and acceptor peptides in the same vesicles.

The observed energy transfer efficiency is affected not only by the ratio of dimers to monomers and the intrinsic efficiency for the particular dye pair as it occurs in the gramicidin dimer, but also by the fact that donors can be paired with donors as well as with acceptors. If we assume that the dimerization constants for all possible dimers [donor–donor, acceptor–donor (two orientations), acceptor–acceptor] are the same, then approximately half of all dimers present should be FRET pairs since the concentrations of donors and acceptors are approximately equal. Single-molecule FRET measurements with these systems would thus be expected to show approximately double the energy transfer efficiency determined from a macroscopic measurement on dimerized gramicidins.

Channel Formation in Black Lipid Membranes.

The single-channel properties of each of the fluorescent gramicidin derivatives were examined in planar lipid bilayers using 1 M CsCl as the electrolyte. Single-channel current recordings are shown in Figure 6. In general, the single-channel properties of all the derivatives are similar to native gramicidin channels measured under the same conditions (see Table 1) (57). This result is expected as the C-terminal modifications are not expected to affect channel function significantly (29). A distinguishing feature of the modified channels, however, is the small steplike current fluctuations superimposed on the main conducting level in each case (Figure 6). Steps in current recording are also observed with gramicidin-EDA of the same average duration (~ 50 ms) and are due to thermal cis–trans isomerization of the carbamate group that is introduced near the channel mouth that alters the position of the protonated amino group relative to the channel mouth (58). The size of the current steps is much smaller for the fluorescent derivatives than for gram-EDA because charges on the fluorescent groups are significantly further from the channel entrance [cf. (59)]. The variation of the step sizes between the different fluorescent derivatives is also expected since isomeriza-

Table 1. Single-Channel Properties of Fluorescent Gramicidin Derivatives

	gram A (<i>n</i> = 250)	gram-R6G (<i>n</i> = 96)	gram-Cy5 (<i>n</i> = 91)	gram-PyMPO (<i>n</i> = 23)	gram-ROX (<i>n</i> = 129)
mean lifetime ^a (s)	0.4	4	2.3	0.8	1.8
conductance ^b (pS)	46	45	45	45	43

^a Estimated as described in (63). ^b Measured at 200 mV, 1 M CsCl, using diphytanoylphosphatidylcholine/decane membranes. Errors are on the order 5%.

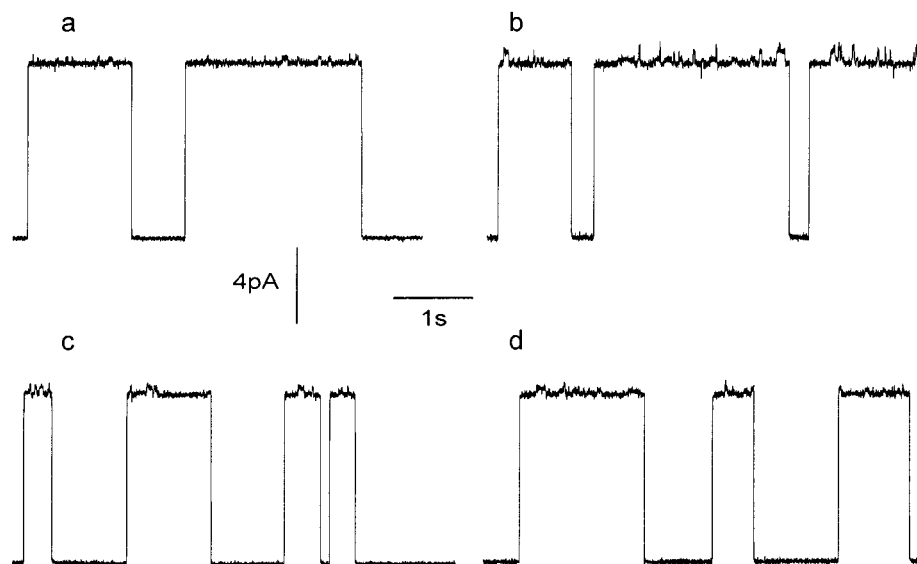


Figure 6. Representative single-channel current recordings of (a) gram-R6G, (b) gram-Cy5, (c) gram-PyMPO, and (d) gram-ROX in diphytanoylphosphatidylcholine membranes. The applied voltage was 200 mV, and 1 M CsCl was used as the electrolyte.

tion of the linkage connecting the fluorescent group to the rest of the peptide is likely to have different effects on ion access in each case. These small fluctuations may be considered signatures of the modified channels that distinguish them from possible gram-EDA or native gramicidin contaminants in the membrane. Isomerization might also be expected to affect FRET efficiency in time-resolved measurements on single molecules, and this feature of the modified channels might also provide an internal calibration for single-molecule optical measurements.

Toward Simultaneous Single-Molecule Optical and Electrical Measurements. Single-molecule electrical measurements with gramicidin and derivatives are straightforward and highly reproducible. The dye conjugates, with the exception of PyMPO, described here have been used successfully in single-molecule optical detection experiments previously, and we have demonstrated that the two pairs can undergo efficient FRET when channels form. Correlating a single-molecule FRET measurement with a channel opening event measured electrically will require that single active channels (dimeric channels) can be observed among nonconducting monomers in a planar bilayer. Single-channel measurements with gramicidin are typically performed at peptide-to-lipid ratios $<1:10^7$ (60). At these densities, individual molecules can be resolved by fluorescence imaging (8, 61). The ratio of dimers to monomers can be increased by altering lipid type or by adding certain detergents to the membranes (62). Asymmetric addition of donor peptides to one side of a planar bilayer and acceptor peptides to the other should ensure that the majority of channel openings occur between FRET pairs since transmembrane flip-flop of gramicidin peptides is slow (33). The fluorescent gramicidin channels presented here thus appear to have properties that should make them suit-

able for attempting simultaneous single-molecule optical and electrical measurements.

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Supporting Information Available: Figure showing mass spectra of gramicidin derivatives (1 page). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Design and Synthesis of Substrate and Internal Standard Conjugates for Profiling Enzyme Activity in the Sanfilippo Syndrome by Affinity Chromatography/Electrospray Ionization Mass Spectrometry

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We describe the design and synthesis of substrate and internal standard conjugates for application in profiling enzyme activity of the enzymes α -D-2-deoxy-2-*N*-sulfonamido-glucosamine sulfamidase, α -D-2-deoxy-2-*N*-acetyl-glucosamine hydrolase, acetyl-coenzymeA: α -D-2-deoxy-2-amino-glucosamine transferase, and α -D-2-deoxy-2-*N*-acetyl-glucosamine-6-sulfate sulfatase. Deficiency of any one of these enzymes results in a single clinical phenotype known as Sanfilippo syndrome. Such substrates have been proven effective in the confirmation of enzyme deficiency by a combination of affinity chromatography (AC) and electrospray ionization mass spectrometry (ESIMS), which forms the foundation for a new analytical technology (ACESIMS) of general interest and application to clinical and biomedical research.

INTRODUCTION

Affinity chromatography has become increasingly popular over the past several decades as a simple yet highly selective method for the purification of a wide assortment of biomolecules (1, 2). In particular, the use of biotin and its corresponding receptor proteins avidin and streptavidin is now quite common in laboratories focused on life science research (3, 4). As a result, there is a growing body of biotin-conjugate chemistry in the literature for the preparation of biotinyl derivatives for a host of applications. In our research (5), we are developing biotinylated enzyme–substrate conjugates as the foundation of a mass spectrometric method to determine clinically relevant enzyme velocities directly in crude cell lysates from children suspected of suffering from enzyme-deficient disease.

The lack of properly functioning enzyme activity due to genetic aberrations in the coding sequence of such enzymes is a common molecular basis for inherited disease (6). Although there are a variety of methods in the clinical laboratory used to biochemically confirm a diagnosis of enzyme deficiency, there exists to date no single, unifying method capable of determining multiple, unique enzyme activities simultaneously, in a single sample (multiplexing). Such a technology could allow for the rapid and prompt screening of newborns for the most common genetic diseases. This would be advantageous and beneficial in the prompt identification and treatment of possible afflictions and would lead to reduced exposure of young patients to repeated biopsies.

Our recent efforts to establish such a technology have been successfully applied to several enzyme-deficient diseases (5, 8). In our approach, biotinylated substrate conjugates are incubated with fibroblast lysate obtained from cultures of skin biopsies from either apparently normal or putatively affected individuals. Action of the enzyme of interest on the substrate portion of the conjugate compositionally modifies the substrate conjugate, resulting in a change in mass of the substrate. A known amount of internal standard is added that is chemically identical to the product generated by enzyme activity but with deuterium incorporated into the conjugate. The mixture of excess substrate, nondeuterated enzyme product, and internal standard is purified from the biological matrix by addition of streptavidin–agarose to the sample and washing with purified water. The biotinylated conjugates are then selectively eluted from the bound receptor by addition of excess free biotin acid. This eluant is transferred to an electrospray-ionization mass spectrometer for analysis. The absolute amount of product formed during the reaction is determined by calculating the ratio of product to internal standard ion currents observed in the mass spectrum. In addition, an enzyme initial velocity can be calculated by determining the moles of product generated during the time in which the initial velocity condition exists.

The design of our substrate conjugates starts with biotin as the molecular handle with which such conjugates can be simply and selectively purified from the biological sample. Biotin is in turn conjugated to sarcosine to form an *N*-methyl biotinamide motif which is resistant to the enzyme biotinidase (7), known to be present in biological samples such as serum. Biotinidase would otherwise cleave our handle from the remainder of the conjugate. Sarcosinyl biotin is further linked to a water-soluble polyether linker. The linker also serves to position the structurally distinct biotin away from the substrate to avoid steric discrimination by the targeted enzyme, avidin or streptavidin. The linker also provides

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a location for facile synthetic incorporation of deuterium for internal standards. The length (i.e., mass) of the linker can also be adjusted to avoid mass redundancy with other conjugate pairs in a multiplex analysis. The amine terminus of the linker is attached to the substrate portion, either by direct conjugation to the substrate or by way of an adapter segment of variable composition for conjugation to a wide variety of substrate substructures.

In the present work, we describe the synthesis of substrate and internal standard conjugates used to profile the enzyme velocities involved in Sanfilippo syndrome. This approach has recently been shown to be successful in the confirmation of diagnoses for several diseases of enzyme deficiency, including the lysosomal storage disorder known as Sanfilippo syndrome (8). In this condition, deficiency of the four heparinoid-degrading enzymes α -D-2-deoxy-2-*N*-sulfonamido-glucosamine sulfamidase, α -D-2-deoxy-2-*N*-acetyl-glucosamine hydrolase, acetyl-coenzymeA: α -D-2-deoxy-2-amino-glucosamine transferase, and α -D-2-deoxy-2-*N*-acetyl-glucosamine-6-sulfate sulfatase results in a Sanfilippo subtype A, B, C, or D, respectively (9–12).

EXPERIMENTAL PROCEDURES

General. Reagent-grade chemicals (including solvents) were obtained from commercial vendors and were generally used without further purification. Anhydrous dimethylformamide and pyridine were purchased from Aldrich Chemical Co. (Milwaukee, WI), and used as received. Triethylamine was frequently redistilled from calcium hydride and stored under argon. HPLC-grade solvents were purchased from Fischer (Fair Lawn, NJ), and water for HPLC was purified at point-of-use by a Milli-Q water system (Millipore, Bedford, MA). Trifluoroacetic acid was purchased from Advanced Chemtech (Louisville, KY), and was added to HPLC solvents as required, followed by 0.2 μ m filtration. Triethylene glycol was purchased from Fluka (Buchs, Switzerland). Methyl *N*-methyl-*N*-biotinyl glycinate (7), 1-fluoro-2-deoxy-2-(*N*-trifluoroacetyl)-3,4,6-tri-*O*-acetyl-glucosamine (13), 1- α -(*p*-nitrophenyl)-2-deoxy-2-(*N*-trifluoroacetyl)-3,4,6-tri-*O*-acetyl-glucosamine (13), and 2,3,5,6-tetrafluorophenyl trifluoroacetate (14) were prepared according to the literature. All syntheses involving highly toxic materials (sodium cyanide etc.) were performed in a fume hood. Due caution is advised in reproducing these syntheses.

Spectral Analyses. ^1H NMR data were obtained on Bruker (Rheinstetten, Germany) spectrometers: AC-200 (200 MHz), AC-300 (300 MHz), or AC-500 (500 MHz). Proton chemical shifts are designated as parts-per-million (ppm) from tetramethylsilane (δ = 0.00 ppm). Mass spectral data were obtained using either a Kratos (Manchester, United Kingdom) HV-4 electrospray ionization-double-focusing (E/B) magnetic sector or Bruker Daltonics (Bremen, Germany) Esquire electrospray-ion trap mass spectrometers with standard, commercial interfaces. Methanol/acetic acid (99.5:0.5) was used as solvent, and contained 5 μM gramicidin S (average, doubly charged molecular weight $(\text{M}+2\text{H}^+)^{2+}$ = 571.6) to verify mass accuracy.

Chromatography. Silica gel chromatography was performed using 230–400 mesh 60 Å silica (Merck, Darmstadt, Germany). Thin-layer chromatography was performed on 60 Å silica with F_{254} , aluminum-backed plates (Merck) and visualized by UV at 254 nm, phosphomolybdic acid, iodine, or ninhydrin, as indicated. Reversed-phase HPLC separations were performed using a Vydac

(Hisperia, CA) semipreparative column (up to 5 mg total loading, 10 μm packing, 10 mm $\times$ 250 mm, catalog no. 218TP1010) at 3.0 mL/min, or a preparative column (up to 300 mg total loading, 10 μm packing, 22 mm $\times$ 250 mm, catalog no. 218TP1022) at 7 mL/min. Solvents were water/0.08% TFA (solvent A) and acetonitrile/0.08% TFA (solvent B), unless otherwise indicated. Common gradient conditions used were: (1) 2% A for 5 min, then a linear gradient from 2% to 35% B for 40 min; and (2) 5% A for 5 min, then a linear gradient from 5% to 55% B for 60 min, unless otherwise specified.

Quantification of Final Conjugates. Quantification of final substrate and internal standard conjugates was performed by ^1H NMR analysis in the presence of an internal standard. In a typical procedure, 6 μmol of dimethylformamide (10 μL of a 0.6 $\mu\text{mol}/\mu\text{L}$ anhydrous dimethylformamide in D_2O stock solution) was added to the conjugate of interest dissolved in 490 μL of D_2O in an NMR tube. Analysis by ^1H NMR was performed on the AC-500, setting the proton relaxation delay to 4 s to properly integrate a broad range of proton relaxation rates. The mole amount of conjugate was determined using the integrals of the aromatic protons on the conjugate of interest and the internal standard. After analysis, the D_2O and dimethylformamide were removed by lyophilization, and the sample was redissolved to the indicated final concentration with purified water and stored in aliquots at -20°C until use.

4,7-Dioxa-1,10-decanedinitrile (1a). To a stirred solution of 2% (w/v) sodium hydroxide (5 mL) and ethylene glycol (5.0 g, 81 mmol) at 0°C was added acrylonitrile (12.8 g, 243 mmol) over 10 min. The mixture was allowed to stir at 0°C for 1 h, followed by stirring overnight at room temperature. Dichloromethane (75 mL) was added, and the organic layer was washed $1\times$ (5 mL) with water and $2\times$ (5 mL each) with a dilute brine solution and dried (MgSO_4). The solvent was removed by rotary evaporation. The oily residue was treated with 25 mL of anhydrous ethanol, and the solvent was removed by rotary evaporation. This was repeated $2\times$ to remove excess unreacted acrylonitrile, followed by removal of solvent by rotary evaporation and drying in vacuo. The resulting clear, colorless oil (13.5 g, 97%) was used without further purification. R_f 0.59, 9:1 dichloromethane/methanol. ^1H NMR (CDCl_3) δ 3.75 (m, 4H), 3.69 (s, 4H), 2.64 (q, 4H). ESI-MS ($\text{M}+\text{Na}^+$): 191.02

4,8-Dioxa-1,11-undecanedinitrile (1b). As for **1a**, using 1,3-propanediol. Yield 95%, R_f 0.63, 9:1 dichloromethane/methanol. ^1H NMR (CDCl_3) δ 3.75 (m, 4H), 3.69 (s, 4H), 2.64 (q, 4H), 1.80 (m, 2H). ESI-MS ($\text{M}+\text{Na}^+$): 205.04.

4,7,10,13-Tetraoxa-1,16-hexadecanedinitrile (1c). As for **1a**, using triethylene glycol. Yield 92%, R_f 0.46, 9:1 dichloromethane/methanol. ^1H NMR (CDCl_3) δ 3.75 (m, 4H), 3.70 (s, 16H), 2.65 (q, 4H). ESI-MS ($\text{M}+\text{Na}^+$): 279.07.

[3,3,8,8- ^2H]-4,7-Dioxa-1,10-decanedinitrile (1a $_d$). Ethylene glycol (0.5 g, 8 mmol) was added to 0.1 g of dry potassium hydroxide in 20 mL of acetonitrile, followed by 1.4 g (24 mmol) of d_3 -acrylonitrile (Cambridge Isotope Laboratories, Andover, MA) with stirring overnight at room temperature. The reaction was filtered, and the solvent was removed by rotary evaporation to yield an oil. Final product was purified by silica chromatography (R_f 0.59, 9:1 chloroform/methanol) to yield 0.9 g (65%) of a colorless oil. ^1H NMR (CDCl_3) δ 3.69 (s, 4H), 2.64 (q, 4H). ESI-MS ($\text{M}+\text{Na}^+$): 195.03.

4,7-Dioxa-1,10-decanediamine (2a). Raney nickel (50 mg) was washed 5 times with anhydrous methanol by inversion and decantation and placed in 20 mL of

anhydrous methanol in a 50 mL screw-cap vial. A small hole (~3 mm) was drilled in the center of the cap, which was covered from the inside with a Teflon-lined rubber septum (2 mm thickness). **1a** (1 g, 6 mmol) was added, and the vial headspace was purged with H₂ gas via an 16-gauge needle piercing the septum. The cap was screwed on tightly, and the entire assembly was charged to 40 psi with H₂ gas through the needle and placed in a hot water bath (80 °C) for 4 h, after which the solid catalyst was removed by filtration over diatomaceous earth. The filtrate was dried by rotary evaporation to yield an oil which was purified by preparative reversed-phase HPLC (preparative column, gradient 0–10 min, 0% B; 10–50 min, 0–18% B, *t_R* = 18.4 min) to yield 94 mg (9%) of a colorless oil. ¹H NMR (D₂O) δ 3.59 (s, 4H), 3.56 (t, 4H), 1.66 (broad m, 8H). ESI-MS (M+H)⁺: 177.02.

[3,3,8,8-²H]-4,7-Dioxa-1,10-decanediamine (**2a_d**). As done previously for **2a**, using **1a_d**. Yield 11%. ¹H NMR (D₂O) δ 3.56 (s, 4H), 2.96 (t, 4H), 1.68 (t, 4H). ESI-MS (M+H)⁺: 181.14.

Diethyl 4,8-Dioxa-1,11-undecanedioate (**3b**). Nitrile **1b** (2 g, 12.0 mmol) was dissolved in 7 mL of 95% ethanol with stirring at 0 °C. Then 1.5 mL of concentrated sulfuric acid was added slowly over 5 min. The reaction was heated to reflux, and allowed to stir at reflux for 24 h. The mixture was cooled to room temperature, and the solvent was removed by rotary evaporation. The resulting material was extracted with 50 mL of methylene chloride, washed 2× with 5 mL of water and 3× with 5 mL of brine. The organic layer was dried (MgSO₄), and solvent was removed to yield an oil. The oil was dissolved in 95% ethanol with stirring at 0 °C. Concentrated sulfuric acid (1 mL) was added over 5 min, and the mixture was returned to reflux for an additional 24 h. The reaction was cooled, extracted, washed, and dried as done previously, and the final product was purified by silica chromatography (gradient dichloromethane/ethyl acetate/acetone) to yield 2.5 g (80%) of product as a colorless oil. *R_f* 0.76, 9:1 dichloromethane/methanol. ¹H NMR (CDCl₃) δ 4.14 (m, 4H), 3.73 (m, 4H), 3.60 (s, 4H), 2.58 (m, 4H), 1.78 (m, 2H), 1.26 (t, 6H). ESI-MS (M+H)⁺: 277.08, (M+Na)⁺: 299.12, (M+K)⁺: 315.11.

Diethyl 4,7,10,13-Tetraoxa-1,16-hexadecanedioate (**3c**). As done previously for **3b**, using nitrile **1c**. Yield 72%, *R_f* 0.59, 9:1 dichloromethane/methanol. ¹H NMR (CDCl₃) δ 4.14 (m, 4H), 3.73 (m, 4H), 3.60 (s, 12H), 2.58 (m, 4H), 1.26 (t, 6H). ESI-MS (M+H)⁺: 351.13, (M+Na)⁺: 373.16, (M+K)⁺: 389.19.

4,8-Dioxa-1,11-undecanediol (**4b**). Lithium aluminum hydride (0.66 g, 16.5 mmol) was slowly added to 100 mL of distilled [lithium aluminum hydride] tetrahydrofuran under argon with stirring at 0 °C. The suspension was allowed to warm to room temperature and stirred for an additional 15 min. **3b** (1.7 g, 6.5 mmol) (previously dried in vacuo in the presence of P₂O₅ and dissolved in 5 mL of distilled [lithium aluminum hydride] tetrahydrofuran) was added dropwise over 10 min. The mixture was stirred an additional 60 min, followed by cooling to 0 °C and dropwise addition of ethanol to quench excess hydride. Salts were precipitated by dropwise addition of saturated sodium sulfate solution with efficient stirring until a white precipitate formed (ca. 6 mL). The organic solvent was decanted, the white precipitate was triturated 4× with 15 mL of tetrahydrofuran, the combined organic extracts were removed by rotary evaporation to yield an oil which was redissolved in 10 mL of dichloromethane and dried (MgSO₄), and solvent was again removed. The final product was purified by silica chromatography

(dichloromethane/ethyl acetate/acetone) to yield 0.9 g (79%) of a colorless oil. *R_f* 0.46, 9:1 dichloromethane/methanol. ¹H NMR (CDCl₃) δ 3.74 (m, 4H), 3.66 (t, 4H), 3.59 (s, 4H), 2.90 (bs, 2H), 1.80 (m, 6H). ESI-MS (M+H)⁺: 193.16, (M+Na)⁺: 215.08.

4,7,10,13-Tetraoxa-1,16-hexadecanediol (**4c**). As done previously for **4b**, using **3c**. Yield 81%, *R_f* 0.33, 9:1 dichloromethane/methanol. ¹H NMR (CDCl₃) δ 3.72 (m, 4H), 3.60 (t, 12H), 3.59 (s, 4H), 2.65 (bs, 2H), 1.82 (m, 4H). ESI-MS (M+H)⁺: 267.21, (M+Na)⁺: 289.16.

4,8-Dioxa-1,11-dichloroundecane (**5b**). **4b** (1.1 g, 4.9 mmol, previously dried in vacuo in the presence of P₂O₅) was dissolved in 25 mL of dry [activated 4A Linde molecular sieves] benzene with stirring under argon. Distilled [CaH₂] pyridine (1.15 g, 14.6 mmol) was added, followed by cooling to 0 °C and dropwise addition of 1.8 g (14.6 mmol) of thionyl chloride in 5 mL of dry benzene. The mixture was warmed to 50 °C and allowed to stir under argon overnight, followed by cooling to 0 °C and addition of 7 mL of 3 M HCl with vigorous stirring. The organic layer was separated, washed 3× with dilute brine, and dried (Na₂SO₄), and solvent was removed by rotary evaporation to yield 1.2 g (86%) of a yellowish oil. The resulting product was used without further purification. *R_f* 0.82, 9:1 dichloromethane/methanol. ¹H NMR (CDCl₃) δ 3.63 (t, 4H), 3.54 (m, 8H), 2.02 (m, 4H), 1.83 (m, 2H). ESI-MS (M+H)⁺: 229.10, (M+Na)⁺: 251.18.

4,7,10,13-Tetraoxa-1,16-dichlorohexadecane (**5c**). As done previously for **5b**, using **4c**. Yield 81%, *R_f* 0.64, 9:1 dichloromethane/methanol. ¹H NMR (CDCl₃) δ 3.65 (t, 4H), 3.54 (m, 16H), 2.01 (m, 4H), 1.83 (m, 2H). ESI-MS (M+H)⁺: 229.10, (M+Na)⁺: 251.18, (M+K)⁺: 267.09.

4,8-Dioxa-1,11-undecanedinitrile (**6b**). Sodium cyanide (0.78 g, 15.5 mmol) in 6 mL of dimethyl sulfoxide was warmed to 80 °C with stirring. After 30 min, 1 g (3.9 mmol) of **5b** was added dropwise as a neat oil. After 2 h, the reaction was cooled slightly and treated with 10 mL of saturated sodium chloride solution, 5 mL of deionized water, and 50 mL of ethyl acetate. After extraction, the organic layer was washed 3× with a brine solution as before and dried (Na₂SO₄), followed by removal of solvent by rotary evaporation. The final product was purified by silica chromatography (gradient dichloromethane/ethyl acetate/acetone) to yield 0.6 g (64%) of product as a colorless oil. *R_f* 0.68, 9:1 dichloromethane/methanol. ¹H NMR (CDCl₃) δ 3.56–3.67 (m, 8H), 2.48 (t, 4H), 1.92 (qint, 4H), 1.81 (qint, 2H). ESI-MS (M+Na)⁺: 233.05.

4,7,10,13-Tetraoxa-1,16-hexadecanedinitrile (**6c**). As done previously for **6b**, using **5c**. Yield 68%, *R_f* 0.55, 9:1 dichloromethane/methanol. ¹H NMR (CDCl₃) δ 3.56–3.67 (m, 16H), 2.44 (t, 4H), 1.92 (qint, 4H). ESI-MS (M+Na)⁺: 307.14.

5,9-Dioxa-1,13-tridecanediamine (**7b**). Lithium aluminum hydride (0.87 g, 20.8 mmol) was carefully added to 150 mL of distilled [lithium aluminum hydride] tetrahydrofuran at 0 °C with stirring under an argon atmosphere. The suspension was heated to gentle reflux under argon for 30 min, followed by addition of 0.5 g (2.6 mmol) of **6b** (previously dried in vacuo in the presence of P₂O₅ and dissolved in 7 mL of distilled [lithium aluminum hydride] tetrahydrofuran) dropwise over 20 min while maintaining reflux by additional heat. After complete addition, the reaction was refluxed an additional 2 h, followed by cooling to 0 °C and dropwise addition of ethanol to quench excess hydride. Salts were precipitated by dropwise addition of saturated sodium sulfate solution with efficient stirring until a white precipitate formed (ca. 8 mL). The organic solvent was decanted, the white precipitate was triturated 4× with 15 mL of tetrahydro-

thian, and the combined organic extracts were evaporated by rotary evaporation to yield an oil which was redissolved in 20 mL of dichloromethane and dried (MgSO_4), and solvent was again removed. Final product was purified by preparative reversed-phase HPLC (gradient 0–10 min, 0% B; 10–50 min, 0–18% B; t_R = 20.5 min) to yield 0.35 g (71%) of a colorless oil. ^1H NMR (D_2O) δ 3.57 (s, 4H), 3.51 (t, 4H), 2.98 (t, 4H), 1.83 (m, 2H), 1.62 (m, 8H). ESI-MS ($\text{M}+\text{H}^+$) $^+$: 219.08.

[1,1,13,13- ^2H]-5,9-Dioxa-1,13-tridecanediamine (**7b_d**). As done previously for **7b**, except lithium aluminum deuteride was used in place of lithium aluminum hydride. Yield 74%. Preparative reversed-phase HPLC, gradient 0–10 min, 0% B; 10–50 min, 0–18% B; t_R = 20.7 min. ^1H NMR (D_2O) δ 3.57 (s, 4H), 3.51 (t, 4H), 1.62 (m, 8H). ESI-MS ($\text{M}+\text{H}^+$) $^+$: 223.11.

5,8,11,14-Tetraoxa-1,18-octadecanedi-amine (**7c**). As done previously for **7b** using **6c**. Yield 64%. Preparative reversed-phase HPLC, gradient 0–10 min, 0% B; 10–50 min, 0–18% B; t_R = 22.2 min. ^1H NMR (D_2O) δ 3.56 (s, 12H), 3.51 (t, 4H), 2.98 (t, 4H), 1.62 (m, 8H). ESI-MS ($\text{M}+\text{H}^+$) $^+$: 293.15.

[1,1,13,13- ^2H]-5,8,11,14-Tetraoxa-1,18-octadecanedi-amine (**7c_d**). As done previously for **7b_d** using **6c**. Yield 68%. Preparative reversed-phase HPLC, gradient 0–10 min, 0% B; 10–50 min, 0–18% B; t_R = 22.5 min. ^1H NMR (D_2O) δ 3.57 (s, 4H), 3.50 (t, 4H), 1.62 (m, 8H). ESI-MS ($\text{M}+\text{H}^+$) $^+$: 297.05.

7-Acetoxycoumarin-4-acetic Acid (**8**). To 100 mg (455 μmol) of 7-hydroxycoumarin-4-acetic acid in 2 mL of acetic anhydride with stirring at room temperature as a slurry was added 20 μL of boron trifluoride/diethyl etherate. A clear, homogeneous solution was observed after 2 min. After stirring for an additional 10 min, the mixture was diluted with 10 mL of water and shaken vigorously for 10 min to hydrolyze any mixed anhydride formed (R_f 0.87, dichloromethane/methanol/acetic acid 9:1:0.1). The resulting suspension was diluted with 30 mL of dichloromethane, washed 2 $\times$ with 10 mL of dilute brine solution, and dried (MgSO_4). The solvent was removed by rotary evaporation to yield a semisolid that was further dried in vacuo in the presence of KOH to yield 122 mg (96%) of product as an off-white solid. R_f 0.53, 9:1:0.1 dichloromethane/methanol/acetic acid. ^1H NMR (d_6 -DMSO) δ 7.74 (d, 1H), 7.29 (d, 1H), 7.17 (dd, 1H), 6.48 (s, 1H), 3.93 (s, 2H), 2.28 (s, 3H).

7-Acetoxycoumarin-4-acetic Acid *tert*-Butyl Ester (**9**). To 183 mg (1.5 mmol) of anhydrous magnesium sulfate in 4 mL of distilled [CaH_2] dichloromethane under argon was added 10 μL of concentrated sulfuric acid with stirring at room temperature. After 60 min, 146 μL (1.5 mmol) of distilled [CaH_2] *tert*-butyl alcohol was added, followed immediately by 50 mg (190 μmol) of **8** in 1 mL of distilled [CaH_2] dichloromethane. The reaction flask was tightly sealed and allowed to stir at room temperature for 15 h. The mixture was diluted with 50 mL of dichloromethane and filtered. Silica gel (1 g) was added to the filtrate, and the solvent was removed by rotary evaporation. The dried silica gel was loaded onto a silica column (dichloromethane), and the product was eluted (5:1 dichloromethane/ethyl acetate). After removal of solvent by rotary evaporation, the resulting product was dried in vacuo to yield 46 mg of a white solid (77%). R_f 0.91, 9:1 dichloromethane/methanol. ^1H NMR (d_6 -acetone) δ 7.77 (d, 1H), 7.16 (m, 2H), 6.41 (s, 1H), 3.88 (s, 2H), 2.29 (s, 3H), 1.43 (s, 9H).

7-Hydroxycoumarin-4-acetic Acid *tert*-Butyl Ester (**10**). To 470 mg of **9** in 8 mL of tetrahydrofuran with stirring at room temperature was added 3 mL of saturated

methanolic ammonia in a tightly sealed flask. The mixture was allowed to stir for an additional 3 h, followed by dilution with 25 mL of dichloromethane, addition of 2.0 g of silica gel, and removal of solvent by rotary evaporation. The resulting silica gel mixture was loaded onto a silica column (dichloromethane), and eluted (5:1 dichloromethane/acetone). Purified fractions were combined, and solvent was removed to yield 390 mg of a white solid (96%). R_f 0.68, 9:1 dichloromethane/methanol. ^1H NMR (d_6 -acetone) δ 9.42 (s, 1H), 7.58 (d, 1H), 6.87 (dd, 1H), 6.76 (d, 1H), 6.17 (s, 1H), 3.80 (s, 2H), 1.41 (s, 9H).

7-O- α -D-(2-Deoxy-2-*N*-trifluoroacetamido-3,4,6-tri-O-acetyl glucopyranos-1-yl)-coumarin-4-acetic Acid *tert*-Butyl Ester (**11**). This compound was prepared as described in the literature (13). 7-Trimethylsilyloxy-4-*tert*-butoxyacetic acid ester was prepared as described for 7-hydroxycoumarin using **10** that was previously dried in vacuo in the presence of P_2O_5 , and used without characterization. R_f 0.89 (α -anomer), 0.44 (β -anomer); 9:1 dichloromethane/methanol; UV. α -Anomer: ^1H NMR (d_6 -acetone) δ 8.75 (d, 1H), 7.77 (d, 1H), 7.26 (m, 2H), 6.36 (s, 1H), 6.03 (d, J = 3.7 Hz, 1H), 5.61 (t, 1H), 5.24 (t, 1H), 4.68 (td, 1H), 4.32 (t, 1H), 4.24 (dd, 1H), 4.14 (dd, 1H), 3.90 (s, 2H), 2.06 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H), 1.47 (s, 9H).

7-O- α -D-(2-Deoxy-2-*N*-trifluoroacetamido-3,4,6-tri-O-acetyl glucopyranos-1-yl)-coumarin-4-acetic Acid (**12**). To 54 mg (82 μmol) of **11** (previously dried in vacuo overnight in the presence of P_2O_5) in 8 mL of distilled [CaH_2] dichloromethane was added 0.5 mL of distilled [P_2O_5] trifluoroacetic acid with stirring under argon. The mixture was warmed to 50 $^\circ\text{C}$ and allowed to stir overnight, followed by removal of solvent by rotary evaporation using a water bath at room temperature (~ 20 $^\circ\text{C}$) in a rotary evaporator previously flushed with dry nitrogen for 45 min. The resulting semisolid was rediluted with 5 mL of distilled dichloromethane and again dried by rotary evaporation. This was repeated twice, followed by addition of 5 mL of dichloromethane and 0.4 g of silica gel, and the solvent was removed by rotary evaporation. The resulting powder was loaded onto a short silica column (dichloromethane), and the final product was eluted (gradient dichloromethane/acetone/methanol). After removal of solvent by rotary evaporation, the final product was dried in vacuo to yield an off-white solid that was triturated in 1 mL of ether and dried by vacuum centrifugation to yield 41 mg (82%) of product. R_f 0.28, 9:1:0.1 dichloromethane/methanol/acetic acid. ^1H NMR (d_6 -acetone) δ 8.70 (d, 1H), 7.75 (d, 1H), 7.20 (m, 2H), 6.35 (s, 1H), 5.96 (d, J = 3.6 Hz, 1H), 5.56 (t, 1H), 5.19 (t, 1H), 4.62 (m, 1H), 4.28 (t, 1H), 4.18 (dd, 1H), 4.09 (dd, 1H), 3.93 (s, 2H), 2.00 (s, 3H), 1.99 (s, 3H), 1.96 (s, 3H).

Conjugate of **12** and **7b** (**13b**). To 5 mg (8.3 μmol) of **12** (previously dried in vacuo in the presence of P_2O_5) in a dry, 4 mL screw-cap vial was added 300 μL of anhydrous dimethylformamide under argon. The vial was agitated by orbital shaking to effect dissolution, followed by vacuum centrifugation to remove solvent. This was repeated, followed by additional drying in vacuo overnight in the presence of P_2O_5 . The film was dissolved in 300 μL of anhydrous dimethylformamide, followed by addition of 7 μL (17 μmol) of anhydrous pyridine and three Linde type-4A molecular sieves (previously dried for 48 h at 150 $^\circ\text{C}$). After orbital shaking for 2 h at room temperature, the solution was treated with 3 μL of tetrafluorophenyl trifluoroacetate and agitated for an additional 45 min. The solution was removed from the vial with a dry syringe and transferred to another 4 mL

screw-cap vial containing 10 mg (50 μ mol) of **7b** (previously dried in vacuo in the presence of P_2O_5), three activated molecular sieves, and 10 μ L of dry, distilled $[CaH_2]$ triethylamine in 200 μ L of anhydrous dimethylformamide under argon. After 2 h of additional agitation, the molecular sieves were washed with 0.5 mL of methanol, and the combined solvents were removed by vacuum centrifugation to yield a crude film which was redissolved in purified water. The pH was adjusted to 3–4 with trifluoroacetic acid, and the product was purified by reversed-phase HPLC [preparative column, gradient (2), t_R = 44.1 min]. The final product was dried by vacuum centrifugation to yield 5.5 mg (85%) of a thin, colorless film. ^1H NMR (d_6 -acetone) δ 8.75 (d, 1H), 7.84 (m, 1H), 7.20 (m, 2H), 6.3 (s, 1H), 5.96 (d, 1H), 5.56 (t, 1H), 5.20 (t, 1H), 4.62 (m, 1H), 4.28 (dd, 1H), 4.1 (m, 1H), 4.10 (dd, 1H), 3.78 (s, 2H), 3.34–3.65 (broad m, 12H), 3.24 (t, 2H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 6H), 1.45 (m, 4H). ESI-MS ($M+H^+$) $^+$: 804.70.

Conjugate of 12 and 7b_d (13b_d). As done previously for **13b**, using **7b_d**. Yield 82%, preparative column, gradient (2), t_R = 44.4 min. ^1H NMR (d_6 -acetone) δ 8.75 (d, 1H), 7.84 (m, 1H), 7.20 (m, 2H), 6.3 (s, 1H), 5.96 (d, 1H), 5.56 (t, 1H), 5.20 (t, 1H), 4.62 (m, 1H), 4.28 (dd, 1H), 4.1 (m, 1H), 4.10 (dd, 1H), 3.78 (s, 2H), 3.34–3.65 (broad m, 8H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 6H), 1.45 (m, 4H). ESI-MS ($M+H^+$) $^+$: 808.73.

Conjugate of 12 and 7c (13c). As done previously for **13b**, using **7c** (81%). Preparative column, gradient (2), t_R = 44.7 min. ^1H NMR (d_6 -acetone) δ 8.75 (d, 1H), 7.84 (m, 1H), 7.20 (m, 2H), 6.3 (s, 1H), 5.96 (d, 1H), 5.56 (t, 1H), 5.20 (t, 1H), 4.62 (m, 1H), 4.28 (dd, 1H), 4.1 (m, 1H), 4.10 (dd, 1H), 3.78 (s, 2H), 3.34–3.65 (broad m, 18H), 3.24 (t, 2H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 4H), 1.45 (m, 4H). ESI-MS ($M+H^+$) $^+$: 878.59.

Conjugate of 12 and 7c_d (13c_d). As done previously for **13b**, using **7c_d**. Yield 84%, preparative column, gradient (2), t_R = 44.9 min. ^1H NMR (d_6 -acetone) δ 8.75 (d, 1H), 7.84 (m, 1H), 7.20 (m, 2H), 6.3 (s, 1H), 5.96 (d, 1H), 5.56 (t, 1H), 5.20 (t, 1H), 4.62 (m, 1H), 4.28 (dd, 1H), 4.1 (m, 1H), 4.10 (dd, 1H), 3.78 (s, 2H), 3.34–3.65 (broad m, 16H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 4H), 1.45 (m, 4H). ESI-MS ($M+H^+$) $^+$: 882.61.

N-Methyl-N-biotinyl Glycine (14). The title compound was prepared by a modification of the method of Wilbur (7). Methyl N-methyl-N-biotinyl glycinate (7) was hydrolyzed in a mixture of 31 mL of methanol and 10 mL of 1 N sodium hydroxide at room temperature with stirring for 1 h. The mixture was diluted with 50 mL of 50% methanol/water and neutralized with cation-exchange resin, hydrogen form (BioRad). The solution was filtered, the resin was washed (3 $\times$ 50 mL) with 50% methanol/water, and the solvents were removed by rotary evaporation to yield 1.6 g (90%) of N-methyl-N-biotinyl glycine as an off-white solid. ^1H NMR (CD_3OD) δ 4.49 (m, 1H), 4.32 (m, 1H), 4.11 (d, 2H), 3.22 (m, 1H), 3.04 (d, 3H), 2.93 (dd, 1H), 2.71 (d, 1H), 2.39 (dt, 2H), 1.69 (m, 4H), 1.49 (m, 2H). ESI-MS ($M+H^+$) $^+$: 316.2.

Tetrafluorophenyl N-Methyl-N-biotinyl Glycinate (15). In a modification of a procedure by Wilbur (7), 2.5 mg (7.4 μ mol) of **14** (previously dried in vacuo in the presence of P_2O_5) was dissolved in 0.2 mL of anhydrous dimethylformamide containing three Linde type-4A molecular sieves (previously dried for 48 h at 150 $^{\circ}C$) by orbital shaking, under argon atmosphere. Then 5 μ L of distilled $[CaH_2]$ triethylamine was added, followed after 2 h of orbital shaking by 1.5 μ L (8.8 μ mol) of tetrafluorophenyl trifluoroacetate. The formation of active ester (15–30 min) was monitored by silica TLC (R_f 0.5, 5:1 dichloro-

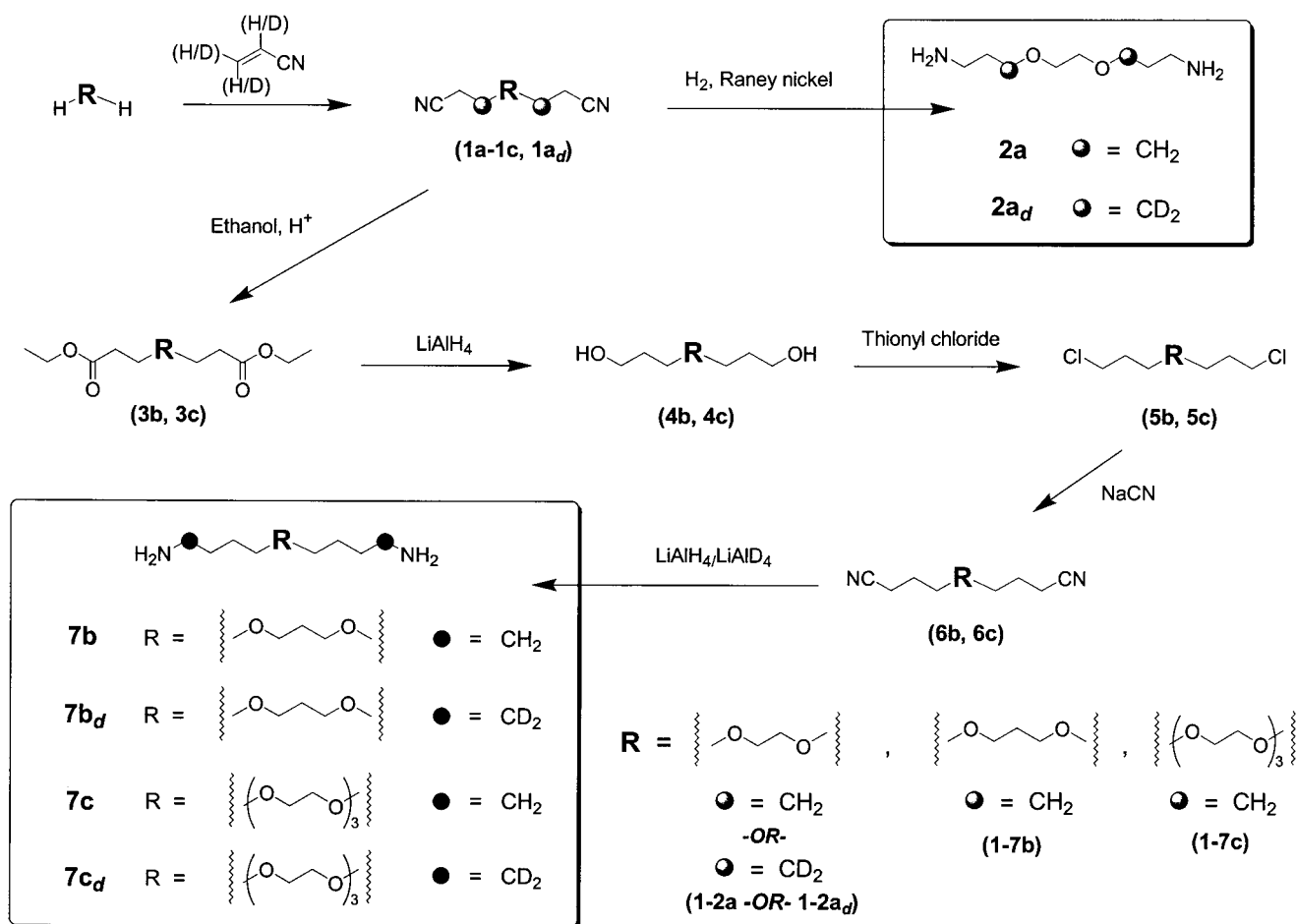
methane/methanol, UV) with brief drying of the spotted TLC plate with a stream of air prior to development. The active ester was used within 1 h and without further purification or characterization.

1-[N-(N-Methyl-N-biotinyl glycine)amido]-5,9-dioxo-13-tridecanamine (16). **7b** (42 mg, 0.2 mmol) was dried in vacuo in the presence of P_2O_5 overnight prior to use, followed by dissolution in 200 μ L of anhydrous dimethylformamide by orbital shaking under argon. Three Linde type-4A molecular sieves (previously dried for 48 h at 150 $^{\circ}C$) were added, followed by 5 μ L (0.03 mmol) of distilled $[CaH_2]$ triethylamine. After 2 h of orbital shaking, **15** [freshly prepared from 10 mg (0.03 mmol) of **14**, in dimethylformamide] was added dropwise by dry syringe, and the reaction was allowed to proceed for 1 h at room temperature while mixing with orbital shaking. The molecular sieves were removed from the reaction vessel and rinsed with 0.5 mL of methanol, and the combined organic solvents were removed by vacuum centrifugation at room temperature. The residue was reconstituted in water, the pH was adjusted to 3–4 with trifluoroacetic acid, and the final product was purified by reversed-phase HPLC (preparative column, gradient 0–5 min, 0% B, 5–60 min, 0–26% B, t_R = 47.2 min) and dried by vacuum centrifugation to yield 13 mg (82%) of a thin, colorless film. ^1H NMR (D_2O) δ 4.49 (m, 1H), 4.32 (m, 1H), 4.08 (d, 2H), 3.55 (m, 8H), 3.41 (t, 2H), 3.22 (m, 1H), 3.04 (d, 3H), 2.93 (dd, 1H), 2.88 (t, 2H), 2.71 (d, 1H), 2.39 (dt, 2H), 1.81 (m, 2H), 1.69 (m, 4H), 1.60 (m, 8H), 1.49 (m, 2H). ESI-MS ($M+H^+$) $^+$: 516.33.

Conjugate of 15 and 13b (17b). To 5.5 mg (6.8 μ mol) of **13b** (previously dried in vacuo in the presence of P_2O_5) in a dry, 4 mL screw-cap vial was added 300 μ L of anhydrous dimethylformamide under argon. The vial was agitated by orbital shaking to effect dissolution, followed by vacuum centrifugation to remove solvent. This was repeated, followed by additional drying in vacuo overnight in the presence of P_2O_5 . The film was dissolved in 200 μ L of anhydrous dimethylformamide, followed by 5 μ L of dry, distilled $[CaH_2]$ triethylamine and three Linde type-4A molecular sieves (previously dried for 48 h at 150 $^{\circ}C$) under argon. The mixture was agitated by orbital shaking for 2 h at room temperature, followed by addition of **15** [prepared from 2.2 mg (7 μ mol) of **14**, in dimethylformamide]. After shaking for an additional 2 h, the molecular sieves were removed from the reaction vessel and rinsed with 0.5 mL of methanol, and the combined organic solvents were removed by vacuum centrifugation at room temperature. The resulting residue was reconstituted in water, the pH was adjusted to 3–4 with trifluoroacetic acid, and the product was purified by reversed-phase HPLC [preparative column, gradient (2), t_R = 52.4 min] and dried by vacuum centrifugation to yield 4.5 mg (62%) of a thin, colorless film. ^1H NMR (d_6 -acetone) δ 8.82 (d, 1H), 7.89 (m, 1H), 7.22 (m, 2H), 6.36 (s, 1H), 6.00 (d, 1H), 5.62 (t, 1H), 5.22 (t, 1H), 4.68 (m, 1H), 4.60 (m, 1H), 4.41 (m, 1H), 4.33 (dd, 1H), 4.24 (m, 1H), 4.15 (dd, 1H), 4.04 (d, 2H), 3.80 (s, 2H), 3.40–3.65 (broad m, 10H), 3.25 (m, 3H), 3.08 (d, 3H), 2.99 (dd, 1H), 2.80 (d, 1H), 2.40 (dt, 2H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 4H), 1.70 (m, 4H), 1.45 (m, 6H). ESI-MS ($M+H^+$) $^+$: 1101.55, ($M+2H^+$) $^{2+}$: 551.62.

Conjugate of 15 and 13b_d (17b_d). As prepared previously for **17b**, using **13b_d**. Yield 70%, preparative column, gradient (2), t_R = 52.5 min. ^1H NMR (d_6 -acetone) δ 8.82 (d, 1H), 7.89 (m, 1H), 7.22 (m, 2H), 6.36 (s, 1H), 6.00 (d, 1H), 5.62 (t, 1H), 5.22 (t, 1H), 4.68 (m, 1H), 4.60 (m, 1H), 4.41 (m, 1H), 4.33 (dd, 1H), 4.24 (m, 1H), 4.15 (dd, 1H), 4.04 (d, 2H), 3.80 (s, 2H), 3.40–3.65 (broad m, 8H), 3.24

Scheme 1



(m, 1H), 3.08 (d, 3H), 2.99 (dd, 1H), 2.80 (d, 1H), 2.40 (dt, 2H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 4H), 1.70 (m, 4H), 1.45 (m, 6H). ESI-MS ($\text{M}+\text{H}^+$): 1105.65, ($\text{M}+2\text{H}^+$): 553.58.

Conjugate of 15 and 13c (17c). As prepared previously for 17b, using 13c. Yield 65%, preparative column, gradient (2), $t_R = 52.7$ min. ^1H NMR (d_6 -acetone) δ 8.82 (d, 1H), 7.89 (m, 1H), 7.22 (m, 2H), 6.36 (s, 1H), 6.00 (d, 1H), 5.62 (t, 1H), 5.22 (t, 1H), 4.68 (m, 1H), 4.60 (m, 1H), 4.41 (m, 1H), 4.33 (dd, 1H), 4.24 (m, 1H), 4.15 (dd, 1H), 4.04 (d, 2H), 3.80 (s, 2H), 3.40–3.65 (broad m, 18H), 3.25 (m, 3H), 3.08 (d, 3H), 2.99 (dd, 1H), 2.80 (d, 1H), 2.40 (dt, 2H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 4H), 1.70 (m, 4H), 1.45 (m, 6H). ESI-MS ($\text{M}+\text{H}^+$): 1175.79, ($\text{M}+2\text{H}^+$): 588.67.

Conjugate of 15 and 13c_d (17c_d). As prepared previously for 17b, using 13c_d. Yield 68%, preparative column, gradient (2), $t_R = 53.0$ min. ^1H NMR (d_6 -acetone) δ 8.82 (d, 1H), 7.89 (m, 1H), 7.22 (m, 2H), 6.36 (s, 1H), 6.00 (d, 1H), 5.62 (t, 1H), 5.22 (t, 1H), 4.68 (m, 1H), 4.60 (m, 1H), 4.41 (m, 1H), 4.33 (dd, 1H), 4.24 (m, 1H), 4.15 (dd, 1H), 4.04 (d, 2H), 3.80 (s, 2H), 3.40–3.65 (broad m, 16H), 3.24 (m, 1H), 3.08 (d, 3H), 2.99 (dd, 1H), 2.80 (d, 1H), 2.40 (dt, 2H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.83 (m, 4H), 1.70 (m, 4H), 1.45 (m, 6H). ESI-MS ($\text{M}+\text{H}^+$): 1179.59, ($\text{M}+2\text{H}^+$): 590.51.

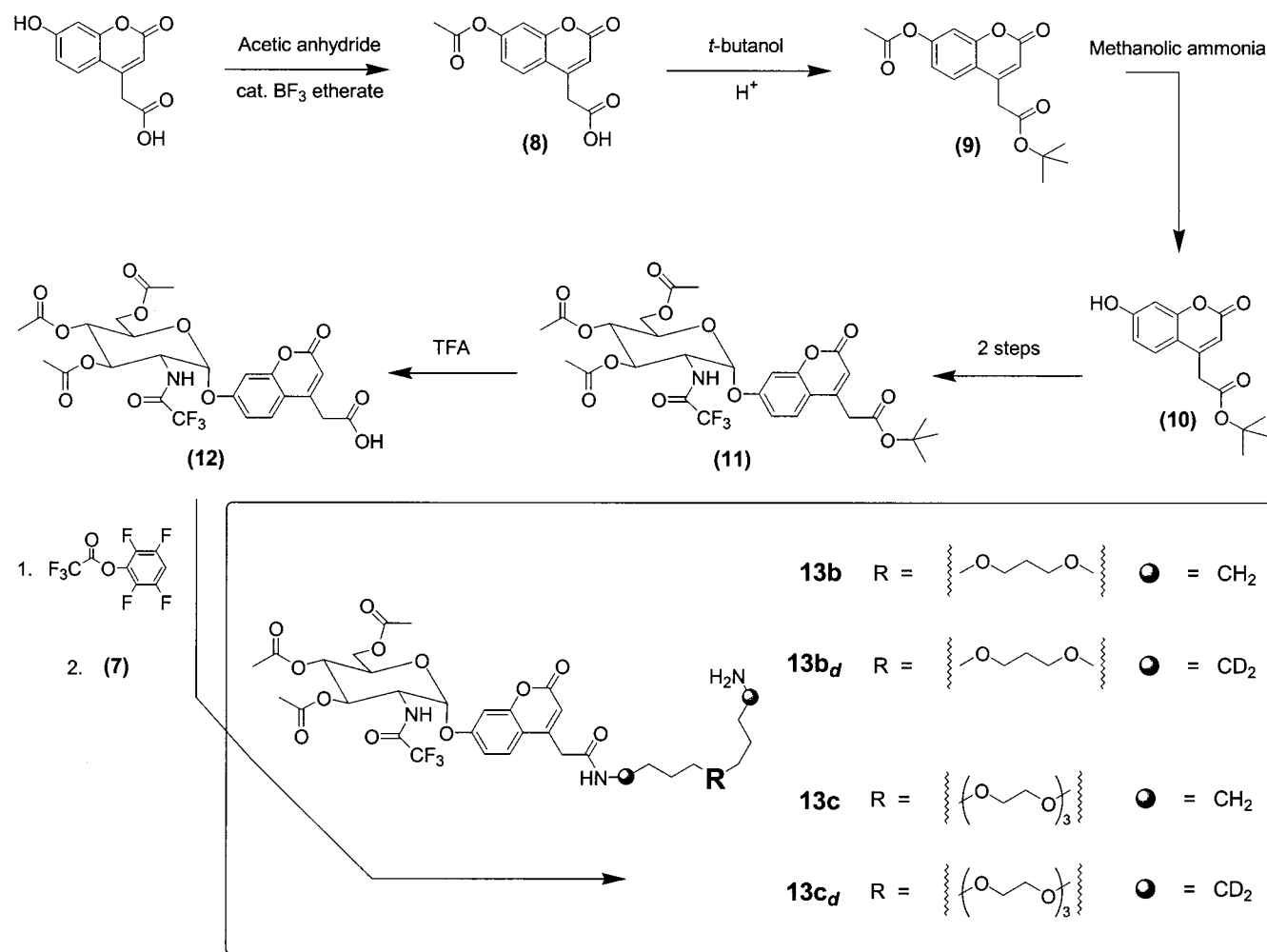
**p-Aminophenyl- α -D-2-deoxy-2-acetamidogluco-
pyranoside (20).** p-Nitrophenyl- α -D-2-deoxy-2-acetamidogluco-
pyranoside (18, 20 mg, 0.07 mmol, Sigma) was added to 5 mg of palladium on activated carbon (previously washed with methanol by inversion and decantation) in 3 mL of methanol with stirring in a 5 mL septum-lined

vial. The septum was pierced by a 16-gauge needle, through which the vial headspace was purged with H_2 gas. H_2 gas was allowed to slowly bubble through the solution for 2 h with stirring. After 45 min, all noncatalyst material was dissolved. The catalyst was removed by filtration over diatomaceous earth (Celite). The solvent was removed by rotary evaporation, and the product was further dried in vacuo to yield 18 mg of an off-white solid (90%). ^1H NMR (D_2O) δ 7.24 (d, 2H), 7.16 (d, 2H), 5.44 (d, 1H), 4.02 (dd, 1H), 3.88 (t, 1H), 3.68 (m, 3H), 3.49 (t, 1H), 1.97 (s, 3H). ESI-MS ($\text{M}+\text{H}^+$): 313.22.

**p-Aminophenyl- α -D-(2-deoxy-2-N-trifluoroacetamido)-
3,4,6-tri-O-acetylglucopyranoside (21).** As done previously for 20, using 19 which was prepared by the method of Voznyi et al. (13). Yield 86%, R_f 0.55, 2:1 dichloromethane/ethyl acetate, UV, ninhydrin. ^1H NMR (CDCl_3) δ 7.35 (d, 2H), 6.92 (d, 2H), 6.77 (d, 1H), 5.69 (d, $J = 3.1$ Hz, 1H), 5.48 (t, 1H), 5.26 (t, 1H), 5.50 (td, 1H), 4.22 (dd, 1H), 4.01 (m, 2H), 2.07 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H). ESI-MS ($\text{M}+\text{H}^+$): 493.29.

**p-Acrylamidophenyl- α -D-2-deoxy-2-acetamidogluco-
pyranoside (23).** 20 (10 mg, 0.03 mmol) was added to 15 mL of methanol and 100 μL of triethylamine was added with stirring. The solution was chilled in an ice bath. Acryloyl chloride (15 mg, 0.17 mmol) was dissolved in 2 mL of distilled [CaH_2] dichloromethane and added dropwise to the stirred solution over 5 min. The reaction was allowed to return to room temperature, followed by 2 h of stirring. The solution was treated with successive anion (hydroxide form) and cation (proton form) exchange resins until neutral pH was obtained with moist pH paper. Solvent was removed by rotary evaporation to yield an off-white

Scheme 2



solid (11 mg, 95%). ^1H NMR (D_2O) δ 7.36 (d, 2H), 7.03 (d, 2H), 6.32 (dd, 1H), 6.23 (d, 1H), 5.78 (d, 1H), 5.50 (d, 1H), 4.02 (dd, 1H), 3.88 (t, 1H), 3.68 (m, 3H), 3.49 (t, 1H), 1.94 (s, 3H). ESI-MS ($\text{M}+\text{Na}^+$) $^+$: 389.14.

p-Acrylamidophenyl- α -D-(2-deoxy-2-*N*-trifluoroacetamido)-3,4,6-tri-*O*-acetylglucopyranoside (**24**). As prepared previously for **23**, using **21**. Yield 87%, R_f 0.63, 9:1 dichloromethane/methanol. ^1H NMR (CDCl_3) δ 7.51 (d, 2H), 7.05 (d, 2H), 6.41 (d, 2H), 6.18 (dd, 1H), 5.75 (dd, 1H), 5.04 (d, 1H), 5.48 (t, 1H), 5.25 (t, 1H), 4.45 (td, 1H), 4.26 (dd, 1H), 4.08 (m, 2H), 2.06 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H).

p-Acrylamidophenyl- β -D-galactopyranoside (**25**). As prepared previously for **23**, using *p*-aminophenyl- β -D-galactopyranoside (**22**, Sigma). Yield 97%. ^1H NMR (*d*-7-dimethylformamide) δ 10.28 (s, 1H), 7.69 (d, 2H), 7.03 (d, 2H), 6.52 (dd, 1H), 6.27 (dd, 1H), 5.69 (dd, 1H), 5.18 (s, 1H), 4.83 (d, J = 10.3 Hz, 1H), 4.79 (s, 1H), 4.77 (s, 1H), 4.55 (s, 1H), 3.93 (t, 1H), 3.77 (m, 1H), 3.70 (m, 1H), 3.58 (m, 1H), 3.39 (s, 2H).

Michael Addition Product of 2a and 23 (26). **23** (20 mg, 0.07 mmol) was added to a stirred solution of 80 mg (0.35 mmol) of **2a** in 5 mL of 0.2 M sodium carbonate, pH 10.5, warmed to 37 °C. The reaction was allowed to continue for 3 days at 37 °C, after which the solution was neutralized with dilute trifluoroacetic acid in water, concentrated by rotary evaporation, and purified by reversed-phase HPLC (preparative column, gradient 0–10 min, 0% B, 10–50 min, 0–19% B, t_R = 33.5 min). The purified product was dried by vacuum centrifugation

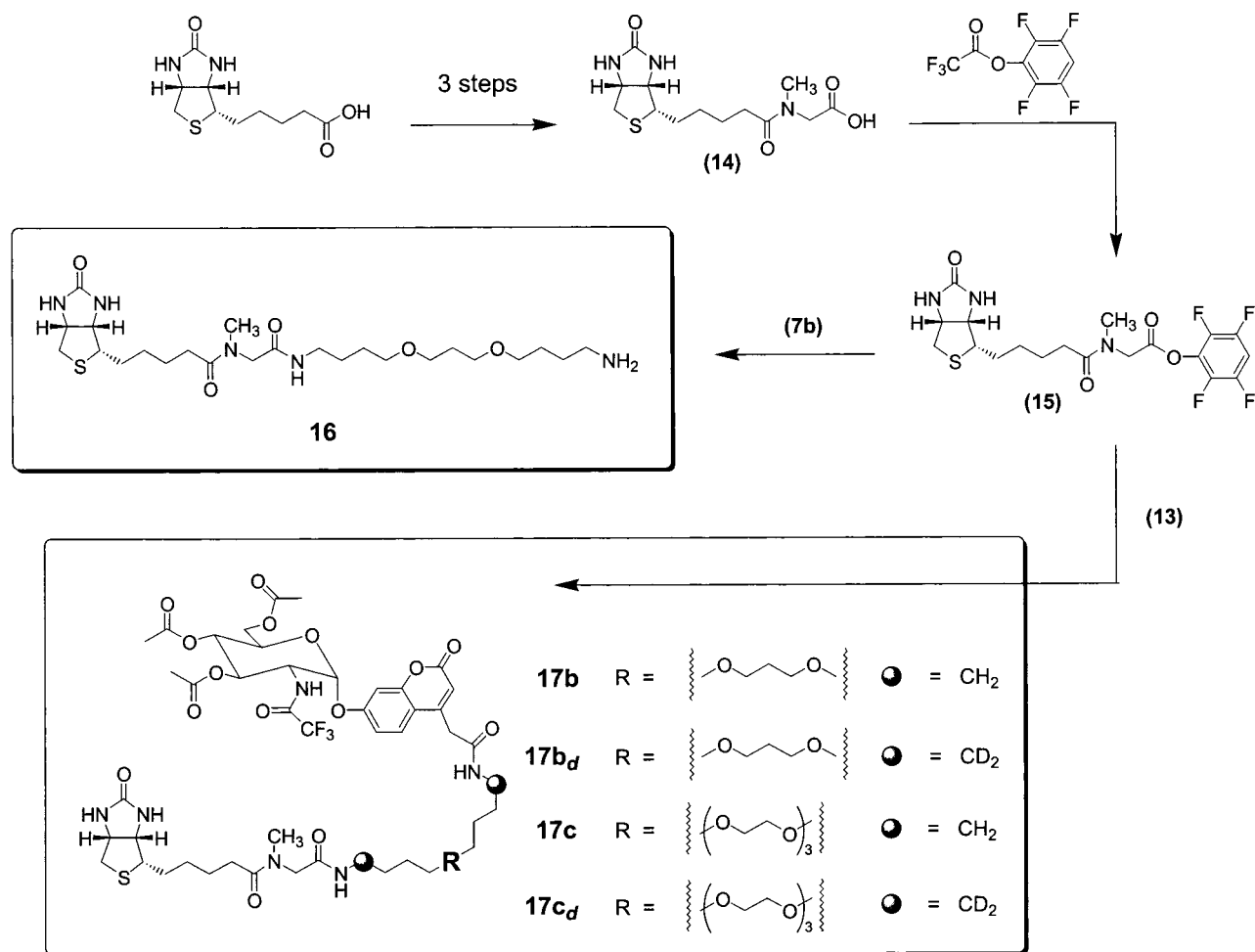
to yield 14.4 mg (38%) as a film. ^1H NMR (D_2O) δ 7.33 (d, 2H), 7.22 (d, 2H), 5.88 (d, 1H), 3.76 (t, 1H), 3.70 (m, 3H), 3.58 (m, 8H), 3.47 (m, 1H), 3.32 (m, 3H), 3.05 (t, 2H), 2.94 (t, 2H), 2.80 (t, 2H), 1.95 (s, 3H), 1.72 (m, 2H), 1.60 (m, 6H). ESI-MS ($\text{M}+\text{H}^+$) $^+$: 543.19, ($\text{M}+2\text{H}^+$) $^{2+}$: 272.09.

Michael Addition Product of 2ad and 25 (27). As done previously for **26**, using **25** and **2ad**. Yield 42%, preparative reversed-phase HPLC, gradient 0–10 min, 0% B, 10–50 min, 0–19% B, t_R = 32.9 min. ^1H NMR (*d*-7-dimethylformamide) δ 10.49 (s, 1H), 7.73 (d, 2H), 7.10 (d, 2H), 4.91 (d, 1H), 3.96 (d, 1H), 3.75 (m, 3H), 3.68 (m, 2H), 3.61 (m, 4H), 3.45 (m, 2H), 3.30 (s, 2H), 3.20 (s, 2H), 3.01 (t, 2H). ESI-MS ($\text{M}+\text{H}^+$) $^+$: 506.23, ($\text{M}+2\text{H}^+$) $^{2+}$: 253.60.

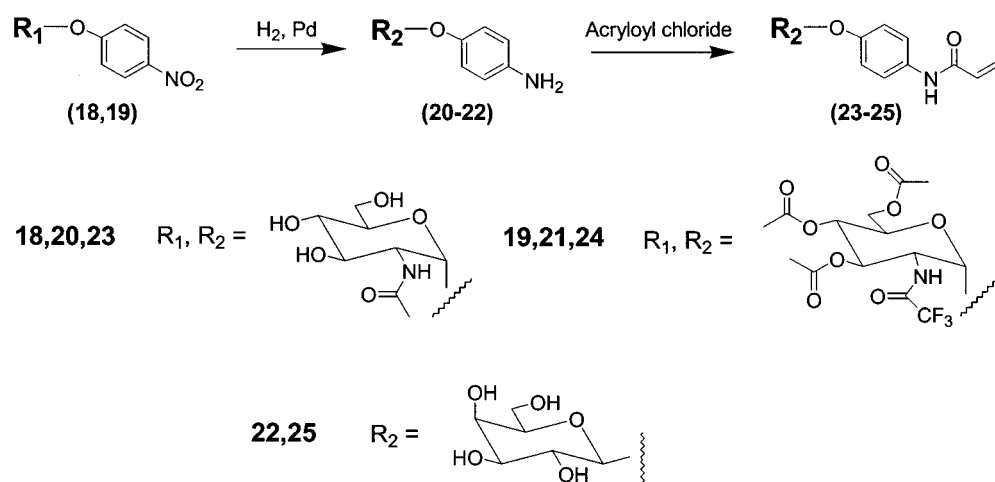
Michael Addition Product of 7ba and 23 (28). As done previously for **26**, using **23** and **7ba**. Yield 36%, preparative reversed-phase HPLC, gradient 0–10 min, 0% B, 10–50 min, 0–19% B, t_R = 33.8 min. ^1H NMR (D_2O) δ 7.33 (d, 2H), 7.22 (d, 2H), 5.88 (d, 1H), 3.76 (t, 1H), 3.70 (m, 3H), 3.58 (m, 8H), 3.47 (m, 1H), 3.32 (m, 3H), 3.05 (t, 2H), 2.94 (t, 2H), 2.80 (t, 2H), 1.95 (s, 3H), 1.72 (m, 2H), 1.60 (m, 6H). ESI-MS ($\text{M}+\text{H}^+$) $^+$: 589.25, ($\text{M}+2\text{H}^+$) $^{2+}$: 295.22.

Michael Addition Product of 16 and 24 (SFC Substrate) (29). As done previously for **26**, using **24** and **16**, except 3 mL of 0.2 M sodium carbonate was used, and **16** was added as a solution in 2 mL of methanol. Yield 32%, preparative reversed-phase HPLC, gradient 0–5 min, 0% B, 5–60 min, 0–22% B, t_R = 40.2 min, broadly eluting.

Scheme 3



Scheme 4

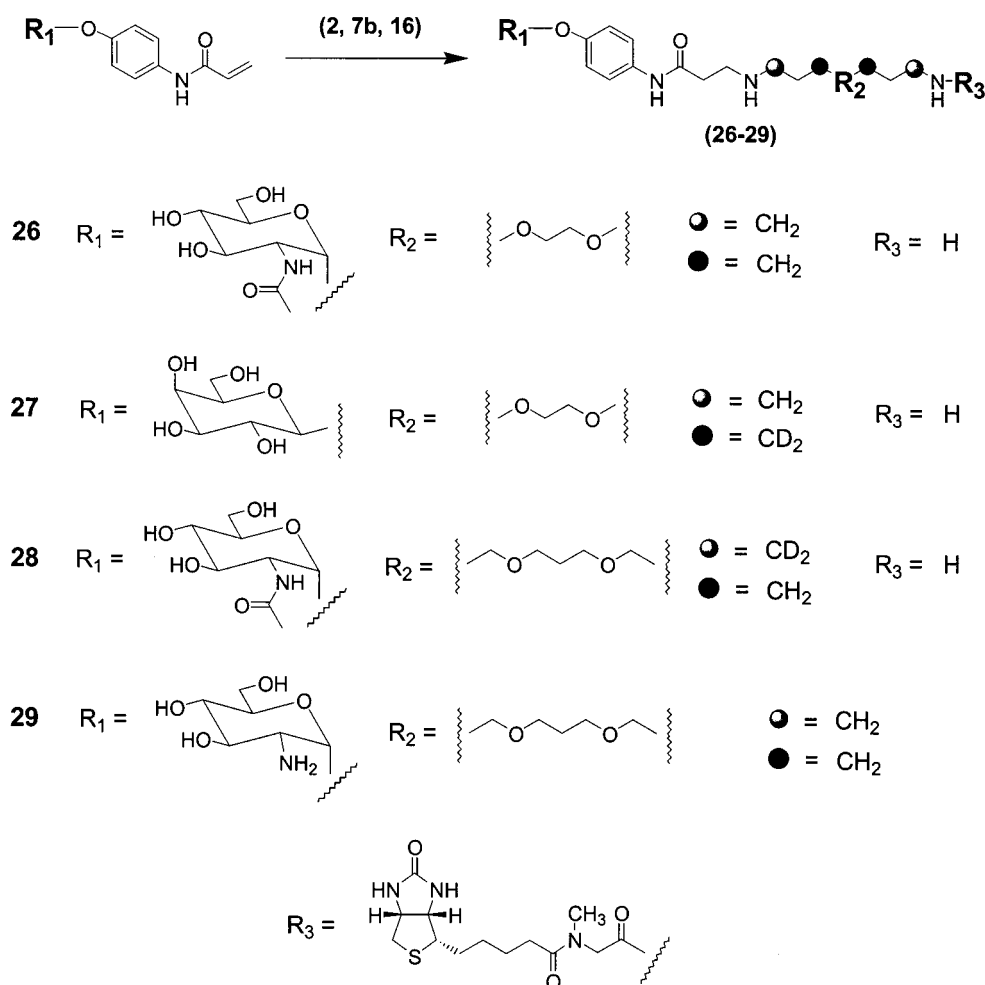


¹H NMR (D₂O) δ 7.40 (d, 2H), 7.18 (d, 2H), 5.82 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.07 (t, 1H), 4.05 (d, 2H), 3.80 (m, 3H), 3.55 (m, 10H), 3.38 (t, 2H), 3.32 (m, 3H), 3.05 (t, 2H), 3.00 (d, 3H), 2.94 (t, 2H), 2.80 (t, 2H), 2.40 (dt, 2H), 1.72 (m, 2H), 1.60 (m, 12H). ESI-MS (M+H)⁺: 840.57, (M+2H)²⁺: 420.72.

Conjugate of 15 and 27 (SFB Substrate) (30). To 3 mg (5.5 μ mol) of **26** previously dried in vacuo overnight in the presence of P₂O₅ was added 200 μ L of anhydrous dimethylformamide and three Linde type-4A molecular sieves (previously dried for 48 h at 150 °C) under argon to redissolve and further dry the film. Dry, distilled

[CaH₂] triethylamine was added (2 μ L), followed after 2 h of orbital shaking by **15** [prepared from 2 mg (5.5 μ mol) of **14**]. After an additional 2 h of orbital shaking, the molecular sieves were removed and washed with 0.5 mL of methanol, and the combined solvents were removed by vacuum centrifugation. The final product was purified by reversed-phase HPLC [preparative column, gradient (1), t_R = 30.5 min] and dried by vacuum centrifugation to yield 4.6 mg of a clear film (62%). After quantitation by ¹H NMR, the conjugate was diluted in purified water to 2.0 mM final concentration and frozen in aliquots at -20 °C until use. ¹H NMR (D₂O) δ 7.45 (d, 2H), 7.18 (d,

Scheme 5



2H), 5.06 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.11 (m, 2H), 4.05 (d, 3H), 3.80 (m, 1H), 3.55 (m, 9H), 3.51 (t, 2H), 3.41 (t, 2H), 3.26 (m, 3H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.90 (t, 2H), 2.77 (dd, 1H), 2.40 (dt, 2H), 2.05 (s, 3H), 1.78 (m, 4H), 1.60 (m, 4H), 1.42 (m, 2H). ESI-MS ($M+H^+$)⁺: 840.66, ($M+2H^+$)²⁺: 420.78.

Conjugate of 15 and 27 (for SFB Internal Standard) (31). As done previously for **30** using **27**. Yield 58%, preparative reversed-phase HPLC, gradient (1), $t_R = 29.6$ min. ¹H NMR (D_2O) δ 7.42 (d, 2H), 7.18 (d, 2H), 5.05 (d, 1H), 4.62 (m, 1H), 4.42 (m, 1H), 4.11 (d, 2H), 3.80 (t, 1H), 3.75 (m, 2H), 3.65 (m, 11H), 3.41 (t, 2H), 3.26 (m, 1H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.90 (t, 2H), 2.77 (dd, 1H), 2.40 (dt, 2H), 1.78 (m, 4H), 1.60 (m, 4H), 1.42 (m, 2H). ESI-MS ($M+H^+$)⁺: 803.55.

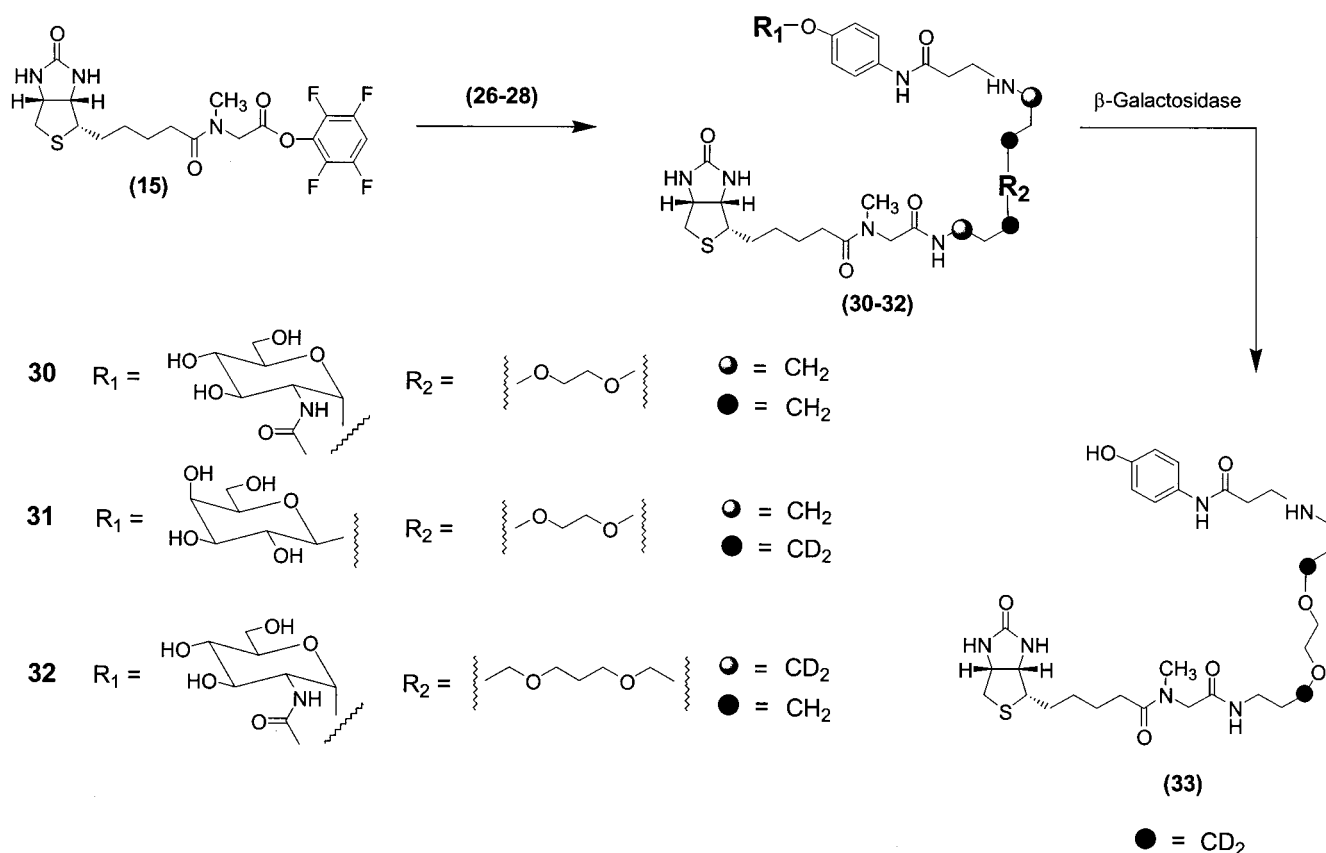
Conjugate of 15 and 28 (SFC Internal Standard) (32). As done previously for **30** using **28**. Yield 68%, preparative reversed-phase HPLC, gradient 0–5 min, 0% B, 5–60 min, 0–22% B, $t_R = 41.8$ min, broadly eluting. After quantitation by ¹H NMR, the conjugate was diluted in purified water to 1.0 mM final concentration and frozen in aliquots at -20°C until use. ¹H NMR (D_2O) δ 7.42 (d, 2H), 7.18 (d, 2H), 5.58 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.11 (m, 2H), 4.05 (d, 3H), 3.80 (m, 1H), 3.55 (m, 9H), 3.41 (t, 2H), 3.26 (m, 1H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.90 (t, 2H), 2.77 (dd, 1H), 2.40 (dt, 2H), 2.05 (s, 3H), 1.78 (m, 6H), 1.60 (m, 8H), 1.42 (m, 2H). ESI-MS ($M+H^+$)⁺: 886.77, ($M+2H^+$)²⁺: 443.64.

SFB Internal Standard Conjugate (33). **31** (1.8 mg) was dissolved in 2 mL of 100 mM Tris-HCl, 10 mM $MgCl_2$ buffer, pH 7.3, with stirring at 37°C . Recombinant β -D-

galactosidase (10 units, Sigma) was added, and after 12 h of additional stirring at 37°C , the mixture was acidified to pH 3–4 with trifluoroacetic acid and concentrated by rotary evaporation to 0.75 mL. The final product was purified by reversed-phase HPLC [semipreparative column, gradient (1), $t_R = 41.3$ min] and dried by vacuum centrifugation to yield 1.3 mg (89%) of a film. After quantitation by ¹H NMR, the conjugate was diluted in purified water to 1.0 mM final concentration and frozen in aliquots at -20°C until use. ¹H NMR (D_2O) δ 7.18 (d, 2H), 6.79 (d, 2H), 5.58 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 3.97 (d, 2H), 3.55 (m, 8H), 3.41 (t, 2H), 3.26 (m, 1H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.90 (t, 2H), 2.77 (dd, 1H), 2.40 (dt, 2H), 1.68 (m, 2H), 1.60 (m, 6H), 1.42 (m, 2H). ESI-MS ($M+H^+$)⁺: 641.51.

Deprotection of Conjugate 17b (34b). To **17b** was added 1 mL of saturated ammonia in methanol and 0.35 mL of 2 M ammonium hydroxide with stirring. The solution was heated to 45°C and allowed to stir overnight, followed by removal of solvent by vacuum centrifugation, redissolution in purified water, and pH adjustment to 3 with dilute trifluoroacetic acid and purification by reversed-phase HPLC [semipreparative column, gradient (2), $t_R = 32.0$ min]. The final product was dried by vacuum centrifugation to yield 3 mg (90%) as a thin, colorless film. ¹H NMR (D_2O) δ 7.71 (d, 1H), 7.26 (d, 1H), 7.19 (dd, 1H), 6.37 (s, 1H), 5.99 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.09 (t, 1H), 4.05 (d, 2H), 3.85 (s, 2H), 3.75 (m, 3H), 3.60 (m, 10H), 3.41 (t, 2H), 3.26 (m, 5H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.77 (dd, 1H), 2.40 (dt, 2H), 1.69

Scheme 6



(t, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS ($M+H^+$): 879.79, ($M+2H^+$): 440.41.

Deprotection of Conjugate 17b_d (34b_d). As done previously for 17b, using 17b_d. Yield 82%, semipreparative column, gradient (2), $t_R = 32.2$ min. ^1H NMR (D_2O) δ 7.71 (d, 1H), 7.26 (d, 1H), 7.19 (dd, 1H), 6.37 (s, 1H), 5.99 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.09 (t, 1H), 4.05 (d, 2H), 3.85 (s, 2H), 3.75 (m, 3H), 3.60 (m, 10H), 3.41 (t, 2H), 3.26 (m, 1H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.77 (dd, 1H), 2.40 (dt, 2H), 1.69 (t, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS ($M+H^+$): 883.77, ($M+2H^+$): 442.42.

Deprotection of Conjugate 17c (34c). As done previously for 17b, using 17c. Yield 92%, semipreparative column, gradient (2), $t_R = 32.6$ min. ^1H NMR (D_2O) δ 7.71 (d, 1H), 7.26 (d, 1H), 7.19 (dd, 1H), 6.37 (s, 1H), 5.99 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.09 (t, 1H), 4.05 (d, 2H), 3.85 (s, 2H), 3.75 (m, 3H), 3.60 (m, 18H), 3.41 (t, 2H), 3.26 (m, 5H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.77 (dd, 1H), 2.40 (dt, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS ($M+H^+$): 953.74, ($M+2H^+$): 477.47.

Deprotection of Conjugate 17c_d (SFA Internal Standard Conjugate) (34c_d). As done previously for 17b, using 17c_d. Yield 88%, semipreparative column, gradient (2), $t_R = 32.8$ min. ^1H NMR (D_2O) δ 7.71 (d, 1H), 7.26 (d, 1H), 7.19 (dd, 1H), 6.37 (s, 1H), 5.99 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.09 (t, 1H), 4.05 (d, 2H), 3.85 (s, 2H), 3.75 (m, 3H), 3.60 (m, 18H), 3.41 (t, 2H), 3.26 (m, 1H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.77 (dd, 1H), 2.40 (dt, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS ($M+H^+$): 957.67, ($M+2H^+$): 479.43.

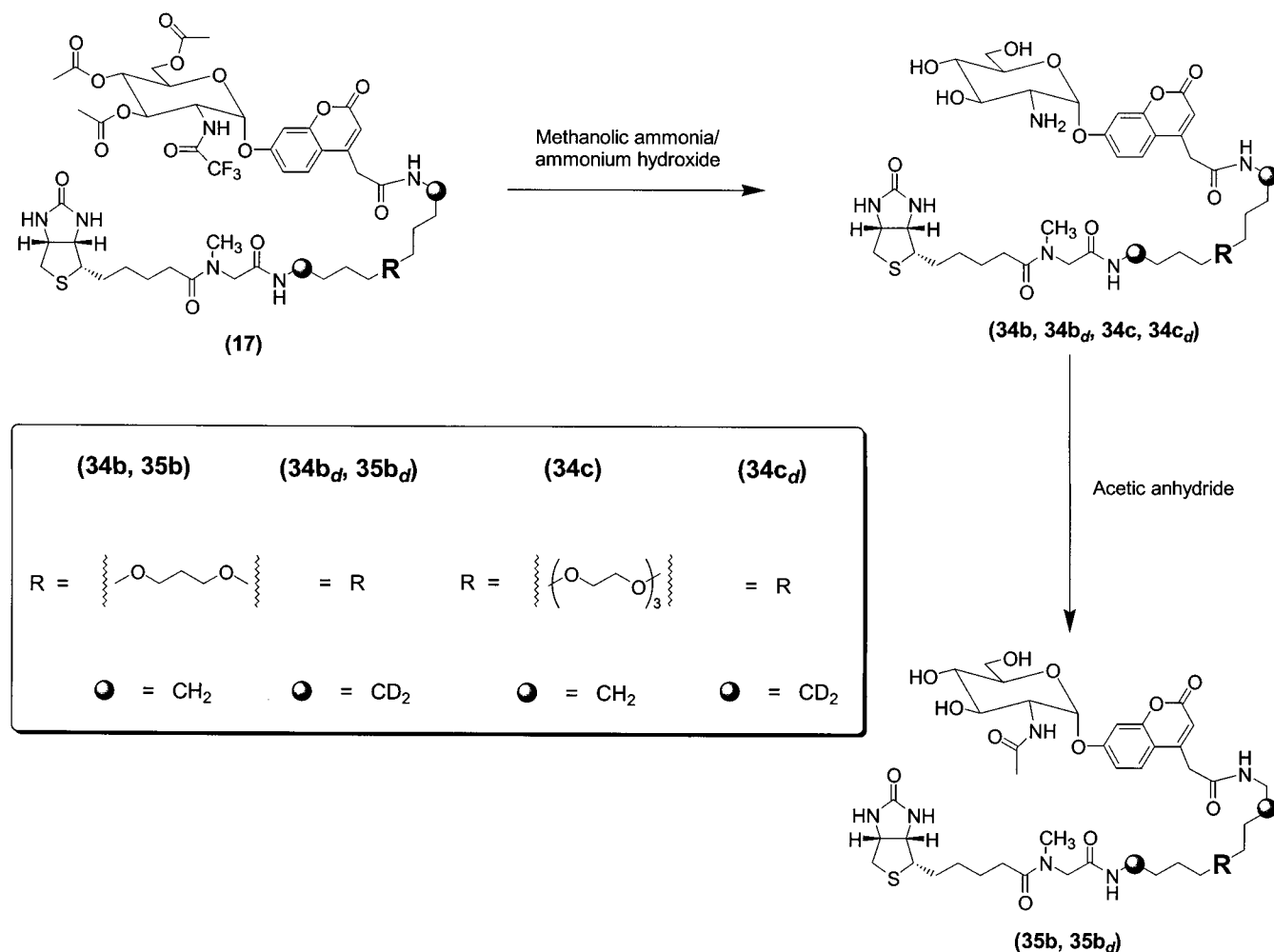
N-Acetylation of 34b (35b). To 3 mg (3.7 μmol) of 34b in 0.75 mL of purified water was added 4 mg (37 μmol) of sodium carbonate with stirring, followed after complete dissolution by 2 μL (18 μmol) of acetic anhydride. The reaction was allowed to stir for 4 h at room temperature, followed by adjustment of the pH to 3 with dilute

trifluoroacetic acid and purification by reversed-phase HPLC [semipreparative column, gradient (2), $t_R = 34.3$ min]. The final product was dried by vacuum centrifugation to yield 3 mg (95%) as a thin, colorless film. ^1H NMR (D_2O) δ 7.67 (d, 1H), 7.12 (m, 2H), 7.19 (dd, 1H), 6.28 (s, 1H), 5.62 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.05 (dd, 1H), 4.00 (d, 2H), 3.88 (t, 1H), 3.75 (s, 2H), 3.68 (s, 2H), 3.59 (m, 1H), 3.52 (m, 1H), 3.45 (m, 8H), 3.23 (m, 5H), 3.00 (d, 3H), 2.88 (dd, 1H), 2.75 (dd, 1H), 2.40 (dt, 2H), 1.97 (s, 3H), 1.69 (t, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS ($M+H^+$): 921.81, ($M+Na^+$): 943.85, ($M+2Na^+$): 483.47.

N-Acetylation of 34b_d (SFD Internal Standard Conjugate) (35b_d). As done previously for 34b, using 34b_d. Yield 94%, semipreparative column, gradient (2), $t_R = 34.7$ min. ^1H NMR (D_2O) δ 7.67 (d, 1H), 7.12 (m, 2H), 7.19 (dd, 1H), 6.28 (s, 1H), 5.62 (d, 1H), 4.57 (m, 1H), 4.38 (m, 1H), 4.05 (dd, 1H), 4.00 (d, 2H), 3.88 (t, 1H), 3.75 (s, 2H), 3.68 (s, 2H), 3.59 (m, 1H), 3.52 (m, 1H), 3.45 (m, 8H), 3.23 (m, 1H), 3.00 (d, 3H), 2.88 (dd, 1H), 2.75 (dd, 1H), 2.40 (dt, 2H), 1.97 (s, 3H), 1.69 (t, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS ($M+H^+$): 925.87, ($M+Na^+$): 947.79.

N-Sulfation of 34c (SFA Substrate Conjugate) (36). To 2.5 mg (2.6 μmol) of 34c in 0.5 mL of purified water was added 3 mg (29 μmol) of sodium carbonate with stirring at room temperature, followed after complete dissolution by 2 mg (13 μmol) of sulfur trioxide-pyridine complex (Lancaster, Lancaster, U.K.). The reaction was allowed to stir for 4 h at room temperature, followed by adjustment of the pH to 3 with dilute trifluoroacetic acid. The mixture was first purified by reversed-phase HPLC using TFA [semipreparative column, gradient (2), $t_R = 42.6$ min], and the correct product fractions were concentrated by vacuum centrifugation to 0.5–1 mL and repurified by reversed-phase HPLC without TFA [semipreparative column, gradient (2), $t_R = 20.5$ min, broadly eluting]. The

Scheme 7



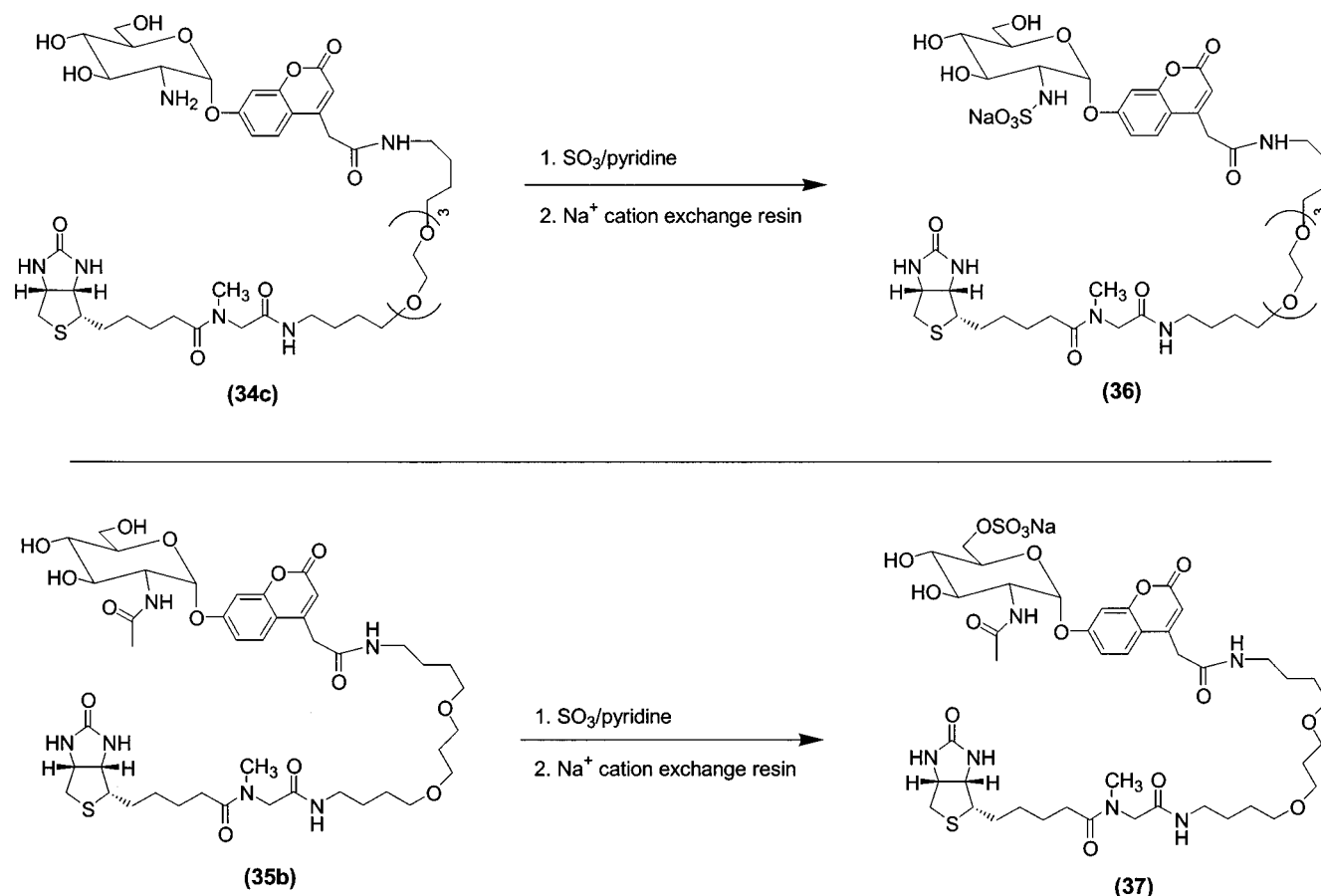
product fractions were again concentrated to 1.0 mL and applied to a short column (300 mg) of AG MP-1 cation-exchange resin, sodium form. The column was washed with 7 mL of purified water, and the combined eluants were concentrated to 1 mL and lyophilized to give 2.0 mg (72%) of an off-white powder. ¹H NMR (D₂O) δ 7.70 (d, 1H), 7.26 (d, 1H), 7.19 (dd, 1H), 6.36 (s, 1H), 6.00 (d, 1H), 4.55 (m, 1H), 4.37 (m, 1H), 4.05 (d, 2H), 3.85 (m, 3H), 3.75 (m, 3H), 3.60 (m, 18H), 3.41 (t, 2H), 3.26 (m, 5H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.77 (dd, 1H), 2.40 (dt, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS (M+H)⁺: 1056.81, (M-Na)⁺: 1032.20.

N-Acetyl-glucosamine-6-O-sulfation of 35b (SFD Substrate Conjugate) (37). 4.5 mg (4.9 μmol) of **35b** was dissolved in 300 μL of anhydrous dimethylformamide under argon. The solvent was then removed by vacuum centrifugation. This was repeated, followed by drying overnight in vacuo in the presence of P₂O₅. The film was redissolved in 400 μL of deuterio-dimethylformamide (d₇) in the presence of three Linde type-4A molecular sieves (previously dried for 48 h at 150 °C) under argon. Then 10 μL of deuterio-pyridine (d₅) was added, and the mixture was allowed to agitate by orbital shaking for 30 min, followed by transfer to an NMR tube (previously dried for 48 h at 150 °C) containing a single activated molecular sieve. A stock solution of 1 μmol/μL sulfur trioxide-pyridine complex in deuterio-dimethylformamide was added in 3 μL aliquots followed by mixing by inversion for 30 min and ¹H NMR analysis to determine the extent of reaction.

The extent of reaction was evaluated by chemical shift change of the *N*-acetyl-glucosamine C-6 methylene protons upon sulfation. These protons shift from a broad singlet at 3.82 ppm to two *pro*-chiral multiplets at 4.06 and 4.17 ppm. Mono-sulfation of the unprotected substrate stops at 55–65% sulfation of the conjugate. Above this level, nonspecific di- and tri-*O*-sulfation occurred. A total of 27 μL (27 μmol) of sulfur trioxide-pyridine solution was used to obtain 60% sulfation.

The reaction solution was rinsed from the NMR tube with methanol. The molecular sieve was washed with methanol, and the combined solvents were removed by vacuum centrifugation. The resultant film was reconstituted with purified water, and the mixture was purified by reversed-phase HPLC without TFA [semipreparative column, gradient (2), *t_R* = 23.0 min, broadly eluting]. The product fractions were combined and concentrated by vacuum centrifugation to 1 mL and applied to a short-column (300 mg) AG MP-1 cation-exchange resin (sodium form). The column was washed with 7 mL of purified water, and the combined eluants were concentrated to 1 mL and lyophilized to yield 2 mg (45%) of an off-white powder. ¹H NMR (D₂O) δ 7.71 (d, 1H), 7.20 (m, 2H), 6.46 (s, 1H), 5.69 (d, 1H), 4.55 (m, 1H), 4.35 (m, 1H), 4.20 (s, 2H), 4.15 (dd, 1H), 4.04 (d, 2H), 3.99 (m, 2H), 3.82 (d, 2H), 3.66 (t, 1H), 3.55 (m, 16H), 3.22 (m, 5H), 3.00 (d, 3H), 2.98 (dd, 1H), 2.77 (dd, 1H), 2.40 (dt, 2H), 2.01 (s, 3H), 1.71 (m, 2H), 1.55 (broad m, 12H), 1.42 (m, 2H). ESI-MS (M+H)⁺: 1023.79, (M-Na)⁺: 999.75.

Scheme 8



RESULTS AND DISCUSSION

Preparation of Deuteratable, Polyether Diamine

Linkers. As described in the Introduction, our enzyme analysis methodology makes use of a linker substructure, both deuterated and nondeuterated, to connect biotin to a particular substrate. Our initial attempts at linker synthesis were based on the use of 4,7,10-trioxa-1,13-tridecane diamine as a linker in a substrate conjugate made previously (5). The octa-deuterio form of that diamine was synthesized by Michael addition of d_2 (O-D) -ethylene glycol and d_3 -acrylonitrile in D_2O . While this reaction produced the desired product in good yield and with little loss of deuterium, the subsequent reduction product was obtained in very low yield (due mostly to elimination of the Michael adduct), and with considerable exchange of the deuterium α to the cyano group. A procedure described previously (15) for the preparation of a polyether dichloride was modified to generate a diamine in good overall yield and with little or no loss of incorporated deuterium (Scheme 1). The diamine linkers used in the present study are prepared from 1,3-propanediol and triethylene glycol. However, identical methods were used to produce polyether diamines from ethylene, diethylene, and tetraethylene glycols. In addition, this procedure allows for a variety of levels of stable isotopic substitution (e.g., using commercially available d_4 -ethylene glycol, d_3 -acrylonitrile, or ^{13}C and/or ^{15}N sodium cyanide) to increase the linker mass by up to +24 amu, in 2 amu increments. Due to issues of scale, we resorted to preparative reversed-phase HPLC purification of the final diamine products, using UV detection at 210 nm. Vacuum distillation may be preferred when linkers are prepared in greater quantity.

Synthesis of Glucosamine-Coumarin Acid Glycoconjugates for Profiling Enzyme Activity in Sanfilippo Subtypes A and D. Our original chemistry to produce substrates for the enzymes relevant to Sanfilippo subtypes A and D was based on the attachment of the linker to the appropriate *p*-acrylamidophenyl glycosides (see below). These conjugates were found to be resistant to enzyme action. Due to the lack of information in the literature concerning the substrate specificity of these enzymes, we opted to conservatively modify the 4-methylumbelliferyl glycoconjugates previously reported as substrates for these enzymes (16, 17). We used glycosides of umbelliferyl-4-acetic acid to further conjugate this substrate substructure to different linkers (Scheme 2), and, ultimately, to sarosinyl biotin (Scheme 3). Synthesis of the protected 7-hydroxy-coumarin-4-*tert*-butoxy acetate **10** and subsequent coupling of the trimethylsilyl ether of **10** with 1-fluoro-2-deoxy-2-(*N*-trifluoroacetyl)-3,4,6-tri-*O*-acetyl-glucosamine gave a mixture of **11** and **12** (partially deprotected by boron trifluoride-etherate in the coupling reaction to form **11**). The remaining **11** was easily deprotected without cleavage of the glycosidic linkage under anhydrous conditions using dry trifluoroacetic acid. The overall yield of the desired α -anomer was consistent with earlier syntheses of analogous 4-methylumbelliferyl glycoconjugates (13) (Scheme 2).

Activation and conjugation of **12** to diamines **7** yielded little or no product with a variety of coupling reagents, including DCC, EDC, and PYBOP under standard coupling conditions. When care was taken to azeotropically dry the starting material, and when activation was performed using 2,3,5,6-tetrafluorophenyl trifluoroacetate in the presence of an inert drying agent such as

molecular sieves, the active ester of **12** was consistently produced in very high yields. Several such successful reactions were performed in *d*-7-dimethylformamide in an NMR tube and monitored by ¹H NMR chemical shift change of the α-carboxylic acid methylene protons from 3.92 to 4.73 ppm upon activation. Although attempts to purify and isolate this activated intermediate were unsuccessful, in situ formation of the active ester and coupling to the diamines **7** consistently produced the desired amide products in at least 85% yield.

Further conjugation of the products **13** to sarcosinyl biotin was performed by a similar route involving careful preparation of tetrafluorophenyl *N*-methyl-*N*-biotinyl glycinate via a minor modification of a procedure by Wilbur (7) (Scheme 3). From this point, removal of protecting groups (Scheme 7) and functionalization of the carbohydrate (Schemes 7 and 8) ultimately afford the desired substrate and internal standard conjugates for profiling enzyme activity in Sanfilippo subtypes A and D (**34cd**, **35b_a**, **36**, and **37**). It should be noted that sulfation of the primary *N*-acetyl glucosamine hydroxyl of **35b** without additional steps to protect the secondary alcohols was performed by addition of sulfur trioxide–pyridine to the substrate in deuterated dimethylformamide in sub-stoichiometric amounts, and the reaction was monitored by ¹H NMR. Although the total amount of sulfating reagent required was in roughly 3-fold excess, addition of this amount in one portion yielded multiply sulfated products. Furthermore, mono-sulfation of the carbohydrate was uniform until roughly 40% of the starting material remained. At this point, di- and tri-sulfation occurred, despite a considerable quantity of unsulfated starting material remaining.

Synthesis of *p*-Amidophenyl Glycoconjugates for Profiling Enzyme Activity Involved in Sanfilippo Subtypes B and C. The commercial availability of some *p*-nitrophenyl glycoconjugates initially led our synthetic efforts. Synthesis of the substrate for Sanfilippo subtype C led us to the chemistry by Voznyi et al. (13) for the preparation of commercially unavailable 2-amino carbohydrate conjugates. Reduction and acrylation of such conjugates by the method of Romanowska (18) was achieved in good yield (Scheme 4). However, the following Michael addition to diamines **2** was much less successful (Scheme 5). Final coupling to sarcosinyl biotin was effected by active ester chemistry described above (Scheme 6).

Application of ACESIMS to Sanfilippo Syndrome. The use of the above substrate and internal standard conjugates for ACESIMS profiling of enzyme velocities involved in the Sanfilippo syndrome will be described elsewhere (ref 8). The ACESIMS methodology has been demonstrated to be rapid, sensitive, and accurate in confirming diagnoses of Sanfilippo subtypes. In addition, the ACESIMS approach has allowed all four Sanfilippo enzymes to be profiled in two duplex incubations, reducing the number of assays required. Owing to the sensitivity of ESI-MS, ACESIMS enzyme velocities have been calculated using 3.5 μg of patient total cell protein (obtained from ~2500 fibroblasts), requiring only 400 ng of substrate conjugate per assay. Although multistep synthesis is required to prepare these ACESIMS conjugates, several thousand assays can be performed per milligram of substrate. In addition, the modular nature of ACESIMS substructures allows for combinations of previously prepared deuterated and nondeuterated linkers, substrates, and sarcosinyl biotin to develop conjugates for a host of clinical and biomedical applications.

ACKNOWLEDGMENT

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Biotin Reagents for Antibody Pretargeting. 5. Additional Studies of Biotin Conjugate Design To Provide Biotinidase Stability

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An investigation was conducted in which the stabilities of four structurally different biotin derivatives were assessed with regard to biotinamide bond hydrolysis by the enzyme biotinidase. The biotin derivatives studied contained an extra methylene in the valeric acid chain of biotin (i.e., homobiotin), or contained conjugated amino acids having hydroxymethylene, carboxylate, or acetate functionalities on a methylene alpha to the biotinamide bond. The biotinidase hydrolysis assay was conducted on biotin derivatives that were radioiodinated at high specific activity, and then subjected to diluted human serum at 37 °C for 2 h. After incubation, assessment of biotinamide bond hydrolysis by biotinidase was readily achieved by measuring the percentage of radioactivity that did not bind with avidin. As controls, an unsubstituted biotin derivative which is rapidly cleaved by biotinidase and an *N*-methyl-substituted biotin derivative which is stable to biotinidase cleavage were included in the study. The results indicate that increasing the distance from the biotin ring structure to the biotinamide bond by one methylene only decreases the rate of biotinidase cleavage, but does not block it. The data obtained also indicate that placing a hydroxymethylene, carboxylate, or acetate alpha to the biotinamide bond is effective in blocking the biotinamide hydrolysis reaction. These data, in combination with data previously obtained, which indicate that biotin derivatives containing hydroxymethylene or carboxylate moieties retain the slow dissociation rate of biotin from avidin and streptavidin [Wilbur, D. S., et al. (2000) *Bioconjugate Chem.* 11, 569–583], strongly support incorporation of these structural features into biotin derivatives being used for in vivo targeting applications.

INTRODUCTION

Radiolabeled derivatives of biotin are being developed for application to "pretargeting" in targeted radiotherapy of cancer (1–5). A design criterion for radiolabeled biotin derivatives used in vivo is that they must be stabilized toward degradation by the enzyme biotinidase (6). Biotinidase is present in serum and tissues of both animals and humans in nanomolar concentrations (6). It is believed that the primary function of this enzyme is to cleave the biotinamide bond linking biotin (vitamin H) and lysine in biocytin (7, 8), such that the essential vitamin can be recycled. Biocytin is released after peptidase degradation of carboxylase enzymes containing biotin (9). An important finding is that biotinidase is not specific for cleaving the biotinamide bond in biocytin (10). This fact led us, and other investigators (11–14), to evaluate the effect of structural changes in biotin derivatives on blocking the biotinamide cleaving action of biotinidase. It was previously reported that an *N*-methylbiotinamide functionality, introduced by conjugation of biotin with *N*-methylglycine (sarcosine), completely blocked biotinidase cleavage of the biotinamide bond (12). Studies in our laboratory confirmed that biotin conjugated with sarcosine, and other *N*-methyl linker molecules, blocked the actions of biotinidase in mouse and human serum (15).

In this investigation, we have extended our previous studies evaluating variations in functional groups in

biotin conjugates for blocking biotinidase activity. The studies were primarily directed at biotin conjugates which contain hydroxymethylene and carboxylate substituents alpha to the biotinamide bond (e.g., **4b** and **5b**). However, two other biotin derivatives were prepared to provide a better understanding of the structural features required for blocking biotinidase cleavage, and two more biotin derivatives were prepared as standards. Similar to the hydroxymethylene and carboxylate derivatives, one of the biotin derivatives tested contained an acetate substituent alpha to the biotinamide bond (**6b**). In another biotin derivative, there was an additional methylene in the valeric side chain of biotin (i.e., homobiotin derivative **3c**). As standards for comparison, an unmodified biotin derivative that is rapidly cleaved by biotinidase (**1c**) and a *N*-methylbiotinamide derivative (**2c**) which is stable to cleavage by biotinidase were included in the study. As in previously studied biotin derivatives, 4,7,10-trioxatridecane-1,13-diamine was incorporated as a linker to aid in water solubilization (16). All of the biotin derivatives tested were radioiodinated to high specific activity to permit evaluation of the biotinidase cleavage. Reported herein are: (a) the syntheses of precursors to the radioiodinated biotin derivatives targeted for testing, (b) syntheses of iodinated HPLC standards, (c) radioiodination methods used, and (d) results obtained from the biotinidase assay.

EXPERIMENTAL PROCEDURES

General. All chemicals purchased from commercial sources were analytical grade or better and were used without further purification unless noted. Most of the reagents used were obtained from Aldrich Chemical Co.

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(Milwaukee, WI). Biotin was obtained from Sigma Chemicals (St. Louis, MO). 4,7,10-Trioxa-1,13-tridecanediamine and sarcosine methyl ester were obtained from Fluka (St. Louis, MO). 2,3,5,6-Tetrafluorophenyl trifluoroacetate (TFP-OTFA)¹ was prepared as previously described (17). *O*-*tert*-Butyl-L-serine methyl ester hydrochloride, L-aspartic acid β -*tert*-butyl ester, and L-aspartic acid α -*tert*-butyl ester were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Solvents for HPLC analysis were obtained as HPLC grade and were filtered (0.2 μ m) prior to use. Silica gel chromatography was conducted with 70–230 mesh 60 Å silica gel (Aldrich Chemical Co.). Microcon-30 centrifugation concentrators were obtained from Amicon (Beverly, MA). Melting points were obtained in open capillary tubes on a Mel-Temp II apparatus with a Fluke 51 K/J electronic thermometer, and are uncorrected.

Radioactive Materials. Standard methods for safely using radionuclides of iodine were employed (18). Na¹²⁵I was purchased from NEN/Dupont (Billerica, MA) as high-pH, high-concentration solutions in 0.1 N NaOH. Measurement of ¹²⁵I was accomplished on the Capintec CRC-15R or a Capintec CRC-6A Radioisotope Calibrator. Radioactive samples were counted in a LOGIC Model 111B Gamma Counter (Abbott Laboratories, Chicago, IL).

Spectral Analyses. ¹H NMR were obtained on either a Bruker AC-200 (200 MHz) or a Bruker AC-500 (500 MHz) instrument. Chemical shifts are expressed as ppm using tetramethylsilane as internal standard (δ = 0.0 ppm). IR data were obtained on a Perkin-Elmer 1420 infrared spectrophotometer. Mass spectral data (both low resolution and high resolution) of most compounds prepared in this study were obtained on a PerSeptive Biosystems Mariner Electrospray Time-of-Flight Mass Spectrometer (ESI-TOF). For analysis, the samples were dissolved in 50:50 MeOH/H₂O and were introduced by an integral syringe infusion pump. Some mass spectral data (low resolution) were obtained on a VG Analytical (Manchester, England) VG-70 SEQ mass spectrometer with associated 11250J Data System. These mass spectral data were obtained by fast atom bombardment (FAB⁺) in a matrix of 2-hydroxyethyl disulfide (2-HEDS) and poly(ethylene glycol) 300 or 600 containing thioglycolate. High-resolution mass spectral data were used for compound identification. Compounds containing Sn isotopes had five matching isotopic masses, but only one is provided as identification. ¹H NMR and HPLC were used to assess the purity of nonradioactive compounds (these data are provided in the Supporting Information).

Chromatography. HPLC separations of nonradioactive compounds were obtained on a Hewlett-Packard quaternary 1050 gradient pumping system with a variable-wavelength UV detector (254 nm) and a Varex ELSD MKIII evaporative light-scattering detector. Analyses of the HPLC data were conducted on Hewlett-Packard HPLC ChemStation software. All reactions were monitored by HPLC. Reversed-phase HPLC chromatography was carried out using an Alltech Altima C-18 column (5 μ m, 250 mm $\times$ 4.6 mm) using a gradient solvent system at a flow rate of 1 mL/min. A gradient of

MeOH and H₂O/0.1% HOAc was used. Starting with 40% MeOH, the initial solvent mixture was held for 2 min; then the gradient was increased to 100% MeOH over the next 10 min, then held at 100% MeOH for 8 min. Retention times (*t*_R) under these conditions for biotin conjugates are provided with the compound experimental data.

HPLC separations of radioiodinated biotin derivatives were conducted using a gradient system which consisted of two Beckman model 110B pumps, a Beckman 420 controller, a Beckman model 153 UV detector (254 nm), and a Beckman model 170 radioisotope detector. The separations were obtained using HPLC conditions described for gradient I, above, except water was used in place of H₂O/0.1% HOAc.

The following compounds were prepared as previously described: **1a**, **1b**, [¹²⁵I]**1c**, **2a**, **2b**, [¹²⁵I]**2c**, (**15**); **7** (**19**); **8a**, **8b** (**15**); **10**, **11**, **12**, **13** (**19**); **20** (**20**); **27** (**21**). Biotin-NHS ester, **9**, was provided as a gift from Quanta BioDesign (Powell, OH).

N-(13-(*p*-Tri-*n*-butylstannylbenzoylamino)-4,7,10-trioxatridecanyl)homobiotinamide, 3a. To a 0.203 g (0.50 mmol) quantity of the TFP ester of homobiotin, **7**, dissolved in 10 mL of DMF was added 70 μ L (50.8 mg, 0.50 mmol) of Et₃N, then 0.307 g (0.51 mmol) of **8a**. The reaction was stirred at room temperature for 1 h, and the DMF was removed under vacuum. The crude residue was loaded onto a silica column and eluted with EtOAc, and then mixtures of EtOAc/MeOH. The product eluted with 4:1 EtOAc/MeOH. Evaporation of the solvent provided 0.380 g (0.44 mmol, 89%) of **3a** as a colorless solid, mp 106–107 °C. ¹H NMR 500 MHz (CD₃OD): 0.88 (t, *J* = 7.0 Hz, 9H), 1.10 (t, *J* = 8.1 Hz, 6H), 1.30–1.45 (m, 12H), 1.53–1.63 (m, 10H), 1.68–1.75 (m, 2H), 1.85–1.90 (m, 2H), 2.16 (t, *J* = 7.3 Hz, 2H), 2.69 (d, *J* = 13.3 Hz, 1H), 2.90 (dd, *J* = 5.1, 12.5 Hz, 1H), 3.15–3.19 (m, 1H), 3.23 (t, *J* = 6.6 Hz, 2H), 3.47 (t, *J* = 6.6 Hz, 4H), 3.52–3.54 (m, 2H), 3.57–3.64 (m, 6H), 4.27 (dd, *J* = 4.4, 8.1 Hz, 1H), 4.46 (dd, *J* = 4.4, 8.1 Hz, 1H), 7.54 (d, *J* = 8.1 Hz, 2H), 7.74 (d, *J* = 8.1 Hz, 2H). HRMS calcd for C₄₀H₇₁N₄O₆SSn (M + H)⁺: 853.4090. Found: 853.4112 (Sn-118). HPLC: *t*_R = 16.9 min.

N-(13-(*p*-Iodobenzoylamino)-4,7,10-trioxatridecanyl)homobiotinamide, 3b. To a 0.244 g (0.60 mmol) quantity of the TFP ester of homobiotin, **7**, dissolved in 10 mL of DMF was added 84 μ L (61 mg, 0.60 mmol) of Et₃N, then 0.268 g (0.61 mmol) of **8b**. The reaction was stirred at room temperature for 1 h, and the DMF was removed under vacuum. The crude residue was loaded onto a silica column and eluted with EtOAc, then mixtures of EtOAc/MeOH. The product eluted with 4:1 EtOAc/MeOH. Evaporation of the solvent provided 0.30 g (0.43 mmol, 72%) of **3b** as a colorless solid, mp 124–125 °C. ¹H NMR 500 MHz (CD₃OD): δ 1.33–1.45 (m, 6H), 1.54–1.62 (m, 4H), 1.73 (t, *J* = 6.6 Hz, 2H), 1.87 (t, *J* = 6.3 Hz, 2H), 2.16 (t, *J* = 7.3 Hz, 2H), 2.69 (d, *J* = 13.2 Hz, 1H), 2.91 (dd, *J* = 5.2, 12.5 Hz, 1H), 3.15–3.18 (m, 1H), 3.23 (t, *J* = 7.0 Hz, 2H), 3.44–3.49 (m, 4H), 3.53–3.63 (m, 8H), 4.28 (dd, *J* = 4.4, 8.1 Hz, 1H), 4.47 (dd, *J* = 4.4, 8.1 Hz, 1H), 7.56 (d, *J* = 8.8 Hz, 2H), 7.83 (d, *J* = 8.1 Hz, 2H). HRMS calcd for C₂₈H₄₄N₄O₆SI (M + H)⁺: 691.2026. Found: 691.2051. HPLC: *t*_R = 12.3 min.

Preparation of Biotin Derivatives 17–19 and 21–23. A 0.24 mmol quantity of a biotin-amino acid derivative (**11**, **12**, or **13**) was combined with 50 μ L (0.36 mmol) of Et₃N in 5 mL of DMF. The mixture was cooled to 4 °C, 69 mg (0.27 mmol) of TFP-OTFA was added, and then the mixture was allowed to stir for 30 min at 4 °C. An additional 40 μ L (0.29 mmol) of Et₃N was added, followed

¹ Abbreviations: Av, avidin; BSA, bovine serum albumin; cpm, counts per minute; DIP, direct insertion probe; EI, electron impact; 2-HEDS, 2-hydroxyethyl sulfide; HOHgBz, hydroxymercuribenzoic acid; *nca*, no-carrier-added; NCS, *N*-chlorosuccinimide; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; rt, room temperature; SAv, streptavidin; *t*-Boc, *tert*-butoxycarbonyl; TFA, trifluoroacetic acid; TFP, tetrafluorophenyl; TFP-OTFA, tetrafluorophenyl trifluoroacetate.

by addition of 0.24 mmol of **8b** or **20**. The reaction mixture was allowed to come to room temperature and was stirred for an additional hour at that temperature. The mixture was concentrated under vacuum, and purified over a silica gel column (2.5 cm $\times$ 22 cm). The column was first eluted with EtOAc, then with increasing amounts (i.e., 10–40%) of methanol in EtOAc.

2-(N-Biotinoyl)-1-(N'-(13'-p-iodobenzoylamino)-4',7',10'-trioxatridecaneamino)-L-serine-O-tert-butyl Ether, 17. This compound was obtained as a colorless oil in 45% yield. ^1H NMR 200 MHz (CD_3OD): δ 1.17 (s, 9H), 1.44 (m, 4H), 1.67 (m, 6H), 1.87 (m, 2H), 2.29 (t, J = 7.3 Hz, 2H), 2.69 (d, 2H), 2.90 (dd, J = 5.1, 12.5 Hz, 1H), 3.30 (m, 6H), 3.55 (m, 10H), 4.30 (m, 1H), 4.39 (m, 1H), 4.48 (m, 1H), 7.56 (d, J = 8.4 Hz, 2H), 7.83 (d, J = 8.8 Hz, 2H). HRMS calcd for $\text{C}_{34}\text{H}_{55}\text{IN}_5\text{O}_8\text{S}$ ($\text{M} + \text{H}$) $^+$: 820.2816. Found: 820.2806. HPLC: t_{R} = 13.2 min.

2-(N-Biotinoyl)-1-(N'-(13'-p-iodobenzoylamino)-4',7',10'-trioxatridecaneamino)-L-aspartate-4-O-tert-butyl Ester, 18. This compound was obtained as a colorless solid in 65% yield, mp 165–167 $^\circ\text{C}$. ^1H NMR 200 MHz (CD_3OD): δ 1.43 (s, 9H), 1.72 (m, 3H), 1.87 (m, 3H), 2.26 (t, J = 6.9 Hz, 1H), 2.59 (m, 2H), 2.79 (d, J = 6.2 Hz, 2H), 2.89 (d, J = 4.7 Hz, 1H), 2.95 (d, J = 4.7 Hz, 1H), 3.31 (m, 8H), 3.51 (m, 4H), 3.61 (m, 8H), 4.30 (m, 1H), 4.48 (m, 1H), 4.66 (m, 1H), 7.56 (d, J = 8.4 Hz, 2H), 7.83 (d, J = 8.4 Hz, 2H). HRMS calcd for $\text{C}_{35}\text{H}_{55}\text{IN}_5\text{O}_9\text{S}$ ($\text{M} + \text{H}$) $^+$: 848.2765. Found: 848.2742. HPLC: t_{R} = 12.9 min.

2-(N-Biotinoyl)-4-(N'-(13'-p-iodobenzoylamino)-4',7',10'-trioxatridecaneamino)-L-aspartate-1-O-tert-butyl Ester, 19. This compound was obtained as a colorless oil in 35% yield. ^1H NMR 200 MHz (CD_3OD): δ 1.44 (s, 9H), 1.71 (m, 3H), 1.89 (m, 3H), 2.24 (t, J = 7.3 Hz, 1H), 2.68 (m, 2H), 2.86 (d, J = 3.3 Hz, 1H), 2.99 (d, J = 3.3 Hz, 1H), 3.30 (m, 8H), 3.49 (m, 4H), 3.62 (m, 8H), 4.30 (m, 1H), 4.41 (m, 1H), 4.63 (m, 1H), 7.57 (d, J = 8.8 Hz, 2H), 7.84 (d, J = 8.8 Hz, 2H). HRMS calcd for $\text{C}_{35}\text{H}_{55}\text{IN}_5\text{O}_9\text{S}$ ($\text{M} + \text{H}$) $^+$: 848.2765. Found: 848.2767. HPLC: t_{R} = 12.8 min.

2-(N-Biotinoyl)-1-(N'-(13'-tert-butoxycarbonylamino)-4',7',10'-trioxatridecaneamino)-L-serine-O-tert-butyl Ether, 21. This compound was obtained as a colorless oil in 82% yield. ^1H NMR 200 MHz (CD_3OD): δ 1.18 (s, 9H), 1.43 (s, 9H), 1.72 (m, 10H), 2.30 (t, J = 6.9 Hz, 2H), 2.70 (d, 1H), 2.94 (dd, J = 5.1, 12.5 Hz, 1H), 3.12 (t, J = 6.9 Hz, 2H), 3.22 (m, 1H), 3.30 (m, 4H), 3.35 (s, 2H), 3.52 (m, 4H), 3.61 (m, 6H), 4.30 (m, 1H), 4.40 (t, J = 5.5 Hz, 1H), 4.49 (m, 1H). HRMS calcd for $\text{C}_{32}\text{H}_{60}\text{N}_5\text{O}_9\text{S}$ ($\text{M} + \text{H}$) $^+$: 690.4112. Found: 690.4117. HPLC: t_{R} = 12.5 min.

2-(N-Biotinoyl)-1-(N'-(13'-tert-butoxycarbonylamino)-4',7',10'-trioxatridecaneamino)-L-aspartate-4-O-tert-butyl Ester, 22. This compound was obtained as a colorless oil in 52% yield. ^1H NMR 200 MHz (CD_3OD): δ 1.44 (s, 18H), 1.74 (m, 8H), 2.09 (t, 2H), 2.54 (m, 2H), 2.79 (d, J = 6.2 Hz, 1H), 2.89 (d, J = 4.7 Hz, 1H), 2.95 (d, J = 4.7 Hz, 1H), 3.11 (t, J = 6.9 Hz, 2H), 3.31 (m, 8H), 3.51 (t, J = 6.2 Hz, 3H), 3.61 (m, 8H), 4.30 (m, 1H), 4.48 (m, 1H), 4.66 (m, 1H). HRMS calcd for $\text{C}_{33}\text{H}_{60}\text{N}_5\text{O}_{10}\text{S}$ ($\text{M} + \text{H}$) $^+$: 718.4061. Found: 718.4045. HPLC: t_{R} = 12.5 min.

2-(N-Biotinoyl)-4-(N'-(13'-tert-butoxycarbonylamino)-4',7',10'-trioxatridecaneamino)-L-aspartate-1-O-tert-butyl Ester, 23. This compound was obtained as a colorless oil in 59% yield. ^1H NMR 200 MHz ($\text{DMSO}-d_6$): δ 1.37 (s, 18H), 1.50 (m, 2H), 1.58 (m, 8H), 2.09 (t, 2H), 2.44 (m, 4H), 2.54 (m, 2H), 2.82 (m, 1H), 2.95 (m, 2H), 3.07 (m, 2H), 3.48 (m, 10H), 4.13 (m, 1H), 4.31 (m, 1H),

4.42 (m, 1H), 6.39 (d, J = 10.6 Hz, 2H), 6.75 (t, 5.9 Hz, 1H), 7.88 (t, J = 5.5 Hz, 1H), 8.04 (d, J = 7.7 Hz, 1H). HRMS calcd for $\text{C}_{33}\text{H}_{60}\text{N}_5\text{O}_{10}\text{S}$ ($\text{M} + \text{H}$) $^+$: 718.4061. Found: 718.4060. HPLC: t_{R} = 12.3 min.

Preparation of 4a, 5a, and 6a. For each of the *p*-iodo compounds **17**, **18**, and **19**, 2 mL of neat TFA was added and stirred for 10 min (the serine compound **17** was stirred for 30 min). The mixture was evaporated and dried on high vacuum overnight (yield $\sim$ 99%). The isolated biotin derivatives were washed several times with ether to remove any remaining TFA.

2-(N-Biotinoyl)-1-(N'-(13'-p-iodobenzoylamino)-4',7',10'-trioxatridecaneamino)-L-serine, 4a. This compound was obtained in nearly quantitative yield as a colorless oil. ^1H NMR 200 MHz (CD_3OD): δ 1.65 (s, 2H), 1.44 (m, 4H), 1.67 (m, 6H), 1.87 (m, 2H), 2.29 (t, J = 7.3 Hz, 2H), 2.69 (d, J = 12.5 Hz, 2H), 2.90 (dd, J = 4.7, 12.5 Hz, 1H), 3.01 (d, 1H), 3.30 (m, 6H), 3.55 (m, 10H), 4.30 (m, 1H), 4.39 (m, 1H), 4.48 (m, 1H), 7.56 (d, J = 8.8 Hz, 2H), 7.83 (d, J = 8.8 Hz, 2H). HRMS calcd for $\text{C}_{30}\text{H}_{47}\text{IN}_5\text{NaO}_8\text{S}$ ($\text{M} + \text{H}$) $^+$: 764.2190. Found: 764.2179. HPLC: t_{R} = 11.4 min.

2-(N-Biotinoyl)-1-(N'-(13'-p-iodobenzoylamino)-4',7',10'-trioxatridecaneamino)-L-aspartate, 5a. This compound was obtained in nearly quantitative yield as a colorless oil. ^1H NMR 200 MHz (CD_3OD): δ 1.72 (m, 3H), 1.87 (m, 3H), 2.26 (t, J = 7.3 Hz, 1H), 2.59 (m, 2H), 2.79 (m, 2H), 2.89 (d, J = 3.3 Hz, 1H), 2.95 (d, J = 3.3 Hz, 1H), 3.31 (m, 8H), 3.51 (t, J = 6.2 Hz, 4H), 3.61 (m, 8H), 4.30 (m, 1H), 4.48 (m, 1H), 4.66 (m, 1H), 7.56 (d, J = 8.4 Hz, 2H), 7.83 (d, J = 8.4 Hz, 2H). HRMS calcd for $\text{C}_{31}\text{H}_{46}\text{IN}_5\text{NaO}_9\text{S}$ ($\text{M} + \text{Na}$) $^+$: 814.1958. Found: 814.1937. HPLC: t_{R} = 11.6 min.

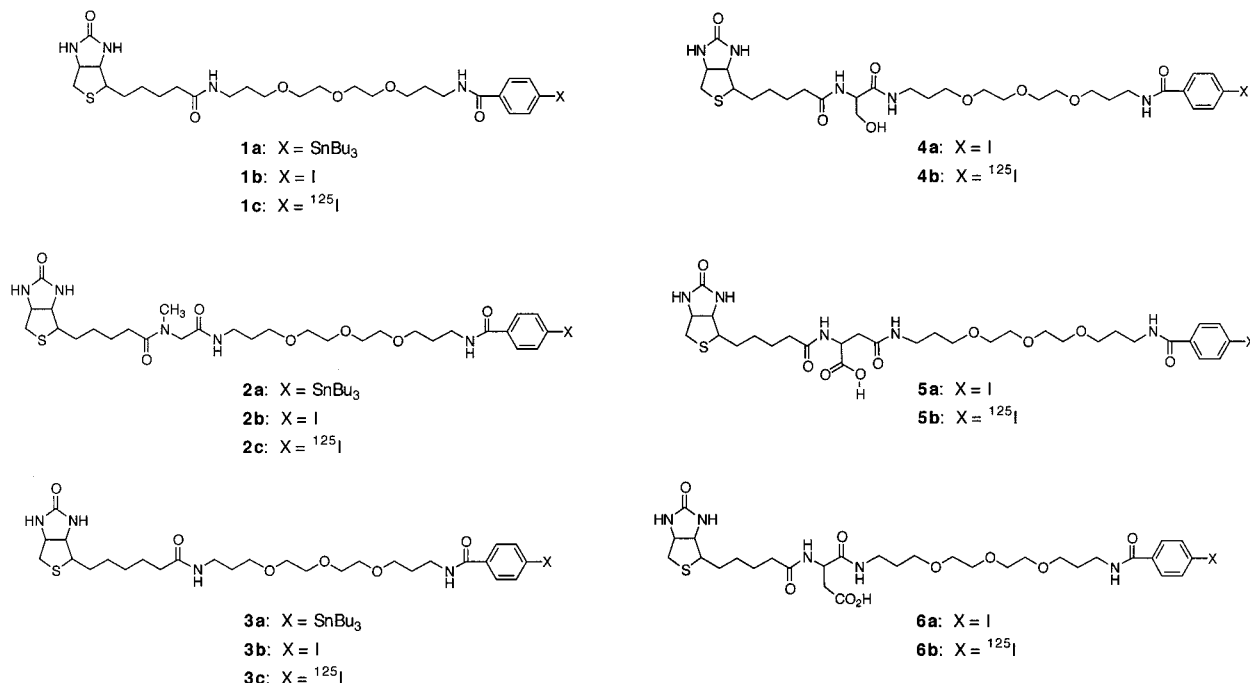
2-(N-Biotinoyl)-4-(N'-(13'-p-iodobenzoylamino)-4',7',10'-trioxatridecaneamino)-L-aspartate, 6a. This compound was obtained in 99% yield as a colorless oil. ^1H NMR 200 MHz (CD_3OD): δ 1.28 (s, 2H), 1.71 (m, 3H), 1.89 (m, 3H), 2.24 (t, 2H), 2.68 (m, 1H), 2.86 (d, J = 4.7 Hz, 1H), 2.99 (d, J = 4.7 Hz, 1H), 3.30 (m, 8H), 3.49 (m, 4H), 3.62 (m, 8H), 4.30 (m, 1H), 4.41 (m, 1H), 4.63 (m, 1H), 7.57 (d, J = 8.7 Hz, 2H), 7.84 (d, J = 8.7 Hz, 2H). HRMS calcd for $\text{C}_{31}\text{H}_{46}\text{IN}_5\text{NaO}_9\text{S}$ ($\text{M} + \text{Na}$) $^+$: 814.1958. Found: 814.1957. HPLC: t_{R} = 11.5 min.

Preparation of [^{125}I]3c. To 50 μL of a 1 mg/mL solution of **3a** was added 1–5 μL of Na^{125}I in 0.1 N NaOH, followed by 10 μL of a 1 mg/mL solution of NCS in MeOH. After 2 min, 10 μL of a 1 mg/mL aqueous sodium metabisulfite solution was added. The reaction mixture was then drawn into a syringe for purification by HPLC. The radioiodinated [^{125}I]3c was obtained from the HPLC effluent in 13% yield.

Preparation of [^{125}I]4b, [^{125}I]5b, and [^{125}I]6b using N-Hydroxysuccinimide *p*-[^{125}I]iodobenzoate, 27. The syntheses were accomplished from the N-*t*-Boc derivatives **21**, **22**, and **23** as depicted in Scheme 3. The steps involved are as follows.

Removal of *t*-Boc Protecting Group in Situ To Prepare 24, 25, and 26. A 100 μg quantity of biotin derivative **21**, **22**, or **23** in 100 μL of MeOH was evaporated to dryness, and 100 μL TFA was added. This solution was stirred for 30 min, and the TFA was evaporated under a stream of argon to yield **24**, **25**, and **26** as TFA salts. The TFA salt residues were diluted with 30 μL of DMF and 20 μL of triethylamine.

Preparation of [^{125}I]27. To 50 μL of HPLC-purified *p*-tri-*n*-butylstannylbenzoate *N*-hydroxysuccinimide ester (**21**) in MeOH/5% HOAc was added 3 μL (1.68 mCi) of Na^{125}I followed by 10 μL of 1 mg/mL NCS in MeOH. After 10 min at room temperature, 10 μL of a 1 mg/mL solution

Table 1. Biotin Derivatives Prepared

of sodium metabisulfite was added to quench the reaction. The mixture was then reduced to dryness under a stream of argon, and 50 μ L of DMF was added.

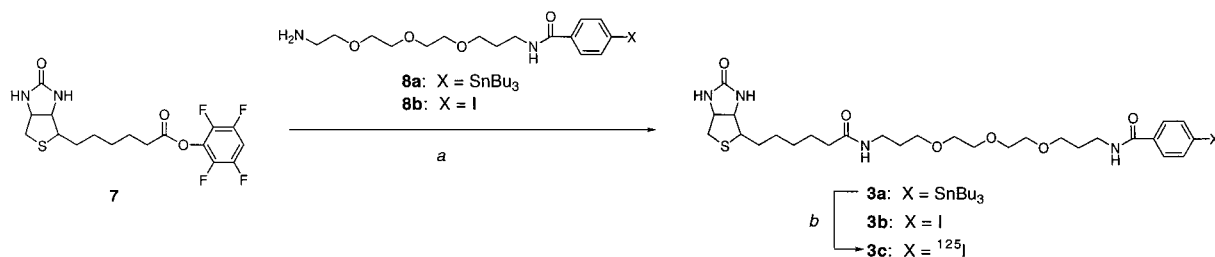
Radiolabeling of Biotin Derivatives. To the basic solution containing **24**, **25**, or **26** in DMF was added 15 μ L of [¹²⁵I]**27**. After 30 min at room temperature, the entire reaction mixture was injected on the HPLC column and eluted. The major radioactivity peaks were collected. The percent of radioactivity in the peak corresponding to the iodo standards (**4a**, **5a**, **6a**) on the HPLC chromatogram was noted ([¹²⁵I]**4b**, 43%; [¹²⁵I]**5b**, 44%; [¹²⁵I]**6b**, 46%). The isolated radiochemical yields were as follows: [¹²⁵I]**4b**, 25%; [¹²⁵I]**5b**, 29%; [¹²⁵I]**6b**, 31%.

Measurement of Biotinidase Cleavage. The same experimental conditions were used as previously reported (15). Briefly, the radiolabeled biotin derivative was diluted to obtain approximately 5000 cpm per 10 μ L in a well counter. To a 0.5 mL aliquot of diluted serum (1:10 with 20 mM phosphate buffer, pH 6.8) was added 10 μ L of the HPLC-purified radiolabeled biotin derivative in MeOH. Each tube was lightly vortexed and placed in a 37 $^{\circ}$ C heating block. After the desired time (e.g., 2 h), the vials were removed, and 40 μ L of 2 mM 4-(hydroxymercuri)benzoic acid (HOHgBz), sodium salt in H₂O, was added to inhibit biotinidase. To that solution was added an excess of avidin (40 μ L; 0.24 mg), and it was incubated at 37 $^{\circ}$ C for another 30 min. The solution was split into two 200 μ L aliquots, and the samples were transferred

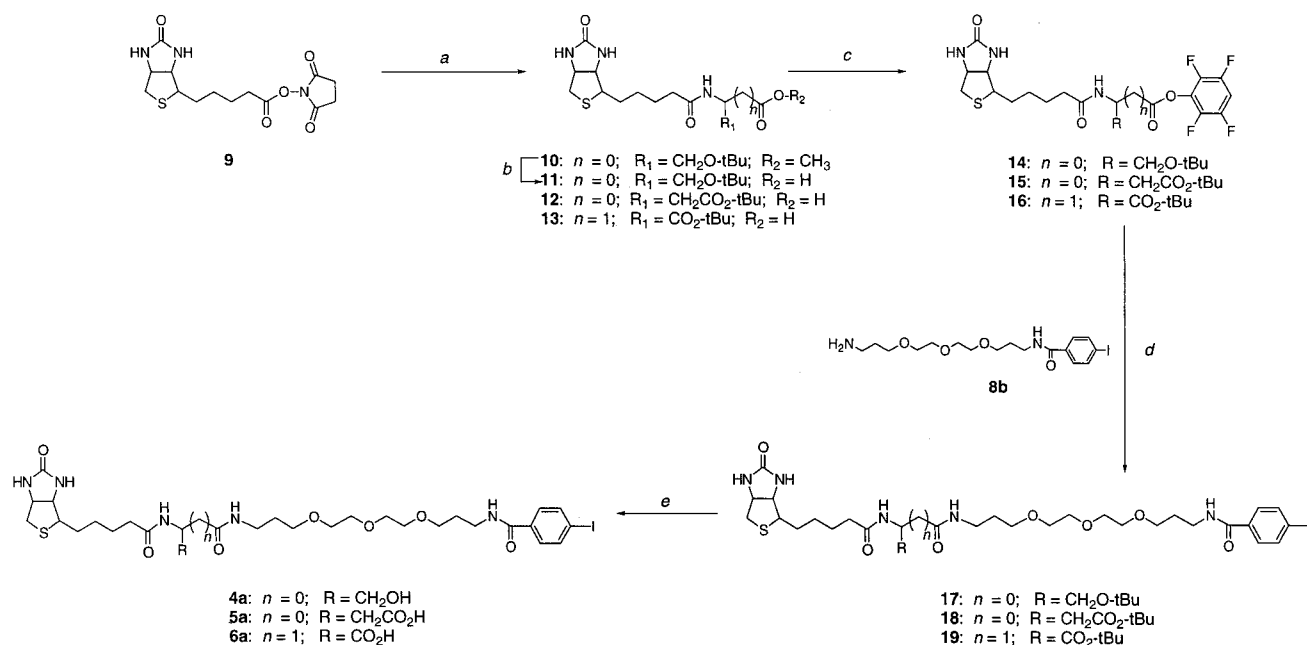
to two Microcon-30 filters and centrifuged at 3000g for 10 min. The material remaining on the filter was washed with 4 $\times$ 100 μ L of phosphate buffer, concentrating by centrifugation each time. The top and bottom (containing filtrate and washes) of the Microcon-30 were counted in a well counter. The ratio of nonbound counts (bottom) to the total counts (top and bottom) was calculated to determine the percent of the biotin derivative that had been cleaved. As a control, this procedure was repeated as above except that the 4-(hydroxymercuri)benzoic acid was added prior to the radiolabeled biotin derivative to inhibit biotinidase activity.

RESULTS

Synthesis and Radioiodination of Biotin Derivatives. The biotin derivatives synthesized for the study are shown in Table 1. Radioiodinated derivatives ([¹²⁵I]**1c**, [¹²⁵I]**2c**, [¹²⁵I]**3c**, [¹²⁵I]**4b**, [¹²⁵I]**5b**, and [¹²⁵I]**6b**) were prepared for evaluation in the biotinidase assay. The arylstannane derivatives (**1a**, **2a**, **3a**) were prepared as precursors for radioiodination, and nonradioactive iodo-phenyl derivatives (**1b**, **2b**, **3b**, **4a**, **5a**, and **6a**) were prepared for use as HPLC standards. Syntheses of homobiotin-stannylbenzoate derivative **3a** and the homobiotin-iodobenzoate derivative **3b** were accomplished using the same synthetic approach as previously described for **1a**, **2a**, **1b**, and **2b** (15). The synthetic route employed is shown in Scheme 1. In the syntheses, homobiotin tetra-

Scheme 1. Synthesis of Homobiotin-Trioxatridecanediamine-Benzoyl Derivatives **3a, **3b**, and [¹²⁵I]**3c****

^a DMF, Et₃N, rt, 1 h, **8a** (89%), **8b** (72%). ^b MeOH, 1% HOAc, NCS, Na¹²⁵I, rt, 5 min, 13% (radiochemical yield).

Scheme 2. Synthesis of Biotin-Amino Acid-Trioxatridecanediamine-*p*-iodobenzoyl Derivatives 4a, 5a, 6a

^{a,b} Reference 19. ^cDMF, Et₃N, 4 °C, TFP-OTFA, 30 min. ^dEt₃N, **8b**, rt, 1 h (35–65%). ^eTFA.

fluorophenyl (TFP) ester, **7**, was generated in situ by reaction of homobiotin with TFP-OTFA (**19**), and was subsequently reacted with one of the substituted benzoyldiaminotrioxatridecane adducts, **8a** or **8b**, to provide **3a** and **3b** in 89% and 72% overall yield, respectively.

Syntheses of iodo-HPLC standards **4a**, **5a**, and **6a** were accomplished as shown in Scheme 2. The biotin-amino acid adducts **10–13** were prepared from reaction of the *N*-hydroxysuccinimide ester of biotin **9** with the corresponding *t*-Bu-protected amino acids, followed by in situ preparation of the tetrafluorophenyl (TFP) esters **14–16** as previously described (19). The *p*-iodobenzoyl adduct of trioxadamine, **8b**, was then reacted with the TFP esters to provide the adducts **17**, **18**, and **19** in 45%, 65%, and 35% yield, respectively. Subsequent conversion of *tert*-butyl ether/ester adducts to the desired **4a**, **5a**, and **6a** was accomplished in neat trifluoroacetic acid (TFA) in nearly quantitative yield.

High specific activity radioiodination was required for the biotinidase assay. Radioiodinated derivatives **1c**, **2c**, and **3c** were obtained by reaction of HPLC-purified stannylbenzoyl derivatives **1a**, **2a**, and **3a** with no-carrier-added (*nca*) Na¹²⁵I in MeOH/1% HOAc using *N*-chlorosuccinimide (NCS) as the oxidant (15). After radiolabeling, the radioiodinated biotin derivatives [¹²⁵I]**1c**, [¹²⁵I]**2c**, and [¹²⁵I]**3c** were purified by HPLC to rid them of radiochemical contaminants that might interfere with interpretation of the biotinidase assay results. In an effort to improve on the initial studies, which employed two HPLC purification steps in the preparation of [¹²⁵I]**1c**, [¹²⁵I]**2c**, and [¹²⁵I]**3c**, an alternate method that had only one HPLC purification step was used to prepare the subsequent radioiodinated derivatives [¹²⁵I]**4b**, [¹²⁵I]**5b**, and [¹²⁵I]**6b**. In the alternate method (Scheme 3), radioiodination was accomplished by preparing *p*-[¹²⁵I]iodobenzoate *N*-hydroxysuccinimide (NHS) ester, [¹²⁵I]**27**, from the stannylbenzoate NHS ester (**21**), followed by reaction with the amino-biotin derivatives **24–26**. The amino-biotin derivatives **24–26** were prepared in situ by removal of the *t*-Boc groups from **21–23** using neat TFA. Although it was anticipated that the desired products would be obtained in high yield using the alternate

approach, this was not found to be the case. Indeed, several products were obtained in the conjugation reactions, such that purification by HPLC gave only moderate (25–31%) yields of radiolabeled products. Identification of the desired radioiodinated products was obtained by HPLC retention time comparison of, and coelution with, the nonradioactive iodo standards. As an example, an HPLC radiochromatogram of the product mixture obtained in the reaction of **25** with [¹²⁵I]**27** is shown in Figure 1A. An HPLC chromatogram of the nonradioactive **5b** using UV detection is shown in Figure 1B. Since the elution of samples through the radioactivity detector is approximately 0.2 min after the UV detector at the flow rate used, a good correlation of the standard and radiolabeled product was obtained. Co-injection of the iodo standard **5b** further confirmed the identity. All isolated radiolabeled biotin derivatives were single peaks after reinjection on the radio-HPLC.

Biotinidase Assay. The isolated radioiodinated biotin derivatives [¹²⁵I]**1c**, [¹²⁵I]**2c**, [¹²⁵I]**3c**, [¹²⁵I]**4b**, [¹²⁵I]**5b**, and [¹²⁵I]**6b** were evaluated in an assay which determined the amount of radioactivity that bound avidin after the samples were incubated in human serum. This biotinidase cleavage assay is based on the fact that hydrolysis of the biotinamide bond results in separation of the biotin moiety from the radioactivity, which can be measured as a lack of radioactivity binding with avidin. Since the active site of biotinidase contains a sulfhydryl moiety, the biotinidase enzymatic activity can be quenched with the addition of hydroxymercuribenzoic acid (HOHgBz) (22). Thus, a comparison of the amount of avidin binding in the absence or presence of HOHgBz provides confirmation that the lack of binding (i.e., free radioactivity) was due to enzymatic activity.

In an initial evaluation (Table 2, assay 1), biotinidase cleavage of the homobiotin derivative [¹²⁵I]**3c** was compared with a biotin derivative, [¹²⁵I]**1c**, which had previously been shown to be rapidly degraded, and a second derivative, [¹²⁵I]**2c**, which had been shown to be highly resistant to degradation (15). In a second evaluation (Table 2, assay 2), biotin derivatives having substituents alpha to the biotinamide bond ([¹²⁵I]**4b**, [¹²⁵I]**5b**, [¹²⁵I]**6b**)

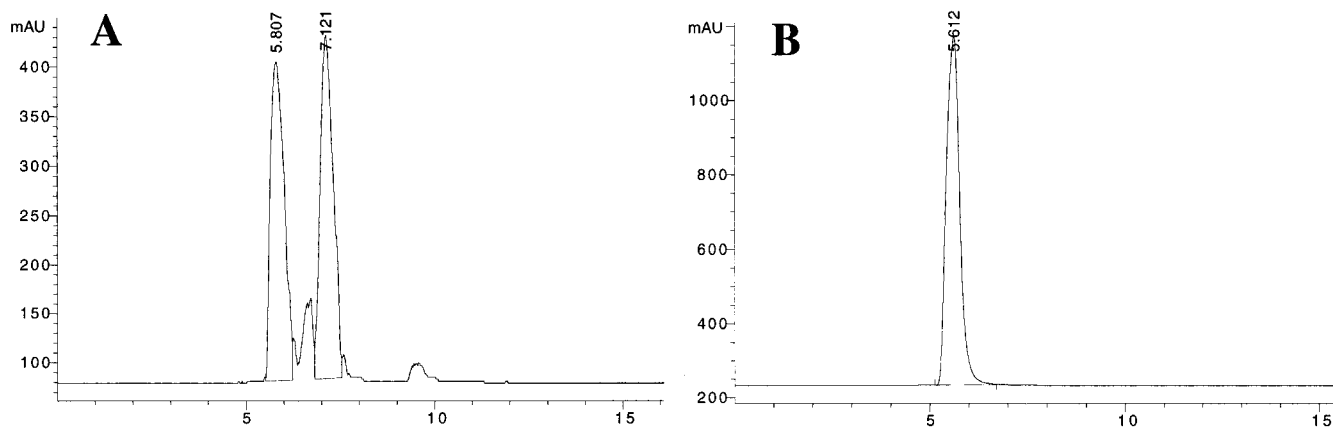
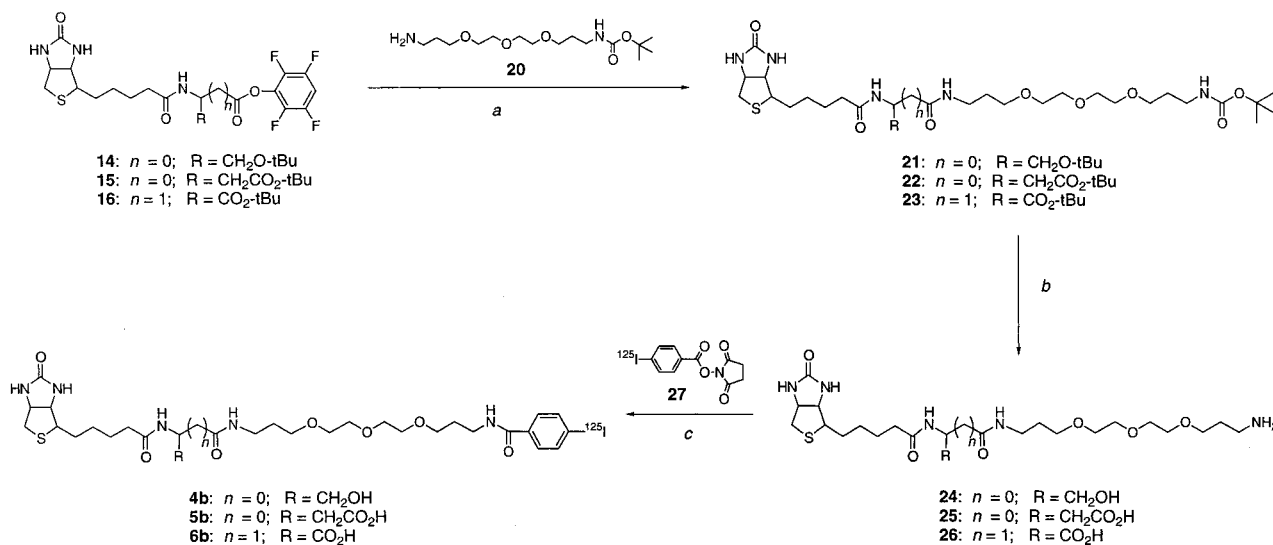


Figure 1. (A) Radiochromatogram of crude product mixture from reaction of **25** with [^{125}I]**27** (Scheme 3). (B) UV chromatogram of purified nonradioactive **5b**.

Scheme 3. Synthesis of Radioiodinated Biotin-Amino Acid-Trioxatridecanediamine-*p*-iodobenzoyl Derivatives [^{125}I]4b**, [^{125}I]**5b**, and [^{125}I]**6b****



^a Et_3N , **20**, rt, 1 h (52–82%). ^b TFA, rt, 10–30 min, 99%. ^c DMF, Et_3N , 30 min, rt, 25–31% (radiochemical yield).

were evaluated. In the analyses, radiolabeled biotin derivatives were incubated in diluted (10%) human serum for 2 h at 37 °C. Following that, HOHgBz was added to quench the reaction, and a phosphate-buffered saline (PBS) solution containing a large excess of avidin was added to that mixture. The biotin derivative was allowed to bind with avidin over a 30 min period at 37 °C. Assessment of the percent of radioactivity that did not bind with avidin was obtained by sizing ultrafiltration separation (Microcon-30). The radiolabeled biotin that was not hydrolyzed remained with the avidin in the top of the Microcon-30, whereas the cleaved material filtered through the membrane. In separate analyses, serum was treated with HOHgBz prior to incubation with the radioiodinated biotin derivative. Duplicate runs were conducted, except for the homobiotin derivative **3c**, which was conducted in triplicate.

DISCUSSION

We are pursuing the development of radiolabeled biotin derivatives for application to the diagnosis and therapy of cancer using the monoclonal antibody-based “pre-targeting method”. During our investigations, and similar investigations of other research groups, it has become apparent that it is essential for application of biotin

derivatives *in vivo* that they be designed to block the enzymatic action of the enzyme biotinidase (8, 10, 23, 24). Although biotinidase stabilization is an essential design feature in new biotin reagents for *in vivo* application, it is also important that biotinidase cleavage be blocked without affecting the high binding affinity of biotin with avidin (Av) and streptavidin (SAv). Following the lead of other investigators (12) in our previous biotin design studies, we found that conversion of the biotinamide bond into a *N*-methylbiotinamide bond blocked cleavage of that bond (15). More recently, we developed a method for evaluating the relative rates of association and dissociation of biotin derivatives from Av and SAv using size-exclusion HPLC (25). Using the HPLC analysis, we evaluated a number of biotin conjugates, including a biotin adduct that contained the *N*-methyl moiety, biotin-sarcosine (19). Unfortunately, it was found that the derivative containing a *N*-methylbiotinamide structure had a more rapid dissociation rate from Av and SAv than biotin conjugates that lacked this structural feature. Not surprisingly, it was found that any alteration of the valeric acid side chain or the biotinamide bond in biotin derivatives resulted in an increased dissociation rate. The studies demonstrated that the dissociation rates were minimally affected when biotin conjugates contained an unaltered biotin moiety linked to molecules (e.g., amino

Table 2. Percentage of Radioiodinated Biotin Derivatives Filtered in the Presence of Avidin after being Incubated in Diluted Human Serum at 37 °C for 2h^a

radioiodinated biotin derivative	group 1 w/o HOHgBz ^b	group 2 with HOHgBz ^c
assay 1		
1c	90.4 ± 0.8	3.8 ± 0.8
2c	5.5 ± 0.0	4.3 ± 0.0
3c	12.3 ± 0.1	3.4 ± 1.6
assay 2		
1c	87.6 ± 1.9 ^d	2.2 ^e
2c	3.0 ± 0.1	1.9 ± 0.0
4b	2.9 ± 0.4	2.8 ± 0.1
5b	1.6 ± 0.0	3.2 ± 0.2
6b	1.9 ± 2.1	1.5 ± 0.1

^a All values represent the percentage of radioactivity filtered through a Microcon-30 centrifugation unit (i.e., not retained by binding with avidin or nonspecifically bound). The activity that filtered through is considered as cleaved from biotin by biotinidase. The values are an average of duplicate assays ± the standard deviation, except for **3c**, which is the average of triplicate assays. Human serum was obtained 1–2 days prior to the assay (refrigerated at 4 °C) and was diluted to 10% of its original composition with 0.9% sterile saline. ^b Assay 1 values represent activity filtered from diluted, but untreated (i.e., without addition of HOHgBz), serum after an excess of avidin was added. ^c Assay 2 values represent activity filtered from diluted and HOHgBz-treated serum after an excess of avidin was added. ^d A similar value (90%) was obtained when this assay was conducted without avidin. Thus, for **1c**, it appears that as much as 10% is retained by nonspecific adherence. ^e One sample was tested.

acids) that had a substituent on a methylene group alpha to the biotinamide bond. As might be expected, large substituents alpha to the biotinamide bond significantly increased dissociation rates, but less sterically bulky groups, e.g., methyl, hydroxymethylene, and carboxylate, had little effect on the dissociation rates. Importantly, previous studies demonstrated that having a methyl substituent alpha to the biotinamide bond decreased the rate of biotinidase cleavage, but did not block it (19).

This investigation was primarily conducted to determine if biotin-amino acid adducts containing the small substituents carboxylate, hydroxymethylene, and acetate were stable to biotinidase cleavage. Prior to the study, we believed there would be a good likelihood of obtaining biotinidase stability. This belief was based on our previous studies and information in the literature relating to structural features of biotin conjugates and stability toward biotinidase cleavage (10, 11, 23, 26–30). Perhaps the most relevant data were reported by Rosebrough, which indicated that an α -carboxylate can block biotinidase activity (11). In that study, the stability toward biotinidase hydrolysis was examined in biotin adducts of deferroxamine, either directly coupled or containing *N*- ϵ -lysyl and cysteinyl linkers. High stability to biotinidase hydrolysis was only demonstrated in the biotin derivative that contained a cysteine linker, which has a carboxylate alpha to the biotinamide bond.

The data on biotinidase cleavage obtained in this study indicate that the substituents hydroxymethylene, carboxylate, and acetate, when placed on a linker molecule alpha to the biotinamide bond, completely block biotinidase activity. These results correlate well with data previously published. The finding that homobiotin is hydrolyzed at a slow rate is consistent with the results reported by Pispas that alterations in the valeric acid chain “almost completely blocked biotinidase activity” (10). However, the increased dissociation rate demonstrated for homobiotin derivatives (19) eliminates it from being an optimal candidate for in vivo use. Foulon et al.

(14, 31) have described the use of norbiotinamine conjugates for blocking the biotinidase activity. Based on our studies with norbiotin derivatives (19), it might be expected that an unfavorable dissociation rate from avidin and SAV would be obtained with these conjugates, making them nonoptimal as well. In this investigation, we included a biotin derivative that contains an acetate moiety alpha to the biotinamide bond (**6b**) even though it was known that this structural feature increased the dissociation rate from Av and SAV, because it was of interest to determine if larger substituents blocked biotinidase. The fact that it completely blocked biotinidase activity suggests that any group larger than a methyl blocks biotinidase activity. The α -acetate might be considered to be isosteric with the *o*-aminobenzoic acid which had been previously shown to block biotinidase activity (26). Although we have not examined the dissociation rate of the biotin-*o*-aminobenzoic acid adduct, it might also be expected to have an increased dissociation rate based on our previous studies.

There are likely to be other structural features of biotin derivatives that will both block biotinidase activity and exhibit the slow dissociation rate with Av and SAV. For example, a linker containing an ethyl group alpha to the biotinamide bond would be a good candidate. We did not evaluate that compound in this study as we felt it would be more lipophilic than desired for in vivo applications. In contrast to our studies, where the evaluation of biotinidase activity of biotin conjugates was based on preparation of targeted high specific activity radioiodinated biotin derivatives, it is likely that optimal structural features may be predictable in the future through molecular modeling techniques. Biotinidase has been isolated, and information on the nature of the binding pocket is being obtained (26, 32). The sequence and cDNA cloning of biotinidase have been completed (33), and more information about its binding pocket is being learned by homology data with other enzymes (34). At this time, we believe that biotin derivatives containing a carboxylate or hydroxymethylene moiety alpha to the biotinamide bond are the best candidates for developing reagents for in vivo application.

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Supporting Information Available: HPLC chromatograms, ¹H NMR spectra, LRMS, and HRMS of the new compounds prepared (**3a**, **3b**, **4a**, **5a**, **6a**, **17**, **18**, **19**, **21**, **22**, **23**) in the research described in this paper (42 pages). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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^{99m}Tc-Labeling of a Hydrazinonicotinamide-Conjugated Vitronectin Receptor Antagonist Useful for Imaging Tumors

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This report describes the ^{99m}Tc labeling of a HYNIC-conjugated vitronectin receptor antagonist (SQ168 = [2-[[[5-[carboonyl]-2-pyridinyl]hydrazono]methyl]benzenesulfonic acid]-Glu(cyclo{Lys-Arg-Gly-Asp-D-Phe})-cyclo{Lys-Arg-Gly-Asp-D-Phe}). The ternary ligand complex [^{99m}Tc(SQ168)(tricine)(TPPTS)] (RP593) was prepared using a non-SnCl₂-containing formulation. The corresponding ^{99m}Tc analogue, [^{99m}Tc]RP593, was also prepared and characterized by HPLC and LC-MS. A HPLC concordance experiment using RP593 and [^{99m}Tc]RP593 showed that the same technetium complex was prepared at both the tracer and macroscopic levels. The LC-MS data is completely consistent with the 1:1:1:1 composition for Tc:SQ168:tricine:TPPTS and provides direct evidence that the two radiometric peaks in the radio-HPLC chromatogram of RP593 are indeed due to the resolution of diastereomers. In an in vitro receptor binding assay, [^{99m}Tc]RP593 was shown to have comparable binding affinity for the vitronectin receptor to that of SQ168 itself.

INTRODUCTION

There is currently a considerable interest in labeling small biomolecules with ^{99m}Tc for the development of target specific imaging agents (1–10). For the last several years, we have been using 6-hydrazinonicotinamide (HYNIC) as a bifunctional coupling agent for the ^{99m}Tc-labeling of small biomolecules (BM), including a GPIIb/IIIa receptor antagonist for thrombus imaging (11–18), a chemotactic peptide (19), and LTB₄ receptor antagonists for imaging infection and inflammation (20–22). It was found that the combination of HYNIC-BM, tricine, and TPPTS (trisodium triphenylphosphine-3,3',3''-trisulfonate) produces a unique and versatile ternary ligand system which forms technetium complexes, [^{99m}Tc(HYNIC-BM)(tricine)(TPPTS)] with high solution stability and only two detectable isomeric forms. As a continuation of our efforts in developing new target-specific diagnostic radiopharmaceuticals, we now describe the ^{99m}Tc-labeling of a vitronectin receptor antagonist (Figure 1: SQ168 = [2-[[[5-[carboonyl]-2-pyridinyl]hydrazono]methyl]benzenesulfonic acid]-Glu(cyclo{Lys-Arg-Gly-Asp-D-Phe})-cyclo{Lys-Arg-Gly-Asp-D-Phe}).

The ternary ligand complex [^{99m}Tc(SQ168)(tricine)(TPPTS)] (Figure 1: RP593) was prepared using a non-SnCl₂-containing formulation (23) and was characterized using a reversed phase HPLC method. The corresponding ^{99m}Tc analogue, [^{99m}Tc]RP593, was also prepared and characterized by HPLC and LC-MS. A HPLC concordance experiment using RP593 and [^{99m}Tc]RP593 showed that the same complex was prepared at both the tracer and macroscopic levels. In an in vitro receptor binding assay, [^{99m}Tc]RP593 was shown to have comparable binding affinity for the vitronectin receptor as SQ168. Apparently, the attachment of the technetium chelate does not have significant effect on the receptor binding affinity. RP593

has been evaluated in two spontaneous tumor models (c-Neu oncomouse and dog) for its potential use as a new radiopharmaceutical for imaging tumors. The biological properties of RP593 have been described in our previous communications (24, 25).

EXPERIMENTAL SECTION

Materials. Succinic acid, TPPTS (trisodium triphenylphosphine-3,3',3''-trisulfonate), and tricine were purchased from Aldrich Chemical Co. TPPTS was purified according to the literature method (26). The cyclic pentapeptide, cyclo(Arg-Gly-Asp-D-Phe-Lys), was prepared according to the literature method (27). Synthesis of SQ168 has been reported as a separate communication (28). Na^{99m}TcO₄ was obtained from a commercial DuPont Pharma ⁹⁹Mo/^{99m}Tc generator, N. Billerica, MA.

Analytical Methods. HPLC methods used a HP 1100 system, a homemade NaI radiometric detector, a Zorbax C₁₈ column (4.6 mm × 250 mm) at a flow rate of 1 mL/min. In method 1, the mobile phase was isocratic at 0–18 min using 90% solvent A (95:5 (v:v) = 0.025 M ammonium acetate buffer (pH = 6.8):acetonitrile) and 10% solvent B (95:5 (v:v) = acetonitrile: 0.025 M ammonium acetate buffer, pH = 6.8), followed by an isocratic wash using 40% solvent A and 60% solvent B at 19–25 min. In method 2, the gradient mobile phase started from 92% solvent A (0.025 M succinate buffer, pH = 5.0) and 8% solvent B (acetonitrile) to 80% solvent A and 20% solvent B at 25 min. The mobile phase was isocratic at 26–31 min using 60% solvent B. The ITLC method used Gelman Sciences silica gel ITLC paper strips and 1:1 mixture of acetone and saline as eluant. Using this ITLC method, the radiolabeled peptide and [^{99m}Tc]pertechnetate migrate while the [^{99m}Tc]colloid remains at the origin.

LC-MS Methods. LC-MS spectral data were collected using a HP1100 LC/MSD system with an API-electrospray interface. The LC-MS method used a Zorbax C₁₈ column (4.6 mm × 150 mm, 3.5 μm particle size) and a mobile phase gradient starting from 92% solvent A (10 mM ammonium acetate buffer, pH 7.0) and 8% solvent

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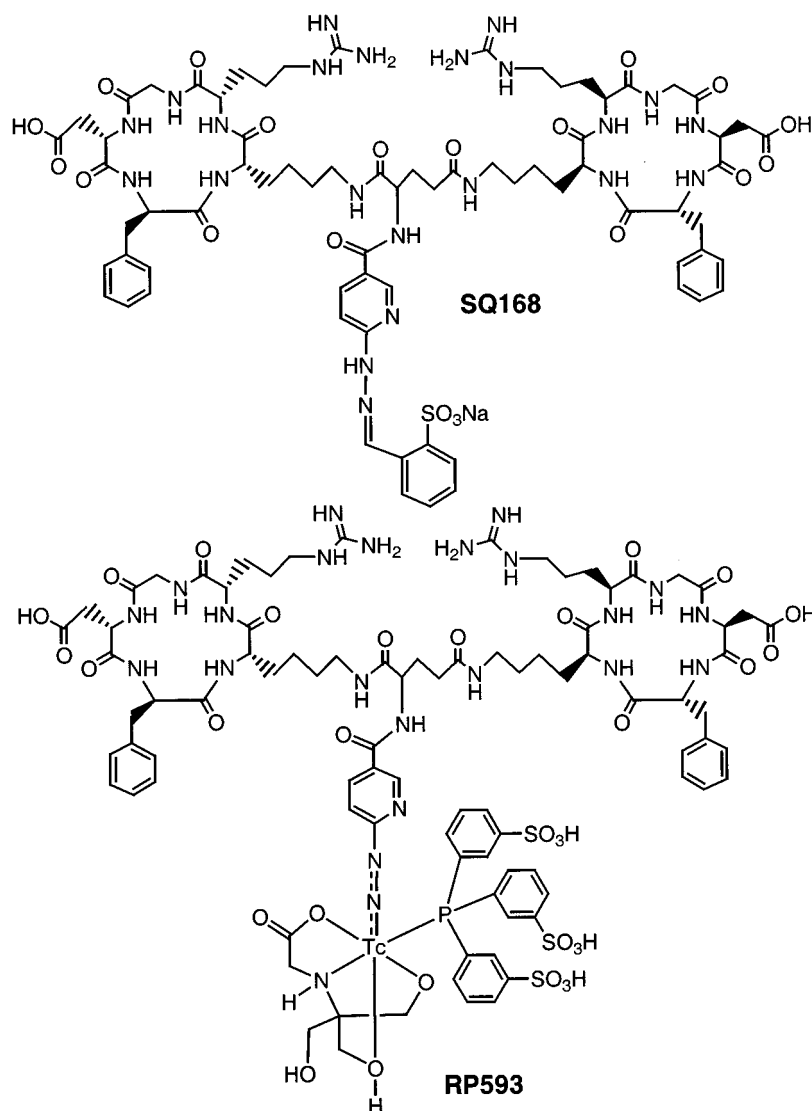


Figure 1. Structures of a HYNIC-conjugated vitronectin receptor antagonist (SQ168) and its ternary ligand technetium complex [^{99m}Tc(SQ168)(tricine)(TPPTS)].

Table 1. LMD Parameters Used for Characterization of RP593

detection mode:	positive
mass range:	600–2000
gain:	1.0
fragmentor:	15 V
gas temperature:	350 °C
drying gas flow:	13 L/min
nebulizer pressure:	60 psig (max.)
V capillary:	4000 V
radio-detector:	NaI
UV detector:	λ = 280 nm

B (methanol) to 100% B at 23 min at a flow rate of 1 mL/min. The MSD parameters are listed in Table 1.

Synthesis of RP593. RP593 was prepared according to the published procedure (13) with a slight modification. To a lyophilized vial containing 5 mg of TPPTS, 6.5 mg of tricine, 40 mg of mannitol, 38.5 mg of disodium succinate hexahydrate, 12.7 mg of succinic acid, and 0.1 mg of pluronic acid (0.1%); pH = 4.8) was added 0.5 mL of SQ168 solution (40 µg/mL in water). The vial was placed into a lead pig. To the vial was added 1.0 mL of Na[^{99m}TcO₄] solution (50 mCi/mL) in saline. The reconstituted vial was heated at 100 °C for 10 min in a lead-shielded water bath. After heating, the vial was placed back into the lead pig and allowed to stand at room

temperature for ~10 min. A sample of the resulting solution was analyzed by the radio-HPLC (method 1) and ITLC.

Solution Stability Studies. For solution stability in the kit matrix, RP593 was first prepared, and samples of the resulting reaction were analyzed by HPLC (method 1) at *t* = 0, 2, 4, and 6 h postlabeling. For HPLC-purified RP593, the two peaks at 9.3 and 12 min were collected into a 25 mL round-bottom flask. Solvents were removed using a rotary evaporator. The residue was dissolved in saline and samples of the resulting solution were analyzed by HPLC (method 1) at 0, 2, 4, and 6 h postpurification.

[⁹⁹Tc]RP593. To a clean 10 mL vial were added 1.5 mg of SQ168, 84 mg of tricine, 49 mg of TPPTS, 1.2 mg of NH₄[⁹⁹TcO₄] in 0.4 mL of H₂O, and 1.5 mL of 25 mM succinate buffer (pH = 5.0). The reaction mixture was heated in a 100 °C water bath for 30 min. After the mixture was cooled to room temperature, the product was separated by HPLC (method 2). The collections of the two peaks due to [⁹⁹Tc]RP593 were combined, and volatiles were removed under reduced pressure. The residue was then desalted using the same HPLC method using water as mobile phase A. Removal of the solvent gave the product, [⁹⁹Tc]RP593.

Immobilized Human Placental $\alpha\beta 3$ Receptor ELISA Assay. The placental $\alpha\beta 3$ receptors were isolated according to the published method (29). The assay conditions were developed and validated using [^{125}I]-vitronectin. Assay validation included Scatchard format analysis ($n = 3$) where receptor number (B_{max}) and K_d (affinity) were determined. Three standards (vitronectin, $\alpha\beta 3$ antibody, LM609, and $\alpha\beta 5$ antibody, P1F6) have been evaluated for IC_{50} determination. Briefly, the method involves immobilizing previously isolated receptors in 96 well plates (EIA/RIA, CORNING Costar) and incubating overnight. The plates are then blocked with 1% bovine serum albumin. During this time, compounds to be tested were prepared in 10 nM biotinylated vitronectin ([B]Vn) solution and were dispensed into a reservoir plate. Blocking buffer is removed, wells were washed, and then compound/[B]Vn mixture was pipetted. Incubation is carried out for 30 min at room temperature. The unbound material was removed, and wells were washed. [B]Vn binding was qualified using goat anti-biotin alkaline phosphatase conjugated antibody (GAB). Unbound GAB was removed, wells were washed, and phosphatase enzyme assay was performed. Enzyme product was detected via spectrophotometry ($\lambda = 405 \text{ nm}$) for subsequent IC_{50} determination. All concentrations of the tested compound were run in triplicate. Four independent measurements were made for [^{99}Tc]RP593. The IC_{50} value was calculated by fitting the percent inhibition values to a regression line.

RESULTS AND DISCUSSION

Angiogenesis, the formation of new blood vessels, is a requirement for malignant tumor growth and metastasis (30–32). The angiogenic process depends on vascular endothelial cell migration and invasion, regulated by cell adhesion receptors. The integrin $\alpha\beta 3$ (vitronectin receptor) is such a cell adhesion receptor and interacts with proteins and peptides containing the RGD tripeptide recognition sequence (33–42). In general, the level of expression of $\alpha\beta 3$ receptor is low on most cell types and is greatly increased in remodeling and growing tissues (43). Many peptide vitronectin receptor antagonists have high binding affinity for the vitronectin receptor and have been shown to be able to inhibit neovascularization, tumor-induced angiogenesis, and tumor growth (40–46). Therefore, these vitronectin receptor antagonists can serve as targeting molecules for diagnosis and radiotherapy of a wide spectrum of tumors.

Recently, Kessler and co-workers (47, 48) reported two ^{125}I -labeled cyclic pentapeptides: 3- ^{125}I -D-Tyr⁴-cyclo-(RGDyV) and 3- ^{125}I -D-Tyr⁴-cyclo-(RGDyK(SAA1)) (SAA = sugar amino acid). It was found that 3- ^{125}I -D-Tyr⁴-cyclo-(RGDyV) has fast hepatobiliary and renal excretion (47). The tumor/muscle and tumor/blood ratios for melanoma in nude mice were 5.5 and 9.5, respectively, at 60 min postinjection. Substitution of leucine with a SAA-functionalized lysine amino acid residue resulted in improved blood retention time, renal excretion, and better target-to-background ratio. A blocking study using 3 mg/kg of the $\alpha\beta 3$ selective cyclo(RGDfV) demonstrated that the localization of radioactivity in the tumor is due to $\alpha\beta 3$ receptor binding (48).

For diagnostic purposes $^{99\text{m}}\text{Tc}$ is more desirable than ^{125}I due to its easy availability, low cost, and ideal nuclear characteristics, which better match the rapid blood clearance and fast tumor accumulation of small peptides. Therefore, a series of RGD-containing peptide conjugates have been prepared for the development of a target-

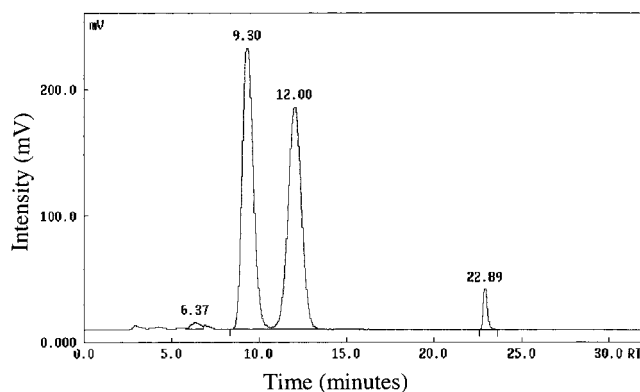


Figure 2. A typical radio-HPLC chromatogram of RP593 (method 1).

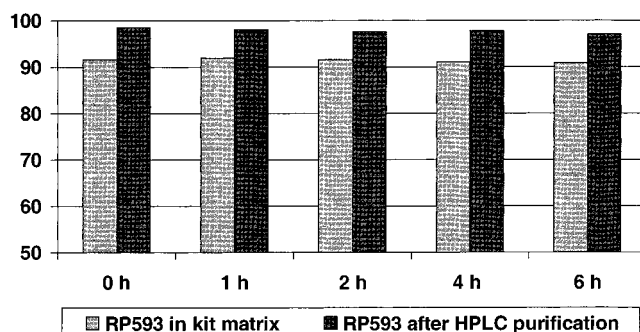


Figure 3. Solution stability of RP593 in the kit matrix and after HPLC purification.

specific tumor imaging agent (24, 25). In the c-Neu Oncomouse tumor model, RP593 shows high tumor uptake and long tumor residence time (3.4% ID/g at 2 h and 1.5% ID/g at 24 h postinjection) and is excreted predominantly via the renal system. As an extension of these efforts, we present the synthesis and characterization of the ternary ligand $^{99\text{m}}\text{Tc}$ complex (Figure 1: RP593) of a HYNIC-conjugated vitronectin receptor antagonist (Figure 1: SQ168).

Synthesis of RP593. In this study, RP593 was prepared according to the published procedure (23) using a non-SnCl₂-containing formulation. The radiolabeling was achieved by adding 1.0 mL of generator eluant (50 mCi of Na[$^{99\text{m}}\text{TcO}_4$]) and heating at 100 °C for 10 min. The radiochemical purity (RCP) for RP593 is usually $\geq 90\%$. The formation of [$^{99\text{m}}\text{Tc}$]colloid is minimal ($<0.5\%$). Very high specific activity ($\sim 10000 \text{ mCi}/\mu\text{mol}$) can be achieved for RP593 without postlabeling purification.

HPLC Characterization. A reverse phase HPLC method was used for characterization of RP593. Figure 2 shows a typical radio-HPLC chromatogram. The small peak at $\sim 23 \text{ min}$ is due to a combination of several radio-impurities. Since most of radio-impurities in the RP593 kit are less than 1.0%, no further characterization was performed. The technetium chelate moiety is chiral, due to the tricine ligand being prochiral and should be formed in an equal mixture of D and L enantiomers. These enantiomers in combination with the chiral centers on the peptide backbone should result in two diastereomers, which are separable using a reverse phase HPLC method. Therefore, it is not surprising that RP593 shows two radiometric peaks in its radio-HPLC chromatogram and the area ratio of these two peaks is always close to 1:1.

Solution Stability Studies. We also examined the solution stability of RP593. Figure 3 shows the plot of RCP vs time for RP593 both in kit matrix and in the

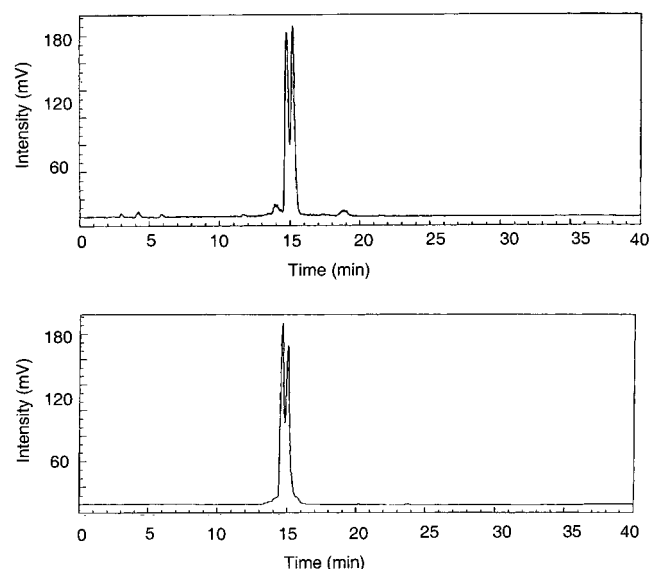


Figure 4. The HPLC concordance (method 2) for RP593 (top) and ^{99m}Tc RP593 (bottom).

dilute solution after HPLC purification. It is clear that RP593 remains stable for at least 6 h both in the kit matrix and after HPLC purification.

Synthesis of ^{99m}Tc RP593. ^{99m}Tc RP593 was prepared in one step by reacting $[\text{NH}_4][^{99m}\text{TcO}_4]$ with SQ168 in the

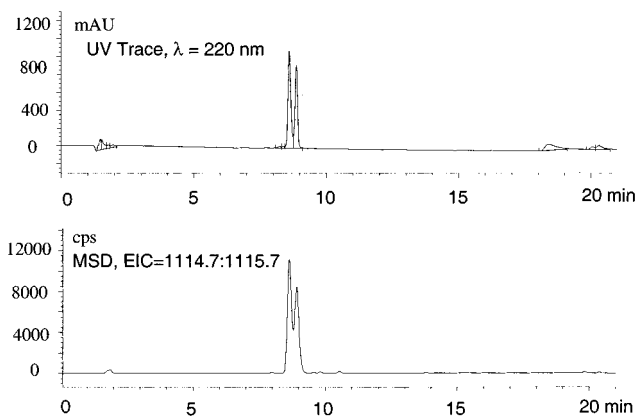


Figure 5. UV (top) and MSD (bottom) profiles for ^{99m}Tc RP593.

presence of excess tricine and TPPTS. Unlike that of RP593, the yield for ^{99m}Tc RP593 is low (<30% by UV). After purification (method 2) and desalting, ^{99m}Tc RP593 was obtained only in milligram quantity, which is not enough for extensive NMR studies. However, sufficient material was obtained for the HPLC concordance experiment, *in vitro* receptor binding assay, and LC-MS characterization.

HPLC Concordance Experiment. The HPLC concordance experiment is a very useful supplementary method for compositional and structural determination

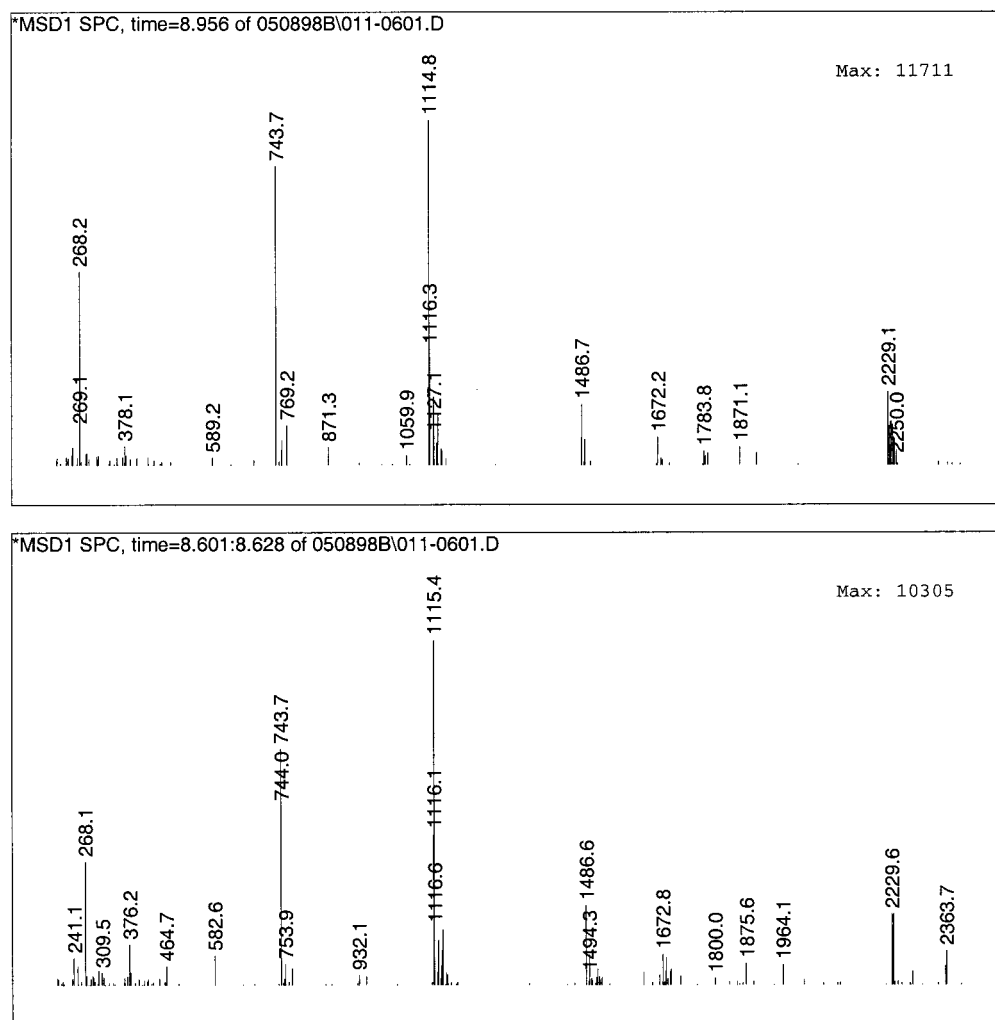


Figure 6. LC-MS spectra of the two isomers of ^{99m}Tc RP593.

of a new radiopharmaceutical. In this study, we used the identical chromatographic conditions for the HPLC analysis of both RP593 and [^{99}Tc]RP593. Figure 4 shows HPLC chromatograms of RP593 (top) and [^{99}Tc]RP593 (bottom). It is clearly demonstrated that the same complexes were prepared at the tracer ($^{99\text{m}}\text{Tc}$) and macroscopic (^{99}Tc) levels.

LC-MS Characterization of [^{99}Tc]RP593. In our previous communication, we reported a simple LC-MS method for the characterization of $^{99\text{m}}\text{Tc}$ radiopharmaceuticals at the tracer level (49). When we used the same MSD parameters for characterization of [^{99}Tc]RP593, only the triprotonated molecular ion, $(M + 3)^{3+}$ ($m/z = 743.8$) and the diprotonated molecular ion, $(M + 2)^{2+}$ ($m/z = 1114.8$) were observed in the LC-MS spectrum. This suggests that the original fragmentor voltage was too high. When the fragmentor voltage was lowered from 30 to 15 V, the monoprotonated molecular ion, $(M + 1)^+$, peak at $m/z = 2229.2$ for $[\text{C}_{89}\text{H}_{115}\text{N}_{23}\text{O}_{31}\text{PS}_3\text{Tc}]^+$ became obvious; but the diprotonated and triprotonated molecular ions remain predominant in the LC-MS spectrum. The observation of the predominant diprotonated and triprotonated molecular ions is also characteristic of the unlabeled HYNIC-BM conjugate, SQ168. The LC-MS data support the proposed structure (Figure 1) and is completely consistent with the 1:1:1:1 composition for Tc:SQ168:tricine:TPPTS. [^{99}Tc]RP593 shows two peaks in both the UV and MSD profiles (Figure 5). Both peaks show almost identical fragmentation patterns and the same molecular ions (Figure 6). This provides direct evidence for the conclusion that the two radiometric peaks in the radio-HPLC chromatogram of RP593 are indeed due to the resolution of diastereomers.

In Vitro Receptor Binding of [^{99}Tc]RP593. In an in vitro binding assay, [^{99}Tc]RP593 was able to effectively compete with 10 nM biotinylated vitronectin ([B]Vn) with IC_{50} of 1.7 ± 0.5 nM ($n = 4$). Compared to the corresponding unlabeled cyclic peptide, which has an IC_{50} of 0.6 ± 0.4 nM ($n = 4$), [^{99}Tc]RP593 remains a high affinity vitronectin receptor antagonist. The $^{99\text{m}}\text{Tc}$ -labeling did not significantly alter the binding affinity even though the molecular weight is significantly increased after radiolabeling.

CONCLUSION

In this report, we described the $^{99\text{m}}\text{Tc}$ labeling of a HYNIC-conjugated vitronectin receptor antagonist. The ternary ligand complex [$^{99\text{m}}\text{Tc}(\text{SQ168})(\text{tricine})(\text{TPPTS})$] (RP593) was prepared in high radiochemical purity and high specific activity using a non-SnCl₂ formulation. RP593 was stable in solution for at least 6 h postlabeling. The corresponding ^{99}Tc analogue, [^{99}Tc]RP593, was also prepared and characterized by HPLC and LC-MS. A HPLC concordance experiment using RP593 and [^{99}Tc]RP593 showed that the same technetium complex was prepared at both the tracer and macroscopic levels. The LC-MS data is completely consistent with the 1:1:1:1 composition for Tc:SQ168:tricine:TPPTS. In an in vitro receptor-binding assay, [^{99}Tc]RP593 had a similar binding affinity (within the experimental error) for the vitronectin receptor to that of the unlabeled cyclic peptide (SQ168).

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Synthesis and Characterization of Two ^{111}In -Labeled DTPA–Peptide Conjugates

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This report describes the synthesis and characterization of two ^{111}In -labeled DTPA–peptide conjugates (DTPA–MA and DTPA–BA). It is surprising to find that $^{111}\text{In}(\text{DTPA–MA})$ and $^{111}\text{In}(\text{DTPA–BA})$ are more hydrophilic than their corresponding ^{90}Y analogues, suggesting a different coordination sphere in ^{111}In and ^{90}Y complexes of the same DTPA–peptide conjugate. By a reversed phase HPLC method, both $^{111}\text{In}(\text{DTPA–MA})$ and $^{111}\text{In}(\text{DTPA–BA})$ showed only one radiometric peak in their respective HPLC chromatogram due to a rapid interconversion of different isomers (particularly *cis* and *trans* isomers for $^{111}\text{In}(\text{DTPA–MA})$; *cis-cis*, *cis-trans*, *trans-cis*, and *trans-trans* isomers for $^{111}\text{In}(\text{DTPA–BA})$). The interconversion of different isomers involves the “wagging” of the diethylenetriamine backbone, “shuffling” of the NO or NO₂ donor sets, and a rapid inversion at the terminal amine–nitrogen atoms.

INTRODUCTION

There is currently a considerable interest in radiolabeled biomolecules (BM) as target-specific radiopharmaceuticals for the diagnosis and treatment of cancer (1–7). DTPA (diethylenetriaminepentaacetic acid) and its analogues have been used as bifunctional chelators (BFCs) for the radiolabeling of various biomolecules, including antibodies, antibody fragments, and small peptides (8–27). This has been reviewed recently (26). While the ^{90}Y -labeled BFC–BM conjugates are used for tumor therapy, the ^{111}In -labeled BFC–BM conjugates are often used as an imageable surrogate for the purpose of dosimetry determination and therapy monitoring (27–36).

In our previous communication (37), we reported two ^{90}Y -labeled DTPA-conjugated cyclic peptides: DTPA–MA and DTPA–BA (Figure 1). It was found that the complex $^{90}\text{Y}(\text{DTPA–MA})$ exists in solution as a mixture of two detectable isomers, which slowly interconvert at room temperature. The interconversion of two isomers involves a rapid exchange of “wrapping isomers” via the “wagging” of the diethylenetriamine backbone, “shuffling” of the two NO₂ donor sets, and the inversion at the terminal amine–nitrogen atom. For the complex $^{90}\text{Y}(\text{DTPA–BA})$, the interconversion of different isomers is much faster due to the weak bonding of two carbonyl–oxygen donors. The complex $^{90}\text{Y}(\text{DTPA–BA})$ shows only one broad radiometric peak in its HPLC chromatogram. The interconversion of different isomers is intramolecular via a partial dissociative mechanism and is consistent with the lack of kinetic inertness of ^{90}Y -labeled DTPA–BM conjugates.

As an extension of our efforts in understanding of the solution chemistry of the radiolabeled DTPA–BM conjugates, we describe the synthesis and characterization of ^{111}In complexes of DTPA–MA and DTPA–BA. The purpose is to compare the ^{111}In -labeled DTPA–BM conjugates with their corresponding ^{90}Y analogues with

respect to hydrophilicity and isomerism. We prepared the ^{111}In complexes by reacting DTPA–MA or DTPA–BA with $^{111}\text{InCl}_3$ in the 0.5 M ammonium acetate buffer (pH = 6.0) at room temperature and characterized them using a reversed phase HPLC method. It was surprising to find that both $^{111}\text{In}(\text{DTPA–MA})$ and $^{111}\text{In}(\text{DTPA–BA})$ are more hydrophilic than their respective ^{90}Y analogues. Unlike the complex $^{90}\text{Y}(\text{DTPA–MA})$, $^{111}\text{In}(\text{DTPA–MA})$ showed only one radiometric peak in its HPLC chromatogram due to a more rapid interconversion of *cis*- and *trans*-isomers.

EXPERIMENTAL SECTION

Materials. Acetic acid (ultrapure), ammonium hydroxide (ultrapure), and diethylenetriaminepentaacetic acid cyclic dianhydride were purchased from either Aldrich or Sigma Chemical Co. and were used as received. $^{90}\text{YCl}_3$ (in 0.05 N HCl) and $^{111}\text{InCl}_3$ (in 0.05 N HCl) were purchased from New England Nuclear, N. Billerica, MA. The DTPA–peptide conjugates, cyclo(Arg–Gly–Asp–D–Phe–Lys)DTPA (DTPA–MA) and [cyclo(Arg–Gly–Asp–D–Phe–Lys)]₂DTPA (DTPA–BA) and their ^{90}Y complexes have been described in our previous communication (37).

Synthesis of $^{111}\text{In}(\text{DTPA–MA})$ and $^{111}\text{In}(\text{DTPA–BA})$. To a lead-shielded clean 5 mL vial containing 50 μg of DTPA–MA or DTPA–BA in 1 mL of 0.5 M ammonium acetate buffer (pH = 6.0) was added 5–10 μL of $^{111}\text{InCl}_3$ stock solution (~ 1 mCi) in 0.05 N HCl. The reaction mixture was allowed to stand at room temperature for 10–30 min. A sample of the resulting solution was then analyzed by HPLC (gradient A for $^{111}\text{In}(\text{DTPA–MA})$ and gradient B for $^{111}\text{In}(\text{DTPA–BA})$) and by ITLC.

The HPLC method used a HP-1100 HPLC system with a UV/visible detector ($\lambda = 230$ nm), an IN–US radio-detector, and a Zorbax C₁₈ column (4.6 mm $\times$ 250 mm, 80 Å pore size). The flow rate was 1 mL/min with a gradient mobile phase. For $^{111}\text{In}(\text{DTPA–MA})$, the gradient started from 95% solvent A (95:5 mixture of 25 mM ammonium acetate buffer, pH 6.8, and acetonitrile) and 5% solvent B (50:50 mixture of 25 mM ammonium acetate buffer, pH 6.8, and acetonitrile) to 90% of solvent

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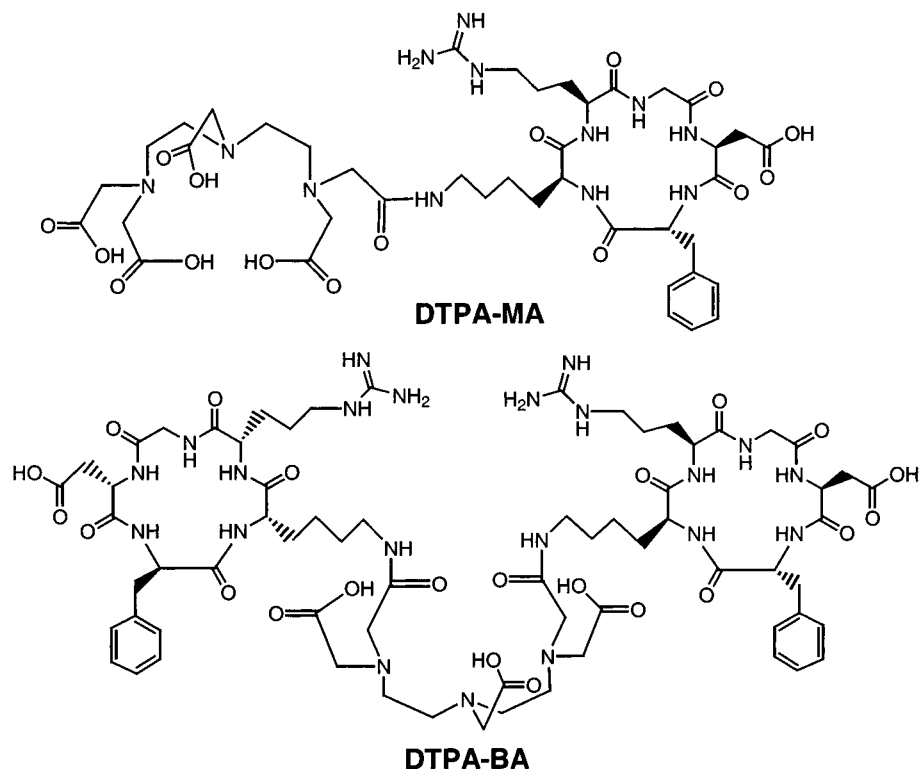


Figure 1. Structures of DTPA-MA (top) and DTPA-BA (bottom).

A and 10% solvent B at 24 min. For $^{111}\text{In}(\text{DTPA-BA})$, the gradient started from 85% solvent A and 15% solvent B to 80% of solvent A and 20% solvent B at 18 min. The ITLC method used Gelman Sciences silica gel ITLC paper strips and 1:1 mixture of acetone and saline as eluant. By this method, $^{111}\text{In}(\text{DTPA-MA})$ and $^{111}\text{In}(\text{DTPA-BA})$ migrate to the solvent front while ^{111}In colloid and ^{111}In -acetate remain at the origin.

RESULTS

Synthesis of $^{111}\text{In}(\text{DTPA-MA})$ and $^{111}\text{In}(\text{DTPA-BA})$.

$^{111}\text{In}(\text{DTPA-MA})$ and $^{111}\text{In}(\text{DTPA-BA})$ were prepared by reacting DTPA-MA and DTPA-BA, respectively, with $^{111}\text{InCl}_3$ in the 0.5 M ammonium acetate buffer (pH = 6.0) at room temperature. Both $^{111}\text{In}(\text{DTPA-MA})$ and $^{111}\text{In}(\text{DTPA-BA})$ could be prepared in high yield (RCP > 95%) by reacting 50 μg of DTPA conjugate with 1.0 mCi of $^{111}\text{InCl}_3$. The ^{111}In -chelation was almost instantaneous for both DTPA-MA and DTPA-BA. This is consistent with the results reported in the literature (22, 23). Both $^{111}\text{In}(\text{DTPA-MA})$ and $^{111}\text{In}(\text{DTPA-BA})$ are stable in solution for at least 24 h without significant decomposition.

HPLC Characterization of $^{111}\text{In}(\text{DTPA-MA})$. For the past decade, HPLC has become a routine technique for quality control of various radiopharmaceuticals. The advantage of radio-HPLC is its capability to determine the radiochemical purity (RCP) of the radiopharmaceutical and to separate different radiolabeled species in the reaction mixture. It is also a very powerful tool for separation of different isomers such as diastereomers. In this study, we used a reversed phase HPLC method to analyze reaction mixtures containing $^{111}\text{In}(\text{DTPA-MA})$. For comparison purpose, we also prepared the complex $^{90}\text{Y}(\text{DTPA-MA})$. Both complexes were analyzed under identical chromatographic conditions. Figure 2 shows typical radio-HPLC chromatograms of $^{90}\text{Y}(\text{DTPA-MA})$ (top) and $^{111}\text{In}(\text{DTPA-MA})$ (bottom). Apparently, the

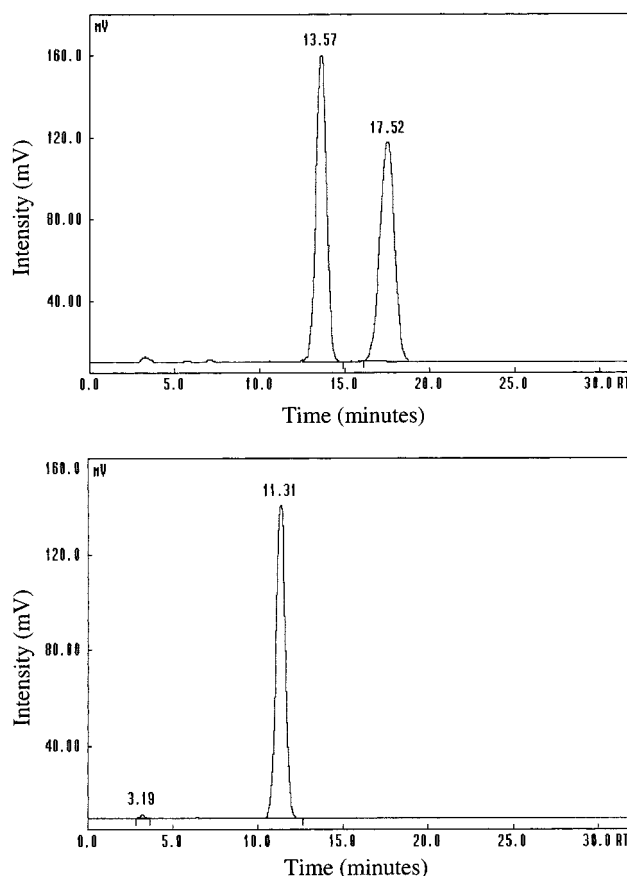


Figure 2. Typical radio-HPLC chromatograms for $^{90}\text{Y}(\text{DTPA-MA})$ (top) and $^{111}\text{In}(\text{DTPA-MA})$ (bottom) using method 1.

hydrophilicity of $^{111}\text{In}(\text{DTPA-MA})$ is higher than that of $^{90}\text{Y}(\text{DTPA-MA})$, reflecting the difference in their coordination chemistry. Unlike $^{90}\text{Y}(\text{DTPA-MA})$, which shows two radiometric peaks due to the resolution of *cis*- and

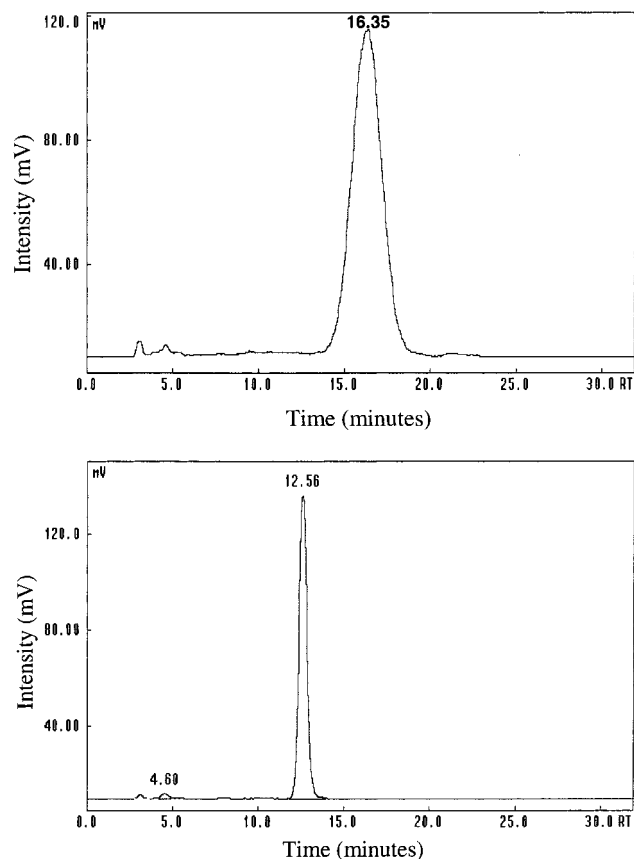


Figure 3. Typical radio-HPLC chromatograms for $^{90}\text{Y}(\text{DTPA}-\text{BA})$ (top) and $^{111}\text{In}(\text{DTPA}-\text{BA})$ (bottom) using method 2.

trans-isomers, $^{111}\text{In}(\text{DTPA}-\text{MA})$ shows only one radiometric peak at ~ 11.5 min. We tried several other reversed phase HPLC methods. In all the cases, $^{111}\text{In}(\text{DTPA}-\text{MA})$ always shows only one radiometric peak, suggesting that the complex $^{111}\text{In}(\text{DTPA}-\text{MA})$ indeed exists as one *detectable* isomer in solution.

HPLC Characterization of $^{111}\text{In}(\text{DTPA}-\text{BA})$. We used a slightly different gradient mobile phase when analyzing $^{111}\text{In}(\text{DTPA}-\text{BA})$ by HPLC due to its higher lipophilicity. We also prepared the complex $^{90}\text{Y}(\text{DTPA}-\text{MA})$. Complexes $^{111}\text{In}(\text{DTPA}-\text{BA})$ and $^{90}\text{Y}(\text{DTPA}-\text{BA})$ were analyzed using the same HPLC method. Figure 3 shows typical radio-HPLC chromatograms of $^{90}\text{Y}(\text{DTPA}-\text{BA})$ (top) and $^{111}\text{In}(\text{DTPA}-\text{BA})$ (bottom). Like $^{111}\text{In}(\text{DTPA}-\text{MA})$, $^{111}\text{In}(\text{DTPA}-\text{BA})$ shows one radiometric peak in its HPLC chromatogram. The retention time of $^{111}\text{In}(\text{DTPA}-\text{BA})$ is ~ 4 min shorter than that of the corresponding yttrium analogue, $^{90}\text{Y}(\text{DTPA}-\text{BA})$, suggesting a higher hydrophilicity for $^{111}\text{In}(\text{DTPA}-\text{BA})$.

DISCUSSION

Previously, we reported the synthesis and characterization of two ^{90}Y -labeled DTPA-conjugated cyclic peptides: DTPA-MA and DTPA-BA (37). It was found that the complex $^{90}\text{Y}(\text{DTPA}-\text{MA})$ exists in solution as a mixture of two *detectable* isomers (Figure 4), which slowly interconvert at room temperature. In the complex $^{90}\text{Y}(\text{DTPA}-\text{MA})$, DTPA-MA is octadentate with three amine-nitrogen, one carbonyl-oxygen, and four carboxylate-oxygen donors binding to the metal center. Although there is a rapid exchange of "wrapping isomers" via the "wagging" of the diethylenetriamine backbone and "shuffling" of the two NO_2 donor sets, the inversion at the terminal amine-nitrogen atoms is slow at room temper-

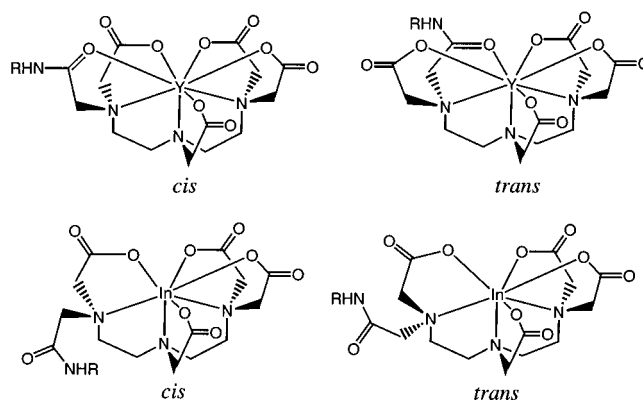


Figure 4. Relative orientations of the acetamide group in $^{90}\text{Y}(\text{DTPA}-\text{MA})$ (top) and $^{111}\text{In}(\text{DTPA}-\text{MA})$ (bottom).

ature so that the *cis* and *trans* isomers in $^{90}\text{Y}(\text{DTPA}-\text{MA})$ is detectable by radio-HPLC. Therefore, the complex $^{90}\text{Y}(\text{DTPA}-\text{MA})$ shows two peaks in its HPLC chromatogram (Figure 2).

In^{3+} has an ionic radius of $0.75 \text{ \AA}$ (38), which is smaller than that of Y^{3+} ($0.87 \text{ \AA}$). The coordination number for In^{3+} is typically 6 or 7. Only a few eight coordinated In^{3+} complexes are known (39, 40). Since In^{3+} is smaller, DTPA-MA is likely heptadentate with three amine-nitrogen and four carboxylate-oxygen donors binding to the metal center while the carbonyl-oxygen remains uncoordinated (Figure 4). The presence of the uncoordinated carbonyl-oxygen makes the complex $^{111}\text{In}(\text{DTPA}-\text{MA})$ more hydrophilic than its eight-coordinated analogue, $^{90}\text{Y}(\text{DTPA}-\text{MA})$. This is consistent with the shorter retention time of $^{111}\text{In}(\text{DTPA}-\text{MA})$ as compared to the average of the two isomers of $^{90}\text{Y}(\text{DTPA}-\text{MA})$. If they were to share the same coordination sphere, the complex $^{111}\text{In}(\text{DTPA}-\text{MA})$ should have the same retention time and chromatographic patterns as $^{90}\text{Y}(\text{DTPA}-\text{MA})$ under identical chromatographic conditions.

There are two possible orientations (*cis* and *trans*) for the acetamide group in $^{111}\text{In}(\text{DTPA}-\text{MA})$ relative to the acetate arm at the central nitrogen atom (Figure 4). The amide carbonyl-oxygen is most likely not bound to the metal center, which makes it easier for the NO donor set to become dissociated momentarily. Once the NO donor set becomes dissociated from the metal ion, it is possible for the terminal amine-nitrogen atom to undergo a rapid inversion. This leads to the rapid interconversion of the *cis*- and *trans* isomers. The presence of a single peak suggests that the complex $^{111}\text{In}(\text{DTPA}-\text{MA})$ exist in solution as only one *detectable* isomer at room temperature. Since $^{111}\text{In}(\text{DTPA}-\text{MA})$ remains stable for at least 24 h at room temperature, it is reasonable to assume that the remaining part of the DTPA-MA is firmly bonded to In^{3+} when the NO donor atoms become dissociated and the terminal amine-nitrogen atom undergoes a rapid inversion.

Like $^{111}\text{In}(\text{DTPA}-\text{MA})$, $^{111}\text{In}(\text{DTPA}-\text{BA})$ also shows a single peak in its HPLC chromatogram due to rapid exchange of different possible isomers (Figure 5). Compared to that of $^{90}\text{Y}(\text{DTPA}-\text{BA})$, the retention time of $^{111}\text{In}(\text{DTPA}-\text{BA})$ is ~ 4 min shorter under identical chromatographic conditions (Figure 3). The peak width (~ 0.6 min) for $^{111}\text{In}(\text{DTPA}-\text{BA})$ is also smaller than that (~ 3 min) of $^{90}\text{Y}(\text{DTPA}-\text{BA})$, suggesting a much faster exchange between different isomers. If these two complexes were to share the same coordination sphere, $^{111}\text{In}(\text{DTPA}-\text{BA})$ should have the same retention time as that of $^{90}\text{Y}(\text{DTPA}-\text{BA})$.

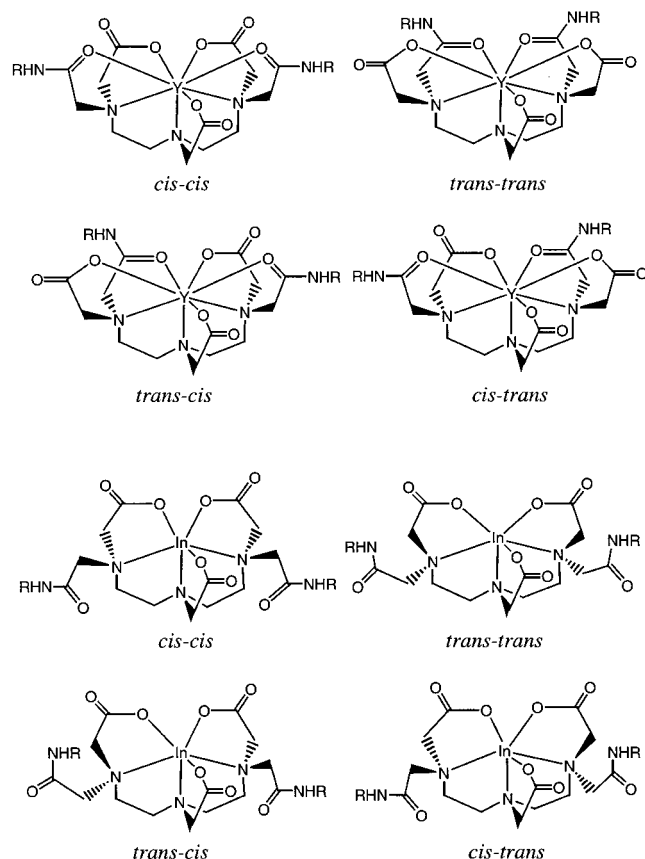


Figure 5. Relative orientations of the two acetamide groups in $^{90}\text{Y}(\text{DTPA-BA})$ (top) and $^{111}\text{In}(\text{DTPA-BA})$ (bottom).

CONCLUSIONS

In this report, we presented the synthesis and HPLC characterization of ^{111}In -labeled DTPA–peptide conjugates (DTPA–MA and DTPA–BA). By a reversed phase HPLC method, we found that complexes $^{111}\text{In}(\text{DTPA-MA})$ and $^{111}\text{In}(\text{DTPA-BA})$ are more hydrophilic than their respective ^{90}Y analogues, $^{90}\text{Y}(\text{DTPA-MA})$ and $^{90}\text{Y}(\text{DTPA-BA})$. Since $^{111}\text{In}(\text{DTPA-MA})$ and $^{90}\text{Y}(\text{DTPA-MA})$ share the same biomolecule, the difference in hydrophilicity strongly suggests that these two complexes have different coordination spheres. These results have significant implications for ^{90}Y - and ^{111}In -labeled DTPA–biomolecule conjugates. For radiolabeled antibodies, the attachment of the metal chelate (radionuclide and BFC) normally does not disturb the tertiary structure of the polypeptide, and the biological properties are not significantly affected. For small biomolecules, however, the metal chelate contributes greatly to the overall size and molecular weight of the radiolabeled biomolecule. Differences in the coordination chemistry may result in biological nonequivalence between the ^{90}Y - and ^{111}In -labeled DTPA–biomolecule conjugates. Thus, the choice of the bifunctional chelator should be those which form ^{90}Y and ^{111}In complexes with the same coordination sphere.

Both $^{111}\text{In}(\text{DTPA-MA})$ and $^{111}\text{In}(\text{DTPA-BA})$ showed one radiometric peak in their respective HPLC chromatograms due to a rapid interconversion of different isomers. The interconversion of different isomers involves the “wagging” of the diethylenetriamine backbone, “shuffling” of the NO or NO₂ donor sets, and a rapid inversion at the terminal amine-nitrogen atoms. Therefore, the presence of different isomeric forms may not impose a significant problem with respect to the difference in

biological properties of these isomers. The most critical issue in using DTPA as a bifunctional chelator is the low kinetic inertness. Once the ^{111}In -labeled DTPA–BM conjugate is injected into a biological system, the presence of biologically important metal ions such as Ca^{2+} , Cu^{2+} , Fe^{2+} , and Zn^{2+} , is expected to have a significant impact on the biological fate of the ^{111}In -labeled DTPA–biomolecule conjugate. These metal ions can compete with ^{111}In for the DTPA–BM conjugate due to their high concentration. Once ^{111}In is dissociated from the DTPA–biomolecule conjugate, it will most likely deposit in bone. It is not surprising that many ^{111}In -labeled DTPA–biomolecule conjugates often show high bone activity. Thus, the design of new BFCs should be focused on those which form indium and lanthanide complexes with high thermodynamic stability and more importantly kinetic inertness.

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Nucleosides and Nucleotides. 208. Alternate-Strand Triple-Helix Formation by the 3'-3'-Linked Oligodeoxynucleotides with the Anthraquinonyl Group at the Junction Point[†]

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The synthesis of 3'-3'-linked oligodeoxynucleotides (ODNs) with the anthraquinonyl group at the junction point is described. The ODNs were synthesized on a DNA synthesizer using a controlled pore glass (CPG) carrying pentaerythritol that has an intercalator at one of the four hydroxymethyl groups. Stability of the triplexes with the target duplexes was studied by thermal denaturation. The 3'-3'-linked ODNs with the anthraquinonyl group enhanced the thermal stability of the triplexes when compared with those without the intercalator and the unmodified nonamer **10**. It was found that the ODNs **12** and **13** carrying the anthraquinonyl groups can form thermally stable triplexes by skipping two or three extra base pairs between two binding domains of the target duplexes. The ability of the 3'-3'-linked ODNs to inhibit cleavage of the target DNA **22** by the restriction enzyme Hind III was tested. It was found that the 3'-3'-linked ODN **16** with the anthraquinonyl group at the junction point inhibited the cleavage by the enzyme more effectively than the nonamer **14** and the 3'-3'-linked ODN **15** without the intercalator.

INTRODUCTION

In the antigene strategy proposed to control gene transcription, an oligopyrimidine-oligopurine sequence of double-helical DNA is recognized by a third-strand oligodeoxynucleotide (ODN) that binds to the major groove of the DNA and forms a local triple helix (triplex) (1). Depending on the orientation of the third strand, two major classes of triplexes are identified. When the third strand consists mainly of pyrimidines, Hoogsteen-type Py-PuPy base triplets (T-AT and C⁺-GC) are formed in which the third strand is parallel to the purine strand of the target duplex (2, 3). When the third strand is predominantly purines, reverse Hoogsteen-type Pu-PuPy base triplets (G-GC and A-AT) are formed in which the third strand is antiparallel to the purine strand of the target duplex (4, 5). However, target sequences in the antigene strategy are very restricted. Since the thermal stability of the triplexes is generally lower than that of the duplexes under physiological conditions, an oligopurine cluster with fairly long chain lengths is required for stable triplex formation. In addition, formation of the pyrimidine motif triplex needs conditions of low pH (pH < 6.0), because unmodified cytosine residues, if present in the third strand, must be protonated to bind with guanine of the G:C duplex (2, 6).

Several approaches have been described to overcome this limitation. If the target duplex is composed of two adjacent and alternating oligopurine-oligopyrimidine tracts, the duplex can form a triplex with a single-strand ODN which pairs with oligopurine sequences on alternate strands by crossover in the major groove (7–24). This so-called “alternate-strand triplex” or “switched triplex” can

be formed in two distinct ways. By combining both Hoogsteen and reverse Hoogsteen motifs (7–14), the natural ODNs with oligopurine-oligopyrimidine sequences such as 5'-oligopurine-oligopyrimidine-3' or 5'-oligopyrimidine-oligopurine-3' can be used as the third strand. In the second approach, only one set of motifs (Hoogsteen or reverse Hoogsteen motif) (15–24) is employed, namely, the 3'- and 3'- or 5'- and 5'-ends of two ODN fragments have to be connected using a suitable linker. The different possibilities for “alternate-strand triplex” formation in the latter case are shown in Figure 1. When the oligopyrimidine sequence follows the oligopurine sequence, the 3'-3'-linked and the 5'-5'-linked ODNs can form the parallel (a) and antiparallel (b) triplexes with the target duplex by Hoogsteen and reverse Hoogsteen hydrogen bonds, respectively. On the other hand, when the oligopurine sequence follows the oligopyrimidine sequence, the 5'-5'-linked and the 3'-3'-linked ODNs can form the parallel (c) and antiparallel (d) triplexes with the target duplex by Hoogsteen and reverse Hoogsteen hydrogen bonds, respectively.

On the other hand, it is known that ODNs carrying intercalators, such as acridine (25–29), oxazolopyridocarbazole (30), benzopyridoindole (31, 32), and perylene (33) enhance the thermal stability of the triplexes. We recently reported the synthesis of branched ODNs with a pentaerythritol at the branch points (34). The ODNs formed thermally stable parallel and antiparallel triplexes with the single strand ODNs. On the basis of this information, we planned a synthesis of a 3'-3'-linked ODN with an intercalator at the junction point. We reasoned that the intercalator would stabilize the triplex by intercalating between the base pairs of the target duplex as shown in Figure 2. Since pentaerythritol has four equivalent hydroxymethyl groups, we envisaged that if we could synthesize the controlled pore glass (CPG) with pentaerythritol carrying an intercalator at one of the four hydroxymethyl groups, we would be able to conveniently

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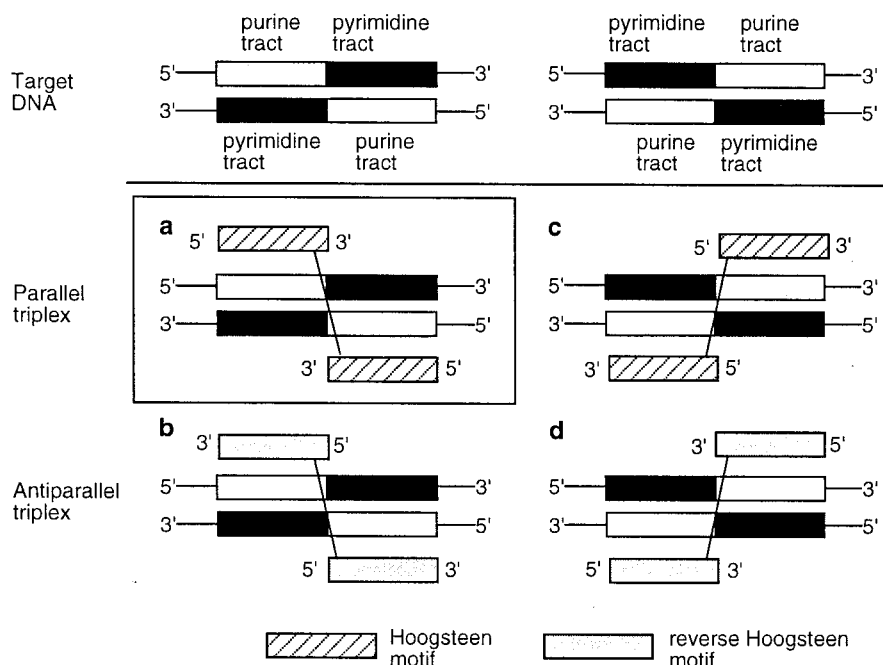


Figure 1. Schematic presentation of "alternate-strand triplex" formation by the 3'-3'- or 5'-5'-linked ODNs.

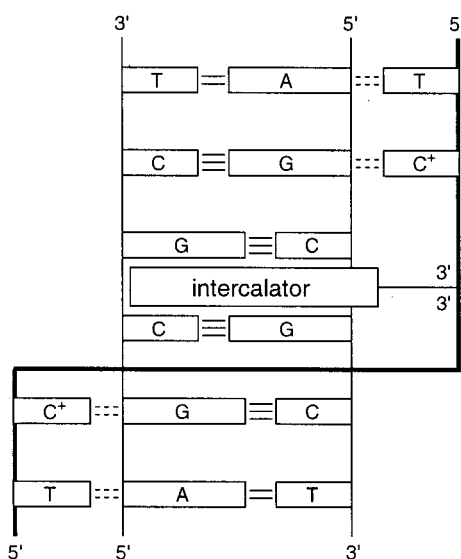
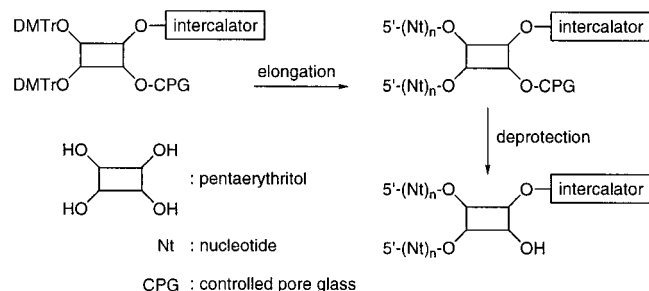


Figure 2. Schematic presentation of "alternate-strand triplex" formation by the 3'-3'-linked ODN with an intercalator at the junction point.

Scheme 1



synthesize the 3'-3'-linked ODN with the intercalator at the junction point as shown in Scheme 1.

In this paper, we report the synthesis of the 3'-3'-linked ODN with the anthraquinonyl group at the junction point. The thermal stability of the triplexes between these ODNs and the target DNAs, and the ability of the 3'-3'-

linked ODNs to inhibit cleavage of a target DNA by a restriction enzyme, are also studied.

RESULTS AND DISCUSSION

Synthesis. The CPG with pentaerythritol that has the anthraquinonyl group was synthesized as shown in Scheme 2. First, 2-[(*tert*-butyldiphenylsilyl)oxymethyl]-2-(hydroxymethyl)-1,3-propanediol (**1**) (**33**) was treated with 2 equiv of 4,4'-dimethoxytrityl chloride (DMTrCl) in pyridine to give the *O*-bis(DMTr) derivative **2** in 87% yield. Compound **2** was converted into a carbonylimidazolid, which was reacted with ethylenediamine or 1,4-diaminobutane to produce **3a** or **3b** in 81 or 76% yield, respectively. Protection of the primary amino group of **3b** with a trifluoroacetyl group followed by desilylation by tetrabutylammonium fluoride (TBAF) gave **5**. Compound **5** was modified to the corresponding 3'-succinate **6**, which was reacted with CPG to give a solid support containing **6** (23 $\mu\text{mol/g}$). At the same time, **3a** or **3b** was treated with anthraquinone-2-carboxylic acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCl) in DMF to afford **7a** or **7b** in 51 or 83% yield, respectively. After removal of the TBDPS groups, **8a** and **8b** were modified to the corresponding 3'-succinates **9a** and **9b**, which were reacted with CPG to give solid supports containing **9a** (20 $\mu\text{mol/g}$) and **9b** (30 $\mu\text{mol/g}$), respectively.

The 3'-3'-linked ODNs **11–13**, **15**, **16**, which consist of thymidine (T) and 5-methyl-2'-deoxycytidine (M), were synthesized on a DNA synthesizer (Figure 3a). The fully protected ODNs (each 1 μmol) linked to the solid support were treated with concentrated NH_4OH at 55 $^\circ\text{C}$ for 16 h. Purification by C-18 HPLC gave the ODNs **11**, **12**, **13**, **15**, and **16** in 52, 41, 44, 31, and 33 OD_{260} units, respectively. These ODNs were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and the observed molecular weights supported their structures.

Studies of Triplex Formation by Thermal Denaturation. The stability of the triplexes was studied by thermal denaturation in a buffer of 0.01 M sodium phosphate (both at pH 6.9 and 6.0) containing 0.5 M NaCl

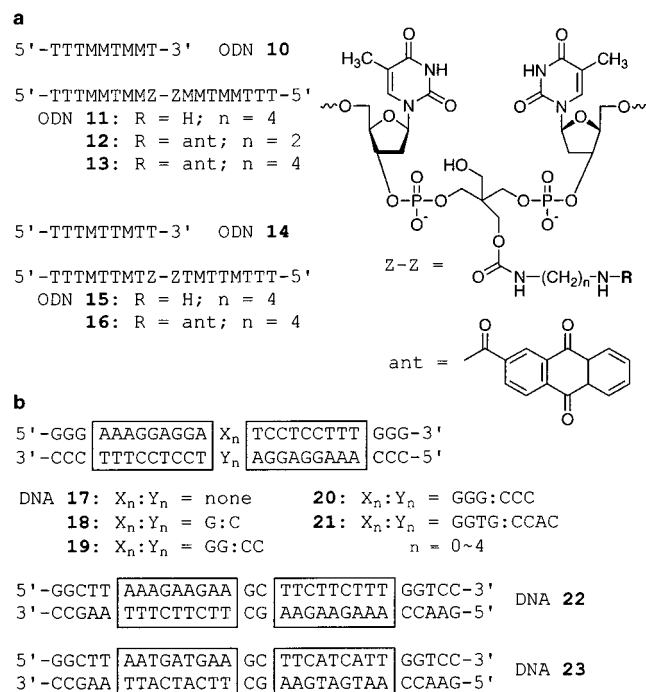
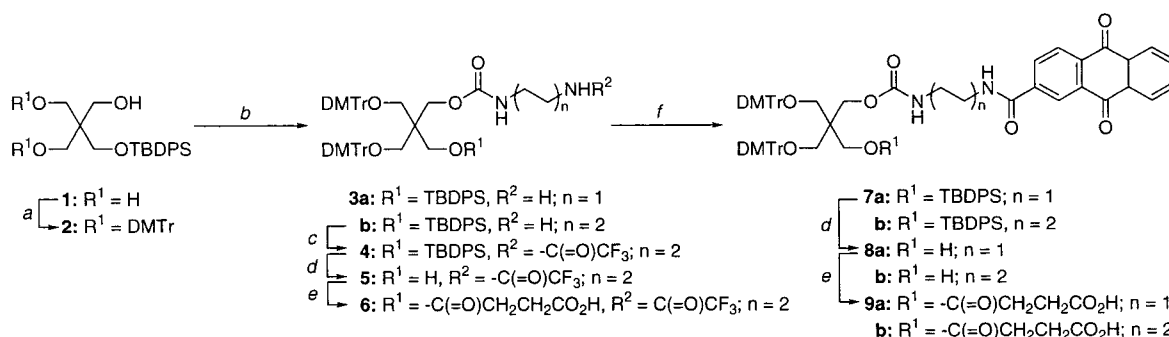
Scheme 2^a

Figure 3. Sequences of ODNs and the target duplexes.

and 0.02 M MgCl_2 . Sequences of target duplexes used in this study are shown in Figure 3b. The DNAs 17–21 have oligopurine sequences, one on each strand of the duplexes, that can be recognized by the ODNs 10–13. The DNAs 18–21 also have extra base pairs between the two binding domains that do not participate in Hoogsteen base pair interactions with the third strands and will be spanned by the linker groups. The sequences of the binding domains are symmetrical. One equivalent of the 3'-3'-linked ODNs or two equivalents of the control ODN 10 to the target DNAs was used for the thermal denaturation.

Figure 4 shows melting profiles of the triplexes between the ODN 13 and the DNA 20 at pH 6.9 (I) and at pH 6.0 (II), and between the ODN 10 and the DNA 17 at pH 6.9 (III), respectively. The T_m s are listed in Table 1. The T_m for the control triplex between the ODN 10 and the DNA 17 was not detected at pH 6.9 and was 18.2 °C at pH 6.0. All 3'-3'-linked ODNs with and without the anthraquinonyl group enhanced the thermal stability of the triplexes at both pH 6.9 and 6.0 as compared with the control ODN 10. The T_m values of the triplexes at pH 6.0 were greater than those at pH 6.9. The ODNs 12 and 13 with intercalators at the junction points more

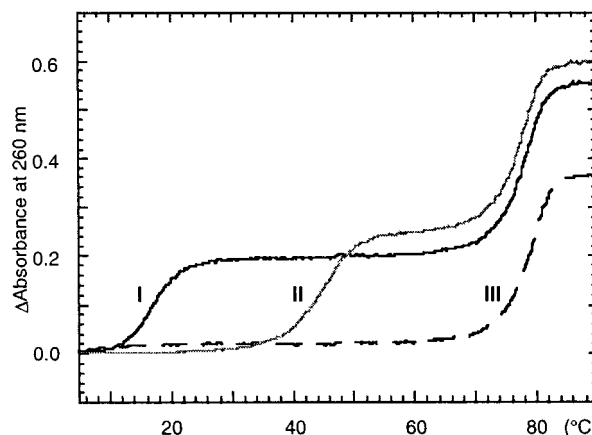


Figure 4. Melting profile of the triplexes between the ODN 13 and the DNA 20 at pH 6.9 (curve I) and at pH 6.0 (curve II), and between the ODN 10 and the DNA 17 at pH 6.9 (curve III), respectively.

Table 1. Hybridization Data^a

	ODN 10		ODN 11		ODN 12		ODN 13	
target DNA	pH 6.9	pH 6.0	pH 6.9	pH 6.0	pH 6.9	pH 6.0	pH 6.9	pH 6.0
DNA 17	ND	18.2	<8	23.0	9.4	36.9	8.1	36.0
DNA 18	—	—	<8	28.8	12.4	39.8	10.1	37.6
DNA 19	—	—	10.1	38.1	17.1	45.0	15.6	43.2
DNA 20	—	—	11.6	38.3	16.6	43.2	16.7	46.0
DNA 21	—	—	8.2	35.0	10.5	37.6	9.3	35.9

^a Experimental conditions are described in the Experimental Procedures. ND: not detected. —: not determined.

efficiently stabilized the triplexes as compared with the 3'-3'-linked ODN 11. Addition of 3 μM sodium anthraquinone-2-sulfonate to a solution containing the ODN 11 and the DNA 18 did not influence the T_m s of the triplexes at either pH 6.9 ($T_m < 8$ °C) or pH 6.0 ($T_m = 28.8$ °C). Thus, it was found that the attachment of the intercalator directly to the ODNs was important for the efficient thermal stabilization of the triplexes.

Thermal denaturation was performed by changing extra base pairs between two binding domains of the target duplexes. With the ODN 11 as the third strand, the highest T_m (11.6 °C at pH 6.9 and 38.3 °C at pH 6.0) was obtained for the DNA 20 which has three extra base pairs between the two binding domains. When the ODN 12 with the *N*-(anthraquinonylcarbonyl)aminoethyl group was used as the third strand, the highest T_m (17.1 °C at pH 6.9 and 45.0 °C at pH 6.0) was obtained for the DNA 19 which has two extra base pairs between the two binding domains. On the other hand, when the ODN 13

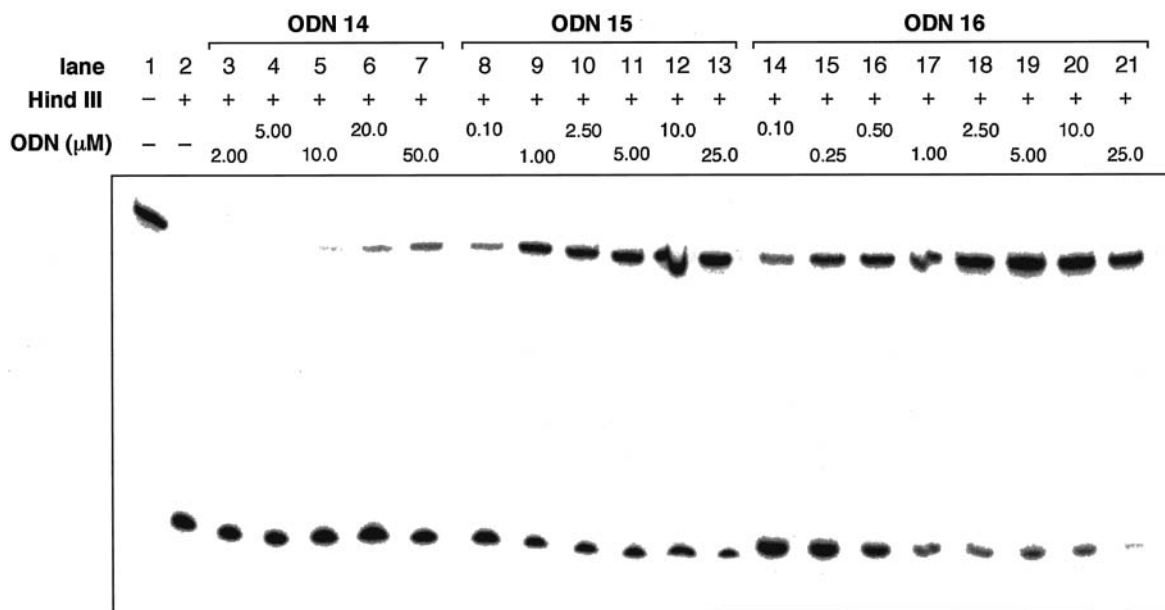


Figure 5. Specific inhibition of Hind III cleavage by the ODNs **14** (lanes 3–7), **15** (lanes 8–13), or **16** (lanes 14–21). Experimental conditions are described in the Experimental Procedures.

with *N*-(anthraquinonylcarbonyl)aminobutyl group was used as the third strand, the highest T_m (16.7 °C at pH 6.9 and 46.0 °C at pH 6.0) was obtained for the DNA **20**. Consequently, the ODNs **11**, **12**, and **13** can form thermally stable triplexes by skipping two or three base pairs between the two binding domains of the target duplexes. The ODN **12** with the ethylenediamine linker was found to be useful for skipping two extra base pairs while the ODN **13** with the diaminobutane linker was useful for skipping three extra base pairs. The difference between the ODNs **12** and **13** is due to the difference in the length of the linker groups between the intercalators and the ODNs.

Inhibition of Restriction Endonuclease Cleavage via Triplex Formation. We next tested the ability of the 3'-3'-linked ODNs to inhibit cleavage of a target DNA by a restriction endonuclease. The restriction enzyme Hind III recognizes the palindromic 6 bp sequence 5'-AAGCTT-3'/3'-AAGCTT-5' and cleaves the two strands in a symmetric way. We designed a 30 bp DNA containing Hind III recognition site as a target duplex (Figure 3b). It involves oligopurine sequences, one on each strand of it, that can be recognized by the third strand. The nonamer **14**, the 3'-3'-linked ODN **15**, and the 3'-3'-linked ODN **16** with the anthraquinonyl group at the junction point were used as the third strands.

The stabilities of the triplexes between the DNA **22** and ODNs **14**, **15**, or **16** were studied by thermal denaturation in a buffer of 10 mM sodium phosphate (pH 6.9)¹ containing 500 mM NaCl and 20 mM MgCl₂. The T_m s for the triplexes between the DNA **22** and ODNs **14**, **15**, or **16** were 20.5, 47.8, or 53.0 °C, respectively.

Inhibition of Hind III cleavage via the triplex formation was studied. A series of experiments was carried out at 37 °C in a 10 mM HEPES buffer (pH 6.9) containing 100 mM NaCl, 10 mM MgCl₂, and 1 mM DTT. Increasing amounts of the third strands were added to the DNA **22**, labeled at one 5'-end by ³²P, and the solutions were preincubated at 37 °C for 30 min before addition of the

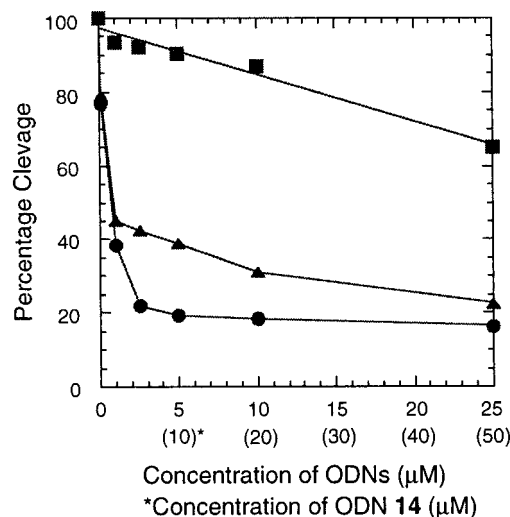


Figure 6. Specific inhibition of Hind III cleavage by triplex formation: plots of percentage cleavage of the DNA fragment versus the concentration of ODN in the reaction mixture. The symbols on the plot indicate the ODNs **14** (■), **15** (▲), and **16** (●).

restriction enzyme. After incubated at 37 °C for 3 h, the products were analyzed by polyacrylamide gel electrophoresis under denaturing conditions (Figure 5). Densities of radioactivity of the gel were visualized by a Bio-imaging analyzer. As shown in Figure 6, cleavage was inhibited in a dose-dependent manner. It was found that the 3'-3'-linked ODN **15** inhibited the cleavage by the enzyme more effectively than the nonamer **14**. Furthermore, the 3'-3'-linked ODN **16** with the intercalator at the junction point inhibited the cleavage more effectively than the ODN **15**.

To show that inhibition was due to the interaction of the ODNs with the target DNA rather than with the restriction enzyme, a parallel experiment was carried out with the DNA **23**, which contains one restriction site for Hind III but no sequence fully complementary to that of the ODN **16**. It was found that the ODN **16** does not inhibit the cleavage of the DNA **23** by the enzyme at all (data not shown). Thus, inhibition of the cleavage was

¹ The enzyme reactions using Hind III are usually carried out at pH 7.5. Since the parallel-type triple-helix is thermally unstable at pH 7.5, the enzyme assay was done at pH 6.9.

found to be not due to the ODNs binding to the enzyme but to triplex formation at the binding site of the enzyme.

CONCLUSION

In this paper, we reported the synthesis of the 3'-3'-linked ODNs with the anthraquinonyl group at the junction point. Stability of the triplex was studied by thermal denaturation. The 3'-3'-linked ODNs with the anthraquinonyl group enhanced the thermal stability of the triplexes as compared with those without the intercalator and the unmodified nonamer **10**. Furthermore, it was found that the ODNs **12** and **13** can form the thermally stable triplexes by skipping two or three extra base pairs between the two binding domains of the target duplexes. We tested the ability of the 3'-3'-linked ODNs to inhibit cleavage of the DNA **22** by the restriction enzyme Hind III. It was found that the 3'-3'-linked ODN **16** with the anthraquinonyl group at the junction point inhibited the cleavage by the enzyme more effectively than the nonamer **14** and the 3'-3'-linked ODN **15** without the intercalator. Thus, the 3'-3'-linked ODN with the intercalator at the junction point may be useful as the antigene ODN to the DNAs, which have the alternating oligopurine-oligopyrimidine sequences on them.

EXPERIMENTAL PROCEDURES

General Remarks. NMR spectra were recorded at 270 or 400 MHz (^1H) and at 100 MHz (^{13}C). Chemical shifts (δ) and coupling constants (J) are reported in ppm downfield from TMS and in Hz, respectively. Mass spectra were obtained by fast atom bombardment (FAB) method. Thin-layer chromatography was done on Merck coated plates 60F₂₅₄. The silica gel used for column chromatography was Merck silica gel 5715.

2-[(*tert*-Butyldiphenylsilyl)oxymethyl]-1,3-O-bis(4,4'-dimethoxytrityl)-2-(hydroxymethyl)-1,3-propanediol (2**).** A mixture of 2-[(*tert*-butyldiphenylsilyl)oxymethyl]-2-(hydroxymethyl)-1,3-propanediol (**1**) (7.49 g, 20.0 mmol) (**34**) and DMTrCl (14.9 g, 44.0 mmol) in pyridine (100 mL) was stirred at room temperature. After 30 min, the mixture was concentrated in vacuo and was taken in EtOAc, which was washed with aqueous NaHCO₃ (saturated) and brine, dried (Na₂SO₄), and concentrated. The residue was purified by column chromatography (SiO₂, 17–20% EtOAc in hexane) to give **2** (17.0 g, 87% as a white foam): FAB-MS m/z 977 (M^+); ^1H NMR (270 MHz, CDCl₃) δ 7.52–6.63 (m, 36H), 3.73 (s, 12H), 3.78–3.71 (m, 6H), 3.67 (d, J = 5.9, 2H), 2.56 (t, J = 5.9, 1H), 0.85 (s, 9H). HRMS calcd for C₆₃H₆₆O₈Si: 978.4523. Found: 978.4547. Anal. Calcd for C₆₃H₆₆O₈Si: C, 77.27; H, 6.79. Found: C, 77.30; H, 6.81.

2-[[*N*-(2-Aminoethyl)carbamoyl]oxymethyl]-2-[(*tert*-butyldiphenylsilyl)oxymethyl]-1,3-O-bis(4,4'-dimethoxytrityl)-1,3-propanediol (3a**).** *N,N'*-Carbonyldiimidazole (608 mg, 3.75 mmol) and DMAP (37 mg, 0.30 mmol) were added to a solution of **2** (1.47 g, 1.50 mmol) in DMF (15 mL), and the mixture was stirred at room temperature. After 24 h, 1,2-diaminoethane (1.34 mL, 20.0 mmol) was added to the mixture, which was stirred at room temperature. After 24 h, the mixture was partitioned between EtOAc and H₂O. The organic layer was washed with brine, dried (Na₂SO₄), and concentrated. The residue was purified by column chromatography (SiO₂, 5% MeOH in CHCl₃ containing 1% Et₃N) to give **3a** (1.30 g, 81% as a white foam): ^1H NMR (400 MHz, CDCl₃) δ 7.52–6.48 (m, 36H), 4.54 (m, 1H), 4.18 (s, 2H), 3.75 (s, 14H), 3.27 (s, 4H), 3.14 (m, 2H), 2.74 (m, 2H), 0.85 (s, 9H); ^{13}C NMR (100 MHz, CDCl₃) δ 158.10,

144.98, 135.97, 135.56, 130.14, 129.39, 128.14, 127.53, 127.45, 126.37, 112.83, 85.70, 77.21, 55.13, 26.81, 19.23. Anal. Calcd for C₆₆H₇₂N₂O₉Si·1/2H₂O: C, 73.78; H, 6.85; N, 2.61. Found: C, 73.69; H, 6.83; N, 2.69.

2-[[*N*-(4-Aminobutyl)carbamoyl]oxymethyl]-2-[(*tert*-butyldiphenylsilyl)oxymethyl]-1,3-O-bis(4,4'-dimethoxytrityl)-1,3-propanediol (3b**).** Compound **2** (4.89 g, 5.00 mmol) was aminobutylcarbamoylated by using 1,4-diaminobutane (5.00 mL, 50.0 mmol) as described in the preparation of **3a** to give **3b** (4.14 g, 76% as a white foam): ^1H NMR (270 MHz, CDCl₃) δ 7.52–6.68 (m, 36H), 4.42 (t, J = 5.3, 1H), 4.17 (s, 2H), 3.74 (s, 14H), 3.27 (s, 4H), 3.10 (m, 2H), 2.66 (m, 2H), 1.44 (m, 4H), 0.85 (s, 9H); ^{13}C NMR (100 MHz, CDCl₃) δ 158.08, 144.98, 135.96, 135.52, 130.11, 129.36, 128.11, 127.50, 127.41, 126.33, 112.80, 85.67, 55.09, 46.07, 26.79, 19.20.

2-[(*tert*-Butyldiphenylsilyl)oxymethyl]-1,3-O-bis(4,4'-dimethoxytrityl)-2-[[*N*-(4-(trifluoroacetamido)butyl)carbamoyl]oxymethyl]-1,3-propanediol (4**).** A mixture of **3b** (1.06 g, 1.00 mmol), ethyl trifluoroacetate (357 μL , 3.00 mmol), and Et₃N (418 μL , 3.00 mmol) in DMF (10 mL) was stirred at room temperature. After 4 h, the mixture was partitioned between EtOAc and H₂O. The organic layer was washed with brine, dried (Na₂SO₄), and concentrated. The residue was purified by column chromatography (SiO₂, 40% EtOAc in hexane) to give **4** (916 mg, 77% as a white foam): FAB-MS m/z 1188 (M^+); ^1H NMR (400 MHz, CDCl₃) δ 7.53–6.69 (m, 36H), 4.25 (t, J = 5.9, 2H), 4.17 (s, 2H), 3.75 (s, 12H), 3.73 (s, 2H), 3.33 (m, 2H), 3.10 (m, 2H), 3.27 (s, 4H), 1.49 (m, 4H), 0.84 (s, 9H). ^{13}C NMR (100 MHz, CDCl₃) δ 158.08, 156.46, 144.95, 135.91, 135.52, 133.20, 130.12, 129.39, 128.08, 127.51, 127.43, 126.37, 112.82, 85.68, 65.11, 63.33, 61.92, 60.36, 55.11, 45.55, 40.08, 39.51, 27.51, 26.77, 25.70, 21.04, 19.19, 14.21. HRMS calcd for C₇₀H₇₆F₃N₂O₁₀Si: 1189.5221. Found: 1189.5210. Anal. Calcd for C₇₀H₇₅F₃N₂O₁₀Si·1/2H₂O: C, 70.15; H, 6.39; F, 4.76; N, 2.34. Found: C, 70.19; H, 6.51; F, 4.92; N, 2.27.

1,3-O-Bis(4,4'-dimethoxytrityl)-2-(hydroxymethyl)-2-[[*N*-(4-(trifluoroacetamido)butyl)carbamoyl]oxymethyl]-1,3-propanediol (5**).** A mixture of **4** (629 mg, 0.530 mmol) and TBAF (1 M in THF, 1.06 mL, 1.06 mmol) in THF (6 mL) was stirred at room temperature for 24 h. The mixture was partitioned between EtOAc and H₂O. The organic layer was washed with brine, dried (Na₂SO₄), and evaporated under reduced pressure. The residue was purified by column chromatography (SiO₂, 33–100% EtOAc in hexane) to give **5** (413 mg, 82% as a white foam): FAB-MS m/z 950 (M^+); ^1H NMR (400 MHz, CDCl₃) δ 7.34–6.75 (m, 26H), 4.46 (t, J = 6.1, 1H), 4.11 (s, 2H), 3.78 (s, 12H), 3.49 (d, J = 6.9, 2H), 3.36 (m, 2H), 3.15 (m, 2H), 3.24 (s, 4H), 2.60 (t, J = 6.9, 1H), 1.52 (m, 4H); ^{13}C NMR (100 MHz, CDCl₃) δ 158.18, 156.85, 144.64, 135.58, 130.05, 127.98, 127.62, 126.54, 117.20, 112.92, 85.93, 77.20, 63.81, 63.36, 61.60, 55.10, 45.41, 40.12, 39.38, 27.21, 26.69. HRMS calcd for C₅₄H₅₇F₃N₂O₁₀: 950.3962, found 950.3976. Anal. Calcd for C₅₄H₅₇F₃N₂O₁₀·3/2H₂O: C, 66.31; H, 6.18; F, 5.83; N, 2.86. Found: C, 66.46; H, 6.12; F, 5.73; N, 2.76.

1,3-O-Bis(4,4'-dimethoxytrityl)-2-[(*O*-succinyl)oxymethyl]-2-[[*N*-(4-(trifluoroacetamido)butyl)carbamoyl]oxymethyl]-1,3-propanediol (6**).** A mixture of **5** (190 mg, 0.200 mmol), succinic anhydride (60 mg, 0.600 mmol), and DMAP (25 mg, 0.200 mmol) in pyridine (2 mL) was stirred at room temperature. After 5 days, the mixture was partitioned between EtOAc and H₂O. The organic layer was washed with aqueous NaHCO₃ (saturated) and brine, dried (Na₂SO₄), and concentrated. The residue was purified by column chromatography (SiO₂,

80–100% EtOAc in hexane, then 0–30% MeOH in EtOAc) to give **6** (204 mg, 97% as a white foam): FAB-MS m/z 1050 (M^+); ^1H NMR (270 MHz, $CDCl_3$) δ 7.34–6.72 (m, 26H), 3.99 (s, 4H), 3.76 (s, 12H), 3.32–3.14 (m, 8H), 2.04 (m, 4H), 1.53 (m, 4H). HRMS calcd for $C_{58}H_{61}F_3N_2O_{13}$ 1050.4125, found 1050.4120.

2-[[N-[2-(2-Anthraquinonylcarbonyl)aminoethyl]-carbamoyl]oxymethyl]-2-[(*tert*-butyldiphenylsilyl)-oxymethyl]-1,3-O-bis(4,4'-dimethoxytrityl)-1,3-propanediol (7a). A mixture of **3a** (532 mg, 0.50 mmol), anthraquinone-2-carboxylic acid (151 mg, 0.60 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCl) (115 mg, 0.600 mmol) in DMF (5 mL) was stirred at room temperature. After 3 days, the mixture was diluted with EtOAc. The organic layer was washed with aqueous $NaHCO_3$ (saturated) and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2 , 40–50% EtOAc in hexane) to give **7a** (329 mg, 51% as a pale yellow foam): FAB-MS m/z 1298 (M^+); ^1H NMR (270 MHz, $CDCl_3$) δ 8.64–7.77 (m, 7H), 7.51–6.67 (m, 36H), 4.54 (m, 2H), 4.23 (s, 2H), 3.71 (s, 14H), 3.55 (m, 2H), 3.37 (m, 2H), 3.29 (s, 4H), 0.84 (s, 9H). HRMS calcd for $C_{81}H_{79}N_2O_{12}Si$: 1299.5402, found: 1299.5370. Anal. Calcd for $C_{81}H_{78}N_2O_{12}Si$: C, 74.86; H, 6.05; N, 2.16. Found: C, 74.67; H, 6.14; N, 2.15.

2-[[N-[4-(2-Anthraquinonylcarbonyl)aminobutyl]-carbamoyl]oxymethyl]-2-[(*tert*-butyldiphenylsilyl)-oxymethyl]-1,3-O-bis(4,4'-dimethoxytrityl)-1,3-propanediol (7b). Compound **3b** (1.09 g, 1.00 mmol) was anthraquinonylcarbonylated as described in the preparation of **7a** to give **7b** (1.11 g, 83% as a pale brown foam): FAB-MS m/z 1325 (M^+); ^1H NMR (270 MHz, $CDCl_3$) δ 8.65–7.81 (m, 7H), 7.53–6.68 (m, 36H), 4.28 (m, 2H), 4.20 (s, 2H), 3.73 (s, 14H), 3.48 (m, 2H), 3.17 (m, 2H), 3.27 (s, 4H), 1.54 (m, 4H), 0.84 (s, 9H). HRMS calcd for $C_{83}H_{81}N_2O_{12}Si$: 1325.5558, found: 1325.5540. Anal. Calcd for $C_{83}H_{82}N_2O_{12}Si \cdot H_2O$: C, 74.08; H, 6.29; N, 2.08. Found: C, 73.96; H, 6.40; N, 2.13.

2-[[N-[2-(2-Anthraquinonylcarbonyl)aminoethyl]-carbamoyl]oxymethyl]-1,3-O-bis(4,4'-dimethoxytrityl)-2-(hydroxymethyl)-1,3-propanediol (8a). Compound **7a** (110 mg, 86 μ mol) was desilylated as described in the preparation of **5** to give **8a** (67 mg, 74% as a pale yellow foam): FAB-MS m/z 1060 (M^+); ^1H NMR (270 MHz, $CDCl_3$) δ 8.62–7.77 (m, 7H), 7.33–6.73 (m, 26H), 4.85 (m, 2H), 4.20 (s, 2H), 3.75 (s, 12H), 3.60 (m, 2H), 3.44 (m, 2H), 3.52 (m, 2H), 3.26 (s, 4H), 2.49 (m, 1H). HRMS calcd for $C_{65}H_{60}N_2O_{12}$: 1060.4146, found: 1060.4160. Anal. Calcd for $C_{65}H_{60}N_2O_{12} \cdot H_2O$: C, 72.34; H, 5.79; N, 2.60. Found: C, 72.35; H, 5.99; N, 2.51.

2-[[N-[4-(2-Anthraquinonylcarbonyl)aminobutyl]-carbamoyl]oxymethyl]-1,3-O-bis(4,4'-dimethoxytrityl)-2-(hydroxymethyl)-1,3-propanediol (8b). Compound **7b** (663 mg, 0.50 mmol) was desilylated as described in the preparation of **5** to give **8b** (489 mg, 90% as a pale yellow foam): FAB-MS m/z 1089 (M^+); ^1H NMR (400 MHz, $CDCl_3$) δ 8.60–7.79 (m, 7H), 7.34–6.74 (m, 26H), 4.55 (t, J = 6.1, 2H), 4.14 (s, 2H), 3.76 (s, 12H), 3.53 (m, 4H), 3.28 (s, 4H), 3.52–3.21 (m, 2H), 2.70 (m, 1H), 1.66–1.59 (m, 4H). HRMS calcd for $C_{67}H_{65}N_2O_{12}$: 1089.4537, found: 1089.4540. Anal. Calcd for $C_{67}H_{64}N_2O_{12} \cdot H_2O$: C, 72.67; H, 6.01; N, 2.53. Found: C, 72.65; H, 6.20; N, 2.36.

2-[[N-[2-(2-Anthraquinonylcarbonyl)aminoethyl]-carbamoyl]oxymethyl]-1,3-O-bis(4,4'-dimethoxytrityl)-2-[(*O*-succinyl)oxymethyl]-1,3-propanediol (9a). Compound **8a** (64 mg, 60 μ mol) was succinylated as described in the preparation of **6** to give **9a** (46 mg, 65%

as a pale brown foam): FAB-MS m/z 1160 (M^+); ^1H NMR (270 MHz, $CDCl_3$) δ 8.59–7.74 (m, 7H), 7.30–6.70 (m, 26H), 4.00 (s, 4H), 3.72 (s, 12H), 3.56–3.29 (m, 4H), 3.21 (s, 4H), 2.36 (m, 4H). HRMS calcd for $C_{89}H_{64}N_2O_{15}$: 1160.4306, found: 1160.4320.

2-[[N-[4-(2-Anthraquinonylcarbonyl)aminobutyl]-carbamoyl]oxymethyl]-1,3-O-bis(4,4'-dimethoxytrityl)-2-[(*O*-succinyl)oxymethyl]-1,3-propanediol (9b). Compound **8b** (161 mg, 148 μ mol) was succinylated as described in the preparation of **6** to give **9b** (116 mg, 66% as a pale yellow foam): FAB-MS m/z 1189 (M^+); ^1H NMR (400 MHz, $CDCl_3$) δ 8.65–7.80 (m, 7H), 7.33–6.72 (m, 26H), 4.69 (m, 1H), 4.04 (s, 2H), 4.03 (s, 2H), 3.78 (s, 12H), 3.50 (m, 2H), 3.24 (s, 4H), 3.20 (m, 2H), 2.50–2.44 (m, 4H), 1.66–1.59 (m, 4H). HRMS calcd for $C_{71}H_{69}N_2O_{15}$: 1189.4697, found: 1189.4680.

Synthesis of the Controlled Pore Glass Support with 6, 9a, or 9b. Aminopropyl controlled pore glass (510 mg, 46 μ mol, 89.8 μ mol/g, CPG Inc.) was added to a solution of **6** (191 mg, 182 μ mol) and WSCI (35 mg, 182 μ mol) in DMF (5 mL), and the mixture was kept at room temperature for 3 days. After the resin was washed with pyridine, 1 mL of a capping solution (0.1 M DMAP in pyridine:Ac₂O = 9:1) was added, and the whole was kept at room temperature for 14 h. The resin was washed with EtOH and acetone and was dried under vacuum. The amount of loaded nucleoside **6** to the solid support is 23 μ mol/g from the calculation of released dimethoxytrityl cation by a solution of 70% $HClO_4$:EtOH (3:2, v/v). In a similar manner, the solid supports with **9a** and **9b** were obtained in 20 and 30 μ mol/g of loading amounts.

Synthesis of ODNs. ODNs were synthesized on a DNA synthesizer (Applied Biosystem Model 392) by the phosphoramidite method. Each oligonucleotide linked to the resin was treated with concentrated NH_4OH at 55 $^{\circ}C$ for 16 h, and the released ODN was purified by reversed-phase HPLC, using a J'sphere ODS M80 column (4.6 $\times$ 150 mm, YMC) with a linear gradient of CH_3CN (from 10 to 35% over 20 min) in 0.1 M triethylammonium acetate (TEAA, pH 7.0) to give a deprotected ODN **11** (52), **12** (41), **13** (44), **15** (31), and **16** (33). The yields are indicated in parentheses as OD units at 260 nm starting from 1 μ mol scale.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Spectra were obtained on a Voyager Elite reflection time-of-flight mass spectrometry (PerSeptive Biosystems, Inc., Framingham, MA) equipped with a nitrogen laser (337 nm, 3-ns pulse). 3-Hydroxypicolinic acid (HPA), dissolved in H_2O to give a saturated solution at room temperature, was used as the matrix. Time-to-mass conversion was achieved by calibration by using the peak representing the C^+ cation of the charged derivative to be analyzed. ODN **11**: calculated mass, 5715.11; observed mass, 5715.03. ODN **12**: calculated mass, 5921.11; observed mass, 5921.05. ODN **13**: calculated mass, 5949.14; observed mass, 5952.77. ODN **15**: calculated mass, 5719.05; observed mass, 5718.70. ODN **16**: calculated mass, 5953.08; observed mass, 5952.99.

Thermal Denaturation. The solution that contained the oligonucleotide (3 μ M) and the target duplex (3 μ M) in a buffer of 0.01 M sodium phosphate (both pH 6.9 and 6.0), 0.5 M NaCl, and 0.02 M $MgCl_2$ was heated at 60 $^{\circ}C$ for 5 min and then cooled gradually to an appropriate temperature and used for the thermal denaturation study. Thermal-induced transitions of each mixture were monitored at 260 nm on a Perkin-Elmer Lambda2S. Sample temperature was increased 0.5 $^{\circ}C$ /min.

Enzymatic Assay. Enzymatic assays were performed in a 10 mM HEPES buffer (pH 6.9) containing 100 mM NaCl, 10 mM MgCl₂, and 1 mM DTT. Various amounts of the third strands were added to the DNA **22** (1 pmol), labeled at one 5'-end by ³²P, and the solutions were preincubated at 37 °C for 30 min. Fifteen units of Hind III (Nippon Gene) was added to each solution, and the mixture (total 10 μL) was incubated at 37 °C for 3 h. Aliquot (3 μL) of the reaction mixture was separated and added to a loading solution (8 M urea, 0.1% xylene cyanol, 0.1% bromophenol blue, 10 μL); then the mixture was heated at 95 °C for 5 min. The solution was analyzed by electrophoresis on 20% polyacrylamide gel containing 7 M urea. Densities of radioactivity of the gel were visualized by a Bio-imaging analyzer (Bas 2000, Fuji Co., Ltd).

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Synthesis and Characterization of a Ruthenium(II)-Based Redox Conjugate for Reagentless Biosensing

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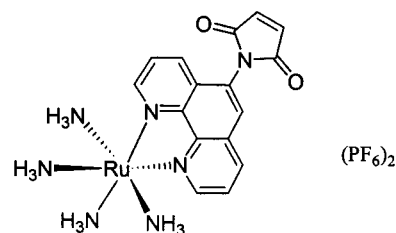
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Synthesis of a novel sulfhydryl-specific, tetraammine Ru(II)polypyridyl complex, $[\text{Ru}(\text{II})(\text{NH}_3)_4(1,10\text{-phenanthroline-5-maleimide})](\text{PF}_6)_2$, which exhibits environment-sensitive electrochemical properties is described. When conjugated to an allosteric site in a genetically engineered mutant of maltose binding protein, the formal potential of the conjugated redox probe is shifted to higher potential upon maltose binding. The magnitude of this potential shift was used to measure maltose affinity of the protein-redox conjugate complex and to monitor maltose concentration in solution. These results are presented in context of reagentless biosensing.

INTRODUCTION

Development of reagentless biosensors is a goal of many scientific communities (1–11). Such sensors combine analyte recognition with a change in an intrinsic property of the sensor upon analyte binding. Recently, fluorescence allosteric signal transduction (FAST) has been demonstrated as a viable approach toward reagentless biosensing (12, 13). Using this approach, an environment-sensitive fluorophore was conjugated to genetically engineered maltose binding protein (MBP) at a site distinct from the binding site for maltose but which underwent local environmental changes upon binding maltose. As a result, fluorescence intensity of the conjugated fluorophore was shown to be dependent upon maltose concentration. Because electrochemical-based detection strategies offer complimentary advantages compared to fluorescence-based strategies (14–18), we were motivated to explore an analogous electrochemical approach. Drawing on Ru(II) polypyridyl chemistry (19), we proposed that a sulfhydryl-specific tetraammine Ru(II)polypyridyl derivative would be a viable redox conjugate for this application based on its expected environmental sensitivity, accessibility of the Ru(II/III) electrochemistry in aqueous media, and insensitivity to oxygen (20). Reported here is the synthesis and structural characterization of a new electroactive sulfhydryl-reactive reagent $[\text{Ru}(\text{II})(\text{NH}_3)_4(1,10\text{-phenanthroline-5-maleimide})](\text{PF}_6)_2$. In addition, environment-sensitive cyclic voltammetry of the unconjugated reagent and maltose-sensitive cyclic voltammetry of the protein-bound conjugate is demonstrated.



EXPERIMENTAL PROCEDURES

Materials. 5-Amino-1,10-phenanthroline (Polysciences, Inc.); maleic anhydride, $[\text{NH}_4](\text{PF}_6)$, acetonitrile, ethyl alcohol, nitrobenzene, dimethylformamide (DMF), $[\text{N}(n\text{-C}_4\text{H}_9)_4](\text{PF}_6)$ (TBAH), and $[\text{Ru}(\text{NH}_3)_5\text{Cl}]\text{Cl}_2$ (Aldrich); and zinc amalgam (GFS Chemicals) were used as received. $[\text{Ru}(\text{NH}_3)_5(\text{H}_2\text{O})](\text{PF}_6)_2$ was prepared as reported (20). The genetically engineered maltose binding proteins were isolated and purified as previously described (12).

Measurements. Electrochemical experiments using a three-electrode configuration in a one-compartment cell were conducted using a potentiostat (EG&G Princeton Applied Research, model 263) using CorrWare software (Scribner Associates, Inc., version 2.1b). For measurements in organic solvents, 0.1 M $[\text{N}(n\text{-C}_4\text{H}_9)_4](\text{PF}_6)$ (TBAH) was used as the electrolyte, Ag/AgNO₃ (0.1 M TBAH and 0.01 M AgNO₃ in CH₃CN) was used as the reference electrode which was calibrated with ferrocene using the Fe(II/III) redox couple at 0.4 V vs SCE, and a platinum-coated niobium wire was used as the counter electrode. Aqueous measurements were conducted using a Ag/AgCl reference electrode and a platinum-coated niobium wire counter electrode. A 1.6-mm diameter platinum disk electrode was used as the working electrode for voltammetry of the unconjugated Ru(II) complex. A 3.0-mm diameter glassy carbon electrode was used as the working electrode for voltammetry of the protein-conjugated Ru complex. Spectroscopic experiments were conducted using a UV–vis spectrophotometer (Cary 4G).

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Synthesis. *1,10-Phenanthroline-5-maleimide*. On the basis of modified literature preparations for similar maleimides (21, 22), 400 mg (2.05 mmol) of 5-amino-1,10-phenanthroline was added to 150 mL of CH_2Cl_2 . The solution was stirred at room temperature, and 1 g (10.2 mmol) of maleic anhydride was slowly added. After 72 h, 380 mg (64% yield) of a yellow precipitate was collected by vacuum filtration, washed with 3×100 mL of CH_2Cl_2 , and air-dried. The product was then added to 20 mL of acetic anhydride containing 2 g of sodium acetate. The solution was stirred and heated to 100 °C for 1 h. After the solution cooled to room temperature, 80 mL of ice-water was added and the solution was stirred until all of the acetic anhydride was consumed. The product was extracted with 3×100 mL CH_2Cl_2 , washed with 3×100 mL of water, and dried over MgSO_4 . The MgSO_4 was filtered off and the CH_2Cl_2 removed by rotoevaporation. The product was redissolved in 10 mL of CH_2Cl_2 , precipitated in 50 mL of hexanes, collected by vacuum filtration, and air-dried. The mass of the final product was 170 mg (30% overall yield). ^1H NMR (400 MHz, CDCl_3): 7.00 (s, 2H); 7.63 (m, 2H); 7.73 (s, 1H); 7.94 (d, 1H); 8.23 (d, 1H); 9.23 (m, 2H). Anal. Calcd for $\text{C}_{16}\text{H}_9\text{N}_3\text{O}_2 \cdot 0.5\text{H}_2\text{O}$: C, 67.60; H, 3.55; N, 14.78. Found: C, 67.75; H, 3.55; N, 14.38.

$[\text{Ru}(\text{NH}_3)_4(1,10\text{-Phenanthroline-5-maleimide})](\text{PF}_6)_2$. On the basis of a modified procedure for the preparation (20) of $[\text{Ru}(\text{NH}_3)_4(\text{bpy})](\text{PF}_6)_2$, 100 mg (0.202 mmol) of $[\text{Ru}(\text{NH}_3)_5(\text{H}_2\text{O})](\text{PF}_6)_2$ was added to 10 mL of argon-purged acetone. After the solution turned dark yellow (due to formation of $[\text{Ru}(\text{NH}_3)_5(\text{CH}_3\text{COCH}_3)]^{3+}$) (20), 63 mg (0.23 mmol) of 1,10-phenanthroline-5-maleimide was added. The solution turned deep purple and was stirred for 3 h at room temperature under Ar. The crude product was precipitated in 60 mL of stirring ether and collected by vacuum filtration. Purification was carried out by a series of precipitations. The crude product was redissolved in 10 mL of acetone and added to 150 mL of stirring CH_2Cl_2 . A red powder (30 mg) containing both the product and impurities (determined by NMR and cyclic voltammetry) was isolated by vacuum filtration. To the supernatant, 50 mL of ether was added and a second precipitate (44 mg of a red powder) was collected by vacuum filtration. Cyclic voltammetry revealed that an electroactive impurity with a redox couple centered at 120 mV vs SCE had been removed; however, NMR revealed ~10% excess free ligand. The precipitate was then redissolved in 2 mL of acetone and added with stirring to 50 mL of CH_2Cl_2 and chilled for 16 h. A red powder was collected by vacuum filtration, washed with 3×15 mL ether, and air-dried. The mass of the final product was 20 mg (20% overall yield). A dark red crystalline material can be obtained by slow evaporation of a concentrated acetone solution of product. ^1H NMR (400 MHz, CD_3CN): 9.43 (m, 2H); 8.43 (d, 1H); 8.15 (d, 1H), 8.13 (s, 1H), 7.88 (m, 1H), 7.83 (m, 1H), 7.17 (s, 2H), 2.92 (s, 6H), 1.48 (s, 6H). Anal. Calcd for $\text{C}_{16}\text{H}_{21}\text{F}_{12}\text{N}_7\text{O}_2\text{P}_2\text{Ru} \cdot 0.5\text{C}_3\text{H}_6\text{O}$: C, 27.53; H, 3.17; N, 12.84. Found: C, 27.14; H, 3.04; N, 12.41.

Protein Labeling. In a typical procedure, the protein concentration was 1 mg/mL in sodium phosphate buffer at pH 7. $[\text{Ru}(\text{NH}_3)_4(1,10\text{-phenanthroline-5-maleimide})](\text{PF}_6)_2$ was dissolved in CH_3CN . The Ru complex in solution was added to a rapidly stirring solution of protein. The organic solvent was kept below 5% of the total volume of the mixture, and the final Ru concentration was 5 to 10 times the molar protein concentration. The Ru complex and Ru-labeled protein were separated using gel filtration chromatography.

Table 1. Spectroscopic and Electrochemical Properties for $[\text{Ru}(\text{II})(\text{NH}_3)_4(1,10\text{-phenanthroline-5-maleimide})](\text{PF}_6)_2$

solvent	DN(23)	λ_{max} , cm^{-1}	E_f Ru(III/II) vs SCE
nitrobenzene	4.4	20330	0.67
acetonitrile	14.1	20410	0.609
EtOH	19	19880	0.458
DMF	26.6	19460	0.291
water	18	20700	0.375

Table 2. Formal Potentials of the Ru(II/III) Couple vs Ag/AgCl of the Ru-MBP(D95C) Conjugate as a Function of 2 Fold Excess of Maltose

[protein], μM	electrolyte ^a	E_f^b (mV)	E_f (maltose) ^c (mV)	ΔE (mV) ^d
37	phosphate	324 ± 1	330 ± 1	6
37	NH_4PF_6	336 ± 1	345 ± 1	9
37	NaClO_4	317 ± 1	331 ± 1	14
75	NaClO_4	316 ± 1	326 ± 1	10
170	NaClO_4	322 ± 1	336 ± 2	14

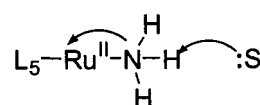
^a 200 mM in addition to 10 mM pH 7 phosphate buffer. ^b Formal potentials measured as the average of 2–4 cyclic voltammograms (10mV/s) ± 1 standard deviation. ^c Formal potentials measured as the average of 2–4 cyclic voltammograms (10mV/s) ± 1 standard deviation with 2-fold excess maltose. ^d The change in formal potential, ΔE , with excess 2-fold maltose.

Maltose Titration. For the detection of maltose, the concentration of Ru-labeled protein was 37–170 μM . A 3.0-mm diameter glassy carbon electrode was used as the working electrode for the voltammetry. The electrochemistry was conducted in a low-volume (200 μL) cell (BAS). Aliquots (1–2 μL) of a 2–10 mM solution of maltose were added to the protein solution. Two to four scans were measured at 10 mV/s for each maltose concentration. The formal potentials were calculated as the average of the cathodic and anodic peak potentials of the Ru(III/II) couple in the Ru-labeled protein and were plotted versus maltose concentration.

Simulations. Cyclic voltammograms were simulated using the DigiSim 2.1 (BAS). Default parameters were used in all simulations with $\alpha = 0.5$ and potential step = 0.005 V. The cyclic voltammograms were simulated at each concentration of maltose using the measured formal potentials of the Ru(III/II) couple for the unbound and maltose-saturated states of the protein (Table 1) and their respective concentrations defined by eq 2. The formal potentials from the simulated cyclic voltammograms were calculated as the average of the cathodic and anodic peak potentials and plotted versus maltose concentration.

RESULTS

To confirm environmental sensitivity of the Ru(II) maleimide complex, its spectroscopic and electrochemical properties were examined in a range of solvents with donor numbers (23) spanning 4.4 kcal/mol (nitrobenzene) to 26.6 kcal/mol (DMF) (Table 1). In Figure 1, the dependency of the formal potential of the Ru(II/III) redox couple, E_f , on solvent donor number (DN) is shown in comparison to that of the Fe(II/III) ferrocene redox couple which is environment-insensitive (24). As expected, the formal potential of the Ru(II/III) redox couple decreases with increasing donor number, reflecting the increase of electron density on Ru(II) metal center as a function of



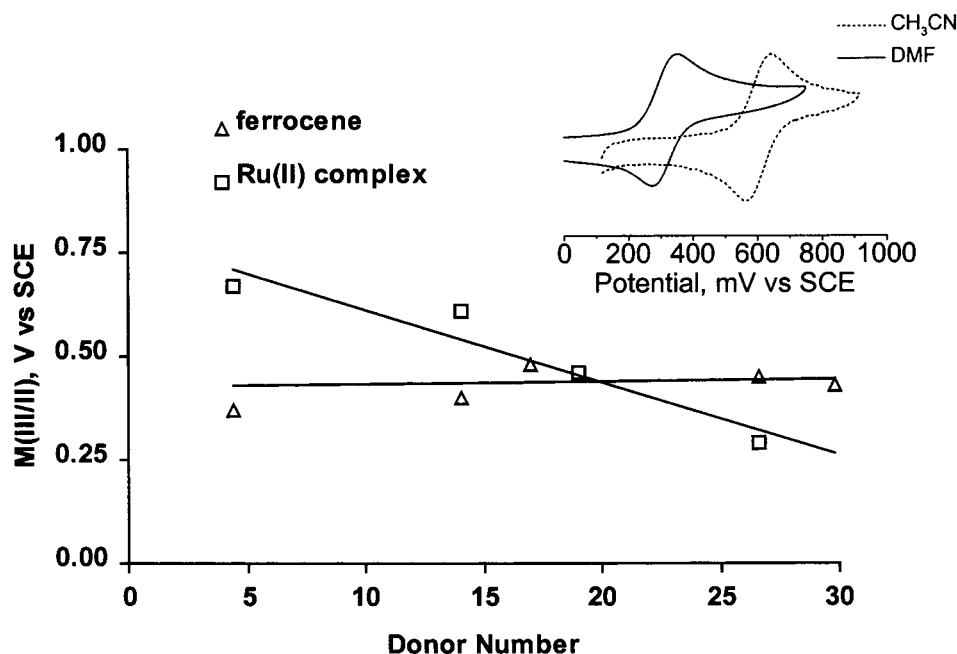


Figure 1. Formal potential vs donor number of the Ru(II/III) redox couple of Ru(II)(NH₃)₄(1,10-phenanthroline-5-maleimide)](PF₆)₂ compared to that of the Fe(II/III) redox couple of ferrocene (27). The solvents listed with donor numbers (kcal/mol) are nitrobenzene (4.4), acetonitrile (14.1), acetone (17), ethanol (19), DMF (26.6), DMSO (29.8). Inset: Cyclic voltammograms of Ru(II)(NH₃)₄(1,10-phenanthroline-5-maleimide)](PF₆) in DMF and CH₃CN 0.1 M in [N(n-C₄H₉)₄](PF₆). 100 mV/s.

the hydrogen bonding (or electron donating) capacity of the solvent environment (12).

In the context of a protein environment (below), it is our supposition that conformational changes in proximity of a conjugated Ru(II) probe may change the extent of hydrogen bonding, resulting in changes in the observed formal potential.

Conjugation of the Ru(II) maleimide complex to the lone cysteine residue of genetically engineered *E. coli* maltose binding protein (MBP) was conducted in pH 7 buffer, with excess Ru complex removed by gel filtration chromatography. The extent of coupling was >95% based on titration of remaining free cysteines using Ellman's reagent and by comparison of the absorbance of the bound Ru complex ($\epsilon_{280} \sim 15000 \text{ M}^{-1} \text{ cm}^{-1}$, and $\epsilon_{490} \sim 5000 \text{ M}^{-1} \text{ cm}^{-1}$) to the absorbance of the protein ($\epsilon_{280} \sim 69000 \text{ M}^{-1} \text{ cm}^{-1}$). Two MBP variants, D95C and S233C, each containing a single cysteine mutation, were investigated. D95C, MBP containing an aspartic acid to cysteine mutation at position 95 (a site distinct from the maltose binding site), had previously demonstrated strong allosteric coupling of maltose binding with changes in fluorescence emission intensity of an environment-sensitive fluorescent conjugate (12). S233C, MBP containing a serine to cysteine mutation at position 233 (a site very close to or partially within the maltose binding site), had also previously demonstrated fluorescent reagentless maltose detection when conjugated with an environment-sensitive fluorophore (12).

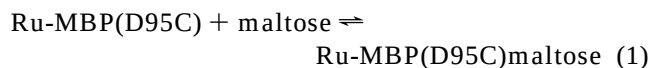
Cyclic voltammetry for both Ru-conjugated MBP mutants was quasi-reversible at 10 mV/s with the ratio of anodic to the cathodic peak current equal to 1 (at a glassy carbon electrode in pH 7 sodium phosphate buffer) (Figure 2) and was stable upon successive scans in air-saturated solutions. The formal potential of the Ru(II/III) couple in Ru-MBP(D95C) changes as a function of maltose concentration in several different supporting electrolytes (Table 1). Potential shifts were not observed for Ru-MBP(S233C) or the unconjugated Ru(II) maleimide complex under similar conditions. In addition,

glucose at concentrations comparable to those used in maltose titrations resulted in no change in formal potential of the Ru(II/III) couple in Ru-MBP(D95C), owing to the small affinity of MBP for glucose (12).

DISCUSSION

As with the fluorescently labeled D95C analog (12), it is hypothesized that the increase in formal potential of the Ru(II/III) couple in Ru-MBP(D95C) results from a change in the local environment of the electrochemically active reporter group due to the conformational change in the protein upon maltose binding. The insensitivity of the formal potential of the Ru(II/III) conjugate in Ru-MBP(S233C) to maltose binding, even though a 2-fold increase in fluorescence intensity was observed in fluorescently labeled S233C upon maltose binding, likely reflects subtle differences in the resulting protein environment of the two probes and underscores the importance of further study in how proteins structurally accommodate conjugated probe molecules.

Assuming binding of maltose to Ru-MBP(D95C) is reversible (eq 1) the fraction of total protein with bound maltose as function of maltose concentration may be calculated from a binding isotherm (25), eq 2,



$$\text{fraction bound} = \frac{([P]_t + [M]_t + K_d) - \sqrt{([P]_t + [M]_t + K_d)^2 - 4[P]_t[M]_t}}{2[P]_t} \quad (2)$$

where $[P]_t$ is the total concentration of protein (bound and unbound), $[M]_t$ is the total concentration of maltose at each titration point, $K_d = 1.5 \mu\text{M}$ (12), and the fraction bound is given by $[\text{Ru-MBP(D95C)maltose}]/[P]_t$. Cyclic voltammograms were simulated over a range of maltose concentrations to explore the possibility that the observed

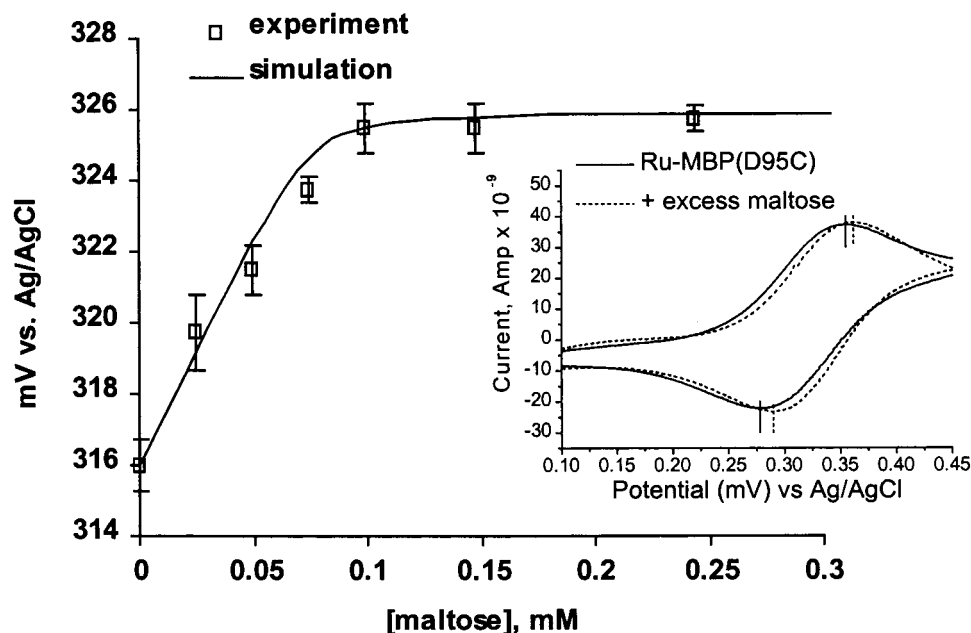


Figure 2. The titration of 75 μM Ru-conjugated MBP(D95C) mutant with maltose in a pH 7, 10 mM phosphate buffer with 0.2 M NaClO_4 . Formal potentials (measured as the average of the cathodic and anodic peak potentials ± 1 standard deviation) plotted vs [maltose]. The line is simulated data obtained as described in the Experimental Section using a binding isotherm (eq 2) with $K_d = 1.5 \mu\text{M}$ (12). Inset: Cyclic voltammogram (background subtracted) of the Ru-labeled MBP(D95C) (75 μM) at a working glassy carbon electrode with scan rate of 10 mV/s in a pH 7 buffer at an ionic strength of 0.2 M NaClO_4 with and without presence of 2-fold excess maltose.

shift in the formal potential could be correlated with the binding of maltose. In the simulation, the concentrations of bound and unbound forms of Ru-MBP(D95C) were determined from eq 2 for each maltose concentration. The cyclic voltammograms were simulated using values for the formal potentials of Ru-MBP(D95C) in both the unbound and maltose-saturated state. As can be seen in Figure 2, the simulated change in formal potential versus maltose concentration overlays the experimentally measured change in formal potential versus maltose concentration. The observable $\Delta E_f(\text{maltose})/\Delta E_f(\text{max})$, where $\Delta E_f(\text{maltose})$ is the change in formal potential of the Ru complex as a function of maltose concentration and $\Delta E_f(\text{max})$ is the maximum change in formal potential over the course of maltose titrations, reflects the fraction of MBP with bound maltose and may be used to quantitatively assess the affinity of MBP for maltose. The maximum change in formal potential as a function of maltose occupancy is somewhat dependent on electrolyte (Table 1), however, and may be due to the sensitivity of Ru complex electrochemistry to hydrogen-bonding and solvation (20).

In conclusion we have demonstrated that the new electroactive sulfhydryl-reactive reagent, $[\text{Ru}(\text{II})(\text{NH}_3)_4-(1,10\text{-phenanthroline-5-maleimide})](\text{PF}_6)_2$, can be implemented in an electrochemical allosteric signal transduction scheme (EAST) complementing fluorescence-based reagentless assay systems. At its current state of development, the EAST technique employed in this study was able to detect maltose at concentrations as low as 10 μM , whereas the fluorescence-based technique (FAST) (12) is able to detect maltose with a detection limit of 0.1 μM .

In existing enzyme-based electrochemical sensors, the main focus has been on the detection of glucose (26). Current enzyme-based electrochemical methods are able to monitor glucose over a wide range of concentrations (e.g., commercially available blood glucose sensors have working glucose concentration ranges of 1–30 mM) with detection limits as low as 0.1–1 μM (26). The predomi-

nant electrochemical detection scheme of glucose biosensors, however, involves the use of glucose oxidase which requires oxygen as an additional substrate and which, as an enzymatic mediator, may also be prone to inaccuracies due to matrix interferences. We are continuing to pursue the goal of reagentless biosensing through further development of the EAST technique and are currently designing new protein redox conjugates that undergo larger changes in formal potential upon analyte binding.

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TECHNICAL NOTES

Photoreduction of Autooxidized Albumin–Heme Hybrid in Saline Solution: Revival of Its O₂-Binding Ability

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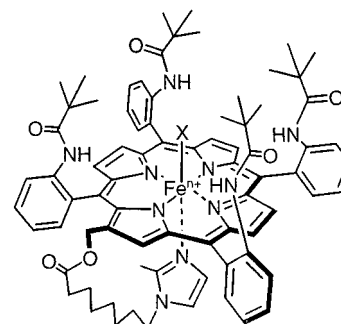
Recombinant human serum albumin (rHSA) incorporating 2-[8-{*N*-(2-methylimidazolyl)}octanoyloxy-methyl]-5,10,15,20-tetrakis($\alpha,\alpha,\alpha,\alpha$ -*o*-pivalamido)phenylporphinatoiron(II)s (Fe(II)Ps) [rHSA–Fe(II)P] is a synthetic hemoprotein which can bind and release O₂ reversibly under physiological conditions (saline solution [NaCl]: 150 mM, pH 7.3) as do hemoglobin and myoglobin. However, the central ferrous ions of Fe(II)Ps are slowly oxidized to O₂-inactive ferric forms. Based on the UV–vis. absorption spectroscopy, the majority of the autooxidized Fe(III)Ps in albumin are determined to be six-coordinate high-spin complexes with a proximal imidazole and a chloride anion, which show ligand-to-metal charge transfer (LMCT) absorption at 330 nm. Interestingly, photoirradiation of this LMCT band under an argon atmosphere led to reduction of the central ferric iron of Fe(III)P, allowing the revival of the O₂-binding ability. The ratio of the photoreduction reached a maximum of 83%, which is probably due to the partial dissociation of the axial imidazole. The same photoirradiation under a CO atmosphere provides the corresponding carbonyl rHSA–Fe(II)P. Laser flash photolysis experiments revealed that the reduction was completed within 100 ns. The quantum yields (Φ) of these photoreductions were approximately 0.01.

INTRODUCTION

The ferrous complexes of protoporphyrin IX in hemoglobin (Hb) are slowly oxidized even in the bloodstream, converting to the O₂-inactive ferric forms. If one can reduce these iron(III) complexes by light irradiation, this becomes the easiest way to revive their O₂-binding abilities. From this point of view, reduction of the porphyrinatoiron(III)s has been of great interest for several decades. The ferric complexes of porphyrins normally coordinate with counteranions and show a ligand-to-metal charge transfer (LMCT) transition in the near UV region. It has been shown that, in organic solvents, photoirradiation of this LMCT absorption led to reduction of the central metal involving a radical dissociation (Bartocci et al., 1980; Brault et al., 1980; Hoshino et al., 1992,1998; Inamura et al., 1985; Ward et al., 1982). Henderikson et al., for example, revealed the clean photoreduction of tetraphenylporphinatoiron(III) halide ([Fe(III)TPP]⁺X[–]) by 355-nm irradiation in benzene (Henderikson et al., 1987). In aqueous media, however, the produced radicals easily react with the formed porphyrinatoiron(II), so that the ferrous complex is not accumulated. Only when hyaluronic acid coexists in phosphate-buffered saline solution, the self-organized fibers of amphiphilic Fe(III)TPP derivative could be reduced by the LMCT irradiation (Komatsu et al., 2000b).

On the other hand, recombinant human serum albumin (rHSA) incorporating eight 2-[8-{*N*-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis($\alpha,\alpha,\alpha,\alpha$ -

o-pivalamido)phenylporphinatoiron(II) (Fe(II)P) [rHSA–Fe(II)P] is a synthetic hemoprotein which can bind and release O₂ under physiological conditions (in aqueous media, pH 7.3, 37 °C) as do Hb and myoglobin (Mb) (Komatsu et al., 1999; 2000a; Tsuchida et al., 1997; 1999). The 5 wt % solution of this albumin–heme hybrid satisfies the physiological requirements for a red blood cell substitute. In fact, administration of this solution into hemorrhagic shock rats showed an increase in the renal cortical O₂-tensions and skeletal tissue O₂-tensions (Tsuchida et al., 2000). Nevertheless, the remaining problem with this rHSA–Fe(II)P is also the relatively short lifetime as an O₂-carrying vehicle. Autoxidation of the central ferrous ion of Fe(II)P takes place slowly through a proton-driven process (Momentau et al., 1994). We report herein for the first time the unique coordination structure of Fe(III)P in albumin and the photoreduction of rHSA–Fe(III)P by LMCT excitation, which allows revival of the O₂-binding ability. Laser flash



n: 2, X: none Fe(II)P
n: 3, X: Cl[–] Fe(III)P

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photolysis experiments also revealed that the reduction was completed within 100 ns.

EXPERIMENTAL PROCEDURES

Materials and Preparation. The synthetic heme, 2-[8-{*N*-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis(*o*-pivalamido)phenylporphyrinatoiron(II) (Fe(II)P) and the rHSA-Fe(II)P solution [rHSA/Fe(II)P = 1/8 (mol/mol), in phosphate-buffered saline (pH 7.4)] was prepared according to our previously reported procedures (Komatsu et al., 2000; Tsuchida et al., 1995). The recombinant human serum albumin (rHSA; 25 wt %) was obtained from the WelFide Corporation (Sumi et al., 1993). FeP concentrations of 20 μ M were normally used for the UV-vis. absorption spectral measurements (Shimadzu V-570 spectrometer) within the range of 300–700 nm. Upon addition of the rHSA-Fe(II)P solution to O₂, the absorption spectrum showed the typical O₂-adduct of Fe(II)P (λ_{max} : 426, 552 nm). After incubation of this dioxygenated rHSA-Fe(II)P at 40 °C for 24 h, all the Fe(II)P sites in rHSA are oxidized, giving rHSA-Fe(III)P with absorption maxima at 330, 423, and 565 nm.

Photoreduction of Fe(III)P Incorporated into rHSA. The photoirradiation of this oxidized rHSA-Fe(III)P solution was performed with a 250-W ultrahigh-pressure Hg arc-lamp (Ushio UCH-250) under an argon or a CO atmosphere in a dark room (25 °C). The Hg lamp was held in a special lamp housing (Koken Kogyo LHX-150), which was connected to a PSX-75 power supply. The light filtered by a U-370 colored filter was irradiated into the rHSA-Fe(III)P solution in a 10-mm cuvette with a constant distance of 7.5 cm. The UV-vis. absorption spectra of the solutions were measured at several intervals. The reduction rates were calculated from the Soret band absorption (A_s) divided by that of the 100% reduced form for the deoxy state (A_{deoxy} at 443 nm) or carbonyl state (A_{carboxy} at 427 nm). The quantum yield (Φ) was determined by the well-established procedure with an oxalight Fe(II) complex (Michaelson et al.).

Transient Absorption Spectroscopy. The transient absorption spectra after the laser flash photolysis were measured using an ICCD detector (ANDOR DH520-18F-WR) with an imaging monochromator (ORIEL MS-257). The excitation laser pulse was generated from the THG (355 nm; pulse width, 5 ns) of a Nd:YAG laser (Spectron SL803G-10). The pulse xenon flash lamp (10 W, 10 Hz, 100 ns pulse width) was used as the monitor light source. The path length of the cuvette was 10 mm, and all measurements were carried out using a two-channel simultaneous detection system (Tokyo Instruments, Ltd.) at 25 °C.

Resonance Raman Spectroscopy. Resonance Raman spectroscopy measurements were performed using a laser Raman spectrometer equipped with a CCD multichannel detector (JASCO NRS-2000). Excitation was carried out with the 457.9 nm line of an NEC GLG2162 Ar⁺ ion laser in a backscattering geometry at 25 °C. The FeP concentrations of 1 mM in special liquid cells were normally used for the measurements.

RESULTS AND DISCUSSION

Coordination Structure of Autooxidized Fe(III)P in Albumin. Although the rHSA-Fe(II)P hybrid can bind molecular O₂ reversibly under physiological conditions, the central ferrous ions of the Fe(II)Ps are slowly oxidized to O₂-inactive ferric forms by a proton-driven process (Momentau et al., 1994). This oxidation, namely

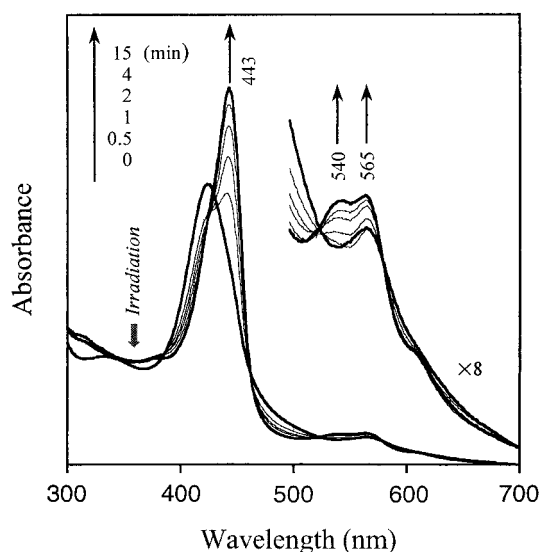


Figure 1. UV-vis absorption spectral changes in photoreduction of rHSA-Fe(III)P in phosphate-buffered saline (150 mM NaCl, pH 7.3) solution under argon atmosphere at 25 °C.

Table 1. Absorption Maxima of rHSA-Fe(III)P Solution with Sodium Halide (150 mM) at 25 °C

	electron negativity	LMCT (nm)	Soret (nm)	Q (nm)
F ⁻	4.0	330	423	566
Cl ⁻	3.0	330	423	565
Br ⁻	2.8	332	422	569
I ⁻	2.5	333	422	569

autoxidation, generally obeyed the first-order kinetics with a half-life of ca. 8 h at 25 °C (Tsuchida et al., 1997). The UV-vis absorption spectrum of the final compound showed maxima at 330, 423, and 565 nm. We have reported that the amphiphilic Fe(III)TPP derivative formed a five-coordinated high-spin complex with a chloride anion (Cl⁻) in phosphate-buffered saline solution (Fleischer et al., 1971; Komatsu et al., 2000b). However, this is not true for rHSA-Fe(III)P. On the basis of the absorption pattern, we determined that the dominant species of Fe(III)P in rHSA is an octahedral six-coordinate high-spin ferric complex with a Cl⁻ and an intramolecularly coordinated imidazole nitrogen (Figure 1, 3) (Shantha et al., 1998; Walker et al., 1976). In general, this type of six-coordination with sterically hindered imidazole, such as 1,2-dimethylimidazole (1,2-Me₂Im), is formed when a large excess of imidazole is present in the solution, because of the extremely low equilibrium constant of 1,2-DMIm to [Fe(III)TPP]⁺Cl⁻; 3.4 M⁻¹ (Walker et al., 1976). However, in albumin, each Fe(III)P molecule is incorporated into the small space of the hydrophobic domain, so that the six-coordination to the ferric complex is presumably formed. We assigned the small absorption band at 330 nm to a ligand-to-metal charge transfer (LMCT) transition between the central ferric ion and the Cl⁻ (Shantha et al., 1998). In the case of the five-coordinate high-spin [Fe(III)TPP]⁺Cl⁻, the LMCT absorption maxima shift to the lower energy with the decreasing electronegativity of the halides (I⁻ < Br⁻ < Cl⁻), while the Soret and Q-bands, which are π - π^* transitions, are not affected (Hendrikson et al., 1987; Komatsu et al., 2000). However, the λ_{max} of the LMCT absorption of the six-coordinated Fe(III)P in rHSA was constant versus the electron negativity of the counteranions ([X⁻]: 150 mM) (Table 1).

We then employed resonance Raman spectroscopy to detect the vibration stretching mode of the porphyrin ring

(ν_2) which is expected in the 1541–1554 cm^{-1} for the six-coordinated high-spin $[\text{Fe}(\text{III})\text{TPP}]^+\text{Cl}^-$ (1,2-Me₂Im) analogue (Parathati et al., 1987). The obvious absorption appeared in this region as double peaks at 1566 and 1547 cm^{-1} (not shown). The latter is likely to be the ν_2 band of the six-coordinate $\text{Fe}(\text{III})\text{P}$ in albumin.

Photoreduction of Autooxidized $\text{Fe}(\text{III})\text{Ps}$ in Albumin. The 365-nm irradiation of the saline solution of rHSA- $\text{Fe}(\text{III})\text{P}$ under argon atmosphere led to reduction of the central ferric ion; the visible absorption spectrum changed to the typical five-*N*-coordinate high-spin $\text{Fe}(\text{II})\text{P}$ complex (λ_{max} : 443, 542, and 565 nm) within 15 min (Figure 1). The isosbestic points (431, 461, 524, and 580 nm) throughout the measurement revealed that no side reactions occurred.

The following two results indicate that the photoreduction of rHSA- $\text{Fe}(\text{III})\text{P}$ occurs through the intramolecular electron transfer initiated by LMCT excitation. (i) In the absence of NaCl (in deionized water), the reduction proceeded by only 20%, suggesting that a Cl^- is essential for this reaction. (ii) The Soret band irradiation also reduced the ferric center, but by less than 40%. Irradiation of the LMCT band causes only the efficient reduction of the autooxidized rHSA- $\text{Fe}(\text{III})\text{P}$. Shantha and co-workers revealed that the coordination of 2-methylimidazole to $[\text{Fe}(\text{III})\text{TPP}]^+\text{Cl}^-$ results in a clean photoreduction of the central ferric ion by 407-nm irradiation (Shantha et al., 1998). They also inferred that a photoreactive state with a λ_{max} at 315–335 nm is responsible for this reduction.

For accumulation of the reduced $\text{Fe}(\text{II})\text{P}$, a radical scavenger should be present in the solution to avoid the back electron transfer. Actually, irreversible photoreduction of the molecular fibers of the amphiphilic $\text{Fe}(\text{III})\text{-TPP}$ derivative occurred only in a phosphate-buffered saline solution containing a small amount of hyaluronic acid (Komatsu et al., 2000). We can therefore assume that the albumin host itself, for instance, the amino acid residues located nearby $\text{Fe}(\text{III})\text{P}$, immediately traps the chloride radicals after the charge separation, then the reaction becomes irreversible. The most probable candidates are tyrosine and tryptophan. It is also remarkable that the photoreduction rate was not affected by the kinds of halide ions (I^- , Br^- , Cl^- , F^-).

Upon exposure of the aqueous solution of the photoreduced rHSA- $\text{Fe}(\text{II})\text{P}$ to O_2 , the UV-vis absorption spectrum changed to that of the dioxygenated complex (λ_{max} : 426, 552 nm). The O_2 coordination reversibly depended on the O_2 -partial pressure. An interesting observation is that the photoreduction rates reached a maximum of 83% (Figure 2). This is probably due to the base elimination in the ferric state; the imidazolyl group once dissociated from the central iron cannot coordinate again to the iron(III) center. This is consistent with our previously reported results on the reduction of the autooxidized rHSA- $\text{Fe}(\text{III})\text{P}$ by ascorbic acid with red blood cells (Tsuchida et al., 2000).

Photoirradiation into the LMCT band of the rHSA- $\text{Fe}(\text{III})\text{P}$ under CO atmosphere provides carbonyl rHSA- $\text{Fe}(\text{II})\text{P}$ (λ_{max} : 427, 540 nm) (Figure 3). CO coordinates with $\text{Fe}(\text{II})\text{P}$ immediately after the photoreduction of the central ferric ion, affording the stable carbonyl complexes. The reduction rate was estimated to be 93% under CO atmosphere. The real concentration of the carbonyl $\text{Fe}(\text{II})\text{P}$ in rHSA should not be as high, around 83%, because the Soret band absorption includes that of the five-coordinated $\text{Fe}(\text{II})\text{P}(\text{CO})$ complex without axial imidazole bonding, which has a large molecular coefficient.

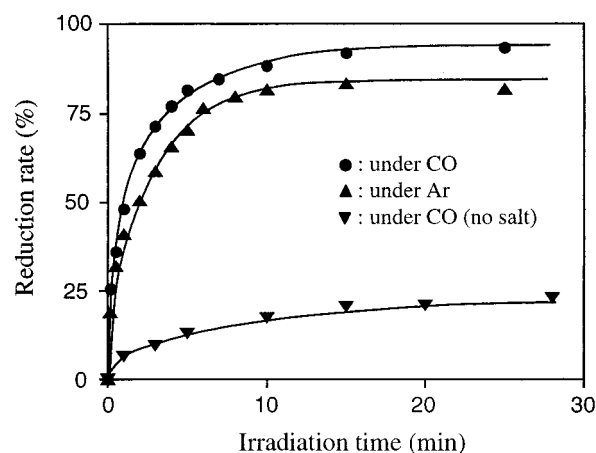


Figure 2. UV-vis absorption spectral changes in photoreduction of rHSA- $\text{Fe}(\text{III})\text{P}$ in phosphate-buffered saline (150 mM NaCl, pH 7.3) solution under CO atmosphere at 25 °C.

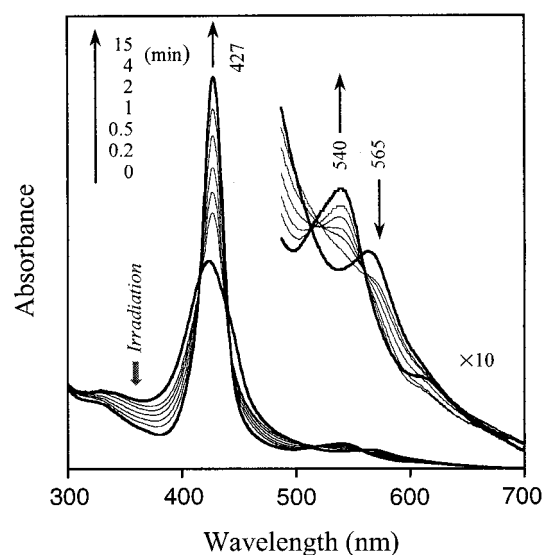


Figure 3. Relationship between the reduction rate and the irradiation time of rHSA- $\text{Fe}(\text{III})\text{P}$ in phosphate-buffered saline (150 mM NaCl, pH 7.3) solution at 25 °C.

Laser flash photolysis at 355 nm (THG of Nd:YAG) of the deaerated aqueous rHSA- $\text{Fe}(\text{III})\text{P}$ solution showed that the photoreduction was finished within the duration of the laser pulse; the transient absorption spectral pattern measured at 100 ns after the pulse was in good agreement with the difference spectrum of the rHSA- $\text{Fe}(\text{II})\text{P}$ solution minus the autooxidized rHSA- $\text{Fe}(\text{III})\text{P}$ solution (Figure 4). The primary reaction step appears to be the homolytic cleavage of the axial Cl^- , and $\text{Fe}(\text{II})\text{P}$ with intramolecularly coordinated axial imidazole is immediately formed. The produced chloride radical should have reacted with the active hydrogen in the amino acid residues of rHSA.

The quantum yields (Φ) of this photoreduction system were determined to be 0.01 under a CO atmosphere and 0.008 in argon. Because the LMCT excitation state (LMCT^*) is located above the S_1 and S_2 states, the photoexcited species has two pathways for decaying (Figure 5). The first is quenching to the S_2 state with a rate constant of k_d , and the second one yields $\text{Fe}(\text{II})\text{P}$ via intramolecular electron transfer. Of course, the former decay is major, and the charge recombination to $\text{Fe}(\text{III})\text{-P}^+\text{Cl}^-$ with a rate constant of k_b is also a facile reaction which returns to the ground state. However, if the

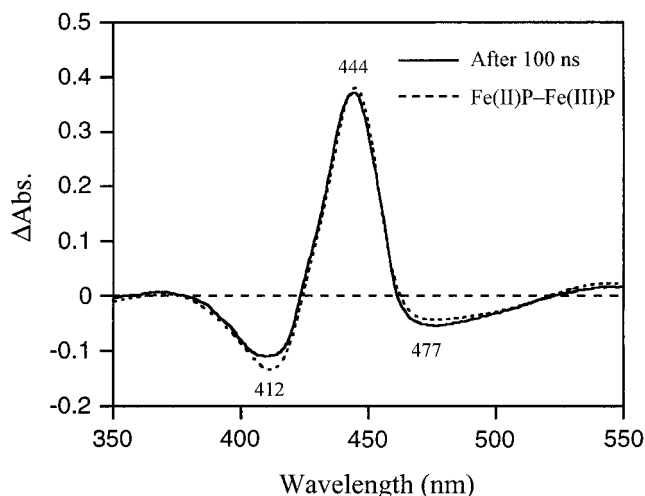


Figure 4. Transient absorption spectrum of rHSA-Fe(III)P in phosphate-buffered saline (150 mM NaCl, pH 7.3) solution at 100 ns after the laser flash photolysis (355 nm) at 25 °C. The spectral pattern is in good agreement with the difference spectrum of rHSA-Fe(II)P minus rHSA-Fe(III)P.

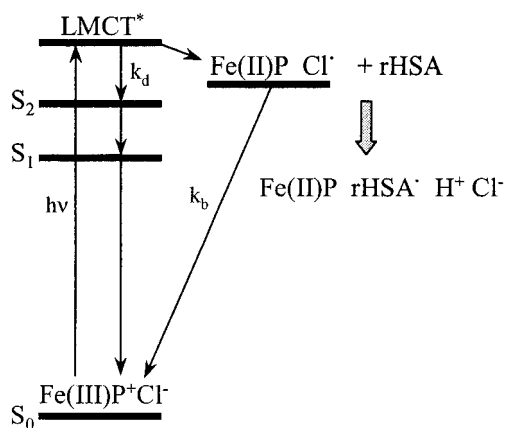


Figure 5. Transient states and interconversion pathways for photoreduction of rHSA-Fe(III)P.

albumin cage traps the chloride radical, the photoinduced electron transfer to the ferric ion becomes irreversible. That is to say, the low quantum yield of this photoreduction is due to the high probabilities of (i) the deactivation of LMCT* to S₂, and (ii) back electron transfer between the ferrous ion and the chloride radical.

CONCLUSIONS

The autooxidized Fe(III)P in the albumin hybrid formed a unique six-coordinate high-spin ferric complex, which shows the LMCT absorption at 330 nm. Photoirradiation of this band under an argon atmosphere led to a clean reduction of Fe(III)P to revive its O₂-binding ability. The partial Fe(III)P which eliminates the axial base could not be reduced, because of the imidazole rebinding is probably interfered by the steric hindrance of the amino acid residues in the albumin interior. The relatively low quantum yield of this photoreduction may increase in the serum solution, because the biological system contains some scavengers for radicals. Picosecond laser flash photolysis experiments to reveal the detailed mechanism of this photoreduction are now in progress.

ACKNOWLEDGMENT

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CORRECTIONS

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Shuang Liu* and D. Scott Edwards

BIFUNCTIONAL CHELATORS FOR THERAPEUTIC LANTHANIDE RADIOPHARMACEUTICALS

The authors sincerely apologize to Dr. Garry E. Kiefer and co-workers for the oversight of their US patent no. 5587451, which was inadvertently omitted in reference to Chart 4 of this manuscript. US patent no. 6013793 disclosed the routes showing condensation of triethylene-tetraamine (TETA) with glyoxal, followed by oxidation with bromine and formation of a dicationic intermediate. In contrast, the monocationic intermediate depicted in Chart 4 is obtained from the condensation of TETA with dimethylformamide dimethyl acetal as described in US patent no. 5587451.

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REVIEWS

Neoglycoconjugates Based on Cyclodextrins and Calixarenes

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INTRODUCTION

Recent years have witnessed not only a growing understanding of the molecular basis of carbohydrate–protein interactions (1), but also a deeper appreciation of their importance in many biological processes. On cell surfaces, carbohydrate–protein interactions are involved in the mediation of important biological processes in which tissue cells, bacteria, and viruses recognize one another. Embryogenesis, cancer metastasis, inflammation, and bacterial and viral infection are all examples of biological events which involve the selective recognition of carbohydrates by proteins. However, isolated carbohydrate–protein interactions are typically very weak (2) with K_d values of the order of 10^{-3} to 10^{-6} M. Nature compensates for the weakness of these isolated interactions by tending to cluster together multiple copies of carbohydrate ligands and their receptors (3) in order to allow for stronger cooperative binding to take place—the so-called multivalent effect (4). This biological phenomenon is an important one to consider when planning how to capitalize on carbohydrate–protein interactions when developing a potentially new class of carbohydrate-based therapeutics.

In view of the important roles played by carbohydrate–protein interactions in disease states, it is hardly surprising that the exploitation of these interactions to develop a new generation of carbohydrate-based therapeutics is already well under way. For example, it is known (5) that the Shiga and cholera toxins, which have an AB_5 structure (6), gain entry to mammalian cells when the B_5 pentamer recognizes carbohydrates on the cell surface.

The recent demonstration by Bundle and co-workers (7) of a pentavalent carbohydrate ligand capable of inhibiting the binding of the B_5 subunit of the *Escherichia coli* O157: H7 Shiga-like toxin I with subnanomolar activity, is one of the most impressive applications of glycoscience to have been described in the literature to date.

The use of carbohydrates to inhibit a particular lectin-mediated process is not the only method by which carbohydrates can find therapeutic applications—indeed, carbohydrate-based cancer vaccines which are based on tumor-associated carbohydrate antigens, are an active area of research at present (8). Another area of contemporary interest is the carbohydrate-mediated delivery of drugs (9). Since cell-surface lectins are often displayed in a very cell-specific manner, it may be possible to use a carbohydrate, which is specifically recognized by a lectin found on the surface of a specific cell type, to direct an existing covalently attached drug molecule to the desired tissues in a selective manner. For example, an earlier study (10) has shown that the antiinflammatory, naproxen, when covalently attached to galactose-derivatized human serum albumin, can be delivered selectively to the liver—in preference to the kidneys—in rat models.

Recent years have witnessed the emergence of another approach by which the power of carbohydrates to target cell surfaces can be exploited in the selective delivery of drugs to their target tissues. Supramolecular chemists have long recognized (11) the ability of certain macrocyclic hosts to complex with complementary guests, including biologically important molecules (12). The macrocyclic hosts that have been studied traditionally by supramolecular chemists almost invariably lack a biologically recognizable component, however. So even if we discount the biological disadvantages associated with

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many macrocycles, such as their lack of aqueous solubility and potential toxicities, they are, at best, limited in their biological potential. The possibility does exist, however, to modify many of these macrocyclic hosts chemically by grafting biorecognizable units—such as carbohydrates—onto their reactive functional groups. Thus, such a unique class of glycoconjugates would possess the potential to sequester a drug molecule noncovalently within their macrocyclic cavities and then rely upon their appended carbohydrate units to direct the glycoconjugate–drug complex to a specific tissue type, dictated by the choice of carbohydrate units displayed on the periphery of the macrocycle. This approach would dispense with one of the problems associated with conventional receptor-mediated glycotargeting—namely, the molecular structure of the drug molecule may have to be modified to allow it to be covalently attached to the glycoprotein, which could have serious consequences on the drug's potency. Even if it is not necessary to alter the structure of the drug molecule, covalently attaching the drug to a linker could still affect the drug's potency. Once the glycoconjugate–drug complex had located the specific target, the drug molecule would be released slowly into the cell membrane and adsorbed by the cell wherein it could fulfill its pharmacological purpose.

It is the prospect of this kind of scenario that has brought about a union of the disciplines practiced by supramolecular and carbohydrate chemists where the aim has been one of designing, synthesizing, and then determining the supramolecular and biochemical properties of these macrocyclic-based “intelligent” drug-delivery systems. This review will concentrate on recent developments in this relatively new field of glycoscience.

Design Considerations. The choice of macrocyclic host—which will become not only the host moiety but a scaffold for the attachment of the carbohydrate residues also—is an extremely important decision that has to be made at the outset by any researcher entering the field. Ideally, the starting macrocycle should be readily available and have appropriately positioned functional groups to allow for the facile attachment of carbohydrate residues. The macrocycle itself should display low pharmacological activity, and ideally, a high degree of biocompatibility. It is also advantageous if there is a well-developed literature dealing with the efficient chemical modification of the macrocycle. Another very important point for consideration is that the macrocycle should display a reasonably predictable propensity to complex with a wide range of guest molecules. The design of the macrocycle-based glycoconjugate must avoid the situation where its carbohydrate appendage(s), or any other part of the molecule for that matter, becomes bound inside the macrocyclic cavity in either an intramolecular or intermolecular sense, thus severely reducing the macrocycles' ability to complex with guest molecules. Indeed, weak complexation of a drug molecule by the macrocycle could result in the two becoming completely dissociated before the complex reaches its target. Therefore, it is advisable for chemists to design macrocyclic-based glycoconjugates which possess suitably strong association constants with their intended guest molecules.

The choice of carbohydrates to be covalently attached to the macrocycle will be determined by the desired lectin target of the neoglycoconjugate, whether for *in vivo* applications or simply model studies. Many of the compounds described in this review have been designed such that their interactions with plant lectins can be studied. Additionally, an emphasis has been placed by many researchers on producing homogeneous compounds which

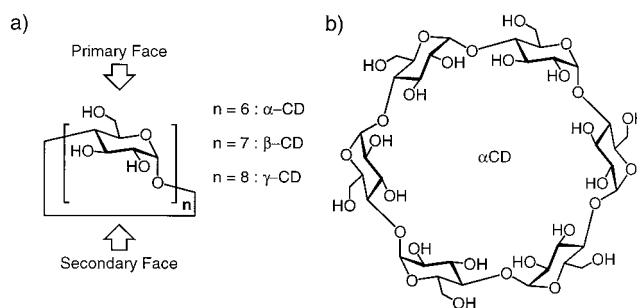


Figure 1. (a) Condensed structural formulas of α -, β -, and γ -cyclodextrin, with the primary and secondary faces indicated. (b) Full structural formula of α -cyclodextrin.

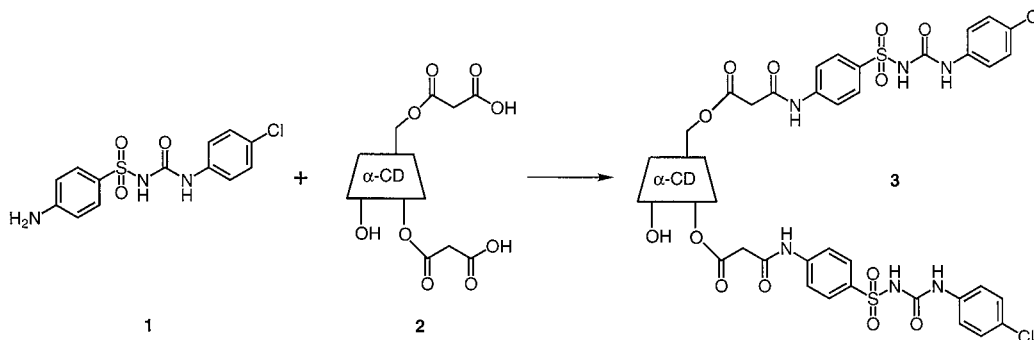
possess a well-defined structure and a precise number of carbohydrate residues. The extra effort required to produce homogeneous compounds is probably worthwhile, since it can be difficult to reproduce accurately the composition of a heterogeneous mixture and evaluate it biologically.

When all the requirements and limitations are taken into consideration, it is perhaps hardly surprising that chemists in this area have chosen “tried-and-tested” macrocycles as the initial compounds for the construction of glycoconjugates. Since cyclodextrins, calixarenes, and calixresorcarenes all have rich and extremely well-documented synthetic and supramolecular chemistries, they have, thus far, been the macrocycles of choice for the construction of these glycoconjugates. This review will cover the research reported for each of these three classes of macrocycles in turn.

Cyclodextrins—Versatile Receptor Molecules. Cyclodextrins (CDs) (13) are cyclic oligosaccharides, composed of six, seven, or eight α -D-glucose units, and giving rise to α -, β -, and γ -CD, respectively. They have an overall shape reminiscent of a truncated cone. On account of their relatively hydrophobic interiors, CDs have the ability to form inclusion complexes with a wide range of substrates in aqueous solution. As CDs are obtained on an industrial scale by the enzymatic degradation of starch by bacterial microorganisms, they are readily available and relatively affordable. This important feature, combined with their inherently low toxicities and pharmacological activities, has meant that CDs are already finding applications in the pharmaceutical industry (14). The formation of inclusion complexes with drug molecules helps to enhance their solubilities, stabilities, and bioavailabilities—for example, to improve its solubility, the antiinflammatory drug, piroxicam, is commonly marketed in many European countries as its β -CD complex.

The physical properties of CDs can be altered upon their chemical modification. CDs contain two classes of free hydroxyl groups—the primary 6-OH groups are situated around the circumference of the narrower “primary face”, and the secondary 2- and 3-OH groups are located on the circumference of the wider “secondary face” (Figure 1). It is these hydroxyl groups, and their associated differences in reactivity, which provide the fundamental basis for the regioselective modification of CDs.

There are a limited number of examples of the chemical modification of CDs with bioactive molecules in an attempt to use the characteristics of the CD to improve the potency of a bioactive molecule. For example, the antitumor sulfonylurea LY237868 (1) has been attached (15) (Scheme 1) covalently to an α -CD derivative 2. The

Scheme 1. Reaction of the Antitumor Sulfonyl Urea 1 with the Modified CD Core 2 To Form the Cluster Compound 3 as a Mixture of Structural Isomers

resulting conjugate **3** is a mixture of isomers which contain approximately 14 sulfonylurea groups per CD molecule. Importantly, the attachment of these sulfonylurea groups to the α -CD core greatly increases the bulkiness of the conjugate, and it is thus less likely to pass through cell membranes. As a consequence, it has been possible to prove that the antitumor sulfonylurea blocks NADH oxidase activity on the external plasma membrane surface of HeLa cells and not in the interior of the cells. However, it is the prospect of utilizing recent advances in glycobiology which has done much to shape current research in this area. It is the possibility of employing CDs adorned with carbohydrate units to target cell surfaces selectively and thus to guide an included drug molecule to a desired site, which has been receiving the attention of many researchers of late.

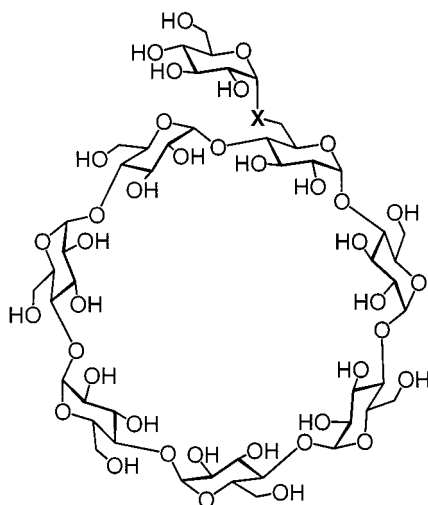
Cyclodextrins Appended with One Carbohydrate. CDs adorned on their primary faces with one or more carbohydrate units have been known (16) since the mid 1960s. These branched CDs, which consist of an α -D-glucopyranosyl unit or a (1 $\rightarrow$ 4)- α -D-glucan connected to the 6-position of one or more of the α -D-glucopyranosyl units of the CD torus, are minor products formed during the action of *Bacillus macerans* cycloamylose glucotransferase on starch during the formation of native CDs. Researchers at the time were interested in these branched CDs, as they potentially offered advantages over native CDs in terms of solubility or substrate complexing ability, as well as being useful in studies of the enzymatic degradation of CDs. However, the tedious purification

procedures required to isolate these compounds prompted (17) the first chemical synthesis of the branched CD derivative **4**. Shortly thereafter, several research groups reported (18) the synthesis of the thio analogues of **4**—namely the 6-S- α -D-glucopyranosyl-6-thiocyclomaltoheptaose derivative **5**.

Although the ability of saccharide-branched CDs to complex with guest molecules had been investigated (19) sometime ago, it was not until the early 1990s that researchers recognized the potential of utilizing the carbohydrate appendage of a CD torus in order to direct the glycoconjugate toward a specific biological site.

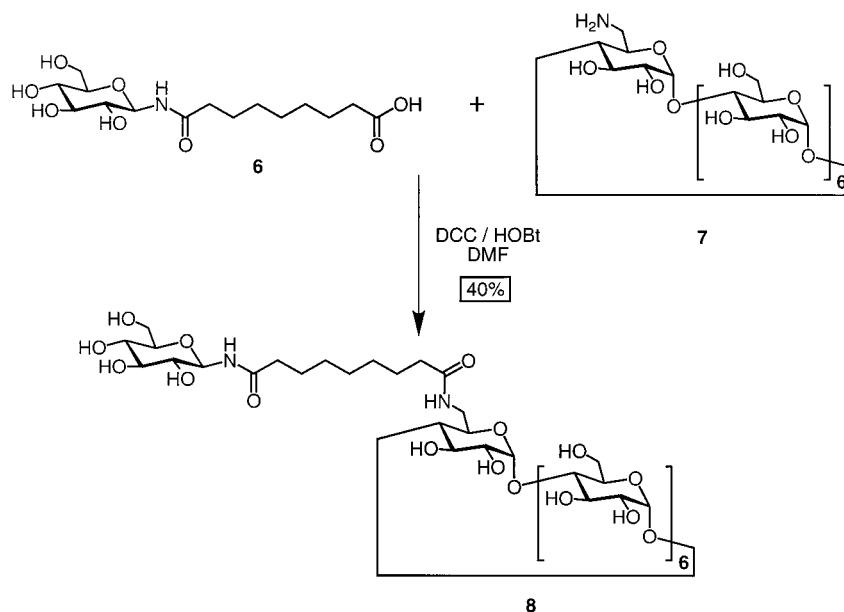
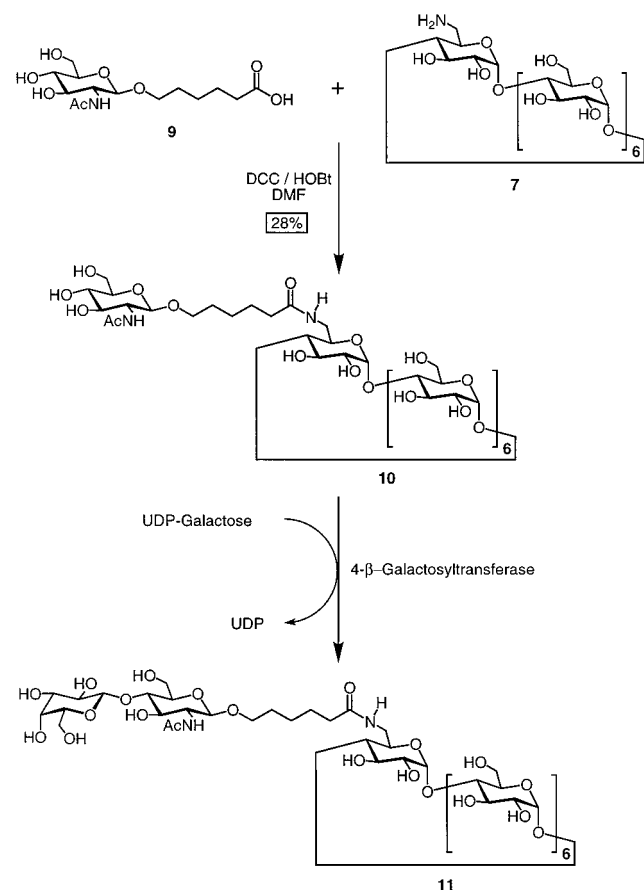
Realizing the potential saccharide-branched CDs could have for the “vectorized transport of drugs”, Parrot-Lopez et al. began to develop synthetic methods to link carbohydrate units onto CD cores. However, the French group recognized an important limitation of the naturally occurring saccharide-branched CDs. Compounds such as **4**, where the carbohydrate appendage is connected directly to the CD torus through an O-glycosidic linkage, will probably not possess sufficient flexibility for the appendage to interact with the receptor. To circumvent this potential pitfall, a saccharide-branched CD was prepared (20) which relies upon a flexible C_9 spacer to attach the carbohydrate unit to the CD core. Thus, a carbohydrate derivative **6** (Scheme 2), containing a β -N-glucosidic linkage and a C_9 linker, terminated by a free carboxylic acid function, was prepared. Standard amide bond-forming chemistry (DCC/HOBt) was then used to attach **6** to mono-6-amino- β -CD (**7**), affording the target compound **8** in a 40% yield. ^1H NMR spectroscopic analyses of **8** revealed very little conformational perturbation of the CD toroidal protons in either $\text{DMSO}-d_6$ or D_2O as solvents, indicating that there is no interaction between the C_9 spacer arm and the hydrophobic cavity of the CD. Compound **8** was also found to have a much improved solubility (200 g L^{-1}) in water relative to that (19 g L^{-1}) of native β -CD. ^1H NMR spectroscopic evidence suggested that **8** can form an inclusion complex with the antihypertensive drug, nifedipine, although no association constant was reported for the complex. The same group also subsequently reported (21) the synthesis of β -N-galactoside, β -N-fucoside, and α -N-mannoside derivatives of **8**. Various biological experiments were then performed using some of these compounds. They indicated (22) that the β -N-galactosyl derivatives possess lower hemolytic properties than does the native β -CD, and that the β -N-galactosyl, β -N-fucosyl, and β -N-acetylglucosylamine derivatives display a certain cytotoxicity toward some human tumor cells.

Later work by Parrot-Lopez and co-workers (23) highlighted the fact that saccharide-branched CDs, with spacer arms between the appended carbohydrate units



4: X = O

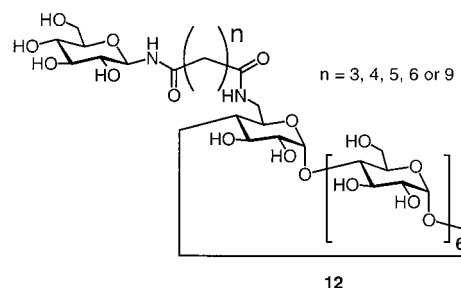
5: X = S

Scheme 2. Synthesis of a CD Derivative Appended with One *N*-glucoside Unit at the End of an Alkyl Chain Spacer Arm**Scheme 3. Chemical–Enzymatic Synthesis of Mono-6-[Gal- β -1,4-GlcNAc- β -(1,6')-hexyl]amido-6-deoxy- β -CD **11****

and the CD torus, could be prepared efficiently, using a chemical–enzymatic strategy. The incorporation of an enzymatic step into the overall synthetic scheme would arguably allow access to these saccharide-branched CDs on a large scale, an issue which would have to be addressed should these compounds ever become of commercial interest. Peptide coupling (Scheme 3) of the GlcNAc derivative **9**, which contains a hexanoic acid

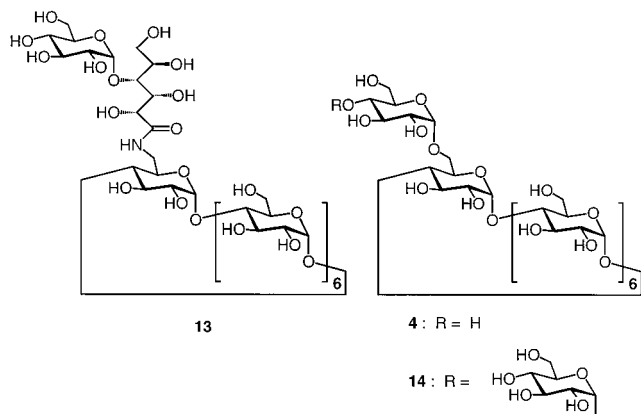
spacer arm, to mono-6-amino- β -CD (**7**) yields the product **10** in 28% yield. Enzymatic coupling of UDP-galactose to **10** in the presence of 4- β -galactosyltransferase affords mono-6-[Gal- β -1,4-GlcNAc- β -(1,6')-hexyl]amido-6-deoxy- β -CD (**11**) in 68% yield. Biological assays indicate that **11** can be recognized by the galactose-specific *Kluveromyces bulgaricus* cell wall lectin (K_b CWL), whereas the simpler GlcNAc derivative **10** is not recognized at all by this lectin.

The effects of the lengths of the spacer arm on the ability of monosaccharide-branched CDs to recognize lectins has been investigated by numerous researchers. Parrot-Lopez and co-workers (24) prepared a series of β -CD derivatives **12**, appended with a single *N*- β -D-



galactosyl unit at the end of a spacer arm of varying length ($n = 3–6$ or 9). The ability of these compounds to be recognized by the K_b CWL in a flocculation assay was then demonstrated. The results suggest that those compounds with short spacer arms are not recognized by K_b CWL as well as those compounds with long spacer arms. Another study, which deals briefly with the effects of spacer arm lengths on lectin recognition, has been performed by Hattori and co-workers (25). In this work, the technique of surface plasmon resonance (SPR) has been used to measure the rates of association and dissociation—information which allows the direct calculation of K_d —of various saccharide-branched CD derivatives with the lectin Concanavalin A (Con A), which is immobilized on a biosensor chip. One important conclusion from this investigation is that the α -glucosylgluconamide- β -CD **13** exhibits a significantly stronger interac-

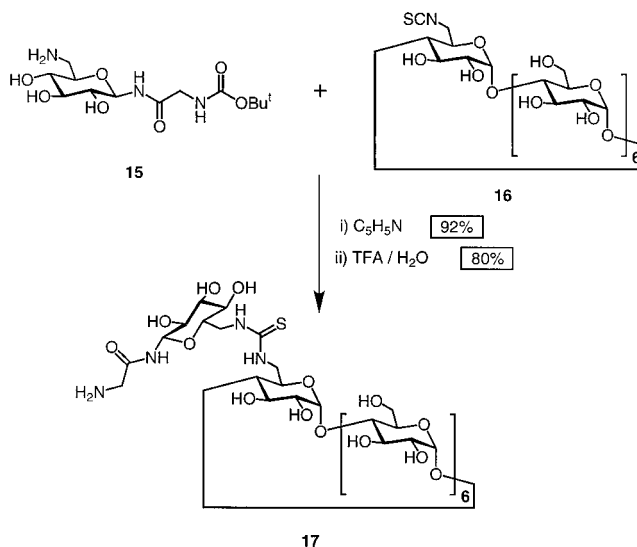
tion with Con A than either of the simpler α -glucosyl- or α -maltosyl- β -CDs **4** and **14**. This result indicates that the carbohydrate appendage has to be spatially far enough removed from the CD core in order to enter into a strong interaction with Con A. However, it also indicates that the crucial terminal carbohydrate appendage must additionally have a certain degree of conformational freedom to help it interact with the Con A, which would explain why the α -glucosylgluconoamide- β -CD **13** experiences a much stronger interaction with Con A than does the α -maltosyl- β -CD **14**, which has a conformationally more restricted terminal α -glucopyranosyl residue. In the study of multivalent carbohydrate-protein interactions, the spacer arm length and relative spatial orientation of the appended carbohydrate ligand is of fundamental importance. Recent investigations (26) have focused upon the effect of these structural characteristics in a more systematic and detailed manner. It is probably fair to conclude that, in the area of saccharide-branched CDs, and indeed saccharide-branched macrocycles, the research carried out to date only begins to scratch the surface of understanding the subtleties of the relative spatial orientation of carbohydrate ligands, relative to their scaffolds and to each other.



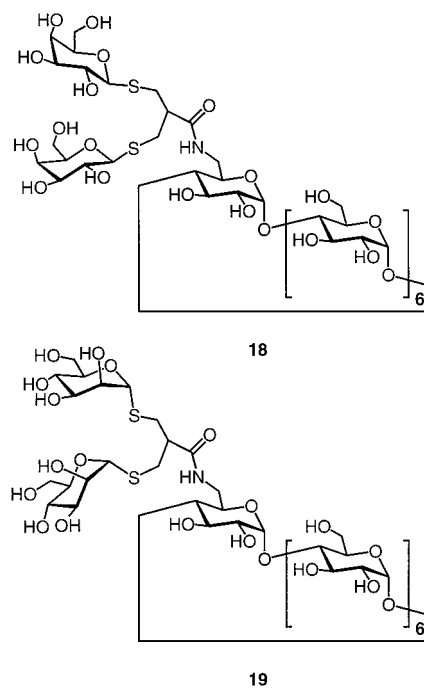
Research performed (27) by the groups of Fernández and Defaye has resulted in the development of a route for the attachment of *N*-glycopeptide entities onto CD cores, utilizing thiourea formation. The glucosylamino acid derivative **15** shown in Scheme 4 was prepared in six steps from 1,2-*O*-isopropylidene- α -D-glucofuranose. Reaction of the free amino function in **15** with mono-6-isothiocyanato- β -CD **16**, followed by removal of the Boc-protecting group, affords the target compound **17** in high yield. The authors hope that this synthetic strategy will be efficient enough to allow the attachment of other more complex *N*-glycopeptide structures units onto CD cores.

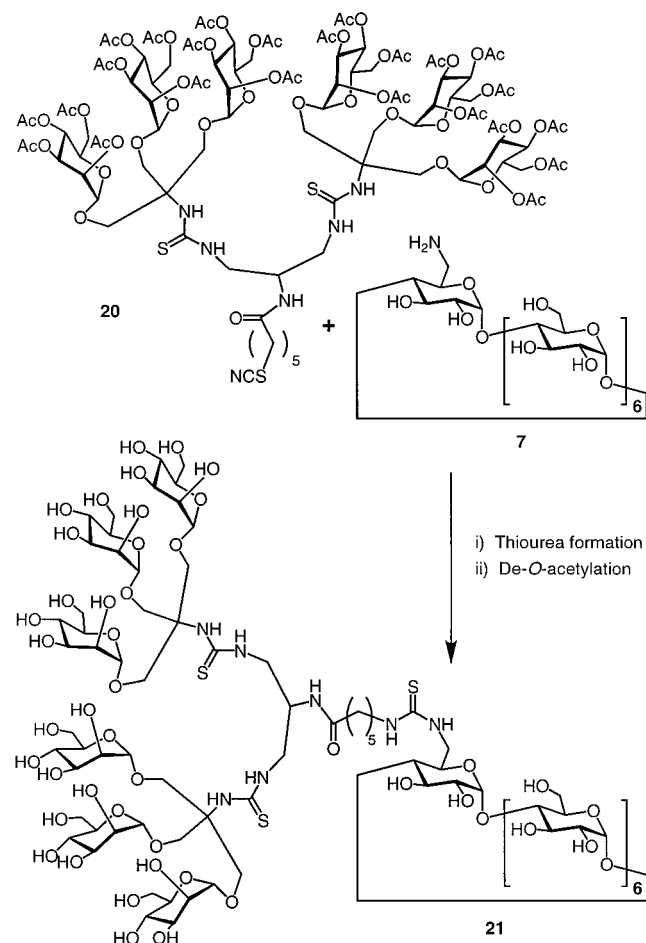
Another interesting example of monofunctionalized CD derivatives has recently been published (28) by the same group. In this investigation, instead of functionalizing the CD core with a single carbohydrate appendage, it has been functionalized with a single dendritic wedge containing up to six carbohydrate units. As these compounds contain multiple copies of their carbohydrate ligands, they now possess the potential to exploit the so-called "multivalent" or "glycocluster" effects (4). Simpler compounds such as **18** and **19**, consisting of β -CD cores and monosubstituted with diantennary galactosyl and mannosyl moieties **19** had previously been described by Driguez and co-workers (29). The synthesis and use of glycodendrimers designed to exploit these effects, have been an active area of research in recent years. This topic has been reviewed elsewhere in detail (30). The synthetic

Scheme 4. The Attachment of a Simple *N*-glycopeptide to a CD Core



strategy of attaching carbohydrate-containing dendritic wedges to CD cores has several advantages. It allows the synthesis of a CD-based cluster containing multiple displays of carbohydrate units, without having to resort to more synthetically challenging perfunctionalizations of the CD core. Additionally, the binding ability of the CD core is also less likely to be perturbed relative to that of the corresponding native β -CD. The Franco-Spanish group have developed an efficient method for the construction of dendrons, substituted with mannosyl residues on their peripheries. Their synthesis, which uses the efficient and clean reaction of amines with isothiocyanates to form thioureas, allows the construction, in a convergent manner, of several mannose-containing dendrons, such as **20** shown in Scheme 5, with an isothiocyanate function at their focal points. Reaction of **20** with mono-6-amino- β -CD (**7**), followed by de-*O*-acetylation, affords the target compound **21**. A series of compounds containing various numbers (1–4 or 6) of mannosyl residues were prepared in a similar manner, and their

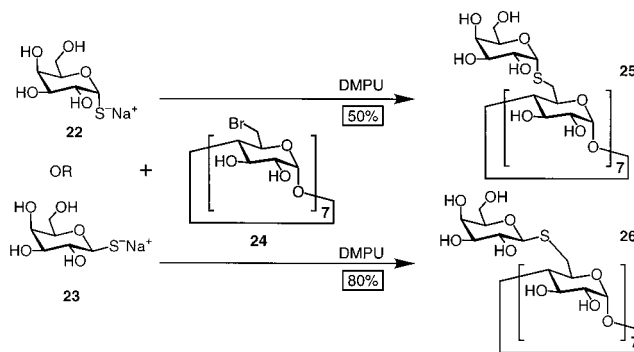


Scheme 5 The Attachment of a Dendritic Wedge Containing Six Mannosyl Residues at Its Periphery to a CD Core

abilities to interact with Con A were determined by an enzyme-linked lectin assay (ELLA). Although the results of this assay are not disclosed by the authors, they do mention that "IC₅₀ values for inhibition of Con A-yeast mannan binding reflected the expected amplification of lectin-binding strength for the higher-valent representatives". It was also observed that these mannosyl-coated β -CD-dendrimer constructs had an ability—which was similar to that observed for monobranched CDs—to solubilize the anticancer drug Taxotère.

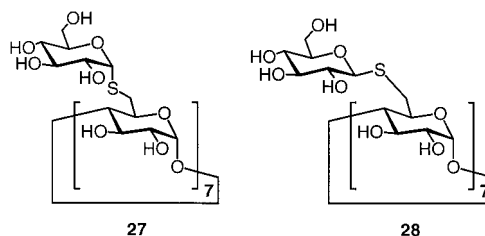
Cyclodextrins Appended with Many Carbohydrates. The previously described example highlights the potential advantages that molecules displaying multiple carbohydrate ligands may have—namely the ability to interact with lectins with much stronger affinities than their monovalent counterparts. Research in the field of multivalent CDs to date has largely focused on the synthesis of persubstituted CDs, where each glucosyl residue of the CD torus has an appended carbohydrate ligand. Although CD derivatives of this type retain their inherent C_n symmetries, their perfunctionalization can pose very formidable synthetic problems.

The first chemical syntheses of CDs, persubstituted with carbohydrate appendages, were performed by Driguez co-workers (31) in 1994. The synthetic approach they utilized expands upon that used by them previously (18b) in the synthesis of the mono-branched CDs 5—i.e., simple nucleophilic displacement of a suitable leaving group in the 6-position(s) of β -CD with a thiolate anion. Reaction of the sodium salts **22** and **23** of both 1-thio- α - and 1-thio- β -D-galactopyranose, respectively, with per-6-bromo- β -CD

Scheme 6. The First Reported Synthesis of CDs Persubstituted on Their Primary Faces with Galactosyl Residues

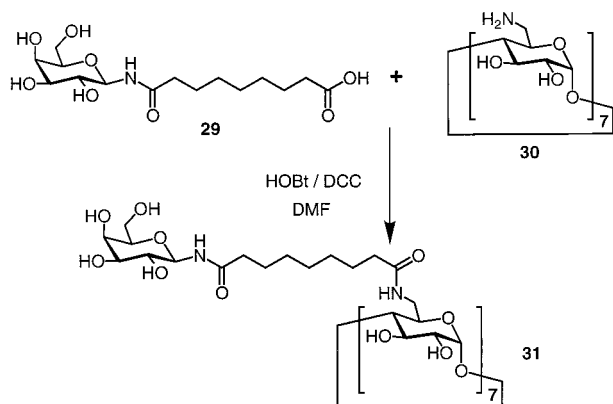
24 affords (Scheme 6) the persubstituted products **25** and **26** in 50% and 80% yields, respectively.

Shortly afterward, Djedaïni-Pilard and co-workers (32) reported a more detailed account of the synthesis and host-guest binding properties of CDs, persubstituted with carbohydrate appendages. The French group was initially interested in comparing the water solubilities of CDs, persubstituted with carbohydrates, relative to those of monosaccharide-branched CDs and native CDs. Increased aqueous solubility could endow the modified CDs with an increased ability to bind drug molecules. Utilizing a strategy similar to that reported independently by Defaye (18a) and Driguez (18b) for the synthesis of **5**, the per-6-thio- α - and per-6-thio- β -D-glucopyranosyl derivatives **27** and **28** of CD were prepared.



Solubility studies indicated that these persubstituted CDs were more soluble than their monobranched counterparts, with the α -anomers being considerably more soluble than the corresponding β -anomers. An extensive NMR spectroscopic study of inclusion complexes formed between both the monosubstituted and persubstituted CD hosts and a series of drug molecules, was then performed. This investigation allowed the determination of association constants (K_a values) and binding orientations of the guests within the CD cavities—and, therefore, an estimation of the effect that the appended carbohydrate moieties have on the complexation ability of the CD cavity—and produced some initially unexpected results. Interestingly, it was found that *p*-nitrophenol, a well-known guest for β -CD, was found to form an exceptionally weak inclusion complex with **27**. This result was explained by the fact that β -CD prefers (33) to bind *p*-nitrophenol through its narrower primary face, suggesting that the bulky carbohydrate groups on the primary face of **27** present enough steric hindrance to prevent the complexation of the guest altogether. A different scenario is found in the complexation of the steroid, prednisolone, where 2D-ROSEY experiments suggest that it is complexed principally by **27** from the secondary face of its CD torus. However, the measured K_a value for the association of prednisolone with **27** in D₂O was only 200 M⁻¹, a value which is considerably

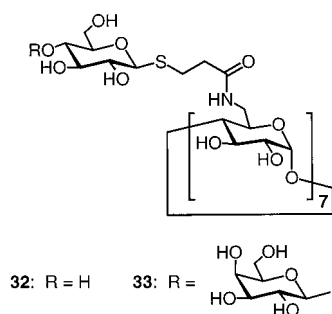
Scheme 7. Attachment of Seven *N*-Galactosyl Residues to a CD Core Using Amide Bond Formation as the Key Step



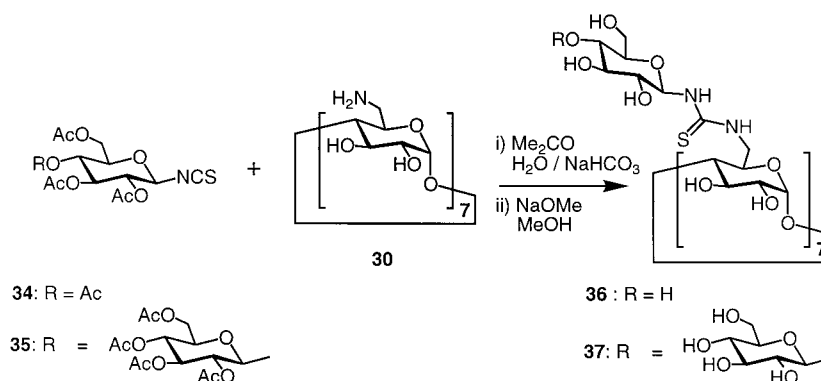
smaller than that (2000 M^{-1}) for the complexation of native β -CD with prednisolone. Molecular modeling of the native β -CD-prednisolone complex, using NMR-derived distance constraints, reveals that hydrogen bonding between the 6-hydroxyl groups of β -CD and the C-3 carbonyl group of prednisolone plays an important role in the stabilization of the complex. The absence of this interaction in the **27**/prednisolone complex may therefore account for the decrease in the K_a value.

Parrot-Lopez and co-workers (24) have expanded their synthetic studies on monobranched CDs such as **8** to investigations of persubstituted β -CD derivatives, adorned with seven *N*- β -D-galactosyl units at the end of a spacer arm. Reaction (Scheme 7) of 8.4 equiv of **29** with per-6-amino- β -CD (**30**), under standard amide bond-forming conditions, yields the fully substituted cluster compound **31**.

Our own research group has also used (34) amide bond formation as the key step in the synthesis of the *S*-glucosyl- and *S*-lactosyl CD-based clusters **32** and **33**,



Scheme 8. Preparation of CD-Based Clusters, Using the Reaction of Isothiocyanates with Primary Amines to Form Thioureas as the Key Step in the Attachment of the Carbohydrate Units to the CD Core



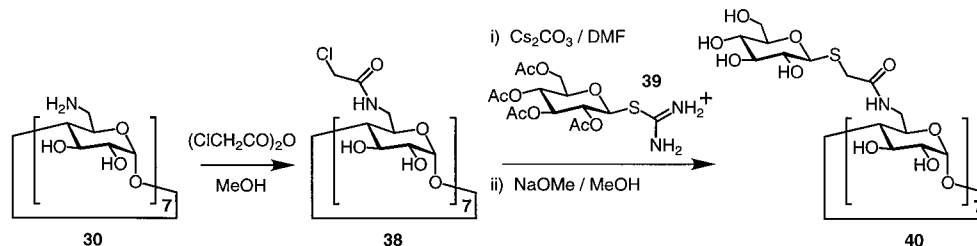
respectively. The lactose cluster **33** was shown to bind the nonsteroidal antiinflammatory, naproxen, in phosphate-buffered saline solution more weakly ($K_a = 165\text{ M}^{-1}$) than does β -CD ($K_a = 374\text{ M}^{-1}$) under identical conditions.

Another research group (35) in the US has utilized amide bond formation as the key step in the attachment of carbohydrate units to CD cores. However, in this research the carboxylic acid functions are located on the primary face of the CD and the amine functions on the appended carbohydrate units. Mixtures containing an average of five carbohydrates per CD and four carbohydrates per CD for the cases of α -CD and β -CD, respectively, were reported.

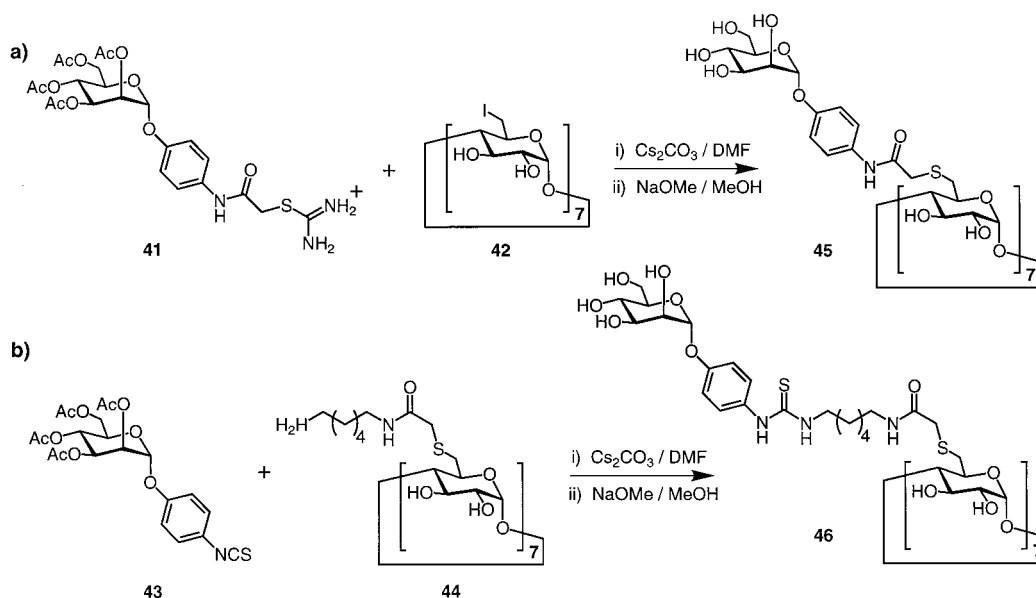
The clean and efficient reaction between amines and isothiocyanates to form thioureas, has been applied (36) by the research groups of Fernández and Defaye in the preparation of persubstituted CDs. The readily prepared carbohydrate isothiocyanates **34** and **35** (Scheme 8) react readily with per-6-amino- β -CD **30** to afford the fully substituted glycoclusters **36** and **37**, respectively, in good yields. The conditions required for this coupling reaction ($\text{Me}_2\text{CO}/\text{H}_2\text{O}/\text{NaHCO}_3$) result in partial deacetylation of the products, which are then fully deacetylated in a further step to afford the free cluster compounds.

Research groups led (37) by Roy and Santoyo-González have performed excellent and extensive investigations into both the syntheses and lectin-binding affinities of perglycosylated β -CD derivatives. In this research, perglycosylated β -CDs, containing four distinct linkage structures between the appended carbohydrate unit and the CD core have been prepared. The first set of CDs synthesized in this study are identical to **28**. The investigation was extended to the synthesis of the corresponding per- β -galactosides, per- β -*N*-acetylglucosamines and per- α -mannosides derivatives, all prepared by slightly differing methods to those described previously. To investigate further, the link between spacer arm length on the lectin binding abilities of the glycoconjugates, the second set of CDs were designed to incorporate a short CH_2CONH linker arm between the CD torus and the appended carbohydrate units. Reaction (Scheme 9) of per-6-amino- β -CD (**30**) with chloroacetic anhydride affords the heptachloro- β -CD derivative **38** in a near quantitative yield. When **38** is treated with the isothiuronium- β -D-glucoside **39**, the desired glycocluster **40** is obtained in excellent yield after de-*O*-acetylation. This synthetic methodology was repeated to obtain the β -galactosyl, β -*N*-acetylglucosamine, and α -mannosyl analogues of **40**. The third and fourth set of derivatives utilize *para*-substituted phenylmannosides only as the carbohydrate units.

Scheme 9. Reaction of Isothiuronium 39—Which Acts as a Latent Sulfur Nucleophile—with the Heptachloro- β -CD 38 to Afford, after Deprotection, the Persubstituted Cluster 40



Scheme 10. Synthesis of α -Mannosyl Clusters with Spacer Arms of Varying Lengths



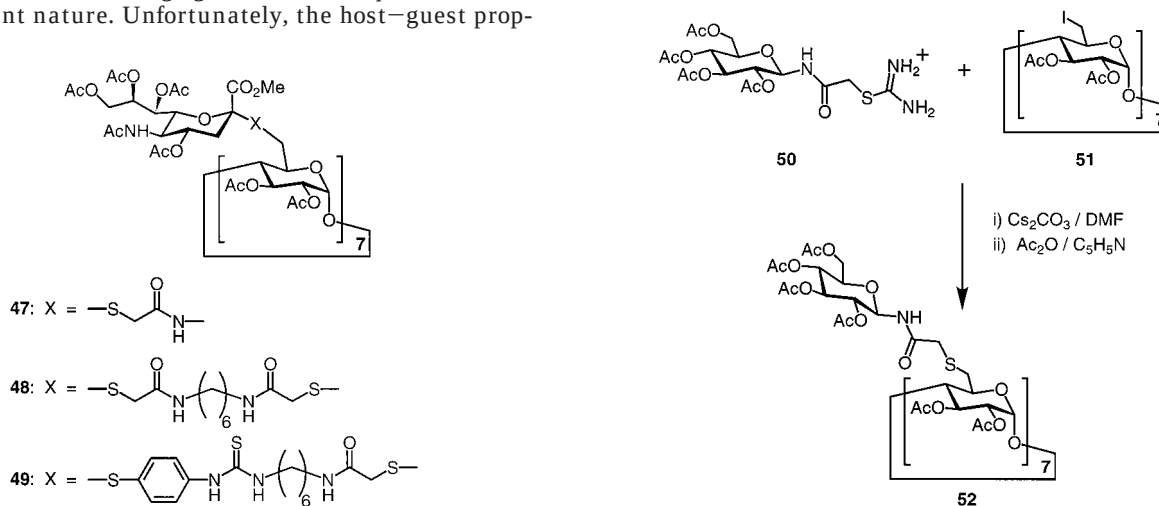
Reaction of **41** (Scheme 10a) with per-6-iodo- β -CD (**42**), or reaction (Scheme 10b) of the isothiocyanate **43** with the extended β -CD core **44**, affords the two mannosyl derivatives **45** and **46**, respectively, with the obvious structural difference between these two compounds being the longer spacer arms present in **46**.

Various assays were then performed on these compounds. They indicated that (1) the cluster compounds are more effective inhibitors than the corresponding simple monosaccharide in competitive inhibition assays, (2) those clusters with longer spacer arms are more effective inhibitors than those with no spacer arms in competitive inhibition assays, and (3) the clusters can act as lectin cross-linking agents as a consequence of their multivalent nature. Unfortunately, the host–guest prop-

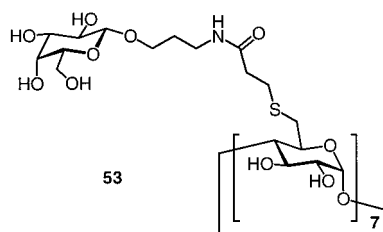
erties of none of these carbohydrate clusters have been, to our knowledge, reported by the authors yet. More recently, Roy and co-workers (38) have described the preparation of the protected persialylated β -CDs **47**, **48** and **49**, using similar synthetic methods to those described above.

Nucleophilic displacement reactions have also proven to be a very effective method for the attachment of carbohydrate units onto CDs. Research groups lead by

Scheme 11. Reaction of the *N*-Glucosyl Isothiuronium 50—Which Acts as a Latent Sulfur Nucleophile—with the Hepta-iodo- β -CD 51 To Afford the Persubstituted Cluster 52



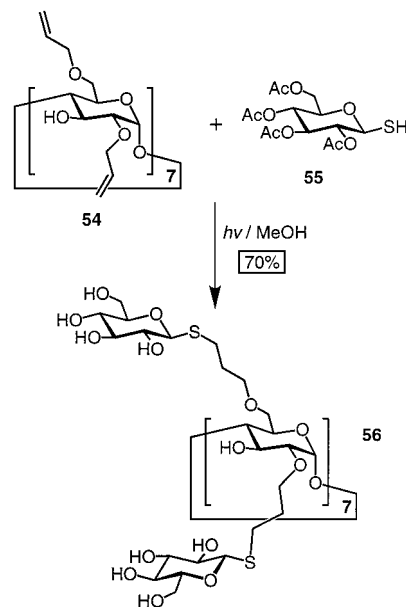
Santoyo-González and Vargas-Berenguel have utilized (39) *N*-glycosyl isothiuronium derivatives—which act as latent thiolate nucleophiles—in the synthesis of CD-based cluster *N*-glycosides. Reaction (Scheme 11) of the isothiuronium derivative **50** with per-2,3-diacetyl-per-6-iodo- β -CD (**51**) in the presence of cesium carbonate affords, after reacylation, the persubstituted derivative **52**. Standard deacetylation is then employed to afford the final deprotected cluster. This synthetic strategy was employed to prepare the corresponding *N*-galactosyl, 2-acetamido-2-deoxy-*N*-glucosyl and *N*-mannosyl derivatives. A variation of this nucleophilic displacement strategy has been used (40) recently to construct clusters, such as the galactosyl cluster **53** which contains longer spacer arms bridging the gap between the CD core and its attached carbohydrate residues. Glucosylamino, lactosyl, and lactosylamino derivatives of this compound were also prepared and their abilities to inhibit the hemagglutination of blood cells by lectins was also reported in this investigation.



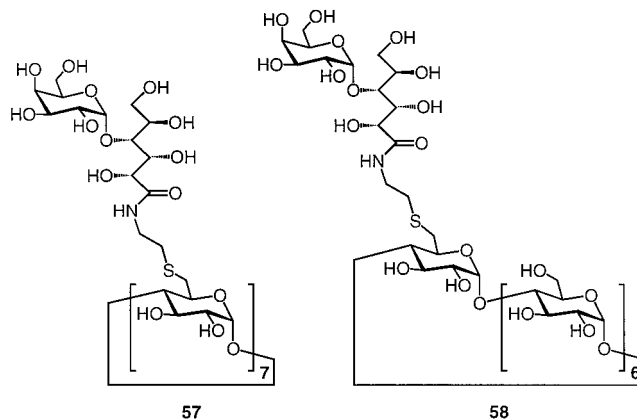
All CD-based carbohydrate clusters synthesized to date have involved the attachment of carbohydrate units onto the primary face of CDs, probably as a consequence of the relative ease of carrying out CD primary face synthetic modifications. The attachment of carbohydrate residues to the secondary face or both faces of CDs could have some potential advantages relative to their primary face-substituted counterparts. In addition, they present a considerable challenge in the preparation of chemically modified CDs. We have devised (41) a method for the perfunctionalization with carbohydrate residues of (1) the primary face, (2) the secondary face, and (3) both the primary and secondary faces, simultaneously, of β -CD. For the key step, our strategy relies upon (1) the well-known photoaddition of thiols to allyl ethers in an anti-Markovnikov fashion to yield thioethers, and (2) the fact that allyl ethers can be attached selectively to either or both the primary and secondary faces of CDs. The power of this methodology is demonstrated by the fact that the reaction (Scheme 12) of per-2,6-diallyl- β -CD (**54**) with an excess of β -D-thioglucose (**55**) in the presence of UV light from an Hg lamp affords, after deprotection, the fully substituted cluster compound **56** in a 70% yield. Derivatives persubstituted on either their primary or secondary faces *only* were also prepared in this straightforward manner.

Hattori and co-workers (42) have synthesized a per-substituted galactosyl cluster **57** and its monovalent analogue **58**. Using SPR-based techniques, they have estimated the binding constants of these two compounds with peanut lectin and found that the multivalent galactosyl cluster has an association constant ($K_a = 130000 \text{ M}^{-1}$) with the lectin approximately 16 times higher than that ($K_a = 8100 \text{ M}^{-1}$) of its monovalent analogue. SPR was also used to measure the association constants of the anticancer drug, doxorubicin with **58** ($K_a = 3100 \text{ M}^{-1}$) and with **57** ($K_a = 62000 \text{ M}^{-1}$). The Japanese group have suggested that the greatly increased K_a value

Scheme 12. Perfunctionalization of Both the Primary and Secondary Faces of a β -CD Core with 14 β -S-Glucosyl Residues



for the binding of doxorubicin with **57** is a consequence of the galactosyl appendages lowering significantly the rate of dissociation of doxorubicin from the CD cavity.



The use of 1,3-dipolar cycloadditions is perhaps an unusual choice of reaction to use as the key synthetic step in the attachment of carbohydrate units to CD cores. Nonetheless, Santoyo-González and co-workers (43) have prepared a galactosyl nitrile oxide derivative **59**, which, when reacted (Scheme 13) with the per-6-propargyl- β -CD derivative **60** in refluxing toluene, affords the cluster compound **61** in 79% yield. The isoxazole rings are formed in a regioselective manner, allowing the isolation of the isomerically pure compound **61**.

Calixarenes Appended with Carbohydrates. Calixarenes (**44**) are macrocyclic compounds commonly derived from the base-induced reaction of phenols with formaldehyde. They can exist in a conformation reminiscent of a basket or vase—indeed, the name calixarene is derived from a type of Greek vase called a calix. Although phenol-derived calixarenes have $[1_n]$ metacyclophane constitutions (Figure 2) and are commonly composed of four, six, or eight arene units, compounds containing other numbers of arene units are also known. All calixarenes discussed within this review contain four arenes per macrocycle and are therefore, broadly referred to as calix[4]arenes. Substituents located on the “wider rim” of the

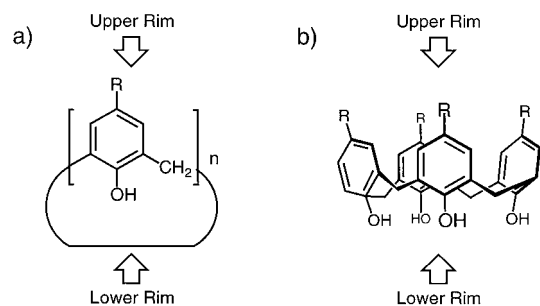
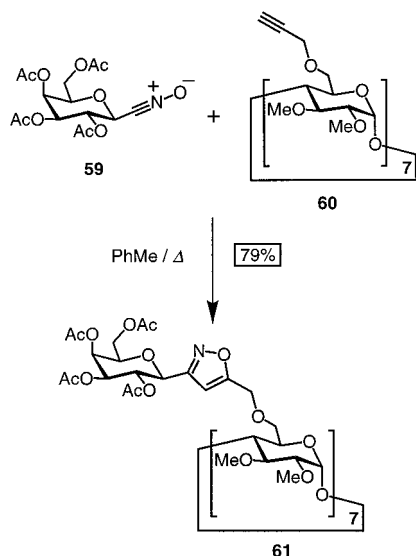


Figure 2. (a) Condensed structural formulas for the calix[n]-arenes with the upper and lower rims of the bowl-shaped macrocycle indicated. (b) The complete structural representation of a calix[4]arene.

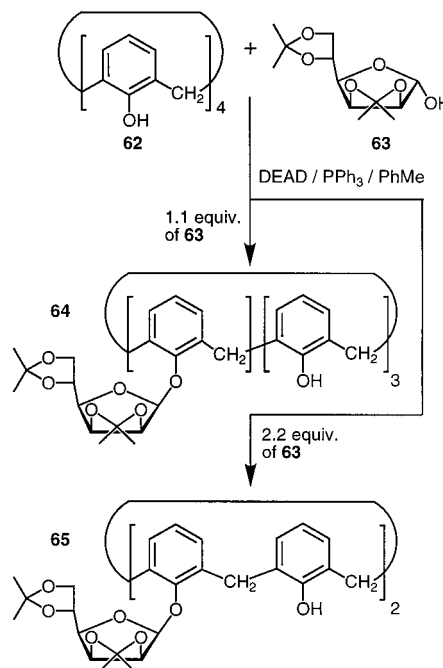
Scheme 13. The Use of 1,3-Dipolar Cycloaddition Reactions To Prepare a Persubstituted CD Cluster



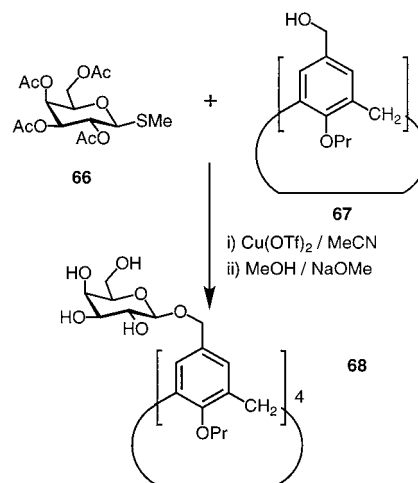
calixarene are said to be situated on the “upper rim” of the macrocycle, while those on the “narrow rim” are said to be situated on the “lower rim” of the macrocycle. They possess an extensive host–guest chemistry, a property which makes them of potential interest to those researchers involved in developing molecular delivery vehicles. A multitude of metal cations, simple arenes, small neutral molecules and even C_{60} are all known examples of guests in both the solution and solid states—for complexation by calixarenes of varying sizes and shapes and with a range of functionalities. The literature of calixarenes has been reviewed extensively, and the reader should consult the monographs and reviews listed in ref 44 for more details.

In a pioneering study, Dondoni, Ungaro and co-workers (45) have developed methods for the attachment of carbohydrates onto calixarenes to form the so-called “calixsugars”. They anticipated that the attachment of carbohydrate substituents would induce water solubility upon the calixarene derivatives, thus opening up the possibility of being able to explore the supramolecular chemistry of calixarenes in water. They also realized that these compounds may have some useful role to play in the study of carbohydrate recognition processes. In their investigations, the Italian consortium developed synthetic routes to O-glycosyl calix[4]arene derivatives, examining how carbohydrates could be attached to both the upper and lower rims of calixarenes. The approach they have developed for the attachment of carbohydrate units to the lower rim of calix[4]arene **62** takes advantage of the

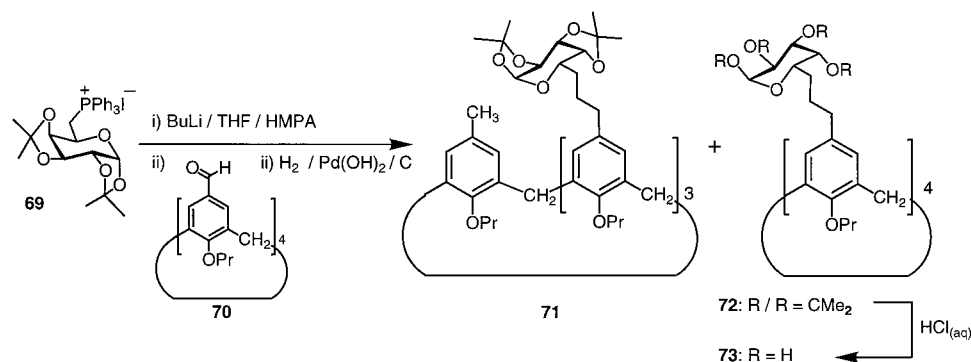
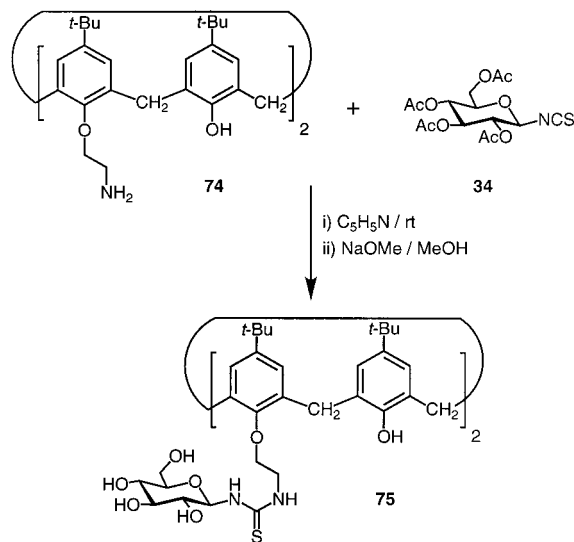
Scheme 14. The Use of the Mitsunobu Reaction in the Formation of Calix[4]arenes Mono- and Disubstituted with Protected Mannosyl Residues



Scheme 15. Preparation of a Tetragalactosyl Calix[4]arene Cluster

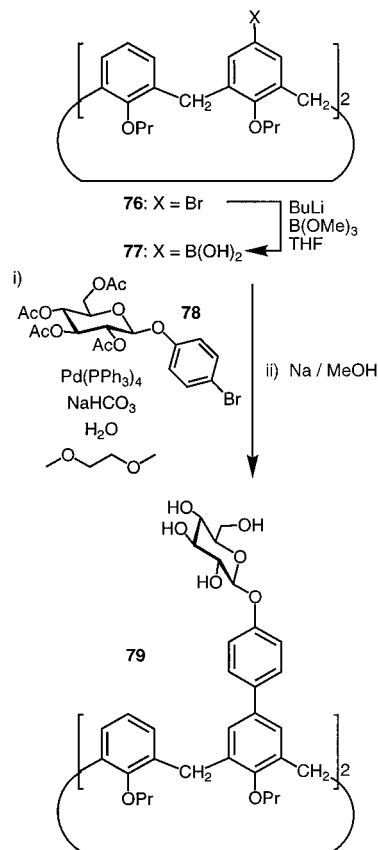


fact that, even although the phenolic hydroxyl groups are poor nucleophiles, they do possess acidic protons. This feature opens up the possibility to couple carbohydrates—unprotected at their anomeric centers—to the phenolic hydroxyl groups using Mitsunobu conditions. Thus, reaction (Scheme 14) of **62** with α -D-mannofuranose diacetone **63** under Mitsunobu conditions affords, in a stereospecific manner, the β -anomers of the mono- or disubstituted products **64** or **65**, depending on the number of equivalents of the acetonide used. Unfortunately, it was not possible to remove the protecting groups cleanly in order to afford the free mannosyl derivatives. Glycosylation of **62** with tetra-O-acetyl- α -D-glucopyranose under similar conditions produced a complex mixture of diastereoisomeric mono- and bis-glycosylated calixarenes—indicating clearly the limitations of using Mitsunobu conditions for the preparation of calixsugars. In an attempt to glycosylate the upper rim of calixarene derivatives, the Italian consortium have chosen calix[4]arene derivatives which are locked into

Scheme 16. The Formation of Tri- and Tetragalactosyl Calix[4]arenes by Wittig Reactions**Scheme 17. Reaction of β -Glucosyl Isothiocyanates with a Diaminocalix[4]arene To Form the Dithiurea Glucosyl Cluster**

cone conformations by four *O*-propyl groups on their lower rims. Hydroxymethyl substituents on the upper rims of these conformationally locked calix[4]arenes act as the glycosyl acceptors for thioglycoside glycosyl donors. After preparing bisglycoside-calix[4]arenes initially—only to find that these deprotected calixsugars were not water soluble—the consortium turned their attention to the synthesis of tetrakisglycoside-calix[4]arenes. Reaction (Scheme 15) of the thiogalactoside tetraacetate **66** with the protected calix[4]arene **67** affords, after deprotection, the tetrakis-*O*-galactosyl calix[4]arene **68**. This compound was found to be not only water soluble but also to act as a host toward charged guests such as D-glucosamine hydrochloride and the dihydrogenphosphate anion. Don-doni and co-workers (46) have also extended their synthetic methodology to prepare upper rim functionalized *O*-ketopyranosyl calix[4]arenes.

Concerned about the limitations imposed upon these calixarene-based carbohydrate clusters by the potentially acid labile glycosidic bonds linking the carbohydrate units to the macrocycle, Don-doni and co-workers (47) have developed another synthetic route in which the carbohydrate appendages are linked to the calixarene core by carbon–carbon bonds. Reaction (Scheme 16) of the diacetonegalactose phosphonium salt **69** with BuLi forms the corresponding phosphorane, which is then reacted with the tetraformylcalix[4]arene **70** to afford, after hydrogenation of the olefins, the tris- and tetragalactosides **71** and **72**, respectively, in a 1:1 ratio. Removal of the protecting groups in **72** by acid hydrolysis affords the free

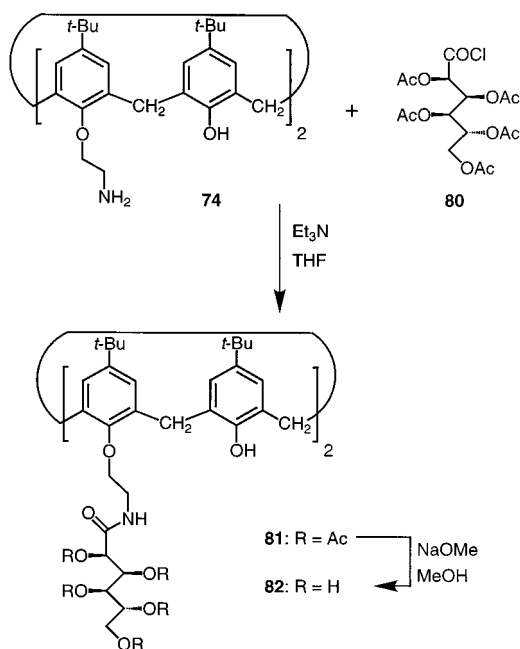
Scheme 18. The Use of Suzuki Cross-Coupling Reactions To Prepare a Di- β -glucosyl Calix[4]arene

carbohydrate cluster **73**, which, however, is not soluble in water—further highlighting the problems of trying to modify calixarenes with appended carbohydrates to induce water solubility. Ribose analogues of these galactosyl clusters were also synthesized by the Italian group.

Santoyo-González and co-workers (48) have used the much tried-and-tested thiourea forming reaction for the attachment of carbohydrate residues to the lower rim of calixarenes. Reaction (Scheme 17) of the diamino-calix[4]arene **74** with the glucosyl isothiocyanate **34** affords, after deprotection, the bisglucosylthiourea-calix[4]arene **75**. This simple synthesis has been extended to the α -D-mannosyl, β -D-galactosyl, and D-lactosyl derivatives as well. No comments were made on the water-solubility of these particular carbohydrate clusters.

Another interesting route to calixarene-based carbohydrate clusters has been reported by Parrot-Lopez and co-workers (49). It relies upon Suzuki-type coupling reactions to attach the carbohydrate units to the upper

Scheme 19. Reaction of 2,3,4,5,6-Pentaacetyl-D-gluconyl Chloride **80 with the Diaminocalix[4]arene **74** To Afford, after Deacetylation, the Disubstituted Cluster **82****



rim of the calixarene scaffold and, at the same time, increase the cavity size of the calixarene. The diboronic acid calix[4]arene **77** can be prepared (Scheme 18) from the dibromide **76** by reaction with $n\text{-BuLi}$ and $\text{B}(\text{OMe})_3$ in THF. Cross-coupling reaction of **77** (2 equiv) with the 4-bromophenyl glycoside **78** using $\text{Pd}(\text{PPh}_3)_4$ as the catalyst afforded the bisglycosylated product **79** in 32% yield after deprotection. Removal of the acetate protecting groups proved to be troublesome, resulting in a yield of only 11% in the deprotection step. The same research group have also developed a "one-pot" synthesis of an analogous calix[4]arene, bearing one maltosyl unit. The water-solubilities of the calixarene-based carbohydrate clusters prepared in this study have not been discussed by the authors.

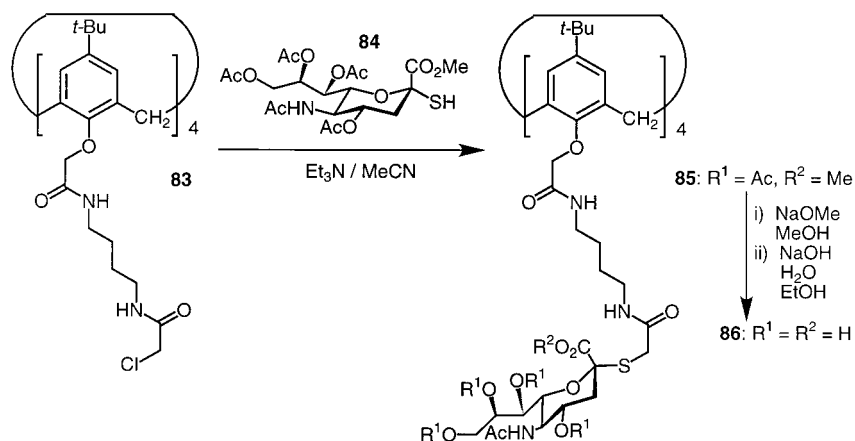
Lhoták and co-workers (50) have prepared some calixarene-based carbohydrate clusters with the aim of developing new molecular receptors, which, they claim, could have interesting properties on account of their chiral polyhydroxylated carbohydrate appendages. Their initial attempts to attach carbohydrate units to the lower rim of calix[4]arenes through amide bond formation

proved successful. Removal of the carbohydrate protecting groups, however, was not so easy. To circumvent this problem, they tried an alternative synthesis. 2,3,4,5,6-Penta-O-acetyl-D-gluconyl chloride **80**, which was prepared from sodium gluconate in two steps, is reacted (Scheme 19) with **74** to afford the protected compound **81** with two amide bonds in a 49% yield. Deacetylation (NaOMe/MeOH) afforded the deprotected compound **82** in an almost quantitative yield. Interestingly, **82** was found to dimerize in solution ($K_{\text{dimerization}} = 6300 \text{ M}^{-1}$ in CDCl_3), a process which presumably is occurring through intermolecular hydrogen bonding between carbohydrate residues. The complexing ability of **82** was investigated in CDCl_3 using 1-O-octyl- α -D-glucopyranoside ($K_a = 650 \text{ M}^{-1}$), 1-O-octyl- β -D-glucopyranoside ($K_a = 1050 \text{ M}^{-1}$), and 1-S-octyl- β -D-glucopyranoside ($K_a = 1000 \text{ M}^{-1}$) as guest molecules. Job plots indicated that 1:1 binding occurs between these guests and the host, although the dimerization of **82** must hamper a precise evaluation of the binding data presented. The authors are of the opinion that these binding constants are similar enough to discount any selectivity of the host toward these guests.

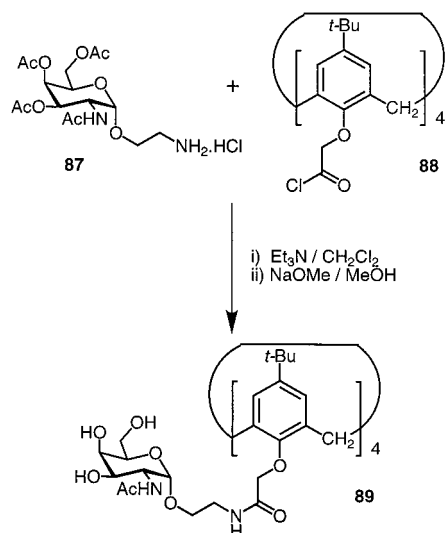
Roy and co-workers have also developed methods for the preparation of calixarene-based carbohydrate clusters. Realizing the lack of a suitable spacer arm between the calixarene scaffold and the carbohydrate appendages, and poor water solubility, in the calixarene-based clusters described so far, the Canadian group have designed compounds which circumvent these problems. In their initial communication (51), the Canadian group synthesized, in six steps, the calix[4]arene derivative **83**, which contains four spacer arms linked to the phenolic oxygen atoms of the calix[4]arenes' lower rim. The *N*-chloroacetyl groups present in **83** have been shown in Roy's laboratory to act as efficient substrates for the attachment of thiolated carbohydrates. Recalling their previous success in the synthesis of sialoside-coated glycodendrimers, they chose to attach α -thiolated sialosides onto the calixarene scaffold. Reaction (Scheme 20) of the thiol **84** with **83** afforded the protected cluster compound **85** in 65% yield. ^1H NMR spectroscopic evidence supports the hypothesis that the calixarene was completely substituted with four sialoside units. Deprotection of **85** afforded the final compound **86** in a quantitative yield. Fortunately, **86** was found to be fairly soluble in water (3 mg/mL). A turbidimetric analysis was performed, which indicated that **86** has a strong cross-linking ability with the lectin, wheat germ agglutinin.

The amphiphilic nature of calixarene-based carbohydrate clusters has been a hindrance to researchers in this

Scheme 20. Preparation of a Calix[4]arene-Based Tetrasialic Acid Cluster

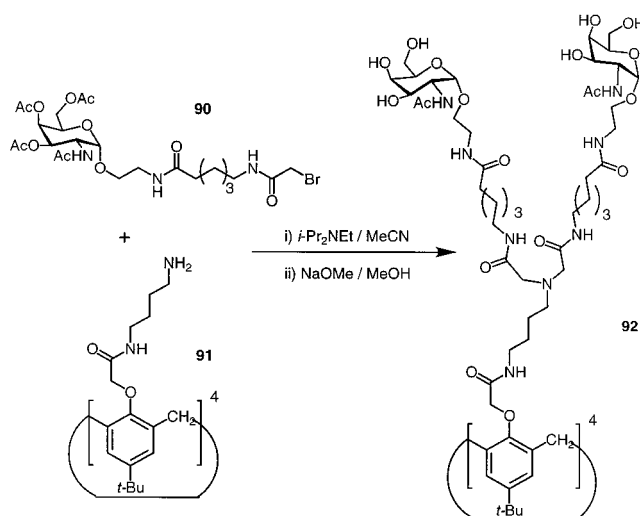


Scheme 21. Attachment of Four α -N-Acetylgalactosyl Residues to the Lower Rim of a Tetraacid Chloride Calix[4]arene To Form the Tetra- α -N-acetylgalactosyl Cluster

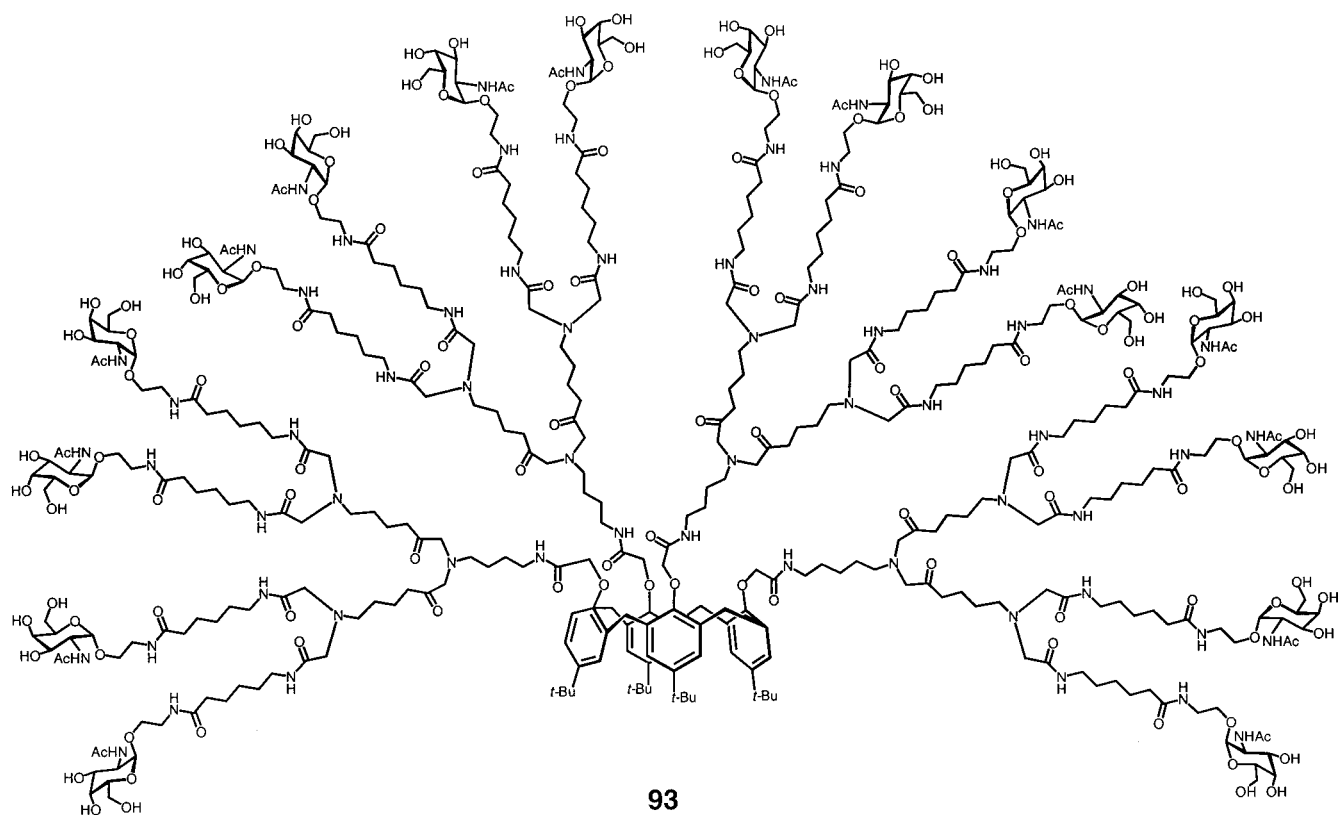


field since it confers upon these compounds, at the very best, poor water solubility, and, more often than not, no solubility in water at all. However, Roy and co-workers (52) have taken advantage of the amphiphilic nature of calixarene-based carbohydrate clusters to prepare a new series of compounds which, on account of their hydrophobic upper rims, can be adsorbed onto the hydrophobic well surfaces in polystyrene microtiter plates. This observation means that amphiphilic carbohydrate cluster compounds of this particular design can be used as antigens in ELISA-type biological assays. The model carbohydrate **87**, chosen in this study, was based on the T_N antigen (GalNAc α 1 $\rightarrow$ O-Ser/Thr) without the O-Ser/Thr aglycon. The T_N antigen corresponds to one of the

Scheme 22. Preparation of an Octa- α -N-acetylgalactosyl Cluster



immunodominant epitopes found in human adenocarcinoma mucins—a class of large, heavily glycosylated glycoproteins associated with some cancerous tumors. Reaction (Scheme 21) of **87** with the readily prepared tetrachlorocalix[4]arene **88** afforded after deprotection, the tetravalent cluster **89**. A semiconvergent approach was then used to synthesize calixarenes with higher valencies. Reaction (Scheme 22) of the bromide **90**—prepared in four synthetic steps from **87**—with the extended calixarene core **91** yields the dendritic octameric cluster **92**. The synthesis has been expanded in an impressive manner to afford the hexadecamer cluster **93**. In all cases, the homogeneities of the cluster compounds were demonstrated by mass spectrometry or by NMR spectroscopy—a technique which was also used to verify that the compounds exist with the calixarene moieties



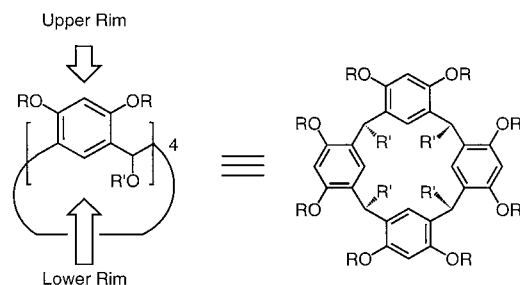


Figure 3. Condensed structural formula for the calix[4]-resorcarenes with the upper and lower rims of the bowl-shaped macrocycle indicated. A complete structural representation of a calix[4]resorcarene is also shown.

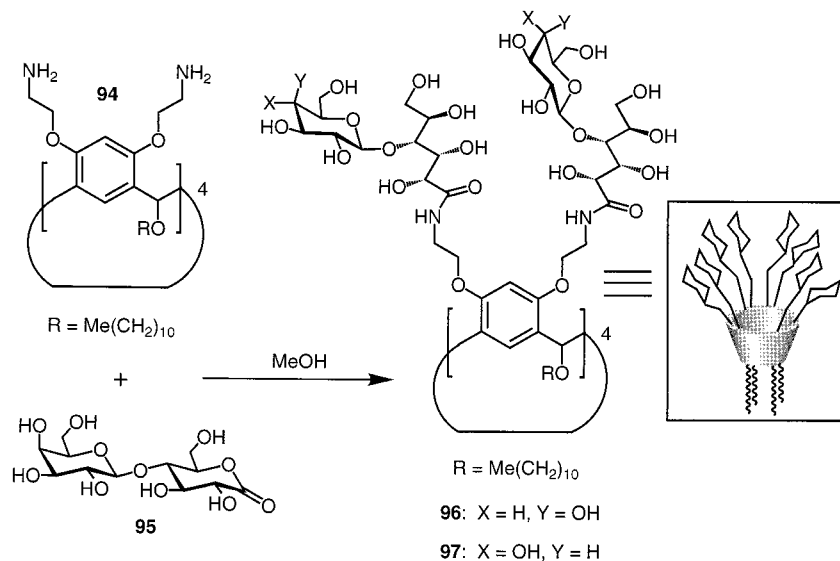
in their cone conformations. The relative lectin-binding properties of **89**, **92**, and **93** were measured with the lectin *Vicia villosa* agglutinin (VVA) which is known to bind α -D-GalNAc derivatives. In a solid-phase competitive inhibition experiment, compound **93** was found to be a very effective inhibitor ($IC_{50} = 13.4 \mu M$ —cf., a monovalent analogue with $IC_{50} = 158.3 \mu M$) of the binding of VVA with asialoglycophorin, which is a natural human blood group serotype.

Calixresorcarenes Appended with Carbohydrates.

Calixresorcarenes are a subdivision of calixarenes. Since the body of work concerning calixresorcarene-based carbohydrate clusters is unique, however, it merits having a separate subsection devoted to it. Calixresorcarenes are cyclo-oligomers (Figure 3) formed from the condensation of resorcinol with aldehydes to form macrocycles with comparable structural and host–guest properties to those of calixarenes. Calixresorcarenes exhibit similar binding and conformational properties to those of calixarenes. Consequently, they have been intensely studied (45, 53) receptors. Calix[4]resorcarenes possess eight resorcinic hydroxyl groups located on their upper rims. They serve as useful handles for the further chemical modification of these receptors. Additionally, they possess four “feet” attached to their lower rims. These feet are determined by the choice of aldehyde utilized in the synthesis of the receptor. These structural features, among others, make calixresorcarenes a useful and readily available choice of receptor for further elaboration with carbohydrate appendages.

Researchers led by Yasuhiro Aoyama have been the pioneers in the study of resorcarene-based carbohydrate clusters. In their first communication (54), this Japanese group reported the synthesis of an octagalactosyl calix-resorcarene-based cluster by reaction (Scheme 23) of the octaamine **94** with lactonolactone (**95**) to afford the galactosyl cluster **96** in a 79% yield. This compound—despite its amphiphilic nature—is soluble in water ($>0.1 M$). Dynamic light scattering experiments indicate that, in aqueous solution, it is monomeric at concentrations of $0.1 mM$, while it aggregates at concentrations of $\sim 1 mM$. When a quartz plate was dipped into an aqueous solution of **96**, electronic adsorption spectroscopy indicated that the galactosyl cluster was adsorbed onto its surface with a packing density of $3.5 nm^2$ per molecule. Additionally, it was also found that compound **96** cannot be rinsed off the plate by repeated washings with water: bases, pyridine, or amines, such as ethanolamine, are required in order to desorb the galactosyl cluster from the quartz plate. These results, supported by other experiments, indicate that compound **96** must be binding to the quartz surface through hydrogen bonding between the hydroxyl functions in the cluster and the silanol and silyl ether functionalities of the quartz surface. Another additional important experimental observation is that galactose and other carbohydrate compounds—when present in a large excess—cannot either inhibit the binding of the galactosyl cluster **96** to the quartz surface, or promote its desorption from the quartz surface. This result indicates that compound **96** must be binding to the quartz surface in a multivalent fashion, i.e., there exists a highly cooperative binding interaction between the hydroxy functions of the carbohydrate appendages of **96** and the many hydrogen bond donor/acceptor sites present on the quartz surface, resulting in the strong binding of the galactosyl cluster to the quartz surface. Additionally, compound **96** was found to form a stable 1:1 complex ($K_a = 2.2 \times 10^5 M^{-1}$) in aqueous solution with 8-anilidonaphthalene-1-sulfonate (ANS) which is located within the resorcarene cavity of **96**. Although ANS is not readily adsorbed onto a quartz surface, it is adsorbed onto this surface in the presence of compound **96**. Experiments have indicated that there is a 1:1 ratio of both ANS and **96** present on the surface. This evidence suggests that

Scheme 23. Formation of an Octagalactosyl Calix[4]resorcarene-Based Cluster **96** from the Octaamino Calix[4]resorcarene **94** and Lactonolactone **95**. Structural Formula for the Glucosyl Derivative (**97**) of **96** Is Also Shown, as Is the Cartoon Representation of Both of These Cluster Compounds



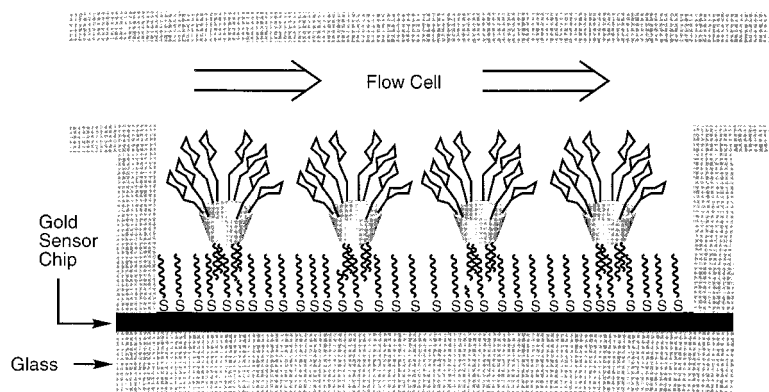


Figure 4. Schematic representation of the galactosyl cluster **96**, immobilized on a hydrophobic surface-plasmon-resonance (SPR) chip. When galactose-binding lectins are passed through the flow cell, they can bind to the immobilized **96**, inducing a refractive index change of the SPR chip.

compound **96** acts as a molecular delivery vehicle and has the ability to deliver guest molecules, with little or no affinity for a quartz surface, onto that same surface. However, since it is possible to remove the guest from the quartz surface by repeated washings, it is likely that the guest molecule is not completely encapsulated between the adsorbed galactosyl cluster and the surface.

The hydrophobicity of the alkyl chains present in the galactosyl cluster has been used (55) to form (Figure 4) a monolayer of compound **96** on a hydrophobic SPR sensor chip. Thus, the interaction of **96** with various compounds, such as lectins and other proteins, can be detected when a solution of these compounds is passed over the **96**-immobilized SPR chip. A strong interaction was detected between the galactosyl cluster and peanut agglutinin (PNA), a lectin which selectively binds galactosyl residues. A very weak interaction was, however, detected between the cluster and Con A, a lectin which selectively binds both mannosyl and glucosyl residues. Conversely, the analogous glucosyl cluster **97** was found to bind Con A but not PNA. The interaction of the clusters **96** and **97** with various monolayers has also been studied (56) using a quartz crystal microbalance. From this work, it has been reported that the clusters have a more favorable interaction with anionic and carbohydrate-based monolayers compared with cationic, zwitterionic, and nonionic monolayers.

Aoyama and co-workers (57) have also shown how these cluster compounds can be used to deliver guests to an immobilized Con A-Sepharose gel, purely as a consequence of carbohydrate-lectin interactions. Compound **96** and its glucosyl analogue **97** both form 1:1 complexes with the dye, eosin Y (**98**) with association constants of $K_a = 7.5 \times 10^5 \text{ M}^{-1}$ and $K_a = 1.8 \times 10^5 \text{ M}^{-1}$, respectively. In a simple series of experiments, the Japanese group have shown (Figure 5) that only the glucosyl cluster **97** can direct the adsorption of the guest dye **98** onto the surface of the Con A-Sepharose gel whereas the galactosyl cluster **96** cannot. Although, in reality, there is a certain amount of nonspecific binding interactions between the clusters and guest with the sepharose, the carbohydrate-lectin mediated binding is sufficiently strong to allow for a significant percentage of the guest in solution to be delivered to the Con A-Sepharose gel surface.

The Fukuoka group (58) have taken the concept of carbohydrate-directed delivery one step further and, in a most impressive piece of work, they have shown how their calixresorcurene-based carbohydrate clusters can be used to deliver guest molecules to cells. Although

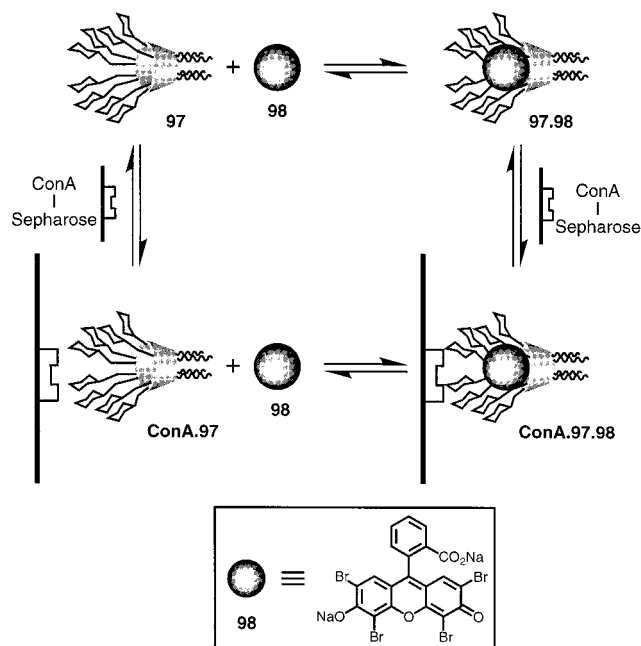
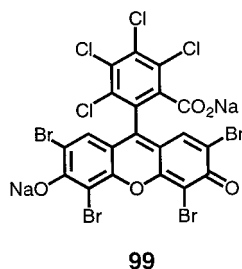


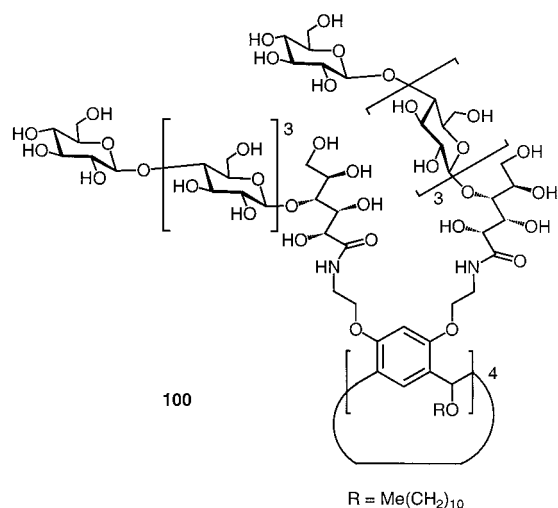
Figure 5. Schematic representation of the use of the glucosyl cluster **97** to deliver the dye eosin Y **98** to the surface of sepharose immobilized Con A. The **97·98** complex forms a ternary complex with the Con A-immobilized on the sepharose. However, as the eosin Y is not completely encapsulated in the ternary complex, it can dissociate from the ternary complex, leaving the ConA·**97** complex.

carbohydrate-cell surface protein interactions are very specific, cells can also show high nonspecific affinities toward hydrophobic molecules. This problem is one which must be overcome if carbohydrate-protein interactions are to be the sole driving force for a delivery system. The Japanese group chose to study molecular delivery to hepatocyte cells (liver cells), which are known to contain a cell surface lectin—the asialoglycoprotein receptor—which specifically binds terminal galactose residues. As potential delivery vehicles, they chose the galactosyl and glucosyl clusters **96** and **97**, and as a suitable guest molecule, the fluorescent dye phloxine B (**99**). Both clusters were shown to form 1:1 complexes with **99** in phosphate-buffered saline solution with association constants of $2.0 \times 10^5 \text{ M}^{-1}$ and $2.1 \times 10^5 \text{ M}^{-1}$, respectively, for **96** and **97**. When a solution of the dye **99** was added to rat hepatoma (liver cancer) cells, these cells were found to fluoresce when viewed under a fluorescence microscope, indicating that the dye has been captured by the

cell as a consequence of nonspecific binding interactions. However, when a solution of **99** and the glucosyl cluster

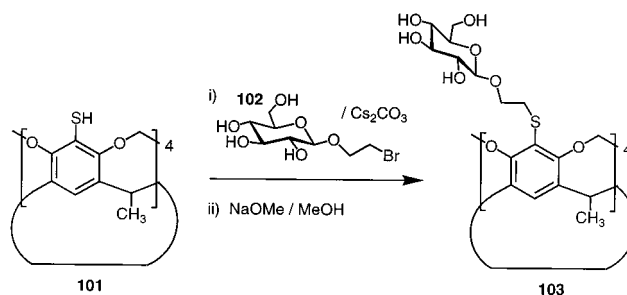


97 is added to the cell (at a concentration where $\sim 100\%$ of the guest is bound by **97**), no fluorescence is detected from the cells, indicating that (1) the dye is so strongly bound by **97** that it is not available for nonspecific binding with the cell, and (2) that the glucosyl cluster is also not bound by the cell in a nonspecific manner. This hypothesis is further supported by the fact that the **96**·**99** complex is bound by the cells as a consequence of the interaction between the terminal galactosyl residues present in the cluster **96** and the asialoglycoprotein receptor on the cell surfaces, causing the cells to fluoresce when viewed under the fluorescence microscope. If mouse spleen cells—cells which lack the asialoglycoprotein receptor—are used in the experiment, then the galactosyl cluster **96** cannot deliver the dye **98** to the cells. Thus, it can be concluded that (1) the clusters **96** and **97** do not bind with the cells through nonspecific interactions, and that (2) the cluster **96** can bind with the hepatocyte cells through interaction with the cell-surface asialoglycoprotein receptor. This work highlights the important role that carbohydrates can play in the masking of hydrophobicity—in compounds such as **96**, it is only because its carbohydrate moieties mask the hydrophobicity of the calixresorcylene core sufficiently well that the carbohydrate-directed delivery of the guest dye to the cell surface is allowed to occur.



Compounds such as **96**, and its larger analogous cluster **100**, which contains 40 glucopyranosyl residues, have been shown (59) to bind various phosphate anions in water. Compound **96** also displays an interesting anion-induced agglutination. Phosphate salts such as $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, D-ribose-5-phosphate and guanosine-5'-monophosphate induce the agglutination of **96**. ^{31}P NMR Spectroscopy was used to estimate the ratio of host:bound salt. In the same investigation, dynamic light scattering

Scheme 24. Formation of a Tetraglucosyl Cavitand-Based Cluster



has been utilized to demonstrate that Na_2HPO_4 can induce **96** to form aggregates which can grow up to μM -sized particles in a matter of minutes. This aggregation phenomena is almost certainly a consequence of hydrogen bonding interactions between the polyhydroxylated carbohydrate appendages of the cluster compounds and the phosphate anions, which, on account of their ability to interact with more than one cluster molecule at a time, can act as a supramolecular “glue”, sticking the clusters together. The Fukuoka group propose that the extended polyhydroxyl appendages in compounds such as **96** and **100** can act as a macrosolvent for anions, allowing these salts to be extracted from water into the unique environment of the cluster compounds.

Another carbohydrate cluster compound prepared by Aoyama and co-workers (60) has been constructed on a cavitand, i.e., a bowl-shaped macrocycle derived from calixresorcarenes. Reaction (Scheme 24) of the tetrathiol cavitand **101** with the bromide **102** affords, after deprotection, the tetraglucose cluster **103**. Maltosyl and maltotriosyl analogues of **103** have been prepared in a similar manner. These three cluster compounds were shown to bind ANS and found to interact with the lectin Con A.

CONCLUSIONS

The field of neoglycoconjugates based on cyclodextrin and calixarene cores is—at least as judged by the sheer number of recent publications—a blossoming one, with many challenges and opportunities available to researchers who can assume multidisciplinary mantles. To date, much effort has been directed toward the syntheses of neoglycoconjugates in feats which are by no means trivial—especially when recognition is granted to synthetic chemists for doing the notoriously difficult synthetic chemistry that surrounds CDs and calixarenes. It appears, however, that the synthetic problems associated with preparing these neoglycoconjugates have, to a large extent, been successfully overcome, thanks to the many varied and ingenious methods of syntheses now available to the talented synthetic chemist. By contrast, investigations of the molecular recognition properties of these neoglycoconjugates, and particularly their abilities to interact with lectins and cells, both in vitro and in vivo, have been few and far between. Thus, it seems obvious that, if the field of CD- and calixarene-based neoglycoconjugates is to continue to blossom, the emphasis in research must now be directed toward the physical and, most importantly of all, the biological evaluations of these compounds. Only when the biological chemistry is as well understood as the synthetic chemistry will the potential of this unique class of bioconjugates be truly realized and appreciated. The rewards for researchers who are willing and able to tackle real scientific issues surrounding

neoglycoconjugates in a multidisciplinary fashion are immense to say the very least.

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COMMUNICATIONS

Synthesis and Terminal Functionalization of a Polymerizable Phosphatidylethanolamine

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We report the design and synthesis of bifunctional phospholipid conjugates, which contain a polymerizable acrylate group and a terminal linker, such as biotin or *N*-(ϵ -maleimidocaproyl) (EMC), to facilitate bioconjugation reactions. The lipid conjugate can be used to generate a multifunctional substrate-supported phospholipid film that is further stabilized via in-situ photocopolymerization.

INTRODUCTION

The generation of model lipid films that mimic cell surfaces have attracted considerable attention due to their potential as tools to probe intermolecular interactions and as bioactive coatings for sensor or medical implant applications (1, 2). Specifically, the fabrication of supported lipid membranes provides a practical method for the immobilization of transmembrane proteins, including those that serve as receptors, channels, or pores, as well as the incorporation of native or synthetic lipopeptides or glycolipids. Typically, phospholipids differing in chemical composition, saturation, and size have been utilized as the primary building blocks of the film structure (3–6). In the past two decades, lipids that contain a polymerizable group at the polar head (7–8), midchain (9), or chain terminus (6, 10–13) have been reported as a mechanism to stabilize vesicles, as well as other lipid assemblies.

We have previously reported a method to prepare stable, substrate-supported phospholipid films via in-situ photopolymerization of an acrylate functionalized phosphatidylcholine (mono-AcrylPC, **1**)¹ assembly (14–16). In the current study, we designed a novel polymerizable lipid, acrylate-functionalized phosphatidylethanolamine (mono-AcrylPE, **2**), in which the amino function can serve as a handle for further modifications. As shown in Figure 1, terminal groups, such as biotinyl and *N*-(ϵ -maleimidocaproyl) (EMC) were introduced by acylation of the amine

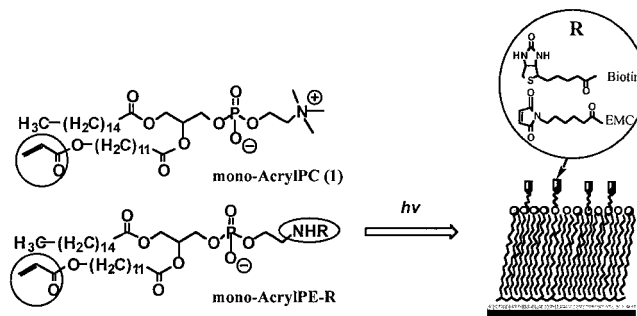


Figure 1. Model of a supported lipid assembly containing bifunctional phospholipid conjugates.

group of phosphatidylethanolamine. These linkers facilitate the incorporation of proteins or other target molecules via specific high affinity (biotin) interaction (17–20) or by covalent (EMC) attachment (21, 22). An example is also provided, in the case of generating a polymerizable lipid conjugate containing a fluorescent dye, which provides a mechanism for direct detection of the lipid membrane (18, 23–25). It is likely that a polymerized thin film composed of both mono-AcrylPC and mono-AcrylPE will be associated with enhanced protein binding efficiency due to inherently greater stability, compared with 2-D planar lipid assemblies that are stabilized solely by noncovalent interactions (14, 26, 27). Herein, we report the synthesis, characterization, and terminal functionalization of mono-AcrylPE (**2**).

EXPERIMENTAL PROCEDURES

General Methods. TLC was performed on Whatman silica gel aluminum-backed plates (F254, 250 μ m thickness) and detected by fluorescence quenching, sulfuric acid (10 vol % in methanol), or phosphomolybdic acid (20 wt % in ethanol). Column chromatography was performed on silica gel (Fisher Chemical, 200–425 mesh). ¹H and ¹³C NMR spectra were recorded at 300 and 75 MHz (Varian INOVA) in CDCl₃, CD₃OD (internal Me₄Si, δ = 0). Mass spectra (EI, FAB) were measured with a JEOL

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¹ Abbreviations: mono-AcrylPC, acrylate functionalized phosphatidylcholine; mono-AcrylPE, acrylate functionalized phosphatidylethanolamine; DCC, dicyclohexylcarbodiimide; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; DMAP, *N,N*-(dimethylamino)pyridine; EY, eosin Y; FITC, fluorescein isothiocyanate; NHS-Biotin, *N*-hydroxysuccinimidobiotin; EMC, *N*-(ϵ -maleimidocaproyl); EMCS, *N*-(ϵ -maleimidocaproyl)succinimide; PMB, *p*-methoxybenzyl.

JMS-SX 102/SX102A/E mass spectrometer. Confocal microscopy studies were performed on a Zeiss LSM510 Laser Confocal Microscope (Carl Zeiss, Inc., Germany) equipped with external argon (for excitation at 458, 488, and 514 nm), HeNe1 (for excitation at 543 nm), and HeNe2 (for excitation at 633 nm) lasers.

Material. Mono-AcryIPC (**1**) was prepared as described by Mara et al. (14). 3-*O*-(4-Methoxybenzyl)-*sn*-glycerol (**3**) was prepared from (+)-1,2-*O*-isopropylidene-glycerol according to the procedure described by Hebert et al (30).

1-*O*-Palmitoyl-3-*O*-(4-methoxybenzyl)-*sn*-glycerol (4**).** A solution of dicyclohexylcarbodiimide (3.7 g, 18.0 mmol) in dichloromethane (10 mL) was added dropwise over a period of 45 min at 0 °C to a solution of 3-*O*-(4-methoxybenzyl)-*sn*-glycerol (**3**) (2.0 g, 9.4 mmol), palmitic acid (2.7 g, 10.4 mmol), and *N,N*-(dimethylamino)pyridine (0.06 g, 0.5 mmol) in dichloromethane (60 mL). The reaction mixture was stirred for 18 h at room temperature under Ar atmosphere. Dicyclohexylurea was removed by filtering through Celite, and the filtrate was evaporated to give a residue, which was purified by column chromatography (SiO₂) using ethyl acetate-*n*-hexane (1:3) as eluent to afford **4** (3.0 g, 71%). ¹H NMR (CDCl₃) δ: 7.24 (d, 2 H, *J* = 9.0 Hz, Ph), 6.88 (d, 2 H, *J* = 9.0 Hz, Ph), 4.48 (s, 2 H, CH₂Ph), 4.14 (m, 1 H, CH), 4.00 (m, 1 H, CH), 3.80 (s, 3 H, OCH₃), 3.51–3.46 (m, 2 H, CH₂), 2.50 (d, 1 H, *J* = 5.1 Hz, OH), 2.31 (t, 2 H, *J* = 7.5 Hz, CH₂CO), 1.62–1.60 (m, 2 H, CH₂), 1.25 (br s, 24 H, CH₂ × 12), 0.87 (t, 3 H, *J* = 7.2 Hz, CH₃). HR-MS (EI) calcd for C₂₇H₄₆O₅: 450.33453; observed 450.33331 *m/z*.

1-*O*-Palmitoyl-2-*O*-(12-acryloyloxy)dodecanoyl-3-*O*-(4-methoxybenzyl)-*sn*-glycerol (5**).** A solution of dicyclohexylcarbodiimide (3.0 g, 13.4 mmol) in dichloromethane (10 mL) was added dropwise over a period of 20 min at room temperature to a solution of **4** (2.5 g, 5.5 mmol), 12-acryloyloxy-1-dodecanoic acid (1.67 g, 6.7 mmol), and *N,N*-(dimethylamino)pyridine (0.06 g, 0.45 mmol) in dichloromethane (30 mL). The reaction mixture was stirred for 18 h at room temperature under Ar atmosphere. Dicyclohexylurea was removed by filtering through Celite, and the filtrate was evaporated to give a residue, which was purified by column chromatography (SiO₂) using ethyl acetate-*n*-hexane (1:4) as eluent to afford **5** (3.0 g, 71%). ¹H NMR (CDCl₃) δ: 7.24 (d, 2 H, *J* = 9.0 Hz, Ph), 6.88 (d, 2 H, *J* = 9.0 Hz, Ph), 6.38 (dd, 1 H, *J* = 17.0, 1.5 Hz, CH=CH₂), 6.11 (dd, 1 H, *J* = 17.0, 10.5 Hz, CH=CH₂), 5.80 (dd, 1 H, *J* = 1.5, 10.5 Hz, CH=CH₂), 5.21 (m, 1 H, CH-2), 4.48 (d, 1 H, *J* = 11.7 Hz, CH₂Ph), 4.42 (d, 1 H, *J* = 11.7 Hz, CH₂Ph), 4.31 (dd, 1 H, *J* = 3.9, 12.0 Hz), 4.16 (dd, 1 H, *J* = 6.0, 9.9 Hz), 3.78 (s, 3 H, OCH₃), 3.54 (d, 2 H, *J* = 5.1 Hz), 2.33–2.24 (m, 4 H, CH₂CO × 2), 1.67–1.55 (m, 6 H, CH₂ × 3), 1.24 (br s, 38 H, CH₂ × 19), 0.89 (t, 3 H, *J* = 6.6 Hz, CH₃). ¹³C NMR (CDCl₃) δ: 173.94, 173.60, 166.61, 130.66, 128.81, 72.26, 64.89, 62.35, 61.52, 53.89, 34.55, 34.45, 34.28, 32.11, 29.88, 29.66, 29.60, 29.55, 29.43, 29.31, 28.77, 26.10, 25.07, 22.30, 14.30. HR-MS (EI) calcd for C₄₂H₇₀O₈-Li: 709.5231; observed 709.5211 *m/z*.

1-*O*-Palmitoyl-2-*O*-(12-acryloyloxy)dodecanoyl-*sn*-glycerol (6**).** To a solution of **5** (2.5 g, 4.2 mmol) in dichloromethane/water (18:1, 20 mL) was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (1.04 g, 4.6 mmol). The reaction mixture was stirred for 16 h at room temperature under Ar atmosphere. The reaction was quenched with NaHCO₃ and extracted with chloroform (20 mL × 3). The combined organic portion was dried over anhydrous Na₂SO₄ and the solvent evaporated to give a residue, which was purified by column chromatography

(SiO₂) using ethyl acetate-*n*-hexane (1:3) as eluent to afford **6** (1.4 g, 55%). ¹H NMR (CDCl₃) δ: 6.39 (dd, 1 H, *J* = 17.0, 1.2 Hz, CH=CH₂), 6.11 (dd, 1 H, *J* = 17.0, 10.8 Hz, CH=CH₂), 5.82 (dd, 1 H, *J* = 1.2, 10.8 Hz, CH=CH₂), 5.07 (m, 1 H, CH-2), 4.31 (dd, 1 H, *J* = 4.2, 12.0 Hz), 4.26 (dd, 1 H, *J* = 4.2, 13.2 Hz), 4.13 (t, 2 H, *J* = 6.9 Hz, CH₂O), 3.73 (t, 2 H, *J* = 4.2 Hz CH₂OH), 2.37–2.23 (m, 4 H, CH₂CO × 2), 1.67–1.60 (m, 6 H, CH₂ × 3), 1.24 (br s, 38 H, CH₂ × 19), 0.87 (t, 3 H, *J* = 7.2 Hz, CH₃). HR-MS (EI) calcd for C₃₄H₆₂O₇Li: 590.0035; observed 590.0021 *m/z*.

Trichloroethoxy 1-*O*-Palmitoyl-2-*O*-(12-acryloyloxy)dodecanoyl-*sn*-glycero-3-phosphoethylcarbamate (7**).** To an ice-cooled solution of dichloro(*N*-β,β,β-trichloroethoxycarbonyl-2-aminoethyl)phosphate (1.5 g, 4.4 mmol) in dichloromethane (15 mL) was added dropwise a solution of **6** (1.2 g, 2.1 mmol) and triethylamine (1.8 mL, 13.5 mmol) in dichloromethane (10 mL). The reaction mixture was stirred for 16 h at room temperature under Ar atmosphere and then washed with water (10 mL × 3). The organic portion was dried over anhydrous Na₂SO₄ and the solvent evaporated to give **7** as a light brown syrup (1.36 g, 71%), which showed high purity in ¹H NMR determination and was molybdenum blue positive on TLC (*R*_f = 0.8 CHCl₃/MeOH/H₂O, 65:25:1). Thus, this compound was used for the next reaction without further purification.

1-*O*-Palmitoyl-2-*O*-(12-acryloyloxy)dodecanoyl-*sn*-glycero-3-phosphoethanolamine (2**).** To a solution of crude **7** (1.0 g, 1.0 mmol) in acetic acid (10 mL) was added zinc dust (2.0 g). The reaction mixture was stirred for 20 h at room temperature. The reaction mixture was diluted with diethyl ether (30 mL) and filtered through Celite. The filtrate was washed with water and aqueous NaHCO₃ and dried over anhydrous Na₂SO₄. The solvent was then evaporated to give a residue, which was purified by column chromatography (SiO₂) using chloroform/methanol/water (65:25:4) as eluent to afford **2** (0.462 g, 62%). ¹H NMR (CDCl₃) δ: 8.51 (br, 3 H, NH₃⁺), 6.39 (dd, 1 H, *J* = 17.4, 1.0 Hz, CH=CH₂), 6.11 (dd, 1 H, *J* = 17.4, 10.5 Hz, CH=CH₂), 5.82 (dd, 1 H, *J* = 1.0, 10.5 Hz, CH=CH₂), 5.21 (m, 1 H, CH-2), 4.37 (dd, 1 H, *J* = 3.0, 12.3 Hz), 4.14 (t, 2 H, *J* = 6.6 Hz, CH₂O), 4.14–4.05 (m, 3 H), 3.94 (br, 2H, CH₂O), 3.16 (br, 2 H, CH₂N), 2.33–2.26 (m, 4 H, CH₂CO × 2), 1.68–1.57 (m, 6 H, CH₂ × 3), 1.24 (br s, 38 H, CH₂ × 19), 0.87 (t, 3 H, *J* = 6.6 Hz, CH₃). ¹³C NMR (CDCl₃) δ: 173.59, 173.29, 166.48, 130.59, 128.83, 70.50, 64.86, 64.63, 64.09, 62.75, 62.44, 40.61, 34.44, 34.26, 32.11, 29.50, 29.41, 28.81, 27.79, 26.14, 25.13, 25.07, 22.87, 14.30. FABMS (*m/z*): 728.6 [M + Na]⁺.

mono-AcrylPE-FITC (8**).** To a solution of **2** (50 mg, 0.071 mmol) in DMF (5 mL) was added triethylamine (0.1 mL, 0.71 mmol). After the solution was stirred for 30 min, a solution of fluorescent isothiocyanate (55 mg, 0.142 mmol) was added. The reaction mixture was stirred for 12 h at room temperature in the dark and then concentrated in a vacuum to give a residue, which was purified by column chromatography (SiO₂) using chloroform/methanol (4:1) as eluent to afford **8** (26 mg, 67%). ¹H NMR (CDCl₃) δ: 8.25 (br, 1 H, Ph), 7.25 (br, 1 H, Ph), 7.16–7.08 (m, 3 H, Ph), 6.68 (m, 4 H, Ph), 6.59 (dd, 1 H, *J* = 15.6, 1.5 Hz, CH=CH₂), 6.09 (dd, 1 H, *J* = 15.6, 10.5 Hz, CH=CH₂), 5.82 (dd, 1 H, *J* = 1.5, 10.5 Hz, CH=CH₂), 5.17 (m, 1 H, CH-2), 4.34 (dd, 1 H, *J* = 3.0, 12.3 Hz), 4.11 (t, 2 H, *J* = 6.6 Hz, CH₂O), 4.13–4.02 (m, 3 H), 3.94 (br, 2H, CH₂O), 3.36 (br, 2 H, CH₂N), 2.27–2.23 (m, 4 H, CH₂CO × 2), 1.66–1.54 (m, 6 H, CH₂ × 3), 1.24 (br s, 38 H, CH₂ × 19), 0.83 (t, 3 H, *J* = 6.6 Hz, CH₃). FABMS (*m/z*): 1096.2 [M + 1]⁺.

mono-AcrylPE-Biotin (9). To a solution of **2** (50 mg, 0.071 mmol) in DMF (5 mL) was added triethylamine (0.1 mL, 0.71 mmol). After the solution was stirred for 30 min, a solution of *N*-hydroxysuccinimidobiotin (36 mg, 0.11 mmol) was added. The reaction mixture was stirred for 24 h at room temperature and then concentrated in a vacuum to give a residue, which was purified by a Sephadex LH-20 column using methanol as eluent to afford **9** (17 mg, 53%). ¹H NMR (CDCl₃-CD₃OD) δ: 7.79 (br, 1 H, NH), 7.00 (br, 1 H, NH), 6.38 (dd, 1 H, *J* = 17.1, 1.0 Hz, CH=CH₂), 6.10 (dd, 1 H, *J* = 17.1, 10.5 Hz, CH=CH₂), 5.80 (dd, 1 H, *J* = 1.0, 10.5 Hz, CH=CH₂), 5.21 (m, 1 H, CH-2), 4.48 (m, 1 H), 4.35 (dd, 1 H, *J* = 3.0, 12.3 Hz), 4.32 (m, 1 H), 4.13 (t, 2 H, *J* = 6.6 Hz, CH₂O), 3.97 (br, 4 H), 3.24 (br, 1 H), 3.13–3.04 (br, 3 H), 2.31–2.20 (m, 5 H, CH₂CO), 1.70–1.57 (m, 6 H, CH₂ × 3), 1.24 (br s, 38 H, CH₂ × 19), 0.85 (t, 3 H, *J* = 6.9 Hz, CH₃). FABMS (*m/z*): 932 [M + 1]⁺.

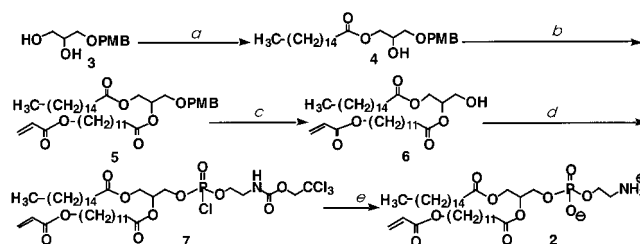
mono-AcrylPE-EMC (10). To a solution of **2** (25 mg, 0.035 mmol) in DMF (5 mL) was added triethylamine (0.05 mL, 0.35 mmol). After the solution was stirred for 30 min, a solution of *N*-(ε-maleimidocaproyl)succinimide (22 mg, 0.11 mmol) was added. The reaction mixture was stirred for 24 h at room temperature and then concentrated in a vacuum to give a residue, which was purified by Sephadex LH-20 column using methanol as eluent to afford **10** (23 mg, 71%). ¹H NMR (CDCl₃) δ: 7.19 (br, 1 H, NH), 6.70 (s, 2 H, CH=CH), 6.39 (dd, 1 H, *J* = 17.4, 1.2 Hz, CH=CH₂), 6.11 (dd, 1 H, *J* = 17.4, 10.5 Hz, CH=CH₂), 5.81 (dd, 1 H, *J* = 1.2, 10.5 Hz, CH=CH₂), 5.21 (m, 1 H, CH-2), 4.37 (dd, 1 H, *J* = 3.0, 12.3 Hz), 4.29 (t, 1 H, *J* = 6.9 Hz), 4.14 (t, 2 H, *J* = 6.9 Hz, CH₂O), 4.07–4.01 (m, 3 H), 3.95 (br, 4 H), 3.15 (br, 5 H), 2.33–2.17 (m, 4 H, CH₂CO × 2), 1.68–1.57 (m, 6 H, CH₂ × 3), 1.24 (br s, 38 H, CH₂ × 19), 0.87 (t, 3 H, *J* = 6.6 Hz, CH₃). FABMS (*m/z*): 907.2 [M + 1]⁺.

RESULTS AND DISCUSSION

Unlike mixed diacyl phosphatidylcholine, mixed diacyl phosphatidylethanolamine could not be prepared directly from commercially available *lyso*-phospholipid because protection and deprotection of the ethanolamine requires additional steps (14, 29). Therefore, in this approach sequential acylation of the primary and secondary hydroxy groups of a 3-protected *sn*-glycerol was performed and followed by deprotection and phosphorylation with an *N*-protected phosphorylating agent. Subsequent global deprotection yielded the expected phospholipid.

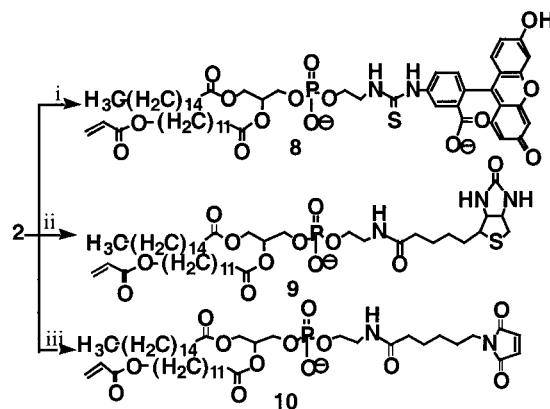
The selective acylation of the primary hydroxy group in **3** (**30**) was performed with palmitic acid in the presence of coupling reagent dicyclohexylcarbodiimide (DCC) and *N,N*-(dimethylamino)pyridine (DMAP) to give monoester **4** and trace diester. Sequential acylation of **4** with 12-acryloxy-1-dodecanoic acid (**6**) provided a protected 1,2-diacyl-*sn*-glycerol (**5**). The *p*-methoxybenzyl (PMB) group was removed by treating it with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) to give 1,2-diacyl-*sn*-glycerol (**6**) in good yield. The phosphorylation of **6** with dichloro(*N*-β,β,β-trichloroethoxycarbonyl-2-aminoethyl)phosphate (**31**) afforded Troc-PE (**7**) which was used for the next reaction without purification. The Troc-protecting group was removed by treating crude **7** with zinc in acetic acid to yield the desired polymerizable mono-AcrylPE (**2**) as a white solid in 62% yield (Scheme 1). These compounds were characterized by NMR spectra. For example, the distinctive multiplet peaks at 5.10–5.00 ppm are characteristic of the proton at the C-2 position on the glycerol backbone of 1,2-diacylglycerol (**5**) (32), and the vinyl group of acrylate was confirmed at 6.38 (dd, 1 H), 6.11

Scheme 1. Synthesis of Acrylate-Functionalized Phosphatidylethanolamine^a



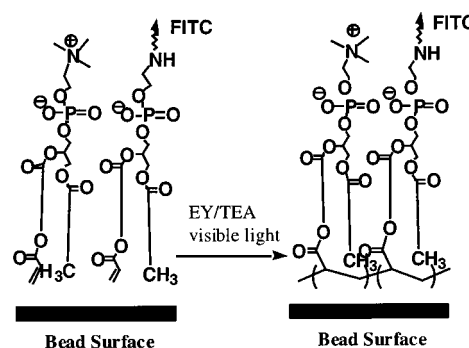
^a Reagent: (a) palmitic acid, DCC, DMAP, CH₂Cl₂, 71%; (b) 12-acryloxy-1-dodecanoic acid, DCC, DMAP, CH₂Cl₂, 79%; (c) DDQ, CH₂Cl₂/H₂O, 55%; (d) dichloro(*N*-β,β,β-trichloroethoxycarbonyl-2-aminoethyl)phosphate, NEt₃, CH₂Cl₂, 71%; (e) Zn/HOAc, 62%.

Scheme 2. Synthesis of Bifunctional Phospholipid Conjugates^a



^a Reagent: (i) FITC, NEt₃, DMF, 67%; (ii) NHS-Biotin, NEt₃, DMF, 53%; (iii) EMCS, NEt₃, DMF, 71%.

Scheme 3. Photopolymerization of Mono-AcrylPE-FITC with Mono-AcrylPC on the Alginate Bead Surface



(dd, 1 H), 5.80 (dd, 1 H) ppm as an AMX spin system. Three additional methylene groups in compound **7** were assigned at 4.73 (2 H), 3.96 (2 H), and 3.47 (2 H) ppm as multiplet peaks. For compound **2**, the NH₃⁺ group was noted as a broad peak at 8.51 ppm (3 H) (33), and the vinyl group of acrylate was confirmed at 6.39 (dd, 1 H), 6.14 (dd, 1 H), 5.81 (dd, 1 H) ppm as an AMX spin system.

Treatment of mono-AcrylPE (**2**) with commercially available fluorescein isothiocyanate (FITC), *N*-hydroxy-succinimidobiotin (NHS-Biotin), and *N*-(ε-maleimidocaproyl)succinimide (EMCS) in the presence of triethylamine provided the desired conjugated lipid-AcrylPE-FITC (**8**), AcrylPE-Biotin (**9**), AcrylPE-EMC (**10**), all in good yield (Scheme 2). ¹H NMR spectra confirmed the anticipated structure of the lipid conjugates. Excluding lipid backbone protons, nine aromatic protons were

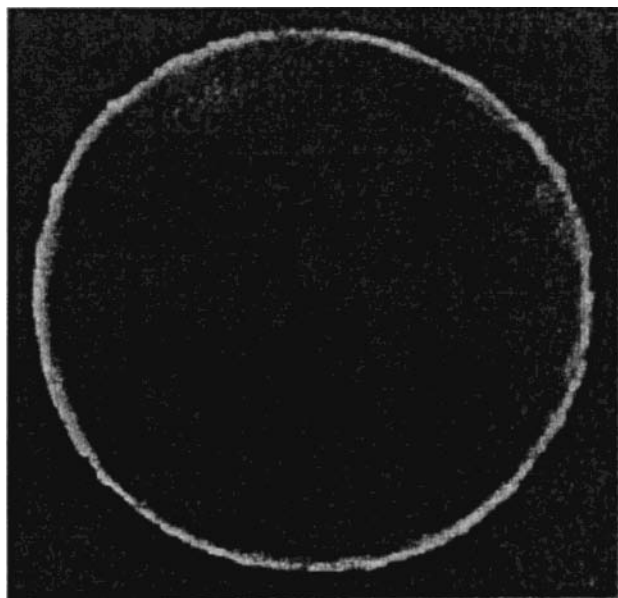


Figure 2. Fluorescent image of mono-AcrylPC and mono-AcrylPE-FITC coated alginate bead.

observed at 8.26 (m, 1 H), 7.40–7.30 (m, 1 H), 7.16–7.04 (m, 3 H), and 6.68–6.59 (m, 4 H) ppm for AcrylPE-FITC (**8**); two amido protons were noted at 7.79 (br, 1 H) and 7.00 (br, 1 H) ppm for AcrylPE-Biotin (**9**), and two olefin protons were observed at 6.67 (s, 2 H) ppm for AcrylPE-EMC (**10**).

Preliminary fabrication of mixed lipid films containing AcrylPE-FITC (**8**) and mono-AcrylPC (**1**) (**7**) was performed on the alkylated surface of alginate beads. Briefly, beads ($d = 300\ \mu\text{m}$) were incubated with a lipid vesicle solution composed of **8** and **1**, followed by photopolymerization using eosin Y (EY)/triethanolamine as coinitiator. The resultant beads were examined by confocal microscopy. As shown in Figure 2, the successful formation of the lipid film was confirmed by the presence of fluorescent activity on the surface of the beads while no such the activity was observed in the interior of the beads.

CONCLUSION

We have successfully developed a synthetic approach for generating bifunctional phospholipid conjugates containing both an acrylate functionality and a terminal linker, such as biotin or the *N*-(ϵ -maleimidocaproyl) functional group. These conjugates will enhance the capacity to generate stable, self-assembled, biologically functional and chemically heterogeneous, membrane-mimetic films.

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ARTICLES

A Dumbbell-like Complex Formation for DNA Target Assay

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A simple and highly sensitive test for the detection of nucleic acid targets is described. It is based upon complex formation between a small-diameter magnetic particle and a larger and nonmagnetic particle through a hybridization reaction, what we have called a dumbbell-like complex. During the different steps, nonreacting nonmagnetic conjugates were eliminated by magnetic separation. At the end of the process, dumbbell complex number was estimated by counting under a microscope. Compared to the already described two-particle tests, our model was able to reach higher sensitivities, with a threshold typically in the amol/mL range (10^6 copies of HIV DNA/mL) without the need for complex instrumentation or genomic amplification reactions.

INTRODUCTION

Assays for the detection of nucleic acid targets useful in pathogen detection, identification, and susceptibility testing are increasingly used in diagnosis. Most of these use genomic amplification techniques such as polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA), and ligase chain reaction (LCR). These methods are able to reach the highest levels of sensitivity, but are known to be very susceptible to contamination and inhibitors. For these reasons, many papers are now dealing with the development of high-sensitivity methods which aim to avoid amplification steps. For example, authors have developed dedicated technologies and instrumentation based on piezoelectric (1) and SPR techniques (2, 3). Watts et al. (4) investigated the potential of a real-time optical sensor for hybridized DNA target quantification. They finally reached a sensitivity limit of 9.2 nM (12.1 ng). In (5), the authors conceived a DNA optical sensor. They used hybridization on a nucleic acid probe–magnetic bead conjugate to specifically extract DNA targets and, finally, used a chemiluminescent enzymatic detection which allowed them to reach the femtomole level. Another example of a DNA optical sensor was given by Piunno et al. (6), who described a hybridization bioassay on an optical fiber. Ethidium bromide was used for detection by fluorescence which allowed them to detect a minimum value of 86 ng of cDNA/mL. Other technologies including voltametric measurements were able to give good sensitivity. For instance, Hashimoto et al. (7) declared they have reached a detection threshold as low as 4×10^4 targets/mL, using a gold electrode modified with DNA probes. Flow cytometry was also used as a highly sensitive method to detect microspheres onto which stained DNA was captured (8), or to perform multiplexed DNA sequence analysis with fluorescent latex particles and oligonucleotides (9). Hakala et al. (10) detected single microparticles by time-

resolved fluorometry after specific capture of photoluminescent europium chelate labeled oligonucleotides. The ultimate sensitivity, i.e., hybridization of two single nucleic acid probes, was reached with techniques such as fluorescence correlation spectroscopy (11) or total internal reflection fluorescence microscopy (12), but analysis parameters (extremely limited volumes, low flow rate, very long analysis time) are not acceptable for medical diagnosis. As can be seen, even when good sensitivity is obtained, all these methods require the use of dedicated or complex instrumentation.

Alternately, the use of magnetic particles in immunoassays is expanding rapidly and is even becoming a generic technique known as ImmunoMagnetic Separation (IMS). Using this simple one-particle capture step, associated with sensitive detection methods such as chemiluminescence (13) or time-resolved fluorescence (14), authors significantly improved the sensitivity of conventional immunoassays. We also demonstrated the feasibility of a very sensitive Scanning Force Microscopic Immunoassay (SFMIA) associated with capture and detection of TSH antigens by the use of antibody–magnetic nanoparticle conjugates (15), which led to the detection of 1 amol of TSH/mL (i.e., 5×10^5 TSH molecules/mL). The presence of magnetic particles associated with a specific biological recognition event may also be evidenced by measuring parameters such as magnetic permeability (16) or magnetoresistance (17) with good sensitivity.

Other simple tests using two particles and aiming to have an acceptable to high level of sensitivity had already been described. Lim et al. (18) recently described an assay based on an agglutination inhibition principle test in tubes, using a large magnetic particle and a smaller colored latex particle. Detection is based upon direct observation by the user, and the sensitivity limit was said to be 16 μ g/mL of antibody (i.e., approximately 0.1 nmol/mL). Another judicious two-particle assay was proposed by Mizutani et al. (19), who studied the detected virus captured on microbeads using antibody-labeled magnetic nanoparticles. The reaction was quantified by the analy-

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Table 1. Characteristics of Oligonucleotides Used for Dumbbell Formation

reference	label	hybridized with	base number	% GC	MT ^a (°C)
biotin-ODN1	5' biotin	target	32	43.7	56.8
HRP-ODN2	5' HRP	target	29	27.6	47.9
target	none	72	41.7	67.6	

^a Melting temperatures (MT) were calculated by Eurogentec (Belgium).

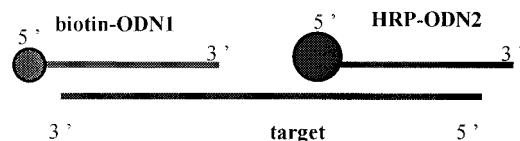


Figure 1. Diagram of oligonucleotide hybridization onto target. Biotin-ODN1 (32 mers) hybridizes the DNA target (72 mers) at its 3' end, and HRP-ODN2 (29 mers) at its 5' end.

sis of interference fringes generated when focusing a laser beam onto concentrated nanoparticles: levels as low as 0.1 pg/mL of p24 antigen can be detected. Earlier, Kim et al. (20) described the same two-particle principle using a turbidimetric detection and obtained a sensitivity of 110 ng/mL of antibody (i.e., approximately 0.1 pmol/mL).

To our knowledge, very little work has been published on the use of the two-particle assay for nucleic acid target detection. Recently, Bains (21) described a simple agglutination format between dense and light particles which led to the detection of 10^5 DNA targets/mL in 6 h, using a sucrose density gradient and ultracentrifugation for the separation and quantification of complexes. Elghanian (22) proposed a selective colorimetric assay, based on the agglutination of modified gold nanoparticles in the presence of a DNA target which enabled detection of 10 fmol/test (3×10^{12} molecules/mL) of an oligonucleotide.

Here, we intend to describe a rapid and very simple two-particle test which has the advantage of using standard instrumentation and can detect DNA targets down to the attomole per milliliter range.

MATERIALS

Chemicals and Biochemicals. 1-Ethyl-[3-(3-dimethylamino)propyl]carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide sodium salt (NHSS) were from Fluka AG (Germany). Ethylene glycol and monoclonal anti-horseradish peroxidase (anti-HRP) antibodies and *o*-phenylenediamine (OPD) enzymatic substrate were obtained from Sigma Chemical Co. (St Louis, MO). Additional chemicals were respectively salmon sperm DNA from Boehringer, Triton X-100 from Sigma, and Tween 20 from Merck.

Oligonucleotides were obtained from Eurogentec (Belgium). The HRP modification was carried out at bio-Mérieux. Table 1 displays characteristics of oligonucleotides used throughout this paper, and Figure 1 visualizes which way the target is hybridized between both labeled nucleic acids.

Particles. Superparamagnetic streptavidin nanoparticles (145 nm diameter) were from Immunicon Corp. (Huntingdon, PA). Carboxylate polystyrene latex particles (850 nm diameter, 10.1% solids content) were obtained from Bangs Laboratories, Inc. (Fishers, IN).

Buffers. Particle coupling buffer consisted of 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 6.8. Phosphate-buffered saline (PBS) was 50 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.4, 0.15 M

NaCl. Particle conjugates blocking buffer was PBS, 40 mM ethanolamine, 0.05% Tween 20, and 0.5% bovine serum albumin. Particle conjugates storage buffer consisted of PBS, 0.05% Tween 20, 0.1% bovine serum albumin, and 0.05% sodium azide. Biotin binding buffer was 50 mM Tris, 100 mM NaCl, pH 7.5, and hybridization buffer was 10 mM Tris, pH 8.0, 1 mM EDTA, 1 M NaCl, 0.14 mg/mL salmon sperm DNA, and 0.05% Triton X-100.

METHODS

Synthesis of Anti-HRP/Bangs Particle Conjugates. EDC (0.1 mg) and NHSS (2 mg) were diluted in 0.4 mL of particle coupling buffer in the presence of 0.05 mg of anti-HRP antibodies. Then 10 mg of Bangs carboxylated polystyrene latex particles was added while stirring and incubated for 1 h at 37 °C. The reaction was stopped by the addition of 0.5 mL of particle conjugates blocking buffer, followed by centrifugation to precipitate microspheres (10 min at 8000 rpm). After resuspension in 0.5 mL of the blocking buffer, centrifugation was repeated once, and anti-HRP latex conjugates were finally resuspended in 0.1 mL of particle conjugates storage buffer. They were stable for several months at +4 °C.

³⁵S Radioactive Labeling of Oligonucleotides. Biotin-ODN1 was labeled with ³⁵S at its 3' end using the labeling kit from Boehringer Mannheim (Terminal Transferase 220582) and (α -³⁵S)ddATP from NEN. After incubation for 1 h at 37 °C, purification was carried out with Sephadex G25 QuickSpin columns also from Boehringer. The specific activity of the resulting 1 pmol/ μ L labeled oligonucleotide solution was measured on a liquid scintillation analyzer (1900TR) from Packard.

Synthesis of Biotin-ODN1/Immunicon Nanoparticle Conjugates. Solutions containing different concentrations of biotin-ODN1 oligonucleotides were obtained by mixing ³⁵S-labeled oligonucleotides with unlabeled (cold) oligonucleotides at known ratios. These solutions were then allowed to react for 1 h with 7×10^8 streptavidin-coated Immunicon particles in 1 mL of hybridization buffer. Excess oligonucleotides were eliminated by a series of rapid magnetizations and resuspensions in hybridization buffer. After resuspension in 0.2 mL of water, particle emission activity was measured. Knowledge of the specific activity, labeled/unlabeled oligonucleotide ratio, and remaining activity on the particles allows the calculation of the binding yield, i.e., the fraction of oligonucleotides retained on the particles. Within a maximal 8% error, no detectable loss of activity was found due to the presence of the particles, as the sum of activities measured in supernatant and on particles matched the total activity of the initial solution.

Formation of Immunicon–Bangs Dumbbells. The principle is described in Figure 2. It is known that hybridization in solution gives better reaction rates than in the solid phase, since the solid support introduces a diffusion limitation which significantly reduces kinetics (23). Hence, targets and ODN were incubated first, before introducing particles. Biotin-ODN1 (10^{12} molecules), HRP-ODN2 (6×10^{11} molecules), and an increasing number of targets (0 – 10^{10} molecules) were incubated in 1 mL of hybridization buffer for 1 h at 37 °C. Then, 7×10^8 particles of Immunicon streptavidin/magnetic particle conjugates were added and allowed to react with hybrids for 1 h at 37 °C.

Solutions were concentrated by magnetic attraction on rare earth magnets (NdFeB, diameter $\times$ height = 12 $\times$

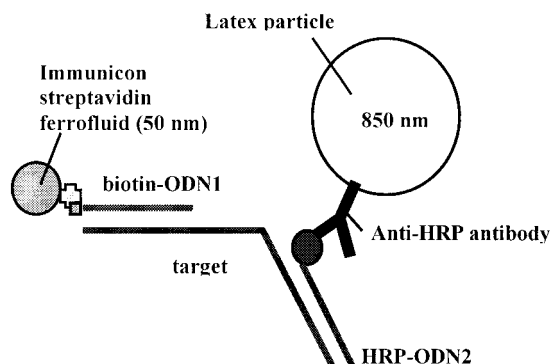


Figure 2. Representative diagram of a dumbbell between one latex particle and one magnetic particle. First, biotin-ODN1, DNA target, and HRP-ODN2 are hybridized. Then, Immunicon magnetic particles are added for capture of hybrids. After magnetic separation, anti-HRP latex particles are incubated with the magnetic complex to form so-called dumbbells, i.e., a two-particle system for DNA capture and detection.

6 mm) for 20 min. Pellets were then resuspended with 6 mL of hybridization buffer.

A total of 10^7 anti-HRP/Bangs particle conjugates diluted in hybridization buffer were added for a final volume of 10 μ L. Incubation was carried out for 1 h at 37 $^{\circ}$ C.

Dumbbells and free magnetic nanoparticles were separated from unbound latex particles by magnetic attraction for 30 min. Pellets were resuspended once with 20 μ L of hybridization buffer and again isolated under a magnetic field. These were finally dispersed in 5 μ L of ethylene glycol. The role of this viscous solvent was to limit the particle's brownian motion, thereby making counting under a microscope easier.

The 5 μ L particle drops (V) were then deposited onto a Malassez cell defining small calibrated volumes (v) of a few picoliters. Such a cell is normally used for counting a number of cells or bacteria and turning it into a volumic concentration. After deposition of coverglasses onto the cell, latex particles were counted with an optical microscope (Olympus) equipped with a $\times 40$ lens, inside 10 compartments giving an average number of particles per compartment (n). The total number of particles (N) in the sample was extrapolated from this value ($N = nV/v$).

Dumbbell Analysis by Transmission Electron Microscopy (TEM). Purified dumbbells obtained with various target concentrations were deposited onto formvar carbon film TEM grids (Ted Pella Inc.) and analyzed on a Philips CM120 microscope under a 80 kV voltage. Controls were carried out to ensure that the drying process did not induce magnetic nanoparticle adsorption onto latexes. Also, samples were analyzed before and after magnetic separation to make sure that this step does not produce false dumbbells.

RESULTS

Characterization of Anti-HRP/Bangs Particle Conjugates by Size Measurement. To check that antibody grafting onto latexes did not induce aggregation, the size of grafted particles was measured by quasi-elastic light scattering using a Zetasizer 3000 (Malvern). No consequent diameter modification was measured before (883 ± 9 nm) and after coupling (905 ± 3 nm). Also, microscopic observation of conjugates revealed the absence of aggregates.

Immobilization Yield of Biotin-ODN1 onto Immunicon Nanoparticles. To avoid competition between free biotin-ODN1 in excess and those hybridized with

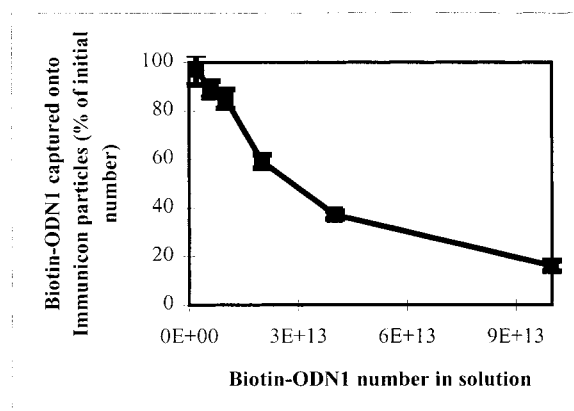


Figure 3. Binding yield of biotin-ODN1 onto streptavidin Immunicon particles as a function of the initial number of biotin-ODN1 molecules in solution estimated using radiolabeled oligonucleotides. Increasing concentrations of ^{35} S-labeled nucleotides are incubated in the presence of magnetic particles. After magnetic separation, the radioactivity is measured directly on the resuspended particle solution, and the number of nucleotides per particle is calculated.

targets, the binding capacity of streptavidin/Immunicon nanoparticles has to be greater than the total number of biotin-ODN1 molecules. In that perspective, the use of solutions containing radiolabeled biotin-ODN1 enabled an estimation of the fraction of ODNs that remained immobilized onto the particles. This binding yield is shown in Figure 3 as a function of the initial number of biotin-ODN molecules in solution.

With an initial number of 10^{12} biotin-ODN1 molecules, this figure indicates that the bonding yield is higher than 97%. Hence, there is no competition between free and hybridized biotin-ODN1 molecules in the dumbbell formation conditions: both are equally immobilized onto streptavidin/Immunicon nanoparticles. This biotin-ODN1/Immunicon particle ratio will thus be used throughout the following experiments.

Dumbbell Isolation Efficiency by Magnetic Separation. First, the efficiencies of magnetic nanoparticle conjugate separation and latex particle conjugate elimination were estimated separately.

Magnetic Nanoparticle Recovery Yield during the Magnetic Separation Step. A total of 100% of the Immunicon nanoparticles was extracted within 10 min when diluted in 1 mL of buffer, by placing the tube against a rare earth permanent magnet. This was determined by measuring the optical density of the particle solution before and after separation at 460 nm.

Latex Particle Elimination Yield. Solutions containing only latex particles at the same concentration as those used for dumbbell formation (10^7 particles in 10 μ L) were placed in the tube. Supernatant was discarded twice, and the remaining particles—later called washing noise—were counted under the microscope. An elimination yield of more than 99% was obtained. Considering the initial number of particles (10^7), the sensitivity of the assay is thus limited to less than 10^5 targets by this washing noise.

Separation of Dumbbells. The efficiency of dumbbell retention, in the presence of two types of particles at the same time, was estimated. The separation duration was extended to 30 min (instead of 10 min for isolated Immunicon particles) because of drag forces exerted on latex particles which are supposed to slow their motion.

To evaluate a possible loss of dumbbells between two separation cycles, the number of latex particles was

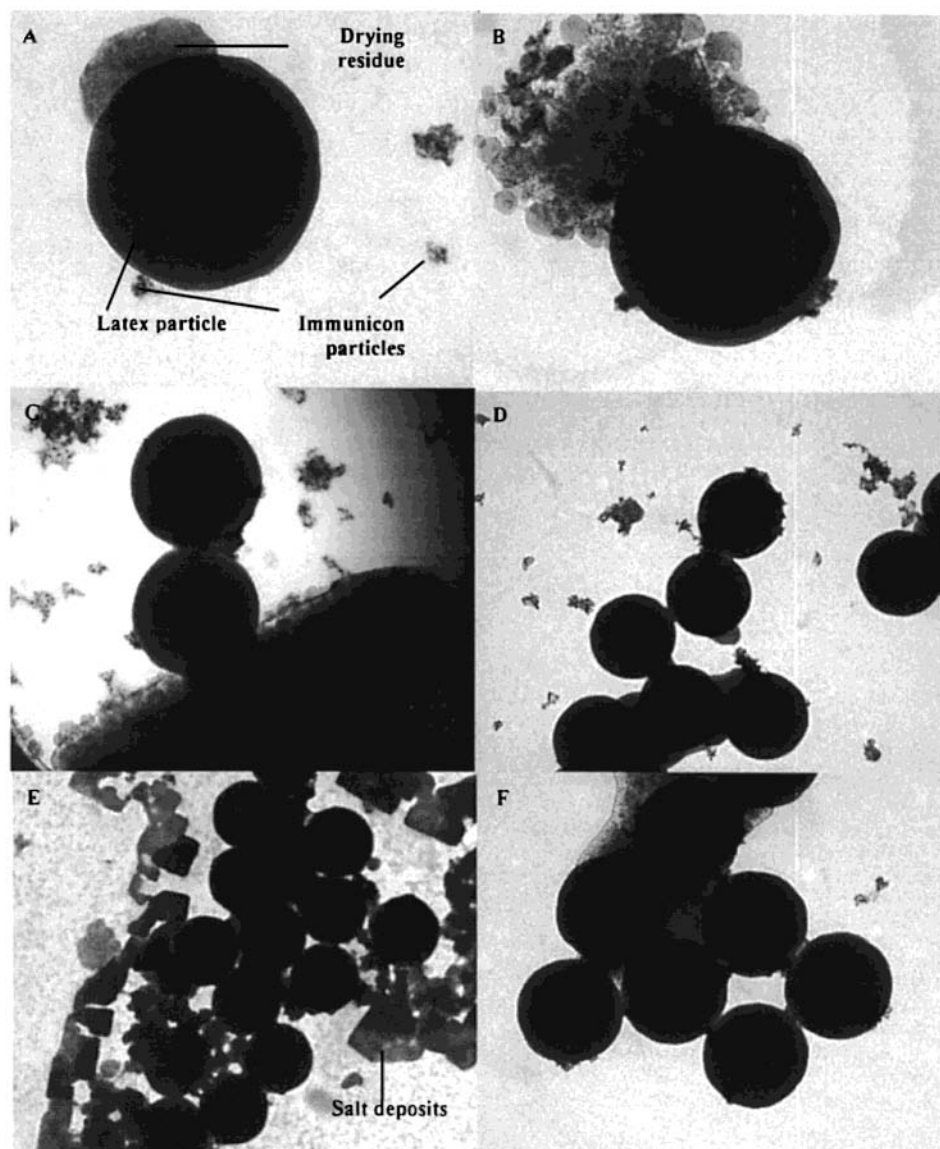


Figure 4. TEM analysis of dumbbells for increasing target numbers. (A) False dumbbells due to the nonspecific adsorption or the drying of a magnetic particle on latex (no magnetic separation or any other process). (B) No target in solution. (C) 10^7 targets. (D) 10^9 targets. (E) 10^{10} targets before magnetic separation. (F) Same sample as (E) (10^{10} targets) after magnetic separation.

evaluated in the same sample after the first and second separation steps. It was shown that as much as 50% of dumbbells can be lost in this way in the presence of 10^8 targets. Two possibilities can explain this loss: First, due to their low magnetic charge, some dumbbells are discarded while sucking up the supernatant, as the magnet can only exert weak magnetic forces. Second, the liquid meniscus also exerts strong capillary forces on the particle's spot and probably carries along the same poorly magnetic complexes.

Dumbbell Study by TEM. Electron microscopy is one of the few methods enabling observation of the structure of dumbbells. Controls ensure that the sample preparation drying step did not induce the formation of a large number of false dumbbells, i.e., nonspecific adsorption of one or several magnetic particles onto one latex. Contrary to the washing noise, these latex particles cannot be eliminated during magnetic washes since they are sensitive to the magnetic field as well as specific dumbbells. For this purpose, latexes, magnetic particles, and oligonucleotides were mixed briefly in the absence of targets, and immediately deposited onto grids. A few

isolated latexes were found to bear magnetic particles as shown in Figure 4A. In this figure, isolated Immunicon particles as well as latex are clearly defined. The structure of the magnetic particles is irregular and varies in shape and size. Some of them are seen on the bottom of the grid, and one seems to be immobilized on the latex. A kind of gel structure that may contain Immunicon particles is also often observed close to the latex. In any case, it was not possible to determine whether this false dumbbell was formed during the short contact time between particles before grid preparation, or during the drying itself. This phenomenon was limited, and the analytical method was not questioned.

Another control was performed to ensure that magnetic attraction did not induce the formation of false dumbbells by forcing the contact between both types of particles. A sample in the presence of 10^{10} targets was analyzed before (E) and after (F) separation. In both cases, latex aggregates are observed, proving that magnetic attraction is not in question. In panel E, salt deposits appear since the saline buffer was not eliminated from the sample as is the case for panel F.

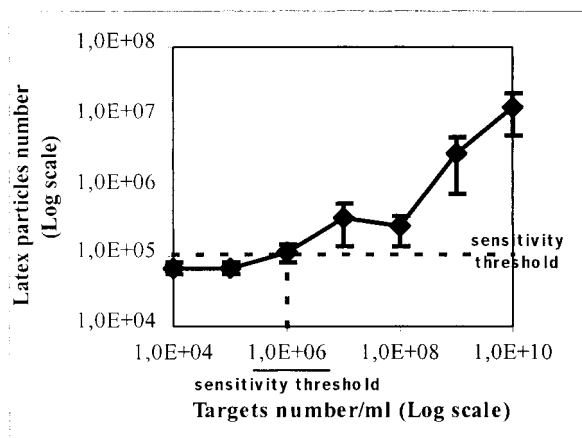


Figure 5. Variation in the number of latex particles as a function of the number of targets in solution. First, biotin-ODN1, increasing concentrations of DNA targets, and HRP-ODN2 are hybridized. Then, Immunicon magnetic particles are added for capture of hybrids. After magnetic separation, anti-HRP latex particles are incubated to form dumbbells. These dumbbells and free magnetic particles are washed twice to eliminate unbound latexes, resuspended in ethylene glycol, and deposited in a Malassez cell for counting under a microscope, and finally particle numbers are determined as a function of initial DNA concentration.

Aggregate size varied as a function of target concentration. Images B (0 target), C (10^7 targets), D (10^9 targets), and F (10^{10} targets) demonstrate the evolution of the dumbbell format into a typical agglutination reaction. As a matter of fact, beyond 10^7 targets, the target/latex particle ratio is greater than 1, which led to the possibility for particles to bind to each other via several targets and several Immunicon particles.

Cluster size distribution is, of course, observed in each sample. As can be seen, latex particles are linked through the intermediate of magnetic nanoparticles. This confirms the specific aspect of the reaction and that these are not aggregates due to simple nonspecific adsorption between latex particles. These clusters are distinguishable from the few observed on the drying control sample. Also, a decrease in the number of isolated Immunicon particles on the bottom of the grid is observed as the target concentration increases, since more of them are involved specifically in dumbbells and immobilized onto latexes.

Assay Sensitivity. Increasing concentrations of targets were assayed, and the resulting dumbbells were counted under an optical microscope after depositing the sample in a Malassez cell. As a matter of fact, only the latex particles are seen and counted under the optical microscope. Magnetic nanoparticles are not visible and thus do not interfere with the measurement. Results are described in Figure 5. A weak error in particle counting can be attributed to the confusion between roughly spherical dusts and true colloids. Furthermore, the standard deviation on each concentration point—an average of 10 measurements on the same sample—is important (20–30%) since it was not possible for the experimenter to evaluate a very large amount of particles that would statistically better represent the sample.

The sensitivity threshold is defined as the minimal target concentration enabling a detection signal higher than that obtained for the background plus 3 times the standard deviation on the noise value to be reached. From this calculation, below 10^6 targets/mL, it was not possible to discriminate background from specific signals. About 7×10^4 latex particles were counted in this sample,

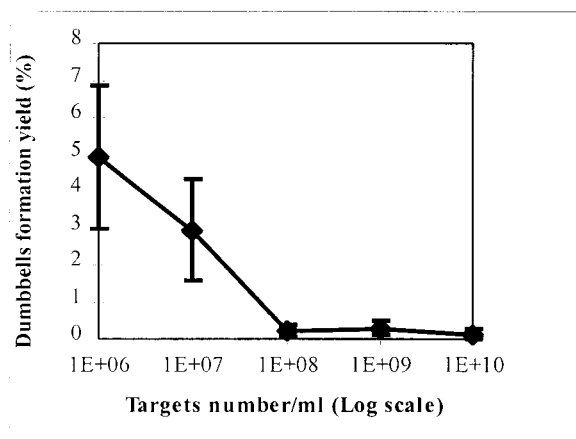


Figure 6. Evaluation of dumbbell formation yield, i.e., the percentage of latex counted at the end of the process over the number of targets. For each target concentration, the particle number obtained from Figure 5 after background subtraction is divided by the number of targets initially introduced in the solution.

representing 0.7% of the initial quantity. This background is essentially due to washing noise (latex particles not eliminated during the washing steps) for 0.1% (estimated as described in the section Dumbbell Isolation Efficiency by Magnetic Separation, by processing latex particles alone) and to false dumbbells (nonspecific adsorption of magnetic nanoparticles onto latex microspheres) for about 0.6%. Nonspecific interactions might be due to electrostatic or hydrophobic interactions between latexes and magnetic particles. TEM was used to ensure that the magnetic separation did not enhance false dumbbell formation to a significant extent.

A significant increase in the number of particles is observed under an optical microscope beyond 10^6 targets/mL, which represents a sensitivity threshold of 2 amol/mL. It is also observed that the presence of latexes becomes detectable by the naked eye beyond 10^8 targets/mL.

The reaction yield, i.e., the ratio between the number of specific dumbbells and the number of targets in solution, was estimated from Figure 5 by subtracting the background from the number of latex particles for each target concentration, and is depicted in Figure 6.

Best yields were obtained for low target concentrations, and did not exceed 5%. Such a result can be explained by method limitations described above. First of all, the capture of hybrids onto latex particles may be limited intrinsically by the affinity of the HRP/anti-HRP antibody system. Furthermore, TEM pictures showed that Immunicon particles are rough and not spherical, which probably enhances steric hindrance during the binding of the two particles. Also, the nonspecific latexes—false dumbbells plus washing noise—limit the sensitivity threshold.

Beyond 10^7 targets, as seen in the TEM section, there is statistically more than 1 target per latex, and the drawing in Figure 2 showing 1 latex and 1 magnetic particle per target is not representative. Clusters of particles are formed, leading to a drop in reaction yield.

DISCUSSION

As demonstrated, this assay reached a very high sensitivity threshold, in the attomole per milliliter range. It is easy to use, does not need complex instrumentation, and avoids tedious amplification techniques.

However, we identified several drawbacks which currently limit the overall sensitivity. Possible approaches to further enhance the assay performance are discussed below.

(a) Capture of hybrids on latex particles may be limited by the affinity of the HRP/anti-HRP antibody system. It would be worthwhile testing other systems that may have a better affinity: other antibody/antigen pairs or DNA-labeled particles.

Furthermore, on TEM pictures, Immunicon particles showed a bumpy structure which may induce steric hindrance and is probably not the most suitable for dumbbell formation. We would probably benefit from working with spherical and smooth magnetic particles. Currently, other commercial particles as well those synthesized in our lab are being tested.

(b) A large proportion of dumbbells—up to 50%—are lost during magnetic separation. The process has to be improved by developing specifically suited systems. Furthermore, particles with higher iron oxide contents would enable more magnetic dumbbells to be obtained, which are better retained on magnets. Nevertheless, attempts with larger and easily isolated particles such as Dynal (2.8 μm diameter) or Seradyn (1 μm diameter) gave no satisfactory sensitivity (detection threshold beyond 10^{10} targets/mL, data not shown). It is believed that the lower diffusion coefficient of larger linked particles limits their motion and, therefore, their binding rate onto latexes. On the other hand, testing with smaller and weakly magnetic particles from Miltenyi (50 nm diameter), used with High Gradient Magnetic Separation systems (24), led to intermediate results (10^7 targets/mL). In conclusion, particles easier to isolate than Immunicon, but whose size does not exceed 200 nm, should be a good choice.

(c) About 0.8% of the latexes initially introduced, resulting from the nonspecific interaction between magnetic nanoparticles and latex particles, are found in the samples in the absence of targets and, thus, restrict sensitivity. The formation of false dumbbells may be limited by working on treatments to reduce nonspecific adsorption of magnetic particles onto latexes.

Another possibility may be to use smaller latexes which are easier to carry in the magnetic fields and to retain on magnets while discarding supernatant. For the moment, assays in this direction show that it is necessary in this case to increase the number of latexes to maintain the same reactive surface, which finally induces an important level of background, which is harmful for sensitivity.

(d) The manual detection counting was shown to induce large standard deviations in the final results. It would be profitable and less tedious for the experimenter to set up an automated system enabling particles to be counted to a larger extent. Furthermore, the use of fluorescent particles would avoid the confusion between dusts and latexes. Besides, an interesting option would be to count particles using systems based on laser light scattering.

CONCLUSION

The assay described in this paper enabled a very high level of sensitivity for DNA target detection (10^6 molecules/mL) to be reached without the use of sophisticated technologies. With the reaction being detectable by the

naked eye beyond 108 targets/mL, applications may be considered for the rapid and sensitive assay of pathogenic material in difficult conditions, i.e., outside the analysis laboratory.

Optimizations are foreseen with the aim of pushing the sensitivity threshold one step beyond. This could allow the simple detection of pathogen nucleic acids, without the use of amplification techniques and their associated drawbacks. Applied to the identification of infectious diseases, this test could also have a high potential in early diagnosis and susceptibility testing. These results and experiments have been included in the bioMerieux patent 99/10675.

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On the Preparation, Characterization, and Enzymatic Activity of Fungal Protease–Gold Colloid Bioconjugates

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We present herein details pertaining to the preparation of bioconjugates of colloidal gold with aspartic protease from the fungus *Aspergillus saitoi* (F-prot) and their characterization and enzymatic activity. Simple mixing of the colloidal gold and protein solutions under protein-friendly conditions (pH = 3) followed by centrifugation (to remove uncomplexed gold nanoparticles and protein molecules) results in the formation of the fungal protease–gold nanoparticle conjugates. The protein–gold nanoparticle bioconjugate was redispersed in buffer solution and indicated the formation of efficient bioconjugates with intact native protein structures. The bioconjugates in solution were characterized by UV–vis spectroscopy, fluorescence spectroscopy, and biocatalytic activity measurements while drop-dried bioconjugate films on Si (111) substrates were characterized by scanning electron microscopy (SEM), energy dispersive analysis of X-rays (EDAX), and X-ray diffraction (XRD) measurements. Microscopy images do show some aggregate formation, but the intactness of the native structure of the enzyme in the bioconjugate material was verified by fluorescence and biocatalytic activity measurements. The enzyme retains substantial biocatalytic activity in the bioconjugate material and was comparable to that of free enzyme in solution.

INTRODUCTION

Colloid chemistry includes the study of a wide range of entities spanning micron-sized to nanometer-sized particles, the properties of the particles being vastly different from that of the bulk material. There has been a great deal of research in different areas of colloid chemistry, viz., protein chemistry, inorganic nanoparticles such as quantum dots, clay materials, interfaces, and membranes, to name a few. Combination of different branches of colloid chemistry is an important goal with exciting spin-offs expected in industrial, medical, and advanced materials applications. Potential applications in the food, chemical, and agriculture industries, markers in electron or standard light microscopy and medicine such as drug delivery systems, efficient immunosensing assays for early detection of diseases, etc., has seen an explosion in the research and design of a new class of materials commonly known as bioconjugates. Formation of bioconjugates by immobilization protocols into different 2D and 3D matrixes is currently a great challenge as far as the stability, accessibility, shelf life, transportation, cost, and ease of manufacturing etc. are concerned. An important requirement for immobilization is that the biomolecule in the bioconjugate material should remain in its natural form and should be able to carry out necessary functions as efficiently as in solution. To date, biomolecules such as proteins have been immobilized on/within various supports such as phospholipid bilayers,¹ self-assembled monolayers,² Langmuir–Blodgett films,³ polymer matrixes,⁴ the galleries of α -zirconium phosphates,⁵ in hydrophobic controlled pore-glasses,⁶ and in

films of thermally evaporated fatty lipids.⁷ All these systems deal with single protein immobilization protocols. In a different approach, Caruso and co-workers have demonstrated that the layer-by-layer (L-B-L) method can be used for the formation of multilayer protein–polymer conjugates.^{8a,b} In this method, electrostatic attraction between alternately deposited charged species of proteins and polyelectrolytes is used to form multilayer planar films.

Studies on using colloidal particles as a versatile and efficient three-dimensional (3-D) template for the immobilization of biomolecules has been recognized since the early 1980s.⁹ Immunomicrospheres, as one class of conjugate-colloids are called, can be defined as specially designed microscopic particles that have antibodies or similar molecules chemically bound to their surfaces.⁹ Appropriately coated colloidal particles can be used to react in a very specific way with antigens, target cells or viruses, depending on the type of antigen adsorbed on the microspheres. Immunomicrospheres have opened up new opportunities in the development of techniques for disease diagnosis and therapy.

A number of groups have studied the adsorption of proteins on both organic and inorganic colloidal particles. As an extension of planar surfaces, Caruso et al. have successfully extended the L-B-L technique to form multilayer protein layers on polymer colloidal systems.^{8c,d} The multilayers of proteins on colloidal particles increases the density of biomolecules and thus, their sensitivity in different applications. The stabilization of colloidal particles of polymers by proteins has been studied by Molina-Bolivar et al.¹⁰ Covalent attachment of immunoglobulins to the surface of polystyrene latex colloidal particles has also been demonstrated.¹¹ Schmitt et al.¹² have reported on a monomolecular layer of bovine serum albumin (BSA) on the surface of polystyrene (PS) latex particles. Elgersma et al. have demonstrated the

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adsorption of BSA on positively and negatively charged polystyrene lattices.¹³

The interaction of noble metal colloidal particles such as gold and silver with proteins/enzymes as well as the study of the enzymatic activity of the nano-bioconjugates has also received attention. Natan and co-workers have studied in great depth cytochrome *c*–gold colloid conjugates as regards stability, protein orientation, and application in surface enhanced Raman scattering (SERS) and found that metal–protein–metal sandwiches offer benefits as reagents for protein SERS.¹⁴ Macdonald and Smith have studied the orientation of cytochrome *c* adsorbed on citrate-reduced silver colloids.¹⁵ Single molecule spectroscopy (SMS) of the hemoglobin molecule adsorbed on 100 nm sized citrate-reduced silver colloidal particles was achieved by SERS.¹⁶ Silver particle aggregation is necessary for this effect, and the protein molecules actually bind the Ag particle clusters together.¹⁶ In the area of metal nanoparticle–enzyme conjugate materials, Crumbliss, Stonehuerner, and co-workers have studied the formation and enzymatic activity of gold nanoparticles complexed with horseradish peroxidase^{17a} and xanthine oxidase^{17b} as well as glucose oxidase and carbonic anhydrase molecules.^{17c} A salient feature of their work is the demonstration that enzyme molecules are bound tightly to gold colloidal particles and retain significant biocatalytic activity in the conjugated form while the enzyme molecules denature on adsorption to planar surfaces of gold.^{17c}

As part of our ongoing investigation into the formation and catalytic activity of enzyme–bioconjugate materials,⁷ we investigate herein the immobilization of fungal protease (F-prot) molecules on 3-D supports such as colloidal gold particles, leading to the formation of efficient bioconjugates. We observe significant catalytic activity of the F-prot molecules in the F-prot–Au colloid bioconjugate material which indicates that the enzyme molecules are immobilized without distortion to the enzyme native structure. The bioconjugates of 35 Å colloidal gold particles with the enzyme F-prot are formed under enzyme-friendly conditions (at pH = 3) where the enzyme shows optimum catalytic activity. The bioconjugate was prepared by simple addition of the F-prot molecules to the colloidal gold solution held at pH = 3. Thereafter, the F-prot gold colloid solution was centrifuged to remove uncoordinated F-prot molecules in solution to yield the purified F-prot–Au colloid bioconjugate material. The purified F-prot–Au colloid bioconjugate material was resuspended in appropriate buffer solution after rinsing several times. UV–vis spectroscopy was used to monitor the surface derivatization before and after centrifugation/resuspension. The bioconjugate material were further characterized by scanning electron microscopy (SEM), energy dispersive analysis of X-rays (EDAX), X-ray diffraction (XRD), fluorescence spectroscopy, and biocatalytic activity measurements. The amount of F-prot on the colloidal gold surface was quantified by fluorescence spectroscopy and used for the determination of specific activity of the enzyme. The specific activities of the enzyme on the surface of colloidal gold and that of free enzyme in solution are comparable, indicating that the enzyme in the bioconjugate is in its active natural conformation. Presented below are details of the investigation.

EXPERIMENTAL SECTION

Chemicals. F-prot and hemoglobin were obtained from Sigma Chemicals and used without further purifi-

cation. Chloroauric acid and sodium borohydride were obtained from Aldrich Chemicals and used as received. All buffer salts were from standard commercial sources and of the highest quality available.

Colloidal Gold Synthesis. Colloidal gold was synthesized by the borohydride reduction of 1.25×10^{-4} M concentrated aqueous solution of chloroauric acid (HauCl_4). This process yields 35 ± 7 Å sized colloidal gold particles as shown previously by us.¹⁸ The colloidal solution was aged for a period of 12 h. The ruby red colored solution yielded an absorbance maximum at 518 nm.¹⁸

Formation of F-prot–Colloidal Gold Bioconjugates. A 10^{-6} M standard solution of the enzyme, F-prot (molecular weight ~ 37000 , pI ~ 9.5) was prepared in glycine·HCl (0.05 M, pH = 3) buffer. Different amounts of this standard solution were added to colloidal gold to yield an F-prot concentration of 10^{-7} , 10^{-8} , and 10^{-9} M in the conjugate solution. The pH of the colloidal gold solution was adjusted to 3 prior to addition of F-prot. This was done since F-prot shows optimum biocatalytic activity at pH = 3. The solution was kept for a period of 12 h at 4 °C and then centrifuged to remove uncoordinated F-prot and free gold particles in solution. The powder so obtained was rinsed several times with buffer solution and then resuspended in the pH = 3 buffer and stored at 4 °C for further experimentation.

UV–Vis Spectroscopy. The formation of the F-prot–gold colloid bioconjugate was studied by UV–vis spectroscopy using a Shimadzu dual-beam spectrophotometer (model UV-1601 PC) operated at a resolution of 1 nm. The absorbance band above 500 nm arising from excitation of the surface plasmon resonance in the gold particles^{18,19} and the resonance at 280 nm due to the π – π^* transition in the tryptophan and tyrosine residues of the enzyme²⁰ were monitored immediately after addition of F-prot to the gold solution as well as after centrifugation/resuspension. Three separate sets of F-prot–gold colloid bioconjugate solutions were prepared and tested for the above UV–vis signatures in order to check the reproducibility of the data.

Scanning Electron Microscopy (SEM) and Energy Dispersive Analysis of X-rays (EDAX). SEM and EDAX measurements were performed on a Leica Stereoscan-440 scanning electron microscopy (SEM) equipped with a Phoenix EDAX attachment. A drop-dried F-prot–gold colloid bioconjugate film (10^{-7} M F-prot in the bioconjugate solution) on Si (111) substrate was used for the analysis. SEM analysis was primarily used to observe the morphology of the bioconjugates in the film.

X-ray Diffraction Studies (XRD). A few drops of the centrifuged/resuspended F-prot–gold colloid bioconjugate solution were placed on 2 cm $\times$ 2 cm Si (111) substrates and dried in air. XRD measurements of these films were made on a Philips PW 1830 instrument operating at 40 kV voltage and a current of 30 mA with Cu K_α radiation. The size of the colloidal gold particles in the bioconjugate film was estimated from the peak width of the (111) Bragg reflection using the Debye–Scherrer formula.²¹

Tertiary Structure Studies. Fluorescence spectroscopy is a powerful tool to study the tertiary structure of proteins and enzymes. The fluorescence of the F-prot–gold colloid bioconjugate solution was studied using a Perkin-Elmer Luminescence Spectrometer (model LS 50B). The tryptophan and tyrosine residues in the enzyme were used for excitation at 280 nm, and the broad emission band having a maximum at about 336 nm was recorded.²² Fluorescence spectra of bare colloidal gold particles in water and that of the buffer used were also recorded as control experiments. As expected, these

controls did not show any emission in the region of interest (ca. 336 nm).

Estimation of Amount of F-prot in the Bioconjugate Composite System. The fluorescence emission intensities at ca. 336 nm²² due to excitation of tryptophan and tyrosine residues in the enzyme (absorption at 280 nm) were recorded for different concentrations of the free enzyme in buffer solution, and a calibration curve was obtained. The amount of F-prot in the different bioconjugate solutions prepared in this study was estimated by comparing the intensity of fluorescence emission at 336 nm in the bioconjugate solution with the calibration curve for this protein. The amount of F-prot estimated in this fashion was then used to calculate the specific activity of the enzyme in the bioconjugate solution.

Biocatalytic Activity Measurements. The biocatalytic activity of the centrifuged/resuspended F-prot–gold colloid bioconjugate solution was determined by reaction with a solution of hemoglobin (5 mg/mL) prepared in glycine·HCl buffer at pH = 3. F-prot is a proteolytic enzyme which acts on proteins such as hemoglobin²³ and has been successfully used by us in studies on thin films of the enzyme immobilized in fatty lipid films.^{7b} The enzyme fungal protease is not a well-studied system, and consequently information on specific chromogenic or fluorogenic substrates is not available. This has prompted us to use hemoglobin as a substrate in estimating the specific activity of the enzyme in the F-prot–gold colloid bioconjugate solution and of the free enzyme in solution. In a typical experiment to estimate the biocatalytic activity of the bioconjugate solution, 1 mL of the solution consisting of 100 μ L of the F-prot–gold colloid bioconjugate material and 0.9 mL of the buffer was reacted with 1 mL of the hemoglobin solution, and the reaction mixture was incubated at 37° C for 30 min. After the incubation time, 1.7 M perchloric acid was added to the reaction solution to precipitate the remaining hemoglobin. After 30 min, the precipitate was removed by centrifugation and the optical absorbance of the filtrate measured at 280 nm. F-prot digests hemoglobin and yields acid soluble products (tryptophan and tyrosine residues) which are readily detected by their strong UV signatures at 280 nm.²⁰ From the amount of F-prot estimated from the fluorescence studies, the specific activity of the enzyme was calculated. For comparison, the biocatalytic activity of similar concentration of free F-prot in solution was recorded. To check the reproducibility of the biocatalytic activity measurements, 10 separate measurements of the F-prot–gold colloid bioconjugate material were performed as mentioned above, and the average specific activity was calculated.

RESULTS AND DISCUSSION

The choice of colloidal gold in the formation of bioconjugates is dictated by a number of advantages which these particles display. The striking ruby red color of colloidal gold makes it an ideal medium for colorimetric determination of molecular recognition processes as has been demonstrated for the prototypical biotin–avidin interaction.²⁴ Colloidal gold has been used extensively by Mirkin, Letsinger and co-workers in the immobilization of oligonucleotides and detection of complementary base sequences by changes in the visible absorption spectra.²⁵ The surface of colloidal gold does not require modification for chemisorption of proteins/enzymes as in the case of organic colloids. The ease of characterization of colloidal gold by observing colorimetric changes upon immobilization of analytes from solution, its biocompatibility, the

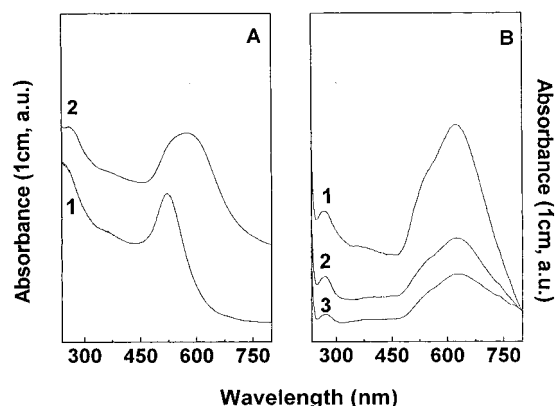


Figure 1. (A) UV–vis spectra of the colloidal gold solution before (curve 1) and after addition of 10^{-6} M F-prot to the colloidal gold solution at pH = 3 (curve 2) to give an equilibrium concentration of 10^{-7} M of F-prot in the bioconjugate solution. (B) UV–vis spectra of F-prot–gold colloid bioconjugate solutions after centrifugation, rinsing and resuspension in pH = 3 buffer. The spectra corresponding to concentrations of F-prot in the starting bioconjugate solutions are curve 1– 10^{-7} M, curve 2– 10^{-8} M, and curve 3– 10^{-9} M.

stability of enzymes on colloidal gold particle surfaces (as also observed by Stonehuerner et al),¹⁷ the simplicity of bioconjugate formation by just addition of proteins/enzymes to the colloidal gold solution, and the fact that the surface does not require premodification has led to the choice of colloidal gold in the formation of bioconjugates of F-prot in this study.

Preparation of F-prot–Colloidal Gold Conjugates. The as-prepared pH of colloidal gold by the borohydride reduction procedure is ca. 9.¹⁵ The enzyme, F-prot, has a pI of about 9.5 and shows optimum catalytic activity at pH = 3,^{7b} and hence the pH of colloidal gold has to be lowered prior to addition of F-prot. As mentioned in the Experimental Section, 10^{-6} M standard solution of F-prot was prepared in glycine·HCl (0.05 M, pH = 3) buffer and added to colloidal gold solution at pH = 3, to form equilibrium F-prot solution concentrations of 10^{-7} , 10^{-8} , and 10^{-9} M, respectively. It is well-known that thiol groups bind to the gold surface via a covalent bond.¹⁸ Cysteine residues on the surface of F-prot molecules may thus bind to the colloidal gold particles via a thiolate linkage. Support for cysteine-mediated binding of enzyme to the gold colloids comes from the report of Sasaki et al.²⁶ who demonstrated that the functional protein, myosin subfragment 1, binds to gold thin films through an Au–S bond involving cysteine residues in the protein. It has been reported in the literature that alkylamine molecules form a weak covalent bond with colloidal gold surfaces.²⁷ Natan and co-workers indicate the role of the NH_2 group in lysine rich pockets for the binding of proteins to the colloidal gold surface.¹⁴ We believe that the binding of fungal protease to colloidal gold is covalent in nature and occurs either via the cysteine or lysine residues or a combination of both. The number of cysteine and lysine residues in the enzyme capable of binding to the gold particle surface would require detailed information regarding the amino acid sequence in F-prot which is not available at the moment.

Figure 1A shows the UV–vis spectra recorded from the gold colloidal solution before (curve 1) and after (curve 2) addition of the F-prot solution (overall F-prot concentration in the solution = 10^{-7} M). The spectra have been displaced vertically for clarity. The surface plasmon resonance due to gold can be clearly seen at about 520

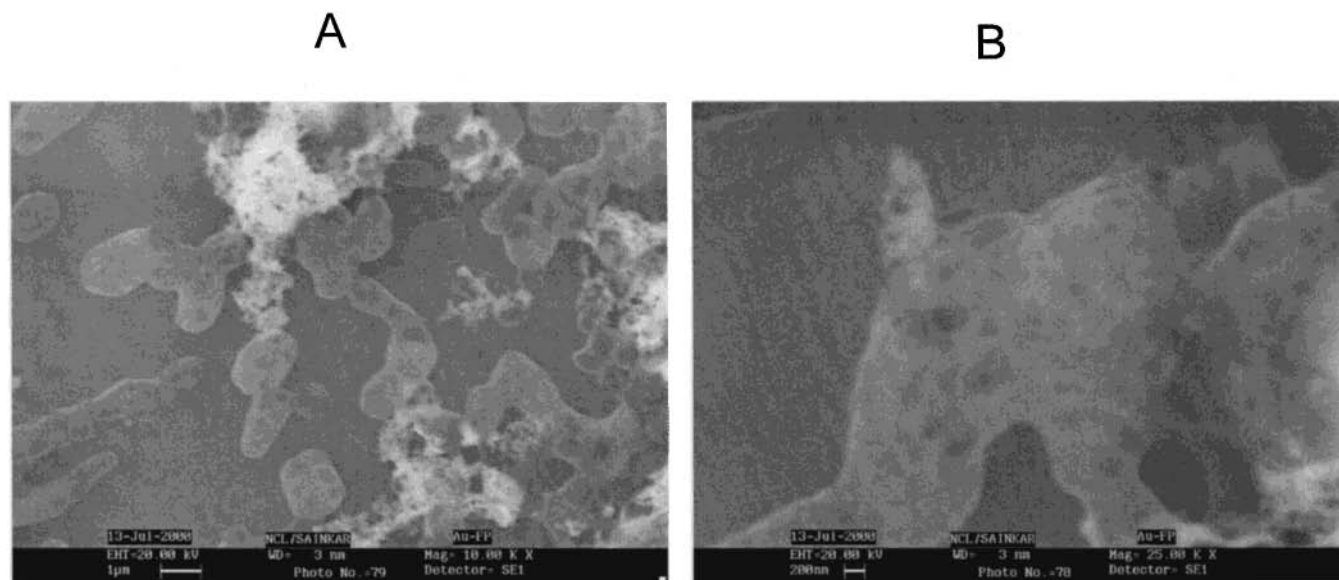


Figure 2. (A) SEM image of a film of the F-prot–gold colloid bioconjugate material drop-dried on a Si (111) substrate (see text for details). (B) SEM image of an aggregate of F-prot and gold colloidal particles in a drop-dried film of bioconjugate material at higher magnification (see text for details).

nm¹⁸ for the as-prepared gold solution (curve 1, Figure 1A). On addition of F-prot, a broadening and red shift of the plasmon resonance is seen (curve 2, Figure 1A) and clearly indicates surface coordination of F-prot with the gold particles. The broadening of the plasmon resonance is also indicative of some degree of aggregation of the gold particles mediated by the protein molecules. The absorbance at about 280 nm seen prominently in the F-prot–gold colloid bioconjugate solution (curve 2, Figure 1A) is due to the π – π^* transitions in the tryptophan and tyrosine residues in the enzyme.²⁰ The spectra for other concentrations of F-prot (10^{-8} and 10^{-9}) gave similar features and are not shown for brevity. The bioconjugate solutions were stored for a period of 12 h at 4 °C before centrifugation and resuspension. UV–vis spectra of the F-prot–gold colloid bioconjugates recorded after centrifugation, rinsing, and resuspension in water for 10^{-7} , 10^{-8} , and 10^{-9} M F-prot in the starting bioconjugate solution are shown as curves 1–3, respectively, in Figure 1B. It is observed that both the surface plasmon resonance intensity from the gold particles as well as the concentration of F-prot molecules in the resuspended bioconjugate solution (as evidenced by the intensity of the absorption at 280 nm) scale with the concentration of F-prot molecules in the starting solution (i.e., the solution prepared prior to centrifugation). Thus, a larger number of gold particles are required to stabilize a greater concentration of F-prot molecules in solution and provides a simple recipe for increasing the loading factor of the enzyme molecules in the bioconjugate material. The magnitude of the absorbance at 280 nm in the case of 10^{-7} M F-prot–gold colloid bioconjugate material before centrifugation is about 0.3 absorbance units which is reduced to 0.06 absorbance units after centrifugation and resuspension. This is also true for other concentrations of F-prot in the bioconjugate material and indicates that the large decrease in intensity of this band is due to removal of uncoordinated F-prot molecules from the respective solutions. Three separate experiments each of bioconjugate formation, rinsing, and resuspension for the three concentrations of F-prot in the bioconjugate material were performed to check the reproducibility of the data. It was found that the curves and the absorbance at 280 nm (corresponding to that of F-prot in the

bioconjugate solution) agreed in the experiments to within 10%.

SEM and EDAX Measurements. Figures 2A and B show the SEM images of F-prot–gold colloid bioconjugate films formed by drop-drying the bioconjugate solution (10^{-7} M F-prot in the bioconjugate solution) on Si (111) substrates. A number of aggregates can be seen in the lower magnification image (Figure 2A) and is likely to be due to enzyme-induced cross-linking of the colloidal gold particles. Figure 2B gives a magnified view of one of the aggregates. On careful observation, it is seen that the aggregate is made up of dark and bright spots. EDAX spot measurements were performed, and the dark regions were found to be deficient in gold particles (but enhanced in protein concentration as evidenced by a large nitrogen signal) while the bright regions were rich in gold particles. Thus the SEM images indicate aggregates within the F-prot–gold colloid bioconjugate material with spatially separated regions of gold particles and protein molecules. We would like to add here that a similar surface morphology was observed by Caruso et al. in multilayer films of a polymer–anti-IgG composite material.²⁸

As mentioned above, EDAX measurements were performed on the above film and the average atomic percentage ratio of gold: nitrogen over the surface of the film was estimated to be 1:5 and that of gold:carbon was recorded to be 1:14. The nitrogen and carbon signals arise from the enzyme molecules and serve as a good indicators for the presence of enzyme in the F-prot–gold colloid bioconjugate material.

XRD Measurements. While formation of aggregates in the F-prot–gold colloid films is clearly seen in the SEM pictures (Figure 2), it was difficult to image the individual gold particles in the aggregates due to limited resolution of the instrument. It is well-known that the size of particles in such nano-structured films may be estimated from the line-broadening of Bragg reflections in the XRD patterns using the standard Debye–Scherrer formula.^{21,29} A few drops of the centrifuged/resuspended F-prot–colloidal gold bioconjugate solution (prepared from the 10^{-6} M F-prot starting solution) were placed on 2 cm $\times$ 2 cm Si (111) substrate and dried in air. Figure 3A shows the XRD pattern recorded from an F-prot–gold colloid

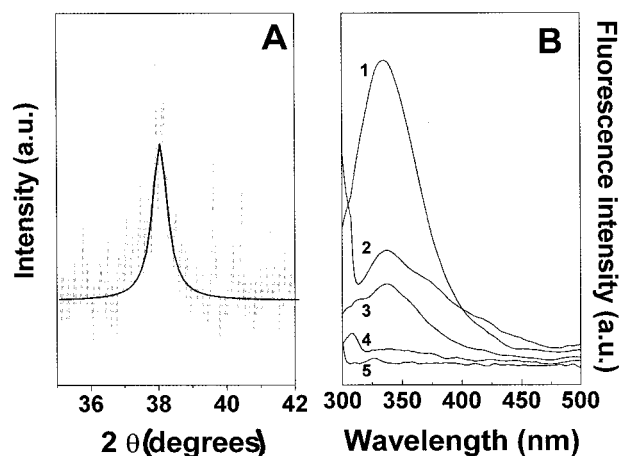


Figure 3. (A) XRD pattern of a film of the F-prot-gold colloidal solution on a Si(111) wafer prepared by a simple drop-drying method (see text for details). The solid line is a Lorentzian fit to the spectra. (B) Fluorescence spectra of F-prot solution at a concentration of 10^{-6} M at pH = 3 (curve 1), F-prot-colloidal gold bioconjugate solution at pH = 3 prepared from the 10^{-6} M concentration starting F-prot solution (curve 2) and F-prot solution at a concentration of 10^{-7} M at pH = 3 (curve 3). Fluorescence spectra from glycine-HCl buffer (0.05 M, pH = 3, curve 4), and bare gold colloidal solution (curve 5) as controls are also shown (see text for details).

bioconjugate film deposited on Si(111) wafer by drop-drying the 10^{-7} M F-prot bioconjugate solution. The (111) Bragg reflection from gold is clearly seen at a 2θ value of ca. 38.2° and has been fit to a Lorentzian (solid line, Figure 3A) to estimate the peak broadening. The size of the gold particles in the F-prot-gold colloidal bioconjugate film was calculated from the fit to be 250 Å. The size of as-prepared colloidal gold particles is about 35 Å,¹⁸ but on forming a bioconjugate with F-prot molecules, the size increases indicating a small amount of sintering of the particles. However, given that the enzyme on the surface of the colloidal gold particles is active (as explained subsequently in the section on biocatalytic activity measurements), enzyme aggregation may be ruled out.

Tertiary Structure Studies. The biocatalytic activity of the immobilized enzyme would, to a large extent, depend on the tertiary structure of the enzyme remaining unperturbed on the surface of colloidal gold. The tertiary structure of an enzyme can be checked monitoring the fluorescence emission from the tryptophan or tyrosine residues in the enzyme. This is a standard procedure, and we have used it to check the tertiary structure of F-prot on the surface of colloidal gold by excitation of the $\pi-\pi^*$ transition in the tryptophan and tyrosine residues at 280 nm.²² Figure 3B shows the fluorescence spectra recorded from 10^{-6} M and 10^{-7} M aqueous solutions of the free enzyme, F-prot, at pH = 3 (curves 1 and 3) as well as the fluorescence spectrum measured from the F-prot-colloidal gold bioconjugate solution prepared from the 10^{-6} M F-prot starting solution (curve 2). The intensity of absorbance maxima in all three solutions occurs at about 336 nm which indicates that the F-prot molecules in the bioconjugate solution are present in their natural configuration maintaining their tertiary structure. Therefore, even though aggregates were seen in the SEM images, fluorescence measurements show that the enzyme molecules in the aggregates maintain their tertiary structure. Control experiments were performed on the fluorescence from the glycine-HCl (0.05 M, pH = 3) buffer (Figure 3B, curve 4) and the bare colloidal gold solution (Figure 3B, curve 5) by excitation at 280 nm. It is observed that for these two controls, there is no

emission in the spectral region where the enzyme contributes (ca. 335 nm) to the signal. This result is important in obtaining a reliable estimate of the concentration of F-prot molecules in the redispersed F-prot-gold colloidal bioconjugate solution, as discussed below.

The fluorescence spectroscopy measurements of Figure 3B were used to quantify the amount of F-prot on the colloidal gold surface in the bioconjugate solution by comparing the intensity of the emission signal at 336 nm with the standard calibration curve. The calibration curve was obtained by plotting the fluorescence intensities of different concentrations of F-prot at pH = 3. The concentration of F-prot in the F-prot-gold colloidal bioconjugate system was calculated to be ca. 3.7×10^{-7} M and is an important number in the estimation of the specific activity of the bioconjugate solution and enables comparison of the activity of the enzyme in the bioconjugate material with that of the free enzyme in solution.

Biocatalytic Activity Measurements. The most important aspect in protein-colloidal bioconjugate systems is the retention of the biocatalytic activity after adsorption onto the nanoparticle surface. A 100 μ L volume of F-prot-gold colloidal bioconjugate solution (starting F-prot concentration in the bioconjugate material = 10^{-6} M) was reacted with hemoglobin as explained in the Experimental Section. The specific activity was calculated using the amount of enzyme estimated from the calibration curve (see Figure 3B and discussion above) and compared with that of free enzyme in solution under identical conditions. The specific activity of free enzyme in solution was 6.1 units, and that of the enzyme immobilized onto colloidal gold surface was 5.5 ± 0.4 units. (One unit of enzyme will produce a change in absorbance at 280 nm of 0.001 per minute at pH = 3.0 and 37 °C measured as acid soluble products using hemoglobin as the substrate).⁷ The value quoted for the specific activity of the enzyme in the bioconjugate solution is a statistical average over 10 separate measurements of freshly prepared solutions of the same concentration F-prot molecules in the bioconjugate. The small standard deviation shows that the results are highly reproducible. A comparison of the specific activities of the free enzyme and enzyme in the bioconjugate solution shows that there is not much decrease in the activity of the enzyme molecules in the bioconjugate system. This is a significant result and shows that the enzymes in the aggregates have not lost their biocatalytic activity and are possibly stabilized by the gold nanoparticles.

Conclusions. F-prot-gold colloidal bioconjugates have been prepared by simple addition of F-prot molecules to the colloidal gold solution. A microscopic analysis of the bioconjugate material showed the presence of aggregates of the enzyme, possibly cross-linked and stabilized by the gold particles. Even though aggregation of the protein was indicated, fluorescence and biocatalytic activity measurements clearly showed that the enzyme on the surface of colloidal gold retains its native confirmation and furthermore, that the specific activity of the enzyme in the bioconjugate was comparable to that of the free enzyme in solution.

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Improvement of Porphyrin Cellular Delivery and Activity by Conjugation to a Carrier Peptide

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The chemical nuclease metalloporphyrin (manganese(III) porphyrin) can cleave DNA irreversibly and can thus constitute a potential antitumor drug. However, these molecules show low permeability to cell surface membranes. We report here the conjugation of an amphipathic carrier peptide to improve considerably its cellular delivery. The metalloporphyrin–peptide conjugate can be internalized by cells within only 5 min of incubation with a yield as high as 80%. Furthermore, the metalloporphyrin–peptide conjugate is able to cleave in vitro high or low molecular weight DNA to the same extent as metalloporphyrin alone without affecting the sequence-specific cleaving activity of the porphyrin. The conjugate is 100-fold more efficient at inducing tumor cells death than the free metalloporphyrin via a mechanism involving genomic DNA cleavage. The results are promising for further therapeutic applications with antitumor drugs such as metalloporphyrin, and also with other existing drugs by using a carrier peptide system in order to improve the cellular uptake of such molecules.

INTRODUCTION

The major barrier to the development of drugs or gene therapies is their poor permeability to the cell membrane. To improve the delivery of these compounds, most research has been directed toward developing viral (1–3) or nonviral vectors (4–6). So far, gene delivery systems for cancer therapy have been restricted to the introduction of genes encoding for a missing protein or the tumor-suppressing protein p53 (7) as well as antisense strategies (8). However, no vectors have been specially designed for carrying small antitumor drugs except for one peptide-based shuttle recently developed for photodynamic therapy using the Chlorin e6 as antitumor agent (9). The efficiency of these drugs is ultimately dependent on their selective uptake and accumulation within cells or tissues. Therefore, the development and the use of optimal delivery shuttles represent an alternative and powerful approach to solve this problem.

We have recently shown that a primary amphipathic peptide Ac-M-G-L-G-L-H-L-L-V-L¹⁰-A-A-A-L-Q-G-A-K-K-K²⁰-R-K-V-Cya (where Ac and Cya are acetyl and cysteamide groups, respectively), where the hydrophobic and the hydrophilic domains correspond to a signal peptide and a nuclear localization sequence respectively, can promote cellular internalization of a covalently linked drug or a short nucleic acid (10, 11). This was first established using lucifer yellow as probe. Peptide-assisted internalization was almost complete within 3 min with a final localization which was mainly nuclear. This was later confirmed using a 26-mer antisense oligonucleotide

complementary to a mRNA encoding β subunits of Ca²⁺ channels (11). These results prompted us to try and carry a molecule which has been described to be biologically active and of potential interest for therapeutic applications and cancer therapy, namely metalloporphyrin (Mn-TMPyP).¹ This molecule has several major advantages: the first one lies in the fact that its cationic manganese derivative cleaves DNA irreversibly (12, 13) by a cleaving process in which no further ligation can be achieved by cellular ligases. Therefore, this manganese(III) porphyrin will become a potential antitumor drug after improvement of their cellular delivery. Moreover, this antitumor alternative approach is supported by results already described for other porphyrin families used for photodynamic or ultrasonic treatments in which they exhibited antitumor effect in vivo (14, 15). In our case, the metalloporphyrin is able to cleave DNA at A-T rich sites (at least three consecutive A-T base pairs) through binding in the DNA minor groove. The cleaving activity of the metalloporphyrin is generated by an oxidative reaction (pseudohydrolysis at the 5'C–H bond of the deoxyribose units of DNA) (16) leading to the appearance of 5' alcohol and 3' phosphate ends. This property also confers to these metalloporphyrins the ability to cleave DNA in vivo by using oxygen from air in combination with a cellular reductive agent similarly to bleomycin (17). Interestingly, porphyrin molecules show another advantage that lies

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¹ Abbreviations used: Mn-TMPyP, manganese(III) derivative of *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrin pentaacetate; trisMePyP-CH₂-I, *meso*-{5-[4-[3-(iodoacetamido)propyloxy]-phenyl]-10,15,20-tris(*N*-methylpyridiniumyl)}porphyrin; Mn-trisMePyP-CH₂-I, manganese(III) derivative of *meso*-{5-[4-[3-(iodoacetamido)propyloxy]-phenyl]-10,15,20-tris(*N*-methylpyridiniumyl)}porphyrin; KHSO₅, potassium hydrogen persulfate; NaBH₄, sodium tetrahydroborate; PS-NLS, peptide signal–nuclear localization sequence.

in the spectroscopic properties of the metal-free form. Although inactive from the biological point of view, they are strongly fluorescent and can thus be used to identify the intracellular domain where the porphyrin localizes.

Therefore, two types of conjugates have been synthesized: (i) a metal-free porphyrin-peptide (trisMePyP-CH₂-S-Peptide) to monitor cellular localization, and (ii) a metal bound porphyrin-peptide (Mn-trisMePyP-CH₂-S-Peptide) to determine cleavage activity. The latter conjugate is expected to be internalized by cells, as well as to cleave DNA and thus inhibit growth of tumor cells.

We present here the synthesis of the conjugates, the cellular localization of the metal-free porphyrin together with a comparison of the activity induced by free and peptide driven metalloporphyrin. We also demonstrate that the inhibition of leukemia cells is effectively due to the cleavage of genomic DNA by these metalloporphyrin-peptide conjugates.

EXPERIMENTAL PROCEDURES

Chemicals. Potassium monopersulfate (in fact, the triple salt 2KHSO₅·KHSO₄·K₂SO₄), NaBH₄, and Hepes were purchased from Sigma. Mn-TMPyP, *meso*-{5-[4-[3-(iodoacetamido)propyloxy]phenyl]-10,15,20-tris(*N*-methylpyridiniumyl)}porphyrin and its manganese derivative, Mn-trisMePyP-CH₂-I were synthesized as previously described (12, 18). T4 polynucleotide kinase and [γ -³²P]dATP were purchased from Boehringer Mannheim and Amersham Pharmacia Biotech, respectively. Oligodeoxynucleotides (called duplex ODN1 and ODN2, see Figure 9) were purchased from Genset SA, France. λ DNA, Bgl I, and EcoR I were purchased from GibcoBRL. Carrier peptides used in this work were synthesized by Fmoc solid-phase methods as already described (19).

Synthesis of the Conjugates. The purified peptide containing a thiol moiety at its C-terminus was dissolved in Hepes buffer (50 mM, pH = 8.2). Then, porphyrin (metal-free or manganese-complexed) owning an iodoacetamide function were added to peptide solution in the same buffer and stirred for 3 h. The corresponding conjugates were recovered after HPLC purification with triethylammonium acetate (20 mM, pH = 7.4) and acetonitrile (95%, 0.08% TFA) as the eluent. The HPLC was performed on Waters equipment (Milford, MA) with a C8 Aquapore RP300 column, semipreparative 220 $\times$ 10 mm, flow 5 mL/min (Brownlee Lab., Applied Biosystem Inc.). The characterization of the final products was achieved by fluorescence, Ellman test, amino acid analysis and mass spectrometry.

Identification of Peptide, Porphyrin, and Conjugates. Amino acids analysis was performed on the carrier peptide on a High Performance Analyzer (Model 7300, Beckman instruments, Fullerton, CA). For the analysis, 50 nmol of peptide was hydrolyzed in 0.2 mL of HCl 5.7 N containing 0.9% (v/v) of phenol at 110 °C under vacuum in sealed glass tubes.

Mass analyses of all compounds were performed on a MALDI-TOF mass spectrometer (Bruker Biflex III) equipped with a N₂ (337 nm; 3ns) laser system (LSI, VSL-337i). The instrument included a single-stage acceleration source, a delayed ion extraction, a two-stage gridless reflector. In all experiments, the ion accelerating voltage was 19 kV. The reflector voltage was set to 20 kV for the acquisition of normal reflector mass spectra. Time-to-mass conversion was achieved by external calibration using angiotensin ([MH]⁺, *m/z* 1046.54) and α -cyano-4-hydroxycinnamic acid (CHCA) ([2M + H]⁺, *m/z* 379.09) as references for the reflector mode and cytochrome *c* for the linear mode. Fifty picomoles (1 μ L of 50 μ M solution)

were used for each analysis. Saturated matrix solution of CHCA was prepared in the solvent system CH₃CN (50%)/H₂O with 0.01% TFA. A mixture of the peptide and matrix solutions (1:1 v/v) was applied to the target and allowed to dry in air before being introduced into the mass spectrometer. The pressure in the analyzer was better than 5 $\times$ 10⁻⁷ mbar.

Cell Culture and Cellular Localization. For this study, three different cellular types were used: human foreskin fibroblasts HS68, fibroblasts NIH 3T3, and murine lymphocytic leukemia L1210 were cultured at 37 °C as described previously (20, 21, 22).

Medium. L1210 lymphocytic cells were grown in nutrient medium RPMI 1640 (Life Sciences) supplemented with 10% of fetal calf serum, L-glutamine (2 mM/ml), and antibiotics such as penicilin (200 U/mL) and streptomycin (50 μ g/mL). DMEM (Dulbecco Modified Eagle's Medium) was used for fibroblast cells supplemented with 10% of foetal calf serum, L-glutamine, and the same antibiotics as mentioned above.

The cellular localization assays were performed on these three different cell types. HS68 and NIH 3T3 were grown on acid-washed coverslips and leukemia L1210 cells in 12-well plates. After a brief wash in PBS (Phosphate Buffer Saline), cells were incubated for 5 min with the porphyrin-peptide conjugate (λ_{exc} : 426 nm, λ_{em} : 660 nm) or 5 min to 2 h with the porphyrin alone (10 μ L at 10⁻⁵ M, dissolved in Hepes, 50 mM, pH 7.2) at 37 °C and then fixed in 3.7% formalin in PBS for 5 min. Coverslips were washed in water and mounted in 15% (w/v) Airvol 205, 33% glycerol in PBS. Hoechst dye (λ_{exc} : 345 nm, λ_{em} : 460 nm) (Sigma) was added for 1 min in the final wash step before mounting. For L1210 cells, localization assays were performed with the porphyrin or the conjugate (5 min at 10⁻⁵ M). Cells were resuspended directly into Airvol after centrifugation of cells (5 min at 1500 rpm). Cells were observed by confocal scanning laser microscopy. Images were acquired using a Leica confocal laser scanning microscope, equipped with a krypton-argon dual wavelength laser. Images were scanned at 512 $\times$ 512 pixel resolution with 12 bits per pixel. DNA staining was visualized with a normal fluorescence light source using a CCD low light sensitivity camera. For visualization of the porphyrin or the conjugate, Rhodamin filters were used.

Cleavage Experiments: λ Phage DNA. DNA of phage λ digested by Hind III (500 ng) dissolved in a solution of Tris (40 mM, pH 8) and NaCl (100 mM) was incubated with metalloporphyrin or the conjugate at room temperature for 15 min in a reaction volume of 7 μ L. Then, KHSO₅ (1 μ L at 10⁻² M) was added and allowed to react for 15 min and was stopped by 1 μ L of Hepes buffer (1 M, pH 8). All samples were subjected to NaBH₄ reduction (1 μ L at 1 M) for 1 h at ambient temperature. Samples were loaded on agarose gel (1.5%) and submitted to electrophoretic migration.

Cleavage Experiments: Oligodesoxyribonucleotides (ODN). ODN I and II (see Figure 7 for the sequences) were 5' end-labeled with [γ -³²P]dATP with T4 polynucleotide kinase for 1 h at 37 °C. Annealing of the complementarity ODNs I and II: 10 μ L of I stock solution (0.1 μ g/ μ L) was mixed with 10 μ L of II stock solution (0.1 μ g/ μ L), 4 μ L of Tris (40 mM, pH 8), EDTA (100 mM), and NaCl (100 mM). This saline buffer solution was heated at 95 °C for 5 min and allowed to slowly cool to ambient temperature before equilibration at 4 °C overnight. The concentration of duplex was 2.33 μ M. A preincubation of metalloporphyrin or the conjugate (at the selected concentration) with duplex (ODN I and II) was performed

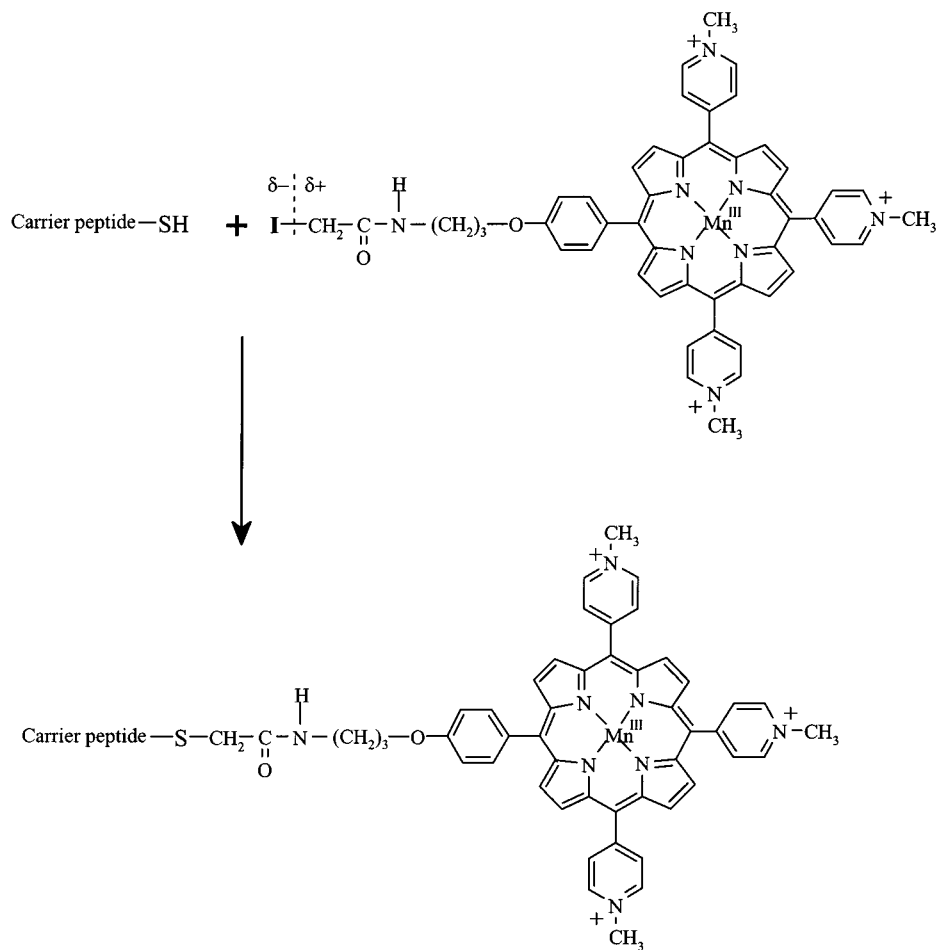


Figure 1. Schema of the synthesis of the conjugates showing the structures of the metalloporphyrin derivatives and conjugates. The manganese derivatives exhibit four positive charges (three for the methylpyridiniumyl units and an additional for the manganese-(III) center).

in a reaction volume of 7 μL for 15 min at room temperature. KHSO_5 (1 μL at 10^{-2} M) was then added and after 15 min of reaction the cleavage reaction was stopped by 1 μL of Hepes buffer (1 M, pH 8). All samples were subjected to NaBH_4 reduction (1 μL at 1M) for 1 h at ambient temperature. The samples were ethanol-precipitated, dried as pellets and dissolved in the loading buffer. The samples were heated 2 min at 95 $^\circ\text{C}$ before loading on 20% denaturing polyacrylamide gel. The gel was dried and exposed for autoradiography.

Inhibition of Cell Growth Rate. The activity of the metalloporphyrin and the metalloporphyrin–peptide conjugate was tested against murine leukemia L1210 cells in vitro by a procedure already described (22). Briefly, metalloporphyrin, peptide or the conjugate were added directly to the culture medium and removed after 15 min with an initial cell density of 5×10^5 . The cells were then washed extensively with fresh medium and re-incubated at 37 $^\circ\text{C}$. After 12, 24, 48, 72, and 96 h of culture, cells were counted using either a cell counter (Coulter Counter ZM, Coulter Electronics Ltd, England) or by the trypan blue exclusion procedure. For the determination of the IC_{50} values, cells were counted after 48 h of culturing.

Analysis of Genomic DNA. L1210 cells were grown in 12-well plates until a cell density of exactly 10^6 cells. Porphyrin, or the conjugate, was then added directly to the culture medium for 15 min at 37 $^\circ\text{C}$ in a concentration range between 5 and 50 μM . After this time, cells were washed with fresh medium and re-incubated at 37 $^\circ\text{C}$ for 48 h. After a brief wash with PBS, cells were lysed in a buffer containing Tris 20 mM pH = 8, EDTA 20 mM, SDS

0.1% and proteinase K 100 $\mu\text{g}/\text{mL}$ (added just before lysis) and submitted to heating overnight at 55 $^\circ\text{C}$. Genomic DNA was isolated by phenol/chloroform/isoamyl alcohol (25/24/1) extraction followed by ethanol precipitation. Samples of DNA were incubated for 1 h at 37 $^\circ\text{C}$ in the presence of Rnase A (Dnase free) in order to eliminate persistent cellular RNA. DNA fragments obtained with this isolation procedure have a size less than 30 Kbp allowing for an agarose gel analysis.

RESULTS

Synthesis of the Conjugates. In this work one carrier peptide belonging to the PS-NLS family (10) has been examined. This peptide family is based on the juxtaposition of two types of sequences, a signal peptide, and a nuclear localization sequence. By combining these two sequences the resulting peptide has an amphipathic character, which allow it to cross cellular membranes. The carrier peptide has been designed in order to carry into cells small drugs and has already been used successfully for the delivery of antisens oligonucleotides via a covalent linkage (11, 23). This carrier peptide has the following sequence Ac-M-G-L-G-L-H-L-L-V-L¹⁰-A-A-A-L-Q-G-A-K-K-K²⁰-R-K-V-Cya. Owing to the presence of the C-terminal cysteamide group (including a free reactive SH) the peptide can be used for postsynthetic modifications, especially a covalent linkage to a cargo. This linkage can be easily achieved by the use of an iodoacetamide derivative of the molecule to be internalized. In this case, the cargo is a metal-free porphyrin (trisMePyP-CH₂-I) or its Mn(III) derivative (Mn-trisMePyP-CH₂-I), (Figure

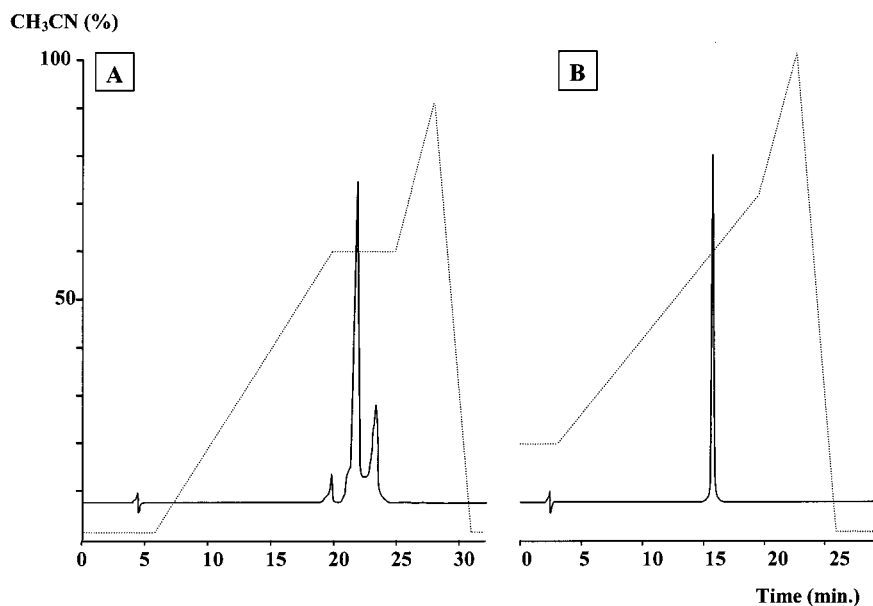


Figure 2. HPLC profiles obtained for the crude conjugate (panel A) and for the purified conjugate (panel B).

Table 1. Characterization of the Porphyrin–Peptide Conjugates (trisMePyP–CH₂–S–Peptide and Mn-trisMePyP–CH₂–S-peptide) by Different Techniques^a

	% of elution (HPLC)	precipitation (ammonium sulfate)	fluorescence properties detected	Ellmann test
peptide	54	no		+++
porphyrin	62	no	porphyrin	—
metalloporphyrin	64	no	—	—
porphyrin–peptide conjugate	60	yes from 10%	porphyrin	—
metalloporphyrin–peptide conjugate	61	yes from 10%		—

^a After purification by HPLC (% of elution in acetonitrile) which already shows different retention times, these compounds have been characterized by ammonium sulfate precipitation, fluorescence, and Ellmann's test. Only the conjugates can be precipitated with ammonium sulfate. TrisMePyP–CH₂–S–Peptide can be detected by fluorescence of the porphyrin moiety while Mn-trisMePyP–CH₂–S–Peptide exhibit no fluorescence. The Ellmann procedure has been used to confirm the absence of any free SH group in the case of the porphyrin and conjugate samples. In these experiments, only the porphyrin with an iodoacetamide function was used.

Table 2. Amino Acid Analysis of the Carrier Peptide after Acidic Hydrolysis

	Gln	Gly	Ala	Val	Met	Leu	His	Lys	Arg
peptide	1 × 1.03	3 × 0.98	4 × 1.01	2 × 0.99	1 × 1	6 × 0.99	1 × 1.01	4 × 1.02	1 × 0.95

1). The peptide is conjugated with each porphyrin molecule for 3 h under basic conditions at room temperature. After purification by HPLC (Figure 2) the conjugates were identified by their absorption spectra in the visible (426 or 466 nm for the Mn(III) derivative). Ellman tests (24) did not indicate the presence of any free SH functions (See Table 1 for details). Further identification was achieved by amino acids analyses which revealed the expected composition of the carrier peptide (Table 2) and by mass spectrometry. Both conjugates showed the expected peaks at $m/z = 3344$ (theoretical 3346) and 3290 (theoretical 3291) for the metal-bound and metal-free compounds, respectively. This is illustrated in Figure 3 where the mass spectra of the two components forming the conjugate (metal bound form) are shown together with that of the final product. Note that the origin of the peak at $m/z = 3500$ (see Figure 3C) is unknown but very probably arises from an impurity found in the starting porphyrin derivative and which could not be removed by HPLC. As to the peak at $m/z = 3289$, it corresponds very probably to the Mn-free form produced by the ionization process.

Cellular Localization. Owing to the very low fluorescence emission yield of the Mn(III) form of porphyrin, these experiments were carried out only with the metal-free form of the porphyrin (trisMePyP–CH₂–I) and its

corresponding conjugate (trisMePyP–CH₂–S–Peptide). The cellular uptake was checked with two types of cells (fibroblasts and L1210). Both types of cells used showed very similar results. In both cases the cellular membranes are poorly permeable to porphyrin and only about 3% of the cells indicated a porphyrin uptake with a cytoplasmic localization after 2 h of incubation (Figure 4). Even after 24 h of incubation only about 20% of the cells show a porphyrin uptake. The influence of the iodoacetamide function was checked by using another porphyrin derivative without this function (trisMePyP), and no differences could be observed under the same experimental conditions.

When the porphyrin is covalently linked to the peptide, an incubation time of 5 min were sufficient for more than 80% uptake of this compound by cells, with the final localization being mainly nuclear (Figure 4). Closer examination of this figure also indicated that, besides the nuclear localization, the conjugate also remained in the cytoplasm.

Growth Inhibition and Cell Death. To determine whether the metalloporphyrin remains active against L1210 cells when linked to the peptide, experiments were also undertaken similarly to previously reported experiments for metalloporphyrin derivative (25). The results

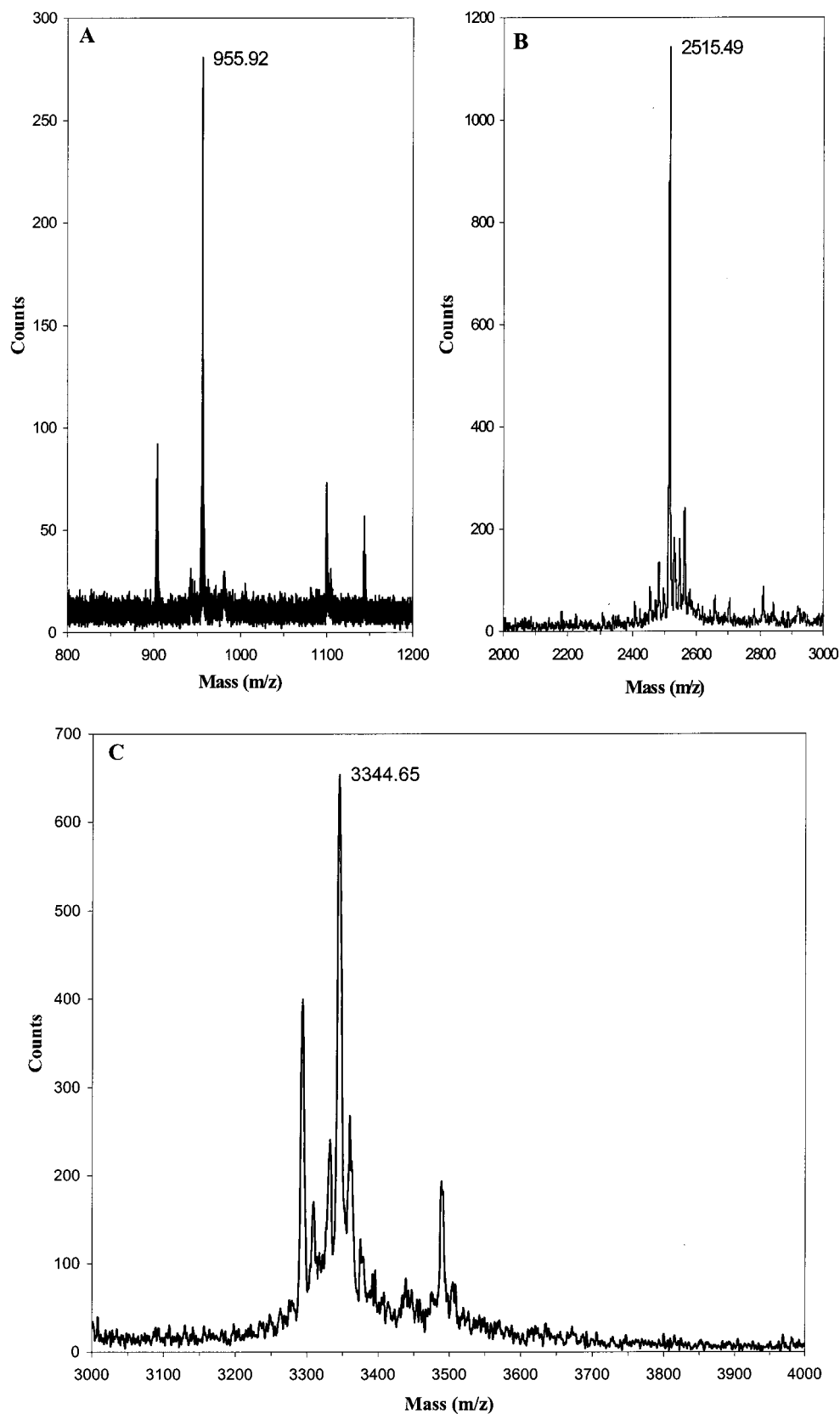


Figure 3. MALDI-TOF mass analysis of the various compounds involved in the synthesis of the metalloporphyrin conjugate. A: Iodoacetamide porphyrin derivative as analyzed in a reflector mode. B: Peptide as analyzed in a linear mode. C: Conjugate as analyzed in a linear mode.

are shown on Figure 5 and indicate that, using our protocol (see methods "inhibition of cell growth rate"), the conjugation of porphyrin to the vector peptide induced, at least, a 100-fold increase of the activity. The IC_{50} (concentration which reduces by 50% after 48 h, the

L1210 cell growth as compared to control) value is 0.6 μM for the conjugate which has to be compared with $>50 \mu M$ for the metalloporphyrin alone. It must also be noted that using free metalloporphyrin to obtain a defined IC_{50} requires incubation throughout the time of experiment

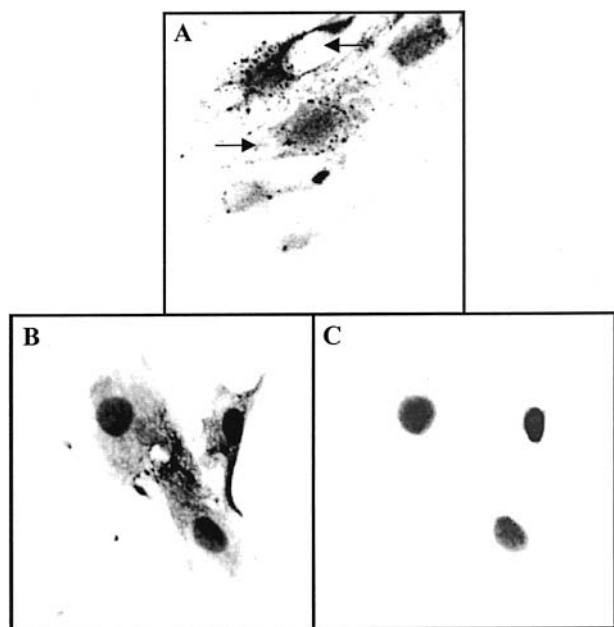


Figure 4. Visualization by confocal microscopy of the fluorescent porphyrin derivative (trisMePyP-CH₂-I) after 5 min of incubation on human fibroblasts (HS68) at 37 °C. (A) porphyrin alone at 5 μ M (arrows indicate the nuclei). (B) porphyrin-peptide conjugate (trisMePyP-CH₂-S-peptide) at 5 μ M. (C) Same section as photo B but revealed by Hoechst 33258 to show the nuclei.

(48 h) leading to an IC₅₀ of 5 μ M. Also note that the peptide alone induced just a slight cell growth inhibition when at concentrations above 5×10^{-5} M. The consequence of conjugation of porphyrin to the peptide (see Figure 5), was also corroborated by following the inhibition of cell growth with time. Indeed, Figure 6 reveals that at a concentration of 0.5 μ M the conjugate inhibited almost totally the cell growth while the peptide or the metalloporphyrin alone did not show any significant difference compared with the reference.

Mechanism of Cell Growth Inhibition. *In Vitro DNA Cleavage.* To elucidate the mechanism of cell growth inhibition and evaluate the possible consequence of

conjugation on cleaving site selection, the effect of the metalloporphyrin- or the conjugate-induced cleavage of various double-stranded DNAs was studied. Figure 7 shows an agarose gel analysis of phage λ DNA (digested by Hind III) after incubation with metalloporphyrin (panel A), the conjugate (panel B), and the free peptide (panel C). Clearly, incubation of the DNA with the free peptide did not modify the migration profile. Comparison of panels A and B indicates that the metalloporphyrin cleavage efficiency is slightly improved when in the conjugate form (500 ng of DNA are fully cleaved with 4 μ M of conjugate while 8 μ M are required for free metalloporphyrin).

For the identification of the cleaving site (manganese porphyrin cleaves preferentially AT rich sites), the same experiment as above was carried out using double-stranded oligonucleotides previously reported (16) (Figure 8). These oligonucleotides have well-defined chemical structure (Figure 9): ODN 1 contains a single AAA site while this site has been modified into AGA for ODN 2. In contrast to the situation described above the cleaving activity of the conjugate appeared to be lower than that of the free form. In addition, it is also seen that when cleavage occurs it concerns the AAA site and that conjugation may improve the specificity for this site since no significant cleavage occurs for ODN 2 with the conjugate contrary to free metalloporphyrin (compare lanes 7 and 14 of Figure 8 panel B).

In Vivo DNA Cleavage. To deepen the understanding of cell growth inhibition, and also to make sure that this inhibition was effectively due to the genomic DNA cleavage, a comparative study with metalloporphyrin, peptide, or conjugate was carried out on L1210 cells. Cancerous cells have been treated by these compounds at three different concentrations, and the genomic DNA has been analyzed using agarose gel electrophoresis (Figure 10). All samples except for the conjugate showed a profile composed of large molecular weight DNA bands (≈ 24 kbp), smears (corresponding to genomic DNA fragments), and an intense band corresponding to low molecular weights DNAs (100 bp). For the metalloporphyrin or peptide alone, no DNA cleaving activity could be

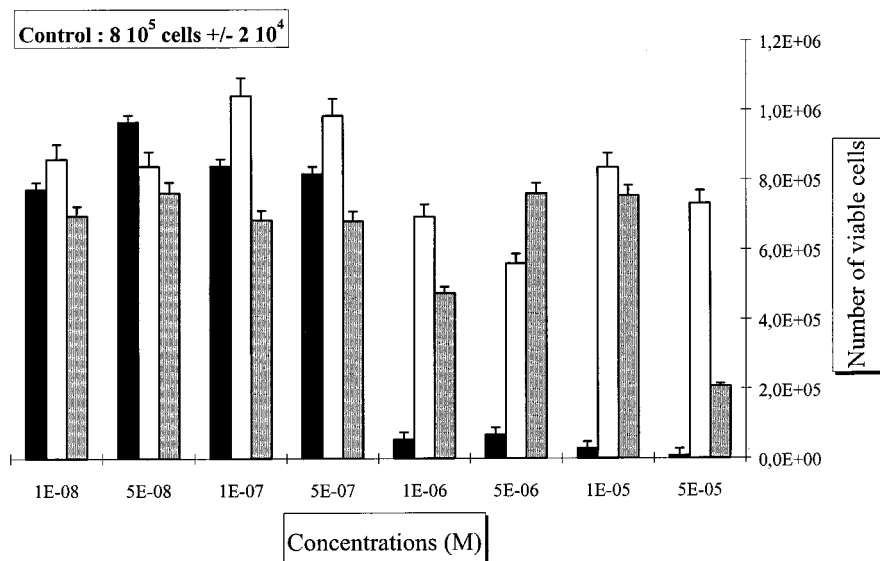


Figure 5. Growth inhibition of cancerous cells (mouse leukemia, L1210) by the metalloporphyrin (Mn-trisMePyP-CH₂-I) and the metalloporphyrin-peptide conjugate (Mn-trisMePyP-CH₂-S-Peptide). After 15 min of incubation on cells at increasing concentrations (from 10^{-8} M to 5×10^{-5} M), cells were washed with PBS and incubated in culture medium for additional 48 h. Then, viable cells were counted with a coulter counter in order to determine the growth inhibition induced by metalloporphyrin (white bars), peptide (grey bars), and the conjugate (black bars).

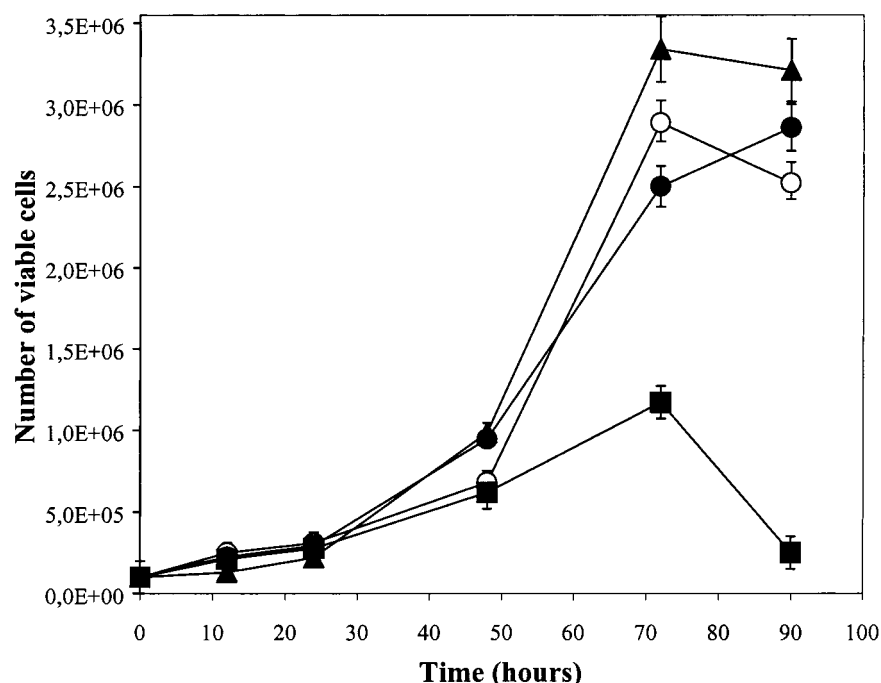


Figure 6. Growth inhibition of cancerous cells (mouse leukemia, L1210) by the metalloporphyrin (Mn-trisMePyP-CH₂-I) and the metalloporphyrin-peptide conjugate (Mn-trisMePyP-CH₂-S-Peptide) at a concentration of 0.5 μ M versus time after 15 min of incubation. After washing with fresh medium cells were reincubated at 37 °C and after 12, 24, 48, 72, and 96 h of culture, cells were counted in order to follow the influence of these compounds during five cycles of cell division (L1210 division cycle $\approx$ 17 h). Symbols used: control (●), peptide (○), metalloporphyrin (▲), and metalloporphyrin-peptide conjugate (■). Initial number of cells for each sample was 10^5 .

detected, except when the peptide was at high concentration. This result is in good agreement with that obtained for the cell growth inhibition. As expected, only the conjugate could induce substantial DNA cleavage, and the profile was plainly different in comparison with peptide or metalloporphyrin (compare lanes from 2 to 7 and lanes from 8 to 10 in Figure 10). Here the appearance of low molecular weights band indicates a strong DNA cleaving activity.

DISCUSSION

In the present study we have shown that covalent linkage of metalloporphyrin to a carrier peptide facilitates its cellular uptake. Within 5 min most of the fluorescence arising from the metal-free porphyrin was localized in the nuclei of more than 80% of the cells. When nonassisted, about 2 h of incubation were required for internalization in a very reduced number of cells (about 3%) with a final localization, which was cytoplasmic and not nuclear. This indicates that, besides an improvement of the cellular uptake, the peptide also governed the final intracellular localization of the drug. It must be noted that this carrier system is actually the fastest one in comparison with the only shuttle used in an antitumor approach, which requires an incubation time of 6 h to deliver the drug to the nucleus (9). The fact that the peptide induced a nuclear localization of the porphyrin improved its biological activity as its target DNA is mostly within the nucleus.

The expected effect of peptide conjugation resulted in greater cell growth inhibition compared with the free form of the metalloporphyrin. Experiments were carried out on L1210 since this cell line has been already used for similar experiments with the metalloporphyrin and metalloporphyrin-ellipticine derivatives. The results presented here appeared to conflict with those previously reported for the IC₅₀ observed in the presence of the free

metalloporphyrin (IC₅₀ = 0.6 μ M). Indeed, in our experiments, nondriven metalloporphyrin appears to be almost inactive against L1210 cells. It must, however, be reminded that the incubation time used for the experiments reported here is considerably lower than that used in reference 25. To obtain the same activity as previously reported for the metalloporphyrin alone, an incubation time of 24 h is required. For the conjugate, 15 min of incubation was sufficient to inhibit cell growth at a concentration of about 0.5 μ M. Concerning the intrinsic toxicity induced by the peptide at high concentrations (>50 μ M), the present result is in line with previous observations made on mollicutes (26) and which were attributed to cell depolarization due to the formation of ionic channels (27).

As to the mechanism inducing cell growth inhibition activity in vitro, high molecular weight DNA cleaving experiments show that conjugation of metalloporphyrin enhances its cleaving activity. In fact, owing to the chemical structure of the vector peptide (it contains five positively charged residues) it appears logical that such a peptide will favor the interactions with nucleic acids. In addition, the metalloporphyrin cleaving activity has been shown to occur at the DNA minor groove (17), and the presence of the peptide increased its activity. These observations favor therefore the idea of the existence of a nonfolded conjugate since an α -helical conformation of the peptide cannot accommodate, for steric reasons, the minor groove.

For double-stranded oligonucleotides, containing or not, only one putative cleaving site, the situation differs from that found for high molecular weight DNA since nonbound metalloporphyrin appeared to be more efficient than the conjugate from the point of view of the cleavage. In addition, cleavage also occurred for the site-free oligonucleotide as already reported but for higher amounts of the cleaving agent. For the conjugate, significant

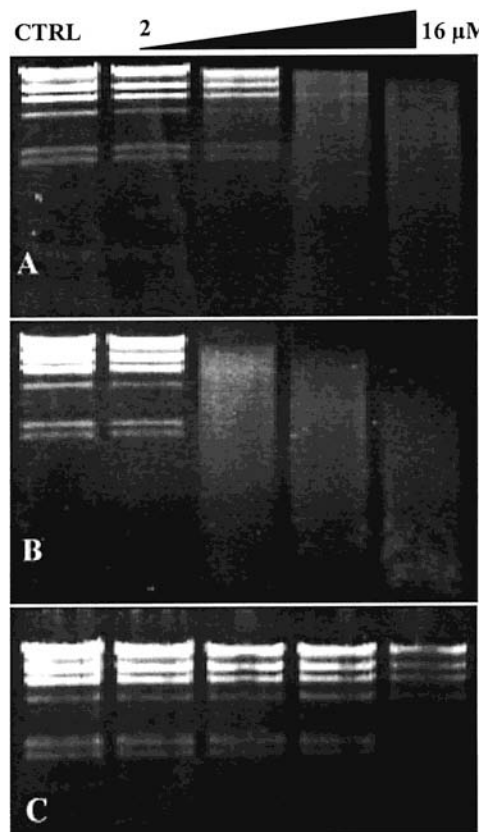


Figure 7. Agarose gel analysis of λ phage/Hind III DNA digestion by the metalloporphyrin Mn-trisMePyP-CH₂-I (A) and the metalloporphyrin-peptide conjugate Mn-trisMePyP-CH₂-S-peptide (B). The peptide alone (C) was checked as control and did not induce any DNA cleavage. Metalloporphyrin or metalloporphyrin-peptide conjugate were used at increasing concentrations (2, 4, 8, and 16 μ M). First, λ /Hind III DNA (500 ng) was composed of six fragments and was fully digested from 4 μ M in conjugate.

cleavage was observed only for the site-containing oligonucleotide. This result rather suggests that conjugation improves the specificity for the cleaving site. Although the reasons of the difference observed in vitro between high molecular weight DNA and oligonucleotides are still unclear, it is tempting to attribute this difference to a protecting role of the peptide. Such a hypothesis is supported by observations carried out on other primary amphipathic peptides containing the same hydrophilic domain as the peptide described here. In that case it was shown that this type of peptide can form complexes with nucleic acids and that the nucleic acid was coated by the peptide preventing thus digestion by nucleases (6, 28). The protective role of the peptide should be more efficient in the case of oligonucleotides rather than high molecular weight DNA. Moreover, in this latter case such a protection certainly also occur but owing to the length of this DNA, the number of specific cleaving sites is exceedingly high favoring thus the cleaving activity of the metalloporphyrin.

For genomic DNA extracted from cells beforehand treated by these compounds, the results we obtained indicate that only the conjugate was able to cleave DNA into cells. Thus, the presence of the peptide linked to the metalloporphyrin is fully needed in order to improve the cell delivery of this antitumor agent. Without this carrier, metalloporphyrin was unable to cross the cellular membrane of cells and therefore cannot act as a DNA cleaving agent. Moreover for the conjugate, the presence of the carrier peptide does not induce a loss of metalloporphyrin

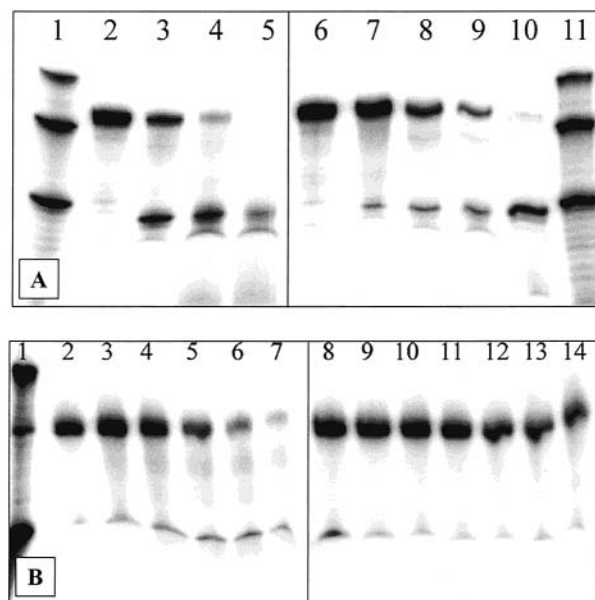


Figure 8. Analysis by polyacrylamide gel electrophoresis of the cleavage of duplex ODN1 and ODN2 by the metalloporphyrin (Mn-trisMePyP-CH₂-I) and the conjugate (Mn-trisMePyP-CH₂-S-peptide) in order to verify the sequence specificity of the cleavage. (A): Duplex ODN1 with the sequence specific 3AT. Lane 1: molecular weights, 45, 36, and 21 bp. Lane 2: ODN1 alone. Lanes 3, 4, and 5: ODN1+ metalloporphyrin (with respectively 0.5, 2, and 8 equiv). Lane 6: ODN1 + carrier peptide. Lanes 7, 8, and 9: ODN1 + metalloporphyrin-peptide conjugate (with respectively 0.5, 2, and 8 equiv). Lane 10: ODN1 + BglI. Lane 11: molecular weights: 45, 36, and 21 bp. (B): Duplex ODN2 without the sequence specific 3AT. 1: molecular weights: 45, 36, and 21 bp. Lane 2: ODN2 alone. Lanes 3, 4, 5, 6, and 7: ODN2 + metalloporphyrin (with respectively 0.5, 1, 2, 4, and 8 equiv). Lane 8: ODN2 alone. Lane 9: ODN2 + carrier peptide. Lanes 10, 11, 12, 13, and 14: ODN2 + metalloporphyrin-peptide conjugate (with respectively 0.5, 1, 2, 4, and 8 equiv).



Figure 9. Sequence of the two duplex oligonucleotides (called ODN 1 and ODN 2) used in this study in order to determinate the sequence-specific cleavage activity of the metalloporphyrin.

activity after the cell delivery step. This result obviously shows the improvement of antitumor drug activity in terms of concentration and time of incubation by using such a carrier. One problem remaining for the development of useful manganese porphyrin as a chemical DNA restriction tool is that the (A-T)₃ cleaver affinity sequence is often encountered on random double-stranded DNA. One way to increase specificity toward a single site on DNA is to covalently link the metalloporphyrin to a sequence recognition agent, such as oligonucleotide or a peptide-nucleic acid (17, 29, 30). However, although these molecules were able to target a specific sequence of DNA in vitro, they still remained poorly permeable to cellular membrane. Future studies should concentrate on using such cleaver agent with a conjugate containing metalloporphyrin, oligonucleotide, and carrier peptide, which may promote cell uptake and highly specific DNA cleavage.

The use of a carrier peptide for drug delivery is rapidly becoming a very useful strategy. Either by complex formation between the carrier and the drug or by covalent linkage of the drug to a carrier peptide, cellular delivery

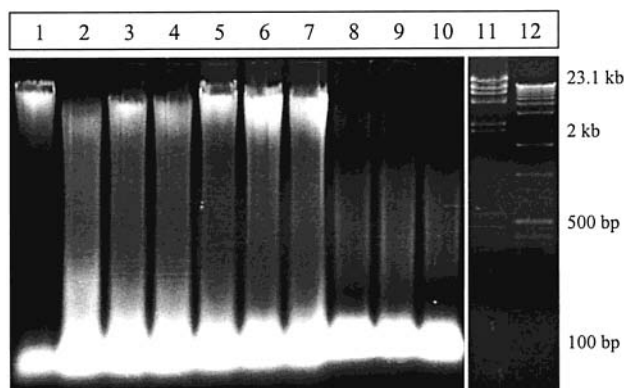


Figure 10. Agarose gel analysis (0.8%) of the genomic DNA extracted from cells beforehand treated or not with metalloporphyrin (Mn-trisMePyP-CH₂-I), peptide or metalloporphyrin-peptide conjugate (Mn-trisMePyP-CH₂-S-Peptide). L1210 cells have been incubated in the presence of the different compounds for 15 min, washed, and put back for growing at 37 °C during 48 h. Cell lysis and genomic DNA extraction have been performed as described in Materials and Methods. Lane 1: DNA extracted from nontreated cells. DNA extracted from cells treated with the peptide (lanes 2–4) or the metalloporphyrin (lanes 5–7) or the metalloporphyrin-peptide conjugate (lane 8–10) at 0.5, 5, and 50 μ M, respectively. Lanes 11 and 12: molecular weight markers corresponding to λ DNA/Hind III and 1 Kb DNA Ladder, respectively.

can be increased markedly. In the present case we have shown that the vector can improve considerably the cellular delivery of metalloporphyrin (by a factor of 100) with a reduced time of incubation (5 min). The increased "transfection" yield of about 80% resulted in higher metalloporphyrin activity. The mechanism of cell growth inhibition is certainly due to an increase of the DNA cleaving activity with a very distinct contrast between the metalloporphyrin alone and the conjugate. The mean reasons of this difference lies in a better cell uptake and peptide-induced improvement of the DNA binding of the metalloporphyrin. It is the first time that this has been shown *in vivo*. Furthermore, the carrier peptide appears to be a very promising strategy to improve the cellular delivery of other existing antitumor drugs including those already used in human therapy.

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Further Studies on the Site-Specific Protein Modification by Microbial Transglutaminase

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A guinea pig liver transglutaminase (G-TGase)-mediated procedure for the site-specific modification of chimeric proteins was recently reported. Here, an alternative method with advantages over the recent approach is described. This protocol utilizes a microbial transglutaminase (M-TGase) instead of the G-TGase as the catalyst. M-TGase, which has rather broad structural requirements as compared to the G-TGase, tends to catalyze an acyl transfer reaction between the γ -carboxamide group of an intact protein-bound glutamine residue and various primary amines. To demonstrate the applicability of the M-TGase-catalyzed protein modification in a drug delivery system, we have utilized recombinant human interleukin 2 (rhIL-2) as the target protein and two synthetic alkylamine derivatives of poly(ethyleneglycol) (PEG12; MW 12 kDa) and galactose-terminated triantennary glycosides ((Gal)₃) as the modifiers. For the M-TGase-catalyzed reaction with PEG12 and (Gal)₃, 1 mol of alkylamine was incorporated per mole of rhIL-2, respectively. Peptide mapping of (Gal)₃-modified rhIL-2 ((Gal)₃-rhIL-2) by liquid chromatography–electrospray ionization mass spectrometry (LC-ESI/MS) suggested that the Gln74 residue in rhIL-2 was site specifically modified with (Gal)₃. The PEG12–rhIL-2 and (Gal)₃–rhIL-2 conjugates retained full bioactivity relative to the unmodified rhIL-2. In pharmacokinetic studies, PEG12–rhIL-2 was eliminated more slowly from the circulation than rhIL-2, whereas (Gal)₃–rhIL-2 accumulated in the liver via hepatic asialoglycoprotein receptor binding. The results of this study expand the applicability of the TGase-catalyzed methodology for the preparation of protein conjugates for clinical use.

INTRODUCTION

Our goal is to develop methods for site-specific protein modification in order to generate protein conjugates for clinical use. The advent of recombinant DNA technology has brought with it the rapid development of protein therapeutics. Cytokines and other biological response modifiers, thrombolytics, adhesion molecules, agonist and antagonist peptide fragments of growth factors, and receptors all have widespread applications. The full exploitation of this new and powerful therapeutic armory requires solutions to several problems. First, most parenterally administered proteins are rapidly cleared from the circulation by the reticuloendothelial system, kidney, spleen, or liver. Second, the targeting efficiency of the administered proteins to the site of action is low. Attaching poly(ethyleneglycol) (PEG, also called polyoxyethylene (POE))¹ or an organ specific targeting device specifically to the proteins can improve the therapeutic utility by controlling the pharmacokinetic profiles in the body (1, 2, 3, 4). In this aspect, the product homogeneity can play an essential role in the licensing aspects of the protein conjugates as therapeutic agents.

The most popular method is the thiol-selective modification of an introduced cysteine residue by using site-directed mutagenesis and either maleimide-based or haloacetic-based reagents (5, 6, 7). A less popular method is the modification of vicinal OH and NH₂ groups on N-terminally introduced serine or threonine residues by using site-directed mutagenesis and aminooxy-function-alizing reagents (8, 9). In addition to the above methods,

we have recently devised a novel method for the site-specific incorporation of alkylamine derivatives into specific glutamine residues by using genetic engineering techniques and guinea pig liver transglutaminase (G-TGase) (10).

This method is superior to the aforementioned procedures, because of its special limitation to glutamine residues for the incorporation and its mild reaction conditions. In fact, thiol-reactive reagents can modify the side chains of histidine and lysine, and the α -amino groups of the peptide to a lesser extent, whereas N-terminal oxidation with periodate can potentially damage the protein. Furthermore, our G-TGase-catalyzed method is applicable to the dual and site-specific incorporation by using a special substrate sequence (TG2; AQQIVM) for G-TGase as the appended tag (11, 12). In fact, a chimeric protein of human interleukin 2 (rTG2-IL-2), in which TG2 was fused to the N-terminus of IL-2, was

¹ Abbreviations: TGase, transglutaminase; M-TGase, microbial TGase; G-TGase, guinea pig liver TGase; IL-2, interleukin 2; hIL-2, human IL-2; rhIL-2, recombinant hIL-2; PEG, poly(ethyleneglycol); PEG12, poly(ethyleneglycol) donor substrate (average MW, 12 kDa); (Gal)₃, galactose-terminated triantennary glycosides; (Gal)₃-rhIL-2, conjugate between (Gal)₃ and rhIL-2; PEG12–rhIL-2, conjugates between PEG12 and rhIL-2; ESI/MS, electrospray ionization mass spectrometry; LC-ESI/MS, liquid chromatography–ESI/MS; ASGP-R, asialoglycoprotein receptor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Gln, glutamine; CTLL, cytotoxic T lymphocyte line; TFA, trifluoroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; RP-HPLC, reversed-phase high-performance liquid chromatography; MW, molecular weight.

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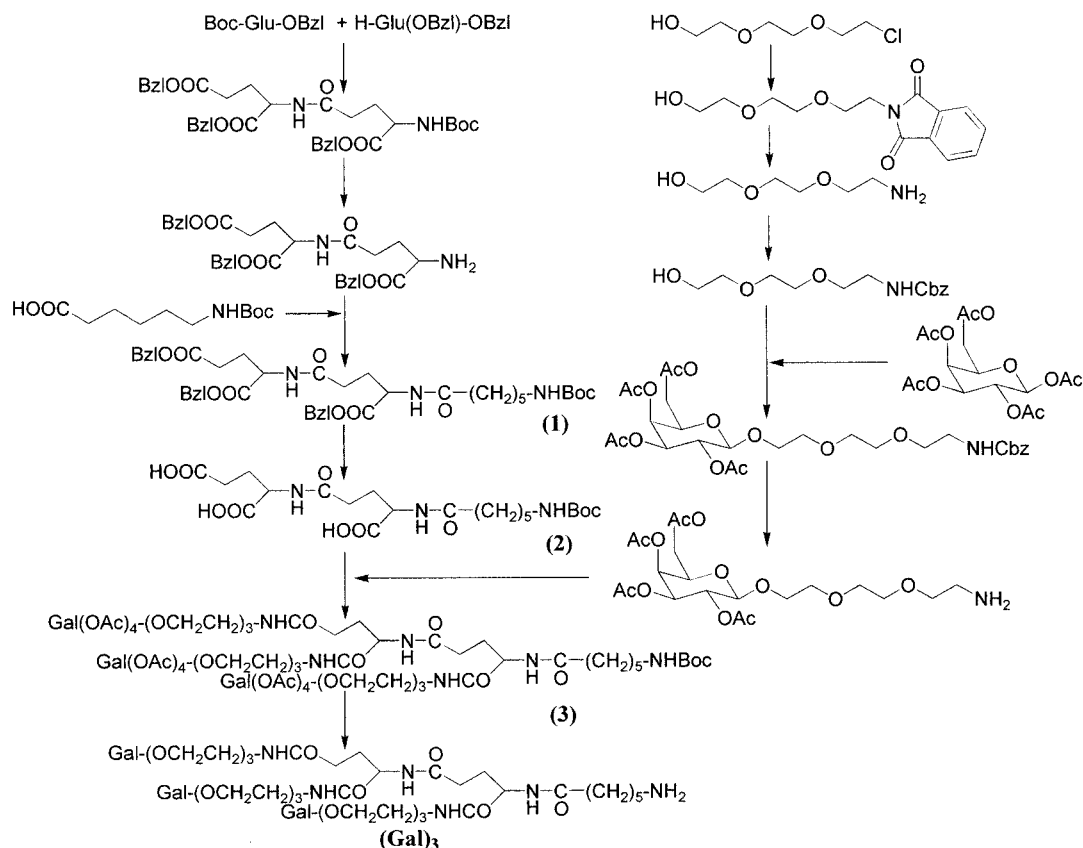


Figure 1. Synthesis of (Gal)₃.

dually and site-specifically modified with alkylamine derivatives of PEG (MW 10 kDa).

However, considering the therapeutic applications of our G-TGase-catalyzed method, one might fear some immunogenicity or antigenicity of the constructed chimeric proteins. To try to overcome the present limitations of our methodology, we have developed a more applicable method for the site-specific incorporation of alkylamines into intact proteins, by using a microbial transglutaminase (M-TGase) in place of G-TGase as the catalyst.

M-TGase, which is produced by the microorganism *Streptovorticillium sp.* strain s-8112, catalyzes the acyl transfer reaction between the γ -carboxyamides of glutamine residues in proteins and various primary amines in the same way as mammalian TGases (13, 14). The amino acid sequence of the M-TGase is very different from those of mammalian TGases. Furthermore, M-TGase has a rather broader substrate specificity for amine acceptor glutamine substrates in proteins than those of the mammalian TGases in our preliminary studies.

To demonstrate the usefulness of the M-TGase for constructing protein conjugates, we have selected recombinant human interleukin 2 (rhIL-2) as a target protein. Although none of the six glutamine residues in rhIL-2 were available as substrates for G-TGase, as described before (10) one reactive Gln residue, which could serve as a substrate for M-TGase, was identified in this paper. For conjugation studies, we have used two model substrates as the circulating carrier and the targeting device. One is a synthetic poly(ethyleneglycol) derivative (PEG12, MW: 12 kDa) with a straight chain alkylamine at one end, which is a useful polymer for prolonging the circulating lifetimes of proteins in vivo, as described before (10, 12). The other is a galactose-terminated triantennary glycoside with a straight chain alkylamine

at one end ((Gal)₃) (15). (Gal)₃ is an artificial ligand for the hepatic asialoglycoprotein receptor (ASGP-R), which is uniquely localized on hepatocytes and recognizes exposed and branched galactose residues on serum glycoproteins (16, 17). Since the uptake of glycoproteins by this receptor is both a high-affinity and high-capacity process, (Gal)₃ would be utilized as a homing device to target proteins specifically around hepatocytes.

We report here the stoichiometrically precise, site-specific incorporation of alkylamine derivatives (PEG12 and (Gal)₃) into intact rhIL-2 by using M-TGase. To demonstrate the modification site in rhIL-2, peptide mapping of (Gal)₃-modified rhIL-2 ((Gal)₃-rhIL-2) by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI/MS) was carried out. We further compared the biological activities and the pharmacokinetic profiles of PEG12-modified rhIL-2 (PEG12-rhIL-2) and (Gal)₃-rhIL-2 to those of unmodified rhIL-2.

MATERIALS AND METHODS

Materials. M-TGase was purified from the culture supernatant of *Streptovorticillium sp.* s-8112 according to the modified method of Ando et al. (14). α -Carboxymethyl- ω -methoxypolyoxyethylene, with an average molecular weight of 12 kDa, was purchased from Nippon Oils & Fats Co., Ltd., Japan. rhIL-2 was purified from inclusion bodies, as described by Tsuji et al. (18). Other chemicals were of reagent grade.

Synthesis of PEG12. The artificial substrate PEG12 was synthesized from α -carboxymethyl- ω -methoxypolyoxyethylene and *N*-Boc-1,5-diaminopentane (Fluka, Buchs, Switzerland) as described (10).

Synthesis of (Gal)₃. The synthesis of (Gal)₃ was carried out according to the modified method of Murahashi et al. (15) as follows (Figure 1).

(a) Synthesis of 2-[2-(2-Aminoethoxy)ethoxy]ethanol. 2-[2-(2-Chloroethoxy)ethoxy]ethanol (36.5 mL, 251 mmol) was added to a solution of potassium phthalimide (50.6 g, 273 mmol) in dimethylformamide (300 mL), and the solution was stirred and heated with an oil bath (100 °C) for 17 h. The suspension was cooled to room temperature and was filtered to remove the precipitate. The filtrate was concentrated under reduced pressure and was extracted with dichloromethane (300 mL). The extract was concentrated under reduced pressure to afford *N*-[2-(2-hydroxyethoxy)ethoxy]ethylphthalimide as a yellow oil: ^1H NMR (300 MHz, CDCl_3) δ 2.58 (br, 1H), 3.51–3.57 (m, 2H), 3.57–3.71 (m, 2H), 3.72–3.80 (m, 2H), 3.87–3.96 (m, 2H), 7.68–7.80 (m, 2H), 7.82–7.90 (m, 2H); ^{13}C NMR (75 MHz, CDCl_3) δ 37.19, 61.72, 67.91, 69.98, 70.32, 72.43, 123.25, 132.06, 133.95, 168.34.

The above product was dissolved in ethanol (1.2 L). To this solution was added 80% hydrazine hydrate (16.8 mL, 277 mmol), and the mixture was mechanically stirred and heated under reflux for 2 h. The reaction mixture was cooled to room temperature and was filtered to remove the precipitate. The filtrate was concentrated under reduced pressure and was extracted with dichloromethane (500 mL). The extract was concentrated under reduced pressure to afford 2-[2-(2-aminoethoxy)ethoxy]ethanol as a yellow oil: yield 34.2 g; ^1H NMR (300 MHz, CDCl_3) δ 2.90 (t, $J = 5.2$ Hz, 2H), 3.18 (br, 3H), 3.53–3.70 (m, 8H), 3.70–3.77 (m, 2H); ^{13}C NMR (75 MHz, CDCl_3) δ 41.33, 61.46, 70.11, 70.35, 72.63, 72.66.

(b) Synthesis of 2-[2-[2-(Benzyloxycarbonylamino)ethoxy]ethoxy]ethanol. 2-[2-(2-Aminoethoxy)ethoxy]ethanol (34.2 g) was dissolved in distilled water (500 mL) and was cooled with an ice–water bath. To this solution were added alternately, in about five equal portions, sodium bicarbonate (19.5 g) and benzyloxycarbonyl chloride (125 mL, 33% solution in toluene), and the resulting mixture was stirred at room-temperature overnight. The reaction mixture was separated from the aqueous layer, which was extracted with ethyl acetate (100 mL) twice. The combined organic phases were washed with saturated aqueous sodium chloride, dried over anhydrous magnesium sulfate, and concentrated under reduced pressure. The residue was purified by column chromatography over silica gel, which was eluted with ethyl acetate to give 2-[2-[2-(benzyloxycarbonylamino)ethoxy]ethoxy]ethanol as a colorless oil; yield 32.1 g; ^1H NMR (300 MHz, CDCl_3) δ 2.58 (br, 1H), 3.35–3.44 (m, 2H), 3.54–3.67 (m, 8H), 3.71 (br, 2H); 5.10 (s, 2H), 5.47 (br, 1H), 7.27–7.42 (m, 5H); ^{13}C NMR (75 MHz, CDCl_3) δ 40.82, 61.65, 66.65, 70.08, 70.28, 70.34, 72.51, 128.05, 128.09, 128.46, 136.52, 156.43.

(c) Synthesis of 1-O-[2-[2-(Benzyloxycarbonylamino)ethoxy]ethoxy]ethyl- β -D-galactopyranose Tetraacetate. Thirty-two grams of 2-[2-[2-(benzyloxycarbonylamino)ethoxy]ethoxy]ethanol and 22 g of β -D-galactopyranose pentaacetate were dissolved in dichloromethane (400 mL), which was cooled in an ice–water bath. To this mixture was added a boron trifluoride diethyl ether complex (13.9 mL) dropwise, and the resulting mixture was stirred at room-temperature overnight. The reaction mixture was washed with saturated aqueous sodium bicarbonate, dried over anhydrous magnesium sulfate, and concentrated under reduced pressure. The crude product was purified by column chromatography over silica gel, which was eluted with ethyl acetate/*n*-hexane (1:1 $\rightarrow$ 2:1, v/v) to give 1-O-[2-[2-(benzyloxycarbonylamino)ethoxy]ethoxy]ethyl- β -D-galactopyranose tetraacetate as a colorless oil; yield 13.7 g; ^1H NMR (300 MHz, CDCl_3) δ 1.98 (s, 3H), 2.04 (s, 6H),

2.14 (s, 3H), 3.40 (dd, $J = 5.2, 10.4$ Hz, 2H); 3.52–3.78 (m, 9H), 3.85 (br t, $J_{\text{app}} = 7.1$ Hz, 1H), 3.95 (m, 1H), 4.06–4.24 (m, 2H), 4.52 (d, $J = 7.9$ Hz, 1H), 5.00 (dd, $J = 3.5, 10.3$ Hz, 1H), 5.11 (s, 2H), 5.20 (dd, $J = 7.9, 10.5$ Hz, 1H), 5.37 (br d, $J_{\text{app}} = 3.5$ Hz, 1H), 7.3–7.4 (m, 5H).

(d) 1-O-[2-(2-(Aminoethoxy)ethoxy)ethoxy]- β -D-galactopyranose Tetraacetate. To a solution of 1-O-[2-[2-(benzyloxycarbonylamino)ethoxy]ethoxy]ethyl- β -D-galactopyranose tetraacetate (13.7 g) in ethanol (300 mL) was added *p*-toluenesulfonic acid monohydrate (4.27 g). The mixture was added to 10% palladium on carbon (1.0 g) under argon and was stirred under atmospheric pressure of hydrogen at room-temperature overnight. After removing the hydrogen under reduced pressure and changing the atmosphere to argon, the reaction mixture was filtered to remove the palladium on carbon and was evaporated. The residue was dissolved in acetonitrile (300 mL), and the solution was evaporated under reduced pressure. This procedure was repeated twice to remove the residual ethanol. The residue was dissolved in acetonitrile (40 mL) again, and 4-methylmorpholine (2.5 mL) was added to this solution to afford a solution of 1-O-[2-(2-(aminoethoxy)ethoxy)ethyl- β -D-galactopyranose tetraacetate (solution A).

(e) Synthesis of *tert*-Butyloxycarbonyl-L-glutamyl-L-glutamic Acid α,γ -Benzyl Ester. Boc-L-Glu-OBzl (4.73 g, Peptide Institute, Inc., Osaka, Japan) and *N*-methylmorpholine (1.54 mL) were dissolved in tetrahydrofuran (100 mL). The solution was cooled with a dry ice–ethanol bath. Ethyl chloroformate (1.35 mL) was added, and the mixture was stirred for 5 min. To the reaction mixture was slowly added a mixture of L-Glu-(OBzl)-OBzl *p*-toulenesulfonate (7.0 g, Peptide Institute, Inc., Osaka, Japan) and *N*-methylmorpholine (1.54 mL) in dimethylformamide (50 mL). The dry ice–ethanol bath was removed and replaced with a sodium chloride–ice bath, and then the reaction mixture was stirred overnight and allowed to warm gradually to room temperature. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure. The residue was dissolved in ethyl acetate and was washed successively with aqueous 10% citric acid, saturated aqueous sodium chloride, saturated aqueous sodium bicarbonate, and saturated aqueous sodium chloride. The organic phase was dried over anhydrous magnesium sulfate and was concentrated under reduced pressure to give 8.85 g of the *tert*-butyloxycarbonyl-L-glutamyl-L-glutamic acid α,γ -benzyl ester as pale yellow crystals; ^1H NMR (300 MHz, CDCl_3) δ 1.40 (s, 9H), 1.86–1.94 (m, 1H), 1.98–2.06 (m, 1H), 2.1–2.3 (m, 4H), 2.3–2.5 (m, 2H), 4.30–4.35 (m, 1H), 4.62–4.64 (m, 1H), 5.00–5.20 (m, 6H), 5.25 (d, $J = 7.0$ Hz, 1H), 6.41 (d, $J = 7.0$ Hz, 1H), 7.2–7.3 (m, 15H).

(f) Synthesis of Compound 1. *tert*-Butyloxycarbonyl-L-glutamyl-L-glutamic acid α,γ -benzyl ester (8.85 g) was dissolved in trifluoroacetic acid (25 mL), stirred for 3 h under room temperature, and then concentrated under reduced pressure. The residue was dissolved in ethanol (200 mL), and the solvent was evaporated under reduced pressure to remove the residual trifluoroacetic acid. After repetition of this procedure, the obtained residue was dissolved in methanol (25 mL) and was neutralized with *N*-methylmorpholine. The solvent was evaporated, and the residue was dissolved in dichloromethane (220 mL) (solution B).

N-Boc-6-aminoheptanoic acid (3.16 g) was dissolved in dichloromethane (220 mL) and was cooled with an ice–water bath. To this solution were added *N*-hydroxysuccinimide (1.73 g), dicyclohexylcarbodiimide (3.1 g), and solution B, and then the resulting mixture was stirred

at room temperature overnight. The precipitate was removed from the reaction mixture by filtration, and the filtrate was washed successively with aqueous 10 % citric acid, aqueous saturated sodium chloride, and aqueous 10 % sodium bicarbonate, dried with anhydrous magnesium sulfate, and was finally concentrated under reduced pressure. The crude product was purified by column chromatography over silica gel, which was eluted with *n*-hexane/ethyl acetate (1:1 → 1:2, v/v) to give compound **1** as a colorless solid; yield 4.24 g; ^1H NMR (300 MHz, CDCl_3) δ 1.30–1.34 (m, 2H), 1.39–1.48 (m, 11H), 1.60–1.64 (m, 2H), 1.93–2.06 (m, 2H), 2.13–2.26 (m, 6H), 2.34–2.48 (m, 2H), 3.06–3.10 (m, 2H), 4.55–4.65 (m, 3H), 5.06–5.20 (m, 6H), 6.50–6.60 (br, 2H), 7.3–7.4 (m, 15H).

(g) Synthesis of Compound 2. Ten percent of palladium on active carbon (900 mg) was added to the solution of compound **1** (4.24 g) in tetrahydrofuran (150 mL) and ethyl acetate (150 mL), and then the mixture was stirred under an atmospheric pressure of hydrogen at room-temperature overnight. The reaction mixture was filtered to remove the catalyst and was concentrated under reduced pressure to give compound **2** as a colorless solid; yield 3.39 g; ^1H NMR (300 MHz, CDCl_3) δ 1.34–1.52 (m, 13H), 1.61–1.67 (m, 2H), 1.91–2.00 (m, 2H), 2.15–2.24 (m, 2H), 2.26 (t, $J = 7.5$ Hz, 2H), 2.35–2.43 (m, 4H), 3.03 (t, $J = 7.0$ Hz, 2H), 4.39 (dd, $J = 5.0, 9.0$ Hz, 1H), 4.44 (dd, $J = 5.0, 9.0$ Hz, 1H).

(h) Synthesis of Compound 3. Compound **2** (3.39 g) was dissolved in dimethylformamide (20 mL) and was cooled with an ice–water bath. To this solution were added *N*-hydroxysuccinimide (2.58 g) and dicyclohexylcarbodiimide (4.63 g), and the mixture was stirred for several minutes with cooling, after which solution A (1-*O*-[2-[2-(aminoethoxy)ethoxy]ethyl]- β -D-galactopyranose tetraacetate in acetonitrile) was added. The resulting mixture was stirred and cooled in a water bath overnight. The precipitate was removed by filtration, and the supernatant was then concentrated under reduced pressure. The residue was dissolved in ethyl acetate (300 mL) and was washed successively with an aqueous 10% citric acid solution, aqueous saturated sodium chloride, and aqueous saturated sodium bicarbonate. The organic phase was dried over anhydrous magnesium sulfate and was concentrated under reduced pressure. The crude product was purified by column chromatography over silica gel, which was eluted with dichloromethane/methanol (20:1 → 15:1, v/v) to give compound **3** as a colorless solid; yield 5.87 g; ^1H NMR (300 MHz, CDCl_3) δ 1.30–1.36 (m, 2H), 1.42 (s, 9H), 1.46–1.52 (m, 2H), 1.60–1.66 (m, 2H), 2.01 (s, 9H), 2.02 (s, 9H), 2.04 (s, 9H), 2.09 (s, 9H), 2.2–2.4 (m, 2H), 3.08–3.12 (m, 2H), 3.5–3.6 (m, 34H), 3.70–3.75 (m, 6H), 3.93–3.98 (m, 6H), 4.10–4.20 (m, 6H), 4.38–4.43 (m, 2H), 4.55–4.57 (m, 3H), 4.72–4.76 (m, 1H), 5.02–5.06 (m, 3H), 5.17–5.22 (m, 3H), 5.39 (d, $J = 3.5$ Hz, 3H), 6.58–6.62 (m, 1H), 7.04–7.08 (m, 1H), 7.12–7.18 (m, 1H), 7.24–7.26 (m, 1H), 7.80–7.85 (m, 1H).

(i) Synthesis of (Gal)₃. Compound **3** (5.87 g) was dissolved in 50% trifluoroacetic acid in dichloromethane (50 mL), stirred for 1 h under room temperature, and then concentrated under reduced pressure. The residue was dissolved in ethanol (200 mL) and was concentrated under reduced pressure to remove the residual trifluoroacetic acid. After repetition of this procedure, the resulting residue was dissolved in dichloromethane (30 mL), cooled with an ice–water bath, and then neutralized with 28% sodium methoxide in methanol. The solvent was evaporated under reduced pressure, and the remaining solution was subjected to column chromatography

over silica gel (dichloromethane/methanol = 30/1 → 5/1, v/v). The obtained product was dissolved in methanol (85 mL) and was cooled with an ice–water bath. To this solution was added 28% sodium methoxide in methanol (320 μL). This mixture was removed from the cooling bath and was stirred at room temperature for 5 h. The resulting mixture was cooled with an ice–water bath and was neutralized with Dowex 50W cation-exchange resin that had been washed with methanol. The resin was then removed by filtration. The filtrate was concentrated under reduced pressure to give 1.95 g of (Gal)₃ as an amorphous solid. The purity of the (Gal)₃ was more than 95% as analyzed by RP-HPLC with an Inertsil ODS-2 column (4.6 $\times$ 150 mm; GL Sciences Inc.), which was developed by a linear gradient of 4–20% acetonitrile containing 0.1% TFA for 30 min at a flow rate of 1 mL/min; ESI-MS; ($M + H$)⁺ 1270.0 (MW 1269.4 Da).

M-TGase-Catalyzed Modification of rhIL-2. The M-TGase-catalyzed modification of rhIL-2 was carried out with the following concentrations of reagents in 0.2 M Tris/HCl (pH 7.5) at 25 °C for 12 h: PEG12 or (Gal)₃, 1.25 mM; protein, 5 μM ; and M-TGase, 0.18 units/mL. At the end of the incubation, the reaction mixtures were analyzed by SDS–PAGE under the same conditions used for rTG1-IL-2 (10). To remove the residual free PEG12 and (Gal)₃, the reaction mixtures were absorbed onto a Sep-Pak C8 cartridge (Millipore Co., Milford, MA) according to the same method used for rTG1-IL-2 (10). The eluted protein fractions were directly applied to a YMC-C8-AP column (4.6 $\times$ 150 mm; YMC Co., Ltd.), which was developed by a linear gradient of 54%–60% acetonitrile containing 0.1% TFA for 30 min at a flow rate of 1 mL/min. Each fraction was collected, and the different protein peaks were separately pooled and analyzed by reversed-phase HPLC (RP-HPLC) and SDS–PAGE. After the analysis, most of the main peak fractions were directly applied to a Molcut L LGC column (Millipore Co., Milford, MA), and the buffer was exchanged with Dulbecco's phosphate-buffered saline (D-PBS). The samples were used for bioactivity and pharmacokinetic studies. The remainder of the main fraction for (Gal)₃-rhIL-2 in the RP-HPLC was used for peptide mapping.

Protease Digestion of Modified and Unmodified rhIL-2. Protease digestions of (Gal)₃-rhIL-2 and rhIL-2 were done according to the modified method for rhIL-2 (18). One hundred micrograms of reduced and S-carboxymethylated (Gal)₃-rhIL-2 and rhIL-2 were each suspended in 180 μL of 0.05 M ammonium hydrogen carbonate buffer (pH 7.9) and were treated with 20 μL of V8 protease solution (Wako Pure Chemical Industries, Ltd., 100 $\mu\text{g}/\text{mL}$) for 22 h at 37 °C. After lyophilization of the reaction mixtures, the dry samples were respectively suspended in 45 μL of 0.05 M ammonium hydrogen carbonate buffer (pH 7.9) and were treated with 5 μL of L-1-(tosylamido)-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma, 400 $\mu\text{g}/\text{mL}$) for 4 h at 37 °C.

ESI-MS Analysis. An LCQ iontrap mass spectrometer (Thermoquest Co., San Jose, CA) was used for all experiments. The MS detector settings are as follows: temperature of the heated capillary, 200 °C; spray voltage, 3 kV; scan range, 300–1850 m/z . The protease-digested peptides of modified and unmodified IL-2 were lyophilized to remove the buffer and were dissolved in 0.1% TFA. The lyophilized peptides were loaded onto a capillary chromatography column (Vydac C₁₈, 0.3 mm i.d. $\times$ 300 mm), and the separated peptides were subjected to an electrospray ion source in the LCQ spectrometer. To achieve a stable electrospray over the course of the gradient elution, 50% MeOH containing 1% acetic acid

was used as a sheath liquid at a flow rate of 3 $\mu\text{L}/\text{min}$. The elution conditions consisted of four successive linear increases of acetonitrile in 0.1% TFA, i.e., 4.5 to 4.5% in 10 min, 4.5 to 63% in 70 min, 63 to 81% in 5 min, and 81 to 81% in 5 min at a flow rate of 3 $\mu\text{L}/\text{min}$. Mass spectra were obtained by scanning the range of m/z 300–1850. The most abundant peptides were further analyzed by collisional-induced dissociation (CID) mass spectrometry.

In Vitro Biological Assays. The bioactivities of PEG12–rhIL-2, (Gal)₃–rhIL-2, and unmodified rhIL-2 were determined by using the IL-2 dependent murine cell line CTLL-2 (19). The amount of IL-2 activity was determined in units/mg by using the rhIL-2 standard (Collaborative Biomedical Products Co., Inc.) and was then expressed as the percent residual bioactivity as compared to the purified rhIL-2. All samples were tested in quadruplicate.

Pharmacokinetics of Modified rhIL-2. The modified and unmodified rhIL-2 were administered intravenously into male mice (C57BL/6, 6W, δ , Charles River Breeding Labs, Japan). At specific time intervals, described in the Results, the mice were bled and sacrificed, and the liver and kidney were excised. Blood was collected in heparin-coated 1.5 mL Eppendorf tubes. Plasma was immediately prepared by centrifugation at 4 $^{\circ}\text{C}$. Each organ was rinsed with ice-cold saline, weighed, and homogenized by adding enough isotonic phosphate buffer containing 0.2% BSA (pH 7.4) to yield a 10% homogenate (w/v). The IL-2 concentrations in plasma, liver, and kidney were determined by an ELISA procedure using the IL-2 Enzyme Immunoassay kit (Cayman Chemical Co., Michigan).

Analytical Methods. The M-TGase enzyme activity was measured by a colorimetric hydroxamate procedure with *N*-carbobenzoyl-L-glutamyl-glycine (20). SDS-PAGE was carried out with the PhastSystem (Amersham Pharmacia Biotech AB, Uppsala, Sweden) using a 20% (w/v) acrylamide gel under reducing conditions, according to the same procedure used for rTG1-IL-2 (10). The concentrations of the unmodified and modified forms of rhIL-2 were quantified by using an extinction coefficient (E_{280}) of $1.2 \times 10^4 \text{ M}^{-1}$. NMR spectra were determined at 300 MHz (^1H) with a spectrometer operating in the Fourier Transform (FT) mode. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as the internal standard.

RESULTS

Synthesis of (Gal)₃. The synthesis of (Gal)₃ includes three stages (Figure 1). The first step involves the preparation of the building block of glutamic acids. The second stage involves the incorporation of the tri(ethylene glycol) spacer into β -D-galactopyranose tetraacetate. In the final stage, the galactopyranosyl-derivatized compounds are introduced into the building block of glutamic acids. The results of the ESI-MS and RP-HPLC analyses of the reaction product indicate that (Gal)₃ is consistent with the assigned structure.

M-TGase-Catalyzed PEG12-Modification of rhIL-2. Figure 2A shows the SDS-PAGE analysis of the PEG12-incorporated product for rhIL-2. In the presence of PEG12 and M-TGase, the rhIL-2 band migrating at about 15 kDa is decreased, and one broad band, migrating at about 35 kDa, is visible (lane 3). The higher molecular weight band that is slightly visible at about 38 kDa could be due to M-TGase (MW 37.8 kDa). Since the time course analysis of the reaction products by SDS-PAGE revealed only one new band (data not

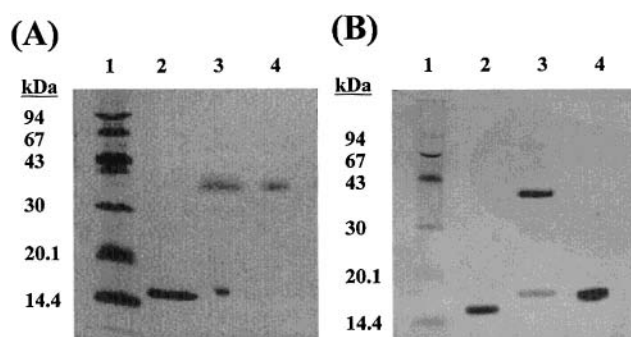


Figure 2. SDS-PAGE analysis of the M-TGase-catalyzed PEG12- (A) and (Gal)₃- (B) modified rhIL-2. (A) Lane 1; MW Marker Lane 2; unmodified rhIL-2 Lane 3; rhIL-2 + PEG12 + M-TGase Lane 4; purified PEG12–rhIL-2 (B) Lane 1; MW Marker Lane 2; unmodified rhIL-2 Lane 3; rhIL-2 + (Gal)₃ + M-TGase Lane 4; purified (Gal)₃–rhIL-2

shown), the band migrating at 35 kDa presumably represents the PEG12-incorporated rhIL-2, with a molar ratio of 1:1. The attachment of one PEG12 molecule to rhIL-2 causes a greater increase in size than that predicted from its molecular mass, presumably due to the bulkiness of the PEG12–rhIL-2 conjugate (PEG12–rhIL-2). After the separation of the modified and unmodified rhIL-2 by RP-HPLC (data not shown), the isolated PEG12–rhIL-2 migrated as a single band at about 35 kDa in lane 4 (yield: 29%).

M-TGase-Catalyzed (Gal)₃-Modification of rhIL-2. Figure 2B shows the SDS-PAGE analysis of the (Gal)₃-incorporated product for rhIL-2. The electrophoretic pattern of the reaction mixture demonstrates that the rhIL-2 band migrating at 15 kDa is almost invisible, and one new band, migrating at 17 kDa, is present (lane 3), indicating that one mole of (Gal)₃ per rhIL-2 is incorporated by M-TGase, similar to the PEG12-modification. The attachment of one (Gal)₃ (MW; 1269 Da) caused a reasonable increase (about 1000 Da) in size, representing the single (Gal)₃-incorporated product of rhIL-2 ((Gal)₃–rhIL-2). The M-TGase band at 38 kDa is stronger than that for the PEG12-modification in Figure 2A, due to differences in the enzyme activity/mg of the M-TGase used for the individual reactions.

To characterize the (Gal)₃–rhIL-2, we carried out the separation of the modified and unmodified rhIL-2 by RP-HPLC (Figure 3). Two separate peaks (a and b) were observed. The large peak that eluted at about 12 min contained M-TGase and (Gal)₃. Peak b corresponds to the unmodified rhIL-2 (data not shown). The protein band from peak a, which migrates at 17 kDa (lane 4 in Figure 2(B)), corresponds to the (Gal)₃–rhIL-2. A fraction of peak a (yield: 20%) was collected and used for characterization. Although the incorporation efficiency of (Gal)₃ was higher than that of PEG12, as determined by the SDS-PAGE analysis of the reaction mixture, the yield of the (Gal)₃–rhIL-2 was inversely lower than that of PEG12–rhIL-2. The reason for the low recovery is assumed to be the insufficiency of the separation between (Gal)₃–rhIL-2 and unmodified IL-2 in the RP-HPLC.

ESI-MS Analysis of (Gal)₃–rhIL-2. Figure 4 shows the LC-ESI/MS chromatography of the protease-digested peptides containing Gln residues of rhIL-2 and (Gal)₃–rhIL-2. The identities of the respective peaks containing Gln residues, together with the theoretical and measured molecular masses, are displayed in Table 1.

A comparison of the chromatography derived from rhIL-2 and (Gal)₃–rhIL-2 reveals that only VT-1 (residues 69–76, VLNLAQSK) was not observed in (Gal)₃–

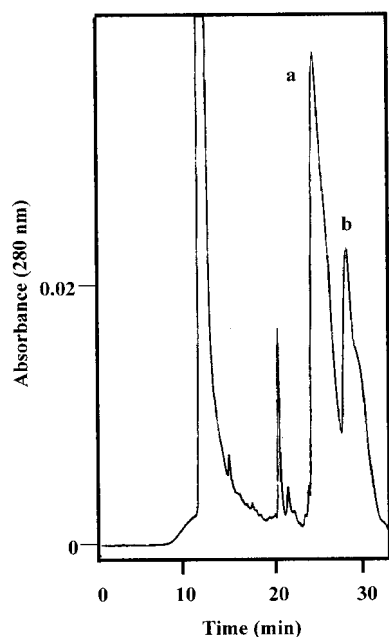


Figure 3. Purification of $(\text{Gal})_3$ -rhIL-2 on the YMC-C8AP column: a, peak containing $(\text{Gal})_3$ -rhIL-2; b, peak containing unmodified rhIL-2.

rhIL-2, and a new peak (VT-1(+ $(\text{Gal})_3$) was observed at 41 min. Since the ESI/MS spectra of the VT-1 (+ $(\text{Gal})_3$) gave a strong peak at m/z 1063.1, as the double-charged protonated molecular ion $[\text{M} + 2\text{H}]^{2+}$, predicted m/z ; 1063.5 Da) of the $(\text{Gal})_3$ -incorporated VT-1 ($(\text{Gal})_3$ ($\text{C}_{52}\text{H}_{96}\text{N}_6\text{O}_{29}$); MW 1269.2 Da, VT-1; MW 871.5 Da) (Figure 5), $(\text{Gal})_3$ was site-specifically incorporated at Gln74 in rhIL-2.

In Vitro Biological Assays. The bioactivities of PEG12-rhIL-2 and $(\text{Gal})_3$ -rhIL-2 are shown in Table 2.

The control, purified rhIL-2, has an activity of 1.43×10^7 units/mg as hIL-2. The bioactivities of PEG12-rhIL-2 and $(\text{Gal})_3$ -rhIL-2 are 1.35×10^7 units/mg (94%) and 1.53×10^7 units/mg (107%), respectively, indicating that both modified forms of rhIL-2 retain full bioactivity relative to rhIL-2.

Pharmacokinetic Studies. The blood plasma curves for PEG12-rhIL-2 and rhIL-2 given intravenously to mice are compared in Figure 6. PEG12-rhIL-2 showed a prolonged circulation half-life in comparison with unmodified rhIL-2. The α and β half-lives of rhIL-2 were 2.2 and 11 min, respectively. For PEG12-rhIL-2, these half-lives were increased to 8.8 and 75 min, respectively. The area under the plasma concentration vs time curve ($\text{AUC}_{0-\text{inf}}$) of PEG12-rhIL-2 (442 ng·h/mL) was about 10 times greater than that of unmodified rhIL-2 (46 ng·h/mL).

Figure 7 shows the tissue distribution of $(\text{Gal})_3$ -rhIL-2 and rhIL-2 after i.v. administration in mice. $(\text{Gal})_3$ -rhIL-2 predominantly accumulated in the liver, at about a 5-fold higher concentration than that of unmodified rhIL-2. When $(\text{Gal})_3$ -rhIL-2 was coadministered with asialoorosomucoid, which is a well-known high-affinity ligand for the hepatic ASGP-R, the liver accumulation of the conjugate was reduced to the same level as that of rhIL-2. This result indicates that the hepatic targeting of $(\text{Gal})_3$ -rhIL-2 is via the ASGP-R.

DISCUSSION

Selective protein drug delivery and targeting seek to achieve the optimal arrival of a drug at its site of action in a manner that is appropriate for the disease and the drug, which would lead to a significant reduction in the possibility of drug side effects. Controlling the circulating lifetimes or tissue distributions of therapeutic proteins is one of the main areas for developing drug delivery

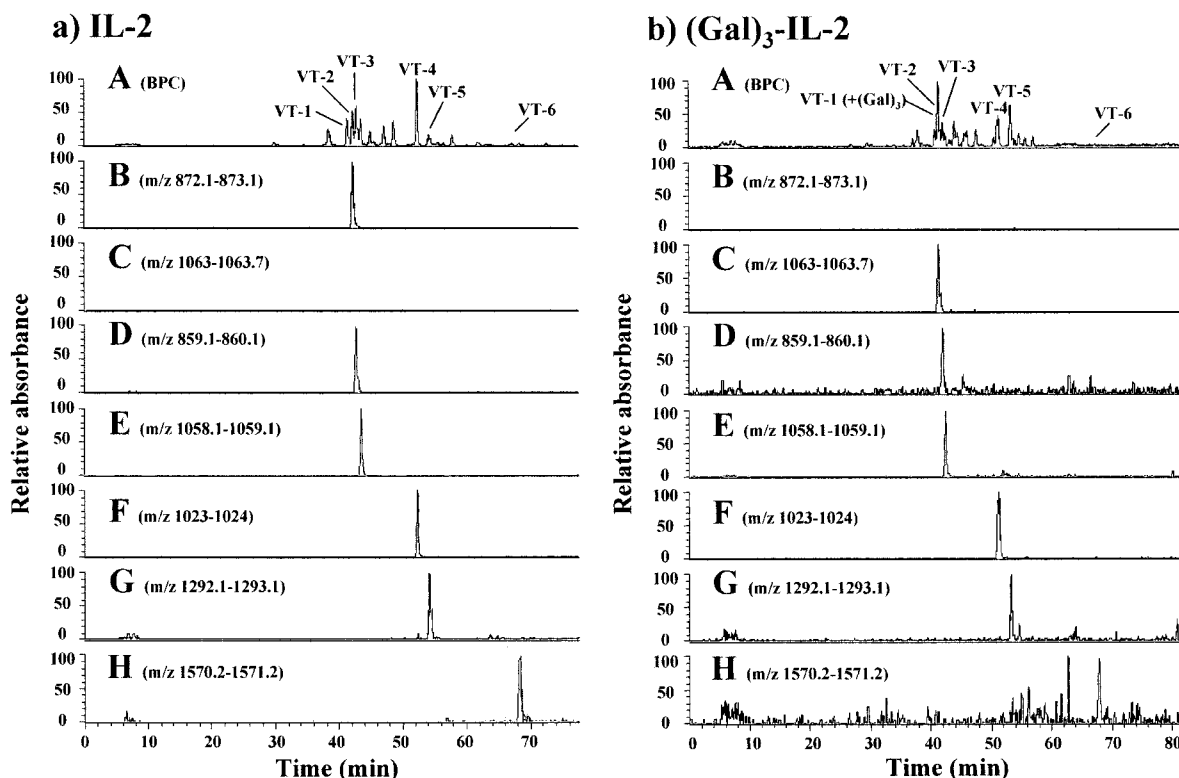
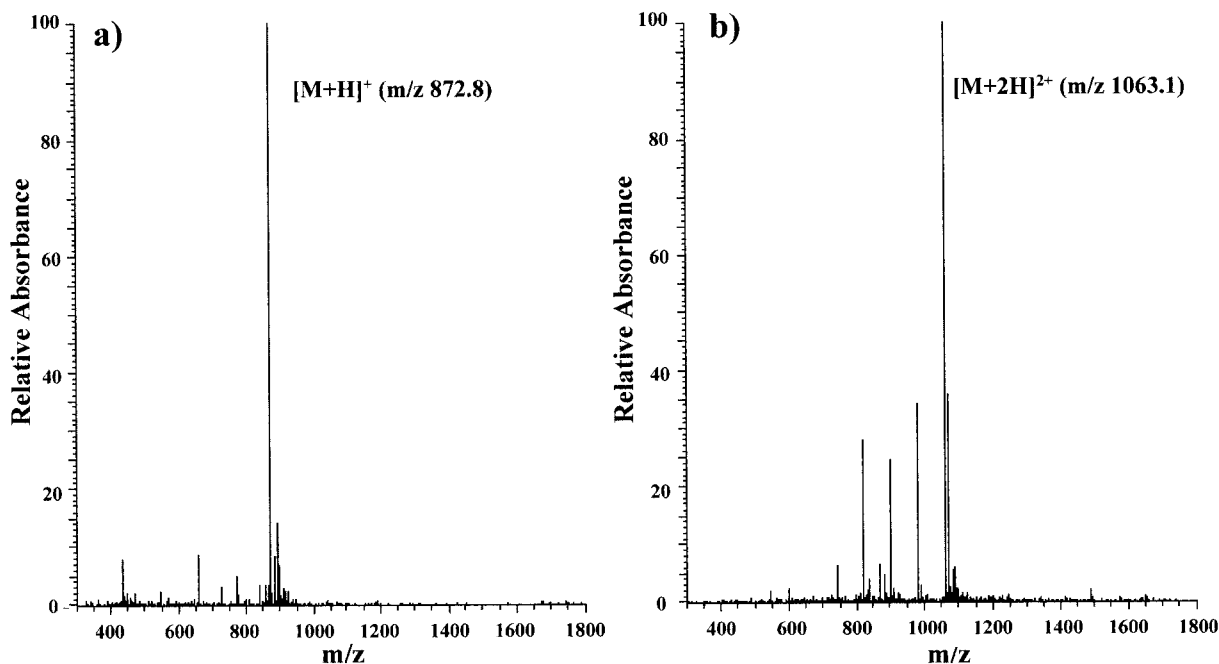


Figure 4. Comparison of LC-ESI/MS chromatography of trypsin- and V-8 protease-digested peptides containing Gln residues of rhIL-2 (a) and $(\text{Gal})_3$ -rhIL-2 (b). A; Base peak chromatogram, B-H; $[\text{M} + \text{H}]^+$ ion chromatograms for the fragments VT-1, VT-1(+ $(\text{Gal})_3$), VT-2, VT-3, VT-4, VT-5, and VT-6, as described in Table 1.

Table 1. Sequence Information for the Observed Fragments Containing Gln Residues of rhIL-2 and (Gal)₃-rhIL-2 Following LC-ESI/MS

fragment	residues	predicted mass (M + H) ⁺ (m/z)	observed mass (M + H) ⁺ (m/z)	sequence ^b
VT-1	69–76	872.5	872.8	VLNLAQSK
VT-1(+Gal) ₃	69–76	1063.5 ^a	1063.1	VLNLAE(-Gal) ₃ SK
VT-2	9–15	859.5	859.7	KTQLQLE
VT-3	55–62	1058.4	1058.8	HLQCLEEE
VT-4	125–133	1023.5	1023.8	CQSIISTLT
VT-5	21–31	1292.7	1293.0	LQMILNGINNY
VT-6	121–133	1570.8	1571.1	WITFCQSIISTLT

^a This value was described as (M + 2H)²⁺. ^b All cysteine (C) residues were carboxymethylated.

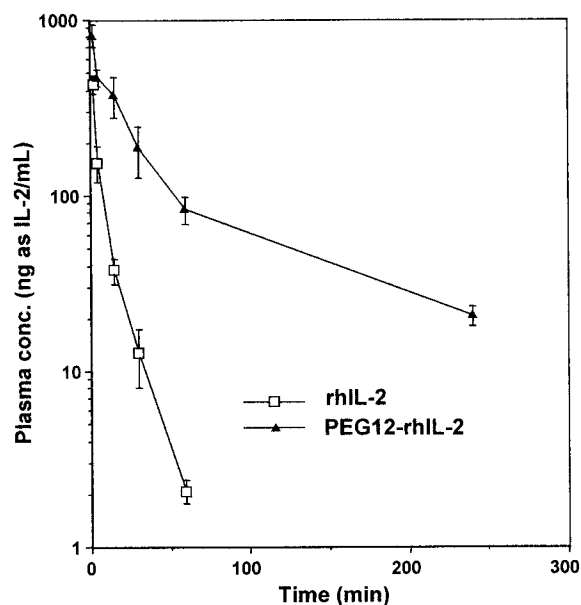
**Figure 5.** ESI/MS spectra from the LC-ESI/MS analysis shown in Figure 4a: mass spectrum of VT-1 of rhIL-2 at 42 min. b: Mass spectrum of (Gal)₃-incorporated VT-1 (VT-1(+Gal)₃) of (Gal)₃-rhIL-2 at 41 min.**Table 2. IL-2 Bioactivity of Modified RhIL-2**

protein	bioactivity ^a	
	units/mg	% activity ^b
rhIL-2	1.43×10^7	100
PEG12-rhIL-2	1.35×10^7	94
(Gal) ₃ -rhIL-2	1.53×10^7	107

^a The amount of IL-2 activity was determined in units/mg by using the IL-2 standard on murine CTLL-2 cells. ^b The activity is expressed as the percent residual bioactivity as compared to the rhIL-2 bioactivity.

systems. By using M-TGase as the catalyst, we have developed a new methodology for the site-specific incorporation of alkylamine derivatives of a hepatic targeting device ((Gal)₃) and PEG (PEG12) into intact proteins and controlling the pharmacokinetic profiles of the protein drugs.

The progress of our application study for the TGase-catalyzed modification of therapeutic proteins depends on the adoption of M-TGase instead of G-TGase as the catalyst. Although a few attempts have been made to characterize the substrate specificity of M-TGase, using synthetic peptide derivatives, the characterizations of the substrate recognition of the enzyme have not considered the tertiary structure (14, 21). From our preliminary studies, M-TGase has lower substrate specificity for the amine acceptor site around the Gln residues in proteins than G-TGase. In the case of the G-TGase-catalyzed modification studies for rhIL-2, there was a need to

**Figure 6.** Plasma concentration time courses of PEG12-rhIL-2 and rhIL-2 in mice. The plasma concentrations were determined by ELISA after intravenous administration (50 μ g as rhIL-2/kg) into C57BL/6 mice (6W, δ). Data are presented as means $\pm$ standard deviations.

construct a chimeric protein of hIL-2, in which the substrate peptide was fused to the N-terminus of hIL-2

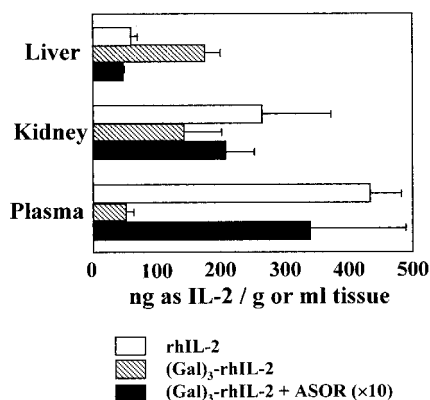


Figure 7. Tissue distributions of (Gal)₃-rhIL-2 and rhIL-2 in mice. Each sample (50 μ g as rhIL-2/kg) was injected intravenously into mice, without or with asialoorosomucoid (1320 μ g/kg). After 2 min of circulation, the uptake by the liver and kidney and the remaining fraction in the serum were determined (percentage of the injected dose). Data are presented as means $\pm$ standard deviations.

(10). On the other hand, we could site-specifically incorporate alkylamine derivatives of PEG or a hepatic targeting device into intact rhIL-2 by using M-TGase. In fact, we have found a lot of other substrate proteins for M-TGase, such as human interleukin 6, human interferon α , and so on (data not shown). The reason for the lower substrate specificity of M-TGase is assumed to be as follows.

It is known that the catalysis of TGase takes place in an acylation-deacylation pathway; this reaction proceeds through the formation of Michaelis-type acyl-enzyme intermediates and the subsequent transfer of acyl-enzyme intermediates (22, 23). Specifically, there is evidence that these enzymes have extended active sites whose interactions with substrate peptides bear directly on their binding affinity for and/or their catalytic efficiency toward glutamine residues (24). These secondary interactions between the target proteins and the active site of TGase are thought to determine the reactivity for TGase. When compared with the structures of the two TGases, by Kanaji et al. (13), the secondary structure of the region around the active site Cys residue of M-TGase is similar to that of G-TGase, whereas the amino acid sequence of the M-TGase is very different from that of G-TGase. However, the molecular weight for M-TGase (37.9 kDa) is much smaller than those of the mammalian TGases, represented by G-TGase (76.6 kDa). Thus, the smaller M-TGase could easily interact with the substrate site of the target proteins, as compared to G-TGase, and the subsequent transfer of the acyl-enzyme intermediates results in the lower specificity toward glutamine residues. This characteristic of M-TGase is assumed to be a special feature among the TGases. To confirm the broad specificity of M-TGase for the Gln substrate in intact proteins, further systematic studies for its reactivity toward various proteins will be necessary.

Our characterization studies of (Gal)₃-rhIL-2 by LC-ESI/MS showed that the Gln74 residue in rhIL-2 was modified with (Gal)₃ in a site-specific manner. In addition, the LC-ESI/MS analysis of PEG12-rhIL-2 also showed that only the VT-1 fragment (residues 69–76) of rhIL-2 was site-specifically modified with PEG12 (data not shown). The consistent results of the incorporation site between (Gal)₃ and PEG12 indicate that only Gln74, out of the six Gln residues in rhIL-2 (Gln11, 13, 22, 57, 74, and 126), can be a substrate for M-TGase.

Table 3. Comparison of the Amino Acid Sequence Surrounding the Gln Residues in RhIL-2

Gln residues	sequence ^{a,b}	location
Gln11	APTSSST KKT Q LQLEHLLLDL	in helix A
Gln13	TSSST KKTQL Q LEHLLLDLQM	in helix A
Gln22	LQLEHLLLDL Q MILNGINNYK	in helix A
Gln57	PKKATELKHL Q CLEELKPLE	in helix B
Gln 74	KPLEEVLNLA Q SKNFHLRPRD	near helix B
Gln126	EFLNRWITFC Q SIISTLT-COOH	in helix D

^a The Gln residues in rhIL-2, as well as the charged amino acids, are printed in boldface. ^b The carboxy terminus of rhIL-2 is indicated by COOH

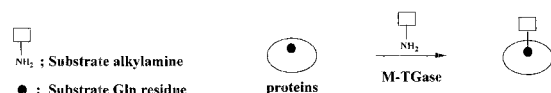
From the stereoview of the hIL-2 mutant (PDB Code: 3INK) represented by a Protein Data Bank program (25), all of the side-chains of the glutamine residues in hIL-2 are located in the exposed region. Since the location of reactive glutamine residues at the surface of the respective proteins is a common feature for TGase-catalyzed modifications, every glutamine residue in hIL-2 has the possibility of being an amine acceptor substrate in this category. Thus, the amino acid sequences surrounding the Gln residues in hIL-2 play a key role for the reactivity. In fact, the reactive Gln residues for TGase are located in a sequence containing a high proportion of charged and polar amino acids (26). Furthermore, in substrate proteins with known three-dimensional structures, i.e., melittin and glucagon, the reactive glutamine residues are located in an α -helix with positively charged amino acids clustered either NH₂- or COOH-terminally, relative to this residue (27, 28).

Table 3 shows the amino acid sequences and the secondary structures surrounding the Gln residues in hIL-2 (29, 30). Comparing the location of each Gln residue, the sequences around Gln57 and Gln74 would be most abundant in polar and (positively) charged amino acids. Thus, the site-specificity at Gln74 appears to be reasonable. Since it is impossible to derive a consensus sequence for the M-TGase-catalyzed modification at present, the reason for the reaction selectivity at Gln74 and not for Gln57 is immediately clear to us. The differences in the amino acid sequence and the peptide flexibility around Gln57 and Gln74 would dramatically affect the binding affinity for the acyl-enzyme intermediate between M-TGase and rhIL-2.

Our pharmacokinetic studies showed that the site-specific introduction of the PEG12 molecule prolonged the circulation lifetime of rhIL-2. To increase the time further, either enhancing the PEG length or adding multiple copies of PEG chains at one site would be necessary. In fact, this type of PEG-protein conjugate, in which one mole of high molecular weight (40 kDa) branched PEG is incorporated per mole of interferon α -2a, has shown sufficient prolongation in its pharmacokinetic profiles (31).

On the other hand, (Gal)₃-rhIL-2 could accumulate in the liver via the hepatic ASGP-R. To achieve hepatic delivery of proteins, many groups have synthesized galactosylated proteins, in which some galactose residues are covalently attached to the lysine side chains of the proteins (32, 33). However, since N-linked oligosaccharides containing multiple terminal Gal residues bind to the hepatic ASGP-R with high affinity, more Gal residues must be incorporated per target protein, which would reduce the bioactivity. Since such conjugates showed product heterogeneity and decreased bioactivity as compared to the unmodified proteins, it was difficult to develop them as therapeutic agents. Thus, the methodology for the hepatic targeting of proteins by using (Gal)₃

1) M-TGase-catalyzed modification of intact proteins



2) G-TGase-catalyzed modification of chimeric proteins

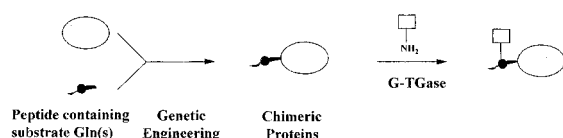


Figure 8. The strategy of the site-specific modification of proteins by the use of M-TGase (i) and G-TGase (ii).

would be generally applicable, as compared to the previous method, as detailed in the following points.

(i) This methodology can achieve the hepatic delivery of proteins with one point of incorporation of the ligand at a substrate Gln residue.

(ii) The incorporation of (Gal)₃ would not affect the function of the proteins.

In addition to the above points, (Gal)₃-mediated targeting has another advantage over the previous hepatic delivery systems. Although there are some reports of the utilization of artificial tri- or tetraantennary ligands for ASGP-R for hepatic targeting devices, these were developed for carrying DNAs, antisense oligonucleotides, cholesterol, and liposomes, but not proteins, into hepatocytes (34, 35, 36, 37). Then, they selected the highest affinity ligands (K_d , nanomolar affinity) for the devices to favor the efficient uptake of the molecules via ASGP-R-mediated endocytosis.

In contrast, (Gal)₃ is a lower affinity ligand for the ASGP-R (K_d , several hundreds nM to μ M affinity) as compared to the previous ligands. This affinity would be sufficient for binding to the ASGP-R after administration, but insufficient for internalization into hepatocytes. In fact, we found that the (Gal)₃ had a special feature that allowed it to escape from ASGP-R-mediated endocytosis after binding to the ASGP-R on hepatocytes in our preliminary studies, whereas the high-affinity ligand was rapidly internalized into the cells (data not shown).

Thus, (Gal)₃-protein conjugates would mainly accumulate around the extracellular region of hepatocytes, not within the cells. Since many endogenous proteins, such as cytokines, are produced and released to act locally, and since these types of molecules have multiple functions in the body, the delivery of systematically administered proteins to the site of action would be necessary to reduce the systemic toxicity. With this viewpoint, our hepatic targeting system is a potential methodology for developing therapeutic proteins toward liver diseases. To demonstrate the utility of the (Gal)₃ ligand as a hepatic targeting device, further studies, such as kinetic studies of the uptake of (Gal)₃-protein conjugates into hepatocytes, will be necessary.

On the basis of the previous observations and the present studies of TGase-catalyzed modification, the strategy of our site-specific modification of proteins can be described as follows (Figure 8). In cases where one would like to introduce alkylamine derivatives into intact proteins, M-TGase would be suitable for their modification (1). Even if the target protein lacks reactive Gln residues, the site-specific introduction is still possible, because a short substrate sequence for G-TGase can be genetically introduced at the terminus of the target

protein (2). This methodology can be generally applied to therapeutic proteins in cases where the incorporation of alkylamine derivatives does not affect the bioactivity of the proteins. Although alkylamine derivatives of PEG and an artificial ligand for the hepatic ASGP-R were employed as the modifier in this study, other functional alkylamines may be introduced to tailor the delivery and targeting of proteins. Furthermore, other kinds of TGases with different substrate specificities could be applicable for constructing protein conjugates.

In conclusion, we have successfully developed a new methodology for the site-specific modification of intact proteins by the use of M-TGase. The high homogeneity of the constructed conjugates and the ability to control the pharmacokinetic profiles will improve the therapeutic indices of proteins.

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***N*-(2-Hydroxypropyl)methacrylamide Copolymer–6-(3-Aminopropyl)-ellipticine Conjugates. Synthesis, in Vitro, and Preliminary in Vivo Evaluation**

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Ellipticine derivatives have potential as anticancer drugs. Their clinical use has been limited, however, by poor solubility and host toxicity. As *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer–anticancer conjugates are showing promise in early clinical trials, a series of novel HPMA copolymer conjugates have been prepared containing the 6-(3-aminopropyl)-ellipticine derivative (APE, NSC176328). Drug was linked to the polymer via GFLG or GG peptide side chains. To optimize biological behavior, HPMA copolymer–GFLG–APE conjugates with different drug loading (total APE: 2.3–7% w/w; free APE: <0.1% w/w) were synthesized. Conjugation of APE to HPMA copolymers considerably increased its aqueous solubility (>10-fold). HPMA copolymer–GG–APE did not liberate drug in the presence of isolated lysosomal enzymes (tritosomes), but HPMA copolymer–GFLG–APE released APE to a maximum of 60% after 5 h. The rate of drug release was influenced by drug loading; lower loading led to greater release. Whereas free APE (35 $\mu\text{g/mL}$) caused significant hemolysis (50% after 1 h), HPMA copolymer–APE conjugates were not hemolytic up to 300 $\mu\text{g/mL}$ (APE-equiv). As would be expected from its cellular pharmacokinetics, HPMA copolymer–GFLG–APE was >75 times less cytotoxic than free drug ($\text{IC}_{50} \sim 0.4 \mu\text{g/mL}$) against B16F10 melanoma in vitro. However, in vivo when tested in mice bearing s.c. B16F10 melanoma, HPMA copolymer–GFLG–APE (1–10 mg/kg single dose, APE-equiv) given i.p. was somewhat more active (highest *T/C* value of 143%) than free APE (1 mg/kg) (*T/C* = 127%). HPMA copolymer–APE conjugates warrant further evaluation as potential anticancer agents.

INTRODUCTION

Ellipticine, 5,11-dimethyl-6*H*-pyrido(4,3-*b*)carbazole (I, Figure 1) [reviewed in (1, 2)], is a potent cytotoxic agent with an IC_{50} in the range of 0.3–0.7 μM toward a variety of malignant cell lines in vitro (3). Its mechanisms of action include DNA intercalation (4), topoisomerase inhibition (5), and alkylation (1). Early clinical development was limited by poor drug solubility and in vivo host toxicities including hemolytic activity, decreased heart rate (1), and hepatotoxicity (6). A 9-hydroxy-2-methylellipticinium acetate derivative (II, Figure 1) did however show interesting clinical activity against thyroid and renal tumors, and bone metastases associated with breast cancer [reviewed in (1)]. These observations led us to propose the ellipticines as interesting candidates for evaluation in the form of polymer–drug conjugates.

Biocompatible, water-soluble polymers provide a useful platform for tumor-selective drug delivery. Conjugation of doxorubicin (7), paclitaxel (8), and camptothecin (9) to HPMA copolymers improves their water solubility and reduces drug toxicity. Additionally, after intravenous (i.v.) administration, such conjugates selectively accumulate in solid tumor tissue by the “enhanced perme-

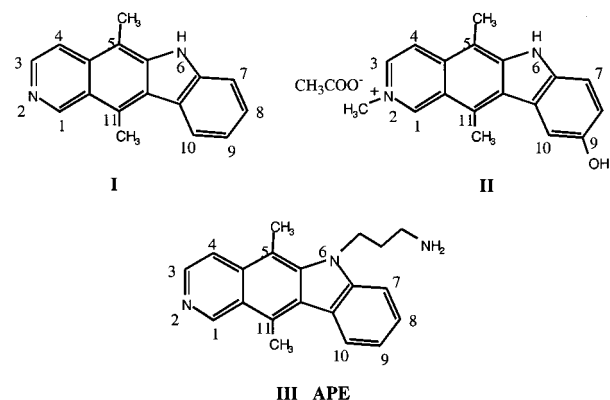


Figure 1. Chemical structure of ellipticine (I), 9-hydroxy-2-methylellipticinium acetate (II), and 6-(3-aminopropyl)-ellipticine (III).

ability and retention” (EPR) effect (10–12). EPR-mediated targeting arises due to preferential extravasation of circulating conjugate as it passes through hyperpermeable, angiogenic, tumor vessels (12), and the absence of an effective tumor lymphatic drainage encourages intratumoral retention. Those factors found previously to be important for the preclinical optimal design of polymer–drug conjugate have been extensively reviewed (13–16). Several conjugates have now progressed successfully into Phase I/II clinical evaluation. The first, HPMA copolymer–GFLG–doxorubicin (PK1, FCE 28068), displayed greatly reduced toxicity compared to free doxorubicin (7), and also showed antitumor activity in

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chemotherapy-refractory patients, even at relatively low doses (80, 120, 180 mg/m² doxorubicin-equiv) (17). The observed PK1 maximum tolerated dose (MTD) was 320 mg/m² doxorubicin-equiv, which is 4–5 times higher than the usual clinical dose of free doxorubicin (60–80 mg/m²) (17).

In this study, a series of HPMA copolymer–6-(3-amino-propyl)-ellipticine dihydrochloride (APE; NSC176328) (III, Figure 1) conjugates were synthesized with the aim of improving the water-solubility of ellipticine and also improving its therapeutic index. Conjugates were prepared by aminolysis of activated HPMA copolymer precursor (18) to give compounds with either a GFLG linker, since this peptide is degraded by the lysosomal thiol-dependent proteases (19, 20), or a potentially nonbio-degradable GG linker. The APE content of the conjugate was varied between 2.3 and 7% w/w to allow investigation of the effect of drug loading on conjugate properties. Hemolytic activity, cytotoxicity, and the rate of APE release in the presence of isolated lysosomal enzymes (tritosomes) were all assessed *in vitro*. Preliminary experiments were also conducted to evaluate the anti-tumor activity of HPMA copolymer–APE in a subcutaneous (s.c.) murine tumor model *in vivo*.

EXPERIMENTAL PROCEDURES

Materials. HPMA copolymers, carrying GG (5 mol %; $M_w \sim 30\,000$ and $M_w/M_n = 1.3\text{--}1.5$) or GFLG (either 5 or 10 mol %; $M_w \sim 30\,000$ and $M_w/M_n = 1.3\text{--}1.5$) peptide side chains, were supplied by Polymer Laboratories, Shrewsbury, U.K. The C-terminal amino acid in both peptide side chains was esterified with 4-nitrophenol, the content of displaceable activated ester being calculated from the extinction coefficient of bound 4-nitrophenol at 274 nm in dimethyl sulfoxide (DMSO) ($\epsilon = 9500\text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$).

APE (NSC176328) was a gift from the National Cancer Institute, Washington, D.C. Dry solvents were supplied by Aldrich, U.K., and dispensed under argon. Chemicals were obtained from Aldrich, U.K., Fischer Chemicals, U.K., or BDH Ltd., U.K., unless otherwise stated.

Synthesis of HPMA Copolymer-GFLG(5 mol %)-APE (1a), HPMA Copolymer-GFLG(10 mol %)-APE (2a), and HPMA Copolymer-GG(5 mol %)-APE (3a) (Method 1). HPMA copolymer (1 equiv, calculated as 4-nitrophenyl ester) and APE (2 equiv) were dissolved in minimal volumes of dry DMSO. Triethylamine (2 equiv) was added to the copolymer solution to neutralize the hydrochloride protons of the APE solution which was added dropwise to the reaction mixture. Aminolysis was allowed to proceed for 5 h. To complete the aminolysis of any unreacted esters, 1-aminopropan-2-ol (2 equiv) was added to the reaction mixture (1 h). The DMSO was removed at room temperature under high vacuum and the residue redissolved in distilled water. The reaction mixture (comprising conjugate, 4-nitrophenol, and unreacted 1-aminopropan-2-ol and unreacted APE) was then dialyzed against distilled water (M_w cutoff 5000, solvent resistant grade, cellulose ester; Spectrapor, USA, Pierce and Warriner, U.K.). For small-scale preparations, an additional purification step was undertaken. The reaction mixture was applied to a PD10 (Sephadex G25) column in water and the void volume fraction collected and freeze-dried. For larger scale preparations, this step was omitted. Overall yields based on polymer weight were 60–75%.

Synthesis of HPMA Copolymer-GFLG(5 mol %)-APE (1b–d) (Method 2). HPMA copolymer-GPLG–p-

nitrophenol ester (5 mol %) (1 g) was dissolved with stirring in dry dimethylformamide (DMF) (25 mL). APE (84 mg, 0.75 equiv) was dissolved in dry DMSO (17 mL). The solutions were mixed, a small aliquot of each (5–10 μL) being retained for thin-layer chromatography (TLC). A dilute solution of dry triethylamine in dry DMF (1:100 v/v, 9.3 mL, 3:1 equiv to APE) was prepared for addition in aliquots (500 μL) at 5 min intervals to the reaction mixture. After each addition, with swirling, samples (10 μL) were taken into a 1 mL cuvette containing Dulbecco's phosphate-buffered saline (pH 7.0), and the initial absorbance at 400 nm was noted to follow the release of 4-nitrophenol (Shimadzu UV-1601 UV-visible spectrophotometer). The reaction mixture, initially pale yellow, decolorizes after the first six aliquots of triethylamine; then the yellow color progressively deepens. The absorbance at 400 nm reaches a plateau as triethylamine addition is completed, consistent with the displacement of the calculated amount of 4-nitrophenol (0.75 equiv). The disappearance of unbound APE was followed by thin-layer chromatography (TLC) [on Kieselgel ALU 60 F₂₅₄ silica gel plates from Merck, using chloroform/methanol/triethylamine 8:1:1 (v/v) as the mobile phase]. The reaction mixture was left overnight in the dark, and then quenched with dilute 1-aminopropan-2-ol (1:100 v/v in dry DMF, 2.2 mL) for 1 h. The solvents were evaporated under high vacuum at 30 °C, and the resulting gum was dissolved in distilled water (60 mL) and dialyzed against three changes of distilled water over 48 h (Spectrapor M_w cutoff 2000, solvent resistant). The contents of the dialysis tubing were freeze-dried to constant weight (750 mg). The content of APE in the preparation (wt %) was determined by UV at 295 nm using APE as a standard.

Determination of Impurities in HPMA Copolymer–APE Conjugates by HPLC. Samples of HPMA copolymer–APE conjugates (1–5 mg) or APE (1–10 μg) were dissolved in 900 μL of water. Doxorubicin (100 μL of a 0.3 g/L stock solution) was added to each sample as an internal standard. The pH of the samples was adjusted to 8.5 with ammonium formate buffer (200 μL , 1 M, pH 8.5), and then a mixture of chloroform/propan-2-ol at a ratio of 80:20 (8 mL) was added. Samples were thoroughly mixed by hand and then centrifuged at 2000g for 10 min. The lower organic layer was carefully removed and placed in glass test tubes. The solvent was evaporated under nitrogen and the dry residue dissolved in 200 μL of methanol/water 60:40 (v/v). APE content was determined by HPLC using a $\mu\text{Bondapak C}_{18}$ (150 mm $\times$ 3.9 mm) column and methanol/water 60:40 (v/v), pH 2.2, adjusted with *o*-phosphoric acid as the mobile phase delivered at 1 mL/min using an LKB Bromma 2150 HPLC pump. A UV detector (Spectroflow 783 Kratos analytical) with a fixed-wavelength filter (307 nm) was used to monitor APE, ellipticine, and doxorubicin.

Determination of the Solubility of APE and HPMA Copolymer-GFLG(5 mol %)-APE (1c) in Phosphate-Buffered Saline (PBS). A stock solution of APE in DMSO was prepared (0.1 mg/mL). To obtain a calibration curve (absorbance at 296 nm), samples were diluted using PBS to give an APE concentration range of 0–5 $\mu\text{g/mL}$. The maximum solubility of APE was determined at 37 °C or at room temperature by preparing a saturated solution in PBS (5 mL) with adjustment of the pH to 7.4 by addition of sodium hydroxide (0.1 M). Either at room temperature or at 37 °C (the saturated solution was left for 10 min to warm, the pH being rechecked) the solution was filtered through a 0.45 μm filter. Triplicate samples (10 μL) were then diluted in PBS (1 mL; 37 °C or room

temperature, respectively), and the absorbance at 296 nm was measured.

In similar experiments, the conjugate **1d** (50 mg) was dissolved in PBS (2 mL) at 37 °C with pH adjustment and equilibration for 10 min. A clear solution was obtained which was nevertheless filtered (to allow for equivalent losses) and treated as before.

Size Exclusion Chromatography (SEC). SEC analysis was performed using two TSK-gel columns in series (G3000 PW followed by G2000 PW) with a guard column (Progel PWXL) and a differential refractometer (Gilson 153 refractive index detector) and a UV-visible spectrophotometer (UV Savern Analytical SA6504) as detectors connected in series. A Tris buffer (Tris, 0.05 M; NaCl, 0.5 M) was used as the mobile phase, delivered at 1 mL/min by a Jasco PU-980 pump. PowerChrom AD Instrument software was used for recording the detector output signal and for analysis of the data.

Evaluation of HPMA Copolymer-APE Conjugate Degradation by Tritosomes (Rat Liver Lysosomal Enzymes). *Preparation of Tritosomes and Determination of Their Activity.* Rat liver lysosomal enzymes were prepared according to the method described by Trouet (21), and their protein content was determined using bicinchoninic acid assay (22). The tritosome protein content of the tritosomes used here was 1.7 mg/mL. Their proteolytic activity was determined by measuring the release of *p*-nitroanilide from *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide (23). Activity was 25 nM min⁻¹ (mg of protein)⁻¹.

Degradation Studies. HPMA copolymer-APE conjugates (at a concentration of 46 µg/mL APE-equiv) to give comparable substrate (peptide spacer-APE concentration) or APE (1–10 µg) were incubated at 37 °C in citrate-phosphate buffer containing 0.2% Triton X-100 (400 µL) in the presence of EDTA (100 µL, 10 mM) and GSH (100 µL, 50 mM). To start the degradation study, tritosomes warmed to 37 °C (200 µL) were added, and the tube was thoroughly mixed. Aliquots of reaction mixture were taken at times 0–10 h, immediately frozen in liquid nitrogen, and stored frozen in the dark until processed by HPLC.

HPLC Evaluation. The samples were mixed with distilled water (800 µL) and ammonium formate buffer (100 µL, 1 M, pH 8.5) and doxorubicin (100 µL of a 0.3 g/L solution) added as an internal standard. The content of free APE was then determined as described above. Throughout this procedure, samples have to be kept on ice because it was noticed that the APE degrades within the organic phase at room temperature to give ellipticine.

Evaluation of Rat Red Blood Cell Lysis. HPMA copolymer-GFLG-APE (**1a**, **1b**), HPMA copolymer-GG-APE (**3a**), APE, and ellipticine (final concentrations between 5 mg/mL and 1 µg/mL) were incubated at 37 °C with rat erythrocytes (2% w/v) for 1 or 24 h. Intact cells and debris were then removed by centrifugation (1500g, 10 min), and the supernatant was removed and used to assess hemoglobin release spectrophotometrically (at 550 nm) using a microtiter plate reader. Data were expressed as the percentage hemolysis observed compared with total hemolysis induced by Triton X-100 (0.5%).

Determination of In Vitro Cytotoxicity. In vitro cytotoxicity of APE and the conjugates was evaluated according to a standard, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), cell viability assay (24) using a B16F10 mouse melanoma cell line. B16F10 cells (10⁴) were seeded in 96-well microtiter plates and then left for 24 h to establish. APE or the HPMA copolymer-GFLG(5 mol %)-APE (**1c**) conjugate was

added in fresh medium (concentration range 0.01–70 µg/mL). Cells grown in medium alone were used as a control. After 67 h of incubation, MTT (20 µL of a 5 mg/mL solution) was added to each well, and the cells were incubated for a further 5 h. After removal of the medium, the precipitated formazan crystals were dissolved in spectroscopic grade DMSO (100 µL), and, after 30 min, the optical density was measured at 550 nm using a microtiter plate reader. Results are expressed as a percentage of cells grown in the absence of drug.

Antitumor Activity of APE and HPMA Copolymer-APE in Vivo. All experiments were conducted according to UKCCCR Guidelines governing experiments with neoplasia.

Male C57BL/6J mice were inoculated s.c. with 10⁵ viable B16F10 murine melanoma cells. Tumors were allowed to reach a palpable size (~50–70 mm² calculated from the product of the two longest diameters), and treatment (saline, free APE, or conjugate **1c** at doses calculated as APE-equiv) was administered by single-dose i.p. Mice were weighed daily, and the tumor growth was also monitored daily until tumor area reached the maximum allowable 289 mm². At this time or if the drugs used showed evidence of toxicity, the animals were humanely killed. Data are expressed as *T/C* values, i.e., the ratio of the mean time to tumor progression of the treated group to the mean time to tumor progression of the control group expressed as a percentage. Animal groups were statistically compared by a Student's *t*-test.

RESULTS AND DISCUSSION

Synthesis and Characterization. Successful conjugation of APE to HPMA copolymer intermediates was achieved using a standard aminolysis reaction (18) (Figure 2). This was predictable as the 3-dimensional structure of APE (Figure 3) shows that the primary amino group is free from any steric hindrance, standing well outside the plane formed by the unsaturated rings. Conjugates were synthesized to contain a range of APE loadings (Table 1). To maximize yield of conjugation, an improved conjugation method was used to prepare conjugates **1b–d**. In the small-scale pilot preparations (1–5 mg of polymer), 4-nitrophenol release (Figure 4) was in molar accordance with that expected. Maintenance of high concentrations of reagents in dry solvents and dropwise addition of the triethylamine to free the 3'-amino group of APE slowly from its hydrochloride salt (such that the copolymer remained in excess during the reaction) were both critical for successful conjugation. Also the use of 0.75 equiv of APE (method 2) instead of 2 equiv (as indicated in method 1) gave greatly improved yields of substitution of the peptide linker. For example, synthesis of conjugate **1a** had a yield of 25.7% compared with a minimum of 62.9% yield for synthesis of conjugates **1b–d**.

During the reaction, the disappearance of free APE and the appearance of conjugate were followed by TLC (*R_f* APE: 0.4, bound APE remains at the origin; *R_f* 4-nitrophenol: 0.9), and after dialysis no free APE was detectable by TLC. The higher sensitivity of the HPLC procedure showed that there was some free APE, and also small amounts of free ellipticine, present (Table 1). However, the amounts of these impurities detected represent <0.1% of the conjugate by weight, and <0.5% of the APE in the preparation was present as free drug—except in conjugate **1a** which contained 2.6% free APE related to the total APE. It is worth noting that conjugates **1b–d** prepared using method 2 contained lower

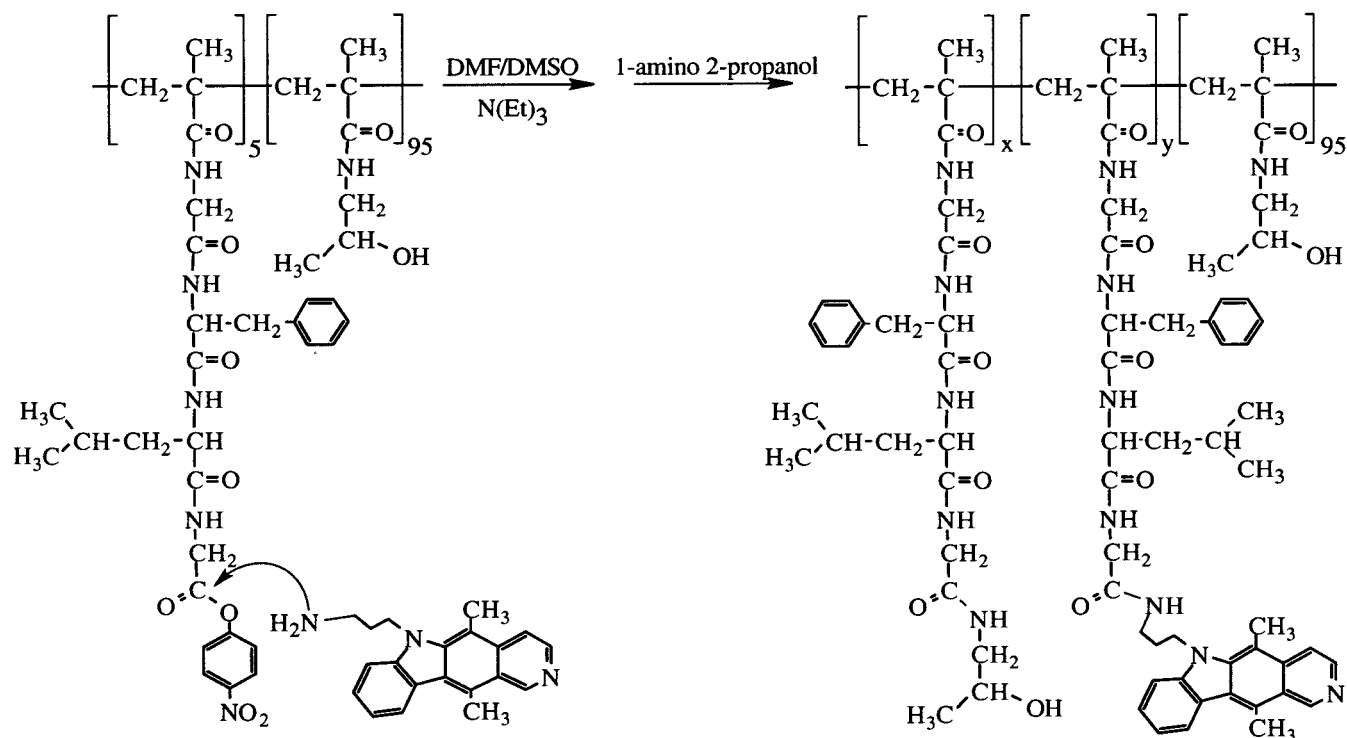


Figure 2. Route of conjugation of APE to HPMA-GFLG(5 mol %)-ONp.

Table 1. Characteristics of the HPMA Copolymer-APE Conjugates

conjugate ^a	code	substitution yield (%)	total APE (w/w %)	free APE (w/w % total APE)	impurities (w/w % conjugate)	
					APE	ellipticine
HPMA-GFLG-APE (5 mol %)	1a	25.7	2.3	<2.60	<0.06	<0.1
HPMA-GFLG-APE (5 mol %)	1b	83.5	7.2	<0.06	<0.01	<0.05
HPMA-GFLG-APE (5 mol %)	1c	62.9	5.5	<0.04	<0.01	<0.05
HPMA-GFLG-APE (5 mol %)	1d	68.9	6	<0.05	<0.01	<0.05
HPMA-GFLG-APE (10 mol %)	2a	47.5	7	<0.14	<0.01	<0.05
HPMA-GG-APE (5 mol %)	3a	45.7	3.8	<0.26	<0.01	<0.05

^a The mol % of peptide side chains in the HPMA copolymer precursor is shown in parentheses.

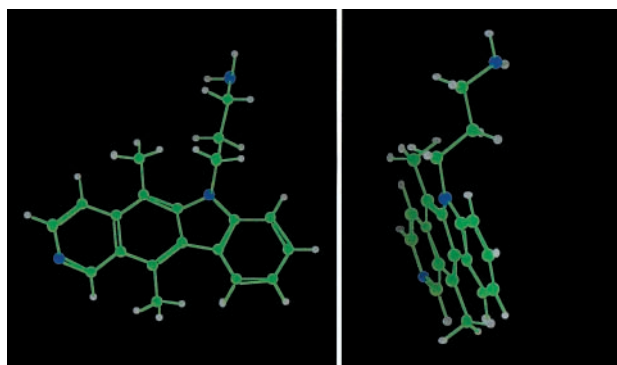


Figure 3. Three-dimensional structure of 6-(3-aminopropyl)-ellipticine.

amounts of free drug than seen when conjugates were synthesized using the original method.

SEC analysis confirmed that APE was conjugated to HPMA copolymers uniformly across the molecular weight range (Figure 5). Both RI and UV detection showed some evidence of aggregation (as indicated by the peak at 11 min). This is not surprising as APE is a hydrophobic drug and its planar aromatic structure would probably promote stacking between the drug molecules present on the polymer backbone. It has already been shown that side-chain chemical modification can influence the solution properties of HPMA copolymers (25).

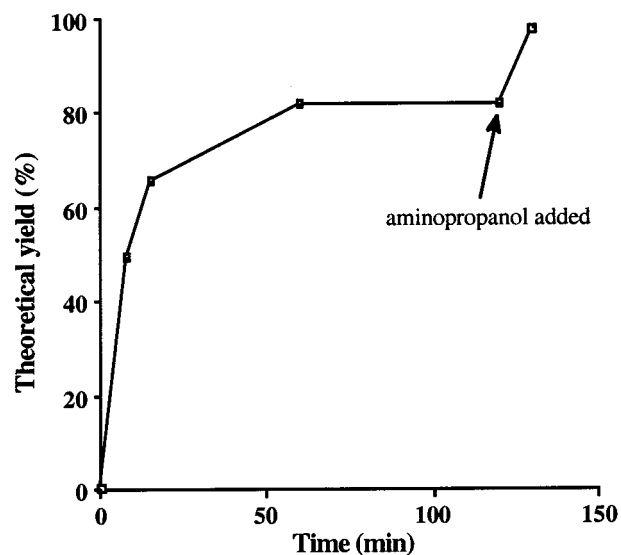


Figure 4. Pilot study of displacement of *p*-nitrophenol from HPMA-GFLG(5 mol %)-ONp treated with 0.75 equiv of APE. Results derived from triplicate measurements of absorbance at 400 nm (SD $\pm$ 1.7%).

A major problem encountered with previous ellipticine derivatives was their limited solubility (26). Here it was found that the maximum solubility of APE at room temperature and 37 °C was 44.5 and 62.4 μ g/mL,

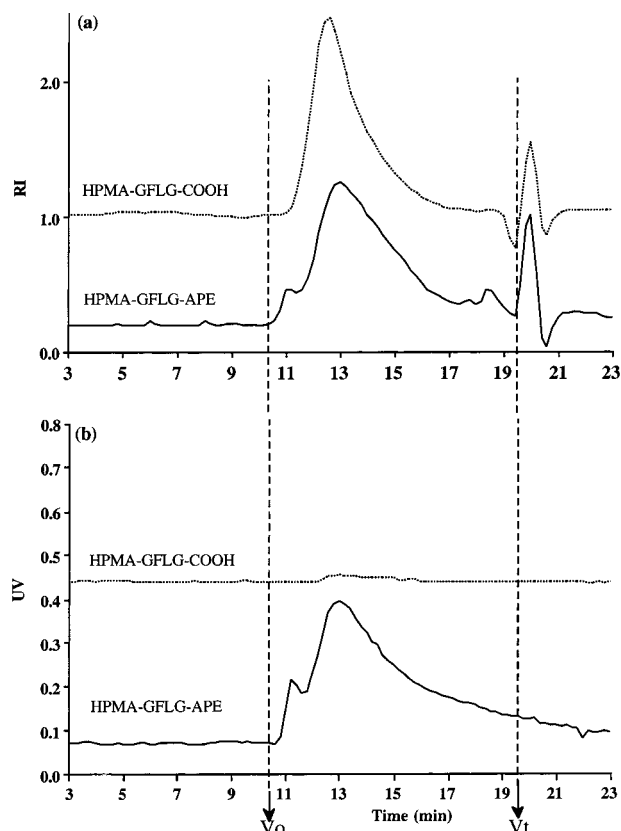


Figure 5. Size exclusion chromatograms of the polymer precursor after hydrolysis of the *p*-nitrophenyl ester and the resulting conjugate with APE, using a refractometer detector (a) and a UV detector (b). V_0 indicates the exclusion volume of the columns and V_t the total elution volume.

respectively. Drug conjugation to HPMA copolymers dramatically improved solubility. For example, the conjugate **1c** was still soluble at 37 °C at an APE-equiv concentration of 625.2 $\mu\text{g/mL}$. The APE concentration in solution increased at least 10-fold by its incorporation into the HPMA copolymer, and this observation is consistent with observations made following conjugation of HPMA copolymer to doxorubicin, camptothecin, or taxol (7–9).

Biological Properties of HPMA Copolymer-APE Conjugates. During incubation with tritosomes, HPMA copolymer-GFLG-APE conjugates (**1a**, **1b**, **2a**) released APE over time to a maximum of ~80% in 10 h (Figure 6). No peptidyl-APE degradation products were seen. Experiments involving HPMA copolymer-GG-APE (**3a**) as substrate or incubation of the above conjugates (**1a**, **1b**, **2a**) without enzyme showed no drug liberation. These results are in agreement with results obtained with HPMA copolymer-GFLG-doxorubicin conjugates. The diglycyl linker is not degraded by tritosomes, while the GFLG spacer released ~30% of doxorubicin after 10 h (20). In the case of HPMA copolymer-APE conjugates, drug loading clearly had an effect on the rate of APE release. The two conjugates (**1a**, **1b**) having a similar APE content (~7 wt %) released the same amount of drug during 10 h incubation (Figure 6). In contrast, conjugate **1a**, having an APE content of only 2.3 wt %, released 80% of APE over the same time interval. Recent studies (27) infer that HPMA copolymer-APE conjugates with a higher APE loading form in solution intramolecular micelles as observed in comparable systems (25), which would therefore be expected to limit enzyme accessibility to the peptidyl side chain. Ongoing studies are trying to

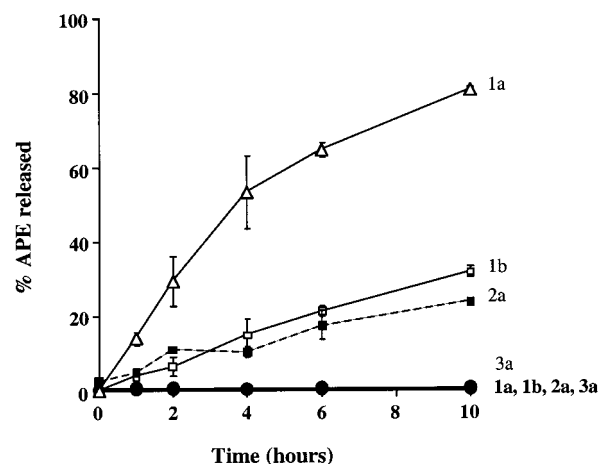


Figure 6. Percent of APE released from the conjugates HPMA-GFLG(5 mol %)-APE (**1a**, **1b**), HPMA-GFLG(10 mol %)-APE (**2a**), and HPMA-GG(5 mol %)-APE (**3a**). The numbers in boldface represent the samples incubated without tritosomes.

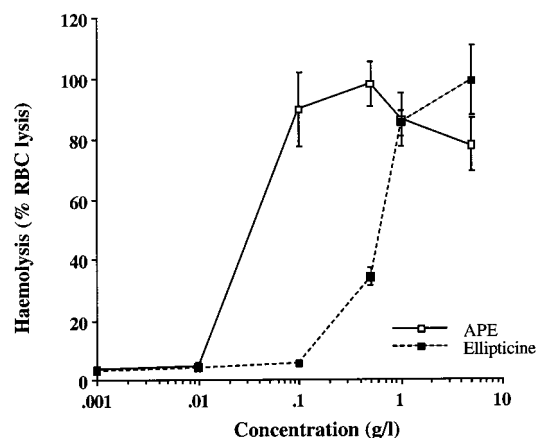


Figure 7. Percent of red blood cell lysis after 1 h incubation at 37 °C with APE and ellipticine at concentrations between 1 $\mu\text{g/L}$ and 5 g/L .

determine the drug loading that will produce an optimum therapeutic index in vivo. Using HPMA copolymer-doxorubicin conjugates and their evaluation in a murine L1210 (i.p.) model, it was shown that a slower doxorubicin release rate led to significantly improved efficacy in vivo (20). However, in the clinical setting, there is still a need to understand better how dose schedule and drug release rates can be best optimized to give maximum therapeutic index in the treatment of solid tumors.

Ellipticines cause significant red blood cell damage [attributed to interaction with negatively charged phospholipids in the membrane (28)], and hemolysis occurs in rhesus monkeys and beagles at doses of 3 mg/kg (28). Here APE and ellipticine caused 50% hemolysis (after 1 h) at 35 and 600 $\mu\text{g/mL}$, respectively (Figure 7). None of the conjugates tested (at concentrations up to 350 $\mu\text{g/mL}$ APE-equiv) demonstrated any in vitro hemolytic activity after either 1 h (Figure 8) or 24 h (data not shown). Polymer-bound APE must be sufficiently well-hidden within the polymer coil to minimize membrane interaction. This property of conjugated drug may lessen the risk of severe hepatic toxicity (as encountered in the Phase I trials of datelliptium) and avoid scheduling difficulties as encountered with elliptinium acetate [these toxicities are reviewed in (1)].

The IC_{50} value of APE was 0.4 $\mu\text{g/mL}$ against the B16F10 cell line (Figure 9). Conjugate **1c** was >75 times less active ($\text{IC}_{50} > 30 \mu\text{g/mL}$ APE-equiv—all cells were

Table 2. Antitumor Activity of APE and HPMA-GFLG(5 mol %)-APE Conjugate in Mice Bearing s.c. B16F10 Melanoma

treatment	dose ^a (mg/kg)	mean time to tumor progression (±SE)	T/C (%)	toxic deaths
saline	—	7.7 ± 0.4	100	0/14
APE	1	9.8 ± 0.6	126.7 ± 8.0 ^b	0/9
APE	5	9.0 ± 0.8	116.7 ± 10.9	0/10
APE	7.5	7.2 ± 0.9	93.3 ± 11.1	1/5
HPMA copolymer-APE	1	11.0 ± 0.7	142.6 ± 9.6 ^c	1/14
HPMA copolymer-APE	5	9.9 ± 1.4	128.0 ± 17.6	0/8
HPMA copolymer-APE	10	8.8 ± 1.1	110.0 ± 13.9	1/5

^a Doses are APE-equiv. ^b $p < 0.01$. ^c $p < 0.001$ (Student's *t*-test).

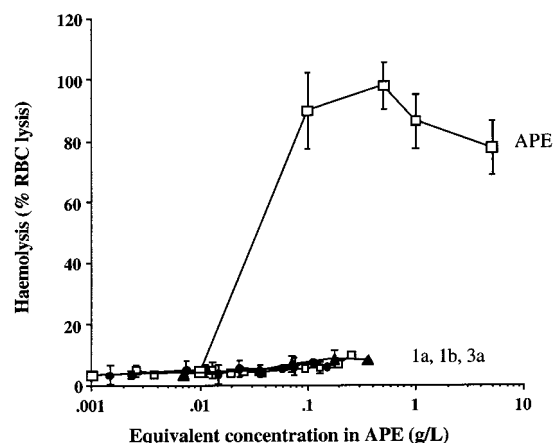


Figure 8. Percent of red blood cell lysis after 1 h of incubation at 37 °C with HPMA-APE conjugates compared to the percent of red blood cell lysis obtained after 1 h of incubation with APE.

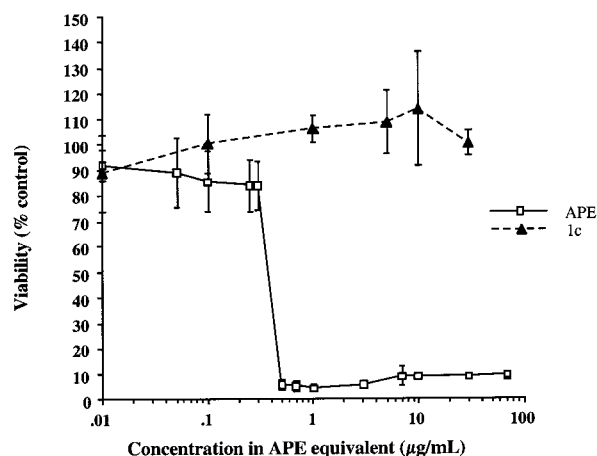


Figure 9. In vitro cytotoxicity on the B16F10 mouse melanoma cell line of APE and HPMA-GFLG (5 mol %)-APE (**1c**) at concentrations of APE between 0.01 and 70 μg/mL.

still viable at this concentration). The HPMA copolymer-APE conjugates (except conjugate **1a**) showed no significant activity in the NCI screen (29) ($t = 48$ h) up to a conjugate concentration of 50.1 μg/mL (~3.5 μg/mL APE-equiv) (NCI Developmental Therapeutics Program, unpublished results). Although conjugate **1a** did show some activity, this was simply attributable to free APE in the conjugate. These results illustrate the impossibility of adequately assessing polymer-drug potential using standard in vitro assays. Whereas free drug present in the culture medium can permeate cells within minutes, the conjugates enter cells slowly via the endocytic route, and, moreover, once internalized, the active drug is also liberated very slowly (30).

A s.c. B16F10 murine model, well standardized in terms of EPR-mediated targeting of PK1 (10), was chosen as a preliminary screen to evaluate the antitumor activity

of HPMA copolymer-APE. Despite lack of body weight loss (results not shown), three toxic deaths were recorded (Table 2) during dose escalation. The highest T/C seen for free APE (127%) was at a dose of 1 mg/kg. At the same dose (1 mg/kg APE-equiv), conjugate **1c** had a T/C = 143% (Table 2). Although the HPMA copolymer-APE showed better antitumor activity than APE for a dose of 1 mg/kg, this was not statistically significant ($p = 0.26$, Student's *t*-test). However, the moderate activity seen is not surprising knowing that a single dose of PK1 (given i.p.) is poorly active in the aggressive s.c. B16F10 tumor model (31). In this study, treatment with PK1 at doxorubicin doses of 5, 10, and 20 mg/kg produced T/C values in the range 98–175%, and i.v. treatment was necessary to obtain higher T/C values. The antitumor activity of the HPMA copolymer-APE conjugates presented here is promising when compared to that of other ellipticine analogues in the B16F10 model (32).

On dose escalation with HPMA copolymer-APE, unexpectedly a T/C value less than that seen for the 1 mg/kg dose was seen (Table 2). This phenomenon could be due to dose-dependent precipitation of the compounds in the peritoneal cavity, an increase of the liver clearance, a preferred accumulation site other than the tumor, or a significant decrease of the release of APE from the conjugate with increasing dose. A dose-dependent bio-distribution study will give an insight into the mechanism of action of the HPMA copolymer-APE conjugates.

The intracellular site of action of ellipticine drugs is not established unequivocally (33–36), but passage through the lysosomes [as indicated by granular cytoplasmic fluorescence and radiolocalization (37, 38)] does not preclude cytotoxicity. The preliminary in vivo studies reported here confirm that HPMA copolymer-APE does show antitumor activity. Further studies are needed to establish the magnitude and spectrum of activity using a panel of different murine and human tumors.

So far, only HPMA copolymer conjugates containing established antitumor agents such as doxorubicin, paclitaxel, camptothecin, and platinates [reviewed in (14)] have progressed to clinical trial. The polymer is proving its safety in man, and drug conjugation can be used to dramatically reduce the toxicity of bound drug; PK1 is ~5 times less toxic than doxorubicin (17). From these observations, it is clear that drug conjugation has the potential to assist the delivery of highly toxic natural product drugs and/or provide improved forms for promising antitumor agents that fail in either preclinical or early clinical development. The ellipticines are an example of the latter. Here HPMA copolymer-APE conjugates displayed improved solubility and reduced hemolytic activity and released APE in the presence of lysosomal enzymes. Preliminary experiments showed that they display antitumor activity in vivo, and although the activity seen was modest, the B16F10 model is particularly aggressive. A recent study on S16020-2 (an ellipticine analogue in clinical trial) using a much higher

multiple dosing regimen (up to 9 doses of 20 mg/kg) resulted in a similar activity ($T/C = 161\%$) (32). HPMA copolymer-Gly-Phe-Leu-Gly-APE warrants further biological evaluation (pharmacokinetics and pharmacology at its maximum tolerated dose) to determine their potential for clinical testing. Additionally, polymer conjugation could, in principle, also be usefully applied to other ellipticine derivatives, notably the potent 9-hydroxyellipticine that has an IC_{50} of $0.06 \mu M$ against L1210 cells in vitro (2 and references cited therein).

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Triple, MPEG-Conjugated, Helix-Forming Oligonucleotides (TRIPEGXs): Liquid-Phase Synthesis of Natural and Chimeric “All-Purine” Sequences Linked to High Molecular Weight Poly(ethylene glycols)

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Long “all-purine” oligonucleotides, up to the 20mer, known to be active as antigene effectors, conjugated to high molecular weight monomethoxy poly(ethylene glycol)s (MPEG)s, were successfully synthesized. Through a liquid-phase, MPEG-supported process, both natural and chimeric sequences containing selected phosphorothioate backbone modifications were obtained, purified, and characterized. To follow their cellular trafficking, a fluorescent probe was linked by soluble supported organic reactions to the 5'-terminus, and the efficiency of the different synthetic procedures for the introduction of a fluorescein moiety was compared. The usefulness of the fluorescent marker was estimated by laser confocal microscopy that ascertains that the MPEG-conjugation enhances the oligonucleotide capacity to cross the cellular membranes and to be accumulated inside the nuclei.

INTRODUCTION

The liquid-phase method, in which a soluble polymer supports the chemical synthesis of simple organic molecules, as well as of large biopolymers, has seen in these last years an explosion of successful applications (1). It is worth recalling that the replacement of insoluble cross-linked resins, such as those employed in the Merrifield-like solid-phase procedures, can solve the problems of converting solution-based reaction protocols for their use under heterogeneous conditions. Moreover, this methodology appears to tolerate an easy scaling-up of the process, as demanded by its commercial development (2). Among the investigated soluble polymers, the monomethoxy poly(ethylene glycol) (MPEG) is by far the most commonly used support, since its first application on peptide synthesis (3). Oligonucleotides were also synthesized through a MPEG-based approach (4), and even very recently a further development of their liquid-phase synthesis was proposed (5), proving the active interest in this methodology. In addition, a modified process was recently set up for the preparation of oligonucleotides chemically linked to high molecular weight MPEGs (6).

The conjugation of polymeric moieties to pharmacologically active molecules represents a promising approach to solve problems due to their *in vivo* lability and poor biodisposability (7). In particular, the oligonucleotides are characterized by a rapid enzymatic degradation and insufficient cellular uptake; however, once conjugated to a MPEG moiety, they are more resistant to nuclease digestion (6, 8). A critical mass value of the MPEG unit

is also observed: significant biological effects are recognized with increased molecular weights of the conjugated polymer (9). It must be stressed that the high viscosity, and poor reactivity, of the reacting polymer hampers an efficient postsynthetic conjugation of high molecular weight MPEG to oligonucleotides. In our case, since the liquid-phase procedure employs large MPEG chains as supports, the same MPEG unit can be left linked to the final oligonucleotide by using a chemical bond (a phosphodiester) that will not be hydrolyzed during the final deblocking procedure (6). This trick allows in principle the introduction of variable-mass MPEG on the synthesized nucleic acid chain.

As indicated by the first biological studies, the MPEG moiety does not impede the formation of the double helix between the linked oligonucleotide and the nucleic acid complementary target; moreover, its presence grants the natural degradative activity of the ubiquitous RNase H on the RNA part of the new duplex, once formed (10). As a further extension of these investigations, the formation of a triple helix between MPEG-conjugated oligonucleotides and a double-helical DNA target is of special interest (11). These MPEG-conjugated, triple helix forming oligonucleotides are dubbed TRIPEGXs. Their biological behavior is actually under investigation in cultured cells (12). As a testing sequence, an “all-purines” AG 13mer was initially preferred, since it recognizes a polypurine-polypyrimidine motif located in the human *bcr* promoter (13); successively, a longer AG 20mer active against the human Ki-ras oncogene that is overexpressed in many human tumors was synthesized (14). The synthesis of oligonucleotide chains bearing multiple guanosine sequences is not easy because their presence gives rise to low-solubility intermolecular structures (15) that make the chromatographic purifications difficult. The presence of a high-mass MPEG can reduce their intermolecular interaction and allows an easier produc-

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tion of such molecules. In addition, the amphiphilic property of this polymer should improve the nuclear internalization of the conjugated oligonucleotide. Both uptake and intracellular distribution of oligonucleotides were examined by confocal laser microscopy of their fluorescein-labeled derivatives (16). Usually, the introduction of a fluorescent moiety is achieved through preformed building blocks or by postsynthetic modification of the oligomers (17–19), both at its extremity as well as along the chain. In our case, since the MPEG unit is linked at the 3'-extremity of the oligonucleotide, an efficient liquid-phase procedure for the postsynthetic modification of the 5'-OH extremity is demanded.

In conclusion, in the present paper we describe the liquid-phase synthesis of "all-purine" sequences conjugated to high molecular weight MPEG, natural or chimeric, i.e., bearing some phosphorothioate modifications selectively located along the backbone, to be used as antigene effectors. To study the oligonucleotide cellular uptake and internalization, further conjugation with a fluorescein derivative was investigated. The compatibility of fluorescein derivatives with the liquid-phase approach was examined, and compared with similar solid-phase approaches (20). Synthesis and purification of these molecules were discussed, and their characterization was reported. An example of the cellular internalization process of these fluorescent MPEG-oligonucleotides is also described.

EXPERIMENTAL PROCEDURES

Synthesis of the MPEG-Conjugated Oligonucleotides. The synthesis of the MPEG-conjugated oligonucleotides was performed following the phosphoramidite-based procedure previously described (6).

A 9000 Da molecular mass monomethoxy poly(ethylene glycol) (MPEG) was used for the 13mer oligonucleotide complementary to the pyrimidinic tract of the *bcr* sequence. In the case of the 20mer complementary to the human Ki-ras S5, a 10 000 Da MPEG was preferred. Both syntheses were performed on 1.0 g of MPEG, corresponding to an approximate 100 μ M scale. The reaction yields were calculated by spectrophotometric analysis of the MPEG-conjugates, using the absorption of the DMT group at 498 nm, as previously reported (6), when a new DMT-bearing molecule was added.

The five thioate substitutions along the anti-Ki ras sequence were obtained by dissolving, after each coupling step, the MPEG-conjugated, growing oligonucleotide in acetonitrile (10% w/v), and reacting it with 10 equiv of diethoxy-dithiocarbonyl-disulfide (DDD) from a 0.5 M solution in acetonitrile. After 15 min under stirring, the product was precipitated with TBME, filtered, washed with ether, and dried under vacuum over KOH pellets.

The fully protected, MPEG-conjugated oligonucleotides were deprotected through the standard two-step ammonia/acetic acid procedure. The final purification was achieved by an anion-exchange HPLC performed on a Mono Q HR 5/5 column. The starting eluent was a 0.05 M NaCl solution, pH 12.0. The MPEG-13mer was eluted with a 0.5 M NaCl solution, pH 12.0, the gradient ranging from 25 to 80% over 30 min. The MPEG-20mer was eluted with a 2.0 M NaCl solution, pH 12.0, the gradient ranging from 0 to 100% over 30 min. Both products were desalted by gel filtration on Sephadex G25-F columns eluted with MilliQ water.

Introduction of the Fluorescein Probe. The introduction of the fluorescein moiety on the MPEG-conjugated oligonucleotide was performed on the 5'-detrity-

lated derivative, OH-free. Three different procedures were investigated:

(a) *Reaction at the 5'-OH Terminal Group with N-Hydroxysuccinimidofluorescein (Fluo-Osu).* Fifty milligrams of 5'-detritylated, MPEG-conjugated oligonucleotide was dissolved in a 50 mL three-neck reactor equipped with self-sealing septa. The product was coevaporated twice from anhydrous acetonitrile, thoroughly desiccated by a rotating pump, and left under an argon atmosphere. Through the septa, 1.0 mL of solution containing 100 mg of Fluo-Osu, freshly prepared (40 equiv), and 20 mg of DMAP was injected; a few drops of anhydrous DMF were added to create a clear solution. The reaction mixture was left under stirring for 72 h, and the product was precipitated, under stirring in an ice bath, by addition of TBME. The MPEG-conjugate was washed with i PrOH and ether, recrystallized from EtOH, and dried under vacuum over KOH pellets.

(b) *Reaction at an NH₂ Group, Introduced at the 5'-Terminal Position, with the Fluo-Osu Derivative.* The 5'-terminal position was previously modified by introduction of an NH₂ group through a commercial "amino-modifier", namely, the 2-[2-(4-monomethoxytrityl)aminoethoxy]ethyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (Glen Research). This synthesis was performed, as during the standard coupling step, by reacting 12 equiv, with respect to the free 5'-OH terminal groups, of the modifying phosphoramidite reagent dissolved in acetonitrile and 50 equiv of 1*H*-tetrazole on the same solvent, in anhydrous conditions, under an argon atmosphere. Fifty milligrams of MPEG-conjugated oligonucleotide in a total of 1.0 mL of anhydrous acetonitrile solution was employed. From the absorbance of the MMT group measured at 472 nm in p-TsOH 0.1 M in acetonitrile ($\epsilon = 17.600$), a 45% yield of reaction was measured.

After oxidation of the new phosphite bond, ammonia deprotection, and removal of the MMT 5'-amino-protecting group with an 80% (v/v) glacial acetic acid solution for 4 h, the MPEG derivative was lyophilized and reacted with 100 equiv of Fluo-Osu, as described in the previous procedure. The product was precipitated with TBME, washed with i PrOH and ether, and dried under vacuum over KOH pellets.

(c) *Reaction at the 5'-OH Terminal Group with a Fluo-Amidite Derivative.* Fifty milligrams of the 5'-detritylated, MPEG-conjugated oligonucleotide was extensively dehydrated, as reported, in a three-neck reactor equipped with self-sealing septa. Under an argon atmosphere, the solid was dissolved in a minimum amount of anhydrous acetonitrile. Successively, 6 equiv of the Fluo-amidite derivative, namely, the 1-DMT-2-(N-thiourea(di-O-pivaloyl-fluorescein)-4-aminobutyl)-propyl-3-O-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (Glen Research), and 25 equiv of 1*H*-tetrazole were added, and left to react under stirring for 15 min in an overall 1.0 mL solution. The product was precipitated, after cooling, with TBME and washed. The final derivative was recrystallized from EtOH to remove all soluble byproducts and residual excess reagent. From the DMT absorption, a 97% condensation yield for the MPEG-13mer and an 89% yield for the MPEG-20mer were measured. The phosphite bond was oxidized by the usual procedure and the product fully deprotected under standard treatment.

Confocal Microscopy. Confocal microscopy experiments were performed following a standard propidium iodide (PI) staining of cells fixed on a glass slide. K562 cells, supplied by Dr. M. Giunta (University of Udine), were seeded in 12-well plates at a density of 0.8×10^5 cells in 600 μ L of RPMI medium supplemented with 10%

heat-inactivated fetal bovine serum 24 h before being added with the oligonucleotides. Cells were treated with free Fluo-13mer or Fluo-13mer-MPEG. After centrifugation, the cells were washed twice with PBS, spun on a glass slide, and fixed with 3% paraformaldehyde (PFA) for 20 min. After washing with 0.1 M glycine containing 0.02% sodium azide to remove PFA and Triton X-100 (0.1% in PBS), the cells were incubated with PI and RNase for 30 min at 37 °C in order to stain the nuclei. Then coverglasses were mounted on the glass slide with Mowiol 4-88 and Dabco (2.5%). Fixed K562 cells were analyzed with a Leica DM IRBE confocal imaging system. Diaphragm and fluorescence detection levels were adjusted to reduce to a minimum any interference between fluorescein and PI channels.

Characterization of the MPEG-Conjugated Oligonucleotide. The spectrophotometric analyses were performed on a Helios β UNICAM spectrophotometer.

^1H and ^{31}P NMR spectra were recorded on a JEOL EX 400 spectrometer, utilizing CDCl_3 (99.96%) as solvent and TMS and 85% phosphoric acid as internal standards.

MALDI-TOF mass spectra were recorded on a Voyager DE mass spectrometer (Perseptive Biosystems, Framingham, MA) equipped with a N_2 laser (337 nm). MALDI conditions are as follows: accelerating voltage, 24 000 V; guide wire, 0.05% of accelerating voltage; grid voltage, 94% of accelerating voltage; delay extraction time, 550 ms; and 256 scans averaged. Samples were mixed with trihydroxyacetate phenone (THAP) or 2,5-dihydroxybenzoic acid (DHB) as a matrix.

To ascertain the purity of MPEG-conjugated derivatives, TLC on silica gel 60 F254 was utilized with the eluant $\text{CHCl}_3/\text{EtOH} = 9:1$ (v/v). The purified compounds were evaluated by an analytical IE HPLC column (eluant: gradient of NaCl, at pH 12.0, from 0.05 to 0.6 M in 38 min for the MPEG-13mer and its shorter derivatives, and from 0.05 to 2.0 M in 25 min for the MPEG-20mer and its shorter derivatives). An FPLC apparatus (Pharmacia) equipped with a Mono Q HR 5/5 column was used. The purity of the longer MPEG-conjugated oligonucleotides was also investigated by the gel electrophoresis technique, in denaturing conditions, on 20% polyacrylamide. Conditions: 2 h of prerunning at 55 °C, 600 V, 25 mA, 15 W; 30 min of running at 55 °C, 600 V, 23 mA, 15 W. Staining with a "stains-all" solution: MPEG-conjugated oligonucleotides appear as blue bands.

Maxam and Gilbert G reaction for footprinting experiments was performed according to the standard procedure (21).

RESULTS AND DISCUSSION

Synthesis and Purification of "All-Purine" MPEG-Conjugated Oligonucleotides. The liquid-phase synthesis of TRIPEGXs was performed by the standard procedures described for similar compounds in a previous paper (6). Concentration, size of support, excess of reagents, and general procedures were followed with meticulousness for a safe comparison with earlier syntheses. A main difference was obviously introduced when a phosphorothioate substitution along the backbone was required. The sulfurizing agent was different from that described in a similar process (22); in this case, the DDD previously used for a large-scale liquid-phase production of fully thioate free oligonucleotides was employed (2). Its efficacy and convenience even in the production of a chimeric phosphate/thioate product were confirmed.

The sequences of the two MPEG-oligonucleotides to be synthesized are reported in Scheme 1. Obviously, the

Scheme 1. Sequences of MPEG-Oligonucleotides Synthesized^a

MPEG-13mer: MPEG-(3')-d(AGGAAGGGGAGGG)-(5')-OH

MPEG-20mer: MPEG-(3')-d(GGpsGAAGAGpsGGApsGAGpsGGGpsA)-(5')-OH

^a ps = phosphorothioate bond.

Table 1. Synthetic Data of the MPEG-Conjugates

	sample	
	MPEG-13mer	MPEG-20mer
average yield (%)	99.5	99.5
overall yield (%)	94.8	90.5
product recovered (%)	48	26
from 1.0 g of MPEG (g)	0.75	0.50

Table 2. PO/PS Ratio of the MPEG-20mer Oligonucleotide Sequences Derived from ^{31}P NMR Analysis of Crude Products (Phosphotriester at -2.8 ppm, Phosphothiotriester at 66.7 ppm)

MPEG-oligomer	no. of PS bonds	PO/PS ratio, calculated	PO/PS ratio, observed
10mer	2	4.0	4.2
14mer	3	3.7	4.0
16mer	4	3.0	3.1
20mer	5	3.0	3.4

conjugated, chimeric anti-Ki-ras 20mer shows the same number and position of thioates as in the free 20mer previously investigated (14). A main drawback was expected in these syntheses due to the presence of long, repeated stretch of guanines along the final sequence. Since it is well-known that guanine-rich chains have a strong propensity to form stable intermolecular aggregates, a positive effect was supposed to be given by a large unit of an amphiphilic polymer linked to the oligonucleotide chain, as in the case of the MPEG moiety. A first, positive response can be observed from the synthetic data reported for the two MPEG-conjugates (Table 1): it is quite clear that, under standard conditions, an almost quantitative yield during all the intermediate steps and a large amount of final, crude derivatives are obtained when a phosphoramidite-activated fluorescein is employed. The estimation of the expected ratio between natural phosphodiester and surrogate thioates in the case of the chimeric molecule, analyzed by ^{31}P NMR, confirmed the efficacy of the procedure within the experimental error (Table 2). In Figure 1 an example of a ^{31}P NMR spectrum of a chimeric, MPEG-conjugated oligonucleotide is reported.

The unfavorable intermolecular interactions of the synthesized derivatives were investigated in detail during the chromatographic purification. The accustomed reverse-phase HPLC was not adopted since the large MPEG component dominates the behavior of the conjugated molecules and gives a false uniform chromatographic pattern even in the presence of a mixture of products. Hence, a strong anionic support was preferred since the charge balance of different oligonucleotides is not suppressed by MPEG. As described in Figure 2, part A, the superimposition of selected products obtained during the overall synthetic production of the MPEG-13mer gives a clear image of the proceeding of the process. In Figure 2, part B, a similar chromatographic procedure, but on a preparative scale, shows that the higher sample concentration demanded by the larger operative scale generates some new late-running peaks, likely due to the aggregation of the single MPEG-conjugated chains. In fact, as reported in the inset, the product collected from those peaks, eluted once again in diluted, analytical conditions, gives a main peak superimposable to that of the pure,

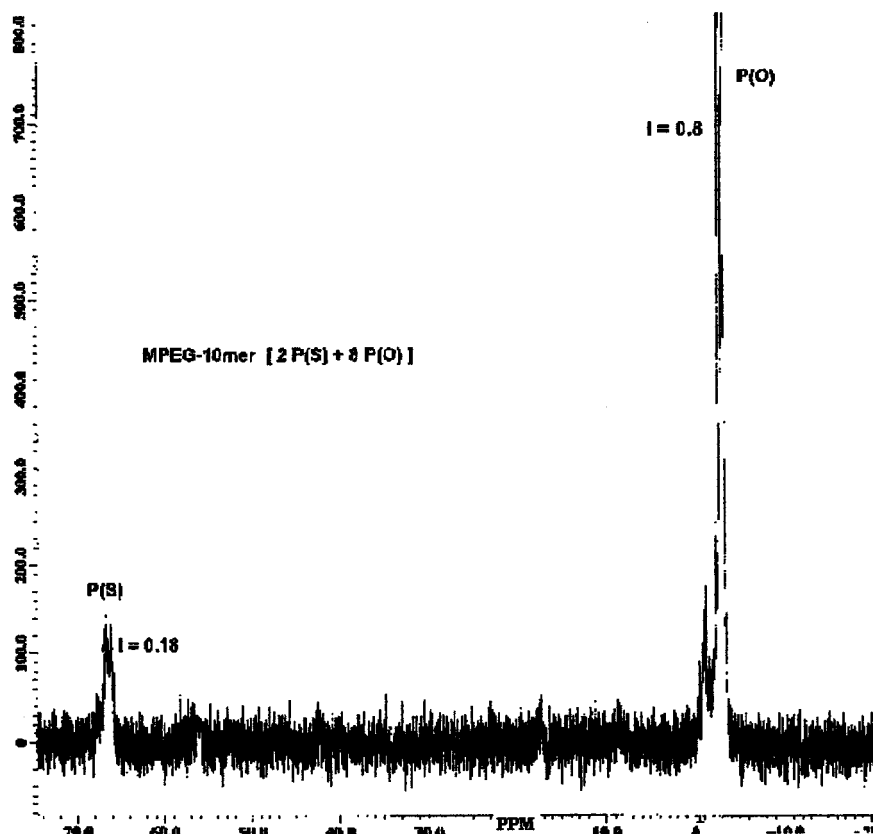


Figure 1. ^{31}P NMR spectrum in CDCl_3 of crude, fully protected, chimeric MPEG-10mer.

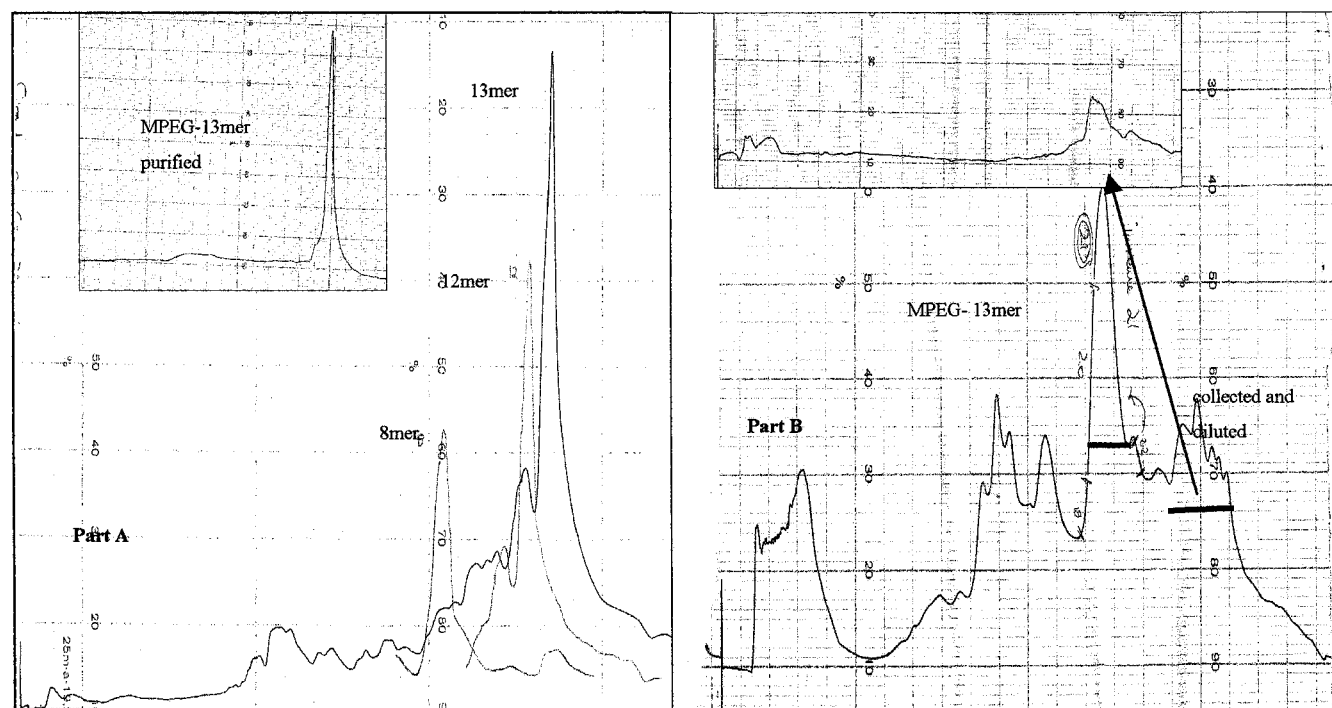


Figure 2. Part A: Analytical IE HPLC (4 mg/mL) patterns of the crude MPEG-13mer and two shorter oligomers. Inset: Analytical IE HPLC of the purified and desalted MPEG-13mer. Part B: IE HPLC purification of the crude MPEG-13mer (24 mg/mL) (the bar indicates the fraction of purified MPEG-13mer collected). Inset: Chromatography of a sample collected from the late-running peak, after dilution.

final product. The same features were given by the MPEG-20mer (noteworthy: the longest oligonucleotide conjugated to a high molecular weight MPEG unit so far obtained!). However, in this case, the chromatographic analysis and purification were made more troublesome due to the presence of the new chiral centers introduced

at the phosphorothioate level, and due to the consequent diastereomeric mixture that produced a multippeak pattern.

Gel electrophoresis analysis on denaturing, high-temperature, conditions (Figure 3) supports our belief in the efficiency of the proposed synthetic method. It is quite

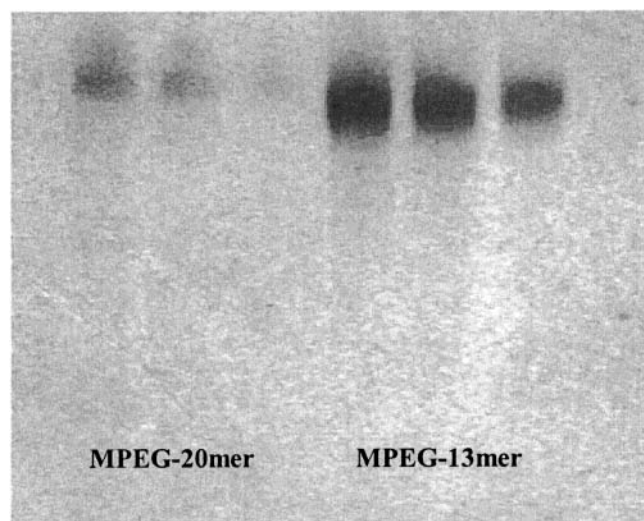


Figure 3. Gel electrophoretic analysis on denaturing conditions of the MPEG-oligonucleotides.

clear that the polydispersity of the supporting MPEG does not allow a full investigation on the detailed composition of the final, purified compound. On the other hand, it ensures that only MPEG-conjugated derivatives are present, in which the nucleic acid component is expected to be homogeneous in length and composition on the basis of the analytical IE HPLC of the purified compounds (Figure 1, part A: inset).

An indirect determination of the final sequence of the TRIPEGXs was obtained by Maxam and Gilbert DMS/piperidine footprinting experiments with 50-mer duplexes containing the *bcr/abl* and Ki-ras target. It should be remembered that DMS reacts with guanine N7 only when this base is not involved in hydrogen bonding. We observed that all the guanines inside the targets were protected from chemical degradation, suggesting that the synthesized MPEG-conjugated oligonucleotides had the expected length and composition (data not shown).

Introduction of a Fluorescein Probe on the MPEG-Oligonucleotides. The purpose of this synthesis was to follow the cellular trafficking of the MPEG-conjugated oligonucleotides and to ascertain their nuclear accumulation, if any, since they are designed to interact with a double-helical, genomic DNA target.

Initially, the reaction of a Fluo-OSu molecule to form an ester bond with the free, terminal 5'-OH group of a simple conjugated dinucleotide was investigated. The same size of the supporting polymeric chain was utilized, namely, 9000 Da molecular mass. Taking advantage of the basic features of the liquid-phase procedure, a large excess of the activated fluorescein derivative was used to overcome the expected low reactivity of the system, but, even after repeated treatments, the final yield did not overcome a few percent reaction. The nucleophilicity of the terminal hydroxy group was clearly too low to permit the introduction of a satisfactory amount of the fluorescent probe. Hence, a more reactive amino group was introduced through a suitable, commercial linker using a phosphoramidite-based procedure. In this case, a good amount, even if not excellent, of linker was found to be present at the 5'-end of the MPEG-oligonucleotide. Successively, the higher nucleophilicity of the amino group allowed the isolation of a derivative bearing a larger amount of fluorescein, activated as previously described. A far better result, nearly quantitatively modified, was obtained by reacting a phosphoramidite-activated fluorescein with the MPEG-oligonucleotide,

Table 3. Yields of Introduction of the Fluorescein Derivatives on the Final, Crude MPEG-Oligonucleotides^a

fluorescein derivative condensation (%)	MPEG-13mer	MPEG-20mer
MPEG-oligonucleotide-(5')-OH + Fluo-OSu	4.5	—
MPEG-oligonucleotide-(5')-NH ₂ + Fluo-OSu	60	—
MPEG-oligonucleotide-(5')-OH + Fluo-amidite	95	90

^a $\epsilon = 11\,600$ at 480 nm in H₂O.

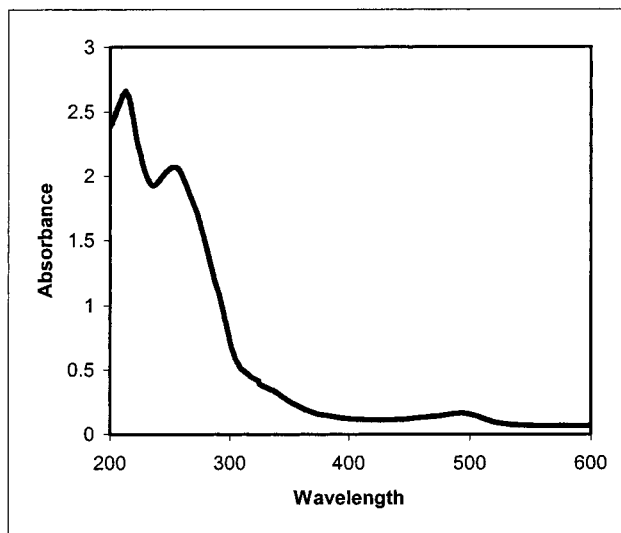


Figure 4. UV/Vis spectrum in water of the MPEG-(3')-20mer-(5')-fluorescein, fully deblocked.

once the 5'-DMT protecting group was removed. In this last reaction, a very high yield was obtained with the standard procedure utilized for the liquid-phase synthesis of the oligonucleotides (6). A further advantage derives from the reduced number of synthetic steps, since any previous introduction of an appropriate linker is avoided. The same reaction was performed on the larger, chimeric MPEG-20mer with similar results. In both cases, it must be reminded that these reactions were always performed on the crude, final mixture deriving from the liquid-phase synthesis of the MPEG-conjugates, since all the shorter, failure sequences were in advance, step-by-step, capped. Consequently, the only reacting chain able to bear a fluorescent probe was exclusively the longer, final sequence. In Table 3 yields of introduction of the fluorescein probes are reported.

A further advantage of this liquid-phase procedure was due to the spectral transparency of the MPEG support that allows an easy evaluation of the proceeding of the reaction through simple UV/Vis analyses. As an example, the UV spectrum of the fluorescent, fully deprotected MPEG-20mer is reported (Figure 4). Unfortunately, the ¹H NMR analysis of these products is difficult due to the low intensities of probes' signals and to their burial under those of the polymer.

Mass Characterization of MPEG-Conjugates. The correct sizes of the final MPEG-conjugated oligonucleotides were tentatively investigated by using MALDI mass spectroscopy. A set of crude, fully protected and deprotected final derivatives were analyzed, to establish if this technique could be convenient for their final characterization. It must be recalled that the electrospray (ESI) technique, proposed for the mass evaluation of similar compounds (23), in this case can be severely limited by the too large a size of the final conjugates. In our hands,

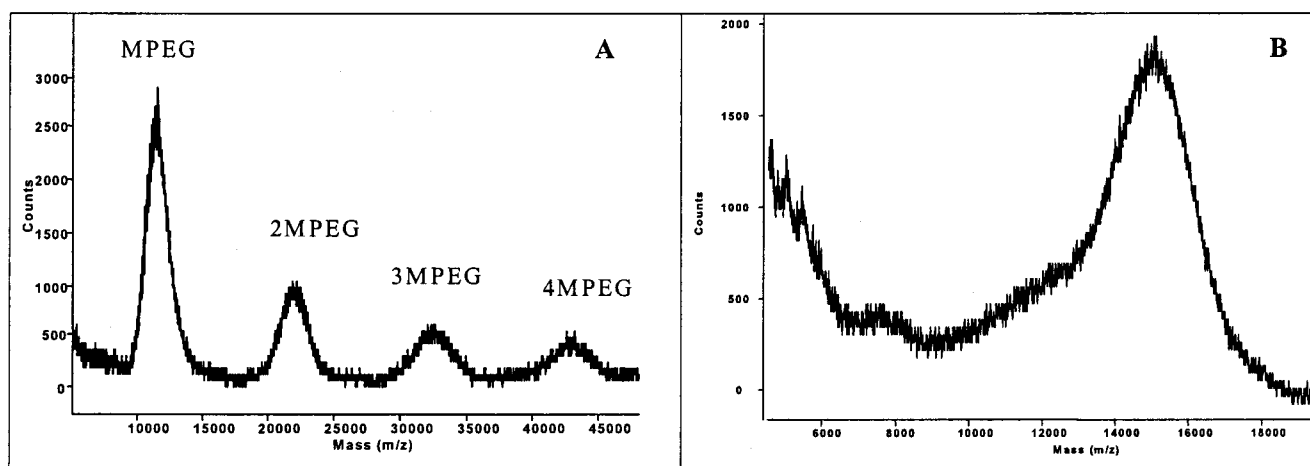


Figure 5. MALDI spectra of (A) starting MPEG and (B) fully protected MPEG-13mer (calculated mass = 14 952 Da).

the MALDI approach has disappointingly given only a partial reply. In particular, it was possible only to establish the mass of the fully protected MPEG-oligo, while any attempt was unsuccessful in the case of MPEG-oligos with charged linkages where only the signal corresponding to the starting MPEG was detected. A possible explanation is that MPEG is more easily ionized than the MPEG-oligos; hence, even a trace of MPEG present in the sample led to spectral suppression.

Typical MALDI spectra are reported in Figure 5.

Efficiency of MPEG-Conjugates as Fluorescent Probes. The fluorescent MPEG-oligonucleotides were tested in selected cell lines for their ability to penetrate the cellular membranes.

In the case of the MPEG-oligonucleotide in which the fluorescein moiety was linked through an ester bond, a low fluorescence intensity was initially observed inside the nucleus which rapidly disappeared, most probably for the hydrolysis of the probe and its removal from the cell.

A similar behavior was observed when the fluorescein molecules were joined to the oligonucleotide through an amide bond, following the previous introduction of an amino-linker at its 5'-extremity. Again, a rapid appearance of fluorescence was noticed inside the nucleus, but its intensity did not further increase, suggesting a reduced stability of the probe's bond.

With the third derivative, in which the chain was modified by a direct phosphorylation of the 5'-OH terminus with the proper fluorescein derivative, an increasing performance was recognized. In fact, a strong fluorescent emission from the nucleus was detected which augmented with time and stabilized at higher values after 72 h from the addition of the fluorescent MPEG-oligonucleotide. To demonstrate the efficiency of these fluorescent, high molecular weight, MPEG-conjugated oligonucleotides, their capacity to penetrate the cellular membranes and to internalize in the nuclei was investigated by laser confocal microscopy on leukemic K562 cells. Figure 6 shows typical confocal images of K562 cells exposed to 10 μ M fluorescein-labeled MPEG-13mer oligonucleotide for 2 and 6 h. The nuclei of the cells have been stained in red with propidium iodide. To evaluate the effect of MPEG on the transport of the 13mer in the cells, we compared the uptake of the unconjugated 13mer oligonucleotide (Fluo-13mer) with the uptake of the conjugated oligonucleotide (Fluo-13mer-MPEG). Panels A and B show that free Fluo-13mer is taken up by K562 cells, as indicated by the green light emitted from the

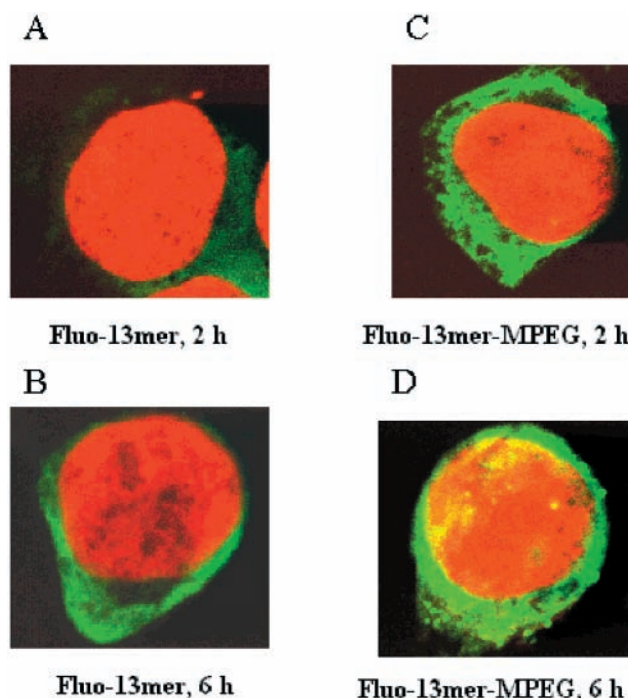


Figure 6. Confocal images of K522 cells exposed to free and MPEG-conjugated 13mer after 2 and 6 h, respectively.

perinuclear region. This suggests that the 13mer oligonucleotide is taken up by the cells and that it remains in the cytoplasm, after incubation times of 2 and 6 h. However, a close inspection of the nucleus in panel B suggests that after 6 h of exposition a small amount of oligonucleotide reached the nucleus, as indicated by the presence of a yellow crown. In fact, the presence of Fluo-13mer (emitting green light) in the nucleus (emitting red light) is detected by the emission of yellow light (green light plus red light). Panels C and D show the confocal images obtained with the MPEG-conjugated 13mer oligonucleotides. It can be seen that after 2 h of incubation Fluo-13mer-MPEG is present in the cytoplasm in large amount as indicated by the intense green fluorescent light color. Comparison of panels A and B with panels C and D indicates that MPEG facilitates the transport of the 13mer oligonucleotides into the cytoplasm of K562 cells. Interestingly, after 6 h of incubation, the nucleus of the cell appears stained in orange and yellow, clearly indicating that Fluo-13mer-MPEG has been internalized in the nucleus. In the case of free Fluo-13mer, after 6 h

of incubation with K562 cells, it was present in the nucleus only in trace amounts. We have extended the confocal analysis to other cell cultures (HeLa, Panc-1) and obtained substantially the same behavior. In conclusion, the conjugation to high molecular weight MPEG enhanced the oligonucleotide capacity not only to penetrate the cellular membranes but also to internalize in the nuclei. Since it is known that MPEG oligonucleotides do not lose their binding property (10), their use as effector molecules is now extensively investigated in our laboratories, and it will be the subject of a forthcoming paper (12).

In conclusion, the liquid-phase approach, based on the use of a soluble inert support, was conveniently utilized even for the production of quite large "all-purine" MPEG-conjugated oligonucleotides designed to act as active anti-gene agents. The demanded phosphorothioate units within the phosphodiester backbone were easily introduced at the desired positions. The synthetic approach adopted for these sequences apparently did not represent a serious obstacle to their efficient large-scale production. The purification of the final product was affordable, despite few drawbacks due to the residual presence of multimeric components resulting from the intermolecular interactions of G-rich sequences. The unique features of the supporting, large MPEG moiety were fully compatible with the further introduction of a fluorescent probe on the final conjugate, extremely useful to follow their cellular destination, and appeared to improve their cellular uptake with a specific nuclear accumulation.

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Toward New Designed Proteins Derived from Bovine Pancreatic Trypsin Inhibitor (BPTI): Covalent Cross-Linking of Two 'Core Modules' by Oxime-Forming Ligation

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A 25-residue disulfide-cross-linked peptide, termed 'oxidized core module' (OxCM), that includes essentially all of the secondary structural elements of bovine pancreatic trypsin inhibitor (BPTI) most refractory to hydrogen exchange, was shown previously to favor nativelylike β -sheet structure [Carulla, N., Woodward, C., and Barany, G. (2000) Synthesis and Characterization of a β -Hairpin Peptide That Represents a 'Core Module' of Bovine Pancreatic Trypsin Inhibitor (BPTI). *Biochemistry* 39, 7927–7937]. The present work prepares to explore the hypothesis that the energies of nativelylike conformations, relative to other possible conformations, could be decreased further by covalent linkage of two OxCMs. Optimized syntheses of six ~50-residue OxCM dimers are reported herein, featuring appropriate monomer modifications followed by oxime-forming ligation chemistry to create covalent cross-links at various positions and with differing lengths. Several side reactions were recognized through this work, and modified procedures to lessen or mitigate their occurrence were developed. Particularly noteworthy, guanidine or urea denaturants that were included as peptide-solubilizing components for some reaction mixtures were proven to form adducts with glyoxylyl moieties, thus affecting rates and outcomes. All six synthetic OxCM dimers were characterized by 1D ^1H NMR; three of them showed considerable chemical shift dispersion suggestive of self-association and mutual stabilization between the monomer units.

INTRODUCTION

An 'oxidized core module' (OxCM),¹ which is a 25-residue disulfide-cross-linked peptide that includes essentially all of the secondary structural elements that in the full protein bovine pancreatic trypsin inhibitor (BPTI: 58 residues, 3 disulfides, Figure 1a) are most refractory to hydrogen exchange, has recently been designed, synthesized, and characterized (1). NMR analysis showed that OxCM favors nativelylike β -sheet structure in rapid equilibrium with populations of non-native β -sheets (1). Based on these encouraging observations, ways were sought to further stabilize nativelylike conformations in OxCM. The working hypothesis is that covalent linkage of two OxCMs may enhance self-association and mutual stabilization, resulting in a more compact, kinetically accessible, and thermodynamically favored structural ensemble resembling a protein native state. This design strategy was reinforced by findings about the full-length protein, i.e., a dimeric form of BPTI exists in solution and undergoes rapid association–dissociation (2, 3). Further, molecular modeling studies on dimeric BPTI indicate that the residues at the hydrophobic monomer–monomer interface are primarily in OxCM (4) (Figure 1b).

The chemistry chosen to link two OxCMs is oxime-forming ligation (5). In its most general form, this method

allows the joining of two unprotected peptide segments in an unambiguous manner by the chemoselective reaction of two unique and mutually reactive functionalities: (a) an (aminoxy)acetyl moiety and (b) any of a variety of carbonyl-containing moieties including aldehydes, methyl ketones, and α -oxo aldehydes (glyoxylyl

¹ Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC–IUB Commission of Biochemical Nomenclature (34). The following additional abbreviations are used: Abu or X, α -amino-*n*-butyric acid; Alloc, allyloxycarbonyl; Aoa or U, (aminoxy)acetyl; Boc, *tert*-butyloxycarbonyl; BPTI, bovine pancreatic trypsin inhibitor; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; Dpr or Z, α,β -diaminopropionic acid; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; ESMS, electrospray mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; FPLC, fast protein liquid chromatography; GdnHCl, guanidine hydrochloride; Gxy or J, glyoxylyl; HBTU, *N*-[[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide]; HOAt, 1-hydroxy-7-azabenzotriazole (3-hydroxy-3*H*-1,2,3-triazolo-[4,5-*b*]pyridine); HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight (mass spectrometry); Mpa or B, β -mercaptopropionic acid; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; OxCM, oxidized core module; PAL, 'peptide amide linker' [5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric acid]; PEG-PS, poly(ethylene glycol)–polystyrene (resin support); Pfp, pentafluorophenyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PyAOP, 7-azabenzotriazol-1-yl-*N*-oxytris(pyrrolidino)phosphonium hexafluorophosphate; SEC, size-exclusion chromatography; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; Tmob, 2,4,6-trimethoxybenzyl; TOCSY, total correlation spectroscopy; TP, model tetrapeptide amide Tyr-Dpr(Gxy)-Ala-Lys-NH₂; Trt, triphenylmethyl or trityl.

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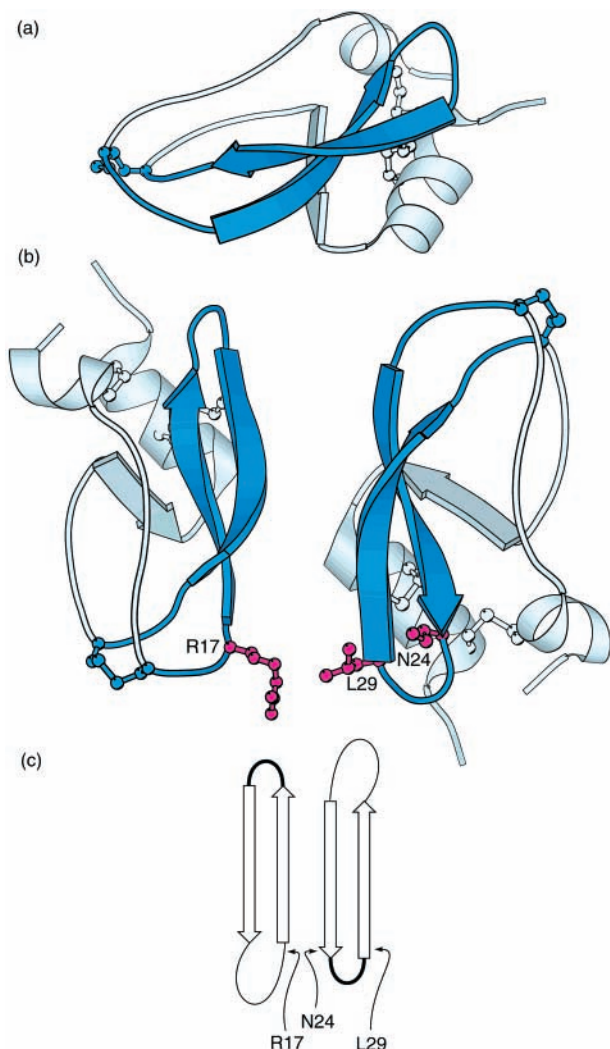


Figure 1. Ribbon representations of (a) crystallographic structure of BPTI, showing positions of the three native disulfides and highlighting in dark blue those residues that correspond to the oxidized core module (OxCM), and (b) a model for the BPTI dimer, reorienting two BPTI structures from (a) in the way proposed by Zielenkiewicz et al. (4), but without repeating those authors' energy minimization calculations. Residues Arg¹⁷ in monomer I (left) and Leu²⁹ (nearer to Arg¹⁷) and Asn²⁴ (further from Arg¹⁷) in monomer II (right) are shown in red. Both diagrams in (a) and (b) were produced using the program MOLSCRIPT (33). (c) Representation of a simpler model for OxCM dimer that, unlike what is shown in (b), ignores the native twist of the β -sheet strands and suggests that Arg¹⁷ in monomer I is spatially closer to Asn²⁴ in monomer II. Block arrows are β -sheet strands (oriented N at tail to C at arrowhead), solid boldface ribbons are β -turns, and solid lines are the flexible loops including the disulfide bond that cyclizes each OxCM.

function). Oxime-forming ligations have been used primarily in the production of peptide dendrimers (5–7), 'template assisted synthetic proteins' (TASPs) (8, 9), and functional proteins such as the heterodimeric transcription factor cMyc-Max (10). When carried out intramolecularly, oxime-forming ligation provides macrocyclic peptides (11, 12). For the present application (Scheme 1), it was observed that the presence of guanidine hydrochloride (GdnHCl) or urea as denaturants in the buffers used to dissolve peptide materials altered the rates and outcomes of the ligations. The underlying chemistries were elucidated in a model tetrapeptide system, and the resultant insights were applied to

optimize conditions for oxime-forming ligations of BPTI core modules.

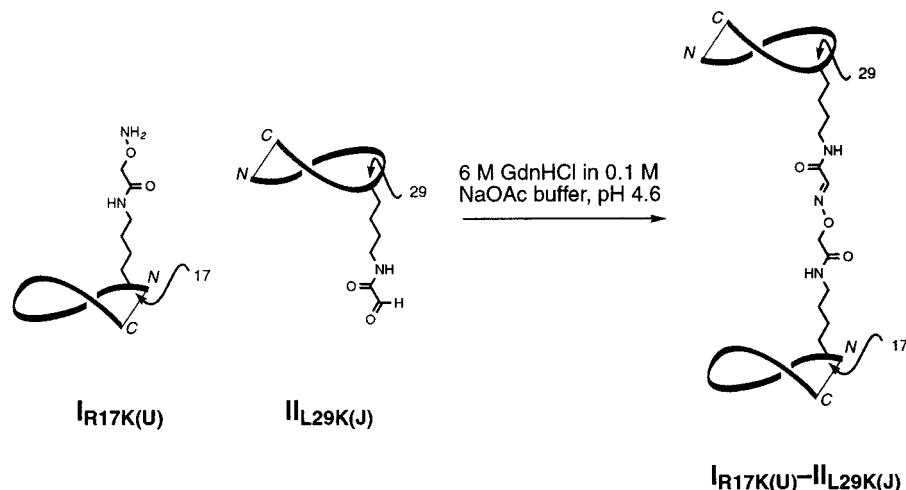
The chosen dimerization chemistry accommodates changes in the position of the cross-link (Figure 2a) and variations in the length of the cross-link (Figure 2b). Six examples of OxCM dimers were synthesized. One-dimensional proton (¹D ¹H) NMR was used as a qualitative criterion to gauge the extent of conformational families present in each OxCM dimer. Three dimers showed considerable chemical shift dispersion, and one of them is likely to be amenable for further structural characterization by multidimensional homo- and hetero-nuclear NMR methods.

EXPERIMENTAL PROCEDURES

General. Materials, instrumentation, and procedures for synthesis, spectroscopy, chromatography, and analysis are detailed in the Supporting Information; some of these follow precedents in earlier publications from our laboratories (13–15), and others are specific for this work. Refer to the Supporting Information for conditions A through I used in reversed-phase analytical or semi-preparative HPLC and conditions J and K for size-exclusion chromatography (SEC). The matrix used for sample analysis by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) was α -cyano-4-hydroxycinnamic acid (4-HCCA; Hewlett-Packard, obtained as solution in methanol), diluted 1:1 with 0.1% TFA in CH₃CN/H₂O (1:1), whereas samples for electrospray mass spectrometry (ESMS) were infused in 0.2% acetic acid in CH₃CN/H₂O (1:1). The final isolated yields were calculated by comparison of amino acid analyses of the purified peptide to the initial loading of the resin. In describing core module monomers, capped monomers, and dimers, the notation presented in Figures 2, 3, and 8, and the accompanying text, is used. When closely related procedures were followed, this section provides representative descriptions while further characterizations are reported in the Supporting Information.

NMR Spectroscopy. NMR samples for the tetrapeptide model studies were 11 mM dissolved in ²H₂O at pH 4.6 (measured at electrode and uncorrected for isotope effects). ²H₂O was from Aldrich, and urea-¹⁵N₂ was from Cambridge Isotope Laboratories, Inc. (Andover, MA). GdnDCl-*d*₆ and urea-¹⁵N₂,*d*₄ were prepared respectively by dissolving GdnHCl or urea-¹⁵N₂ in an excess of ²H₂O, lyophilization, and repeating the solution/lyophilization cycle 3 more times. Experiments were performed at 25 °C on Varian 300, 500, 600, or 800 MHz Inova instruments, and the pulse sequences used were obtained from the Varian library (macros: gCOSY, gHMQC, and gHMBC). ¹H/¹H-gCOSY were acquired using 2K × 333 data points and a spectral width of 12 ppm in both dimensions. ¹H/¹³C-gHMQC and ¹H/¹³C-gHMBC were acquired using 2K × 512 data points and a spectral width of 12 ppm in the ¹H dimension and 240 ppm in the ¹³C dimension. ¹⁵N/¹H-gHMQC and ¹⁵N/¹H-gHMBC were acquired using 2K × 128 data points and a spectral width of 12 ppm in the ¹H dimension and 200 ppm in the ¹⁵N dimension. A shifted sine bell or a Gaussian function was applied to both dimensions during data processing.

NMR samples for the capped monomers were 0.3–0.5 mM, pH 4.5, and for the OxCM dimers were 0.2–0.4 mM, pH 2. The lyophilized pure peptides or proteins were dissolved in water at the desired pH and adjusted when required with aliquots of 0.1 N ²HCl/²H₂O or 0.1 N NaO²H/²H₂O. ¹H NMR spectra were obtained in 90:10 H₂O/²H₂O on a Varian 600 MHz Inova instrument.

Scheme 1. Synthesis of $I_{R17K(U)}-II_{L29K(J)}$ Dimer by Oxime-Forming Ligation To Connect Corresponding OxCM Monomers^a

^a See later in this paper (Figures 2 and 3 and accompanying text) for explanations of schematic and nomenclature conventions.

Proton chemical shifts of capped monomers were measured from an internal DSS standard defined as 0 ppm. NMR data acquisition and processing followed methods described previously (1).

The hydrogen isotope exchange rate for the most downfield amide proton resonance of $I_{R17K(U)}-II_{L29K(J)}$ was obtained by measuring peak integrals vs time in a series of 1D spectra at pH 2 and 5 °C (Supporting Information Figure 2). The pseudo-first-order rate constant was determined from nonlinear least-squares fit of an exponential rate equation to experimental data.

Peptide Synthesis. Starting with Fmoc-PAL-PEG-PS resin (13, 16) (1 g, 0.24 mmol/g, 0.24 mmol), the core module monomer linear sequences were assembled by automated Fmoc solid-phase synthesis using an Applied Biosystems Pioneer automated peptide synthesizer. Side-chain protecting groups were provided by appropriate *tert*-butyl (tBu) ethers and esters for Thr, Tyr, Asp, and Glu; *tert*-butoxycarbonyl (Boc) for Lys (when not part of cross-link); 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg; 2,4,6-trimethoxybenzyl (Tmob) for Asn and Gln; and trityl (Trt) for Cys. Washes between chemical steps were carried out with DMF. Fmoc removal was achieved with piperidine/DMF (1:4, 5 min). Couplings of all residues other than Cys were 1 h, in DMF, as mediated by HBTU/HOBt/DIEA (4:4:8 equiv with respect to peptide-resin; 3.5 min preactivation) using 4 equiv of Fmoc-amino acids. Fmoc-Cys(Trt)-OPfp was used for 1 h, in DMF, to introduce Cys with minimal racemization (17). As expected, completed peptide-resins had a lower substitution (i.e., 0.12 mmol/g), due to weight gain from the peptide portion.

Peptide Synthesis of Core Module Monomers: $I_{R17K(U)}$, $I_{R17K(UG)}$, and $I_{R17Z(U)}$ (Supporting Information Scheme 1), and $II_{L29K(J)}$, $II_{L29K(JG)}$, $II_{L29Z(J)}$, and $II_{N24K(J)}$ (Supporting Information Scheme 2 and Figure 4). $I_{R17K(U)}$. In the linear core module sequence, Arg¹⁷ was replaced by Fmoc-Lys(Alloc)-OH. A portion of the completed peptide-resin (1 g, 0.12 mmol/g, 0.12 mmol) was washed with CH₂Cl₂ (10 × 0.5 min), and the *N*^ε-Alloc protecting group of Lys¹⁷ was cleaved under an argon atmosphere at 25 °C by adding Me₂NH·BH₃ (84.8 mg, 1.44 mmol, 12 equiv) in CH₂Cl₂ (4.7 mL), followed 1 min later by Pd(PPh₃)₄ (27.7 mg, 0.024 mmol, 0.2 equiv) in CH₂Cl₂ (0.8 mL). After 10 min treatment, the peptide-resin was drained and washed with CH₂Cl₂ (3 × 0.5 min),

and the deprotection procedure was repeated twice. The peptide-resin was then washed successively with CH₂Cl₂ (8 × 0.5 min), 0.2% TFA in CH₂Cl₂ (2 × 1 min), DIEA/CH₂Cl₂ (1:19) (3 × 1 min), and CH₂Cl₂ (5 × 0.5 min). An aliquot of the peptide-resin at this stage was cleaved, and analyzed by HPLC (*t*_R 16 min, condition D). The identity of the peptide in the HPLC peak was confirmed by MALDI-TOF analysis; calcd monoisotopic mass: 2826.4, found: 2827.6 [M+H]⁺.

The peptide-resin (1 g) with the Alloc group removed was placed into a column of the Applied Biosystems Pioneer synthesizer. Next, Boc-Aoa-OH (8 equiv) was incorporated onto the *N*^ε-amino side chain of Lys¹⁷ using PyAOP/HOAt/DIEA (8:8:16 equiv with respect to peptide-resin). Boc-Aoa-OH, PyAOP, and HOAt were weighed as solids into the same vial, and were dissolved by addition of equal volumes of 1 M DIEA-DMF and neat DMF; after a 3.5 min preactivation period, this mixture was added to the peptide-resin to initiate coupling. Two more such couplings were required to complete the incorporation of Boc-Aoa-OH.

Upon completion of on-resin steps, the dried protected peptide-resin was cleaved portion-wise with freshly prepared Reagent R [TFA/thioanisole/1,2-ethanedithiol/anisole (90:5:3:2)]. Peptide-resin (450 mg, 58.4 μmol of peptide) was treated with cleavage cocktail (5 mL) for 1 h at 25 °C. The filtrate was collected and set aside at 25 °C, while the remaining peptide-resin was cleaved twice more, 1 h each, with fresh cocktail (5 mL). The separate filtrates were concentrated under a stream of N₂, and Et₂O (~25 mL, freshly distilled from Na) was added at 0 °C with thorough agitation. The precipitated peptide was collected by low-speed centrifugation, washed with cold freshly distilled Et₂O (3 × 25 mL), and dissolved in ~100 mL of H₂O/CH₃CN (9:1) for lyophilization. The crude peptide (126 mg, 74% cleavage yield) was characterized by analytical HPLC (*t*_R 26.3 min, >90% purity; condition A), and the identity of the main HPLC peak was confirmed by MALDI-TOF analysis; calcd monoisotopic mass: 2899.45, found: 2900.32 [M+H]⁺. When the precipitated peptide was washed with reagent grade Et₂O from a can (not distilled), a byproduct was observed by HPLC (*t*_R 21 min; condition D) and the identity of this HPLC peak was confirmed by MALDI-TOF; found: 2940.1 [M+H]⁺ (40 amu above the expected mass).

To form the disulfide bridge, the crude reduced peptide (60 mg, 20.7 μ mol) was dissolved in 6 mL of 0.01 N aqueous HCl, and then ~300 mL of a mixture of dimethyl sulfoxide/6 M GdnHCl in 0.1 M sodium phosphate buffer, pH 7 (1:18), was added. The reaction was monitored by analytical HPLC (t_R 26.3 min and t_R 21.8 min for reduced and oxidized, respectively; condition A). After oxidation had gone to completion in 12 h at 25 °C, the mixture was concentrated over a 1 day period using an Amicon ultrafiltration cell (YM1 membrane, MWCO ~1000) (final volume ~25 mL). Crude oxidized peptide was loaded in five batches onto semipreparative HPLC (condition E). Purified peptide (10.2 mg, 6%) was characterized by analytical HPLC (t_R 21.8 min; condition A), amino acid analysis (Asp, 2.02; Thr, 0.93; Glu, 1.02; Gly, 3.02; Ala, 1.99; Val, 0.97; Ile, 1.35; Leu, 1.02; Tyr, 2.86; Phe 1.97; Lys, 2.92; Arg, 1.03), and MALDI-TOF; calcd monoisotopic mass: 2897.43, found: 2898.29 [M+H]⁺.

For synthesis and characterization of I_{R17K(U)} and I_{R17Z(U)}, see Supporting Information.

II_{L29K(J)}. In the linear core module sequence, Leu²⁹ was replaced by Fmoc-Lys(Alloc)-OH. Alloc deprotection was carried out in the same way as already described with the procedure for I_{R17K(U)}. Coupling of Fmoc-Ser(tBu)-OH toward II_{L29K(J)} was difficult, but accomplished by the same procedures used to add Boc-Aoa-OH toward I_{R17K(U)}. The N^α-Fmoc group of Ser was removed immediately before cleavage. The dried protected peptide-resin was cleaved essentially as described for I_{R17K(U)}, except that Reagent K [TFA/phenol/thioanisole/H₂O/1,2-ethanedithiol (82.5:5:5:5:2.5)] was used instead of Reagent R. The crude peptide (130 mg, 75% cleavage yield) was characterized by analytical HPLC (t_R 21 min, >90% purity; condition A), and the identity of the main HPLC peak was confirmed by ESMS analysis; calcd average mass: 2958.46, found: 2957.7 ± 1.3. When Reagent R was used to cleave the peptide-resin, a byproduct (~25% of major) was observed. This was analyzed and isolated by HPLC (t_R 22.8 min; condition A) and identified by ESMS; found: 3054.2 ± 0.6 (96 amu above the expected mass).

There follows a description of experimental details for three different scenarios matching Figure 4 and the accompanying text discussion.

Figure 4a. Crude reduced peptide was loaded in six batches, each of 20 mg/5 mL of 0.01 N aqueous HCl, onto semipreparative HPLC (condition F) (25 mg, 19% purification yield). To form the disulfide bridge, the pure reduced peptide (15 mg, 5 μ mol) was dissolved in 4 mL of 0.01 N aqueous HCl, ~200 mL of a mixture of dimethyl sulfoxide/2 M GdnHCl in 0.1 M sodium phosphate buffer, pH 7 (1:18), was added, and the reaction was stirred for 24 h at 25 °C. The reaction was monitored by analytical HPLC (t_R 22.8 min and t_R 18.5 min for reduced and oxidized, respectively; condition A). The identity of the oxidized peptide in the HPLC peak was confirmed by MALDI-TOF analysis; calcd monoisotopic mass: 2954.46, found: 2955.3 [M+H]⁺. Directly following disulfide bond formation and retaining the same milieu, the Ser residue branched off the Lys side chain was oxidized by adding aqueous sodium periodate (2 mL, 5 mM, 2 equiv). After 10 min reaction in the dark at 25 °C, the reaction was quenched with aqueous ethylene glycol (2 mL, 10 mM, 4 equiv). The resultant glyoxylyl peptide was characterized by analytical HPLC [t_R 18.6 min (P1) and 19 min (P2); ratio 2:9; condition A]. The reaction mixture (200 mL) was placed in an Amicon ultrafiltration cell (YM1 membrane, MWCO ~1000) that was part of an Amicon diafiltration setup. During diafiltration, H₂O was introduced into the cell while the buffer components (GdnHCl,

sodium phosphate) and undesired species (DMSO, periodate, ethylene glycol, and formaldehyde generated by the serine oxidation) were removed from the reaction mixture. After 600 mL of H₂O (3 times the volume of the reaction mixture) had passed through the cell, the mixture was concentrated (final volume ~50 mL) and finally lyophilized. Lyophilized peptide (17 mg, 10%) was characterized by analytical HPLC [t_R 19 min (P2); condition A], amino acid analysis (Asp, 1.99; Thr, 0.94; Glu, 1.01; Gly, 3.09; Ala, 1.99; Val, 0.86; Ile, 0.98; Tyr, 2.58; Phe, 1.92; Lys, 2.93; Arg, 2.03), ESMS; calcd average mass: 2925.39, found: 2942.6 ± 0.5 [18 amu (H₂O) above expected with glyoxylyl end group], 2924.7 ± 0.7 [expected], 2906.7 ± 0.2 [18 amu (H₂O) below expected with glyoxylyl end group], and by MALDI-TOF; calcd monoisotopic mass: 2923.43, found: 2924.1 [M+H]⁺.

Figure 4b. To form the disulfide bridge, the crude reduced peptide (60 mg, 20.2 μ mol) was dissolved in 6 mL of 0.01 N aqueous HCl, ~300 mL of a mixture of dimethyl sulfoxide/6 M GdnHCl in 0.1 M sodium phosphate buffer, pH 7 (1:18), was added, and the reaction was stirred for 24 h at 25 °C. The crude oxidized peptide was characterized by analytical HPLC (t_R 18.5 min, >90% purity; condition A), and the identity of the main peak was confirmed by MALDI-TOF analysis; calcd monoisotopic mass: 2954.46, found: 2955.3 [M+H]⁺. Directly following disulfide bond formation and retaining the same milieu, the Ser residue branched off the Lys side chain was oxidized by adding aqueous sodium periodate (3 mL, 13.5 mM, 2 equiv). After 10 min reaction in the dark at 25 °C, the reaction was quenched with aqueous ethylene glycol (3 mL, 27 mM, 4 equiv). The glyoxylyl peptide was characterized by analytical HPLC [t_R 18.6 min (P1) and 19 min (P2), ratio 1:1; condition A]. After periodate oxidation, the mixture was concentrated over a 1 day period using an Amicon ultrafiltration cell (YM1 membrane, MWCO ~1000) (final volume ~25 mL). Crude glyoxylyl peptide was loaded in five batches onto semipreparative HPLC (condition G) to provide separately analytical HPLC pure P1 and P2 which were each characterized by ESMS; calcd average mass: 2925.39, found: for P1, 2943.6 ± 1.2 [18 amu (H₂O) above], 2924.9 ± 0.5 [expected], 2906.4 ± 0.2 [18 amu (H₂O) below]; and for P2, 2942.6 ± 0.5 [18 amu (H₂O) above], 2924.7 ± 0.7 [expected], 2906.7 ± 0.2 [18 amu (H₂O) below]. Purified P1 and P2 were indistinguishable by MALDI-TOF; calcd monoisotopic mass: 2923.43, found: 2924.2 [M+H]⁺, and by amino acid analysis (Asp, 2.00; Thr, 0.80; Glu, 0.98; Gly, 3.08; Ala, 1.96; Val, 0.96; Ile, 1.62; Tyr, 2.78; Phe 1.98; Lys, 2.88; Arg, 2.08). Including fractions that had both components, the final overall isolated yield of P1 plus P2 was 34 mg (20%). When either pure P1 or pure P2 was dissolved in various solvent milieus, reequilibration occurred as described in the text.

Figure 4c. The same procedures as in the previous paragraph were followed, except that 6 M GdnHCl was replaced by 8 M urea. Purified peptide (34 mg, 20%) was characterized by analytical HPLC [t_R 19 min (P3); condition A], amino acid analysis (Asp, 2.20; Thr, 0.85; Glu, 1.15; Gly, 3.27; Ala, 2.32; Val, 0.81; Ile, 1.59; Tyr, 3.25; Phe 2.21; Lys, 3.31; Arg, 2.16), ESMS; calcd average mass: 2925.39, found: 2984.4 ± 1.4 [60 amu (urea) above], 2925.0 ± 0.9 [expected], 2906.7 ± 0.5 [18 amu (H₂O) below], and MALDI-TOF; calcd monoisotopic mass: 2923.43, found: 2924.7 [M+H]⁺.

For synthesis and characterization of II_{L29K(JG)}, II_{L29Z(J)}, and II_{N24K(J)}, see Supporting Information.

Tyr-Dpr(Ser)-Ala-Lys-NH₂. Starting with Fmoc-PAL-PEG-PS resin (1 g, 0.24 mmol/g, 0.24 mmol), the title

precursor to model tetrapeptide TP was assembled by Fmoc solid-phase synthesis using an Applied Biosystems Pioneer automated peptide synthesizer. As also done for synthesis of $\text{II}_{\text{L29K(J)}}$, the dipeptide building block Fmoc-Dpr(Boc-Ser(*t*Bu))-OH was incorporated at the third cycle. The dried protected peptide-resin (500 mg, 120 μmol) was cleaved using Reagent K. Crude reduced peptide was loaded onto semipreparative HPLC (condition H). Fractions with the desired peptide were pooled and lyophilized to provide a white powder (47 mg, 71%), pure by analytical HPLC (t_{R} 7.3 min; condition C), with the expected amino acid analysis (Ser, 0.80; Ala, 1.00; Tyr, 0.98; Lys, 0.95) and ESMS; calcd average mass: 552.62, found: 554.2 $[\text{M}+\text{H}]^+$.

Tyr-Dpr(Gxy)-Ala-Lys-NH₂. Purified Tyr-Dpr(Ser)-Ala-Lys-NH₂ (10 mg, 18.1 μmol) was dissolved in 10 mL of 0.1 M sodium phosphate buffer, pH 7, and then aqueous sodium periodate (0.1 mL, 0.36 M, 2 equiv) was added. After 10 min reaction in the dark, the reaction was quenched with aqueous ethylene glycol (0.1 mL, 0.72 M, 4 equiv). The crude glyoxylyl peptide, characterized by analytical HPLC [t_{R} 7.30 min (T1); condition C], was loaded in two batches onto semipreparative HPLC (condition H). Fractions with the desired peptide were pooled and lyophilized to provide a white powder (29 mg, 43%), characterized further by amino acid analysis (Ala, 1.00; Tyr, 0.70; Lys, 0.97) and ESMS; calcd average mass: 521.57, found: 540.2 $[\text{M}+\text{H}_2\text{O}+\text{H}]^+$, 522.2 $[\text{M}+\text{H}]^+$, 504.3 $[\text{M}-\text{H}_2\text{O}+\text{H}]^+$.

Corresponding oxidation experiments were carried out in the presence of GdnHCl or urea. In 2, 6, and 8.5 M GdnHCl in 0.1 M sodium phosphate buffer, pH 7, the peptide products were recognized by analytical HPLC [t_{R} 7.3 min (T1) and t_{R} 7.6 min (T3); ratio 4:1, 3:2, and 1:1, respectively; condition C] and characterized by HPLC/ESMS; calcd average mass: 521.57, found: for T1, 540.2 $[\text{M}+\text{H}_2\text{O}+\text{H}]^+$, 522.2 $[\text{M}+\text{H}]^+$, 504.3 $[\text{M}-\text{H}_2\text{O}+\text{H}]^+$; and for T3, 539.8 $[\text{M}+\text{H}_2\text{O}+\text{H}]^+$, 522.3 $[\text{M}+\text{H}]^+$, 504.1 $[\text{M}-\text{H}_2\text{O}+\text{H}]^+$. In 8 M urea in 0.1 M sodium phosphate buffer, pH 7, peptide product was recognized by analytical HPLC [t_{R} 7.4 min (T2); condition C] and characterized by HPLC/ESMS; calcd average mass: 521.57, found: 582.2 $[\text{M}+\text{urea}+\text{H}]^+$, 540.2 $[\text{M}+\text{H}_2\text{O}+\text{H}]^+$, 522.2 $[\text{M}+\text{H}]^+$, 504.3 $[\text{M}-\text{H}_2\text{O}+\text{H}]^+$.

Oxime-Forming Ligation Reactions. Capping of Monomers. (A) $\text{I}_{\text{R17K(U*)}}$. $\text{I}_{\text{R17K(U)}}$ (2.9 mg, 1 μmol) was dissolved in 1 mL of 0.01 N aqueous HCl, and acetone (10 μL , 136 μmol) was added. The reaction was complete at 25 °C within 30 s, as shown by analytical HPLC (t_{R} 25.1 min; condition A). Crude capped monomer was purified by semipreparative HPLC (condition I) to give 2 mg of $\text{I}_{\text{R17K(U*)}}$ as a white solid after lyophilization (70%, based on $\text{I}_{\text{R17K(U)}}$). Purified peptide was characterized by amino acid analysis (Asp, 2.00; Thr, 0.92; Glu, 1.07; Gly, 3.02; Ala, 2.00; Val, 0.96; Ile, 1.44; Leu, 1.01; Tyr, 2.71; Phe, 1.87; Lys, 2.91; Arg, 0.96) and MALDI-TOF; calcd monoisotopic mass: 2937.47, found: 2938.22 $[\text{M}+\text{H}]^+$.

For characterization of $\text{I}_{\text{R17K(U*G)}}$ and $\text{I}_{\text{R17Z(U*)}}$, see Supporting Information.

(B) $\text{II}_{\text{L29K(J*)}}$. $\text{II}_{\text{L29K(J)}}$ (2.9 mg, 1 μmol) was dissolved in 0.87 mL of 6 M GdnHCl in 0.1 M NaOAc buffer, pH 4.6, and then, $\text{CH}_3\text{ONH}_2\cdot\text{HCl}$ (25–30 wt % solution in water) (0.13 mL, 400 μmol) was added, and the reaction mixture was stirred for 24 h at 25 °C. Crude capped monomer was purified by semipreparative HPLC (condition G) to give 1.5 mg of $\text{II}_{\text{L29K(J*)}}$ as a white solid after lyophilization (50%, based on $\text{II}_{\text{L29K(J)}}$). Pure peptide was characterized by analytical HPLC (t_{R} 20.3 min; condition A), amino acid analysis (Asp, 2.04; Thr, 0.92; Glu, 1.01; Gly, 2.98; Ala,

1.99; Val, 0.97; Ile, 1.35; Tyr, 2.78; Phe, 1.97; Lys, 2.96; Arg, 2.03), and ESMS; calcd average mass: 2954.43, found: 2953.6 $\pm$ 1.4.

For characterization of $\text{II}_{\text{L29K(J*G)}}$, $\text{II}_{\text{L29Z(J*)}}$, and $\text{II}_{\text{N24K(J*)}}$, see Supporting Information.

Dimer Formation. $\text{I}_{\text{R17K(U)}}-\text{II}_{\text{L29K(J)}}$. Reaction was initiated by combining purified $\text{I}_{\text{R17K(U)}}$ (2.9 mg, 1 μmol) and $\text{II}_{\text{L29K(J)}}$ (P1 or P2) (2.9 mg, 1 μmol) in 1 mL of 6 M GdnHCl in 0.1 M NaOAc buffer, pH 4.6, and maintaining the reaction for 3 days at 25 °C while monitoring by analytical HPLC (Supporting Information Figure 1). Crude dimer was loaded in two batches for SEC (condition J) to give 3.3 mg of $\text{I}_{\text{R17K(U)}}-\text{II}_{\text{L29K(J)}}$ as a white solid after lyophilization (57%, based on $\text{I}_{\text{R17K(U)}}$). Pure peptide was characterized by analytical HPLC (t_{R} 29.2 min; condition B), amino acid analysis (Asp, 3.82; Thr, 1.67; Glu, 1.98; Gly, 5.94; Ala, 3.85; Val, 1.88; Ile, 3.11; Leu, 1.10; Tyr, 5.02; Phe, 3.65; Lys, 5.45; Arg, 2.86), and ESMS; calcd average mass: 5806.76, found: 5806.3 $\pm$ 1.6.

Contrasting to the optimal conditions to carry out the oxime-forming ligation given in the previous paragraph, when the reaction was initiated by combining $\text{I}_{\text{R17K(U)}}$ (2.9 mg, 1 μmol) and $\text{II}_{\text{L29K(J)}}$ (P3) (2.9 mg, 1 μmol) in 1 mL of 8 M urea in 0.1 M NaOAc buffer, pH 4.6, the reaction was extremely slow due to the low reactivity of P3 (see Figure 7, dotted line and accompanying text). Moreover, during that lengthy time period, $\text{I}_{\text{R17K(U)}}$ was mostly converted to the corresponding carbamylated byproduct, recognized by analytical HPLC (t_{R} 27.1 min; condition B) and identified by MALDI-TOF; found: 2941.09 $[\text{M}+\text{H}]^+$ (42.6 amu above the expected mass for $\text{I}_{\text{R17K(U)}}$).

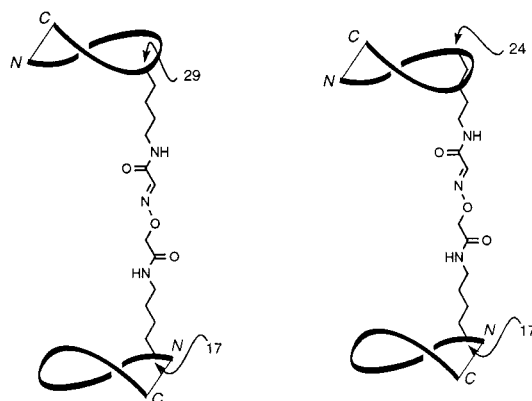
For characterization of $\text{I}_{\text{R17Z(U)}}-\text{II}_{\text{L29K(J)}}$, $\text{I}_{\text{R17Z(U)}}-\text{II}_{\text{L29Z(J)}}$, $\text{I}_{\text{R17K(U)}}-\text{II}_{\text{L29K(JG)}}$, $\text{I}_{\text{R17K(UG)}}-\text{II}_{\text{L29K(JG)}}$, and $\text{I}_{\text{R17K(U)}}-\text{II}_{\text{N24K(J)}}$, see Supporting Information.

RESULTS AND DISCUSSION

Dimer Design Considerations: Positioning and Length of Cross-Link between Monomers. The general rationale for creating and studying dimers of OxCM has already been stated. Presented herein is an iterative design strategy that is integrated with the available chemical options dictated by the central choice of oxime formation in solution as the means to link two monomers (Scheme 1). Since an entirely rigorous nomenclature would be cumbersome, several intuitive pictorial and written shorthands are used to describe the target molecules (Figures 2 and 3).

The disulfide-bridged OxCM monomer comprises ~25 residues, and these are numbered to comply with the corresponding residues in native BPTI; e.g., the *N*-terminal Mpa and *C*-terminal Cys of OxCM are respectively 14 and 38 (Figure 3). Solid-phase methods to assemble such molecules, to cleave them from the support, and to produce the spanning disulfide bridge have already been described (1). As 2 monomers are linked to form a dimer of ~50 residues, symmetry is lost: the monomer which will be modified to include an (amino-oxy)acetyl (Aoa or U) residue is referred to as I, and the monomer which will eventually provide a glyoxylyl (Gxy or J) functionality is referred to as II. The choice of where to position the I–II cross-link is suggested by modeling studies of Zielenkiewicz et al. (4), which propose a large BPTI dimer interface involving numerous intermolecular hydrophobic contacts but only three intermolecular hydrogen bonds. The hydrophobic residues of BPTI involved in this antiparallel self-complementary association are primarily the ones also found in the OxCM. Inspection

(a)



(b)

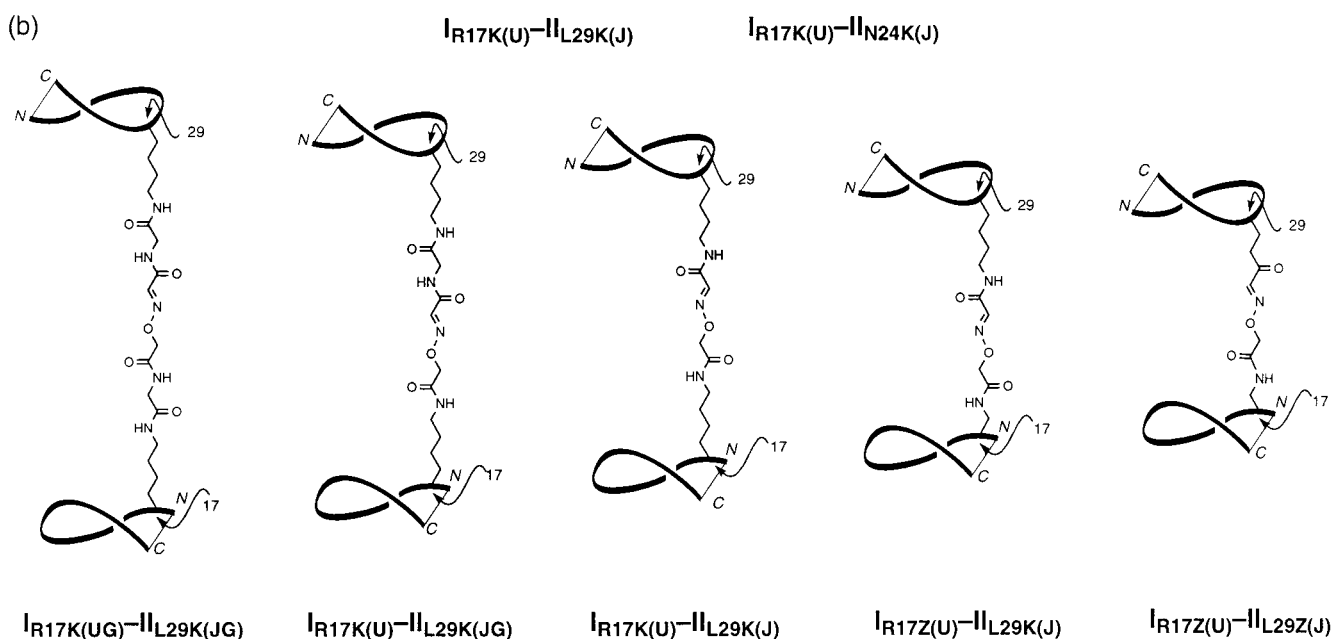


Figure 2. Schematic representation of, and nomenclature system for, the six OxCM covalent dimers synthesized in this work, emphasizing (a) different positions of the cross-link and (b) different length cross-links between the monomers. See accompanying text for details. Each solid boldface ribbon depicts an OxCM, and the spanning disulfide bond between Mpa¹⁴ and Cys³⁸ is indicated by the solid line connecting the *N* and *C* termini. Arrows indicate residue numbers that are connected; the type I monomer is drawn directly beneath the type II monomer in all representations. All atoms in the cross-links, starting with the β -carbons, are shown. The dimer $I_{R17K(U)}-II_{L29K(J)}$ is drawn twice so that it can be compared to other dimers addressing points (a) and (b).

of the BPTI dimer model reveals that Arg¹⁷ in one monomer is near Leu²⁹ in the other monomer (Figure 1b). A simpler model that ignores the nativelike twist of the β -sheet strands suggests that Arg¹⁷ in one monomer is near Asn²⁴ in the other monomer (Figure 1c). Each of these residues (i.e., Arg¹⁷, Asn²⁴, Leu²⁹) can be replaced in the linear sequence by an orthogonally protected $N^{\alpha,\omega}$ -diamino C^{α} -monoacid, i.e., Lys or Dpr (Figure 3c–e). At the appropriate stage of the overall solid-phase synthesis process (see Supporting Information Schemes 1 and 2 and later text for details), the N^{ω} -amino group can be exposed selectively, and extended further to accomplish one or more of the following: (i) vary the linker length, i.e., by coupling a protected Gly residue (this is in addition to control of length by the earlier choice of Dpr vs Lys); (ii) for monomer I, ultimately introduce the Aoa functionality by coupling the corresponding protected building block; and (iii) for monomer II, couple a protected Ser derivative which serves as a precursor to the Gxy moiety that is established later by a periodate oxidation step carried out in solution.

Based on these principles and strategies, six OxCM dimers were assembled (Figure 2). All dimers replaced Arg¹⁷ in monomer I that would contribute the Aoa component of the oxime linkage. In one set of studies (Figure 2a), the ‘matching’ residue that was replaced in monomer II was either Leu²⁹ or Asn²⁴. In brief (details later), NMR studies show that the dimer with the oxime linkage between residue 17 in monomer I and residue 24 in monomer II, i.e., $I_{R17K(U)}-II_{N24K(J)}$, has less chemical shift dispersion than the dimer with the oxime linkage between residue 17 in monomer I and residue 29 in monomer II, i.e., $I_{R17K(U)}-II_{L29K(J)}$. Therefore, a further set of studies (Figure 2b) kept constant the *preferred* cross-linkage site between residue 17 in monomer I and residue 29 in monomer II, but varied from 10 to 22 atoms the cross-linking length (counting atoms between, but not including, the α -carbon of position 17 in I and the α -carbon of position 29 in II).

Synthesis of Core Module Monomers Suitable for Oxime-Forming Ligation. (A) Assembly, Cleavage, and Disulfide Formation in Unprotected OxCM Monomers

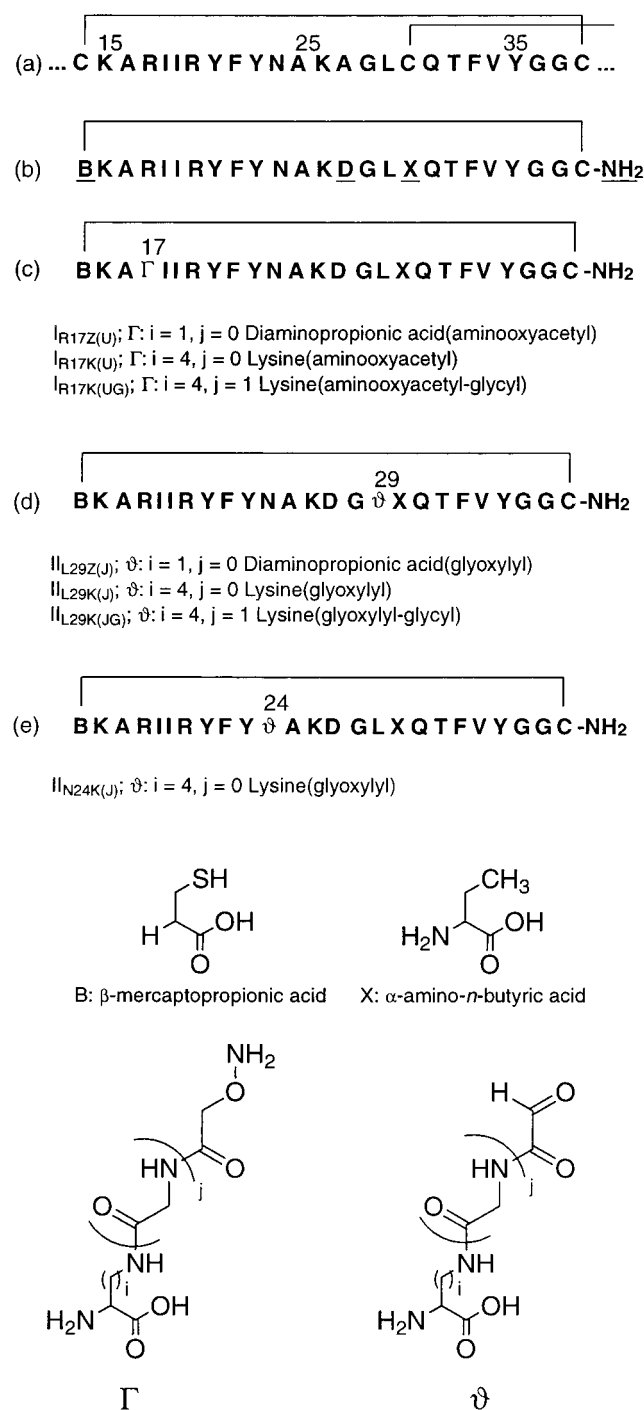


Figure 3. Amino acid sequences for (a) residues 14–38 in native BPTI, (b) OxCM [ref (1)]; underlined residues are different from the native sequence], (c) monomers of type I, replacing Arg¹⁷, (d) monomers of type II, replacing Leu²⁹, and (e) monomer of type II, replacing Asn²⁴. More detailed chemical structures drawn at the bottom include B (β -mercaptopropionic acid), X (α -amino-*n*-butyric acid), and the cross-linking substituents Γ and ϑ , for (c), (d), and (e). The glyoxylyl endgroup of ϑ is drawn in its aldehyde form to explain its reactivity in oxime-forming ligation; NMR experiments reported later prove that this endgroup exists in the hydrated dihydroxyacetyl form (see Figure 5a for accurate structure in a model peptide).

Containing the (Aminoxy)acetyl Functionality. Linear protected sequences were assembled by automated step-wise Fmoc solid-phase peptide chemistry starting with Fmoc-PAL-PEG-PS supports (Supporting Information Scheme 1). Leading to the eventual elaboration of the cross-link, the synthetic strategy next required on-resin

removal of an *N*^ε-Alloc protecting group from Lys¹⁷. Application of Pd(Ph₃)₄ (0.1 equiv) and Me₂NH·BH₃ (6 equiv) in CH₂Cl₂ (18) worked smoothly, and set the stage for addition of Boc-Aoa-OH to the liberated amino group. Couplings were sluggish and required repeat treatments; the best conditions used Boc-Aoa-OH (8 equiv) mediated by PyAOP (8 equiv) and DIEA (16 equiv) in DMF for 3 × 1 h to achieve a negative ninhydrin test result. The aforementioned series of steps gave monomer *I*_{R17K(U)} (see previous section for explanation of nomenclature); inter-polymer of one coupling/deprotection cycle of Fmoc-Gly-OH gave monomer *I*_{R17K(UG)}. Preparation of a third monomer of interest, i.e., *I*_{R17Z(U)}, was expedited because Fmoc-Dpr(Boc-Aoa)-OH, a commercially available building block, could be incorporated directly during the linear assembly.

Cleavages and manipulations of these peptides presented new challenges, due to the ready reactivity of the Aoa functionality with aldehydes and ketones to provide stable oxime derivatives. During routine work, peptides are precipitated from acidolytic cleavage cocktails by addition of ether; they may also become exposed to acetone either from glassware last washed with this solvent or from the dry ice/acetone baths used to shell-freeze aqueous samples before lyophilization (10). Often, a major byproduct was observed, present at a level comparable to or even higher than the desired Aoa-containing product. These byproducts all show a mass (ESMS) 40 amu above the expected, corresponding to addition of acetone (or propionaldehyde) and elimination of H₂O. Fortunately, such byproducts were eliminated entirely when the ether used to precipitate the peptide was freshly distilled from sodium, and when precautions were taken to avoid exposure to incidental or residual acetone. Cleavages were carried out conveniently using TFA/thioanisole/1,2-ethanedithiol/anisole (90:5:3:2) (Reagent R) (13), and provided the peptides with all free side chains including two thiols from the termini (Mpa¹⁴ and Cys³⁸-NH₂).

For OxCM, disulfide bond formation was carried out by dissolving the peptide in DMSO/2 M GdnHCl in 0.1 M phosphate buffer, pH 7 (1:18), for 24 h at 25 °C (1). However, the substitution of Arg¹⁷ by Γ (Figure 3) changes the solubility characteristics of the peptide, and a precipitate is observed as the oxidation proceeds under the previously optimized conditions. GdnHCl sometimes aids in solubilizing peptides as well as creating a denaturing environment that favors disulfide formation (19). Several GdnHCl concentrations (1–6 M) were tested, and the best results were obtained with DMSO/6 M GdnHCl in 0.1 M phosphate buffer, pH 7 (1:18), for 12 h at 25 °C. Other conditions, which varied pH (6 or 7) and/or used different percentages of DMSO without GdnHCl, were less satisfactory because disulfide product precipitated. Procedures concluded by concentration of reaction mixtures and peptide purification by reversed-phase HPLC. The overall isolated yield was typically 6%, based on the starting PAL-PEG-PS resin.

(B) Assembly, Cleavage, Disulfide Formation, and Periodate Oxidation of Unprotected OxCM Monomers Containing the Glyoxylyl Functionality. Again, linear protected sequences were assembled by automated step-wise Fmoc solid-phase peptide chemistry starting with Fmoc-PAL-PEG-PS supports (Supporting Information Scheme 2). Toward monomer *II*_{L29K(J)}, the *N*^ε-Alloc protecting group of Lys²⁹ was removed as already described, and Fmoc-Ser(*t*Bu)-OH was coupled as mediated by PyAOP/HOAt/DIEA. The *N*^ε-Fmoc group of Ser was removed from the resultant peptide-resin immediately

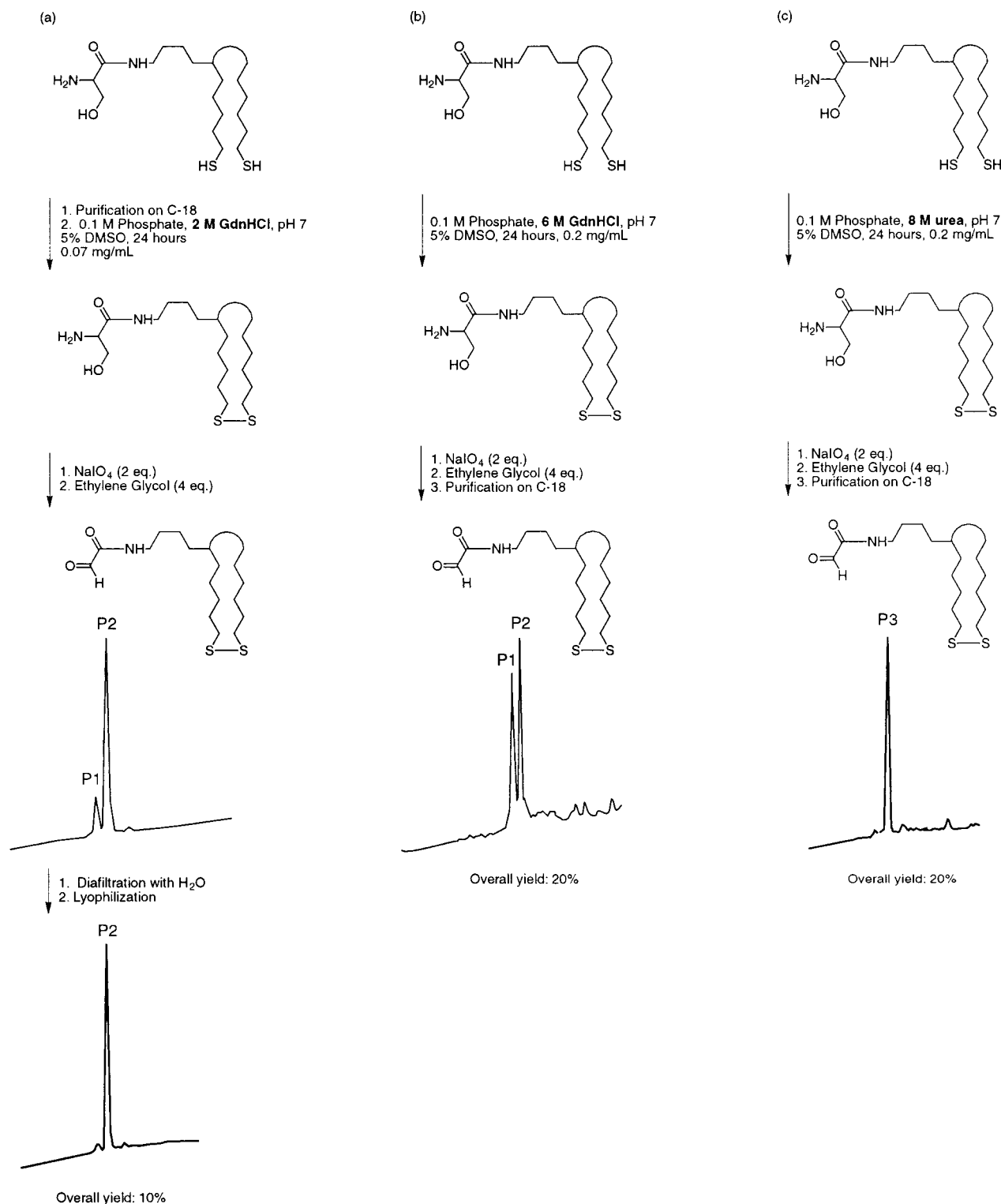


Figure 4. Chemistries and chromatographic analyses of three different scenarios that were followed to generate the disulfide bond and the glyoxylyl functionality in monomers of type II. Elucidation of accurate structures for P1, P2, and P3, based in part on studies with a model peptide TP, is covered extensively in the accompanying text.

before acidolytic cleavage. Toward monomer II_{L29K(JG)}, an additional coupling/deprotection cycle incorporated Fmoc-Gly-OH onto the N^ε-amine of Lys²⁹, prior to addition of Fmoc-Ser(*t*Bu)-OH. Finally, preparation of the II_{L29Z(J)} monomer featured direct incorporation of commercially available Fmoc-Dpr(Boc-Ser(*t*Bu))-OH.

The cleavage and further processing of these modified monomers revealed several unanticipated problems, which were systematically elucidated and circumvented. Recall

that a central part of the plan was to eventually oxidize the Ser residue with periodate in order to generate the glyoxylyl functionality. Acidolytic cleavage with Reagent R (composition given in the previous section) was invariably accompanied by formation of a byproduct, at a level typically 25% that of desired, which was shown by MALDI-TOF to have a mass 96 amu above what was expected. Moreover, the byproduct (as part of crude mixture) did not react further upon periodate treatment.

This information was consistent with a tentative conclusion that the byproduct is an *O*-trifluoroacetyl ester of the *N*-terminal serine residue, although an *N*-trifluoroacetyl species formed by $O \rightarrow N$ migration cannot be ruled out (20). Consistent with this hypothesis, and of obvious practical import, the trifluoroacetyl byproduct was not formed when cleavage was carried out with TFA/phenol/thioanisole/ H_2O /1,2-ethanedithiol (82.5:5:5:5:2.5) (Reagent K) (21). This result is attributed to the presence in the latter cocktail of water, which either scavenges any activated trifluoroacetyl species or hydrolyzes any ester that is produced.

Because of concerns about incompatibility of free thiols with the oxidation conditions planned for converting Ser to the glyoxylyl moiety (22), the disulfide-forming step was carried out next in the overall process. In turn, solubility considerations as already described made it necessary to add substantial levels of denaturants (GdnHCl or urea), along with the DMSO (5% v/v) used as the oxidizing reagent. Three scenarios were followed (Figure 4). In each case, peptide was first dissolved fully in 0.01 N aqueous HCl, next the mixture of pH 7 phosphate buffer, denaturant, and DMSO was added, and then disulfide formation was carried to completion (24 h, single major peak by HPLC). Subsequently, in the same milieu, $NaIO_4$ (2 equiv) was added, and 10 min later, oxidation was quenched with ethylene glycol (4 equiv) (23). HPLC analysis showed three products (P1, P2, and P3 in Figure 4), of which only P2 was later proven to correspond to the expected structure. The highest relative level of P2, i.e., ~80%, occurred when the crude peptide had been purified first, and then dissolved in 2 M GdnHCl (Figure 4a). When the crude peptide was used directly, the concentration of GdnHCl required for solubilization was higher, i.e., 6 M, and consequently the relative level of P2 dropped to ~50% (determined after the periodate step; Figure 4b). The byproduct in the experiments with GdnHCl was P1, for which the following properties were deduced: (i) the most intense ESMS peak corresponds to the aldehyde, and two other peaks, half the intensity of the major, correspond to the hydrated and dehydrated forms of the aldehyde; (ii) the unique MALDI-TOF signal corresponds to the aldehyde form; (iii) the byproduct was quite unreactive for the oxime-forming ligation step (see 'Synthesis of OxCM Dimers'); and (iv) the byproduct was converted to the desired P2, which has the anticipated good reactivity, by extensive diafiltration with H_2O (Figure 4a), albeit with reduction in the overall yield due to solubility problems. In contrast, ESMS data on the desired P2 showed that the most intense peak corresponds to the hydrated form of the aldehyde, while the intensities of the free aldehyde and the dehydrated form were about 1/3 and 1/6 respectively; MALDI-TOF analysis of P2 again showed only the aldehyde form. Finally, in experiments carried out in the presence of 8 M urea, neither P1 nor P2 formed, but the exclusive unwanted product was P3. Purified P3 had the following characteristics: (i) the most intense ESMS peak corresponds to the peptide aldehyde having reacted with urea (60 amu above what was expected), with the aldehyde form and the dehydrated forms also present each at about 1/4 intensity; (ii) the unique MALDI-TOF signal corresponds to the aldehyde form; and (iii) P3 was exceptionally unreactive for the oxime-forming ligation step (see 'Synthesis of OxCM Dimers').

Model Studies To Determine Structures of Byproducts Obtained after Periodate Oxidation in the Presence of Denaturants. To understand the formation of unexpected byproducts P1 and P3 after oxidation

Table 1. Effects of Denaturants on the Distribution of Products from Periodate Oxidation at pH 7 of Model Peptide H-Tyr-Dpr(Ser)-Ala-Lys-NH₂^a

milieu (aqueous buffer, denaturant)	T1	T2	T3
0.1 M phosphate, pH 7	100	0	0
2 M GdnHCl in 0.1 M phosphate, pH 7	82	0	18
6 M GdnHCl in 0.1 M phosphate, pH 7	63	0	37
8.5 M GdnHCl in 0.1 M phosphate, pH 7	49	0	51
8 M urea in 0.1 M phosphate, pH 7	0	100	0

^a Products are designated in order of HPLC elution, and relative amounts are expressed in percent so as to add up to 100%. As noted in the text, TP products T1, T2, and T3 correlate to type II monomer-derived products P2, P3, and P1, respectively.

of a Ser residue in the presence of GdnHCl or urea, the model peptide H-Tyr-Dpr(Ser)-Ala-Lys-NH₂ (corresponding to BPTI residues 23–26, with replacement of Asn²⁴) was synthesized and evaluated. Mirroring the results with the full 25-residue OxCM type II monomers, treatment of the model with periodate produced two products (T1, T3) with varying HPLC-reported ratios depending on the GdnHCl concentration (2–8.5 M) that was used, and a unique product (T2) when urea (8 M) was used (Table 1). In the absence of denaturant, only T1 formed, suggesting that this corresponds to the desired H-Tyr-Dpr(Gxy)-Ala-Lys-NH₂. Further supporting the correlations P1/T3, P2/T1, and P3/T2, HPLC/ESMS analysis was inconclusive about the true structures of T1 and T3, but supportive of T2 being a urea adduct.

The Gxy-containing T1 was readily purified, and then redissolved in each of three different milieus for NMR analysis: (a) ^2H_2O at pH 4.6, (b) 6 M GdnDCl-*d*₆ in ^2H_2O at pH 4.6, and (c) 8 M urea- $^{15}N_2d_4$ in ^2H_2O at pH 4.6. Milieu (a) allowed structural conclusions about pure T1, whereas milieu (b) promoted a rapid equilibration to a 3:2 ratio of T1 to T3 (confirmed by HPLC), and T1 was converted quantitatively to T2 within 12 h in milieu (c). From the NMR data and detailed interpretations that follow, it is concluded (for the tetrapeptide structure) that in T1, the terminal Gxy residue exists as the corresponding hydrate, i.e., a dihydroxyacetyl residue; that an amino group of guanidine reacts with the terminal aldehyde of Gxy to form a covalent hemiaminal adduct (two diastereomers) as exemplified in T3; and that the amino group of urea reacts in an analogous fashion as exemplified in T2 (Figure 5 shows all of the proposed structures, including numbering of key atoms in the positions of interest). It is highly likely that these structural inferences generalize to systems (P1, P2, and P3 in Figure 4) for which the observations were originally made. It is instructive to compare the NMR-established structural conclusions with the more ambiguous information from mass spectrometry; more specifically, MALDI-TOF suggested only the expected aldehyde structures (without the proper hydration), whereas the more "gentle" ESMS technique was clear only for the urea adduct, hinted at the aldehyde hydrate (major peak, but unhydrated and dehydrated species also noted), and entirely missed the guanidine adduct (unhydrated as major, and hydrated as well as dehydrated aldehyde forms as minor, all present). Since the environments of the rapidly equilibrating species in these samples change as they are prepared for and then analyzed by mass spectrometry, these findings are not especially surprising.

Supporting the structural conclusions, consider first the 1D ^1H NMR spectra (Figure 6), backed up by $^1H/^1H$ -gCOSY experiments which allowed assignments of all nonexchangeable hydrogen resonances (Supporting Information Table 1). The most significant differences are

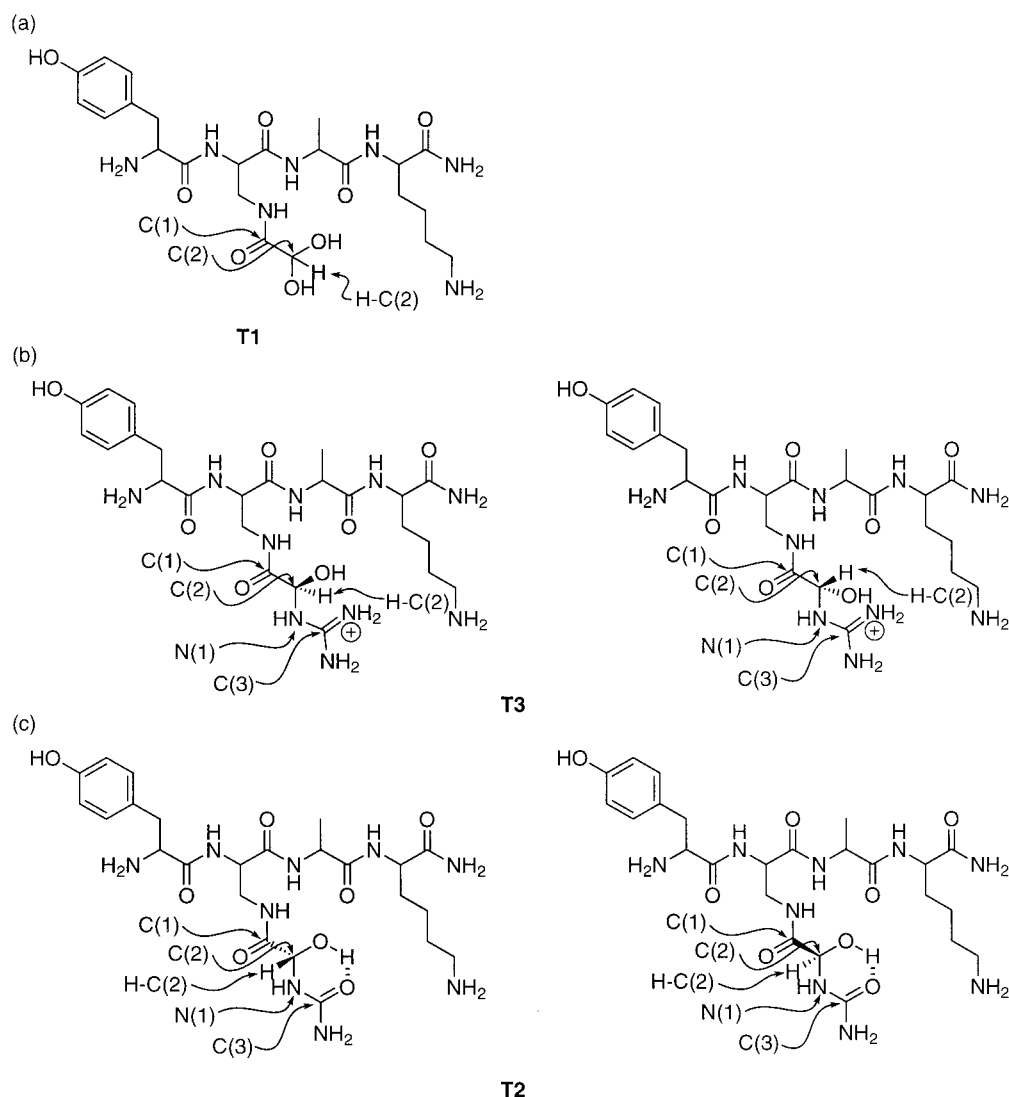


Figure 5. Structures of (a) T1, the hydrated Gxy-containing model tetrapeptide, (b) T3, the two hemiaminal diastereomers obtained by reversible reaction of guanidine with the terminal aldehyde of the Gxy residue in T1, and (c) T2, the two hemiaminal diastereomers obtained by reversible reaction of urea with the terminal aldehyde of the Gxy residue in T1. Arrows identify key atoms in the structural identifications (see also Table 2).

between 5.4 and 5.8 ppm. In the absence of denaturants (Figure 6a), a unique singlet at 5.52 ppm is assigned to the H-C(2) proton of the dihydroxyacetyl residue [the former H-C(α) of Ser]. In an $^1\text{H}/^{13}\text{C}$ -HMQC experiment, the ^1H signal at 5.52 ppm correlates to a ^{13}C signal at 89.6 ppm, again consistent with a hydrated aldehyde (24) (Figure 5a). When spectra are recorded in the presence of GdnHCl- d_6 (Figure 6b), the singlet at 5.52 ppm was supplemented by two more signals, of equal height, at 5.71 and 5.72 ppm. The latter two signals had consistent chemical shifts regardless of what field strength (300, 500, 600, or 800 MHz) was used for spectral acquisition; hence, they are derived from two unique chemical environments rather than from splitting of a single resonance. Further information obtained from $^1\text{H}/^{13}\text{C}$ -HMQC and $^1\text{H}/^{13}\text{C}$ -HMBC spectra (Table 2) reprise the observations about T1 already noted, and provide a way to characterize structures of the two new species corresponding to T3. Thus, the ^1H signals at 5.71 and 5.72 ppm *both* correlated in $^1\text{H}/^{13}\text{C}$ -HMQC to the *same* ^{13}C signal at 76.8 ppm, consistent with a hemiaminal, and in $^1\text{H}/^{13}\text{C}$ -HMBC to *two* ^{13}C signals at 172.5 and 159.6 ppm. While the 172.5 ppm signal is assigned to the C(1) carbon (originally C=O of Ser), the 159.6 ppm peak is

particularly conclusive because it is derived from guanidine. The ^{13}C chemical shift is close to, but different from, 157.2 ppm, which is the shift of the carbon from GdnHCl itself (24). These data establish the covalent connectivity of the adduct (Figure 5b). The two close, but separate, ^1H peaks in the 1D NMR spectra are now readily explained by the chirality of C(2) after nucleophilic attack by a guanidine nitrogen on the Gxy aldehyde.

Spectroscopic examination of the Gxy-containing peptide in the presence of urea (Figure 6c) reveals similarities as well as differences from the same peptide in the presence of GdnHCl. The absence of a signal at 5.5 ppm is taken to mean that the aldehyde function of the model tetrapeptide had reacted quantitatively with urea. As before, ^1H signals at 5.57 and 5.61 ppm were assigned to the H-C(2) proton, correlated by $^1\text{H}/^{13}\text{C}$ -HMQC to the same ^{13}C signal at 76 ppm and by $^1\text{H}/^{13}\text{C}$ -HMBC to two ^{13}C signals at 175.3 ppm [assigned to the C(1) carbon] and 162.7 ppm [similar to the shift of urea itself at 163.5 ppm (24)]. Also as before, the two ^1H shifts are independent of field strength and explained by the chirality of C(2). The availability of [^{15}N]urea allowed correlation in $^1\text{H}/^{15}\text{N}$ -HMBC of the ^1H signals at 5.57 and 5.61 ppm, respectively, to ^{15}N signals at 100.8 and 101.0 ppm

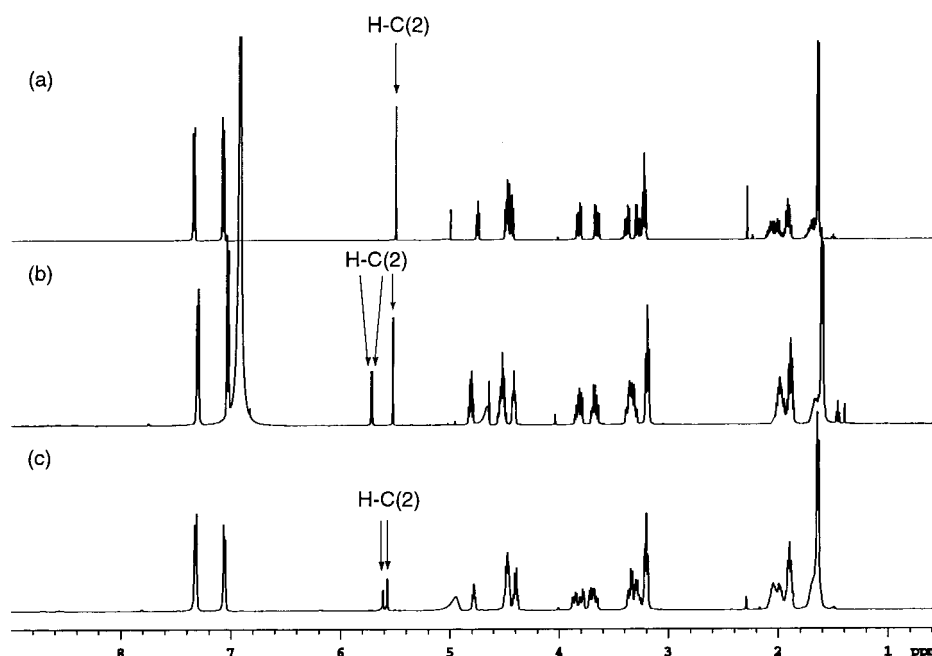


Figure 6. ^1H NMR spectra, recorded at 500 MHz, of the purified Gxy-containing model tetrapeptide redissolved in (a) $^2\text{H}_2\text{O}$ at pH 4.6, (b) 6 M GdnDCl- d_6 in $^2\text{H}_2\text{O}$ at pH 4.6, and (c) 8 M urea- $^{15}\text{N}_2, d_4$ in $^2\text{H}_2\text{O}$ at pH 4.6. The proton resonance(s) of H-C(2) in each milieu is/are indicated in the spectra. Full ^1H assignments are compiled in Supporting Information Table 1. The residual H_2O signal was suppressed by use of presaturation techniques. The signal around 6.9 ppm in (b) corresponds to residual GdnHCl. In (c), the signal of residual protons from urea is not observed because urea exchanges rapidly with water, and the urea signal is saturated concomitant with H_2O suppression.

Table 2. Diagnostic ^1H , ^{13}C , and ^{15}N Chemical Shifts for H-Tyr-Dpr(Gxy)-Ala-Lys-NH $_2$, Recorded at 500 MHz in $^2\text{H}_2\text{O}$ at pH 4.6 in the Absence and Presence of Denaturants^a

residue	position	$^2\text{H}_2\text{O}$		6 M GdnDCl- d_6		8 M urea- $^{15}\text{N}_2, d_4$	
Gxy-derived	H-C(2)	5.52	5.52 ^b	5.71	5.72	5.57	5.61
	C(1) ^c	^d	^d	172.5	172.5	175.3	175.3
	C(2) ^e	89.6	89.7 ^b	76.8	76.8	76.0	76.0
denaturant-derived	N(1) ^f					101.0	100.8
	C(3) ^c			159.6	159.6	162.7	162.7

^a Refer to Figure 5 to define the atoms in the Ser to Gxy conversion, and the atoms derived from denaturants. Sample preparations are defined in the legend to Figure 6, and the ^1H chemical shifts in the first row of this table overlap with that figure. ^b These signals, which overlap those observed in $^2\text{H}_2\text{O}$ in the absence of denaturant, are attributed to the original species present in $^2\text{H}_2\text{O}$, and not to the Gdn adduct. ^c Chemical shifts of C(1) and C(3) were obtained from $^1\text{H}/^{13}\text{C}$ -HMBC experiments. ^d No correlation was observed between the H-C(2) proton and C(1) in the $^1\text{H}/^{13}\text{C}$ -HMBC experiment. Two bond coupling constants are small, and their observation depends on electronic properties of the bond. ^e Chemical shifts of C(2) were obtained from $^1\text{H}/^{13}\text{C}$ -HMQC experiments. ^f Chemical shifts of N(1) were obtained from $^1\text{H}/^{15}\text{N}$ -HMBC experiments.

(compared to ^{15}N of urea itself at 76 ppm), further supporting the urea adduct structure (Figure 5c). It is also plausible that additional stabilization could be provided by formation of a hydrogen bond involving the oxygen of $\text{O}=\text{C}(3)$ and the hydrogen of $\text{OH}-\text{C}(2)$; note this gives a six-membered ring.

The observations described in this and the preceding section are of considerable practical importance, given the subsequent goal to apply glyoxylyl-containing peptides for oxime-forming ligation reactions. In aqueous media, the glyoxylyl moiety itself exists in the dihydroxyacetyl (hydrated) form; this follows classical expectations about aldehyde chemistry and will not affect its ultimate reactivity with strong nucleophiles. Formation under mild aqueous conditions of the described *intermolecular* adducts of glyoxylyl functions with guanidine or urea is unprecedented and surprising due to the low nucleophilicities of guanidino or amide nitrogens. The high concentrations of denaturants do drive the equilibrium toward adduct, although in the case of guanidine, enough adduct reverts to the glyoxylyl species so that reactions with Aoa-containing products can proceed (albeit much slower; see next section). A review of organic literature

databases showed numerous examples of related transformations (i.e., amides or carbamates react with glyoxylyl or simpler aldehyde derivatives), although invariably under nonaqueous conditions with heating and/or long reaction times. The recent report by Rose et al. (25) of a rapid *intramolecular* cyclization (six-membered ring forms, specifically facilitated by structural constraint of the Pro residue), involving irreversible nucleophilic attack in aqueous media by a peptide amide nitrogen on an *N*-terminal glyoxylyl function, provides a somewhat analogous precedent to what was found in the present work.

Synthesis of OxCM Dimers: Oxime-Forming Ligation. At this stage of the work, methodologies were on hand to obtain both families of functionalized monomers that needed to be connected by an oxime-forming ligation, but the identification of covalent adducts between glyoxylyl moieties and either GdnHCl or urea denaturants was a source of concern. To create the desired OxCM dimers, it was necessary to react the functionalized monomers under acidic aqueous conditions at pH 4.6, based on literature precedents (5–7, 10). Several strategies were explored to combine $\text{I}_{\text{R17K(U)}}$ with

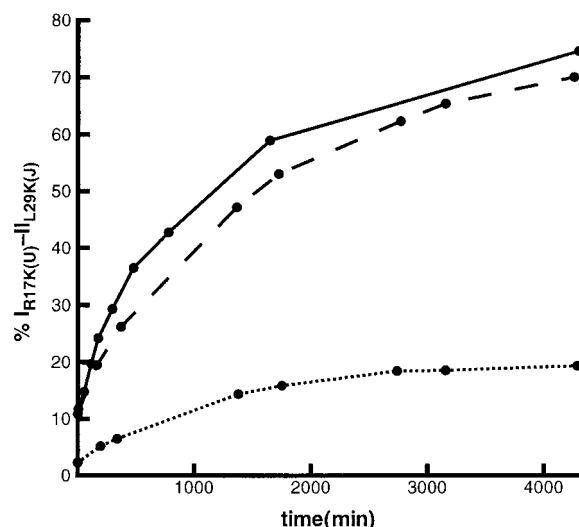


Figure 7. Oxime-forming reactions connecting I_{R17K(U)} and II_{L29K(J)} (1 mM each) as a function of time and denaturant. The denaturant used to solubilize components in the reaction mixture was 6 M GdnHCl (solid line), 8.5 M GdnHCl (dashed line), and 8 M urea (dotted line). The ordinate, expressed as a percentage, was calculated (based on HPLC areas) as the amount of I_{R17K(U)}-II_{L29K(J)} formed divided by the total amount of all species (product, starting materials, adducts of II with either GdnHCl or urea, and adduct of I with cyanate).

II_{L29K(J)}, in the context that the use of denaturants could not be avoided due to the need to keep peptide materials in solution. Later, the best procedures were extended to prepare the other dimers.

In one experiment, the Ser-containing precursor of II_{L29K(J)} was oxidized with periodate in 6 M GdnHCl in 0.1 M phosphate buffer, pH 7, generating a 1:1 ratio of P1 and P2 as already indicated (Figure 4). These two products were separated by semipreparative reversed-phase HPLC and separately dissolved in 6 M GdnHCl in 0.1 M NaOAc buffer, pH 4.6, for reaction with I_{R17K(U)}. There was an early burst of initial reactivity corresponding to P2 (free glyoxylyl) vs P1 (guanidine hemiaminal), i.e., 10% vs 2% of I_{R17K(U)}-II_{L29K(J)} formed after 3 min. However, within 1 h both reaction mixtures had equilibrated, so that regardless of whether pure P1 or pure P2 had been present at the outset, these materials were now in a 1:1 ratio. From this point on, the kinetics of the ligations were essentially the same in both reactions (Figure 7, solid line). The experiment was repeated at higher GdnHCl concentration, i.e., 8.5 M. In this case, equilibration within 1 h gave P1 and P2 in a ratio of 1.4:1, and the overall kinetics were slightly slower (Figure 7, dashed line). Experiments in the other direction, i.e., with lower GdnHCl concentration, were not feasible due to solubility problems. These data are consistent with the idea that the higher the concentration of GdnHCl, the more guanidine hemiaminal adduct (P1) is formed, and the slower the reaction. In another experiment, the peak obtained from periodate oxidation in the presence of 8 M urea, i.e., P3 (Figure 4c), was reacted with I_{R17K(U)} in 8 M urea in 0.1 M NaOAc buffer, pH 4.6. This reaction was the slowest of the experiments carried out (Figure 7, dotted line), and was accompanied by slow formation of a I_{R17K(U)}-derived byproduct which by MALDI-TOF was 42.6 amu higher than the starting monomer. It is known that in aqueous solution, urea is in equilibrium with ammonium cyanate (26, 27), and that cyanate reacts with amino groups to yield carbamyl derivatives (26). It is reasonable to propose that, over the long time course of reaction in the presence of urea [where the urea adduct

of II_{L29K(J)} is particularly unreactive], the Aoa moiety of I_{R17K(U)} eventually reacts with the slowly accumulating cyanate.

From these experiments, optimized conditions were established for the oxime-forming ligation. Equivalent amounts of purified I_{R17K(U)} and II_{L29K(J)} (the latter obtained from periodate oxidation in 6 M GdnHCl in 0.1 M phosphate buffer, pH 7) were combined in 6 M GdnHCl in 0.1 M NaOAc buffer, pH 4.6. The course of the reaction was followed by analytical reversed-phase HPLC (Supporting Information Figure 1); after 3 days, no appreciable further product was formed, and product was isolated in 57% yield after SEC purification. Five further dimers (Figure 2) were created by the identical protocols with comparable yields.

Evaluation of Conformational Integrity of OxCM Monomers Modified for Incorporation into Dimers.

Having developed chemical means to combine two OxCM monomers and form the corresponding dimers, it was also necessary to check that the antiparallel β -sheet conformations sampled by OxCM are not significantly perturbed by the structural modifications that later facilitate cross-linking. Accordingly, each monomer was appropriately 'capped', and characterized by a spectroscopic criterion the reliability of which had been established in earlier work (1). Monomers of type I, with an (aminooxy)-acetyl functionality, were treated with acetone, whereas monomers of type II, with a glyoxylyl functionality, were treated with CH₃ONH₂·HCl (Figure 8; note the '*' convention to indicate oxime capping).

For each capped monomer, the C α H chemical shifts were determined as a function of residue number, and the deviation from literature random coil values (28, 29) was calculated. The baseline experiment (Figure 8, red traces) was the previously reported analysis of OxCM (1), in which residues 25–28 (a turn in native BPTI) show a negative deviation (upfield shift), and residues 18–24 and 29–35 (strands in native BPTI) show positive deviations (downfield shift). I_{R17K(U*)}, I_{R17K(U*G)}, II_{L29K(J*)}, II_{L29K(J*G)}, and II_{L29Z(J*)} have C α H chemical shift deviations similar to OxCM both in the strands and in the turn (Figure 8), consistent with the conclusion that the structures of these monomers do not differ significantly from that observed in OxCM. However, I_{R17Z(U*)} has C α H chemical shift deviations similar to OxCM for turn residues but smaller C α H chemical shift deviations for strand residues. The latter result indicates that replacement of Arg¹⁷ in OxCM by the Dpr(Aoa) unit is a sufficiently drastic change to impact strand conformation. A more dramatic example for a poorly tolerated substitution was revealed with II_{N24K(J*)}, where surrounding the modified site at residue 24, residue 23 which was in a strand for OxCM now appears to be in a turn, and residue 25 which was in the OxCM turn now is indistinguishable from random.

Structural Characterization of the OxCM Dimers.

Each synthesized OxCM dimer was evaluated by ¹H NMR and size-exclusion chromatography. Synthetic dimers were dissolved to a concentration of 0.3 mM in CH₃CN/10 mM aqueous NaCl (1:19) at pH 2, and applied at 3 °C to a size-exclusion chromatography column made of cross-linked agarose and dextran. The salt and CH₃CN were included to suppress, respectively, ionic and hydrophobic interactions between the packing material and the proteins, whereas the pH was selected for consistency with what pilot NMR studies had shown to be most promising. Under these conditions, all synthesized proteins elute as a single peak in the order expected by their molecular weights, without any evidence for noncovalent association (i.e., they were physically monomeric, with

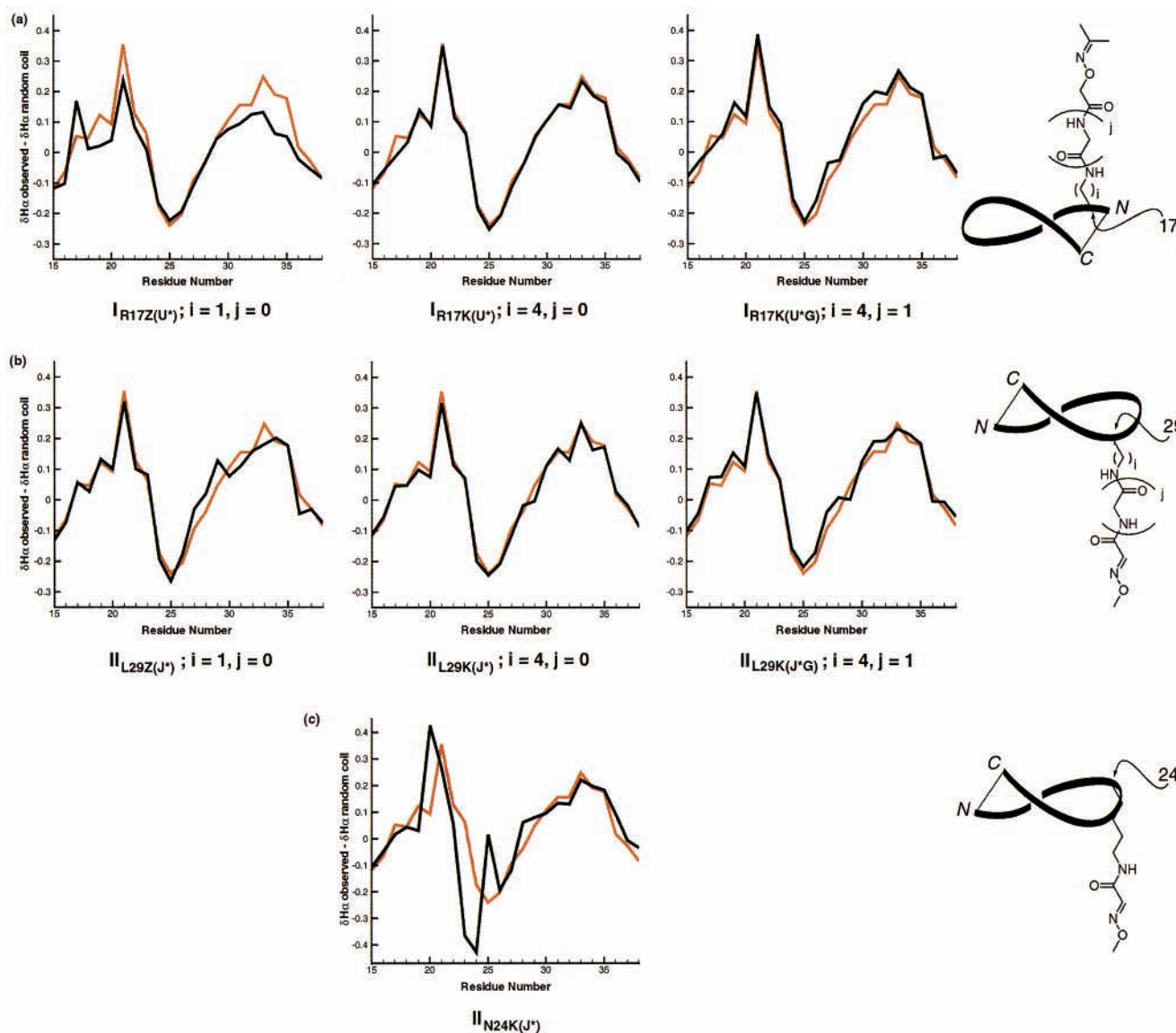


Figure 8. Along the right side are shown schematic representations, all drawn according to the same conventions used earlier (Figure 2), of capped monomers of (a) type I, replacing Arg¹⁷, and with acetone used to cap (aminoxy)acetyl functions; (b) type II, replacing Leu²⁹, and with CH₃ONH₂·HCl to cap glyoxylyl moieties; and (c) type II, replacing Asn²⁴, again capped with CH₃ONH₂·HCl. Each panel compares the CαH chemical shift deviations, obtained at pH 4.5 and 3 °C, of OxCM (red trace) to a capped monomer (black trace) defined and named underneath, with an asterisk (*) used to indicate the presence of the appropriate oxime cap.

the preceding term not to be confused with the “monomer/dimer” terminology used to describe the chemical constructs of this work) (Supporting Information Table 2).

The potential for folding of each protein variant was qualitatively assessed by 1D ¹H NMR spectra, recorded at 5 °C and pH 2 (Figure 9). Well-folded proteins have relatively narrow line widths, while conformational heterogeneity and increased internal mobility on the millisecond to microsecond time scale, which characterize molten globule and aggregated states, result in broad and/or heterogeneous line widths. Spectra of well-folded proteins are also characterized by pronounced chemical shift dispersion, which arises from the variety of unique, local magnetic environments present in a compact protein.

To evaluate the best position for the cross-link, I_{R17K(U)}–II_{N24K(J)} was compared to I_{R17K(U)}–II_{L29K(J)}. The former is characterized by broad proton line widths and negligible chemical shift dispersion (Figure 9a), whereas the latter shows narrower line widths and considerable chemical shift dispersion (Figure 9b). The problems with I_{R17K(U)}–

II_{N24K(J)} may have been anticipated by the NMR studies on II_{N24K(J*)} (see Figure 8c and accompanying discussion in previous section) which showed that replacement of Asn²⁴ changes the structure of the monomer itself. In addition, the present working model for the antiparallel BPTI dimerization interface shows Arg¹⁷ to be closer to Leu²⁹ (Figure 1b). Asn²⁴, which on the basis of a simpler two-dimensional representation (Figure 1c) was predicted to be close to Arg¹⁷, is now believed to be on the opposite side due to the natively like ‘twist’ of the β-sheets.

With the I₁₇–II₂₉ optimal cross-link position established, variations in the length of the cross-link were considered next. Although intuition suggested that self-association between covalently joined monomers might be improved if they were closer, experiments showed better results with longer cross-links. 1D ¹H NMR of I_{R17Z(U)}–II_{L29Z(J)} (Figure 9c) and I_{R17Z(U)}–II_{L29K(J)} (Figure 9d) show broad line widths and reduced chemical shift dispersion. These results can be explained in part by the NMR studies on I_{R17Z(U*)} (Figure 8a, left), which showed a decrease in structure for the strand residues with

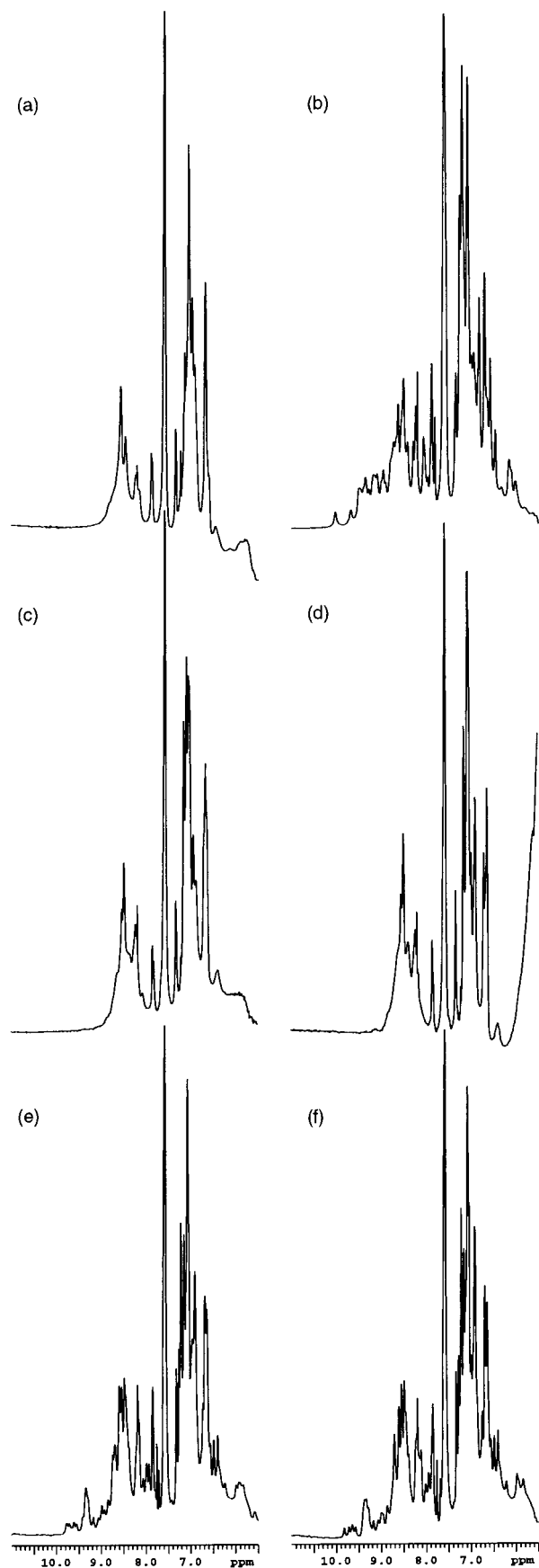


Figure 9. Aromatic and amide regions of 1D ^1H NMR spectra at 600 MHz, obtained at pH 2 and 5 $^\circ\text{C}$, are shown for OxCM dimers (a) $\text{I}_{\text{R17K(U)}}\text{-II}_{\text{N24K(J)}}$, (b) $\text{I}_{\text{R17K(U)}}\text{-II}_{\text{L29K(J)}}$, (c) $\text{I}_{\text{R17Z(U)}}\text{-II}_{\text{L29Z(J)}}$, (d) $\text{I}_{\text{R17Z(U)}}\text{-II}_{\text{L29K(J)}}$, (e) $\text{I}_{\text{R17K(UG)}}\text{-II}_{\text{L29K(JG)}}$, and (f) $\text{I}_{\text{R17K(U)}}\text{-II}_{\text{L29K(JG)}}$.

respect to OxCM. In addition, unwanted steric interactions between monomers may be a factor. 1D ^1H NMR spectra of $\text{I}_{\text{R17K(UG)}}\text{-II}_{\text{L29K(JG)}}$ (Figure 9e) and $\text{I}_{\text{R17K(U)}}\text{-II}_{\text{L29K(JG)}}$ (Figure 9f) show narrower line widths and similar chemical shift dispersion, by comparison to $\text{I}_{\text{R17K(U)}}\text{-II}_{\text{L29K(J)}}$, demonstrating the advantages of a longer cross-link.

Preliminary Characterization of OxCM Dimers

$\text{I}_{\text{R17K(U)}}\text{-II}_{\text{L29K(J)}}$ and $\text{I}_{\text{R17K(UG)}}\text{-II}_{\text{L29K(JG)}}$. The first dimer that gave evidence of folded structure beyond what is inherent in the OxCM monomer was $\text{I}_{\text{R17K(U)}}\text{-II}_{\text{L29K(J)}}$. Strong evidence in further support of this conclusion comes from 1D $^1\text{H}/^2\text{H}$ isotope exchange studies at pH 2 and 5 $^\circ\text{C}$, monitored in the region 7.5–10.5 ppm where the amide protons resonate (Supporting Information Figure 2). While a subset ($\sim 36\%$) of the protons exchange within a day, the majority of the amides (particularly the more downfield ones) are still observable after 6 days. The most downfield NH at pH 2 and 5 $^\circ\text{C}$ (labeled 'a' in Supporting Information Figure 2) was sufficiently resolved from the amide envelope to allow determination of the hydrogen isotope exchange rate ($k_{\text{obs}} \sim 10^{-4} \text{ min}^{-1}$, $t_{1/2} \sim 110 \text{ h}$). By comparison, exchange of strand residues in OxCM ($t_{1/2} \sim 1 \text{ h}$, pH 4.1 and 6 $^\circ\text{C}$) is 2 orders of magnitude faster, and exchange in the most protected regions of a partially folded protein such as $[\text{14-38}]_{\text{Abu}}$ (30) ($t_{1/2} \sim 10 \text{ h}$, pH 4.6 and 1 $^\circ\text{C}$) is 1 order of magnitude faster.

Of additional promise, the 1D ^1H NMR spectrum of $\text{I}_{\text{R17K(U)}}\text{-II}_{\text{L29K(J)}}$ in $^2\text{H}_2\text{O}$ at pH 2 at 5 $^\circ\text{C}$ shows a characteristic singlet at the unusually upfield position of 0.35 ppm. This signal is likely due to a methyl group from an aliphatic residue side chain that is in a structured environment. As the temperature was increased to 60 $^\circ\text{C}$, the upfield signal decreased in a cooperative transition with an apparent $T_{\text{m}} \sim 39 \text{ }^\circ\text{C}$. By comparison, the transition of $[\text{14-38}]_{\text{Abu}}$ at pH 6 occurs at $\sim 19 \text{ }^\circ\text{C}$ (31).

Efforts to characterize the structure of the $\text{I}_{\text{R17K(U)}}\text{-II}_{\text{L29K(J)}}$ dimer further were stymied by the fact that relatively few cross-peaks were observed in the TOCSY spectra. These findings, while disappointing, reflect a situation preceded in the literature for partially folded proteins (32). One possible explanation recognizes multiple conformations in chemical exchange, leading to relatively short T_2 relaxation times which in turn broaden peaks to the extent that they are not recognized.

The iterative development of dimers to date culminates with $\text{I}_{\text{R17K(UG)}}\text{-II}_{\text{L29K(JG)}}$, the 1D ^1H NMR spectrum (Figure 9e) of which shows narrow line widths and good dispersion. TOCSY spectra show considerably more cross-peaks, allowing for optimism on the prospects for further structural analysis.

Conclusions. Optimized syntheses of six OxCM dimers via oxime-forming ligation have been worked out. Issues that have been investigated include practical aspects of the preparation and manipulation of (aminoxy)acetyl-functionalized peptides, as well as the effects of using denaturants (GdnHCl or urea) in the buffers to dissolve glyoxyl-functionalized peptides, or their precursor peptides, for further steps. In the latter regard, it is recommended to avoid the use of urea entirely, and to use GdnHCl only at the lowest possible concentration consistent with peptide solubility.

Three of the dimers, $\text{I}_{\text{R17K(U)}}\text{-II}_{\text{L29K(J)}}$, $\text{I}_{\text{R17K(U)}}\text{-II}_{\text{L29K(JG)}}$, and $\text{I}_{\text{R17K(UG)}}\text{-II}_{\text{L29K(JG)}}$, were judged by 1D ^1H NMR spectroscopy to show evidence of compact structure. Among them, $\text{I}_{\text{R17K(UG)}}\text{-II}_{\text{L29K(JG)}}$ seems to be particularly suited for further structural characterization. Such stud-

ies may ultimately validate the proposed strategy to create new proteins by combining core modules.

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Supporting Information Available: Detailed general materials, instrumentation, and procedures for synthesis, spectroscopy, chromatography, and analysis. Additional information about syntheses and characterizations of peptides not covered in the representative text descriptions. Figures documenting HPLC analysis to monitor oxime-forming ligation, and $^1\text{H}/^2\text{H}$ isotope exchange of the OxCM dimer $\text{I}_{\text{R17K(U)}}-\text{II}_{\text{L29K(J)}}$. Detailed schemes outlining syntheses and cleavages of OxCM monomers of type I and type II. Tables showing complete ^1H NMR assignments for the model peptide TP, and presenting elution behavior of the OxCM dimers on a SEC column (12 pages). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Towards New Designed Proteins Derived from Bovine Pancreatic Trypsin Inhibitor (BPTI): Covalent Cross-linking of Two 'Core Modules' by Oxime-forming Ligation.

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General. Fmoc-PAL-PEG-PS supports (initial loading 0.17–0.24 mmol/g) for peptide synthesis were obtained from Applied Biosystems (Foster City, CA). Protected Fmoc-amino acid derivatives and coupling reagents were from Applied Biosystems or Advanced Chemtech (Louisville, KY), S-Trt-mercaptopropionic acid was from Peptide International (Louisville, KY), Boc-Aoa-OH, Fmoc-Dpr(Boc-Aoa)-OH, Fmoc-Dpr(Boc-Ser(*t*Bu))-OH, and Fmoc-Asn(Tmob)-OH were from Novabiochem (San Diego, CA), and Fmoc-Gln(Tmob)-OH was from Bachem Bioscience (Philadelphia, PA). TFA, piperidine, and DIEA were obtained from Fisher (Pittsburgh, PA). GdnHCl and urea 'ultrapure reagents' were from Gibco BRL (Rockville, MD), and DMSO, sodium periodate, ethylene glycol, and $\text{CH}_3\text{ONH}_2\cdot\text{HCl}$ (25–30 wt % solution in water) 'ACS grade reagents' were from Aldrich (Milwaukee, WI). Et_2O was freshly distilled from Na.

Amino acid analyses were performed on a Beckman 6300 analyzer with a sulfated polystyrene cation-exchange column (0.4 cm x 21 cm). Peptide or protein samples were hydrolyzed in 6 N aqueous HCl plus one drop of liquefied phenol (to prevent degradation of Tyr), for 90 min at 155 °C. Cys was not quantitated and is therefore not reported. The Ile values were consistently low, due to incomplete hydrolysis of the peptide bond between Ile¹⁸ and Ile¹⁹.

Analytical HPLC of the core module monomers, capped monomers, and of the model tetrapeptide and its derivatives, was performed using a Vydac C₁₈ reversed-phase column (5 μm , 300 Å; 0.46 x 25 cm), and analytical HPLC of the core module dimers was performed using a Waters YMC C₄ reversed-phase column (5 μm , 200 Å; 0.46 x 25 cm), all on a Beckman instrument, configured with two 112 pumps and a 165 variable wavelength detector. UV detection was at 220 nm.

Condition A: linear gradients of 0.1% TFA in CH_3CN and 0.1% aqueous TFA were run, at 1.2 mL/min flow rate, from 1:9 to 2:3 over 30 min, then to 4:1 over the next 10 min.

Condition B: linear gradients of 0.1% TFA in CH_3CN and 0.1% aqueous TFA were run, at 1.2 mL/min flow rate, from 1:4 to 7:13 over 40 min, then to 4:1 over the next 10 min.

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Condition C: linear gradients of 0.1% TFA in $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ (3:2) and 0.1% aqueous TFA were run, at 1.2 mL/min flow rate, from 0:1 to 1:1 over 20 min, then to 4:1 over the next 10 min.

Condition D: linear gradients of 0.1% TFA in $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ (3:2) and 0.1% aqueous TFA were run, at 1.2 mL/min flow rate, at 1:3 for 5 min, then to 1:1 over 30 min, then to 4:1 over the next 10 min.

Semipreparative HPLC was carried out on a Waters DeltaPrep 3000 using a Vydac semipreparative C_{18} reversed-phase column (10 μm , 300 Å; 1 x 25 cm). UV detection was at 220 nm.

Condition E: linear gradients of CH_3CN and 0.1% aqueous TFA were run, at 5 mL/min flow rate, from 0:1 for 10 min, then to 19:81 over the next 5 min, then to 27:73 over the next 120 min, and finally to 4:1 over the next 10 min.

Condition F: linear gradients of CH_3CN and 0.1% aqueous TFA were run, at 5 mL/min flow rate, from 4:21 for 5 min, then to 9:16 over the next 45 min, and finally to 4:1 over the next 10 min.

Condition G: linear gradients of CH_3CN and 0.1% aqueous TFA were run, at 5 mL/min flow rate, from 0:1 for 10 min, then to 17:83 over the next 5 min, then to 1:3 over the next 120 min, and finally to 4:1 over the next 10 min.

Condition H: linear gradients of $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ (3:2) and 0.1% aqueous TFA were run, at 4 mL/min flow rate, from 0:1 to 3:17 over 20 min, then to 4:1 over the next 10 min.

Condition I: linear gradients of CH_3CN and 0.1% aqueous TFA were run, at 5 mL/min flow rate, from 0:1 for 10 min, then to 11:39 over the next 5 min, then to 3:7 over the next 120 min, and finally to 4:1 over the next 10 min.

Size exclusion chromatography (SEC) was performed using two Superdex Peptide HR 10/30 columns assembled in series, in an FPLC System (Pharmacia).

Condition J: injections of 500 μL were eluted with 0.1% TFA in $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ (3:7), at 0.2 mL/min.

Condition K: injections of 25 μL were eluted with $\text{CH}_3\text{CN}-10\text{ mM}$ aqueous NaCl (1:19) at pH 2, at 0.2 mL/min.

MALDI-TOF mass spectrometric data were collected with a Bruker Biflex III instrument, equipped

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with a N_2 -laser (337 nm, 3 nanosecond pulse length) and a microchannel plate (MCP) in the reflectron mode, positive polarity, with an accelerating potential of 19 kV. Each spectrum was the accumulation of 200 laser shots. External calibration was performed using human angiotensin II (monoisotopic mass 1046.5 $[M+H]^+$; Sigma) and adrenocorticotrophic hormone (ACTH) fragment 18-39 (monoisotopic mass 2465.2 $[M+H]^+$; Sigma). The matrix used for samples and standards was α -cyano-4-hydroxycinnamic acid (4-HCCA; Hewlett-Packard, obtained as solution in methanol), diluted 1:1 with 0.1% TFA in CH_3CN-H_2O (1:1). ESMS data were collected using a ThermoFinnigan LCQ Classic ion trap mass spectrometer equipped with an ESI source in full scan mode from 50-2000 m/z with a spray voltage setting of 4.0 kV and a heated capillary temperature of 200 °C. Samples were infused at a flow rate of 5 $\mu L/min$ in 0.2% acetic acid in CH_3CN-H_2O (1:1), using the syringe pump on the instrument. Deconvolution of multiply charged protein peaks was performed using ThermoFinnigan's BioMASS add-on tool in the Xcalibur software. Molecular masses were calculated with the GPMW Version 4.0 program.

Peptide synthesis of core module monomers: $I_{R17K(U)}$, $I_{R17K(UG)}$, and $I_{R17Z(U)}$ (Supporting Information Scheme 1), and $II_{L29K(J)}$, $II_{L29K(JG)}$, $II_{L29Z(J)}$, and $II_{N24K(J)}$ (Supporting Information Scheme 2 and Figure 4).

$I_{R17K(U)}$. The synthesis and characterization of this molecule is described in the main text.

$I_{R17K(UG)}$. The only difference between the procedure for this molecule, and for $I_{R17K(U)}$ described in the main text Experimental, was the extra incorporation of Fmoc-Gly-OH preceding Boc-Aoa-OH. As summarized in the main text, three repeat PyAOP/HOAt/DIEA (8:8:16 equiv with respect to peptide-resin)-mediated couplings in DMF, 1 h each, were required for the Fmoc-Gly-OH incorporation to go to completion. Pure peptide was characterized by analytical HPLC (t_R 22.8 min; condition A), amino acid analysis (Asp, 2.04; Thr, 0.93; Glu, 1.05; Gly, 4.07; Ala, 1.98; Val, 0.92; Ile, 1.17; Leu, 0.99; Tyr, 2.46; Phe 1.91; Lys, 2.87; Arg, 1.07), and MALDI-TOF; calcd monoisotopic mass: 2954.46, found: 2955.1 $[M+H]^+$.

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I_{R17Z(U)}. In the linear core module sequence, Arg¹⁷ was replaced by Fmoc-Dpr(Boc-Aoa)-OH. Cleavage, disulfide bond formation, and purification followed as already described for I_{R17K(U)}. Pure peptide was characterized by analytical HPLC (*t_R* 22.5 min; condition A), amino acid analysis (Asp, 1.83; Thr, 0.91; Glu, 1.04; Gly, 3.14; Ala, 1.71; Val, 1.02; Ile, 1.18; Leu, 1.04; Tyr, 2.53; Phe 1.85; Lys, 1.83; Arg, 0.93), and MALDI-TOF; calcd monoisotopic mass: 2855.39, found: 2856.03 [M+H]⁺.

II_{L29K(J)}. The synthesis and characterization of this molecule is described in the main text.

II_{L29K(JG)}. The only difference between the procedure for this molecule, and for II_{L29K(J)} (Figure 4b) described in the main text Experimental, was the extra incorporation of Fmoc-Gly-OH preceding Fmoc-Ser(*t*Bu)-OH. As summarized in the main text, three repeat PyAOP/HOAt/DIEA (8:8:16 equiv with respect to peptide-resin)-mediated couplings in DMF, 1 h each, were required for the Fmoc-Gly-OH incorporation to go to completion. Semipreparative HPLC (condition G) provide *separately* analytical-HPLC pure P1 and P2 [*t_R* 18.4 min (P1) and *t_R* 18.8 min (P2); condition A]. Purified P1 and P2 were indistinguishable by amino acid analysis (Asp, 1.98; Thr, 0.91; Glu, 1.00; Gly, 3.99; Ala, 2.00; Val, 1.01; Ile, 1.37; Tyr, 2.86; Phe, 1.92; Lys, 2.79; Arg, 2.02) and MALDI-TOF; calcd monoisotopic mass: 2980.45, found: 2981.3 [M+H]⁺.

II_{L29Z(J)}. In the linear core module sequence, Leu²⁹ was replaced by Fmoc-Dpr(Boc-Ser(*t*Bu))-OH. Cleavage, disulfide bond formation, and purification followed as already described for II_{L29K(J)} (Figure 4b). Semipreparative HPLC (condition G) provide *separately* analytical-HPLC pure P1 and P2 [*t_R* 18.8 min (P1) and 19.2 (P2) min; condition A]. Purified P1 and P2 were indistinguishable by amino acid analysis (Asp, 2.01; Thr, 0.93; Glu, 1.00; Gly, 3.12; Ala, 1.99; Val, 0.94; Ile, 1.24; Tyr, 2.67; Phe 1.93; Lys, 1.94; Arg, 2.07) and MALDI-TOF; calcd monoisotopic mass: 2881.38, found: 2882.1 [M+H]⁺.

II_{N24K(J)}. The procedure for this molecule was essentially as described for II_{L29K(J)} (Figure 4b), except that Asn²⁴ was replaced by Fmoc-Lys(Alloc)-OH and Leu²⁹ was added normally. Semipreparative HPLC (condition G) provide *separately* analytical-HPLC pure P1 and P2 [*t_R* 20.8 min (P1) and 21.8 min (P2); condition A]. Purified P1 and P2 were indistinguishable by amino acid analysis (Asp, 1.01; Thr,

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0.86; Glu, 1.02; Gly, 3.09; Ala, 1.98; Val, 0.96; Ile, 1.58; Leu, 1.00; Tyr, 2.57; Phe, 1.97; Lys, 2.97; Arg, 1.97) and MALDI-TOF; calcd monoisotopic mass: 2922.47, found: 2923.12 $[M+H]^+$.

Oxime-forming ligation reactions

Capping of monomers

I_{R17K(U*)}. The synthesis and characterization of this molecule is described in the main text.

I_{R17Z(U*)}. Pure peptide was characterized by analytical HPLC (t_R 27 min; condition A), amino acid analysis (Asp, 2.01; Thr, 0.92; Glu, 0.99; Gly, 3.01; Ala, 2.02; Val, 0.95; Ile, 1.11; Leu, 1.00; Tyr, 2.85; Phe, 1.96; Lys, 2.05; Arg, 1.05), and MALDI-TOF; calcd monoisotopic mass: 2895.42, found: 2896.09 $[M+H]^+$.

I_{R17K(U*G)}. Pure peptide was characterized by analytical HPLC (t_R 25.5 min; condition A), amino acid analysis (Asp, 1.99; Thr, 0.86; Glu, 1.03; Gly, 3.95; Ala, 2.02; Val, 0.99; Ile, 1.63; Leu, 1.03; Tyr, 2.78; Phe, 1.95; Lys, 2.91; Arg, 1.05), and MALDI-TOF; calcd monoisotopic mass: 2994.49, found: 2996.3 $[M+H]^+$.

II_{L29K(J*)}. The synthesis and characterization of this molecule is described in the main text.

II_{L29K(J*G)}. Pure peptide was characterized by analytical HPLC (t_R 21.3 min; condition A), amino acid analysis (Asp, 2.00; Thr, 1.03; Glu, 1.13; Gly, 3.89; Ala, 2.05; Val, 1.07; Ile, 1.40; Tyr, 2.89; Phe 2.01; Lys, 2.76; Arg, 1.87), and MALDI-TOF; calcd monoisotopic mass: 3009.47, found: 3010.9 $[M+H]^+$.

II_{L29Z(J*)}. Pure peptide was characterized by analytical HPLC (t_R 20.1 min; condition A), amino acid analysis (Asp, 1.96; Thr, 0.83; Glu, 1.06; Gly, 2.96; Ala, 1.97; Val, 0.98; Ile, 1.59; Tyr, 2.56; Phe, 1.87; Lys, 1.96; Arg, 2.06), and ESMS; calcd average mass: 2912.35, found: 2912.6 ± 1.4 .

II_{N24K(J*)}. Pure peptide was characterized by analytical HPLC (t_R 24.6 min; condition A), amino acid analysis (Asp, 1.14; Thr, 1.01; Glu, 1.25; Gly, 3.00; Ala, 1.78; Val, 1.13; Ile, 1.15; Leu, 1.15; Tyr, 2.45; Phe, 1.96; Lys, 2.56; Arg, 1.95), and MALDI-TOF; calcd monoisotopic mass: 2951.49, found: 2953.2 $[M+H]^+$.

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Dimer formation

I_{R17K(U)}-II_{L29K(J)} The synthesis and characterization of this molecule is described in the main text.

I_{R17Z(U)}-II_{L29K(J)} Pure peptide was characterized by analytical HPLC (t_R 29.1 min; condition B), amino acid analysis (Asp, 3.95; Thr, 1.82; Glu, 2.03; Gly, 5.95; Ala, 3.96; Val, 1.93; Ile, 2.56; Leu, 1.06; Tyr, 5.55; Phe, 3.90; Lys, 4.89; Arg, 2.83), and ESMS; calcd average mass: 5764.68, found: 5764.1 ± 1.8 .

I_{R17Z(U)}-II_{L29Z(J)} Pure peptide was characterized by analytical HPLC (t_R 29.0 min; condition B), amino acid analysis (Asp, 3.96; Thr, 1.87; Glu, 2.01; Gly, 5.97; Ala, 3.94; Val, 1.92; Ile, 2.46; Leu, 1.06; Tyr, 5.57; Phe, 3.91; Lys, 3.91; Arg, 2.87), and ESMS; calcd average mass: 5722.66, found: 5719.8 ± 1.5 .

I_{R17K(U)}-II_{L29K(JG)} Pure peptide was characterized by analytical HPLC (t_R 28.6 min; condition B), amino acid analysis (Asp, 3.91; Thr, 1.63; Glu, 2.01; Gly, 7.26; Ala, 3.91; Val, 1.88; Ile, 2.91; Leu, 0.93; Tyr, 4.30; Phe, 3.78; Lys, 5.47; Arg, 2.81), and ESMS; calcd average mass: 5862.87, found: 5863.56 ± 0.64 .

I_{R17K(UG)}-II_{L29K(JG)} Pure peptide was characterized by analytical HPLC (t_R 28.8 min; condition B), amino acid analysis (Asp, 4.15; Thr, 1.98; Glu, 2.21; Gly, 7.46; Ala, 4.00; Val, 2.02; Ile, 2.90; Leu, 1.15; Tyr, 5.83; Phe, 3.93; Lys, 5.79; Arg, 2.85), and ESMS; calcd average mass: 5919.92, found: 5920.31 ± 0.48 .

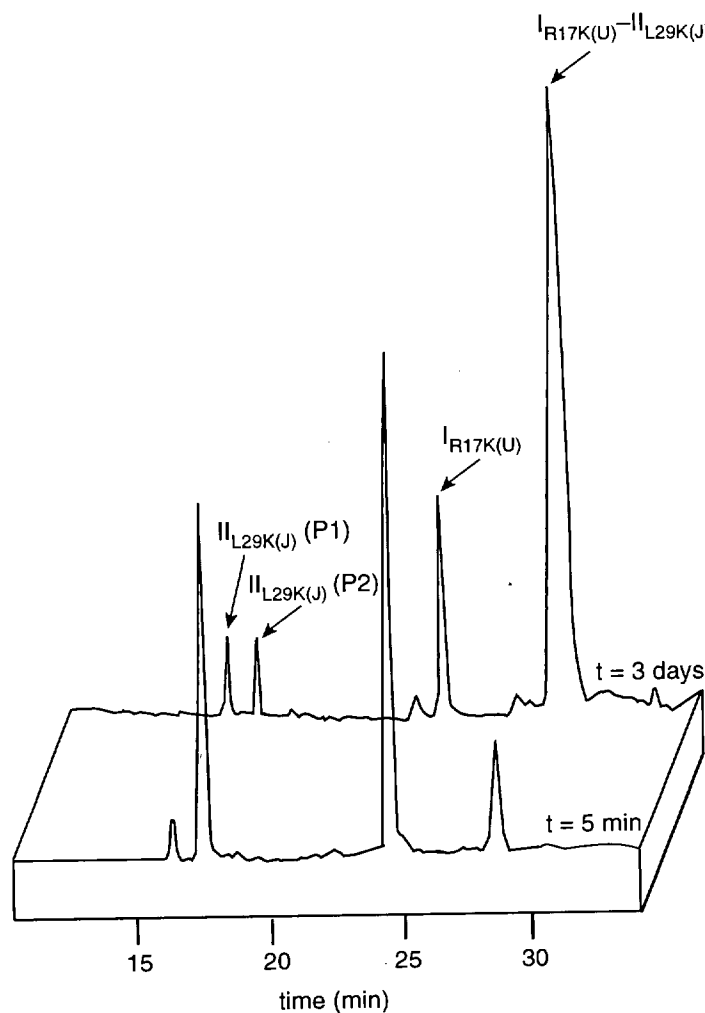
I_{R17K(U)}-II_{N24K(J)} Pure peptide was characterized by HPLC (t_R 29.2 min; condition B), amino acid analysis (Asp, 2.99; Thr, 1.61; Glu, 2.01; Gly, 6.15; Ala, 4.02; Val, 1.89; Ile, 3.35; Leu, 1.99; Tyr, 5.08; Phe, 3.91; Lys, 5.91; Arg, 2.87), and ESMS; calcd average mass: 5805.82, found: 5805.00 ± 0.78 .

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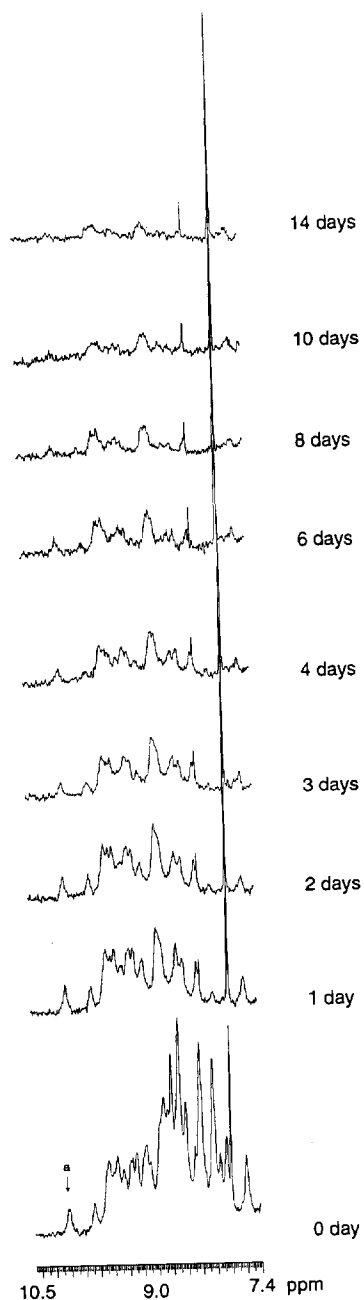
Supporting Figure 1. RP-HPLC analysis (C_4 column; condition B) to document oxime-forming reaction to produce OxCM dimer, carried out in 6 M GdnHCl in 0.1 M NaOAc buffer, pH 4.6. Front panel, at start of reaction (5 min), and rear panel, at point where, no appreciable further product was formed (3 days). Peaks in the rear panel are labeled by the system introduced in Figure 2 of the main text; the peaks in the front panel are not explicitly labeled but correspond. Also, see Figure 4 for definitions of P1 and P2.

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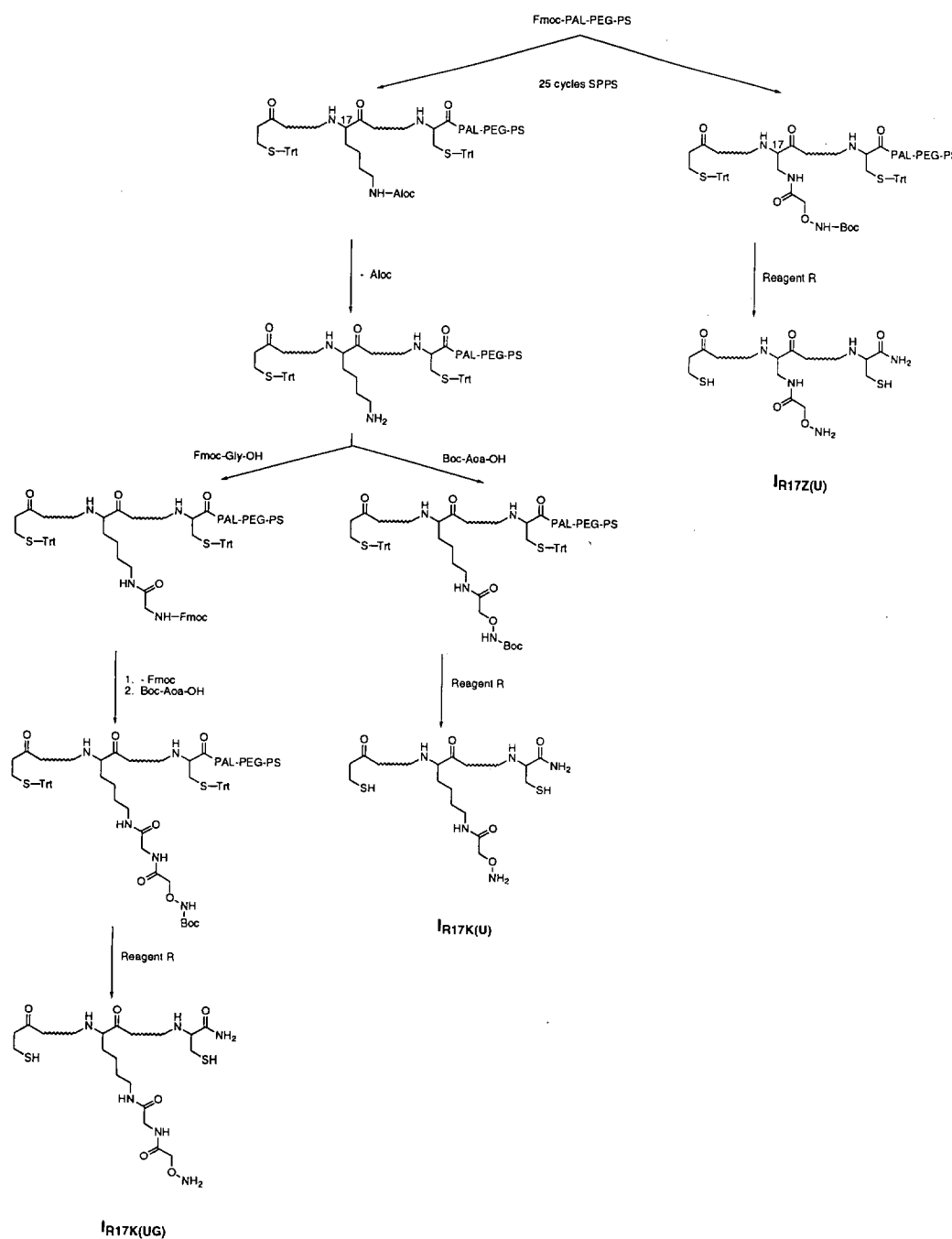
Supporting Figure 2. $^1\text{H}/^2\text{H}$ isotope exchange at pH 2 and 5 $^\circ\text{C}$, of $\text{I}_{\text{R17K(U)}}\text{-II}_{\text{L29K(I)}}$, monitored by a series of 1D ^1H NMR spectra at 600 MHz. Amide resonance 'a', indicated by arrow, was sufficiently resolved from the remaining envelope to allow calculation of hydrogen isotope exchange rate.

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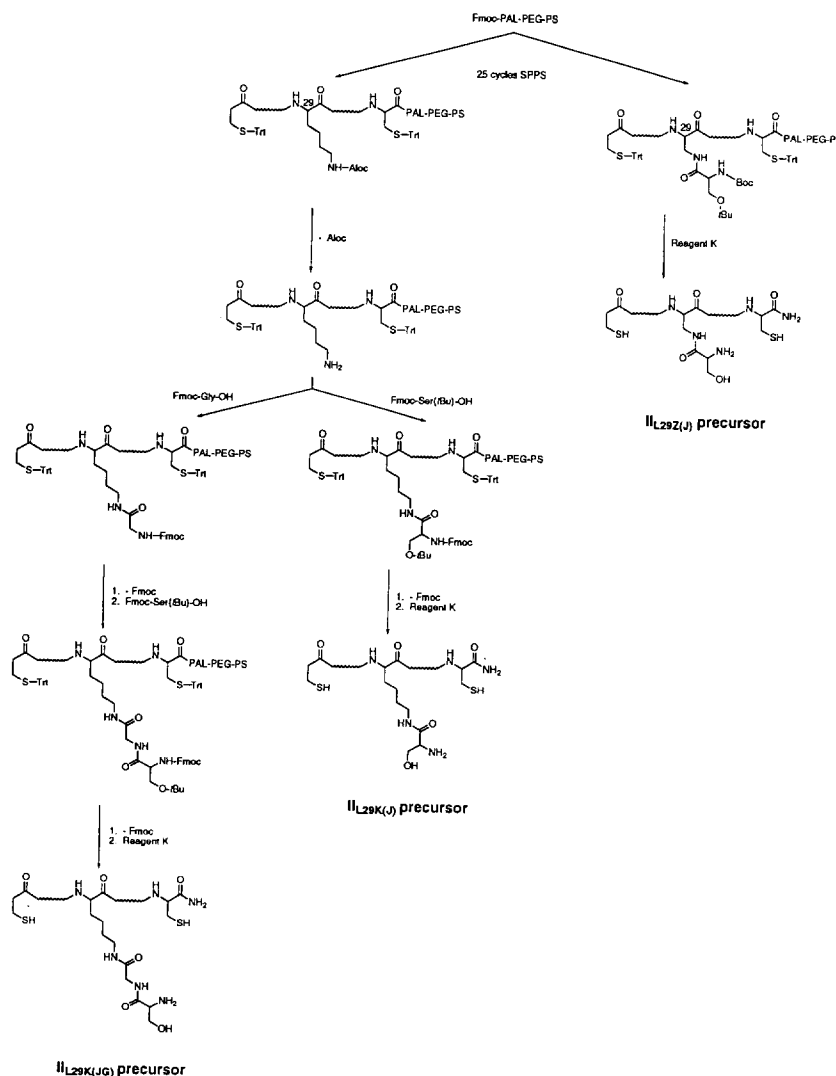
Supporting Scheme 1. Synthesis and cleavage of monomers of type I containing the (aminoxy)acetyl functionality.

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Supporting Scheme 2. Synthesis and cleavage of monomers of type II containing the glyoxylyl functionality.^{(a),(b)}



(a) The schemes are drawn up to the point of reduced precursors with a free Ser residue at the *N*-terminus of the branch. Further manipulations to form the disulfide and create the Gxy moiety are discussed in the main text.

(b) $\text{II}_{\text{N24K(J)}}$ (not shown in the Scheme) was prepared as $\text{II}_{\text{L29K(J)}}$, with the only difference that Fmoc-Lys(Alloc)-OH was incorporated at position 24.

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Supporting Table 1. ^1H chemical shifts for H-Tyr-Dpr(Gxy)-Ala-Lys-NH₂, recorded at 500 MHz in $^2\text{H}_2\text{O}$ at pH 4.6 in the absence and presence of denaturants

Residue	Position	$^2\text{H}_2\text{O}$	6 M GdnDCl- d_6	8 M urea- $^{15}\text{N}_2\text{d}_4$
Tyr	C α H	4.47	4.42	4.41
	C β H	3.33, 3.40	3.33, 3.37	3.30, 3.36
	C δ H	7.10	7.03	7.07
	C ϵ H	7.37	7.31	7.33
Dpr (Gxy)	C α H	4.77	4.81	4.79
	C β H	3.70, 3.83	3.71, 3.79	3.70, 3.83
	C ϵ H ^(a)	5.52	5.52, 5.71, 5.72	5.57, 5.61
Ala	C α H	4.50	4.54	4.50
	C β H	1.66	1.61	1.66
Lys-amide	C α H	4.50	4.52	4.48
	C β H	2.05, 2.08	1.97, 2.01	2.00, 2.07
	C γ H	1.69, 1.72	1.65	1.66, 1.69
	C δ H	1.94	1.89	1.92
	C ϵ H	3.25	3.20	3.22

^(a) Main text, Figures 5, 6, and Table 2 refer to this as H-C(2).

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Supporting Table 2. SEC chromatography of OxCM dimers^(a)

	Molecular Weight	Retention time (mL)
BPTI ^(b)	6512.7	20.86
I _{R17K(UG)} -II _{L29K(JG)}	5919.9	22.64
I _{R17K(U)} -II _{L29K(JG)}	5862.9	22.90
I _{R17K(U)} -II _{L29K(J)}	5806.8	24.06
I _{R17K(U)} -II _{N24K(J)}	5805.8	24.23
I _{R17Z(U)} -II _{L29K(J)}	5764.7	24.30
I _{R17Z(U)} -II _{L29Z(J)}	5722.7	24.56
Gly ^(b)	75.1	37.84

^(a) The proteins were eluted in CH₃CN-10 mM aqueous NaCl (1:19) at pH 2 and 3 °C.^(b) BPTI and Gly are used as standards.

Synthesis and Characterization of Membrane-Active GALA-OKT9 Conjugates

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Cellular processing of immunotoxins is inefficient, limiting the overall effectiveness of current immunotoxin therapies. Specifically, translocation of ribosome-inactivating toxins across intracellular membranes is agonizingly slow. In one strategy to improve immunotoxin efficacy, membrane-active peptides are attached to immunotoxins to facilitate transfer of the toxic moiety across a cellular membrane to the cytosol. pH-sensitive peptides are of particular interest, as the membrane activity can be localized to the endosomal/lysosomal pathway, reducing nonspecific interactions at the cell surface. In this study, GALA, a pH-sensitive peptide that forms multimeric pores in membranes, was chemically attached to OKT9, an anti-transferrin receptor mAb. Conjugates were tested by measuring release of encapsulated dyes from liposomes to determine the extent to which the membrane-lytic properties of GALA were retained. The most significant feature affecting the lytic properties of GALA-OKT9 conjugates was the number of attached GALA per OKT9. Conjugates with a single GALA per OKT9 caused almost no leakage while conjugates with two or three GALA per OKT9 caused significant leakage in a concentration-dependent manner. Invariably, GALA-OKT9 conjugates were significantly less active than unconjugated GALA, attributable to a decrease both in partitioning and in surface aggregation. No improvement in membrane-lytic activity was achieved by using a longer, more flexible poly(ethylene glycol) cross-linker. Attachment of GALA via C- versus N-terminal linkage had no effect on membrane-lytic properties. Size-selective release of high molecular weight dextrans was almost identical for conjugated and unconjugated GALA, suggesting that GALA forms the same pore structure regardless of conjugation state.

INTRODUCTION

Immunotoxins are chimeric molecules with both targeting and toxic capabilities, of interest for treatment of diseases such as cancer, graft-vs-host disease, and AIDS. Targeting ability is derived from monoclonal antibodies, stable hormones, or carrier proteins, while toxic activity is typically conferred by plant or bacterial toxins. Ideally, immunotoxins bind with high specificity and affinity only to the targeted cell, while avoiding healthy tissue.

Even if immunotoxins bind tightly to the surface of targeted cells, cell death is not assured. Commonly used toxins (e.g., diphtheria toxin, gelonin, ricin, *Pseudomonas* exotoxin A) act at the ribosomal level to inactivate protein synthesis. Thus, the toxin must be correctly routed to the ribosome for cell death to occur. For immunotoxins targeted to specific cell-surface receptors, this involves receptor-mediated endocytosis into an appropriate intracellular vesicle, followed by translocation of the toxin across the vesicular membrane to the cytosol. Inefficient intracellular trafficking, e.g., immunotoxins traveling to lysosomes, results in a large reduction in utilization of the targeted toxin (1). In one study, it was estimated that ~10 million transferrin–gelonin immunotoxins are processed through a cell for each one that actually reaches the cytosol and inactivates protein synthesis (2). The slowest step by far appears to be translocation across the membrane (2, 3), and it has been suggested that strategies which improve toxin translocation rates could result in gains in immunotoxin efficacy (4).

Another delivery system that encounters membrane barriers is gene therapy. There are two main strategies for gene therapy: (1) lipofection, in which translocation occurs across the plasma membrane followed by transport into the nucleus; and (2) polyfection, in which internalization into an endosomal vesicle occurs, then translocation across the endosomal membrane, followed by transport to the nucleus (5). Molecules used to facilitate transport to the cell and across membranes include polycations, lipid-based DNA complexes, targeting ligands, and endosomolytic agents (5, 6). Further improvements in membrane translocation of gene therapy products are also needed.

In one strategy for improving membrane translocation of internalized macromolecules, membrane-disrupting peptides are linked to macromolecules via chemical conjugation or genetic fusion. pH-sensitive peptides are of particular interest, since membrane activity can be localized to the acidic endosomal/lysosomal system. The hypothesis is that perturbation of intracellular membranes by the peptide would lead to enhanced translocation of the attached macromolecule. A few studies in which this strategy was implemented to improve immunotoxin efficacy have been reported, with variable success. Decreases in IC_{50} (concentration required to inhibit protein synthesis by 50%) of 2–20-fold were observed for some peptide-modified immunotoxins relative to unmodified immunotoxins (7–9), but in other cases no effect or even a reduction in efficacy was observed (8, 9). Lorenzetti et al. compared the ability of three peptide–toxin fusion proteins to destabilize liposomes with their activity in protein synthesis inhibition assays, and observed no

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strong correlation (9). In fact, the peptide–toxin combination that was most effective at destabilizing liposomes proved to be least effective at killing cells. Thus, although this strategy is appealing, greater mechanistic insight is needed to fulfill its promise. Specifically, the structure–activity relationship between peptide–conjugate design, membrane destabilization, and macromolecule translocation remains undefined.

In this report, we describe how the activity of a membrane-destabilizing peptide is affected by conjugation to a carrier protein. We chose as a model system the synthetic pH-sensitive peptide GALA chemically conjugated to an anti-transferrin antibody, OKT9. GALA converts from a random coil monomer at neutral pH to an amphipathic α -helix at pH 5 (10). When contacted with large unilamellar vesicles at acidic pH, GALA partitions into the lipid bilayer, forms pores of 8–12 monomers, and allows leakage of encapsulated small molecules. GALA also causes phospholipid flip-flop in large unilamellar vesicles and fusion of small vesicles (10–13). These properties make GALA an attractive candidate for investigation. In fact, GALA has been used successfully to improve delivery of genes (14) and antisense oligodeoxynucleotides (15), and may improve intracellular retention of Fabs in cancer cells (16). However, no detailed biophysical studies have been undertaken to establish the extent to which GALA's activity was retained upon attachment to macromolecules. The goals of this work are as follows: (1) to determine how cross-linker length, peptide:antibody ratio, and peptide orientation relate to the membrane activity of GALA-OKT9 conjugates; and (2) to compare membrane interaction properties of unconjugated versus conjugated GALA.

MATERIALS AND METHODS

Materials. GALA has the following sequence: WEAALAEALAEALAEHLAEALAEALAEALAA (10). A cysteine was added to the N- or C-terminus of GALA to add a chemically reactive sulfhydryl group. GALA, cys-GALA, and GALA-cys were purchased from American Peptide Co. (Sunnyvale, CA). Purity was greater than 90% by reverse-phase HPLC,¹ and identity was confirmed by mass spectroscopy. Hybridoma serum-free media (SFM) was purchased from Gibco (Gaithersburg, MD). *N*-Succinimidyl-3-(2-pyridyldithio)propionate (SPDP) was purchased from Pierce Chemical Co. (Rockford, IL), while maleimido-PEG₃₄₀₀-succinimidyl propionate (PEG) was purchased from Shearwater Polymers, Inc. (Huntsville, AL). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dibromostearoyl-*sn*-glycero-3-phosphocholine (OBPC) were purchased from Avanti Polar-Lipids, Inc. (Alabaster, AL), aliquoted into 5 or 10 mg vials, dried under nitrogen, vacuum-desiccated for at least 2 h, and then stored under nitrogen at –20 °C. Neutral Texas-Red 3000 and 10 000 MW dextran were

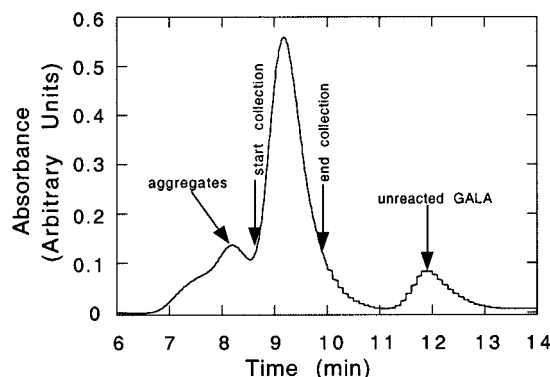


Figure 1. Purification of an OKT9-GALA conjugate mixture. A representative HPLC chromatograph of an OKT9-SPDP-GALA conjugate mixture on a Zorbax GF-250 column. Conjugate used in testing was purified from aggregated conjugate and unreacted GALA.

purchased from Molecular Probes (Eugene, OR). All other reagents were purchased from Sigma (St. Louis, MO), unless otherwise stated.

Conjugate Production and Purification. mAb OKT9, a murine anti-transferrin receptor antibody, was purified on a protein G affinity column (Pharmacia Biotech, Piscataway, NJ) from supernatant of the hybridoma cell line CRL8021 (American Type Culture Collection, Rockville, MD) and then dialyzed into PBSA [3 mM KH₂PO₄, 7 mM K₂HPO₄·3H₂O, 140 mM NaCl, and 0.02% (w/v) NaN₃, pH 7.0]. To synthesize conjugates with disulfide linkers, 2 mg of SPDP was dissolved in DMSO, and then added in varying molar ratios to 2–3 mg of OKT9 (~1 mg/mL) in PBSA. SPDP and OKT9 were reacted for 30 min at 25 °C. Excess SPDP was removed by overnight dialysis in a 10K MWCO Slide-A-Lyzer (Pierce, Rockford, IL) against 1 L of PBE (0.1 M Na₂PO₄/NaHPO₄, pH 7.4, + 1 mM EDTA) at 4 °C. Alternatively, SPDP-modified OKT9 was separated from unreacted SPDP on a Zorbax GF-250 (Mac Mod Analytical, Chadds Ford, PA) size exclusion HPLC (running buffer PBE), and then concentrated to ~1 mg/mL by ultrafiltration with a YM10 filter (MWCO 10K, Millipore, Bedford, MA). To synthesize conjugates with PEG linkers, PEG was dissolved in ~1 mg/mL OKT9 and incubated for 1 h at 25 °C. Excess PEG was removed by Zorbax GF-250 size exclusion HPLC (running buffer PBE). After HPLC purification, PEG-modified OKT9 was concentrated to ~1 mg/mL by ultrafiltration. Modified OKT9 was mixed with a 5 molar excess of cys-GALA or GALA-cys and reacted for 4 h at 25 °C. OKT9-GALA conjugates were purified from unconjugated GALA by Zorbax GF-250 size exclusion HPLC (running buffer PBE). Approximately 75% of the conjugate peak was collected, to eliminate aggregates (Figure 1). Conjugate was stored at 4 °C overnight at a concentration of ~0.15 mg/mL and tested the following day. Conjugate concentration was determined by optical density measurements at 280 nm using an extinction coefficient of 1.4 mL/(mg·cm). As a control, sham-conjugation of OKT9 and cysteine-modified GALA, in the absence of cross-linker, was carried out under identical conditions.

The degree of SPDP modification was determined by quantifying the number of pyridine-2-thione groups released from OKT9 upon incubation with DTT (17). SPDP modification ranged from 0.8 to 3.4 SPDP per OKT9. The degree of PEG modification was determined from shifts in the HPLC retention time of PEG-modified OKT9 vs unmodified OKT9. Column calibration was

¹ Abbreviations: EDTA, ethylenediaminetetraacetic acid disodium salt; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; mAb, monoclonal antibody; MWCO, molecular weight cutoff; PEG, maleimido-PEG₃₄₀₀-succinimidyl propionate; OBPC, 1,2-dibromostearoyl-*sn*-glycero-3-phosphocholine; PBE, phosphate buffer with EDTA (0.1 M Na₂PO₄/NaHPO₄, pH 7.4, + 1 mM EDTA); PBS, phosphate-buffered saline (3 mM KH₂PO₄, 7 mM K₂HPO₄·3H₂O, 140 mM NaCl, pH 7.0); PBSA, phosphate-buffered saline with azide [3 mM KH₂PO₄, 7 mM K₂HPO₄·3H₂O, 140 mM NaCl, and 0.02% (w/v) NaN₃, pH 7.0]; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SDS, sodium dodecyl sulfate; SFM, serum-free media; SPDP, succinimidyl-3-(2-pyridyldithio)propionate.

determined using OKT9 (150 kDa), bovine serum albumin (68 kDa), and ovalbumin (43 kDa). A total of 0.5–4 PEG groups were attached per OKT9. The number of GALA attached per OKT9 was assumed equal to the degree of modification by the cross-linker, as the conjugation reaction was run with a 5-fold molar excess of cysteine-modified GALA.

Liposome Production. POPC and OBPC liposomes were prepared by extrusion. Briefly, lipids were rehydrated to a concentration of 10 mg/mL in an aqueous calcein (50 mM) solution at pH 7.0 in Tris buffer (1 mM EDTA and 20 mM Tris), or at pH 5.0 in citrate buffer (1 mM EDTA, 100 mM NaCl, 5.9 mM $C_6H_5O_7Na_3$, 4.5 mM $C_6H_8O_7$, pH adjusted with NaOH), or in an aqueous solution of neutral Texas-red 3000 or 10 000 MW dextran (0.167 mM) in citrate buffer, pH 5.0. After 30 min, the hydrated lipid suspension was subjected to 4–5 freeze–thaw cycles in an ethanol–dry ice bath and warm water bath, and then passed through a 0.1 μ m polycarbonate filter (Avanti Polar Lipids) 21 times. Liposomes were separated from unencapsulated calcein by Sephadex G-50 size exclusion chromatography or from unencapsulated dextran by Sephacryl S-400 size exclusion chromatography. Lipid concentration was determined using a Stewart assay (18). Liposome diameter was 100–150 nm, as measured by dynamic light scattering.

Calcein Leakage Assay. Leakage of encapsulated calcein from liposomes was used to determine the lytic ability of conjugates. Calcein is a self-quenching dye; release of encapsulated calcein from liposomes into buffer causes a reduction in concentration and a corresponding increase in fluorescence intensity. Fluorescence measurements were collected using a model M-3 Alphascan spectrofluorometer (Photon Technology International, South Brunswick, NJ) with excitation at 490 nm and emission at 520 nm. Conjugate was dialyzed into pH 5.0 citrate buffer using a 10K MWCO Slide-A-Lyzer for 3 h, with two buffer changes. Samples were prepared by mixing calcein-encapsulated liposomes (final lipid concentration 10 μ M) with varying molar ratios of conjugate, 1% (v/v) Triton-X 100 (100% leakage), or citrate (pH 5.0) buffer or PBS (pH 7.0) (0% leakage). Samples were briefly vortexed, and then left quiescent for 16 h at room temperature, which was sufficient time to allow equilibration (data not shown). Fluorescent intensity (FI) measurements were normalized as

$$\% \text{ leakage} = \frac{(FI_{\text{sample}} - FI_{\text{buffer}})}{(FI_{\text{triton}} - FI_{\text{buffer}})} \times 100 \quad (1)$$

Partitioning Assay. The partition coefficient of GALA-OKT9 conjugates (containing three GALA per OKT9 linked by SPDP) in liposomes was determined by incubating ^{125} I-labeled conjugate with dense liposomes, separating the lipid phase from the aqueous phase by centrifugation, and measuring the radioactivity associated with each phase. To facilitate centrifugal separation, dense liposomes were made with brominated lipids (OBPC) (19). Following the manufacturer's instructions, Iodobeads (Pierce, Rockford, IL) were used to radiolabel conjugate and antibody with $Na^{125}I$ (DuPont, New England Nuclear, Boston, MA). To deter nonspecific binding to tubes, 0.6 mL tubes were preincubated with 50 μ L of a 40 mg/mL bovine serum albumin/citrate buffer. Samples were prepared by mixing ~ 0.5 mg/mL ^{125} I-labeled conjugate or ^{125} I-labeled antibody in PBS, pH 7.0, with OBPC liposomes in citrate, pH 5.0, and then adding 450 μ L to the coated tubes (final lipid:antibody/conjugate

ratios of 1000 or 3000, final lipid concentration 10 μ M, final pH 5.0). After incubation for 16 h, samples were centrifuged at 7000g for 30 min followed by 16000g for 30 min. Centrifuged samples were separated by removing 250 μ L of supernatant (SN) from the remaining 250 μ L solution plus pelleted material (P). Radioactivity associated with SN and P was measured in a gamma counter. The partition coefficient, K_p , was calculated as

$$K_p = \frac{P_L}{P_W} = \frac{(CPM_P - CPM_{SN})}{2 \cdot CPM_{SN}} \quad (2)$$

where P_L = moles of peptide in the lipid phase per volume of solution and P_W = moles of peptide in the aqueous phase per volume of solution.

Model of GALA-Induced Calcein Leakage from Liposomes. GALA pore formation was modeled as a two-step process: (1) partitioning of the peptide into lipid phase and (2) surface aggregation. The equilibrium constant for partitioning between lipid and aqueous phases, K_p , is defined as

$$K_p = \left(\frac{P_L}{P_W} \right) \frac{1}{L_{\text{tot}}} = K'_p \frac{1}{L_{\text{tot}}} \quad (3)$$

where L_{tot} = moles of lipid per volume solution (10^{-5} M). It was assumed that peptides reversibly aggregate on the lipid surface, and that aggregates containing M or more peptides will leak. Total leakage, L_{∞} , is (20, 21)

$$L_{\infty} = \sum_{i=M}^N z_i A_i \quad (4)$$

where A_i = fraction of liposomes containing i peptides, z_i = probability that a liposome containing i peptides will include an aggregate of M or more peptides, and N = maximum allowable number of peptides per liposome. A_i is calculated from (20)

$$A_0 = (1 - T)^N \quad (5)$$

$$A_{i+1} = A_i \left(\frac{N-i}{i+1} \right) \left(\frac{T}{1-T} \right) \quad (6)$$

$$T = \frac{P_L}{NG_0} \quad (7)$$

subject to

$$\sum_{i=0}^N A_i = 1 \quad (8)$$

where G_0 = molar concentration of liposomes. z_i is calculated from (21)

$$z_i = P_i^{M-1} (M - MP_i + P_i) \quad (9)$$

$$P_i = \frac{1}{K_i + \sqrt{K_i^2 - 1}} \quad (10)$$

$$K_i = 1 + \frac{1}{4iK_s} \quad (11)$$

where K_s = the equilibrium constant for surface aggregation. We assumed $M = 10$ (20) and determined K_s and K_p from a least-squares fit to the leakage data. (Adjusting

M to 8 or 12 peptides affected the absolute values of K_S and K_P but did not change the relative ordering; data not shown.) For simplicity we neglected the effects of liposomal size distribution on leakage. Parameters were determined using the general purpose multiresponse parameter estimation program GREG (22, 23).

Dextran Leakage Assay. Leakage of 3000 or 10 000 MW dextrans from liposomes was used to determine whether conjugates formed size-selective pores. All work with Texas-red dextran was done with minimal light exposure to minimize photobleaching. Conjugates with three GALA per OKT9 cross-linked with SPDP were used in these experiments. Conjugate was dialyzed into pH 5.0 citrate buffer using a 10K MWCO Slide-A-Lyzer for 3 h, with two buffer changes. Samples were prepared by mixing dextran-encapsulated liposomes (final lipid concentration 10 μ M) with varying molar ratios of conjugate, 0.2% (w/v) SDS, or buffer. Conjugate samples were prepared at 50, 500, and 2000 lipid:conjugate ratios to span calcein leakage ranges from 20 to 100%, while cys-GALA samples were prepared at 200, 500, 2000, and 7000 lipid:peptide ratios to span calcein leakage ranges from 50 to 100%. Samples were briefly vortexed and left quiescent for 16 h at room temperature. Then 500 μ L aliquots were placed in a Microcon-100 (100K MWCO, Millipore, Bedford, MA) filtration unit and centrifuged at 5000*g* for 15 min to separate released dextran. The Microcon unit was removed, and 10 μ L of a 10% (w/v) SDS solution was added to retentate to lyse liposomes; then 500 μ L of citrate buffer was added. The filter was reversed and centrifuged for 10 min at 3000*g* to release all retained dextran. To account for SDS background fluorescence, filtrate samples were mixed with 10 μ L of 10% (w/v) SDS. The fluorescence intensity (FI) of each fraction was measured with excitation at 595 nm and emission at 610 nm. Control experiments in which buffer alone or SDS was added in place of the conjugate were conducted, and corrections were made for the SDS contribution to FI of each dextran sample. The fraction, f , of released dextran was determined as

$$f = \left(\frac{FI_{\text{filtrate}}}{FI_{\text{filtrate}} + FI_{\text{retentate}}} \right) \quad (12)$$

Results were normalized according to

$$\% \text{ leakage} = \frac{(f_{\text{sample}} - f_{\text{buffer}})}{(f_{\text{SDS}} - f_{\text{buffer}})} \times 100 \quad (13)$$

Dynamic Light Scattering. Dynamic light scattering was used to determine the size of liposomes before and after addition of conjugate at acid pH. Liposome samples were prepared by mixing various concentrations of conjugate with a constant 10 μ M lipid in pH 5.0 citrate buffer and then incubated for 16 h. Samples were filtered through a 0.45 μ m filter into dust-free light-scattering cuvettes, and then placed in a temperature-controlled bath (25 $^{\circ}$ C) of decahydronaphthalene. Dynamic light scattering measurements were collected with a Lexel (Fremont, CA) argon laser and a Malvern (Southborough, MA) 4700 C system as described in detail elsewhere (24). Three replicate measurements were taken at a 90 $^{\circ}$ scattering angle. Data were analyzed using CONTIN.

RESULTS

Effect of Conjugation Method on Retention of GALA Lytic Activity. At pH 5, GALA causes leakage of small molecules from egg phosphatidylcholine lipo-

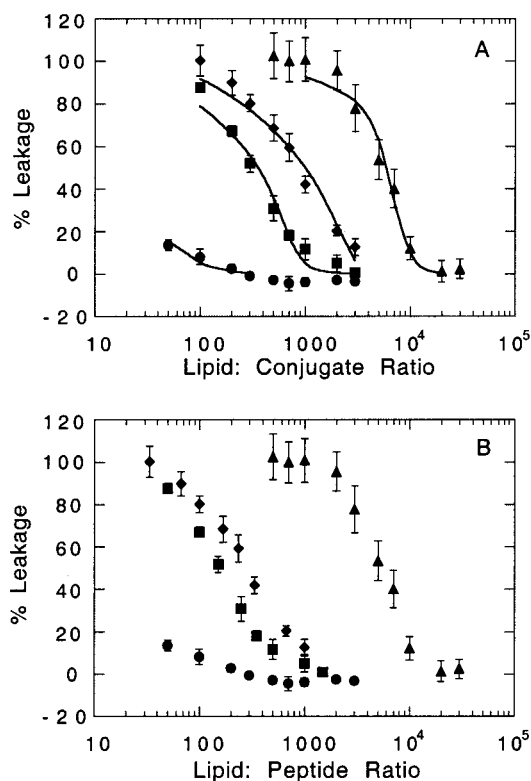


Figure 2. Effect of derivatization on conjugate-induced leakage. Conjugate was mixed with calcein liposomes (final concentration 10 μ M lipid) and left quiescent for 16 h; then fluorescent measurements were obtained with excitation at 490 nm and emission at 520 nm. Percent leakage was calculated per eq 1. (A) OKT9-GALA conjugates were synthesized with 1 (●), 2 (■), and 3 (◆) GALA per OKT9 using a SPDP cross-linker. The data represent the average of 1–3 preparations of conjugate with 3–4 data points per lipid:conjugate ratio for each conjugate. Cys-GALA (▲) data are a single representative set of data with 3–4 data points per lipid:peptide ratio. Error bars are standard deviation of the average leakage from each separate conjugate preparation. Lines show model fits of the data. (B) The lipid:conjugate molar ratio was divided by the integer number of GALA per OKT9 to give the lipid:peptide ratio.

somes (12). A panel of conjugates was synthesized and tested to determine the extent of GALA's lytic activity retained upon attachment to OKT9. Effects of three variables were examined: number of GALA per OKT9, cross-linker type, and C- or N-terminal placement of the cysteine on GALA.

GALA-OKT9 conjugates with SPDP cross-linkers were synthesized with one, two, or three GALA per OKT9. Conjugates with a single GALA per OKT9 caused little leakage (Figure 2A), reaching only $14 \pm 3\%$ leakage at a 50:1 lipid:conjugate ratio. Conjugates with two or three GALA per OKT9 caused significant calcein leakage in a concentration-dependent manner, with the extent of leakage increasing with extent of derivatization (Figure 2A). Approximately 100% leakage was reached at ~ 100 lipid:conjugate ratio. Conjugated GALA was substantially less effective than unconjugated GALA (Figure 2A). For example, at least 7-fold greater concentration of GALA-OKT9 conjugate was required to cause 50% leakage compared to unconjugated GALA. At equivalent final leakage values, rates of leakage for conjugated and unconjugated GALA were similar (data not shown), demonstrating that the kinetics of GALA's interaction with liposomes are unchanged upon conjugation. Neither GALA nor OKT9-GALA caused leakage at pH 7, indicating that GALA did not lose its pH sensitivity upon conjugation (data not shown). Thus, GALA retains pH

Table 1. Model Fit Parameters^a

	$K_P (\times 10^3 \text{ M}^{-1})$	K_S
GALA	200 ± 100	$> 3^b$
OKT9-SPDP-3GALA	28 ± 0.6	0.8 ± 0.01
OKT9-SPDP-2GALA	7 ± 2	1.0 ± 0.5
OKT9-SPDP-1GALA	0.2 ± 2	0.1 ± 0.7
OKT9-PEG-3GALA	8 ± 1	0.7 ± 0.2
OKT9-PEG-2GALA	6 ± 4	0.3 ± 0.2
OKT9-PEG-1GALA	2 ± 1	0.2 ± 0.01

^a OKT9 concentration, not GALA concentration, was used to fit OKT9-GALA conjugate data. Error bars indicate 95% confidence intervals. ^b Solution becomes insensitive to K_S at large K_S (see eq 11).

sensitivity, but only a fraction of its lytic activity, when multiple peptides are attached to an antibody.

The activity of conjugates was compared on a per-peptide basis (Figure 2B). Differences in activity among conjugates with one, two, or three GALA persist, showing that the greater efficacy of conjugates with greater degrees of derivatization observed in Figure 2A is not due solely to the increased number of peptides. The steepness of the curves, an indication of the extent of cooperative self-association, differs between GALA and OKT9-GALA conjugates. For example, the ratio of peptide concentration required to produce 90% versus 10% leakage (an indication of the sharpness of the transition) is ~ 5 for GALA but ~ 15 for OKT9-GALA.

To provide a more quantitative analysis of differences between unconjugated and conjugated GALA, data were fit to eqs 3–11. The model captured the lytic behavior of both unconjugated and conjugated GALA (Figure 2A). Coupling of GALA to OKT9 significantly reduced both the partition coefficient, K_P , and the surface aggregation constant, K_S (Table 1). The decrease in K_P , determined by evaluation of leakage data, could be due to an actual loss of partitioning into the membrane phase, or to a loss in the ability of membrane-bound conjugated GALA to form pores. To distinguish between these two possibilities, an independent measure of K_P was obtained by direct measurement of partitioning of conjugates with three GALA per OKT9 cross-linked via SPDP. The K_P for these conjugates was determined to be $(1.1 \pm 0.3) \times 10^4 \text{ M}^{-1}$, in reasonably good agreement with the K_P of $(2.8 \pm 0.06) \times 10^4 \text{ M}^{-1}$ from the fit to leakage data, demonstrating that the decrease in K_P was due to a true decrease in partitioning. The presence of GALA was essential for partitioning into the lipid; OKT9 alone did not associate with the lipid phase (data not shown). Thus, the reduction in lytic activity observed upon conjugation of GALA to OKT9 is due to a combination of decreased partitioning and decreased surface aggregation.

We hypothesized that retention of GALA activity upon conjugation could be improved by increasing the length and/or flexibility of the cross-linker. To test this, OKT9-GALA conjugates were synthesized with a cross-linker containing a poly(ethylene glycol) chain with an estimated end-to-end length of 60 Å, compared to a spacer arm length of 6.8 Å for SPDP. As with SPDP-cross-linked conjugates, PEG-cross-linked conjugates with three GALA per OKT9 caused the most leakage while conjugates with a single GALA per OKT9 caused the least (Figure 3). The PEG-cross-linked conjugates were slightly less effective than the SPDP cross-linker: conjugates with two GALA per OKT9 cross-linked with SPDP had similar lytic activity as conjugates with three GALA per OKT9 cross-linked with PEG. Values for K_P and K_S were determined by fitting the data to eqs 3–11. This analysis suggests

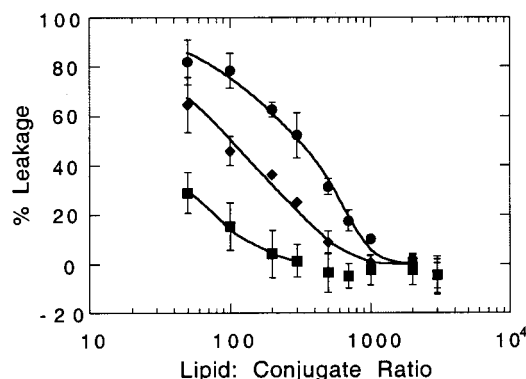


Figure 3. Effect of linker on conjugate-induced leakage. Samples were prepared as described in Figure 2. OKT9-GALA conjugates were made with 1 (●), 2 (■), or 3 (◆) GALA per OKT9 using a PEG cross-linker. Actual number of PEG linkers per OKT9 varied from 0.5 to 1.6 for “1”, 1.8–2.8 for “2”, and 2.4–3 for “3”. The data points represent 2–3 replicate conjugate preparations with 3–4 replicates at each lipid:conjugate ratio. Error bars show standard deviation of the average leakage from each conjugate preparation. Solid lines show model fit to data.

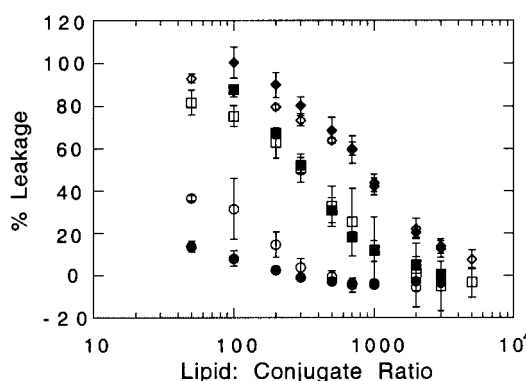


Figure 4. Effect of GALA cysteine placement on conjugate leakage ability. Samples were prepared as in Figure 2. OKT9-GALA conjugates were synthesized with either N-terminally modified GALA (closed symbols) or C-terminally modified GALA (open symbols) with either 1 (●), 2 (■), or 3 (◆) GALA per OKT9. Data points represent the average of 1–3 replicate conjugate preparations with 3–4 replicates at each lipid:conjugate ratio. Error bars show standard deviation of the average leakage from each conjugate preparation.

that the decreased lytic ability of PEG-cross-linked conjugates, like SPDP-cross-linked conjugates, relative to unconjugated GALA was due to reductions in both partitioning and surface aggregation.

When inserted in a membrane, GALA has a preferred orientation: 75% N-terminally and 25% C-terminally inserted (25). To determine if conjugated GALA retained a preferred orientation, OKT9-GALA conjugates synthesized with C- or N-terminal placement of cysteine on GALA were compared. Conjugates with two or three GALA per OKT9 produced identical leakage curves regardless of peptide orientation (Figure 4). Conjugates with a single GALA per OKT9 showed slight preference (increased lytic activity) for N-terminal insertion, the preferred peptidyl insertion. Thus, cross-linking of GALA at the C- or N-terminus does not strongly affect the lytic ability of GALA-OKT9 conjugates, which suggests that the preferred orientation observed in unconjugated GALA has little or no consequence for the lytic ability of conjugated GALA.

Mechanism of Enhanced Membrane Permeability. Membrane-inserted GALA self-associates into oligo-

mers of 8–12 monomers, forming pores with a hydrated channel 5–10 Å in diameter (20). Discrete pore formation is therefore believed to be the mechanism by which GALA allows leakage of liposomal contents. A similar mechanism could explain leakage with OKT9-GALA conjugates; in fact, a pore formation model was able to fit calcein leakage data. Alternatively, leakage could be due to liposomal break-up. To determine whether the observed leakage of calcein upon interaction with conjugates was due to catastrophic loss of liposome integrity, the size of liposomes in the presence of high concentrations of GALA or OKT9-GALA conjugate was measured by dynamic light scattering. Neither hydrodynamic diameter nor number concentration of liposomes (as measured by total scattering intensity) changed with addition of lytic quantities of GALA or OKT9-GALA conjugates (data not shown). These data demonstrate that neither micelle formation nor fusion of liposomes occurred to any detectable level and therefore complete liposome disruption could not explain the leakage data.

Alternatively, if pores of defined size were formed, then leakage of liposomal contents would be sensitive to the molecular size of the encapsulated markers. Size selectivity of pores was determined by measuring leakage of encapsulated 3000 and 10 000 MW dextran by conjugates with three GALA per OKT9 cross-linked with SPDP. For 3000 MW dextran, little to no leakage was observed until calcein leakage reached roughly 35% (Figure 5A). Above this, dextran leakage increased with increasing calcein leakage (decreasing lipid:peptide or conjugate ratio), reaching 60% dextran leakage at full calcein leakage. In contrast, essentially none of the 10 000 MW dextran was released (Figure 5B). Remarkably, the size selectivities for conjugated and free GALA were virtually identical. These data indicate that OKT9-GALA conjugates form size-selective pores similar to GALA pores. Interestingly, if GALA concentration is increased to 4–10-fold greater than that required for 100% calcein leakage, ~40% leakage of 10 000 MW dextran is observed (data not shown). This observation, together with the nonlinear shape of the data in Figure 5A, indicates that the average pore size increases as the GALA:lipid or OKT9-GALA:lipid ratio increases.

DISCUSSION

Synthesis of Membrane-Lytic Conjugates. The objectives of this work were to synthesize GALA-OKT9 conjugates that retained membrane activity and to compare the membrane-lytic activity of conjugates to unconjugated GALA. Three design parameters were evaluated: number of GALA per OKT9, choice of cross-linker, and orientation of attached GALA. The most significant parameter affecting retention of lytic activity was the number of GALA per OKT9. As shown in Figure 2A, conjugates containing two or three GALA per OKT9 caused dramatically increased leakage compared to conjugates with only one GALA, which were essentially inactive. Conjugates with 4–5 GALA per OKT9 contained a large fraction of aggregated protein, and thus were not further evaluated (data not shown). Thus, the optimum extent of derivatization is 2–3 GALA per OKT9.

Lytic ability was significantly reduced for conjugated GALA relative to unconjugated GALA (Figure 2A,B). A possible explanation could be that partitioning of conjugated GALA into the membrane and/or surface aggregation is hindered by the bulky antibody. We attempted to overcome this problem by synthesizing a GALA-OKT9 conjugate containing a long, flexible spacer. GALA-PEG-

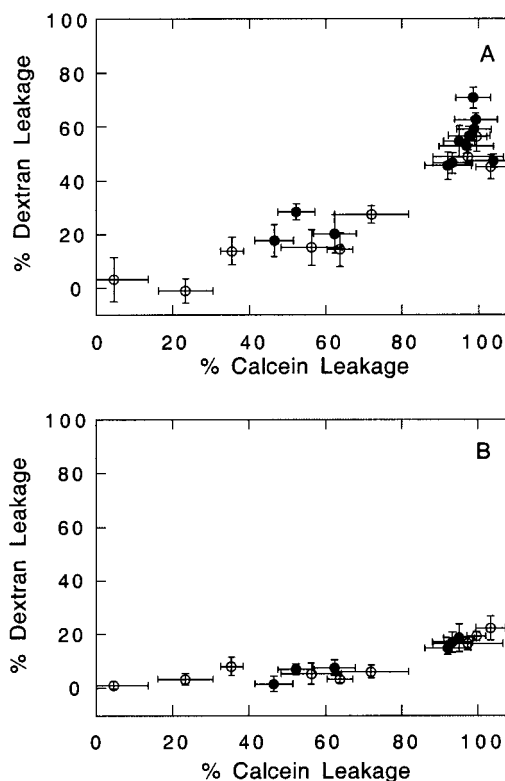


Figure 5. Leakage of higher molecular weight compounds. Conjugate was mixed with liposomes (final concentration 10 μ M lipid) containing either 3000 or 10 000 MW Texas-Red-labeled dextran, left quiescent for 16 h, and then centrifuged in a 100 kDa MWCO Microcon to separate released dextran from encapsulated dextran. Fluorescent measurements were obtained with excitation at 595 nm and emission at 610 nm. Percent leakage was calculated with eq 1 using the fraction of released dextran as in eq 2. GALA-OKT9 conjugates with 3 SPDP per antibody made with cys-GALA were tested. Data represents individual data points of 3–4 replicates for 3 conjugate and 3 cys-GALA preparations. Error bars show standard deviation of the average leakage from a single conjugate or cys-GALA preparation. Cys-GALA leakage data (●) and conjugate leakage data (○) are shown for (A) 3000 MW dextran or (B) 10 000 MW dextran.

OKT9 conjugates performed no better than conjugates made with a short SPDP cross-linker (Figure 3). This could suggest that steric hindrance caused by close proximity of OKT9 and GALA is not a significant influence on GALA-OKT9 lytic activity, or alternatively that addition of the PEG linker was not sufficient to eliminate the steric hindrance effects of OKT9 on GALA.

Membrane-inserted GALA has a preferred orientation in liposomes, with 75% N-terminally inserted into liposomes (25). Other systems also show differential activity based on orientation. For example, chimeric toxins of restrictocin and a single-chain region of anti-(transferrin receptor) antibody have 3–12-fold increased cellular activity when restrictocin is attached with a C-terminal linkage compared to an N-terminal linkage (26). These results suggested that the orientation of conjugated GALA might affect the conjugate's lytic ability; however, no significant effect of orientation was observed. Nicol et al. (25) suggested two alternative interpretations of the preferred orientation of GALA in membranes: (1) all pores consist of 75% N-terminally inserted GALA and 25% C-terminally inserted GALA or (2) 75% of pores consist exclusively of N-terminally inserted GALA and 25% of pores consist exclusively of C-terminally inserted GALA. Based on our data, when GALA is forced into a single conformation, it can still cause leakage of liposomal

contents. Although this does not directly elucidate the behavior of unconjugated GALA, it suggests that unconjugated GALA could indeed form pores of exclusively one orientation.

Of the design parameters investigated, the most significant modifier of lytic ability is the number of GALA per OKT9. To our knowledge, no other study has specifically addressed this issue. If this proves to be generally true, then chemical conjugation would be preferable to genetic fusion as the mode of attachment for membrane-active peptides to proteins. Chemical conjugation provides a means to produce a protein studded with several copies of the active peptide; such a molecule cannot be produced easily by genetic means.

There are at least two plausible mechanisms by which increased derivatization might lead to improved lytic activity. If placement of multiple copies of GALA on a single macromolecule facilitates peptide self-association by rendering the reaction effectively intramolecular, one would expect K_s to increase with the extent of derivatization. As shown in Table 1, however, K_s is insensitive to the number of GALA per OKT9. Thus, increased activity cannot be attributed to intramolecular interactions of multiple GALA on a single mAb. Alternatively, conjugate association with liposomes could increase with the number of GALA per OKT9, perhaps due to an increase in the hydrophobicity of the conjugate. Under this mechanism, one would expect to see K_p increase with GALA content; in fact, this is exactly what is observed.

Mechanism of Interaction of GALA-OKT9 Conjugates with Liposomes. It is fairly well established that GALA inserts into membranes and forms oligomeric pores of defined size by an all-or-none mechanism (20), but it was not clear a priori that GALA-OKT9 conjugates would do the same. Kinetics of calcein leakage, liposome stability, and size selectivity of released molecules were investigated to determine how GALA-OKT9 conjugates interact with liposomes compared to unconjugated GALA. When compared at similar equilibrium extents of leakage, kinetics of calcein leakage for GALA and GALA-OKT9 conjugates were similar. Incubation of conjugates with liposomes did not change liposomal size, demonstrating that the conjugates did not cause liposomal fusion, micelle formation, or other catastrophic changes in liposome structure. Rather, leakage of liposomal contents must occur via formation of stable or transient structures in the lipid bilayer. GALA and GALA-OKT9 conjugates demonstrate similar size-selective release of encapsulated molecules, allowing passage of molecules up to 3000 MW, while generally excluding passage of 10 000 MW molecules. This strongly suggests that GALA and GALA-OKT9 conjugates form pores of similar diameter [~ 1 nm, (20)]. This is a very intriguing finding since GALA is attached to an antibody with a hydrodynamic diameter of ~ 11 nm; one might expect that GALA self-association must be disturbed in order to accommodate multiple bulky antibodies. This result suggests that GALA-GALA association in pores is under rather rigid geometric constraints. Some leakage of 10 000 MW dextran occurs with both GALA and GALA-OKT9 conjugates at high concentrations. This is indicative of a distribution of pore sizes, wherein the average size increases with increasing number of monomers per liposome, as proposed by Nicol et al. (27). Thus, the geometric constraints, although strong, are not absolute. Although it is established that GALA causes leakage via an all-or-none mechanism (20), we have no direct evidence of a similar mechanism with OKT9-GALA. Given that OKT9-GALA mimics unconjugated GALA with

respect to kinetics of leakage, liposome stability, and size selectivity, it is likely that OKT9-GALA causes leakage via an all-or-none mechanism as well.

Although unconjugated GALA and GALA-OKT9 conjugates appear to form similar structures in liposomes with identical size selectivity, there is still a substantial loss of activity upon conjugation. By fitting the data to a simple leakage model, we surmised that this loss is due to both decreased partitioning and decreased surface aggregation. The decreased partitioning of GALA-OKT9 conjugates is a true decrease in conjugate association with liposomes, because the value for K_p extracted from fitting the leakage data was consistent with that obtained by independent partitioning experiments. This indicates that the decrease in K_p established from the leakage data is due to a true decrease in partitioning of GALA-OKT9 conjugates into the membrane and not due to unproductive association of OKT9-GALA conjugates with lipid (i.e., association that does not lead to pore formation). In the conjugates, K_p increased substantially with the number of GALA per OKT9 but was not improved by the use of a long, flexible spacer arm. This leads us to speculate that reduced partitioning of conjugates is caused not by steric constraints but by reduced hydrophobicity of GALA-OKT9 conjugates relative to unconjugated GALA. Surface aggregation is reduced across-the-board for conjugates relative to GALA. The bulkiness of the OKT9 moiety might hinder GALA self-association; alternatively, self-association might occur just as readily, but be unproductive because the bulky antibody blocks some pores. Improved GALA-OKT9 conjugates could be generated by increasing partitioning and/or surface aggregation. Conjugates with a greater number of GALA, or a smaller targeting protein (e.g., Fab fragment), might have greater hydrophobicity and therefore increased partitioning. Attachment of a preformed GALA multimer to a single site on OKT9 might effectively increase surface aggregation.

To summarize, we have successfully synthesized membrane-active conjugates and shown that multiple GALA per OKT9 are required for membrane activity. These conjugates apparently form pores of defined size and relatively small diameter. Thus, it seems unlikely that, after internalization by a targeted cell, GALA-OKT9 conjugates would facilitate rampant transport of globular proteins across endosomal/lysosomal membranes via pores. However, this does not necessarily imply that GALA-OKT9 conjugates cannot facilitate translocation of proteins across these membranes. For example, GALA, as one component of a transfection mixture, successfully increased the transport of DNA to the cytosol (28, 29). The mechanism by which this transport occurred is unknown, but is hypothesized as destabilization of intracellular membranes. This hypothesis seems reasonable, especially since anti-endosomal acidification agents significantly reduced transport (28). Therefore, it seems likely that our membrane-active GALA-OKT9 conjugates could also destabilize endosomal/lysosomal membranes, facilitating translocation of co-internalized toxins to the cytosol. Experiments to test this hypothesis are underway.

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Prolonged in Vivo Residence Times of Antibody Fragments Associated with Albumin

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Antibody fragments can be expressed at a high level in microbial systems, but they may have limited therapeutic value because they are rapidly eliminated from the body. We demonstrate here that site-specific conjugation or binding of bacterially derived Fab' to the long-lived protein serum albumin allows full retention of the antibody's binding characteristics while imparting the albumin's longevity in vivo. In rats the area under the curve for Fab' conjugated to rat serum albumin was 17-fold greater than for the control of Fab' conjugated to cysteine. Again, a bispecific F(ab')₂ with specificity for rat serum albumin showed an area under the curve about 8-fold greater than did a F(ab')₂ without specificity to albumin. Genetic fusions of scFv to albumin were similarly long-lived and could be expressed in yeast to provide the basis of a cost-effective production system.

INTRODUCTION

Antibodies are useful therapeutic agents because of their great specificity in binding to other molecules. Large volumes of low cost antibody are required to satisfy the large markets of common or chronic diseases. The ability of cultured cells or transgenic organisms to supply a large market with immunoglobulin at low cost is unproven so far. Expression of whole immunoglobulin G (IgG1)¹ has been reported in fungi (1, 2), but not at high level or on an industrial scale. Furthermore, the nonmammalian glycosylation pattern of IgG produced in fungi would be expected to show problems of immunogenicity and compromised function when injected into man.

Bacterial systems are a source of low-cost immunoglobulin fragments, such as Fab', F(ab')₂, or single chain Fv (scFv) (3). These molecules lack the Fc domain and its functions, for example, the interaction with FcRn that gives a long lifetime in vivo (4): the half-life in circulation in mammals of Fab' or F(ab')₂ is about 1% that of whole IgG (5), and the β -phase half-life (the time taken for half of the molecules in circulation to be eliminated) of Fab' is about 5% that of whole IgG (6). This rapid elimination of immunoglobulin fragments from the body can be disadvantageous where prolonged presence of the molecule is required, for instance, in order to provide prolonged therapeutic cover for inhibition of inflammatory cytokines. Extension of the half-life of antibody fragments such as Fab' or F(ab')₂ has been achieved by in vitro conjugation to one or more molecules of poly(ethylene glycol) (6, 7). In the present work, we have investigated an alternative approach to improving in vivo half-life that employs a natural polymer, namely serum

albumin. Albumin is abundant in both vascular and extravascular compartments and has a half-life of about 19 days (8) in man (similar to that of IgG1 at about 21 days (5)), though it is less in other species (about 2 days in rats, for example (8)). Albumin does not possess the noted ability of antibodies to specifically bind ligands, particularly those of high molecular weight.

We have investigated the potential of association of immunoglobulin fragments with albumin in three different ways: chemical cross-linking of the separate, low-cost components, albumin, and Fab'; genetic fusion, which would allow a one-step synthesis of the combination; noncovalent binding of an antibody species to endogenous albumin, an approach that could lead to a relatively small, low-cost protein therapeutic. The concept was validated since all three approaches were successful: each albumin-associated immunoglobulin fragment retained its antigen-binding capability while showing extended in vivo half-life.

EXPERIMENTAL SECTION

Preparation of Proteins. Rat serum albumin (RSA) was obtained from Sigma, St. Louis, MO (code A6272). Anti-RSA IgG F(ab')₂ was generated by pepsin (Sigma, St. Louis, MO) digestion (9) of IgG that was prepared from rabbit antiserum (ICN Biomedicals, Costa Mesa, CA) by affinity chromatography on (i) Gammabind plus Sepharose (following the instructions of the manufacturer, Amersham Pharmacia Biotech, Little Chalfont, UK, with elution of adsorbed protein by a buffer of acetic acid, 0.5 M, made to pH 3 by addition of sodium hydroxide), and then (ii) RSA-Sepharose (prepared by linking RSA to CNBr-activated's instructions). For RSA-Sepharose affinity chromatography, the sample (at pH 7) was loaded onto the column, which was preequilibrated in phosphate-buffered saline, and the RSA-binding component eluted in acetate buffer (0.5 M acetic acid, brought to pH 3 by addition of sodium hydroxide). Fractions eluted from each column were immediately neutralized by addition of trizma (2 M, pH 8). Control F(ab')₂ was

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¹ Abbreviations: AUC, area under the curve; BMH, 1,6-bismaleimidohehexane; FcRn, neonatal Fc receptor; HSA, human serum albumin; IgG, immunoglobulin G; RSA, rat serum albumin; SDS PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; scFv, single chain Fv; TNF, tumour necrosis factor.

made by the same method, from rabbit anti-keyhole limpet haemocyanin antiserum. Recombinant anti-human Tumor Necrosis Factor α (TNF) and anti-cell surface marker $F(ab')_2$ and Fab' molecules were produced in *E. coli*, as described previously (6). Thiols were assayed as described previously (6).

Preparation of Conjugates. *Bispecific $F(ab')_2$.* Freshly reduced anti-RSA Fab' was incubated for 2 h at 21 °C in PE buffer (sodium phosphate, 0.1 M, pH 6, 2 mM ethylenediaminetetraacetate) with an 80-fold molar excess of 1,6-bismaleimidohexane (BMH, Perbio Science UK Ltd, Tattenhall). Excess BMH was removed by Sephadex G25M size exclusion chromatography ("PD10", Amersham Pharmacia Biotech, Little Chalfont, UK) in PE buffer, as per manufacturer's instructions. The derivatized anti-RSA Fab' was then incubated for 20 h at 21 °C with freshly reduced anti-TNF Fab' at a molar ratio of anti-RSA Fab':anti-TNF Fab':1:3.1. Purification of the bispecific anti-RSA–anti-TNF $F(ab')_2$ heterodimer was purified by affinity chromatography on RSA immobilized on Sephadex (as described above), followed by size exclusion chromatography in a buffer of 0.2 M sodium phosphate, pH 7, on Zorbax GF250 (Agilent Technologies, Palo Alto, CA), using an HP1090 HPLC (formerly Hewlett-Packard, currently Agilent Technologies, Palo Alto, CA), to remove anti-RSA Fab'. Overall yield (nonoptimized) was about 9%. Control monospecific anti-TNF $F(ab')_2$ homodimer was made in an analogous way and purified by gel filtration on Zorbax GF250 in a buffer of 0.2 M sodium phosphate, pH 7.

RSA-Fab'. RSA was reduced either by (a) incubation for 50 min at 37 °C in 5 mM 2-mercaptoethylamine (Sigma, St Louis, MO) in PE buffer, or (b) incubation for 40 min at 37 °C in a 3-fold molar excess of dithiothreitol (Sigma, St Louis, MO) in sodium acetate, 0.1 M, pH 5.9. Reduced RSA was reacted in PE buffer with a 21-fold excess of BMH in PE buffer for 100 min, 21 °C. Approximately 80% of reduced RSA reacted with BMH under these (nonoptimized) conditions. Derivatized RSA was reacted for 2 h at 21 °C with freshly reduced Fab' in the molar ratio, derivatized RSA:reduced Fab':1:1.4. The reaction mixture was first subjected to chromatography on Gammabind plus Sepharose (described above), pre-equilibrated in PE buffer. Adsorbed protein was eluted by acetic acid, 0.5 M, made to pH 3 by addition of sodium hydroxide. Eluted fractions were immediately neutralized by addition of 2 M trizma, pH 8. As expected of this protein G matrix, nonconjugated Fab' bound and was eluted by the pH 3 buffer, whereas the nonconjugated RSA did not bind at all and emerged in the flow-through. Conjugation of a single Fab' to one RSA molecule clearly affected its binding to the protein G on the matrix, for the conjugate emerged in the flow-through, just slightly later than (and overlapping with) the unconjugated RSA. More free RSA was removed from the conjugate by both: (i) a further cycle of chromatography on Gammabind plus (as above); (ii) gel permeation chromatography on a Zorbax GF250 HPLC column of size (described above). Overall yield (nonoptimized) was about 5%.

Control anti-TNF Fab'-cys conjugate was prepared by a similar approach: (i) derivatization of Fab' by BMH; (ii) incubation with a 22-fold molar excess of cysteine (Sigma, St Louis, MO). Purification was by chromatography on a 1 mL volume Mono S column using an FPLC apparatus (Amersham Pharmacia Biotech). Adsorbed protein were then eluted in a gradient of 0 to 250 mM sodium chloride in sodium acetate, 50 mM, pH 4.5. Overall yield (nonoptimized) was about 28%.

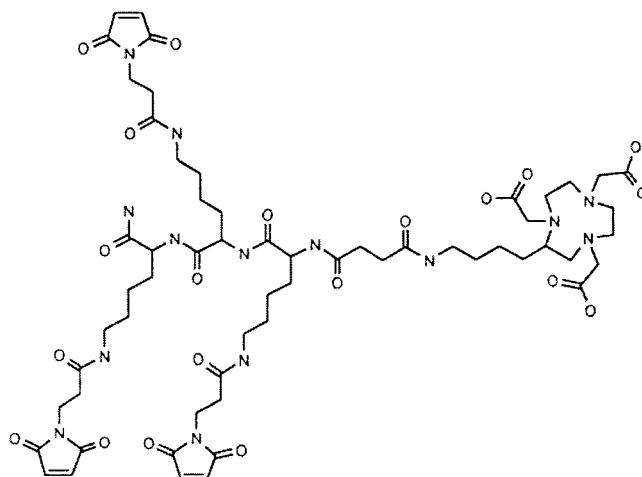


Figure 1. Structure of trimaleimide cross-linking agent.

Bivalent Conjugate RSA- $F(ab')_2$. This conjugate was made by use of the trimaleimide cross-linking agent N_α -[1,4,7-tris(carboxymethyl)-1,4,7-triaza-2-cyclononyl]butyl-aminosuccinoyl- N_ω -(maleimidopropanoyl)lysyl- N_ω -(maleimidopropanoyl)lysyl- N_ω -(maleimidopropanoyl)lysyl- N_ω -(maleimidopropanoyl)lysyl-L-lysine, illustrated in Figure 1. It was synthesized according to the method described previously (10), starting from 2-(4-aminobutyl)-1,4,7-triazacyclononane-1,4,7-triyltriacetic acid (11) and N6-[3-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)-1-oxopropyl]-L-lysyl-N6-[3-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)-1-oxopropyl]-L-lysyl-N6-[3-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)-1-oxopropyl]-L-lysine (trifluoroacetate). Anti-cell surface marker Fab' PE buffer was reacted with this trimaleimide cross-linking agent for 3 h at 21 °C at a molar ratio cross-linker:Fab':1:2.56. Monomer, dimer, and trimer Fab'conjugates were resolved by chromatography on SP-Sepharose HP (Amersham Pharmacia Biotech, Little Chalfont, UK), being eluted by a gradient of 0 to 250 mM sodium chloride in 50 mM acetate, pH 4.5. The dimer (approximately 30% of the total material) was then reacted with freshly reduced RSA at molar ratio of 1:1, for 24 h at 21 °C. The RSA- $F(ab')_2$ was finally prepared by affinity chromatography on Gammabind plus Sepharose to remove free albumin, then on Blue Sepharose, to remove unconjugated $F(ab')_2$. The yield of this (nonoptimized) procedure was about 12%.

A control molecule of anti-cell surface marker $F(ab')_2$ (containing no RSA) was prepared by conjugating two freshly reduced Fab' molecules with BMH, in a manner analogous to conjugation of anti-TNF Fab' (see above).

Preparation of scFv-HSA Fusion Proteins. Oligonucleotides were designed that coded for fusions of scFv with the N-terminus of mature Human Serum Albumin (HSA), either directly (scFv-HSA) or via linkers positioned to physically separate the scFv from the albumin moiety. The linkers were a sequence of 3 repeats of (glycine)₄-serine (scFv-G4S-HSA); a sequence derived from the flexible upper (N-terminal) end of the human IgG1 hinge region, (scFv-UHL-HSA); or the C-domain from human Ig-kappa light chain (scFv-C_K-HSA). The orientation of variable domains in the scFv was V_L-V_H, except for scFv-C_K-HSA, in which the order was V_H-V_L. The scFv sequence was immediately preceded by a spacer of (glutamic acid-alanine)₂ as recommended by Sreerishna et al. (12) to alleviate possible problems of steric hindrance of pro-sequence-processing protease. An "EasySelect Pichia Expression" kit (Invitrogen, Carlsbad), with the plasmid pPIC, was used as per manufacturer's

instructions to express each type of fusion protein in shake flask culture. Each fusion protein was secreted into 30–40 mL of medium. Supernatant from each culture was adjusted to pH 7 by addition of 2 M sodium hydroxide, and applied to Blue Sepharose (Amersham Pharmacia Biotech, Little Chalfont, UK). Each construct bound and was eluted by sodium thiocyanate, 0.2 M in phosphate-buffered saline. Each protein was concentrated and the buffer exchanged to phosphate-buffered saline by use of an Amicon stirred cell concentrator (Millipore, Bedford, MA).

Analytical Procedures. Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS PAGE) utilized precast SDS gels (Novex, San Diego, CA), run as per manufacturer's instructions. Protein bands were quantified after staining by Coomassie BBG (13). Protein sequencing was performed on an Applied Biosystems (Warrington, UK) model 470A or Procise 492 protein sequencer. A combination of approximations of molecular weight (by SDS PAGE) and quantity (by N-terminal sequencing) allowed determination of the stoichiometry of components in conjugates.

Surface plasmon resonance study of interactions with ligand was performed a BIACORE 2000 (Biacore AB, Stevenage, UK) on a CM5 Sensor Chip, with kinetic parameters calculated by use of BIAevaluation 3.0 software.

scFv-HSA fusion protein was bound to the chip surface by amine coupling chemistry to approximately 400 Response Units. The ligand, recombinant human TNF α , was passed across the immobilized molecule at concentrations between 0 nM to 120 nM. RSA-Fab' conjugate was captured by goat anti-human IgG F(ab')₂ fragment specific antibody (Jackson ImmunoResearch Labs Inc, West Grove) that was immobilized on the chip surface, prior to application of ligand. For the bispecific conjugate, anti-RSA-anti-TNF, affinity for TNF was assayed by capture of the conjugate by AffiniPure F(ab')₂ fragment, goat anti-rabbit IgG, F(ab')₂ fragment specific (Jackson ImmunoResearch Labs Inc, West Grove) that was immobilized on the chip surface, prior to application of the TNF α ligand. To assay the affinity of the bispecific conjugate for RSA, the ligand RSA was immobilized on the chip surface, and conjugate solution was passed over that.

RSA-F(ab')₂ and F(ab')₂ control were fluoresceinated prior to Scatchard analysis of binding, in which they were incubated with cells for 3 h at 4 °C for 3 h, before analysis by fluorescence activated cell sorting, using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes).

Pharmacokinetic Analysis of the Conjugate. Proteins were labeled at the ϵ -amino groups of lysyl residues, using ¹²⁵I-labeled Bolton and Hunter reagent (Amersham International, Little Chalfont, UK) as follows. A 300 μ g amount of protein dissolved in 300–370 μ L of 0.1 M borate, pH 8, was mixed with 20 μ L of Bolton and Hunter solution in propan-2-ol (containing 9 MBq of ¹²⁵I) and incubated at 21 °C for 15 min. The reaction was then quenched by addition of 60 μ L solution of glycine, 1 M, in borate, 0.1 M, pH 8. After approximately 5 min reaction at 21 °C, the labeled protein was separated from other reagents by chromatography on Sephadex G25M using a PD10 column (Amersham Pharmacia, used as per manufacturer's instructions). In doing so, the buffer was exchanged for phosphate buffered saline. The specific activity of each preparation was calculated from estimates of protein concentration (estimated by absorption at 280 nm) and of radioactivity and was typically in the

range 0.45 to 0.54 μ Ci/ μ g. The radiolabeled samples were used directly after labeling.

A 20 μ g amount of ¹²⁵I-labeled or 180 μ g of unlabeled protein in solution in phosphate-buffered saline was injected into a tail vein of male Wistar rats (approximately 250 g each). Blood samples were taken periodically from the tail artery, with plasma prepared by heparinization and centrifugation. Radioactivity was detected in whole blood by gamma counting (using a Cobra II auto gamma, from Canberra Packard, Ontario). The percent injected dose (%ID) was calculated for each individual rat, based on standards and expressed as %ID/mL total blood volume. Data were analyzed by WinNonlin software (Pharsight Corp., Mountain View, CA). For this, a two-compartment model was used, except for Fab'-cys monitored by ELISA, where too few data were acquired due to rapid elimination and where a one-compartment model was used instead.

Phosphorimaging and quantification of radiolabeled samples on SDS PAGE utilized a Cyclone system with Optiquant software (Canberra Packard, Ontario). This enabled examination of the quality of radiolabeled protein in plasma throughout the experiments.

ELISA's used microtiter plates coated with the antigen recombinant human TNF α (Strathmann Biotech GMBH, Hannover). Detection was by rabbit anti-RSA (Cappel, obtained from ICN, Costa Mesa, CA) followed by goat anti-rabbit immunoglobulin Fc-peroxidase conjugate (Jackson ImmunoResearch Labs Inc, West Grove), or by goat anti-human kappa light chain (Southern Biotechnology Associates, Inc., Birmingham, AL) followed by donkey anti-goat immunoglobulin (H+L)-peroxidase conjugate (Jackson ImmunoResearch Labs Inc, West Grove). Quantification was by absorption at 630 nm, generated from 3,2',5',5'-tetramethylbenzidine (R and D Systems Europe, Abingdon, UK; 120 μ M in 10 mM acetate, pH 6) as a result of the activity of peroxidase.

Anti-TNF activity was assayed by the inhibition of killing of monolayered mouse L929 cells by recombinant human TNF α (Strathmann Biotech GMBH, Hannover, NH) in normal RPMI 1640 plus glutamine and 10% (v/v) foetal calf serum (both from Life Technologies, Paisley, UK). Cell survival was quantified as follows: the brown color produced by surviving cells from 50 μ g/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma, St Louis, MO) in the culture medium at 37 °C for 4 h was extracted (by a solution of SDS, 20%, w/v, in 50% v/v in water, adjusted to pH 4.7 by addition of 50% acetic acid in 1 M hydrochloric acid) and quantified by absorbance at 570 nm; absorption at 630 nm was subtracted, and compared to standards from known amounts of TNF added to samples of cells, in the absence of TNF-neutralizing activity.

RESULTS

Characterization of Conjugates. The cysteinyl residue at position 34 of albumin is commonly engaged in formation of a mixed disulfide with molecules such as glutathione or free cysteine, but suitable reduction conditions can liberate that thiol without disrupting disulfides present elsewhere in the molecule (8). Reduction of albumin in such conditions increased the amount of free thiol from 0.15 in the original albumin preparation to between 0.95 and 0.97 mol of thiol per mol of albumin. Albumin dimers were not formed during derivatization by BMH, because the free cysteinyl 34 with which the BMH is presumed to react lies within a cleft in the albumin structure, and the 16.1 angstrom spacer arm of

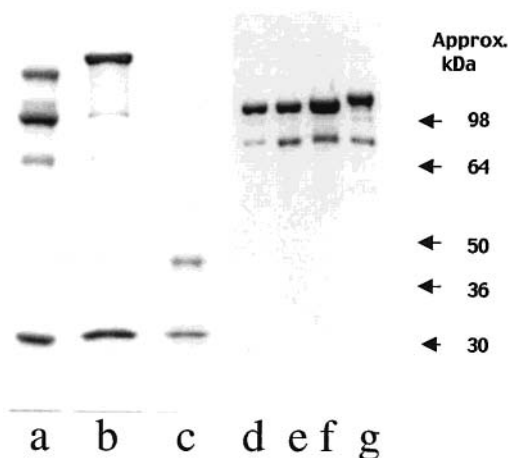


Figure 2. Reduced SDS PAGE of conjugates and fusion proteins. Stain: Coomassie Brilliant Blue G in perchloric acid. Approximate apparent molecular weights (indicated) were determined by co-electrophoresis of standard proteins. Samples: a = RSA-anti-TNF Fab' conjugate; b = RSA-anti-TNF F(ab')₂ conjugate; c = bispecific anti-TNF-anti-RSA F(ab')₂; d = scFv-HSA fusion protein; e = scFv-G4S-HSA fusion protein; f = scFv-UHL-HSA fusion protein; g = scFv-C_K-HSA fusion protein.

the bifunctional BMH was too short to reach from within the cleft of one albumin molecule and penetrate the depth of the equivalent cleft in a second albumin molecule.

Again, the reducing conditions used here produced Fab' that contained only one free cysteinyl residue, near the C-terminus of the Fd chain (12). It was therefore possible to cross-link specific thiols in albumin and Fab' molecules, to make conjugates with defined stoichiometry. Methods for production of the various conjugates were not optimized, but provided sufficient material for subsequent experiments.

SDS PAGE (Figure 2) and N-terminal sequencing of preparations and of protein bands on blots from SDS PAGE confirmed that 1:1 molar ratios were achieved in conjugates of anti-TNF Fab' with RSA, and anti-TNF Fab' with anti-RSA Fab'. Similarly, one anti-TNF F(ab')₂ was covalently attached to one RSA molecule. Fab' molecules were conjugated through their Fd chain, as seen by generation of free light chain by reducing conditions in SDS PAGE, seen as a band of about 30 kDa apparent molecular weight. The H chain dimer from the bispecific F(ab')₂ appeared as a band of 40 to 50 kDa apparent molecular weight or as a species of very approximately 150 kDa apparent molecular weight when linked to RSA. The H chain-RSA from the Fab-RSA conjugate migrated as an approximately 100 kDa band but was accompanied by lesser a quantity of H chain-RSA dimer material (as can sometimes be seen upon electrophoresis of preparations of albumin alone – not shown), and some (putative) free albumin at an apparent molecular weight of about 65–70 kDa.

Four types of scFv-HSA fusion proteins were secreted into the medium of *Pichia pastoris* cultures. A single step of Blue Sepharose chromatography of culture supernatant provided fusion proteins of between 75% and 91% purity (see Figure 2), with mature HSA as the other component (presumably arising from proteolysis of linker). Yields of prepared proteins as $\mu\text{g/mL}$ of culture supernatant (mean of two experiments) were: scFv-HSA, 9.0 $\mu\text{g/mL}$; scFv-G4S-HSA, 8.5 $\mu\text{g/mL}$; scFv-UHL-HSA, 7.5 $\mu\text{g/mL}$; scFv-C_K linker, 7.8 $\mu\text{g/mL}$. Neither the nature of the linker nor the order of the variable domains in the scFv portion significantly affected levels of expression of

Table 1. Ligand-Binding Characteristics of Conjugates and Fusion Proteins^a

	k_a , $10^5 \text{ M}^{-1} \text{ s}^{-1}$	k_d , 10^{-4} s^{-1}	K_D , 10^{-10} M
IgG	3.63	1.41	3.88
F(ab') ₂	2.79	0.56	2.01
RSA–Fab' conjugate	3.88	1.65	4.25
scFv-HSA	7.67	1.11	1.45
scFv-G4S-HSA	6.58	1.32	2.29
scFv-UHL-HSA	6.64	1.52	2.29
scFv-C _K -HSA	7.09	0.95	1.34
anti-RSA–anti-TNF F(ab') ₂			
vs RSA	0.23	6.08	260.00
vs TNF	5.05	0.40	0.78
F(ab') ₂ (i)	–	–	120
(ii)	–	–	30
RSA–F(ab') ₂ (i)	–	–	80
(ii)	–	–	30

^a Data were generated by surface plasmon resonance analysis, except for F(ab')₂ and RSA-F(ab')₂, which were determined by Scatchard analysis of binding to ligand on cell surfaces, which was biphasic. (i and ii) were the data for the two phases.)

Table 2. Pharmacokinetics of Albumins, Conjugates, Fusions, and Other Immunoglobulins in Rats

	assay	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	AUC (0– ∞)
RSA	¹²⁵ I	4.1	49.1	2637 h*%id
F(ab') ₂	¹²⁵ I	1.4	15.9	213 h*%id
Fab'-cys	¹²⁵ I	1.0	31.4	94 h*%id
RSA–Fab' conjugate	¹²⁵ I	4.6	39.6	1609 h*%id
bispecific F(ab') ₂	¹²⁵ I	9.2	42.5	1672 h*%id
HSA	¹²⁵ I	0.8	14.8	1272 h*%id
scFv-HSA	¹²⁵ I	2.3	16.6	1164 h*%id
scFv-G4S-HSA	¹²⁵ I	1.0	15.2	1282 h*%id
scFv-UHL-HSA	¹²⁵ I	3.0	16.5	1225 h*%id
Fab'-cys	ELISA1	nd	0.7	4 h* $\mu\text{g/mL}$
RSA–Fab' conjugate	ELISA1	1.3	21.6	824 h* $\mu\text{g/mL}$
RSA–Fab' conjugate	ELISA2	0.8	19.5	780 h* $\mu\text{g/mL}$
RSA–Fab' conjugate	L929	2.7	24.7	975 h* $\mu\text{g/mL}$

^a Data were analyzed using WinNonlin and a two-compartment model, except for Fab'-cys when a one-compartment model was used. AUC (0– ∞): area under the plasma concentration curve, or plasma residence time. ¹²⁵I: proteins monitored by gamma counting. ELISA1: TNF-binding material detected by anti-L chain. ELISA2: TNF-binding material detected by anti-albumin. L929: quantified by neutralization of TNF activity. For ¹²⁵I assay, $n = 5$ –7, others $n = 2$. Data shown for RSA and F(ab')₂ are means of four and two experiments, respectively.

fusion protein. N-Terminal protein sequencing revealed that the (Glu-Ala)₂ sequence lying N-terminal to the scFv domain was not removed by aminopeptidase activity (cf. ref 12).

Anti-TNF Fab' retained ligand binding activity in all conjugates and fusions (Table 1, and ELISA and L929 assay data, below). Similarly, affinity for cell surface ligand was retained by both the RSA-F(ab')₂ and the corresponding control F(ab')₂ conjugate. Scatchard analysis showed the presence of both low and high affinity binding sites (Table 1) with 40 000 and 20 000 RSA-F(ab')₂ binding sites per cell, respectively, and 125 000 and 50 000 F(ab')₂ binding sites per cell, respectively. Thus, association of Fab' with albumin did not decrease ligand binding affinity, though the 67 kDa albumin moiety hindered access to some binding sites present on a cell surface.

Pharmacokinetic Analysis of the Conjugate in Rat Plasma. As judged by phosphorimaging, all ¹²⁵I-labeled conjugates were stable in vivo (that is, the quality of the labeled protein remained constant throughout the experiment), with one exception. The exception was the ¹²⁵I-RSA-Fab' conjugate, in which some instability was

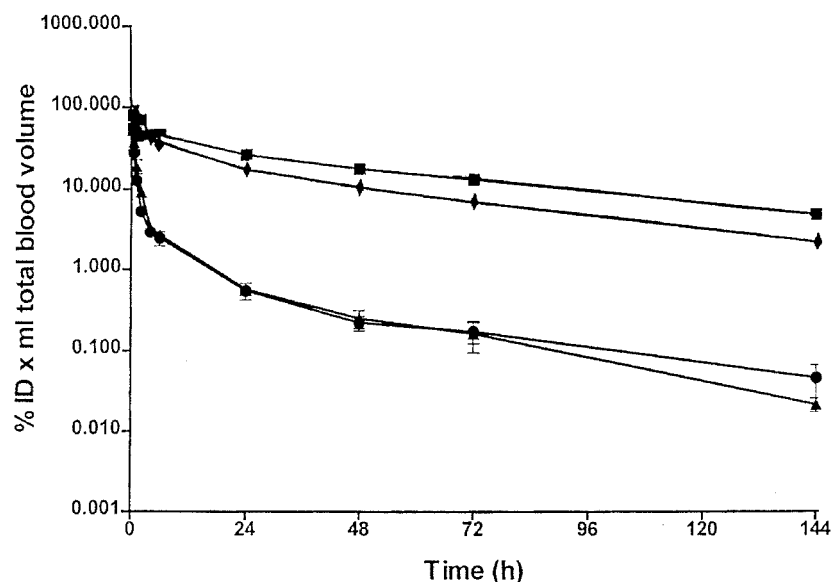


Figure 3. Pharmacokinetics of ^{125}I -labeled RSA, conjugates, and other immunoglobulins in rats. ^{125}I detected in plasma samples by gamma-counting. (■) RSA; (◆) RSA-Fab'; (●) Fab'-cys; (▲) F(ab')₂. For each, $n = 6$. Error bars: sem.

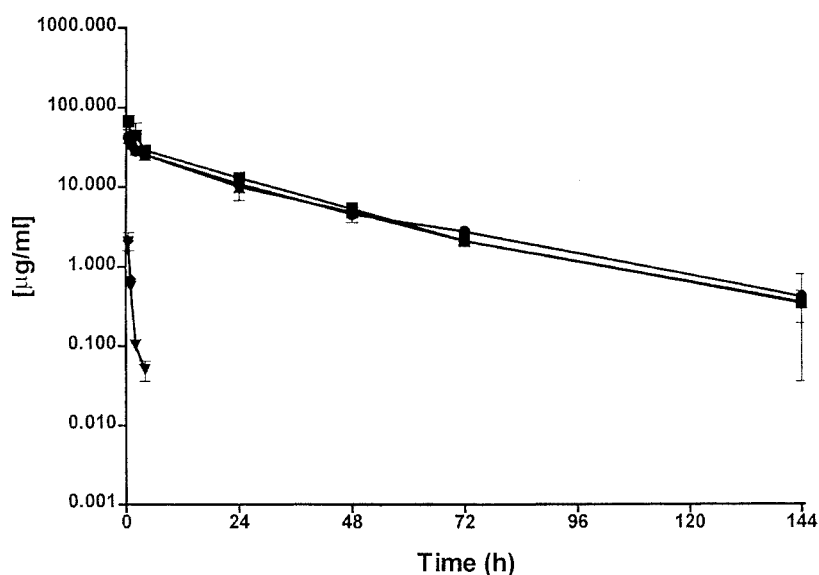


Figure 4. Pharmacokinetics of unlabeled RSA-Fab' conjugate and Fab'-cys in rats. Proteins quantified in plasma samples by ELISA in which TNF-binding protein was detected by anti-L chain: (▲) RSA-Fab'; (▼) Fab'-cys. Proteins quantified by ELISA in which TNF-binding protein was detected by anti-albumin: (●) RSA-Fab'. Protein quantified by assay of neutralization of L929 cell killing by TNF α : (■) RSA-Fab'; (◆) Fab'-cys. Results for the three types of assay of RSA-Fab' overlay each other, and results for the two types of assay for Fab'-cys likewise overlay each other. For each, $n = 2$. Error bars = sem.

noted in vivo, whereby a species was generated with an apparent molecular weight similar to that of RSA on SDS PAGE. Experiments (not shown) indicated that this instability was artificial, coinciding with the presence of the covalently attached ^{125}I or to some other aspect of the labeling process. Nevertheless, phosphorimage analysis showed that even after 144 h in vivo the intact conjugate still represented about 30% of all labeled protein present. Data from phosphorimage analysis of rat plasma samples were used to correct gamma counting data from in vivo experiments with ^{125}I -labeled RSA-Fab', to reflect the quantity of ^{125}I in intact, labeled conjugate only. In rat plasma the intact ^{125}I -RSA-Fab' conjugate had a residence time, or an area under the curve (AUC), that was 17-fold greater than the Fab'-cys control in the same experiment in vivo (Figure 3 and Table 2). The longevity of the RSA-Fab' conjugate in vivo was confirmed by experiments using conjugate that was not labeled, by ELISA format or by L929 survival assay (Figure 4, Table

2). By these methods, the RSA-Fab' AUC was approximately 200-fold greater than that of the Fab'-cys, which was very rapidly eliminated.

Similarly, binding to endogenous albumin by ^{125}I -bispecific F(ab')₂ caused a 6-fold increase in AUC over that of the control in the same experiment, unispecific anti-TNF F(ab')₂, which did not bind albumin (Figure 5, Table 2). The residence time for the bispecific F(ab')₂ was approximately 70% that of RSA in the same experiment, though their $t_{1/2\beta}$ values were similar. The loss of bispecific F(ab')₂ in the first few minutes of the experiment was probably not due to failure to bind to endogenous albumin, for experiments (not shown) with anti-RSA Fab' showed that in vitro incubation with plasma prior to injection did not alter the residence time in vivo.

The residence times of three ^{125}I -labeled fusion proteins tested (scFv-HSA, scFv-G4S-HSA, and scFv-UHL-HSA) were similar to those of HSA and approximately 12-fold greater than those of ^{125}I -Fab'-cys control in the same

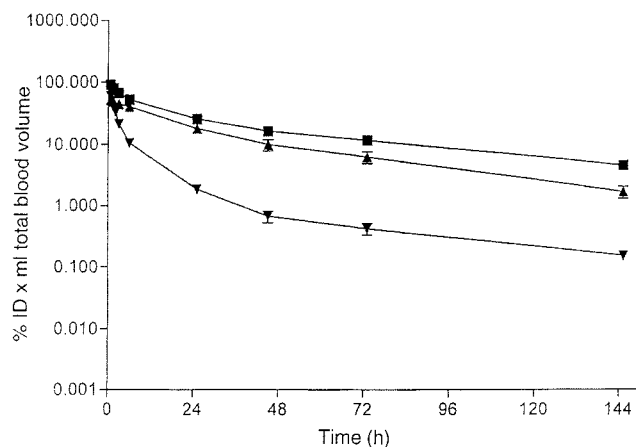


Figure 5. Pharmacokinetics of ^{125}I -labeled RSA, bispecific and unspecific $\text{F}(\text{ab}')_2$ in rats. ^{125}I detected in plasma samples by gamma counting. (■) RSA; (▲) anti-RSA-anti-TNF bispecific $\text{F}(\text{ab}')_2$; (▼) anti-TNF unspecific $\text{F}(\text{ab}')_2$. For each, $n = 5\text{--}7$. Error bars = sem.

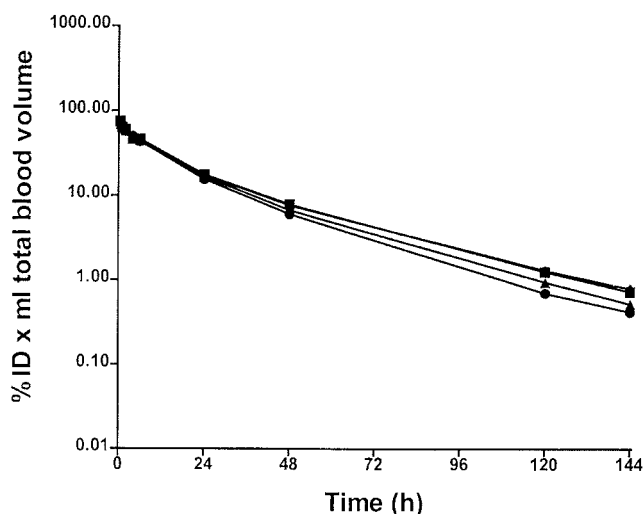


Figure 6. Pharmacokinetics of ^{125}I -labeled HSA and fusion proteins in rats. ^{125}I detected in plasma samples by gamma counting. (◆) HSA; (●) scFv-HSA; (■) scFv-G4S-HSA; (▲) scFv-UHL-HSA. For each, $n = 6$. Error bars = sem, but are small and not readily visible on the plot. The four curves overlay each other.

experiment (Figure 6, Table 2). In rats the residence time of scFv-HSA fusions was shorter than of RSA-Fab' conjugate, but this was taken to be because the albumin moiety dictates the longevity, and the heterologous HSA was only about half as persistent as the homologous RSA.

DISCUSSION

Data presented here show that association of immunoglobulin fragments with serum albumin, whether by conjugation, fusion or noncovalent binding, results in extended persistence in plasma *in vivo*, approaching that of albumin itself. The albumin moiety determined longevity, and in man the $t_{1/2\beta}$ of serum albumin is about 19 days in man (8) (compared to 2 to 2.5 days in rat (8)). Accordingly, in man an immunoglobulin–albumin molecule might persist for about the same time as would whole IgG1 ($t_{1/2\beta}$ = about 21 days in man (5)). Extended residence time of an agent *in vivo* can be beneficial in providing extended therapeutic cover. This goal of extending the half-life of Fab' has previously been achieved by attachment of poly(ethylene glycol), which gave 7- to 21-fold increase in AUC, depending on the amount of the

polymer that was attached (7). We show here that albumin provides an alternative to poly(ethylene glycol) as a conjugation partner. Any effect due to the albumin moiety, administered as part of a therapeutic agent, would be expected to be negligible: the role of albumin is in transportation of various molecules (such as steroid hormones, bile pigments and fatty acids) in circulation and in extravascular fluids and is normally present in plasma at about 50 mg/mL, in great excess over any administered therapeutic. In the present work defined conjugates were made *in vitro* by cross-linking through a specific site in each component, particularly a site in the Fab' molecule near its C-terminus, such that the Fab' ligand-binding properties are not adversely affected (6). Both Fab'(s) and albumin components can be produced at low cost by microbial systems and therefore provide the basis of an economic therapeutic molecule.

Additionally, we have illustrated an alternative possibility, namely that of genetically engineering novel albumin–immunoglobulin fusion proteins and expressing them in yeast cells. This approach has the extra advantage, compared to conjugation of immunoglobulin to albumin or PEG, that no *in vitro* conjugation step is required in the production process, which is therefore simpler. Expression of these fusion molecules in yeast was not fully optimized here, but it has considerable potential if it can approach the level of albumin production in yeast, which can be at a level of grams per liter (15, 16, 17). This level of expression makes industrial production of pharmaceutical grade protein economically feasible (16). The potential problems of antigenicity that surround yeast oligosaccharides when injected into mammals are avoided by excluding glycosylation sites from the fusion protein sequence—glycosylation is not required for prolonged serum half-life in this case, unlike for whole IgG (18).

Bispecific immunoglobulin that can bind to endogenous serum albumin provides a third strategy. Once injected, anti-albumin Fab' binds rapidly to endogenous albumin, whose concentration in plasma is about 0.6 mM. From the observed association and dissociation rates for anti-RSA (Table 1) it may be calculated that at equilibrium in serum only 4.4×10^{-3} % of bispecific $\text{F}(\text{ab}')_2$ would remain unbound to albumin. We have exemplified the bispecific molecule approach with a molecule made by chemical conjugation of components produced economically in microbial systems, but other forms of bispecific antibodies such as those reviewed by Cao and Suresh (19) would be expected to function in the same way.

Possibilities exist for further elaboration of the principles described here. These include use of alternative proteins to albumin, generation of both polyvalent and polyspecific immunoglobulins (by either chemical cross-linking or genetic fusion, or a combination of both), and engineering of additional function into the protein or the cross-linking agents. For instance, the trimaleimide cross-linker used here includes a macrocycle moiety capable of chelating any of a range of metal ions such as might be used for therapeutic or diagnostic purposes. The macrocycle illustrated (Figure 1) is able to chelate Indium.

In summary, we have shown that association of fragments or derivatives of immunoglobulin with albumin provides the basis of a system for production of a potentially low cost but long-lived therapeutic immunoglobulin.

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Synthesis and Binding Properties of Oligo-2'-deoxyribonucleotides Covalently Linked to a Thiazole Orange Derivative

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Thiazole orange label was coupled to the eighth phosphate of a pentadeca-2'-deoxyriboadenylate via a phosphoramidate linkage using different linkers. The stereoisomers were separated, and their absolute configurations were determined. Finally, the thiazole orange moiety was also linked to the tenth phosphate of icosathymidylates in both the α and the β series via a phosphoramidate linkage. Once again, the thiazole orange–icosathymidylate conjugates were obtained as pure stereoisomers. The binding properties of these oligo-2'-deoxyribonucleotide–thiazole orange conjugates with their complementary sequences were studied by absorption spectroscopy. The covalent attachment of the thiazole orange derivatives to the oligoadenylates stabilizes the complexes formed with both the DNA and RNA targets. On the contrary, when the thiazole orange is tethered to the oligo- α -thymidylate or oligo- β -thymidylate, no significant stabilization of the duplexes formed with poly r(A) can be observed.

INTRODUCTION

The rapidly increasing rate of sequencing has opened the way to greater progress in various research areas such as gene organization and function including research on the underlying genetic causes of inherited and acquired diseases with possible applications for their prevention and treatment, the detection of pathogens for clinical diagnoses, and finally food technology, agricultural, environmental, and forensic sciences. Base-pairing properties of nucleic acids are used, in most methods which involve the hybridization of a single-stranded oligonucleotide labeled probe, to detect a complementary target sequence in a complex nucleic acid mixture. The most commonly used hybridization assays employ a solid phase in order to facilitate the separation of bound from unbound analytes (1, 2). In contrast, assays that process in a homogeneous solution avoid problems such as nonspecific adsorption associated with the heterogeneous assays, but the separation of hybridized and free probes is required. The separation step can be eliminated by a probe whose signal changes upon hybridization (3, 4). Such probes could be used inside cells to detect their complementary sequences *in situ* directly on their RNA targets. The development of fluorescent or luminescent labeled oligonucleotides that display a modified signal upon hybridization with their target sequences has been the subject of intense research. Among the reported systems, molecular beacons are based on the quenching/dequenching of fluorescence. This system involves stem-loop-forming oligo-2'-deoxyribonucleotides bearing a fluorescent dye at one end and a quencher at the other end (5). Upon hybridization to the target sequence, the stem is dissociated and the distance between the fluorescent dye and the quencher is largely increased, generating an increase in fluorescence. Other methods utilize a change in the fluorescent emission wavelength, rather than in

the intensity alone. Among these are the fluorescence resonance energy transfer (FRET) between a donor and an acceptor dye (6, 7), and the luminescence resonance energy transfer (LRET) (8) between a sensitized lanthanide metal and an acceptor dye. Another example of the color-changing strategy involving a two-chromophore system is the utilization of excimer-forming chromophores such as pyrene (9, 10). The last strategy is based on modification of the signal generated by a single fluorescent or luminescent DNA binding agent linked to oligonucleotides upon hybridization with their target sequences. A minor groove binder, Hoechst 33258 (11, 12), and intercalating agents such as ruthenium complex (13) and oxazole orange (14, 15) have been covalently tethered to oligonucleotides, and fluorescent or luminescent, in the case of the ruthenium complex, signal increases were observed upon hybridization of these labeled probes. Following our previous work (16) and one report (17) concerning the fluorescent properties of thiazole orange-linker derivatives in interaction with single-stranded and double-stranded targets, we now describe the synthesis and binding properties of oligo-2'-deoxyribonucleotides covalently linked to thiazole orange. [During the course of this work, results on the fluorescent properties of thiazole orange–peptide nucleic acid conjugates were published (18–20).] Since the homodimeric thiazole orange (TOTO) was reported to intercalate in double-stranded DNA (21) and our previous work on oligo-2'-deoxyribonucleotide–acridine conjugates showed stronger stabilization of the duplex when the acridine moiety, an intercalator, is linked to an internucleotidic phosphate (22), we chose to tether the thiazole orange residue to an internucleotidic position of the oligonucleotide sequence.

EXPERIMENTAL PROCEDURES

General Methods. All solvents used were dried, distilled, and stored as described in ref 23. All chemicals were used as obtained unless otherwise stated. Cystamine dihydrochloride, 3-methylbenzothiazole-2-thione,

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over 30 min, to a stirred solution of 1,11-dibromo-3,6,9-trioxaundecane **1** (1 g, 2.84 mmol) in anhydrous (freshly distilled) refluxing dioxane (3 mL) contained in a two-necked round-bottomed flask equipped with a reflux condenser and a dropping funnel and protected from the light. After 3 h of heating, the mixture was left under stirring for 16 h at room temperature, and then concentrated by evaporation. The residue was dissolved with a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ mixture (97:3, v/v) (1 mL) and purified on a silica gel column using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ mixture (97:3, v/v, to 93:7, v/v) to give a colorless oil: yield 60% (0.315 g, 0.68 mmol); $R_f = 0.31$ using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (95:5, v/v) as eluent; ^1H NMR (CDCl_3) δ 2.98 (s, 3H, CH_3), 3.40–3.79 (m, 12H, CH_2O), 4.18 (t, 2H, $J = 4.77$ Hz, CH_2Br), 5.58 (t, 2H, $J = 4.75$ Hz, NCH_2), 7.90–7.95 (m, 2H, Ar), 8.16–8.18 (m, 1H, Ar), 8.30 (d, 1H, Ar, $J = 7.62$ Hz), 8.66 (d, 1H, Ar, $J = 9.03$ Hz), 10.2–10.5 (m, 1H, Ar); mass analysis, ESI, polarity positive; calculated for $\text{C}_{18}\text{H}_{25}\text{O}_3\text{NBr}$: $\text{M}+\text{H} = 384.3$, found 384.4.

Synthesis of *N*-(10-Iododecyl)-lepidine **3.** This compound was obtained following the procedure used for the preparation of *N*-(11-bromo-3,6,9-trioxaundecyl)-lepidine **2** except that 1,11-dibromo-3,6,9-trioxaundecane **1** was replaced by 1,10-diiododecane. Starting from lepidine (0.162 g, 1.17 mmol) and 1,10-diiododecane (1.12 g, 2.84 mmol), *N*-(10-iododecyl)-lepidine **3** was obtained with 65% yield (0.304 g, 0.74 mmol): $R_f = 0.23$ using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (95:5, v/v) as eluent; ^1H NMR (CDCl_3) δ 1.22–1.28 [m, 8H, $(\text{CH}_2)_4$], 1.29–1.33 (m, 2H, CH_2), 1.42–1.50 (m, 2H, CH_2), 1.73–1.79 (m, 2H, CH_2), 2.03–2.09 (m, 2H, CH_2), 2.99 (s, 3H, CH_3), 3.40–3.79 (m, 12H, CH_2O), 3.17 (t, 2H, $J = 7.5$ Hz, CH_2I), 5.24 (t, 2H, $J = 7.5$ Hz, NCH_2), 7.94–8.0 (m, 2H, Ar), 8.16–8.19 (m, 1H, Ar), 8.34 (d, 1H, Ar, $J = 8$ Hz, $J = 9.03$ Hz), 10.15 (d, 1H, Ar, $J = 6$ Hz); mass analysis, ESI, polarity positive; calculated for $\text{C}_{20}\text{H}_{29}\text{NI}$: $\text{M}+\text{H} = 410$, found 410.3.

Synthesis of *S*,3-Dimethylbenzothiazole-2-thiol **4.** *S*,3-Dimethylbenzothiazole-2-thiol **4** was obtained as reported in ref 27: yield 92% (5.97 g, 18.48 mmol); $R_f = 0.51$ using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (80:20, v/v) as eluent; mp = 152–154 °C; ^1H NMR ($\text{DMSO}-d_6$) δ 3.12 (s, 3H, SCH_3), 4.10 (s, 3H, NCH_3), 7.71 (t, 1H, $J = 7.8$ Hz), 7.82 (t, 1H, $J = 7.8$ Hz), 8.18 (d, $J = 8$ Hz, 1H), 8.40 (d, $J = 8$ Hz, 1H); mass analysis, ESI, polarity positive; calculated for $\text{C}_9\text{H}_{10}\text{NS}_2$: $\text{M}+\text{H} = 197$, found 196.6.

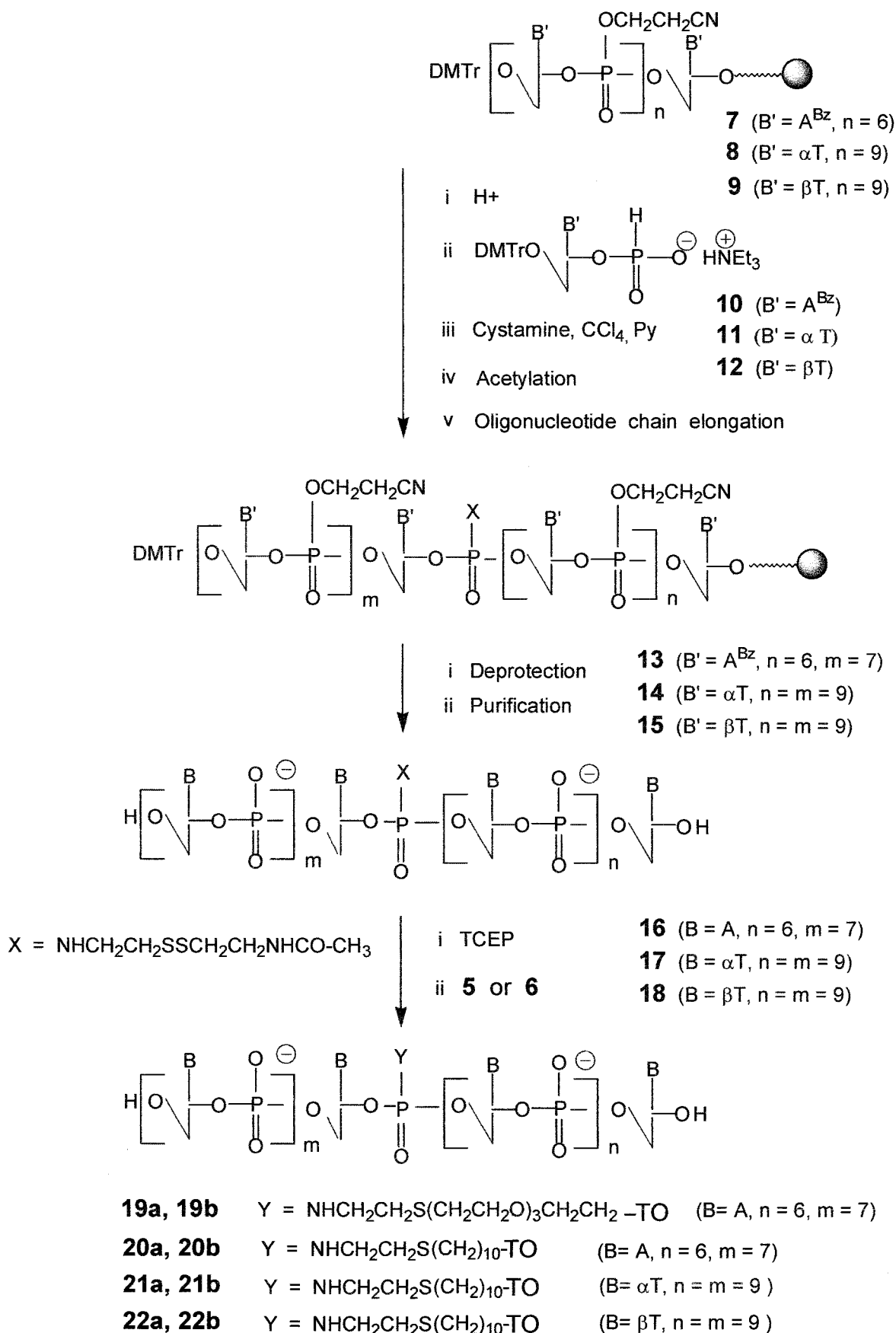
Synthesis of *N*-(11-Iodo-3,6,9-trioxaundecyl)-thiazole Orange **5.** *N*-(11-bromo-3,6,9-trioxaundecyl)-lepidine **2** (0.300 g, 0.65 mmol) and *S*,3-dimethylbenzothiazole-2-thiol **4** (0.303 g, 0.94 mmol) were dissolved in warm ethanol (4 mL) under stirring, and then triethylamine (200 μL , 1.5 mmol) was added to the solution. The red-colored mixture was protected from the light and left under stirring for 1 h at room temperature. The solvent was removed by evaporation, and the residue was purified on a silica gel column using a gradient of MeOH (0–3%) in CH_2Cl_2 to give a red powder: yield 35% (0.120 g, 0.22 mmol); $R_f = 0.77$ using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (80:20, v/v) as eluent; ^1H NMR (CDCl_3) δ 3.43–3.67 (m, 12H, CH_2O), 3.98 (t, 2H, $J = 5.16$ Hz, CH_2I), 4.00 (s, 3H, NCH_3), 4.71 (m, 2H, NCH_2), 6.72 (s, 1H, CH), 7.24–7.35 (m, 4H, Ar), 7.40–7.50 (m, 1H, Ar), 7.64–7.86 (m, 4H, Ar), 8.64 (d, $J = 7.5$ Hz, 1H, Ar); mass analysis, ESI, polarity positive; calculated for expected product $\text{C}_{26}\text{H}_{30}\text{BrN}_2\text{O}_3\text{S}$: $\text{M}+\text{H} = 530$, found 577.2, corresponds to $\text{C}_{26}\text{H}_{30}\text{IN}_2\text{O}_3\text{S}$. (These results indicate that halogen exchange occurred. This can be explained by the fact that the reaction between **2** and **4** to give **5** is performed in basic conditions in the presence of triethylamine.) mp = 117–118 °C.

Synthesis of *N*-(10-Iododecyl)-thiazole Orange **6.** This

compound was obtained following the procedure used for the preparation of *N*-(11-iodo-3,6,9-trioxaundecyl)-thiazole orange **5** except *N*-(11-bromo-3,6,9-trioxaundecyl)-lepidine **2** was replaced by *N*-(10-iododecyl)-lepidine **3**. Starting from 0.383 g (1 mmol) of *N*-(10-iododecyl)-lepidine **3** and 0.325 g (1 mmol) of *S*,3-dimethylbenzothiazole-2-thiol **4**, 0.208 g of 10-iododecylthiazole orange **6** was obtained: yield 62% (0.208 g, 0.29 mmol); $R_f = 0.46$ using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (90:10, v/v) as eluent; ^1H NMR (CD_3OD) δ 1.22–1.45 (m, 12H, $(\text{CH}_2)_6$), 1.75–1.78 (m, 2H, CH_2), 1.84–1.95 (m, 2H, CH_2), 3.15 (t, 2H, $J = 4.2$ Hz, CH_2I), 3.98 (s, 3H, NCH_3), 4.52 (t, 2H, $J = 4.5$ Hz, NCH_2), 6.72 (s, 1H, CH), 7.21–7.32 (m, 2H, Ar), 7.38–7.40 (m, 1H, Ar), 7.41–7.48 (m, 1H, Ar), 7.57–7.61 (m, 1H, Ar), 7.65–7.70 (m, 2H, Ar), 7.75–7.78 (m, 1H, Ar), 8.65 (d, $J = 4.8$ Hz, 1H, Ar), 8.90 (d, $J = 4.2$ Hz, 1H, Ar); mass analysis, ESI, polarity positive; calculated for $\text{C}_{28}\text{H}_{34}\text{IN}_2\text{S}$: $\text{M}+\text{H} = 558.1$, found 557.1; mp = 164–165 °C.

Synthesis of Pentadeca-2'-deoxyriboadenylates **19a, **19b**, **20a**, and **20b** Bearing a Thiazole Orange Derivative at the Eighth Internucleotidic Phosphate and Icosa- α -thymidylates **21a** and **21b** and Icosa- β -thymidylates **22a** and **22b** Bearing a Thiazole Orange Derivative at the Tenth Internucleotidic Phosphate (Scheme 2).** **Preparation of 5'-O-(4,4'-Dimethoxytrityl)- α -thymidine-3'-H-phosphonate **11**.** This synthesis was adapted from ref 28. To a stirred solution of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (1.39 g, 6.86 mmol) in a pyridine/ $\text{ClCH}_2\text{CH}_2\text{Cl}$ (90:10, v/v) mixture cooled to 0 °C was added slowly a solution of 5'-O-(4,4'-dimethoxytrityl)- α -thymidine, previously dried by coevaporation with anhydrous pyridine and kept under vacuum overnight (2.5 g, 4.6 mmol) in an anhydrous pyridine/ $\text{ClCH}_2\text{CH}_2\text{Cl}$ (50:50, v/v) mixture. The reaction was monitored by TLC on silica gel with $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NEt}_3$ (90:9.5:0.5, v/v/v) as eluent. After 15 min, the starting material ($R_f = 0.54$) was fully transformed into a new compound having a lower R_f (0.31). The reaction mixture was poured into an $\text{NEt}_3/\text{H}_2\text{O}$ (20:80, v/v) mixture (100 mL) under vigorous stirring for 5 min. The reaction mixture was then extracted with CH_2Cl_2 (50 mL), twice. The organic phase was then washed with a saturated NaCl solution (15 mL), and the latter was back-extracted with CH_2Cl_2 (50 mL). The organic phases were pooled, dried over Na_2SO_4 , and concentrated under reduced pressure. The residue was purified on a silica gel column using a gradient of methanol in dichloromethane in the presence of triethylamine, $\text{CH}_2\text{Cl}_2/\text{NEt}_3$ (99.5:0.5, v/v) to $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NEt}_3$ (94.5:5:0.5, v/v/v), as eluent to give a pale yellow solid: $R_{f11} = 0.62$ using $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{NEt}_3$ (79.5:20:0.5, v/v/v) as eluent; yield 40%, 1.3 g, 8.4 mmol; mass analysis, ESI, polarity negative; calculated for $\text{C}_{31}\text{H}_{32}\text{N}_2\text{O}_9\text{P}$: $\text{M}+\text{H} = 608$, found 607.3.

Preparation of Oligonucleotides **16, **17**, and **18** Bearing a Linker at the Central Phosphate Group and Separation of Their Diastereoisomers.** The oligo-2'-deoxyribonucleotides were assembled using classical phosphoramidite chemistry on a CPG support at a micromolar scale except that at the site selected for the introduction of the linker arm, a coupling step using H-phosphonate chemistry was manually performed. The CPG support involving α -thymidine, the phosphoramidite, and the H-phosphonate derivatives of α -thymidine was not commercially available. The first two were available in the laboratory, while the last one (compound **11**) was obtained as described above. The syntheses were performed as follows. At the end of the chain assembly of the hepta-2'-deoxyriboad-

Scheme 2. Synthesis of the Oligo-2'-deoxyribonucleotide-Thiazole Orange Conjugates **19a**, **19b**, **20a**, **20b**, **21a**, **21b**, **22a**, and **22b**

enylate **7**, the deca- α -thymidylate **8**, and the deca- β -thymidylate **9**, an additional detritylation step was

performed. N^6 -Benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyadenosine-3'-H-phosphonate **10** (0.033 g, 0.04 mmol) and

5'-O-(4,4'-dimethoxytrityl)- α -thymidine-3'-H-phosphonate **11** [or 5'-O-(4,4'-dimethoxytrityl)- β -thymidine-3'-H-phosphonate **12**] (0.04 g, 0.056 mmol) in a pyridine/CH₃CN (50:50, v/v) mixture (0.4 mL) and pivaloyl chloride (0.02 g, 0.166 mmol) in a pyridine/CH₃CN (50:50, v/v) mixture (0.5 mL) were then added simultaneously to the hepta-2'-deoxyriboadenylate, the deca- α -thymidylate, or the deca- β -thymidylate bound to the support. After a 2.5 min reaction, the solution was removed and the support washed with an anhydrous pyridine/CH₃CN (50:50, v/v) mixture (1 mL, 3 times). Then cystamine (0.165 g, 0.87 mmol) solubilized by a pyridine/CCl₄ (50:50, v/v) mixture (1 mL) was added. After 1 h, the solution was removed and the support washed with an anhydrous pyridine/CH₃CN (50:50, v/v) mixture (1 mL) 3 times and then with anhydrous CH₃CN (1 mL) 3 times. The support was treated with a mixture of capping solutions used on the synthesizer (0.5 mL each) for 10 min, washed with CH₃CN (1 mL, 4 times), and dried. The oligonucleotide chain assemblies were completed via phosphoramidite chemistry to give the fully protected pentadeca-2'-deoxyriboadenylate **13**, icoso- α -thymidylate **14**, and icoso- β -thymidylate **15** bound to the support bearing the linker at the central phosphates of the oligonucleotide chains. At the end of the chain assembly, an additional detritylation step was performed to deblock the 5'-terminal hydroxyl function. The deprotection step was completed by overnight concentrated ammonia treatment at 50 °C in the case of the oligo-2'-deoxyriboadenylates and at room temperature in the case of the oligothymidylates (in both cases, the support was discarded after a 2 h treatment). Purification was performed by ion exchange chromatography on a DEAE column (8 μ M, 100 mm $\times$ 10 mm, Waters) using a linear gradient of NaCl (0.075–0.525 M over 30 min) in 25 mM Tris-HCl, pH 7, buffer containing 10% MeOH. After the desalting steps, reversed-phase chromatography analyses were performed on a Lichrospher 100 RP (5 μ M) column (125 mm $\times$ 4 mm) from Merck with a linear gradient of CH₃CN (17.4–18.4% over 15 min) in 0.1 M aqueous triethylammonium acetate, pH 7, with a flow rate of 1 mL/min. Two roughly equivalent peaks with very close retention times (Rt) were obtained for both oligo-2'-deoxyribonucleotides with Rt_{16a} = 8 min, 24 s and Rt_{16b} = 9 min in the case of the oligo-2'-deoxyriboadenylates. In the case of oligothymidylate isomers, analyses were performed on the same column with the same buffer and flow rate but with different gradients of CH₃CN: 5–35% over 50 min for oligo- α -thymidylate isomers (Rt_{17a} = 19 min, 46 s; Rt_{17b} = 21 min, 40 s) and 12.5–20% over 30 min for oligo- β -thymidylate isomers (Rt_{18a} = 18 min, 52 s; Rt_{18b} = 19 min, 40 s).

Synthesis of Oligonucleotides 19a, 19b, 20a, 20b, 21a, 21b, 22a, and 22b by Coupling Thiazole Orange-Linker Derivatives 5 and 6 with the Oligonucleotides Bearing a Thiol Function Obtained after Treatment of Oligo-2'-deoxyribonucleotides 16a, 16b, 17, and 18 with a Reducing Agent. Solutions of **16a** and **16b**, d⁵(Ap)₇Ap*[NH-(CH₂)₂S-S-(CH₂)₂NHCOCH₃]A(pA)₆^{3'} (15 OD each), in 5% aqueous bicarbonate buffer, pH 9 (800 μ L), were degassed by bubbling argon. An aqueous tris(2-carboxyethyl)-phosphine (TCEP) solution (0.06 mg in 5 μ L of H₂O) was then added, and the solutions were vortexed for a few minutes. Finally, a DMF solution of thiazole orange-linker derivative **5** or **6** (1.5 mg in 800 μ L) was added. The solutions were vortexed and heated at 35–40 °C. The coupling reactions were controlled by reversed-phase analyses as follows. The oligonucleotides were recovered by centrifugation of an aliquot of the reaction mixture. The pellets were dissolved in water, and the red solutions

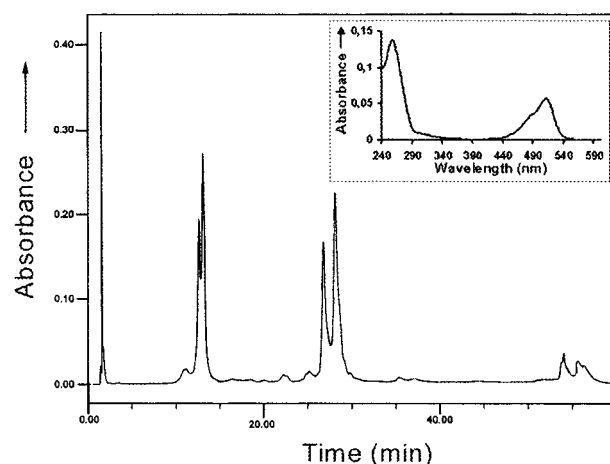
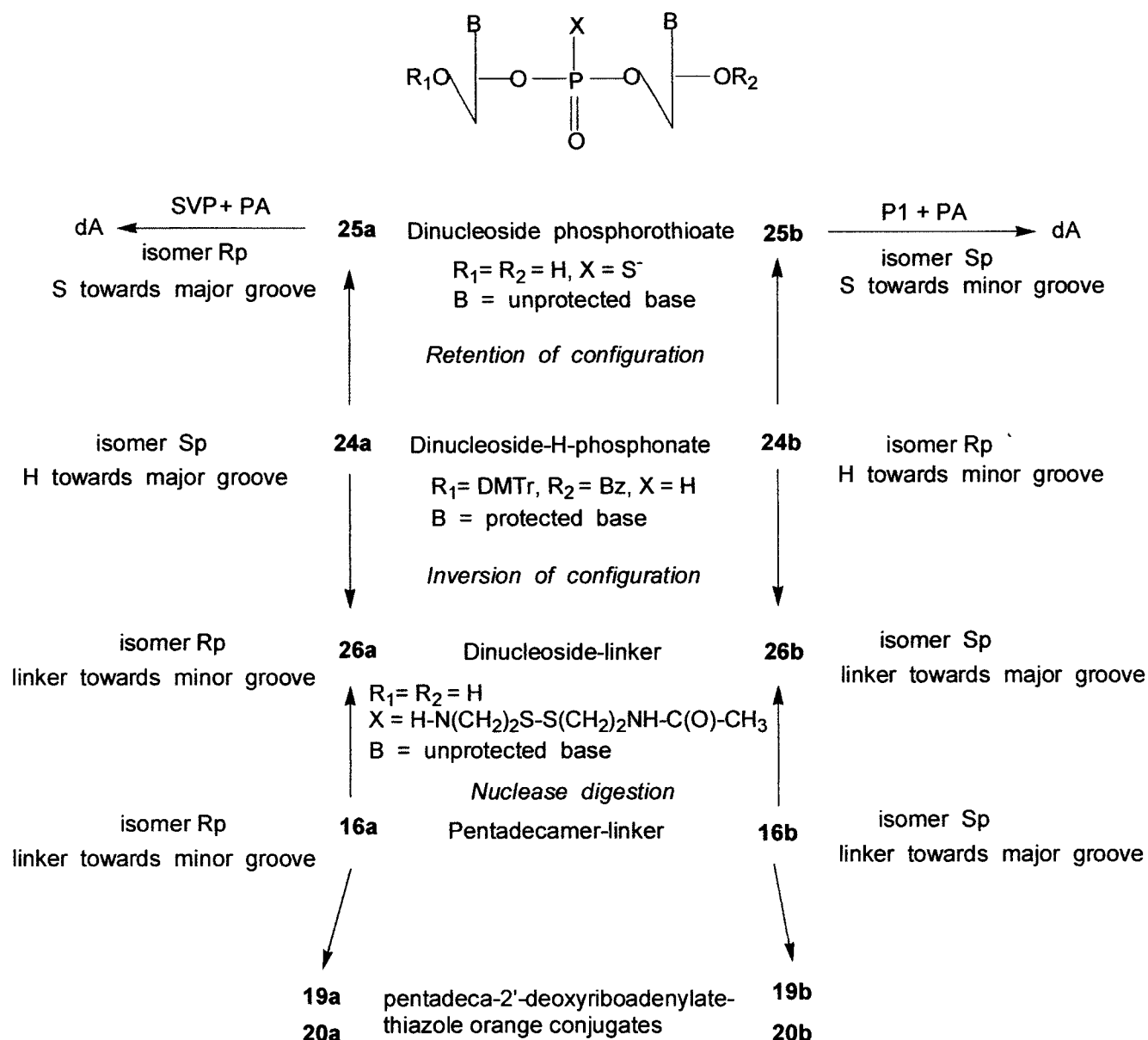


Figure 1. HPLC reversed-phase analysis of the coupling reaction between the pentadeca-2'-deoxyriboadenylate **16** (isomer mixture) and the thiazole orange-linker derivative **5** after 2 h incubation. Pentadeca-2'-deoxyriboadenylate-thiazole orange conjugates **19a** and **19b** are eluted with Rt_{19a} = 26 min, 48 s and Rt_{19b} = 28 min, 10 s, respectively. The inset shows the absorption spectrum, recorded between λ = 240 and λ = 600 nm, of the conjugate **19a**. The analysis was performed on a Lichrospher 100RP-18 (5 μ m) column (125 $\times$ 4 mm) using a linear gradient of CH₃CN in 0.1 M aqueous triethylammonium acetate buffer (pH 7) with a flow rate of 1 mL/min (12–36% over 45 min).

were analyzed by reversed-phase chromatography on the Lichrospher column described above with a linear gradient of CH₃CN (12–36% over 45 min) in 0.1 M aqueous triethylammonium acetate, pH 7, with a flow rate of 1 mL/min (Rt_{19a} = 19 min, 1 s; Rt_{19b} = 20 min, 5 s; Rt_{20a} = 30 min; Rt_{20b} = 31 min). The coupling reaction could be performed on the mixture of isomers of oligo-2'-deoxyriboadenylate (**16a** + **16b**), but separate coupling reactions on each isomer are required for determination of the stereochemistry of oligo-2'-deoxyriboadenylate-thiazole orange conjugates. Reversed-phase analysis of the coupling reaction between the oligo-2'-deoxyriboadenylate **16** and the thiazole orange-linker derivative **5**, after 2 h of incubation, is shown in Figure 1. The inset shows the absorption spectra, recorded between λ = 240 nm and λ = 600 nm, of the oligo-2'-deoxyriboadenylate-thiazole orange conjugates with a hydrophilic linker, **19a**. Usually the reaction was completed after 5–6 h of incubation: yield 50% after purification; mass analysis, ESI, polarity negative. Pentadeca-2'-deoxyriboadenylate-thiazole orange conjugates with a polymethylene linker: compounds **20a** and **20b**: calculated mass for C₁₈₀H₂₁₉O₇₂N₇₈S₂P₁₇: M–H = 5125.08, found 5125.26 $\pm$ 0.63 for compound **20a** and 5125.23 $\pm$ 0.63 for compound **20b**.

Solution of icoso- α -thymidylate **17** (20 OD, 0.11 μ mol) in 5% aqueous NaHCO₃, pH 9, buffer (800 μ L) was degassed by bubbling argon. Then an aqueous tris(2-carboxyethyl)phosphine (TCEP) solution (0.06 mg, 0.22 μ mol in 1 μ L of H₂O) was added, and the solutions were vortexed for a few minutes. After verification of the cleavage of the disulfide bridge by reversed-phase chromatography [Rt_{17a} = 17 min, 22 s; Rt_{17b} = 17 min, 42 s; and for the thiol derivatives, Rt = 17 min, 9 s; Rt = 17 min, 22 s on the Lichrospher column described above with a linear gradient of CH₃CN (0–24% over 15 min) in 0.1 M aqueous triethylammonium acetate, pH 7, with a flow rate of 1 mL/min], a DMF solution (1 mL) of thiazole orange-linker derivative **6** (1.5 mg, 2.2 $\times$ 10^{–6} mol) was added. The solutions were vortexed and heated at 35–40 °C. The coupling reactions were controlled by reversed-

Scheme 3. Isomer Identification



phase analyses as follows. The oligonucleotides were recovered by centrifugation. The pellets were dissolved in water, and the solutions were analyzed by reversed-phase chromatography on a Lichrospher column (the same as above) with a linear gradient of CH_3CN (0–40% over 50 min) in 0.1 M aqueous triethylammonium acetate, pH 7, with a flow rate of 1 mL/min. Two peaks corresponding to the R_p and S_p isomers were obtained ($R_{t_{21a}} = 34$ min, 40 s; $R_{t_{21b}} = 36$ min, 2 s). The coupling reactions were nearly complete after 5–6 h of reaction. The oligonucleotide–thiazole orange isomers **21a** and **21b** were purified by using a more flat gradient: yield 50% after purification; mass analysis, ESI, polarity negative. Icosa- β -thymidylate–thiazole orange conjugates **21a** and **21b**: calculated mass for $\text{C}_{230}\text{H}_{298}\text{O}_{167}\text{N}_{43}\text{S}_2\text{P}_{19}$: $M-H = 6509.74$, found mass 6509.64 ± 1.93 .

The icosa- β -thymidylate–thiazole orange conjugates **22a** and **22b** were obtained by using the isomer mixture of the icosa- β -thymidylate-linker derivative **18** and the thiazole orange-linker derivative **6**, and proceeding as reported for the preparation of the icosa- α -thymidylate–thiazole orange conjugates **21a** and **21b**. HPLC reversed-phase analyses were performed on a Lichrospher column

(the same as above) with a linear gradient of CH_3CN (20–30% over 30 min) in 0.1 M aqueous triethylammonium acetate, pH 7, with a flow rate of 1 mL/min. Two peaks corresponding to the R_p and S_p isomers were obtained ($R_{t_{22a}} = 12$ min, 42 s; $R_{t_{22b}} = 19$ min, 50 s).

Isomer Identification (Scheme 3). Preparation of Dinucleosides $d\text{-}5'\text{DMTrA}^{Bz}p^*(H)A^{Bz}Bz^{3'}$ **24a** and **24b**. To an anhydrous stirred pyridine solution (5 mL) of a mixture of N^6 -benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyriboadenosine-3'-H-phosphonate **10** (0.412 g, 0.5 mmol) and N^6 -benzoyl-3'-O-benzoyl-2'-deoxyriboadenosine **23** (0.230 g, 0.5 mmol), previously dried by coevaporation with anhydrous pyridine (20 mL, 3 times) and kept under vacuum overnight, was added pivaloyl chloride (0.180 g, 1.5 mmol) at room temperature. After 15 min of stirring, the reaction mixture was diluted with CH_2Cl_2 (50 mL), and the organic phase was washed with a 5% aqueous NaHCO_3 solution (50 mL). The aqueous phase was back-extracted with CH_2Cl_2 (50 mL), twice. The organic phases were then pooled, dried over Na_2SO_4 , filtered off, and concentrated under reduced pressure. The residue was solubilized with acetone and coevaporated with toluene (10 mL), 3 times, and then purified on a silica gel column

(ref. 109336 from Merck) using an ethyl acetate/acetone mixture (60:40, v/v) containing 0.2% acetic acid as eluent until the first isomer was eluted and then an ethyl acetate/acetone mixture (50:50, v/v) containing 0.2% acetic acid as eluent for the elution of the second isomer. After removal of the solvents by evaporation, the residual acetic acid was removed by co-evaporation with CH_3CN (3 mL), 3 times. Products corresponding to each pure dinucleoside-H-phosphonate isomer (0.2 g each) were obtained as pale yellow solids: $R_{24a} = 0.61$ and $R_{24b} = 0.53$ using $\text{CH}_2\text{Cl}_2/\text{iPrOH}$ (90:10, v/v) as eluent; $R_{24a} = 0.56$ and $R_{24b} = 0.53$ using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (90:10, v/v) as eluent; mass analysis, ESI, polarity positive; calculated mass for $\text{C}_{62}\text{H}_{55}\text{O}_{12}\text{N}_{10}\text{P}_{19}$: $M+H = 1163.4$, found mass 1163.3.

Preparation of Dinucleosides $d\text{-}^5\text{Ap}^*(S)\text{A}^3'$ 25a and 25b. To pyridine solutions (1 mL) of each pure dinucleoside-H-phosphonate (**24a** and **24b**) (0.02 g, 17.2 mmol), previously dried by co-evaporation with anhydrous pyridine (5 mL, 3 times), was added, at room temperature, under stirring, and in an argon atmosphere, a pyridine/ CS_2 (50:50, v/v) solution of S_8 (4.25 mg/mL) (2 mL) (32). After 1 h of stirring, the reaction mixtures were evaporated to dryness, and the residues were co-evaporated with toluene (5 mL), 3 times. The residues were solubilized with ethyl acetate (6–7 mL). The reaction mixtures were filtered and then washed with water (1–2 mL). The organic phases were concentrated to dryness, and an 80% solution of acetic acid in water (5 mL) was added. After 30 min, acetic acid was removed by evaporation. The residues obtained were solubilized with a 28% aqueous ammonia solution (4–5 mL) and left overnight at 50 °C. The reaction mixtures were evaporated to dryness. The residues were solubilized with water (7–8 mL), and the aqueous phases were extracted with ethyl acetate (3×1 mL): $R_{25a} = 0.53$ and $R_{25b} = 0.61$ using $\text{iPrOH}/\text{NH}_4\text{OH}/\text{H}_2\text{O}$ (70:20:10, v/v/v) as eluent; $R_{25a} = 0.17$ and $R_{25b} = 0.31$ using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (90:10, v/v) as eluent. The presence of the sulfur atom was confirmed by the obtention of pink-colored spots on TLC plates by spraying with a 2,6-dibromo-4-benzoquinone-*N*-chloroimine (DBPNC) solution in ethanol (2 mg/mL) and heating; mass analysis, ESI, polarity positive; calculated for $\text{C}_{20}\text{H}_{25}\text{N}_{10}\text{O}_7\text{SP}$: $M+H = 580.52$, found 581.1.

Preparation of Dinucleoside $d\text{-}^5\text{Ap}^*[\text{NH}(\text{CH}_2)_2\text{S-S}(\text{CH}_2)_2\text{-NHCOCH}_3]\text{A}^3'$ 26a and 26b. Both pure isomers **24a** and **24b** of the dinucleoside-H-phosphonate N^6 -benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyriboadenosyl- N^6 -benzoyl-3'-*O*-benzoyl-2'-deoxyriboadenosine-3'-H-phosphonate (0.035 g each, 0.03 mmol) were treated, separately, with cystamine (0.09 g, 0.6 mmol) solubilized by an anhydrous pyridine/ CCl_4 (50:50, v/v) mixture (1 mL) for 10 min over an argon atmosphere. The reaction mixture was concentrated to dryness and solubilized with CH_2Cl_2 (7–8 mL). The organic phase was washed with water (1–2 mL), dried over Na_2SO_4 , and concentrated to dryness. The residue was co-evaporated with anhydrous pyridine (3 mL, 3 times), and during the last evaporation, 1 mL of pyridine was kept in the flask. Acetic anhydride (0.93 g, 0.9 mmol) was added under stirring at room temperature. The reaction was monitored by TLC analysis using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (90:10, v/v) as eluent. When the reaction was completed, the reaction mixture was evaporated to dryness, co-evaporated with toluene, and purified by flash chromatography using a gradient of MeOH in CH_2Cl_2 (0–20%). An 80% acetic acid solution in water (3 mL) was then added. After 30 min of reaction at room temperature, the solution was evaporated to dryness and co-evaporated with toluene (3 mL), twice. The residue was

then treated with a 28% solution of aqueous ammonia in a MeOH/ H_2O (80:20, v/v) mixture at 50 °C overnight. After removal of the ammonia solution by evaporation, the residue was solubilized with water (3 mL), and the aqueous phase was extracted with ethyl acetate (0.5 mL), 3 times. Purification by reversed-phase chromatography was performed on a Lichrospher 100 RP (5 μM) column (125 mm $\times$ 4 mm) from Merck with a linear gradient of CH_3CN (10–35% over 50 min) in 0.1 M aqueous triethylammonium acetate, pH 7, with a flow rate of 1 mL/min: $R_{t26a} = 19$ min, 30 s; $R_{t26b} = 19$ min, 55 s; mass analysis, ESI, polarity positive; calculated for $\text{C}_{26}\text{H}_{37}\text{N}_{12}\text{O}_8\text{S}_2\text{P}$: $M+H = 741.76$, found 741.1.

RESULTS AND DISCUSSION

Synthesis of the Oligonucleotide-Thiazole Orange Conjugates. One report shows that the homodimeric thiazole orange TOTO bisintercalates in DNA with the linker in the minor groove (21). Since our previous work on oligo-2'-deoxyribonucleotide-acridine conjugates showed that stronger stabilization (therefore better intercalation) was observed when the intercalating residue was linked to an internucleotidic phosphate (22), we chose the same position for the linking of the thiazole orange moiety. Substitution at an internucleotidic position introduces a chirality at the phosphorus atom with the formation of two diastereoisomers (Figures 2 and 3). These oligonucleotides can be separated as pure isomers. When these oligonucleotides are hybridized with their complementary sequences, the substituent of one isomer protrudes in the direction of the minor groove, while the substituent of the other isomer points in the direction of the major groove. The CPK model indicates that a linker involving 14 atoms fixed on an internal phosphorous atom and on the nitrogen atom of the quinolinium ring of the thiazole orange residue would be suitable for the intercalation of the thiazole orange residue by either the minor or the major groove. We chose to attach the linker via a phosphoramidate linkage at the central phosphate of a pentadeca-2'-deoxyriboadenylate (Figure 2), an icoso- α -thymidylate, and an icoso- β -thymidylate (Figure 3) and to separate both isomers in order to study the influence of the stereochemistry at the phosphorus atom on the interactions of the oligonucleotide-thiazole orange conjugates with their complementary sequences. A linker involving a masked thiol function such as cystamine that can withstand the conditions required for the deprotection and purification steps of the oligonucleotide was chosen (29). After in situ cleavage of the disulfide bridge, the released thiol function was selectively reacted with a halogenoalkyl group linked at the nitrogen atom of the quinolinium ring of the thiazole orange. Of the 2 different linkers fixed on the thiazole orange, 1 was hydrophilic, derived from tetraethylene glycol (compound 5), and the other was hydrophobic with 10 methylene groups (compound 6). These thiazole orange-linker derivatives, 5 and 6, were reacted with each pure S_p and R_p isomer of the pentadeca-2'-deoxyriboadenylate 16 while only the thiazole orange derivative involving a polymethylene linker, 6, was reacted with the mixture of the S_p and R_p isomers of icoso- α -thymidylate 17 and with the mixture of the S_p and R_p isomers of icoso- β -thymidylate 18. S_p and R_p isomers of the icoso- α -thymidylate-thiazole orange conjugates and of the icoso- β -thymidylate-thiazole orange conjugates were then separated by reversed-phase chromatography.

Preparation of Thiazole Orange-Linker Derivatives 5 and 6. The preparation of thiazole orange-linker derivatives 5 and 6 (Scheme 1) was achieved by a

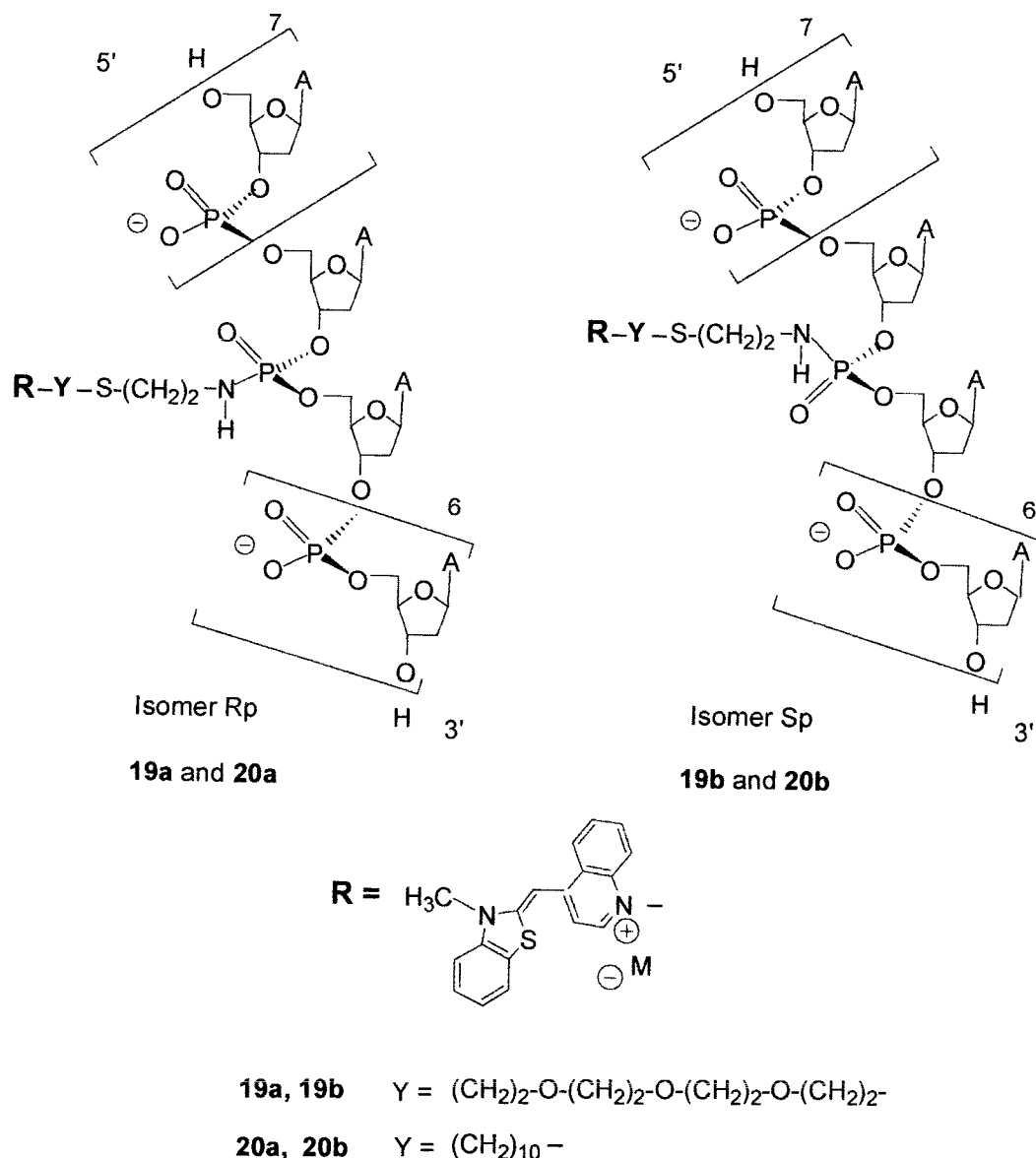
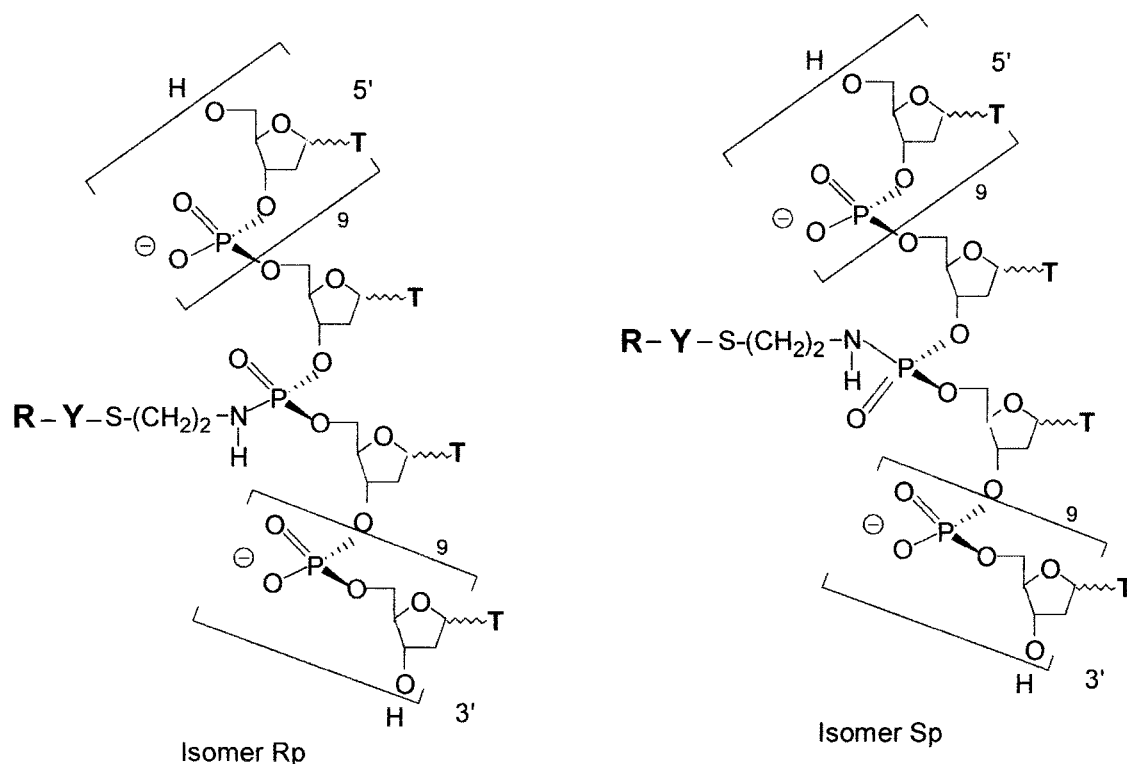


Figure 2. Structures of the pentadeca-2'-deoxyriboadenylate-thiazole orange conjugates **19a**, **19b**, **20a**, and **20b**.

procedure adapted from the literature (27, 30). The lepidine-linker derivatives **2** and **3** were obtained by reaction of lepidine with 1,11-dibromo-3,6,9-trioxaudecane **1** and 1,10-diiododecane, respectively. 1,11-Dibromo-3,6,9-trioxaudecane **1** was obtained by treatment of tetraethylene glycol with triphenylphosphine and BrCCl_3 . Reaction of the lepidine-linker derivatives **2** and **3** with *S*,3-dimethylbenzothiazole-2-thiol **4** (obtained by reaction of 3-methylbenzothiazole-2-thione with methyl iodide) led to the expected thiazole orange-linker derivatives **5** and **6**, respectively.

Synthesis of Oligo-2'-deoxyribonucleotide-Thiazole Orange Conjugates 19a, 19b, 20a, 20b, 21a, 21b, 22a, and 22b. This was achieved following Scheme 2. At the end of the chain assembly of hepta-2'-deoxyriboadenylate **7**, deca- α -thymidylate **8**, and deca- β -thymidylate **9**, an additional detritylation step was performed, and the fixation of the cystamine on the central phosphate of the oligo-2'-deoxyribonucleotide was achieved by performing an H-phosphonate coupling step, manually, including an oxidation step using a mixture of CCl_4 and cystamine in pyridine, at a position preselected for the attachment of the linker (31). Acetylation of the terminal primary amino function of the linker was performed in

order to prevent its phosphitilation during the assembling of the second parts of the oligonucleotide chains via phosphoramidite chemistry. This led to the fully protected pentadeca-2'-deoxyriboadenylate **13**, icoso- α -thymidylate **14**, and icoso- β -thymidylate **15** bound to the support and bearing the linker at the central phosphates. After deprotection and purification by ion-exchange chromatography, reversed-phase analyses of pentadeca-2'-deoxyriboadenylate **16**, icoso- α -thymidylate **17**, and icoso- β -thymidylate **18** bearing the linker at their central phosphate via a phosphoramidate linkage exhibited two roughly equivalent peaks with very close retention times (see Experimental Procedures). The pentadeca-2'-deoxyriboadenylate isomers **16a** (fast eluted) and **16b** (slowly eluted) were separated by reversed-phase chromatography (see Experimental Procedures for the conditions used) while the mixture of isomers was used in the case of icoso- α -thymidylate **17** and icoso- β -thymidylate **18** for the coupling step with the thiazole orange derivative **6**. Determination of the configuration was made for pentadeca-2'-deoxyriboadenylates **16a** and **16b** after their digestion by the nuclease P1 or snake venom phosphodiesterase followed by alkaline phosphatase treatment. Each hydrolysate was then compared with pure S_p or R_p



21a and 21b, T in α -anomeric position

22a and 22b, T in β -anomeric position

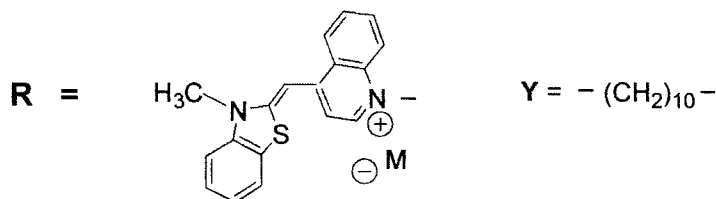


Figure 3. Structures of the icoso- α -thymidylate–thiazole orange conjugates **21** and **21b**, and the icoso- β -thymidylate–thiazole orange conjugates **22a** and **22b**.

isomer samples of the dinucleoside $d\text{-}5'\text{Ap}^*[\text{NH}(\text{CH}_2)_2\text{S}(\text{CH}_2)_2\text{NHCOCH}_3]\text{A}^{3'}$, whose stereochemistry was determined following the method reported in Scheme 3 and discussed in the next paragraph. The thiazole orange derivatives **5** and **6** were coupled with the released thiol function obtained after in situ treatment of pure isomers **16a** and **16b** of the pentadeca-2'-deoxyriboadenylate with a reducing agent (TCEP) to give the pentadeca-2'-deoxyriboadenylate–thiazole orange conjugates **19a** and **19b**, **20a** and **20b**, respectively. The same reaction was performed with the thiazole orange derivative **6** and a mixture of the isomers of icoso- α -thymidylate **17** or icoso- β -thymidylate **18** to give a mixture of the isomers of icoso- α -thymidylate–thiazole orange conjugates **21a** and **21b** and of icoso- β -thymidylate–thiazole orange conjugates **22a** and **22b**, respectively. The latter were then separated by chromatography.

Determination of the Configuration of Isomers 19a, 19b, 20a, and 20b of the Pentadeca-2'-deoxyriboadenylate–Thiazole Orange Conjugates. This was achieved as described in Scheme 3. The fully protected dinucleoside-H-phosphonate **24** was obtained following a procedure adapted from the literature (32) except that the benzoyl group was used for the protection of the 3'-

hydroxyl function. Dinucleoside isomers **24a** and **24b** were purified and separated by silica gel chromatography. A part of both pure dinucleoside-H-phosphonate isomers **24a** and **24b** was separately transformed (with retention of configuration at the phosphorus center) into pure dinucleoside phosphorothioates by treatment with S_8 in a pyridine/ CS_2 mixture. After deprotection and purification by reversed-phase chromatography, aliquots of both pure dinucleoside phosphorothioate isomers **25a** and **25b** were treated separately with P1 endonuclease and snake venom phosphodiesterase (SVP) and then with phosphatase alkaline (PA). Treatment with P1 + PA led to the full degradation of the S_p isomer into dA while the R_p isomer remained unchanged. On the contrary, treatment with SVP + PA led to full degradation of the R_p isomer into dA while the S_p isomer remained unchanged (33). These results showed that only dinucleoside phosphorothioate **25b** which comes from dinucleoside-H-phosphonate **24b** was cleaved by P1 + PA treatment while only dinucleoside phosphorothioate **25a** which comes from dinucleoside-H-phosphonate **24a** was cleaved by SVP + PA treatment. The remaining part of both pure dinucleoside-H-phosphonate isomers **24a** and **24b** was separately transformed (with inversion of configuration

at the phosphorus center) (34) into the dinucleoside d-⁵Ap*[NH(CH₂)₂S-S(CH₂)₂NHCOCH₃]₃A^{3'} **25a** and **25b** by treatment with cystamine in a CCl₄/pyridine mixture followed by an acetylation step. After deprotection, reversed-phase purification of the dinucleoside gave nearly equivalent peaks which correspond to diastereoisomers **25a** and **25b**. Both pure dinucleoside isomers **25a** and **25b** d-⁵Ap*[NH(CH₂)₂S-S(CH₂)₂NHCOCH₃]₃A^{3'} were used as a reference during analysis of the mixture obtained after nuclease degradation by either P1 + PA or SVP + PA of each pure isomer of the pentadecamers d-⁵(Ap)₇Ap[NH(CH₂)₂S-S(CH₂)₂NHCOCH₃]₃(Ap)₆A^{3'} **16a** and **16b**, thus allowing the determination of their configuration and subsequently those of all the pentadeca-2'-deoxyriboadenylate-thiazole orange conjugates described (**16a** gives dA + **25a**, and **16b** gives dA + **25b**). We then verified that the elution order for pentadeca-2'-deoxyriboadenylate-thiazole orange conjugates **19a** and **19b** was the same as that for oligonucleotides **16a** and **16b**. The same result was observed for oligo-2'-deoxyriboadenylates **20a** and **20b** with the hydrophobic linker. The coupling of **16a** with **5** and **6** gave **19a** and **20a**, respectively, while the coupling of **16b** with **5** and **6** gave **19b** and **20b**, respectively. From these results and in accordance with a literature report (35), we were able to conclude that the fast-eluted pentadeca-2'-deoxyriboadenylate-thiazole orange conjugates **19a** and **20a** have the *R_p* configuration while the slow-eluted conjugates **19b** and **20b** have the *S_p* configuration. When the conjugates with the *R_p* configuration are hybridized with their target sequences, the linker is directed toward the minor groove of the duplex while in the case of the conjugates with the *S_p* configuration the linker is directed toward the major groove.

Interaction with the Complementary Sequence.

The stabilizing effect of the thiazole orange residue on the hybridization of the thiazole orange-oligonucleotide conjugates with their complementary sequences was determined by absorption spectroscopy studies carried out between $\lambda = 240$ nm and $\lambda = 600$ nm in the case of the thiazole orange-oligonucleotide conjugates and between $\lambda = 240$ nm and $\lambda = 350$ nm in the case of the corresponding unmodified oligonucleotides used as references.

Pentadeca-2'-deoxyriboadenylate-Thiazole Orange Conjugates 19a, 19b, 20a, and 20b. To determine the influence of the nature of the linker and of the stereochemistry at the phosphorus atom bearing the thiazole orange residue, modified oligo-2'-deoxyriboadenylates **19a**, **19b**, **20a**, and **20b** were hybridized with an oligo-2'-deoxyribonucleotide, ⁵d-CACT₁₅CAC^{3'}, involving the complementary sequence. The unmodified ⁵d-(A₁₅)^{3'} was used as a reference. The spectra of all four oligo-2'-deoxyriboadenylate-thiazole orange conjugates (**19a**, **19b**, **20a**, and **20b**) were almost identical. For a typical UV-visible spectrum of a pentadeca-2'-deoxyriboadenylate-thiazole orange conjugate, see the inset in Figure 3. The spectra contain one absorption band in the visible region between $\lambda = 400$ nm and $\lambda = 600$ nm (where only the thiazole orange absorbs light) with $\lambda_{\text{vis max}}^{\text{vis}} \approx 511$ nm and a shoulder at $\lambda = 485$ nm. The other absorption band in the UV range corresponds to the absorbance of the pentadeca-2'-deoxyriboadenylate and the thiazole orange with a $\lambda_{\text{uv max}}^{\text{uv}} \approx 257$ nm. A small additional band was also present at $\lambda \approx 305$ nm.

Adding increasing concentrations of ⁵d-CACT₁₅CAC^{3'} to a 0.1 μ M solution of ⁵d-(A₁₅)^{3'} in 10 mM sodium cacodylate, pH 7, buffer containing 0.1 M NaCl at 3 °C led to a sharp break when the absorbance at 260, 270,

Table 1. *T_m* Values for Oligo-2'-deoxyribonucleotides β -⁵'d-(A₁₅)^{3'}, α -⁵'d-(T₂₀)^{3'}, and β -⁵'d-(T₂₀)^{3'} and Oligo-2'-deoxyribonucleotide-Thiazole Orange Conjugates **19a**, **19b**, **20a**, **20b**, **21a**, **21b**, **22a**, and **22b** Hybridized with Their Complementary Sequences

	β - ⁵ 'd-(CACA ₁₅ CAC) ^{3'}	poly r(U)		poly r(A)
β - ⁵ 'd-(A ₁₅) ^{3'}	32.5 ^a	26.4 ^b	α - ⁵ 'd-(T ₂₀) ^{3'}	49.5 ^c
19a	41 ^a	—	21a	50 ^c
19b	40 ^a	—	21b	49 ^c
20a	40.5 ^a	35 ^b	β - ⁵ 'd-(T ₂₀) ^{3'}	34 ^d
20b	38 ^a	33 ^b	22a	36 ^d
	—	—	22b	35.5 ^d

^a Oligonucleotide concentrations are 0.1 μ M, each strand, in 10 mM sodium cacodylate, pH 7, buffer containing 0.1 M NaCl.

^b Oligonucleotide concentrations are 1 μ M (with U/A = 2) in 10 mM sodium cacodylate, pH 7, buffer containing 0.1 M NaCl.

^c Oligonucleotide concentrations are 1 μ M (with T/A = 1) in 66.5 mM, pH 7, phosphate buffer. ^d Oligonucleotide concentrations are 1 μ M (with T/A = 1) in 10 mM sodium cacodylate, pH 7, buffer containing 0.1 M NaCl.

or 280 nm was plotted versus the A/T concentration ratio (data not shown). The break occurred at a 1:1 ratio, indicating the formation of a double helix. We considered that the presence of thiazole orange in the conjugates should not have changed the stoichiometry of their binding with their complementary targets. For all the oligo-2'-deoxyriboadenylate-thiazole orange conjugates (**19a**, **19b**, **20a**, and **20b**) studied, the addition of 1 equiv of the complementary sequences at 3 °C induced a change of the absorption spectra between $\lambda = 240$ and $\lambda = 600$ nm (data not shown). Only changes observed between $\lambda = 400$ and $\lambda = 600$ nm will be discussed. Addition of 1 equiv of ⁵d-CACT₁₅CAC^{3'} to oligo-2'-deoxyriboadenylate-thiazole orange conjugates **19a**, **19b**, **20a**, and **20b** induces a blue shift of the λ_{max} of the visible spectra of thiazole orange 3, 4, 4, and 4 nm, respectively, without a significant change in intensity.

In all cases, the melting temperatures of the duplexes formed by the oligo-2'-deoxyriboadenylates and their complementary sequences increased when the thiazole orange derivative was linked to the oligonucleotides with similar $\Delta T_m = +8.5$ and $+7.5$ °C for oligonucleotide-thiazole orange conjugates **19a** and **19b** with the hydrophilic linker, respectively (Table 1). In the case of oligonucleotide-thiazole orange conjugates **20a** and **20b** involving the polymethylene linker, ΔT_m differences of +8 and +5.5 °C were observed, respectively. The greatest stabilization was observed for oligonucleotide-thiazole orange conjugate **20a** (with the *R_p* configuration) with the linker directed toward the minor groove. This result is consistent with literature data indicating that the homodimeric thiazole orange TOTO intercalates with the nitrogen atom of the quinolinium ring in the minor groove (36). The lower stabilization observed with the second isomer **20b** could be explained by the fact that the linker was directed toward the major groove and intercalation of thiazole orange is only possible if the linker fold-back is in the direction of the minor groove. Being so, the linker may be too short to permit full intercalation. In the case of conjugates **19a** and **19b**, the linker length is a little longer than it is for conjugates **20a** and **20b**. Therefore, in both cases full intercalation is possible, giving nearly the same *T_m* values.

To test the ability of the thiazole orange moiety to intercalate into DNA/RNA hybrids, we have studied the interaction of the conjugates **20a** and **20b** involving the polymethylene linker with poly r(U). Adding increasing concentrations of poly r(U) to a 0.1 μ M solution of ⁵d-

(A₁₅)^{3'} or **20b** in 10 mM sodium cacodylate, pH 7, buffer containing 0.1 M NaCl at 3 °C led to a sharp break when the absorbance at 260 nm was plotted versus the A/U concentration ratio (data not shown). The break occurred at a 1:2 ratio, indicating the formation of a triple helix. These results indicating the formation of a triplex with both the unmodified 5'-d-(A₁₅)^{3'} and the conjugate **20b** are consistent with the intercalation of the thiazole orange. Thus, when the isomer **20b** (*R_p* configuration) is hybridized with poly r(U) forming a duplex, unless being intercalated, the thiazole orange is directed toward the major groove and should prevent the binding of a second strand of poly r(U) in the major groove to form a triplex.

Addition of 2 equiv of poly r(U) to conjugates **20a** and **20b** induces a red shift of the $\lambda_{\text{vis}}^{\text{max}}$ value (≈ 5 nm) of the visible spectra of thiazole orange in both cases. No significant change in intensity of the spectra was observed, but the general shape was less broad on the left part of the visible band of the spectra. These changes are reversed when increasing the temperature. The melting of the triple helices formed between the conjugates **20a** or **20b** (or the unmodified pentadecadeoxyriboadenylate) and poly r(U) showed only one transition. Once again, the presence of the thiazole orange stabilized the duplexes (Table 1) with $\Delta T_m = +8.6$ and $+6.6$ °C for oligonucleotide-thiazole orange conjugates **20a** and **20b**, respectively. These results are in accordance with intercalation of the thiazole orange in DNA/RNA complexes.

Icosa- α -thymidylate-Thiazole Orange Conjugates 21a and 21b and Icosa- β -thymidylate-Thiazole Orange Conjugates 22a and 22b. In the same way, the stability of the complexes formed by the thiazole orange-icosa- α -thymidylate conjugate isomers **21a** and **21b** and the thiazole orange-icosa- β -thymidylate conjugate isomers **22a** and **22b** with their target poly r(A) was compared to those of the complexes formed between the corresponding icosathymidylates and poly r(A) (Table 1). In the case of the thiazole orange-icosa- α -thymidylate conjugates **21a** and **21b**, the studies were performed in the buffer used for microinjections in cells for in situ hybridization experiments, whose results will be discussed elsewhere, while in the case of thiazole orange-icosa- β -thymidylate conjugates **22a** and **22b** the buffer used was the same as for the studies carried out with oligoadenylates.

The spectra of the two icosa- α -thymidylate-thiazole orange conjugates **21a** and **21b** were almost identical. Adding increasing concentrations of α -T₂₀ to a poly r(A) solution (20 μ M) in 66.5 mM phosphate, pH 7, buffer at 3 °C led to a sharp break when the absorbance at 260, 270, or 280 nm was plotted versus the A/T concentration ratio (data not shown). The break occurred at a 1:1 ratio, indicating the formation of a double helix. As in the case of the oligo-2'-deoxyriboadenylate-thiazole orange conjugates **19a**, **19b**, **20a**, and **20b**, we considered that the presence of thiazole orange in the conjugates should not have changed the stoichiometry of their binding with their complementary targets.

In the presence of 1 equiv of the complementary sequence poly r(A), differences could be observed for the two isomers. While no change of the $\lambda_{\text{vis}}^{\text{max}}$ value (508 nm) was observed for compound **21a**, the general shape of the spectrum was less broad as compared to that for compound **21a** alone. In the case of the other isomer (compound **21b**) in the presence of 1 equiv of the target sequence, the spectrum was also less broad as compared to that for the conjugate alone and also there was a small red shift of the $\lambda_{\text{vis}}^{\text{max}}$ value (≈ 2 nm). But in the latter case the change could be observed on the left part of the

visible band of the spectrum, while for the first isomer (**21a**) changes were observed on both the left and right sides of the spectrum. No notable variations in intensity were observed in the spectra of compounds **21a** and **21b** upon hybridization with the target sequence. Spectral modifications observed between $\lambda = 400$ and 600 nm when 1 equiv of conjugates **21a** or **21b** was mixed with 1 equiv of poly r(A) at 3 °C were, however, reversed upon a temperature increase to 70 °C.

In the case of duplexes formed between icosa- α -thymidylate-thiazole orange conjugates **21a** and **21b** with poly r(A), the presence of thiazole orange did not induce any stabilizing effect (Table 1). These results indicate that intercalation of thiazole orange is unlikely to occur. This may probably be due to the difference in the structure of the duplex formed between the icosa- α -thymidylate and the poly r(A). Oligo- α -thymidylates have been reported to hybridize with poly r(A) in the antiparallel orientation, forming a type A duplex (37), but to our knowledge examples of intercalation in such duplexes have never been reported. To address this point, we have synthesized and studied the hybridization properties of the icosa- β -thymidylate-thiazole orange conjugate isomers **22a** and **22b**. The unmodified icosa- β -thymidylate was used as reference. The spectra of the icosa- β -thymidylate-thiazole orange conjugate isomers **22a** and **22b** were almost identical. The stoichiometry for the binding with poly r(A) was also equal to 1. But once again, no significant change of the visible absorption spectra of the thiazole orange was observed upon the mixing of conjugates **22a** and **22b** with 1 equiv of poly r(A), and melting studies of the duplexes indicated that hardly no stabilization ($\Delta T_m = +2$ and $+1.5$ °C for oligonucleotide-thiazole orange conjugates **22a** and **22b**, respectively) was induced by the presence of the thiazole orange (Table 1). Another explanation for this absence of stabilization could be that intercalation of TOTO was reported to occur with the quinolinium ring placed between purine bases and the thiazole ring between pyrimidine bases (36). Linkage of the thiazole orange on the pyrimidine strand (via the quinolinium ring) compared to the purine strand should change possible intercalation interactions.

We have reported the synthesis and binding properties of oligo-2'-deoxyribonucleotide-thiazole orange conjugates. The thiazole orange label was coupled to the eighth phosphate of a pentadeca-2'-deoxyriboadenylate via a phosphoramidate linkage. Two different linkers, either hydrophilic or more hydrophobic, were used to tether the oligonucleotide to the nitrogen atom of the quinolinium ring of the thiazole orange moiety. Substitution at the internucleotidic position introduces a chirality at the phosphorus atom with the formation of two stereoisomers. The latter were separated, and their absolute configurations were determined. Finally, the thiazole orange moiety was also tethered to the tenth phosphate of an icosa- α -thymidylate using the hydrophobic polymethylene linker. Once again, the thiazole orange-icosa- α -thymidylate conjugates were obtained as pure isomers. The binding properties of these oligo-2'-deoxyribonucleotide-thiazole orange conjugates with their complementary sequences were studied by absorption spectroscopy. In the case of the pentadeca-2'-deoxyriboadenylate series, the presence of the label induces a stabilization of the complexes formed both with the DNA and with the RNA targets. This is consistent with the intercalation of the thiazole orange moiety. In contrast, the stability of the duplexes formed between the icosa- α -thymidylate derivatives and poly r(A) is similar to that of the corresponding

unmodified duplex, indicating the absence of intercalation of the thiazole orange. Studies carried out with the corresponding icoso- β -thymidylate derivatives indicated also a lack of stabilization by covalent linking of the thiazole orange in the presence of poly r(A). These results could be explained by the fact that in this case the thiazole orange is linked to the pyrimidine strand via the quinolinium ring when intercalation of the TOTO was reported to take place with the benzothiazole ring between the pyrimidine bases. Work to address this question will be developed in the near future.

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A Ribonuclease H–Oligo DNA Conjugate That Specifically Cleaves Hepatitis B Viral Messenger RNA

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Ribonuclease H (RNaseH) recognizes and efficiently cleaves the RNA strand of DNA–RNA hybrids, but has no inherent sequence selectivity. However, the formation of DNA–RNA hybrids does require specific sequence recognition. On the basis of this concept, we wondered whether antisense oligonucleotides complementary to target RNA covalently linked to RNase H could be used to direct specific cleavage events mediated by RNase H. The aim of this research was to couple a DNA oligonucleotide to RNase H to confer specificity of ribonuclease activity toward hepatitis B viral (HBV) mRNA. A modified 13-base oligonucleotide that is specific for the DR1 region of HBV mRNA was conjugated to modified *E. coli* RNase H using a water soluble cross-linker. A 1200 base fragment of HBV RNA including the DR1 region was synthesized as a substrate using T7 RNA polymerase. Incubation of the RNase H–oligonucleotide conjugate with the RNA transcript resulted in cleavage of the HBV mRNA transcript in a concentration dependent manner. Eighty-five percent of substrate was cleaved under optimal conditions. Controls consisting of RNase H alone, oligonucleotide alone, and incubation of the conjugate with an unrelated mRNA substrate resulted in no cleavage activity. RNase H coupled to an HBV antisense oligonucleotide can specifically cleave target HBV transcripts.

INTRODUCTION

Ribonuclease H (RNase H) is an enzyme found in a variety of organisms ranging from viruses to mammalian cells and functions as an endonuclease that can recognize and cleave the RNA strand of a DNA–RNA hybrids (1–4). *E. coli* RNase H1 is one of the best characterized (3, 5). It requires divalent cations and generates products with 5'-phosphate and 3'-hydroxyl termini (6, 7). However, RNase H activity lacks sequence specific recognition (1–3). Coaddition of antisense molecules with the enzyme has been used to direct specificity to RNase H cleavage (8–11). However, to be biologically useful, this requires that the antisense oligos and enzyme be present in the same intracellular compartment as the target and in effective concentrations. With this goal in mind, the aim of this research was to determine whether an antisense oligo DNA could be chemically coupled to RNase H to specifically target RNase H activity to the 3' direct repeat 1 (DR-1) region of the HBV¹ mRNA.

MATERIALS AND METHODS

RNase H Purification. An RNase H overproducer mutant strain (rnh gene transformed into MIC 1066, an RNase H deficient strain of *E. coli*) was a gift from Robert Crouch, NIH. RNase H was purified according to the method of Ma et al. (1). In brief, an overnight culture

was diluted in LB plus 100 μ g/mL ampicillin and grown until the OD₅₅₀ reached 1.0. Isopropylthio- β -D-galactosidase (IPTG) (Gibco, Grand Island, NY) was added to a final concentration of 1 mM, and the culture was grown for an additional 5 h. Cells were harvested by centrifugation at 1500g, 15 min at 4 °C. Cell pellets were resuspended in 0.2 M KCl, 50 mM Tris HCl, pH 7.9, 5 mM MgCl₂, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride and disrupted by sonication on ice using 5 $\times$ 15 s bursts at amplitude 40. Cell lysates were clarified by centrifugation, diluted with 50 mM Tris HCl, pH 7.9, 0.1 mM EDTA to an ionic strength equivalent to 50 mM NaCl, 50 mM Tris HCl, pH 7.9, 0.1 mM EDTA. Clarified cell lysates were loaded onto a DEAE-Sepharose column, 5 $\times$ 6 cm (Amersham Pharmacia Biotech, Uppsala, Sweden), equilibrated with 50 mM Tris HCl, pH 7.9, 0.15 M NaCl, 0.1 mM EDTA. A DEAE-sepharose column was connected in series to a P-11 phosphocellulose column, 2.5 $\times$ 20 cm (Whatman International Ltd, Maidstone, England) equilibrated with the same buffer. RNase H was eluted using 50 mM Tris HCl, pH 7.9, 0.1 mM EDTA, 0.75 M NaCl (1). Aliquots of eluted fractions were run on a 15% SDS–PAGE. Fractions found to contain RNase H were pooled and desalted using a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden) and concentrated through P10 centricon concentrators (Amersham Pharmacia Biotech, Uppsala, Sweden). A Bradford assay was performed to determine protein concentration (12).

Conjugation of RNase H and Oligonucleotide. A modified 13-base oligonucleotide (GCAGAGGTGAAGC) specific for the DR1 site on HBV adw strain (13) was synthesized by Keck Oligonucleotide Synthesis Facility at Yale University. The 5'-guanosine was amino-modified at the 6-carbon to provide a substrate for the *N*-(γ -maleimidobutyryloxy) sulfosuccinimide ester, sulfo-GMBS (Pierce Chemical Co, Rockford, IL), linkage, and all other

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¹ Abbreviations: HBV, hepatitis B virus; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; IPTG, isopropylthio- β -D-galactosidase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

residues were phosphorothioate modified. The oligonucleotide, 360 nM, was incubated with a 50-fold molar excess of sulfo-GMBS in PBS pH 7.4 for 1 h at 25 °C. RNase H, 90 nM, was incubated with 100 mM dithiothreitol (DTT) (Sigma, St. Louis, MO) in PBS pH 7.4 at 25 °C for 1 h. Both were purified by elution through a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden) with PBS pH 7.4. The peaks from the DR1 oligonucleotide-GMBS and the reduced RNase H were pooled and incubated overnight at 4 °C. Conjugate was purified by passing through a HiTrap Q column (Amersham Pharmacia Biotech, Uppsala, Sweden) using a stepwise NaCl gradient, 0.3 M to 1.0 M NaCl (1). Concentrations were determined by Bradford assay and spectrophotometer readings at A_{260} . Conjugation and purity were checked by SDS-PAGE, agarose gels, and radiolabeling experiments. Activity was determined by cleavage assays as described below.

SDS-PAGE and Agarose Gels. To visualize the protein and determine the efficacy of purification, 5 μ g of RNase H and conjugate (containing the equivalent to 5 μ g RNase H) were run on a 10% SDS-PAGE. Bands were visualized by Coomassie Blue staining. To detect DNA in samples of conjugate, 5 μ g of free oligonucleotide and conjugate (containing the equivalent of 5 μ g of oligo DNA) were run on a 1% agarose gel in 0.045 M Tris-borate, 1 mM EDTA buffer, and stained with ethidium bromide to visualize DNA.

Radiolabeling. To follow conjugation products and extent of purification, the 3'-OH ends of both oligonucleotides alone and conjugates were labeled with γ - ^{32} P ATP using terminal deoxynucleotidyl transferase, Promega, Madison, WI (14). Samples with a specific activity of 2000 cpm/ng were run on a denaturing 12% polyacrylamide gel containing 8 M urea and autoradiographed (15).

Cleavage Assays. HBV and control β -globin RNA templates were synthesized from plasmid DNA using T7 polymerase (Gibco Life Technologies, Grand Island, NY). The HBV RNA template was created from an adw R9 plasmid (gift from Dr. T. J. Liang, NIH) resulting in a fragment of 1200 bases, which included the DR1 site. A control transcript consisted of a similar size fragment from nonrelated pLMR3 rabbit β -globin plasmid DNA (gift from Dr. Gordon Carmichael, University of Connecticut). One microgram (0.25 pmol) of RNA transcript was incubated with 2.5 μ mol of RNase H alone, oligonucleotide alone, or a mixture of 2.5 μ mol unconjugated RNase H plus 2.5 μ mol oligonucleotide, or 5 μ mol of conjugate in reaction buffer consisting of 40 mM Tris pH 7.5, 4 mM $MgCl_2$, 1 mM DTT, 150 mM NaCl, plus anti-RNase (Ambion, Austin, TX), which does not inhibit RNase H (Ambion Manufacturer's note).

To assess the effects of inhibitory agents on cleavage activity of the conjugate, 0.25 pmol of HBV transcript was incubated with 5 μ mol of oligo-RNase H conjugate alone or plus 25 μ M EDTA (ethylenediaminetetraacetic acid), 25 μ M EGTA [ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid] or 25 μ M NEM (N -ethylmaleimide). To determine concentration dependence, cleavage assays were performed using increasing amounts of conjugate with constant amounts of substrate. A comparison of conjugated versus unconjugated RNase H activity was made using 5 μ mol of conjugate versus decreasing amounts of unconjugated RNase H and oligonucleotide in equimolar ratios. As a control for nonspecific cleavage, conjugate was added to a nonrelated β -globin RNA transcript. All cleavage assays were performed by incubation for 1 h at 37 °C and run on 2.2 M formaldehyde, 1% agarose gels in MOPS buffer (0.02 M MOPS

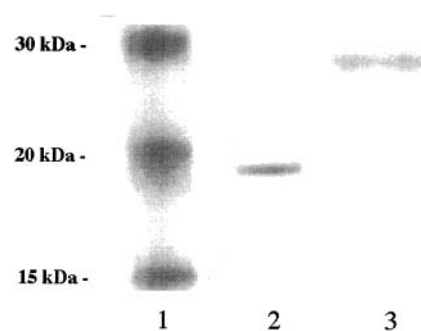


Figure 1. 10% SDS-PAGE of purified mutant RNase H and oligo-RNase H conjugate prepared as described in the Materials and Methods section. Lane 1, molecular weight marker; lane 2, 5 μ g of mutant RNase H; and lane 3, 5 μ g of oligo-RNase H conjugate.



Figure 2. An autoradiogram of an RNase H conjugate composed of a ^{32} P-oligo linked to mutant RNase H and analyzed on a 12% polyacrylamide gel in 8 M urea as described in the Materials and Methods section. Lane 1, 7.5 ng of ^{32} P-oligo alone; lane 2, 7.5 ng of ^{32} P-oligo-RNase H conjugate. Both samples had a specific activity of 2000 cpm/ng.

[3-(N -morpholino)propanesulfonic acid (Sigma, St. Louis, MO), 8 mM sodium acetate, 1 mM EDTA]. RNA bands were visualized by ethidium bromide staining and quantitated by densitometry (16).

RESULTS

From 3 L of LB culture medium, a yield of 32 mg of RNase H was obtained. SDS-PAGE analysis of purified protein revealed a band of approximately 18000 Da, which corresponds to the published size of mutant RNase H (1), Figure 1, lane 2. The oligonucleotide DNA-RNase H conjugate migrated at a position corresponding to approximately 29000 Da in the SDS-PAGE gel, lane 3.

To visualize the oligonucleotide DNA component of the RNase H conjugate, and to determine whether the sample had any contaminating free DNA, the conjugate was radiolabeled, electrophoresed, and autoradiographed. Figure 2 shows that the conjugate, lane 2, produced a labeled band migrating much slower than free oligonucleotide, lane 1, as similarly seen on the SDS-PAGE in Figure 1. Contaminating free oligonucleotide was not

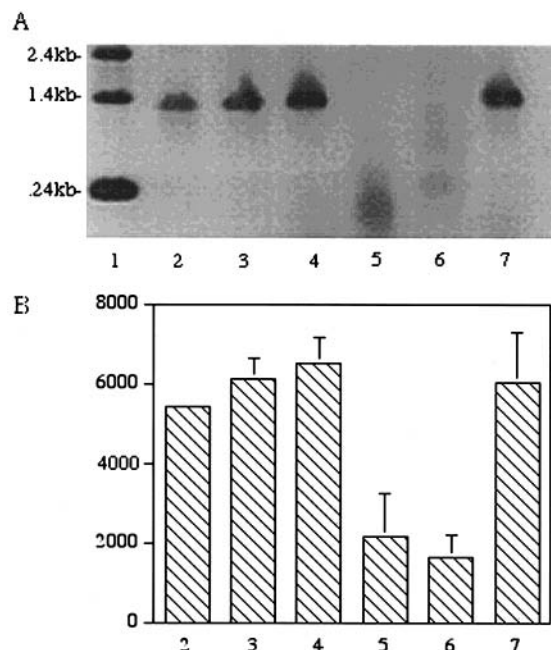


Figure 3. Panel A. Formaldehyde-agarose gel of cleavage of 0.25 pmol of 1200 bp HBV transcript stained with ethidium bromide. Lane 1, RNA molecular weight markers; lane 2, transcript alone; lane 3, transcript plus 2.5 μ mol of RNase H; lane 4, transcript plus 2.5 μ mol of oligo; lane 5, transcript plus 2.5 μ mol of free oligo plus 2.5 μ mol of RNase H; lane 6, transcript plus 5 μ mol of oligo-RNase H conjugate; and lane 7, 0.25 pmol of β -globin transcript plus 5 μ mol of oligo-RNase H conjugate. Panel B. A graph of densitometric analyses of residual substrate HBV RNA present in the corresponding lanes of which Panel A shows a representative. All assays were performed in triplicate and the results expressed as means $\pm$ SD in arbitrary densitometric units.

seen in the conjugate sample, indicating that within the limits of detectability, the purification process successfully eliminated uncoupled oligo DNA. From the protein and DNA assays of the conjugate, it was calculated that the RNase H and oligonucleotide were linked in molar ratio of one to one.

To determine whether the conjugated oligonucleotide DNA-RNase H conjugate was functional, cleavage activity was studied using HBV mRNA substrates containing the target DRI sequence. In Figure 3, lane 2, the HBV substrate transcript is shown without exposure to any enzyme. Lane 3 shows that exposure of the HBV substrate to RNase H alone failed to result in any detectable cleavage. Similarly, lane 4 shows that incubation of HBV substrate with oligonucleotide DNA alone had no effect. However, lane 6 shows that transcripts incubated with conjugate were nearly completely digested with little intact substrate remaining. This result is similar to the positive control containing unconjugated RNase H and oligonucleotide mixed in the same proportions as provided by the conjugate and incubated under identical conditions, lane 5. A control, unrelated transcript having no HBV sequences, incubated with conjugate in the same proportions as in lane 6, showed no detectable cleavage, lane 7. Densitometry revealed that incubation of conjugated oligonucleotide DNA-RNase H or the mixture of enzyme plus free oligonucleotide DNA decreased substrate by more than 85%.

As controls for nonspecific degradation, digestions of substrate HBV mRNA with oligonucleotide DNA-RNase H conjugate were carried out in the presence of inhibitors. Figure 4 shows that addition of 25 μ M EDTA, a magnesium chelator, to the incubation mixture containing RNA

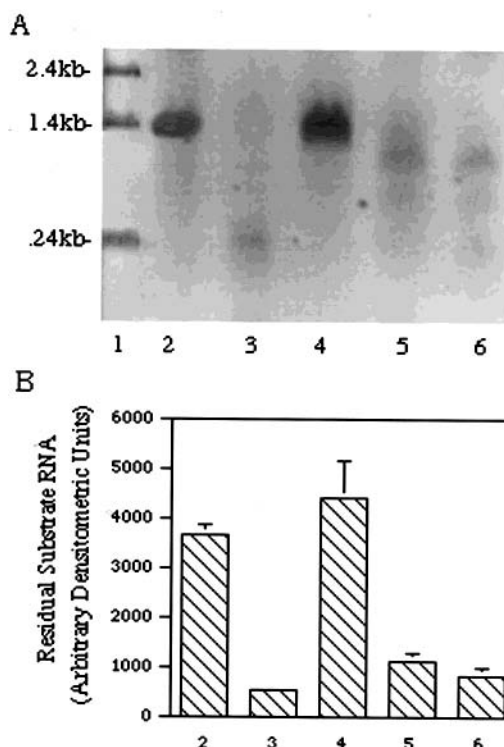


Figure 4. Panel A. Formaldehyde-agarose gel of 0.25 pmol of HBV transcript cleavage products in the presence of chelating and alkylating agents, and stained with ethidium bromide. Lane 1, RNA molecular weight markers; lane 2, transcript alone; lane 3, transcript plus 5 μ mol of oligo-RNase H conjugate; lane 4, same as lane 3 plus 25 μ M EDTA; lane 5, same as lane 3 plus 25 μ M EGTA; and lane 6, same as lane 3 plus 25 μ M NEM. Panel B. A graph of densitometric analyses of residual substrate HBV RNA present in the corresponding lanes of gels which Panel A shows a representative. All assays were performed in triplicate and the results expressed as means $\pm$ S. D. in arbitrary densitometric units.

substrate prevented cleavage of HBV transcript by the conjugate, lane 4. In contrast, addition of EGTA, a calcium chelator, lane 5, or NEM, which interferes with cysteine residues, lane 6, had no significant inhibition of cleavage activity. These results are consistent with the known requirement of the RNase H enzyme for magnesium, but not calcium for cleavage activity (1,3,17). The mutant RNase H1 used in these conjugation experiments is known not to be susceptible to inhibition by sulfhydryl reagents and, therefore, is not affected by NEM (18). However, it has been shown that NEM does inhibit native RNase H1 by the steric effects of sulfhydryl modification (7). In the current studies, RNase H1 was mutated to eliminate all but one cysteine and that remaining cysteine was modified by linkage to the oligonucleotide DNA, therefore eliminating the availability of cysteine residues for interference by NEM. Furthermore, the data argue against significant contamination with RNase H2 or other RNases susceptible to inhibition by sulfhydryl reactive agents.

The dependence of cleavage activity on conjugate concentration is shown in Figure 5. The gel shows that with a constant amount of starting substrate RNA of 0.25 pmol, decreasing the amounts of oligonucleotide-RNase H conjugate from 5 to 0.625 μ mol resulted in decreasing amounts of substrate cleaved, lanes 2-6, respectively. Quantitatively, this represented a decrease from 68% at 5 μ mol to 24% at 0.625 μ mol as shown in panel B. Additional bands can be seen in this gel and correspond with the predicted sizes resulting when the HBV tran-

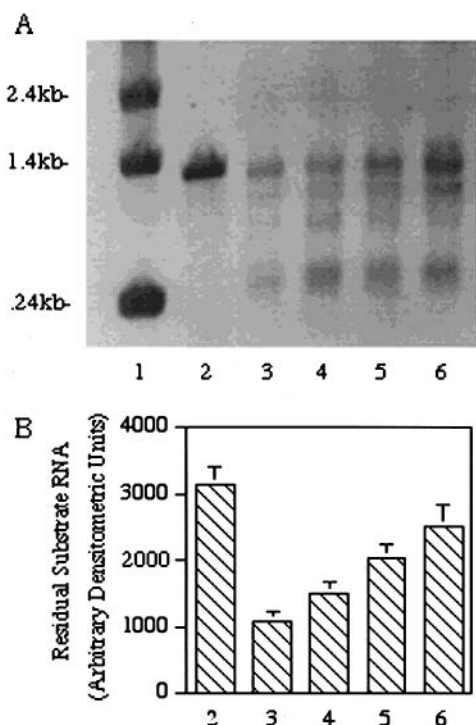


Figure 5. Cleavage of 0.25 pmol of HBV transcript by varying amounts of conjugates assayed by agarose gels stained with ethidium bromide. Panel A: Lane 1, RNA molecular weight markers; lane 2, transcript alone; lane 3, transcript plus 5 μ mol of oligo-RNase H conjugate; lane 4, transcript plus 2.5 μ mol of conjugate; lane 5, transcript plus 1.25 μ mol of conjugate; and lane 6, transcript plus 0.625 μ mol of conjugate. Panel B: A graph of densitometric analyses of residual substrate HBV RNA present in the corresponding lanes of gels which Panel A shows a representative. All assays were performed in triplicate and the results expressed as means $\pm$ SD in arbitrary densitometric units.

script is cleaved, 180, 230, and 772 nucleotides. In most studies these bands were not seen, most likely due to degradation of the smaller more unstable fragments.

To estimate the activity of the conjugated RNase H relative to the unconjugated enzyme, HBV transcript, 0.25 pmol, was incubated with conjugate, 5 μ mol, or with varying amounts of free enzyme plus free oligo in equimolar amounts relative to each other. Figure 6 shows that the cleavage activity of 5 μ mol of conjugated enzyme, lane 3, was equal to the activity seen with between 0.25 μ mol, lane 5, and 0.025 μ mol, lane 4, of mixed enzyme and free oligonucleotide. By this calculation, the conjugate was approximately 5% as active as a mixture of free enzyme and oligo on an equimolar basis.

DISCUSSION

Although a DNA virus, HBV undergoes replication through a pregenomic RNA intermediate that serves not only as a template for replication, but also as a messenger RNA species (19). Because of its importance in the viral life cycle, cleavage of this intermediate could prevent synthesis of new virus. An agent that could result in sequence specific destruction of this entity could be valuable not only in the study of the HBV replication, but also, potentially as a therapeutic agent. The data presented in this report suggest that chemical linkage of the oligonucleotide to RNase H can confer HBV sequence specificity and still maintain enzymatic activity. Because the enzyme and oligonucleotide DNA are chemically linked, the two components are brought into physical proximity, and therefore have the potential to be

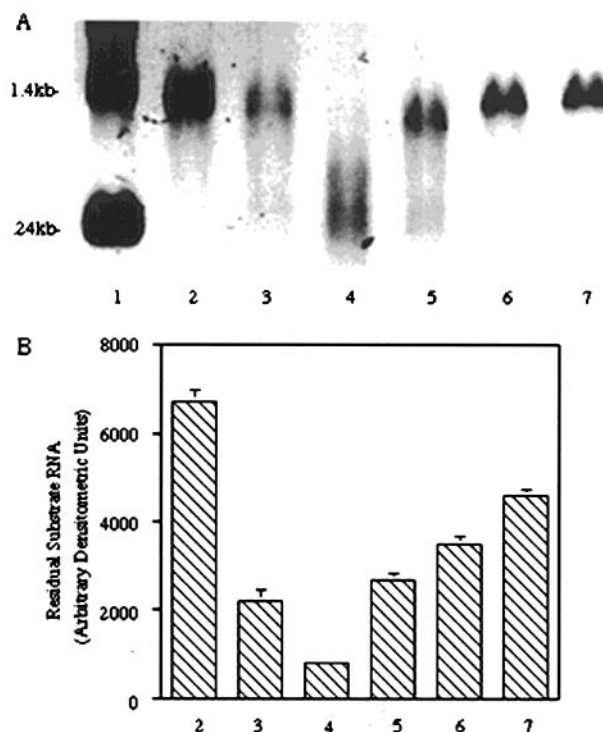


Figure 6. Cleavage of 0.25 pmol of HBV transcript with varying concentrations of free oligo and mutant RNase H, assayed by agarose gels stained with ethidium bromide. Panel A: Lane 1, RNA molecular weight markers; lane 2, transcript alone; lane 3, transcript plus 5 μ mol of oligo-RNase H conjugate; lane 4, transcript plus 2.5 μ mol of free oligo and 2.5 μ mol of RNase H; lane 5, transcript plus 0.25 μ mol of free oligo and 0.25 μ mol of RNase H; lane 6, transcript plus 0.025 μ mol of free oligo and 0.025 μ mol of RNase H; and lane 7, transcript plus 2.5 nmol of free oligo and 2.5 nmol of RNase H. Panel B: A graph of densitometric analyses of residual substrate HBV RNA present in the corresponding lanes of gels which Panel A shows a representative. All assays were performed in triplicate and the results expressed as means $\pm$ SD in arbitrary densitometric units.

delivered together into intracellular compartments. In addition, the chemical linkages created between oligonucleotide-cross-linker-RNase H are noncleavable (20) and should remain stable within intracellular compartments.

E. coli and human RNase H1 share many of the same enzymatic properties including the requirement of divalent cations. The sites of cation binding, substrate binding, and enzyme activity are highly conserved in all RNase H molecules (4). Several lysine groups in the molecule's substrate binding site are thought to interact with the phosphate groups on the substrate, and the site of this interaction is thought to be in the minor groove (1, 3). The cysteine 13 residue was chosen for disulfide linkage of antisense DNA as it is close to the active site on the enzyme, but is not essential for cleavage activity (1). The other cysteine residues, also not necessary for cleavage activity, were mutated to serine and alanine in order not to interfere with sulfo-GMBS linkage of the oligonucleotide. A restriction site was added for cloning purposes to create the mutant RNase H. This molecule was shown to have 90% activity of wild type (1). Ma et al. showed that these modifications do not interfere with binding to the target mRNA.

The antisense oligonucleotide used in current system was modified in two ways. The first was the formation of phosphorothioate linkages to inhibit degradation by cellular nucleases. Heteroduplexes consisting of RNA and

phosphorothioate-linked DNA have been shown to be suitable substrates for RNase H activity (3, 21). The second was an addition of an amino group to the 5' carbon. This provided a functional group for modification by a coupling agent for conjugation of the oligonucleotide to the mutated RNase H without interfering with the sequence specificity and binding activities. The mutations in RNase H eliminated other potentially reactive cysteines, thus permitting specific modification and coupling of the oligonucleotide to the cysteine 13 residue in the enzyme.

The length of the antisense oligonucleotide DNA is also an important consideration. The minimum RNA-DNA hybrid that has been found to result in cleavage is six nucleotides. Sequences greater than six but less than nine have been shown to be cleaved but at slower rates (3). However, the longer the length of an oligonucleotide the more likely the possibility of nonspecific binding and cleavage (2). On the basis of this information, a 13-mer sequence was selected to be used in the current studies.

The cleavage of the HBV transcript by the conjugated ribonuclease H was expected to result in fragments of 180, 230, and 772 nucleotides, based on the complementary sites predicted to be recognized by the oligonucleotide. Some of the smaller fragments were barely or not detectable in some of the cleavage assays in this current study. It is likely that the resulting cleavage fragments were unstable, and were degraded quickly as has been reported in other studies on antisense oligonucleotides against other target RNAs (22).

Successful cleavage was seen when the conjugate was incubated with the HBV RNA, but not with the rabbit β -globin transcript, indicating that the observed cleavage was not due to nonspecific degradation of the target. The fact that neither RNase H nor oligonucleotide alone resulted in cleavage activity confirmed that neither had intrinsic nonspecific nuclease activity or were contaminated with another RNase, and that the presence of both RNase H and DNA together with the RNA substrate was required for cleavage activity.

The RNase H activity in the conjugate was found to be less than that of RNase H alone, estimated to be approximately 5% of unconjugated RNase H. This is similar to but higher than activity reported by Ma et al. for a conjugated RNase H against mouse β -globin message in which activity of the conjugate was only 0.3% that of free enzyme mixed with oligonucleotide DNA (1). The difference in results may be due to the differing affinities of the oligonucleotide DNA of their respective target RNA sequences.

Experiments combining the catalytic properties of RNase H and the binding specificity of antisense DNA have been used previously to obtain sequence-specific cleavage of the target template (1, 9–11). Most of these studies involved mixing of antisense DNA with the RNase H to obtain specificity of cleavage. However, to be of practical value, both components need to be delivered to the same compartment in effective concentrations. The data in the current studies indicate that it is possible to specifically couple RNase H in an equimolar ratio of enzyme to oligonucleotide, virtually ensuring simultaneous codelivery of both components at that fixed molar ratio, and represents the first demonstration that such a chimeric molecule can specifically cleave an HBV target mRNA.

To be useful, delivery of RNase H-antisense oligonucleotide into cells must occur. This could be achieved using lipofectamine, a polycationic lipid, which has been suc-

cessful in delivering nucleic acids (23), and proteins into cultured cells without loss of biological activity (24). Alternatively, for specific delivery into liver cells, liver-specific carrier consisting of liver cell recognition component and nucleic acid binding component may be employed to bring RNase H-antisense oligonucleotide into liver cells (23).

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Transformation from Block-Type to Graft-Type Oligonucleotide-Glycopolymer Conjugates by Self-Organization with Half-Sliding Complementary Oligonucleotides and Their Lectin Recognition

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Block-type oligonucleotide-glycopolymer conjugates bearing α -mannosides and β -galactosides were prepared by coupling 5'-thiol-modified oligonucleotides with iodoacetamidated glycopolymers that were synthesized by telomerization. The conjugates minimally affected the DNA conformation and melting behavior of the duplex. Their self-organization via hybridization with the half-sliding complementary oligonucleotides produced graft-type conjugates or macromolecular gapped DNA duplexes grafted with glycopolymers at regular intervals, which was confirmed using size exclusion chromatography and electrophoresis. The binding affinity of block-type and self-organized graft-type conjugates to lectins was investigated using fluorometry. The affinity of the graft-type duplex assembly bearing mannosides to Con A was approximately 2 times stronger than that of block-type single-stranded or double-stranded conjugates with full complementary oligonucleotides. The organization strategy of DNA-glycopolymer conjugates might be useful for constructing novel glyco-clusters and also for developing a new methodology for gene therapy.

INTRODUCTION

The control of the well-defined structures of materials is a major goal of chemistry, materials science, and nanotechnology. Self-organization strategies are increasingly recognized as an efficient way to produce nanometer-scaled structural and functional materials. ODNs¹ have attracted recent attention as a "molecular glue" for supramolecular architectonics based on the self-organization strategy (1–10). ODNs are promising construction materials to arrange functional components in controlled space and direction, because DNA forms a linear, rigid double-strand helix by complementary hydrogen-bonded base-pairing. Thus, oligonucleotides offer several advantages over nonbiologic linker molecules. The size and stability of DNA-based supramolecules can be controlled through the sequence and length of the ODNs and also by changing parameters of the aqueous environments, such as concentration, solvent, temperature, pH, and electrolyte.

Oligosaccharide chains, especially as glyco-clusters, on cell surfaces participate in biologic molecular recognition and signal transduction through carbohydrate-binding proteins (11, 12). Various glycoconjugate polymers (13–26), dendrimers (27–29), calixarenes (30), nanospheres (31), and transition metal complexes (32, 33) have been developed as glyco-cluster models. Construction of glyco-clusters by the self-organization strategy is very promising for the development of a new field of glyco-clusters, but there have been only a few reports on its use (34–36).

There are several self-organized glyco-clusters in nature. In aggrecans (the major proteoglycan of cartilage), for example, many glycosaminoglycan chains (keratan and chondroitin-sulfates specifically) are attached to a protein core, and the resulting aggrecan molecules are noncovalently bound to a hyaluronan backbone through small proteins linked at regular intervals (37, 38). The noncovalent association of these proteoglycans strongly influences the properties of tissues, matrix–cell interaction, cell–cell adhesion, and diffusion of large molecules in the matrix (39, 40). Artificially self-organized glyco-clusters were prepared, for example, via incorporation of glycopolymer-carrying lipids into liposomes (34) and also as self-assembled monolayers of glycopolymers on the surface of silver colloids (35). The lectin affinities of these assemblies of glycopolymers were stronger than those of the glycopolymers themselves, and depended on the surface density of the glycopolymer chain. The distance among the glycopolymers, however, could not be controlled using these methods.

ODNs are useful as a scaffold for constructing glyco-clusters using self-organization, because ODNs produce periodic and space-controlled assemblies by hybridization due to their strict sequence-specificity and the rigid linear conformation of the duplexes. We recently synthesized

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¹ Abbreviations: ODNs, oligodeoxynucleotides; hscODN, half-sliding complementary ODN; SEC, size exclusion chromatography; FITC, fluorescein isothiocyanate; **5-Gal**, 2-(*N*-(methacryloyl)amino)ethyl β -D-galactopyranoside; **5-Man**, 2-(*N*-(methacryloyl)amino)ethyl α -D-mannopyranoside; poly(Gal)*n*-NH₂ and poly(Man)*n*-NH₂, their respective glycopolymers carrying an amino function at the terminal of the polymer chain; poly(Gal)-*n*-CH₂-I and poly(Man)-*n*-CH₂-I, their respective iodoacetamido glycopolymers; ODN-Gal *n* conjugate and ODN-Man *n* conjugate, their respective conjugates where "*n*" stands for the degree of polymerization of the glycopolymers.

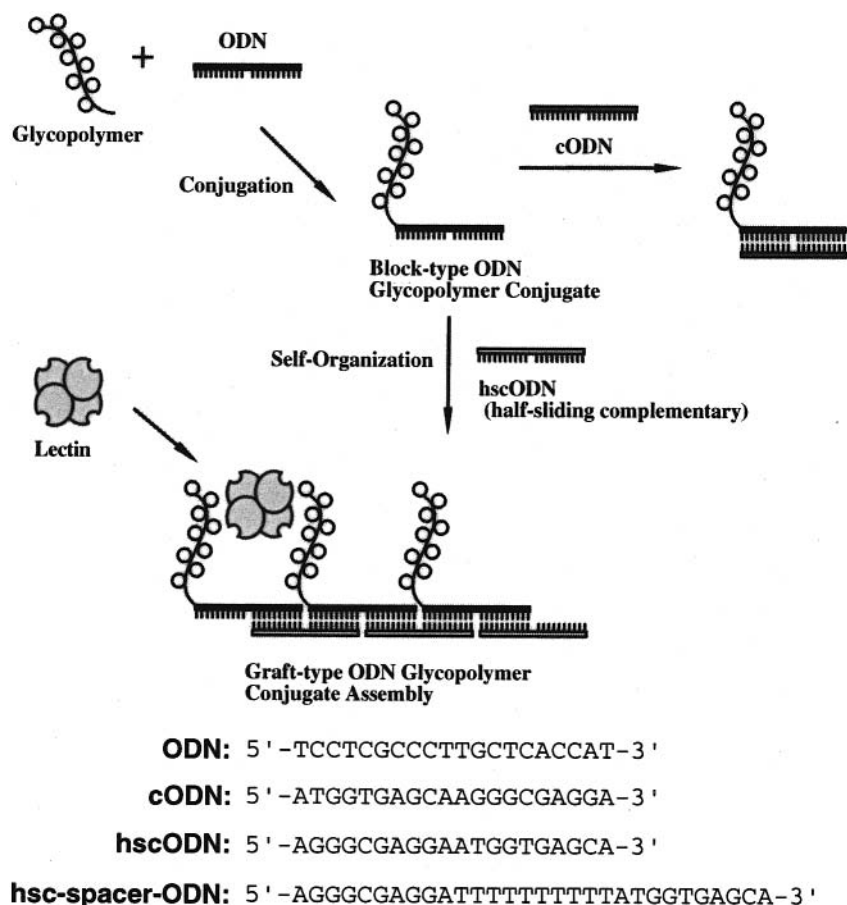


Figure 1. Schematic illustration of transformation from block-type to graft-type ODN-glycopolymer conjugates by self-organization assisted with the half-sliding complementary ODN.

site-specific galactosylated ODNs in an automated solid-phase synthesizer (41) and constructed glyco-clusters via self-organization with hybridization of the conjugate using half-sliding complementary ODN (36). In half-sliding complementary n -mer ODN, the respective right- and left-half sequences are designed to be complementary to the left- and right-half sequences of the target n -mer ODN. The hybridization produced nanometer-sized macromolecular assemblies, and the resulting periodic glyco-clustered ODNs were cooperatively recognized by the specific RCA₁₂₀ lectin.

In the present paper, we propose an easy method for constructing periodically clustered glycopolymers using self-organization of block-type ODN-glycopolymer conjugates via hybridization with half-sliding complementary (hsc) ODN (Figure 1). The block-type ODN-glycopolymer conjugates were prepared by conjugation of artificial iodoacetamidated glycopolymer with 5'-thiol-modified ODN. The self-organization of block-type conjugates in the presence of hscODN produced the graft-type conjugate assembly, which was confirmed using size exclusion chromatography (SEC) and electrophoresis, and characterized by CD spectroscopy and melting behavior. Enhanced binding affinity of the assembly to lectins was estimated using fluorometry.

EXPERIMENTAL PROCEDURES

Instruments. ¹H NMR spectra were recorded on Varian Gemini-200 and Gemini-500 NMR spectrometers. Absorption spectra were determined using a JASCO V-530 UV/VIS spectrophotometer. CD spectra were determined in a 10 mm quartz cell with a JASCO J-720 L

spectrophotometer. Fluorescence spectra were recorded on a JASCO FP-777 spectrophotometer equipped with a thermal controller. Mass spectra were obtained on a JEOL JMS-AX505HA mass spectrometer using the electron impact method operating in the positive mode. Infrared spectra were recorded with a JASCO Fourier transform IR-230 spectrophotometer. Optical rotation was determined with a JASCO DIP-1000 digital polarimeter using a water-jacketed 1 dm cell at 25 °C. SEC was recorded at 40 or 25 °C with JASCO Gulliver SB-804HQ to SB-803HQ columns and an RI detector (RI-930). Reverse-phase HPLC was recorded at 25 °C with a Shiseido Capcell Pak C18 UG120 (4.5 × 150 mm) and a JASCO Gulliver UV/VIS detector (UV-975). TLC was performed with a Merck TLC plate precoated with silica gel 60.

Materials. The following five types of ODNs were purchased from BEX Co., Ltd. (Japan): 5'-TCCTCGC-CCTTGCTCACCAT-3', 5'-TCCTCGCCCTTGCTCACCAT-3' modified with 6-mercaptohexyl phosphate at the 5'-end, 5'-ATGGTGAGCAAGGGCGAGGA-3', 5'-AGGGCG-AGGAATGGTGAGCA-3', and 5'-AGGGCGAGGATTTTTTTTTTATGGTGAGCA-3'. FITC-labeled *Ricinus communis* agglutinin (RCA₁₂₀, from *Ricinus communis*) and FITC-labeled concanavalin A (Con A, from Jack Bean, *Canavalia ensiformis*) were purchased from Sigma Co., Ltd. 2-Bromoethyl 2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranoside (42), 2-bromoethyl 2,3,4,6-tetra-*O*-acetyl-α-*D*-mannopyranoside (42), and *N*-iodoacetoxy succinimide (43) were prepared as previously reported. 2,2'-Azo-2-

amidinopropane dihydrochloride (AAPD, Wako Pure Chemical, Osaka) was purified by recrystallization from water.

2-Azidoethyl 2,3,4,6-Tetra-O-acetyl- β -D-galactopyranoside (2-Gal) (44). 2-Bromoethyl 2,3,4,6-tetra-O-acetyl- β -D-galactopyranoside (42) (**1-Gal**, 3.25 g, 7.15 mmol) was added to a solution of sodium azide (696 mg, 10.7 mmol) in DMF (33 mL). The mixture was heated at 65 °C. TLC (ethyl acetate/hexane = 1:1 in volume): R_f of **1-Gal** = 0.57 and **2-Gal** = 0.51. After 1 h, the mixture was diluted with chloroform and washed with water, and the organic layer was treated with MgSO_4 . The filtrate was concentrated in vacuo, chromatographed on a silica gel column (eluent, ethyl acetate/hexane 1:1 in volume), and concentrated in vacuo to afford pure **2-Gal** as a crystalline solid (2.98 g, 100% yield).

2-(N-(Methacryloyl)amino)ethyl 2,3,4,6-Tetra-O-acetyl- β -D-galactopyranoside (4-Gal). Palladium carbon (1.05 g) was added to a solution of **2-Gal** (4.20 g, 10.1 mmol) in THF (420 mL). The mixture was stirred under a hydrogen atmosphere at room temperature for 40 min. TLC (ethyl acetate/hexane = 1:2 in volume): R_f of **2-Gal** = 0.74 and **3-Gal** = 0. Palladium carbon was removed by filtration on Celite. The filtrate was used for the next step without further purification.

Methacryloyl chloride (1.97 mL, 40.3 mmol) was added to the reaction mixture and triethylamine (17.2 mL, 80.6 mL) in THF at 0 °C and stirred magnetically at room temperature for 30 min. TLC (ethyl acetate/hexane = 1:2 in volume): R_f of **4-Gal** = 0.25. The solution was concentrated in vacuo, chromatographed on a silica gel column (eluent, ethyl acetate/hexane 1:2 in volume), and concentrated in vacuo to afford pure **4-Gal** as a colorless syrup (3.71 g, 80% yield). $[\alpha]_D^{25} = +0.1^\circ$ (c 0.10 in chloroform). FAB-MS: m/z 460 ($M^+ + H$). ^1H NMR (CDCl_3): δ 6.21 (1H, br-m, $-\text{C}=\text{CHH}$), 5.71 (1H, br-m, $-\text{C}=\text{CHH}$), 5.39 (1H, dd, $J_{4,5} = 1.0$ Hz, H-4), 5.37 (1H, m, amido), 5.24 (1H, dd, $J_{2,3} = 10.0$ Hz, H-2), 5.01 (1H, dd, $J_{3,4} = 3.4$ Hz, H-3), 4.55 (1H, d, $J_{1,2} = 7.8$ Hz, H-1 of β -anomer), 4.15–4.12 (2H, m, H-6), 3.92–3.40 (5H, m, H-5 and $-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}$), 2.15, 2.04, 1.99, 1.96 (12H, 4s, $-\text{OCOCH}_3$), 1.56 (3H, s, $-\text{CH}_3$). IR (KBr): 2940, 1760, 1660, 1630, 1530 cm^{-1} . Anal. Calcd for $\text{C}_{20}\text{H}_{29}\text{O}_{11}\text{N}$: C, 52.29; H, 6.18; N, 3.05. Found: C, 52.26; H, 6.21; N, 3.01.

2-(N-(Methacryloyl)amino)ethyl β -D-Galactopyranoside (5-Gal). Sodium methoxide (6.6 mg) was added to a stirred solution of **4-Gal** (3.65 g, 7.95 mmol) in methanol (35 mL), and the mixture was stirred for 6 h. The mixture was neutralized with Amberlyst 15E ion-exchange resin, filtrated, and concentrated. Finally, the product was dissolved into water and freeze-dried, affording pure **5-Gal** as a white powder (2.30 g, 100% yield). mp: 61–63 °C. $[\alpha]_D^{25} = +4.0^\circ$ (c 0.10 in H_2O). FAB-MS: m/z 292 ($M^+ + H$). ^1H NMR (D_2O): δ 5.64 (1H, br-m, $-\text{C}=\text{CHH}$), 5.39 (1H, br-m, $-\text{C}=\text{CHH}$), 4.34 (1H, d, $J_{1,2} = 7.4$ Hz, H-1), 4.02–3.4 (10H, m, H-2 ~ H-6 and $-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}$), 1.86 (3H, s, $-\text{CH}_3$). IR (KBr): 3360, 2950, 1660, 1610, 1540 cm^{-1} . Anal. Calcd for $\text{C}_{12}\text{H}_{21}\text{O}_7\text{N}$: C, 49.48; H, 7.27; N, 4.81. Found: C, 49.44; H, 7.32; N, 4.77.

2-Azidoethyl 2,3,4,6-Tetra-O-acetyl- α -D-mannopyranoside (2-Man) (44). Compound **2-Man** was prepared in a similar manner as **2-Gal** (97% yield).

2-(N-(Methacryloyl)amino)ethyl 2,3,4,6-Tetra-O-acetyl- α -D-mannopyranoside (4-Man). Compound **4-Man** as a colorless syrup was prepared in a similar manner as **4-Gal** (82% yield from **2-Man**). $[\alpha]_D^{25} = +39.4^\circ$ (c 0.10 in chloroform). FAB-MS: m/z 460 ($M^+ + H$). ^1H NMR (CDCl_3): δ 6.22 (1H, br-s, $-\text{C}=\text{CHH}$), 5.72 (1H, br-

m, $-\text{C}=\text{CHH}$), 5.37 (1H, m, amido), 5.34 (1H, dd, $J_{3,4} = 10.0$ Hz, H-3), 5.27 (1H, t, $J_{4,5} = 10.0$ Hz, H-4), 5.25 (1H, dd, $J_{2,3} = 3.5$ Hz, H-2), 4.83 (1H, d, $J_{1,2} = 2.0$ Hz, H-1 of α -anomer), 4.26 (1H, dd, $J_{5,6b} = 5.0$ Hz, $J_{6a,6b} = 12.0$ Hz, H-6b), 4.12 (1H, dd, $J_{5,6a} = 2.5$ Hz, H-6a), 3.96 (1H, ddd, H-5), 3.84–3.50 (4H, m, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{Br}$), 2.16, 2.10, 2.05, 2.00 (12H, 4s, $-\text{OCOCH}_3$), 1.56 (3H, s, $-\text{CH}_3$). IR (KBr): 2940, 1760, 1660, 1630, 1530 cm^{-1} . Anal. Calcd for $\text{C}_{20}\text{H}_{29}\text{O}_{11}\text{N}$: C, 52.29; H, 6.18; N, 3.05. Found: C, 52.25; H, 6.23; N, 3.00.

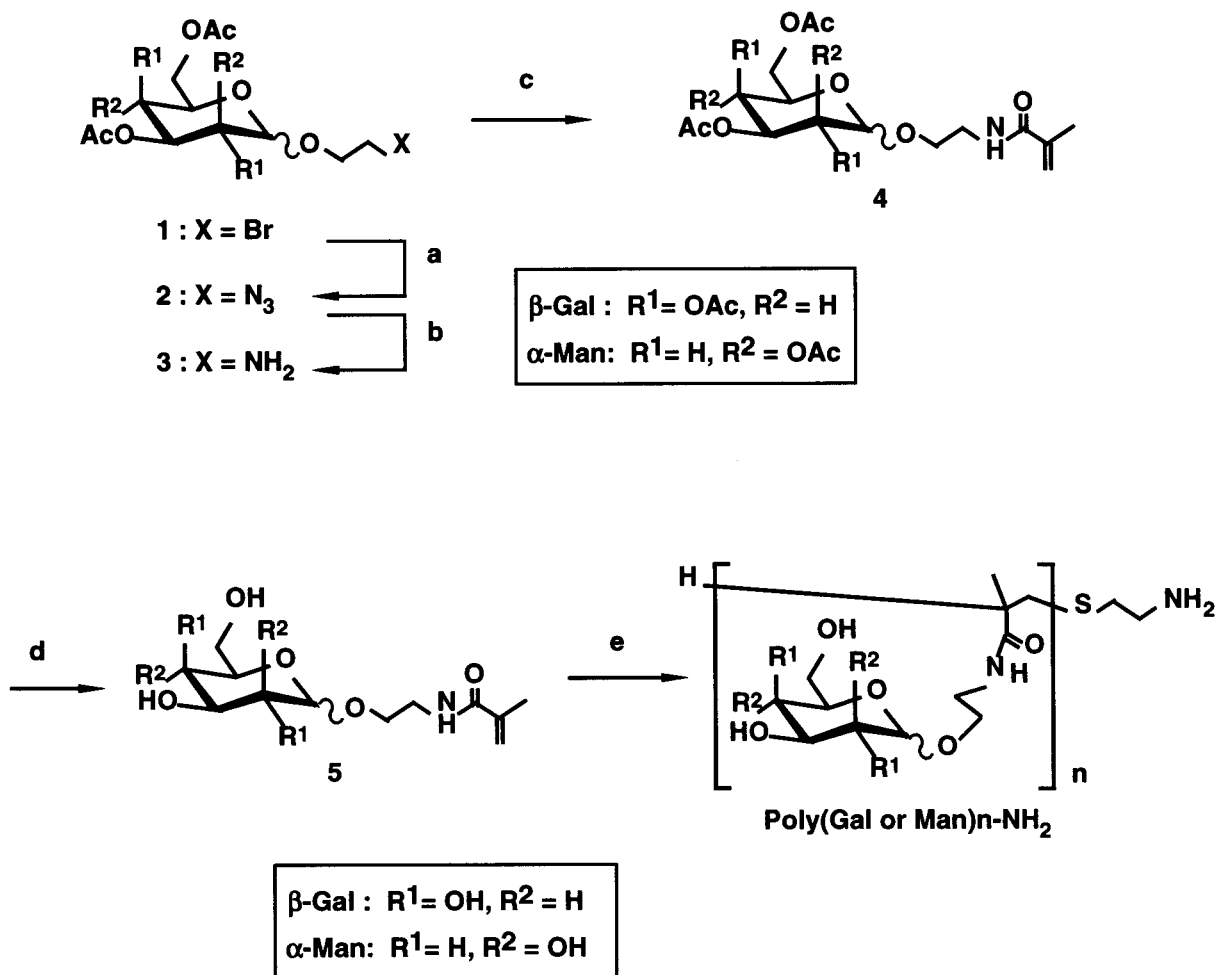
2-(N-(Methacryloyl)amino)ethyl α -D-Mannopyranoside (5-Man). Compound **5-Man** as a hygroscopic solid was prepared in a similar manner as **5-Gal** (99% yield). $[\alpha]_D^{25} = +39.7^\circ$ (c 0.10 in H_2O). FAB-MS: m/z 292 ($M^+ + H$). ^1H NMR (D_2O): δ 5.53 (1H, br-s, $-\text{C}=\text{CHH}$), 5.30 (1H, br-m, $-\text{C}=\text{CHH}$), 4.71 (1H, d, $J_{1,2} = 1.5$ Hz, H-1 of α -anomer), 3.77 (1H, dd, $J_{2,3} = 3.5$ Hz, H-2), 3.71–3.28 (9H, m, H-3 ~ H-6 and $-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}$), 1.78 (3H, s, $-\text{CH}_3$). IR (KBr): 3440, 2930, 1650, 1610, 1540 cm^{-1} . Anal. Calcd for $\text{C}_{20}\text{H}_{29}\text{O}_{11}\text{N}$: C, 52.29; H, 6.18; N, 3.05. Found: C, 52.24; H, 6.23; N, 3.02.

Telomerization (45, 46). Prescribed amounts of the monomer, AAPD, and cysteamine were charged in a glass ampule and dissolved in water. The ampule was placed in a carbon dioxide/methanol bath, and the solution was frozen and degassed. The procedure was repeated 3 times. The ampule was sealed under a reduced pressure and maintained at 60 °C. The ampule was chilled, and the product was reprecipitated from its aqueous solution into acetone. The precipitate was dissolved in water, and the solution was chromatographed through Sephadex G-25 with detection at 254 nm. Fractions containing the polymer were collected and freeze-dried to give a white powdery polymer.

The degree of polymerization was estimated from the ^1H NMR area ratios at 4.35 ppm for the anomer proton of galactoside (or at 4.75 ppm for the anomer proton of mannoside) and at 3.02 ppm for the methylene protons of the cysteamine moiety, and also estimated from SEC based on pullulan standards. ^1H NMR (D_2O): δ 4.35 (br-s, H-1 of β -anomer), 3.88–3.10 (br-m, H-2 ~ H-6 and $-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}$), 3.02 (br-m, $\text{S}-\text{CH}_2-\text{CH}_2-$), 2.68 (br-m, $\text{S}-\text{CH}_2-\text{CH}_2-$), 1.92–1.50 (br-m, $-\text{CH}_2-$), 1.10–0.70 (br-m, $-\text{CH}_3$).

Iodoacetylation of Glycopolymers (47). The glycopolymer bearing the terminal amino group [poly(Gal)37- NH_2 , 4.8 μmol , 50 mg] was dissolved in 1.0 mL of 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES)/NaOH buffer (pH 6.0), and then the iodoacetic acid *N*-hydroxysuccinimide ester (43) (93.4 μmol , 26.4 mg) in 200 μL of DMF was added to the solution. The progress of the reaction was monitored by the disappearance of the terminal amino groups of the glycopolymers, which was detected with ninhydrin after an aliquot of the reaction mixture was separated with TLC (ethyl acetate/acetic acid/methanol/water = 3:4:3:1 in volume): R_f of terminal amino and iodoacetamido glycopolymers = 0. After 3 h reaction at room temperature in the dark, the solution was chromatographed through a Sephadex G-25 column with detection at 254 nm. Fractions containing the product were collected and freeze-dried to obtain a white powdery polymer [poly(Gal)37- $\text{CH}_2\text{-I}$, 49.2 mg, 97%]. The iodoacetamido glycopolymer was stored in a freezer (–20 °C) and used in the following step. ^1H NMR (D_2O): δ 4.35 (br-s, H-1 of β -anomer), 3.88–3.10 (br-m, H-2 ~ H-6 and $-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}$ and $\text{S}-\text{CH}_2-\text{CH}_2-\text{N}$ and $-\text{CH}_2\text{-I}$), 1.92–1.50 (br-m, $-\text{CH}_2-$), 1.10–0.70 (br-m, $-\text{CH}_3$).

Conjugation of 5'-Thiol-Modified ODN with Iodoacetamido Glycopolymers (48). Triethylamine

Scheme 1. Synthesis of Galactoside and Mannoside Glycopolymers Bearing the Amino Terminal^a

^a Reagents and conditions: (a) NaN₃, DMF, 65 °C, 1 h. (b) H₂ gas, Pd/C, THF, rt, 40 min. (c) Methacryloyl chloride, triethylamine, THF, rt, 30 min. (d) Sodium methoxide, methanol, rt, 6 h. (e) Cysteamine, AAPD, water, 60 °C.

(1.6 μL) and iodoacetamido glycopolymer [poly(Gal)37-CH₂-I or poly(Man)42-CH₂-I, 18 μL of a 31.7 mM solution in water, 571 nmol] were added to a solution of ODN with a terminal thiol group (81.5 nmol) in 63.6 μL of water. The progress of the reaction was monitored using analytical reverse-phase HPLC on a Shiseido Capcell Pak C18 UG120 with a linear gradient of acetonitrile (2–30%) in 0.1 M ammonium acetate buffer (pH 7.0) over 30 min. After 2–3 h, the solution was loaded onto an anion exchange chromatograph with Qiagen resin (Qiagen GmbH, Germany), washed with water and 0.05 M aqueous NaCl, and then eluted with 0.5 M aqueous NaCl. The appropriate fractions were collected, and the product was precipitated into ethanol and centrifuged at 14 000 rpm for 15 min, to afford pure conjugates (block-type ODN-glycopolymer conjugates). Isolated yield from starting ODN was 80% for ODN-Gal37 conjugate and 75% for ODN-Man42 conjugate, which was determined from the UV absorbance in H₂O using OD₂₆₀ = 32.7 μg/mL.

Estimation of Organization of ODN by Gel Electrophoresis. Double-stranded ODN samples were prepared by mixing ODN-glycopolymer conjugates with equimolar amounts of full complementary ODN or half-sliding complementary ODN in PBS buffer (pH 7.4) at 25 °C for 1 h. The double-strand samples (total 10 μL) were loaded onto a 3% agarose gel, and electrophoresis was performed in 0.5 × TBE buffer (90 mM Tris and 90 mM boric acid) at 25 °C at 4 V/cm. DNA bands were stained in a 6 μg/mL ethidium bromide solution and

visualized by transillumination with ultraviolet light (310 nm) using a DC40 camera equipped with 1D Image Analysis Software MAC Ver 2.0 (Kodak Digital Science). The length of DNA relative to the *Hinf*I restriction enzyme digest of φX174 was determined by agarose gel electrophoresis.

Estimation of Organization of ODN by SEC. Double-stranded ODN samples were prepared by mixing the conjugates with equimolar amounts of full complementary ODN and half-sliding complementary ODN at 3.4 × 10⁻³ M-base concentrations in PBS buffer (pH 7.4) at 25 °C. After 1 h, SEC analysis of the double- and single-strand samples (20 μL) was performed. The eluent was 0.1 M phosphate buffer (pH 7.0) for glycopolymers and ODN-glycopolymer conjugates, and PBS buffer (pH 7.4) for organized ODN-glycopolymer conjugates. The flow rate was 0.5 mL/min. Pullulan was used as the molecular weight standard.

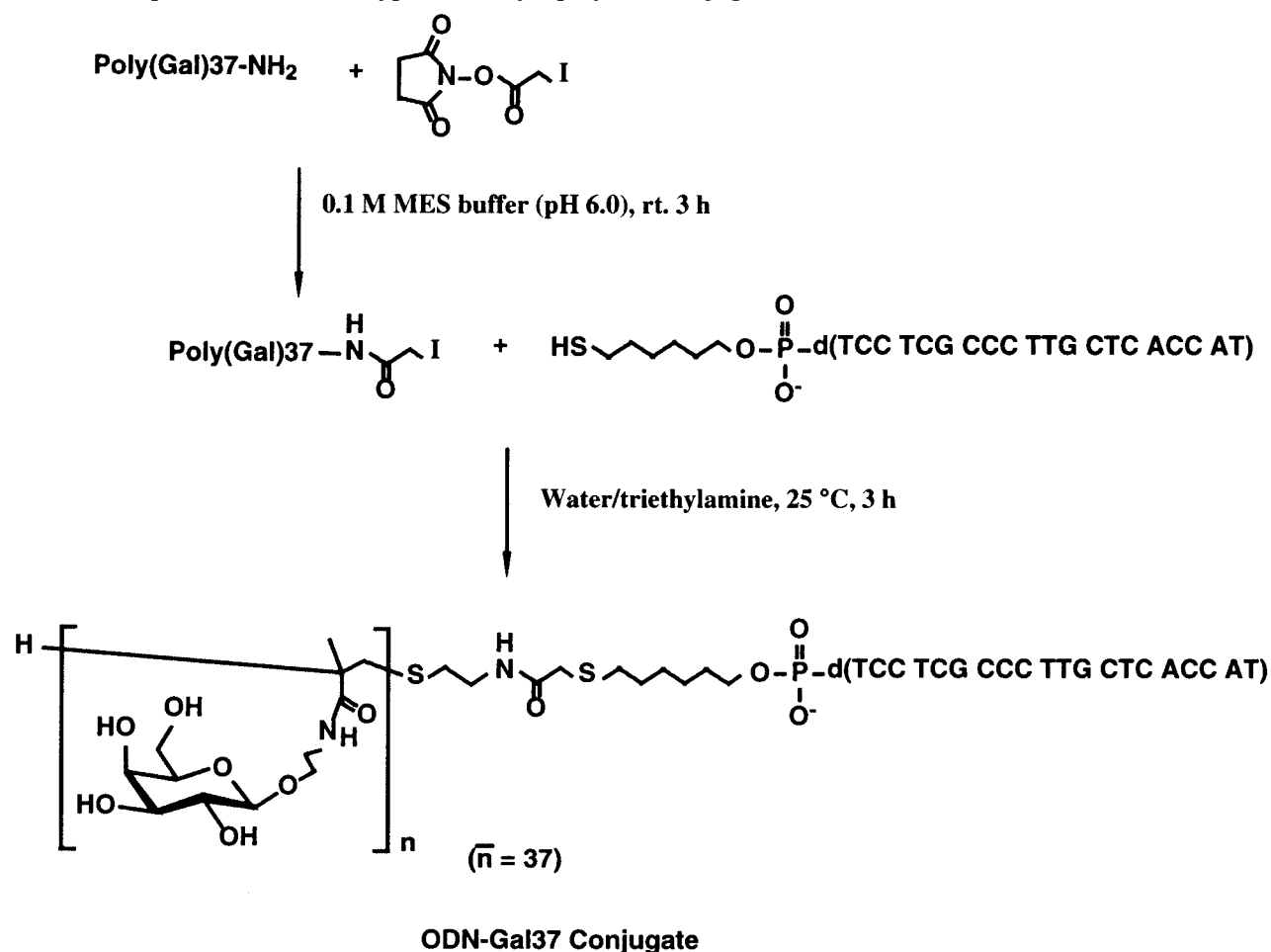
CD Spectra. Single-strand ODN samples were prepared by dissolving the conjugates in PBS buffer (pH 7.4) at 22.5 μM-base concentrations. Double-stranded ODN samples were prepared by mixing the conjugate with an equimolar amount of full complementary or half-sliding complementary ODN at 45.0 μM-base concentrations for 1 h. CD spectra of double strands were recorded at 25 °C under equilibrium conditions.

Melting Behaviors. Double-stranded ODN samples were prepared by mixing the conjugates with an equimolar amount of full complementary or half-sliding comple-

Table 1. Telomerization of Galactoside and Mannoside Monomers^a

entry	monomer (mmol)	cysteamine (mol %)	time (min)	yield (%)	SEC ^b			¹ H NMR ^c	
					M_n	M_w/M_n	DP	M_n	DP
1	5-Gal (0.515)	6.7	150	50	8200	1.5	28	10700	37
2	5-Gal (0.515)	10.0	150	64	6800	2.1	23	9900	34
3	5-Gal (0.309)	50.0	150	11	1500	3.5	5	7700	26
4	5-Man (2.06)	6.7	180	80	10800	2.0	37	ND	
5	5-Man (2.06)	6.7	150	78	8600	1.9	30	12300	42

^a Telomerization was performed with 2,2'-azo-2-amidinopropane dihydrochloride (AAPD, 2.9 mol % to the monomer) as the initiator and cysteamine as the telogen in water at 60 °C. ^b Using 0.1 M phosphate buffer (pH 7.0) eluent and pullulan standard. ^c DP was estimated from the ¹H NMR area ratios of signals for the anomer proton and the methylene protons of cysteamine moiety.

Scheme 2. Preparation of Block-Type ODN-Glycopolymer Conjugates

mentary ODN at 45.0 μ M-base concentrations in PBS buffer (pH 7.4). The absorbance was recorded at 260 nm as a function of increasing temperature at 2.0 °C/2 min on a spectrophotometer equipped with a thermal controller.

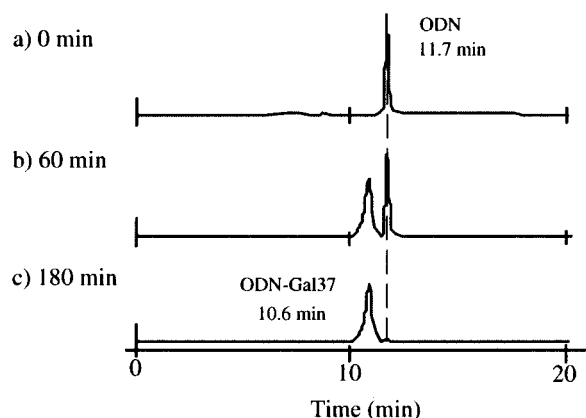
Fluorescence Assay of Lectin Binding. Aliquots (1 μ L) of a stock solution of 1.5 mM-sugar (to RCA₁₂₀) or 0.3 mM-sugar (to Con A) sample were added every 25 min to a solution of FITC-labeled RCA₁₂₀ lectin (14.7 nM, 600 μ L) in PBS buffer (pH 7.4) or to a solution of FITC-labeled Con A lectin (327 nM, 600 μ L) in PBS (pH 7.0) containing 0.1 mM Ca²⁺ and 0.1 mM Mn²⁺. Fluorescence spectra (500–600 nm) were recorded with excitation at 490 nm at 25 °C.

RESULTS AND DISCUSSION

Preparation of Block-Type ODN-Glycopolymer Conjugates. Our strategy for the molecular design of block-type ODN-glycopolymer conjugates applies a highly

efficient coupling of iodoacetamidated glycopolymer derivatives to commercially available ODN, modified with thiol at the 5'-terminal (43, 47, 48). Scheme 1 shows the synthesis of glycopolymers bearing an amino group at the terminal. Bromoethyl peracetylated β -galactosides and α -mannosides (42) were transformed to the methacrylamide monomers via a convenient synthetic route. The yields of β -galactoside monomers (**5-Gal** from **1-Gal**) and α -mannoside monomers (**5-Man** from **1-Man**) were 80% and 79%, respectively. The formation of **5-Gal** and **5-Man** was confirmed by ¹H NMR spectroscopy. Telomerization (45, 46) of **5-Gal** and **5-Man** was then performed with cysteamine as a telogen and 2,2'-azo-2-amidinopropane dihydrochloride (AAPD) as an initiator in water at 60 °C to produce glycopolymers bearing an amino group at the terminal, as summarized in Table 1. The telomerization proceeded homogeneously to give a viscous solution, and a powdery glycopolymer was obtained by reprecipitation from water into acetone, fol-

A) Coupling reaction profiles



B) A mixture of reference samples

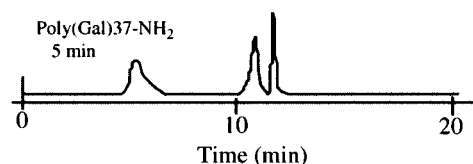


Figure 2. HPLC profiles for (A) the coupling reaction of 5'-thiol-modified ODN (20mer) with poly(Gal)37-NH₂ to give ODN-Gal37 conjugate. (B) Mixture of 5'-thiol-modified ODN (20mer), poly(Gal)37-NH₂, and isolated ODN-Gal37 conjugate. Shiseido Capcell Pak C18 UG120 column (4.5 × 150 mm); flow rate 1.5 mL/min; detected at UV 260 nm; temperature 25 °C; eluted with a linear gradient of 0.1 M ammonium acetate (pH 7.0) containing 2–30% acetonitrile over 30 min. The poly(Gal)-NH₂ sample was injected at a concentration approximately 100 times that of the ODN sample.

lowed by gel filtration chromatography. The formation of glycopolymers was confirmed using ¹H NMR spectroscopy. The average molecular weights of the glycopolymers were estimated by the ¹H NMR intensity of the cysteamine moiety and the anomer proton and by SEC. Because the ¹H NMR data were more reliable, the average degrees of polymerization were employed respectively for galactoside (*n* = 37) and mannoside (*n* = 42) glycopolymers in the following experiments.

Scheme 2 illustrates a simple synthetic route to block-type ODN-glycopolymer conjugates. The terminal amino group of the glycopolymer was iodoacetylated with *N*-iodoacetoxy succinimide in MES buffer. The introduction of the iodoacetamide group was quantitative, which was confirmed as follows: No residual amino groups were detected using the ninhydrin reagent, and ¹H NMR signals assignable to the cysteamine moiety were observed. Commercially available 5'-thiol-modified ODN (20mer) was coupled with 7 equiv of iodoacetamido glycopolymer in the presence of triethylamine for 2–3 h. The reaction profile was monitored by the absorbance of the nucleobases at 260 nm in reverse-phase HPLC (Figure 2A). There were no byproducts derived from ODN, suggesting that the coupling proceeded quantitatively in 180 min. The resulting conjugate was purified by anion exchange chromatography and by reprecipitation with ethanol. The isolated yields were 80% for the ODN-Gal37 conjugate and 75% for the ODN-Man42 conjugate. Figure 2B shows the results of the HPLC analysis of a mixture of the reactants and product, where a large amount of poly(Gal)-NH₂ sample (approximately 100 times that of ODN) was injected and was detectable at 260 nm. The peak of the conjugate was broader than that of ODN, and appeared between the retention times

A) ODN

B) Poly(Gal)37-NH₂

C) ODN-Gal37

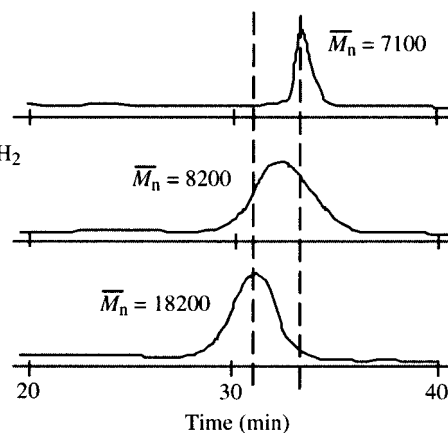


Figure 3. SEC chromatogram of (A) 5'-thiol-modified ODN (20mer), (B) poly(Gal)37-NH₂, and (C) ODN-Gal37 conjugate. Shodex OH Pak SB-804 HQ to SB-803 HQ; RI detector; eluent 0.1 M phosphate buffer (pH 7.0); flow rate 0.5 mL/min; temperature 40 °C.

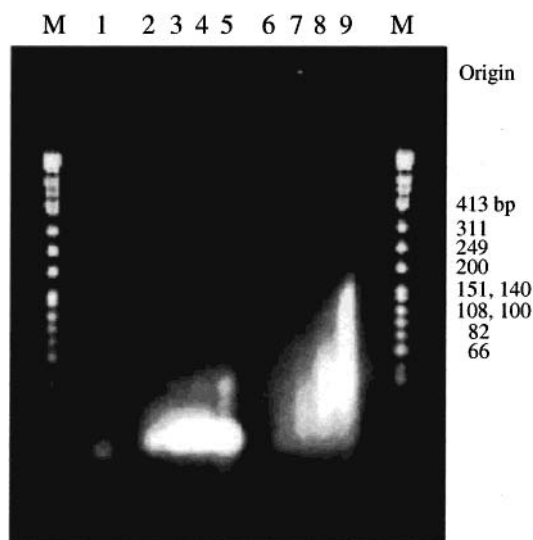


Figure 4. Gel electrophoresis analysis for self-organization of ODN with half-sliding cODN. Lane 1: single-strand ODN (3.3 mM-base). Lanes 2–5: ODN/full cODN duplex (0.7, 1.7, 3.3, and 6.7 mM-base, respectively). Lanes 6–9: ODN/hscODN duplex (0.7, 1.7, 3.3, and 6.7 mM-base, respectively). M: double-stranded DNA marker.

of ODN and the glycopolymer. In SEC analysis (Figure 3), the apparent molecular size of the conjugate was higher than those of glycopolymer and ODN alone.

Organization of ODN-Glycopolymer Conjugates.

Gel electrophoresis and SEC were performed to confirm whether self-organization of the block-type ODN-glycopolymer conjugates was accomplished by hybridization with hscODN to afford the graft-type conjugate assembly. Agarose gel electrophoresis (Figure 4) demonstrates that the ODN/hscODN bands (lanes 6–9) were broader and had shifted to a higher molecular weight than ODN/full cODN bands (lanes 2–5). The tendency was even more remarkable with an increase in the concentration, and the ODN/full cODN bands (lanes 2–5) were minimally shifted even at high concentrations (6.7 mM). ODN could be self-organized with the half-sliding complementary ODN to produce a high molecular weight assembly, the molecular size of which depended on the concentration. However, gel electrophoresis of the ODN-glycopolymer conjugates exhibited mobile bands that were too broad to examine the organization (data not shown).

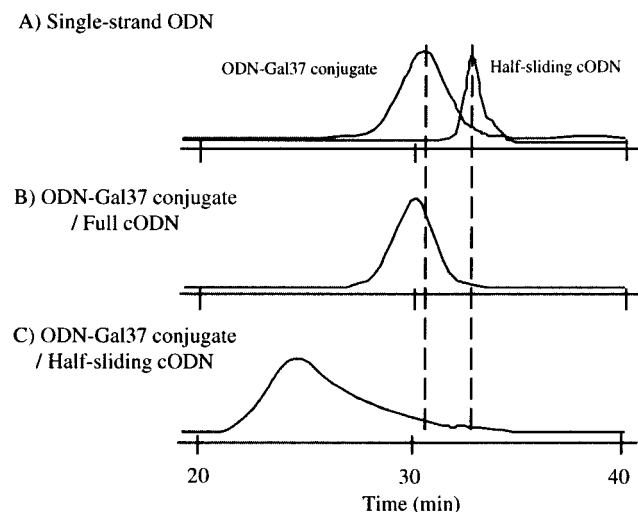


Figure 5. SEC analysis of (A) single-strand ODN-Gal37 conjugate and hscODN, (B) duplex of ODN-Gal37 conjugate with full cODN, and (C) duplex of ODN-Gal37 conjugate with hscODN. Shodex OH Pak SB-804 HQ to SB-803 HQ; RI detector; flow rate 0.5 mL/min; temperature 25 °C; eluted with PBS buffer, pH 7.4; pullulan standard. The concentration of ODN at the injection was 1.7 mM-base for single-strand ODN and 3.4 mM-base for double-strand ODN.

Figure 5 compares SEC of the single-strand ODN-Gal37 conjugate and its duplexes with full cODN and with hscODN. The peak of the conjugate/full cODN was shifted slightly to a higher molecular size ($M_n = 23\,300$) than those of the single-strand conjugate ($M_n = 17\,500$) and full cODN ($M_n = 4700$), indicating the formation of the full-matching double-stranded complex. The peak of the conjugate/hscODN occurred at a much higher molecular size ($M_n = 71\,300$), suggesting that the block-type ODN-glycopolymer conjugates were hybridized with hscODN to form the graft-type conjugate assembly.

As shown in Figure 6, the duplexes of unmodified ODN, ODN-Gal37, and ODN-Man42 with full cODN (A) and with hscODN (B) produced almost the same CD pattern, which was shifted slightly from the B-type to the A-type ODN duplex. This finding suggests that the glycopolymer moiety of the conjugates minimally affected the conformation of the duplex with full cODN and the self-organized duplex with hscODN.

The melting curves of the duplexes of ODN-glycopolymer conjugates with their full cODN (Figure 7A) and with hscODN (Figure 7B) in PBS buffer (pH 7.4) were almost the same as the corresponding curves of the duplexes of unmodified ODN. The glycopolymer moiety in the conjugates also minimally affected the stability of the ODN duplex. The result suggests that the glycopolymer chains dangle outside from the duplexes and interact minimally with the duplexes. Melting temperatures of the organized duplex with hscODN were approximately 40 °C, which was lower and broader than those with full cODN (68 °C). The 1:1 mixture between the conjugates and hscODN sufficiently formed the organized duplex at 25 °C.

Binding Affinity to Lectins. Binding affinity of the conjugates to lectins was estimated quantitatively by fluorimetry using FITC-labeled lectins to evaluate the effects of organization of the conjugates. Because the duplex of the conjugate/hscODN was sufficiently stable at 25 °C, the binding assay was performed at 25 °C with FITC-labeled RCA₁₂₀ (*Ricinus communis*, Gal β specific) and Con A (Jack Bean, *Canavalia ensiformis*, Man α or Glc α specific). The addition of an increasing amount of ODN-Man42/full cODN and ODN-Man42/hscODN de-

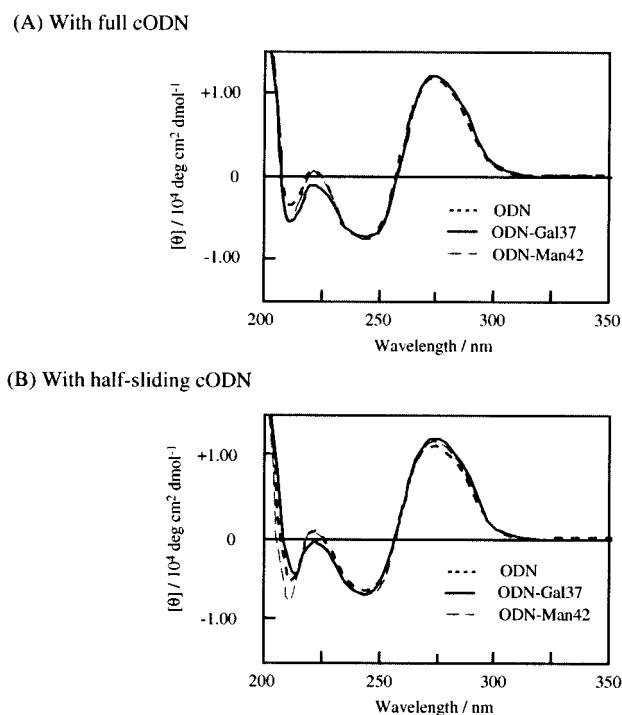


Figure 6. CD spectra of the duplexes of ODN-glycopolymer conjugates with (A) full cODN and (B) hscODN at a concentration of 45 μ M-base in PBS (pH 7.4) at 25 °C.

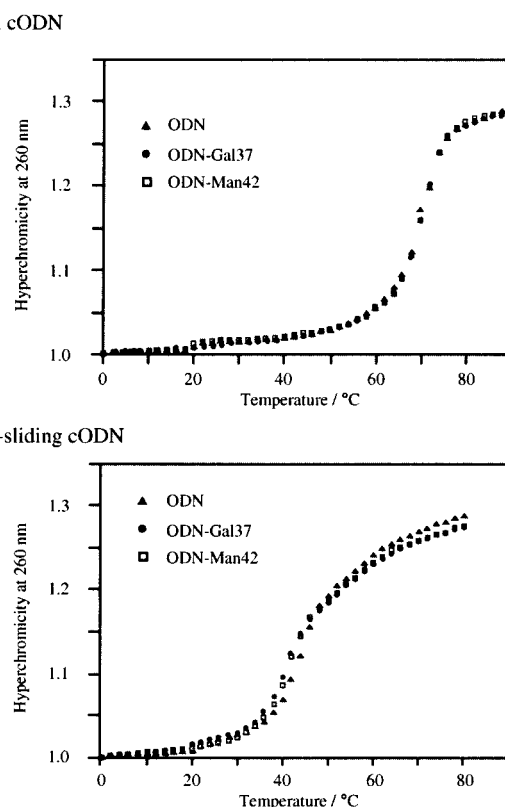


Figure 7. Melting behaviors of ODN-glycopolymer conjugates with (A) full cODN and with (B) hscODN. The melting curves were recorded at a heating rate 2 °C/2 min at a concentration of 45.0 μ M-base in PBS buffer (pH 7.4).

creased the fluorescence intensity at 520 nm (excitation at 490 nm) without causing any turbidity. The decrement of fluorescence intensity ($\Delta F/F_0$) was plotted against the concentration per sugar unit of the conjugate (Figure 8A). Equation 1 was applied to the saturation to obtain a

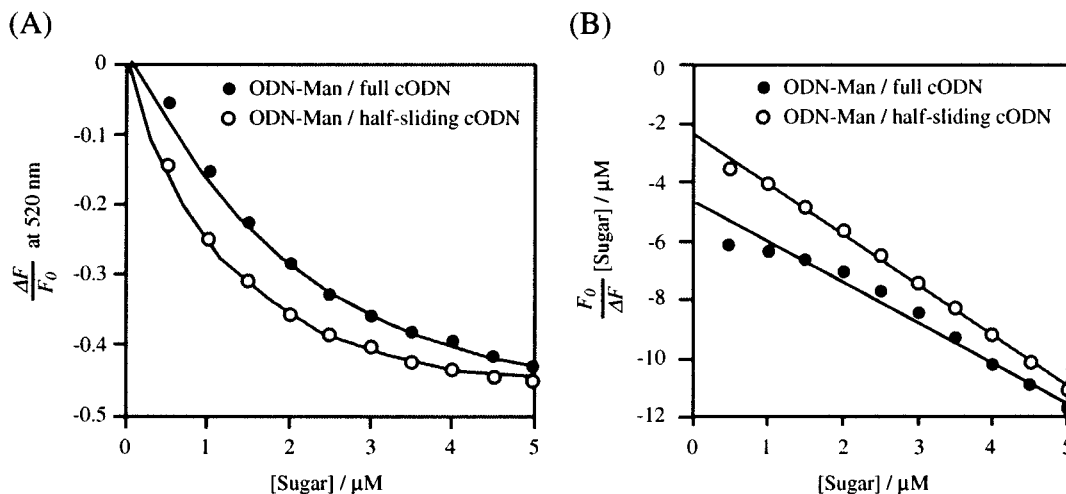


Figure 8. (A) Dependence of fluorescence intensity ($\Delta F/F_0$ at 520 nm, Ex 490 nm) of FITC-Con A on the concentration of the sugar moiety of the duplexes of ODN-Man42 conjugate with full cODN and with hscODN. Initial concentration of lectin = 327 nM in PBS containing 0.1 mM Mn^{2+} and 0.1 mM Ca^{2+} (pH 7.4) at 25 °C. (B) Linearized plot according to eq 1 for interaction of ODN-Man42 conjugate/full cODN and ODN-Man42 conjugate/hscODN with Con A.

Table 2. Association Constants (K_a) in Binding of ODN-Glycopolymer Conjugates to Lectins

compound	Con A $K_a/(\text{M-sugar})^{-1}$	RCA ₁₂₀ $K_a/(\text{M-sugar})^{-1}$
ODN-Man42/full cODN	3.0×10^5	$<10^2$
ODN-Man42/hscODN	7.3×10^5	$<10^2$
ODN-Man42/hsc-spacer-ODN	7.2×10^5	$<10^2$
ODN-Man42	4.1×10^5	$<10^2$
poly(Man)42-NH ₂	4.1×10^5	$<10^2$
ODN-Gal37/hscODN	$<10^2$	3.1×10^5
ODN-Gal37	$<10^2$	3.7×10^5
poly(Gal)37-NH ₂	$<10^2$	2.1×10^5

linear relationship (Figure 8B). Apparent affinity constants (K_a) were calculated from the intercept and slope as summarized in Table 2.

$$\frac{F_0}{\Delta F}[\text{sugar}] = \frac{F_0}{\Delta F_{\max} K_a} + \frac{F_0}{\Delta F_{\max}}[\text{sugar}] \quad (1)$$

Both Poly(Man)42-NH₂ and ODN-Man42 conjugate were bound to Con A with a similar affinity constant, $K_a = 4.1 \times 10^5 (\text{M-sugar})^{-1}$, which was approximately 10 times higher than that of the corresponding mannoside monomer. The cluster effect (14, 49, 50) was observed in the Poly(Man)42-NH₂ and ODN-Man42 conjugates, and the affinity to the lectin was not disturbed by the block-type conjugation of ODN. In addition, the hybridization of the ODN-Man42 conjugate with full cODN did not increase the affinity [$K_a = 3.0 \times 10^5 (\text{M-sugar})^{-1}$], but the hybridization of the ODN-Man42 conjugate with hscODN increased the affinity approximately 2 times [$K_a = 7.3 \times 10^5 (\text{M-sugar})^{-1}$]. It is important to note that the affinity was enhanced by the organization between the ODN-Man42 conjugate and hscODN. The effect was not as striking as we expected, in that a large amplification of affinity should be caused by the organization. On the other hand, the organized ODN-Gal37/hscODN duplex was bound to RCA₁₂₀ lectin with a high affinity constant, $K_a = 3.1 \times 10^5 (\text{M-sugar})^{-1}$, which was similar to those of poly(Gal)37-NH₂, ODN-Gal37, and ODN-Gal37/full cODN duplex to RCA₁₂₀. The organization effect of the ODN-Gal37 to RCA₁₂₀ was not expressed under these conditions.

The small organizing effect on the lectin affinity is discussed below. Kitano et al. reported that the binding ability of glycopolymers on Ag colloids to Con A was

increased by only approximately 2.5 times that of the glycopolymers at 25 °C because of the large steric hindrance (35). Therefore, it is possible that the present small organization effect is due to steric hindrance based on the short distance between glycopolymer chains along the ODN duplex: the calculated distance between carbohydrate recognition domains of ConA was approximately 6 nm, and the calculated distance between glycopolymers along the B-type duplex of 20 nucleobases was approximately 6.8 nm. To inspect the steric effect, thymine 10mer was inserted into the middle part of the hsc-ODN sequence as a spacer (the ODN is termed hsc-spacer-ODN). However, the binding affinity of the resulting ODN-Man42/hsc-spacer-ODN assembly to ConA was not improved [$K_a = 7.2 \times 10^5 (\text{M-sugar})^{-1}$]. It is possible that the length of the spacer may not be optimized or that the organized ODN-Man42/hsc-spacer-ODN assembly may be less stable than the organized ODN-Man42/nonspacer hsc-ODN assembly since the spacer thymine 10mer cannot form duplex but remains as single chain. At present, we assume that the large cluster effect is attained by the glycopolymers alone and, hence, the organization effect is not detectable.

CONCLUSION

We propose an easy method for transformation from block-type ODN-glycopolymer conjugates to the graft-type conjugate assembly by self-organization to construct periodically clustered glycopolymers. The block-type ODN-glycopolymer conjugates bearing galactosides and mannosides were prepared effectively by coupling of terminal-iodoacetamido glycopolymer with 5'-thiol-modified ODN and then self-organized by hybridization with hscODN. The conjugation and organization of the glycopolymers with ODN did not affect the conformation and melting behavior of ODN. The graft-type conjugate assembly ODN-Man42/hscODN was bound to ConA approximately 2 times stronger than the ODN-Man42/full cODN and the ODN-Man42 alone, but the effect was not as large as expected. On the other hand, the organization effect of the ODN-Gal37 with hscODN on the binding to RCA₁₂₀ was not expressed under these conditions. We expect that the proposed self-organization strategy to assemble glycopolymer conjugates might be useful for a new method of gene therapy and for novel neoglycoconjugates.

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Synthesis of Ring- and Side-Chain-Substituted *m*-Iodobenzylguanidine Analogues

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With the goal of developing MIBG analogues with improved targeting properties especially for oncologic applications, several radioiodinated ring- and side-chain-substituted MIBG analogues were synthesized. Except for 3- ^{131}I -iodo-4-nitrobenzylguanidine and *N*-hydroxy-3- ^{131}I -iodobenzylguanidine, the radioiodinated analogues were prepared at no-carrier-added levels from their respective tin precursors. The radiochemical yields generally were in the range of 70–90% except for 3-amino-5- ^{131}I -iodobenzylguanidine for which a radiochemical yield of about 40% was obtained. While the silicon precursor *N*¹,*N*²-bis(*tert*-butyloxycarbonyl)-*N*¹-(4-nitro-3-trimethylsilylbenzyl)guanidine did not yield 3- ^{131}I -iodo-4-nitrobenzylguanidine, its deprotected derivative, *N*¹-(4-nitro-3-trimethylsilylbenzyl)guanidine was radioiodinated in a modest yield of 20% providing 3- ^{131}I -iodo-4-nitrobenzylguanidine. Exchange radioiodination of 3-iodo-4-nitrobenzylguanidine gave 3- ^{131}I -iodo-4-nitrobenzylguanidine in 80% radiochemical yield. No-carrier-added ^{131}I -NHIBG was prepared from its silicon precursor *N*¹-hydroxy-*N*³-(3-trimethylsilylbenzyl)guanidine in 85% radiochemical yield.

INTRODUCTION

Despite its wide applications in oncology and cardiology, the radiopharmaceutical *m*-iodobenzylguanidine (MIBG¹) remains a less than ideal agent (Shapiro, 1991; Wafelman et al., 1994). Although MIBG is an effective diagnostic agent for neuroblastoma and pheochromocytoma (Gefland, 1993), its therapeutic efficacy is less than desired (Sisson et al., 1990). To augment the therapeutic effect of ^{131}I -MIBG, its use in a tandem with other modalities has been attempted (Gaze et al., 1995; Mas-trangelo et al., 1995). Both higher peak uptake in tumor and prolonged tumor retention of an endoradiotherapeutic agent are important for maximizing therapeutic effects. Although high uptake of radioiodinated MIBG in a number of neuroblastoma cell lines has been demonstrated, the ability of these cells to retain the radioactivity for more than a few hours was rather poor (Lashford et al., 1991; Mairs et al., 1991; Smets et al., 1989; 1990; 1991). A short biological half-life of MIBG in tumor has been observed in clinical settings as well (Sisson and Wieland, 1986), and attempts have been made to boost MIBG retention by pharmacological intervention (Blake et al., 1988).

Maximizing tumor-to-normal tissue ratios is an objective that needs to be given important consideration in order to reduce normal tissue toxicity and to improve the therapeutic outcome. Achieving higher tumor-to-normal tissue ratios rapidly is essential for diagnostic applications as well. One of the drawbacks of MIBG-aided diagnosis is that an elapsed period of 1–2 days is necessary to provide sufficient target-to-background ratios for tumor detection (Sisson and Shulkin, 1999). We have hypothesized that improvements in the selective tumor uptake and retention probably can be made by altering the structure of the MIBG molecule. For example, in SK-N-SH cells in vitro and in mouse target tissues in vivo, 4-fluoro-3- ^{125}I -iodobenzylguanidine (FIBG) was retained to a greater extent than MIBG itself (Vaidyanathan et al., 1997a). While tumor uptake depends in part on binding to norepinephrine transporter (Mairs et al., 1994; Glowinski et al., 1993; Hadrich et al., 1999), tumor retention to a certain degree is dictated by the stability of the compound. MIBG is known to be generally resistant to metabolism; however, tumor retention of radioactivity might be increased as well if MIBG was rendered less susceptible to deiodination by structural alterations. Indeed, one of the contributing factors for the enhanced retention of FIBG described above might be its higher resistance to deiodination.

In the current study, a number of ring- and side-chain-substituted MIBG derivatives have been synthesized with the goal of maximizing tumor-to-normal tissue ratios. Due to the encouraging results obtained with FIBG, we were interested in the properties of the corresponding chloro-analogue. Analogues with singly and multiply substituted polar groups such as nitro, amino, methoxy, and hydroxyl at different positions were also prepared. This paper deals with the syntheses of these novel analogues, and their biological evaluation is described in the accompanying paper.

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¹ Abbreviations: BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; CIBG, 4-chloro-3-iodobenzylguanidine; DHIBG, 3,4-dihydroxy-5-iodobenzylguanidine; DIAD, diisopropyl azodicarboxylate; FIBG, 4-fluoro-3-iodobenzylguanidine; HIBG, 4-hydroxy-3-iodobenzylguanidine; HIMBG, 4-hydroxy-3-iodo-5-methoxybenzylguanidine; *m*AIBG, 5-amino-3-iodobenzylguanidine; MIBG, *m*-iodobenzylguanidine; *m*INBG, 5-iodo-3-nitrobenzylguanidine; MOM, methoxymethyl; nca, no-carrier-added; NCS, *N*-chlorosuccinimide; NET, norepinephrine transporter; NHIBG, *N*¹-hydroxy-*N*³-3- ^{131}I -iodobenzylguanidine; *p*AIBG, 4-amino-3-iodobenzylguanidine; *p*INBG, 3-iodo-4-nitrobenzylguanidine; TPP, triphenylphosphine.

MATERIALS AND METHODS

General. All chemicals were purchased from Aldrich Chemical Co. unless otherwise noted. Sodium [$^{131/125}$ I]iodide in 0.1 N NaOH was supplied by DuPont-New England Nuclear (North Billerica, MA).

Melting points were determined on a Haake Buchler apparatus and were uncorrected. High-pressure liquid chromatography (HPLC) was performed using one of two systems: (1) a Beckman System Gold HPLC equipped with a Model 126 programable solvent module, a Model 168 diode array detector, a Model 170 radioisotope detector, and a Model 406 analog interface module; or (2) a Perkin-Elmer Series 4 Liquid Chromatograph connected to a Perkin-Elmer LC-95 UV/visible spectrophotometer detector and a Perkin-Elmer LCI-100 Laboratory Computing Integrator. Methods were programmed using a Perkin-Elmer 6312 display terminal. For reversed-phase chromatography, a Waters μ Bondapak C18 (10 μ m, 3.9 $\times$ 300 mm) column was used. Normal-phase HPLC was performed using a 4.6 $\times$ 250 mm (10 μ m) Partisil silica column (Alltech, Deerfield, IL). Gas chromatography was performed using a SRI 8610C gas chromatograph (SRI Instruments, Torrance, CA) equipped with an FID detector. The samples were analyzed using a RESTEK 15 m MXT-1 column and using helium as the carrier gas at 5 psi; different temperature programs were used for different samples. Analytical TLC was performed on aluminum-backed sheets (Silica gel 60 F₂₅₄), and normal-phase column chromatography was performed using Silica gel 60; both obtained from EM Science (Gibbstown, NJ). Column chromatographic fractions were collected either manually, by using a Gilson model 203 micro fraction collector (Middleton, WI) or by using an ISCO Foxy 200 fraction collector (Lincoln, NE), and the products were identified by TLC. In some cases, an ISCO UA-6 UV-Vis detector was placed between the column outlet and the fraction collector to identify fractions. Preparative thick layer chromatography was performed using 20 $\times$ 20 cm, 1000 μ m plates (Whatman, Clifton, NJ). Before applying the sample, the plates were run in ethyl acetate to remove any adsorbed impurities. One plate per approximately 50 mg of sample was used. Radio-TLC was initially analyzed using a System 200 Imaging Scanner (BioScan, Washington, D.C.) and then cut into strips and counted using an automatic gamma counter (LKB 1282, Wallac, Finland). ^1H NMR (300 MHz) and ^{13}C NMR (75 MHz) were obtained on a GE Midfield GN-300 or on a Varian Mercury 300 spectrometer. Chemical shifts are reported in δ units; solvent peaks were referenced appropriately. Mass spectra were obtained on a Hewlett-Packard GC/MS/DS Model HP-5988A instrument, or on JEOL SX-102 high-resolution mass spectrometer. IR spectra were obtained on a MB-100 IR spectrometer (BOMEM, Québec, Canada). Elemental analyses were provided by Galbraith Laboratories (Knoxville, TN) or by Microanalysis Laboratory, University of Illinois (Urbana, IL).

4-Chloro-3-iodobenzyl Alcohol (2). Sodium borohydride (76 mg, 2 mmol) was added to a homogeneous mixture of **1** (Trans World Chemicals; 566 mg, 2 mmol), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP; Fluka; 974 mg, 2.2 mmol), and diisopropylethylamine (349 mg, 2.6 mmol) in THF (10 mL). The mixture was stirred at room temperature for 20 min. The THF was evaporated from the mixture, and 1 N HCl (5–10 mL) was added to the residue. The resultant aqueous suspension was extracted repeatedly with EtOAc. The combined organic layers were washed with 1 N NaOH and dried with Na₂SO₄. The mixture was

adsorbed onto silica gel and chromatographed using 20% EtOAc/hexane to obtain 397 mg (74%) of an oil: ^1H NMR (CDCl₃): 1.82 (br s, 1H), 4.63 (s, 2H), 7.26 (m, 1H), 7.40 (m, 1H), 7.86 (s, 1H). GCMS m/z : 286 (M + NH₄)⁺, 268 (M⁺). Anal. Calcd for C₇H₆ClIO: C, 31.32; H, 2.32. Found: C, 31.84; H, 2.32.

4-Chloro-3-(trimethylstannyl)benzyl Alcohol (3). A mixture of **2** (135 mg, 0.5 mmol), bis(triphenylphosphine)palladium dichloride (100 mg, 0.14 mmol), and hexamethylditin (900 mg, 2.75 mmol) in dioxane (10 mL) was refluxed under argon for 1 h. Dioxane was evaporated, and a suspension of the dark residue in hexane was passed through a plug of silica gel. Very nonpolar byproducts were removed by washing with hexane, and the more polar components of the reaction mixture including **3** were eluted from the silica gel with ethyl acetate. EtOAc was removed by rotary evaporation, and the residue was purified by preparative TLC using 20% EtOAc/hexane (four plates were used) to yield 38 mg (25%) of an oil: ^1H NMR (CDCl₃): 0.37 (s, 9H), 1.66 (t, 1H), 4.66 (d, 2H), 7.26–7.37 (m, 3H). GCMS m/z : cluster peaks at 307 (MH⁺). Anal. Calcd for C₁₀H₁₅ClOSn: C, 39.33; H, 4.95. Found: C, 39.41; H, 5.16.

N¹,N²-Bis(tert-butyloxycarbonyl)-N¹-[4-chloro-3-(trimethylstannyl)benzyl]guanidine (4). Diisopropyl azodicarboxylate (DIAD; 203 mg, 1 mmol) was added dropwise to a solution of **3** (153 mg, 0.5 mmol), *N,N'*-bis(tert-butyloxycarbonyl)guanidine (259 mg, 1 mmol), and TPP (275 mg, 1.1 mmol) in THF (ca. 5 mL), and the mixture was stirred overnight at room temperature. The THF was evaporated, and the residual oil was chromatographed using 10% EtOAc/hexane to obtain 184 mg (67%) of a thick oil: ^1H NMR (CDCl₃): 0.35 (s, 9H [^{119}Sn –H, d]), 1.39 (s, 9H), 1.49 (s, 9H), 5.19 (s, 2H), 7.15–7.42 (m, 3H), 9.40 (br d, 2H). MS (FAB⁺) m/z : cluster peaks at 548 (MH⁺), 492, 436. HRMS (FAB⁺) calcd for C₂₁H₃₅ClN₃O₄¹¹⁸Sn (MH⁺): 546.1334. Found: 546.1352. Anal. Calcd for C₂₁H₃₄ClN₃O₄Sn: C, 46.14; H, 6.27; N, 7.69. Found: C, 46.76; H, 6.59; N, 7.27.

N¹,N²-Bis(tert-butyloxycarbonyl)-N¹-(4-chloro-3-iodobenzyl)guanidine (5). This was prepared from **2** following a procedure similar to that used for the preparation of **4**. The crude mixture was chromatographed using a stepwise gradient of 20% CHCl₃/hexane to 100% CHCl₃ to obtain 160 mg (63%) of a solid: mp 146–147 °C. ^1H NMR (CDCl₃): 1.40 (s, 9H), 1.50 (s, 9H), 5.15 (s, 2H), 7.25 (d, 1H), 7.40 (d, 1H), 7.80 (s, 1H), 9.35 (br d, 2H). MS (FAB⁺) m/z : 510 (MH⁺), 454, 398. HRMS (FAB⁺) calcd for C₁₈H₂₆ClIN₃O₄ (MH⁺): 510.0657. Found: 510.0643. Anal. Calcd for C₁₈H₂₅ClIN₃O₄: C, 42.41; H, 4.94; N, 8.24. Found: C, 42.72; H, 4.66; N, 8.43.

N¹-(4-Chloro-3-iodobenzyl)guanidine (6). Stannic chloride (75 mg, 0.3 mmol) was added dropwise to a solution of **5** (25 mg, 0.05 mmol) in anhydrous EtOAc (0.5 mL), and the mixture was stirred at room temperature for 3 h. EtOAc and SnCl₄ were evaporated on a rotary evaporator initially using a water-aspirator and eventually with a vacuum pump. Ether (5 mL) was added to the residue with stirring, and the mixture was left stirring overnight at 4 °C. The resultant white precipitate was filtered, washed with ether, and dried to yield 14 mg (82%) of a solid: mp 262–268 °C (decomp). ^1H NMR (CD₃OD): 4.37 (s, 2H), 7.33 (d, 1H), 7.51 (d, 1H), 7.88 (s, 1H). MS (FAB⁺) m/z : 310 (MH⁺). ^{13}C NMR (CD₃OD): 44.4, 98.7, 129.6, 130.4, 138.1, 138.8, 140.0, 158.5. HRMS (FAB⁺) calcd for C₈H₁₀ClIN₃ (MH⁺): 309.9608. Found: 309.9610 $\pm$ 0.0009 ($n = 4$).

3-Chloro-4-(trimethylsilyl)toluene (8). Chlorotrimethylsilane (7.6 mL, 60 mmol) was added via a syringe

to a stirred mixture of **7** (Trans World Chemicals; 10 g, 40 mmol) and magnesium turnings (1 g, 42 mmol) in ether (40 mL), and then the mixture was slowly heated to reflux. After the initial vigorous reaction subsided, the mixture was refluxed for an additional 1 h. Saturated NH_4Cl was added to quench the reaction, and the aqueous layer was extracted twice with ether. The pooled ethereal layer was washed with brine, dried, and distilled under water-aspirator vacuum to obtain 2.7 g (34%) of an oil. Gas chromatographic analysis indicated the presence of minor impurities, but the product was used without further purification for the next step. GCMS m/z : 199 (MH^+), 183, 91.

3,4-Bis(trimethylsilyl)toluene (9). Sodium (690 mg, 30 mmol) was taken up in a mixture of anhydrous toluene (3 mL) and xylene (4.7 mL) and melted by heating the mixture to reflux. To this finely divided suspension of sodium were added dropwise **8** (2.7 g, 13.6 mmol) and chlorotrimethylsilane (1.9 mL, 15 mmol) simultaneously to maintain a steady reflux. The reaction mixture was heated for an additional 1 h at reflux. The precipitate was filtered through a Celite bed, and the filtrate was concentrated. The residual oil was chromatographed using pentane to get 1.2 g (37%) of an oil: ^1H NMR (CDCl_3): 0.35 (s, 9H), 0.36 (s, 9H), 2.34 (s, 3H), 7.18 (d, 1H), 7.46 (s, 1H), 7.59 (d, 1H). GCMS m/z : 237 (MH^+), 221, 166, 149.

4-Nitro-3-(trimethylsilyl)toluene (10). A solution of 70% nitric acid (0.56 g) in acetic anhydride (2.4 mL) was added dropwise to a solution of **9** (418 mg, 1.8 mmol) in acetic anhydride (2.9 mL), and the mixture was heated at 100 °C for 6 h, cooled, and added to 0.4 N NaOH (50 mL). The aqueous solution was extracted with ether, and the pooled ethereal extract was washed, dried, and evaporated to get an oil. The oil was chromatographed using pentane to obtain 120 mg (32%) of a pale yellow solid: mp 46–48 °C. IR (NaCl): cm^{-1} 2926, 1522, 1345, 845. ^1H NMR (CDCl_3): 0.35 (s, 9H), 2.45 (s, 3H), 7.31 (d, 1H), 7.46 (s, 1H), 8.09 (d, 1H). GCMS m/z : 227 ($\text{M} + \text{NH}_4$) $^+$, 194. Anal. Calcd for $\text{C}_{10}\text{H}_{15}\text{NO}_2\text{Si}$: C, 57.38; H, 7.22; N, 6.69. Found: C, 57.20; H, 7.36; N, 6.57. That this compound is not the isomeric 3-nitro-4-trimethylsilyl-toluene is shown by the fact that chemical shifts of aromatic protons are closer to those predicted (Chem-Draw software) for the 4-nitro derivative than for the 3-nitro isomer.

4-Nitro-3-(trimethylsilyl)benzyl Bromide (11). A mixture of **10** (157 mg, 0.75 mmol), NBS (133 mg, 0.75 mmol), and a grain of benzoyl peroxide in CCl_4 (10 mL) was refluxed for 1 h in the presence of an incandescent light. The insolubles were filtered and washed, and the CCl_4 was evaporated from the filtrate. The residue was chromatographed with pentane to obtain 97 mg (34%) of a pale yellow oil: ^1H NMR (CDCl_3): 0.37 (s, 9H), 4.52 (s, 2H), 7.56 (d, 1H), 7.66 (s, 1H), 8.16 (d, 1H). GCMS m/z : 305 and 307 ($\text{M} + \text{NH}_4$) $^+$, 272 and 274 ($\text{M} - \text{CH}_3$) $^+$. Anal. Calcd for $\text{C}_{10}\text{H}_{14}\text{BrNO}_2\text{Si}$: C, 41.67; H, 4.90; N, 4.86. Found: C, 41.53; H, 4.87; N, 4.63.

N^1, N^2 -Bis(*tert*-butyloxycarbonyl)- N^1 -(4-nitro-3-trimethylsilylbenzyl)guanidine (12). To a heterogeneous mixture of sodium hydride (60% dispersion; 9.6 mg, 0.24 mmol) in DMF (silylation grade, Pierce; 1 mL) was added N^1, N^2 -bis(*tert*-butyloxycarbonyl)guanidine (63 mg, 0.24 mmol), and the reaction mixture was stirred at room temperature for 5 min, resulting in a homogeneous solution. A solution of **11** (72 mg, 0.25 mmol) in DMF (1 mL) was added dropwise and the progress of reaction was monitored by TLC. After 30 min, the reaction mixture was partitioned between water and EtOAc, and

the organic layer was washed with brine, dried, and concentrated. Preparative TLC of the crude product using 10% EtOAc/hexane gave 51 mg (46%) of a yellow solid: mp 104–106 °C. IR (KBr): cm^{-1} 3386, 2973, 1717, 1609, 1519, 1293, 1244, 1147, 1121, 848. ^1H NMR (CDCl_3): 0.34 (s, 9H), 1.36 (s, 9H), 1.49 (s, 9H), 5.26 (s, 2H), 7.40 (d, 1H), 7.68 (s, 1H), 8.18 (d, 1H). MS (FAB^+) m/z : 467 (MH^+), 411, 355. Anal. Calcd for $\text{C}_{21}\text{H}_{34}\text{N}_4\text{O}_6\text{Si}$: C, 54.06; H, 7.34; N, 12.01. Found: C, 54.10; H, 7.60; N, 11.71.

N^1 -(4-Nitro-3-trimethylsilylbenzyl)guanidine (13). Compound **12** (20.1 mg, 0.04 mmol) was dissolved in 10% (v/v) H_2SO_4 /dioxane (0.5 mL), and the mixture was stirred at room temperature for 2 h. The solvents were evaporated, and the residue in a small amount of water was loaded onto an activated C18 solid-phase cartridge (tC18 ENV; Waters). The cartridge was washed with water (4 $\times$ 5 mL), and the product was eluted with MeOH (~5 mL) in almost quantitative yield. ^1H NMR (CD_3OD): 4.19 (s, 2H), 7.55 (d, 1H), 7.70 (s, 1H), 8.20 (d, 1H). MS (FAB^+) m/z : 267 (MH^+). HRMS (FAB^+) calcd for $\text{C}_{11}\text{H}_{19}\text{N}_4\text{O}_2\text{Si}$ (MH^+): 267.1277. Found: 267.1280 $\pm$ 0.0003 ($n = 3$).

3-Iodo-4-nitrotoluene (15). A solution of sodium nitrite (110 mg, 1.6 mmol) in water (0.5 mL) was added with stirring to a cold slurry of 5-methyl-2-nitroaniline (221 mg, 1.47 mmol) in concentrated HCl (0.7 mL) and ice (1 g). The mixture was stirred for 20 min at 0 °C, the resultant cold solution was added dropwise to a stirred solution of KI (2.44 g, 14.6 mmol) in water (3 mL) at room temperature, and the resultant mixture was stirred overnight. The reaction mixture was extracted with EtOAc, and the organic layer was washed with 1 N NaOH, 1 N HCl, and brine, dried, and concentrated. Column chromatography with 1% EtOAc/hexane gave 380 mg (98%) of an oil. An analytical sample was obtained by further purification by preparative TLC using the same solvent: ^1H NMR (CDCl_3): 2.39 (s, 3H), 7.27 (d, 1H), 7.82 (d, 1H), 7.89 (s, 1H). GCMS m/z : 281 ($\text{M} + \text{NH}_4$) $^+$, 264 (MH^+), 155. Anal. Calcd for $\text{C}_7\text{H}_6\text{INO}_2$: C, 31.96; H, 2.30; N, 5.33. Found: C, 32.14; H, 2.39; N, 5.30.

3-Iodo-4-nitrobenzyl Bromide (16). The title compound was prepared from **15** (269 mg, 1.02 mmol) by a procedure similar to that used for the preparation of **11**. Chromatography using 5% EtOAc/hexane provided 45 mg (30%) of a solid: mp 61–63 °C. ^1H NMR (CDCl_3): 4.41 (s, 2H), 7.52 (d, 1H), 7.85 (d, 1H), 8.05 (s, 1H). Anal. Calcd for $\text{C}_7\text{H}_5\text{BrINO}_2$: C, 24.59; H, 1.47; N, 4.10. Found: C, 24.76; H, 1.52; N, 4.07.

N^1, N^2 -Bis(*tert*-butyloxycarbonyl)- N^1 -(3-iodo-4-nitrobenzyl)guanidine (17). The guanidination of **16** (87 mg, 0.25 mmol) was performed by a procedure similar to that used for the preparation of **12** from **11**. Chromatography using a gradient of 5–10% EtOAc/hexane yielded 74 mg (90% based on the starting material consumed) of a yellow solid: mp 142–144 °C. ^1H NMR (CDCl_3): 1.41 (s, 9H), 1.49 (s, 9H), 5.16 (s, 2H), 7.43 (d, 1H), 7.85 (d, 1H), 8.01 (s, 1H), 9.40 (br d, 2H). MS (FAB^+) m/z : 521 (MH^+). HRMS (FAB^+) calcd for $\text{C}_{18}\text{H}_{26}\text{IN}_4\text{O}_6$ (MH^+): 521.0897. Found: 521.0899. Anal. Calcd for $\text{C}_{18}\text{H}_{25}\text{IN}_4\text{O}_6$: C, 41.55; H, 4.84; N, 10.77. Found: C, 42.25; H, 5.03; N, 10.90.

N^1 -(3-Iodo-4-nitrobenzyl)guanidine (18). This was prepared quantitatively from **17** (52 mg, 0.1 mmol) in a similar fashion as **6** from **5**: mp 258–259 °C (decomp). IR (KBr): cm^{-1} 3443, 3355, 1660, 1518, 1352. ^1H NMR (CD_3OD): 4.49 (s, 2H), 7.5 (d, 1H), 7.85 (d, 1H), 8.10 (s, 1H). ^{13}C NMR (CD_3OD): 44.4, 87.3, 126.2, 128.8, 141.0,

143.8, 154.1, 158.5. MS (FAB⁺) *m/z*: 320 (M⁺). HRMS (FAB⁺) calcd for C₈H₁₀IN₄O₂ (MH⁺): 320.9848. Found: 320.9847.

3-Iodo-5-nitrobenzoic Acid (20). A heterogeneous mixture of 3-amino-5-nitrobenzoic acid (3.64 g, 20 mmol) in 10 mL of concentrated HCl was stirred at 0 °C as a solution of NaNO₂ (1.66 g, 24 mmol) in 10 mL of ice-water was added. The resultant homogeneous orange solution was stirred at ca. 0 °C for 1 h, then added slowly with stirring to a solution of KI (8.3 g, 50 mmol) in 30 mL of water at 0 °C. The reaction mixture was slowly warmed to room temperature over 2 h. The aqueous solution was extracted with EtOAc, and the organic layer was washed with water, aqueous saturated sodium metabisulfite, and brine, dried, and concentrated to give 5.3 g of a dirty brown solid. Chromatography with 20% MeOH/EtOAc afforded 5.2 g (89%) of a brown solid: IR (KBr): cm⁻¹ 3481, 3389, 1704, 1632, 1584, 1532, 1480, 1355. ¹H NMR (CD₃OD): 8.59 (t, 1H), 8.67 (dd, 1H), 8.74 (dd, 1H). ¹³C NMR (CD₃OD): 94.1, 124.4, 135.6, 136.5, 144.91, 149.7, 166.1. HRMS (FAB⁻) calcd for C₇H₃INO₄ (M - H)⁻: 291.9107. Found: 291.9115.

3-Iodo-5-nitrobenzyl Alcohol (21). A solution of **20** (1.5 g, 5.1 mmol) in 10 mL of anhydrous THF was stirred and refluxed for 3.5 h with 2 M BH₃-THF (13 mL, 25.6 mmol) and B(OMe)₃ (5.7 mL, 51.2 mmol) under a flow of argon. Excess borane was removed by addition of MeOH, and the solvents were evaporated. The resultant precipitate was reconstituted in EtOAc, washed with water and brine, dried, and concentrated by rotary evaporation to give 1.5 g of the crude product mixture. Chromatographic separation using a stepwise gradient of 50% to 75% EtOAc/hexane provided 720 mg (51%) of a pale yellow solid: IR (CCl₄): cm⁻¹ 2977, 2866, 1742, 1537, 1348, 1239, 1119. ¹H NMR (CDCl₃): 4.79 (d, 2H), 8.04 (td, 1H), 8.19 (ddd, 1H), 8.46 (dd, 1H). ¹³C NMR (CDCl₃): 63.1, 94.2, 120.7, 131.1, 141.1, 144.4. MS (FAB⁻) *m/z*: 278.93 (M⁻). HRMS (FAB⁻) calcd for C₇H₄INO₃ (M - H)⁻: 277.9314. Found: 277.9324. Anal. Calcd for C₇H₆INO₃: C, 30.13; H, 2.17; N, 5.02. Found: C, 30.03; H, 2.26; N, 4.92.

3-Nitro-5-tri-*n*-butylstannylbenzyl Alcohol (22). Compound **21** (100 mg, 0.36 mmol) in 2 mL of anhydrous toluene was refluxed with (Ph₃P)₂PdCl₂ (60 mg, 0.09 mmol) as hexabutyliditin (414 mg, 0.72 mmol) was added by syringe, and the ensuing black heterogeneous mixture was stirred and refluxed under a flow of argon for 5 h. The reaction mixture was cooled to room temperature, filtered over Celite to remove suspended particles, and washed with EtOAc. The filtrate was concentrated to provide 464 mg of a brown oil, which was purified by column chromatography using a hexanes-EtOAc gradient to give 115 mg (73%) of a brown oil: IR (NaCl): cm⁻¹ 3397, 2915, 2856, 1523, 1463, 1343. ¹H NMR (CDCl₃): 0.89 (t, 3H), 1.13 (m, 2H), 1.36 (m, 2H), 1.57 (m, 2H), 4.81 (d, 2H), 7.74 (ddd, 1H), 8.14 (td, 1H), 8.20 (dd, 1H). ¹³C NMR (CDCl₃): 10.0, 13.7, 27.4, 28.9, 29.1, 30.6, 63.6, 121.0, 129.1, 140.3, 144.9, 147.6. Anal. Calcd for C₁₉H₃₃NO₃Sn: C, 51.61; H, 7.52; N, 3.17. Found: C, 51.48; H, 7.31; N, 3.21.

N¹,N²-Bis(*tert*-butyloxycarbonyl)-N¹-(3-nitro-5-tri-*n*-butylstannylbenzyl)guanidine (23). The benzyl alcohol **22** (111 mg, 0.25 mmol) in 2 mL of anhydrous THF was stirred at room temperature under argon with *N*,*N'*-bis(*tert*-butyloxycarbonyl)guanidine (131 mg, 0.5 mmol) and TPP (331 mg, 1.25 mmol) as DIAD (247 mL, 1.25 mmol) was added, and the resulting yellow heterogeneous mixture was stirred for 3 h under a flow of argon. The solvent was evaporated, and the resulting solids were reconstituted in 15 mL of EtOAc, washed with 2 × 15 mL

of water and 20 mL of brine, dried, and concentrated. Chromatographic purification of the crude product yielded 44 mg of a mixture, composed of the protected guanidine and diisopropylhydrazine dicarboxylate, which was further purified by preparative TLC using 5% EtOAc/hexane to give 18 mg (11%) of a colorless solid: IR (NaCl): cm⁻¹ 3386, 2963, 2924, 1721, 1613, 1528, 1363, 1147. ¹H NMR (CDCl₃): 0.78 (t, 3H), 1.02 (m, 2H), 1.21 (m, 2H), 1.33 (m, 2H), 1.30 (s, 9H), 1.40 (s, 9H), 5.13 (s, 2H), 7.66 (d, 1H), 8.00 (dd, 1H dd), 8.07 (d, 1H), 9.3 (br d, 2H). ¹³C NMR (CDCl₃): 10.0, 13.7, 27.3, 28.0, 28.3, 29.0, 122.2, 129.1, 141.5. MS (FAB⁻) *m/z*: cluster peaks at 683.2 (M⁻). HRMS (FAB⁻) calcd for C₃₀H₅₁N₄O₆Sn (M - H)⁻: 681.2831. Found: 681.2806. Anal. Calcd for C₂₁H₃₆N₄O₄Sn: C, 52.72; H, 7.67; N, 8.20. Found: C, 53.61; H, 7.63; N, 7.86.

N¹,N²-Bis(*tert*-butyloxycarbonyl)-N¹-(3-iodo-5-nitrobenzyl)guanidine (24). A solution of **21** (139 mg, 0.5 mmol) in 5 mL of anhydrous THF was stirred at room temperature under argon with *N*¹,*N*²-bis-*tert*-butyloxycarbonyl-S-methylisothiurea (259 mg, 1 mmol) and TPP (656 mg, 2.5 mmol) as DIAD (492 mL, 2.5 mmol) was added, and the resulting yellow heterogeneous mixture was stirred for 5 h under a flow of argon. The solvent was evaporated, and the resulting solids were dissolved in EtOAc, washed with water and brine, dried, and concentrated to give 439 mg of crude products. Purification by column chromatography provided 156 mg (60%) of a pale yellow solid: ¹H NMR (CDCl₃): 1.45 (s, 9H), 1.53 (s, 9H), 2.38 (s, 3H), 4.78 (s, 2H), 8.0 (t, 1H), 8.20 (dd, 1H), 8.47 (dd, 1H). HRMS (FAB⁺) calcd for C₈H₉IN₄O₂ (MH⁺): 320.9848. Found: 320.9848 ± 0.0013 (*n* = 2).

The above product (130 mg, 0.24 mmol) in 1.5 mL of MeOH was refluxed in liquid NH₃ (ca. 2 mL) for 2.5 h. Rotary evaporation of the reaction mixture gave 125 mg (100%) of a pale yellow oil: ¹H NMR (CDCl₃): 1.47 (s, 9H), 1.51 (s, 9H), 5.14 (s, 2H), 8.16 (s, 1H), 8.27 (s, 1H), 8.44 (s, 1H), 9.3 (br d, 2H). MS (FAB⁻) *m/z*: 519.0 (M⁻). HRMS (FAB⁻) calcd for C₁₈H₂₄IN₄O₆ (M - H)⁻: 519.0741. Found: 519.0735.

N¹-(3-Iodo-5-nitrobenzyl)guanidine (25). A solution of **24** (125 mg, 0.24 mmol) in 4 mL of CH₂Cl₂ and 1 mL of TFA was stirred and heated at 65 °C in a sealed flask for 4 h. Evaporation of the solvents afforded 67 mg (64%) of the deprotected benzyl guanidine as an oil: IR (NaCl): cm⁻¹ 3347, 2503, 1673, 1616, 1533, 1352, 1203, 1141. ¹H NMR (CD₃OD): 4.89 (s, 2H), 8.08 (td, 1H), 8.18 (dd, 1H), 8.49 (dd, 1H). ¹³C NMR (CD₃OD): 44.3, 94.6, 122.2, 132.4, 142.0, 142.9, 149.9, 158.2.

3-Bromo-5-methoxy-4-methoxymethoxybenzaldehyde (27). To a solution of 5-bromovanillin (1.16 g, 5 mmol) in anhydrous THF (20 mL) was added dropwise a 1M solution of potassium *tert*-butoxide (5 mL) followed by MOM-chloride (0.5 mL, 6.6 mmol). The reaction mixture was stirred for about 4 h at room temperature and the solvents were evaporated. The residual material was partitioned between water and EtOAc, and the combined organic layer was washed with brine, dried, and concentrated. Chromatography using 30% EtOAc/hexane yielded 1.1 g of an oil: ¹H NMR (CDCl₃): 3.54 (s, 3H), 3.81 (s, 3H), 5.18 (s, 2H), 7.27 (d, 1H), 7.55 (d, 1H), 9.73 (s, 1H). GCMS *m/z*: 303 and 305 (M + C₂H₅)⁺, 292 and 294 (M + NH₄)⁺, 275 and 277 (M⁺). Anal. Calcd for C₁₀H₁₁BrO₄: C, 43.66; H, 4.03. Found: C, 43.95; H, 4.24.

3-Bromo-5-methoxy-4-methoxymethoxybenzyl Alcohol (28). To a stirred solution of **27** (1.05 g, 3.9 mmol) in anhydrous CH₂Cl₂ (15 mL) was added dropwise a 1 M solution of DIBAL in CH₂Cl₂ (5.4 mL), and the mixture was stirred for 1–2 h. Methanol (10 mL) and water (10

mL) were added to the cooled reaction mixture, and the resultant gelatinous material was filtered through a bed of Celite and washed alternately with a total volume of 200 mL of CH_2Cl_2 and 50 mL of water. The layers of the filtrate were separated, and the CH_2Cl_2 layer was dried and concentrated to obtain 1.06 g (100%) of an oil: ^1H NMR (CDCl_3): 2.21 (br s, 1H), 3.64 (s, 3H), 3.82 (s, 3H), 4.57 (s, 2H), 5.13 (s, 2H), 6.85 (d, 1H), 7.10 (d, 1H). GCMS m/z : 294 and 296 ($\text{M} + \text{NH}_4^+$), 278 and 280 (MH^+), 259 and 261, 215 and 217. Anal. Calcd for $\text{C}_{10}\text{H}_{13}\text{BrO}_4$: C, 43.34; H, 4.73. Found: C, 43.50; H, 5.06.

N^1, N^2 -Bis(*tert*-butyloxycarbonyl)- N^1 -(3-bromo-5-methoxy-4-methoxymethoxybenzyl)guanidine (29). The title compound was prepared from **28** (522 mg, 1.9 mmol) by a protocol similar to that used for the preparation of **4**, **5**, and **23**. Chromatography using a 10–30% EtOAc/hexane gradient afforded 506 mg (51%) of a solid: mp 131–133 °C. ^1H NMR (CDCl_3): 1.45 (s, 9H), 1.49 (s, 9H), 3.64 (s, 3H), 3.82 (s, 3H), 5.03 (s, 2H), 5.15 (s, 2H), 7.12 (m, 2H), 9.34 (br d, 2H). MS (FAB^+) m/z : 518 and 520 (MH^+), 462 and 464, 406 and 408. Anal. Calcd for $\text{C}_{21}\text{H}_{32}\text{BrN}_3\text{O}_7$: C, 48.65; H, 6.22; N, 8.11. Found: C, 48.31; H, 6.35; N, 7.94.

3-Iodo-5-methoxy-4-methoxymethoxybenzyl Alcohol (30). To a stirred solution of **28** (278 mg, 1 mmol) in THF (5 mL) under argon at -78°C was added dropwise *n*-BuLi (1.6 M in hexanes; 1.45 mL, 2.32 mmol) over a period of 5 min, and the reaction mixture was stirred at -78°C for an additional 15 min. To the resultant mixture at -78°C was then added gradually 2-iodo-1,1,1-trifluoroethane (550 μL , 5.6 mmol) over a period of 5 min. The reaction mixture was allowed to warm gradually to room temperature and left stirring overnight. Solvents were evaporated from the reaction mixture, and the residual material was partitioned between EtOAc and water. Workup and silica gel chromatography using 40% EtOAc/hexane gave 88 mg (27%) of an oil: ^1H NMR (CDCl_3): 1.79 (br s, 1H), 3.67 (s, 3H), 3.84 (s, 3H), 4.60 (s, 2H), 5.15 (s, 2H), 6.92 (d, 1H), 7.36 (d, 1H). GCMS m/z : 342 ($\text{M} + \text{NH}_4^+$), 324 (M^+), 307, 263.

The R_f values on an analytical TLC plate (50% EtOAc/hexane) were the same (~ 0.4) for both **28** and **30**. However, their retention times on a reversed-phase HPLC column were marginally different. The t_R for **28** was 22.1 min versus 23.0 min for **30** when eluted on a μ Bondapak C18 column with a 0.1% TFA in water (A) 0.1% TFA in acetonitrile (B) gradient (5% B for 10 min and then to 80% B in 20 min) at a flow rate of 1 mL/min.

N^1, N^2 -Bis(*tert*-butyloxycarbonyl)- N^1 -(3-iodo-5-methoxy-4-methoxymethoxybenzyl)guanidine (31). Compound **31** was prepared from **30** (88 mg, 0.28 mmol) by a protocol similar to that for **29**. Chromatography with 10% EtOAc/hexane gave 113 mg (74%) of a waxy solid: mp 107–109 °C. ^1H NMR (CDCl_3): 1.47 (s, 9H), 1.49 (s, 9H), 3.66 (s, 3H), 3.81 (s, 3H), 5.01 (s, 2H), 5.14 (s, 2H), 7.18 (d, 1H), 7.38 (d, 1H), 9.32 (br d, 2H). HRMS (FAB^+) calcd for $\text{C}_{21}\text{H}_{33}\text{IN}_3\text{O}_7$ (MH^+): 566.1363. Found: 566.1372 ± 0.0013 ($n = 2$). Anal. Calcd for $\text{C}_{21}\text{H}_{32}\text{IN}_3\text{O}_7$: C, 44.61; H, 5.70; N, 7.43. Found: C, 44.87; H, 5.88; N, 7.45.

N^1 -(3,4-Dihydroxy-5-iodobenzyl)guanidine (32). A solution of BBr_3 (1 M in CH_2Cl_2 ; 0.5 mL, 0.5 mmol) was added to **31** (33.8 mg, 0.06 mmol), and the mixture stirred at room temperature for 30 min. The CH_2Cl_2 was evaporated under a stream of argon, and residual BBr_3 was removed by coevaporation with 2×1 mL of EtOAc. The residual mixture was taken in 1–2 mL of water and passed through two activated C18 solid-phase cartridges (tC18 ENV; Waters) connected in series. The cartridge was washed with water, and the product was eluted with

1 mL portions of EtOH. The second EtOH fraction, containing a majority of the product (HPLC), was concentrated to yield 6 mg (33%) of an oil. An analytical sample was obtained by semipreparative reversed-phase HPLC: ^1H NMR (CD_3OD): 4.20 (s, 2H), 6.73 (s, 1H), 7.11 (s, 1H). MS (FAB^+) m/z : 330 ($\text{M} + \text{Na}^+$), 308 (MH^+). ^{13}C NMR (CD_3OD): 45.1, 84.5, 115.4, 129.3 (C1 and C6), 146.8, 147.0, 158.6. HRMS (FAB^+) calcd for $\text{C}_8\text{H}_{11}\text{IN}_3\text{O}_2$ (MH^+): 307.9896. Found: 307.9897 ± 0.0002 ($n = 3$). The structure was further confirmed by heteronuclear multi bond connectivity (HMBC, Bax and Summers, 1986) and heteronuclear multiple quantum coherence (HMQC, Summers et al., 1986) NMR correlation experiments.

N^1 -(4-Hydroxy-3-iodo-5-methoxybenzyl)guanidine (33). Stannic chloride (10 mg, 0.04 mmol) was added to a solution of **31** (24 mg, 0.04 mmol) in EtOAc (0.1 mL), and the reaction mixture was stirred at room temperature for 3 h. The EtOAc was evaporated, and ether (0.5 mL) was added with stirring resulting in a precipitate. The precipitate was washed with 2×0.5 mL of ether, the ether was decanted, and the precipitate was dried under vacuum for several hours to yield **33** as a crystalline solid in quantitative yield: mp, decomposes. ^1H NMR (CD_3OD): 3.83 (s, 3H), 4.27 (s, 2H), 6.90 (s, 1H), 7.23 (s, 1H). MS (FAB^+) m/z : 322 (MH^+). ^{13}C NMR (CD_3OD): 45.2, 56.7, 83.6, 111.9, 130.2 (C1 and C6), 130.5, 147.9, 148.6, 158.5. HRMS (FAB^+) calcd for $\text{C}_9\text{H}_{13}\text{IN}_3\text{O}_2$ (MH^+): 322.0052. Found: 322.0050 ± 0.0008 ($n = 2$). The structure was further confirmed by HMBC/HMQC NMR experiments. The title compound can be also prepared by treatment of **31** with TFA at room temperature.

3-Methoxy-4-methoxymethoxy-5-(trimethylstannyl)benzyl Alcohol (34). A solution of *n*-BuLi (1.6 M in hexanes; 750 μL , 1.2 mmol) was added to a solution of **28** (142 mg, 0.52 mmol) in THF (2.5 mL), and the mixture was stirred at -78°C for 15 min. A solution of trimethyltin chloride (1 M in hexanes; 1.2 mL, 1.2 mmol) was added, and the mixture was brought to room temperature gradually and stirred overnight. Evaporation of solvents, aqueous workup, and column chromatography with 40% EtOAc/hexane gave 51 mg (27%) of an oil: ^1H NMR (CDCl_3): 0.32 (s, 9H [$^{119}\text{Sn}-\text{H}$, d]), 3.54 (s, 3H), 3.85 (s, 3H), 4.65 (s, 2H), 5.10 (s, 2H), 6.94 (m, 2H). MS (FAB^+) m/z : cluster peaks at 362 (M^+) and 345. Anal. Calcd for $\text{C}_{13}\text{H}_{22}\text{O}_4\text{Sn}$: C, 43.25; H, 6.14. Found: C, 43.98; H, 6.56.

N^1, N^2 -Bis(*tert*-butyloxycarbonyl)- N^1 -(3-methoxy-4-methoxymethoxy-5-trimethylstannylbenzyl)guanidine (35). The title compound was prepared from **34** (51 mg, 0.14 mmol) by a procedure similar to that used for **31**. Chromatography using 10% EtOAc/hexane yielded 81 mg (95%) of an oil: ^1H NMR (CDCl_3): 0.30 (s, 9H [$^{119}\text{Sn}-\text{H}$, d]), 1.45 (s, 9H), 1.49 (s, 9H), 3.53 (s, 3H), 3.82 (s, 3H), 5.08 (s, $2 \times 2\text{H}$), 6.95 (d, 1H), 7.22 (d, 1H), 9.40 (br d, 2H). MS (FAB^+) m/z : cluster peaks at 604 (MH^+); HRMS (FAB^+) calcd for $\text{C}_{24}\text{H}_{42}\text{N}_3\text{O}_7^{120}\text{Sn}$ (MH^+): 604.2045; Found: 604.2052 ± 0.0006 ($n = 2$). Anal. Calcd for $\text{C}_{24}\text{H}_{41}\text{N}_3\text{O}_7\text{Sn}$: C, 47.86; H, 6.86; N, 6.98. Found: C, 48.25; H, 7.03; N, 7.00.

N^1 -Hydroxy- N^3 -(3-iodobenzyl)guanidine (38). A solution of CNBr (220 mg, 2.1 mmol) in methanol (0.7 mL) was added dropwise with stirring to a cooled mixture of 3-iodobenzylamine (obtained by the basification of its commercially available hydrochloride salt) (414 mg, 1.8 mmol) and NaOAc (323 mg, 3.9 mmol) in methanol (3.5 mL). The reaction mixture was stirred at 5°C for 2–3 h and at ambient temperature overnight. The mixture was filtered through a frit funnel, and the filtrate was adsorbed onto silica gel and chromatographed using 30–50% EtOAc/hexane to obtain 146 mg (32%) of a white

crystalline solid: mp 49–50 °C; ^1H NMR (CDCl_3): 3.68 (br s, 1H), 4.21 (d, 2H), 7.13 (t, 1H), 7.32 (d, 1H), 7.69 (m, 2H). GCMS m/z : 276 ($\text{M} + \text{NH}_4^+$), 259 (MH^+), 234, 217.

Since **37** was not very stable, it was carried over to the next step immediately. To a solution of $\text{NH}_2\text{OH}\cdot\text{HCl}$ (70 mg, 1 mmol) and **37** (146 mg, 0.57 mmol) in 1 mL of DMF was added Na_2CO_3 (200 mg, 1.9 mmol) in small portions. The mixture was stirred at room temperature for 2 h and partitioned between EtOAc and water. The organic phase was washed with brine, dried, and concentrated to obtain a chromatographically homogeneous oil in quantitative yield: ^1H NMR (CD_3OD): 4.15 (s, 2H), 7.07 (t, 1H), 7.29 (d, 1H), 7.58 (d, 1H), 7.70 (s, 1H). MS (FAB^+) m/z : 292 (MH^+), 276. HRMS (FAB^+) calcd for $\text{C}_8\text{H}_{11}\text{IN}_3\text{O}$ (MH^+): 291.9947; Found: 291.9940 $\pm$ 0.0000 ($n = 2$).

3-(Trimethylsilyl)benzylamine (40). To a solution of 3-bromobenzylamine (obtained from its hydrochloride salt; 393 mg, 2.1 mmol) in THF (12 mL) at -78°C was added dropwise $n\text{-BuLi}$ (1.6 M in hexanes; 3.9 mL, 6.3 mmol) over a period of 5 min. The resultant pink solution was stirred for 45 min at -78°C , and Me_3SiCl (795 μL , 6.3 mmol) was added dropwise. The mixture was gradually warmed to room temperature and stirred overnight, worked up, and chromatographed using 80/20/1 $\text{CHCl}_3/\text{MeOH}/\text{HOAc}$ yielding 144 mg (38%) of a waxy solid: mp 191–193 °C. ^1H NMR (CDCl_3): 0.28 (s, 9H), 1.47 (s, 2H), 3.88 (s, 2H), 7.29–7.46 (m, 4H). GCMS m/z : 180 (MH^+).

N^1 -Hydroxy- N^1 -(3-trimethylsilylbenzyl)guanidine (42). Compound **41** was prepared from **40** (370 mg, 2.1 mmol) by a procedure similar to that for **37**. Chromatography using a 25–100% EtOAc/hexane gradient afforded 196 mg (46%) of an oil: ^1H NMR (CDCl_3): 0.26 (s, 9H), 3.76 (br s, 1H), 4.25 (d, 2H), 7.32–7.52 (m, 4H). GCMS m/z : 222 ($\text{M} + \text{NH}_4^+$), 205 (MH^+), 163.

Compound **42** was prepared from **41** (176 mg, 0.86 mmol) in 94% yield by the same protocol used for **38**: mp 128–130 °C. ^1H NMR (CD_3OD): 0.26 (s, 9H), 4.19 (s, 2H), 7.42–7.64 (m, 4H). MS (FAB^+) m/z : 238 (MH^+). Anal. Calcd for $\text{C}_{11}\text{H}_{19}\text{N}_3\text{OSi}$: C, 55.66; H, 8.07; N, 17.70. Found: C, 55.82; H, 8.24; N, 17.66.

3-Amino-5-iodobenzyl Alcohol (43). A mixture of **21** (280 mg, 1 mmol), indium (836 mg, 7.3 mmol), saturated NH_4Cl (1.2 mL), and EtOH (4 mL) was refluxed for 1 h. The reaction mixture was worked up as per the literature protocol (Moody and Pitts, 1998), and the resultant oil was purified by silica gel chromatography using 1:1 EtOAc:hexanes to yield 83 mg (33%) of an off-white solid: mp 65–67 °C. IR (KBr): cm^{-1} 3417, 3296, 3193, 1598, 1568, 1262, 1099, 1026, 800. ^1H NMR (CDCl_3): 1.62 (br s, 1H), 3.69 (br s, 2H), 4.55 (s, 2H), 6.62 (dd, 1H), 6.96 (dd, 1H), 7.08 (d, 1H). GCMS (CI): m/z : 250 (MH^+), 232, 123. HRMS (EI^+) calcd for $\text{C}_7\text{H}_8\text{INO}$ (M^+): 248.9651; Found: 248.9653 $\pm$ 0.0011 ($n = 4$).

N^1 , N^2 -Bis(*tert*-butyloxycarbonyl)- N^1 -(3-amino-5-iodobenzyl)guanidine (44). The title compound was prepared from **43** (198 mg, 0.8 mmol) by a Mitsunobu protocol as described earlier. Chromatography using 30% EtOAc/hexane gave a solid in quantitative yield: mp 179–180 °C (dec). ^1H NMR (CDCl_3): 1.37 (s, 9H), 1.49 (s, 9H), 3.64 (br s, 2H), 5.02 (s, 2H), 6.51 (m, 1H), 6.91 (m, 1H), 6.96 (m, 1H), 9.40 (br d, 2H). MS (FAB^+) m/z : 491 (MH^+). HRMS (FAB^+) calcd for $\text{C}_{18}\text{H}_{28}\text{IN}_4\text{O}_4$ (MH^+): 491.1155; Found: 491.1149 $\pm$ 0.0014 ($n = 4$). Anal. Calcd for $\text{C}_{18}\text{H}_{27}\text{IN}_4\text{O}_4$: C, 44.09; H, 5.55; N, 11.43. Found: C, 45.55; H, 6.16; N, 12.04.

3-Amino-5-iodobenzylguanidine (45). Compound **44** (25 mg, 0.05 mmol) was treated with TFA (100 μL) at

room temperature for 15 min. TFA was evaporated and the residue was triturated thrice with EtOAc and dried under high vacuum to yield **45** as an oil in quantitative yield: ^1H NMR (CD_3OD) 4.34 (s, 2H), 6.84 (s, 1H), 7.22 (m, 2H). MS (FAB^+) m/z : 291 (MH^+). HRMS (FAB^+) calcd for $\text{C}_8\text{H}_{12}\text{IN}_4$ (MH^+): 291.0107; Found: 291.0111 $\pm$ 0.0005 ($n = 4$).

N^1 , N^2 -Bis(*tert*-butyloxycarbonyl)- N^1 -[3-amino-5-(trimethylstannyl)benzyl]guanidine (46). A mixture of **44** (20 mg, 0.04 mmol), $(\text{Ph}_3\text{P})_2\text{PdCl}_2$ (25 mg, 0.04 mmol), and $(\text{Me}_3\text{Sn})_2$ (130 mg, 0.4 mmol) in anhydrous dioxane (0.5 mL) was refluxed for 1 h. The reaction mixture was worked up as described for **3**, and the crude product was purified by preparative TLC using 20% EtOAc in hexanes (the TLC plate was dried after the first run and subjected to a second development to ensure separation between starting material and product) to obtain 19 mg (83%) of a pale amber-colored oil: ^1H NMR (CDCl_3): 0.24 (s, 9H [$^{119}\text{Sn}-\text{H}$, d]), 1.35 (s, 9H), 1.49 (s, 9H), 3.60 (br s, 2H), 5.07 (s, 2H), 6.53 (m, 1H), 6.68 (dd, 1H), 6.76 (d, 1H), 9.40 (br d, 2H). MS (FAB^+) m/z : cluster peaks around 529 (MH^+). HRMS (FAB^+) calcd for $\text{C}_{21}\text{H}_{36}\text{N}_4\text{O}_4^{120}\text{Sn}$ (M^+): 528.1763; Found: 528.1772 $\pm$ 0.0009 ($n = 4$). Anal. Calcd for $\text{C}_{21}\text{H}_{36}\text{N}_4\text{O}_4\text{Sn}$: C, 47.84; H, 6.88; N, 10.09. Found: C, 47.70; H, 6.68; N, 10.09.

N^1 -(4-Chloro-3-[^{131}I]iodobenzyl)guanidine (6a). To **4** (0.25 mg) in a $1/2$ -dram vial was added ^{131}I (~ 1 mCi in 1–2 μL of 0.1 N NaOH) followed by a solution of NCS in HOAc (0.4 mg in 20 μL). The vial was capped and heated in an oil bath at 70 °C for 30 min. Trifluoroacetic acid (20 μL) was then added to the mixture, and the vial was heated for an additional 5 min. The solvents were evaporated, and the residual activity reconstituted in methanol (25 μL) and injected onto a reversed-phase HPLC column eluted with 20/80 $\text{CH}_3\text{CN}/\text{water}$ containing 0.1% TFA at a flow rate of 1 mL/min. The product activity ($t_R = 15$ –16 min) was isolated in about 70% radiochemical yield.

N^1 -(3-[^{131}I]iodo 4-nitrobenzyl)guanidine (18a). (A) *Attempted Radioiododesilylation of 12.* To ^{131}I in 0.1 N NaOH (1 μL , 100–200 μCi) in a $1/2$ -dram vial was added a solution of NCS in TFA (0.4 mg in 10 μL) followed by **12** (0.2 mg) in TFA (10 μL). The vial was vortexed and maintained at room temperature or heated at 70 °C for 5–30 min. The contents were injected onto a reversed-phase HPLC column eluted with 15/85 THF/0.2 M $\text{NH}_4\text{H}_2\text{PO}_4$ at a flow rate of 1 mL/min. Although other unidentified radioactive peaks were observed, no activity peak corresponding to **17a** ($t_R = 26$ –27 min) was noticed.

(B) *Radioiododesilylation of 13.* To 0.2 mg of **13** in a $1/2$ -dram vial was added about 1 μL of ^{131}I in 0.1 N NaOH (50–100 μCi) followed by NCS in TFA (0.4 mg in 10 μL). The reaction mixture was left at room temperature for 30 min, and injected onto a reversed-phase HPLC column eluted with 25/75 $\text{CH}_3\text{CN}/\text{water}$ containing 0.1% TFA at a flow rate of 1 mL/min. About 18% of the injected activity eluted with a retention time (7 min) corresponding to that of **18a**. Conducting the reaction at 70 °C reduced the radiochemical yield of the product, presumably due to deiodination.

(C) *Exchange Radioiodination of 18.* In accordance with a reported procedure (Mock and Weiner, 1988), a Reacti vial fitted with a vent tube and containing 1–5 μL of ^{131}I in 0.1 N NaOH (~ 1 mCi), $(\text{NH}_4)_2\text{SO}_4$ in water (10 mg in 1 mL), and **17** (0.2 mg) was heated at 150 °C for 30 min. To ensure that radioiodination had occurred, an aliquot of the reaction mixture was injected onto a reversed-phase column eluted with 15/85 $\text{CH}_3\text{CN}/\text{water}$ containing 0.1% TFA at a flow rate of 1 mL/min. HPLC analysis

indicated that ca. 88% of the injected activity was associated with a peak coeluting with **18** ($t_R = 16$ – 17 min). The product activity from the bulk of the reaction mixture was isolated in about 75% yield using a C18 solid-phase cartridge (see below).

N^1 -(3-[^{131} I]Iodo-5-nitrobenzyl)guanidine (25a**).** A solution of **23** (0.2 mg) in 10 μ L of AcOH was added to a $1/2$ -dram vial containing ^{131} I in NaOH (~ 0.5 mCi in 1–2 μ L) and 0.4 mg NCS in 10 μ L of AcOH, capped and vortexed, and heated at 75 $^\circ$ C for 15 min. TFA (10 μ L) was added and vortexed, and the vial was heated again at 75 $^\circ$ C for 5 min. The contents of the vial were then injected onto a reversed-phase HPLC column eluted with a 85:15 water/ CH_3CN solvent system containing 0.1% TFA at a flow rate of 1 mL/min. The principal radioactive peak ($t_R = 16$ – 17 min) corresponding to **25** was isolated in 45–50% radiochemical yield. HPLC analyses indicated that extended heating of the reaction mixture resulted in deiodination of the product.

N^1, N^2 -Bis(*tert*-butoxycarbonyl)- N^1 -(3-[^{131} I]iodo-5-methoxy-4-methoxymethoxybenzyl)guanidine (31a**).** To 0.2–0.3 mg of **35** in a $1/2$ -dram vial was added 1 μ L of ^{131} I in 0.1 N NaOH (0.5–1.0 mCi) followed by a 3:1 (v/v) solution of HOAc:30% H_2O_2 . The mixture was sonicated for 30 s and injected onto a silica HPLC column eluted with 5/95 EtOAc/hexanes containing 0.2% HOAc at a flow rate of 1 mL/min. The activity corresponding to **31a** ($t_R = 15$ – 16 min) was isolated in about 80% radiochemical yield.

N^1 -(3,4-Dihydroxy-5-[^{131} I]iodobenzyl)guanidine (32a**).** About 100 μ Ci of **31a** (HPLC fractions) was taken in a $1/2$ -dram vial, and the solvents were evaporated to dryness. To the residual activity was added TFA (100 μ L), and the vial was vortexed and maintained at room temperature for 15 min. TFA was evaporated, and the residue was triturated with 2×100 μ L of EtOAc. To the residual activity was added anhydrous dichloromethane (80 μ L) followed by BBr_3 (25 mg), and the reaction was allowed to proceed at room temperature for 15 min. The solvent was evaporated and the residue triturated with 2×100 μ L of EtOAc. The activity was dissolved in water (50 μ L) and injected onto a reversed-phase HPLC column. The column was eluted with a solvent mixture consisting of 0.1% (w/v) TFA in each of water (A) and acetonitrile (B) at 1 mL/min. For the first 10 min, the solvent contained 3% of B which was then linearly increased to 90% over a period of 15 min. About 40% of the injected activity was associated with a peak corresponding to **32a** ($t_R = 12$ min); another 30–40% of the injected activity was associated with a nonpolar peak ($t_R = 19$ min) which is presumably **33a**.

N^1 -(4-Hydroxy-3-[^{131} I]iodo-5-methoxybenzyl)guanidine (33a**).** To 100 μ Ci of **31a** in 25 μ L of EtOAc in a $1/2$ -dram vial was added SnCl_4 (5 μ L), and the solution was vortexed and maintained at room temperature for 10 min. The EtOAc was evaporated, and the residue was triturated with 2×25 μ L of EtOAc. The activity was redissolved in methanol (25 μ L) and injected onto a reversed-phase column eluted with a gradient method as above starting from an initial solvent composition of 5% B. About 80–90% activity eluted at a retention time (17 min) corresponding to that for **33a**.

N^1 -Hydroxy- N^3 -(3-[^{131} I]iodobenzyl)guanidine (38a**).** To a $1/2$ -dram vial containing ^{131} I (~ 0.5 mCi) in 1–2 μ L of 0.1 N NaOH was added NCS (0.4 mg in 10 μ L of TFA) and **42** (0.2 mg in 10 μ L of TFA). The vial was vortexed and maintained at room temperature for 10 min. The contents of the vial were injected onto a reversed-phase HPLC column eluted with 85/13.5/1/0.5 water/THF/

triethylamine/ H_3PO_4 at a flow rate of 1.2 mL/min. The product activity ($t_R = 16$ – 17 min) was isolated in about 85% radiochemical yield.

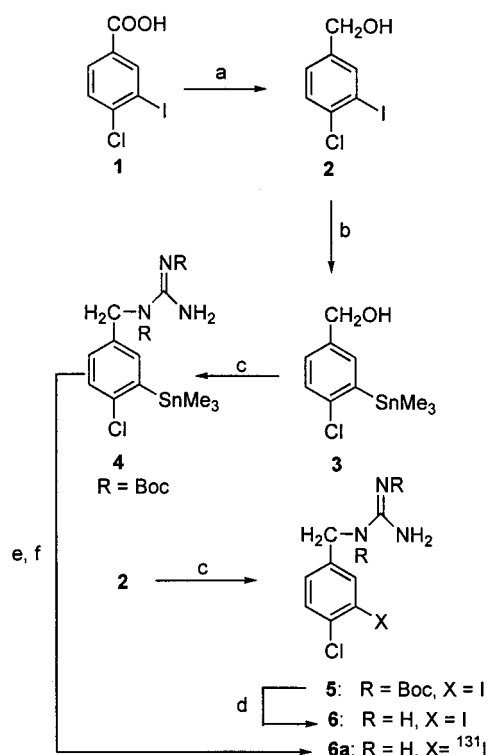
3-Amino-5-[^{131} I]iodobenzylguanidine (45a**).** About 1 mCi of ^{131} I in 1 μ L of 0.1 N NaOH was added to a $1/2$ -dram vial containing **46** (0.2 mg) in CHCl_3 (20 μ L). A 1:3 mixture of 30% H_2O_2 :HOAc (10 μ L) was added to the above mixture, and the vial was sonicated for 30 s. Most of the solvents were evaporated under a flow of argon, and the residual mixture was treated with TFA (0.1 mL) at room temperature for 5–10 min. The product **45a** was isolated in 35–40% radiochemical yield by reversed-phase HPLC ($t_R = 11$ min). For this, a C18 column (μ Bondapak) was eluted initially with 99.9:0.1 water:acetonitrile each containing 0.1% (w/v) TFA at a flow rate of 1 mL/min; after 15 min, the acetonitrile content was linearly increased to 90% over 15 min. All the above radioiodinated guanidine derivatives except **32a** and **45a** were concentrated from the HPLC fractions by solid-phase extraction and reconstituted in appropriate media for biological evaluations, as has been reported earlier (Vaidyanathan et al., 1997a). Concentration by solid-phase extraction of **45a** was performed by a slightly modified protocol (Wafelman et al., 1993). While a regular C18 cartridge was used in most cases, for very polar compounds such as **32a** and **45a** a bigger cartridge (tC18 ENV; Waters) was used.

RESULTS AND DISCUSSION

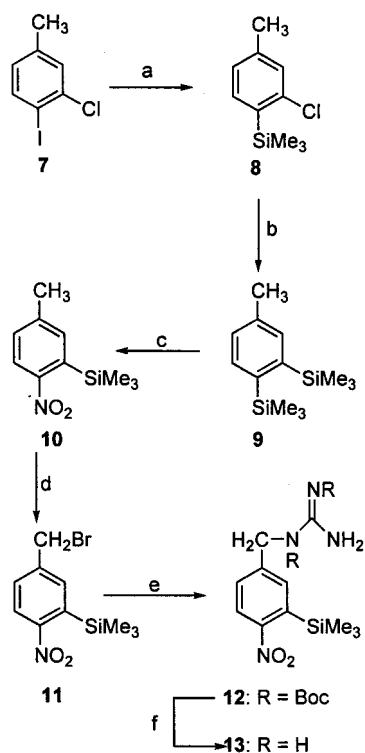
From an extensive structure–activity study of MIBG analogues, it has been shown that ring substituents in general are better tolerated than are modifications to the guanidinomethyl functionality (Wieland, 1986). We anticipated that derivatives with polar substituents might yield higher tumor-to-normal tissue ratios, because these analogues may be expected to clear faster from normal tissues. Efforts to synthesize MIBG analogues with highly polar groups such as sulfonic and phosphonic acid are underway; however, in the current study, we were able to prepare other analogues of varying polarity.

FIBG, the MIBG analogue with a fluorine at the 4-position had shown increased uptake and retention in tumor cells, as well as lower levels of deiodination (Vaidyanathan et al., 1997a). Although substituting fluorine in FIBG with a chlorine is expected to decrease its polarity, we wished to investigate whether a chlorine-substituted analogue will have a higher tumor uptake without concomitant increases in normal tissue uptake. The synthetic approaches used in the preparation of the unlabeled 4-chloro-3-iodobenzyl guanidine (**6**; CIBG), and the tin precursor **4**, from which **6a** could be obtained at a no-carrier-added level, are presented in Scheme 1. Commercially available 4-chloro-3-iodobenzoic acid was converted to **2** by NaBH_4 -mediated reduction of the active ester, formed in situ by its treatment with BOP (McGeary, 1998). Compound **2** was converted to the protected guanidine **5** by using the Mitsunobu protocol (Dodd and Kozikowski, 1994), and **6** was obtained from **5** by treatment with SnCl_4 (Miel and Rault, 1997). Attempts to convert **5** to **4** either by palladium-catalyzed stannylation (Wigerinck et al., 1993), or by initial Li/I exchange and subsequent stannylation were not successful. However, it was possible to convert **2** to **3**, from which **4** was obtained. Radioiodination of **4** with NCS in acetic acid and subsequent in situ deprotection with TFA resulted in **6a** with a radiochemical yield of about 70%.

The π value, an indicator of polarity, is typically negative for a nitro group (–0.28, 0.14, and 0.71 for NO_2 ,

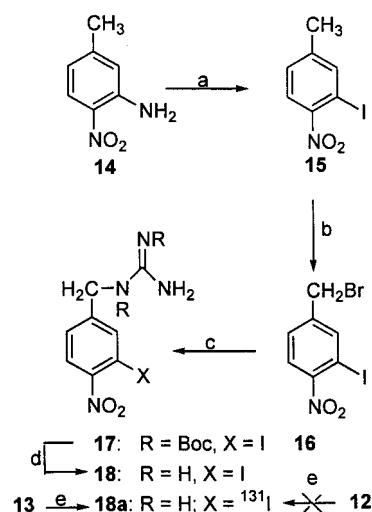
Scheme 1^a

^a (a) BOP, DIPEA; NaBH₄, THF; (b) (Ph₃P)₂PdCl₂, (Me₃Sn)₂, dioxane; (c) *N,N'*-bis-Boc-guanidine, NaH, DMF; (d) SnCl₄; (e) ¹³¹I, NCS, HOAc; (f) TFA.

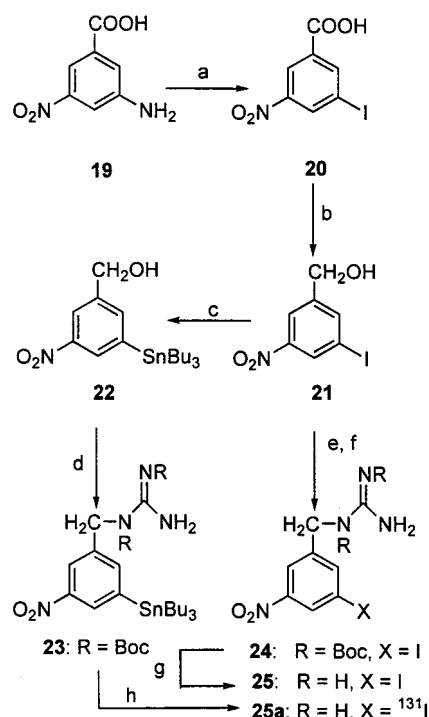
Scheme 2^a

^a (a) Mg, Me₃SiCl, Et₂O; (b) Na, Me₃SiCl, toluene, xylene; (c) 70% HNO₃, Ac₂O; (d) NBS, benzoyl peroxide, CCl₄; (e) *N,N'*-bis-Boc-guanidine, NaH, DMF; (f) H₂SO₄, dioxane.

F, and Cl, respectively; Fujita et al., 1964; Hansch, 1995), and nitro-substituted derivatives are often more polar than their parent molecules. To investigate the effect of a 4-NO₂ substituent on the resulting MIBG analogue, a

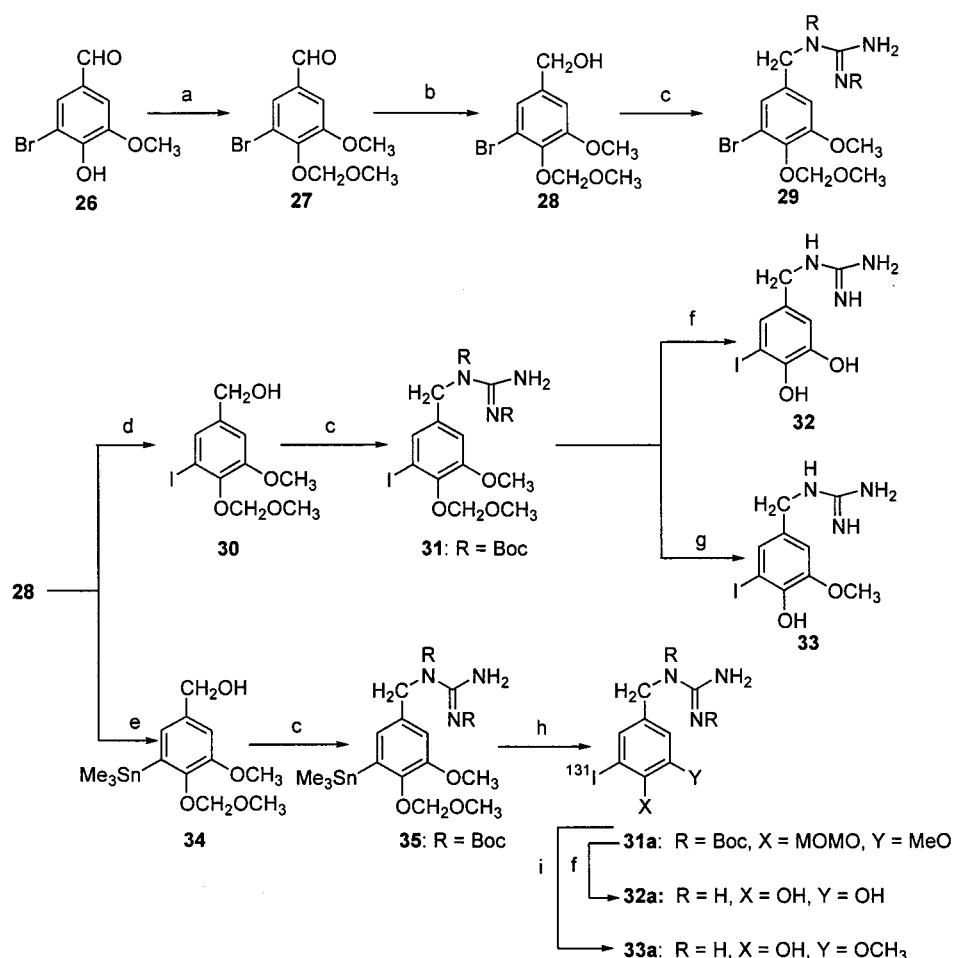
Scheme 3^a

^a (a) NaNO₂, HCl, KI; (b) NBS, benzoyl peroxide, CCl₄; (c) *N,N'*-bis-Boc-guanidine, NaH, DMF; (d) SnCl₄, EtOAc; (e) ¹³¹I, NCS, TFA.

Scheme 4^a

^a (a) NaNO₂, HCl, KI; (b) BH₃/THF, B(OMe)₃; (c) (Ph₃P)₂PdCl₂, (Bu₃Sn)₂, toluene; (d) *N,N'*-bis-Boc-guanidine, TPP, DIAD, THF; (e) *N,N'*-bis-Boc-S-methylisothiourea, TPP, DIAD, THF; (f) liquid NH₃; (g) TFA, CH₂Cl₂, 65 °C; (h) 1. ¹³¹I, NCS, HOAc, 2. TFA.

precursor for 3-[¹³¹I]iodo-4-nitrobenzyl guanidine (*p*[¹³¹I]-INBG) was sought (Scheme 2). Radioiodination of aryl stannanes are more facile in comparison to aryl silanes. However, due to the success we have had in the preparation of n.c.a. [¹³¹I]MIBG from a corresponding silicon precursor (Vaidyanathan and Zalutsky, 1993), **12** was initially chosen as a precursor for the preparation of radioiodinated *p*-INBG. The intermediate **10** was prepared from 3-chloro-4-iodotoluene in three steps using literature procedures (Clark et al., 1951; Eaborn et al., 1964; 1969). Benzylic bromination of **10** yielded **11** which was converted to the protected guanidine **12** by a method reported earlier (Vaidyanathan and Zalutsky, 1997b). A

Scheme 5^a

^a (a) MOMCl, potassium *tert*-butoxide; (b) DIBAL, CH₂Cl₂; (c) *N,N'*-bis-Boc-guanidine, DIAD, TPP, THF; (d) BuLi, CF₃CH₂I, THF; (e) BuLi, Me₃SnCl, THF; (f) BBr₃; (g) SnCl₄, EtOAc; (h) ¹³¹I, H₂O₂, HOAc, sonication; (i) TFA.

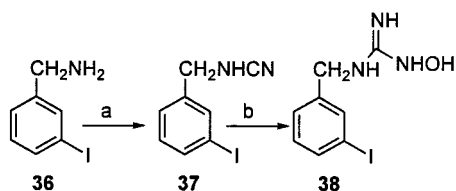
different synthetic route was taken to prepare unlabeled *p*-INBG (Scheme 3). The key intermediate, the benzyl bromide **16**, was prepared from **14** in two steps and converted to the protected guanidine **17** as above. The guanidine **18** was obtained by the SnCl₄-mediated deprotection of **17**.

Radioiodination of **12** and subsequent TFA treatment did not show a peak on HPLC corresponding to that of **18a**. Our prior unpublished results have shown that TFA sometime fails to remove both Boc groups from bis-Boc guanidines. To ensure that the absence of a peak corresponding to **18a** under reversed-phase HPLC conditions did not result from this partial deprotection, **12** was deprotected to **13** before being subjected to radioiodination. Although a peak corresponding to **18a** was observed by HPLC, the radiochemical yield was poor. Before undertaking the synthesis of a tin precursor to **18a**, an exchange radioiodination method (Mock and Weiner, 1988) was used to prepare **18a** in order to check its suitability as an MIBG analogue.

One of the problems associated with *p*INBG was its *in vivo* instability (see following article). This may be due to the presence of a strong electron-withdrawing group *ortho* to the iodine which may facilitate the nucleophilic displacement of iodine. To investigate the effect of switching the NO₂ group to the *meta*-position on the stability, and to examine the overall suitability of *m*INBG as a MIBG analogue, the isomeric (3-iodo-5-nitro)benzylguanidine (*m*INBG; **25**; Scheme 4) and its tin precursor **23** were synthesized. Compound **21** was prepared in two

steps from **19**. Although **24** could have been prepared directly from **21** by its coupling with bis-Boc-guanidine under Mitsunobu conditions, the *S*-methylisothiourea derivative was first prepared serendipitously and then converted quantitatively to **24** by treatment with liquid ammonia. It is often difficult to isolate products from Mitsunobu reactions by chromatography due to the coelution of starting materials and byproducts (O'Neil et al., 1998; Falconer et al., 1999; Kiankarimi et al., 1999). While this was generally the case for the purification of our diprotected guanidines, chromatographic separation of the *S*-methylisothiourea intermediate from the byproducts was much easier, at least in this specific instance, affording the pure product in a greater isolated yield. Treatment of **24** with TFA in CH₂Cl₂ yielded **25**. No-carrier-added *m*[¹³¹I]INBG was prepared from **23** in 50% radiochemical yield. The lower radiochemical yield may be a result of the chemical instability of **25a** under these reaction conditions. Accordingly, use of protracted reaction times and higher temperatures resulted in higher amounts of radioiodide, presumably from the deiodination of **25a**.

The natural substrate of the NET is norepinephrine, which has two hydroxy substituents at the 3- and 4-positions of its benzene ring. The potential of a "norepinephrine-like" benzylguanidine analogue with two hydroxyl groups has been suggested previously (Wieland, 1986). We wanted to investigate whether an MIBG derivative with two ring hydroxyl groups would retain affinity for NET. A *nc*a synthesis of 3,4-dihydroxy-5-[¹³¹I]-

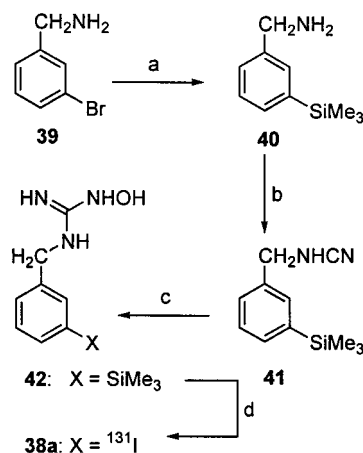
Scheme 6^a

^a (a) CNBr, NaOAc, MeOH; (b) NH₂OH·HCl, Na₂CO₃, DMF.

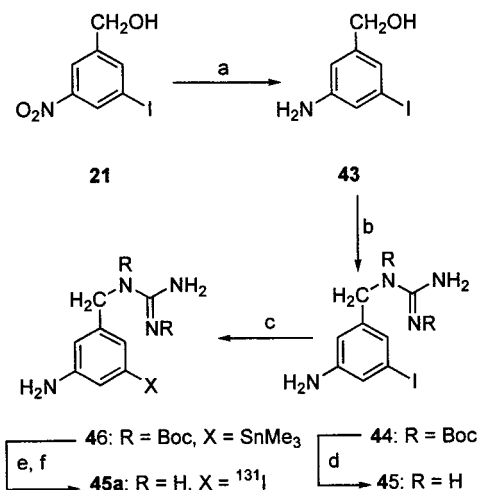
iodobenzyl guanidine ([¹³¹I]DHIBG; **32a**) was accomplished following an approach depicted in Scheme 5. The syntheses of unlabeled DHIBG and that of a suitable tin precursor were commenced from commercially available bromovanillin, **26**. To make the chemical manipulations facile, the 4-OH group of **26** was protected with a methoxymethoxy (MOM) group. The resultant **27** was reduced to the benzyl alcohol **28** which in turn was converted to **29**. Neither attempted stannylation of **29** to **35**, either by the palladium-catalyzed reaction or by the lithiation pathway, nor the conversion of **28** to **34** via the modified Stille protocol, were successful. However, **34** could be prepared by the lithium/bromine exchange of **28** and subsequent stannylation of the intermediate anion. Compounds **30** and **34** were readily converted to their respective guanidine derivatives, **31** and **35**, under Mitsunobu conditions. Both the methoxy and the MOM group as well as the Boc groups of **31** were removed simultaneously by treatment with BBr₃ to afford **32**. A radiochemical yield of about 80% was obtained for the conversion of **35** to **31a** under mild reaction conditions. Compound **31a** was smoothly converted to **33a** by its treatment with either SnCl₄ or TFA in 80–90% radiochemical yields. Treatment of **31a** with BBr₃ at room temperature gave **32a** but in meager radiochemical yields. In situ treatment of **33a**, formed by TFA treatment of **31a**, with BBr₃ increased the radiochemical yield of **32a** to some extent (ca. 40%). The potential for enhancing the radioiodination yield of **32a** by using alternative demethylating agents exists but was not investigated.

As indicated earlier, structural alterations at the side chain of MIBG have been less tolerated (Wieland, 1986). Except when the radioiodine was at the ortho position, both *N*¹-mono- and *N*¹,*N*²-dialkylated MIBG derivatives had insignificant target uptake in the dog adrenal medulla. Introduction of an amino group on the guanidine function had deleterious effects as well; however, a *N*-hydroxy-substituted MIBG derivative has not been studied. A number of *N*-hydroxyguanidines have been developed as potential replacements for the anti-hypertensive guanethidine; two of these had potencies similar to that of guanethidine (Balley and DeGrazia, 1973). MIBG itself was derived by combination of the guanidine group of guanethidine and the benzyl portion of bretylium, another neuron blocking agent. On the basis of these facts, we were interested in the development of the *N*-hydroxy-modified MIBG. The synthetic pathways for the preparation of *N*¹-hydroxy-*N*³-(3-iodobenzyl)guanidine (NHIBG; **38**) and its silicon precursor **42** are presented in Schemes 6 and 7, respectively. These were prepared from their corresponding benzylamines following a literature procedure (Balley and DeGrazia, 1973). Compound **40** (Vaidyanathan and Zalutsky, 1993) was prepared from **39** via a lithiation/silylation procedure. Radioiodination of **42** to **38a** was achieved in 80–90% radiochemical yield.

The MIBG derivative with an amino group at the 4-position (*p*AIBG) was better than the parent compound

Scheme 7^a

^a (a) BuLi, THF, Me₃SiCl; (b) CNBr, NaOAc, MeOH; (c) NH₂OH·HCl, Na₂CO₃, DMF; (d) ¹³¹I, NCS, TFA.

Scheme 8^a

^a (a) In, aq NH₄Cl; (b) *N,N'*-bis-Boc-guanidine, DIAD, TPP; (c) (Me₃Sn)₂, (Ph₃P)₂PdCl₂, dioxane; (d) TFA; (e) ¹³¹I, H₂O₂, HOAc, sonication; (f) TFA.

with respect to its human myocardial uptake. However, the thyroid uptake was also higher, suggesting its higher degree of dehalogenation (Shulkin et al., 1986), probably due to the presence of an amino group *ortho* to the carbon carrying the iodine. We wanted to investigate whether the isomeric compound, wherein the amino group has been switched to the *meta* position (*m*AIBG, **45**), would exhibit less dehalogenation. The synthesis of **45** and its tin precursor were readily achieved as shown in Scheme 8. Compound **43** was prepared from the nitro derivative **21** by metallic indium-mediated reduction. Guanidinylation of **43** via the Mitsunobu protocol, followed by TFA-mediated deprotection of Boc groups from the resultant guanidine derivative **44** delivered the target benzylguanidine. The tin precursor **46** was obtained by the stannylation of **44** using the modified Stille reaction. Both **44** and **46** exhibited very similar polarity, and hence it was difficult to separate [¹³¹I]**44** from **46** by normal-phase HPLC. For this reason, **46** was directly converted to **45a** in one-pot reaction and the latter isolated by reversed-phase HPLC. Based on a few attempts, the radiochemical yield for this conversion is about 40%.

In summary, this paper describes the syntheses of various MIBG analogues and their tin or silicon precursors. Methods for the preparation of radioiodinated derivatives have been described as well. In vitro and in

vivo evaluation of these analogues is the subject of the article which follows.

ACKNOWLEDGMENT

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Biological Evaluation of Ring- and Side-Chain-Substituted *m*-Iodobenzylguanidine Analogues

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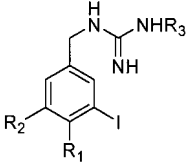
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A number of ring- and side-chain-substituted *m*-iodobenzylguanidine analogues were evaluated for their lipophilicity, in vitro stability, uptake by SK-N-SH human neuroblastoma cells in vitro, and biodistribution in normal mice. As expected, the lipophilicity of *m*-iodobenzylguanidine increased when a halogen was introduced onto the ring and decreased with the addition of polar hydroxyl, amino, and nitro substituents. Most of the derivatives showed reasonable stability up to 24 h in PBS at 37 °C. While *N*¹-hydroxy-*N*³-3-[¹³¹I]iodobenzylguanidine and 3,4-dihydroxy-5-[¹³¹I]iodobenzylguanidine generated a more nonpolar product in addition to the free iodide, 3-[¹³¹I]iodo-4-nitrobenzylguanidine decomposed to a product more polar than the parent compound. The specific uptake of 4-chloro-3-[¹³¹I]iodobenzylguanidine, 3-[¹³¹I]iodo-4-nitrobenzylguanidine, and *N*¹-hydroxy-*N*³-3-[¹³¹I]iodobenzylguanidine by SK-N-SH human neuroblastoma cells in vitro, relative to that of *m*-[¹²⁵I]iodobenzylguanidine, was 117 ± 10%, 50 ± 4%, and 12 ± 2%, respectively. The specific uptake of the known *m*-iodobenzylguanidine analogues 4-hydroxy-3-[¹³¹I]iodobenzylguanidine and 4-amino-3-[¹³¹I]iodobenzylguanidine was 80 ± 4% and 66 ± 4%, respectively. None of the other *m*-iodobenzylguanidine derivatives showed any significant specific uptake by SK-N-SH cells. Heart uptake of 4-chloro-3-[¹³¹I]iodobenzylguanidine in normal mice was higher than that of *m*-[¹²⁵I]iodobenzylguanidine at later time points (11 ± 1% ID/g versus 3 ± 1% ID/g at 24 h; *p* < 0.05) while uptake of 3-[¹³¹I]iodo-4-nitrobenzylguanidine and of *N*¹-hydroxy-*N*³-3-[¹³¹I]iodobenzylguanidine in the heart was lower than that of *m*-iodobenzylguanidine at all time points. In accordance with the in vitro results, none of the other novel *m*-iodobenzylguanidine derivatives showed any significant myocardial or adrenal uptake in vivo.

INTRODUCTION

Since its development about 2 decades ago, radioiodinated MIBG¹ (Table 1) has been used extensively in a number of oncologic and cardiologic applications; however, its properties are far from ideal for these applications (Wafelman et al., 1994a). As described by these authors, there are a number of compounding factors responsible for the sub-optimal performance of MIBG. Our laboratory has been investigating radiochemical variables with the goal of improving the clinical efficacy of MIBG. For example, we developed a no-carrier-added synthesis of radioiodinated MIBG (Vaidyanathan and Zalutsky, 1993, 1995), and a few centers around the world are evaluating the clinical utility of nca [¹²³I]MIBG (Farahati et al., 1997; Samnick et al., 1999; Owens et al., 2000). We also have evaluated an analogue of MIBG labeled with the alpha particle emitter ²¹¹At which might

Table 1. Structures of MIBG and Analogues



abbreviation	R ₁	R ₂	R ₃
MIBG	H	H	H
CIBG	Cl	H	H
FIBG	F	H	H
<i>p</i> AIBG	NH ₂	H	H
HIBG	OH	H	H
<i>m</i> INBG	H	NO ₂	H
<i>p</i> INBG	NO ₂	H	H
NHIBG	H	H	OH
HIMBG	OH	OCH ₃	H
DHIBG	OH	OH	H
<i>m</i> AIBG	H	NH ₂	H

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¹ Abbreviations: ABG, 4-aminobenzylguanidine; CIBG, 4-chloro-3-iodobenzylguanidine; DHIBG, 3,4-dihydroxy-5-iodobenzylguanidine; DMI, desipramine; FIBG, 4-fluoro-3-iodobenzylguanidine; HIBG, 4-hydroxy-3-iodobenzylguanidine; HIMBG, 4-hydroxy-3-iodo-5-methoxybenzylguanidine; *m*AIBG, 5-amino-3-iodobenzylguanidine; MIBG, *m*-iodobenzylguanidine; *m*INBG, 5-iodo-3-nitrobenzylguanidine; nca, no-carrier-added; NET, norepinephrine transporter; NHIBG, *N*¹-hydroxy-*N*³-3-iodobenzylguanidine; *p*AIBG, 4-amino-3-iodobenzylguanidine; *p*INBG, 3-iodo-4-nitrobenzylguanidine.

be useful for the treatment of metastatic diseases (Vaidyanathan and Zalutsky, 1992). Currently we are looking at the possibility of developing an MIBG analogue that will yield higher tumor retention and tumor-to-normal tissue ratios.

Because polar compounds are generally eliminated rapidly via the urine, MIBG derivatives containing polar substituents may yield higher tumor-to-normal tissue ratios, provided that such analogues are taken up efficiently by the tumor. Although many compounds with diverse structural features are substrates for the norepinephrine transporter, subtle changes in the structure

of aralkylguanidines can result in significant alterations in their accumulation in adrenergic-rich tissues (Wieland, 1986; Wieland et al., 1984). Because polar substituents such as OH and NH₂ were not included in earlier structure-activity studies of aralkylguanidines (Short and Darby, 1967; Fielden et al., 1965), it was speculated that such substituents may be counterproductive (Lee et al., 1986). Indeed, our attempt to make MIBG more polar by replacing its benzene ring with a pyridine has yielded a compound that was not taken up by human neuroblastoma cells in vitro (Vaidyanathan et al., 1998). In contrast, while substituting a hydroxyl group at the 4-position of MIBG (HIBG) did not affect its adreno-medullary uptake in dogs significantly, the 4-amino derivative (*p*AIBG) had a higher uptake (Lee et al., 1986) than MIBG. Also, in patients, AIBG sequestered in pheochromocytoma tumor to a degree similar to that of MIBG (Shulkin et al., 1986).

HIBG and *p*AIBG have not been evaluated in tumor cells or xenografts containing upregulated norepinephrine transporter. It should be pointed out that there are differences in the norepinephrine uptake and storage systems not only between tumors and sympathetically innervated tissues, but also within different nontumor target tissues such as heart and salivary gland. While MIBG is stored in vesicles in tissues such as adrenals and heart, and in pheochromocytoma, SK-N-SH human neuroblastoma cells lack storage vesicles; the storage in this case seems to be cytoplasmic (Clerc et al., 1993; Sisson and Sulkin, 1999). The uptake of [¹³¹I]MIBG in heart is diminished by endogenous norepinephrine; in contrast, its uptake in parotid glands was little affected (Sisson and Wieland, 1986). Thus, the aim of the present study was to evaluate a number of newly developed MIBG analogues, as well as HIBG and *p*AIBG, in the SK-N-SH neuroblastoma cell line.

As described in the preceding paper (Vaidyanathan et al., 2001), the synthesis of several new analogues of MIBG has been developed. In the current study, these compounds have been evaluated with respect to their stability, lipophilicity, and uptake in SK-N-SH human neuroblastoma cells in vitro and in normal mouse tissues in vivo. Our results show that a number of these novel compounds completely lost their affinity for NET. Unfortunately, the others which retained the affinity do not seem to offer better in vivo properties than MIBG itself.

MATERIALS AND METHODS

General. All chemicals and reagents are obtained from Aldrich or Sigma unless otherwise noted. The details of the preparation of all new tracers used in this study are described in the preceding publication (Vaidyanathan et al., 2001). Except for *p*[¹³¹I]INBG, which was prepared by an exchange radioiodination, all radioiodinated MIBG analogues used in this study were *nca* preparations. Previously reported protocols were used for the syntheses of 4-hydroxybenzylguanidine hemisulfate and 4-hydroxy-3-iodobenzylguanidine hemisulfate (HIBG) (Lee et al., 1986). No-carrier-added (*nca*) [¹²⁵I]MIBG and radioiodinated FIBG were prepared as reported before (Vaidyanathan and Zalutsky, 1993; Vaidyanathan et al., 1997a). Details concerning the various analytical methods such as NMR, HPLC, etc., used to chemically characterize the compounds reported in this paper, are described in the preceding article (Vaidyanathan et al., 2001).

4-Aminobenzylguanidine Nitrate (ABG). A mixture of 4-aminobenzylamine (244 mg, 2 mmol) and 3,5-dimethylpyrazole-1-carboxamide nitrate (403 mg, 2

mmol) in ethanol (10 mL) was refluxed for 3 h. The solvent was evaporated, and the residual solid was washed with ethyl acetate. Recrystallization of the solid from ethanol/ether afforded 318 mg (70%) of a crystalline solid: mp 139–141 °C. ¹H NMR (CD₃OD) 4.50 (s, 2H), 7.4–7.58 (m, 3H). MS (FAB⁺) *m/z*: 165 (MH⁺).

4-Amino-3-iodobenzylguanidine (*p*AIBG). An HPLC standard of the title compound was prepared as follows: A solution of Iodogen (150 μg) in chloroform (30 μL) was taken in a half-dram vial, and the solvent was evaporated. To this vial was added a solution of ABG (0.5 mg) in acetate buffer, pH 4.5 (1 mL), followed by a solution of sodium iodide (0.45 mg) in the same buffer (420 μL). The mixture was incubated at room temperature for 30 min, and the product was isolated by semipreparative HPLC (*t*_R = 7.5 min). For HPLC, a C18 column (μ Bondapak) was eluted with 99.5:0.5 mixture of water/acetonitrile containing 0.1% (w/v) TFA. MS (ESI) *m/z*: 291 (MH⁺).

[¹³¹I]HIBG and *p*[¹³¹I]AIBG. The title compounds were prepared following protocols reported in the literature (Lee et al., 1986) and isolated by reversed-phase HPLC. For HPLC, a C18 column (μ Bondapak) was eluted with 99.7:0.3 water/acetonitrile containing 0.1% (w/v) TFA at a flow rate of 1 mL/min. The retention times of *p*[¹³¹I]AIBG and [¹³¹I]HIBG under these HPLC conditions were 9 and 15 min, respectively. Concentration of the tracers was performed by solid-phase extraction using bigger (tC18 ENV; Waters) cartridges by a method similar to that described in the preceding paper (Vaidyanathan et al., 2001). After eluting the initial solvent from the HPLC fractions, the cartridge was washed with 5 mL of water and 0.5 mL of 5 mM NaOAc, pH 4.5, in that order before eluting the tracer with methanol or ethanol.

Octanol–Saline Partition Coefficient. The lipophilicity of the MIBG analogues was compared to that of MIBG by determining their octanol–PBS (pH 7.4) partition coefficients. The assay was performed in a paired-label format, in most cases directly comparing the analogue with MIBG. Both octanol and PBS were pre-saturated with each other. About 500 000 counts of each tracer in 10 μL of PBS were added in triplicate to glass tubes containing a mixture of 2 mL each of octanol and PBS. The contents of the tubes were thoroughly mixed by vortexing, and the layers were separated by centrifugation using a microcentrifuge. Duplicate samples (50 μL) of each layer from each tube were withdrawn and counted for radioactivity. The aqueous layer was discarded, and fresh PBS (1.9 mL) was added and the process repeated. The assay was repeated a third time with 1.8 mL of fresh PBS. The partition coefficient was calculated as the ratio of counts in octanol to that in PBS. The mean and standard deviation from 18 measurements for each tracer were calculated.

In Vitro Stability. To determine the influence of various substituents on the degree of dehalogenation, the stability of some of the radioiodinated derivatives was determined. About 100–200 μCi of activity was taken in PBS (100 μL) and incubated at 37 °C. Aliquots were taken periodically over a period of about 24 h, and the percent of total activity that was associated with the intact tracer was determined by reversed-phase HPLC. The HPLC system used for each tracer was the same as reported earlier for the purification and isolation of the compound (Vaidyanathan et al., 2001).

Cells and Culture Conditions. The human neuroblastoma cell line SK-N-SH (Uptake-1 positive; Biedler et al., 1973) was purchased from the American Type

Culture Collection (Rockville, MD). The incubation medium (JRH Biosciences, Lenexa, KS) was made by mixing 440 mL of RPMI 1640, 50 mL of Serum Plus, 5 mL of penicillin-G/streptomycin (5000 units of penicillin and 5000 μ g of streptomycin in 1 mL of 0.85% saline), and 5 mL of glutamine (200 mM in saline). The cells were grown at 37 °C in a humidified incubator containing 5% CO₂. Cell viability was evaluated prior to each binding experiment by trypan blue dye (Kaltenbach et al., 1958), and was 95–98% for all studies.

Paired-Label In Vitro Uptake of MIBG Analogues by SK-N-SH Cells in Comparison to That of MIBG. SK-N-SH cells were plated in 6-well plates at an initial density of 5×10^5 cells per well in 3 mL of medium, and incubated for 24 h. The medium was removed by aspiration, and 100 nCi each of radioiodinated MIBG and one of the MIBG analogues² in 3 mL of medium was added to each well and incubated for 2 h at 37 °C. The specificity and energy dependence of tracer uptake were determined using DMI and ouabain, respectively. The initial medium was removed, and the cells were incubated with 3 mL each of 1.5 μ M DMI and 1 mM ouabain, respectively, for 30 min. The medium was removed, and tracers were added as above and incubated for 2 h at 37 °C. The energy dependence of tracer uptake was ascertained by determining the effect of temperature as well. For this, the original medium was removed, and fresh medium that was cooled to 4 °C was added followed by tracers (100 nCi each) in 10 μ L of the medium, and the cells were incubated for 2 h at 4 °C. Finally, the effect of norepinephrine and MIBG was ascertained by removing the original medium and co-incubating the cells with the tracers (100 nCi in 10 μ L each) and 3 mL each of 50 μ M norepinephrine and 10 μ M MIBG, respectively, at 37 °C for 2 h. At the end of the 2 h incubation period, the cells were solubilized by incubation with 500 μ L of 0.5 N NaOH for 30 min at room temperature and then removed with cotton swabs. The cell-bound activity was counted along with input standards using a dual-channel gamma counter. Each set of experiments was performed 3–6 times. The entire experiment was conducted twice in some cases.

Paired-Label Exocytosis of [¹³¹I]FIBG and [¹²⁵I]-CIBG from SK-N-SH Cells. In a previous study, we demonstrated that washout of FIBG from SK-N-SH cells in vitro was considerably slower than that of MIBG (Vaidyanathan et al., 1997a). To investigate whether a chlorine substituent yielded a similar effect, a paired-label study was performed comparing the washout of [¹³¹I]FIBG and [¹²⁵I]CIBG from SK-N-SH cells. Cells were added to 6-well plates at a density of $\sim 4 \times 10^5$ cells per well per 3 mL of medium and incubated at 37 °C for 24 h. After the 24 h period, the medium was replaced with fresh medium containing $\sim 1 \mu$ Ci of each tracer in a total volume of 3 mL per well. After incubating the cells with the activity for 2 h at 37 °C, the medium was removed and supplemented with 3 mL of either fresh medium or 1.5 μ M desipramine in medium. The cell-bound activity was determined at 0, 2, 4, 8, 24, 48, 72, and 96 h after the initial uptake. The medium was aspirated at the end of each period, and the cells were washed twice with 0.5 mL each of PBS. The cells were solubilized by incubating with 500 μ L of 0.5 N NaOH for 30 min and removed with cotton swabs. The cell-bound activity was counted using a dual-channel gamma counter. For each time point, the assay was performed in quadruplicate.

Table 2. Octanol–Saline Partition Coefficients of MIBG and Analogues

tracer	partition coefficient (mean $\pm$ SD)
MIBG	2.61 $\pm$ 0.07
4-chloro-3-iodobenzylguanidine	8.36 $\pm$ 1.43
4-fluoro-3-iodobenzylguanidine	3.01 $\pm$ 0.20
4-amino-3-iodobenzylguanidine	0.32 $\pm$ 0.02 ^a
4-hydroxy-3-iodobenzylguanidine	0.60 $\pm$ 0.03 ^a
3-iodo-5-nitrobenzylguanidine	2.08 $\pm$ 0.14
3-iodo-4-nitrobenzylguanidine	1.13 $\pm$ 0.03
<i>N</i> -hydroxy-3-iodobenzylguanidine	3.82 $\pm$ 0.17
4-hydroxy-3-iodo-5-methoxybenzylguanidine	0.60 $\pm$ 0.04
3,5-dihydroxy-5-iodobenzylguanidine	0.38 $\pm$ 0.02 ^a
3-amino-5-iodobenzylguanidine	0.68 $\pm$ 0.03 ^a

^a In these cases, the partition coefficient values of these compounds increased substantially with each equilibration step, while that of MIBG from the paired-label format remained fairly constant; values shown are from the first equilibration.

Biodistribution Studies in Normal Mice. These studies were performed for all tracers except [¹³¹I]HIBG and *p*[¹³¹I]AIBG in a paired-label format comparing the new tracer directly to MIBG. The study utilized male BALB/c mice weighing about 25 g with groups of five mice for each time point. Tracers (1–5 μ Ci) in 100 μ L of PBS were injected via the tail vein, and groups of animals were killed at 1, 4, and 24 h postinjection. The specificity of uptake was determined by pretreating a group of mice with DMI 30 min before injecting the tracers, and the tissue distribution was performed 1 h later. The mice were killed by an overdose of halothane, and tissues of interest were isolated, washed, blot-dried, and weighed. The tissues, along with 5% dose standards, were counted for ¹³¹I and ¹²⁵I using a dual-label program in an automated gamma counter. The uptakes were expressed as percent injected dose per gram of tissue (%ID/g) unless otherwise specified. The statistical significance in the difference between the uptake of ¹²⁵I and ¹³¹I in each tissue was calculated by the paired Student's *t*-test using the Excel computer program (Microsoft).

RESULTS

Syntheses of ABG and *p*AIBG. ABG was prepared from readily available 4-aminobenzylamine in more than 70% yield. An HPLC sample of *p*AIBG was obtained by the microscale iodination of ABG. Both were characterized by standard spectral techniques.

Octanol–Saline Partition Coefficient. The relative lipophilicities of the MIBG analogues were obtained by measuring their octanol–saline partition coefficients using their radioiodinated analogues (Table 2). As expected, halogen substitution increased lipophilicity. While the effect was only minimal with fluorine (3.01 $\pm$ 0.20 for FIBG vs 2.61 $\pm$ 0.07 for MIBG), substituting chlorine at the 4-position resulted in an almost 4-fold increase in the partition coefficient. The only other derivative with a higher partition coefficient than that of [¹²⁵I]MIBG was [¹³¹I]NHIBG. This corroborates the earlier claim that conversion of a guanidine to *N*-hydroxy-substituted guanidine results in increased lipophilicity (Balley and De-Grazia, 1973). While a nitro group at the para position decreased the lipophilicity by more than a factor of 2, meta substitution of the nitro group did not yield a similar effect. Partition coefficients for *p*[¹³¹I]AIBG, *m*[¹³¹I]-AIBG, [¹³¹I]HIBG, and [¹³¹I]DHIBG increased to a considerable extent with each equilibration step, while values for [¹²⁵I]MIBG from paired-label experiments

² If MIBG was labeled with ¹³¹I, the analogue was labeled with ¹²⁵I, and vice versa.

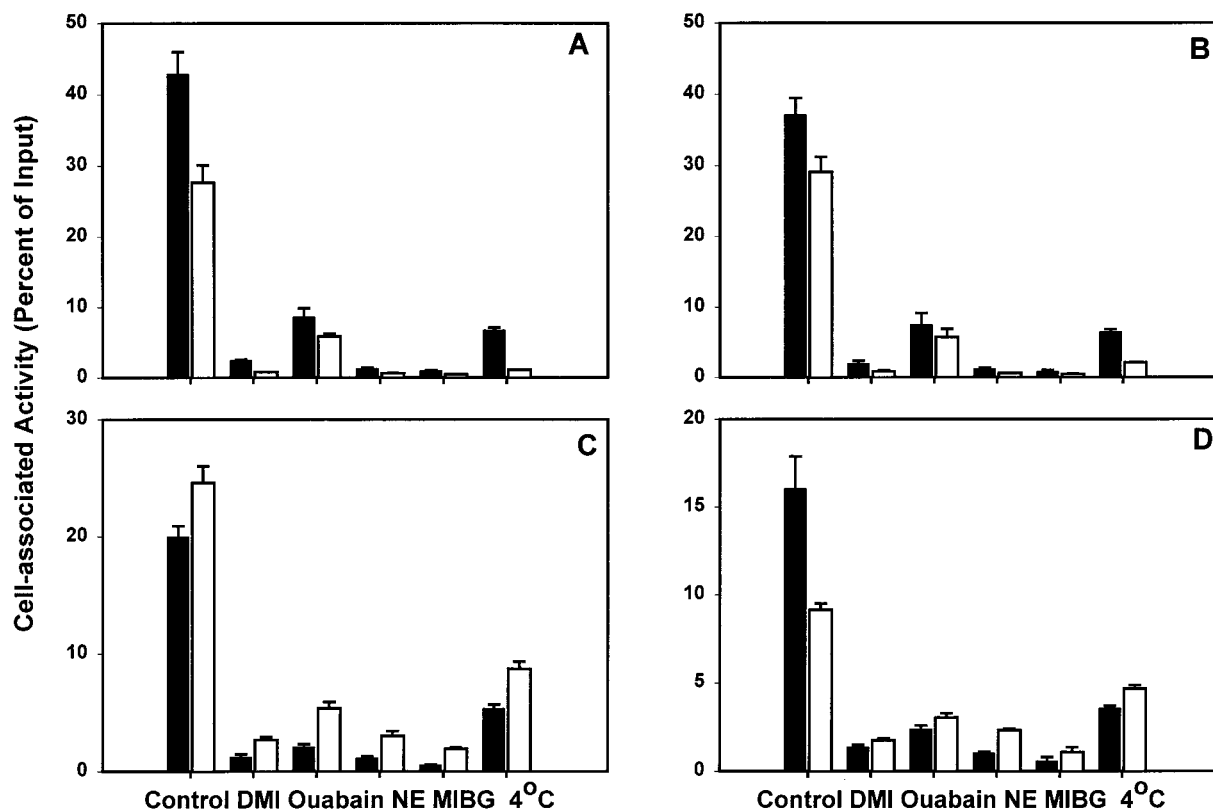


Figure 1. Uptake of [^{131}I]HIBG (A), $p[^{131}\text{I}]$ AIBG (B), [^{131}I]CIBG (C), and ex- $p[^{131}\text{I}]$ INBG (D) by SK-N-SH human neuroblastoma cells in vitro (open bars). Results obtained for [^{125}I]MIBG from respective paired-label assays are also shown (filled bars). The effect of various Uptake-1-blocking conditions is also presented. Cells were incubated as described in the text in the absence or presence of various Uptake-1 inhibitors or at 4 °C, and the cell-associated activity was determined as a percent of input radioactivity.

remained fairly constant. For these compounds, values from the first equilibration are shown in Table 2. $p[^{131}\text{I}]$ -AIBG was the most hydrophilic (0.32 ± 0.02) of all the compounds studied. Switching the amino group from the 4- to 5-position increased lipophilicity. While [^{131}I]HIBG had a partition coefficient of 0.60 ± 0.03 , introduction of another hydroxyl group reduced the partition coefficient to 0.38 ± 0.02 , and capping one of the hydroxyl groups in [^{131}I]DHIBG increased the lipophilicity back to 0.60 ± 0.04 .

In Vitro Stability. In vitro stabilities of radioiodinated MIBG derivatives were determined by HPLC after incubating them in PBS at 37 °C for various periods of time. [^{131}I]FIBG, [^{131}I]CIBG, $p[^{131}\text{I}]$ INBG, $m[^{131}\text{I}]$ AIBG, and [^{131}I]HIMBG were stable up to 24 h with less than 5% of free iodide formed. While there was about 5–10% of free iodide in the $p[^{131}\text{I}]$ AIBG and [^{131}I]HIBG preparations even at the beginning of incubation, no further deiodination was seen up to 4 h at 37 °C. [^{131}I]DHIBG deteriorated steadily with time with only about 30–35% of the total activity associated with the intact molecule at 4 h. While another third of the activity was free iodide, the remainder was an unidentified nonpolar compound. At 24 h, most of the activity corresponded to free iodide. Although little deiodination was seen in the case of $m[^{131}\text{I}]$ INBG, a radioactive byproduct more polar than the parent compound accrued with time. For up to 4 h, the [^{131}I]NHIBG remained intact except for the formation of a small amount of a slightly more nonpolar byproduct. Three radioactive peaks were observed at 24 h with the majority of the activity associated with the above byproduct. A commensurate amount of the activity was associated with the intact molecule, and only 5–10% of the total activity corresponded to free iodide.

Table 3. Uptake of Various MIBG Analogues by SK-N-SH Cells in Vitro

MIBG analogue	% specific uptake ^a
MIBG	100.00 $\pm$ 0.00
4-hydroxy-3-iodobenzylguanidine	66.42 $\pm$ 3.92
4-amino-3-iodobenzylguanidine	80.48 $\pm$ 4.05
4-chloro-3-iodobenzylguanidine	116.73 $\pm$ 4.80
3-iodo-4-nitrobenzylguanidine	50.40 $\pm$ 3.77
3-iodo-5-nitrobenzylguanidine	1.24 $\pm$ 0.12
3,5-dihydroxy-5-iodobenzylguanidine	0.08 $\pm$ 0.03
4-hydroxy-3-iodo-5-methoxybenzylguanidine	0.69 $\pm$ 0.24
3-amino-5-iodobenzylguanidine	0.27 $\pm$ 0.10
N-hydroxy-3-iodobenzylguanidine	12.34 $\pm$ 1.14

^a Specific uptake was obtained by subtracting uptake in the presence of DMI from that of control values. Each value is then normalized to the same value for MIBG obtained from paired-label assays; mean $\pm$ SD ($n = 3$ –6).

Uptake of MIBG Analogues by SK-N-SH Cells in Vitro. The degree of uptake, and the influence of various interventional agents and inhibitory conditions, for four analogues is shown in Figure 1. The specific uptake, defined as the difference between control values and those obtained in the presence of DMI, for each new analogue as well as for $p[^{131}\text{I}]$ AIBG and [^{131}I]HIBG is presented in Table 3. The 4-chloro derivative had a specific uptake of 117% that of [^{125}I]MIBG. The specific uptakes of $p[^{131}\text{I}]$ AIBG and [^{131}I]HIBG were 80% and 60% that of [^{125}I]MIBG, suggesting that these derivatives retain the biological characteristics of MIBG not only with respect to the canine adrenomedullary uptake in vivo reported previously (Lee et al., 1986), but also with respect to the uptake in SK-N-SH human neuroblastoma cells in vitro. The switching of the amino group to the 5-position resulted in the complete loss of specificity (specific uptake of $m[^{131}\text{I}]$ AIBG was 0.27% that of [^{125}I]-

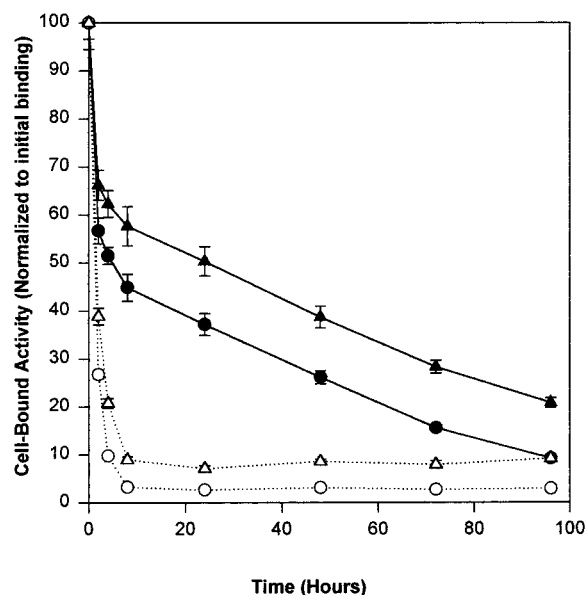


Figure 2. Paired-label exocytosis of [^{125}I]CIBG ($\blacktriangle$, without DMI; $\triangle$, with DMI) and [^{131}I]FIBG ($\bullet$, without DMI; $\circ$, with DMI) as a function of time. Cells were allowed to take up the tracers at 37 °C for 2 h. The medium containing the tracers was replaced with fresh medium without or with DMI, and the cell-associated activity was determined periodically.

MIBG). The 4-nitro derivative had a specific uptake about half that of [^{125}I]MIBG. As with the amino derivatives, switching the nitro group to the 5-position yielded a compound with a specific uptake of about 1% that of MIBG. Both dihydroxy- and hydroxy-methoxy-substituted derivatives did not have any significant uptake. While the *N*-hydroxy derivative showed some uptake, it was not substantial. The uptake of certain derivatives such as [^{131}I]CIBG and *p*[^{131}I]INBG was not reduced to the same level as [^{125}I]MIBG by some of the Uptake-1-blocking conditions, especially incubation at 4 °C.

Washout of CIBG from SK-N-SH Cells in Comparison to That of FIBG. Figure 2 shows the results obtained from a paired-label study of retention of [^{125}I]CIBG and [^{131}I]FIBG by SK-N-SH cells. The amount of initially bound [^{125}I]CIBG activity retained was consistently higher than that of [^{131}I]FIBG at all time points with the difference increasing with time. Even at 96 h, the specifically retained [^{125}I]CIBG activity was about 2-fold higher than that for [^{131}I]FIBG.

Biodistribution. Detailed biodistribution data are presented for those novel MIBG derivatives that demonstrated a reasonable uptake in SK-N-SH human neuroblastoma cells in vitro. Figure 3 shows the %ID/g in selected normal mouse tissues obtained from a paired-label biodistribution of [^{131}I]CIBG and [^{125}I]MIBG (see Supporting Information for uptake in other tissues). The heart uptake of [^{131}I]CIBG ($12.9 \pm 2.6\%$ ID/g) was less than that for [^{125}I]MIBG ($16.6 \pm 2.1\%$ ID/g) at 1 h ($p < 0.05$); however, the $^{131}\text{I}/^{125}\text{I}$ ratio in the heart increased with time, reaching >3 at 24 h. The uptake of both [^{131}I]CIBG and [^{125}I]MIBG was similar in the adrenals, the other Uptake-1 target tissue, and the difference in uptake was statistically significant only at 4 h ($9.09 \pm 5.03\%$ ID/g vs $7.49 \pm 3.98\%$ ID/g; $p < 0.05$). The uptake of [^{131}I]CIBG in a number of other tissues was higher than that of [^{125}I]MIBG, a finding similar to that observed for [^{131}I]FIBG (Vaidyanathan et al., 1997a). Compared to FIBG, one notable difference of CIBG was in its thyroid uptake. [^{131}I]CIBG activity in thyroid was 2–3-fold higher ($p < 0.05$)

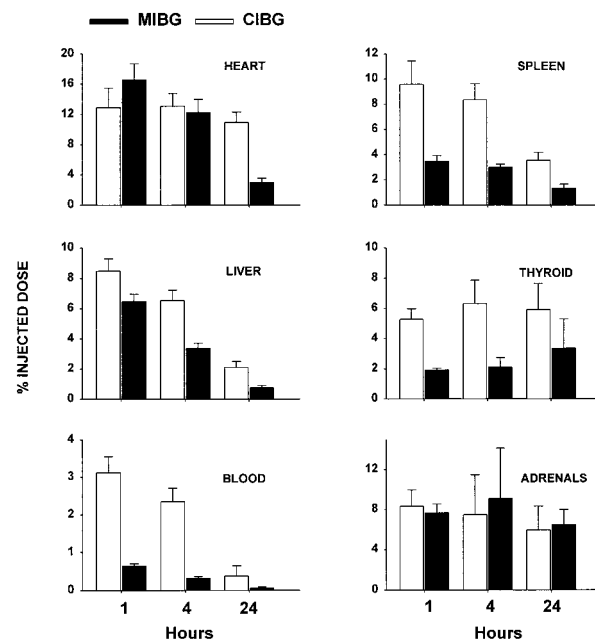


Figure 3. Paired-label uptake (%ID/g) of radioactivity in heart, spleen, liver, thyroid, blood, and adrenals at 1, 4, and 24 h after intravenous administration of [^{131}I]CIBG ($\square$) and [^{125}I]MIBG ($\blacksquare$).

than that seen for [^{125}I]MIBG, suggesting that the former was more susceptible to in vivo deiodination; in contrast, [^{131}I]FIBG was less susceptible to deiodination than [^{125}I]MIBG. Also, unlike FIBG, the blood activity from [^{131}I]CIBG was considerably higher than that of [^{125}I]MIBG. At 1 and 4 h, blood levels were $3.12 \pm 0.43\%$ ID/g and $2.35\% \pm 0.36\%$ ID/g, respectively, for [^{131}I]CIBG compared to $0.64 \pm 0.06\%$ ID/g and $0.32 \pm 0.05\%$ ID/g, respectively, for [^{125}I]MIBG.

The heart uptake of *p*[^{131}I]INBG was consistently lower than that of [^{125}I]MIBG (14.6 ± 2.6 , 11.7 ± 2.3 , and $3.4 \pm 0.6\%$ ID/g versus 20.5 ± 2.4 , 15.9 ± 2.6 , and $3.8 \pm 0.8\%$ ID/g at 1, 4, and 24 h, respectively; $p < 0.05$ for 1 and 4 h), and a similar trend was seen in adrenals as well (Figure 4). The uptake of *p*[^{131}I]INBG in a number of other normal tissues was significantly higher than that for [^{125}I]MIBG. For example, the uptake of *p*[^{131}I]INBG in spleen, lungs, and blood was 3–40-fold higher over the 24 h period. In addition, the $^{131}\text{I}/^{125}\text{I}$ ratio in the thyroid reached a value of more than 6 at 24 h, suggesting that *p*[^{131}I]INBG is very susceptible to in vivo deiodination. The biodistribution of *m*[^{131}I]INBG was quite different from that of the para-isomer. Heart uptake of *m*[^{131}I]INBG was only about one-tenth or less that of [^{125}I]MIBG at all time points. For example, at 1 h, the heart uptake of *m*[^{131}I]INBG was $1.89 \pm 0.29\%$ ID/g compared to $15.44 \pm 1.84\%$ ID/g for [^{125}I]MIBG ($p < 0.05$). Although the value of adrenal uptake for *m*[^{131}I]INBG at 1 h was roughly half that for [^{125}I]MIBG, no significant activity was left in the adrenals at 24 h ($0.24 \pm 0.02\%$ ID/g vs $6.86 \pm 0.5\%$ ID/g for [^{125}I]MIBG). The uptake in a number of other tissues such as liver, spleen, and lung was significantly less than that of [^{125}I]MIBG. The thyroid uptake of *m*[^{131}I]INBG was 2–5-fold lower than that of [^{125}I]MIBG, suggesting that, unlike the para-isomer, this derivative was less likely to undergo in vivo deiodination.

Except in the heart, adrenals, thyroid, and the brain, the uptake of [^{131}I]NHIBG was similar to [^{125}I]MIBG in most tissues at all time points (Figure 5). The heart uptake of [^{131}I]NHIBG (12.2 ± 1.0 and $8.0 \pm 1.1\%$ ID/g) was about half and two-thirds ($p < 0.05$) that of [^{125}I]MIBG (22.2 ± 2.6 and $12.0 \pm 2.1\%$ ID/g) at 1 and 4 h,

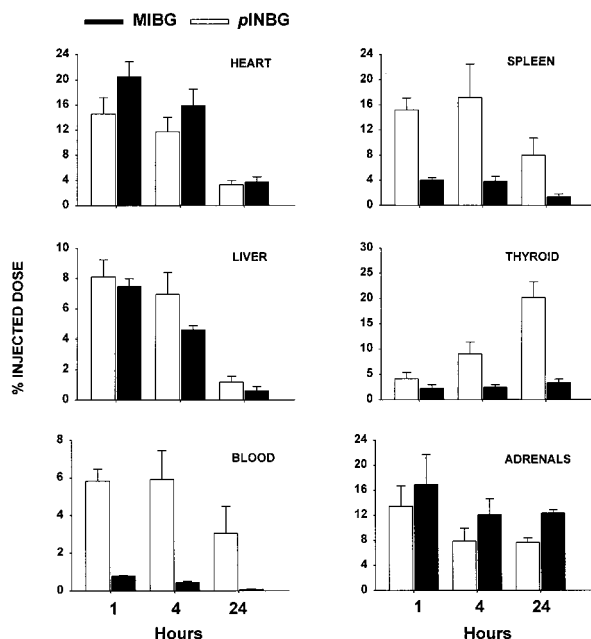


Figure 4. Paired-label uptake (%ID/g) of radioactivity in heart, spleen, liver, thyroid, blood, and adrenals at 1, 4, and 24 h after intravenous administration of $p[^{131}\text{I}]$ INIBG (□) and $[^{125}\text{I}]$ MIBG (■).

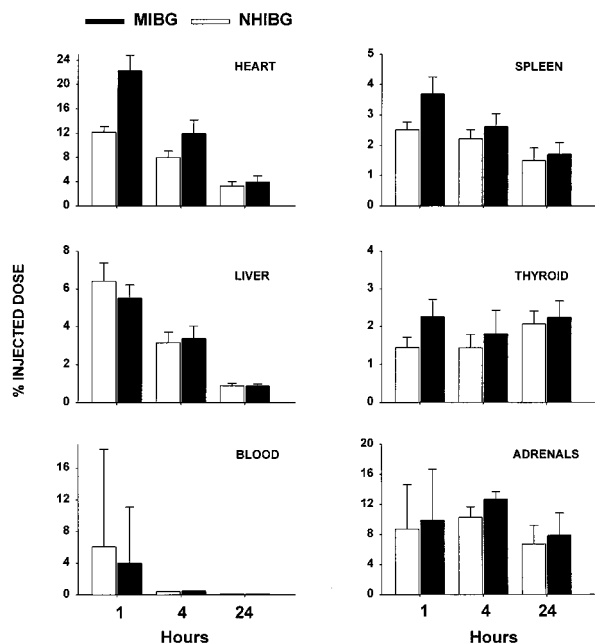


Figure 5. Paired-label uptake (%ID/g) of radioactivity in heart, spleen, liver, thyroid, blood, and adrenals at 1, 4, and 24 h after intravenous administration of $[^{131}\text{I}]$ NHIBG (□) and $[^{125}\text{I}]$ MIBG (■).

respectively, and at 24 h, the heart uptake of both tracers was similar. The adrenal uptake of $[^{131}\text{I}]$ NHIBG was lower ($p < 0.05$ except at 1 h) than that of $[^{125}\text{I}]$ MIBG, but the differences were not very substantial. Although not to a great degree, the thyroid uptake of $[^{131}\text{I}]$ NHIBG was less than that of $[^{125}\text{I}]$ MIBG at all time points ($p < 0.05$ only at 1 h). At 1 and 4 h, the brain uptake of $[^{131}\text{I}]$ -NHIBG was about 4-fold higher than that of $[^{125}\text{I}]$ MIBG, possibly due to its higher lipophilicity.

Neither heart nor adrenals had any significant uptake of $[^{131}\text{I}]$ DHIBG at any time points. The heart uptake of $[^{131}\text{I}]$ DHIBG at 1, 4, and 24 h was $1.12 \pm 0.07\%$ ID/g, $0.35 \pm 0.06\%$ ID/g, and $0.06 \pm 0.01\%$ ID/g, respectively,

compared to $25.78 \pm 5.08\%$ ID/g, $14.76 \pm 4.24\%$ ID/g, and $4.24 \pm 0.35\%$ ID/g for $[^{125}\text{I}]$ MIBG. Similarly, the adrenal uptake was also less than 10% that of $[^{125}\text{I}]$ MIBG at all time points. A number of other tissues also sequestered $[^{131}\text{I}]$ DHIBG to a considerably low degree. Thyroid uptake of $[^{131}\text{I}]$ DHIBG was almost 3-fold higher than that of $[^{125}\text{I}]$ -MIBG at 24 h, suggesting the in vivo instability of the former. While $[^{131}\text{I}]$ HIMBG did not have any significant uptake in heart and adrenals, most of the $[^{131}\text{I}]$ HIMBG-associated activity was eliminated via the urine within the first hour. The uptake of $m[^{131}\text{I}]$ AIBG in major organs including the target organs, heart and adrenals, was considerably lower than that of $[^{125}\text{I}]$ MIBG. The stomach and intestines exhibited a higher uptake of $m[^{131}\text{I}]$ AIBG at the early time points. Thyroid uptake of $m[^{131}\text{I}]$ AIBG was 2–3-fold lower than that of $[^{125}\text{I}]$ MIBG, suggesting its inertness to deiodination in vivo.

The specificity of the uptake of the various MIBG analogues in heart and adrenals was determined by measuring the effect of DMI on the uptake in these tissues at 1 h postinjection. As shown in Table 4, DMI reduced the heart uptake of $[^{131}\text{I}]$ CIBG to 58% of the control values; in comparison, $[^{125}\text{I}]$ MIBG uptake was reduced to 48% of its control values, and these reductions are statistically significant ($p < 0.05$). In adrenals, the uptake of $[^{131}\text{I}]$ CIBG and co-injected $[^{125}\text{I}]$ MIBG actually increased in animals pretreated with DMI. While the difference in $[^{131}\text{I}]$ CIBG uptake was statistically significant ($p = 0.03$), that of $[^{125}\text{I}]$ MIBG was not. The heart accumulation of $p[^{131}\text{I}]$ INIBG was reduced to 32% of the control values, and that of co-injected $[^{125}\text{I}]$ MIBG was reduced to 45%. Adrenal uptake of $p[^{131}\text{I}]$ INIBG was also reduced by DMI pretreatment, but the difference was not statistically significant. While DMI did not have any effect on the $[^{131}\text{I}]$ DHIBG uptake in heart, the uptake actually increased in the case of $m[^{131}\text{I}]$ INIBG. Considering the very low myocardial uptake of $m[^{131}\text{I}]$ INIBG, this increase may not have any significance. While the adrenal uptake of both $m[^{131}\text{I}]$ INIBG and $[^{131}\text{I}]$ DHIBG was reduced in the DMI-treated animals, the differences were not statistically significant. The heart uptake of $[^{131}\text{I}]$ -NHIBG was specific as shown by the fact that its uptake was reduced to about 48% of the control values when the mice were pretreated with DMI. As seen with $[^{131}\text{I}]$ CIBG, DMI-treatment increased the adrenal uptake of $[^{131}\text{I}]$ -NHIBG; however, the increase was not statistically significant. DMI did not have any effect on the heart uptake of $[^{131}\text{I}]$ HIMBG. Although the absolute heart uptake of $m[^{131}\text{I}]$ AIBG was considerably lower than that of $[^{125}\text{I}]$ MIBG, it seems to be mediated by the Uptake-1 pathway as it was reduced to roughly half its value by DMI, and the difference was statistically significant.

DISCUSSION

The purpose of this study was to determine the effects of ring- and side-chain substitution in MIBG on the biological properties of the compound. A number of MIBG derivatives were prepared, and most of them, with the exception of CIBG and NHIBG, had polar substituents on the ring (Vaidyanathan et al., 2001). CIBG was developed to investigate whether the encouraging results obtained with FIBG could be extended by substituting fluorine with chlorine. The canine adrenomedullary concentration of FIBG was similar to that of MIBG at 0.5 h but increased with time up to 24 h; however, by 72 h, FIBG uptake was less than that of MIBG (Wieland, 1986). On the other hand, uptake of FIBG in SK-N-SH neuroblastoma cells in vitro and mouse heart in vivo at 72 h and beyond was significantly higher than that of

Table 4. Heart and Adrenal Uptake of Various MIBG Analogues in Control Mice and in Those Treated with DMI

tracer	%ID/g ^a					
	heart			adrenals		
	control	DMI	<i>p</i>	control	DMI	<i>p</i>
CIBG	21.1 ± 6.3 (24.7 ± 6.4)	12.3 ± 2.8 (12.0 ± 2.3)	0.02 (0.00)	8.5 ± 3.9 (8.1 ± 3.2)	13.8 ± 2.4 (9.8 ± 0.8)	0.03 (0.29)
<i>p</i> -INBG	14.1 ± 1.6 (22.7 ± 2.0)	4.5 ± 0.5 (10.3 ± 2.0)	0.00 (0.00)	9.3 ± 2.0 (10.7 ± 2.2)	7.24 ± 2.0 (6.9 ± 2.0)	0.10 (0.02)
<i>m</i> -INBG	1.9 ± 0.3 (15.4 ± 1.8)	2.4 ± 0.3 (9.1 ± 1.1)	0.04 (0.00)	6.8 ± 2.1 (11.3 ± 3.8)	6.6 ± 1.2 (7.5 ± 1.0)	0.87 (0.17)
DHIBG	1.1 ± 0.1 (25.8 ± 5.1)	1.1 ± 0.2 (11.6 ± 2.2)	0.99 (0.00)	1.9 ± 1.3 (11.6 ± 4.0)	1.7 ± 0.5 (9.8 ± 2.6)	0.76 (0.42)
HIMBG	1.0 ± 0.1 (19.5 ± 1.5)	1.1 ± 0.2 (10.5 ± 1.3)	0.36 (0.00)	1.7 ± 0.5 (15.4 ± 7.2)	1.1 ± 0.2 (8.5 ± 0.8)	0.05 (0.07)
<i>m</i> -AIBG	2.8 ± 0.7 (20.3 ± 4.0)	1.5 ± 0.3 (8.1 ± 1.7)	0.00 (0.00)	6.0 ± 2.1 (14.1 ± 4.2)	4.5 ± 1.1 (8.5 ± 1.1)	0.19 (0.02)
<i>N</i> -HIBG	12.1 ± 1.0 (22.2 ± 2.6)	5.9 ± 0.5 (8.2 ± 0.6)	0.00 (0.00)	8.7 ± 5.9 (9.9 ± 6.8)	10.2 ± 1.4 (11.3 ± 1.4)	0.31 (0.31)

^a Mean ± SD (*n* = 5); values in parentheses are those for MIBG obtained from a paired-label study.

MIBG (Vaidyanathan et al., 1997a). Another of the analogues selected for study was NHIBG, an MIBG derivative with a hydroxyl group on the guanidine side chain. Although the hydroxyl group is polar, modifying the guanidine moiety with a hydroxyl substituent in fact reduces the polarity of guanidine derivatives (Baley and DeGrazio, 1973). Nevertheless, we were interested in studying the effect of an *N*-hydroxy substituent on the biological characteristics of MIBG.

The effect of various substituents on lipophilicity was generally as expected. While introduction of fluorine at the 4-position of MIBG did not alter the lipophilicity substantially, substituting a chlorine increased the lipophilicity of MIBG 4-fold. Introduction of polar substituents such as hydroxyl, nitro, and amino onto the ring indeed reduced the lipophilicity of MIBG. In the case of INBG and AIBG, switching the position of the amino or nitro group from the para to the ortho position resulted in an increased lipophilicity.

Based on studies of the in vitro stability of MIBG and its few analogues, it has been concluded that free iodide is the major decomposition product (Lee et al., 1986; Wafelman et al., 1994b). Very small amounts of *m*-iodobenzylamine and a compound more nonpolar than MIBG, among other impurities, have been detected in the case of [¹³¹I]MIBG (Wafelman et al., 1994b). Most of the derivatives exhibited an in vitro stability comparable to that of MIBG. Although both *p*[¹³¹I]AIBG and [¹³¹I]-HIBG were purified by reversed-phase HPLC, analytical HPLC of a freshly prepared sample of these showed the presence of free iodide. As reported before for other radiotracers (Larsen et al., 1997), this may be due to radiolytic degradation during the Sep-pak concentration. The in vitro stability of the above benzylguanidine derivatives has been studied at 4 °C for extended periods of time (Lee et al., 1986). When *p*AIBG was labeled with ¹²⁵I, less than 1% of radioiodide was formed up to 5 days. On the other hand, 5% of free iodide was generated from *p*[¹³¹I]AIBG at 30 h. [¹²⁵I]HIBG was less stable than *p*[¹²⁵I]AIBG and gave rise to about 2% radioiodide at 42 h. In the current study, the stability was studied for only a short duration; however, a higher, more biologically relevant temperature was used. No extensive deiodination was seen under these conditions.

[¹³¹I]DHIBG, a [¹³¹I]HIBG analogue with an additional hydroxyl group, degraded completely during the 24 h period. A considerable amount of an unidentified, nonpolar product was the major component at 4 h. Both [¹³¹I]-DHIBG and this nonpolar product deiodinated completely

by 24 h. A peak more nonpolar than the parent compound was also seen by HPLC in the case of [¹³¹I]NHIBG. While *p*[¹³¹I]INBG remained fairly intact, a considerable amount of the meta isomer decomposed to a product more polar than the parent benzylguanidine.

The specificity of the various MIBG analogues for NET was determined by paired-label uptake assays using SK-N-SH human neuroblastoma cells (Vaidyanathan and Zalutsky, 1993; Vaidyanathan et al., 1994, 1995, 1997a). Because the uptake is dependent on the passage number of the cells (Lashford et al., 1991; Vaidyanathan et al., 1995), the uptake of the [¹²⁵I]MIBG control varied from assay to assay. Only *nca* [¹³¹I]CIBG showed specific uptake comparable to that of [¹²⁵I]MIBG; in fact, its specific uptake (116.7 ± 9.6%) was higher than that of [¹²⁵I]MIBG. This is similar to the result observed for both ¹⁸F- and ¹³¹I-labeled FIBG (Vaidyanathan et al., 1994, 1997a), and may be due in part to the increased lipophilicity as a result of the introduction of a halogen substituent.

The specific uptake of *p*[¹³¹I]INBG prepared by exchange radioiodination (specific activity = ~5 mCi/mg) was 50.4 ± 3.6% that of [¹²⁵I]MIBG. The lower uptake could not be due to the presence of the carrier, because no saturation in uptake was seen at this activity concentration when [¹³¹I]MIBG of similar specific activity was used (Vaidyanathan and Zalutsky, 1993). Switching the -NO₂ group to the 5-position resulted in complete destruction of NET specificity. This lack of specificity indicates that NET may have some steric requirements for its substrates. [¹³¹I]NHIBG had a specific uptake 12.3 ± 2.0% that of [¹²⁵I]MIBG, corroborating the earlier observation that alterations in the guanidine function are detrimental to NET-mediated uptake.

It was disappointing to note that [¹³¹I]DHIBG, the MIBG analogue structurally similar to norepinephrine, did not have any significant uptake in SK-N-SH cells. Although norepinephrine is also very polar, the lack of specificity of [¹³¹I]DHIBG for NET may be a result of its poor trans-membrane transport due to its very high polarity. The MIBG analogue with one hydroxyl group at the 4-position (HIBG) has been shown to be an excellent analogue of MIBG with respect to its adreno-medullary uptake in dogs (Lee et al., 1986). It was anticipated that capping of the 3-hydroxyl group in DHIBG with a group such as methyl (HIMBG) might enhance its specific uptake; however, this was not the case. It is unclear whether this is an intrinsic property

of HIMBG or whether it was rapidly converted to DHIBG within the cells.

Like HIBG, the 4-amino-substituted MIBG (*p*AIBG) has also been shown to be an excellent MIBG analogue (Lee et al., 1986). While it is easy to introduce iodine at the ortho and para positions of hydroxyl and amino groups of phenols and anilines, respectively, the resultant iodophenols and iodoanilines are more susceptible to deiodination. We developed a radiosynthesis of 3-amino-5-iodobenzylguanidine anticipating it to be more stable, and thus a better analogue than *p*[¹³¹I]AIBG. However, it did not show any specificity for NET in the SK-N-SH cells. Based on the lack of specificity of various MIBG derivatives in which both 3- and 5-positions were occupied, it may be fair to conclude that the presence of substituents at both meta positions in MIBG may be counterproductive. It has been reported that while 3,4-diiodobenzylguanidine exhibited high adrenomedullary uptake despite its *in vivo* instability, relocating the 4-iodo substituent to the 5-position resulted in substantial reduction in the adrenomedullary uptake (Wieland et al., 1984; Wieland, 1986).

Based on the above results, it is tempting to speculate that polar substituents in the ring of MIBG adversely affect its NET specificity. While HIBG and *p*AIBG exhibit heart and adrenomedullary uptake in dogs and monkeys, no studies have been done to demonstrate their specificity of uptake in tumor cells such as SK-N-SH. As described earlier, there are variabilities in MIBG uptake from tissue to tissue. To determine the specificity of these two compounds for potential oncologic applications, we investigated their uptake in SK-N-SH neuroblastoma cells. The results (Figure 1 and Table 3) clearly demonstrate that, although not to the same level as MIBG, these two compounds are taken up by SK-N-SH cells to a considerable degree, suggesting that polar groups such as hydroxyl and amino at the 4-position do not destroy the specificity of uptake in this cell line. The lower uptake of [¹³¹I]HIBG compared to that of *p*[¹³¹I]AIBG is somewhat similar to their behavior in dog adrenal medulla. It may be possible that, once within the cells, [¹³¹I]HIBG is deiodinated to a higher degree than *p*[¹³¹I]AIBG.

One of the motivations for investigating CIBG is the enhanced retention of FIBG seen in SK-N-SH cells *in vitro* (Vaidyanathan et al., 1997a). From a paired-label study, it was clear that SK-N-SH cells retained [¹²⁵I]CIBG specifically to an even higher degree than [¹³¹I]FIBG. Thus, potentially higher tumor doses might be delivered by [¹³¹I]CIBG compared to [¹³¹I]FIBG. At least part of the enhanced retention may be as a result of the higher lipophilicity of CIBG. It may also be possible that the halogen substituent alters the rate and the pathway of metabolism of MIBG. For example, MIBG is metabolized to HIBG, albeit to a small degree, within pheochromocytoma in humans (Mangner et al., 1986). It is not clear how the presence of a 4-substituent would alter this pathway. It is known that microsomal cytochrome P-450 hydroxylates *o*- and *m*-fluoroanilines at the para position. On the other hand, it defluorinates as well as *o*-hydroxylates *p*-fluoroaniline (Rietjens and Vervoort, 1989). Studies are in progress to determine *in vitro* metabolism of MIBG and its fluoro and chloro analogues.

A number of the novel analogues were further evaluated by determining their biodistribution in normal mice. Thyroid uptake of [¹³¹I]CIBG was 2–3-fold higher than that for [¹²⁵I]MIBG, suggesting that, contrary to the *in vitro* stability results, chlorine at the 4-position makes MIBG more prone to deiodination *in vivo*. While fluorine substitution at the 4-position increased the stability of

[¹³¹I]MIBG (Vaidyanathan et al., 1997a), it had a destabilizing effect on the astatinated derivative (Vaidyanathan et al., 1997b). These results are somewhat enigmatic and cannot be easily rationalized. Another important difference in the biodistribution of [¹³¹I]CIBG was its higher blood uptake. Because bone marrow is a critical organ with respect to the radiation dose it can receive, it is not desirable to have persistent activity in the circulation. Most of the MIBG in the circulation was found to be in the erythrocyte fraction, and its movement from erythrocyte to plasma is not clearly understood (Sisson and Wieland, 1986). Because more lipophilic compounds are sequestered into erythrocytes rapidly (Zimmerman et al., 1987; Zou et al., 1998), it is reasonable to suggest that CIBG is taken up and probably retained by erythrocytes to a greater extent than MIBG and FIBG.

The uptake of *p*[¹³¹I]INBG in tissues such as spleen, blood, thyroid, and lungs was considerably higher than that of [¹²⁵I]MIBG. The uptake in these tissues was similar to that seen for free iodide (Garg et al., 1990). This suggests that, although it was found to be stable *in vitro*, *p*[¹³¹I]INBG underwent rapid deiodination *in vivo*, and that the observed biodistribution may be that of a combination of free iodide and intact molecule. The isomeric compound *m*[¹³¹I]INBG was developed to hopefully augment the inertness to deiodination. Paradoxically, while it was more unstable than *p*[¹³¹I]INBG *in vitro*, *in vivo* results suggested it to be more stable. Nonetheless, results from both *in vitro* experiments using SK-N-SH cells and that from *in vivo* in mouse showed it to be a poor analogue of MIBG.

While the uptake of [¹³¹I]NHIBG in adrenals and a number of other tissues was comparable to that of [¹²⁵I]MIBG, it had a lower heart uptake at early time points. This corroborates the results obtained from *in vitro* experiments and suggests that introduction of a hydroxyl group on the guanidino function results in the diminution of NET specificity. Results shown for [¹³¹I]DHIBG, [¹³¹I]HIMBG, and *m*[¹³¹I]AIBG in Table 4 clearly demonstrate that, as seen in the *in vitro* system, these MIBG derivatives are not suitable substrates for NET. This suggests that this transporter possibly accepts aromatic substrates with one vacant meta position.

In conclusion, among a number of ring- and side-chain-substituted, radioiodinated MIBG analogues developed, only [¹³¹I]CIBG showed higher uptake by SK-N-SH human neuroblastoma cells *in vitro* than [¹²⁵I]MIBG. However, its biodistribution data in normal mice suggest that it may not have any advantage over MIBG. While polar substituents are tolerated at the 4-position, it appears that the 5-position needs to be left unsubstituted. We are currently taking a prodrug approach to developing MIBG analogues with masked polar substituents. Hopefully with this strategy, tumor uptake may not be hindered, and at the same time faster normal tissue clearance of the deprotected metabolites may occur.

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Supporting Information Available: Tables (5, 6, and 7) containing numerical data of tissue uptake for CIBG, *p*INBG, and NHIBG for several mouse tissues (4 pages). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Pretargeting with Amplification Using Polymeric Peptide Nucleic Acid

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One goal of this investigation was to develop a polymer conjugated with multiple copies of peptide nucleic acid (PNA) and with pharmacokinetic properties suitable for applications in vivo. The second goal was to establish whether the multiple copies of PNA on the polymer could be targeted by hybridization in vitro and in vivo with ^{99m}Tc -labeled complementary PNA (cPNA). If successful, this approach could then be considered in further investigations as an alternative to existing pretargeting approaches because of the potential for signal amplification in the target. A 80 KDa poly(methyl vinyl ether-*alt*-maleic acid) (PA) polymer was conjugated with multiple copies of PNA and with multiple copies of poly(ethylene glycol) (PEG) by reacting the NHS derivative of PA with the amine derivatives of PNA and PEG. Using ^{99m}Tc -MAG₃-cPNA, targeting of PNA-PA-PEG was studied in vitro and in vivo in inflammation and tumor mouse models, in both cases relying upon nonspecific diffusion for localization. In addition, cPNA-avidin was considered as a clearing agent with biotinylated PNA-PA-PEG. About 80 PNAs could be conjugated to PA provided that about 200 PEGs were also conjugated to raise the aqueous solubility of the PNA-PA-PEG polymer lowered by the addition of the PNAs. About 70% of the PNAs on this polymer in vitro either in solution or attached to beads could be successfully targeted with ^{99m}Tc -cPNA. In both the inflammation and tumor mouse models, between 35 and 60% of these PNAs could be targeted in the lesions. The advantage of amplification was evident when less favorable results were obtained with PNA-PA-PEG conjugated with only six PNAs. We conclude that amplification can be achieved in vivo using polymers of PNA followed by radiolabeled complementary PNA and that the application of pretargeting using polymers of PNA for amplification can improve localization.

INTRODUCTION

Since pretargeting was first introduced (1), an improvement in target/nontarget radioactivity ratios over conventional imaging has been documented in many animal and patient imaging studies. With few exceptions (2, 3), pretargeting has been achieved through the use of (strept)avidin and biotin (4–7). Mathematical modeling of pretargeting has shown clearly the importance to pretargeting of high affinities between the molecular pair employed (8). (Strept)avidin and biotin admirably satisfy this criteria as a result of an affinity of approximately 10^{-15} M (9).

Despite the obvious success of pretargeting applications with (strept)avidin and biotin, use of this molecular pair is not without its difficulties. Unless properly designed, biotin analogues carrying the radiolabel are subject to biotinadase degradation (10). Streptavidin has been found to be immunogenic when conjugated to antibodies (11). Finally, endogenous biotin (vitamin H), present in all living tissues, can saturate the biotin binding sites of (strept)avidin located in a tumor and thus prevent targeting by radiolabeled biotin (12, 13).

Complementary oligonucleotides such as deoxyribonucleic acids (DNAs) may offer an alternative to (strept)avidin and biotin for pretargeting that avoids these difficulties. Depending upon chain length and base sequence, the affinities for duplex formation of comple-

mentary DNAs in serum environments may approach or even exceed that of (strept)avidin for biotin. Furthermore, single-strand DNAs have been administered repeatedly to patients in large dosages in connection with antisense chemotherapy without evidence of immunogenicities or serious toxicities (14). Finally, there will be no interference from endogenous substances.

Native DNA with its phosphodiester backbone is generally considered unsuitable for pretargeting and other in vivo applications due to rapid degradation by nucleases (15, 16). Fortunately, numerous chemically modified oligonucleotides have been prepared and shown to be stable to nucleases (17). One such modification that, in our view, may be particularly attractive as an alternative to (strept)avidin and biotin for pretargeting, has been named "peptide nucleic acid" (PNA) (18, 19). This synthetic oligomer consists of (2-aminoethyl) glycines to which the nitrogenous based (nucleobases) are attached via methylenecarbonyl linkers. PNAs are reported to be stable to proteases as well as nucleases (20), to show hybridization affinities greater than that of DNA (21) and to be nontoxic (20).

This laboratory has previously considered PNAs for pretargeting applications (22). In that study, pretargeting was investigated in both an inflammation and tumor mouse model in which streptavidin saturated with biotinylated PNAs was administered first and allowed sufficient time to accumulate by nonspecific diffusion in the target. At that point, the radiolabeled complementary PNA was administered. For both models, pretargeting was successfully accomplished to judge by improved

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lesion accumulation and improved lesion/normal tissue ratios relative to controls.

One property of (strept)avidin and biotin for pretargeting provides the potential of signal amplification by a factor of 4 when the radioactivity is attached to biotin since (strept)avidin possesses four biotin binding sites. However, among the advantages of using oligonucleotides for pretargeting is the potential of amplification far in excess of four. This could be achieved if the first administration consisted of a polymer to which multiple copies of oligonucleotides were bound. There would then be the potential that multiple copies of the radiolabeled complementary oligonucleotide within a second administration could bind with the resulting amplification of the radioactivity localized in a target containing the polymer (23).

Amplification, as defined above, obviously requires a multivalent oligonucleotide polymer. In studies preliminary to this report, we considered a number of polymers in a search for platforms upon which a suitable number of PNAs could be attached. These preliminary studies helped to define more clearly the properties needed in a polymer useful for pretargeting PNA applications: the polymer must be large enough to carry sufficient numbers of PNAs; the PNA polymer should be water soluble and stable both in saline and serum; the pharmacokinetic properties must be favorable, and the PNAs must be arranged on the polymer such that binding is not unduly hindered sterically. One of the polymers we considered was found to possess suitable properties and became the focus of this investigation.

Poly(methyl vinyl ether-*alt*-maleic acid) (PA), as used in this research is a 80 KDa (number average) linear polymer in which carboxylate groups alternate with methyl ether groups. The ethers and the negatively charged carboxylates provide aqueous solubility while the carboxylates are required for conjugation to the amine-derivatized PNAs. There are approximately 920 carboxyl groups per PA polymer molecule. In the course of preliminary studies, it was observed that conjugation of PA with PNA to the desired degree considerably reduced the aqueous solubility of the polymer unless the number of PNAs per polymer was reduced. Since reducing the number of PNAs could render the polymer less suitable for pretargeting with amplification, the PNA-PA was also conjugated with multiple copies of poly(ethylene glycol) (PEG) to improve water solubility. Ultimately, the polymer used in this investigation was conjugated with an average of 80 PNAs and 200 PEGs per PA molecule. The molecular weight was calculated to be 1400 KDa.

Pretargeting studies by their nature are complex. Among the variable are at least two dosages as well as two time periods, that between injections and that between the last injection and sacrifice or imaging. The complexity increases further if a clearing agent is used. Additional variables unique to this investigation include the nature of the polymer, its molecular weight, the number of PNAs and PEGs attached, and the length and base sequence of both the PNA and the radiolabeled complementary PNA. This report describes our first pretargeting study using PA-PEG conjugates as platforms for PNA designed to display suitable pharmacokinetics in vivo, to localize nonspecifically (i.e., by passive diffusion) in mouse models of focal infection/inflammation and tumor and to provide there a target for ^{99m}Tc labeled complementary PNA (cPNA) within a second administration. As is described, in the course of this investigation, one clearing agent was also considered to improve upon the pharmacokinetics of the radiolabel.

MATERIALS AND METHODS

The 15-base single-stranded PNAs were provided as a gift (Michael Egholm, PerSeptive Biosystems, Framingham, MA). Both PNA and cPNA were derivatized with a primary amine on the amino terminus (i.e., 5' equivalent) end via a 17-member ethylene-ether linkage. The cPNA was also prepared occasionally with a biotin group on this end via the same linker. The linker and base sequence of PNA was $\text{NH}_2(\text{CH}_2)_2\text{O}(\text{CH}_2)_2\text{OCH}_2\text{CONH}(\text{CH}_2)_2\text{O}(\text{CH}_2)\text{OCH}_2\text{CO-TGT-ACG-TCA-CAA-CTA-CONH}_2$ and that for cPNA was (biotin)- or $(\text{NH}_2)-(\text{CH}_2)_2\text{O}(\text{CH}_2)_2\text{OCH}_2\text{CONH}(\text{CH}_2)_2\text{O}(\text{CH}_2)\text{OCH}_2\text{CO-TAG-TTG-TGA-CGT-ACA-CO NH}_2$ as previously described (19). The expected molecular masses were 4337.23, 4634.57, and 4408.27 Da, respectively, and were observed by mass spectrometry to be 4336.90, 4633.48 and 4407.65 Da. Purity was established by reverse phase HPLC (in all cases showing a single peak) and mass spectrometry (showing one predominant peak). Avidin and methoxypolyoxyethylene amine (MW 5000) were purchased (Sigma Chemical Co., St. Louis, MO). Poly(methyl vinyl ether-*alt*-maleic acid) (PA, number average MW ca. 80 KDa), anhydrous dimethyl formamide (DMF), *N*-methyl-2-pyrrolidinone (NMP), *N*-hydroxysuccinimide (NHS), dicyclocarbodiimide (DCC), and diisopropylethylamine (DIEA) were purchased (Aldrich Chemical Co., Milwaukee, WI). All reagents were used as received without further purification. NHS-MAG₃ was synthesized according to published procedures (24). ^{99m}Tc -pertechnetate was obtained from a ^{99}Mo - ^{99m}Tc radionuclide generator (Dupont, Billerica, MA).

Preparation of MAG₃-Coupled cPNA. Because of different base sequences, PNA is more soluble in aqueous solution than is cPNA. As a consequence, in this investigation PA was always conjugated with PNA while the radiolabel was usually attached to cPNA. Amine-derivatized cPNA was conjugated with NHS-MAG₃ as previously described (24). Briefly, a solution of cPNA was prepared at a concentration of 2–5 mg/mL in 0.25 M sodium bicarbonate–1 M sodium chloride–1 mM EDTA, pH 8.5, with 10–20% (v/v) of acetonitrile occasionally added to improve the solubility of cPNA. A solution of NHS-MAG₃ in anhydrous DMF (50 mg/mL) was then added dropwise to the stirred cPNA solution until a MAG₃ : cPNA molar ratio of 20:1 was reached. This solution was then incubated at room temperature for 30 min. The conjugated oligomer was purified on a 0.7 × 20 cm P4 column (BioRad, Melville, NY) eluted with 0.25 M ammonia acetate buffer, pH 5.2. Fractions off the P4 column were collected, and the absorbency of each was measured (U-2000, Hitachi Instruments, Danbury, CT) using an extinction coefficient at 260 nm determined in this laboratory of 35 $\mu\text{L}/\mu\text{g}$. The modified cPNA was stored at –20 °C for further use.

Radiolabeling of cPNA-MAG₃. A fresh 50 mg/mL sterile solution of sodium tartrate was prepared in 0.5 M sodium bicarbonate, 0.25 M ammonium acetate, 0.18 M ammonium hydroxide, pH 9.2. The high pH of the tartrate solution was necessary to adjust the final pH to approximately 7.6. In addition, a 1 mg/mL solution of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in 10 mM HCl was prepared just prior to use. To MAG₃-coupled cPNA (about 10–50 μg) contained in a sterile test tube was added the tartrate solution to a final concentration of about 7 $\mu\text{g}/\mu\text{L}$. To this was added sufficient ^{99m}Tc -pertechnetate solution to provide about 100 $\mu\text{Ci}/\mu\text{g}$ of PNA. The stannous ion solution was added immediately thereafter such that 3–4 μg of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ was added for each 10 μL of ^{99m}Tc -pertechnetate solution. Higher activities of ^{99m}Tc required proportionately larger

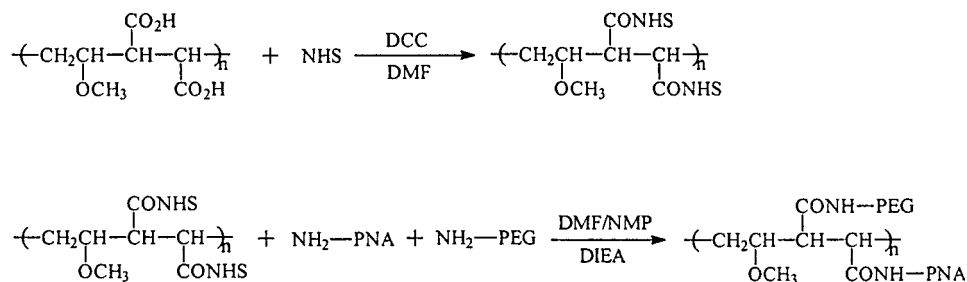


Figure 1. Schematic illustrating the preparation of NHS-PA and its conjugation with PNA and PEG.

volumes of the tin solution. After 40 min at room temperature, the labeled cPNA was purified on a 0.7×20 cm P4 gel filtration column using sterile pH 7.2 PBS eluant. Radioactivity and absorbency at 260 nm were used to identify and quantitate peak fractions. Preparations were routinely analyzed by size exclusion HPLC using a 1×30 cm Superose 12 column (Pharmacia, Piscataway, NJ).

Control labelings were performed in which the native, unconjugated cPNA was subjected to the identical labeling procedure to assess the extent of nonspecific labeling. In all cases, nonspecific radiolabeling was less than 5%.

PA Conjugation. Figure 1 illustrates the method of PNA and PEG conjugation to PA. As the first step, the NHS derivative of PA was prepared. With stirring, 20 mg of PA (0.230 mmol carboxyl groups) and 26 mg of NHS (0.226 mmol) were dissolved in 1 mL of anhydrous DMF, and the solution was cooled to 0°C . To this was added 48 mg of DCC (0.233 mmol) in 1 mL of anhydrous DMF. The molar ratio of NHS and DCC to carboxylate groups on PA was therefore about 1:1. Immediately after the addition of DCC, the solution was put on ice and kept at 0°C for 24 h. The dicyclohexylurea precipitated slowly and was filtered off at the end of the reaction. The NHS-PA solution at a concentration of 10 mg/mL was stored at -20°C and was used without further purification.

Typically, about 200 μg of the amine-derivatized PNA (0.046 μmol) was dissolved in 150 μL of anhydrous DMF and 150 μL of anhydrous NMP. To this solution were added 466 μg methoxypolyoxyethylene amine (Me-PEG-NH₂, MW 5000, Sigma, 0.093 μmole) and 3 μL anhydrous DIEA. After thorough vortexing, 100 μg of the NHS derivatized PA in dry DMF (1.15 μmol carboxyl groups) was added, and the solution thoroughly vortexed again. After 2 h at room temperature, the unreacted NHS ester was hydrolyzed by the addition of water to the reaction mixture. Early studies showed that ^{99m}Tc -labeled PA-PNA polymer localized in the liver of normal mice to an unacceptable extent ($>40\%$ id/g). This unfavorable biodistribution was considered to be due to the decreased aqueous solubility of PA when conjugated with 80 PNAs per molecule. Rather than reducing the number of PNA groups, which could adversely influence amplification, the biodistribution properties of the polymer were improved by conjugating the PA with poly(ethylene glycol) (PEG) as shown in Figure 1. The conjugation of PA with PNA and PEG was accomplished simultaneously. The molar ratio of PNA to PA was usually 100:1. In most cases, the molar ratio of PEG to PA was 200:1.

The average number of accessible PNA groups per PA was estimated using an aliquot of the conjugated polymer solution prior to purification. After adding tracer radio-labeled cPNA, the areas under the polymer peak and under the free PNA-cPNA duplex peak were compared in the radioactivity HPLC profile (Figure 2). The synthesized polymer was purified over a Sephadex G100

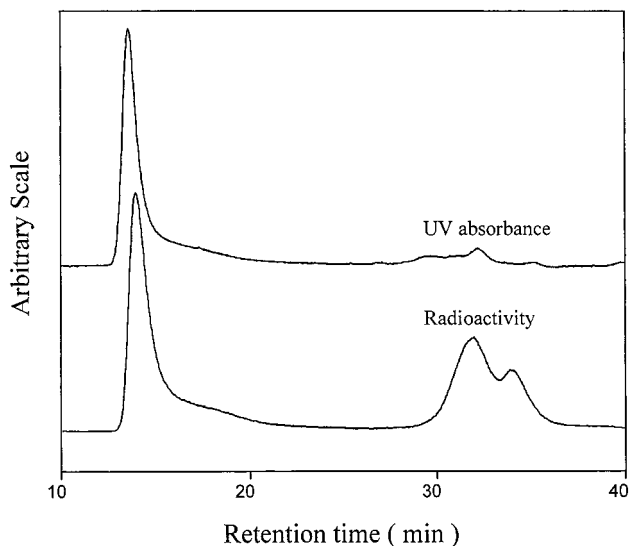


Figure 2. Size exclusion HPLC profiles of conjugated but unpurified PNA-PA-PEG after the addition of ^{99m}Tc -cPNA. Both UV and radioactivity traces are presented.

column (1×30 cm) using sterile water as eluant. The conjugated PA was stored in sterile water solution at -20°C .

The average number of PEGs per PA molecule was estimated using an aliquot of the conjugated polymer solution prior to purification. Using TNBS (trinitrobenzene sulfonic acid) (25), the number of free amine groups present due to unreacted PEG was quantitated and corrected for the contribution of free amine groups by PNA (the nitrogenous bases of PNA do not contribute in this assay). An aliquot of the reaction mixture containing a total of about 200 μg of PEG was removed to a glass tube and the solvent completely evaporated away with dry nitrogen. The residue was dissolved in 1 mL of 0.1 M sodium bicarbonate. To this solution was added 0.5 mL of 0.01% TNBS in 0.1 M sodium bicarbonate. After vortexing, the solution was incubated at 37°C for 2 h. After adding 0.5 mL of 10% SDS and 0.25 mL of 1 M HCl, the absorbance was measured at 335 nm. Free PEG was used to construct a standard curve. Under these conditions, the conjugation of PA with PEG was virtually quantitative.

Surface Plasmon Resonance Measurements. Recent improvements in instrumentation for biomolecular interaction analysis (BIA) has made it easier to measure rates of association and dissociation directly. Thus, BIA may be used to measure the rate of binding of an analyte on a surface to which its partner has been immobilized (26). The detection principle relies on surface plasmon resonance, an optical phenomenon that arises when light illuminates thin conducting films under specific conditions. Surface plasmon resonance forms the basis of BIA and permits the generation of sensorgrams in which the

refractive index changes due to this binding are measured in resonance units (RUs) (27).

In this investigation, surface plasmon resonance was used to estimate the association rate constants for duplex formation between immobilized cPNA and PNA both free and attached to PA polymer. In addition, the association rate constant between immobilized cPNA and PNA-PA-PEG with an average of 6 and 80 PNAs per PA was measured. The analysis was performed on a BIAcore-1000 (BIAcore, Piscataway, NJ) instrument operating at room temperature. Biotinylated-cPNA was added to a streptavidin-dextran-coated sensor chip only until a response of about 200 RUs was reached. The absence of mass transfer effects was confirmed by running separately one concentration of free PNA and one concentration of each PA polymer at three different flow rates (10, 30, and 75 $\mu\text{L}/\text{min}$) and demonstrating identical RU responses in all cases. Solutions of free PNA and both PA polymers were prepared at six concentrations (0.0 to 5.0 μM with respect to PNA) in the same running buffer (10 mM HEPES, 150 mM sodium chloride, 3.4 mM $\text{Na}_2\text{-EDTA}$, 0.005% P20, pH 7.4). Each analysis consisted of two measurements. A sample was first applied to a control surface consisting of an identical streptavidin-dextran-sensor chip but to which biotinylated PNA rather than cPNA had been added. Subtraction of the sensorgram obtained with the control surface from that obtained with the test surface at each concentration corrected for bulk refractive index changes. Following subtraction, the resulting sensorgrams were analyzed using instrument software (BIAevaluation 3.0, BIAcore) by assuming 1:1 Langmuir interaction.

Radiolabeling PNA-PA-PEG. To determine the bio-distribution of PNA-PA-PEG, the polymer was radiolabeled with $^{99\text{m}}\text{Tc}$ by adding a trace amount (about 10 μCi) of $^{99\text{m}}\text{Tc}$ -cPNA to 16 μg of the polymer in saline. The solution was repeatedly vortexed and incubated at room temperature for 20 min before administration.

In Vitro Stability of PNA-PA-PEG. Size exclusion HPLC was used to assess the stability of PNA-PA-PEG in serum. The polymer was incubated in fresh human serum at 37 $^{\circ}\text{C}$ at a concentration of 40 $\mu\text{g}/\text{mL}$, and aliquots were removed at different intervals over 24 h. To each aliquot, $^{99\text{m}}\text{Tc}$ -labeled cPNA was added in the identical fashion to radiolabel the polymer by hybridization.

Clearing Agents. Avidin conjugated with biotinylated cPNA was added as a clearing agent to reduce levels of the polymer in blood and in most normal tissues just prior to the administration of the radiolabeled cPNA. The cPNA-avidin was prepared by adding 200 μg of avidin to 40 μg of biotin-cPNA in 400 μL saline. The solution was gently vortexed, incubated at room temperature for 1 h and used without purification.

In Vitro Studies. The concept of pretageting with amplification was tested in vitro using streptavidin-coated magnetic beads 0.96 μm in diameter (Bangs Laboratories, Inc., Fishers, IN). The in vitro study consisted of three parts: involving beads without PNA (nonspecific), beads with PNA but without the addition of PNA-PA-PEG (specific without amplification), and beads with cPNA but with the addition of PNA-PA-PEG (specific with amplification).

In Vivo Biodistribution Studies. Normal CD-1 male mice (mean weight 26 g, Charles River, Wilmington, MA) were injected via a tail vein with 0.15 mL of 50 mM PBS containing 16 μg (10–12 μCi) of labeled PNA-PA-PEG. At 3 h, the animals were anesthetized with metofane (Schering-Plough, Omaha, NE) and sacrificed by cervical

dislocation. Following sacrifice, animals were dissected to provide samples of tissue for counting in a NaI (TI) well counter along with a standard of the injectate. Results were expressed as percent of injected dose per gram of tissue (% id/g).

To prepare the inflammation mouse model, a clinical isolate of *Escherichia coli* (American Type Culture Collection, Rockville, MD) was grown in LB medium (Sigma) to a density of 10^8 organisms/mL. Male CD-1 mice (Charles River) weighing approximately 30 g were anesthetized by inhalation of metofane and inoculated subcutaneously in the posterior right thigh with 0.1 mL of the bacterial suspension. Swelling was apparent after 14 h. At this time 16 μg of PNA-PA-PEG was administered through a tail vein. After 3 h, each animal received 2 μg (about 100 μCi) of radiolabeled cPNA by the same route. Two control groups of animals were used. One group (control 1) received in identical fashion the radiolabeled cPNA without having previously received the PNA-PA-PEG polymer. Another group (control 2) received in identical fashion PNA-PA-PEG but received $^{99\text{m}}\text{Tc}$ -labeled PNA instead of labeled cPNA. The anesthetized animals were imaged at 3 h post administration of the radioactivity and were then killed by cervical dislocation. Along with other tissues, the entire infected right leg and, for comparison, the whole left leg, was removed for counting.

To prepare the mouse tumor model, ACHN cells (American Type Culture Collection, Manassas, VA) were grown in minimal essential medium (Gibco, Grand Island, NY). The cells were removed from the culture flask by trypsinization and then washed in the culture media. Swiss male nu/nu mice (Taconic Labs, Germantown, NY) were each injected subcutaneously in the posterior left thigh with 3×10^6 cells each in 0.1 mL of medium. Two weeks later, when the tumors were approximately 0.6 cm in diameter, each animal received 16 μg of PNA-PA-PEG and, 3 h later, received 2 μg of $^{99\text{m}}\text{Tc}$ -cPNA. The animals were imaged 3 and 18 h later. Control animals received the radiolabeled cPNA without the prior administration of PNA-PA-PEG. After 18 h, all mice were sacrificed and tissues obtained for counting.

When under investigation, the clearance agent was administered in different dosages but always 2 h after the administration of PNA-PA-PEG polymer and 1 h before the administration of $^{99\text{m}}\text{Tc}$ -cPNA.

Imaging Studies. Scintigrams were acquired using a portable large field of view scintillation camera (Elscent APEXD 409M, Hackensack, NJ) equipped with a parallel-hole, medium-energy collimator and under computer control (Elscent APEX F1). Animals were anesthetized with nembutal (Abbott Laboratories, North Chicago, IL) and were then positioned directly on the collimator. Typically, several animals were imaged simultaneously at 3 h post administration of the radiolabel.

RESULTS

Conjugation Efficiencies. The HPLC profiles of PNA-PA-PEG, after conjugation but before purification are presented in Figure 2 for both radioactivity and UV detection at 260 nm. In both profiles, the peaks at 32–34 min are due to free PNA while that at 14 min is due to PNA-PA-PEG. A value of 80 for the average number of PNAs per PA was calculated on the basis of the ratio of UV peak areas. In this research, the PA-PEG polymer was also conjugated at a 10:1 PNA to PA molar ratio. By applying the same analysis, a value of 6 PNAs/PA was determined for this polymer. At 260 nm, the absorbance of PA and PEG are negligible.

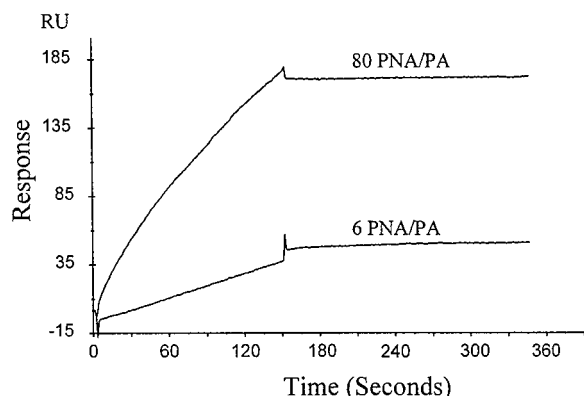


Figure 3. Typical room-temperature sensograms after subtraction for the hybridization of PNA-PA-PEG with cPNA immobilized on the sensor chip. Traces are presented at one concentration for PA conjugated with 80 and with 6 PNAs per polymer.

The HPLC profiles presented in Figure 2 were also used to determine the number of PNAs conjugated to PA that are accessible in solution. The ^{99m}Tc -cPNA added to unpurified PNA-PA-PEG distributes between free PNAs and PNAs accessible on the polymer according to their relative concentrations. Since the free PNA is unquestionably accessible, the radioactivity under the 32–34 min peaks was used to calculate the number of counts per accessible PNA. This value was then applied to the radioactivity of the 14 min peak. In this way the number of PNAs on PNA-PA-PEG accessible in solution was estimated to be about 60. The average number of PEGs per PA molecule was determined as described above to be about 200.

Surface Plasmon Resonance. Figure 3 presents typical sensograms for the hybridization of PNA-PA-PEG to immobilized cPNA obtained at room temperature and after subtraction showing association during injection followed by dissociation. Curves are presented at one concentration for both PNA-PA-PEG conjugated with 80 and 6 PNAs/PA. The higher response observed for PA conjugated with 80 PNAs compared to 6 PNAs was expected and confirms that a higher number of PNAs are available for hybridization in the case of this polymer.

As was observed previously for DNAs (28), the slow dissociation rate of these oligomers is below the detection limits of the method. Accordingly, it was not possible to measure an equilibrium constant although, as before, it was possible to measure the association rate constants. For the 80 and 6 PNAs/PA, respectively, the association rate constant was measured as 1.5 and $1.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ($N = 3$). Because each polymer presumably attaches to several immobilized cPNAs on the sensor chip, the absolute value of the association rate constant based on 1:1 Langmuir binding may be unreliable. Nevertheless, that these values are of the same order of magnitude suggests that the kinetics of binding probably is the same for both polymers. Furthermore, since the association rate constant for free PNA was measured as $1.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, the hybridization of the polymer may therefore have been about 10 fold faster than that of free PNA. A faster association may be expected for polymer with multiple PNAs. Finally, these values suggest that the method used to conjugate PNA to PA probably have not chemically altered the nitrogenous bases of the oligomer.

Stability of PNA-PA-PEG in Serum. The results presented in Figure 4 demonstrate that PNA-PA-PEG is stable in 37°C serum environments. The addition of ^{99m}Tc -cPNA to aliquots of serum containing PNA-PA-PEG

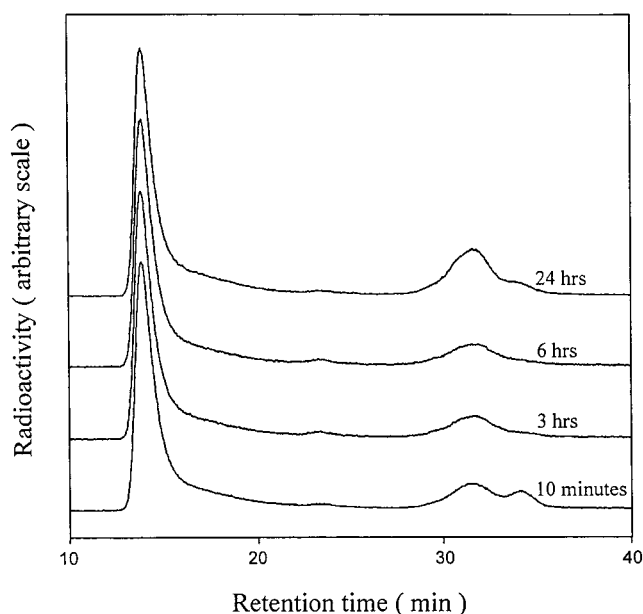


Figure 4. Size exclusion HPLC radiochromatograms of ^{99m}Tc -cPNA added at different times to a 37°C serum incubate of PNA-PA-PEG.

shows essentially no change in the distribution of the radiolabel between hybridized polymer and free ^{99m}Tc -cPNA even after 24 h of incubation.

In Vitro Amplification Studies. The concept of pretargeting with amplification was tested in vitro using streptavidin-coated magnetic beads. Radiolabeled cPNA was added identically to beads prepared in three different manners: beads without PNA (nonspecific binding), beads with PNA but without the addition of PNA-PA-PEG (specific binding and including nonspecific binding), and beads with cPNA and the prior addition of PNA-PA-PEG (specific binding with amplification). Table 1 summarizes the protocol and lists the results.

About $50 \mu\text{L}$ of packed magnetic beads with a total biotin capacity of greater than 100 pmol of biotinylated PNA, according to the manufacturer, was washed and resuspended with the aid of a magnetic holder. Over a period of 20 min at room temperature with periodic vortexing, the beads were bound with $1.0 \times 10^{-3} \mu\text{g}$ (0.22 pmol) of either biotin-derivatized PNA or biotin-derivatized cPNA. Since this addition would occupy only 0.3% of the biotin binding sites, complete PNA or cPNA binding was assumed. After additional washes, the beads were resuspended. To the cPNA beads only was added $0.4 \mu\text{g}$ of PNA-PA-PEG (i.e. 0.29 pmol PA, 23 pmol of PNA) followed by another 20 min incubation at room temperature. The 0.29 pmol of polymer may be expected to saturate the 0.22 pmol of cPNA on the beads leaving about (0.22×80) or 18 pmol of PNA on the beads. These beads were then washed as before and resuspended. To each of the three samples of beads was added $0.12 \mu\text{g}$ ($24.2 \mu\text{Ci}$, 25 pmol) of ^{99m}Tc -cPNA. After incubation for 30 min at room temperature with periodic vortexing, the beads were thoroughly washed for counting in a dosage calibrator and, after sufficient decay, in a NaI(Tl) well counter.

These results show that nonspecific binding is an important contributor to what is referred to above as "specific". Thus, true "specific" binding (minus nonspecific) amounted to only a little more than $0.1 \mu\text{Ci}$ ($0.35 - 0.23$). This result is reasonable since about $0.12 \mu\text{g}$ of labeled cPNA was added to beads containing about $10^{-3} \mu\text{g}$ of PNA for a theoretic maximum binding of only $(10^{-3}/$

Table 1. Experimental Protocol and Results for in Vitro Pretargeting with Amplification Using Three Different Conditions. The Radioactivity on the Beads Follows the Identical Addition of Labeled CPNA, $N = 3$

	nonspecific	specific	specific with amplification
beads	native beads	PNA-beads	cPNA-beads
first addition	nothing	nothing	PNA-PA-PEG
second addition	^{99m}Tc -cPNA	^{99m}Tc -cPNA	^{99m}Tc -cPNA
mean (range) radioactivity (μCi)	0.23 (0.22–0.23)	0.35 (0.33–0.37)	15.1 (15.0–15.3)

Table 2. The Biodistribution of PNA-PA-PEG with 0, 100, and 200 PEGs per Polymer Molecule in Normal Mice at 3 h. %id/g (s.d.), $N = 5$

tissue	0 PEGs/ polymer	100 PEGs/ polymer	200 PEGs/ polymer
liver	44 (0.7)	35 (0.4)	13 (1.7)
heart	1.2 (0.2)	1.1 (0.1)	3.5 (0.5)
kidney	5.1 (0.3)	3.5 (0.3)	5.5 (0.8)
lung	1.4 (0.2)	1.0 (0.04)	4.7 (0.7)
spleen	15 (0.8)	11 (2.3)	7.5 (1.5)
stomach	0.8 (0.3)	0.7 (0.1)	1.1 (0.3)
sm. int.	0.7 (0.1)	0.8 (0.2)	1.7 (0.3)
lg. int.	0.8 (0.3)	1.1 (0.1)	1.1 (0.1)
blood	1.1 (0.2)	0.6 (0.1)	25 (1.5)
muscle	0.3 (0.02)	0.3 (0.1)	0.8 (0.2)

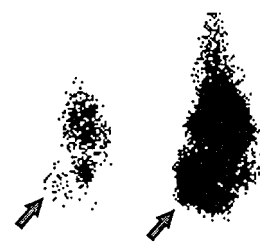
$0.12 \times 24 \mu\text{Ci}$) or about $0.20 \mu\text{Ci}$. In the case referred to above as “specific with amplification”, the 25 pmol of labeled cPNA was added to beads which should contain 18 pmol of PNA if amplified by the presence of 80 PNAs per PA. If 18 pmol of ^{99m}Tc -cPNA was now to bind (i.e., saturation of PNA on PA), there will be about $[(24 \mu\text{Ci}/25 \text{ pmol}) \times 18 \text{ pmol}]$ or $17 \mu\text{Ci}$ on the beads. This value is in agreement with the $15 \mu\text{Ci}$ observed and suggests that the radiolabeled cPNA has access to PNA on PA-PEG and the accessible PNAs on each immobilized PNA-PA-PEG molecule is about $[(15/17) \times 80]$ or 70. Since a value of 60 accessible PNAs per polymer molecule was determined from the UV and radioactivity HPLC profiles, the value of 70 PNAs per polymer molecule measured in the bead study suggests that the PNAs on immobilized polymer are equally accessible to the polymer in solution.

Normal Mouse Biodistributions. The biodistributions of PNA-PA-PEG with 80 PNAs but with 0, 100 and 200 PEG per polymer are shown in Table 2. The molecular weights of the polymers were calculated to be 427, 927 and 1427 KDa, respectively.

The results of Table 2 show that when PA is conjugated with 80 PNAs, the polymer must also be conjugated with more than 100 PEGs to achieve what may be a satisfactory biodistribution. Otherwise, liver levels are excessively high at about 40% id/g, blood clearance is rapid with blood levels showing only about 1% id/g at 3 h. However, when the polymer is conjugated with 200 PEGs, the liver level drops to 13% and the blood level rises to 25%.

Influences on Inflammation Imaging of PNAs per Polymer. To investigate the influence of the number of PNAs per polymer, PA-PEG was conjugated with either 80 or 6 PNAs. The molecular weights were calculated as 1427 and 1106 KDa, respectively. Figure 5 presents representative images of inflammation mice at 3 h post administration of ^{99m}Tc -cPNA in animals receiving the polymer conjugated with 6 PNAs (left) vs 80 PNAs (right). Table 3 lists the biodistribution results.

In the above table, differences in tissue values between the two polymers are significant in all cases ($P < 0.005$). These results show clearly the advantage of increasing the number of PNAs per PA. While the accumulation of radioactivity increased in all tissues sampled (for example, the target in the infected thigh increased about

**Figure 5.** Whole body images of two infected mice at 3 h post administration of ^{99m}Tc -cPNA and 6 h post administration of PNA-PA-PEG with 6 (left image) and 80 (right image) PNAs per polymer molecule. The location of the inflammation in the thigh is indicated by the arrows.**Table 3. The Biodistribution of ^{99m}Tc in the Infected Mouse Model 3 h Post Administration of Radiolabeled CPNA and 6 h Post Administration of PNA-PA-PEG. Results Are Presented for Polymer Conjugated with 6 and 80 PNAs. %id/g (s.d.), $N = 5$**

tissue	6 PNA/PA	target/ nontarget	80 PNA/PA	target/ nontarget
liver	1.5 (0.2)	0.5	7.8 (1.1)	0.7
heart	0.45 (0.05)	1.8	2.5 (0.6)	2.2
kidneys	2.1 (0.2)	0.4	4.2 (1.2)	1.3
lung	1.0 (0.2)	0.8	6.4 (1.5)	0.9
spleen	2.1 (0.5)	0.4	9.8 (1.6)	0.6
stomach	1.0 (0.2)	0.8	1.6 (0.3)	3.5
sm. int.	0.38 (0.05)	2.1	1.5 (0.2)	3.7
lg. int.	0.75 (0.11)	1.1	1.3 (0.1)	4.3
blood	9.4 (1.0)	0.1	11.1 (0.9)	0.5
normal thigh	0.3 (0.1)	2.7	1.2 (0.2)	4.7
infected thigh	0.8 (0.2)	—	5.6 (0.5)	—

Table 4. Biodistribution of ^{99m}Tc in Infected Mice 3 h Post Administration of Radiolabeled cPNA and 6 h Post Administration of PNA-PA-PEG. Results Presented for Study Animals as well as Two Controls (see text). %id/g (s.d.), $N = 5-6$

tissue	study mice	control mice 1	control mice 2
liver	5.8 (0.5)	0.6 (0.1)	0.4 (0.3)
heart	1.8 (0.2)	0.08 (0.02)	0.1 (0.1)
kidney	2.9 (0.3)	2.0 (0.3)	2.2 (0.9)
lung	5.4 (0.5)	0.2 (0.1)	0.2 (0.3)
spleen	5.5 (0.7)	0.6 (0.1)	0.3 (0.2)
stomach	0.9 (0.2)	0.5 (0.1)	0.9 (0.1)
sm. int.	1.2 (0.2)	0.2 (0.1)	0.3 (0.4)
lg. int.	0.6 (0.2)	0.4 (0.1)	0.7 (0.2)
blood	15.6 (1.0)	0.22 (0.05)	0.3 (0.3)
normal thigh	0.8 (0.1)	0.09 (0.02)	0.09 (0.01)
infected thigh	3.2 (0.4)	0.08 (0.01)	0.13 (0.03)

7-fold), the target/nontarget ratio are more favorable in all cases. The 22% difference in molecular weight between the two polymers is unlikely in itself to alter the biodistribution to the extent shown.

Infection Studies without Clearance Agents. Table 4 presents biodistribution results in mice infected in one thigh with *E. coli* and obtained 3 h. post iv administration of labeled cPNA and 6 h post iv administration of PNA-PA-PEG. Results are presented for the study animals and for two sets of control animals. One set of animals (control 1) did not receive the first administration of PNA-PA-PEG while the second set (control 2) received the PNA-PA-PEG in the identical manner but received radiola-



Figure 6. Two pairs of images of infected mice receiving PNA-PA-PEG followed by radiolabeled cPNA 3 h later. Whole body images obtained at 3 h post administration of ^{99m}Tc -cPNA. In each pair, the control animals is on the left. The location of the inflammation in the thigh is indicated by the arrows.

beled PNA instead of radiolabeled cPNA in the second injection. The PA was conjugated with 200 PEGs and 80 PNAs.

With the exception of stomach and large intestines, tissue radioactivity levels are significantly higher in the study animals compared to the control animals. This increase is related to the presence in infected and normal tissues of PNA-PA-PEG in concentrations sufficient to bind the radiolabeled cPNA in the second injection.

These values may be used to calculate the percentage of PNAs on PA in the lesion which are targeted with radiolabeled cPNA. From the biodistribution of radiolabeled PNA-PA-PEG in infected mice (data not presented) about 4% id/g of the polymer accumulates in the target at 3 h and, as such, about 35% of the PNAs on PA were targeted in each gram of lesion. This value represents a decrease from the 60–70% of PNAs targeted on the polymer when in solution or when bound to beads *in vitro* but is still considerable for *in vivo* targeting.

The mean inflamed to normal thigh ratios from the results presented in Table 4 are 4.0, 0.89, and 1.4 for the study and two groups of control animals, respectively. The differences between study and each control animals are significant. Thus, this ratio has been more than doubled by the prior administration of the polymer. Moreover, the mean uptake in the infected thigh of animals receiving the polymer has been increased a factor of 40 over the infected thighs of control animals not receiving the polymer. Finally the inflamed thigh to tissue radioactivity ratios are higher for the study animals in all tissues compared to the control animals, except for blood.

The two pairs of whole body images in Figure 6 are representative of the infected animals. Both control animals show radioactivity in bladder with faint accumulation in the target thigh. In each pair, the image on the left is of the study animals showing similar activity in bladder, increased activity in liver and, in particular, increased uptake in the infected thigh.

Tumor Studies without Clearing Agents. Table 5 presents biodistribution results in nude mice implanted in one thigh with the ACHN tumor. The results were obtained at 18 h post administration of labeled cPNA and 21 h post iv administration of PNA-PA-PEG. Results are presented for both the study and control animals (animals not receiving PNA-PA-PEG).

Tissue radioactivity levels are again significantly higher in most tissues for the study animals compared to the control animals. This increase is again related to the presence of PNA-PA-PEG in concentrations sufficient to bind the radiolabeled cPNA in the second injection.

The mean tumored to normal thigh ratios from the results presented in Table 5 are 14.6 and 2.0 for the study and control animals, respectively. As in the study of the

Table 5. the Biodistribution Results in Tumored Nude Mice 18 h Post Administration of Radiolabeled CPNA and 21 h Post Administration of PNA-PA-PEG. ID%/gm (s.d.), $N = 5$

tissue	study mice	control mice
liver	4.0 (0.6)	0.2 (0.04)
heart	0.4 (0.1)	0.01 (0.01)
kidneys	0.6 (0.1)	0.31 (0.03)
lung	0.4 (0.1)	0.02 (0.01)
spleen	3.9 (0.5)	0.06 (0.02)
stomach	0.3 (0.1)	0.3 (0.2)
sm. int.	0.5 (0.1)	0.08 (0.03)
lg. int.	3.7 (0.8)	1.8 (0.5)
blood	1.1 (0.2)	0.03 (0.02)
muscle	0.1 (0.02)	0.01 (0.003)
tumor	1.9 (0.4)	0.02 (0.01)



Figure 7. Two pairs of images of tumored mice receiving PNA-PA-PEG and radiolabeled cPNA 3 h later. Whole body images obtained at 3 h (left images) and 18 h (right images) post administration of ^{99m}Tc cPNA. In each pair, the control animal not receiving the polymer is on the left. The location of the tumor in the thigh is indicated by the arrows.

infected mice, prior administration of the PNA-PA-PEG has significantly improved the uptake ratio. Moreover, the mean uptake in the tumored thighs has been increased a factor of 90 over that in the tumored thighs of control animals not receiving the PNA-PA-PEG polymer. Finally, the tumored thigh to tissue radioactivity ratios are higher for the study animals compared to the control animals in all tissues.

These values may be used to calculate the percentage of PNAs on PA in the tumor that are targeted with radiolabeled cPNA. From the biodistribution of radiolabeled PNA-PA-PEG in tumored mice (data not presented), at 3 h post administration, about 2.5% id/g of the polymer accumulates in the target and, as such, about 58% of the PNAs on PA were targeted by radiolabeled cPNA. This is compared to the 35% targeting in the inflammation model and is equal to the 60–70% of PNAs targeted on the polymer *in vitro*.

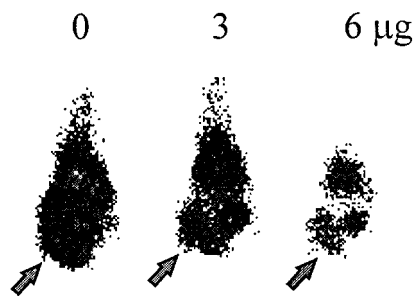
The whole body images of Figure 7 were obtained at 3 and 18 h post administration of radiolabeled cPNA and are representative of the tumored animals. On the left of each pair is the image of a control animal not receiving the polymer.

Inflammation Studies with Clearing Agents. Avidin-cPNA was prepared by mixing avidin with biotin-cPNA as described above. Mice were administrated IV either 0, 1, 3, 6, or 15 μg of avidin-cPNA 2 h after the iv injection of PNA-PA-PEG and 1 h before the administration of radiolabeled cPNA. The results are presented in Table 6 while Figure 8 presents representative images of animals receiving 0, 3, and 6 μg of this clearing agent. Under these circumstances, a 6 μg dosage appears to be optimum in terms of improving the target/nontarget ratios without excessively decreasing accumulation in the target.

Tumored Studies with Clearing Agent. Mice were administrated IV either 0 and 6 μg of avidin-cPNA 2 h after the first injection of PNA-PA-PEG and 1 h before the administration of radiolabeled cPNA. Table 7 presents the biodistribution results.

Table 6. Biodistribution at 3 h Post Administration of Radiolabeled CPNA and 6 h Post Administration of PNA-PA-PEG to Infected Mice Receiving Varying Amount of Avidin-cPNA as Clearance Agent. %id/g (s.d.), *N* = 5

tissue	dosage of Avidin-cPNA				
	0 μ g	1 μ g	3 μ g	6 μ g	15 μ g
liver	10.7 (0.9)	7.7 (0.3)	7.7 (1.2)	3.2 (0.3)	1.6 (0.2)
heart	1.7 (0.1)	1.7 (0.3)	1.14 (0.02)	0.5 (0.1)	0.13 (0.01)
kidney	3.7 (0.2)	4.1 (0.3)	3.6 (0.3)	3.8 (1.4)	1.6 (0.2)
lung	4.1 (0.4)	4.8 (0.3)	3.0 (0.5)	1.3 (0.3)	0.64 (0.05)
spleen	9.8 (1.0)	14.3 (0.6)	10.9 (2.0)	4.2 (0.3)	2.6 (0.4)
stomach	1.9 (0.3)	1.6 (0.3)	1.6 (0.4)	1.5 (0.2)	0.9 (0.2)
sm. int.	1.7 (0.2)	1.4 (0.1)	1.4 (0.3)	0.8 (0.2)	0.17 (0.02)
lg. int.	0.8 (0.2)	0.8 (0.1)	0.8 (0.2)	0.6 (0.3)	0.52 (0.04)
blood	10.4 (0.9)	12.2 (0.2)	7.2 (0.9)	1.3 (0.3)	0.42 (0.02)
normal thigh	1.1 (0.1)	1.03 (0.03)	0.8 (0.1)	0.3 (0.2)	0.11 (0.02)
inflamed thigh	3.3 (0.5)	3.7 (0.2)	2.7 (0.4)	2.3 (0.3)	0.45 (0.05)

**Figure 8.** Whole body images of infected mice 3 h post administration of ^{99m}Tc cPNA and 6 h post administration of PNA-PA-PEG. Each mouse received the indicated dosage of avidin-cPNA as clearance agent 2 h earlier. The location of the inflammation in the thigh is indicated by the arrows.**Table 7. Biodistribution at 3 h Post Administration of Radiolabeled CPNA and 6 h Post Administration of PNA-PA-PEG to Tumor-bearing Mice Receiving Varying Amount of Avidin-cPNA as Clearance Agent. %id/g (s.d.), *N* = 5**

tissue	dosage of avidin-cPNA	
	0 μ g	6 μ g
liver	8.1 (0.6)	1.8 (0.2)
heart	1.5 (0.1)	0.2 (0.02)
kidney	3.1 (0.2)	2.0 (0.3)
lung	2.8 (0.1)	0.5 (0.1)
spleen	7.4 (2.1)	0.8 (0.2)
stomach	1.9 (0.2)	1.4 (0.2)
sm. int.	1.5 (0.2)	0.6 (0.4)
lg. int.	0.9 (0.2)	0.3 (0.03)
blood	11.8 (0.6)	1.0 (0.05)
muscle	0.4 (0.2)	0.07 (0.01)
Tumor	2.9 (0.6)	0.8 (0.3)

The use of a 6 μ g dosage of avidin-cPNA as clearing agent reduced the radioactivity levels in all tissues including the tumor, such that tumor/normal tissue ratios were improved only in liver, heart, lung, spleen, blood, and muscle. In contrast to the use of the same dosage in the infected mice where the lesion lost only about 30% of its radioactivity, in the tumor-bearing mice, the lesion lost more than 70%. Clearly, the optimal dosage of clearing agent must be determined for each model system.

DISCUSSION

Since the goal of this investigation was to determine whether polymers of PNA could be targeted with radiolabeled cPNA to improve the localization of radioactivity using a pretargeting strategy, it was often necessary in the course of this study to select among a number of possibilities. Apart from the selection of the polymer scaffold itself, the selection of PNA as oligomer was significant. By surface plasmon resonance measure-

ments, the association rate for hybridization of this 15 base PNA pair was found to be similar to that for a pair of 18 base uniformly phosphorothioate DNAs (29). Accordingly, both DNAs and PNAs were considered to display the hybridization kinetics required for in vivo targeting. However, PNA was selected because of its stability in vivo and its high binding affinity toward its complement. Furthermore, the high charge of DNA was considered a disadvantage compared to the electrically neutral PNAs if charge neutrality permits closer packing on polymers. In fact, much greater difficulties were experienced in conjugating uniform phosphorothioate DNAs to PA and other polymers, and this was attributed to charge repulsion. It may be calculated that the average spacing of PNA groups on the PNA-PA-PEG polymer used in this investigation was 3.5 nm. This is closer than that used in DNA microarrays where about 10 nm separates each DNA (30). Even at this larger spacing, charge repulsion effects and steric hindrance are occasionally encountered (FR Ortigao, Hybain, private communication, 2000). Thus the short 3.5 nm spacing might be expected to severely restrict targeting. However, PA is a linear molecular with a backbone permitting free rotation. This fact may help explain that over 70% of the PNAs on the polymer were targeted in solution and even when the polymer was immobilized on beads. That targeting was 35–58% in vivo is only slightly less impressive considering the barriers which exist in vivo compared to the in vitro situation.

The selection of PNA introduced one difficulty as the poor aqueous solubility of PNA and, in particular, of cPNA, limited the number of PNAs that could be conjugated to PA. Biodistributions of radiolabeled PA, even with limited numbers of PNA, showed rapid blood clearance and excessive accumulation in liver (data not presented), properties which were only exasperated by increasing the number of PNAs per polymer to the required levels. Fortunately, the large number of carboxylate groups on PA (about 920) over that needed for PNA conjugation permitted the addition to this polymer of sufficient numbers of PEGs to increase circulation time and decrease liver accumulation. As shown in Table 2, liver accumulation at 3 h in normal mice was 44 id%/g with no PEG on PA molecule, while this value fell to 13 id%/g at 200 PEGs/PA.

An additional consideration was the number of PNAs per polymer molecule. Fortunately, the addition of PEGs permitted up to 80 PNAs per PA to be attached without adversely influencing the biodistribution of PA. That the number of PNAs is an important determinant to the success of pretargeting with this polymer is evident from the biodistribution results of PAs conjugated with 6 vs 80 PNAs (Table 3 and Figure 5). The absolute uptake

was 7 fold higher and the target/nontarget ratios were more favorable in all tissues when the polymer conjugated with the higher number of PNAs was administered.

Most pretargeting studies employ an intermediate administration of a clearing agent to remove as much of the first injectate remaining in circulation as possible before the radioactivity is administered. This process can significantly improve the target/nontarget radioactivity ratios (7). Therefore a number of clearing agents were considered in this investigation. With biotin conjugated to PNA-PA-PEG, it was possible to use either avidin or streptavidin as a clearing agent since both proteins are tetravalent for biotin and are therefore capable of cross-linking biotinylated PA. Accumulation in the infected thigh was unchanged by either clearing agent as was expected since neither protein should interfere with the target. However, liver, kidney, and spleen levels decreased only in the case of streptavidin, but by much less than desired (data not presented).

Avidin-cPNA was also investigated since cPNA on this potential clearing agent will hybridize to PNA-PA-PEG in circulation while the avidin should encourage clearance of the complex into the liver. However, avidin-cPNA can also reduce the number of available PNAs in the target. Any clearing agent containing cPNA may hybridize to PNA-PA-PEG in the target, thereby reducing the number of PNAs available to hybridize ^{99m}Tc -cPNA. Table 6 presents biodistribution results showing the effect of dosage of avidin-cPNA. The infected thigh target shows minimal decrease with increasing dosage of this clearing agent until 15 μg are reached. However all normal tissues except kidneys show dosage-related decreases within this range. The images of Figure 8 also show this improvement.

Thus, the results of this research show that pretargeting may be accomplished with PNA polymers. Using the hyperperfusion of denatured capillaries at sites of inflammation and tumor in mice, we have observed that the PNA-PA-PEG polymer of this investigation accumulates in both lesion types. Thereafter, the polymer may be targeted with a subsequent administration of radiolabeled cPNA. This investigation is similar to those conducted in this laboratory previously using PNA but with streptavidin-PNA as the first injectate followed by ^{99m}Tc -cPNA without a clearing agent (22). Previously, in both the inflammation and tumored mouse models, it was necessary to inject a large dosage of both the streptavidin-PNA and radiolabeled cPNA (150 and 50 μg , respectively) to achieve positive pretargeting. By contrast, in this research, only 16 μg of the PNA-PA-PEG and 2 μg of the radiolabeled cPNA were required to achieve superior ratios even in the absence of a clearing agent. With the use of 6 μg of avidin-cPNA in the inflammation model, almost all target/nontarget ratios were further improved (Table 6).

In conclusion, the results of this investigation demonstrate that PA may be conjugated with a large number of PNAs and, when conjugated with sufficient PEGs as well, the polymer displays pharmacokinetic properties suitable for in vivo applications. In particular the conjugated PA was shown to accumulate by nonspecific diffusion in lesion of both inflammation and tumored mouse models. We were also able to demonstrate that the PNAs on PA may be successfully targeted in vitro and, in particular, in vivo by ^{99m}Tc -labeled cPNA. Finally, the advantage of including amplification in pretargeting applications was also shown. Although a number of polymer scaffolds were considered preliminary to this work, it is inconceivable that the ideal polymer was found

in PA. Furthermore, although PNAs have very attractive properties, other oligomers may eventually be shown to be superior for this application. Clearing agents other than those studied herein could be considered. Thus, this report may be viewed as describing only the first step to the use of oligomer polymers for pretargeting. Nevertheless, the goal of demonstrating that pretargeting using polymers of PNA for amplification can improve localization was realized.

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A General Synthesis of Structurally Diverse Building Blocks for Preparing Analogues of C-Linked Antifreeze Glycoproteins

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A synthetic methodology to afford unusual glycoconjugate building blocks useful for the solid-phase synthesis of C-linked antifreeze glycoprotein (AFGP) analogues is described. Such compounds are urgently required in order to elucidate the molecular mechanism by which AFGPs function. All reactions are general in nature and accommodate structural variation in the carbohydrate moiety, polypeptide backbone, and amino acid side chain.

INTRODUCTION

Biological antifreezes are a diverse class of compounds that have the unusual ability to inhibit the growth of ice crystals in organisms inhabiting subzero environments. Consequently, these organisms are protected against cryoinjury. Antifreeze glycoproteins (AFGPs¹) are one class of biological antifreezes found in Arctic and Antarctic teleost fish. The structure of a typical AFGP is shown in Figure 1. The core unit consists of a L-threonyl-L-alanyl-L-alanyl tripeptide where the secondary hydroxyl group of the threonine is glycosylated with the β -D-galactosyl-(1,3)- α -D-N-acetylgalactosamine subunit. There are eight classes of AFGP and each differs in molecular weight (1–3). For instance, AFGP 1–4 are 20–33 kDa while AFGP 5–8 are in the 19–2.2 kDa range. Minor variations in primary structure are often found in low molecular weight AFGPs (i.e., AFGP 7–8) (4–6).

During the last 30 years, the macromolecular mechanism of action for AFGPs has been extensively investigated and is largely regarded as an adsorption-inhibition phenomenon in which the biological antifreeze binds to the surface of a growing ice crystal (7, 8). After adsorption, ice growth occurs between adjacent AFGPs, and the surface grows outward in a convex fashion. As the radius of curvature increases, it becomes energetically unfavorable to add water molecules to the ice lattice, and consequently a localized freezing point depression is observed. This is referred to as the Kelvin Effect (9). The difference between the melting and freezing points is defined as thermal hysteresis (TH).

In contrast to the macromolecular mechanism, a definitive molecular mechanism of action has not emerged and remains a source of debate among experts in the field. This has restricted the use of AFGPs in many medical, industrial, and commercial applications (10–13). Additional complications include the fact that isolation/purification of AFGPs from natural sources remains a costly and laborious process (14) while the chemical and

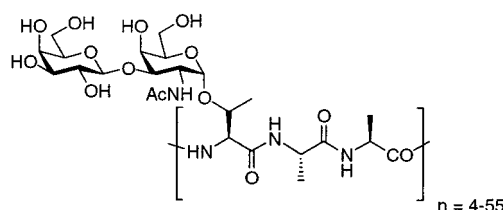


Figure 1. A typical antifreeze glycoprotein (AFGP).

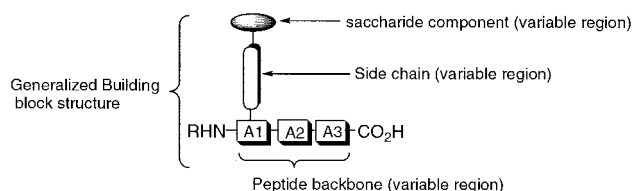


Figure 2. Generalized building block structure.

biological instability of natural AFGPs make them unsuitable for most applications (15).

The chemical synthesis of AFGP and AFGP analogues is an attractive alternative to the difficult isolation from natural sources. With the many recent advances in peptide and carbohydrate chemistry, the rational design of chemically and biologically stable AFGP analogues possessing enhanced activity should be feasible. However, before such compounds can be designed, a thorough understanding of how AFGPs function at the molecular level must be attained (14). In an attempt to address these issues, our laboratory has been the first to synthesize low molecular weight C-linked AFGP analogues (16). While this may seem like a dramatic structural modification, recent studies have demonstrated that C-linked glycoconjugates bind substrates with nearly identical conformations and affinities as O-linked glycoconjugates (17, 18). C-linked structures are attractive and offer significant advantages with respect to natural O-linked glycoconjugates as they are stable to a wide range of chemical and biological conditions. Our approach centers on the preparation of glycosylated tripeptide building blocks (Figure 2) that are assembled into C-linked AFGP analogues using conventional solid-phase synthesis (SPS).

We recently reported the synthesis of a C-linked AFGP analogue with a molecular weight of 1.5 kDa using this strategy (Figure 3) (19, 20). An attractive advantage of the solid-phase approach is that C-glycopeptide polymers

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¹ Abbreviations: AFGP, antifreeze glycoprotein; SPS, solid-phase synthesis; NMR, nuclear magnetic resonance; CDI, 1,1-carbonyldiimidazole; DIPEA, *N,N*-diisopropylethylamine; TBS, *tert*-butyldimethylsilyl.

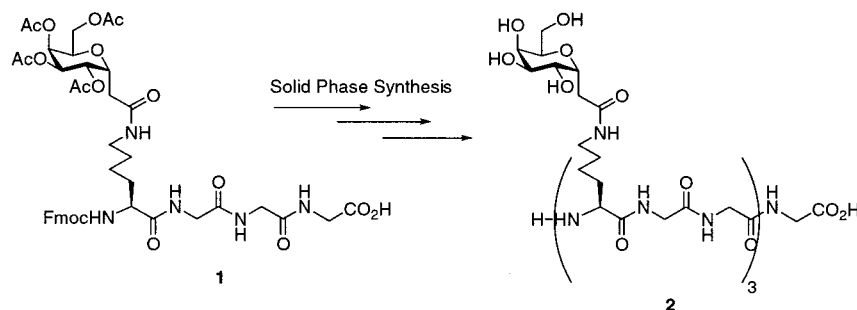


Figure 3. Solid-phase synthesis of a C-linked AFGP mimic.

of a predetermined length are easily prepared. In contrast, solution-phase polymerization approaches to AFGP often produce mixtures of varying molecular weight (21). This article describes the rationale for and preparation of several structurally diverse glycoconjugate building blocks necessary to synthesize C-linked AFGP analogues using a linear solid-phase approach.

EXPERIMENTAL PROCEDURES:

General Methods. Infrared (IR) spectra were recorded using a Perkin-Elmer 1600 Series FTIR. Unless otherwise noted ^1H and ^{13}C NMR spectra were recorded using a Bruker AM 360 or a Bruker AC 300 in CDCl_3 . Chemical shift values are reported in ppm downfield from TMS as an internal standard. Multiplicities are reported as s, singlet; bs, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet. Low-resolution mass spectra were obtained using a Hewlett-Packard series 1100 MSD instrument equipped with an API-ES ionization chamber. High-resolution mass spectra were obtained using a Micromass QToF-II high-resolution mass spectrometer and sodium iodide as an internal standard. Chromatographic separations were performed using 230–400 mesh silica gel from Natland Inc. All solvents were dried and distilled prior to utilization; THF was distilled over sodium–benzophenone, and methylene chloride was distilled over calcium hydride.

2-(2,3,4,6-Tetra-O-acetyl- α -D-galactopyranosyl)acetaldehyde (3). Ozone was bubbled into a solution of C-allylated β -D-galactose pentaacetate (866 mg, 2.33 mmol) in dry dichloromethane (12 mL) under nitrogen at -78°C until the solution turned blue in color. Nitrogen was then bubbled through the solution until it turned colorless. Triphenylphosphine (1.5 g, 5.7 mmol) was then added, and the solution was then allowed to warm to room temperature over a period of 3 h. Dichloromethane was removed under reduced pressure, and the residue was purified by column chromatography (ethyl acetate/hexanes 1:1) to afford 632 mg (73% yield) of **3** as a colorless oil. IR (CH_2Cl_2): 1738 cm^{-1} ; ^1H NMR (360 MHz, CDCl_3) δ : 9.64 (1H, s), 5.15 (1H, dd, $J = 5.8, 2.8\text{ Hz}$), 5.02 (1H, dd, $J = 8.8, 4.7\text{ Hz}$), 4.96 (1H, dd, $J = 8.8, 3.1\text{ Hz}$), 4.62 (1H, m), 4.04 (1H, dd, $J = 11.1, 7.5\text{ Hz}$), 3.9 (1H, m), 3.84 (1H, dd, $J = 11.2, 4.6\text{ Hz}$), 2.5 (2H, m), 1.87 (3H, s), 1.81 (3H, s), 1.78 (6H, s); ^{13}C NMR (90 MHz, CDCl_3) δ : 198.4, 169.6, 169.3, 169.1, 169.0, 68.8, 67.2, 66.6, 66.4, 61.0, 41.0, 19.9.

Benzyl Fluorenemethyloxycarbonyl-L-lysine[(2,3,4,6-tetra-O-acetyl- α -D-galactopyranosyl)ethyl]glycylglycinate (5). Aldehyde **3** (100 mg, 0.47 mmol) was dissolved in 10 mL of dry THF under nitrogen atmosphere at room temperature. Compound **4** (60 mg, 0.85 mmol) was dissolved in 3 mL of dry THF and added to the solution of **3**. Sodium triacetoxyborohydride (100 mg, 0.47 mmol) was then added, and the solution was stirred

at room temperature for 12 h. The solution was concentrated under reduced pressure and the residue dissolved in methanol and stirred for 20 min. This solution was concentrated under reduced pressure and the residue purified by column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 10:1) to afford 80 mg (55% yield) of **5** as a colorless oil. IR (CH_2Cl_2): $3366, 1744, 1672\text{ cm}^{-1}$; ^1H NMR (360 MHz, CDCl_3) δ : 7.62 (2H, d, $J = 7.4\text{ Hz}$), 7.47 (2H, br s), 7.21 (9H, m), 6.50 (1H, br s), 5.30 (1H, br s), 5.10 (2H, br s), 4.91 (3H, br s), 4.37 (1H, m), 4.22 (4H, br s), 4.07 (3H, br s), 3.91 (6H, br s), 3.03 (2H, br s), 2.93 (2H, br s), 1.95 (14H, m), 1.73 (4H, br s), 1.47 (2H, br s); ^{13}C NMR (90 MHz, CDCl_3) δ : 175.5, 173.17, 170.9, 169.8, 169.7, 169.6, 169.5, 156.6, 143.7, 141.1, 135.1, 128.4, 128.3, 128.0, 127.6, 127.0, 125.1, 119.8, 69.8, 68.6, 68.3, 67.5, 67.2, 67.0, 66.9, 60.9, 54.7, 47.9, 47.0, 45.1, 42.9, 41.2, 31.3, 25.3, 23.6, 22.2, 20.9, 20.7, 20.7, 20.6. LRMS (electrospray, MeOH, positive ion mode, m/z) calcd. for $\text{C}_{48}\text{H}_{58}\text{N}_4\text{O}_{15}$ (M^+) 930.38; found 931.40 ($\text{M}^+ + \text{H}$). HRMS calcd for $\text{C}_{43}\text{H}_{38}\text{N}_4\text{O}_{15}$ ($\text{M}^+ + \text{H}$) 931.3977, found 931.3976.

Benzyl Fluorenemethyloxycarbonyl-L-lysine[bis-[(2,3,4,6-tetra-O-acetyl- α -D-galactopyranosyl)ethyl]glycylglycinate (6). Aldehyde **3** (120 mg, 0.32 mmol) was dissolved in 3 mL of dry THF and allowed to stir at room temperature under nitrogen atmosphere. Compound **4** (100 mg, 0.15 mmol) was dissolved in 6 mL of dry THF and added to the solution of **3**. Sodium triacetoxyborohydride (100 mg, 0.47 mmol) and 0.1 mL of acetic acid were added directly to this solution, and the mixture was allowed to stir at room temperature for 12 h. The solvent was removed under reduced pressure, and the resulting residue was redissolved in methanol and stirred for 20 minutes. This solution was then concentrated and the residue was redissolved in ethyl acetate. The ethyl acetate solution was washed successively with saturated sodium bicarbonate, water, and brine and the solution dried over MgSO_4 and concentrated under reduced pressure. The crude product was then purified by column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 30:1) to afford 117 mg (63% yield) of **6** as a colorless oil.

IR (CH_2Cl_2): $1733, 1655\text{ cm}^{-1}$; ^1H NMR (360 MHz, CDCl_3) δ : 7.71 (2H, d, $J = 7.4\text{ Hz}$), 7.56 (2H, t, $J = 6.2\text{ Hz}$), 7.35 (2H, t, $J = 7.2\text{ Hz}$), 7.26 (7H, m), 7.14 (1H, br s), 5.81 (1H, br s), 5.38 (2H, br s), 5.26 (1H, s), 5.20 (3H, br s), 5.06 (2H, s), 4.36 (2H, br s), 4.26 (4H, m), 4.16 (2H, t, $J = 6.9\text{ Hz}$), 4.00 (8H, m), 2.42 (4H, br s), 2.34 (2H, br s), 2.07 (6H, s), 2.02 (6H, s), 2.00 (6H, s), 1.99 (6H, s), 1.80 (2H, br s), 1.64 (2H, br s), 1.54 (2H, br s), 1.38 (4H, br s); ^{13}C NMR (90 MHz, CDCl_3) δ : 172.4, 170.6, 169.9, 169.8, 169.4, 169.0, 156.4, 143.8, 141.1, 135.1, 128.4, 128.3, 128.1, 127.6, 126.9, 124.9, 124.9, 119.8, 70.1, 68.3, 67.9, 67.8, 67.4, 66.9, 61.4, 61.2, 54.9, 49.6, 47.0, 42.8, 41.0, 32.0, 26.6, 25.4, 23.1, 20.6, 20.5, 20.5, 20.2. HRMS calcd for $\text{C}_{64}\text{H}_{80}\text{N}_4\text{O}_{24}$ ($\text{M}^+ + \text{Na}$) 1311.5062, found 1311.5069.

Benzyl *tert*-Butoxycarbonyl-L-alaninyl-L-alaninate (10). Boc-L-Ala-OBn (842 mg, 3.0 mmol) was dissolved in 10 mL of dry dichloromethane at stirred at room temperature. Trifluoroacetic acid (5 mL) was added, and the solution was stirred for 1 h after which time the mixture was concentrated under reduced pressure. The resulting residue was then added to a flask containing a dichloromethane solution of t-Boc-Ala-OH (586 mg, 3.0 mmol) and 1,1'-carbonyldiimidazole (CDI) (535 mg, 3.1 mmol) which had been stirring for 10 min under nitrogen. The entire solution was allowed to react for 1 h at room temperature and then washed successively with 5% aqueous hydrochloric acid, saturated sodium bicarbonate, and brine. The combined organic extracts were then dried over magnesium sulfate and concentrated. The crude product was then purified by flash chromatography (ethyl acetate/hexanes 1:3) to afford 517 mg (96% yield) of **10** as a colorless oil. IR (CH₂Cl₂): 3311, 1738, 1661 cm⁻¹; ¹H NMR (360 MHz, CDCl₃) δ : 7.29 (5H, br s), 7.04 (1H, br s), 5.37 (1H, d, *J* = 7.4 Hz), 5.14 (1H, d, *J* = 12.3 Hz), 5.08 (1H, d, *J* = 12.3 Hz), 4.56 (1H, quintet, *J* = 7.1 Hz), 4.21 (1H, br s), 1.39 (9H, s), 1.35 (3H, d, *J* = 7.2 Hz), 1.28 (3H, d, *J* = 7.2 Hz); ¹³C NMR (90 MHz, CDCl₃) δ : 172.4, 172.4, 155.3, 135.2, 128.4, 128.2, 127.9, 79.7, 66.8, 49.7, 47.9, 28.1, 18.3, 17.8.

Benzyl Fluorenylmethoxycarbonyl-L-lysine(*tert*-butyloxycarbonyl)-L-alaninyl-L-alaninate (11). Compound **10** (0.624 g, 1.78 mmol) was dissolved in dry dichloromethane, and trifluoroacetic acid (8 mL) was added. The solution was stirred for 2 h at room temperature under nitrogen atmosphere after which time the reaction mixture was concentrated under reduced pressure. The resulting deprotected dipeptide was then added to a solution of Fmoc-Lys(Boc)OH (0.84 g, 1.8 mmol) and CDI (0.33 g, 2 mmol) dissolved in 10 mL of dichloromethane. The solution was stirred overnight at under nitrogen atmosphere and then washed successively with 5% HCl, water, and brine. The combined organic layers were dried using MgSO₄, filtered, and concentrated under reduced pressure. Column chromatography was performed using hexanes:EtOAc (1:2) as eluant and afforded 1.0 g (74% yield) of the desired tripeptide **11**. IR (CH₂Cl₂): 3288, 1738, 1683, 1644 cm⁻¹; ¹H NMR (360 MHz, CDCl₃) δ : 7.68 (2H, d, *J* = 7.4 Hz), 7.52 (2H, d, *J* = 7.3 Hz), 7.26 (9H, m), 7.03 (1H, dd, *J* = 11.1, 3.0 Hz), 5.85 (1H, br d, *J* = 5.8 Hz), 5.11 (1H, d, *J* = 12.2 Hz), 5.05 (1H, d, *J* = 12.3 Hz), 4.52 (2H, m), 4.31 (3H, m), 4.11 (3H, m), 3.03 (2H, br s), 1.74 (2H, br s), 1.51 (2H, br s), 1.37 (11H, br s), 1.31 (3H, d, *J* = 5.9 Hz), 1.21 (3H, d, *J* = 7.1 Hz); ¹³C NMR (360 MHz, CDCl₃) δ : 172.4, 170.4, 156.7, 156.6, 144.5, 142.3, 135.2, 128.8, 128.7, 128.4, 127.9, 127.2, 125.2, 120.1, 67.6, 54.3, 49.3, 48.6, 47.3, 32.2, 29.8, 28.6, 22.5, 18.7, 18.1. LRMS (electrospray, MeOH, positive ion mode, *m/z*) calcd. for C₃₉H₄₈N₄O₈ (M⁺) 700.34; found 723.31 (M⁺ + Na).

Benzyl Fluorenylmethoxycarbonyl-L-lysine[2-(2,3,4,6-tetra-*O*-*tert*-butyldimethylsilyl- α -D-galactopyranosyl)acetyl]-L-alaninyl-L-alaninate (12). Tripeptide **11** (370 mg, 0.528 mmol) was dissolved in CH₂Cl₂ (3 mL), and 2 mL of trifluoroacetic acid was added. The solution was stirred at room temperature, under nitrogen atmosphere for 2 h, and then concentrated under reduced pressure. A solution of **14** (110 mg, 0.161 mmol) and CDI (40 mg, 0.242 mmol) dissolved in 10 mL of dichloromethane was stirred at room temperature, under nitrogen atmosphere for 1 h. Then, the deprotected tripeptide (0.37 g, 0.528 mmol) and *N,N*-diisopropylethylamine (0.2 mL) were added to the reaction mixture, and the solution was stirred overnight at room temperature. The crude

reaction mixture was washed successively with 5% HCl, water, and brine. The organic extract was dried over MgSO₄, filtered, and concentrated under reduced pressure. Purification of the crude product was accomplished using CH₂Cl₂/MeOH (50:1) and afforded 120 mg (57% yield) of the desired glycosylated tripeptide as a colorless oil. IR (CH₂Cl₂): 3288, 1716, 1644 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ : 7.74 (2H, d, *J* = 7.4 Hz), 7.61 (2H, m), 7.31 (9H, m), 6.85 (1H, br d, *J* = 7.7 Hz), 5.89 (1H, br d, *J* = 4.6 Hz), 5.15 (1H, d, *J* = 12.3 Hz), 5.12 (1H, d, *J* = 12.4 Hz), 4.51 (1H, m), 4.46–4.12 (6H, m), 3.73 (2H, m), 2.70 (1H, m), 2.42–2.07 (1H, m), 1.50 (1H, br s), 1.35 (3H, d, *J* = 6.0 Hz), 1.22 (3H, s), 0.88 (44H, m), 0.06 (24 H, m); ¹³C NMR (360 MHz, CDCl₃) δ : 174.5, 172.5, 167.8, 156.3, 143.9, 141.3, 138.0, 132.4, 130.9, 127.0, 125.2, 121.0, 119.9, 107.7, 79.5, 73.1, 68.1, 66.9, 58.6, 47.1, 38.7, 31.9, 28.9, 25.7, 23.7, 23.0, 18.9, 18.5, 12.0, 10.9.

2-(2,3,4,6-Tetra-*O*-*tert*-butyldimethylsilyl- α -D-galactopyranosyl)acetic Acid (14). Ozone was bubbled into a solution of **13** (4.5 g, 6.8 mmol) dissolved in dry dichloromethane and cooled to -78 °C. Once the solution turned blue in color, nitrogen was bubbled through the solution until the blue color disappeared. Triphenylphosphine (2.0 g, 7.6 mmol) was added, and the solution was then allowed to warm to room temperature over a period of 3 h. This solution was then concentrated under reduced pressure, and the residue was redissolved in 10 mL of *tert*-butyl alcohol and 10 mL of 2-methyl-2-butene. Monobasic potassium phosphate (9.3 g, 68 mmol) and sodium chlorite (6.1 g, 68 mmol) were dissolved in 20 mL of water and added to the above solution. After stirring for 4 h at 0 °C, the solution was extracted with ethyl acetate. The organic extracts were washed successively with water and brine, and the solution was then dried over magnesium sulfate and concentrated under reduced pressure. The crude product was purified by column chromatography (neat dichloromethane) to afford 3.6 g (78% yield) of **14** as a white solid. IR (CH₂Cl₂): 1722 cm⁻¹; ¹H NMR (360 MHz, CDCl₃) δ : 4.23 (3H, m), 4.02 (1H, dd, *J* = 13.4, 6.8 Hz), 3.74 (1H, t, *J* = 3.0 Hz), 3.70 (1H, dd, *J* = 12.0, 2.2 Hz), 3.51 (1H, d, *J* = 3.7 Hz), 2.70 (1H, dd, *J* = 16.6, 9.7 Hz), 2.38 (1H, dd, *J* = 16.6, 3.9 Hz), 0.89 (9H, s), 0.87 (18H, s), 0.85 (9H, s), 0.03 (24H, m); ¹³C NMR (90 MHz, CDCl₃) δ : 174.0, 79.2, 73.1, 72.8, 66.7, 63.3, 58.4, 36.0, 26.3, 25.9, 25.8, 25.6, 18.2, 18.0, 17.8, -4.5, -4.6, -4.99, -5.1, -5.3, -5.5. HRMS calcd for C₃₂H₇₀O₇-Si₄ (M⁺ + H) 679.4277, found 679.4262.

Benzyl Fluorenomethoxycarbonyl-L-lysine[2-(2,3,4,6-tetra-*O*-*tert*-butyldimethylsilyl- α -D-galactopyranosyl)acetyl]-L-prolylglycinate (15). Fmoc-Lys(Boc)-Pro-Gly-OBn (0.1582 g, 0.22 mmol) was dissolved in dry dichloromethane (5 mL), and TFA (0.15 mL) was stirred at room temperature for 1 h. The solution was concentrated under reduced pressure. Compound **14** (0.1513 g, 0.22 mmol) and CDI (0.0392 g, 0.25 mmol) were added to dry dichloromethane (5 mL). The resulting solution was stirred at room temperature, under nitrogen atmosphere. After 1 h, the deprotected tripeptide was dissolved in dry dichloromethane (3 mL) followed by the addition of diisopropyl ethylamine (0.3 mL). The solution was stirred for 12 h at room temperature and then washed successively with saturated ammonium chloride, water, and brine. The solution was dried over magnesium sulfate, filtered, and concentrated under vacuum. The crude product was purified by column chromatography (CH₂Cl₂:MeOH 30:1) and furnished 146 mg (60% yield) of **15** as a colorless oil. IR (CH₂Cl₂): 3333, 1716, 1644 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ : 7.65 (2H, d, *J* = 7.3 Hz), 7.48 (2H, d, *J* = 6.6 Hz), 7.27 (5H, m), 5.04 (2H, s),

4.25 (4H, m), 4.18 (2H, d, $J = 7.8$ Hz), 4.09 (6H, m), 3.91 (6H, m), 3.62 (8H, br s), 3.38 (2H, d, $J = 3.7$ Hz), 1.39 (8H, m), 1.19 (1H, s), 0.80 (36H, m), -0.05 (24H, m). ^{13}C NMR (360 MHz, CDCl_3) δ : 172.5, 172.0, 171.0, 169.4, 155.9, 143.7, 143.6, 141.1, 135.0, 134.6, 130.7, 128.6, 128.4, 128.4, 128.3, 128.2, 128.1, 127.5, 126.9, 124.9, 120.3, 119.8, 78.9, 73.6, 73.4, 72.9, 66.9, 66.8, 66.5, 63.1, 58.1, 52.0, 47.0, 41.2, 36.2, 29.5, 28.2, 25.9, 25.8, 25.8, 25.7, 25.6, 25.5, 25.5, 25.5, 25.4, 18.1, 17.9, 17.8, 17.8, 17.7, -3.7 , -4.5 , -4.6 , -4.7 , -4.8 , -5.0 , -5.0 , -5.1 , -5.2 , -5.2 , -5.7 . LRMS (electrospray, MeOH, positive ion mode, m/z) calcd. for $\text{C}_{67}\text{H}_{108}\text{N}_4\text{O}_{12}\text{Si}_4$ (M^+) 1274.72; found 1274.71 (M^+). HRMS calcd for $\text{C}_{67}\text{H}_{108}\text{N}_4\text{O}_{12}\text{Si}_4$ ($\text{M}^+ + \text{Na}$) 1295.6939, found 1295.6940.

Benzyl Fluorenemethyloxycarbonyl-L-ornithine-[2-(2,3,4,6-tetra-O-*tert*-butyldimethylsilyl- α -D-galactopyranosyl)acetyl]glycylglycinate (16). A solution of the Fmoc-Orn(Boc)-Gly-Gly-OBn (0.1037 g, 0.16 mmol) and dry dichloromethane (5 mL) and TFA (0.12 mL) was stirred at room temperature for 1 h, and then concentrated under reduced pressure. Compound **14** (0.1070 g, 0.16 mmol) and CDI (0.028 g, 0.17 mmol) were dissolved in dry dichloromethane (10 mL) and stirred at room temperature, under nitrogen atmosphere. After 1 h, the deprotected tripeptide was dissolved in dry dichloromethane (3 mL) and added to this reaction mixture, followed by diisopropyl ethylamine (0.27 mL). The solution was stirred overnight at room temperature, under nitrogen, and then washed successively with saturated ammonium chloride, water, and brine. The solution was dried over magnesium sulfate and concentrated under vacuum. The crude product was purified using column chromatography (CH_2Cl_2 :MeOH 30:1) to yield 87 mg (53%) of **16**. IR (CH_2Cl_2): 3311, 1738, 1677 cm^{-1} ; ^1H NMR (300 MHz, CDCl_3) δ : 7.65 (2H, d, $J = 7.44$ Hz), 7.49 (2H, m), 7.28 (10H, m), 4.98 (2H, s), 4.23 (15H, m), 3.91 (8H, m), 3.66 (6H, m), 3.36 (2H, d, $J = 3.77$ Hz), 2.71 (2H, dd, $J = 7.5$ Hz), 2.25 (2H, dd, $J = 4.8$ Hz), 0.81 (36H, m), -0.05 (24H, m); ^{13}C NMR (360 MHz, CDCl_3) δ : 172.9, 172.6, 172.3, 169.6, 169.3, 169.1, 169.0, 167.5, 156.4, 143.7, 143.6, 141.1, 136.3, 130.7, 128.4, 128.4, 128.3, 128.2, 128.2, 128.2, 128.1, 128.1, 127.5, 127.5, 126.9, 125.0, 125.0, 124.9, 119.8, 119.7, 115.8, 79.7, 73.0, 72.4, 67.0, 66.9, 66.8, 66.8, 66.7, 63.5, 58.3, 58.2, 47.0, 41.0, 41.0, 36.9, 28.2, 28.1, 25.9, 25.9, 25.8, 25.7, 25.5, 25.5, 25.4, 18.1, 17.9, 17.9, 17.8, 17.7, 17.7, -4.5 , -4.5 , -4.6 , -4.7 , -4.8 , -4.9 , -5.0 , -5.1 , -5.4 , -5.5 , -5.6 , -5.7 . HRMS calcd for $\text{C}_{63}\text{H}_{102}\text{N}_4\text{O}_{12}\text{Si}_4$ ($\text{M}^+ + \text{Na}$) 1241.6469, found 1241.6465.

RESULTS AND DISCUSSION

Building block **1** has been at the center of our preliminary work. The structure of **1** is different than that of the core repeating tripeptide unit in Figure 1 and serves to function as a structural analogue for the tripeptide unit in low molecular weight AFGP 7–8 which has the threonine substituted with an arginine residue (5, 6). The amide bond between the C-linked galactose and ϵ -amino group of the lysine side chain mimics the terminal guanidinium group of arginine. This may seem like a dramatic structural modification since lysine is not native to AFGPs; however, recent results have shown that polypeptides containing repeating L-lysine residues possess weak antifreeze protein-specific activity (22). In addition to the above modification, the α -D-galactosyl- β -D-N-acetylgalactosamine found in AFGP has been truncated in **1**. This is based upon early structure–function studies where the *cis*-3,4-dihydroxy group in the terminal

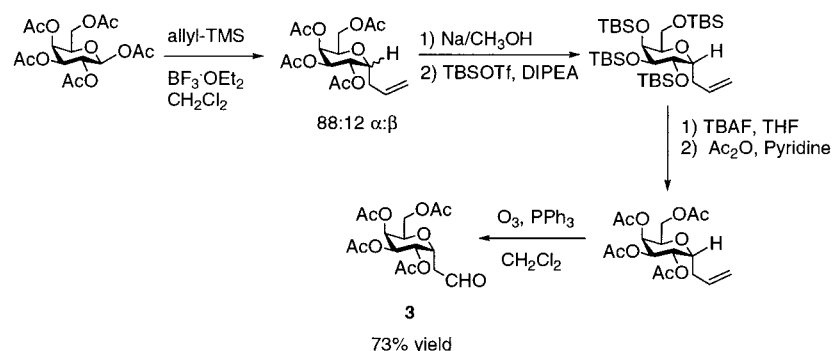
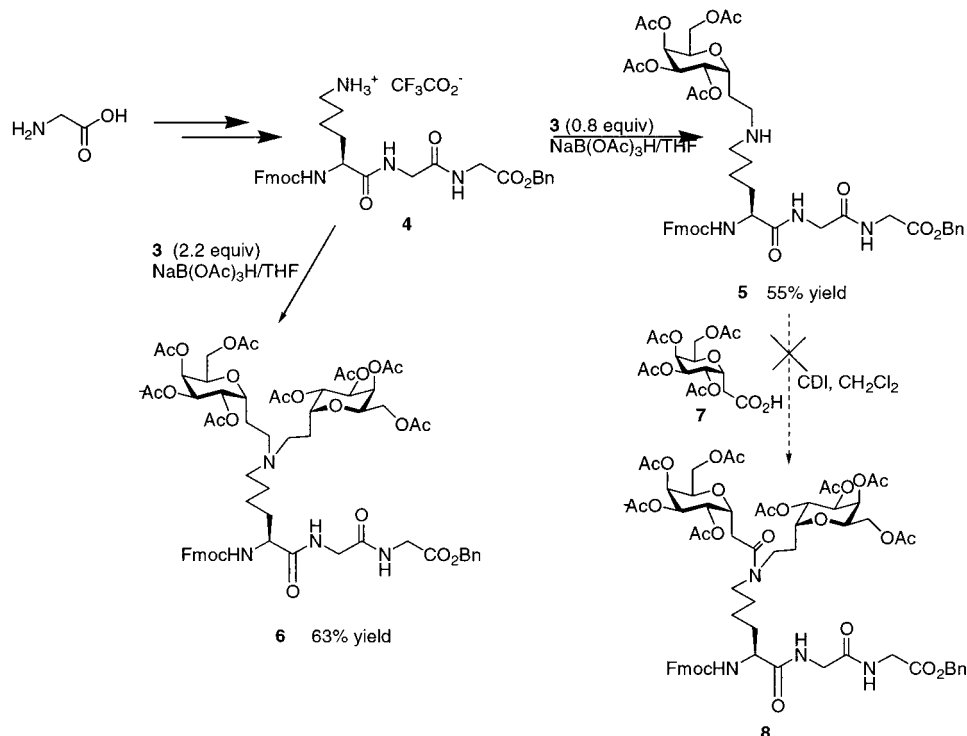
galactose residue was shown to be especially important for antifreeze protein-specific activity (23). This result is reminiscent of biologically significant oligosaccharides where in most cases, only the terminal residue(s) are necessary for tight interactions with a receptor (24).

Traditionally, the driving force for adsorption of AFGP onto the ice surface is thought to involve hydrophilic interactions (hydrogen bonds) between specific hydroxyl groups of the carbohydrate moiety and the ice surface. While hydrogen bond formation is a very general phenomenon, molecular modeling studies of potential interactions between the α -D-galactosyl- β -D-N-acetylgalactosamine moiety and the ice surface imply that only two hydroxyl groups are ideally positioned to hydrogen bond to the ice lattice (25). This suggests that AFGP 8 (with four glycosylated tripeptide units) will form only eight hydrogen bonds with the ice surface. Given that individual hydrogen bonds are relatively weak in nature, it is difficult to rationalize the irreversible binding of AFGP to ice, and consequently it has been proposed that the hydroxyl groups are actually incorporated *into* the ice lattice. While the above hypothesis is based upon the notion that hydrophilic interactions are a dominant force at the protein–ice interface, recent evidence has shown that hydrogen bonding might be only a secondary interaction and hydrophobic interactions might predominate (26, 27).

The assessment of AFGP analogues with structurally diverse carbohydrate moieties will lend valuable insight into hydrophilic interactions and the role they play in the molecular mechanism of action. Consequently, our laboratory has undertaken the preparation of C-linked AFGP analogues in which both the structure and orientation of a C-linked carbohydrate may be easily modified. One way to modify orientation of the saccharide residue is to alter the linkage between the saccharide and tripeptide. We are also interested in preparing AFGP analogues possessing multivalent saccharide derivatives of the native AFGP saccharide moiety. Multivalent saccharide derivatives have found numerous therapeutic applications as effective inhibitors of various carbohydrate protein interactions (28–30).

As we wish to synthesize C-linked AFGP building blocks possessing different carbon-linked carbohydrate structures, the preparative methodology must be high yielding and general in nature. To accomplish this, we employ a general and highly stereoselective allylation reaction (Scheme 1) (31). Unfortunately, the resulting olefin was produced as an 88:12 mixture of α : β anomers, and such a mixture proved very difficult to separate. However, replacement of the acetate protecting groups with the less polar *tert*-butyldimethylsilyl (TBS) ethers allowed for clean separation of the desired α -anomer (19). After exchange of the TBS protecting groups with acetates, the resulting olefin was subjected to ozonolysis and furnished aldehyde **3** in 73% yield after purification. This sequence of reactions has been successfully utilized to prepare both the α - and/or β -D-mannose and glucose derivatives of **3**.

As previously discussed, one way to alter orientation of the carbohydrate residue in our building block is to utilize different covalent linkages between the carbohydrate and amino acid side chain. Consequently, we chose to modify the amide linkage in **1**. To accomplish this we sought to employ sodium cyanoborohydride in a reductive amination protocol reported by Campbell et al. (32) where reaction of tripeptide **4** (19) with aldehyde **3** was expected to produce glycosylated tripeptide **5** (Scheme 2). However, this approach failed to produce even trace quantities of

Scheme 1. Synthesis of C-Linked Saccharide**Scheme 2. Synthesis of Mono- and Divalent Building Blocks**

5. Alternatively, sodium triacetoxy borohydride worked very well. As illustrated in Scheme 2, reaction of tripeptide **4** with 0.8 equiv of **3** in the presence of sodium triacetoxy borohydride furnished **5** in 55% yield. Two-dimensional NMR studies (NOESY and ROESY) confirmed that the C-linked galactose residue in **5** adopts a *rigid* orientation (relative to peptide backbone) that is different than in **1**.

The glycosylated tripeptide building block **5** is a precursor to structurally diverse divalent building blocks. As illustrated in Scheme 2, reaction of **4** with 2.2 equiv of **3** and sodium triacetoxy borohydride (2.2 equiv) furnished divalent tripeptide **6** in 63% yield after purification. We also sought to synthesize divalent derivatives possessing both the amide and amine linkages between the saccharide residue and polypeptide backbone as represented by compound **8** in Scheme 2. However, reaction of **5** with 1.2 equiv of carboxylic acid **7** (**19**) using 1,1'-carbonyldiimidazole (CDI) failed to produce any trace of the desired product even at elevated temperatures.

This reaction also failed in the presence of uronium coupling agents such as HBTU (*O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate) and HATU (*O*-(7-aza-benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate).

Studies of the solution structure of AFGP **8** have implied the existence of a polyproline type II helix (33, 34). While this structural motif is believed to be essential for antifreeze activity, definitive evidence to support this hypothesis has not been published. Thus, a preparative methodology that affords structural diversity in the polyamide backbone is highly desirable. Of potential interest are polypeptide backbones that possess the L-alanyl-L-alanyl dipeptide of native AFGP and derivatives that incorporate L-proline residues. Since the glycylglycine dipeptide residue in the tripeptide sequence of **1**, **5**, **6**, **8** is structurally dissimilar to the L-alanyl-L-alanyl dipeptide found in native AFGP, we prepared the glycosylated L-lysyl-L-alanyl-L-alanine polypeptide backbone.

Unfortunately, initial attempts to synthesize L-alanine benzyl ester **9** using standard procedures failed to produce **9** in high optical purity, and we were forced to employ an alternate approach. As illustrated in Scheme 3, reaction of commercially available *N*-tert-butoxycarbonylalanine with benzyl alcohol (2.5 equiv) in the presence of CDI furnished **9** as the trifluoroacetate salt in 82% yield after removal of the *tert*-butyl carbamate with trifluoroacetic acid. Analysis of the product by polarimetry confirmed that no racemization had taken place. Subsequent reaction of **9** with *N*-tert-butoxycar-

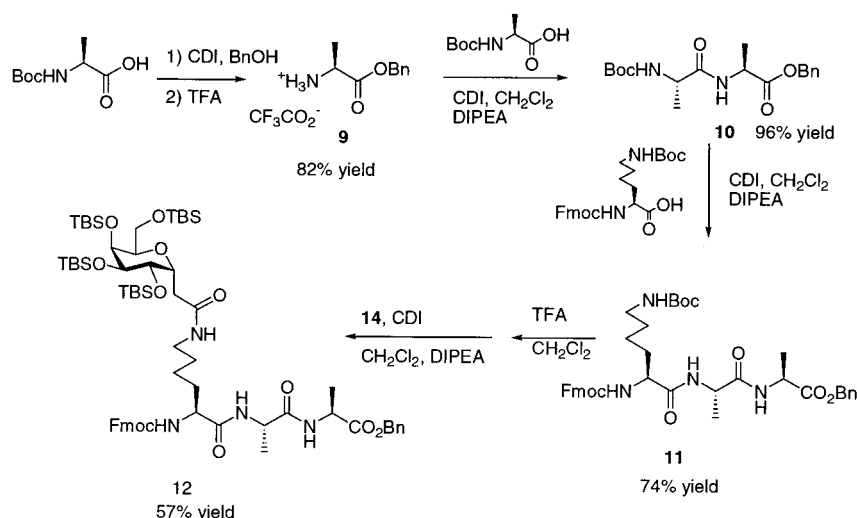
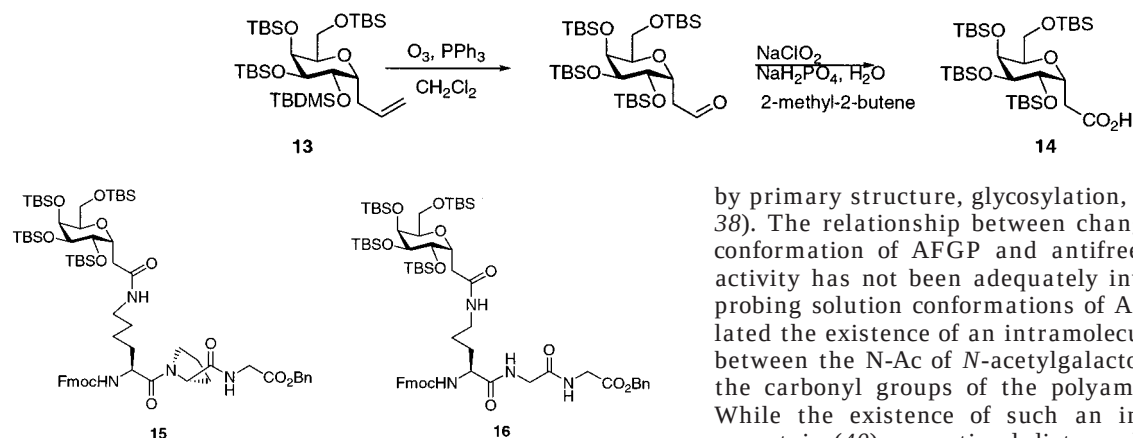
Scheme 3. Synthesis of Monovalent Building Block**Scheme 4. Synthesis of C-Linked Saccharide**

Figure 4. Other structurally diverse C-linked AFGP building blocks.

bonylalanine using CDI and *N,N*-diisopropylethylamine (DIPEA) in dichloromethane yielded dipeptide **10** in 96% isolated yield. Repetition of this sequence using Fmoclys(Boc)-CO₂H furnished tripeptide **11** in 74% yield and a final CDI-mediated coupling between galactose derivative **14** and the deprotected derivative of **11** furnished the glycosylated tripeptide **12** in 57% yield after purification. The C-linked galactose derivative **14** was prepared as outlined in Scheme 4 (35) from **13** (19). Building block **12** is a structural analogue of the glycosylated tripeptide in low molecular weight AFGP where the L-threonine residue is substituted with arginine (5, 6). This preparative methodology affords endless structural variations in the polypeptide backbone using other natural amino acids or even synthetic amino acid derivatives that may serve as a peptide scaffold. To further demonstrate this, building block **15** (with L-proline incorporated into the polyamide backbone, Figure 4) was easily prepared in a convergent fashion using procedures and reagents analogous to that described in Schemes 3 and 4. This is important because it has been observed that one or both L-alanine residues may be substituted with L-proline in AFGP 7–8 (4). The reason for this substitution is not understood.

It is likely that the structure and length of the amino acid side chain bearing the carbohydrate residue affects conformation and ultimately antifreeze activity. This is a complex issue since protein conformation is governed

by primary structure, glycosylation, and solvation (36–38). The relationship between changes in the solution conformation of AFGP and antifreeze protein-specific activity has not been adequately investigated. Studies probing solution conformations of AFGP 8 have postulated the existence of an intramolecular hydrogen bond between the N-Ac of *N*-acetylgalactosamine and one of the carbonyl groups of the polyamide backbone (39). While the existence of such an interaction remains uncertain (40), an optimal distance between the carbohydrate residue and polyamide backbone may exist, and close proximity of the carbohydrate residue may have a profound effect upon the solution conformation of AFGP. Consequently, structurally diverse tripeptide units possessing different amino acid side chain lengths will be useful to study possible intramolecular interactions and probe solution conformations. Our preparative methodology can easily accommodate these structural modifications as evidenced by the convergent synthesis of **16** (Figure 4) in 66% overall yield (two steps).

CONCLUSIONS

In summary, we have presented a preparative methodology for the synthesis of structurally diverse C-linked glycoconjugates useful for the solid-phase synthesis of chemically and biologically stable AFGP analogues. The approach centers on the preparation of carbon-linked glycosylated building blocks and permits structural modification of the saccharide component, polyamide backbone, and amino acid side chain. These building blocks are currently being assembled into discrete C-linked AFGP analogues. The *in vitro* assessment for antifreeze protein-specific activity will provide valuable insight into the structural features necessary to inhibit ice crystal growth.

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REVIEWS

Cellular Delivery of Impermeable Effector Molecules in the Form of Conjugates with Peptides Capable of Mediating Membrane Translocation

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Most molecules that are not actively imported by living cells are impermeable to cell membranes, including practically all macromolecules and even many small molecules whose physicochemical properties prevent passive membrane diffusion. The use of peptide vectors capable of transporting such molecules into cells in the form of covalent conjugates has become an increasingly attractive solution to this problem. Not only has this technology permitted the study of modulating intracellular target proteins, but it has also gained importance as an alternative to conventional cellular transfection with oligonucleotides. Peptide vectors derived from viral, bacterial, insect, and mammalian proteins endowed with membrane translocation properties have now been proposed as delivery vectors. These are discussed comprehensively and critically in terms of relative utility, applications to compound classes and specific molecules, and relevant conjugation chemistry. Although in most cases the mechanisms of membrane translocation are still unclear, physicochemical studies have been carried out with a number of peptide delivery vectors. Unifying and distinguishing mechanistic features of the various vectors are discussed. Until a few years ago speculations that it might be possible to deliver peptides, proteins, oligonucleotides, and impermeable small molecules with the aid of cellular delivery peptides not only to target cells *in vitro*, but *in vivo*, was received with scepticism. However, the first studies showing pharmacological applications of conjugates between macromolecules and peptide delivery vectors are now being reported, and therapies based on such conjugates are beginning to appear feasible.

INTRODUCTION

Biological membranes are natural barriers central to compartmentalization in living systems. They prevent unchecked influx and efflux of solutes from cells and

cellular organelles. All biological membranes contain a phospholipid bilayer as the basic structural unit. Phospholipids consist of hydrophobic tails and hydrophilic heads. Under physiological conditions they aggregate spontaneously into a bilayer, where the fatty acyl chains are sequestered within the hydrophobic interior and the polar heads face the aqueous surfaces. Disposed within the fluid but discrete bilayer structure are proteins and

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protein complexes that act as specific pumps and channels to permit active transport of solutes. More primitive channels are found in bacterial membranes, nuclei, and mitochondria. These function essentially as molecular sieves with defined size exclusion limits.

The ability to introduce membrane-impermeable molecules into mammalian cells has been an invaluable tool for the study of intracellular processes. Both physical and chemical methods have been developed to this end. Microinjection, electroporation, freeze-thaw techniques, etc., belong to the former, whereas the latter involve transient cell membrane permeabilization with the aid of solvents such as toluene or dimethyl sulfoxide, hypotonic buffers, and various detergents. The main problem with these methods is the fact that they are not readily reproducible, they are not quantitative, and they generally compromise cellular integrity. Furthermore, such methods are obviously only relevant for *in vitro* cell biology applications but not suitable for *in vivo* use. One of the main constraints in the design of drugs whose interaction targets are located within host cells is thus membrane permeability. Only compounds within a narrow range of molecular size, net charge, and polarity are able to diffuse effectively through the lipid bilayers of biological membranes and can thus reach their pharmacological target (1). The vast majority of molecules, including many natural products and biomolecules, i.e., compounds often possessing inherent biochemical selectivity and potency, are thus apparently precluded from therapeutic application.

The conventional mode of cellular entry for hydrophilic macromolecules is endocytosis. This process involves absorption of the macromolecules to the plasma membrane or to membrane-associated receptors, followed by energy-dependent formation of vesicles. The endocytic machinery of the cell then tags and directs the internalized molecules to appropriate compartments for either destruction or recycling. In general, molecules that enter cells via the endocytic route are not released to the cytoplasm in unaltered form, if at all. Liposomes have been used widely in order to enhance the cellular delivery of biomolecules via the endocytic route. Liposomal delivery, as well as liposomes in general, have been reviewed very comprehensively elsewhere (2, 3). Efficient cellular uptake via endocytosis is generally observed but the efficiency is then limited downstream by insufficient endosomal escape, restricted diffusion, especially of high-molecular weight substances, in the gellike cytosol, dissociation of the complexes, or nuclear entry. Enhanced endosomal escape has been achieved through modification of liposomes, i.e., pH-sensitive liposomes (4, 5), or association with helper lipids such as DOPE, or complexation with endosome-disrupting or destabilizing additives, i.e., PEG (5, 6), cholesterol (7), or lytic peptides (8–10). The nuclear barrier may be crossed efficiently by attachment of nuclear localization signals (11). Although numerous encouraging strategies to overcome various limitations of liposomal delivery are being developed, there is still a need for further improvement and consideration of alternative delivery strategies. For this reason, delivery systems addressing their molecular cargo to cells through endocytosis are only partly or not at all suitable if the molecular targets are cytosolic or nuclear, as is usually the case. Many of the peptide vectors described below, however, can effect cell entry of their molecular conjugates through mechanisms best described as penetration (12). Although poorly understood, these mechanisms appear to be energy-independent and address conjugates to the cytosol and/or the nucleus. No

distinct membrane receptors seem to be involved and membrane translocation is nonselective in terms of cell type. A recent study compared the cellular uptake of a number of different delivery peptides by a combination of high-performance liquid chromatography and confocal laser-scanning microscopy (13). The presence of both endocytic and nonendocytic modes of uptake were observed. Contrary to current opinion, the authors propose a general ability of peptides to cross plasma membranes and suggest that effective delivery with certain peptides may be due to prevention of wash-out in certain cases, e.g., enhancement of binding to intracellular components. While there is probably insufficient evidence at present to support this hypothesis, it highlights the fact that the mechanisms of cell membrane translocation by delivery vector peptides are far from clear. An additional complication stems from the fact that each vector-effector conjugate will probably behave differently, depending on the nature of different effector molecules.

Infective organisms such as viruses and bacteria possess proteins endowed with properties to penetrate living cells indiscriminately (14). For example, the HIV TAT protein, the HBV VP22 protein, pore-forming toxins such as tetanus toxin (15), anthrax toxin (16), cytolysins (17), diphtheria toxin (18), etc., can gain entry into cells in essentially receptor-independent fashion. Peptides derived from some of these proteins, particularly those of viral origin, have been developed as delivery vectors and these are discussed below.

Secreted proteins, some membrane proteins, and proteins of membrane-enveloped organelles of eukaryotic cells are partially or fully transported across membranes and contain signal sequences which direct them to the correct subcellular compartment. Most signal sequences are *N*-terminal extensions of the functional proteins and as a rule are removed proteolytically after translocation. Surprisingly, no sequence homology has been found for sequences directing proteins to the same subcellular compartment, and only general features such as charge distribution and hydrophobicity seem to be common. The secreted proteins of prokaryotes and proteins destined for the endoplasmic reticulum of eukaryotes generally contain a signal sequence consisting of a few charged residues, followed by an uninterrupted stretch of 7–16 apolar residues. Mitochondrial signal sequences, and those of many bacterial proteins consist of 12–30 apolar, polar, and charged residues, arranged in a pattern potentially allowing formation of secondary structures with polar/charged and apolar faces (helical amphiphilicity) or ends (segmental amphiphilicity). Mammalian and insect host defense peptides, including e.g., melittin, protegrins, defensins, tachyplesins, cecropins, etc., as well as pore-forming proteins and peptides in general (19), also fall within this class of substances. Numerous studies on such peptides have shown that they are capable of forming amphiphilic structures and that this propensity correlates with membrane activity (20). Both classes of signal peptides have led to the development of vector systems, and these are discussed more fully below. Amphiphilic peptides have gained some importance in cellular delivery of oligonucleotides, particularly as an endosomal entrapment escape device.

Yet other endogenous mammalian proteins possess the innate ability to cross cell membranes due to their function in intercellular signaling. At least in some cases such proteins do not appear to rely on membrane receptors or transporter systems. The best-documented case falling within this category concerns homeoproteins. Because much pioneering work on peptide-based cellular

Table 1. Summary of the Best-Characterized Delivery Peptide Vectors

sequence	name (origin of sequence)	literature reference
RQIKIWFAQNRRMKWKK	pAntp(43–58); Penetratin	67
kkwkmrrnqfwkvqr	retro-inverso pAntp(43–58)	69
RRWRRWRRWRRWRR	W/R Penetratin	70
RRMKWKK	pAntp(52–58)	73
GRKKRRQRRRPPQ	HIV TAT	124
YGRKKRRQRRR	HIV TAT	136
rrrrrrr	r7	138
GALFLGWLGAAGSTMGA	gp41 fusion sequence	168
GALFLGFLGAAGSTMGAWSQPKSKRKV	MPG (gp41 fusion sequence – SV40 NLS)	196
MGLGLHLLVLAALQGA	<i>Caiman crocodylus</i> Ig(v) light chain	168
MGLGLHLLVLAALQGAWSQPKKKRKV	<i>Caiman crocodylus</i> Ig(v) light chain – SV40 NLS	168
PLSSIFSRIGDP	PreS2-TLM	145
GWTLSAGYLLGKINLKALAALAKKIL	Transportan	225
RGGRLSYRRRFSTSTGR	SynB1	52
AAVALLPAVLLALLAP	MPS (kaposi FGF signal sequence)	166
AAVLLPVLLAAP	MPS (kaposi FGF signal sequence)	178
VTVALGALAGVGVG	MPS (human integrin β_3 signal sequence)	234
VAYISRGGVSTYYSDTVKGRFTRQKYNKRA	P3	222
KLALKLALKALKAALKLA	Model amphiphilic peptide	215
WEAKLAKALAKALAKHLAKALAKALAKACEA	KALA	214

delivery vectors was originated with this system, a separate section is devoted to this topic.

APPLICATIONS OF VECTOR CONJUGATES

Polypeptides. Only the very shortest of peptides, such as di- and tripeptides, can enter cells nonspecifically, either using general peptide transporter systems (21), or by diffusion (22). Although very short peptides may be rendered permeable by derivatization with membrane-active lipids such as myristic acid (23, 24), native polypeptides cannot traverse membrane barriers, unless they participate in specific cellular or subcellular processes, in which case appropriate and highly specific receptor and transporter systems exist. While some of the methods developed in the context of gene transfer are also applicable to cellular delivery of polypeptides, e.g., transient chemical cell permeabilisation (25) or liposomal delivery (4, 26, 27), it is the recent development of peptidic cell delivery vectors that has made possible the routine intracellular administration of polypeptides.

Traditionally, delivery vector-derivatized polypeptides have been used as tools in order to elucidate signaling pathways and generally to probe protein–protein interactions in cellular systems. Perhaps the most surprising finding with vectorized polypeptides has been their ability to internalize into cells and there to exert their biological function almost irrespective of molecular mass. Thus, short effector peptides containing only a few amino acid residues can be transported across membranes, as well as entire proteins. For example, the 20-kDa p16^{INK4a} tumor suppressor and the 120-kD β -galactosidase proteins have been shown to penetrate into cells using the pAntp and TAT peptide vectors (refer to Table 1), respectively (28, 29).

Oligonucleotides. Apart from receptor-mediated approaches (30, 31) and viral transfection methods, which have gained therapeutic importance for gene therapy but are not discussed here, carrier-mediated oligonucleotide and gene delivery falls into two categories. The first depends on the reversible association of condensing molecules with oligonucleotides. The carriers that have been used most frequently are composed of polymers, particularly polylysines (32) and polyethyleneimine (33), lipids (34–36), or cationic peptides that bind to anionic sites on the oligonucleotides (34). In all these cases, noncovalent association between the carriers and the oligonucleotides leads to compaction and condensation of the oligonucleotides into small particles that gain entry

into cells by nonspecific fluid-phase pinocytosis (37). Some synthetic peptide-based carriers falling within this category have been used successfully, e.g., AlkCWK₁₈ (38), and others (39, 40). Because pinocytotic uptake usually leads to endosomal trapping of the oligonucleotide complexes, helper molecules such as chloroquine (facilitator of endocytic exit through limitation of DNA degradation by lysosomal enzymes (41, 42)) and glycerol (enhanced uptake of particles and labilization of vesicular membranes (43)) are being used, and peptides capable of rupturing endosomal membranes at the low pH of this compartment have been developed. These are discussed under the amphiphilic peptide vectors below. The second category of carrier-mediated oligonucleotide delivery concerns vectors capable of mediating direct cytosolic delivery of oligonucleotides across the plasma membrane. Several peptide vectors in this category have been described and are referred to specifically below. Early examples using antisense oligonucleotides and pAntp vector conjugates showed not only successful cellular delivery of such constructs, but also clear antisense effects: inhibition of neurite outgrowth due to suppression of amyloid precursor protein neosynthesis (44) and cell death due to down-regulation of Cu/Zn superoxide (45), respectively.

Nanoparticles. Intracellular magnetic labeling of cells with ferro/superparamagnetic substances can be achieved, e.g., by fluid phase endocytosis, receptor-mediated endocytosis, or phagocytosis. The cellular uptake of dextran-coated superparamagnetic iron oxide particles was shown to be over 100-fold more efficient when the particles were derivatized with an HIV TAT-delivery vector peptide (46). In this approach, a monodispersed superparamagnetic iron oxide colloid was cross-linked and aminated. The pendant amino groups were first derivatized with the heterobifunctional cross-linker *N*-succinimidyl 3-(2-pyridyldithio)propionate, followed by disulfide exchange using a cysteinylated TAT(48–57) vector peptide. The resulting peptidyl conjugate particles had an average size of 41 nm and contained ca. 6.7 TAT peptides. Internalization of these particles into lymphocytes was very efficient, resulting in up to 10⁷ particles/cell, permitting NMR imaging techniques. This technology has recently been applied to the in vivo tracking and recovery of progenitor cells (47).

Organic Molecules. Not only biomolecules, but also many small-molecule natural products, as well as numerous synthetic drug candidates, are membrane-

impermeable due to unfavorable physicochemical properties. Here too, administration in vectorized form may be useful. Furthermore, effective therapeutic application of many organic molecules is prevented because of their poor solubility, absorption, or distribution. It is conceivable that some of these difficulties could be overcome with drug–vector peptide conjugates. One such problem is the delivery of neuropharmaceuticals across the blood–brain barrier. Various strategies, including peptide–vector approaches, have been proposed for the brain delivery of drugs (48). Thus it was shown that the anticancer agent doxorubicin was able to cross the blood–brain barrier in a brain perfusion model (49), as well as after parenteral administration to mice, when covalently linked to Pegelin (50, 51) or pAntp vector peptides (52).

VECTORS

Some of the more prominent vector peptides are summarized in Table 1. Although various different membrane translocation mechanisms are implicated, depending on the vectors, certain low-order structural similarities between the peptides can be discerned. Apart from the highly lipophilic signal peptide-derived vectors, amphiphilicity and the preponderance of positively charged amino acid residues (Arg and Lys) are noted. Recent systematic approaches to investigate the latter observation demonstrated that natural Arg-rich peptides, including RNA-binding and DNA-binding peptides derived from all types of organisms (viruses and yeasts to human), as well as synthetic polyarginines, can translocate across membranes and are able to deliver proteins (53). Furthermore, selected Lys- and Arg-rich peptides derived from a phage-displayed library were capable of delivering β -galactosidase *in vitro* and *in vivo* (54). Many of the vectors also possess oligonucleotide-binding properties, as indeed do most proteins with nuclear localization signals (55, 56) and in many cases DNA-binding and translocation capacities appear to be interdependent. Few studies have been reported where different vectors have been applied to the same delivery problem, and it is thus difficult to assess accurately the relative advantages and disadvantages of the various vector systems. In one study, three vector systems (Penetratin, MPS, and model amphiphilic peptide; refer to Table 1) were compared for the delivery of SH3 domain blocker peptides (57). Here Penetratin was found to be the superior vector, with the other two peptides either reducing biochemical affinity of the blocker peptide in vectorized form and the vector displaying cellular toxicity, respectively.

Homeoprotein Vectors. The homeobox encodes the 60-amino acid sequence of the homeodomain in the products of many developmental genes. These products are often regulatory proteins, which affect the expression of other developmental genes through binding to cognate DNA sequences. The fact that a synthetic peptide (pAntp) encompassing the entire homeodomain of the *Drosophila* homeoprotein antennapedia was sufficient to bind to specific DNA had already been demonstrated (58), when it was discovered that this peptide was also capable of penetrating nerve cells in culture and to accumulate in their nuclei through an energy-independent mechanism (59). Initially, α -2,8-polysialic acid (PSA) chains specific to the neural cell adhesion molecule were proposed as the neuronal cell surface receptors (60). However, the ability of pAntp to penetrate efficiently into a large number of cells devoid of PSA suggested the existence of a more general cell internalization mechanism (61). Even lymphocytes, which are notoriously difficult to transfect, take up the pAntp peptide efficiently (62). After delivery

into cells, pAntp conjugates are generally localized to the cytosol and the nucleus. However, mitochondrial delivery is also possible with the aid of chimaeric pAntp – mitochondrial signal sequence vectors (63).

Homeodomains are composed of three α -helices, with a β -turn between helices 2 and 3 (64) and are highly conserved among homeoproteins and across species (65). The region of the Antp homeodomain responsible for internalization of the protein by cells was mapped to the third helix (66), and this finding led to the demonstration that a 16-residue peptide corresponding to that helix is necessary and sufficient for the translocation of biological membranes (67). Subsequent structure–activity studies based on that peptide showed that cell internalization was receptor-independent and correlated in some way with a proposed bioactive amphipathic structure (68). Thus membrane translocation was equally efficient at 4 °C and 37 °C for the native sequence, as well as *retro*- and *inverso*-peptide isomers. The same was independently shown to be true for *retro-inverso* analogues of the same sequence (69). An analogue derived from pAntp(43–58), and consisting entirely of Arg and Trp residues (70–72), has also been studied, and the exclusive presence of cationic and hydrophobic groups in this peptide support the notion that amphipathicity is involved in the mechanism of action. The minimal sequence necessary and sufficient for functional membrane translocation of pAntp peptides, previously thought to correspond to pAntp(43–58), has recently been mapped to the C-terminal heptamer encompassing residues 52–58 (73). A pAntp-derived vector, designed from known structure–activity relationships and incorporating peptidomimetic elements was also reported recently (74). The fact that several homeoproteins and homeodomains investigated to date appear to internalize into cells suggests that additional peptides, mechanistically related to pAntp(43–58), may yet be discovered (12, 65, 75, 76).

Although pAntp(43–58) adopts amphipathic helical structure in various solvents and in the presence of SDS micelles (77), helicity does not appear to be a prerequisite for activity, since Pro-containing substitution analogues presumably incapable of helical structures nevertheless traversed membranes (78). In fact it has been shown recently that the pAntp(43–58)'s secondary structure in membrane-mimicking environments is dominated by β -structures (79, 80). The peptide exhibits high surface activity in the presence of phospholipids, and translocation properties appear to be linked with the disorder induced by the peptide in charged lipid layers. The cellular uptake mechanism appears to involve only peptide–membrane lipid interactions (81), and the translocation does not apparently involve pore formation (82, 83). However, although models have been proposed (72, 84), the exact mechanism responsible for membrane translocation by pAntp peptides remains largely elusive.

The question of how certain proteins without a signal sequence can be secreted has been raised in connection with homeoproteins (84). A detailed study (85, 86) with the homeoprotein Engrailed, which, when expressed in cells, is secreted into the culture medium, revealed a short sequence (termed Δ 1) that appears to be necessary for nuclear export and secretion. The sequence in question is positioned N-terminally of the cellular import sequence (helix 3) and partly overlaps with it. This situation is suggestive of an import–export model that may apply to other homeoproteins, and messenger proteins in general. Furthermore, these findings may lead to the discovery of vector peptides capable not only of cellular import, but also cellular trafficking.

The use of pAntp vectors in order to probe protein–protein interactions with short peptide sequences in the context of whole-cell function has been very extensive, while protein transduction has been analyzed to a much lower extent (28, 76). Thus peptides involved in regulation of cell proliferation (28, 57, 87–102), cell migration (103, 104), cellular immune processes (105), and peptidic active-site antagonists and other modulators of cellular processes (106–110), have all been delivered to cells in vitro, and, in some cases, in vivo, and effector mechanism-based functional read-outs have generally been obtained. Applications involving successful cellular delivery of antisense oligonucleotides (44, 111, 112) and antisense peptide nucleic acids (PNAs) (113, 114), leading to antisense target-related effects, have also been reported, again including in vivo applications.

HIV TAT Vectors. The human immunodeficiency virus (HIV) encodes several regulatory proteins not present in other retroviruses. One of these is the 86-residue TAT protein, which *trans*-activates certain viral genes and is essential to viral replication. Independent studies using bacterially expressed (115) and chemically synthesized (116) TAT protein, respectively, showed that this protein could enter cells when added to the medium of cells in culture. Furthermore, the protein located to the nucleus and was capable of *trans*-activating viral promoters. The domain responsible for translocation was shown to encompass the basic domain of the TAT protein. A peptide extending from residues 37–72 enabled cellular delivery of protein cargoes such as β -galactosidase, horseradish peroxidase, RNase A, a *Pseudomonas* exotoxin A fragment not only in vitro, but also in vivo (117, 118). Similarly, a vector based on the sequence 37–62 was successfully used to deliver an antibody Fab fragment to cells (119, 120).

A peptide containing the TAT sequence including residues 38–60 was shown to adopt an α -helical conformation under certain conditions; in particular, the sequence 38–45 appeared capable of forming an helix with amphipathic characteristics, whereas the basic sequence 49–57 was unstructured (121). The latter sequence was demonstrated to contain a nuclear localization sequence (NLS) (122). Furthermore, using a β -galactosidase-TAT cellular expression vector, it was demonstrated that the TAT-NLS was able to confer nuclear entry and binding to nuclear components through a novel import pathway (123). A detailed study (124) involving peptides derived from the TAT(37–60) sequence showed that the amphipathic helix was in fact not required for cellular peptide uptake. In contrast, the whole basic domain from the TAT sequence, which appears unstructured in solution (125), was necessary for cell internalization. The minimal sequence necessary and sufficient for membrane translocation was TAT(48–57). Although intact HIV-1 TAT protein contains an RGD cell adhesion site (126) and enters cells by energy-dependent absorptive endocytosis (127), uptake of TAT peptide constructs does not apparently involve classical receptor-, transporter-, endosome- or adreptive-endocytosis-mediated processes. However, unlike the inverted micelle model of membrane transduction favored for pAntp, a mechanism involving direct penetration of the lipid bilayer caused by the localized positive charge of the vector, in which the momentum of the molecule drives the covalently attached cargo into the cytoplasm, has been proposed (128). On the other hand, a similar mechanism is indicated by studies with equine TAT protein which suggest that the structural elements responsible for the cell membrane transduction

properties of TAT and homeoproteins could be related by evolution (129).

Short TAT peptide vectors have been used widely in order to introduce various functional peptides and oligonucleotides into cells (112, 130–132). Furthermore, TAT peptide vectors have been used particularly successfully for cellular transduction with functional proteins. An interesting example is the design of a transducing, modified, apoptosis-promoting caspase-3 protein, in which endogenous proteolytic cleavage sites were substituted with HIV protease-specific ones (133). The application of this TAT peptide-caspase construct to HIV-infected cells led to apoptosis, while remaining without effect on uninfected cells. Although entire proteins can be delivered into living cells using TAT peptide vectors (118, 128, 134–136), membrane transduction appears to be a stringent process that involves partial or complete unfolding of the protein cargo (137). For example, although delivery of correctly folded TAT-GFP fusion protein into cells was efficient, it was accompanied by significant loss of GFP fluorescence emission (128).

Very recently, a detailed structure–activity relationship study (138) based on the HIV TAT(49–57) sequence RKKRRQRRR (139) revealed that while further truncation of the 9mer sequence led to abolition of membrane-translocation properties, the sequence could be *retro*-, *enantio*-, and *retroenantio*-modified with beneficial effects. Furthermore, of the nine residues only Gln⁵⁴ was particularly sensitive to Ala substitution. Replacement of the two Lys and the Gln residues with Arg, i.e., formation of an Arg₉ homopolymer, afforded a peptide with improved cell internalization properties. This could be further enhanced by using D-Arg residues exclusively (r9). Of the D-Arg homopolymers the 9mer had optimal properties but here truncation down to the 7mer (r7) was tolerated in terms of internalization properties. Corresponding Lys, Orn, and His oligomers were less effective at entering cells (140). Comparison of r9 with HIV TAT(47–59) and pAntp(43–58) showed that the former entered cells significantly faster than the native TAT sequence and the pAntp sequence. Peptoid analogues of the HIV TAT sequence (141, 142), i.e., peptidomimetics containing *N*- rather than *C* α -substituted α -amino acid monomers, were also studied. Here similar findings as with the Arg homopolymers were made. Additionally, cellular uptake was proportional to the length of the alkyl chain in the *N*-alkylguanidine monomer side chains. In this study the energetics of cellular uptake of the peptides and peptoids were assessed not only in terms of temperature-dependence, but also by performing penetration assays in the presence or absence of sodium azide. While temperature did not apparently influence membrane translocation (consistent with results obtained elsewhere with many different vectors), azide, an inhibitor of oxidative phosphorylation (143), was found to inhibit cell internalization.

An application of the r7 vector, conjugated through a pH-sensitive linker to cyclosporin A, has been reported (144). Cyclosporin A is ineffective for topical applications due to its poor skin-penetration properties. In contrast, it was found that the r7 conjugate was transported efficiently into mouse and human skin, reached dermal T lymphocytes, and inhibited cutaneous inflammation.

HBV PreS2 Vector. A permeable peptide, referred to as PreS2-TLM (translocation motif), was recently reported (145). The presence of a masked fusion peptide in the PreS2 surface antigen of hepatitis-B virus had previously been postulated (146), when it was observed that proteolytic digestion of viral particles enabled

infection of otherwise nonpermissive cells (147). The PreS2-TLM corresponds to an amphipathic α -helix between residues 41 and 52 of the PreS2 protein. The peptide appears to be able to penetrate a variety of cells, including plant cells, by an energy-independent mechanism. Once inside a cell, it distributes evenly over the cytosol. Recombinant PreS2 protein, when infused into the portal vein of anesthetized rats, was detected in various liver cells, including cell layers distant from capillaries (145). Interestingly, in comparison to the TAT-peptide, Penetratin, and other peptides rich in positively charged residues, PreS2-TLM contains only one negatively and one positively charged amino acid. Therefore, this peptide may follow a different translocation mechanism.

HSV VP22 Vector. A 38-kDa structural protein, VP22, from herpes simplex virus-1 has the remarkable property of intercellular transport (148). When expressed in a subpopulation of cells, the protein spreads to every cell in a monolayer, where it concentrates in the nucleus and binds chromatin. VP22 is also a target for phosphorylation in the nucleus after viral infection, presumably as a result of casein kinase II activity (149). Apparently, intercellular trafficking occurs via a nonclassical Golgi-independent mechanism that may involve the actin cytoskeleton. The utility of VP22 as a transport vector was demonstrated using fusion proteins with a 12-residue epitope tag or the 27-kDa jellyfish green fluorescent protein (GFP). In the former case, transport properties were found using either endogenous synthesis, or by application to the medium and uptake. The GFP fusion protein was expressed in COS-1 cells and was observed to move to surrounding untransfected cells in the same way as wt-VP22. Although intercellular transport of GFP-VP22 constructs was discussed controversially (150), recent reports have confirmed intercellular transport, not only to proliferating cells, but also to terminally differentiated cells (151–154). Furthermore, transport was quantitated (155) and *in vivo* delivery demonstrated (156). VP22-mediated protein transport has also been used in order to deliver thymidine kinase (TK) to neuroblastomas in live mice, rendering the tumors sensitive to the TK-activated prodrug ganciclovir and causing tumor regression (157). Functional application relevant to gene therapy was shown with a chimaeric protein consisting of VP22 linked to the tumor suppressor protein p53 (158). Not only was spreading between cells seen using this protein, but induction of apoptosis in p53-negative human osteosarcoma cells, resulting in a widespread cytotoxic effect. A mutant VP22 protein lacking the C-terminal 34 residues was found to be synthesized in transfected cells at similar levels as wt-VP22; however, it was not detected in the nuclei of surrounding cells. This indicates that the sequence responsible for imparting membrane permeability to the protein is located in the C-terminus and that, as has been found in many similar cases, a short permeable peptide may be derived from the sequence of this protein. As far as can be ascertained, this has not as yet been achieved. Identification of such peptides would be desirable as immunogenicity is likely to be a problem with full-length VP22 chimaeras (134). Viral VP22 is known to be a T-cell antigen (159) and VP22-reactive T-cells may play a role in the control of recurrent HSV infection. Nevertheless, VP22-mediated delivery of transgene products is now considered a promising strategy for successful gene therapy, overcoming current problems associated with limited delivery (160).

Apolar Signal Peptide Vectors. Although hydrophobic signal sequences lack primary sequence identity,

the critical characteristic which correlates with membrane activity appears to be residue-average hydrophobicity in the h region (161), and neither the positively charged N-terminal segment nor the C-terminal signal peptidase cleavage site are necessary for membrane translocation (162). The h region, comprising 7–16 nonconserved residues, is thus the dominant structure determining membrane-translocating signal sequence function (163, 164) and has been identified in numerous signal peptides (165). Synthetic peptides corresponding to the h region of signal sequences from Kaposi FGF (166), integrin β_3 (167), and *Caiman crocodylus* Ig(v) light chain (168) have been developed as delivery vectors. Kaposi FGF and β_3 -integrin derived delivery peptides are also known as MPS (membrane permeable sequences) in the literature.

As with other peptide vectors, cell permeability is not limited to particular cell types, and membrane translocation appears to be independent of ATP, since cells depleted of their metabolic ATP pool were nevertheless able to internalize vector peptide (166). However, the mechanism of membrane translocation here is clearly different to the cases discussed above, since transport of apolar signal peptide vectors is temperature-dependent (166). Presumably membrane translocation in this case is dependent in some way on membrane fluidity. Again the precise mechanism remains unclear, although it is clear that import is not dependent on caveolae (164). Conformational analysis of a delivery vector constructed by fusing the signal sequence of *caiman crocodylus* Ig(v) light chain (169) and the NLS of the SV40 large T antigen (170) showed that the peptide adopted a disordered structure in water, α -helical structure in the presence of trifluoroethanol or detergent micelles, but mainly antiparallel β -sheet form in the presence of lipids, regardless of the nature of the phosphate headgroup, and embedded in the lipid cores (171). Unlike other apolar signal peptide vectors, this particular peptide was observed to internalize efficiently into cells at low temperature.

The apolar signal peptide vectors have been applied extensively and successfully in dissecting intracellular signaling pathways (172, 173), particularly those concerning intracellular integrin signaling (166, 174) and blocking of signaling to the nucleus through specific NLSs (167). Recent studies with the latter approach also suggest potential *in vivo* utility of apolar signal peptide vectors (175–177). As with the HIV TAT peptide, large macromolecular cargo can be imported into cells. Thus a 12mer apolar signal peptide vector was fused to the 41-kDa protein glutathione S-transferase, and the resulting construct was imported into cells efficiently (178).

Although several examples of functional molecular cargoes being imported into living cells using these vectors are documented (164, 179), a question remains concerning their universal applicability. This question concerns intracellular targeting and localization, since hydrophobic signal peptides generally direct the polypeptides they form part of to the endoplasmic reticulum (180). At least in one case (181), where antisense oligonucleotides were fused with these vectors, efficient membrane translocation was observed, but no appreciable antisense effect was obtained. Examination of the cellular distribution revealed endosomal trapping of the conjugates. Interestingly, this was the case regardless of whether a NLS was appended to the vector sequence. In a different approach (182), the K-FGF vector was conjugated with a polycationic linker, which was then complexed electrostatically with an oligonucleotide. In this case cytosolic and nuclear accumulation of the

construct after application to cell cultures was shown, although no functional read-out was reported.

Fusion Peptide from HIV gp41. Because of their role in cell fusion, the hydrophobic amino-terminal regions of transmembrane proteins of various enveloped viruses, including the orthomyxoviruses, paramyxoviruses, and retroviruses, are known as fusion peptides (183). Viral fusion reactions fall into two classes, low pH-dependent (see below) and pH-independent. Viruses with low pH-dependent activity fuse with membranes of acidic endosomes, whereas those with pH-independent activity fuse with both plasma membranes and endosomes. Thus, cells expressing the fusion proteins of simian virus 5, respiratory syncytial virus or HIV fuse at neutral pH. A fusion peptide consisting of 28 contiguous hydrophobic amino acid residues was shown to be present in the HIV gp41 glycoprotein (184), and its function in syncytium formation was demonstrated (185). Certain mutations in the conserved *N*-terminal fusion domain of gp41 (186, 187), as well as antibodies specific for this domain, inhibited HIV infection, syncytium formation, and HIV cytopathy (188, 189). Furthermore, the ability of synthetic gp41 fusion peptides to perturb and lyse artificial lipid bilayers has been demonstrated and the membranotropic ability shown to be dependent on peptide length, sequence, and temperature (190–192). Using human erythrocyte membranes it was shown that at subhaemolytic doses *N*-terminal gp41 fusion peptides bind to erythrocytic lipids, whereas at doses of $\geq 5 \mu\text{M}$ haemolysis takes place (193).

The potential utility as a cellular delivery vector of a gp41-derived peptide (residues 1–17), also containing the NLS of SV40 large T-antigen (170, 194), was suggested (195). Until recently it has been assumed that peptide vectors had to be tethered covalently to their cargo in order to effect cellular delivery. This concept has now been challenged. The fact that cellular transfection with oligonucleotides can be mediated by cationic liposomes and other cationic amphiphilic species is well established (37). Here, packaging and compaction resulting from electrostatic and hydrophobic interactions with anionic oligonucleotides is important. Similar effects have recently been shown with the above gp41/SV40T vector peptide, when noncovalently complexed with oligonucleotides (196). Some of the claimed advantages over traditional transfection methods of this system are as follows: delivery does not follow the endosomal pathway, it is not cytotoxic, and it is not affected by serum proteins. More surprisingly, noncovalent association of the vector with a hydrophobic peptide has recently been shown to permit cellular delivery (197). High-affinity interaction between the peptide and the vector, presumably involving the hydrophobic *N*-terminus of the latter, resulting in vector–peptide complexes (ca. 20:1 ratio), were demonstrated. It remains to be seen how general this promising approach is in terms of polypeptide delivery.

Amphiphilic Peptide Vectors. Lipid-enveloped viruses infect their host cells by fusing with either the plasma membrane or the endosome membrane after endocytosis of the virion. The former process is mediated by hydrophobic fusion sequences (see above), which are normally expressed on the surface of the fusion proteins, whereas the fusion sequence of, e.g., influenza virus haemagglutinin (HA), is exposed only at the low pH of endosomes, at pH 5–6 (198, 199). The low pH results in a change of conformation of HA leading to the exposure of the amino terminus, which consists of an amphiphilic helix ending in a short hydrophobic sequence of about 10 residues. Short synthetic peptides containing the first

17–23 residues of the HA2 subunit of HA also display pH-dependent lytic properties, with little activity at neutral pH but with >100 -fold increase in transfection efficiency at pH 5. The lytic properties of these anionic peptides are only revealed as the Asp and Glu side-chain carboxyl groups are protonated, allowing the peptides to adopt amphiphilic helical structures (200, 201). Oligonucleotide–peptide conjugates containing such HA peptides have been used successfully in order to overcome the frequent phenomenon of endosomal trapping after transfection (39, 202, 203). While certain anionic peptides cause membrane fusion at acidic pH, corresponding cationic peptides may have the same effect at alkaline pH. Thus amphiphilic anionic and cationic peptides formally derived from the influenza virus HA fusion segment, have been proposed as vehicles for oligonucleotide transfection (8, 204, 205). A study examining a large number of influenza fusion sequences, as well as many other different lytic peptides (19), led to the design of the amphipathic endosomolytic vector peptide JTS-1 (GLFE-ALLELLESLWELLEA), whose application for gene delivery, in conjunction with the synthetic poly-Lys (40, 206, 207) substitute YKAK₈WK, has been advocated (208). Many other naturally occurring and designed amphiphilic lytic peptides, including, e.g., ones derived from the wasp venom component mastoparan (209), from the *C*-terminus of the HIV-1 gp41 protein (210), and from synthetic ion channel peptides (119), are known and have been studied as delivery vectors (134). In many of these constructs containing endosomolytic peptides, DNA-binding and compacting sequences, and NLSs have also been incorporated (11, 211, 212).

A particularly interesting system is represented by the so-called GALA (213) and KALA peptides. The latter peptide (WEAKLAKALAKALAKHLAKALAKALKACEA (214)) undergoes a pH-dependent random coil to amphiphilic α -helical conformational change as the pH increases from 5.0 to 7.5. KALA is efficient at causing membrane leakage from neutral and negatively charged liposomes and causes complete leakage of entrapped contents over the pH range of 4.0–8.5. Furthermore, KALA binds to oligonucleotides and plasmid DNA and assists their nuclear delivery when complexes are prepared at a 10/1 $\pm$ charge ratio. In fact such complexes mediated transfection of a variety of cell lines. It seems likely that here direct plasma membrane translocation to the cytosol, as well as endocytic uptake and release, may be involved. For a similar model α -helical amphiphilic peptide (KLALKLALKALKALKLA, (215, 216)), nonendocytic uptake mechanisms were in fact demonstrated. Further examples of designed cationic amphiphilic α -helical delivery peptides are known (217).

Not only do peptide-based oligonucleotide delivery systems now offer a viable alternative to traditional *in vitro* lipofection methods (33, 34, 36, 218), but synthetic peptide-based gene delivery systems consisting of Lys-rich oligonucleotide binding regions and pH-sensitive endosomolytic motifs have been developed for *in vivo* gene delivery and expression (38). In general it should be remembered, however, that amphiphilic peptides are generally cytolytic and cannot therefore be expected to provide delivery vectors permitting delivery of effector molecules at high therapeutic indices.

Anti-DNA Antibody-Derived Peptide Vectors. The fact that certain anti-DNA Abs are capable of entering living cells has been known for some time (219–221). Studying naturally occurring polyreactive anti-DNA mAbs derived from the spleen cells of a nonimmunized lupus mouse, Avrameas et al. (222) found that many

hybridomas cloned secreted cell-penetrating IgG mAbs. These Abs, or their Fab' and (Fab')₂ fragments, were capable of penetrating various different cell types through an energy-dependent mechanism, entering the nucleus, and accumulating there. This process did not apparently alter either cell viability or metabolism and internalization was mediated in some way by mAb binding to cell surface structures. Interestingly, only polyreactive anti-DNA mAbs could penetrate cells. They appear to represent clones of natural polyreactive autoantibodies that escape normal control and which are expanded in lupus disease. Penetrating mAbs or their fragments were effective vectors for the delivery of haptens, oligonucleotides, and polypeptides ($389 > M_r > 40000$).

Sequencing of three mAbs revealed high homology in the V_H CDR2. A synthetic peptide corresponding to one of these CDR2 sequences did not bind antigen or translocate membranes. A peptide corresponding to the V_H CDR3, however, had some penetration properties. When both sequences were linked in the form of a peptide, full DNA-binding, polyreactivity, and efficient cell penetration properties were observed. This peptide vector (P3) was capable of carrying molecular cargo into cells in the same way as the parent mAb. It has been speculated that by combining the CDR2 and CDR3 of different penetrating anti-DNA mAbs or their mimotopes (223), peptide vectors with various properties may be designed (222).

Incubation of cells with complexes between penetrating mAb and plasmid DNA encoding a tumor antigen resulted in expression of the antigen in no more than 0.1% of cells. It is unclear whether this is due to misrouting of the internalized plasmids or hindrance to internalization arising from structural alterations imposed on the mAbs through plasmid binding. The latter is more probable, as site-directed mutagenesis studies of penetrating anti-DNA mAbs have shown that the same residues are involved in DNA binding and mediation of cell entry (221). Vector P3 was also ineffective in oligonucleotide delivery. Extension of the peptide with 19 Lys residues to achieve DNA condensation (38), however, afforded an efficient gene delivery vehicle. For example, efficient transfection of cells with plasmids carrying the genes of the green fluorescent protein or luciferase was reported (224).

Transportans. Transportan is a 27-residue peptide vector (225). Its sequence is composed of 12 residues derived from the neuropeptide galanin, connected, via a Lys residue, with 14 residues corresponding to the wasp venom peptide mastoparan. The former represents the smallest active galanin-receptor ligand with agonist properties. Mastoparan has the ability to penetrate membranes, where it creates short-lived pores by translocating into the inner leaflet; it inhibits Golgi transport and facilitates the opening of mitochondrial permeability pores. Transportan appears to enter cells in an energy-independent manner unrelated to endocytosis. Like other peptide vectors, it does not seem to be selective for particular cell types. It first localizes to the outer membrane, followed by redistribution into the nuclear membrane and uptake into the nuclei and concentration in the nucleoli. Because of its derivation from bioactive peptides, it can be expected that this vector may have undesirable biological effects. Thus it lowers GTPase activity in Bowes cell membranes with an apparent IC₅₀ of 50 μ M, although apparently not exhibiting cytotoxic effects at concentrations below 20 μ M. An improved version, termed transportan 2, containing an inactive mastoparan analogue, has been proposed to overcome these problems (226). The utility of transportans as

delivery vectors has not as yet been examined extensively. Nevertheless, comparative studies with the Antp vector (227) and using cellular and in vivo delivery of PNAs suggest promise (114). A truncation analysis of transportan has also been reported recently (228).

PHARMACOLOGY IN LIVING ANIMALS

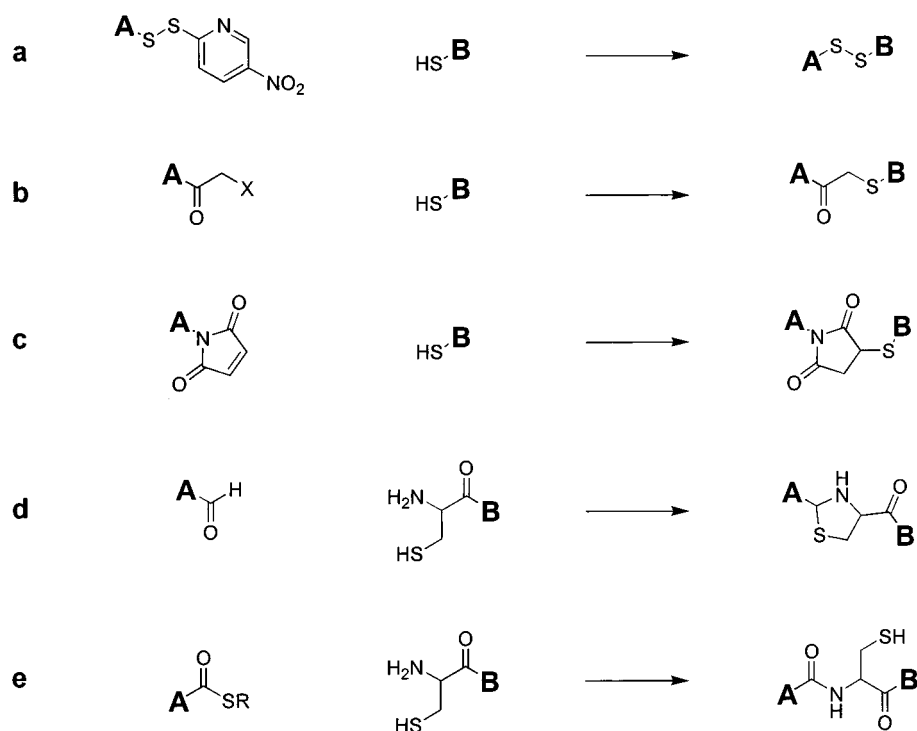
The utility of peptidic delivery vectors in the context of cell biology has been demonstrated extensively. However, the ultimate aim of the technology is to provide novel therapeutic modalities for macromolecules. Although the first report on peptide vector-mediated systemic delivery of polypeptides into whole animals dates back to 1994 (118), comparatively few in vivo studies with vector conjugates have been reported since then and more detailed pharmacological studies are required. In the case of the 11mer TAT vector it was demonstrated that a fusion protein with the 120-kD β -galactosidase protein could be delivered in vivo (29). After intraperitoneal injection of this construct into mice, specific galactosidase activity could be developed in all tissues, including the brain. In fact several in vivo protein transduction applications have been reported using this vector; the subject was reviewed recently (135). The pAntp delivery peptide system, as well as several other vectors discussed above, have also been used for in vivo applications (105, 106, 114, 229).

The main concern regarding therapeutic utility concerns the rapid and highly efficient uptake of vector conjugates by tissues. For example, it was shown that intraperitoneal administration of 0.6 nmol of a HIV TAT peptide-derived vector to mice resulted in complete transduction into all cells present in whole blood after only 30 min exposure, with no additional increases at 60 min postadministration (230). Pharmacokinetically this situation suggests that targeting to tissues in vivo will be difficult and that high doses may be required. Little appears to be known about the inherent toxicity of peptidic delivery vectors; however, cationic peptides such as polylysines are known to be toxic. Thus the potential for rate-limiting toxicity, if high conjugate doses are required, clearly exists. A further area requiring examination concerns the peptidic nature of the delivery vectors in relation to immunogenicity. Since the majority of applications will presumably require repeated administration of conjugates, avoiding the induction of an immune response to peptidic conjugates will be important. One of the advantages with the peptide vectors is the fact that they appear to be able to cross the blood-brain barrier (49, 114). Furthermore, topical applications have recently been shown to hold promise (106, 144).

VECTOR CONJUGATION METHODS

Polypeptide-Vector Conjugates. In the majority of vector peptide applications, the effector parts of the conjugates are peptides themselves. In these cases the two sequences are usually synthesized contiguously. This approach is practically feasible for conjugates up to about 50 amino acid residues in length. For longer constructs, genetic engineering methods or chemoselective conjugation can be used. The latter approach is also sometimes preferred for shorter constructs when a modular approach is desired. Apart from ligation techniques, protein conjugation methods are not discussed here as there are excellent treatises on this subject (231). Methods that have found application in the preparation of permeable polypeptide conjugates are summarized in Scheme 1, and some are discussed in more detail below in the context

Scheme 1. Chemoselective Conjugation Methods



of oligonucleotide-peptide conjugation. Of particular importance are peptide heterodimers formed through disulfide bond formation (Scheme 1a). Specific and efficient methods for the synthesis of such heterodimers by titration of thiol-activated peptides with thiol-containing peptides have been developed (232). In principle the same considerations as for polypeptide constructs apply for peptide nucleic acid (PNA)-peptide hybrids. These can be obtained by synthesis in tandem (233) or through conjugation (114).

Ligation of an aldehyde group in one reactant with a 1,2-aminothiol function in the second reactant is site-specific and rapid, resulting in a conjugate containing a stable thiazolidine ring (Scheme 1d). This method has been used to prepare functionally active permeable peptides, and it was found that the thiazolidine-linked conjugates were equipotent with those containing a conventional amide linkage (234). The required C-terminal peptide aldehydes were elaborated by periodate oxidation of a 1,2-diaminopropanediol or 1,2-amino alcohol group.

Oligonucleotide-Peptide Conjugates. The fact that thiol groups can readily be introduced during solid-phase oligonucleotide synthesis at either the 3'-end by using appropriate thiol linkers (235, 236), or 5'-terminally (237-240), has often been exploited in order to obtain conjugates. Thus thiolated oligonucleotides have been conjugated with cysteinyl vector peptides through disulfide bond formation (44, 112, 114, 168, 181, 241). To favor formation of the heterodimeric conjugate in such reactions, disulfide-exchange between the free thiol in one reaction partner with a pyridylsulfenyl-activated form in the other is usually employed (242, 243) (Scheme 1a). Maleimide-thiol chemistry (244) provides the required selectivity in order to obtain unambiguous oligonucleotide-peptide hybrids from unprotected precursors in most cases (Scheme 1c). Coupling of thiolated oligonucleotides with maleimido-peptides has thus been used successfully for the synthesis of thioether-conjugated hybrids (245, 246). Specifically, thiolated oligodeoxy-

nucleotides were shown to react selectively and efficiently with maleimide-derivatized 18mer TAT and 22mer C-terminal gp41 vector peptides (247). Alternatively, 5'-amino-modified oligonucleotides can be maleimide-functionalized and then conjugated with Cys-containing peptides (248, 249) or with thiol-peptides obtained from SPPS using a disulfide bond-based linker (250). The latter method was applied to prepare oligonucleotide conjugates with a fusogenic peptide (203). Similar thioether conjugation can be achieved using bromoacetyl-instead of maleimide-functionalized peptides (251) (Scheme 1b).

Nonselective covalent attachment of peptides to DNA can also be achieved, e.g., by using a method based on the DNA alkylating moiety cyclopropylpyrroloindole (CPI). A bromoacetyl-CPI derivative is first alkylated with a cysteinyl peptide, and the resulting complex is then conjugated to DNA through *N*-alkylation of purine bases.

Unsatisfactory reactivity during macromolecular condensations in solution frequently derives from limited and incompatible solubility of macromolecular substrates. This situation has been shown to be ameliorated to a large extent by employing solid-phase segment condensation methods (252). This approach is applicable to the synthesis of peptide-oligonucleotide hybrids: for example, protected solid phase-bound oligonucleotides can be derivatized 5'-terminally with an aminoalkyl group, followed by amidation with suitably side-chain protected *N*^α-Fmoc-blocked peptide acids. The protected immobilized hybrids are then liberated from the support through appropriate deprotection and cleavage (253). A linker-free version using a 3'-terminal aminothymidine building block, followed by stepwise Fmoc-based SPPS, has also been reported (254). Although various approaches toward total stepwise solid-phase synthesis of oligonucleotide-peptide hybrids have been taken (255-258), this attractive method of synthesis cannot as yet be regarded as mature enough for general application.

Native ligation techniques (259), originally devised for the splicing of unprotected peptide segments in the

chemical synthesis of proteins, have also been adapted to the conjugation of peptides with oligonucleotides (Scheme 1e). In one such approach (260), oligodeoxynucleotides are equipped with a *S*-*tert*-butylsulfenylcysteine phosphoester at the 3' end during solid-phase synthesis. After cleavage and deprotection, the product is condensed with a peptidyl benzyl thioester. The latter is also obtained through solid-phase assembly using an activated thiosuccinate to introduce the thioester moiety. The ligation reaction is catalyzed by the water-soluble reducing agent tris(2-carboxyethyl)phosphine, and an auxiliary thiol nucleophile is employed in order to facilitate thiol exchange. The reaction proceeds through nucleophilic attack of the oligonucleotide Cys thiol group on the peptide thioester. The resulting thioester ligation product undergoes rapid intramolecular rearrangement to the amide, due to the favorable geometry of the Cys amino and thioester carbonyl groups. A similar approach was also reported (261), in which the oligonucleotide is equipped 5'-terminally with a guanosine-5'-phosphorothioate. A phenyl thioester group was then introduced via *S*-alkylation of the terminal phosphorothioate moiety with phenyl α -bromothioacetate. The 5'-thioester oligonucleotide product was then transthioesterified with a stoichiometric amount of a peptide containing *N*-terminal Cys. As in the preceding example, facile intramolecular attack of the free peptidyl α -amino group on the newly formed thioester bond led to rearrangement to the stable amide-bonded conjugate. In effect, the ligation mechanism provides entropy activation for the eventual amide bond formation (262) and is therefore particularly attractive for condensation of macromolecular species. Ligations can be carried out in aqueous or mixed aqueous/organic media, even in the presence of powerful solubilizing agents such as urea or guanidinium hydrochloride. Furthermore, due to the near-complete chemoselectivity of native ligation, fully deprotected peptide and oligonucleotide components can be employed.

Template-directed ligation of peptides to oligonucleotides has also been reported (263): An activated peptide thioester was reacted with a 5'-thiolated oligodeoxynucleotide¹, resulting in a thioester linked peptide-oligodeoxynucleotide¹. This was then brought into close proximity with a 3'-amino-modified oligodeoxynucleotide² by hybridization to adjacent sites on a complementary oligodeoxynucleotide template. The nucleophilic amine then attacked the thioester carbonyl group of the peptide-oligodeoxynucleotide¹, forming an amide bond between the oligodeoxynucleotide² and the peptide, and releasing the 5'-thiol-oligodeoxynucleotide¹. This method relies on a template to promote the ligation reaction and to confer specificity for a particular combination of oligonucleotides and peptide. Because here ligation is carried out in an addressable format, different combinations of oligonucleotides and peptides should be able to be joined simultaneously. The full scope of this approach does not appear to have been explored to date.

Selective conjugation of biosynthetic oligonucleotides with peptides is more difficult. In the case of RNA, periodate oxidation of the 2',3'-cis diol group of the 5'-terminal ribose and coupling of, for example, peptide hydrazides or thiosemicarbazides to the dialdehyde product would appear to offer a possibility (264, 265). It has been shown that modified nucleotides can be incorporated enzymatically into both RNA and DNA (266, 267). It should therefore be possible similarly to introduce peptides, suitably modified with TTP or UTP moieties. A synthetic route for the preparation of peptides carrying

such a group, linked through an acylphosphoramidate linkage, appears to be available (268).

CONCLUSIONS

While the *in vitro* and *in vivo* cellular delivery of membrane-impermeable therapeutic agents, including inherently selective macromolecules such as proteins and oligonucleotides, now seems feasible, the next question to address will be how such agents might be delivered in a tissue- and cell type-selective manner. Here too, significant advances have been made (134, 269, 270), again using peptide-based approaches. In particular, targeted nonviral systems for gene transfer have shown promise (271–273). In fact it may soon prove possible to combine cellular delivery and targeting functions using truly modular and discriminating delivery vector systems.

Despite these prospects, more detailed answers to many mechanistic questions are still required. Thus it remains unclear in most cases if and to what extent target cells are damaged by the delivery vectors. Very little is as yet known about the ability of delivery vector conjugates to penetrate into tissues, e.g., tumors. Also, intrinsic cell selectivity has not been addressed in sufficient detail. Generally absorption, distribution, metabolism, immunogenicity, and cellular fate of delivery peptide vector conjugates will need to be studied in depth before the current promise of these agents can be realized for therapeutic applications.

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COMMUNICATIONS

Kinetics of Coupling Reactions That Generate Monothiophosphate Disulfides: Implications for Modification of RNAs

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The inclusion of guanosine-5'-monothiophosphate (GMPS) in an in vitro transcription reaction facilitates enzymatic synthesis of an RNA transcript with a monothiophosphate group at the 5' end. A kinetic study of the modification reactions that generate monothiophosphate disulfide linkages with either 5'-GMPS alone or 5'-GMPS-primed RNA as the substrate revealed that the second-order rate constants increased as the pH was decreased. For example, when the reaction pH was lowered from 8 to 4, the k_2 value for the coupling reaction between N-(6-[biotinamido]hexyl)-3'-(2'-pyridyldithio)-propionamide (biotin-HPDP) and GMPS increased 67-fold from 1.84 to 123 M⁻¹ s⁻¹. In addition to discussing a possible mechanism for coupling reactions that involve GMPS and disulfides, we also indicate conditions that are likely to be optimal for modification of the nucleophilic sulfur in 5'-GMPS-primed RNAs.

INTRODUCTION

Impetus for the design of simple strategies for site-specific incorporation of biochemical and biophysical probes in RNAs is fueled by the increasing realization that the role of RNA and RNA–protein interactions is pervasive in various cellular and developmental processes. Advances in solid-phase oligonucleotide synthesis of small RNAs have facilitated the site-specific introduction of functional groups that lend themselves to subsequent chemical modification. For instance, a 2' amino group in an RNA oligonucleotide can be converted to a thiol (by treatment with an activated ester and a reducing agent), which in turn can be modified to incorporate a probe of choice (4). However, for large RNAs that cannot be synthesized chemically, a commonly employed approach has been to include guanosine-5'-monothiophosphate (GMPS) in an in vitro transcription reaction to enable enzymatic synthesis of an RNA transcript whose 5' end contains the monothiophosphate group (10). The nucleophilic sulfur at the 5' end can be subsequently modified to introduce a photoaffinity cross-linking agent or a spin label or an affinity tag (3, 7, 9). Despite recent reports of successful modifications of 5'-GMPS-primed RNAs, the kinetics and mechanism of the coupling reactions remain uncharacterized. In this report, we have examined the kinetics and pH-dependence of the modification reactions that generate monothiophosphate disulfide linkages using either 5'-GMPS or 5'-GMPS-primed RNA as the substrate.

RESULTS AND DISCUSSION

Spectrophotometric measurement of the kinetic rates for the coupling reactions with GMPS was made possible by choosing 2-thiopyridyl derivatives (such as 2-PDS and biotin-HPDP, Figure 1) as modifiers. The modification reactions were carried out at 24 (±2) °C under pseudo-first-order conditions with the pyridine disulfide in at least 30-fold excess. Plots of log[GMPS] versus time were linear over two half-lives and the calculated second-order rate constants (k_2) did not exhibit any significant variation when the concentrations were varied over a 3-fold range. When k_2 values were examined as a function of pH, an increase in the rate was observed as the pH of the reaction was decreased (Figure 2). For instance, the second-order rate constant for the reaction between GMPS and 2-PDS in 20% DMF (v/v) showed an 18-fold increase as the pH was decreased from 8 to 5.8 (Figure 2). Similarly, the coupling reaction between biotin-HPDP and GMPS was accelerated 67-fold (from 1.84 to 123 M⁻¹ s⁻¹) as the reaction pH was lowered from 8 to 4 (Figure 2).

These findings with GMPS as a substrate prompted us to pursue the biotin-HPDP modification reaction with a 5'-GMPS-primed precursor tRNA (ptRNA). The rate of biotinylation of 5'-GMPS-primed ptRNA was enhanced 2 orders of magnitude upon lowering the pH from 8 to 4 (Figure 2). However, since this conclusion is based on 2-thiopyridone release and not based on direct quantitation of biotin content in the RNA, we decided to employ an independent measure of biotinylation. The modification reaction was performed at different pH values, and the putative biotinylated ptRNAs were transferred to a nylon membrane where-in they were probed with a streptavidin–horseradish

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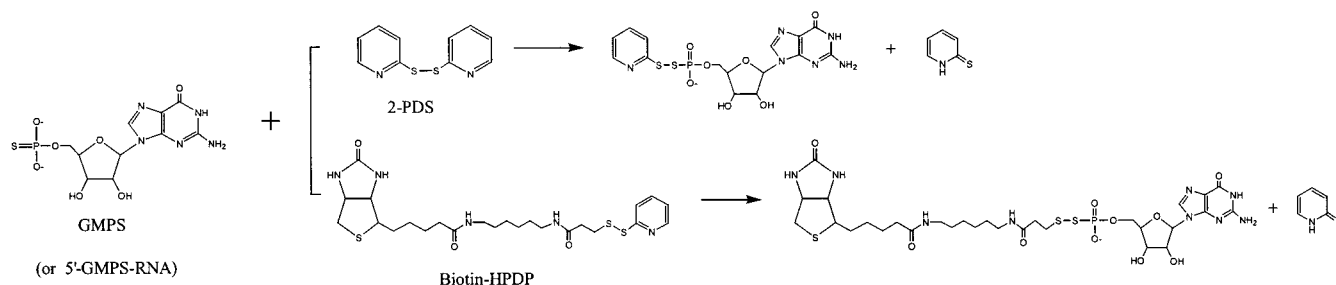


Figure 1. Generation of disulfide linkages using GMPS as the substrate. The coupling reactions between GMPS (synthesized as described elsewhere (1)) and either 2,2'-pyridine disulfide (2-PDS, Sigma) or N-(6-[biotinamido]hexyl)-3'-(2'-pyridyldithio)-propionamide (biotin-HPDP, Pierce) are illustrated. A variation on the latter reaction involves the modification of a 5'-GMPS-primed RNA with biotin-HPDP. The ptRNA^{Gln} substrate was synthesized in vitro by incubating 38 $\mu\text{g/mL}$ BstN I-linearized pUC-pGln (8) with 4.8 mM GMPS, 1.2 mM GTP, 6 mM ATP, 6 mM CTP, 6 mM UTP, and 400 U T7 RNA polymerase (Ambion) for 5 h at 37 $^{\circ}\text{C}$ in 200 μL of 200 mM HEPES, 40 mM DTT, 2 mM spermidine, and 28 mM MgCl_2 . After DNase I treatment and phenol/chloroform extractions, the RNA in the aqueous phase was washed over a Microcon YM-10 membrane to remove unutilized rNTPs and GMPS.

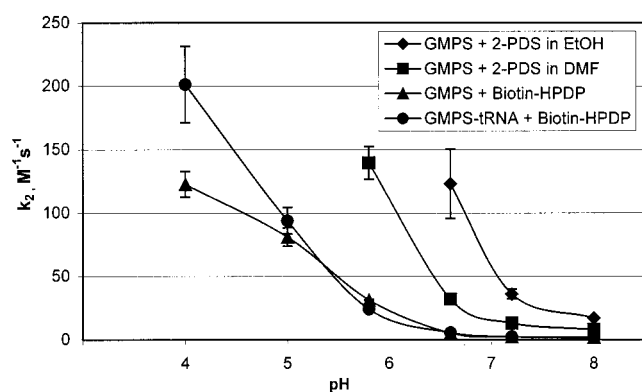


Figure 2. The pH dependence of second-order rate constants for the various GMPS-coupling reactions (see text for details). Inset provides a key to the symbols used in the figure. The averages were calculated using second-order rate constants obtained from at least two independent experiments. 2-PDS and biotin-HPDP were dissolved in either 50% (v/v) ethanol or DMF and diluted appropriately for the modification reactions. The reactions of GMPS with either 2-PDS or biotin-HPDP were performed in 0.1 M phosphate or citrate or acetate buffer. Reactions were initiated by rapid mixing (Sigma cuvette mixer) of stock GMPS. Kinetics of the coupling reactions were measured using a BioSpec-1601 spectrophotometer (Shimadzu) by following the appearance of 2-thiopyridone at 343 nm (using $\epsilon = 7060 \text{ M}^{-1} \text{ cm}^{-1}$) for reactions in either 20% (v/v) ethanol or 20% (v/v) DMF.

peroxidase (HRP) conjugate. Increased biotinylation of ptRNA with decreasing pH would result in greater binding of the HRP-linked streptavidin. In the presence of a chemiluminescent substrate for HRP, the signal produced would be proportional to the amount of RNA that was biotinylated. The chemiluminescence signal is at least 5-fold more intense when the GMPS-primed ptRNA was modified with biotin-HPDP at pH 4 than at pH 8 (Figure 3). Since a 70-min incubation period was used for these ptRNA modification reactions, the 100-fold difference in second-order rate constants between pH 4 and 8 manifests itself as a much smaller (5-fold) apparent difference between the two pH values (Figures 2 and 3). Although this explanation could be validated by performing the tRNA modification reaction for a much shorter time (e.g., only 5 min), this was not technically feasible due to the inability to terminate the reaction prior to loading the products on the polyacrylamide gel.

The pH-rate profiles for the reactions that we have studied provide some clues with regard to the mechanism of the GMPS-modification reactions. The reactions are first-order in hydrogen ion concentration over the pH range that we studied. The first pK_a of GMPS is not known with accuracy, but it is probably around zero; the second pK_a is about 5.2 (6). The first pK_a for 2-PDS is 0.35 and the second pK_a is 2.37 (5). Since the rates of the coupling reactions are very slow at pH 7 and above (Figure 2), a significant reaction between the dianionic form of GMPS and the neutral form of 2-PDS (or similar 2-thiopyridyl derivatives) is excluded. The rates do increase remarkably with a decrease in pH and suggest that the most significant contribution to the reaction rate is probably due to the GMPS monoanion attacking either (or both) the monocation or the dication of 2-PDS. Moreover, the increased rates at acidic pH values could be attributed to the fact that protonation of both the ring nitrogens in 2-PDS not only enhances the electrophilic nature of both the sulfurs but creates a better leaving group in the protonated thiopyridone (2, 5).

It is instructive to compare the predominant pathways for the reaction of 2-PDS with either a thiol (e.g., 2-mercaptoethanol) or GMPS. The former reaction involves the nucleophilic attack of the thiolate anion on the unprotonated disulfide at pH values near and above neutrality. As expected, the pH-rate profile reveals that when the pH is decreased from pH 10 to pH 5, the rate decreases sharply probably due to a reduction in the thiolate anion concentration (2, 5). However, between pH 5 and 1, a plateau is observed in the pH-rate profile. In this pH range, protonation of the pyridine rings results in enhanced reactivity of 2-PDS and offsets the drop in rate due to decreased thiolate concentration. Although the increased electrophilicity of the protonated 2-PDS prevents the rate from falling further, it is unable to cause an overall increase in the rate; hence, the plateau. In contrast, we find that the reaction of 2-PDS with GMPS displays an increase as the pH is decreased from 8 to 4 (Figure 2). This is due principally to the increased electrophilicity of 2-PDS at acidic pH values.

A practical outcome of this study is the optimization of reaction conditions for modifications involving 5'-GMPS-primed RNAs and 2-thiopyridyl derivatives coupled to different probes. While others have performed GMPS-coupling reactions at near-neutral pH values and succeeded in $\sim 70\%$ modification (9), we believe that by

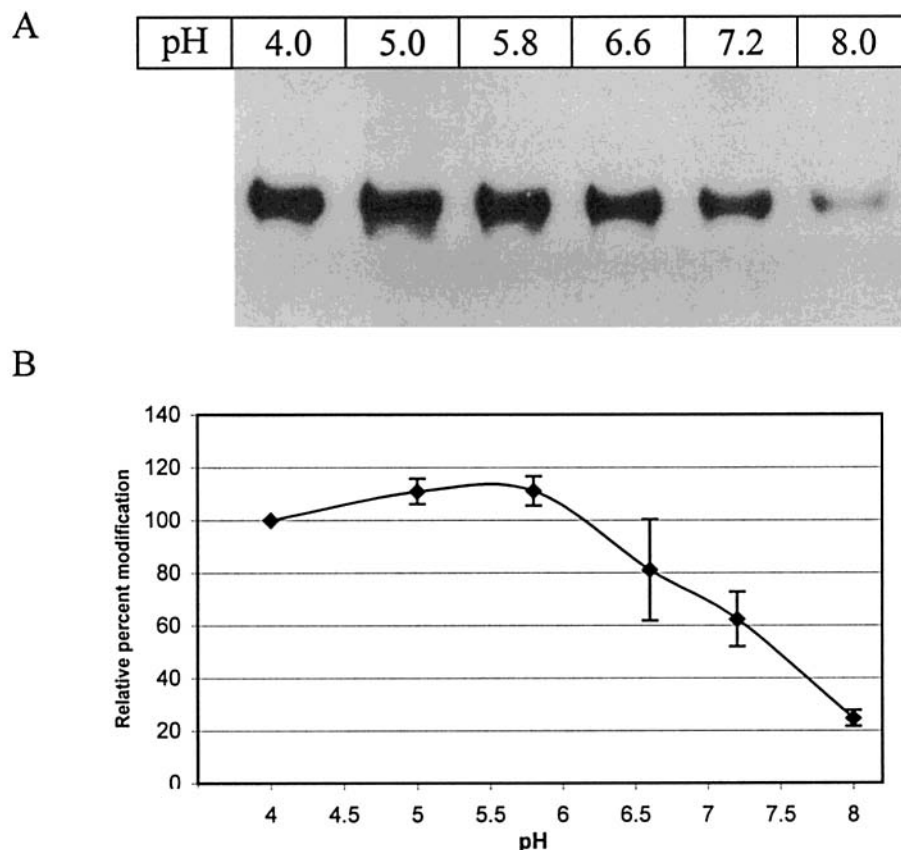


Figure 3. RNA modification and Northern blot analysis. Panel A, the pH dependence of biotinylation of GMPS-primed tRNA as detected by chemiluminescence (see below). Panel B, quantitation of the data shown in panel A. Multiple reactions (20 μ L each) were set up with 10 μ M GMPS-primed tRNA and 300 μ M biotin-HPDP in 0.1 M buffer of varying pH in the range 4 to 8. The RNA samples were then electrophoresed in a 7 M urea/ 10% polyacrylamide gel and electroblotted to a Zeta-probe membrane (Bio-Rad). After blocking the membrane for 1 h in 50 mL of nonfat dry milk [5% (w/v)]-supplemented TBST buffer (10 mM Tris, 150 mM NaCl, 0.1% Tween 20), the membrane was washed thrice with TBST and incubated for 1 h in 30 mL of TBST containing 30 μ L of 0.1 mg/mL peroxidase-conjugated streptavidin. After washing the membrane thrice in 50 mL of TBST, 3 mL of luminol/enhancer solution (SuperSignal West Dura Extended Duration Substrate) was added directly to the membrane for 1 min. The membrane was exposed to an X-ray film to obtain the photographic record displayed in Panel A. The chemiluminescence generated by the peroxidase was quantitated by using a FluorChem 8000 (Alpha Innotech) to obtain the data shown in Panel B.

lowering the pH of the reaction, the efficiency can be easily made to approach 100% and the reaction time can be shortened considerably (e.g., 5 min at pH 5 instead of 70 min at pH 8). Moreover, longer incubations at high pH are undesirable since both the 2-thiopyridyl substrates and the monothiophosphate disulfide product are unstable at alkaline pH values (9).

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ARTICLES

A Derivative of Diethylenetriaminepentaacetic Acid for Europium Labeling of Proteins

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Preparation of a reagent that will incorporate diethylenetriaminepentaacetic acid (DTPA) into proteins under mild conditions and make a strong europium chelate is described. Aminoacetaldehyde diethyl acetal was reacted with DTPA dianhydride, and mono- and disubstituted products as well as unsubstituted DTPA were separated by gel filtration. The monosubstituted product, after conversion into the corresponding aldehyde by mild acid hydrolysis, is conjugated to protein or other amino-containing compounds via reductive amination at neutral pH. Although the DTPA–Eu-labeled proteins are themselves not fluorescent, a strong fluorescence of europium can be generated easily by the dissociation–enhancement mechanism. A direct measurement of lectin–ligand interaction using Eu-labeled ligand and lectin immobilized on 96-well plate illustrates that the assay utilizing Eu fluorescence is as sensitive as the radioactive assays.

INTRODUCTION

Radioactive labeling has been an indispensable tool in the investigation of biological activities that requires high sensitivity and specificity. In recent years, however, the use of fluorescence of lanthanide chelates (e.g., europium and terbium) has been gaining favor as a viable alternative to the radioactivity, since lanthanide fluorescence has high sensitivity (detection level in the sub-pmol range) and its fluorescence is more specific than that of conventional fluorescent probes (1, 2). The higher specificity and sensitivity result from the fact that the fluorescence emission of these lanthanide ions is narrow-banded with a long Stokes shift and a very long fluorescence decay time (3). The latter two characteristics allow detection of lanthanide fluorescence in the presence of background fluorescence derived from biological samples and common plastic ware, especially if the time-resolved fluorescence measurement is made.

The obvious advantage of lanthanide is that there is no need for special handling, including the waste disposal, such as that required for radioactive materials. However, the major disadvantage of lanthanide labeling is the requirement for incorporating a chelating agent, a structure much larger than radioisotopes, which may affect the property of the compound labeled. For this reason it is prudent to aim for minimum level of

incorporation of a chelater (ideally <1 mol/mol on the average) into biological macromolecules, such as proteins.

Diethylenetriaminepentaacetic acid (DTPA) and related polyaminocarboxylic acids and derivatives thereof are proven chelaters of lanthanide ions. Simple polyaminocarboxylate–lanthanide chelates are not fluorescent. To potentiate the fluorescence of lanthanide for measurement, two strategies are generally used. One way is to incorporate a suitable aromatic group close to the chelater which will deliver the captured light to lanthanide so as to make it fluorescent. The second way is to use DELFIA (dissociation-enhanced lanthanide fluorescence immunoassay) technique, in which at the final stage of fluorescence measurement the lanthanide is displaced from the polyaminocarboxylate chelate and transferred into a micellar chelating environment, which will make it highly fluorescent. The former method is required when a lanthanide is used for certain experiments, e.g., fluorescence resonance energy transfer (FRET), and for which a number of synthetic compounds based on DTPA and related polyaminocarboxylates have been reported (4–7). The second method requires an extra enhancement step, but the incorporation of DTPA group into proteins is as simple as reacting them directly with commercially available DTPA dianhydride (8). The shortcomings of reacting directly with the dianhydride are (1) difficulty in controlling the extent of DTPA incorporation; (2) possibility of conjugation via both anhydride groups, which will cause undesirable cross-linking and incorporation of a lanthanide chelate that is unstable in the acidic pHs; and (3) possible change in protein stability due to neutralization of the amino group utilized in conjugation. A monofunctional reagent, *p*-isothiocyanatobenzyl DTPA, is available commercially (Wallac, Gaithersburg, MD). However, this reagent introduces an aromatic group as well as neutralizes the positive charge of the amino group

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¹ Abbreviations used: BCA, bicinchoninic acid; BSA, bovine serum albumin; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DTPA, diethylenetriaminepentaacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TPTZ, 2,4,6-tripyridyl-*s*-triazine; TFA, trifluoroacetic acid; RCA-I, *Ricinus communis* agglutinin I.

when it reacts with proteins. Both of these facts may introduce undesirable characteristics to the protein modified (9). For these reasons, we developed a nonaromatic reagent that has a single functional group through which proteins can be derivatized under mild conditions.

MATERIALS AND METHODS

Materials. DTPA dianhydride and aminoacetaldehyde diethyl (and dimethyl) acetal are from Aldrich (Milwaukee, WI). Europium nitrate and 2,4,6-tripyridyl-s-triazine (TPTZ) are from GFS chemicals (Columbus, OH). Bicinchoninic acid (BCA) protein assay reagent and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) are from Pierce (Rockford, IL) and from Research Organic (Cleveland, OH), respectively. Human immunoglobulin G (IgG) is from Sigma Chemical Co. (St. Louis, MO), and bovine serum albumin (BSA) is from Pentex Division of Bayer (Kankakee, IL). *Ricinus communis* agglutinin (RCA-I) is from E-Y Labs, Inc. (San Mateo, CA). The lanthanide fluorescence enhancing solution (10) is from Wallac (Gaithersburg, MD). The neoglycoproteins were prepared as described (11). Lac₄₀-BSA is a BSA derivative containing, on the average, 40 mol of lactosyl residues per mol of BSA, and likewise Man₃₅-BSA contains ca. 35 mol/mol of mannosyl groups on the average.

General Methods. ¹H NMR spectra were recorded using a Bruker AMX 300 spectrometer, and FAB-MS spectra were taken with 70-S gas chromatograph/mass spectrometer (VG Instruments) using nitrobenzoic acid as matrix. Acetal group was determined by neocuproine method (12) after mild acid hydrolysis. For hydrolysis, a sample solution (<5 μL) was mixed with 0.2 mL of 50 mM HCl and heated in a boiling water bath for 20 min. After cooling, the hydrolysate was neutralized with 0.2 mL of 50 mM NaOH, and the neocuproine assay was carried out. The protein content was determined using micro-BCA assay as described (13).

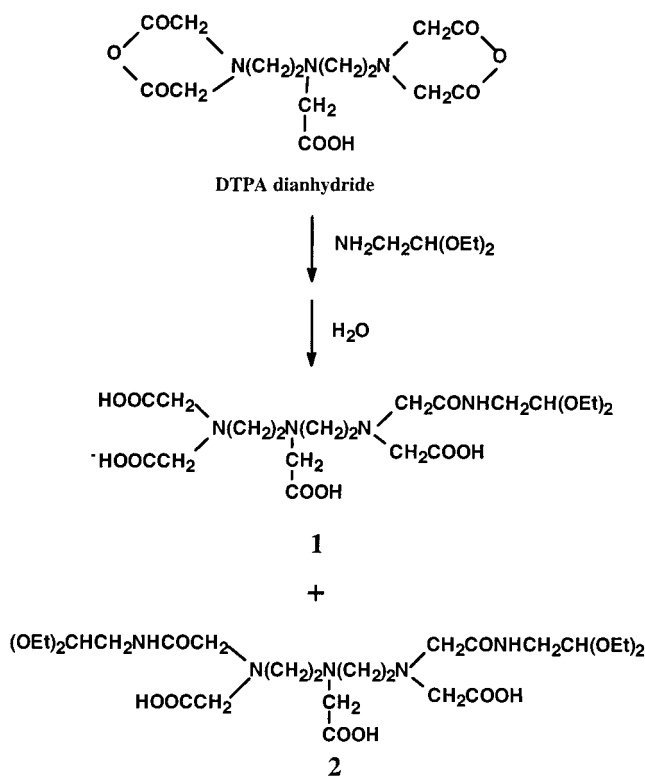
Determination of DTPA Derivatives. The TPTZ method was originally designed for determination of an oxidant such as periodate ion (14). We adapted the method for determination of DTPA derivatives, utilizing their ability to compete ferrous ion off from the TPTZ-Fe²⁺ complex (deep blue color) (15). The decrease in the blue color (*A*_{593 nm}) is proportional to the amount of the DTPA derivative present. For monitoring of column effluent, an appropriate volume of column fraction was added to 3 mL of 2-fold-diluted TPTZ stock solution (which will give *A*_{593 nm} of 0.95–1). The tubes were incubated at 37 °C for 0.5 h and *A*_{593 nm} was determined.

Determination of Europium Content. Fluorescence determination of europium chelate was carried out in a 96-well microtiter plate. To each well containing sample (0.2–1 pmol of europium in <20 μL) was added 0.2 mL of enhancing solution, and the plate was tapped for a few seconds to mix the content of the well. After 20 min at room temperature, the fluorescence was read in a fluorometer (1420 Victor multilabel counter, Wallac) using the time-resolved fluorescence mode set for europium.

RESULTS

Preparation of Mono[2-(diethoxy)ethylamido]-DTPA (1) (Scheme 1). DTPA dianhydride (0.89 g, 2.5 mmol) was suspended in 18 mL of dry DMSO. To this suspension was added, while being stirred, a solution of aminoacetaldehyde diethyl acetal (363 mL, 2.5 mmol) in 2 mL of DMF a few drops at a time. The cloudiness completely dissipated by the end of addition. The mixture

Scheme 1



was kept for several hours at room temperature and then was evaporated under vacuum to reduce the volume by ~1/3. To this was added 5- to 6-fold volume of toluene, and the resulting cloudy solution was left standing at room-temperature overnight. A clear solution was then decanted off, and the precipitate was washed with toluene and ether and dried. The residue was dissolved in 5 mL of water and left standing for a few hours at room temperature and then in the cold overnight. The crystalline DTPA formed was filtered and washed with cold water. The filtrate was evaporated to ~5 mL and fractionated on a column (2.5 × 140 cm) of Sephadex G-15 equilibrated in 0.1 M acetic acid, collecting 6-mL fractions. The effluent was assayed by TLC on silica gel using 2-propanol–acetic acid–water (2:1:1) as eluant and by the TPTZ method. Both the desired product (**1**) and the disubstituted product (**2**) can be detected on the TLC plates by charring after spraying with 15% sulfuric acid in 50% ethanol. *R_f* of the di- and monosubstituted products are 0.26 and 0.19, respectively. The column fractions were also examined by TPTZ method, especially to detect the presence of DTPA. On a molar basis, the capacity of **1** to compete for TPTZ is similar to DTPA, while that of **2** was about one-half of DTPA. This means that the presence of five or four carboxylate groups makes little difference in the Fe²⁺-complexing capacity, while the presence of only three carboxylate group (disubstituted) greatly diminishes the capacity to complex. As expected, the compounds were eluted in the order of di-, mono-, and unsubstituted DTPA with some overlap between compounds. The column fractions having more than one component were refractionated on the same G-15 column. After two rounds of fractionation, the yield of the desired product, **1**, was ~30% and that of **2**, ~10%.

This preparation scheme was also carried out using dimethyl acetal of aminoacetaldehyde instead of diethyl acetal with similar yield, but the separation on TLC plates is clearer when the diethyl acetal is used.

Characterization of Products. The content of acetal in the two products was determined using neocuproine method after a mild acid hydrolysis. (β -D-Galactopyranosylthioglycolyl)aminoacetaldehyde dimethyl acetal (Gal-AAA) (16), a synthetic product that had been characterized sufficiently, was used as standard. Calculated on the weight basis, both products **1** and **2** had the acetal content that is 84% of the expected amount based on 1 and 2 mol of acetal per mol of the compound, respectively. We suspected that this low color yield (A_{460}) might be due to the presence of polycarboxylate interfering in the color development. Indeed when Gal-AAA was assayed in the presence of DTPA (in the amount comparable to that present in **1** and **2**), its molar absorbance was suppressed by 13%. With this correction factor, the acetal content of **1** and **2** would be 97% of the expected.

NMR spectra in D_2O were taken of the dimethoxyethyl derivatives. As expected from the molecular symmetry, the spectrum of the disubstituted product was much simpler than that of the monosubstituted product. Both products registered correct ratios of methine signal (triplet at 4.6 ppm) to methylene signals, which was about 1:18 for the mono- and about 1:10 for the disubstituted product. ^1H NMR of monosubstituted product: δ 3.269–3.303 (m, 2 H, CH_2); 3.325–3.385 (m, 2 H, CH_2); 3.456 (s, 6 H, CH_3); 3.534–3.578 (m, 2 H, CH_2); 3.727 (s, 2 H, CH_2); 3.873 (s, 2 H, CH_2); 3.978 (s, 4 H, CH_2); 4.016 (s, 2 H, CH_2); 4.604 (t, 1 H, OCHO). There are probably 4 unresolved Hs in 3.423–3.468. Disubstituted product: δ 3.352–3.368 (d, 2 H, CH_2); 3.393–3.410 (d, 2 H, CH_2); 3.457 (s, 12 H, CH_3); 3.47 (s, 4 H, CH_2); 3.771 (s, 2 H, CH_2); 3.78 (s, 4 H, CH_2); 3.91 (s, 4 H, CH_2); 4.586–4.620 (t, 2 H, OCHO). There are probably four unresolved Hs in 3.45–3.465. FAB-MS spectra of the same two products taken with nitrobenzoic acid as matrix gave peaks for the mass of 481.2 (M + H) and 503.2 (M + H) for mono- and disubstituted product, respectively.

Eu-DTPA Labeling of Proteins and Poly-L-lysine Using Compound 1. An appropriate amount of **1** (1–10-fold molar excess over the protein to be labeled) was dissolved in 0.5 mL of water, and an equal volume of TFA was added. The tube was heated at 55 °C for 2 h. The hydrolysate was evaporated after adding ~5 volumes of absolute ethanol, and this process was repeated three times. The residue was kept in a vacuum desiccator over fresh NaOH pellets overnight. The exposed aldehyde group was used to link to the protein amino group via reductive amination. The reaction was carried out for 24 to 48 h at room temperature using 5 mg of protein in 1 mL of 0.1 M HEPES buffer, pH 7.4, with up to 10-fold molar excess of hydrolyzed **1** (see below for detail) and with enough pyridine borane added to make it ~25 mM in concentration. After the reaction, the mixture was passed through a small Sephadex G-50 column (1 $\times$ 47 cm) equilibrated in 5 mM HEPES buffer at pH 7.4 to separate the excess reagent from the protein. The protein peak was located by micro-BCA method. Fractions containing protein were combined and lyophilized to a small volume (<1 mL). A 10-fold molar excess of $Eu(NO_3)_3$ was added, and the protein solution was refractionated on a smaller Sephadex G-50 column (1 $\times$ 19 cm) in 10 mM HEPES buffer, pH 7.4. The protein peak was located by analyzing the fractions for Eu fluorescence. The peak fractions were combined and lyophilized to dryness and then dissolved in a desired volume of water.

When IgG and BSA were modified using 5–10-fold molar excess of reagent over protein, the incorporation of Eu-DTPA was around 1–3 mol/mol. In the case of

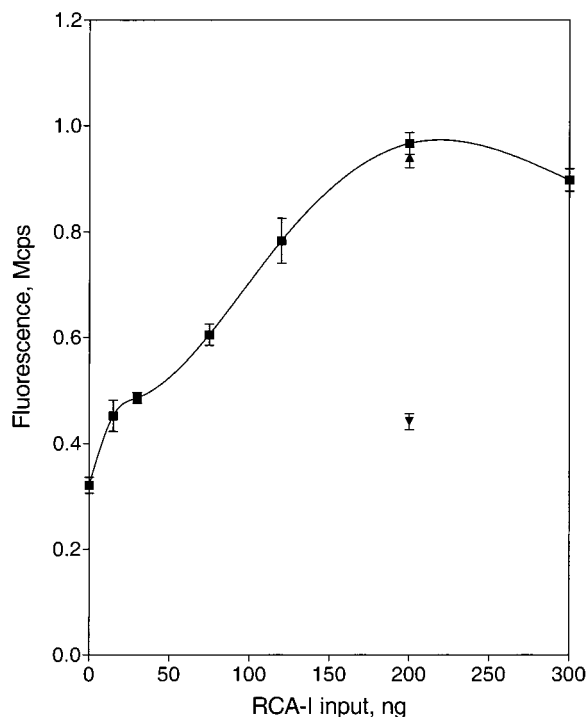


Figure 1. Binding of Eu-Lac₄₀-BSA to RCA-I immobilized on microtiter wells. The bound ligand in terms of Eu fluorescence (y axis) was plotted against the amount of RCA-I used per well. ■, bound amount in the absence of inhibitor; ▼, bound amount in the presence of 6.2 μ M Lac₄₀-BSA; ▲, bound amount in the presence of 5.1 μ M Man₃₅-BSA.

Lac₄₀-BSA, which has most of its readily accessible amino groups already being used up in the lactoside incorporation, the incorporation of Eu-DTPA was only 0.4 mol/mol with the reagent-to-protein ratio of 10. In contrast, when the amino group is in a large excess as in poly-L-lysine of DP 240, the incorporation of Eu-DTPA was quantitative, meaning that the DTPA reagent was totally incorporated into polylysine.

Binding of Eu-Lac₄₀-BSA to a Galactose-Recognizing Lectin (RCA-I) Immobilized on Microtiter Plates. RCA-I solutions (100 μ L) in 25 mM HEPES buffer, pH 8.2, containing 0.15 M NaCl were placed in wells of Immulon 2 removawell strips (Dynatech Labs, Inc., Chantilly, VA) and left in the cold for 24 h. The liquid is removed by suction and replaced with 5% BSA in the same buffer. After 2 h at room temperature, the BSA solution was removed, and the wells were washed once with incubation buffer (25 mM HEPES buffer, pH 7.2, containing 0.15 M NaCl and 1% BSA). Incubation mixtures (100 μ L) containing Eu-Lac₄₀-BSA at 400 nM with or without potential inhibitor (Lac₄₀-BSA or Man₃₅-BSA) at ~5 μ M were added to the wells (in triplicate) and incubated for 24 h in the cold with rocking motion. After removal of the mixture, wells were washed once with incubation buffer that contained 0.05% Tween 20 instead of 1% BSA. To each well was added 0.2 mL of the enhancing solution, and fluorescence was determined as described in Methods. Fluorescence in million counts per second (Mcps) was plotted against amount of RCA-I added to the well, as shown Figure 1. The figure shows that there was an RCA-I dependent binding of Eu-Lac₄₀-BSA, which leveled off at about 200 ng of RCA-I per well. The binding was inhibited by unlabeled Lac₄₀-BSA, but not by Man₃₅-BSA.

DISCUSSION

Tracer labeling technique is a powerful method for measurement of biological activities. In the labeling process, it is important to strive for as low an incorporation of tracer as practical, in order not to cause change in the characteristics of the biomolecule to be labeled. This is particularly important when the incorporated label is a lanthanide (i.e., lanthanide ion + chelator) instead of a radioactive element, since the incorporated group is very much larger.

DTPA is a frequently used chelator for europium that contains five carboxylic acid groups clustered in close proximity, through which efficient chelation of europium ion is achieved. One of its carboxylic acids can be used for modification of proteins and other amino-containing compounds without affecting the chelating efficiency much. However, the loss of the second carboxylate group will compromise the stability of Eu-DTPA complex considerably (6). DTPA dianhydride is a convenient, commercially available reagent for labeling of amino-containing compounds, such as proteins. However, it has two major drawbacks. The more serious drawback is its potential for reacting with two amino groups of the macromolecule to be labeled, so as to cause intra- and intermolecular cross-linking, and to produce in the process an inferior chelator of europium having only three carboxylic acid groups. Although one may rarely encounter this complication for labeling of proteins, it posed a grave problem for us when we attempted to introduce DTPA into polylysines, because the amino group is present in a large excess of DTPA dianhydride needed for labeling. In one of the labeling experiments, polylysine actually became insoluble, presumably due to intermolecular cross-linking. In another case, although the labeled polylysine stayed soluble, the incorporated Eu was easily lost during protein-ligand binding assays if the pH was below 6. At pH 4, less than 10% of Eu remained associated with polylysine at the end of the assay, whereas europium on a similarly labeled neoglycoprotein remained totally associated. The second drawback is difficulty in controlling the amount of DTPA incorporated. Typically DTPA dianhydride is added to a protein solution in mildly alkaline medium (e.g., 10% NaHCO₃) in 10 to 50-fold molar excess (8). The large excess is necessary because anhydride is hydrolyzed rapidly in a mildly alkaline medium. To attain a low but sufficient level of DTPA incorporation under such conditions is not easy.

To overcome these problems, we prepared a monovalent labeling reagent, **1**, that will provide a single potentially reactive group and will ensure the presence of four free carboxylate groups in the labeled protein. Compound **1** was prepared by *N*-acylating an acetal-containing compound with DTPA dianhydride. This reaction as well as the formation of benzylhydrazide by reacting benzyl hydrazine with the dianhydride proceeded well, producing both mono- and disubstituted products. However, the conversion of the acetal in **1** to aldehyde did not proceed as easily as of expected, presumably due to the presence of carboxylate groups in proximity. For deacetalization of **1**, a more forcing condition, i.e., 50% TFA at 55 °C for 2 h, than that employed for a similar acetal (50% TFA at room temperature, 5–10 min) (11) had to be used. Similarly, the hydrazide group of DTPA monohydrazide (obtained from the mono-benzylhydrazide by hydrogenation) was quite unreactive when used in a reductive amination reaction (not shown). In contrast, the reductive amination proceeded well when the hydrolyzed **1** was used to

label proteins. Presumably the added distance away from the cluster of carboxylate allowed the reductive amination to proceed. The efficiency of incorporation of DTPA by this reagent depended on availability of the amino group, from being quantitative for a polylysine which possesses a large excess of amino group to somewhat sluggish for Lac₄₀-BSA which has only a few reactive amino groups remaining, since most of the reactive amino groups had been consumed in the lactose incorporation. Still, a suitable level of incorporation required no more than 10-fold excess of reagent even for unreactive proteins.

We have used Eu-labeled proteins and small ligands in the investigation of a number of lectin-ligand interactions. To illustrate the sensitivity of assays based on Eu fluorescence measured in the time-resolved mode, we present an example of a lectin (RCA-I)-ligand (Eu-Lac₄₀-BSA) interaction carried out in microtiter wells. As shown in Figure 1, the binding can be measured directly by Eu fluorescence without relying on any enhancement mechanism such as enzyme-linked color production. Although the Eu-labeled ligand used here contained on the average 0.4 mol of Eu per mol of protein, the fluorescence signal generated was more than sufficient for the measurement, indicating that the Eu fluorescence method rivals radioactive method in sensitivity. In fact, in our experience a protein with low incorporation of DTPA-Eu (<1 mol/mol) generally gave lower nonspecific binding when used as ligand in the lectin-ligand binding assays and longer shelf life when stored as solution.

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In Vitro Selection of RNA Aptamers Carrying Multiple Biotin Groups in the Side Chains

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In vitro selection or the systematic evolution of ligands by an exponential enrichment (SELEX) technique using a biotin-carrying nucleotide monomer was used for the development of a new type of molecular recognition sensor. Cytidine triphosphate (CTP) carrying the biotinyl group at the *N*⁴-position was applied for the technique. A pool of random sequence RNAs containing biotinyl groups in the side chains was prepared, and the RNAs binding to adenosine 5'-triphosphate (ATP) were selected. The selected nonnatural RNAs were used for an assay of ATP. Since they carried multiple biotin groups in the side chains, the sensitivity was high.

INTRODUCTION

Two methods, namely antibody production (1) and molecular imprinting (2), have been used to synthesize molecules sensing a target molecule. On the other hand, in vitro selection, also known as the systematic evolution of ligands by exponential enrichment (SELEX), was devised in 1990 for the identification of oligonucleotides, which are highly specific to target molecules. (3, 4) The selected oligonucleotides were named as aptamers and have been used as a new type of molecular sensor (5). These aptamers offer several potential advantages over traditional antibody-based reagents because they are not derived from living organisms and can be reproducibly, precisely, and quickly synthesized by automated processes. Recently, chemically modified nucleic acids were used in SELEX to expand the scope of the potential molecules (6–24). In the present study, cytidine 5'-triphosphate carrying a biotinyl group at the *N*⁴-position {*N*⁴-[*N*-(*N*-biotinyl- ϵ -aminocaproyl)-6-aminoethyl]cytidine 5'-triphosphate, Biotin-CTP} was subjected to the SELEX protocol, and a biotin-carrying RNA aptamer was synthesized and used as a new molecular recognition element. The molecular recognition element was expected to have a high sensitivity because it carried many biotin groups as probes.

EXPERIMENTAL PROCEDURES

Chemicals. Biotin-CTP was purchased from the Life Technologies Co. (Rockville, MD). Custom synthesis of DNA oligomers was performed by the Sawady Technology Co. (Tokyo, Japan). AmpliTaq DNA polymerase was obtained from the Perkin-Elmer Co. (Foster City, CA). T7 RNA polymerase and RNasin were purchased from Promega Co. (Madison, WI). Dithiothreitol (DTT) was purchased from the New England Biochemicals Co. (Beverly, MA). [α -³²P]GTP was purchased from the Am-

ersham Pharmacia Biotech. Co. (Buckinghamshire, UK). Reverse transcriptase Superscript II and a Photogene streptavidin/alkaline phosphatase conjugate were purchased from the Life Technologies Co. (Rockville, MD). ATP-immobilized gel and nucleoside 5'-triphosphates (NTPs) were purchased from the Sigma Co. (St. Louis, MO). Biotinylated nylon membrane was purchased from the Pall BioSupport Co. (East Hills, NY).

Transcription Reaction. A synthetic DNA pool containing a random region of 59 bases (5'-TAG-GGA-ATT-CGT-CGA-CGG-ATC-C-N₅₉-CTG-CAG-GTC-GAC-GCA-TGC-GCC-G-3') (80 ng) was amplified by 10 PCR cycles (94 °C, 15 s; 55 °C, 15 s; 72 °C, 15 s) in 800 μ L of reaction solution containing AmpliTaq DNA polymerase [Perkin-Elmer Co. (Foster City, CA)], 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 μ M dNTPs, 0.5 μ M 5'-primer (P1) containing the T7 promoter sequence (5'-TAA-TAC-GAC-TCA-CTA-TAG-GGA-ATT-CGT-CGA-CGG-AT-3'), and 0.5 μ M 3'-primer (5'-CGG-CGC-ATG-CGT-CGA-CCT-G-3'). The amplified template was precipitated with ethanol, and the resulting pellet was dissolved in 30 μ L of RNase-free water. The DNA was transcribed with T7 RNA polymerase (4 μ L, 1200 U) in a reaction mixture (100 μ L) consisting of 80 mM Tris-HCl (pH 7.9), 12 mM MgCl₂, 4 mM spermidine, 20 mM DTT, 20 μ M [α -³²P] GTP, 0.4 U/ μ L RNasin, 1 mM of Biotin-CTP, and 1 mM other unmodified NTPs. This gave an approximate yield of 1 μ g of random RNA. After incubation at 37 °C for 4 h, RQ1 RNase-free DNase (Promega, 2U) was added to the reaction solution to digest the DNA template. After incubation at 37 °C for 45 min, a small aliquot (10 μ L) of the solution was analyzed by 7 M urea/8% denaturing polyacrylamide gel electrophoresis (PAGE). Dot blotting was carried out as described below. The transcripts were transferred to a Biotinylated nylon membrane using the electroblotting method. The transcripts were then detected with a Photogene streptavidin-alkaline phosphatase conjugate according to the protocol provided by the manufacturer.

Reverse Transcription. The transcription product (5 μ L) was mixed with 5 μ L RNase-free water and 2 μ L

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of 10 μ M 3'-primer P2. The mixture was incubated at 70 °C for 10 min and then chilled on ice. To this mixture were added 4 μ L of a concentrated reverse transcription buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM MgCl₂), 2 μ L of 0.1 M DTT, and 1 μ L of 20 mM dNTPs. Reverse transcriptase Superscript II (Life Technologies Co.) (200 U) was added to the mixture solution. After incubation at 42 °C for 50 min, the reaction was stopped by incubation at 70 °C for 15 min. Subsequently, the cDNA was amplified by PCR. Five microliters of the reverse-transcription reaction solution was added to a PCR solution (100 μ L). After 10 PCR cycles on the same thermal condition as described, small aliquots (10 μ L) were analyzed by 3% NuSieve GTG agarose gel electrophoresis.

In Vitro Selection. The randomly sequenced RNA pool was prepared as described in the transcription experiment section and purified by denaturing PAGE on an 8% gel (100 mm $\times$ 100 mm in size, 2 mm thick). After excision of the band and elution into 0.6 mL of an elution buffer (0.5 M ammonium acetate; 10 mM magnesium acetate; 1 mM EDTA, pH 8.0; 0.1% SDS), the recovered RNA was precipitated with ethanol. The ³²P-labeled RNA pool (1 μ g) was dissolved in a binding buffer (0.3 M NaCl; 5 mM MgCl₂; 20 mM Tris-HCl, pH 7.6; 5% DMSO; 0.5% Triton X-100), and the solution was incubated at 85 °C for 5 min. The solution was applied onto a column (0.5 mL) packed with an ATP-immobilized gel, and the solution and the gel were equilibrated for 30 min at 15 °C. Unbound RNAs were washed away with 40 column volumes of the binding buffer. The bound RNAs were eluted with six column volumes of a binding buffer containing 3 mM ATP and precipitated with ethanol in the presence of glycogen (200 μ g). The resulting RNA pellet was dissolved in 30 μ L of RNase-free water.

The collected RNAs were amplified by RT-PCR, as described in the RT-PCR, and transcribed with [α -³²P]-GTP and Biotin-CTP, as described in the transcription experiments. The labeled RNA pool was again applied onto a column packed with ATP-immobilized gel, and bound RNAs were eluted. The amount of ³²P-labeled RNAs was measured by Cerenkov counting. These processes were repeated.

Binding Assay. The RNAs (1 μ g) were added to the ATP-immobilized gel (0.1 mL). After washing with the binding buffer, 0.1 mL of GTP (3 mM), CTP (3 mM), UTP (3 mM), or ATP (0, 1, 2, 3 mM) was added to the gel to investigate the ligand specificity and the sensitivity for ATP. After incubation with the gel for 20 min, each of the eluted RNAs (5 μ L) was spotted onto a Biotinylated nylon membrane (1 cm $\times$ 1 cm). After each membrane dried for 15 min, it was irradiated with UV (365 nm) for 30 s and was incubated in 800 μ L of blocking solution [3 wt % bovine serum albumin in TBS-Tween 20 buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05 v/v Tween 20)] at 65 °C for 1 h. Subsequently the membrane was incubated in TBS-Tween 20 buffer (800 μ L) containing streptavidin-alkali phosphatase (0.083 μ g/mL, GIBCO) for 10 min at room temperature. The membrane was washed with the TBS-Tween 20 buffer twice (800 μ L $\times$ 2), was placed in a microplate well, and was incubated with a final buffer (800 μ L, 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂) containing 10 mM *p*-nitrophenyl phosphate for 1.5 h at 37 °C. The absorbance (405 nm) of supernatant of each well was measured, and the amount of eluted RNAs was determined.

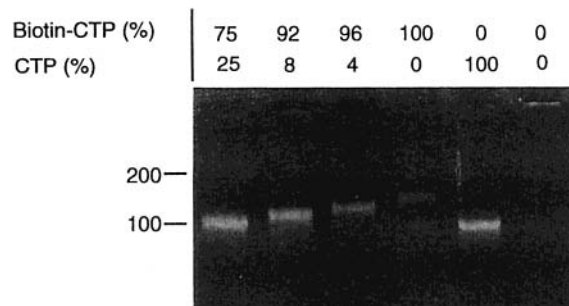


Figure 1. 8% Denaturing PAGE analysis of the transcription products (10 μ L) at various concentrations of modified Biotin-CTP and CTP. The gel was stained by ethidium bromide. The faint band at around a 100 base length corresponds to the DNA template.

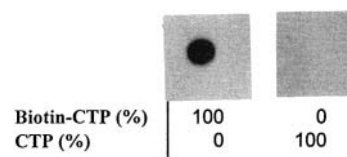


Figure 2. Dot blotting of the transcription products in the presence of Biotin-CTP or CTP. The biotin residue is detected with a Photogene kit.

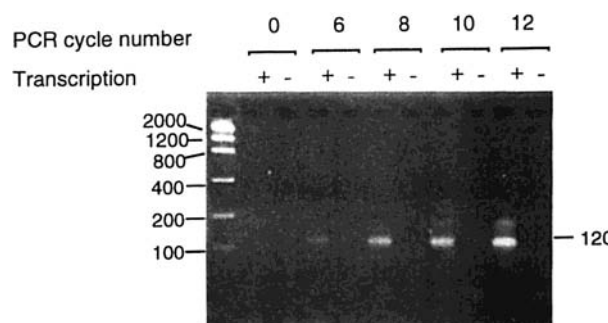


Figure 3. Agarose gel electrophoresis analysis of RT-PCR products from the transcripts containing Biotin-CTP.

RESULTS AND DISCUSSION

For each round, the SELEX process requires the transcription of RNA, the isolation and reverse transcription of the target-bound RNA, and PCR amplification of the cDNA. To introduce modifications to RNA, a modified nucleotide triphosphate must be a substrate for T7 RNA polymerase. The modified RNA, on the other hand, must be a substrate for reverse transcriptase for cDNA synthesis. The use of Biotin-CTP in place of CTP in the transcription reaction gave biotin-incorporated transcripts (Figure 1). The biotin-incorporated transcripts migrated more slowly in gel electrophoresis than normal transcripts. The yield of biotin-modified transcripts was lower than that of normal transcripts. The presence of the biotinyl group in the biotin-incorporated transcripts was confirmed by dot blotting (Figure 2).

Another requirement for the SELEX protocol is that the modified RNA must serve as a template for reverse transcriptase to produce cDNA. When the biotin-incorporated transcripts were reverse-transcribed to cDNA, they gave the full-length of cDNA (Figure 3). The band density increased with the increase of the PCR cycle. Comparing the efficiency of the reverse transcription reaction in the presence of Biotin-CTP with that of CTP, both were almost the same. The sequential analysis of the product cDNA demonstrated that the cDNA was the same as that of the original synthetic DNA.

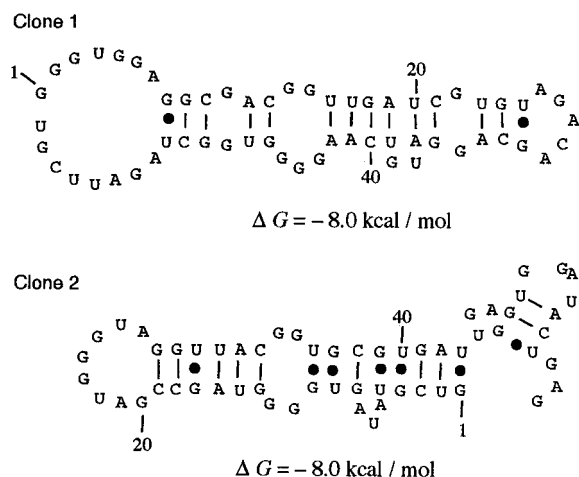


Figure 4. Secondary structure of cloned RNAs. The symbols — and • represent Watson-Crick base-pairing and Wobble base-pairing, respectively. Biotin-CTP was used instead of CTP.

These results demonstrated that Biotin-CTP is applicable to the SELEX experiment, although the bulky biotin group existed.

In Vitro Selection. The selection of aptamers for ATP was started from a biotin-modified random sequence pool of RNA molecules, which were prepared by transcription reaction using synthetic DNA with random sequences as templates. The diversity was theoretically considered to be 10^{12} – 10^{13} according to the estimation by Ellington and Szostak (3). The transcripts were added to the ATP-immobilized agarose gel. After being washed with the binding buffer, the bound RNA molecules were eluted using soluble ATP. The collected RNA molecules were reverse-transcribed and amplified by PCR. The amplified cDNAs were again transcribed to RNA molecules using Biotin-CTP again, and the transcripts were added to ATP-immobilized gel. These processes were repeated. At the final round, counter-SELEX was carried out using soluble GTP. When measuring the binding ratio of RNA to ATP-immobilized gel by dividing the amount of RNA molecules eluted by soluble ATP with that of all RNA molecules added, it was found that the ratio increased with the increase of the selection round (1st, 0%; 2nd, 3%; 3rd, 7%; 4th, 7%; 5th, 8%; 6th, 12%; and 7th, 31%). After eight rounds of selection process, 42% of RNA molecules were found to bind to the ATP-immobilized gel.

At this point, PCR-amplified DNA was cloned, and 32 clones were sequenced, resulting in only two different sequences (Figure 4). The sequential variety of the selected biotin-modified RNA, which was lower than that of normal RNA, might be explained in terms of the lower yield of transcription products of biotin-modified RNA as compared with that of normal RNA. Both of the biotin-modified RNAs had approximately 10% of the Biotin-CTP component. This result indicated that the biotin residue suppressed the incorporation of Biotin-CTP in the transcripts.

The requirement of the biotin residues for affinity binding was tested by synthesizing normal transcripts with unmodified CTP. The transcripts did not bind to the ATP-immobilized gel (Figure 5). This result suggested that the presence of the biotin groups was critical for the high affinity of the biotin-modified RNA. The biotin groups should contribute to adopt conformations suitable for recognizing ATP or to interact directly with ATP.

After selected RNAs were bound on the ATP-immobilized gel, known concentrations of nucleotide triphos-

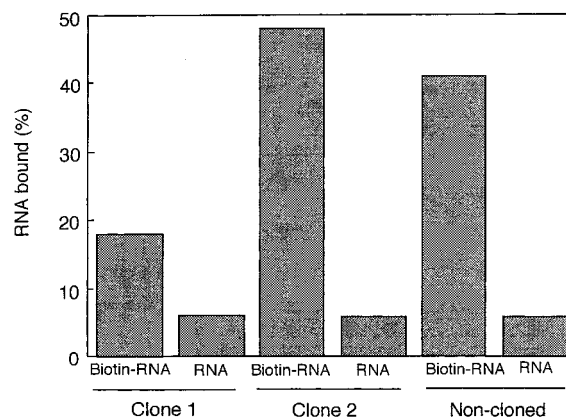


Figure 5. Binding affinity of RNAs transcribed from Biotin-CTP or CTP.

Table 1. Elution Percentage of RNAs Bound to ATP-Immobilized Gel by Nucleotide 5'-Triphosphates^a

	ATP	CTP	UTP	GTP
noncloned	100	10	7	20
clone 1	100	7	5	7
clone 2	100	2	4	3

^a The percentage of RNAs eluted by ATP was taken as 100%.

phate were added to elute the RNAs, and the eluted RNAs were measured by conventional biotin-avidin interaction (Table 1). The selected RNAs were eluted only by ATP. It was indicated that the triphosphate region is not important for the recognition of RNAs. When adenosine monophosphate (AMP), adenosine diphosphate (ADP), or 3'-deoxyadenosine triphosphate (dATP) was added to the clone 2 bound to ATP-immobilized gel, the clone was eluted (ADP, 85%; AMP, 90%; and dATP, 80%). These results indicated that the RNAs recognized the ATP at the base moiety.

When streptavidin was added to the clone 2 bound to the ATP-immobilized gel, no significant amount of clone was eluted from the gel. However, the biotin of the clone could be detected with a conventional biotin-streptavidin detection kit. It was considered that the bound clone had a folded structure to inhibit streptavidin from invading the biotin residue in the binding state but that the biotin of the eluted clone binds the streptavidin after the denaturation (unfolding) for detection (Figure 6).

The dissociation constants of both clones 1 and 2 were 10 and 15 μM , respectively. The dissociation constants of normal RNA or DNA selected for ATP ranged from 14 to 6 μM according to the reports of Sassanfar and Szostak (25) and Huizenga and Szostak (26), respectively. The dissociation constants of biotin-carrying RNA aptamers in the present study were almost the same order of those of normal oligonucleotide aptamers. Dieckmann et al. (27) reported that several highly conserved nucleotides in the binding pockets could be substituted while retaining binding under NMR conditions. Figure 7 shows the result of sensing the concentration of ATP by clone 2. The clone 2 RNA bound to the ATP-immobilized gel was released from the gel with the increase of ATP. This result demonstrates that the selected RNAs quantitatively recognize ATP and can therefore be used as a molecular sensor for ATP. All of the six biotin residues on the aptamer were not recognized by the streptavidin. The sensitivity of the assay using the aptamer carrying multiple biotin residues had about three times as much as that labeled with single biotin. Considering the development of a new biotin detection system (28), the

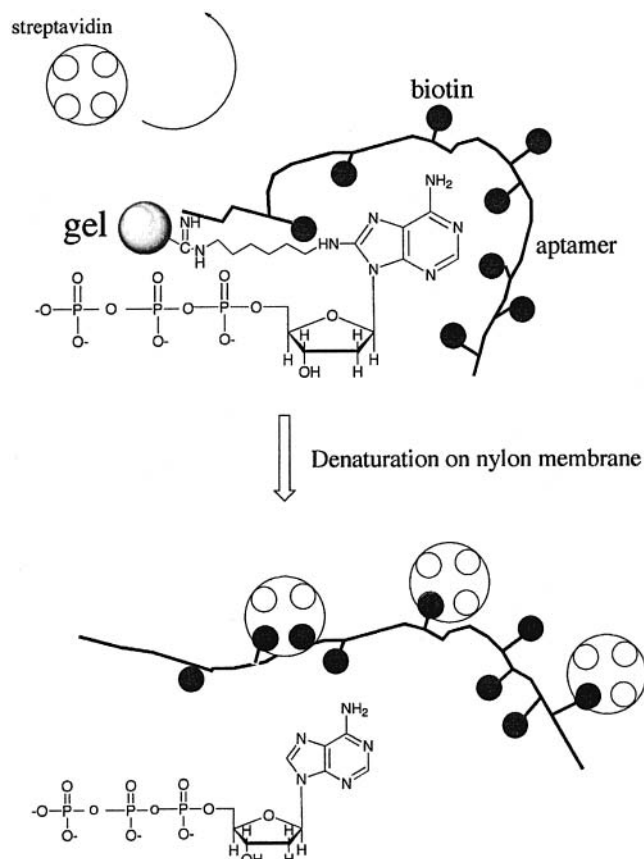


Figure 6. Schematic drawing of interaction of biotin-carrying RNA aptamer with target molecule, ATP. Streptavidin did not bind the biotin of aptamer bound to the ATP-immobilized gel but bound to the eluted aptamer.

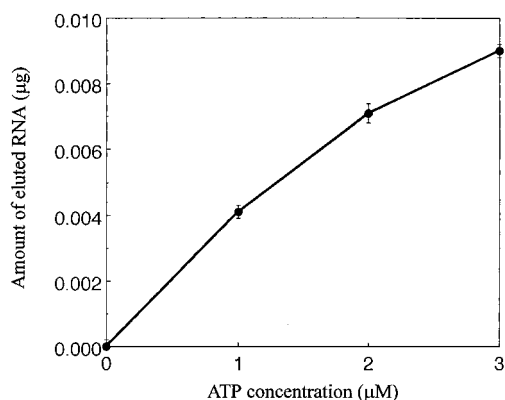


Figure 7. Binding assay of ATP concentration by using aptamers carrying multiple biotin groups in the side chains. $n = 4$. Bars represent standard deviations.

aptamers carrying multiple biotin groups will offer a new detection system with higher sensitivity.

Recently, aptamers have been used for various analytical purposes (7). The present study develops a new methodology to synthesize a chemosensor instead of using antibodies. For biotin-carrying RNAs, it is a great advantage not to have to worry about denaturation by the labeling or modification process in comparison with antibodies. In addition, these RNAs carry a number of probes in a chain to increase sensitivity in comparison with other aptamers consisting of natural nucleotides. Taking into consideration the convenience of RNA preparation in vitro, this type of RNA sensor will replace the traditional antibody sensors, which are produced from animals.

ACKNOWLEDGMENT

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Kinetic Evaluation of a Metalated Diglycine Conjugate as a Functional Mimetic of Phosphate Ester Hydrolase

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The crucial role of phosphate ester hydrolysis in various biological processes has spurred vigorous research activities to understand mechanisms of phosphate ester hydrolysis and to develop model systems that assist the above-mentioned reaction in a catalytic fashion. In the present study, we describe a novel, metalated peptide conjugate **4** possessing phosphate ester hydrolyzing activity against a phosphate monoester, diester, and a RNA chemical model. The design of conjugate **4** is inspired by the ATCUN binding tripeptide motif of serum albumin and involves tethering of two diglycine units by a flexible 1,3-diaminopropane linker. Detailed kinetic investigations of phosphate ester hydrolysis using model substrates and efforts to decipher underlying mechanisms are presented.

INTRODUCTION

The amino terminal Cu (II) and Ni (II) (ATCUN) binding motif has been known for the last 40 years (1), and its presence in serum albumin has been implicated for the transport of small molecules and metal ions (2). This motif occurs naturally in human, bovine and rat serum albumin, neuromedins C and K, human sperm protamine P2a, and histatins (2). Gly–Gly–His (GGH) tripeptide sequence represents Cu (II) coordinating motif in albumin, and structural features of this motif include a free amino terminus, a histidine residue at the C-terminus, and two intervening peptide nitrogens. The metalated tripeptide motif (Cu (II)-GGH-*N*-methyl amide) has been characterized through X-ray crystallography (3), and the metal ion was tetracoordinated with the participation of NH₂-terminal nitrogen, two peptide nitrogens, and a histidyl nitrogen, in a slightly distorted square planar arrangement. The arrangement of coordinating atoms is depicted in Figure 1.

Pauling and co-workers were the first ones to report the antitumor activity of copper (II)-GGH tripeptide/ascorbate system in Ehrlich ascites tumor cells (4). This activity was presumably manifested through the generation of reactive hydroxyl radicals (5), which could lead to DNA strand cleavage as demonstrated for other Fenton-type of reagents used for DNA footprinting (6). GGH and related tripeptide motifs capable of coordinating to Cu (II) have been engineered into larger peptide segments for the design of artificial nucleases. Examples include attaching of GGH motif to the NH₂-terminus of DNA binding segment of Hin recombinase (7), to a zinc finger protein Sp1 (8), and to the basic domain of Fos (9), to study their DNA cleavage activity. Considerable efforts have been invested in the design of GGH tripeptide variants through combinatorial approaches to develop a formal structure–nuclease activity relationship database (10). It has been proven that the prerequisite of having a GGH motif at the N-terminus is not valid. It could reside within a much longer peptide, undergo metalation, and exhibit nucleic acid cleavage activities.

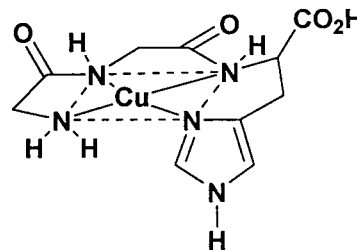


Figure 1. Binding of copper to the Gly–Gly–His tripeptide unit of the ATCUN motif.

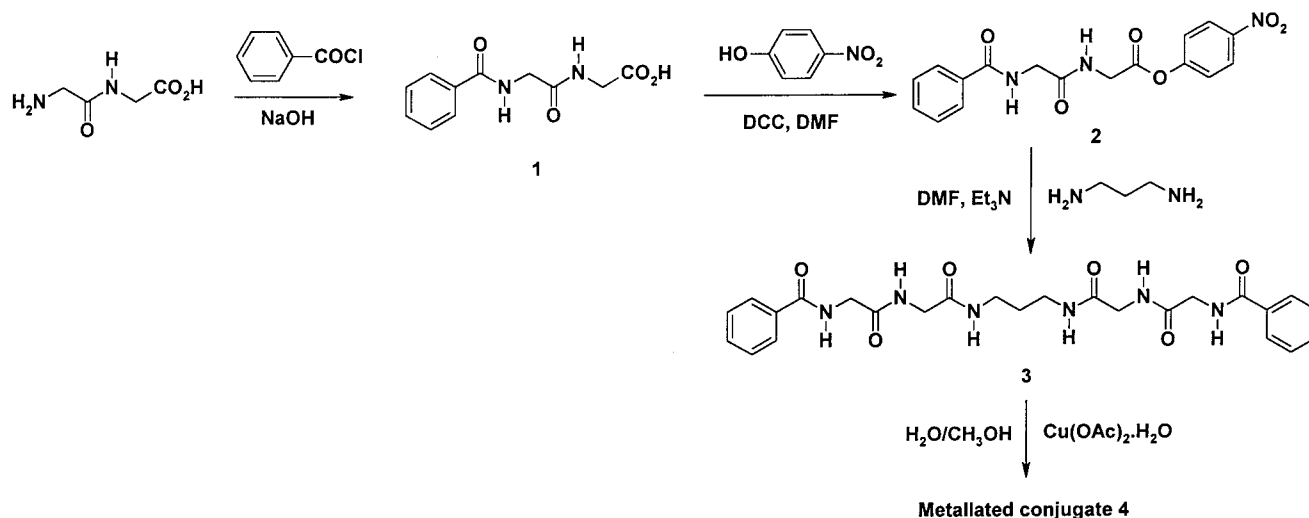
This motif has also been conjugated to a wide array of small molecules to generate new DNA binding and cleaving analogues (11). Metalated GGH motif not only causes nucleic acid damage but is also capable of cross-linking proteins through oxidative pathways. Several proteins have been cross-linked in the presence of oxidants such as oxone and magnesium monoperoxyphthalate, using copper or nickel as the cofactor (12).

We have tried to further prune the GGH motif to the diglycine level to investigate whether a dipeptide conjugate alone, lacking the C-terminus histidine residue, will support phosphate ester hydrolysis and DNA cleavage. Our strategy of using metalated *bis*-diglycine conjugate has drawn inspiration from several literature reports regarding crystal structures of metal ions complexed to this dipeptide, and moreover, a bis derivative of GGH tripeptide, appended to an anthraquinone moiety, has previously shown activity in DNA cleavage assays (11b). Interestingly, diglycine has been found to be a versatile ligand for a variety of metal ion complexation, as exemplified by numerous crystal structures containing Cu, Sr, Se, Te, Nd, Eu, V, Sn, and Na (13).

In the present study, we have synthesized a *bis*-diglycine derivative, where the two dipeptide units are tethered by a flexible diaminopropyl linker, through amide bonds (Scheme 1). The resulting bis compound **3** was spectroscopically characterized, metalated with copper (II), and subjected to exhaustive kinetic analysis using three commonly used activated phosphate ester substrates: *p*-nitrophenyl phosphate (pNPP), *bis*-(*p*-nitrophenyl phosphate) (bNPP), and 2-hydroxypropyl

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Scheme 1. Synthesis of Metalated Conjugate 4



p-nitrophenyl phosphate (hNPP). Pseudo-first-order rate constants and Michaelis–Menten kinetic parameters were determined for the three phosphate ester substrates. As metalated peptide **4** was completely insoluble in aqueous buffer solutions, this study could be considered as an example of heterogeneous catalysis of phosphate ester hydrolysis (14).

EXPERIMENTAL SECTION

Glycylglycine (Aldrich), dicyclohexyl carbodiimide and $\text{Cu}(\text{OAc})_2 \cdot \text{H}_2\text{O}$ (Lancaster), benzoyl chloride and methanol (S. D. Fine Chemicals, India), and *p*-nitrophenyl phosphate (pNPP) and *p*-nitrophenol (SRL, India) were used as supplied. Phosphodiester substrate, *bis*-(*p*-nitrophenyl phosphate) (bNPP, Fluka) was converted to its sodium salt prior to use. RNA model substrate, 2-hydroxypropyl *p*-nitrophenyl phosphate (hNPP) was synthesized as described in the literature (15), and the final compound was characterized by ^1H , ^{13}C , and ^{31}P NMR and by FAB mass spectral analysis. 1,3-Diaminopropane (Fluka) and triethylamine (S. D. Fine Chemicals, India) were distilled prior to use. NMR spectra were recorded using a JEOL 400 MHz spectrometer in $\text{DMSO}-d_6$ (Aldrich) and tetramethylsilane (TMS, Aldrich) was used as an internal standard. Elemental analysis and FAB mass spectra was recorded at RSIC, Lucknow, while EI mass spectra (EIMS) and AAS values were recorded at FEAT Laboratory, IIT-Kanpur.

N-Benzoylglycylglycine (1). Glycylglycine (10 g, 75 mmol, 1 equiv) was dissolved in 10% NaOH solution (50 mL) and cooled to 0 °C. Benzoyl chloride (11.64 g, 83 mmol, 1.1 equiv) was then added to this solution with vigorous stirring. The stirring was continued for an hour at room temperature. The reaction mixture was then cooled in an ice-bath and acidified with concentrated HCl. The precipitated product was filtered and washed with acetone (3 × 10 mL) and CCl_4 (3 × 10 mL). The product was recrystallized from hot water (8.5 g, 47% yield). mp: 205–206 °C (lit. 206.5 °C) (16); ^1H NMR: δ (ppm) 3.76 (2H, d), 3.91 (2H, d), 7.5 (3H, m), 7.9 (2H, m), 8.2 (1H, t), 8.7 (1H, t).

N-Benzoylglycylglycine *p*-Nitrophenyl Ester (2). **1** (4.74 g, 20 mmol, 1 equiv) and *p*-nitrophenol (3.05 g, 22 mmol, 1.1 equiv) were dissolved in dry DMF (50 mL) and cooled in an ice-bath. DCC (4.14 g, 20 mmol, 1 equiv) was added in portions, and the solution was stirred for

30 min in an ice-bath, followed by 4 h at room temperature. The reaction mixture was filtered, and solvent was evaporated under reduced pressure. The residue was washed with cold methanol (5 × 10 mL) and was recrystallized from hot methanol (5.1 g, 71% yield). mp: 181–183 °C; ^1H NMR: δ (ppm) 3.96 (2H, d), 4.18 (2H, d), 7.48 (5H, m), 7.9 (2H, m), 8.3 (2H, m), 8.5 (1H, t, D_2O exchangeable), 8.84 (1H, t, D_2O exchangeable); EIMS: m/z 358 ($\text{M}+1$)⁺, 219, 162, 105.

***N',N''*-bis-(*N*-Benzoylglycylglycine)-1,3-propane Diamine (3).** Product **2** (2.35 g, 6.5 mmol, 2 equiv) from the previous reaction was dissolved in dry DMF (35 mL). A mixture of 1,3-diaminopropane (0.242 g, 6.5 mmol, 1 equiv) and triethylamine (0.66 g, 6.5 mmol, 2 equiv) in dry DMF (5 mL) was then added to the above solution with vigorous stirring at room temperature for 2 h. The reaction mixture was filtered, and the residue was washed with methanol (5 × 10 mL) and acetone (5 × 10 mL). Product was recrystallized thrice from hot DMF (1.41 g, 84% yield). mp: 255–257 °C; ^1H NMR: δ (ppm) 1.56 (2H, m), 3.07 (4H, m), 3.68 (4H, d), 3.89 (4H, d), 7.5 (6H, m), 7.76 (2H, t, D_2O exchangeable), 7.88 (4H, m), 8.22 (2H, t, D_2O exchangeable), 8.82 (2H, t, D_2O exchangeable); ^{13}C NMR: δ (ppm) 29.03, 36.26, 42.1, 42.9, 127.3, 128.2, 131.3, 133.8, 166.7, 168.7, 169.34; EIMS: m/z 510 (M^+), 291, 162, 77; anal. calcd: C, 58.82; H, 5.88; N, 16.47; anal. found: C, 59.16; H, 6.08; N, 16.55.

Cu (II)-metalated *bis*-Dipeptide 4. A mixture of **3** (0.34 g, 0.67 mmol, 1 equiv) and $\text{Cu}(\text{OAc})_2 \cdot \text{H}_2\text{O}$ (1.08 g, 5.4 mmol, 8 equiv) were stirred in methanol–water (43:13) for 48 h, at room temperature. After this time, the precipitate so formed was filtered and washed with warm DMSO (4 × 10 mL), methanol (4 × 10 mL), followed by acetone (4 × 10 mL). The pale blue solid so obtained was air-dried and subjected to atomic absorption spectroscopic analysis to determine its copper content.

KINETICS

All hydrolytic reactions were performed in duplicate in centrifuge tubes thermostated at 40 °C, unless otherwise specified. The assay mixture contained 3 or 5 mL of substrate solution of appropriate concentration prepared in 0.01 M *N*-ethylmorpholine (NEM) buffer (pH 8.0) in 50% aqueous methanol. The reference cell contained substrate without **4** to correct for background hydrolysis. The amount of metalated dipeptide used

for kinetic analysis were either 0.1 mg/mL or 1 mg/3 mL of buffered substrate solution, and the concentration of the catalyst corresponded to the amount of copper present in dipeptide conjugate **4**. Control experiments proved that incubation of unmetalated peptide conjugate **3** and copper acetate alone, with phosphate ester substrates, did not accelerate hydrolysis. Initial velocities were determined as a function of time-dependent release of *p*-nitrophenolate anion ($\epsilon_{400} = 1.65 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$), with a Shimadzu UV-160 spectrophotometer. Michaelis–Menten parameters were determined using corresponding Lineweaver–Burk plots, while the pseudo-first-order rate constants were derived from $\ln A_{\infty}/A_{\infty} - A_t$ vs time plots. All hydrolytic reactions were performed at least over five half-lives of each substrate.

RESULTS AND DISCUSSION

The goal of the present study was to design a metallopeptide conjugate, based on the ATCUN motif found in serum albumins, to study its ability to facilitate hydrolytic cleavage of phosphate ester substrates, over corresponding uncatalyzed reactions. It is hoped that these studies will serve as model observations for the development of metalated dipeptide conjugates as a new class of nucleolytic reagents. Consequently, we have synthesized a *bis*-derivative of glycylglycine containing a 1,3-diaminopropane tether, using the active ester method (Scheme 1). Benzoyl protecting group was purposefully introduced and retained in the final product to make the derivative insoluble in common solvents as our long-term aim is to generate novel reagents that effect heterogeneous catalysis of chemical and biochemical reactions (17). Conjugate **3** was characterized by spectroscopic methods and was subjected to metalation using copper acetate monohydrate.

Successful incorporation of copper in conjugate **4** and its actual content was determined by atomic absorption spectroscopy and was found to be 268 mg/g of *bis*-dipeptide. Alternatively, amount of copper incorporated in conjugate **4** was ascertained by digesting a known amount of sample in 8 M HNO_3 , followed by spectrophotometric analysis. A standard curve was prepared by using known concentrations of copper acetate in 8 M HNO_3 , and the amount of copper present in conjugate **4** was determined to be 241 mg/g of *bis*-dipeptide. These data together indicate a stoichiometry of >2 copper atoms per dipeptide conjugate.

Conjugate **4** was insoluble in common organic solvents and water, including 50% aqueous methanol in which all the kinetic measurements were performed. The latter solvent system was chosen because of its known ability to support phosphate ester hydrolytic reactions and, moreover, for studying activation of water *vis-à-vis* alcohol hydroxyl group (17).

The catalytic potential of derivative **4** was evaluated using three different phosphate monoester, phosphodiester, and RNA model substrates. These nonnatural, model substrates included *p*-nitrophenyl phosphate (pNPP), bis-(*p*-nitrophenyl phosphate) (bNPP), and 2-hydroxypropyl *p*-nitrophenyl phosphate (hNPP). Remarkable rate enhancements were observed for conjugate **4** assisted cleavage of all phosphate ester substrates, when compared to the respective uncatalyzed reactions. Out of the three substrates studied by us, the results were most striking for the phosphodiester substrate, bNPP, where a 10^6 -fold acceleration was obtained over the uncatalyzed reaction. This rate enhancement is similar to previously reported synthetic catalysts used for the

Table 1. Pseudo-First-Order Rate Constants for pNPP, bNPP, and hNPP Hydrolysis Catalyzed by Conjugate **4^a**

catalyst	substrate ^b	k_{obs} (min^{-1})	k_{uncat} (min^{-1})	k_{rel}
4 ^c	pNPP	7.15×10^{-4}	4.92×10^{-7}	1.45×10^3
4 ^d	bNPP	2.19×10^{-3}	7.8×10^{-10}	2.81×10^6
4 ^c	hNPP	5.85×10^{-3}	1.98×10^{-6}	2.95×10^3

^a All reactions were performed in duplicate in 5 mL of 0.01 M NEM buffer in 50% aqueous methanol [pH 8.02, 30 °C (hNPP) or 40 °C (pNPP and bNPP)]. ^b Concentrations of pNPP, bNPP, and hNPP were 3, 8.4, and 1 mM, respectively. ^c Bis peptide conjugate **4** weight was taken as 0.1 mg/1 mL of buffer, corresponding to 0.42 mM of Cu^{2+} , if **4** were to be completely soluble in buffer. ^d Bis peptide conjugate **4** weight was taken as 1 mg/5 mL of buffer, corresponding to 0.84 mM of Cu^{2+} , if **4** were to be completely soluble in buffer.

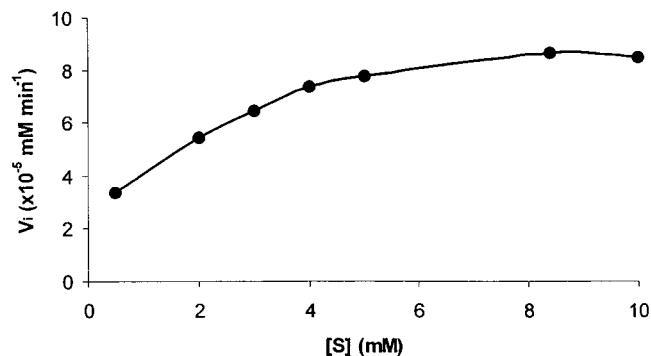


Figure 2. Saturation kinetics profile of pNPP hydrolysis by **4**.

Table 2. Michaelis Constants, Maximal Velocities, and Turnover Numbers of Conjugate **4 Catalyzed pNPP, bNPP, and hNPP Hydrolyses^a**

substrate ^b	K_m (mM)	V_{max} (mM min^{-1})	k_{cat} (min^{-1})
pNPP	1.78	1.04×10^{-4}	1.24×10^{-4}
bNPP	1.39	3.34×10^{-5}	2.38×10^{-5}
hNPP	1.37	1.52×10^{-3}	1.08×10^{-3}

^a All reactions were performed in duplicate with dipeptide conjugate **4** in 0.01 M NEM buffer prepared in 50% aqueous methanol [pH 8.02, 30 °C (hNPP) or 40 °C (pNPP and bNPP)]. The amount of **4** was 1 mg/5 mL for pNPP, while it was 1 mg/3 mL for bNPP and hNPP. ^b pNPP and bNPP concentrations, [S] = 1–5 mM; hNPP concentration, [S] = 0.2–0.5 mM.

hydrolysis of this substrate (17c and references therein). Dipeptide conjugate **4** also assisted cleavage of a monoester and an RNA model substrate, thus demonstrating its versatility as a synthetic phosphate ester hydrolase. For phosphate monoester substrate (pNPP), about 1500-fold rate enhancement was observed as compared to the uncatalyzed reaction, while the cleavage rate enhanced nearly 3000-fold for the RNA model compound (hNPP), over corresponding uncatalyzed reactions (18). Pseudo-first-order rate constants (k_{obs}) for individual substrates are mentioned in Table 1. Saturation curve for this catalytic system was determined using substrate concentration vs initial velocity profile by employing pNPP as the substrate. As indicated by Figure 2, good Michaelis–Menten behavior was observed for conjugate **4** catalyzed cleavage of pNPP. We have also performed detailed kinetic characterization of conjugate **4** by determining Michaelis–Menten constants (K_m), maximal velocities (V_{max}), and the turnover number (k_{cat}), from respective Lineweaver–Burk plots $1/[S]$ vs $1/V_i$ (Figures 3–5). The values of these kinetic parameters for individual substrates are listed in Table 2.

All the phosphate ester cleavage experiments were performed at pH 8. Several literature reports have demonstrated that the effect of pH on phosphoester

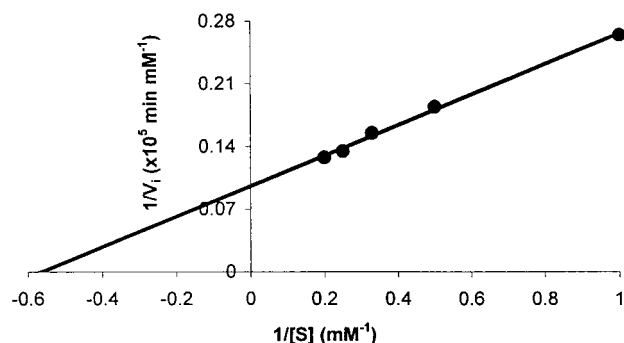


Figure 3. Lineweaver-Burk plot of pNPP hydrolysis by 4.

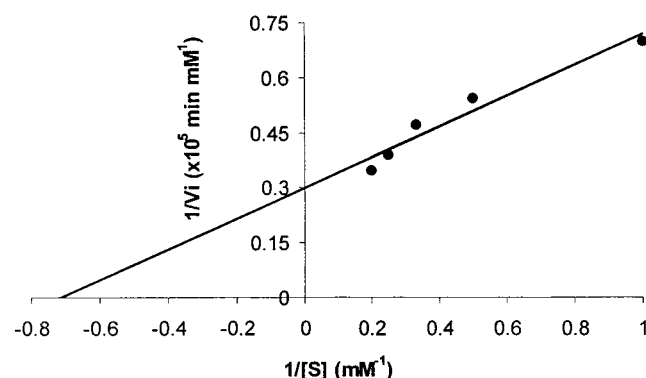


Figure 4. Lineweaver-Burk plot of bNPP hydrolysis by 4.

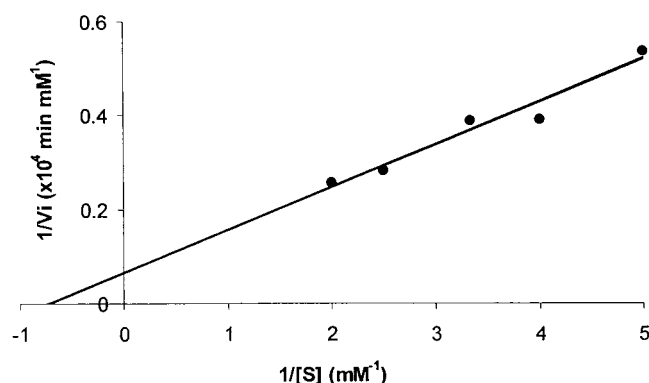


Figure 5. Lineweaver-Burk plot of hNPP hydrolysis by 4.

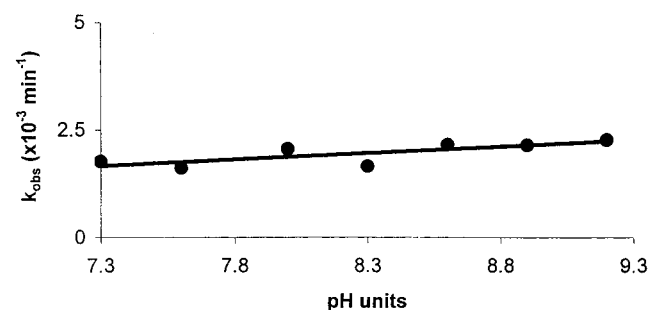


Figure 6. pH versus k_{obs} profile of pNPP hydrolysis by 4.

cleavage rate may provide clues regarding catalytic mechanism (19). In the present study, we have sought to understand the mechanism of phosphate monoester cleavage by studying effect of pH on the rate of pNPP cleavage. Pseudo-first-order rate constants were recorded at seven different pH values ranging from 7.3 to 9.2, and, as evident from Figure 6, k_{obs} values virtually remained constant thus indicating the lack of influence of pH on pNPP cleavage assisted by conjugate 4. This result

Table 3. Inhibition of Conjugate 4 Assisted pNPP Hydrolysis by D-Mannitol^a

D-mannitol ^b (M)	k_{obs} (min ⁻¹)	k_{rel}
no mannitol	9.65×10^{-4}	1
0.1	2.45×10^{-4}	0.25
0.2	9.93×10^{-5}	0.10

^a All reactions were performed in duplicate with conjugate 4 (1 mg/5 mL) in 0.01 M NEM buffer prepared in 50% aqueous methanol (pH 8.02, 30 °C). ^b pNPP concentration, [S]: 2 mM.

Table 4. Effect of Solvent on Conjugate 4 Catalyzed pNPP Solvolysis^a

solvent used	k_{obs} (min ⁻¹)	K_{rel} ^b
ethylene glycol (100%)	1.76×10^{-4}	1.0
water-ethylene glycol (1:1)	3.21×10^{-4}	1.82
water (100%)	9.57×10^{-4}	5.44

^a All reactions were performed in duplicate in 3 mL of 0.01 mM NEM buffer in respective solvent systems (pH 8.0, 30 °C). The reference cell contained substrate without 4 to correct for background hydrolysis. Weight of conjugate 4 used was 1 mg/3 mL for each reaction. The substrate concentration was fixed at 2 mM. ^b Relative rates are reported with respect to k_{obs} obtained for pure ethylene glycol.

indirectly suggests a free radical-induced mechanism rather than a hydrolytic phosphoester bond cleavage. Interestingly, a free radical based mechanism of DNA cleavage by metalated ATCUN peptide motif is already documented (10c).

Such indirect evidence of free radical-assisted phosphate monoester cleavage prompted us to perform this reaction in the presence of a hydroxyl radical scavenger, such as D-mannitol (20). We surmised that a decrease in the catalytic rate, in the presence of radical scavengers, would provide more evidence for free radical-assisted phosphate ester cleavage. Accordingly, pNPP cleavage experiments were performed in the presence of 0.1 and 0.2 M D-mannitol. A 10-fold decrease in k_{obs} was observed in the presence of 0.2 M mannitol, and correspondingly, the decrease was about 4 times in the presence of 0.1 M mannitol, when compared to the control reaction. These observations further indicate a crucial role for reactive species in cleaving pNPP and other phosphate esters. However, a possible role of D-mannitol in copper ion coordination, thereby leading to a reduction in the observed rate, cannot be ruled out at this stage.

As all of our cleavage kinetic experiments were performed in 50% aqueous methanol, we also became interested in delineating the effect of aqueous medium versus alcoholic medium, on phosphate ester bond cleavage. Preferential activation by conjugate 4 was determined by performing cleavage reactions in water, ethylene glycol, and 50% aqueous ethylene glycol, using pNPP as a substrate. Ethylene glycol was used instead of methanol to ensure optimum solubility of pNPP. Pseudo-first-order rate constants were determined for the three systems (Table 4). As compared to the rate of solvolysis in pure ethylene glycol, about a 2-fold increase in k_{obs} was found for 50% aqueous ethylene glycol system, while the rate enhancement was over 5-fold if the reaction was performed in water alone. These observations indicate enhanced cleavage efficiency of conjugate 4 in water when compared to ethylene glycol. It can be surmised that in mixed aqueous solvent systems, ethylene glycol (or methanol) quenches reactive species that are generated due to conjugate 4, leading to a decrease in k_{obs} for pNPP hydrolysis. This is in accordance with the potential of ethanol and other alcohols to act as hydroxyl radical scavengers. Keeping the possible role of reactive free

radical species and preferential catalytic activation in aqueous medium by conjugate **4** in mind, it will be of interest to apply this system to compare cleavage of DNA versus intramolecular cleavage of RNA substrates.

One of our long-term goals is to devise recyclable catalysts possessing nucleolytic activity. We have achieved some success in this direction with nucleobase containing resins (17), and therefore, it was of considerable interest for us to extend this design paradigm for peptide conjugates as well. As mentioned before, we purposefully introduced benzoyl groups to render the peptide conjugate insoluble in aqueous medium for subsequent recycling experiments. In a typical recycle experiment, conjugate **4** was filtered, washed copiously with 50% aqueous methanol, methanol, and acetone, and air-dried. Recycled conjugate assisted the cleavage of pNPP, albeit with a reduced initial rate, demonstrating its reusability toward catalysis of phosphate ester hydrolysis (data not shown).

Hydrolytic reactions were also performed in the presence of two different concentrations of EDTA to determine if leached out copper ions were responsible for the catalytic activity and for the generation of reactive species. The initial rate of hydrolysis, using pNPP as the substrate, did not appreciably change in the presence of 0.1 and 1 mM EDTA, when compared to the initial hydrolytic rate obtained in the absence of EDTA (data not shown). This observation suggests that the acceleration of phosphate ester hydrolysis is probably due to copper species coordinated to **4**, rather than free copper ions in solution. The precise mode of copper ion coordination remains unclear at the present time and would require crystal structure data to unequivocally establish the coordination geometry. For this reason, we are now trying to generate soluble peptide conjugates to determine their crystal structure and to predict the geometry of copper ion binding within the *bis*-dipeptide motif, through detailed EPR and magnetic moment measurements.

In summary, we have crafted a novel and catalytically competent metalated dipeptide conjugate possessing the ability to cleave phosphate esters in a highly efficient fashion. Michaelis–Menten kinetic parameters have been determined for this conjugate and initial efforts have also been made to probe for the mechanism of cleavage. Differential solvent effects and the reusable nature of such *bis*-dipeptides merit further attention and are worthy of being developed as potential nucleolytic reagents. Future efforts in this direction will also aim for engineering specificity in such constructs for site-specific cleavage of nucleic acids.

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Physicochemical Characterization of Poly(ethylene glycol)-Modified Anti-GAD Antibodies

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Monoclonal antibodies against glutamic acid decarboxylase (anti-GAD) were modified with poly(ethylene glycol) (PEG), and the resulting conjugates were characterized. Monoclonal anti-GAD antibodies were purified from ATCC HB184 hybridoma cells by either cell culture supernatant or ascites fluid from BALB/c mice. Polyclonal rabbit IgG antibodies were also used as a model protein. Polyclonal rabbit IgG or purified anti-GAD was modified by PEG (MW = 5000 or 20000 Da) through either the lysine residues or through the carbohydrate moiety. Lysine modification was performed in PBS (pH 7.4) or 0.1 M borate (pH 9.2) by adding a molar excess (5–80) of a succinimidyl activated propionic acid terminated mPEG (SPA-PEG) while stirring at room temperature. Carbohydrate modifications were performed in PBS (pH 6.2) by first oxidizing the antibody with sodium periodate followed by incubation with hydrazide-terminated PEG followed by reduction with sodium cyanoborohydride. The degree of modification was assessed by ^1H NMR or TNBS (trinitrobenzenesulfonic acid). Circular dichroism (CD) spectra were obtained for lysine-modified rabbit IgG at various degrees of modification ranging from 5 to 60 PEG per antibody. Binding was assessed using an ELISA method with GAD or rabbit anti-mouse-IgG (H+L) coated plates. The TNBS and ^1H NMR analysis of the modified antibody showed reasonably similar results from 5 to 60 PEG per antibody. The ^1H NMR method showed greater sensitivity at low modifications (below 20:1) and was fairly linear up to about 60 PEG per antibody. The CD spectra of the polyclonal rabbit IgG showed only small differences at variously modified antibody. The binding affinity of anti-GAD is lower for all PEG modifications with respect to unmodified anti-GAD. Modifications at pH 7.4 show lower binding to GAD than modifications at pH 9.2. Binding to GAD or anti-mouse-IgG is decreased as the degree of modification is increased. Lysine modifications showed lower binding to GAD or anti-mouse-IgG than carbohydrate modifications. Binding to GAD or anti-mouse-IgG is lower for PEG20000-modified anti-GAD with respect to PEG5000-modified anti-GAD.

INTRODUCTION

Type I diabetes, or insulin-dependent diabetes mellitus (IDDM), is an autoimmune disorder in which the patient's immune system selectively attacks and destroys the insulin-producing β -cells of the pancreas (1). Type I diabetes is an irreversible condition that usually occurs before adulthood in a patient's life. The autoimmune process in Type I diabetes is characterized by macrophage infiltration into the pancreas followed by T-cell and macrophage activation which then leads to β -cell destruction and loss of insulin production (1–4). Once the β -cells are completely destroyed, no further insulin production is observed, and exogenous insulin must be administered to maintain normal glucose levels.

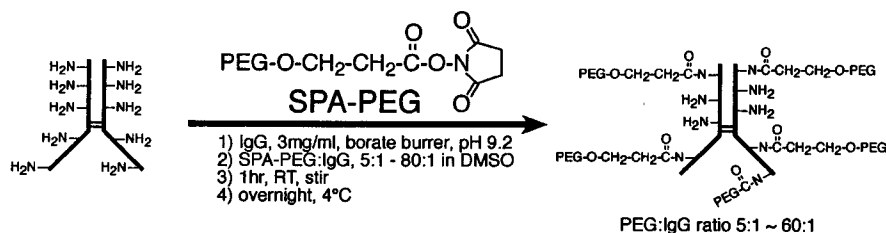
There is a strong genetic component to Type I diabetes which seems to be linked to certain major histocompatibility complex (MHC or HLA) genes, and individuals with this tissue type show an increased susceptibility toward developing Type I diabetes (5–8). The MHC molecules present on antigen-presenting cells (APCs), such as macrophages, phagocytes, and B-lymphocytes, serve as sites of antigen presentation to T-cells of the immune system. When APCs phagocytize cellular debris,

they breakdown the ingested proteins into small peptides which are then presented on the cell surface in association with these MHC molecules. Here, they can interact with the T-cells which will either recognize the complex as being native or foreign. In the case of Type I diabetes, T-cells recognize some of the peptide sequences from β -cell proteins as being foreign which cause the T-cells to become activated. This eventually leads to β -cell destruction and the onset of clinical diabetes.

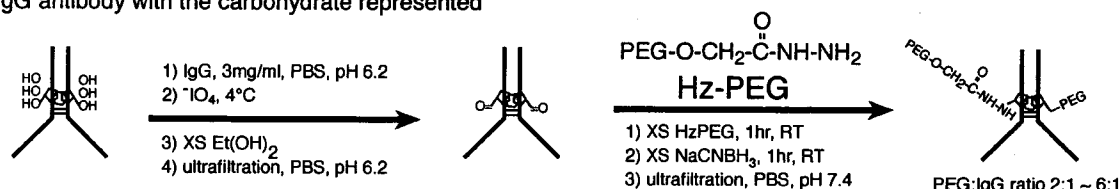
There have been many proteins studied which are suspected to be important in the initiation and progression of the disease (9–12). Among the most intensely studied autoantigens is the protein glutamic acid decarboxylase (GAD) which can be found at appreciable levels in the β -cells of the pancreas. It is thought that GAD is one of the most important autoantigens responsible for Type I diabetes (13–15). In fact, studies using the NOD (nonobese diabetic) mouse model show that diabetes can be eliminated if GAD production in the β -cells is eliminated (16).

It has been shown that antibodies bound to proteins can affect the resulting peptides produced by enzymatic cleavage (17–19). In this manner, it would seem possible to alter the specific GAD peptides presented to T-cells by using antibodies against GAD (anti-GAD). By binding to the GAD, the anti-GAD antibodies will alter how the APCs process GAD which may lead to a difference in the

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A) Lysine ConjugationIgG antibody with representative lysines ($-\text{NH}_2$)**B) Carbohydrate Conjugation**

IgG antibody with the carbohydrate represented

**Figure 1.** Schematics of the modification reactions of antibodies by PEG through (A) lysine and (B) carbohydrate.

specific peptides presented by these cells and thereby alter the immune response to GAD.

In our laboratory, we have studied the effect of the administration of monoclonal anti-GAD antibodies in the progression of Type I diabetes in the NOD mouse model (20). We have found that weekly injections of anti-GAD antibodies can reduce the incidence of diabetes from greater than 80% in untreated NOD mice to less than 20% in treated mice.

PEG modification of proteins has been shown to improve a number of properties of proteins. Typically, PEG-modified proteins will show an increased resistance to enzymatic attack (21–23), a reduced immunogenic response (24–26), an increase in plasma half-life (25–28), and an increase in solution stability (29–31).

Of particular interest is the alteration of the enzymatic degradation of PEG-modified proteins. Thus, it is thought that PEG modification of an antibody against a protein may have a greater effect at altering the resulting enzymatic peptide sequences than the antibody alone which may further decrease the incidence of diabetes.

In this report, we describe the synthesis and characterization of PEG-modified, monoclonal anti-GAD. We studied the effect of the degree of modification (number of PEG chains per antibody), the site of modification (lysine residues versus carbohydrate moiety), reaction conditions (low pH versus high pH for lysine modification), and PEG molecular weight on the antibody structure, the binding affinity toward GAD, and the ability of other antibodies to recognize the anti-GAD-PEG conjugates.

EXPERIMENTAL PROCEDURES

Antibody Production. Ascites fluid was produced by injecting hybridoma cells (HB184, ATCC) intraperitoneally into pristane-treated BALB/c mice. The monoclonal antibody, anti-GAD, was purified from ascites fluid by ammonium sulfate precipitation followed by diethylaminoethyl (DEAE) cellulose chromatography. Purity was gauged by native PAGE. Polyclonal rabbit IgG was obtained from Sigma and used without further purification.

Antibody Modification. The reaction scheme for the antibody modifications is shown in Figure 1. The purified antibody was exchanged into the appropriate buffer for

each modification by ultrafiltration (50 kDa MWCO). Lysine modifications were done in either a phosphate buffer at pH 7.4 or in a borate buffer at pH 9.2. Antibody concentrations were 3 mg/mL. Succinimidyl propionate activated PEG (SPA-PEG) (MW = 5 or 20 kDa) (Shearwater Polymers), see Figure 1, was dissolved in DMSO and added to the anti-GAD buffered solutions in a dropwise manner while stirring. The solutions were stirred at room temperature for 1 h and incubated at 4 °C overnight. As a model, polyclonal rabbit IgG was modified with molar excesses SPA-PEG (MW = 5000 Da) of 5, 10, 20, 30, 40, 60, or 80. Carbohydrate modifications (32–33) were done in a phosphate buffer at pH 6.2. The carbohydrate was first oxidized by addition of sodium periodate (NaIO_4) in a 1000 molar excess with respect to anti-GAD concentration. After stirring 1 h at 4 °C, the oxidation was quenched with ethylene glycol, and the reaction byproducts were removed by ultrafiltration (50 or 100 kDa MWCO). A 50 molar excess of hydrazide-PEG (Shearwater Polymers) was added to the oxidized antibody at pH 6.2. After stirring at room temperature for 1 h, an excess of sodium cyanoborohydride (NaBH_3CN) was added and stirred at room temperature for another hour. The modified antibodies were then purified by ultrafiltration (50 or 100 kDa MWCO) against PBS, pH 7.4.

^1H NMR Analysis. The modified antibody was exchanged into phosphate buffered D₂O by ultrafiltration (50 kDa MWCO). The molar amount of antibody in 400 μL was calculated by UV absorbance at 280 nm based on an extinction coefficient of 1.38 mL/mg/cm and an antibody molecular weight of 150000 Da. Antibody concentrations were between 3 and 5 mg/mL. Stock solutions of acetone, DMSO, and sodium benzoate were made in D₂O at known concentrations. Known amounts of the standards were added to the solutions, and the total volume is brought up to 700 μL with D₂O. The amount of each standard added to each sample is based on the estimated number of PEG chains attached to the antibody such that the integrations of the PEG peak and the reference peaks will be close. For example, if the final molar concentrations of acetone and DMSO were 76 times larger than the estimated molar concentration of PEG5000 in the sample, the integrated peaks should be close to the same. Spectra were gathered on a Bruker 200 MHz NMR. The acetone (2.06 ppm), DMSO (2.56

ppm), PEG (3.54 ppm) and aromatic benzoate (7.31–7.72 ppm) peaks were integrated. The ^1H NMR spectrum was taken, and the integration of the methylene ($-\text{CH}_2-$) hydrogen peak of the PEG was compared to the acetone and DMSO peaks to calculate the PEG concentration. The number of PEGs per anti-GAD is determined by dividing the concentration of PEG by the concentration of anti-GAD.

TNBS Assay. For TNBS analysis (34–35), the modified antibody solutions were diluted to about 3 mg/mL in 600 μL in 0.1 M borate buffer pH 9.2. To keep the antibody soluble throughout the reaction, DMSO was added to 33%. Absorbance blanks consisting of buffer and DMSO were also made. To each solution was added 8 μL of a 1 M TNBS solution. The solutions were mixed well and incubated at room temperature for 6 h. To stop the reaction, 4 μL of 12 N HCl was added to each solution. Dilutions of the samples were made by measuring between 30 and 120 μL of the sample and the bringing this volume to 120 μL by the addition of the blank solution. This was done to keep the background constant in all the dilutions. Then 1080 μL of 0.1 N HCl was added to each solution. The absorbance blank was made by adding 120 μL of the blank solution to 1080 μL of 0.1 N HCl. The absorbance at 345 nm was then taken for each sample.

CD Spectroscopy. Lysine-conjugated (PEG5000) rabbit IgG was used at conjugation ratios ranging from 0 to 56 PEG per IgG. The antibody concentration of each was brought to 0.5 mg/mL in PBS (pH 7.4), as determined spectrophotometrically, ϵ_{280} (mL/mg/cm) = 1.38. The samples were degassed under vacuum pressure for 20 min prior to the CD measurements. The molar ellipticity was read between 200 and 250 nm using a 0.1 cm path length cell.

Binding Affinity. The binding of the modified anti-GAD to GAD as well as an anti-mouse-IgG antibody was assessed by ELISA. Glutamic acid decarboxylase (GAD) from E-coli (Sigma, EC 4.1.1.15) or rabbit-anti-mouse-IgG (H+L) (Zymed) was used to coat 96-well ELISA plates by incubating each well with a 2 $\mu\text{g}/\text{mL}$ GAD or 5 $\mu\text{g}/\text{mL}$ anti-mouse-IgG solution at pH 9.5. After 2 h, the solution was removed, and the plates were incubated with a 1% BSA in PBS, pH 7.4 solution for 1 h. Dilutions of the anti-GAD (modified or unmodified) were made with PBS, 1% BSA, pH 7.4, and incubated in the plates for 2 h. The plates were then incubated with a 1:100 dilution of alkaline phosphatase-coupled anti-mouse-IgG antibody (Zymed) for 2 h. The plates were then washed four times with 125 $\mu\text{L}/\text{well}$ of PBS, 0.05% Tween20. A solution of *p*-nitrophenyl phosphate (Pierce) in a diethanolamine buffer, pH 9.8, was added. After sufficient color developed, the absorbance at 405 nm of each well was measured by an automated ELISA plate reader.

RESULTS

The degree of modification was assessed by a TNBS method. The number of lysines was previously determined by amino acid analysis to be approximately 86, data not shown, which yields a total number of free amines about 90 due to the four N-terminal amines per antibody. The absorbances at 345 nm of the TNBS samples were normalized to the unmodified sample. Thus, the approximate number of free amines per antibody in each sample is 90 multiplied by the normalized absorbance at 345 nm. Thus, the number of PEG chains conjugated per antibody is the difference between 90 and the number of free amines.

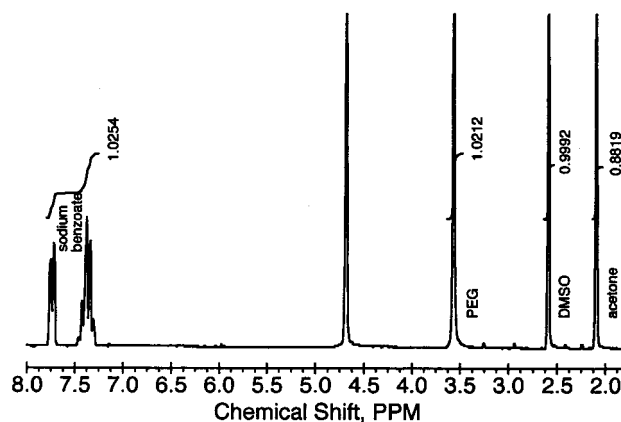


Figure 2. ^1H NMR spectrum of a PEG5000-modified antibody with acetone, DMSO, and sodium benzoate standards.

For ^1H NMR analysis, it is important that all unreacted PEG be removed before analysis. Ultrafiltration was used in this study to remove the unreacted PEG. The molecular weight cutoff of the ultrafiltration membrane used was 50 kDa. Permeability of the PEG (MW = 5000) through the membrane was confirmed by analyzing the ultrafiltrate for PEG, data not shown.

To quantitate the PEG peak of an ^1H NMR spectrum, it is necessary to reference peaks of known concentration. Integration standards (acetone, DMSO, and sodium benzoate) were added to the PEG-antibody conjugate solution at known concentrations. In this manner, the integration of the PEG peak can be used to calculate its concentration based on the integrations of the reference peaks. Figure 2 shows a typical ^1H NMR spectrum of a solution of a PEG-modified antibody with the three reference standards. This clearly shows that PEG (3.54 ppm) and the three reference standards of acetone (2.06 ppm), DMSO (2.56 ppm), and sodium benzoate (7.27–7.73 ppm) are easily resolved from each other.

The integration of an ^1H NMR peak is proportional to the number of resonant hydrogens at the particular chemical shift. Each peak can be defined as the product of the concentration and the number of resonant hydrogens. Thus, the concentration of PEG can be easily solved since the concentrations of the standards and the antibody are known.

When the calculated PEG ratios are plotted versus the PEG reaction ratio, as shown in Figure 3, the plot reveals a linear relationship between the measured PEG conjugation ratio with respect to the activated PEG reaction ratio for the NMR calculations. When a linear regression is performed, it shows a slope of 0.73 with an R^2 of 0.996 when the fit is forced through zero. This indicates that approximately 73% of the activated PEG added to the reaction conjugated to the antibody under the reaction conditions used.

For comparison, the TNBS analysis results are plotted with the NMR analysis in Figure 3. In contrast to the NMR analysis, the TNBS analysis shows a nonlinear trend of PEG conjugation. The results of the two techniques match only at two points, 40 and 60 PEG reaction ratio. Since the TNBS method relies on an indirect method of subtraction, it seems to have difficulty detecting conjugation at lower reactions ratios (<20) since it involves the difference of two large numbers to obtain a small number. However, the direct detection by NMR easily measures the PEG at all conjugations.

The direct detection of PEG by NMR also allows the quantification of PEG conjugated by any method. Except

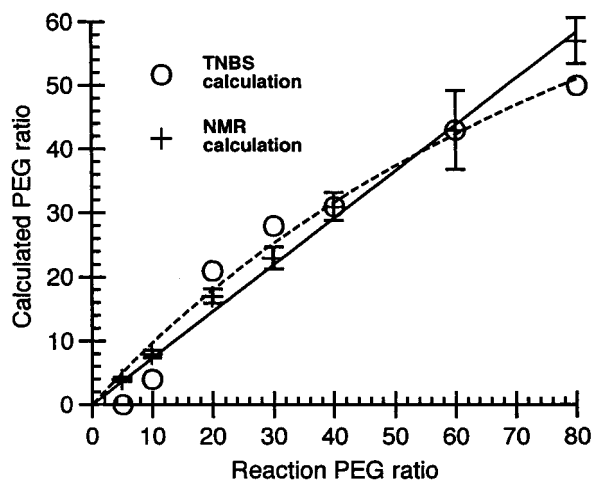


Figure 3. Plot of the calculated number of PEG per antibody versus the molar excess of activated PEG in the reaction mixture.

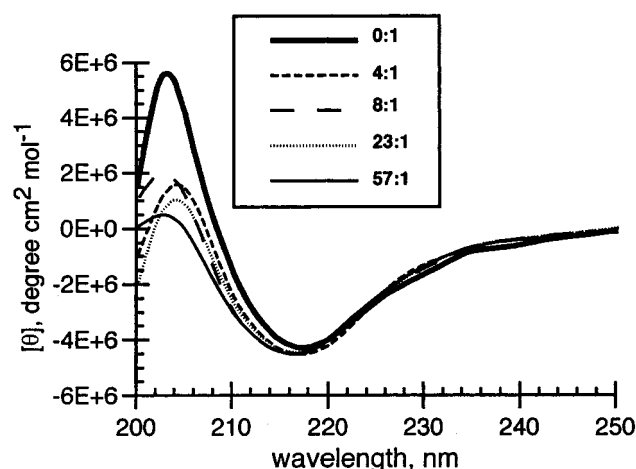


Figure 4. CD spectra of PEG-modified polyclonal rabbit IgG.

for the absolute values of the various peak integrations, the NMR spectrum of a carbohydrate-modified antibody was indistinguishable from the lysine-modified antibody depicted in Figure 2. The number of PEG chains attached per antibody were typically between two and six for the carbohydrate modifications. This is consistent with the number of aldehydes generated by periodate oxidation, as determined by a Lucifer Yellow assay (36), data not shown.

The CD spectra were obtained for a range of modified polyclonal rabbit IgG antibody. Figure 4 shows the obtained spectra. The spectra shows the very characteristic trough (37) of the β -sheet secondary structure element, which is the most predominant structural element of antibodies (38, 39). The spectra suggest a high degree of structural homology among the modified antibodies with very little change in the structure even at a high modification of 57 PEG5000 chains per antibody.

Monoclonal anti-GAD antibody was modified by PEG5000 and PEG20000 by either the lysine residues or by the carbohydrate moiety. The importance of the modification pH during lysine modification was studied by performing the modification at pH 7.4 or 9.2. Table 1 shows the number of coupled PEG chains to each anti-GAD molecule for the various modifications studied.

By varying the reaction pH of lysine-modified anti-GAD-PEG, the importance of the particular amines undergoing reaction can be examined. At low pH, the

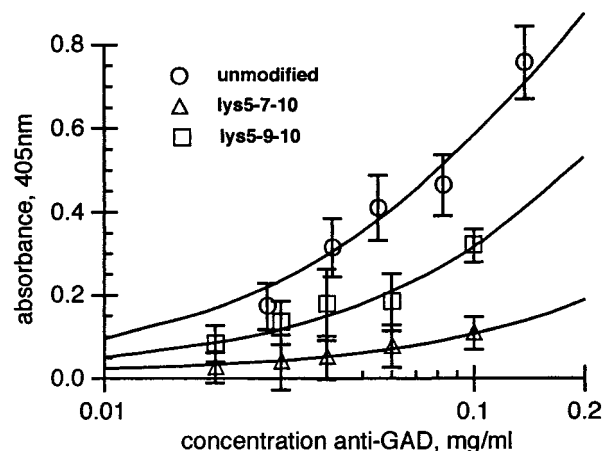


Figure 5. Effect of modification pH on the binding of modified anti-GAD antibodies to GAD; solid line, k varied; dashed line $A_{\max}$ varied.

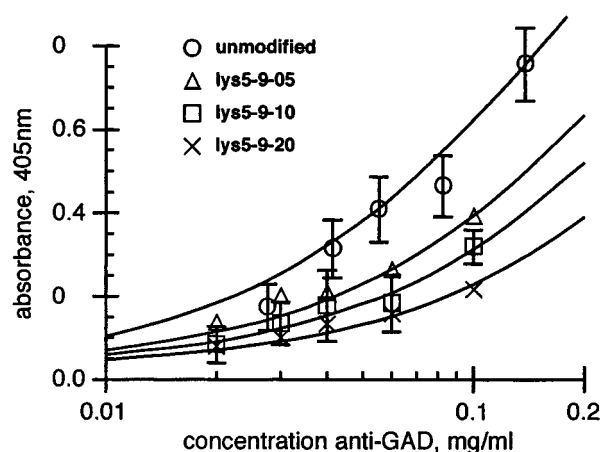


Figure 6. Effect of degree of modification on the binding of anti-GAD to GAD; solid line, k varied; dashed line $A_{\max}$ varied.

Table 1. The Modification Conditions and PEG to Anti-GAD Ratios for the Modified Anti-GAD Samples

sample id	modification conditions	PEG MW, Da	no. of PEG
Lys5-7-10	Lys, pH 7.4	5000	10
Lys5-9-05	Lys, pH 9.2	5000	5
Lys5-9-10	Lys, pH 9.2	5000	10
Lys5-9-20	Lys, pH 9.2	5000	20
Lys20-9-05	Lys, pH 9.2	20000	5
Carb5	carbohydrate	5000	3
Carb20	carbohydrate	20000	5

reaction will favor amines of lower pK_a ; at high pH, the reaction will be more random. Figure 5 shows that the binding of lysine-modified anti-GAD is affected by the modification pH. A low modification pH of 7.4 shows a much greater loss of binding than high modification pH of 9.2 when the degree of modification is constant at about 10 PEG per anti-GAD. This indicates that there may be some primary amines with a lower pK_a which have a great effect on binding when modified by PEG.

It is assumed that as the number of PEG chains attached to the anti-GAD antibody was increased, there would be a greater coverage of the antibody by the PEG, and that this would reduce the binding of anti-GAD to GAD. Figure 6 confirms that the binding of lysine-modified anti-GAD to GAD decreases with increasing degree of modification (from 5 to 20 PEG per anti-GAD).

Modification of anti-GAD antibodies through its carbohydrate moiety is in stark contrast to modification

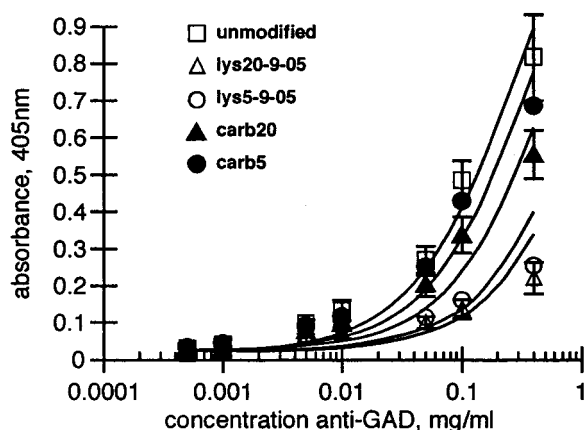


Figure 7. Comparison of lysine and carbohydrate modification methods and PEG molecular weight on the binding of anti-GAD to GAD; solid line, k varied; dashed line $A_{\max}$ varied.

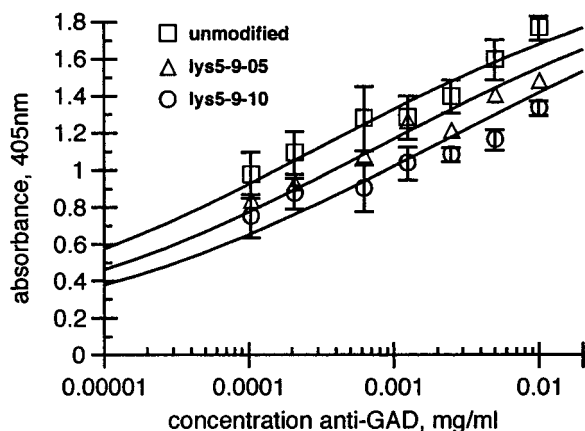


Figure 8. Comparison of the effect of degree of modification on the binding of the modified anti-GAD to anti-mouse IgG (H+L) antibodies.

through its lysine residues. The carbohydrate is localized to a single region well away from the antigen binding region, and the lysine residues are distributed, more or less, randomly throughout the antibody. Figure 7 shows that the binding of the carbohydrate-modified anti-GAD to be quite close to the unmodified anti-GAD and higher than a lysine-modified anti-GAD of similar degree of modification.

It was suspected that the molecular weight of PEG would be an important factor in the binding of modified anti-GAD. The longer chains of PEG20000 should have a greater influence than PEG5000. The effect of PEG molecular weight can be seen in Figure 7. The PEG20000-modified anti-GAD samples show lower binding affinities than their PEG5000 counterparts. It appears that the site of modification influences the binding to a greater extent than molecular weight. PEG attached only to the carbohydrate will affect binding much less than PEG attached randomly over the antibody.

The ability of anti-mouse-IgG antibodies to recognize and bind to anti-GAD-PEG antibodies is a good measure of how well the PEG chains can shield or hide the surface of antibody. Figure 8 shows the effect of degree of modification on the binding of anti-mouse antibodies to PEG-modified anti-GAD. As expected, the binding of anti-mouse-IgG antibodies is reduced as the number of PEG chains attached is increased.

The site of modification is also an important parameter in PEG shielding. The random attachment of PEG

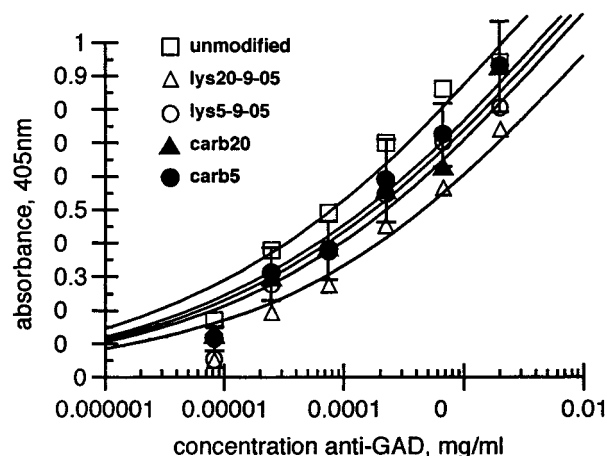


Figure 9. Comparison of the effect of modification site and PEG molecular weight on the binding of the modified anti-GAD to anti-mouse IgG (H+L) antibodies.

through the lysines is expected to provide a more effective coverage than the localized attachment through the carbohydrate. Thus, the effect of PEG molecular weight should have a greater effect with the lysine modification than the carbohydrate modification. Figure 9 shows the effect of molecular weight and site of modification on the binding of anti-mouse antibodies to PEG-modified anti-GAD. The carbohydrate-modified anti-GAD-PEG samples appear to be recognized more by the anti-mouse-IgG antibodies than the lysine-modified samples. The molecular weight of the PEG also has a greater influence on the lysine-modified than the carbohydrate-modified anti-GAD.

To quantitate the effect of the PEG on binding, the ELISA data were fitted to the following equation (40):

$$A = A_{\max} \frac{(k \times \text{concn})^a}{1 + (k \times \text{concn})^a} + b$$

This is a generalized form of the Langmuir equation which was adapted by Nisonoff and Pressman from an equation by Sips (40) and includes an ' a ' parameter to account for multiple binding sites. In general, for cooperative binding, such as hemoglobin/oxygen, ' a ' will be greater than one, and for nonspecific, multiple binding site binding, such as polyclonal antibodies, ' a ' will be less than 1. Thus, for the binding data from the anti-mouse-IgG-coated plates, ' a ' was fitted to account for the range of affinities of the polyclonal anti-mouse-IgG antibodies. For the binding data from the GAD-coated plates, ' a ' was fixed at 1 due to the singular specificity of the monoclonal anti-GAD antibody to the GAD protein. In this equation, A represents the absorbance of the sample at 405 nm, and $A_{\max}$ is the calculated absorbance plateau and is related to the maximum number of antibodies that can bind in each well and will be referred to as binding activity; k is the affinity constant for the antibody; concn is the concentration of the antibody; b accounts for background absorbance. Within each data group, b and either $A_{\max}$ or k was assumed to be the same for all series, and k or $A_{\max}$, respectively, was fitted for each series.

For each plot and curve-fit, the affinity constant or the absorbance maximum for unmodified anti-GAD was designated as k° or $A_{\max}^\circ$, respectively. Thus, to easily compare across data sets, k or $A_{\max}$ for each sample was divided by the k° or $A_{\max}^\circ$ fitted to the unmodified anti-GAD. In this manner, the binding activity of each

Table 2. The Relative Binding of PEG-Modified Anti-GAD Relative to Unmodified Anti-GAD with Calculated 90% Confidence Intervals

sample	k/k° , GAD	$A_{\max}/A_{\max}^\circ$, GAD	k/k° or $A_{\max}/A_{\max}^\circ$, anti-mouse
unmodified	1	1	1
Lys5-7-10	0.06 ± 0.05	0.11 ± 0.08	—
Lys5-9-05	0.3 ± 0.2^a	0.40 ± 0.13^a	0.84 ± 0.13^b
Lys5-9-10	0.32 ± 0.07	0.40 ± 0.07	0.75 ± 0.05
Lys5-9-20	0.21 ± 0.06	0.28 ± 0.08	—
Lys20-9-05	0.08 ± 0.03	0.24 ± 0.04	0.66 ± 0.13
Carb5	0.71 ± 0.18	0.84 ± 0.06	0.88 ± 0.14
Carb20	0.43 ± 0.11	0.67 ± 0.05	0.84 ± 0.14

^a Average of curve fits from Figures 6 and 7. ^b Average of curve fits from Figures 8 and 9.

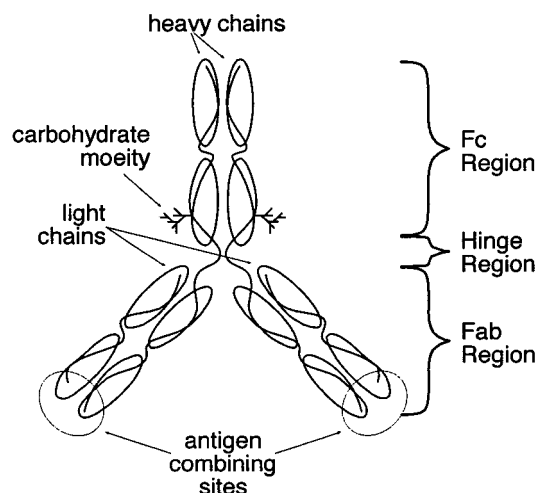
modified anti-GAD can be seen as a fraction of the unmodified anti-GAD. As seen in Table 2, there were noticeable differences in the relative binding calculated by these two methods. However, with each method, it can be seen that the binding affinity of all carbohydrate-modified anti-GAD is higher than any lysine-modified anti-GAD, regardless of PEG molecular weight. The effect of PEG molecular weight appears also to have a greater effect on lysine-modified anti-GAD with respect to the carbohydrate-modified anti-GAD. The lysine-modified anti-GAD shows an additional loss of approximately 73% of its binding affinity or 40% of its binding activity when PEG20000 is used over PEG5000. However, the carbohydrate-modified anti-GAD shows an additional loss of about 39% of its affinity or 20% of its binding activity when PEG20000 is used over PEG5000. When the lysines are used for modification, it appears to be critical that a high pH be used. Otherwise, the binding may be further reduced by 81% (affinity) or 73% (activity), with respect to modification at pH 9.2.

Interactions with anti-mouse-IgG antibodies, which bind to various sites throughout the anti-GAD antibody, are also more affected by molecular weight for lysine-modified versus carbohydrate-modified anti-GAD. There was no difference in the calculated loss in binding with either curve-fitting method used, as seen in Table 2. The binding affinity of anti-GAD to anti-mouse IgG (H+L) antibodies are very close, regardless of molecular weight, when the carbohydrate is used for conjugation. However, when the lysines are used for conjugation, the binding affinity of PEG20000-modified anti-GAD is reduced about 21% with respect to PEG5000-modified anti-GAD.

DISCUSSION

To accurately characterize PEG-modified antibodies, it is important to confirm the PEG modification and to accurately quantify its conjugation. In most studies using the lysine groups for conjugation, the degree of PEG modification is typically measured by a TNBS assay. However, in the case of low modification ratios where the overall number of lysines per molecule are high, this method can be unreliable. For example, at reaction feed ratios (PEG:antibody) below 20:1, the TNBS method shows a conjugation efficiency of 0–40%. Above 20:1, the conjugation efficiency ranges between 62 and 105%, with an average of 84%. Thus, in this case, where the desired modification ratios are below 20:1, a better method for determining this ratio is needed.

Using a polyclonal rabbit IgG as a model antibody system, an ¹H NMR method for quantitating the conjugated PEG was developed. The principle of the method relies on the strong singlet peak of PEG at 3.54 ppm. As seen in Figure 2, the peaks from the antibody protein

**Figure 10.** A schematic structural representation of a typical IgG antibody.

itself could not be used to quantify the PEG peak, since the protein did not exhibit a strong enough peak above the baseline to integrate when compared to the PEG peak. Thus, standards were added to the PEG–antibody solution at known concentrations. In this manner, the peaks of the standards could be used to calculate the concentration of the PEG. The standards acetone (2.06 ppm), DMSO (2.56 ppm), and sodium benzoate (7.27–7.73 ppm) were chosen such that their peaks would not interfere with the PEG peak. As seen in Figure 2, the reference peaks are easily resolved from the PEG peak.

The amount of each reference sample added was calculated based on the expected amount of PEG in the solution. In this manner, the actual amount of PEG is simply the integration of the PEG peak divided by the average of the integration of the reference peaks multiplied by the expected amount of PEG.

As seen in Figure 3, the results of the ¹H NMR analysis of the PEG5000-modified polyclonal rabbit IgG show ratios very close to those calculated using the TNBS method. When a linear curve fit was applied, a slope of 0.73 was obtained with an R^2 of 0.996, indicating a very good fit. Thus, the average conjugation efficiency as calculated from the NMR measurements is 73% which was equally valid at low modifications up to relatively high modifications.

The other great advantage of this technique is that it is a direct measure of the PEG in solution and thus can be applied to PEG-modified proteins regardless of the conjugation method employed. In this case, we successfully used this method to calculate the PEG:antibody ratios for lysine-modified and carbohydrate-modified antibodies.

As seen in Figure 10, IgG antibodies are comprised of four chains: two identical chains of about 50000 Da and two identical chains of about 25000 Da, known as the heavy and light chains, respectively. The four chains associate to form a rough T or Y configuration. The antibody structure is further labeled as having an Fc and Fab segments. The Fc represents the stem of the T or Y, and the Fab represents the two arms of the T or Y and contains the antigen region at the ends of the arm. Each arm of the antibody contains two domains of antiparallel β -sheets. Since, the antibody is comprised almost entirely of the β -sheet secondary structure element, CD spectroscopy can be easily used to measure perturbations in the antibody structure.

As can be seen in Figure 4, the shape of the CD spectra curves closely follow that expected for a β -sheet (37), as seen by the symmetric trough between 210 and 230 nm. At all modification ratios, there is a clear predominance of the β -sheet conformation. There does not appear to be any trend toward a lessening of the β -sheet structure as the modification ratio is increased. Even at its highest modification, at 57 PEG5000 chains per antibody, there is no noticeable change in the trough depth or shape. This indicates that the antibody structure is quite stable and resistant to change upon PEG modification.

The function of antibodies is to bind to antigens. Thus, it is critical that the binding of a modified antibody to retain as much binding activity as possible. In this report, we investigated the effect of PEG on the binding of anti-GAD, a monoclonal antibody against GAD. We investigated the influence of the reaction conditions, the degree of modification, the molecular weight of PEG, and the site of conjugation on the binding activity.

When the lysines are used for PEG conjugation, the modification pH affects the reactivity of the primary amine on the lysine side chain. The pK_a of the ϵ -amine of a lysine in a protein can be expected to be between 9 and 10, whereas, the N-terminal amine of a protein chain can be expected to have a pK_a of around 8 (41). When the solution/reaction pH is equal to the pK_a of an amine, there is an equal number of protonated and unprotonated amines. Thus, since the pK_a of the side-chain amine of the lysines is expected to be between 9 and 10, there should be between 40 and 45 unprotonated amines per antibody, based on 86 lysines and four N-terminal amines, when the solution pH is between 9 and 10. In order for a primary amine to undergo a reaction, it must be unprotonated. Therefore, the pH should be elevated to promote the reaction at the lysines.

At a pH between 9 and 10, there should be approximately 10 reactive lysines for every reactive N-terminal-amine, since there are four N-terminal amines per antibody. Thus, reactions at lysine residues are more likely than at the N-terminal amines at an elevated pH. At a neutral reaction pH, the reaction of the lysines will be much slower. At pH 7.4, the number of unprotonated lysine amines will be approximately 100 times lower than at pH 9.4. However, since the pK_a of the N-terminal amines is about 8, there should still be an unprotonated N-terminal amine at pH 7.4. Thus, we can expect the reactive-lysine:N-terminal-amine ratio to be closer to 1:1. Thus, at pH 7.4, reactions at the N-terminal amines will be equally likely as the lysine ϵ -amines. Thus, it can be expected that N-terminal amines will be modified in addition to lysine amines at a low pH, but at higher pH's, there will many fewer N-terminal amines modified at a pH greater than 9.

The antigen combining region of antibodies is located proximal to the N-terminal amines, and thus a reaction of an N-terminal amine would introduce a massive steric impedance to antigen binding. This is demonstrated in Figure 5. Based on the curve fits, the calculated binding constants show a loss of about 73% of its activity or 81% of its affinity of the pH 7.4-modified with respect to the pH 9.2-modified anti-GAD. Thus, as expected, it appears likely that modification at a low pH will modify an N-terminal amine. Assuming that the loss is due to modified N-terminal amines, this would indicate a loss of activity. According to the binding activity calculations, this would indicate that at least 3 in 8 overall (1 to 2 per antibody) N-terminal amines are modified when a reaction pH 7.4 is used.

When the lysines are used to conjugate the PEG, the PEG chains appear to be randomly attached throughout the antibody. As the modification ratio is increased, the coverage of the antibody by PEG chains will increase. This applies to the area near the antigen combining region as well. Attachment of PEG chains near the antigen combining region will increase the steric hindrance to antigen binding. This can be seen in Figure 6. When the number of PEG chains attached is increased from 5 to 20 per antibody, the binding activity decreases. The specific loss in binding can be approximated by applying a curve fit to the data. When this is done, the loss is approximately 55, 68, and 79% in affinity or 48, 60, and 72% in activity for 5:1, 10:1, and 20:1 modified anti-GAD, respectively.

The carbohydrate moiety of IgG antibodies is located in the Fc region near the hinge of the antibody which is distal to the antigen combining site. Thus, when PEG was conjugated through the carbohydrate, there is a localized attachment of the PEG with minimal interference to the antigen binding. As seen in Figure 7, carbohydrate-modified anti-GAD showed a much higher binding affinity to GAD than lysine-modified anti-GAD. Based on the curve fit, the lysine-modified shows an approximate 30–60% loss of binding affinity with respect to carbohydrate-modified anti-GAD.

The molecular weight of the PEG also has an effect on antigen binding. The effect of PEG with molecular weights of 5000 and 20000 Da was studied. Anti-GAD was modified through the lysine as well as through the carbohydrate with the two molecular weights. The degree of modification was kept between three and five PEG chains per anti-GAD. As seen in Figure 7, the PEG20000 has a greater effect on binding than PEG5000. On the basis of the calculated constants, there is a loss of 40% (carbohydrate-modified) to 73% (lysine-modified) in binding affinity or 20% (carbohydrate-modified) to 40% (lysine-modified) in binding activity of PEG20000-modified anti-GAD with respect to PEG5000-modified anti-GAD.

Binding of modified anti-GAD to antibodies against mouse antibody was studied. These anti-mouse antibodies are polyclonal and are directed against epitopes on both the heavy and light mouse IgG chains. Thus, these antibodies will bind to many sites throughout the anti-GAD protein. This can be a measure of the ability of the PEG at preventing nonspecific-type binding to the anti-GAD. As seen in Figure 8, the degree of modification affects the binding to anti-mouse antibodies when the PEG is conjugated to lysines. A loss of about 16% can be seen in the 5:1 modified sample, and a loss of about 25% can be seen in the 10:1 modified sample. This clearly demonstrates the effect of the greater coverage by the PEG at higher modification ratios. The effect of PEG molecular weight and site of modification can be seen in Figure 9. As expected, the carbohydrate conjugated anti-GAD-PEG show higher binding than the lysine-conjugated anti-GAD-PEG samples. Both the PEG5000 and PEG20000 carbohydrate-modified samples are very close to the PEG5000-modified lysine sample. The molecular weight does not seem to have as much of an effect on the binding to anti-mouse antibodies as it does with the lysine-modified samples. The PEG20000 lysine-modified anti-GAD shows a loss of about 21% of the binding activity toward anti-mouse antibodies with respect to PEG5000-modified anti-GAD. This is in contrast to the carbohydrate-modified anti-GAD, which shows only a small decrease of about 5%, at most, of the PEG20000 versus PEG5000-modified anti-GAD.

These effects can be easily explained by the localization of the PEG at the carbohydrate for the carbohydrate-modified samples. This gives a lower overall coverage of the anti-GAD by the PEG than can be achieved with the lysine-conjugated samples. However, the 95% confidence error bars are relatively wide in these figures, and thus, while differences and trends can be seen, the precision of the calculated numbers may not be reflective of actual values, especially in the carbohydrate- and lysine-PEG5000 curves in Figure 9.

Binding is a function of the degree of modification. The fewer the number of PEG polymer chains attached to the antibody, the more its binding is preserved. Binding is a function of modification pH when the lysine residues are used to couple the PEG. Modification at higher pH values leads to a greater preservation of binding. This is most likely due to the lower pK_a value of the N-termini amino groups. Their lower pK_a values suggest that they will more likely be modified at a lower pH than the lysine ϵ -amine. Since the N-termini are proximal to the binding site on the antibody, their modification would be detrimental to binding to the antigen. Binding is a function of modification site. The carbohydrate-modified anti-GAD shows a much greater binding than the lysine-modified anti-GAD due to the localized manner in which the PEG is attached to the carbohydrate of the antibody.

ACKNOWLEDGMENT

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Sequence-Specific DNA Cleavage by Dipeptides Disubstituted with Chlorambucil and 2,6-Dimethoxyhydroquinone-3-mercaptoacetic Acid

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Two dipeptides, each containing a lysyl residue, were disubstituted with chlorambucil (CLB) and 2,6-dimethoxyhydroquinone-3-mercaptoacetic acid (DMQ-MA): DMQ-MA-Lys(CLB)-Gly-NH₂ (DM-KCG) and DMQ-MA-β-Ala-Lys(CLB)-NH₂ (DM-BKC). These peptide–drug conjugates were designed to investigate sequence-specificity of DNA cleavage directed by the proximity effect of the DNA cleavage chromophore (DMQ-MA) situated close to the alkylating agent (CLB) inside a dipeptide moiety. Agarose electrophoresis studies showed that DM-KCG and DM-BKC possess significant DNA nicking activity toward supercoiled DNA whereas CLB and its dipeptide conjugate Boc-Lys(CLB)-Gly-NH₂ display little DNA nicking activity. ESR studies of DMQ-MA and DM-KCG both showed five hyperfine signals centered at $g = 2.0052$ and are assigned to four radical forms at equilibrium, which may give rise to a semiquinone radical responsible for DNA cleavage. Thermal cleavage studies at 90 °C on a 265-mer test DNA fragment showed that besides alkylation and cleavage at G residues, reactions with DM-KCG and DM-BKC show a preference for A residues with the sequence pattern: 5'-G-(A)_n-Pur-3' > 5'-Pyr-(A)_n-Pyr-3' (where $n = 2-4$). By contrast, DNA alkylation and cleavage by CLB occurs at most G and A residues with less sequence selectivity than seen with DM-KCG and DM-BKC. Thermal cleavage studies using N7-deazaG and N7-deazaA-substituted DNA showed that strong alkylation and cleavage at A residues by DM-KCG and DM-BKC is usually flanked on the 3' side by a G residue whereas strong cleavage at G residues is flanked by at least one purine residue on either the 5' or 3' side. At 65 °C, it is notable that the preferred DNA cleavage by DM-KCG and DM-BKC at A residues is significantly more marked than for G residues in the 265-mer DNA; the strongest sites of A-specific reaction occur within the sequences 5'-Pyr-(A)_n-Pyr-3'; 5'-Pur-(A)_n-G-3' and 5'-Pyr-(A)_n-G-3'. In pG4 DNA, cleavage by DM-KCG and DM-BKC is much greater than that by CLB at room temperature and at 65 °C. It was also observed that DM-KCG and DM-BKC cleaved at certain pyrimidine residues: C40, T66, C32, T34, and C36. These cleavages were also sequence selective since the susceptible pyrimidine residues were flanked by two purine residues on both the 5' and 3' sides or by a guanine residue on the 5' side. These findings strongly support the proposal that once the drug molecule is positioned so as to permit alkylation by the CLB moiety, the DMQ-MA moiety is held close to the alkylation site, resulting in markedly enhanced sequence-specific cleavage.

INTRODUCTION

Alkylating agents such as chlorambucil (CLB) have been widely used in clinical practice against chronic lymphocytic leukemia for more than 30 years (2). Chlorambucil is also widely used clinically against lymphoma, Hodgkin's disease, and testicular cancer and is effective against other types of cancer in combination therapy (3–9). Chlorambucil, melphalan, and other alkylating agents are known to alkylate DNA preferentially at the N7 position of guanine nucleotides, resulting in intra- or interstrand cross-linking of the double helix and thus interfering with DNA replication (10). In a recent paper we reported the DNA binding and sequence specificity of some cyclic peptide–chlorambucil conjugates (11). Apart from DNA alkylation at the guanine-N7 position, no propensity to cause DNA cleavage was observed with these cyclic peptide conjugates. They displayed similar sequence specificity as regards DNA alkylation to that

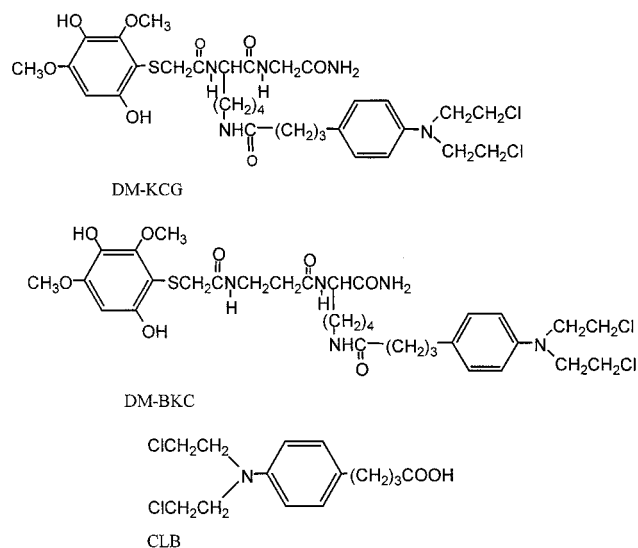
seen with CLB alone. The specificity of alkylation for both CLB and its peptide conjugates appears to be 5'-Pyr-G-Pur-3' > 5'-Pyr-G-Pyr-3' > 5'-Pur-G-Pyr-3' = 5'-Pur-G-Pur-3'. However, there are some apparent differences in the selectivity of alkylation between the peptide derivatives and CLB at certain guanine-containing sequences. We concluded that the spacing of the two CLB moieties in these cyclic peptides can impose certain sequence selectivity over and above that of CLB (11).

The design and synthesis of sequence-specific DNA cleaving agents has been and remains an important research topic in chemistry and molecular biology. Various agents have been reported to have sequence-specific DNA cleaving ability, including antibiotics, triple-helix forming oligonucleotides, and peptides (12–19). In this work we report the synthesis and DNA cleavage properties of two dipeptides containing a lysyl residue disubstituted at the ε-amino and α-amino positions, respectively, with chlorambucil (CLB), and 2,6-dimethoxyhydroquinone-3-mercaptoacetic acid (DMQ-MA, **1**), which possesses significant DNA cleaving activity (23). Two disubstituted dipeptides were synthesized: DMQ-MA-Lys-

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Chart 1^a

^a Structures of DM-KCG, DM-BKC, and CLB.

(CLB)-Gly-NH₂ (DM-KCG) and DMQ-MA-β-Ala-Lys(CL B)-NH₂ (DM-BKC) (Chart 1). The peptide conjugates were designed such that the average intramolecular separation between the CLB and DMQ-MA groups in DM-BKC is greater than that of DM-KCG. We anticipated that the spacing between the two cytotoxic groups might impose certain differences in binding affinity and alkylation sequence specificity on DNA. Previous reports have shown that DMQ-MA and its derivatives can nick supercoiled DNA efficiently in the absence of L-ascorbic acid, suggesting that radicals such as the semiquinone might be involved (23). Radicals such as the semiquinone and hydroxyl radicals are potent biological oxidants and can readily attack DNA. We envisaged that once the CLB moiety of the dipeptide derivative is positioned suitably to alkylate the N7 position of a guanine nucleotide, the DMQ-MA group would lie in close proximity to the DNA backbone, thus facilitating the cleavage of the strand/strands by various forms of radicals. These considerations led us to investigate the efficiency of DNA cleavage and DNA sequence specificity directed by the proximity effect of the CLB and DMQ-MA moieties in this series of peptide–drug conjugates.

EXPERIMENTAL SECTION

Drugs. Chlorambucil, DM-KCG, DM-BKC, and DMQ-MA were dissolved in methanol to yield a 1 mM stock solution. Appropriate concentrations of drugs were made by dilution with phosphate-buffered saline (PBS, pH 7.4).

Chemicals. All of the protected amino acid derivatives were purchased from Bachem California (Torrance, CA) and Sigma Chemical Co. (St. Louis, MO) or synthesized in our laboratory according to published procedures. Ammonium persulfate, tris base, acrylamide, bis-acrylamide, ultrapure urea, boric acid, sodium citrate, tetramethylethylenediamine, and dimethyl sulfate were from BDH. X-ray film was from Fuji Photo Film Co. Ltd. Formic acid, piperidine, and formamide were from Aldrich. Bromophenol blue and xylene cyanol were from Sigma. Melting points were determined on a Mel-Temp apparatus (Cambridge, MA) and were uncorrected. Medium-pressure column chromatography was performed using Merck 230–400 mesh silica gel. TLC was per-

formed on Merck silica gel 60-coated aluminum sheets. Optical rotations were determined on a Rudolph Autopol II instrument. Low-resolution and high-resolution mass spectra were taken from a JEOL JMX-HX 110 instrument operating in the FAB mode. Elemental analyses were performed at the Chemistry Department, Cheng-Kung University.

Biochemicals. Radiolabeled nucleoside triphosphates, [γ -³²P]ATP and [α -³²P]dATP, were obtained from NEN Life Science Products at a specific activity of 6000 Ci/mmol. Taq polymerase (Promega), T4 polynucleotide kinase (Promega), AMV reverse transcriptase (Promega), and the restriction endonucleases EcoRI (Roche) and PvuII (New England Biolabs) were used according to the supplier's recommended protocol in the activity buffer provided. The primers, 5' AACAGCTATGACCATG (M13/pUC reverse sequencing primer) or 5' GTTTTCCCACTCAGGAC (M13/pUC forward sequencing primer) bearing a 5'-OH were obtained from the Department of Biochemistry, University of Cambridge, England. The pG4 template was a kind gift of Dr. N. E. Møllegaard (Center for Biomolecular Recognition, Department of Medical Biochemistry and Genetics, The Panum Institute, University of Copenhagen, Denmark). All other chemicals were analytical grade reagents, and all solutions were prepared using doubly deionized, Millipore-filtered water.

Boc-Lys(Z)-Gly-NH₂ 2. This compound was prepared by coupling Boc-Lys(Z) (1.0 g, 2.63 mmol) with glycylamide (0.43 g, 2.63 mmol) by the DCC/HOBt method in DMF at pH 7.5. After sonicating at room temperature for 2 h, the reaction mixture was filtered and the filtrate evaporated in vacuo. The residue was redissolved in EtOAc and washed successively with citric acid (10%, 3 × 10 mL), saturated NaHCO₃ (3 × 10 mL), and water (3 × 10 mL), dried (MgSO₄), and evaporated in vacuo to give a white solid. Medium-pressure chromatography using stepwise elution (CHCl₃/MeOH, 100:0, 99:1, 98:2, 97:3, 96:4, 95:5, 94:6) afforded a white solid (1.0 g 88%). TLC [(CHCl₃/MeOH, 90:10), *R_f* 0.45; mp 102–103 °C; [α]_D³⁰ –7.5 (MeOH/CHCl₃, 1:1, *c* 0.03). Anal. (C₂₁H₃₂N₄O₆·0.5 H₂O) C, H, N.

Boc-Lys(CL B)-Gly-NH₂ 3. This compound was prepared by coupling CLB-Pfp with the TFA salt of dipeptide 2 in DMF at pH 7.5. Medium-pressure chromatography using stepwise elution (CHCl₃/MeOH, 100:0, 99:1, 98:2, 97:3, 96:4, 95:5, 94:6) afforded a white solid (0.77 g 82%). TLC [(CHCl₃/MeOH, 90:10), *R_f* 0.52; mp 82–83 °C; [α]_D³⁰ –10.8 (MeOH/CHCl₃, 1:1, *c* 0.03). Anal. (C₂₇H₄₃N₅O₅Cl₂·H₂O) C, H, N.

DMQ-MA-Lys(CL B)-Gly-NH₂ 4. The dipeptide 3 was deblocked with TFA/CH₂Cl₂ and reacted with DMQ-MA-Pfp in DMF at pH 7.0 by sonication for 2 h. The reaction mixture was evaporated in vacuo to give a crude brown solid. Medium-pressure chromatography using stepwise elution (CHCl₃/MeOH, 100:0, 99:1, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7) afforded an orange solid (0.33 g 65%). TLC [(CHCl₃/MeOH, 90:10), *R_f* 0.51; mp 81–82 °C; [α]_D³⁰ –17.3 (MeOH/CHCl₃, 1:1, *c* 0.03). HRFABMS 730.2437 [MH]⁺.

Boc-β-Ala-Lys(Z)-NH₂ 5. This compound was prepared by coupling Boc-β-Ala (0.49 g, 2.6 mmol) with the TFA salt of Boc-Lys(Z)-NH₂ (1.0 g, 2.6 mmol) by the DCC/HOBt method in DMF at pH 7.5. After sonicating at room temperature for 2 h, the reaction mixture was filtered and the filtrate evaporated in vacuo. The residue was redissolved in EtOAc and washed successively with citric acid (10%, 3 × 10 mL), saturated NaHCO₃ (3 × 10 mL), and water (3 × 10 mL), dried (MgSO₄), and evaporated in vacuo to give a white solid. Medium-pressure chro-

matography using stepwise elution (CHCl₃/MeOH, 100:0, 99:1, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7) afforded a white solid (1.0 g 86%). TLC [(CHCl₃/MeOH, 90:10), *R_f* 0.46; mp 120–122 °C; [α]_D³⁰ –11.02 (MeOH/CHCl₃, 1:1, *c* 0.03). Anal. (C₂₂H₃₄N₄O₆) C, H, N.

Boc- β -Ala-Lys(CLB)-NH₂ **6**. This compound was prepared by coupling CLB-Pfp with the TFA salt of dipeptide **5** in DMF at pH 7.5. Medium-pressure chromatography using stepwise elution (CHCl₃/MeOH, 100:0, 99:1, 98:2, 97:3, 96:4, 95:5) afforded a white solid (0.59 g 78%). TLC [(CHCl₃/MeOH, 90:10), *R_f* 0.56; mp 136–137 °C; [α]_D³⁰ –37.73 (MeOH/CHCl₃, 1:1, *c* 0.03). Anal. (C₂₈H₄₅N₅O₅-Cl₂) C, H, N.

DMQ-MA- β -Ala-Lys(CLB)-NH₂ **7**. The dipeptide **6** was deblocked with TFA/CH₂Cl₂ and reacted with DMQ-MA-Pfp in DMF at pH 7.0 by sonication for 2 h. The reaction mixture was evaporated in vacuo to give a crude brown solid. Medium-pressure chromatography using stepwise elution (CHCl₃/MeOH, 100:0, 99:1, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7) afforded an orange solid (0.195 g 63%). TLC [(CHCl₃/MeOH, 90:10), *R_f* 0.62; mp 102–104 °C; [α]_D³⁰ –10.03 (MeOH/CHCl₃, 1:1, *c* 0.03). HRFABMS 744.2533 [MH]⁺.

Electron Spin Resonance Spectroscopy Studies. The ESR spectra were obtained on a Bruker EMX-10 EPR spectrometer; microwave power 20 mW; modulation frequency 100 kHz; sweep time 4.0 G/min; receiver gain 8.93×10^5 ; time constant 2.62 s. Drugs were dissolved in 0.5X PBS (containing 2.5 μ M ferrous sulfate) at a final concentration of 40 μ M in a volume of 0.4 mL. The spectrum of DMQ-MA was taken without UV irradiation whereas that of DMQ-KCG was taken by irradiating with a 500 W mercury lamp.

Preparation, Purification, and Labeling of DNA Fragments. Plasmid pBluescript was isolated from *E. coli* by a standard sodium dodecyl sulfate–sodium hydroxide lysis procedure and purified by banding in CsCl–ethidium bromide gradients. Ethidium bromide was removed by several 2-propanol extractions followed by exhaustive dialysis against Tris–EDTA buffer. The purified plasmid was then precipitated and resuspended in appropriate buffer prior to digestion with *Eco*RI and *Pvu*II. After precipitation, the DNA was resuspended in AMV reverse transcriptase buffer. The DNA was then 3'-end labeled with [α -³²P]dATP in the presence of AMV reverse transcriptase. This yielded two appropriately sized fragments, a 117-mer and a 265-mer which were then separated on a 6% polyacrylamide gel. After electrophoresis for about 90 min, the gel was exposed to X-ray film for about 5 min. After developing the film, the bands were located, excised, crushed, and soaked overnight in TN buffer (pH 7.0) at 37 °C. This suspension was filtered through a Millipore 0.22 μ m filter, and the DNA was precipitated with ethanol. Following washing with 70% ethanol and vacuum-drying of the precipitate, the purified DNA was resuspended in TN buffer (pH 7.0) to 4800 Cerenkov cpm/ μ L.

Primer Labeling. The 5'-OH M13/pUC reverse sequencing primer was 5'-end labeled with [γ -³²P]ATP in the presence of T4 polynucleotide kinase.

Polymerase Chain Reaction (PCR). The protocol used to incorporate N7 deazaadenosine and/or N7 deazaguanosine residues into DNA was comparable to that previously described (25). PCR reaction mixtures contained 10 ng of pG4 template, 0.1 μ M of the M13/pUC forward sequencing 5'-OH primer and M13/pUC reverse sequencing 5'-³²P primer, 250 μ M of each appropriate dNTP (dATP or N⁷dATP, dGTP or N⁷dGTP, dCTP, dTTP according to the desired DNA), and 1 unit of *Taq*

polymerase in a volume of 50 μ L containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.1% Triton X-100, and 1.5 mM MgCl₂. To prevent unwanted primer–template annealing before the cycles began, the reactions were heated to 60 °C before adding the *Taq* polymerase. After an initial denaturing step of 3 min at 94 °C, 30 amplification cycles were performed. Parallel reactions were pooled and several extractions with water-saturated *n*-butanol were performed to reduce the volume prior to loading the samples on to a 6% nondenaturing polyacrylamide gel. After electrophoresis for about 90 min, the DNA was isolated as described for the 3'-end labeled DNA.

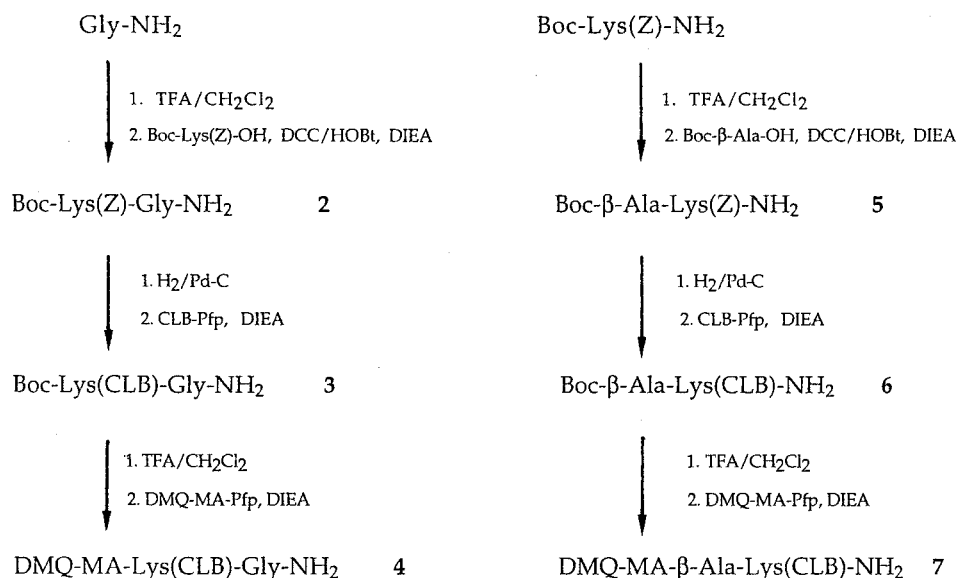
Alkylation Studies. 1 μ L of the labeled DNA fragment (117-mer, 265-mer or pG4) was added to 20 μ L of the appropriate concentration of drug dissolved in 10 μ L of PBS (pH 7.4). The samples were then incubated for 2 h at 37 °C, ethanol-precipitated, washed, and resuspended in the appropriate solvent for the assay.

Thermal Cleavage Assay. To determine whether the N3 or N7 of adenines or guanines were alkylated, the samples were resuspended in 100 μ L of 1.5 mM sodium citrate buffer containing 15 mM NaCl (pH 7.2), heated to 90 °C for 30 min, and then cooled on ice. The samples were then ethanol-precipitated. After a further ethanol precipitation, the samples were lyophilized and resuspended in 80% formamide containing tracking dyes, heated at 90 °C for 4 min, and then cooled on ice.

DNA Cleavage at Lower Temperatures in the Presence and Absence of Ferrous Ions. To determine the influence that the presence of iron(II) had on the level of cleavage by the compounds, the samples were resuspended in PBS containing 10 μ M ferrous sulfate solution. They were heated at 65 °C or room temperature for 30 min or for specified periods of time and then ethanol-precipitated. After cleavage treatments, the samples were lyophilized, washed twice with 50 μ L of water, lyophilized again, and finally resuspended in 4 μ L of an 80% formamide solution containing tracking dyes. Samples were heated at 90 °C for 4 min and chilled in ice for 4 min prior to electrophoresis.

Electrophoresis and Autoradiography. The products of DNA cleavage were resolved by polyacrylamide gel electrophoresis under denaturing conditions (0.3 mm thick, 8% acrylamide containing 8 M urea). After electrophoresis (about 2.5 h at 60 W, 1600 V in TBE buffer, BRL sequencer model S2), gels were soaked in 10% acetic acid for 15 min, transferred to Whatman 3MM paper, dried under vacuum at 80 °C, and placed against a Molecular Dynamics 425E PhosphorImager storage screen overnight (24). Baseline-corrected scans were analyzed by integrating all the densities between two selected boundaries using ImageQuant for the Macintosh version 1.2 software. Each resolved band was assigned to a particular bond within the DNA fragment by comparison of its position relative to sequencing standards. For the 5'-end labeled pG4 fragments, G and G+A tracks were generated by treatment of the radiolabeled DNA with dimethyl sulfate and formic acid, respectively, followed by piperidine-induced cleavage at the sites of reaction (11). For the 3'-end labeled 117-mer and 265-mer DNA fragments, G+A tracks were made. Briefly, to 2 μ L of the treated DNA were added 20 μ L of water and 5 μ L of an 80% formamide solution containing tracking dyes. The sample was then heated at 90 °C for 45 min with the Eppendorf tube cap opened, cooled on ice for 4 min, and then loaded on the gel.

Scheme 1



RESULTS AND DISCUSSION

A. Synthesis of DMQ-MA-Lys(CLB)-Gly-NH₂ (DM-KCG) and DMQ-MA-β-Ala-Lys(CLB)-NH₂ (DM-BKC).

The strategy for the synthesis of CLB and DMQ-MA disubstituted dipeptides is shown in Scheme 1. The dipeptides **2** and **5** were synthesized by coupling the corresponding protected amino acids using the DCC/HOBt procedure. For the synthesis of the dipeptide derivatives **3** and **6**, we avoided conjugating the DMQ-MA moiety to the dipeptide in the first step since the DMQ-MA moiety is prone to decomposition during the removal of the lysyl ϵ -amino Z protecting group of the dipeptides by catalytic hydrogenation or by treatment with HBr/HOAc in the following step. Thus, the lysyl Z protecting group of the dipeptides **2** and **5** were deblocked by catalytic hydrogenation, and the CLB moiety was then conjugated using DCC/HOBt. Subsequent removal of the α -Boc protecting groups of **3** and **6** by TFA followed by acylation with DMQ-MA pentafluorophenyl ester at neutral pH afforded the target peptides **4** and **7**.

B. Agarose Gel Electrophoresis and ESR Studies.

In common with other DMQ-MA conjugates, the dipeptide conjugates DM-BKC and DM-KCG brought about efficient nicking of supercoiled DNA whereas chlorambucil produced little DNA cleavage even at 100 μ M. The DNA nicking effects of DM-KCG and CLB on pBluescript SKII plasmid are illustrated in Figure 1. Similar results were obtained with DM-BKC (not shown). It is interesting to note that DM-KCG and DM-BKC can quite efficiently nick supercoiled DNA in the absence of ferrous ions and L-ascorbic acid. However, ferrous ions exert little dose-dependent enhancement of DNA nicking induced by either of the drugs, although addition of Fe²⁺ ions alone often produced a significant (though rather variable) amount of background nicking.

Since the generation of hydroxyl radicals by DMQ-MA and its conjugates in the absence of L-ascorbic acid is very slight (21, 22), we reasoned that other radicals such as the semiquinone could be responsible for the DNA nicking process. ESR studies of DMQ-MA in PBS showed five hyperfine signals centered at $g = 2.0052$ (lower trace, Figure 2). Similarly, DM-KCG also showed identical ESR signals at $g = 2.0052$, but notably weaker (upper trace, Figure 2), due to its much lower aqueous solubility. It was also reported that 2,6-dimethoxybenzoquinone which

is much less soluble than its derivative DMQ-MA, has very weak ESR spectrum assigned to semiquinone signals (27). We propose that these five hyperfine signals of DMQ-MA and DM-KCG correspond to radical structures III and IV of the equilibrium and resonance forms shown in Figure 3. The semiquinone (radical structure II), being stabilized by four resonance forms, may be responsible for the DNA nicking process.

C. DNA Cleavage Studies. Thermal Cleavage Studies. This methodology detects alkylation sites and cleavage sites by incubating DNA with different concentrations of drug at 37 °C for 2 h and then incubating at 90 °C in 1.5 mM sodium citrate buffer containing 15 mM NaCl (pH 7.2) for 30 min (26). In the 117-mer DNA fragment, the DNA cleavage pattern of DM-KCG (Figure 4b) and DM-BKC (results not shown) was similar to that of CLB (Figure 4a). However, the intensity of the cleaved bands produced by both conjugate drugs was considerably greater than that of CLB. Besides cleavage at guanine residues, all three drugs also show a propensity to attack a consecutive sequence of four adenines: A42–A45 (Figure 4).

In the 265-mer DNA fragment once again all/most G residues show alkylation and cleavage. However, there are some indications that the substitutions cause the compounds to interact differently with DNA. For example, DM-KCG produces strong alkylation and cleavage at a consecutive sequence of three adenines: A53–A54–A55 whereas A56 was only relatively weakly cleaved. By contrast, CLB exhibits strong alkylation and cleavage at all four adenines: A53–A54–A55–A56 (Figure 4). Other notable sites of alkylation and cleavage by DM-KCG occur at positions A74, A86, A107–G108, A110–A111, whereas DNA alkylation and cleavage by DM-BKC was seen at positions A74–A75, A79, A81, A86, A92, A110–A111 (results not shown). Hence DM-BKC and DM-KCG seem to show a preference for A residues within the sequence pattern: 5'-Pur-(A)_n-G-3' > 5'-Pyr-(A)_n-Pyr-3' (where $n = 1-3$). This is in marked contrast to CLB which alkylates DNA at most G and A residues with little sequence specificity.

Thermal Cleavage Using N7-Deaza-guanosine DNA and N7-Deaza-adenosine DNA. Having established that the substitutions alter the sequence specificity of DM-KCG and DM-BKC, we decided to further probe

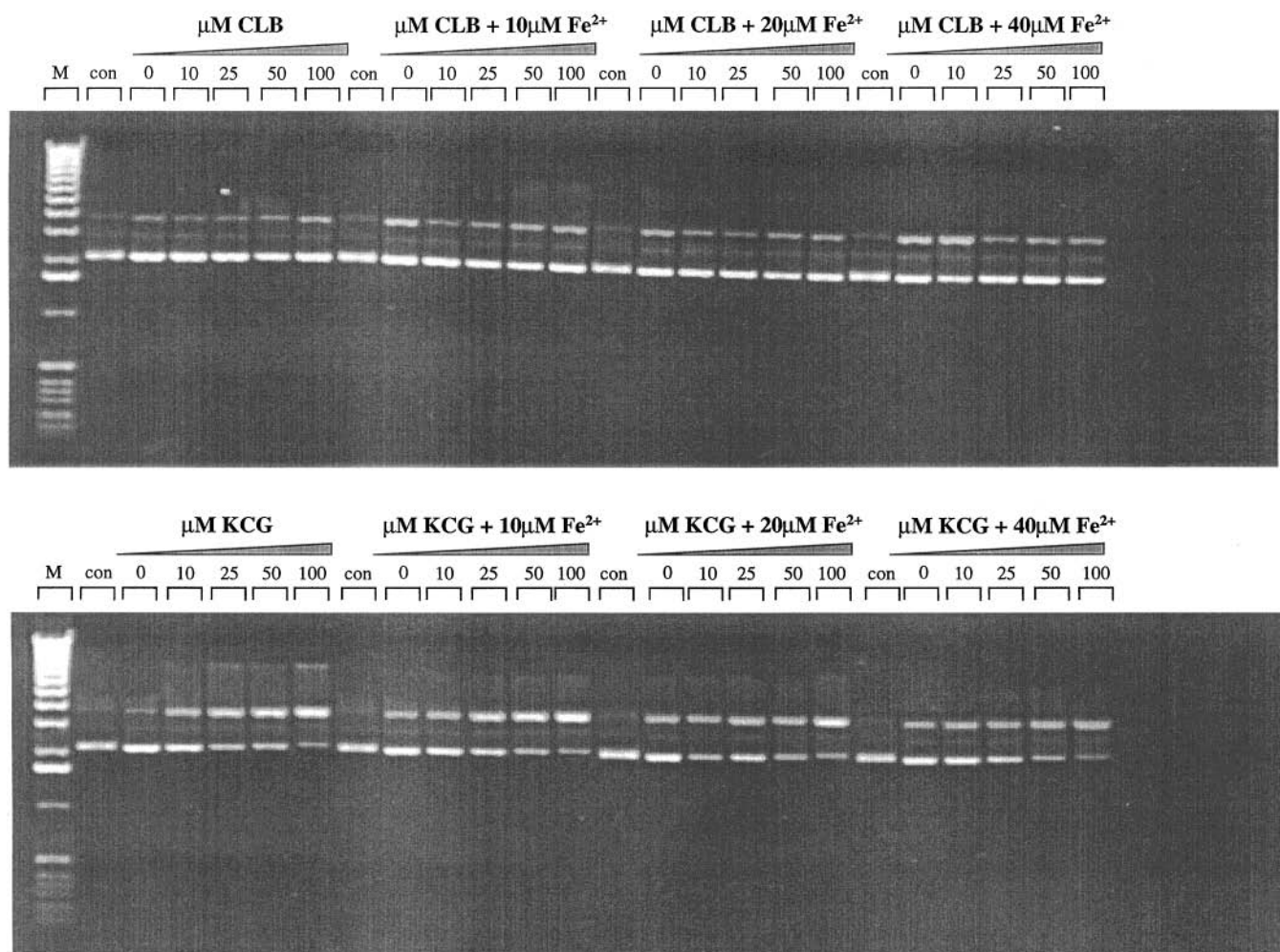


Figure 1. DNA cleavage of plasmid pBluescript DNA induced by DM-KCG and chlorambucil (CLB) in the presence or absence of ferrous ions. Samples were incubated at 37 °C for 10 min and then at 65 °C for 30 min. The upper band represents the open-circular form, and the lower band the supercoiled form.

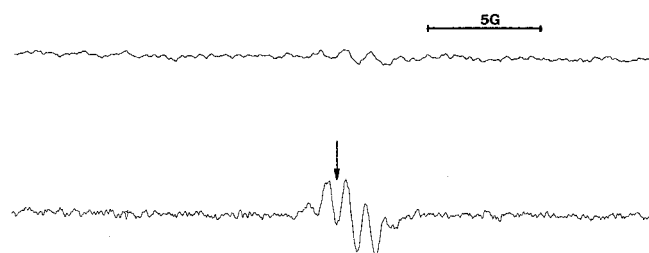


Figure 2. ESR spectra obtained by adding DMQ-MA to PBS (lower spectrum) or DM-KCG to PBS containing 2.5 μM ferrous sulfate (upper trace). The spectrum of DMQ-MA was taken without UV irradiation whereas that of DMQ-KCG was taken by irradiating with a 500 W mercury lamp. Microwave power 20 mW; modulation frequency 100 kHz; sweep time 3.6 G/min; receiver gain 8.93×10^5 ; time constant 2.62 s. $g = 2.0052$.

the molecular interactions involved by incorporating modified nucleotides into another DNA fragment, pG4. The pG4 DNA fragment was substituted with either N7-deaza-guanosine residues in place of guanositines or N7-deaza-adenosine residues in place of adenosine. When N7-deaza-guanosine residues were substituted in the DNA, all alkylation reactions at guanosine residues by DM-BKC (Figure 5) and DM-KCG (results not shown) were abolished. This indicates that the N7 position is crucial for alkylation at guanosine residues. Conversely, when N7-deaza-adenosine residues were substituted in the DNA, all of the bands corresponding to alkylation at

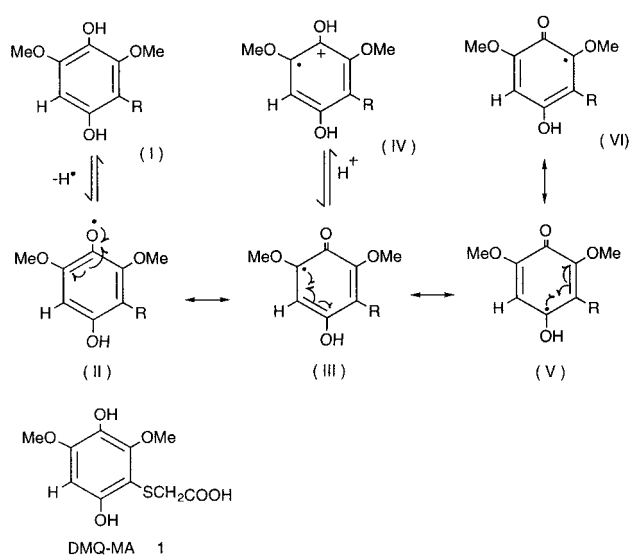


Figure 3. Proposed equilibrium and resonance forms of DMQ-MA or its conjugates in aqueous solution. R represents the side-chain of DMQ-MA or its conjugates.

adenosine residues disappeared, showing that the N7 position is required for alkylation at adenosine residues.

The alkylation and cleavage by DM-BKC (Figure 5) in N7-deazaG DNA shows a preference for A residues with the following sequence specificity: 5'-AAG-3' > 5'-CAG-

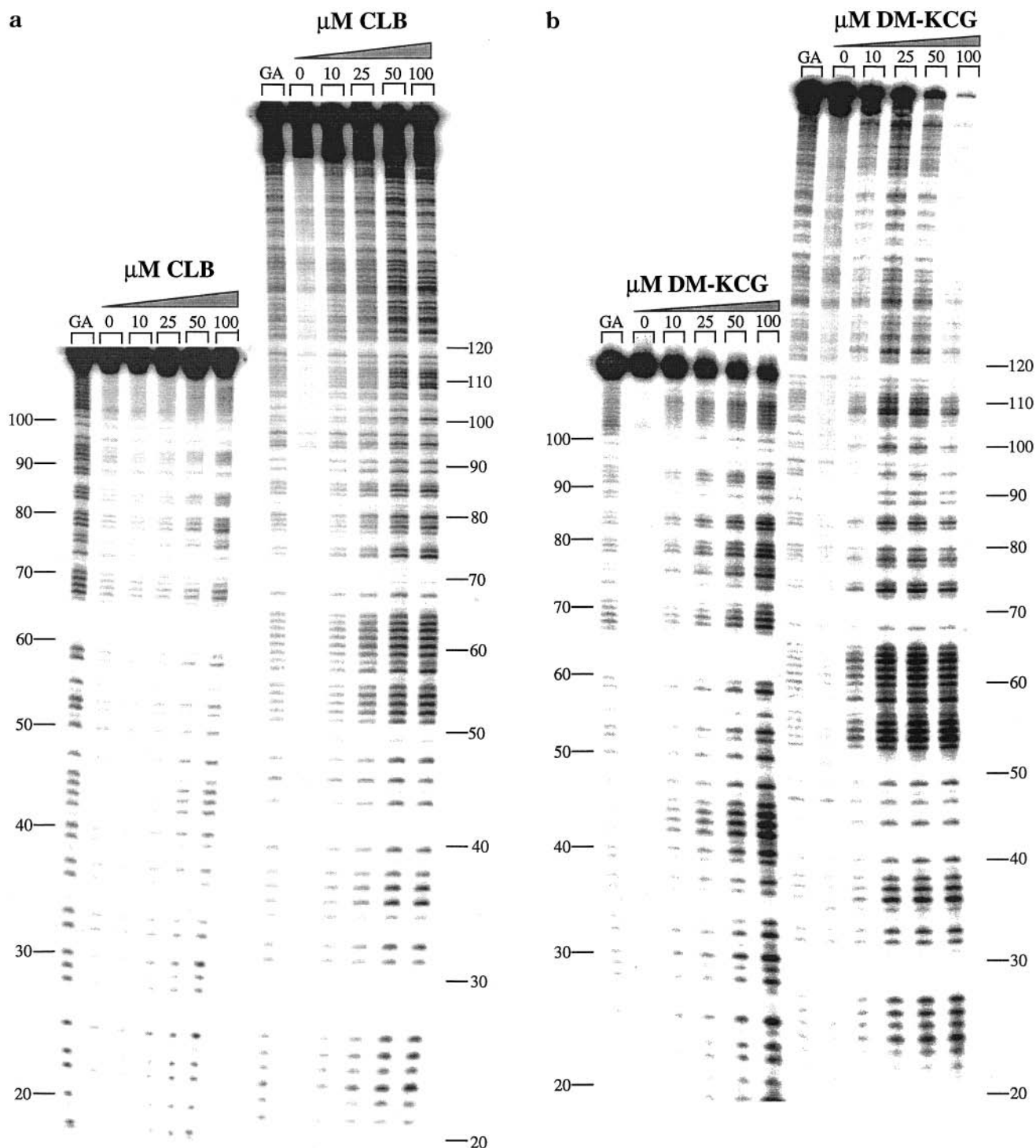


Figure 4. (a) Autoradiograph showing thermal cleavage of 117-mer or 265-mer DNA incubated with chlorambucil (CLB) at different drug concentrations for 2 h at 37 °C and then at 90 °C for 30 min in 1.5 mM sodium citrate buffer containing 15 mM NaCl (pH 7.2). GA represents the Maxam–Gilbert purine specific sequencing track. The autoradiograph on the right-hand panel and left-hand panel corresponds to the 265-mer and 117-mer DNA, respectively. (b) Autoradiograph showing thermal cleavage of 117-mer or 265-mer DNA incubated with DM-KCG under the same conditions.

3' > 5'-TAG-3' = 5'-GAG-3' = 5'-TAC-3' > 5'-CAA-3' > 5'-CAT-3' > 5'-GAC-3'. This result shows that strong alkylation and cleavage of A residues requires flanking at the 3' side by a G residue. However, since the 3' flanking G will be deazaG, alkylation at the N7 of G is not possible. Thus cleavage seen at an A residue is caused by alkylation of the drug at the same A residue or by, for example, the O6 of the 3' flanking G residue. By contrast, alkylation and cleavage by DM-BKC in N7-deazaA DNA shows a preference for G residues with the

following sequence specificity: 5'-AGG-3' = 5'-GGT-3' = 5'-AGG-3' > 5'-AGC-3' > 5'-AGA-3' = 5'-TGC-3' > 5'-CGC-3'. This indicates that strong alkylation and cleavage at G residues requires flanking by at least one purine residue at either the 5' or 3' side. Alkylation and cleavage by DM-KCG in N7-deazaG DNA was found to be similar to that seen for DM-BKC, showing a preference for A residues with the following sequence specificity: 5'-AAG-3' > 5'-TAG-3' = 5'-GAG-3' = 5'-CAG-3' > 5'-CAA-3' = 5'-TAC-3' >> 5'-GAC-3' > 5'-CAT-3'. Once more, strong

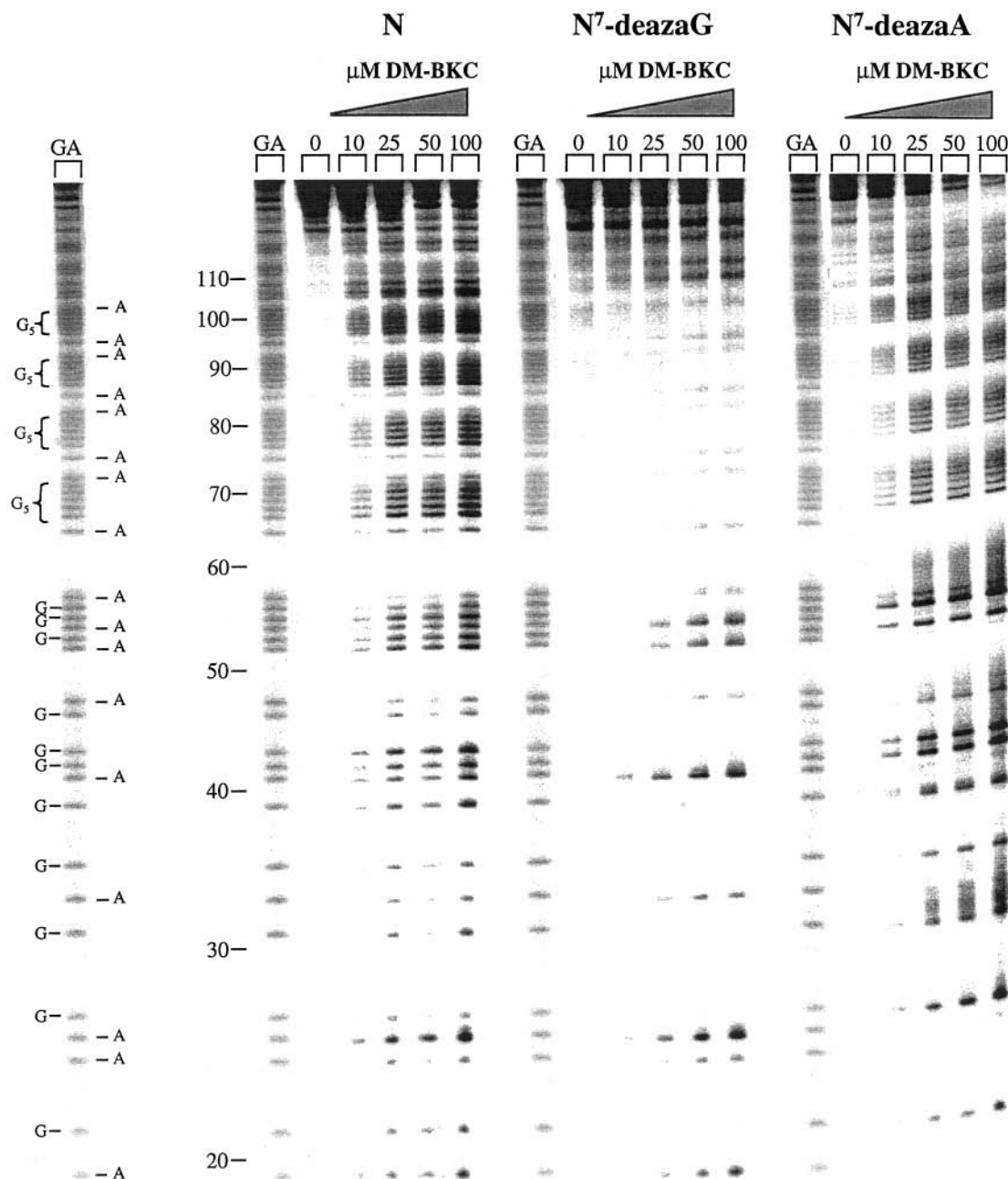


Figure 5. Autoradiograph showing thermal cleavage of pG4 DNA containing normal nucleotides, N7-deazaG for G (N7-deazaA DNA) or N7-deazaA for A (N7-deazaG DNA) in the presence of increasing concentrations of DM-BKC. The substituted DNA was incubated with different concentrations of DM-BKC for 2 h at 37 °C and then at 90 °C for 30 min in 1.5 mM sodium citrate buffer containing 15 mM NaCl (pH 7.2).

alkylation and cleavage at A residues requires flanking by a G residue at the 3' side. The rationale for this sequence specificity is similar to that described for DM-BKC. Moreover, alkylation and cleavage by DM-KCG in N7-deazaA DNA was also similar to that seen for DM-BKC (results not shown).

DNA Cleavage at Lower Temperatures in the Presence and Absence of Ferrous Ions. Since it is known that DMQ-MA and its peptide conjugates can nick DNA in the presence or absence of ferrous ions and that this can be influenced by the temperature (23), we monitored the time course of DNA cleavage by DM-KCG (Figure 6), DM-BKC, and CLB (results not shown) at 65 °C in the presence and absence of ferrous ions. The cleavage bands produced from the 265-mer DNA fragment by CLB were much weaker than those produced

by DM-KCG and DM-BKC both at room temperature and at 65 °C (results not shown).

Initial inspection of the autoradiograph (Figure 6) reveals that the presence of iron has little influence on either the specificity of the cleavage by DM-KCG (Figure 6) and DM-BKC (results not shown) or the magnitude of that cleavage. However, increasing the incubation time at 65 °C does appear to exert some effect on the level of cleavage by these compounds. The strongest bands cleaved in the 117-mer DNA by DM-KCG and DM-BKC were seen at positions A42-A43-A44-A45. Therefore DM-KCG and DM-BKC show a preference for A residues with the following sequence pattern: 5'-Pyr-(A)_n-Pyr-3' (Figures 6a and 6b). The cleavage pattern for the remainder of the sequence is similar to that seen following thermal cleavage at 90 °C (Figure 4). A similar result is seen with

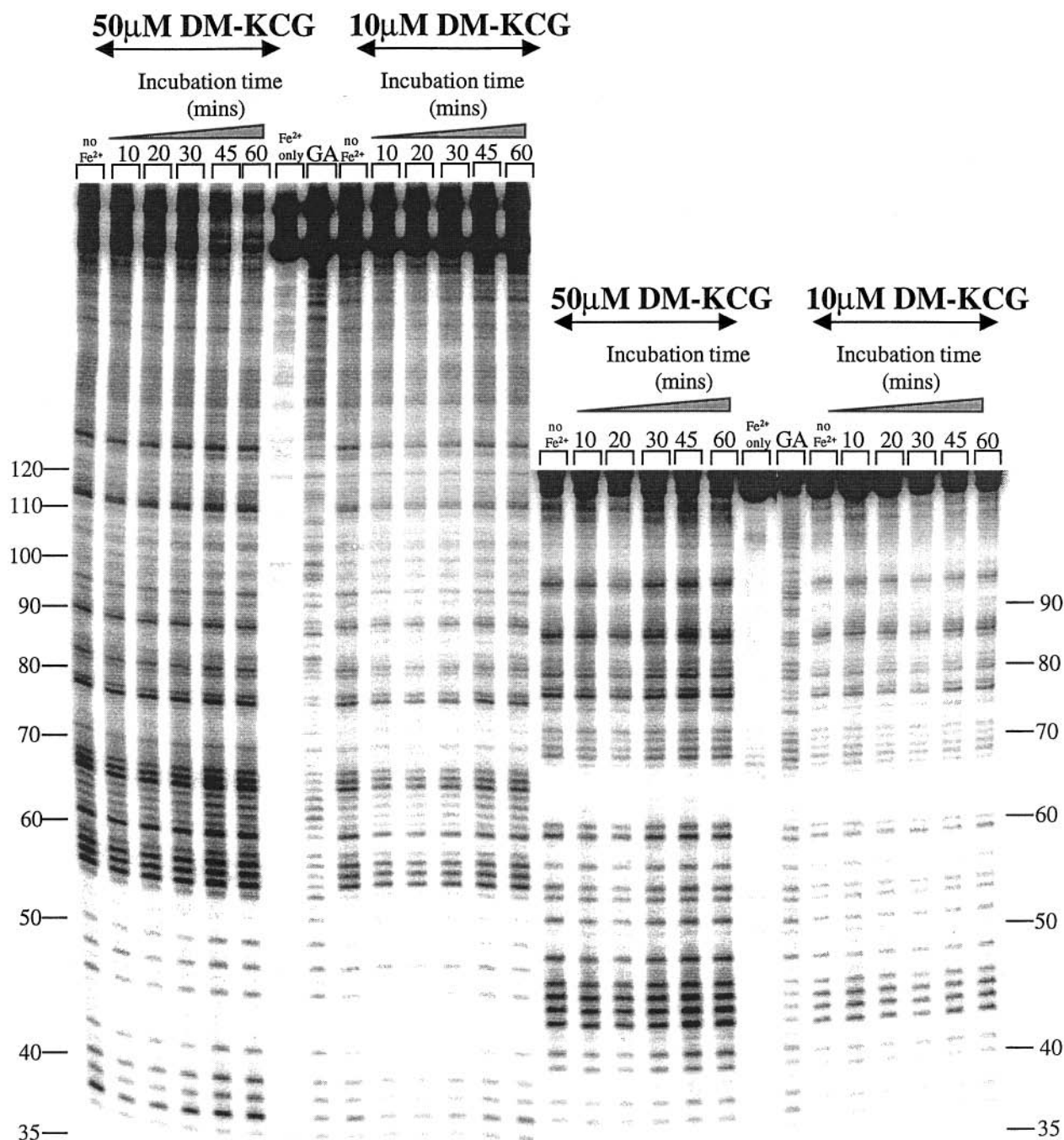


Figure 6. Autoradiograph showing cleavage of 117-mer and 265-mer DNA incubated with DM-KCG in the presence or absence of ferrous ions (10 μ M) at 37 $^{\circ}$ C for 2 h and then incubated at 65 $^{\circ}$ C up to 60 min.

the 265-mer fragment. The strongest bands cleaved are seen at positions A53-A54-A55, indicating a preference for A residues with the sequence pattern: 5'-Pur-(A) $_n$ -G-3'. There was also strong cleavage at positions A63-A64-A65, indicating a preference for A residues with the sequence pattern: 5'-Pyr-(A) $_n$ -G-3'. The other bands that were cleaved significantly correspond to positions A58-A59 (sequence pattern: 5'-Pur-(A) $_n$ -Pyr-3'); A74-A75 (sequence pattern: 5'-Pyr-(A) $_n$ -Pyr-3'); A78-A79-A80 (sequence pattern: 5'-Pur-(A) $_n$ -Pyr-3'); A86-A87 (sequence pattern: 5'-Pyr-(A) $_n$ -G-3'); and A110-A111-A112 (sequence pattern: 5'-Pur-(A) $_n$ -Pyr-3').

At 65 $^{\circ}$ C, it is noticeable that guanine residues G81, G85, G90, and G96 in the 265-mer DNA were barely cleaved. In addition guanine residues G52, G60-G61-G62 were only slightly cleaved (Figures 6). By contrast, all of these guanine residues were strongly cleaved by CLB at

90 $^{\circ}$ C. Taken together these data indicate that DNA cleavage by DM-KCG and DM-BKC at A residues is significantly enhanced compared to that seen at G residues.

Using pG4 DNA we further examined the influence of temperature on the cleavage by both DM-BKC and DM-KCG. From this it is clear that the levels of DNA cleavage by DM-KCG and DM-BKC at room temperature and 65 $^{\circ}$ C greatly exceed those by CLB (Figures 7 and 8). For CLB, almost no DNA cleavage was observed up to base number 100 at room temperature, and little was seen up to base number 70 at 65 $^{\circ}$ C. When the reaction was performed at 65 $^{\circ}$ C in the presence of 10 μ M Fe $^{2+}$, some DNA cleavage was seen with CLB. However, the levels of cleavage were always much lower than those seen with DM-KCG and DM-BKC under all experimental conditions (Figure 8).

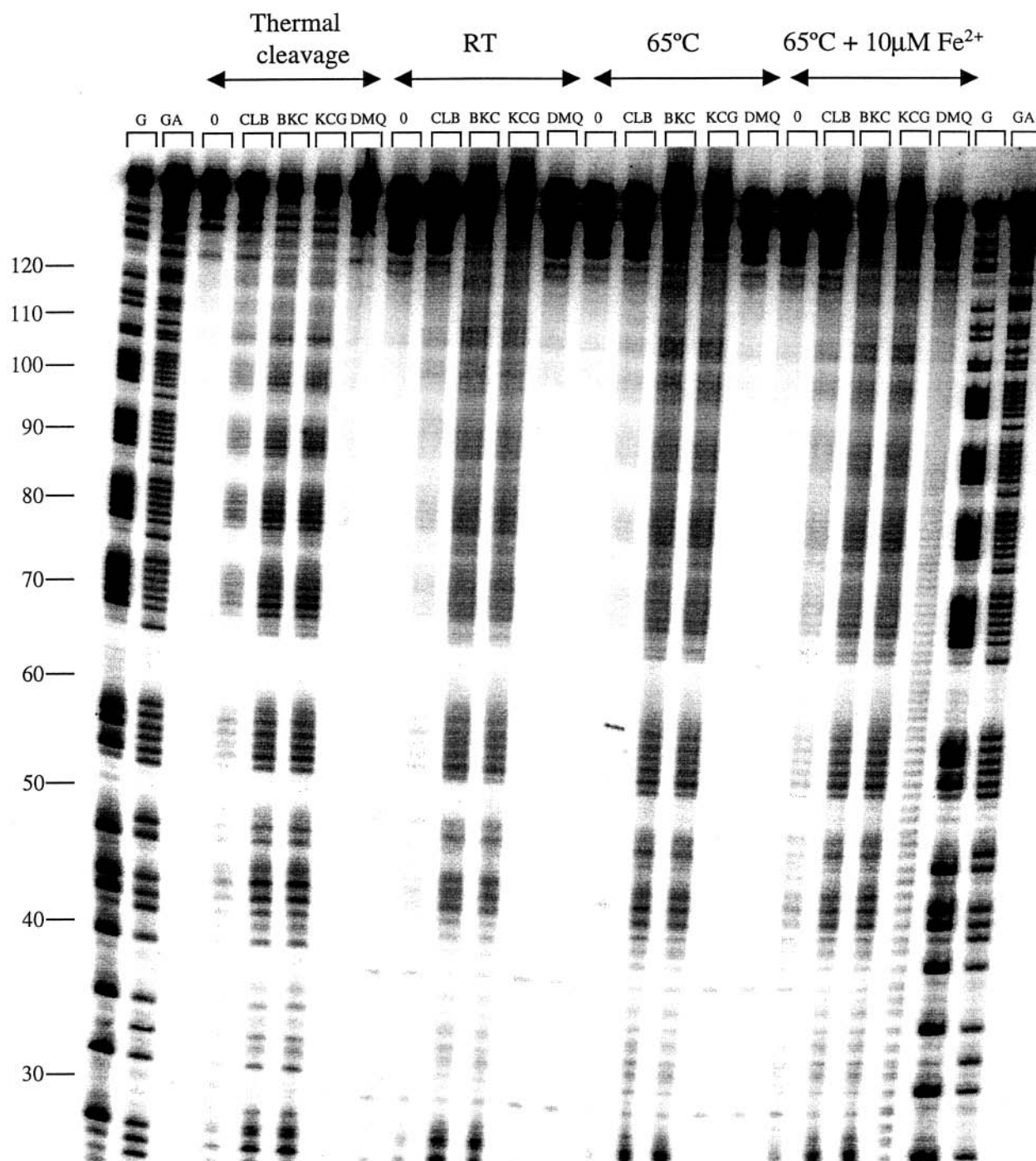


Figure 7. Autoradiograph showing cleavage of pG4 DNA incubated with CLB, DM-KCG, DM-BKC, or in the absence of drug at 37 °C for 2 h and then incubated at various temperatures as shown for 30 min. The conditions for thermal cleavage are identical to that shown in Figure 5.

At 65 °C, the strongest bands cleaved by DM-BKC and DM-KCG correspond to consecutive guanine residues. In addition, it is noteworthy that DM-KCG and DM-BKC at 100 μ M also produced cleavage at some pyrimidine residues in the pG4 DNA under each experimental condition. However, the levels of cleavage were much less than for purine residues. For example, cleavage at pyrimidine residues C40, T66, C32, T34, C36 is evident under the four different experimental conditions (Figure 7). These are considered to be sequence-specific cleavage effects since only a few pyrimidine residues flanked by two purine residues on both the 5' and 3' sides or by one purine residue on the 5' side are affected. This ability to cleave pyrimidine residues is attributable to the DMQ-

MA moiety of the peptide conjugates. It demonstrates sequence specificity of DNA cleavage directed by a proximity effect of the DNA scissoring chromophore (DMQ-MA) situated close to the alkylation chromophore (CLB) inside a dipeptide moiety. The conjugation of DMQ-MA to CLB is mandatory for this sequence selectivity since in the presence of DMQ-MA and iron, only random nonselective cleavage effects are seen (Figure 7).

SUMMARY AND CONCLUSIONS

This paper reports efforts to investigate the sequence-specificity of DNA cleavage directed by a DNA cleavage chromophore (DMQ-MA) in close proximity to an alkylation agent (CLB) in dipeptide conjugates. In summary,

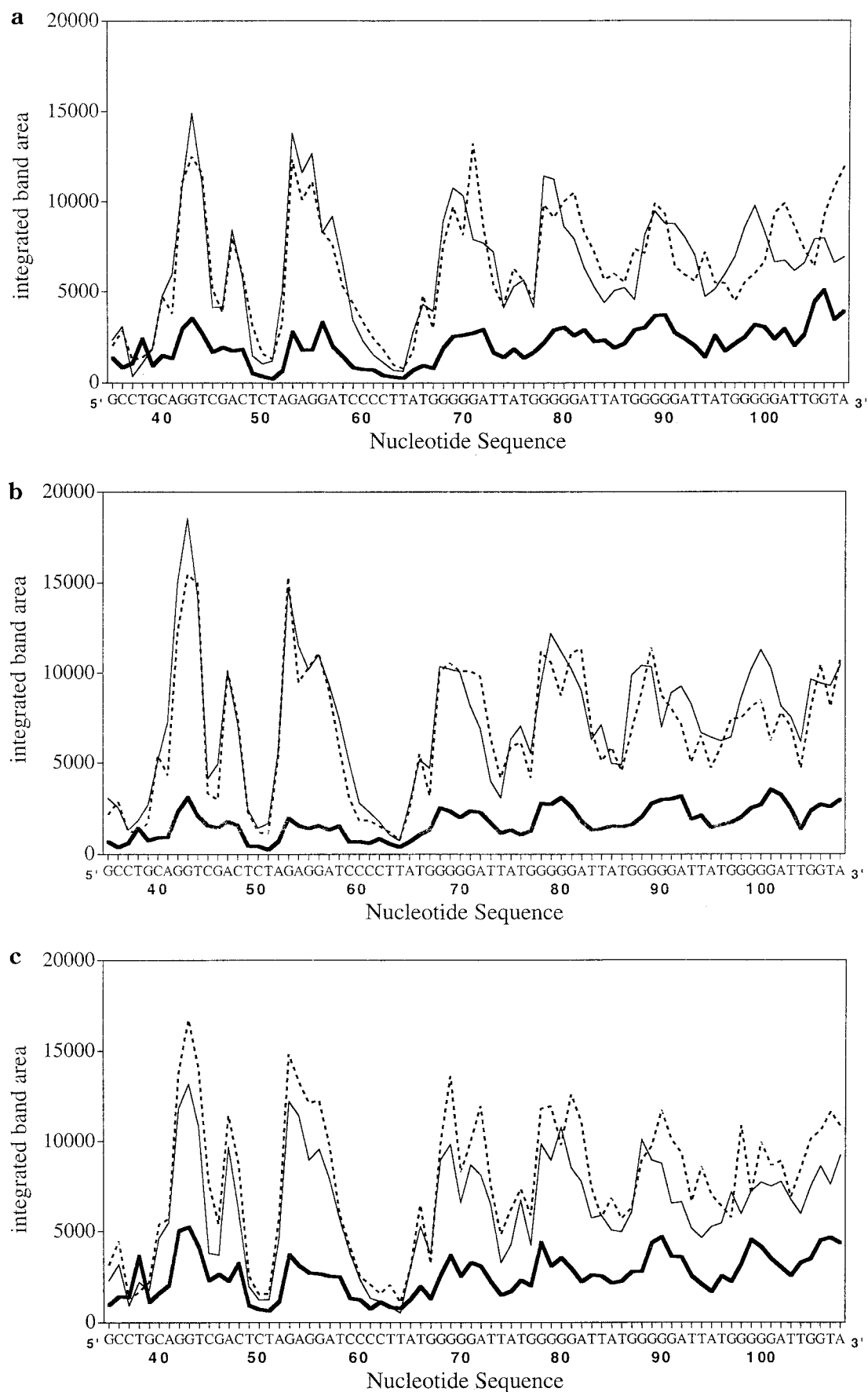


Figure 8. Comparison of band intensities produced by CLB, DM-KCG, and DM-BKC on the cleavage of pG4 DNA at room temperature (a), at 65 °C (b), and at 65 °C in the presence of 10 μM Fe²⁺ (c). The thick solid line represents CLB, the light solid line DM-BKC, and the dashed line DM-KCG.

fragment EcoRI-PvuII of pBS (117 bp)

10	20	30	40
TTCGCCCTAT	AGTGAGTCGT	ATTACAATTC	ACTGGCCGTC
AAGCGGGATA	TCACTCAGCA	TAATGTTAAG	TGACCGGCAG
50	60	70	80
GTTTTACAAC	GTCGTGACTG	GGAAAACCTT	GGCGTTACCC
CAAAATGTTG	CAGCACTGAC	CCTTTTGGGA	CCGCAATGGG
90	100	110	117
AACTTAATCG	CCTTGCAGCA	CATCCCCCTT	TCGCCAG-3'
TTGAATTAGC	GGAACGTCGT	GTAGGGGGAA	AGCGGTC-5'

fragment EcoRI-PvuII of pBS (265 bp)

10	20	30	40
TTCGAGCTCG	GTACCCGGGG	ATCCTCTAGA	GTCGACCTGC
AAGCTCGAGC	CATGGGCCCC	TAGGAGATCT	CAGCTGGACG
50	60	70	80
AGGCATGCAA	GCTTTTGTTC	CCTTTAGTGA	GGGTTAATTT
TCCGTACGTT	CGAAAACAAG	GGAAATCACT	CCCAATTAAA
90	100	110	120
CGAGCTTGGC	GTAATCATGG	TCATAGCTGT	TTCCTGTGTG
GCTCGAACCG	CATTAGTACC	AGTATCGACA	AAGGACACAC
130	140	150	160
AAATTGTTAT	CCGCTCACAA	TTCCACACAA	CATACGAGCC
TTTAACAATA	GGCGAGTGTT	AAGGTGTGTT	GTATGCTCGG
170	180	190	200
GGAAGCATAA	AGTGTAAGC	CTGGGGTGCC	TAATGAGTGA
CCTTCGTATT	TCACATTTCG	GACCCACGG	ATTACTCACT
210	220	230	240
GCTAACTCAC	ATTAATTGCG	TTGCGCTCAC	TGCCCCGCTTT
CGATTGAGTG	TAATTAACGC	AACGCGAGTG	ACGGGCGAAA
250	260	265	
CCAGTCGGGA	AACCTGTCGT	GCCAG-3'	
GGTCAGCCCT	TTGGACAGCA	CGGTC-5'	

PG4 sequence (159 bp)

10	20	30	40
AACAGCTATG	ACCATGATTA	CGCCAAGCTT	GCATGCCTGC
TTGTCGATAC	TGGTACTAAT	GCGGTTCGAA	CGTACGGACG
50	60	70	80
AGGTCGACTC	TAGAGGATCC	CCTTATGGGG	GATTATGGGG
TCCAGCTGAG	ATCTCCTAGG	GGAATACCCC	CTAATACCCC
90	100	110	120
GATTATGGGG	GATTATGGGG	GATTGGTACC	GAGCTCGAAT
CTAATACCCC	CTAATACCCC	CTAACCATGG	CTCGAGCTTA
130	140	150	159
TCACTGGCCG	TCGTTTTACA	ACGTCGTGAC	TGGGAAAAC-3'
AGTGACCGGC	AGCAAAATGT	TGCAGCACTG	ACCCTTTGTG-5'

Figure 9. Nucleotide sequence of 117-mer DNA fragment, 265-mer DNA fragment and PG4 DNA fragment.

thermal cleavage studies have shown that although the gross sequence preferences of DM-KCG and DM-BKC in the 117-mer DNA are similar to those of CLB, the intensity of cleavage produced by both drugs is much greater than for CLB. In the 265-mer DNA, besides alkylation and cleavage at G residues, DNA alkylation and cleavage by DM-KCG and DM-BKC is also seen at A residues within the sequence pattern: 5'-Pur-(A)_n-G-3' > 5'-Pyr-(A)_n-Pyr-3' (where *n* = 1–3). By contrast, DNA alkylation and cleavage by CLB occurs at most G and A residues with much less sequence specificity than seen with DM-KCG and DM-BKC.

Thermal cleavage studies using N7-deazaG and N7-deazaA DNA exposed to DM-BKC and DM-KCG showed that strong alkylation and cleavage at A residues requires G residues flanking the 3' side whereas strong alkylation

and cleavage at G residues requires flanking by at least one purine residue on either the 5' or 3' side.

From agarose gel electrophoretic studies it is clear that DM-KCG and DM-BKC exhibit efficient nicking of supercoiled DNA at concentrations as low as 10 μM whereas CLB causes little DNA nicking even at 100 μM. The addition of ferrous ion has little effect on the enhancement of DNA nicking by these drugs, showing that the presence of iron is not mandatory for DNA cleavage. In addition, cyclic peptide-CLB conjugates (11) and Boc-Lys(CLB)-Gly-NH₂ (data not shown) are incapable of nicking supercoiled DNA. These studies indicate that the conjugation of DMQ-MA (a DNA cleavage agent) (23) to the dipeptide is required for DNA nicking activity. ESR studies of DMQ-MA and DM-KCG both showed five hyperfine signals centered at *g* = 2.0052, corresponding

to two equilibrium radical forms. We propose that the semiquinone structure II is responsible for cleaving the DNA strand (Figure 3).

DNA cleavage studies at lower temperatures in the presence or absence of ferrous ions provide us with more valuable information. At 65 °C and at room temperature, DNA cleavage by CLB was much weaker than that seen with DM-KCG and DM-BKC. With the 265-mer DNA, very few G residues are cleaved by DM-KCG and DM-BKC at 65 °C. By contrast, all of these G residues were strongly cleaved by CLB at 90 °C during thermal cleavage. It is notable that DM-KCG and DM-BKC cleave more readily at A residues than G residues. At 65 °C, the strongest levels of cleavage by DM-KCG and DM-BKC are seen at A residues within the sequences 5'-Pyr-(A)_n-Pyr-3'; 5'-Pur-(A)_n-G-3' and 5'-Pyr-(A)_n-G-3'. The structural differences between DM-KCG and DM-BKC did not alter the sequence specificity of DNA cleavage since the cleavage pattern appears to be quite similar under all experimental conditions studied.

In the pG4 DNA, DNA cleavage by DM-KCG and DM-BKC is much greater than that by CLB at room temperature and at 65 °C. Almost no DNA cleavage is seen with CLB. At 65 °C, the strongest cleavage by DM-KCG and DM-BKC occurs at consecutive G residues. It is noteworthy that DM-KCG and DM-BKC at 100 μM also cleaved some pyrimidine residues: C40, T66, C32, T34, and C36. This must be due to sequence proximity effects since these pyrimidine residues are flanked by two purine residues on both the 5' and 3' sides or by a guanine residue on the 5' side. These findings strongly support the proposal that once the drug molecule is positioned so as to permit alkylation by the CLB moiety, the DMQ-MA moiety is placed close to the alkylation site resulting in the sequence-specific cleavage observed. Whether productive alkylation by the CLB moiety is a necessary prerequisite for nearby radical cleavage by the DMQ-MA moiety we cannot tell, but mutual positioning-enhancement by the functional substituents seems not to be in doubt.

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Inert Phosphorescent Nanospheres as Markers for Optical Assays

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A simple encapsulation technique is presented to produce highly phosphorescent, inert nanospheres that are suitable luminescent markers. It is based on the coprecipitation of phosphorescent ruthenium(II)-tris(polypyridyl) complexes and polyacrylonitrile (PAN) derivatives from a solution in *N,N*-dimethylformamide. The beads precipitate in the form of very small aggregates of spherical shape and a typical particle diameter of less than 50 nm. This process allows the encapsulation of phosphorescent and fluorescent dyes in an individual nanosphere provided that they are sufficiently lipophilic. Quenching by oxygen is negligible due to the use of PAN. The nanospheres were characterized with respect to their spectral properties (quantum yields of the luminophores, brightness, luminescence decay time), stability in aqueous buffered suspensions, and in terms of size, shape, and surface charge of the particles, as well as storage stability, quenching by oxygen, and dye leaching.

INTRODUCTION

Micro- and nanospheres are popular tools for the use as markers in optical luminescence and bioanalysis to increase the sensitivity of assays (1, 2). Intrinsic background fluorescence of samples is a problem often faced in optical analysis. When trying to eliminate background luminescence, two approaches are feasible. On one hand, NIR dyes can be used since most samples show no fluorescence in this region. On the other hand, phosphorescent dyes with long decay times can be employed. Here, the background fluorescence decays comparatively fast and can be eliminated by the measuring arrangement. The latter pathway shall be discussed here.

Basically, three groups of phosphorescent dyes are known that are potentially useful for labeling biomolecules. The first and most important group includes the rare earth metal chelates such as Eu(III)- or Tb(III)-complexes (3–7). They are excitable in the UV-region only, for instance, with a xenon flash lamp, and their lifetimes can be as long as 1 ms. Despite the advantage that the phosphorescence of those chelates is hardly quenched by oxygen, they suffer from the fact that they must be excited by UV-light sources. Nevertheless, they are very useful in single-shot assays. The second group comprises the Pt- and Pd-complexes of the porphyrins with lifetimes between 40 μ s and 1 ms. Unfortunately, these are strongly quenched by oxygen and other notorious quenchers. The third type of long-lived luminescent dyes includes the ruthenium, osmium, and rhenium complexes with polypyridyl ligands. Their lifetimes are between 100 ns and 10 μ s (8–11). The latter two groups of luminophores can be excited by semiconductor light

sources in the visible region. The third group is cationic, but their counteranions can easily be exchanged. Yet, those compounds are prone to luminescence quenching by both oxygen and oxidative or reductive quenchers, but the quenching is less crucial than for the Pt- or Pd-porphyrins.

One efficient way to prevent quenching consists of shielding the dyes from quenching gases or ions. This is accomplished by encapsulation of the dyes in micro- or nanospheres of a material that is impermeable to oxygen. Polystyrene and poly(methyl methacrylate) are common materials to produce micro- and nanospheres. However, they are permeable to oxygen so that some quenching still occurs.

It was shown recently that ruthenium(II) complexes dissolve in films of polyacrylonitrile (PAN), and these are not at all quenched by oxygen. Such materials were applied to design luminescent temperature sensors (12). Thin films of polyacrylonitrile are also used as gas-impermeable barriers in food technology.

By taking advantage of those findings, we have encapsulated luminescent transition metal complexes in beads made of polyacrylonitrile and various copolymers. We present a simple technique for manufacturing phosphorescent nanospheres in high quantities and in a reproducible manner. The resulting nanospheres are practically not quenched by oxygen. The spheres were characterized in terms of spectra, photophysical properties, storage stability in aqueous suspensions, and in terms of particle size, shape and surface charge, quenching by oxygen, and dye leaching.

MATERIAL AND METHODS

Chemicals and Reagents. All chemicals and solvents used were of analytical grade and used without further purification. Doubly distilled water was used throughout. Polyacrylonitrile (MW 150 000) was purchased from Polyscience (Warrington, PA). Poly-(acrylonitrile-co-acrylic acid) containing 5% (w/w) of acrylic acid and the copolymers of Table 1 were obtained from Optosense GmbH (Wörth a. d. Isar, Germany). TransFluoSpheres

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Table 1. Matrix Composition of Polyacrylonitrile Copolymers

polymer no.	main monomer (= acrylonitrile, -CN) (% (w/w))	type of comonomer (reactive groups)	comonomer (% (w/w))
1	100.0		
2	95.0	acrylic acid (-COOH)	5.0
3	90.0	acrylic acid (-COOH)	10.0
4	87.0	acrylic acid (-COOH)	13.0
5	76.9	acrylic acid (-COOH)	23.1
6	95.0	ethylene glycol (-OH)	5.0
7	83.4	acrylic acid (-COOH), ethylene glycol (-OH)	8.3, 8.3
8	87.0	acrylic acid (-COOH), sulfonic acid (-SO ₃ H)	4.3, 8.7
9	90.0	primary amine (ester, -CO(CH ₂) ₂ NH ₂)	10.0

T-8865 were purchased from Molecular Probes (Eugene, OR). The preparation of ruthenium(II)-tris-4,7-diphenyl-1,10-phenanthroline dichloride [Ru(dpp)₃Cl₂] was described elsewhere (13–15).

Preparation of Polyacrylonitrile Nanospheres. A total of 118.5 mg of polymer **1** and 1.19 mg of Ru(dpp)₃Cl₂ were dissolved in 25 mL of DMF. While the sample was stirred vigorously, 125 mL of water was added dropwise with a dropping funnel upon which the solution turned slightly opalescent due to the spontaneous formation of the nanospheres after addition of the initial roughly 20 mL of water. Then, 1 mL of a saturated aqueous solution of sodium chloride (360 g/L at 20 °C) was pipetted to the flask at once to precipitate the finely dispersed nanospheres. The DMF/water mixture was centrifuged at 3000 rpm for 15 min, and the colored residue was washed twice with 50 mL of a 5% (w/w) sodium chloride solution and then three times with 50 mL of water, the wash solutions always being colorless. The residue was taken up in 50 mL of water and heated to 70 °C for 15 min. They were centrifuged and then taken up in 50 mL of phosphate buffer (pH 7.00, ionic strength = 20 mM). After sonification for 1 h, the suspensions were stored in the dark at 10 °C.

Preparation of Poly(acrylonitrile-co-polymer) Nanospheres. A total of 118.5 mg of polymers **2–9** (for samples **2** and **5–11**), 59.3 mg of polymer **2** (for sample **3**), or 23.7 mg of polymer **2** (for sample **4**), and 1.19 mg (for samples **2** and **5–11**), 0.59 mg (for sample **3**), or 0.24 mg (for sample **4**) of Ru(dpp)₃Cl₂ were dissolved in 25 mL of DMF. While the sample was stirred vigorously, 125 mL of a 1 mM solution of sodium hydroxide (for samples **2–7** and **9–10**), 125 mL of water (for sample **8**), or 125 mL of 1 mM hydrochloric acid (for sample **11**) were added dropwise with a dropping funnel. After addition of roughly 20 mL of either sodium hydroxide (samples **2–7** and **9–10**), water (sample **8**), or hydrochloric acid (sample **11**), spontaneous formation of nanospheres occurred. Except for samples **8** and **11**, where the suspensions turned turbid, all other solutions remained clear. Then, hydrochloric acid was added to samples **2–10** and sodium hydroxide solution to sample **11** until the pH was 4.0 for samples **2–10** and 9.0 for sample **11**. This caused the precipitation of finely dispersed nanospheres. The DMF/water mixtures were centrifuged at 3000 rpm for 15 min, and the colored residues were washed twice with 50 mL of 10⁻⁵ M hydrochloric acid (for samples **2–10**) or 50 mL of 10⁻⁵ M sodium hydroxide (for sample **11**), and finally three times with 50 mL of water. All washing solutions remained colorless. The residues were taken up in 50 mL of water and heated to 70 °C for 15 min. They were

centrifuged and then taken up in 50 mL of phosphate buffer (pH 7.00, ionic strength = 20 mM). After sonification for 1 h, the suspensions were stored in the dark at 10 °C.

Instrumentation and Measurements. Absorption spectra were acquired on a Perkin-Elmer Lambda 14 P spectrophotometer, and fluorescence excitation and emission spectra on a Perkin-Elmer LS 50 B spectrofluorometer. Corrected fluorescence emission spectra were obtained with an Aminco Bowman series 2 luminescence spectrometer and used for calculating quantum yields with the following expression:

$$\Phi_x = \Phi_R \left(\frac{A_R(\lambda_R)}{A_x(\lambda_x)} \right) \left(\frac{I(\lambda_R)}{I(\lambda_x)} \right) \left(\frac{n_x^2}{n_R^2} \right) \left(\frac{D_x}{D_R} \right)$$

where Φ are the quantum yields, $A(\lambda)$ is the absorbance/cm of the solution at the wavelength of excitation λ , $I(\lambda)$ is the relative intensity of the exciting light at wavelength λ , n is the average refractive index of the solution, and D is the integrated area of the corrected emission spectrum. Subscripts x and R refer to the unknown and reference (ruthenium(II)tris(2,2-bipyridyl) chloride hexahydrate) solutions, respectively. Because of the similarity of both unknown and reference emission spectra, no conversion of wavelength to frequency is needed for the calculation of quantum yields. Since the voltage of the detector was kept constant during those quantum yield measurements and since all solutions were aqueous, the following simplifications can be made:

$$I(\lambda_R) \approx I(\lambda_x) \text{ and } n_x \approx n_R$$

Luminescence frequency spectra (1–500 kHz) were acquired on an ISS K2 multifrequency phase fluorometer. All other decay time measurements were performed at a modulation frequency of 45 kHz with a fiber-optic setup as described previously (16). Apparent decay times τ were calculated by a simple mathematical formalism from phase angles θ obtained by a single frequency measurement:

$$\tau = \frac{\tan \theta}{2\pi\nu}$$

A bifurcated fiber bundle with glass fibers (NA 0.46, $d = 2$ mm) was connected to a thermostated ($T = 25$ °C) cell with the fiber bundle tip being dipped into the stirred sample solution.

RESULTS AND DISCUSSION

Choice of Matrix. Polyacrylonitrile and its derivatives are attractive polymeric matrixes for the encapsulation of phosphorescent dyes in micro- and nanospheres (17, 18). They display an extraordinarily poor permeability for gases and ionic as well as neutral chemical species. Hence, they can protect luminescent dyes against potential luminescence quenchers such as oxygen. Polyacrylonitrile polymers are soluble in DMF, swell in DMF/water mixtures, and act themselves as solvents for lipophilic dyes. In addition to plain polyacrylonitrile, we have employed functional copolymers such as the ones listed in Table 1. Both anionic and cationic charges were introduced into the polymer and no significant change was observed in the permeability of the copolymers for oxygen.

Polyacrylonitrile is amphiphilic in a sense that it is both hydrophilic and lipophilic. This makes polyacrylo-

nitrile and its functional copolymers soluble in DMF and, in turn, opens the way to produce nanospheres by the precipitation process. On dropwise addition of water to a diluted solution of polyacrylonitrile in DMF, a stable dispersion of nanoscale polyacrylonitrile aggregates is formed. Surprisingly, the nanospheres do not tend to aggregate and sediment.

If the spheres are precipitated from DMF solutions containing Ru(dpp), the dye is coprecipitated with the spheres. This is an elegant way to stain nanospheres in a defined manner. Solvents other than water may also be used to precipitate the polyacrylonitrile nanospheres provided (a) that the solvent is miscible with DMF and (b) that the polymer is not soluble in the binary mixture. Since polyacrylonitrile and its copolymers are soluble in DMF only (they swell in dimethyl sulfoxide), the nanospheres may be suspended in almost any other solvent.

Choice of PAN-Copolymer. Among the various polyacrylonitrile-based copolymers investigated as encapsulation matrixes (polymers 2–9), polyacrylonitrile with an acrylic acid content of 5% (w/w) proved to be the best choice as far as a preferable combination of low oxygen quenching, high quantum yields, long decay times, and a small nanosphere diameter is concerned. With increasing amount of acrylic acid comonomer, the apparent decay time gets shorter and the quantum yield gets smaller as a result of a more polyelectrolyte character of the nanospheres. When still decreasing the content of acrylic acid, the nanosphere suspensions become more instable due to too little electrostatic repulsion of the surface of the nanospheres. Using other copolymers than acrylic acid-containing ones to introduce active groups such as hydroxyl, sulfonic acid, or primary amine groups (polymers 6–9) goes along with a higher oxygen quenching and less stable nanosphere suspensions. The active groups on the surface of the nanospheres ($-\text{COOH}$, $-\text{OH}$, $-\text{SO}_3\text{H}$, $-\text{NH}_2$) depending on the different comonomers used enable the covalent coupling of biomolecules by conventional methods. This will be demonstrated in subsequent work.

Choice of Dye. The requirements to meet for ideal dyes are an extremely good solubility in both PAN (copolymer matrix) and DMF (solvent), insolubility in water for precipitation of the nanospheres, and a positive charge to cross-link them with the negatively charged matrix. The long-lived phosphorescent ruthenium(II)-tris-pyridyl complexes were selected as dyes since they exhibit those positive features. They yield bright luminescent nanospheres with a large Stokes' shift of about 150 nm ($\lambda_{\text{ex}} = 465 \text{ nm}$, $\lambda_{\text{em}} = 613 \text{ nm}$). Because of their positive charge, it is possible to cross-link them with copolymers containing negatively charged groups. Furthermore, the dyes can be made lipophilic by using proper ligands. The lipophilic dyes are then extracted quantitatively into the nanospheres during the preparation process because they are very well soluble in the polymer. Even in a lipophilic environment, e.g., if proteins are present in the sample, no dye leaching in aqueous solutions is occurring. The high quantum yields ($\Phi > 40\%$) and the large extinction coefficients ($\epsilon \approx 30\,000 \text{ L mol}^{-1} \text{ cm}^{-1}$) of the ruthenium dyes are further advantages. They are excitable with a blue argon ion laser at 488 nm or cheap bright blue light emitting diodes (LEDs) at 450 or 470 nm. The use of phosphorescent nanospheres eliminates background fluorescence, leading to a higher sensitivity. Last but not least, the complexes are stable against the loss of ligands, and its emission spectrum is broad enough to overlap with the absorbance spectra of various luminescence acceptor dyes.

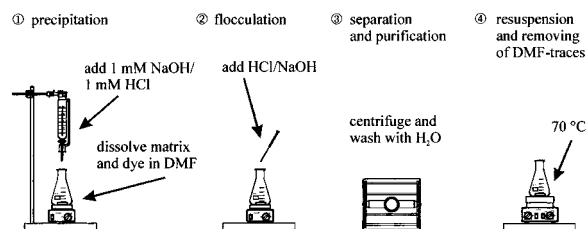


Figure 1. Schematic of the process for making poly-(acrylonitrile-co-polymer) nanospheres.

Among the several ruthenium(II)-tris-polypyridyl complexes, the ruthenium(II)-tris-4,7-diphenyl-1,10-phenanthroline complex with chloride as counterion $[\text{Ru}(\text{dpp})_3\text{Cl}_2]$ is most suitable as indicator dye since it is very easily prepared, and when incorporated into a PAN-matrix, its quantum yield is the highest and its decay time is the longest, even though the free dye has the highest oxygen quenching of all ruthenium complexes. This shows the superb shielding effect of the matrix.

Choice of Ligand. When using transition metal complexes other than $\text{Ru}(\text{dpp})_3^{2+}$, the apparent decay time does not cover the desired long microsecond range anymore. Exchanging the diphenyl-phenanthroline (dpp) ligand against phenanthroline (phen) or bipyridyl (bipy) makes the resulting complexes $\text{Ru}(\text{phen})_3^{2+}$ and $\text{Ru}(\text{bipy})_3^{2+}$ water-soluble. Consequently, they cannot be extracted into the polymer matrix. Actually, ruthenium complexes with ligands such as diphenyl-bipyridyl show an even lower oxygen cross-sensitivity than diphenyl-phenanthroline but unfortunately a shorter apparent decay time, as well.

Choice of Counterion. Chloride is preferred as a counterion for $\text{Ru}(\text{dpp})_3^{2+}$ since the resulting nanosphere suspensions are much more stable due to a cross-linking of the matrix. The chloride ions easily exchange with the carboxylic groups on the copolymer surface and thus build a three-dimensional network with the double positive charged ruthenium dye by electrostatic interaction (compare Ca^{2+} as cross-linking agent in alginate fibers). Otherwise, individual polymer chains might be washed out.

There are a number of important details in the preparation process (compare Figure 1 for the copolymer approach) of the phosphorescent nanospheres that need to be mentioned. The size, surface charge, and reactive groups of the nanospheres can be varied distinctively by the preparation process.

In contrast to the widely used emulsion polymerization, the particles (polymers 1–9) were prepared by the precipitation polymerization technique. By using those prepolymerized particle systems, it is provided that all nanospheres can be made of the same stock polymer which leads to reproducible properties of the nanospheres.

Matrix Concentration. The maximum amount of matrix with respect to its organic solvent that still yields clear solutions is 1.0% (w/w) matrix/DMF, but a concentration of 0.5% (w/w) proved to be the optimal choice (compare samples 2–4 with 0.50, 0.25, and 0.10% (w/w) matrix/DMF, respectively). A higher percentage leads to larger nanosphere diameters and finally makes the precipitation polymerization impossible since larger, jelly-like aggregates are formed at the dropping location that cannot be redissolved anymore.

Dye Loading. The concentration of incorporated phosphorescent dye is aimed to be as high as feasible to yield higher luminescence intensities. The amount of ruthenium complex (for chemical structure compare

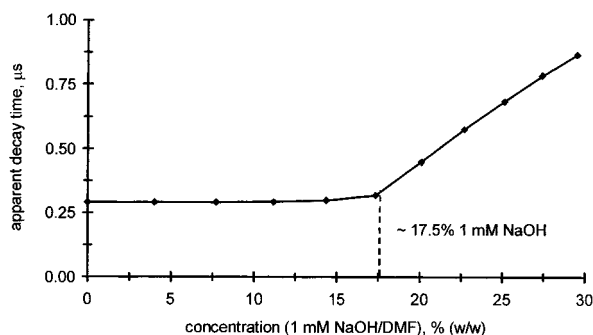


Figure 2. Dependence of the apparent decay time on the addition of sodium hydroxide solution to a DMF solution of the matrix during the precipitation polymerization process (sample 2).

insert in Figure 3) within the nanospheres can be up to 10% (w/w) dye/matrix for polymer 2. If this is exceeded, the apparent decay time becomes shorter and the nanosphere suspensions become instable. This is probably due to a compensation of the repulsing negative carboxylic groups on the surface by the double positively charged ruthenium dye. Then aggregates are formed immediately, and no dispersions are obtained anymore.

Precipitation Details. The amount of doubly distilled water (when starting with polymers 1 and 6), 10^{-3} M sodium hydroxide (for polymers 2–5 and 7–8), or 10^{-3} M hydrochloric acid (for polymer 9) added to the DMF solution of the matrix and the dye has to be present in at least the 5-fold amount as compared with the volume of organic solvent to extract all of the DMF out of the nanospheres. Adding a 1 mM solution of sodium hydroxide to polymers with acidic comonomer components or 1 mM hydrochloric acid to polymers with basic comonomer components leads to a higher concentration of those reactive comonomer groups on the surface of the nanospheres. This way the hydrophilic parts (e.g., carboxyl or amine groups) are primarily on the outside and the PAN main monomer mostly on the inside of the nanospheres. The resulting suspensions are more stable due to electrostatic repulsion of the deprotonated carboxyl groups and the protonated amine groups.

The reason for using doubly distilled water is to avoid flocculated systems that would form immediately in the presence of electrolytes. In contrast to other solvents such as ethanol, for example, water has the advantage that the ruthenium dyes are insoluble here and a complete extraction of the lipophilic dyes into the nanospheres is guaranteed.

The system PAN/DMF/water allows the precipitation of the nanospheres at a defined point of time, controlled by the amount of water in the DMF solution. Moreover, stable dispersions are obtained without any content of emulsifiers such as sodium lauryl sulfate (SDS) that would otherwise have to be removed in a complicated process (e.g., by dialysis). During the precipitation polymerization process, at a characteristic concentration of between 10 and 20% (w/w) 10^{-3} M sodium hydroxide solution in the DMF solution of the copolymer matrix, the apparent decay time increases sharply (Figure 2 for polymer 2). This is due to the completion of the formation of the nanospheres and a subsequent extraction of DMF out of the nanospheres. Since the decay time measurement was performed in an air-saturated solution what is actually seen is the oxygen quenching which decreases drastically after that point. Now the polymer gets more and more rigid with more water added. Only up to this concentration of roughly 10–20% (w/w) water/DMF,

depending on the copolymers used, are the polymers soluble in the water/DMF mixture. Afterward, when adding more water, the nanospheres precipitate, which can be observed by the slightly opalescent appearance of the solution. Continuously measuring the apparent decay time of the solution allows one to monitor the preparation process and to quantify the amount of water needed to precipitate a specific polymer.

When precipitating the nanospheres with hydrochloric acid (polymers 1–8) or sodium hydroxide solution (polymer 9), the pH should be lowered to approximately 4.0 for the flocculation of the nanospheres with acidic surface groups (polymers 1–8) and to a pH of roughly 9.0 for nanospheres with basic surface groups (polymer 9). Only then it is provided that the carboxylic groups (polymers 1–5 and 7–8) on the surface of the nanospheres are all protonated and the amine groups (polymer 9) are deprotonated. Associates are then formed due to the neutral surface. Since this process is fully reversible, when adjusting the pH back to 7.0, the nanospheres can be suspended once again due to electrostatic repulsion. This shows the importance of the pH value at all preparation stages and the possibility to precipitate or resuspend the nanospheres as needed. It should be mentioned that the nanospheres can be only resuspended from freshly prepared associates. After several days, there is a tendency to irreversibly form large and stable aggregates. During the washing steps, no leaching of the dye out of the polymer matrix was observed.

To resuspend the nanospheres, it is sufficient to treat them in an ultrasonic bath for 1 h. The heating of the aqueous suspensions to about 70 °C is meant to remove traces of DMF that might still be present in the suspensions even after the washing steps. Residues of DMF in the nanospheres result in a higher gas permeability and therefore a higher level of oxygen quenching.

Buffer Composition and Storage Conditions. The tendency of the nanospheres to aggregate and their stability in general was also examined. This is of importance for the use of the nanospheres as labels. Nanospheres from pure PAN show a strong tendency to form aggregates. It was found that when kept in phosphate buffer solution at pH 7.00 with an ionic strength (adjusted with NaCl) of at least 20 mM, the nanospheres made of PAN copolymers were stable over a period of several months. No sedimentation in buffered systems was observed. The higher the ionic strength gets, the more repulsion among the nanospheres is created in addition to the one originating from the deprotonated acidic groups (polymers 1–8) or the protonated amine groups (polymer 9) on the surface, leading to even more stable solutions. An ionic strength of up to 100 mM was successfully applied, as well. The resulting suspensions are clear and stable over months if stored in the dark at 10 °C and thus prove the superb dispersibility of the nanospheres in an aqueous buffered environment.

Photophysical Characterization. Figure 3 shows the excitation and emission (aerated/deaerated) spectra of the incorporated ruthenium dye $\text{Ru}(\text{dpp})_3\text{Cl}_2$ in a buffered aqueous suspension. Since the excitation spectrum is very broad, various excitation light sources can be applied. $\text{Ru}(\text{dpp})$ can be excited with a bright blue (470, 450 nm) and blue-green (505 nm) LED or with the argon ion laser at 488 nm. Even the violet diode lasers (404 nm) are a potential light source. The excitation and emission spectra show almost no overlapping and a very large Stokes' shift of about 150 nm ($\lambda_{\text{ex}} = 465$ nm, $\lambda_{\text{em}} =$

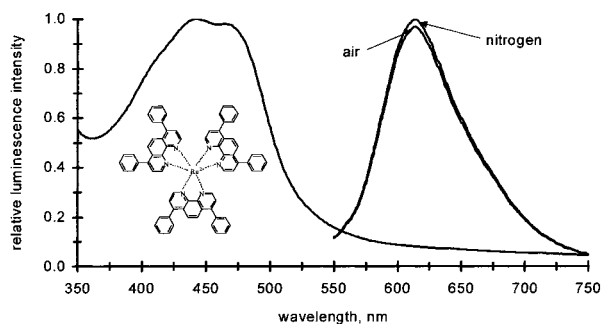


Figure 3. Spectral characteristics (absorbance and emission spectra, $\lambda_{\text{ex}} = 488$ nm) of encapsulated Ru(dpp)_3^{2+} dye (sample 2).

Table 2. Brightness of the Nanospheres (Sample 2) as Compared to the TransFluoSpheres T-8865 ($\lambda_{\text{em}} = 605$ nm)

light source	λ_{ex} (nm)	luminescence intensity ^a
blue diode laser	404	4.1
blue LED	450	3.4
blue LED	470	1.4
blue argon ion laser	488	0.7
blue-green LED	505	0.2

^a TransFluoSpheres = 1.0.

613 nm). In the emission spectra in Figure 3, one can also notice the negligible amount of quenching caused by oxygen.

The brightness of the nanospheres was compared to the luminescence intensity of the carboxylate modified microspheres (TransFluoSpheres T-8865, $\lambda_{\text{em}} = 605$ nm) from Molecular Probes (Table 2). Those TransFluoSpheres have comparable spectral properties to the $\text{Ru(dpp)}_3\text{Cl}_2$ dye and are known to be extremely strong fluorescent. To examine the luminescence intensity, an aqueous solution of both micro- and nanospheres of identical polymer concentration (w/w) was measured. It can be seen that when excited with the blue diode laser (at 404 nm) or blue LEDs (at 450 or 470 nm), the nanospheres have an up to 4-fold intensity as compared to the TransFluoSpheres microspheres due to their broad excitation spectrum. Only at the longer wavelengths of the blue argon ion laser (488 nm), for which the TransFluoSpheres were specifically designed, or the blue-green LED (505 nm), the TransFluoSpheres show a higher brightness. Nevertheless, if the blue LEDs are the preferred light source, the presented nanospheres are much brighter.

As expected, with increasing dilution of the nanosphere suspensions, no change in the decay behavior of the dyes was observed. This also indicates that the dye is not washed out of the matrix. A concentration of 0.5% (w/w) matrix/DMF of the original polymer solution during the preparation process proved to be the best choice because a lower ratio means shorter apparent decay times, higher oxygen cross-sensitivity, lower quantum yields, and a higher consumption of DMF.

In the luminescence frequency spectra (Figure 4) for sample 2, one can see the dependence of the phase angle and the modulation on the applied modulation frequency. An evaluation of the data points to a biexponential fit as the best choice (and not a monoexponential one), which is typical for incorporated dyes. The apparent decay time varies between 6 and 7 μs for the main component (~95%) and 1–2 μs for a second component (~5%). The minor component results from surface-bound dye which

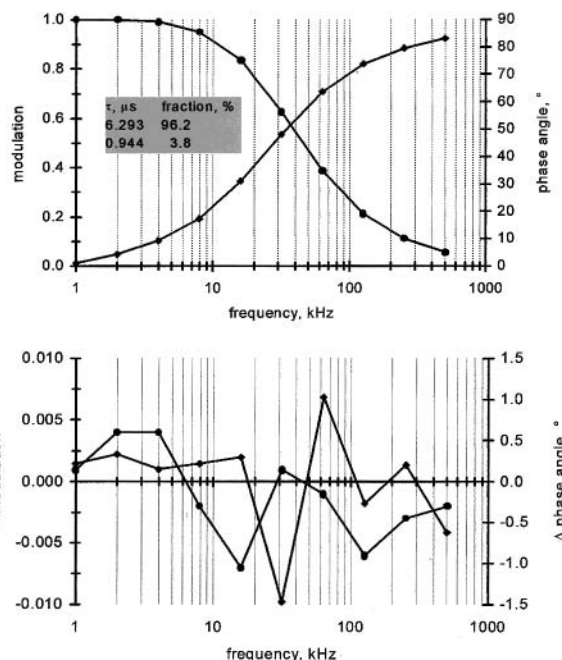


Figure 4. Luminescence frequency spectra (top) and error distribution (bottom, $\xi^2 = 7.1$) displaying the modulation (●) and phase angle (◆) for sample 2.

can be quenched by oxygen. Even though ξ^2 (7.1) is relatively high, a systematic error can be ruled out because the errors are evenly distributed over the whole frequency range.

Physical Characterization. Table 3 summarizes the type of suspension, the average diameter d (by dynamic-light scattering at 90°), the apparent decay time τ (air/ N_2), the oxygen cross-sensitivity $\Delta\tau$ (change of τ air/ N_2), and the quantum yield ϕ for all of the nanospheres investigated.

The most important characteristic of the new nanospheres is their very low oxygen cross-sensitivity as can be seen in Table 3. Whereas free $\text{Ru(dpp)}_3\text{Cl}_2$ dye in water (sample 0) has a tremendously high oxygen quenching $\Delta\tau$ of about 75% and a quantum yield ϕ of below 0.30 in nitrogen, the incorporated dye in the proper polymers (e.g., polymer 2) has a quenching rate of only 3–5% and a quantum yield of up to 0.40. The apparent decay time of the free ruthenium complex in water varies between 1.20 μs in air and 4.70 μs in nitrogen, but can be as high as 6.00 μs in apolar organic solvents. The extremely low oxygen quenching of the incorporated dye proves the excellent shielding effect of the PAN copolymers and thus shows that the matrix itself does not act as a quencher. With decreasing nanosphere diameter, the oxygen quenching increases due to a higher surface-to-bulk ratio. Increasing the amount of carboxylic groups in the polymer by using more acrylic acid comonomer unfortunately leads to a higher quenching, as well, since the nanospheres become more hydrophilic and can take up more water. But since carboxylic functional groups are essential for stabilizing the suspensions, for cross-linking the nanospheres with the positive ruthenium dye and for the covalent coupling of biomolecules to the nanosphere surface, a compromise is necessary.

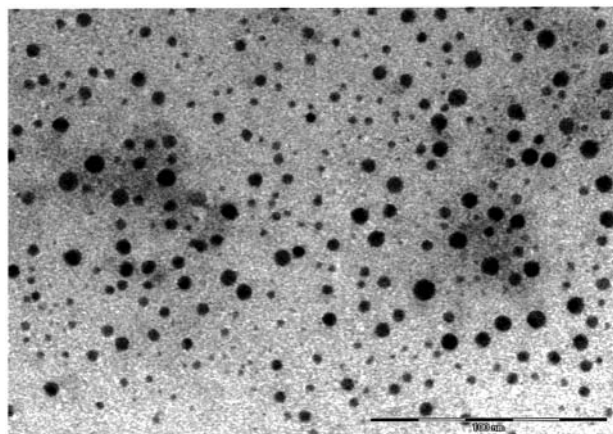
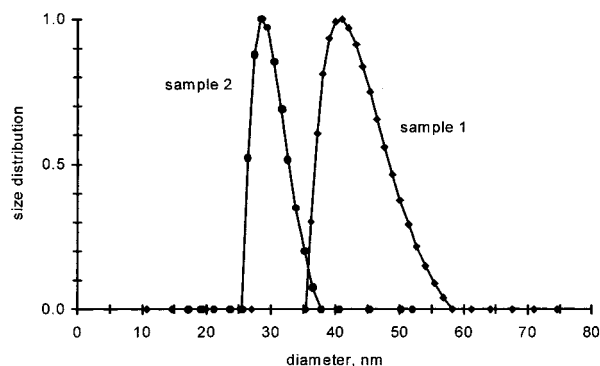
For all of the polyacrylonitrile polymers 1–9, membranes (200 μm films on Mylar polyester foils) were also measured. The oxygen cross-sensitivity was always less than 2%.

Transmission electron microscopic pictures of the nanospheres showed a nearly circular shape and a

Table 3. Characterization of Polyacrylonitrile Nanospheres

sample no.	polymer no.	c (matrix/DMF) (% (w/w)) ^a	suspension	d (nm)	τ , air (μ s)	τ , N ₂ (μ s)	$\Delta\tau$ (%)	ϕ
0 ^b					1.20	4.70	-74.5	0.30
1	1	0.50	clear	43.4	6.01	6.20	-3.0	0.39
2	2	0.50	clear	29.8	5.78	6.11	-5.5	0.32
3	2	0.25	clear	19.8	5.57	6.08	-8.5	0.31
4	2	0.10	clear	21.7	4.22	4.63	-9.0	0.26
5	3	0.50	clear	32.3	5.70	6.10	-6.5	0.43
6	4	0.50	clear	6.1	5.46	5.91	-7.5	0.39
7	5	0.50	clear	2.8	5.32	5.93	-10.5	0.38
8	6	0.50	turbid	instable	6.01	6.24	-4.0	
9	7	0.50	clear	18.7	5.79	6.18	-6.5	
10	8	0.50	clear	18.5	5.36	5.98	-10.5	0.34
11	9	0.50	turbid	instable	5.55	5.84	-5.0	

^a c (Ru(dpp)₃Cl₂/matrix) = 1.0% (w/w) for all samples. ^b free Ru(dpp)₃Cl₂ in water.

**Figure 5.** Transmission electron microscopic picture of nanospheres (sample 2).**Figure 6.** Radii of nanospheres (samples 1 and 2) by dynamic light-scattering at a detection angle of 90°.

diameter of roughly 10 nm (Figure 5 for sample 2). Static and dynamic light-scattering (Figure 6 for samples 1 and 2) along with laser Doppler anemometry experiments revealed a polydisperse coil with a diameter of the nanospheres of down to about 5 nm (for sample 7) and a zeta potential (ζ = -54.0 mV for sample 2) confirming the negative surface charge resulting from the carboxylic groups.

Even though the light-scattering distributions point to a not extremely monodisperse shape of the nanospheres, the very simple preparation technique fully compensates this handicap. The nanosphere diameter can be influenced by the preparation process. Both increasing the amount of carboxylic groups in the copolymer and increasing the concentration of the matrix in DMF leads to larger nanospheres. Some matrixes (e.g., polymers 6 and 9) show a higher tendency to aggregate and a rather polydisperse size distribution.

CONCLUSION

Polyacrylonitrile derivatives are attractive matrixes for the encapsulation of organic phosphorescent dyes since they have a poor permeability for gases and dissolved ionic and neutral chemical compounds. Thus, they are shielded efficiently against luminescence quenching, e.g., caused by molecular oxygen, and therefore, they show constant decay times and quantum yields in samples of variable and unknown composition. In addition, many lipophilic dyes are well soluble in these materials and will be not washed out into the sample.

The nanospheres have a very high surface-to-bulk ratio, which is evidence for a highly branched structure. Polyacrylonitrile with a acrylic acid content of 5% proved to be the best choice as encapsulation matrix. Suspensions of such phosphorescent nanospheres are only poorly quenchable by oxygen, show no tendency to sedimentation, and have an activated surface for the coupling of biomolecules or chemically responsive indicators. In case of using the ruthenium(II)-tris(4,7-diphenyl-1,10-phenanthroline) complex as phosphorescent dye, bright luminescent nanospheres with a large Stokes shift were obtained. No dye leaching was observed in aqueous solutions. They can be excited with either the blue argon ion laser or bright blue LEDs.

The new nanospheres can be used as bright phosphorescent markers in immuno- or DNA-sensing or as nanoprobe to measure intracellular chemical parameters. Furthermore, they are excellent phosphorescence standards and are useful to design phosphorescent chemical sensors.

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Enhanced Biorecognition and Internalization of HPMA Copolymers Containing Multiple or Multivalent Carbohydrate Side-Chains by Human Hepatocarcinoma Cells

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N-(2-Hydroxypropyl)methacrylamide (HPMA) copolymers containing pendant saccharide moieties (galactosamine, lactose, and triantennary galactose) were synthesized. The relationship between the content of saccharide moieties and three-dimensional arrangement of galactose residues and their biorecognition and internalization by human hepatocarcinoma HepG₂ cells was investigated. The results obtained clearly indicated preferential binding of the trivalent galactose and the lactose-containing copolymers to these cells. The higher the saccharide moieties content in HPMA copolymers, the higher the levels of binding. The biorecognition of the glycosylated HPMA copolymers by HepG₂ cells was inhibited by free lactose. The data on the internalization and subcellular trafficking of HPMA copolymer conjugates obtained by confocal fluorescence microscopy correlated well with the flow cytometric analysis of their biorecognition by target cells. Structural features of the glycosides responsible for the specific recognition of the HPMA copolymers have been identified. The results underline the potential of glycosylated HPMA copolymers for delivery of pharmaceutical agents to hepatocarcinoma cells.

INTRODUCTION

The extensive participation of saccharides in recognition processes has led to the concept of targetable glycosylated drug delivery systems (1–3). Carbohydrate specific cell surface receptors play essential roles in various biological recognition processes, including intercellular recognition, adhesion, cell growth regulation, and cell differentiation (4, 5). Receptors expressed at the surface of cells, which mediate endocytosis of their ligands, are attractive targets for delivering bioactive macromolecules to specific cells (6, 7). Several cell surface lectins have been discovered that sort their ligands into the cell. For example, it was recognized that liver parenchymal cells have cell surface receptors specific for galactose (7), phagocytic cells have receptors specific to mannose (8), and L1210 leukemia cells have receptors specific to fucose (9). The galactose binding receptor of hepatocytes has been widely investigated, and its specific interactions with asialoglycoproteins (ASGP¹) (10) and neoglycoproteins (11) have been documented. The asialoglycoprotein receptor (ASGP-R), also known as the hepatic lectin, is a recycling endocytotic receptor that recognizes terminal galactose (Gal) and *N*-acetylgalactosamine (GalNAc) (10). The ASGP-R is composed of three or more subunits that project their CRD (carbohydrate recognition domain) extracellularly (1). The affinity of a ligand for this receptor is highly dependent on the valency of sugar residues, as well as on their three-dimensional arrangement (12). Schwartz et al. have previously described the presence of the galactose-recep-

tor on human hepatoma cell line HepG₂, suggesting approximately 150000 binding sites per cell (13, 14).

Glycosylated polymers, in which saccharide residues are displayed from side-chains as pendant groups, appear to increase binding affinity (15). Saccharide-substituted polymer derivatives have diverse potential uses, such as multivalent inhibitors of cell or virus binding, specific binding to cell surface, artificial antigens, and drug delivery agents (16, 17). The chemically well-defined, water-soluble carbohydrate containing synthetic copolymers, the “glycopolymers”, can be readily synthesized for various biological assays (18). Most studies of carbohydrate-substituted polymers have focused on the polyacrylamide backbone (19–21), polylysine (22), or HPMA copolymers (23, 24). These glycopolymers may have lower molecular weight and higher sugar densities compared with synthetic glycoproteins. Water-soluble polylactoside copolymers have increased binding properties and have demonstrated enhanced binding affinity to plant lectins

¹ Abbreviations: AIBN, 2,2'-azobis(isobutyronitrile); AH, amino-hexyl; AHT, (6-amino-hexanamido)tris(hydroxymethyl)methane; ASGP, asialoglycoprotein; ASGP-R, asialoglycoprotein receptor; Cbz, benzyloxycarbonyl; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DPBS, Dulbecco's phosphate-buffered saline; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; FBS, fetal bovine serum; FITC, fluorescein-5-isothiocyanate; Gal, galactose; GalN, galactosamine; GalNAc, *N*-acetylgalactosamine; Hex, hexanoyl; HPMA, *N*-(2-hydroxypropyl)methacrylamide; Lac, lactose; MA, methacryloyl; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MEM- α , minimum essential medium; Mw, weight average molecular weight; ONp, *p*-nitrophenoxy; P, HPMA copolymer backbone; PNp, *p*-nitrophenol; SEC, size-exclusion chromatography; TLC, thin-layer chromatography; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TriGal, tris-based galactoside; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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(25). Poly-L-lysine substituted with mannose and glucosyl residues displays a high specificity toward membrane lectins (26). It is expected that sugar residues exposed on macromolecular delivery systems will mediate targeting to corresponding receptors expressed on certain organs or cells.

During the past decade, targetable *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugates have been designed, developed, and evaluated for their potential use in cancer therapy (reviewed in ref 27). Phase 1 clinical trial data indicate their great potential for applications in oncology (28, 29). Various targeting moieties, including carbohydrates (30, 31) have been used to promote HPMA copolymer conjugate recognition at the plasma membrane. It has been found that HPMA copolymers containing side chains terminating in *N*-acylated galactosamine behaved similarly to the ASGP and were biorecognizable *in vivo*. Their biorecognition by the ASGP-R of hepatocytes was dependent on the number of saccharides displayed on the polymer (23). The polymeric backbone of HPMA copolymers could be designed for a desired property such as biodegradation and stability (27). The design, activity, and mechanism of action of HPMA copolymer drug conjugates have been reviewed in detail (32). Incorporation of certain glycoside moieties (targeting residues) into HPMA copolymer conjugates has been used to facilitate targeting of anthracyclines to the vicinity of tumor cells. One of the most successful polymer-drug-saccharide conjugate systems reported are the HPMA copolymer–adriamycin (ADR)–sugar conjugates, in which both ADR and sugar were attached to the polymer backbone via lysosomally cleavable oligopeptide spacers (33). The conjugates containing galactosamine moieties were reported to accumulate in the liver selectively (34).

Our knowledge of the ligand binding preferences of the ASGP-R may be exploited for targeting molecules to hepatocytes (1). However, one of the main difficulties associated with using carbohydrates to target lectins is their low binding affinity. The interactions of individual saccharide groups are generally of low affinity (35), and dissociation constants (K_d) are generally in the mM to μ M range. To obtain adequate binding affinities for some proteins, multiple carbohydrate moieties are often needed. Many naturally occurring glycoproteins contain multi-antennary structures (36). When the density and relative spatial arrangements of the carbohydrate residues incorporated are appropriate, glycoconjugates can induce a marked enhancement of binding affinity toward proteins due to the multivalent recognition of ligand (37). The incorporation of numerous biorecognizable moieties into one macromolecule can enormously enhance its rate of uptake by cells, by enhancing adherence to the plasma membrane being internalized. Such polyvalent interactions can collectively be much stronger than the corresponding monovalent interactions (38). The most comprehensive description of an enhancement in binding affinity for a series of polymeric sialosides was reported by Sigal et al. (17). The inhibitory properties of polyacrylamides containing pendant sialoside groups illustrate the value of cooperative polyvalent interaction in the design of potent inhibitors of virus-mediated agglutination of erythrocytes. Similar polyvalency effect (cooperative binding) was observed for synthetic glycosylated systems as well (23, 24). Thus, it may be possible to generate synthetic glyconjugates, organized as multivalent (cluster) structures that compensate for weak-binding interactions (39, 40). Classical studies by Lee et al., (41) have established the “glycoside cluster effect”, which is defined

as an “affinity enhancement over and beyond what would be expected from the concentration increase for the determinant sugar in a multivalent ligand” (42). Triantennary glycopeptide with three terminal galactose residues showed approximately a 10^5 -affinity enhancement over a monovalent ligand for the human hepatic lectin expressed on the human hepatoma cell line HepG₂. In the same study, data from partial structures of the triantennary glycopeptide suggested that the affinity increased about 1000-fold per galactoside residue.

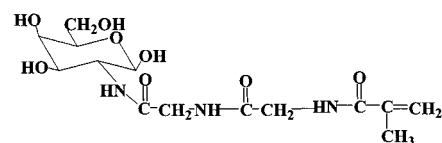
This paper describes the synthesis of novel HPMA copolymers bearing pendant saccharide moieties of different structure and content, which facilitates targeting. The saccharides were chosen based on their specific interactions with the asialoglycoprotein receptor of hepatocytes. The ultimate aim of our research is to investigate the influence of the structure and content of the targeting saccharide group on the specific recognition of saccharide-containing HPMA copolymers by hepatocarcinoma cells. The purpose of this characterization is to identify saccharide moieties with improved attachment properties. The influence of multiple sugar residues attached to individual monomers composing HPMA copolymers (clustering) and the degree of polymer substitution (monitored as sugar concentration: 10–30 mol %) were assessed with regard to delivery efficacy to hepatocarcinoma cells.

EXPERIMENTAL SECTION

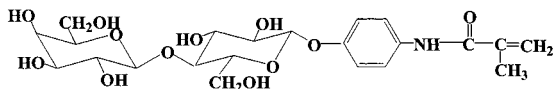
Materials. All sugars were of D configuration and in the pyranose form. 2,2'-Azobis(isobutyronitrile) (Polysciences, Warrington, PA) was recrystallized from methanol. Solvents were freshly distilled. D-(+)-Galactosamine hydrochloride, D-(+)-galactose, and β -D-lactose, and Dulbecco's phosphate-buffered saline (DPDS) were obtained from Sigma, St. Louis, MO. Palladium-on-charcoal (Aldrich, Milwaukee, WI) and 6-(benzyloxycarbonamido)-hexanoic acid (Bachem, King of Prussia, PA) were used without further purification. 2,3,4,6-Tetra-O-acetyl- β -D-galactopyranosyl bromide (43) and 4-bromo-2,3,6,2',3',4',6'-hepta-O-acetyl- β -lactoside (44) were prepared by the published methods indicated. MEM- α medium and FBS (fetal bovine serum) were obtained from HyClone (Logan, UT). Monensin (sodium salt) was purchased from Calbiochem, La Jolla, CA.

Cell Line. The human hepatocarcinoma cell line HepG₂ was purchased from the American Type Culture Collection (ATCC, Rockville, MD). HepG₂ cells were cultured in MEM- α medium supplemented with 10% FBS. Cells were grown at 37 °C in a humidified atmosphere of 5% (v/v) CO₂ in air.

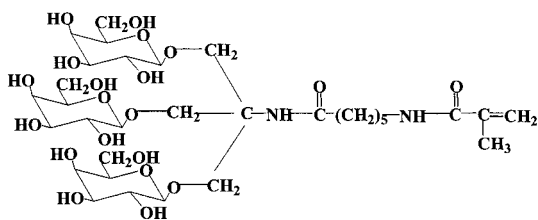
Methods. The intermediary and final products were characterized by classical methods (SEC, UV spectrophotometry, ¹H NMR, elemental analysis) and by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Colorimetric quantification of natural sugars by the phenol–sulfuric-acid assay and of *N*-acetylated sugars by the Morgan-Elson assay were conducted by the published procedures (45). Amino groups were quantified by modified TNBS assay (46) using glycine as the standard. The TLC examination of reaction mixtures and products was performed on silica gel layers (F₂₅₄) precoated on aluminum sheets. Carbohydrate-containing compounds were visualized by spraying the sheets with 15% sulfuric acid in 50% ethanol and heating on a hot plate at ~150 °C. Amino-containing components were detected by spraying the sheets with 0.5% ninhydrin in ethanol and heating the plates briefly. Aromatic groups were detected by exposing the plate to iodine vapor in an enclosed chamber for a few minutes.



N-Methacryloyl glycyglycylgalactosamine (MA-GG-GalN) [3].



4-Methacryloylamidophenyl- β -lactoside (MA-Ph-Lac) [6].



N-methacryloyl-6-(aminohexanamido)-tris-(β -D-galactopyranosyloxymethyl) methane (MA-AHT-(Gal)₃) [9].

Figure 1. Scheme of the synthesized monomers.

Monomer Synthesis. *N*-(2-Hydroxypropyl)methacrylamide [HPMA] [1] and 5-[3-(methacryloylaminopropyl)-thioureidyl] fluorescein [MA-AP-FITC] [2] were prepared according to previously described procedures (47 and 48, respectively).

N-Methacryloyl glycyglycylgalactosamine (MA-GG-GalN) [3]. *N*-Methacryloyl glycyglycine *p*-nitrophenyl ester (MA-Gly-Gly-ONp) and *N*-methacryloyl glycyglycylgalactosamine [MA-Gly-Gly-GalN] were prepared as previously described (49). Briefly, MA-Gly-Gly-ONp was synthesized by reacting methacryloyl chloride (MA-Cl) with glycyglycine (Gly-Gly-OH) in a basic solution to yield methacryloyl glycyglycine (MA-Gly-Gly-OH) (yield 82%), which was then coupled with *p*-nitrophenol (PNp) in DMF in the presence of DCC to give MA-Gly-Gly-ONp in 79% yield. MA-Gly-Gly-GalN was prepared by reacting MA-Gly-Gly-ONp (3.21 g, 0.01 mol) with galactosamine (2.80 g, 0.013 mol) at 0 °C, in dry DMSO (20 mL), in the presence of triethylamine (1.5 mL, 0.015 mol). The reaction mixture was continuously stirred at 20 °C for 6 h. The obtained methacryloyl glycyglycylgalactosamine was purified and crystallized to give 2.31 g (65%) of MA-GG-GalN; mp 179–181 °C (dec). Its ¹H NMR (200 MHz, D₂O) spectrum and elemental analysis were in agreement with the structure given (32): δ 1.8 (s, 3H), 3.5–3.7 (m, 4H), 3.9 (m, 6H), 5.1 (d, 1H), 5.3 (s, 1H), 5.7 (s, 1H). Anal. Calcd for C₁₄H₂₃N₃O₈ (361): C, 46.53; H, 6.41; N, 11.64. Found: C, 46.37; H, 6.51; N, 11.69. The structure of MA-GG-GalN is shown in Figure 1.

4-Aminophenyl 2,3,6,2',3',4',6'-hepta-O-acetyl- β -lactoside [4] was prepared as previously described by Roy et al. (25). Briefly, transformation of lactose into acetobromolactose through the intermediary of octa-O-acetyl-lactose was carried out using 45% w/v HBr in glacial acetic acid (44) to give acetobromo-lactose in 55% overall yield. The 2,3,6,2',3',4',6'-hepta-O-acetyl- β -lactopyranosyl bromide was then glycosylated with PNP using

phase transfer-catalyzed (PTC) condition, in the presence of tetrabutylammonium hydrogen sulfate (TBAHS), to result in 4-nitrophenyl 2,3,6,2',3',4',6'-hepta-O-acetyl- β -lactoside in 53% yield. Transformation of the nitro group into the amino one was performed by using 10% Pd/C in the presence of ammonium formate. A solution of 4-nitrophenyl β -lactoside (241.5 mg, 0.319 mmol) and ammonium formate (400 mg) in 40 mL of MeOH containing 10% Pd/C (50 mg) was gently warmed for 5–10 min in a stoppered round-bottom flask. After cooling, the reaction mixture was filtered and rinsed with methylene chloride. TLC in hexane/EtOAc (1:1) indicated a complete conversion of the nitro into amino. The reaction product gave a positive response in a ninhydrin test. The solvents were evaporated, and the residue was dissolved in methylene chloride. The organic phase was washed with water and dried with sodium sulfate. The residue obtained from the solvent gave pure 4-aminophenyl-2,3,6,2',3',4',6'-hepta-O-acetyl- β -lactoside (240 mg, 96%), which failed to crystallize from ethanol/methanol. ¹H NMR spectrum and elemental analysis of intermediary and compounds are identical to the given structures (13,24). mp 104–124 °C. Anal. Calcd for C₃₂H₄₁NO₁₈: C, 52.82; H, 5.68; N, 1.92. Found: C, 52.19; H, 5.74; N, 1.89. ¹H NMR (200 MHz, CDCl₃): δ 6.74 (d, 2 H), 6.55 (d, 2 H), 5.29–4.76 (6dd, 6H), 4.43 (d, 2 H), 4.07–4.02 (m, 3 H), 3.85 (m, 2 H), 3.63 (m, 1 H), 2.10–1.89 (7s, OAc).

4-Methacrylamidophenyl 2,3,6,2',3',4',6'-hepta-O-acetyl- β -lactoside [5] was synthesized according to the procedure described by Roy et al. (25). Compound [4] (460 mg, 0.632 mmol) and triethylamine (385 μ L, 3.82 mmol) in CH₂Cl₂ (44 mL) were cooled to 0 °C. Methacryloyl chloride (89 μ L, 1.25 equiv) in CH₂Cl₂ (16.5 mL) was added dropwise, while the temperature was kept at 0 °C for a further 30 min, after which it was allowed to reach room temperature. MeOH (2 mL) was then added, and the reaction mixture was stirred for a further hour. Water (60 mL) was then added, and the organic phase was washed successively with 0.5 M HCl, saturated NaHCO₃, and water. The dried (Na₂SO₄) organic phase was filtered and evaporated to dryness. The residue was purified by silica gel column chromatography with CHCl₃/EtOAc to give pure 4-methacrylamidophenyl 2,3,6,2',3',4',6'-hepta-O-acetyl- β -lactoside, (480 mg, 95%), which failed to crystallize. Anal. Calcd For C₃₆H₄₅NO₁₉ (795): C, 54.34; H, 5.66; N, 1.76. Found: C, 54.85; H, 6.32; N, 1.50. ¹H NMR (200 MHz, CDCl₃): δ 7.41 (d, 2 H), 6.89 (d, 2 H), 5.71 (s, 1 H), 5.40 (s, 1 H), 5.28–4.91 (m, 4 H), 4.43 (d, 2 H), 4.05–3.81 (m, 5 H), 2.09–1.90 (7s, OAc), 1.53 (t, 3 H), 1.19 (t, 3 H), 0.82 (t, 2 H).

4-Methacryloylamidophenyl- β -lactoside (MA-Ph-Lac) [6] was prepared by de-O-acetylation of 5 in methanol containing a catalytic amount of sodium methoxide. Compound 5 (480 mg, 0.603 mmol) was dissolved in warm MeOH (15 mL) containing 0.5 M sodium methoxide (40 μ L). After a few hours at room temperature, an additional 15 mL of MeOH containing sodium methoxide was added, and the solution was warmed again. The solution was stirred overnight at room temperature. Ether (25 mL) was added to the cold solution. Filtration yielded with pure 6 (336 mg, 67%). mp 208–212 °C. Anal. Calcd for C₂₂H₃₁NO₁₂ (501): C, 52.69; H, 6.19; N, 2.73. Found: C, 51.73; H, 6.32; N, 2.65. ¹H NMR (200 MHz, CDCl₃): δ 7.25 (d, 2 H), 7.01 (d, 2 H), 5.67 (s, 1 H), 5.42 (s, 1 H), 5.01 (d, 1 H), 4.34 (d, 1 H), 3.80–3.20 (m, 11 H), 1.67 (s, 3 H), 1.03 (t, 1 H). The structure of MA-Ph-Lac is shown in Figure 1.

6-(Aminohexanamido)tris(2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyloxymethyl)methane, (AHT-

[Gal(OAc)₄]₃ [7]. According to the method reported by Lee et al. (49), Tris was coupled with Cbz-aminoheptanoic acid in absolute ethanol in the presence of 2-ethoxy-*N*-(ethoxycarbonyl)-1,2-dihydroquinoline (EEDQ) to afford a 65% yield of [6-(benzyloxycarbonamido)hexanamido]-tris(hydroxymethyl)methane (Cbz-AHT). Transformation of galactose into 2,3,4,6-tetra-*O*-acetyl- β -D-galactopyranosyl bromide was carried out using 45% w/v HBr in glacial acetic acid (43) to effect a 97% yield of 1-Br-Gal-(OAc)₄. The acetylated sugar was glycosylated by coupling with Cbz-AHT, as described by Polidori et al. (51), in acetonitrile in the presence of mercuric cyanide (catalyst) followed by sonication under nitrogen atmosphere to give a 66.5% yield of the [6-(benzyloxycarbonamido)hexanamido]tris(2,3,4,6-tetra-*O*-acetyl- β -D-galactopyranosyloxymethyl)methane (Cbz-AHT-[Gal(OAc)₄]₃) conjugate. Hydrogenolysis in 60% acetic acid in the presence of 10% palladium on carbon was performed, to give 74% yield of AHT-[Gal(OAc)₄]₃. ¹H NMR analysis (CDCl₃) data indicated the removal of the benzyl group, showing the presence of aminoheptanoyl group at 5.6 ppm and the presence of acetylated galactose in correct ratios. Mass spectrum analysis (+FAB): 1225.7 (M + H⁺). Furthermore, colorimetric analysis for the amino group (TNBS method) and galactose (phenol-sulfuric acid method) indicated the ratio of these groups to be 1:3. The ¹H NMR spectrum and MS analysis of intermediary and final product are in agreement with the given structures (45, 52, 53).

***N*-Methacryloyl-6-(aminohexanamido)-tris(2,3,4,6-tetra-*O*-acetyl- β -D-galactopyranosyloxymethyl)methane (MA-AHT-[Gal(OAc)₄]₃) [8].** The monomer in the acetylated form was synthesized by coupling AHT-[Gal(OAc)₄]₃ with methacryloyl chloride (MA-Cl) in CH₂-Cl₂ in the presence of triethylamine at 0 °C. A solution of AHT-[Gal(OAc)₄]₃ (20 mg, 0.0163 mmol) and triethylamine (20 μ L, 11 equiv) in CH₂Cl₂ (1.0 mL) was cooled to 0 °C. Methacryloyl chloride (2 μ L, 1.1 equiv) in CH₂-Cl₂ (0.5 mL) was added dropwise while the temperature was allowed to reach room temperature. The reaction mixture was stirred for 2 h. Water (2 mL) was then added, and the organic phase was successively washed with 0.5 M HCl, saturated NaHCO₃, and water. The dried (Na₂SO₄) organic phase was filtered and evaporated to dryness. MA-AHT-[Gal(OAc)₄]₃ was obtained in 65% yield. TLC in EtOAc:acetic acid:H₂O (3:2:1) indicated complete consumption of the amino-compound. ¹H NMR spectra (200 MHz, CDCl₃) confirmed the appropriate functional groups (acetylated galactose, aminohexanoyl, oxymethylene, and double bond at 5.4 and 5.5 ppm). Mass spectrum analysis (+FAB): 1316 (M + Na⁺).

***N*-Methacryloyl-6-(aminohexanamido)tris(β -D-galactopyranosyloxymethyl)methane (MA-AHT-(Gal)₃) [9].** De-*O*-acetylation of MA-AHT-[Gal(OAc)₄]₃ was performed in methanol containing a catalytic amount of sodium methoxide. MA-AHT-[Gal(OAc)₄]₃ (20 mg, 0.0154 mmol) was dissolved in dry methanol (800 μ L) containing 0.5 M sodium methoxide (20 μ L). After 24 h stirring at room temperature, an additional amount of dry methanol (800 μ L) containing 0.5 M sodium methoxide (20 μ L) was added, and the reaction mixture was stirred at room temperature for a further 24 h. Sixty-percent acetic acid (200 μ L) was then added (deionization), evaporated, and coevaporated with EtOH/water. The TLC in mixed solvents of EtOAc:acetic acid:H₂O (3:2:1) and EtOAc:2-propanol:H₂O (5:3:2) indicated a conversion of the per-*O*-acetylated derivative into a more polar product, MA-AHT-(Gal)₃ in a yield of 74%. ¹H NMR spectra (200 MHz, D₂O): δ 5.44 (s, 1H, =CH₂), 5.68 (s, 1H, =CH₂), 4.7–5.0

Table 1. Characteristics of HPMA Copolymer Conjugates Containing Pendant Saccharide Moieties

polymer	polymer no.	targeting moiety	mol % sugar ^a	mol % FITC ^b	approx Mw of conjugate (Da) ^c
P-Gal	10a	GalN	14.8	2.04	15000
	10b	GalN	20.4	2.42	16000
	10c	GalN	29.1	2.64	16000
P-Lac	11a	lactose	11.1	1.58	18000
	11b	lactose	19.9	1.76	20000
	11c	lactose	27.6	1.90	19500
P-TriGal	12a	tri-galactose	8.47	2.23	14500
	12b	tri-galactose	13.6	2.34	14000
	12c	tri-galactose	24.4	2.52	14000
P-F	13	—	—	2.51	20000

^a The contents of galactose and lactose residues were determined spectrophotometrically using the phenol-sulfuric acid assay; the content of galactosamine was determined by the Morgan–Elson assay. ^b The content of FITC was determined spectrophotometrically using $\epsilon = 82000 \text{ M}^{-1} \text{ cm}^{-1}$ (FITC; 495 nm, pH 9.1 (0.1 M sodium borate)). ^c Weight average molecular weights (Mw) of copolymers were estimated by SEC using a Superose 12 column, FPLC system, PBS buffer pH 7.3, calibrated with poly(HPMA) fractions.

(m, 3H, α -H), 3.5–4.5 (m, 24H, sugar), 3.28 (m, 2H, CH₂N), 2.30 (t, 2H, CH₂O), 1.95 (s, 3H CH₃), 1.5–1.7 (m, 2H, CH₂), 1.2–1.4 (m, 2H, CH₂), 0.8–1.1 (m, 2H, CH₂). Mass spectrum analysis (+FAB): 788.3 (M + H⁺), 809.8 (M + Na⁺). The structure of MA-AHT-(Gal)₃ is shown in Figure 1.

Polymer Synthesis. Polymer glycoconjugates containing fluorescein were synthesized by radical precipitation copolymerization as described below. The polymers are of the general structure shown in Figure 2. The characterization of polymers and the method of preparation are shown in Table 1. All the polymerizations were conducted using free radical copolymerization of three monomers: HPMA, MA-AP-FITC, and the saccharide-containing monomer (either MA-GG-GalN, or MA-Ph-Lac, or MA-AHT-(Gal)₃). Three copolymers of each saccharide moiety (i.e., galactosamine, lactose, or trivalent-galactose) were synthesized. The monomer feed concentrations were 3 mol % of MA-AP-FITC; 10 mol %, 20 mol %, and 30 mol % of the saccharide-containing monomer, and 87 mol %, 77 mol % and 67 mol % of HPMA, respectively. The concentration of monomers, with respect to initiator and solvent, was in the weight ratio of 12.5:1.0:86.5, respectively. The monomer mixture of the control copolymer, which did not contain carbohydrate moiety, was composed of 98 mol % of HPMA to make up the lack of carbohydrate-containing monomer. The polymerization was initiated using AIBN.

Synthesis of P-(AP-FITC)-GalN [10a–c]. Galactose-containing HPMA copolymers were synthesized by copolymerization of HPMA, MA-GG-GalN, and MA-AP-FITC in acetone/DMSO (7:3). All monomer mixtures contained 3 mol % (17.7 mg) of MA-AP-FITC; the contents of MA-GG-GalN were 10 mol % (40 mg), 20 mol % (80 mg), and 30 mol % (120 mg), and of HPMA were 87 mol % (138 mg), 77 mol % (122.2 mg), and 67 mol % (106.4 mg), respectively. MA-GG-GalN and MA-AP-FITC were dissolved in DMSO, HPMA, and AIBN were dissolved in acetone. The solutions were mixed, transferred to an ampule, bubbled for 10 min with nitrogen and sealed, and the mixture was polymerized in a water bath at 50 °C for 40 h while stirring. After polymerization, the ampules were cooled to room temperature and opened, and the volume was reduced under vacuum. The viscous liquid thus obtained was precipitated into a large excess of acetone. The precipitated polymer was dissolved in distilled water, dialyzed against water for 24 h, and

etone. Yields: 97% (271 mg). The characterization of copolymer is shown in Table 1.

Flow Cytometric Analysis. Cell suspensions were prepared by trypsinization of cell monolayers. Cells (1×10^6) were seeded in 24-well plates in 2 mL of MEM- α medium supplemented with 10% FBS. The cells were incubated separately with 500 ng/mL of the saccharide-containing HPMA copolymers (P-Gal, P-Lac, and P-TriGal) for 24 h at 37 °C under 5% CO₂ atmosphere. Control cells were incubated with the same concentration of the FITC-labeled HPMA copolymer without sugar moiety (P-F). Inhibition experiments were performed with the same concentrations of the saccharide-containing copolymers, but in the presence of 150 mM lactose in the incubation medium. After incubation, the polymeric solutions were removed from the well, and the cells were washed twice with 1 mL of cold PBS containing 1mM CaCl₂, 1mM MgCl₂ and 0.5% BSA. Cells were harvested by trypsinization with 0.25% (w/v) trypsin and further incubated for 30 min at 4 °C in PBS containing 50mM monensin. Cell suspensions were immediately analyzed by flow cytometry using fluorescence activated cell sorter (FACS, Becton Dickinson). Cells were gated according to light-scattering parameters.

Confocal Microscopy. The intrinsic fluorescence of FITC was also used to monitor the subcellular fate of the saccharide-containing HPMA copolymer conjugates (P-Gal, P-Lac and P-TriGal) with different contents of pendant saccharide moieties. After trypsinization, 2×10^4 cells were seeded on a N1 coverslip (Corning) and cultured for 24 h. The culture medium was replaced with medium containing polymer conjugates (20 μ g/mL) and cultured for 24 h at 37 °C. Unbound conjugates were removed by washing the cell layer three times with cold PBS. Cells were fixed with 3% paraformaldehyde for 10 min at room temperature and washed again. The fate of glycoconjugates in cells was analyzed on a BioRad (Hercules, CA) MRC-600 laser scanning confocal imaging system with a Zeiss (Oberkochen, Germany) Axioplan microscope with a plan-apo objective (60x, NA=1.4, oil) and a krypton/argon laser. Images were acquired with a Fluor filter block (excitation at 488 nm, emission collected with a 515 nm barrier filter). Settings were adjusted so that control (untreated) cells always yielded dark images. Fluorescent images were scaled to 256 Gy levels. Quantitative analysis was performed using Image-Pro Plus 4.0 (Media Cybernetics). Fluorescence intensities were expressed in arbitrary units per square micron.

RESULTS

The saccharide-containing monomers (MA-GG-GalN, MA-Ph-Lac, and MA-AHT-(Gal)₃) copolymerized with HPMA and small amounts of MA-AP-FITC gave high molecular weight compounds soluble in water. Analysis of the sugar content in the copolymers (Table 1) confirmed that the saccharide-containing monomer units present in the polymerization mixture were polymerized to give a final product in which the ratio of monomer units was close to their initial ratio in the reaction mixture. SEC analysis of the final preparations confirmed that the weight average molecular weight (Mw) of all copolymers was similar and did not alter under the influence of saccharide (substitution) attachment. These data indicate that the monomers synthesized did not contain minute amounts of dimethacryloylated impurities. The latter would act as cross-linking (branching) agents increasing the molecular weight with increasing content of saccharide-containing monomers in the polymerization mixture.

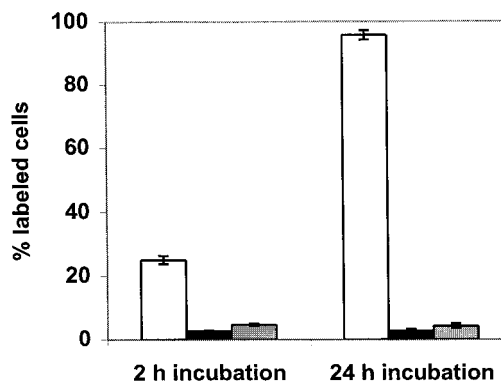


Figure 3. Flow cytometry analysis of the binding of 10% P-Gal (10 μ g/mL) to HepG₂ cells, in the presence of 150-mM lactose (solid bars) and the absence of lactose (open bars), after 2 and 24 h incubation at 37 °C. HPMA copolymer (P-F), which did not contain sugar moiety served as control (gray bars).

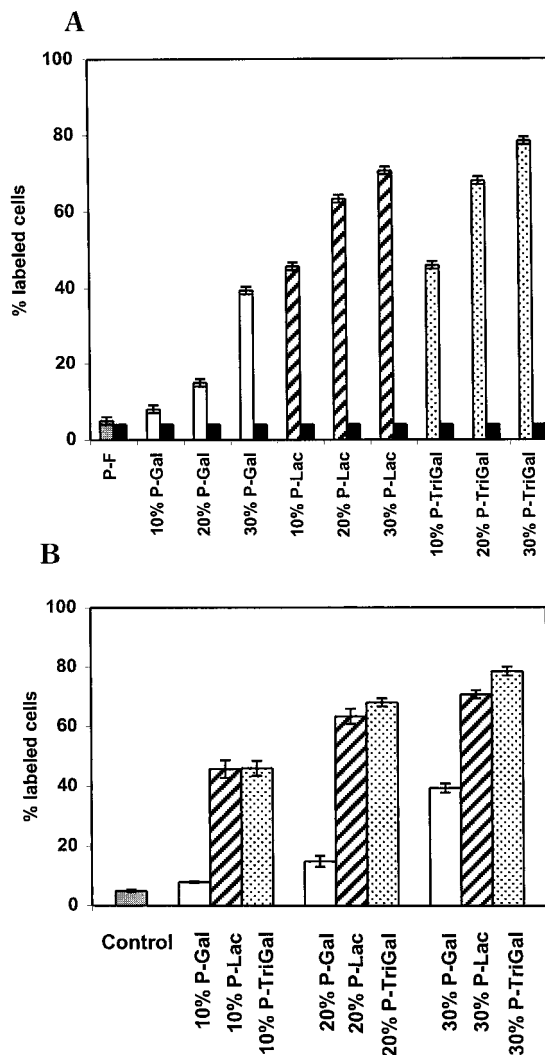


Figure 4. Flow cytometry analysis of the binding of the saccharide-containing HPMA copolymers (500 ng/mL) to HepG₂ cells after 24 h incubation at 37 °C. Upper panel (4A): Binding in the presence (solid bars) and absence of free lactose (150-mM). In all studies P-F (gray bars, left) served as control. (P-Gal, open bars; P-Lac, hatched bars; P-TriGal, dotted bars). Lower panel (4B): Binding dependency on the type and the content of the sugar moiety in the copolymer.

The results of binding of the fluorescein-labeled glycoconjugates to HepG₂ cells are shown in Figures 3 and 4. The total amount of cell-associated conjugate increased

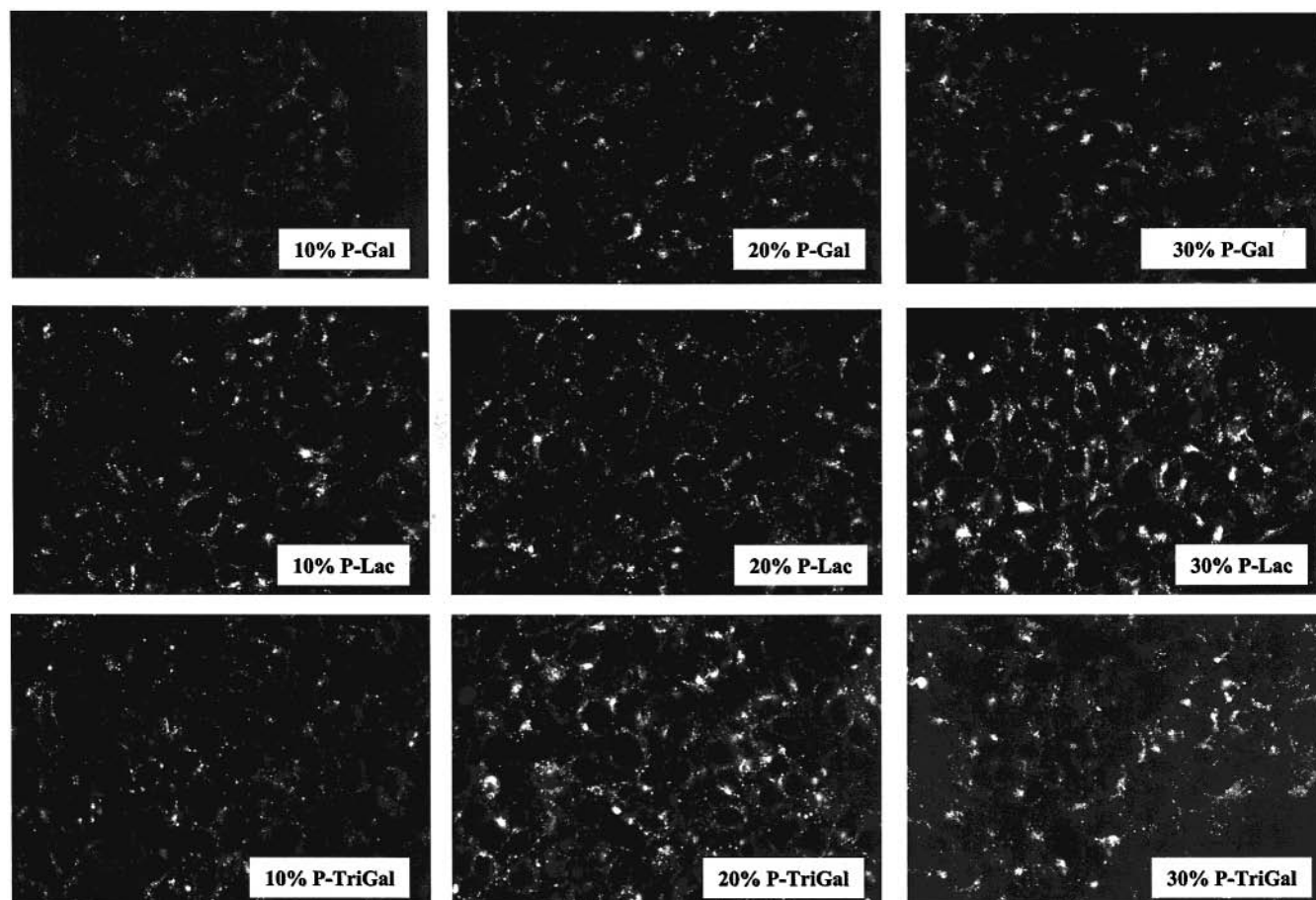


Figure 5. Confocal fluorescence images of HepG₂ cells incubated with the saccharide-containing HPMA copolymer conjugates, after 24 h incubation at 37 °C. Internalized glycoconjugates were transported into the lysosomal compartment of the cells. Upper three images (left to right): 10% P-Gal, 20% P-Gal, and 30% P-Gal; Centered three images (left to right): 10% P-Lac, 20% P-Lac, and 30% P-Lac. Lower three images (left to right): 10% P-TriGal, 20% P-TriGal, and 30% P-TriGal.

when the incubation period was raised (Figure 3). At 37 °C the fluorescence intensity of all cells incubated with the glycoconjugates was greater than that of the cells incubated at 4 °C, suggestive of an endocytic process (data not shown). The cell-associated fluorescence of HepG₂ cells after incubation with the HPMA copolymer conjugates for 24 h at 37 °C is summarized in Figure 4. The flow cytometry analysis revealed strong binding of the copolymers to HepG₂ cells. The fluorescence intensity indicated that their biorecognition by hepatocarcinoma cells was dependent on the type and the content of the pendent saccharide moieties in the copolymer. The increased glycosylation level of copolymers with galactose and lactose, caused an increase in cell binding. In general, the binding of the trivalent galactoside and the lactoside-containing copolymers was found to be greater than that of the galactoside-containing copolymers (Figure 4). In the lowest sugar concentration (10 mol %), a 5.6-fold difference in binding intensity was observed between the trivalent-galactoside- and the monovalent-galactoside-containing copolymer. Twenty mol % concentration resulted in a difference of 4.5-fold, whereas in the highest concentration (30 mol %) the difference was only 2 times greater between the trivalent and monovalent galactoside-containing copolymers. The specificity of lectin–glycopolymers interactions was verified by an inhibition assay. The incubation of cells with 150 mM lactose (competitive inhibitor) completely abolished cell binding of copolymers and indicated sugar specificity of the process involved (Figures 3 and 4). In addition, copoly-

mers with no sugar bound very weakly to the cells, ruling out nonspecific reactivity (Figure 3).

The uptake and subcellular trafficking of FITC-labeled HPMA copolymer conjugates by HepG₂ cells were followed by confocal fluorescence microscopy. Cells were incubated with the HPMA copolymer conjugates for 24 h at 37 °C, before optical sections were taken. Confocal images indicated the lysosomotropism of the conjugates, i.e., internalized glycoconjugates were transported into the lysosomal compartment of the cells (Figure 5). The internalization rate of conjugates was dependent on the type and the content of the pendant carbohydrate moiety in the copolymers. The higher the contents of each sugar moiety in the HPMA copolymer, the brighter the image. Furthermore, the internalization rates correlated well with the biorecognition of the saccharide moieties by the cells, as detected by flow cytometry, i.e., the trivalent galactoside- and the lactoside-containing copolymers were internalized more efficiently than the galactoside-containing copolymers (Figure 6). The fluorescence images of cells stained with the untargeted copolymer P-F (which did not contain sugar) were dimmer than the targeted polymer at the same polymer concentration, indicating no presence of the nontargeted copolymer inside the cells.

DISCUSSION

HPMA copolymers designed in this study can be used as drug carriers to achieve optimal targeting to hepatocarcinoma cells. The incorporation of sugar residues into HPMA copolymers results in effective biorecognition by

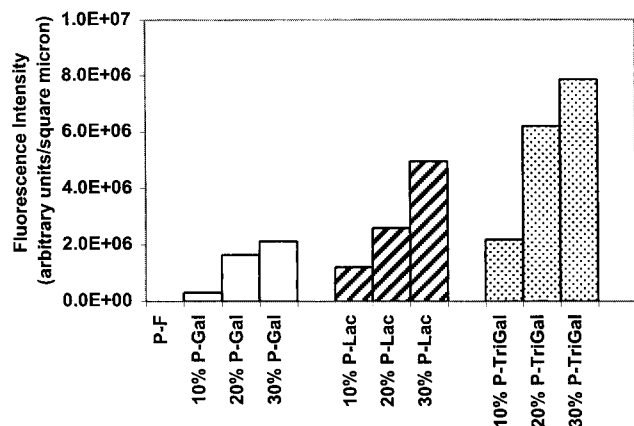


Figure 6. Fluorescence intensity of HepG₂ cells incubated with the saccharide containing HPMA copolymer-FITC conjugates, after 24 h at 37 °C. The fluorescence intensity is expressed in arbitrary units per square micron.

liver cells (52). The copolymers were evaluated for their potential to induce a marked enhancement of binding affinity toward hepatocytes.

The copolymers used in this study were synthesized by radical copolymerization of HPMA, MA-AP-FITC, and the saccharide-containing monomers. From the similarity in the structure of the polymerizable parts of the comonomers, it was expected that the copolymerization parameters would be close (49) and consequently the monomer units randomly distributed along the polymer backbone. In addition, the distribution of chemical composition of the resulting copolymers was expected to be narrow, despite the high polymerization yields.

Of the mammalian lectins discovered to date, the ASGP-R has been studied in greatest detail (10, 13, 14, 53, 54). Although the ASGP-R binds and internalizes most galactose-terminated ligands, binding affinity is strongly influenced by the ligand structure. Lee et al. demonstrated that monovalent ligands such as galactose, lactose, and monoantennary galactosides bind the ASGP-R with a millimolar dissociation constant (12, 41). Ligands containing a cluster of three galactose residues bind to the receptor with a nanomolar dissociation constant which appear to be the affinity threshold needed to achieve appreciable targeting of glycoconjugates under physiological conditions (37). This relationship between sugar-clustering and enhanced binding affinity is related to the structure of the ASGP-R, which is composed of three subunits, that each of them contains a galactose binding site (1, 37). Although small polyvalent ligands often display enhanced binding affinity for target proteins, glycosylated polymers could achieve better biorecognition just by increasing the concentration of the biorecognizable sugar moiety along the polymeric chain (15).

To learn more about the relationship between the structural features of glycosylated polymers containing multiple glycoside residues and their biorecognition, we synthesized HPMA copolymers containing side-chains terminated with galactosamine, lactose, or trivalent-galactose. The relationship between the content of saccharide moieties, the three-dimensional arrangement of galactose residues, and their biorecognition and internalization by human hepatocarcinoma cells was investigated.

The observed strong binding of saccharide-containing HPMA copolymers to hepatocarcinoma cells in these

studies supports the hypothesis that the saccharide groups serve as ligands for hepatic lectins. Furthermore, it was demonstrated that efficient internalization of the saccharide-containing polymers by hepatocarcinoma cells occurred. This internalization increased with increasing the content of the sugar residues bound to the polymer. The endocytosis of the glycoconjugates was not clearly defined in the flow cytometry experiments. However, when considered together with the confocal data, there is a strong evidence to suggest that receptor-mediated endocytosis was responsible for the increased internalization of the glycopolymers. The internalization rates were dependent on the type of sugar bound to the polymer. Inhibition studies in the presence of a competitive inhibitor (free lactose) revealed a complete blockage of copolymer binding, indicating the sugar specificity of the process involved. Moreover, the binding intensity of the monovalent galactoside containing copolymer to HepG₂ cells is rather weak. However, the trivalent-galactoside-bearing copolymer displayed stronger binding intensities, which were attributed to the clustering of galactose residues. To achieve biorecognition of monosaccharides at levels comparable to multiantennary structures, HPMA copolymers must contain a high content (above 20 mol %) of randomly distributed carbohydrate moieties at side chain termini. About 30 mol % of galactose in the copolymer was needed to achieve binding similar to the 10% trivalent-galactose. Nevertheless, the enhancement in binding affinity observed in these studies was somehow less dramatic than expected (as described by Lee (37) and Whitesides (38)). The increase in valency was only weakly reflected in the reactive potency of the glycoconjugates to enhance binding affinity. This factor did not exceed 5.6-fold binding enhancement of the triantennary over the monoantennary galactoside-containing copolymer. A possible explanation could be that in these binding studies the polymer concentration was relatively high. At high polymer concentrations, receptor saturation may occur and consequently decrease the efficiency of targeting to the cells (55). If that was the case, it could explain why high concentrations of the lactoside- and trivalent-galactoside-containing copolymers maintained, more or less, similar binding intensities.

Our findings are consistent with the data reported by Vansteenkiste et al., indicating that dextran substituted with trigalactose units was captured by the liver only 1.6-times faster than the mono-galactose-substituted dextran (56). Interestingly we found that the HPMA copolymer substituted with triantennary galactose exhibits a larger binding intensity difference between the triantennary- and monoantennary-substituted copolymers than the substituted-dextran, at the same concentrations (5.6-fold difference for HPMA compared with 1.6-fold difference for dextran). This difference may be attributed to the influence of the polymer backbone on the recognition, i.e., HPMA copolymer backbone is more flexible than dextran, thus the chain may have a conformation in which several sugar moieties from the same chain interact with one receptor. The fact that the ratio of tri- to mono-binding decreases with increasing contents of sugar in the copolymer also supports this point.

Affinity studies of ligands containing glycoside residues were traditionally been undertaken using glycoconjugates labeled with radioactive probes. This approach allows binding affinities to be tested even at the nanomolar concentration range. Perhaps incubation studies at these low concentrations may be more indicative of the extent of copolymer uptake by receptor mediated endocytosis, and will exhibit more pronounced clustering effect.

In conclusion, the binding of the saccharide-containing HPMA copolymers to HepG₂ cells was demonstrated by flow cytometry. While the results of the binding experiments could not distinguish endocytosis from surface adherence, the use of confocal microscopy did show the presence of the glycoconjugates inside the cells. The receptor-mediated endocytosis is suggested since the nontargeted copolymer could hardly be detected inside the cells under similar conditions. The data are in agreement with the results of Omelyanenko et al. (50) who observed in human ovarian carcinoma cells that subcellular organelles containing HPMA copolymer conjugates were morphologically similar to organelles containing a lysosomal marker, Neutral Red. The relative rate or extent of endocytosis of the glycoconjugates was dependent on the type and the content of the sugar moiety. Our results suggest that the copolymers containing multivalent carbohydrate clusters may be considered as a targetable delivery system with enhanced binding affinity toward hepatocytes. It appears that high sugar concentrations facilitate multiple binding domains and therefore enhance the binding of the polymer to recognition sites on hepatocytes. Since the ASGP-R is expressed on HepG₂ cells, it is reasonable to speculate that the increased binding of the saccharide-containing HPMA copolymers reported here is due to endocytosis mediated by the asialoglycoprotein receptor.

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Enhancing Sequence-Specific Cleavage of RNA within a Duplex Region: Incorporation of 1,3-Propanediol Linkers into Oligonucleotide Conjugates of Serinol–Terpyridine

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The syntheses and RNA cleavage efficiencies of a new series of oligonucleotide conjugates of Cu(II)–serinol–terpyridine and 1,3-propanediol are reported. These reagents, termed ribozyme mimics, were designed such that they would yield *multiple* unpaired RNA residues directly opposite the site of the RNA cleavage catalyst upon ribozyme mimic–RNA duplex formation. This design effect was implemented using the 1,3-propanediol linker **3**, which mimics the three-carbon spacing between the 5′- and 3′-hydroxyls of a natural nucleotide. Incorporation of one or more of these 1,3-propanediol linkers at positions directly adjacent to the serinol–terpyridine modification in the ribozyme mimic DNA strand resulted in cleavage at multiple phosphates in a complementary 31-mer RNA target sequence. The linkers effectively created artificial mismatches in the RNA–DNA duplexes, rendering the opposing RNA residues much more susceptible to cleavage via the transesterification/hydrolysis pathway. The RNA cleavage products produced by the various mimics correlated directly with the number and locations of the linkers in their DNA strands, and the most active ribozyme mimic in the series exhibited multiple turnover in the presence of excess 31-mer RNA target.

INTRODUCTION

As the impact of scientific research at the interface of chemistry and biology continues to expand, so does our recognition of the potential that exists in the area of biological mimicry by synthetic reagents. One such area of interest today is the development of synthetic mimics of ribozymes (or ribozyme mimics). Since the earliest reports of sequence-specific RNA cleavage by this unique class of bioconjugates (1–5), much effort has been devoted to the establishment of structure–activity relationships for these reagents (2, 6–15). One of the principal goals is to achieve catalytic cleavage of specific RNA substrates with efficiencies approaching and/or exceeding those of natural ribozymes and ribonucleases (16). A ribozyme mimic is typically synthesized by the covalent attachment of an RNA cleavage catalyst to a synthetic DNA strand (or DNA analog) via a linker arm. The catalyst is usually a metal complex or nitrogen-containing organic molecule that catalyzes the scission of RNA phosphodiester bonds via transesterification and/or hydrolysis reactions. The DNA probe serves as the recognition domain of the ribozyme mimic, and the catalyst serves as the active site. Through the convenience of automated DNA synthesis and the specificity of complementary Watson–Crick base pairing, the DNA strand can be custom-tailored to direct the RNA cleaving capability of the catalyst toward any RNA target of known base sequence.

The central concept behind ribozyme mimic development is that upon selectively binding to its complementary RNA sequence, the mimic will deliver the attached RNA cleavage catalyst across the major or minor groove

of the DNA–RNA duplex and within sufficient proximity of the RNA phosphodiester backbone to effect the RNA transesterification reaction. Theoretically, the ribozyme mimic should disengage from the resultant RNA product fragments and subsequently bind to and cleave another complementary RNA target sequence. This would in effect create a catalytic cycle of specific RNA destruction.

Our approach over the last decade (1, 13, 16, 17), coupled with Magda's recently proven strategy (6) for achieving catalytic activity with ribozyme mimics, encourages placement of the catalyst at an internal position within the ribozyme mimic DNA strand rather than simply tethering the catalyst to the end of the sequence. This is because cleavage of the RNA target within the duplex region is more favorable for product release, given that cleavage outside of the duplex region will be plagued by product inhibition. One of the major concerns with this particular strategy, however, is the ability of ribozyme mimics to cleave the RNA residues within the double-stranded regions of DNA–RNA hybrids (18). RNA in a DNA–RNA duplex is relatively inert to cleavage by metal ions and complexes in comparison to its single-stranded form (19). This is likely a consequence of the conformational rigidity that is characteristic of duplexed RNA. In fact, this pronounced difference in cleavage susceptibility between single- and double-stranded RNA has been exemplified by pioneering efforts from several research groups. For example, Kolasa et al. demonstrated that when a short DNA oligonucleotide was annealed to a segment of t-RNA^{Phe}, the complementary RNA sequence was protected from cleavage by Eu³⁺- and La³⁺-based RNA cleavage catalysts (18). The results of this study led the authors to conclude that in the design of ribozyme mimics, it may be more advantageous to place the catalyst at the end of the DNA strand (proximate to single-stranded RNA) rather than at an internal position where the RNA is duplexed by DNA and inert to cleavage. In a related study, Hegg et al. investigated the

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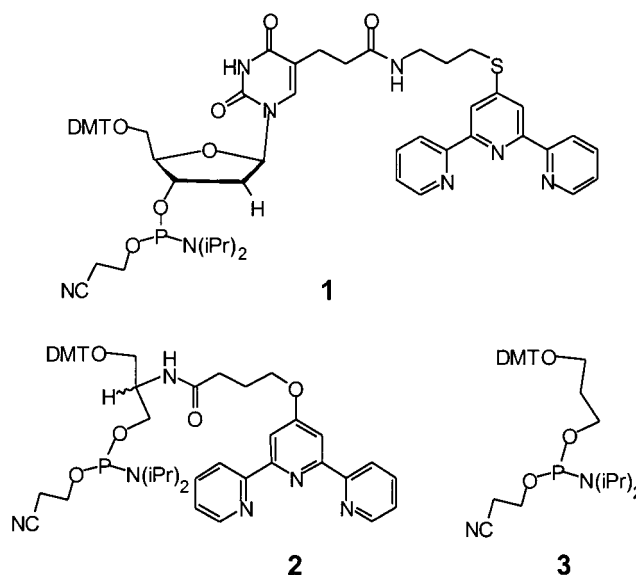
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cleavage of RNA hairpin structures by a macrocyclic Cu^{2+} complex (20). Cleavage occurred in both the single- and double-stranded regions of the RNA hairpins; however, the amount of cleavage in double-stranded regions was not as pronounced as that observed in single-stranded regions. In another relevant study, Huesken et al. showed that RNA bulges in DNA–RNA duplexes are highly susceptible to cleavage by Mg^{2+} ion and La^{3+} hexadentate-Schiff base macrocycles (21). The authors concluded that the single-stranded RNA in the bulged region has a high degree of conformational freedom, which allows the RNA to adopt the in-line conformation required for the transesterification reaction. On the basis of these findings, the same research group designed ribozyme mimics that would place lanthanide-based RNA cleavage catalysts directly opposite induced bulges in the RNA target strand (11). These mimics were found to be 13 times more reactive than those that formed perfect duplexes with the RNA target. Most recently, this group reported ribozyme mimics that function with multiple turnover (12). In that study, lanthanide metal complexes were covalently linked to the 5'-ends of 2'-methoxyethyl (MOE) oligonucleotides. The key to this approach was that the oligonucleotide portions of the ribozyme mimics were designed such that upon binding to the RNA target, a bulge would be formed in the RNA strand near the center of the DNA–RNA duplex region. The attached catalyst could subsequently be folded back to preferentially cleave the single-stranded RNA in the bulged region. This creative design yielded catalytic turnover with the 2'-MOE conjugates, but no evidence of catalytic activity was observed with conjugates comprised of unmodified DNA probes.

In an analogous context, we demonstrated that the extent of sequence-specific RNA cleavage by $\text{Cu}(\text{II})$ terpyridine-based ribozyme mimics could be substantially increased using the concept of minimal nucleotide replacement (13). In that study, serinol–terpyridine modification **2** was incorporated in place of a single nucleotide residue in the ribozyme mimic DNA strand. The serinol–terpy effectively served as an artificial abasic site, which (upon DNA–RNA duplex formation) left an unpaired RNA residue directly opposite the site of the modification. The increased level of conformational freedom provided by the serinol–terpyridine modification rendered its opposing RNA residue much more susceptible to cleavage than when in the fully base-paired state and resulted in a greater than 3-fold increase in overall RNA cleavage efficiency in comparison to our first-generation, fully duplexed ribozyme mimics based on modification **1**.

The work described in this report represents the next logical step in our group's efforts to design ribozyme mimics that exploit the enhanced cleavage susceptibility of single-stranded RNA over double-stranded RNA. We have designed a new series of ribozyme mimics that yield *multiple* unpaired RNA residues directly opposite the site of the RNA cleavage catalyst ($\text{Cu}(\text{II})$ -terpy) upon duplex formation. This design effect was achieved using the 1,3-propanediol linker **3**, which, like serinol, mimics the three-carbon spacing between the 5'- and 3'-hydroxyls of a natural nucleotide. Incorporation of single and multiple linkers at positions adjacent to a serinol–terpyridine modification in the DNA strand resulted in perturbations to the opposing RNA backbone that allowed for the cleavage of multiple phosphodiester linkages by metal-catalyzed transesterification and/or hydrolysis reactions. The most active ribozyme mimic in the series exhibited cleavage of multiple equivalents of its complementary



RNA substrate, revealing a catalytic rather than stoichiometric mode of action for this reagent.

EXPERIMENTAL PROCEDURES

Synthesis of Ribozyme Mimics. The parent DNA sequence and the conjugates containing the serinol–terpyridine or 1,3-propanediol linker modifications (Figure 2) were prepared by automated DNA synthesis on an ABI Model 380B DNA synthesizer. The modified phosphoramidite building blocks **2** and **3** were placed on two of the free auxiliary ports of the synthesizer and incorporated at appropriate positions in the synthesis protocols. The detailed synthesis of phosphoramidite **2** has been previously reported by our group (13). Linker phosphoramidite **3** was purchased from Glen Research and used as received. All oligonucleotides were purified by UV-shadow excision from preparative 20% denaturing polyacrylamide gels, followed by elution from the gel fragment and ethanol precipitation. The structures of probes 1–7 were confirmed by negative-ion MALDI-TOF mass spectrometry (Table 1). The concentrations of the oligonucleotide conjugates were determined by UV absorbance at 260 nm utilizing molar extinction coefficients (ϵ) calculated with the Oligo Analyzer program (version 2.0) from Integrated DNA Technologies (IDT). [ϵ (L/mol·cm): probe 1, 283,700; probe 2, 276,500; probe 3, 269,000; probe 4, 276,500; probe 5, 266,600; probe 6, 269,300; probe 7, 252,500].

Preparation of 3'-End-Labeled 31-mer RNA Target Sequence. The synthetic 31-mer RNA sequence (5'-AAACCAACCCUUCAGAGACUCUGUCGACGGG-3') was purchased from IDT. The RNA was 3'-end labeled using α - ^{32}P -dATP (Amersham) and poly(A) polymerase (USB) according to standard procedures (22). Note that this procedure adds a single dA residue to the 3' end of the RNA, increasing the total length to 32 nucleotides. The labeled RNA was phenol extracted, ethanol precipitated, and purified by excision from a 20% denaturing polyacrylamide gel. The RNA was then eluted from the gel fragment, ethanol precipitated, and diluted to the appropriate concentrations with RNase-free water. RNA concentrations were determined by UV absorbance at 260 nm using the molar extinction coefficient calculated using the Oligo Analyzer program (version 2.0) from IDT [ϵ (L/mol·cm): 302 500].

Table 1. MALDI-TOF Mass Spectral Analysis of Conjugates

probe	calcd mass [M - H] ⁻¹	obsvd mass [M - H] ⁻¹
1	9719	9728
2	9568	9556
3	9402	9427
4	9568	9562
5	9402	9397
6	9417	9407
7	9084	9078

Sequence-Specific RNA Cleavage Reactions. RNA cleavage reactions were carried out in a total volume of 10 μ L containing 10 mM (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) (HEPES) buffer (pH 7.4), 0.1 M NaClO₄ (to control ionic strength), 250 nM 31-mer RNA, 5 μ M ribozyme mimic, and 5 μ M CuSO₄. The ribozyme mimic and CuSO₄ were premixed and added to a solution of the RNA, buffer, and NaClO₄. The reactions tubes were tightly sealed and completely submerged in a 45 °C water bath for 40 h. Quenching of the reactions was achieved through the addition of 10 μ L gel-loading buffer. Aliquots of 10 μ L were run on 20% denaturing polyacrylamide sequencing gels, alongside partial RNase T1 digests and base hydrolysis lanes for mapping of the RNA cleavage sites. The electrophoresis images were quantified using a Molecular Dynamics PhosphorImager and the ImageQuant software package (Version 5.0).

Catalytic Cleavage of RNA with Probe 6. The sequence-specific RNA cleavage reactions with probe 6 in the presence of excess RNA target were carried out in a total volume of 10 μ L containing 10 mM (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) (HEPES) buffer (pH 7.4), 0.1 M NaClO₄, 125 nM probe 6, 125 nM CuSO₄, and either 250 nM, 500 nM, 1.0 μ M, or 1.25 μ M of 31-mer RNA. The ribozyme mimic and CuSO₄ were premixed and added to a solution containing the RNA, buffer, and NaClO₄. The reactions tubes were tightly sealed and completely submerged in a water bath at 45 °C for 40 h, after which the reactions were quenched by the addition of 10 μ L gel-loading buffer. Aliquots of 10 μ L were run on 20% denaturing polyacrylamide sequencing gels, and the electrophoresis images were quantified using a Molecular Dynamics PhosphorImager and the ImageQuant software package (Version 5.0).

RESULTS AND DISCUSSION

The structures of the ribozyme mimics employed in this study are detailed in Figure 1. Figure 2 is a representative autoradiograph of a 20% denaturing polyacrylamide gel obtained after treatment of the 3'-³²P-end labeled 31-mer RNA target with probes 1–7.

In these experiments, ribozyme mimics containing from zero to four linkers surrounding the serinol-terpyridine modification were tested for sequence-specific RNA cleavage activity in the presence of Cu²⁺ ion. For each probe, the serinol-terpy modification was located at position 16 (from the 5'-end) of the 31-mer DNA sequence. The 1,3-propanediol linkers were placed to the 3'-side, to the 5'-side, and to both sides of the modification simultaneously. The ribozyme mimic and RNA target were incubated in the presence of Cu²⁺ for 40 h at 45 °C and pH 7.4. For electrophoretic analyses, RNA cleavage lanes were run alongside partial RNase T1 digests and base hydrolysis lanes to allow mapping of the exact sites of cleavage produced by the different mimics. The relative RNA cleavage percentages of the probes are denoted in Figure 2.

The ribozyme mimics employed in this study have structural differences in their respective DNA strands that yield unique RNA cleavage patterns for each reagent (Figure 1). The patterns are clearly governed by the locations of the 1,3-propanediol linkers in the DNA sequences. Probe 1, which contains a single serinol terpyridine modification in the middle of the DNA strand (16th position), cleaves the RNA phosphodiester linkages directly opposite the expected location of the catalyst upon RNA–DNA duplex formation. The serinol-terpy modification of the DNA strand is located directly opposite nucleotide rA¹⁶ in the complementary RNA strand, and the major site of cleavage is observed at this position as expected. There is also a minor cleavage band at residue rG¹⁵ to the 5' side of the cleavage site. These results were consistent with our previous observations using these types of reagents (13).

Cleavage of the RNA target with probe 2, which contains a single three-carbon linker to the 3'-side of the serinol-terpy in the DNA strand (directly across from rG¹⁵), yielded slightly different results. The major site of cleavage remained at nucleotide rA¹⁶; however, the number of minor cleavage bands increased toward the 5'-side of the RNA. A minor cleavage band was observed at nucleotide rA¹⁴ in addition to the minor band at rG¹⁵. There was also a slight increase in the amount of cleavage at position rG¹⁵ in comparison to probe 1.

Incorporation of two linkers to the 3'-side of the serinol-terpy residue in the DNA strand (probe 3) results in an even further extension of the number of minor cleavage bands to the 5'-side of the major cleavage site (rA¹⁶) in the RNA strand. In this case, the amount of cleavage at rA¹⁴ increased, but the amount of cleavage at rG¹⁵ did not (in comparison to probe 2).

For comparison, we investigated the effects of placing the linker to the 5'-side of the serinol-terpy modification in the DNA strand. Cleavage of the RNA target with probes 4 and 5, which contain one and two linker(s) to the 5'-side of the serinol-terpy respectively, yielded results similar to those obtained with the analogous probes 2 and 3. Nucleotide rA¹⁶ was the major site of cleavage, and rG¹⁵ was a minor site of cleavage in each case. Probe 4, which was designed to place a linker across from nucleotide rG¹⁷ in the RNA strand, led to an additional minor cleavage band at position rG¹⁷. Probe 5, which was designed to place linkers directly opposite positions rG¹⁷ and rA¹⁸ yielded two additional minor cleavage bands at these locations as expected.

To provide further corroboration of the utility of this approach, we prepared ribozyme mimics having linkers on both the 3'- and 5'- sides of the serinol-terpy modification in the DNA strand. Probe 6 consisted of one linker to the 3'-side of the modification, in addition to one linker to the 5'-side of the modification. Interestingly, cleavage of the RNA target with this reagent revealed a shift in the major site of cleavage from nucleotide rA¹⁶ (as observed with probes 1–5) to nucleotide rG¹⁵—a one-nucleotide shift toward the 5'-end of the RNA sequence. Minor cleavage bands were also observed at nucleotides rA¹⁴, rA¹⁶, rG¹⁷, and rA¹⁸. These cleavage products essentially represented a combination of the cleavage products obtained separately with probes 2 and 4, except that the major site of RNA cleavage was shifted by one nucleotide. A similar phenomenon was observed with probe 7, which contains two linkers to the 3'-side of the serinol-terpy as well as two linkers to the 5'-side of the serinol-terpy. In this case, the major site of cleavage was shifted even further to the 5'-side of the RNA to position rA¹⁴. Minor cleavage bands were also observed at nucle-

Probe 3	Probe 2	Probe 1	Parent Strand (PS)	RNA Target	Probe 4	Probe 5	Probe 6	Probe 7	Control Probe
3'	3'	3'	3'	5'	3'	3'	3'	3'	3'
T	T	T	T	A	T	T	T	T	T
T	T	T	T	A	T	T	T	T	T
T	T	T	T	A	T	T	T	T	T
G	G	G	G	C	G	G	G	G	G
G	G	G	G	C	G	G	G	G	G
T	T	T	T	A	T	T	T	T	T
T	T	T	T	A	T	T	T	T	T
G	G	G	G	C	G	G	G	G	G
G	G	G	G	C	G	G	G	G	G
A	A	A	A	U	A	A	A	A	A
A	A	A	A	U	A	A	A	A	A
G	G	G	G	C	G	G	G	G	G
S	S	C	T	A	X	X	X	S	S
X	X	X	C	G	S	S	S	S	S
C	C	C	C	A	T	G	G	G	G
T	T	T	T	C	G	A	A	A	A
G	G	G	G	U	A	A	A	A	A
A	A	A	A	C	G	G	G	G	G
A	A	A	A	U	A	A	A	A	A
C	C	C	C	G	C	C	C	C	C
A	A	A	A	U	A	A	A	A	A
G	G	G	G	C	G	G	G	G	G
C	C	C	C	A	T	T	T	T	T
T	T	T	T	C	G	G	G	G	G
G	G	G	G	G	C	C	C	C	C
C	C	C	C	G	C	C	C	C	C
C	C	C	C	G	C	C	C	C	C
5'	5'	5'	5'	dA*	5'	5'	5'	5'	5'
				3'					

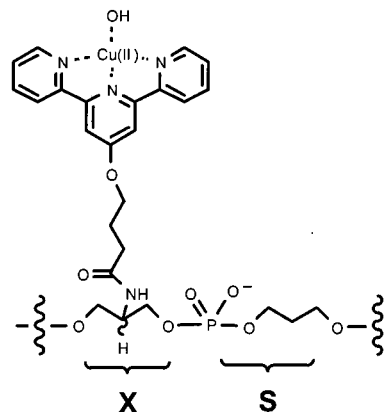


Figure 1. Structures of the ribozyme mimics and 31-mer RNA target sequence employed in this study. The “X” indicates the location of the serinol–terpyridine modification in the DNA strand, while the “S” represents the 1,3-propanediol linker modification. The 31-mer RNA target sequence is shown in **bold** text.

otides rC¹³, rG¹⁵, rA¹⁶, rG¹⁷, and rA¹⁸, which represents a combination of the cleavage products obtained with probes 3 and 5 separately. In this instance, however, the major site of cleavage was shifted by *two* nucleotides toward the 5′-end of the RNA target. The observed shifts in the major site of cleavage with probes 6 and 7 may offer some structural insights into the orientation of the serinol–terpyridine modification in the DNA–RNA duplex. When the degree of flexibility is equal on both sides of the serinol–terpy modification, cleavage occurs preferentially toward the 5′-side of the RNA strand, suggesting a preference for delivery of the catalyst across the larger major groove of the DNA–RNA duplex.

In addition to the sequence-specific RNA cleavage reactions using modified probes as described above, control experiments were conducted with the parent and control probes (Figure 1). Both of these probes lack the serinol–terpyridine modification in their DNA strands and hence possess no RNA cleavage capabilities. The parent probe is simply the full complement of the 31-mer RNA target sequence employed in this study. As

expected, no cleavage of the RNA target was effected by this probe in the presence of Cu(II) under our standard assay conditions (Figure 2, lane 4). The control probe, which contains four consecutive 1,3-propanediol linkers in the center of the DNA strand, also effected no significant cleavage of the RNA target (<3%) in the presence of excess Cu(II) (data not shown). These control experiments explicitly prove that the RNA cleavage products obtained with ribozyme mimics 1–7 are the direct result of the serinol–terpyridine modification in the DNA strand and not merely the effect of free Cu(II) ions on the induced single-stranded regions of the RNA target.

To further define the RNA cleavage efficiencies of this new series of ribozyme mimics, the most active probe in the series, probe 6, was examined for its ability to cleave multiple equivalents of complementary 31-mer RNA target. The experiments were designed such that the probe concentration was held constant at 125 nM, while the concentration of RNA target was increased from 250 nM (a 2-fold excess) to 1.25 μ M (a 10-fold excess).

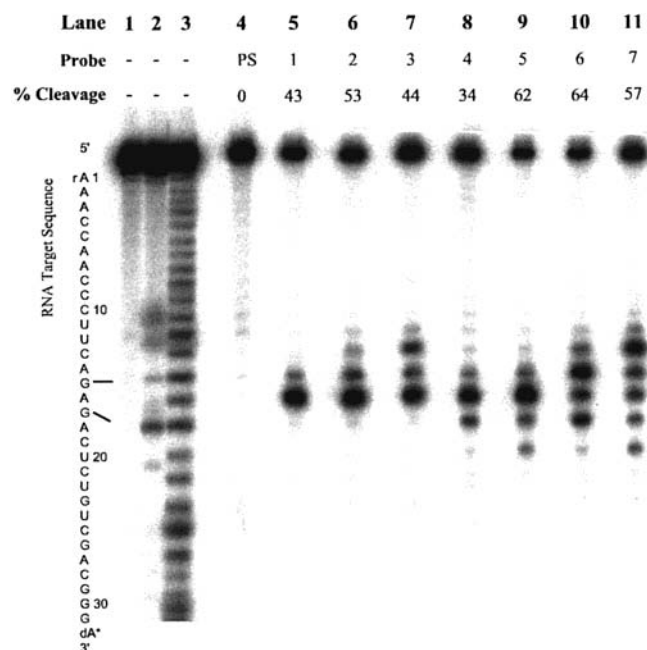


Figure 2. Autoradiograph of the sequence-specific cleavage of 31-mer RNA with probes 1–7. Lane 1: 31-mer RNA starting material; lane 2: RNase T1 digest of 31-mer RNA (rG-specific); lane 3: NaOH hydrolysis of 31-mer RNA; lanes 4–11: Incubation of 31-mer RNA in the presence of the parent strand (PS) and probes 1–7. The reactions in lanes 4–11 were carried out at 45 °C in a total volume of 10 μ L containing the following: 0.1 M NaClO₄, 10 mM HEPES (pH 7.4), 250 nM RNA, 5 μ M probe, and 5 μ M CuSO₄. The probes and CuSO₄ were premixed, and the reactions were stopped after 40 h with gel loading buffer. The cleavage percentage for each probe is shown at the top of the gel and is reported as the average of three experimental runs.

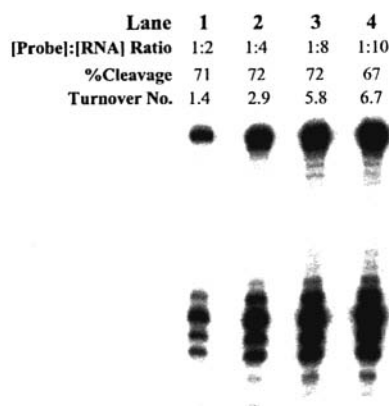


Figure 3. Autoradiograph of a 20% denaturing polyacrylamide gel showing the results of cleavage reactions involving probe 6 in the presence of excess 31-mer RNA target. The reactions were carried out in a total volume of 10 μ L containing 10 mM HEPES buffer (pH 7.4), 0.1 M NaClO₄, 125 nM probe 6, 125 nM CuSO₄, and the concentrations of 31-mer RNA target specified below. The reaction tubes were submerged in a water bath at 45 °C for 40 h and quenched by the introduction of gel loading buffer. Lane 1: 125 nM probe 6 with 250 nM RNA; lane 2: 125 nM probe 6 with 500 nM RNA; lane 3: 125 nM probe 6 with 1.0 μ M RNA; lane 4: 125 nM probe 6 with 1.25 μ M RNA.

Quantification of the cleavage results from these experiments (Figure 3) revealed that up to 6.7 equiv of RNA were cleaved by probe 6 in the presence of 10 equiv of RNA substrate (Figure 3, lane 4). In addition, as the concentration of excess RNA decreased, so did the number of turnovers (Figure 3, lanes 1–3). The findings of this study reveal that probe 6 functions in a catalytic rather than stoichiometric mode of action under these

conditions. This represents a major discovery, considering that there have been only two additional reports of catalytic activity with ribozyme mimics to date (6, 12). Interestingly, each of the prior studies also encompassed the idea of increasing the degree of flexibility in the target RNA strand, thereby highlighting the importance of this strategy in the design of ribozyme mimics that function with multiple turnover.

CONCLUSIONS

A new series of ribozyme mimics has been designed to cleave multiple phosphates in the RNA backbone. This was achieved by increasing the total number of unpaired RNA residues directly opposite the site of the RNA cleavage catalyst in the ribozyme mimic–RNA duplex. The RNA residues were forced into the unpaired state by replacing their complementary nucleotides with the 1,3-propanediol linker **3**, which lacks the sugar ring and the nucleobase of a normal deoxynucleoside. Incorporation of one or more of these linkers at positions directly adjacent to the serinol–terpyridine modification in the ribozyme mimic DNA strand resulted in cleavage at multiple phosphates in the RNA target sequence. The linkers effectively created artificial mismatches, which distorted the phosphodiester backbone of the RNA target and increased its susceptibility to cleavage by Cu(II)–serinol–terpyridine. The RNA cleavage products produced by these ribozyme mimics correlated directly with the number and locations of the linkers in the DNA strands. A major finding of this study was that the most active ribozyme mimic in the series, probe 6, cleaved multiple equivalents of complementary RNA target in the presence of an excess of the target. The results of this study will serve as important additions to the growing set of selection rules for the design of highly efficient mimics of ribozymes. We are currently combining this strategy with other approaches to make fully optimized ribozyme mimics that may ultimately have utility as catalytic antisense therapeutics and as tools for molecular biology.

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Note Added in Proof: For recent, complementary studies, see (1) Putnam, W. C., Daniher, A. T., Trawick, B. N., and Bashkin, J. K. (2001) Efficient new ribozyme mimics: direct mapping of molecular design principles from small molecules to macromolecular, biomimetic catalysis. *Nucleic Acids Res.* 29 (10), 2199–2204. (2) Osiek, T. A., and Bashkin, J. K. (2001) Probing the Importance of Electrostatic Interactions of Ce(III) with the Phosphodiester Backbone During Transesterification Using Methylphosphonates, *New J. Chem.* 25, 541–544. (3) Osiek, T. A., Putnam, W. C., and Bashkin, J. K. (2000) in *Ribozyme mimics: the evolution of gene-specific chemotherapy* (P. F. Torrence, Ed.) pp 385–405, Wiley, New York.

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A pH-Sensitive Polymer That Enhances Cationic Lipid-Mediated Gene Transfer

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The efficient release of nonviral gene carriers from endosomes is an important step for the successful delivery of DNA into the cell nucleus. A synthetic pH-sensitive anionic polymer, poly(propylacrylic acid) (PPAA), was designed to aid in endosomal escape of nonviral vectors and improve the transfection efficiencies with these vectors. Transfection of NIH3T3 fibroblasts with ternary physical mixtures of the cationic lipid DOTAP, pCMV β plasmid DNA, and PPAA showed marked enhancement of both gene expression levels and fraction of cells transfected compared to binary control mixtures of DOTAP and DNA. PPAA also significantly improved the serum-stability of DOTAP/DNA vectors. The DOTAP/DNA/PPAA vectors maintained high levels of transfection in media containing up to 50% serum. The striking enhancement of transfection efficiency with cationic lipid/DNA/PPAA mixtures, along with the enhanced serum-stability, suggests that PPAA may provide significant improvements for the *in vivo* intracellular delivery of drugs such as DNA, oligonucleotides, proteins, and peptides.

INTRODUCTION

Gene therapy represents a promising approach for the treatment of a wide variety of diseases. DNA delivery systems based on attenuated viruses have shown the greatest successes to date (1), but numerous concerns over the safety of these vectors (2–6) have led to the development of nonviral vectors for use in gene therapy. The efficacy achieved with these nonviral vectors, however, is significantly lower than that achieved with viral vectors. The low transfection efficiencies are due to the multiple biological barriers encountered during their delivery from extracellular locations into the cell nucleus (7). One of the major problems with systemic nonviral gene delivery is that these particles are typically unstable in the circulation. Furthermore, if the particles are able to reach the target cell and are endocytosed, the endosomes are generally trafficked to lysosomes, where degradation of the DNA occurs.

A number of biological organisms have evolved surface proteins to solve similar trafficking problems, and a variety of viral-based, fusogenic peptides have been studied as pH-dependent membrane-disruptive components in gene delivery systems to enhance transport from the endosome to the cytoplasm. For example, fusogenic peptides derived from the N-terminal domain of hemagglutinin (INF peptides, from the influenza virus) (8, 9) and the rationally designed GALA-based peptides developed by Szoka (10–12) undergo a conformational change at acidic pHs from a negatively charged, random coil to a hydrophobic, amphipathic α -helical structure. This structural transition leads to the insertion of the peptides into phospholipid bilayers and subsequent pore formation and disruption of these bilayers (13, 14). Significantly

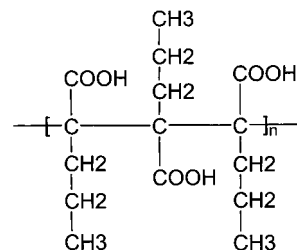


Figure 1. Chemical structure of poly(propylacrylic acid).

higher levels of gene expression have been reported when INF peptides or GALA were added to polyplex carriers (8, 9, 12), while only moderate enhancements were observed upon addition of the peptides to lipoplexes (15–18).

While these results show the potential of incorporating additives that enhance endosomal release into gene carrier systems, the peptides may exhibit intrinsic limitations of immunogenicity (19–21), stability, and/or cost. The development of synthetic, pH-sensitive polymers that can mimic the function of fusogenic peptides may provide an alternative to peptide-based pH-dependent membrane destabilization in drug delivery. Tirrell and co-workers previously detailed the pH-dependent mechanism of vesicle disruption of poly(ethylacrylic acid) (PEAA) (22, 23). We have synthesized other polymers belonging to the alkyl acrylic acid family of polymers that exhibit greater pH-sensitive membrane disruption than PEAA (24, 25). In this study, we show that the incorporation of a particularly active polymer, poly(propylacrylic acid) (PPAA) (Figure 1), into lipoplex carriers can significantly enhance gene expression levels and provide serum stability in an *in vitro* cell culture model.

EXPERIMENTAL SECTION

Synthesis of Poly(propylacrylic acid). Poly(propylacrylic acid) (PPAA) was synthesized according to the protocols published previously (24). Briefly, polymeriza-

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tion of propylacrylic acid was performed using 2,2'-azobisisobutyronitrile (AIBN) as the initiator and cystamine as a chain transfer agent. Polymerization reactions were performed at 60 °C for 24 h. The resulting polymer was dissolved in methanol and precipitated using diethyl ether. The average molecular weight of PPAA was 61 kD as determined by GPC using PEG standards on Ultra-hydrogel 250 and 1000 columns (Waters Corporation, Milford, MA). The pH-sensitive membrane-disrupting activity of the PPAA produced was confirmed by red blood cell hemolysis assays as described previously (24, 25).

In Vitro Transfection. NIH3T3 mouse fibroblast cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO₂. The cells were seeded into 12-well cell culture plates the day prior to the transfection experiments at a density of 7.5×10^4 cells/well. These cells were transfected with ternary physical mixtures of particles consisting of PPAA, the cationic lipid dioleyltrimethylammonium propane (DOTAP) (Roche Molecular Biochemicals, Indianapolis, IN), and the pCMV β plasmid DNA (CLONtech, Palo Alto, CA) which encodes for β -galactosidase. Control mixtures consisted of the DOTAP and plasmid DNA without PPAA. The DOTAP was first mixed with pCMV β in HEPES-buffered saline (HBS, pH 7.4), incubated for 30 min, with subsequent addition of PPAA. Mixtures were prepared with theoretical charge ratios ranging from 1.0 to 1.9 ($\pm$). The charge ratio was determined as the total positive to negative charge, calculated using the assumption of 33% PPAA ionization at pH 7.4 as determined from titration experiments (data not shown). For cell transfections, 0.7 mL of DMEM with variable amounts of FBS and 0.3 mL of solutions containing ternary mixtures of DOTAP/DNA/PPAA or binary control mixtures of DOTAP/DNA were added to each well. All formulations were tested in triplicate. The cells were incubated with these transfection mixtures for 4 h at 37 °C. The media was removed after this incubation period and replaced with 2 mL DMEM/10%FBS. The cells were then incubated for an additional 48 h prior to assaying for reporter gene activity.

Quantitation of Transfection Efficiency. The amount of β -galactosidase activity present within the transfected cells was quantified by utilizing the β -Galactosidase Enzyme Assay System (Promega, Madison, WI). Cell extracts were collected after lysis of the cells with the addition of Reporter Lysis Buffer. *o*-Nitrophenyl- β -D-galactopyranoside (ONPG) was added to the extracts, and the amount of β -galactosidase activity was measured spectrophotometrically with a plate reader at 415 nm. Concurrently, the total protein produced by the cells was determined by measuring the protein content in the cell extracts by the BCA method (Pierce, Rockford, IL). The transfection efficiency was also monitored by histological staining of the NIH3T3 cells. NIH3T3 cells were cultured in two-well glass chamber slides (Nalge Nunc, Rochester, NY) and transfected using the procedure described above. The cells were then fixed with 2% formaldehyde/0.2% glutaraldehyde in PBS for 30 min following the 48 h incubation after transfection. After fixation, the cells were stained with X-gal (1 mg/mL X-gal, 2 mM MgCl₂, 5mM K₄Fe(CN)₆, and 5mM K₃Fe(CN)₆) and 20 μ g/mL 4',6-Diamidino-2-phenylindole (DAPI) for 2 h at 37 °C. The chambers were then removed and the slides mounted. A Nikon Eclipse E800 upright fluorescence microscope was used to examine the slides for β -galactosidase activity.

Cellular Uptake of Particles Using [³⁵S]-Labeled Plasmid DNA. The uptake of DOTAP vectors containing radiolabeled plasmid DNA with and without PPAA was

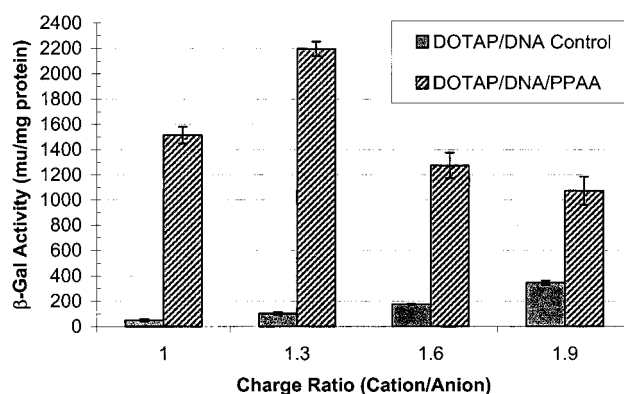


Figure 2. β -Galactosidase gene expression levels following transfection of NIH3T3 cells with either DOTAP/DNA or DOTAP/DNA/PPAA formulations in serum-free conditions. Both formulations were prepared at theoretical charge ratios ranging from 1.0 to 1.9. Each well of cells was transfected with particles containing 1.6 mg/mL of plasmid DNA. All formulations were tested in triplicate.

examined. The pCMV β plasmid DNA was labeled with [³⁵S]-dCTP (NEN, Boston, MA) by nick translation (26) using the Nick Translation System (Promega, Madison, WI). Purification of labeled plasmid DNA from unincorporated nucleotides and enzymes was performed by centrifugation with Microspin S-400 HR columns (Amersham Pharmacia, Piscataway, NJ). Uptake studies were performed by incubating NIH3T3 cells with either DOTAP/DNA or DOTAP/DNA/PPAA particles incorporating [³⁵S]-labeled plasmid DNA for 4 h at 37 °C. The cells were washed twice with PBS and then trypsinized from the substrate surface. The trypsinized cells were centrifuged to recover the cells and then lysed with a solution containing 0.3N NaOH/0.1% Triton X-100. The level of radioactivity within the cells was determined by liquid scintillation counting.

RESULTS

Transfection Enhancement in Serum-Free Conditions. Addition of PPAA to DOTAP/DNA lipoplexes significantly enhanced transfection efficiencies in NIH3T3 mouse fibroblast cells at all charge ratios tested (Figure 2). The level of β -galactosidase gene expression with ternary DOTAP/DNA/PPAA carriers was increased ca. 20-fold over control DOTAP/DNA vectors prepared with a particle charge ratio of 1.3 ($\pm$). A control experiment was performed incorporating another synthetic polymer, poly(acrylic acid) (PAA), which is also a carboxylic polyanion similar to PPAA but does not contain the repeating propyl groups along the backbone. Results from these transfections showed that an enhancement in gene expression was observed only with formulations containing PPAA (data not shown). This finding indicates that the enhancement of transfection by PPAA is due specifically to the unique properties of the PPAA polymer, which contains the repeating propyl group that confers the unique pH-dependency and hydrophobic membrane destabilizing properties. Histological staining of NIH3T3 cells with X-gal showed that the increase in gene expression levels correlated with an increase in the number of cells transfected with DOTAP lipoplexes containing PPAA (Figure 3).

Serum Stabilization of Lipoplexes with PPAA. Cationic lipoplexes are often inactivated by interactions with serum proteins (27, 28), so the ability of PPAA to influence the serum stability of DOTAP/DNA lipoplexes

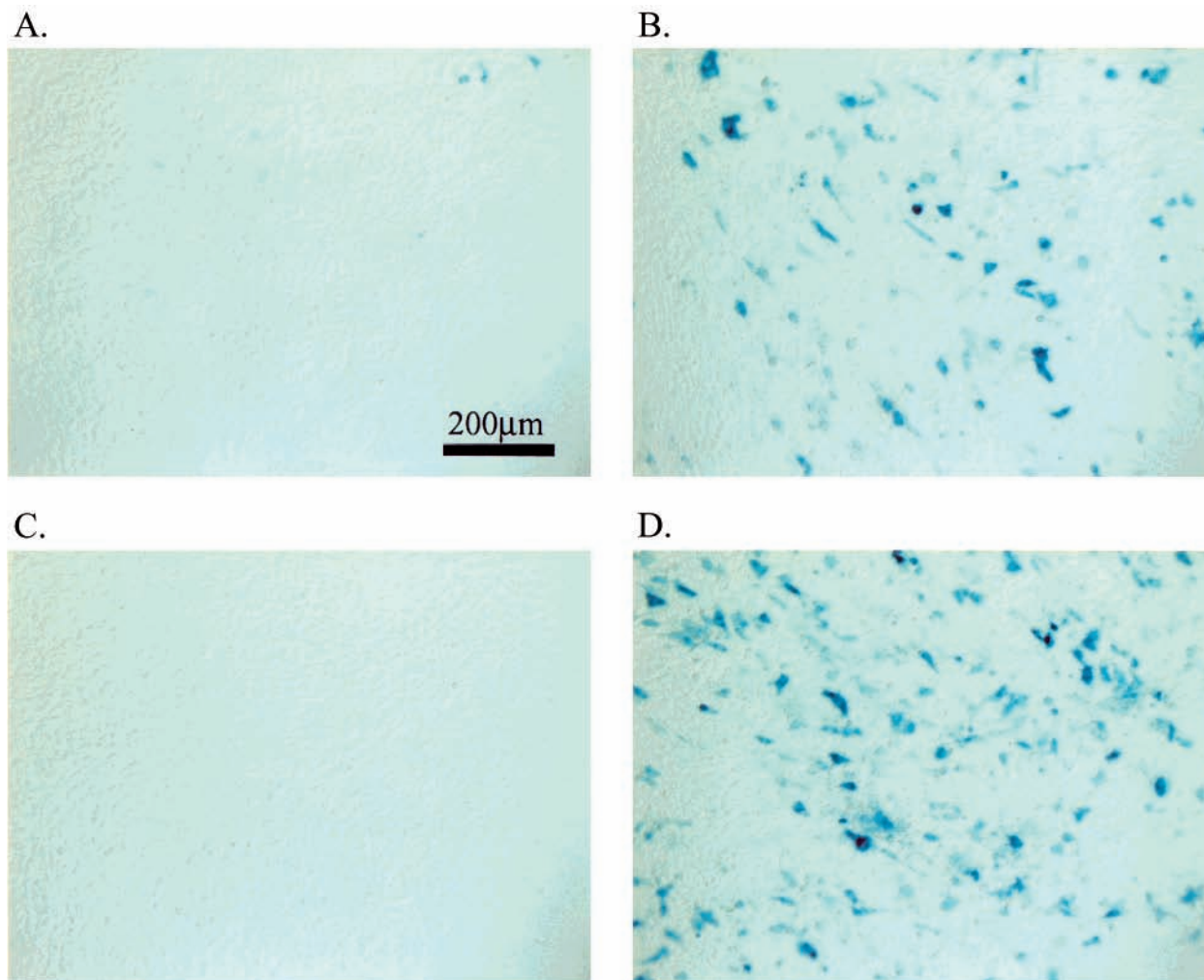


Figure 3. Histological staining of NIH3T3 cells with X-gal. Cells were transfected with (A) DOTAP/DNA 1.9 ($\pm$ charge ratio) particles in serum-free DMEM. (B) DOTAP/DNA/PPAA 1.3 ($\pm$) particles in serum-free DMEM. (C) DOTAP/DNA 1.9 ($\pm$) particles in DMEM/10% FBS. (D) DOTAP/DNA/PPAA 1.3 ($\pm$) particles in DMEM/10% FBS.

was tested. Cells were transfected with the DOTAP/DNA lipoplex with and without added PPAA, using formulations that provided the highest gene expression in serum-free conditions (at $\pm$ charge ratios for DOTAP/DNA = 1.9 and for DOTAP/DNA/PPAA = 1.3) and using media containing increasing levels of serum up to a maximum of 50% FBS. The DOTAP/DNA control formulation was inactivated in media containing as little as 10% FBS, while the DOTAP/DNA/PPAA particles exhibited high levels of transfection throughout the entire range of serum levels tested (Figure 4). Close to 70% of the gene expression level found in serum-free conditions was retained with DOTAP/DNA/PPAA particles at 50% FBS, and thus expression at serum levels simulating whole blood remained over 2-fold higher than the peak expression level of DOTAP/DNA particles observed in serum-free conditions (0% FBS). Histological staining for β -galactosidase in the presence of 10% FBS confirmed the absence of transfected cells, reiterating the inability of DOTAP alone to mediate transfection in the presence of serum. In contrast, a significant number of stained cells were evident after transfection with DOTAP/DNA/PPAA (Figure 3), confirming that high levels of transfection are retained in serum conditions upon addition of PPAA to the particles.

Quantitation of Particle Uptake with [^{35}]S-Labeled pCMV β . To measure the levels of plasmid uptake into cells, transfection studies were performed utilizing

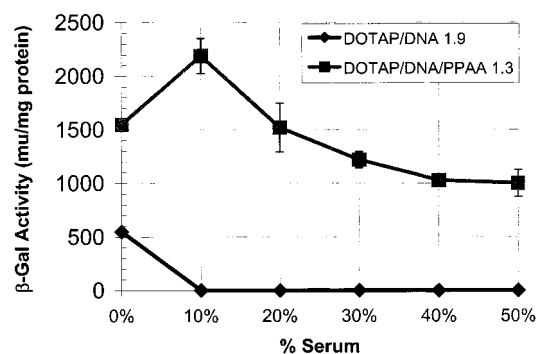


Figure 4. Effects of variable serum concentration on gene transfer efficiency. The ability of DOTAP/DNA and DOTAP/DNA/PPAA formulations, prepared at the optimal charge ratios as determined from serum-free conditions, were assessed in media containing FBS levels up to 50% of the media. Each formulation was tested in triplicate at each serum level.

a [^{35}]S-labeled plasmid DNA that was incorporated into the DOTAP lipoplex particles. The quantitative uptake of these particles was investigated in the presence of both serum-free DMEM and DMEM containing 10% FBS. The presence of serum proteins within the media reduced overall particle uptake with all formulations. The uptake of DOTAP/DNA formulations was reduced 10-fold, whereas uptake was reduced only 2-fold with DOTAP/DNA/PPAA particles (Table 1). Therefore, the differences in trans-

Table 1. Radioactive Uptake of [³⁵S]-labeled Plasmid DNA Incorporated into Transfection Formulations into NIH3T3 Cells^a

particles	media	particle uptake
DOTAP/DNA 1.9	serum-free DMEM	6.4 ± 0.23%
	DMEM/10% FBS	0.70 ± 0.077%
DOTAP/DNA/PPAA 1.3	serum-free DMEM	11.4 ± 1.1%
	DMEM/10% FBS	4.9 ± 0.41%

^a Percentages represent the fraction of radioactivity found within cells compared to the total radioactivity exposed to the cells. Each percentage represents the uptake from triplicate wells.

fection efficiencies with DOTAP/DNA/PPAA and DOTAP/DNA particles in the presence of serum, as measured both by level of expression and number of transfected cells, correlates with greater intracellular uptake of DNA from particles containing PPAA. The addition of PPAA also enhances intracellular uptake in the absence of added serum, although to a lesser extent than that observed in the presence of serum.

DISCUSSION

Our group has been designing and characterizing synthetic polymers that enhance the endosomal release of biotherapeutics based on strategies utilized by biological organisms such as viruses and toxins. These organisms utilize fusogenic proteins or peptides that contain amino acids with pendant hydrophobic alkyl groups as well as others containing ionizable carboxyl moieties that become protonated within endosomes, leading to secondary structural changes within the peptides that promote membrane disruption at low pH. The alkylacrylic acid family of polymers also contains pendant alkyl groups along with carboxyl moieties. PEAA has previously been shown to destabilize model lipid bilayers at pHs within the range for endosomal acidification (pH 5.5–6.5). PPAA differs from PEAA by the addition of one methylene group to the pendant hydrophobic side chain in each monomer subunit. The increased hydrophobicity gained by the additional methylene group in PPAA, relative to PEAA, results in the enhanced association of PPAA with red blood cell membranes at low pH, which leads to enhanced hemolysis as well as a higher pH sensitivity of this disruption (24, 25). PPAA also exhibits no cytotoxic effects when added to NIH3T3 mouse fibroblasts at concentrations up to at least 100 µg/mL (highest concentration tested, unpublished data). This information suggests the potential for using PPAA for the intracellular delivery of therapeutic molecules.

In the study reported here, we have tested the ability of PPAA to enhance cell transfections by incorporating PPAA into nonviral lipoplex gene delivery vectors. Ternary physical mixtures of particles consisting of DOTAP, pCMVβ plasmid DNA, and PPAA were prepared for transfection. These particles were formed by the electrostatic interactions between the positively charged DOTAP liposomes and the negatively charged DNA and PPAA. The carrier nanoparticles containing PPAA showed greatly enhanced gene expression levels and a higher percentage of transfected cells compared to control carriers lacking PPAA. Incorporation of PPAA into these lipoplex particles also provided an unexpected protection of these particles against serum inactivation upon exposure of up to 50% serum levels. Radioactive uptake experiments indicated that vectors containing PPAA maintained high levels of particle uptake even in the presence of serum. This high level of particle uptake may be due to an ability of the added PPAA to provide steric

stabilization of the lipoplex particles, as is well established with other hydrophilic polymers such as poly(ethylene glycol) (PEG) (29–31) and poly[N-(2-hydroxypropyl)methacrylamide] (pHPMA) (32). Therefore, PPAA in its hydrophilic state (i.e., pH 7.4) is likely inhibiting serum protein adsorption and/or nanoparticle disruption.

While the exact molecular mechanism of PPAA enhancement is currently unknown, the results presented here suggest that the polymer is increasing the cytoplasmic delivery of the plasmid DNA. Studies examining the interaction of PEAA with model liposomal bilayers have shown that the polymer undergoes a conformational transition from a hydrophilic extended coil to a more hydrophobic globule state at low pH, which leads to membrane association and destabilization (22). More recently, PEAA has been shown to form ion-selective membrane channels in lipid vesicle membranes, which has been shown to mediate the release of model, vesicle-entrapped drugs from liposomes (33, 34). Our studies showing that DOTAP/DNA nanoparticles incorporating PAA did not exhibit higher transfection efficiency demonstrates that the enhancement is not due to a nonspecific effect of adding a polyanionic polymer to the DOTAP/DNA carriers. Therefore, the pendant propyl groups in PPAA are required to confer the proper hydrophobic/hydrophilic balance essential for pH-dependent membrane disruption and the endosomal release of nanoparticle gene delivery systems. The overall ability of the PPAA-containing particles to transfect cells in media containing 50% serum suggests that PPAA may provide both serum stabilizing and endosomal escaping properties to lipoplex carriers in systemic or local in vivo delivery applications for gene therapy.

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BC0100408

Cellular Internalization of a Cargo Complex with a Novel Peptide Derived from the Third Helix of the Islet-1 Homeodomain. Comparison with the Penetratin Peptide

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Cellular translocation into a human Bowes melanoma cell line was investigated and compared for penetratin and pIsl, two peptides that correspond to the third helices of the related homeodomains, from the Antennapedia transcription factor of *Drosophila* and the rat insulin-1 gene enhancer protein, respectively. Both biotinylated peptides internalized into the cells with similar efficacy, yielding an analogous intracellular distribution. When a large cargo protein, 63 kDa avidin, was coupled to either peptide, efficient cellular uptake for both the peptide–protein complexes was observed. The interactions between each peptide and SDS micelles were studied by fluorescence spectroscopy and acrylamide quenching of the intrinsic tryptophan (Trp) fluorescence. Both peptides interacted strongly and almost identically with the membrane mimicking environment. Compared to penetratin, the new transport peptide pIsl has only one Trp residue, which simplifies the interpretation of the fluorescence spectra and in addition has a native Cys residue, which may be used for alternative coupling reactions of cargoes of different character.

INTRODUCTION

Homeodomains are approximately 60 amino acids long DNA binding regions of transcription factors (Müller et al., 1988). It has been shown that several homeodomain-containing transcription factors exhibit cell-penetrating properties (Chatelin et al., 1996; Prochiantz, A., 1999; Han et al., 2000). For the Antennapedia homeodomain protein of *Drosophila*, it was found that the fragment corresponding to the third helix (residues 43–58) of the homeodomain penetrates cell membranes (Derossi et al., 1994, 1996, 1998). The peptide with the corresponding sequence was named “penetratin”, and this was the first homeoprotein-derived cell-penetrating peptide (CPP) (Table 1). The cellular internalization of penetratin occurs at 4 °C and is unsaturable, suggesting that it does not require a chiral receptor protein (Derossi et al., 1994). To reveal the mechanism by which penetratin is internalized, modified (with deletions, additions, replacements or inversions) penetratin analogues have been synthesized and studied (Brugidou et al., 1995; Derossi et al., 1996; Fischer et al., 2000; Thorén et al., 2000; Drin et al., 2001), but a detailed knowledge of the mechanisms of membrane penetration is still lacking.

As previous studies demonstrate, the homeodomains have similar and well-organized secondary and tertiary

structures (Ippel et al., 1999). The homeodomain proteins consist of three α -helices. The third helix contains highly conserved amino acid motifs and is also amphipathic. It has been discussed whether the amphipathic α -helical conformation is essential for the membrane-translocating properties (Dathe et al., 1996; Magzoub et al., 2001).

The present study concerns a novel peptide sequence, pIsl, derived from the homeodomain of the rat transcription factor Islet-1 (Isl-1) (Karlsson et al., 1990) and homologous to penetratin. The Islet-1 protein was initially shown to function as an insulin-1 gene enhancer. Later, Isl-1 protein has been shown to possess other important regulatory functions, e.g., in motor neuron differentiation (Pfaff et al., 1996), regulation of amylin (Wang and Drucker, 1996), proglucagon expression (Wang and Drucker, 1995), and estrogen receptor modulation (Gay et al., 2000). The sequence corresponding to the third helix (residues 45–60) of the homeodomain of Isl-1 (pIsl) is similar, although not identical to penetratin (Table 1). The pIsl sequence has only one Trp residue, which is a simplification for studies with optical spectroscopy, and it has a native Cys residue, which may be useful in coupling reactions of cargoes.

We demonstrate that pIsl and penetratin peptides internalize efficiently into human Bowes melanoma cells. As shown before, penetratin can translocate relatively large molecules, coupled to it, across the cell membrane (Allinquant et al., 1995; Pooga et al., 1998; Derossi et al., 1998). Here, the biotin-labeled pIsl peptide and penetratin are complexed to fluorescently labeled avidin, a biotin binding protein with a molecular weight of about 63 kDa (Pugliese et al., 1993). The cell translocation of the two complexes is studied and compared. The intrinsic Trp fluorescence in either peptide is used to characterize the interaction of the peptide with negatively charged SDS

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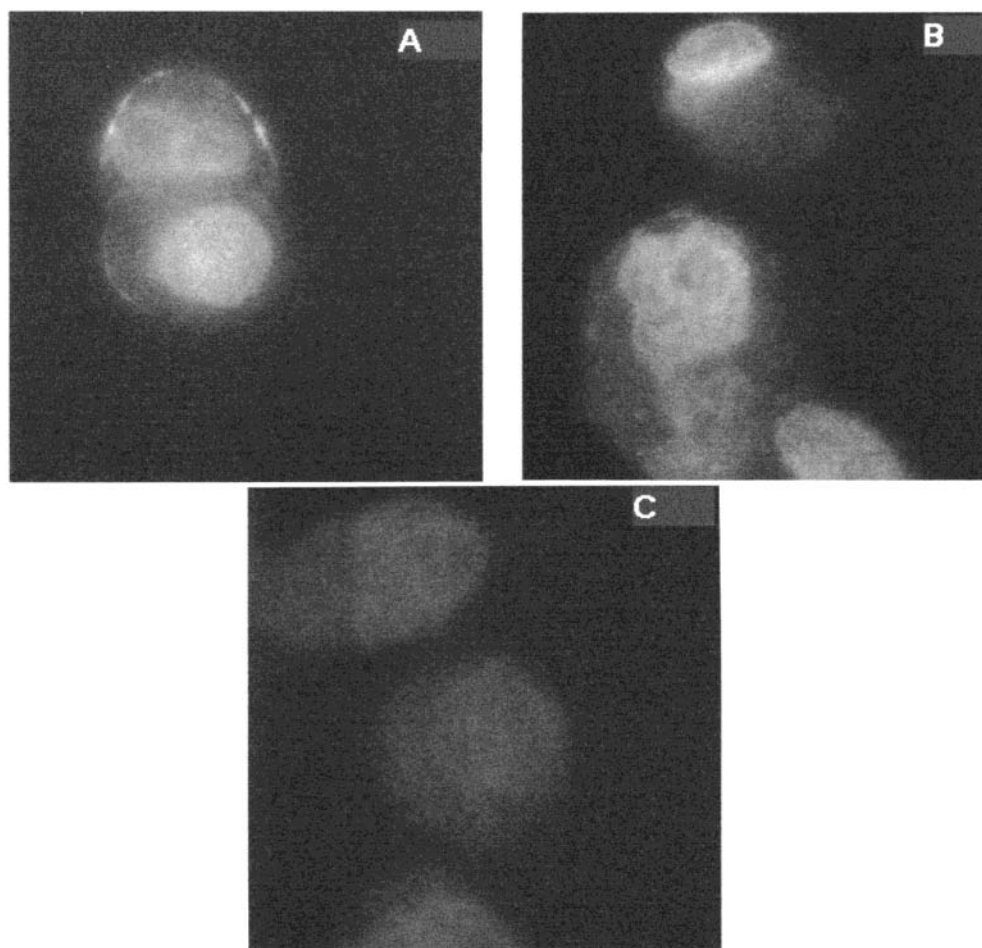
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Table 1. Comparison of the Penetratin and pIsl Internalization into Bowes Cells as well as Acrylamide Quenching Constants Calculated from the Tryptophan Fluorescence Spectra in Water and SDS Micelles

name	peptide sequence	localization		quenching constant (M^{-1})	
		cytoplasm	nucleus	water	SDS micelles
penetratin	RQIKIWFQNRRMKWKK	+	+	35	25
pIsl	RVIRVWFQNKRCDDKK	+	+	39	22

**Figure 1.** Localization of biotinyl-pIsl (A), biotinyl-penetratin (B), and natural background (C) in Bowes melanoma cells. Concentration of peptides was 10 μM , incubation time 1 h at 4 $^{\circ}C$. Staining was done using 150 nM streptavidin-FITC.

micelles, which may be considered as a simple solvent model for a biomembrane.

MATERIALS AND METHODS

Peptide Synthesis. The peptides (Table 1) were synthesized using the *t*-Boc/DCC/HOBt strategy of solid-phase peptide synthesis on a PE Applied BioSystems stepwise peptide synthesizer model 431A. The N-terminal biotin was coupled manually using TBTU/HOBt activation, to increase the coupling yield. Cleavage from resin was performed with liquid HF. Highly pure peptides were obtained by purification on a reversed-phase HPLC C_{18} column. Molecular weights were determined using a plasma desorption time-of-flight mass spectrometer (Bio-Ion) and always corresponded to calculated values. Both peptides were C-terminally amidated.

Cells. The human Bowes melanoma cell line was cultivated in a minimal essential medium with Earl-salts (MEM), supplemented with 10% foetal calf serum, 2 mM L-glutamine, 5 IU/mL penicillin, and 5 $\mu g/mL$ streptomycin, in air enriched with CO_2 to 5% at 37 $^{\circ}C$. For the penetration assay the cells were grown on round glass coverslips in a 24 well plate to approximately 50% confluence.

Cellular Penetration of Biotin-Labeled Peptides.

The serum-containing medium was replaced by a serum-free medium, and an aqueous stock solution of the peptide was added directly into the medium surrounding the cells, to reach the concentration of 10 μM . For detection of a natural level of intracellular biotin and estimation of nonspecific staining, water instead of the peptide solution was added, and in all other respects these negative controls were handled identically to other coverslips. The cells were incubated with peptides for 1 h at 37 $^{\circ}C$ in 5% CO_2 enriched air or 4 $^{\circ}C$ in air. Thereafter, the cells were washed three times in PBS, fixed, and permeabilized with methanol for 10 min at $-20^{\circ}C$, washed with PBS, and incubated for 1 h in a 5% (w/v) solution of fatfree milk in PBS in order to block nonspecific binding sites. The peptides were visualized by staining with 150 nM streptavidin-FITC (Amersham-Pharmacia Biotech) for 1 h at room temperature. The cell nuclei were visualized by staining the DNA with Hoechst 33258 (0.5 $\mu g/mL$) for 5 min. Thereafter, the coverslips were washed three times in PBS and mounted in 20% glycerol in PBS. The images were obtained by a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss Inc., Germany).

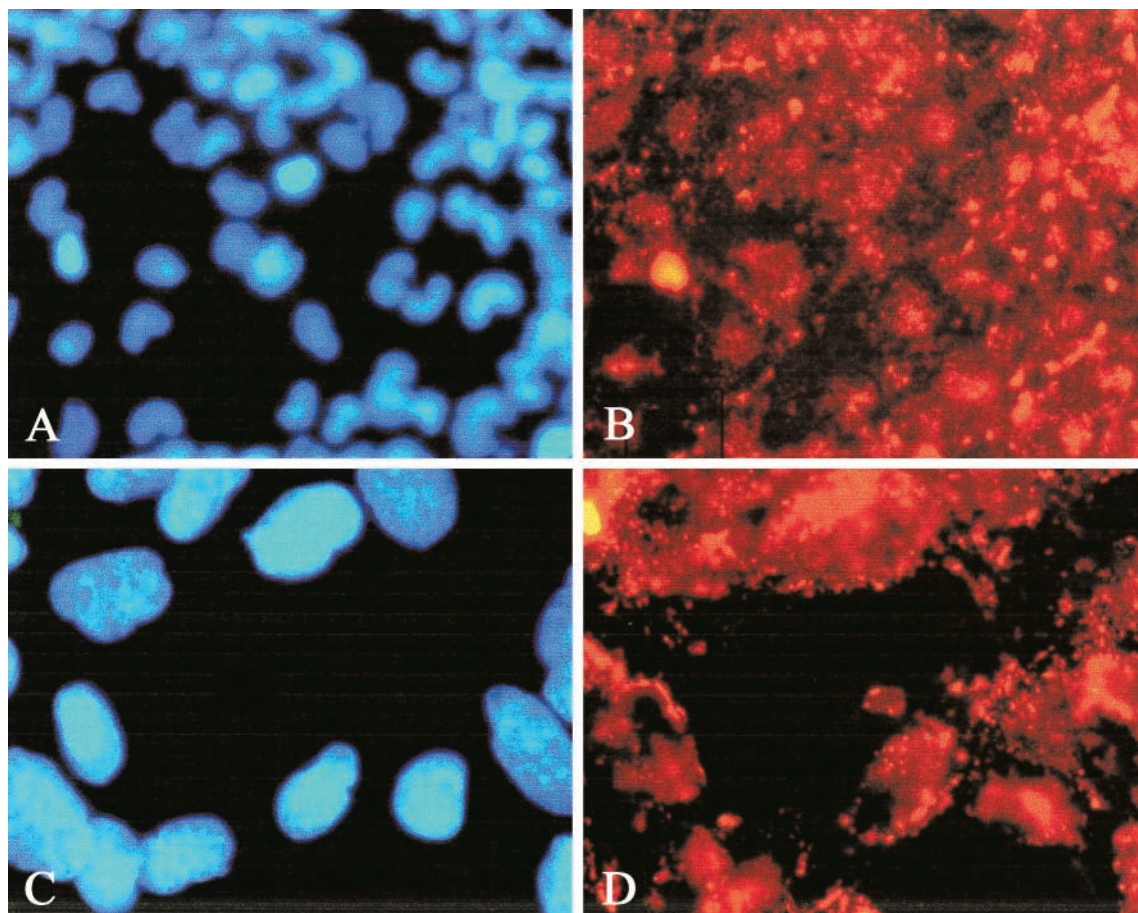


Figure 2. Cellular penetration of biotinyl-peptide-avidin-TRITC complexes. Bowes melanoma cells were incubated for 2 h with 5 μ M biotinyl-pIsl-avidin-TRITC or biotinyl-penetratin-avidin-TRITC in serum free medium at 37 °C. Nuclei of pIsl treated cells: (A) Hoechst 33258 staining showing DNA, (B) TRITC fluorescence showing avidin transported by pIsl. Nuclei of penetratin treated cells: (C) Hoechst 33258 staining showing DNA, (D) TRITC fluorescence showing avidin transported by penetratin.

Cellular Penetration of Peptide-Cargo Complexes. The cells for protein transport experiments were grown on round glass coverslips in 24-well plates to about 50% confluence. A solution of biotinylated pIsl or penetratin complexed with avidin-TRITC in serum-containing medium was added to the cells. The Bowes cells were incubated for 2 h at 37 °C with 5 μ M of peptide-protein complex. The coverslips with cells were washed two times with PBS, fixed with the mixture of 4% paraformaldehyde and 1% glutaric aldehyde in PBS and permeabilized with methanol for 15 min at -20 °C. Nonspecific binding was blocked with 5% BSA in PBS. After final rinsing with PBS, the prepares were mounted in Slow Fade mounting medium (Molecular Probes, Inc.). The localization of the peptide-avidin complexes was examined and photographed by a Vanox AH2-NAS microphotograph system (Olympus) or by a laser confocal scanning microscope MRC-1024 (Bio-Rad).

Fluorescence Spectroscopy. Fluorescence spectra were recorded on a Perkin-Elmer LS 50B Luminescence Spectrometer equipped with FL WinLab software. All measurements were made in 4 $\times$ 10 mm cuvettes at an ambient temperature of 20 °C. Spectra were recorded in water or a 100 mM solution of sodium dodecyl sulfate (SDS, Kebo), at pH of about 3, well above the critical micellar concentration. The fluorescence was excited at 280 nm, and the emission was recorded from 300 to 500 nm. Emission spectra were recorded with 10 nm excitation and emission bandwidths.

For the fluorescence quenching experiments, acrylamide (Sigma) from a 1 M stock solution was added to

the peptide solution, resulting in concentrations between 4 and 100 mM. Quenching constants K_{SV} were determined by a linear regression with the Stern-Volmer equation for a dynamic process (Lakowicz, 1999):

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \quad (1)$$

where F and F_0 are the fluorescence intensities in the presence and the absence of acrylamide, respectively, and $[Q]$ is the molar concentration of acrylamide in the solution.

RESULTS

Cellular Penetration. The membrane penetration efficacy of pIsl and penetratin peptides was estimated and compared. Bowes melanoma cells were incubated with 10 μ M solution of each biotinylated peptide and stained with streptavidin-FITC after fixing the cells. Both peptides yielded identical high intensities of intracellular fluorescence that strongly exceeded the registered natural background (Figure 1). No obvious differences in cellular distribution were observed between pIsl and penetratin, as demonstrated in Figures 1A and 1B. Both peptides were able to penetrate into the cells also at 4 °C (data not shown). The observations show that pIsl, with a sequence analogous to that of penetratin, translocates into the cells in a nonendocytotic manner, because at temperatures below 18 °C endocytosis is abolished. The results also suggest that both peptides penetrate through

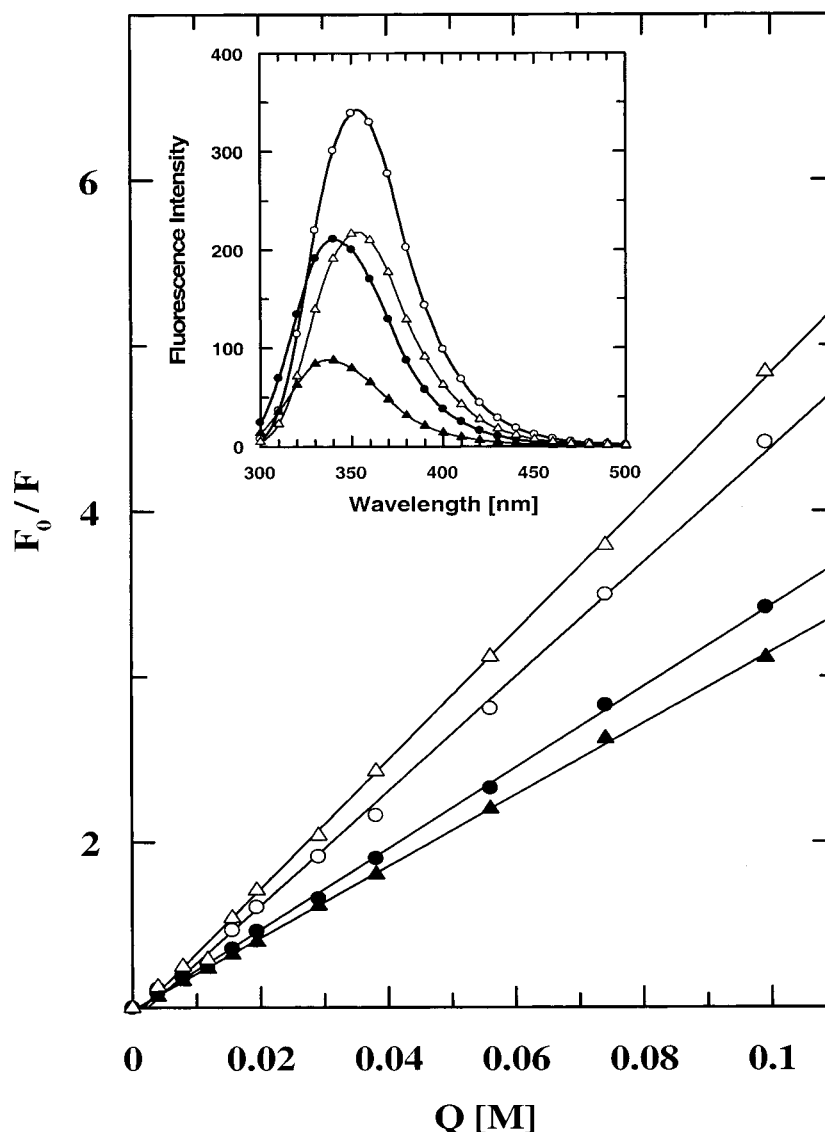


Figure 3. Stern–Volmer plots for the acrylamide quenching of the tryptophan fluorescence from the two homeopeptides in water (open symbols), or 100 mM SDS micelles (filled symbols). The concentration of pIsl (Δ $\blacktriangle$) and penetratin ($\circ$ $\bullet$) was 10 μ M. Insert: The fluorescence emission spectra (excitation at 280 nm) of pIsl and penetratin in buffer and in 100 mM SDS micelles.

the cell membranes by the same mechanism, and their intracellular localization is similar.

Delivery of Cargo Protein over the Plasma Membrane. The property of avidin (MW about 63 kD) to form a very strong and specific complex with biotin was used to demonstrate the ability of pIsl, as well as penetratin, to carry large molecules into the cells. The cell nuclei are shown in Figure 2A and C by staining with the DNA-binding substance Hoechst 33258. The strong complexes between the biotinylated pIsl or penetratin and TRITC-labeled avidin were preformed by incubating the components together in a minimal volume for 5 min at room temperature. This solution was added directly into the culture medium over the cells. Incubation of Bowes cells for 30 min or longer with these complexes resulted in accumulation of fluorescent particles in the cells (Figures 2B and 2D), confirming that avidin–TRITC was conveyed into cells also by the pIsl peptide as a noncovalent complex. With both transport peptides, the cargoes are clearly present both in the cytoplasm and in the nucleus of the cells (Table 1).

Spectroscopic Studies. Transport peptides have the common feature to interact strongly with biomembrane model systems. The intrinsic Trp fluorescence of the

nonbiotinylated peptides was used to study this interaction. Penetratin contains two Trp residues and pIsl only one (Table 1). Figure 3 (insert) shows the fluorescence emission spectra of penetratin and pIsl in buffer (pH 7) and in 100 mM solution of SDS, a negatively charged detergent that forms micelles in an aqueous solution. In this membrane mimetic solvent both peptides show a 15–20 nm (“blue”) shift of the Trp fluorescence emission peak to shorter wavelengths as compared to the aqueous solutions, indicating that the Trp residues interact with a less polar environment than water, i.e., SDS micelles. In a highly hydrophobic environment the wavelength shift of Trp fluorescence could reach 30–35 nm (Lakowicz, 1999). Therefore, the present data suggest a partial shielding from the aqueous solvent.

To evaluate the accessibility of Trp residues to the solvent, acrylamide was used as a neutral hydrophilic fluorescence quenching agent. Figure 3 shows the quenching graphs for the two peptides in water and in SDS micelles. In all cases the graphs obey the linear Stern–Volmer equation. The calculated quenching constants are 35 and 39 M^{-1} in aqueous solution and of 25 and 22 M^{-1} in micellar solution, for penetratin and pIsl, respectively (Table 1). The values observed for the two peptides are

not significantly different within the accuracy of the measurements. It should be noted that the quenching behavior in the current case is also not significantly dependent on the number of Trp residues.

DISCUSSION

The present study is the first to demonstrate that a sequence homologous to penetratin taken from a different homeodomain of a transcription factor is also able to carry a large cargo protein through cell membranes. The pIsl peptide efficiently penetrates into the cells in culture and can be therefore considered as a new member of the homeoprotein-derived class of CPPs. We have compared the transport and membrane interaction properties of two homeodomain-derived peptides, penetratin and pIsl, which have somewhat different sequences and net charges (Table 1), but with identical positions of the positive charges. Recently, it was demonstrated that only the C-terminus of penetratin, RRMKWKK, is obligatory for cellular penetration (Fischer et al., 2000). Corresponding sequences are present in many of the known homeoproteins. In the transcription factor PDX-1 the same motif is present, and it was demonstrated to be a nuclear localization signal (NLS) for proteins (Moede et al., 1999). However, in that study the tagged cargo was introduced into the cells by a transfection technique.

The results suggest that the translocation property of the CPPs is mainly dependent on the presence of the positive charges in the C terminus. Moreover, L-Ala-scanning showed that all basic amino acid residues in the RRMKWKK fragment are necessary for efficient translocation (Fischer et al., 2000). Substitution of any charged residue in this motif by L-Ala decreases the penetration yield by 60% or more, whereas replacing Trp does not cause any such drastic drop in the penetration yield. Our results support the previous observations on penetratin analogues that the Trp⁵⁶ of penetratin is not a major determinant for cellular penetration (Fischer et al., 2000). In a solution with SDS micelles the single Trp of pIsl shows similar fluorescence properties as the two Trp residues in penetratin do. These results suggest that both Trp residues in penetratin are in an environment of similar polarity and accessibility from the solvent as the one Trp residue in pIsl. In both peptides, the Trp residues are partially shielded from the interaction with the aqueous solvent.

The detailed secondary structures induced in a cell-penetrating and transporting peptide by the interaction with a membrane do not seem to be directly related to the penetration properties (Derossi et al., 1996; Scheller et al., 2000; Drin et al. 2001; Magzoub et al., 2001). Instead, the strength of the interaction with the charged membrane and possibly the overall topology of the peptide relative to the membrane surface may be decisive factors for the translocation process. How the cargo will then then become accommodated in a nonlytic manner in the translocation process remains a challenging phenomenon.

The presentation of the pIsl peptide as a novel CPP and comparing its properties with penetratin gives further understanding of the cell-penetrating mechanisms of CPPs. Additionally, the native Cys residue in the pIsl sequence may be used for various coupling reactions, which should make pIsl useful as an alternative for cellular delivery of different hydrophilic cargo molecules.

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Novel Polymer–DNA Hybrid Polymeric Micelles Composed of Hydrophobic Poly(D,L-lactic-co-glycolic Acid) and Hydrophilic Oligonucleotides

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Biodegradable poly(D,L-lactic-co-glycolic acid) (PLGA) was chemically conjugated to oligonucleotide (ODN) to form an amphiphatic structure which is similar to an A–B type block copolymer. A terminal end of PLGA was activated and reacted with primary amine-terminated ODN. The ODN/PLGA conjugates self-assembled in aqueous solution to form a micellar structure by serving PLGA segments as a hydrophobic core and ODN segments as a surrounding hydrophilic corona. Critical micelle concentration was determined by a spectrofluorometric method. Atomic force microscopic observation revealed that the micelle size was around 80 nm. These micelles could release ODN in a sustained manner by controlled degradation of hydrophobic PLGA chains. Compared to unconjugated ODN, the ODN/PLGA micelles could be more efficiently transported within cells, presumably by endocytosis. This study proposes a potential delivery method of ODN into cells by forming hybrid ODN/PLGA micelles.

INTRODUCTION

Recently, much attention has been drawn to various amphiphatic block polymers, which can self-associate to form micelles in aqueous milieu. They can be utilized as potential drug carriers (1–6). Most polymeric micelles have a core–shell structure composed of hydrophobic segments as an internal core and hydrophilic segments as a surrounding corona (7). The size of polymeric micelles, approximately less than 100 nm in diameter, not only makes them ideal drug delivery carriers for escaping from renal exclusion and the reticuloendothelial system, but also provides them with enhanced endothelial cell permeability in the vicinity of solid tumors (8, 9). In particular, amphiphatic A–B type diblock copolymers demonstrated a fairly good thermodynamic stability in physiological solution by virtue of their relatively low critical micelle concentration (cmc) (10). Various polymeric micelles have been used to deliver anticancer drugs for passive targeting to solid tumors in vivo. For example, diblock copolymers composed of poly(aspartic acid) and poly(ethylene glycol) (PEG) have been used for delivering doxorubicin to solid tumors in a target specific manner by the “enhanced permeation and retention (EPR)” mechanism (11). More recently, a variety of biodegradable polymeric micelles, based on diblock copolymers composed of aliphatic polyesters and PEG, were synthesized. Poly(D,L-lactic-co-glycolic acid) (PLGA) has been most popularly used as a constituent of the diblock copolymers because it can be hydrolyzed in vivo into lactic and glycolic acid forms, which can be metabolized further into carbon dioxide and water (12).

Oligonucleotides (ODNs) represent short DNA molecules, which have been tested for treating various genetic diseases. Oligomeric antisense DNA and RNA have been used to block the expression of specific proteins

by hybridization with specific (target) mRNA sequences. The antisense effect of ODNs has been demonstrated in both in vitro cell culture (13, 14) and in vivo studies (14–16), and their therapeutic potentials have been proposed (17). However, one of the most serious problems is to efficiently deliver them into cells (18, 19). To obtain the most potent antisense effect, the formulation of ODN with suitable carriers must be developed to facilitate cellular uptake. A variety of nonviral vectors such as cationic liposomes (20) and synthetic polymers (19) have been used for delivering antisense ODNs into mammalian cells.

In this study, we conjugated *c-myc* antisense ODN as a model ODN to biodegradable PLGA to make an amphiphilic structure, which could form micelles in an aqueous phase, like A–B type diblock copolymers. The ODN/PLGA micelles were used as vectors for the delivery of antisense ODNs, which can facilitate the cellular uptake of ODN via endocytosis. Characteristics and cellular uptake behaviors of the ODN/PLGA polymeric micelles were investigated.

MATERIALS AND METHODS

Materials. Poly(D,L-lactic-co-glycolic acid) (PLGA5010) having a lactic/glycolic molar ratio of 50/50 was obtained from Wako Chemicals (Japan). This polymer (nominal MW 10000) has a free hydroxyl and a carboxyl group at its terminal ends. Oligodeoxynucleotide (antisense *c-myc* ODN, CACGTTGAGGGGCAT), which has a primary amine group at the end of its 5′ phosphate, was synthesized, modified, and purified by Bioneer (Korea). Dicyclohexyl carbodiimide (DCC), *N*-hydroxysuccinimide (NHS), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

Conjugation of Oligonucleotide to PLGA. PLGA (1.0 g, 0.1 mmol) dissolved in 5 mL of DMSO was activated by DCC (61.9 mg, 0.3 mmol) and NHS (34.5

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mg, 0.3 mmol) (PLGA/DCC/NHS stoichiometric molar ratio: 1:3:3). The activated polymer was precipitated in cold ethanol followed by drying under reduced pressure. ODN (1.0 mg, 0.2 μ mol) dissolved in 100 μ L of 5 mM sodium borate buffer (pH 8.0) was added to DMSO (0.9 mL). Insoluble salt particulates were removed from the buffer solution by centrifugation at 14000 rpm for 10 min. The supernatant solution was then dropped into the DMSO solution (1 mL) containing the activated PLGA (2 mg, 0.2 μ mol) (stoichiometric ratio: 1:1). The reaction mixture was incubated at room temperature for 12 h and then dialyzed against deionized water for the removal of unreacted ODN and the generation of ODN/PLGA micelles. The concentration of ODN in the micelles was determined by measuring absorbance at 260 nm using a Shimadzu UV-Vis spectrophotometer (UV-1601, Japan). The formation of self-assembled micellar structure was characterized by gel permeation chromatography using Bio-Rad Bio-columns SEC 250 (300 $\times$ 7.8 mm) in an aqueous mobile phase with a flow rate of 1 mL/min. Elution profiles of micelles and ODN were detected by absorbance at 260 nm.

Characterization of ODN/PLGA Micelles. Effective hydrodynamic diameter of the micelle was measured by dynamic light scattering at 25 $^{\circ}$ C using a dynamic light scattering photometer (Zeta Plus, Brookhaven Instrument Co., New York) equipped with He-Ne laser at a wavelength of 632 nm. The measurement was carried out in triplicate.

Measurement of Critical Micelle Concentration (cmc). The critical micelle concentration was determined by a fluorescence probe technique using pyrene as a fluorescence probe, as described previously (21, 22). Pyrene dissolved in acetone was added to deionized water to make a concentration of 12×10^{-7} M, and acetone was subsequently removed by stirring for 5 h at room temperature. The final concentration of pyrene was adjusted at 6×10^{-7} M. The concentration of ODN/PLGA conjugate was varied from 0.05 to 500 mg/mL. Combined solution of pyrene solution and ODN/PLGA conjugate was equilibrated at room temperature in a dark room for 1 day before measurement. Fluorescence spectra were monitored at room temperature using a Shimadzu spectrofluorometer (RF-5301PC, Japan) at an excitation and emission wavelength of 339 and 390 nm, respectively. The spectra were accumulated with an integration of 2 s/nm.

Atomic Force Microscopy (AFM). Five microliters of ODN/PLGA micellar solution (100 μ g/mL) was dropped on a clean glass surface and air-dried at room temperature. The AFM analysis was carried out in a constant tapping mode by using Nanoscope III (Digital Instruments). A standard Nanoprobe silicon single-crystal lever was used as a cantilever and a constant force mode was used with a scan speed at 0.5 Hz. The scanned image was collected from a $2.5 \times 2.5 \mu$ m area.

In Vitro ODN Release. Five milliliters of the micelle solution containing 300 μ g of ODN/PLGA conjugate in dry weight basis was put into a dialysis membrane (molecular cutoff: 50000). The dialysis bag was placed in a 50 mL conical tube filled with 4 mL of Tris-EDTA buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), which was maintained at 37 $^{\circ}$ C with gentle shaking (100 rpm). The buffer was collected and replaced at desired interval times. The collected sample was filtered and stored at 4 $^{\circ}$ C until analyzed at 260 nm.

Fluorescence Labeling of Micelles. A fluorescence-labeled oligonucleotide having a complementary sequence to antisense *c-myc* ODN (sense *c-myc* ODN) was used to

label the ODN incorporated in micelles by hybridization between sense and antisense ODNs. The sense ODN (ATGCCCCCTCAACGTG) having a primary amine group at its 5' end, was labeled by using rhodamine-NHS in PBS (pH 7.6) at room temperature for 12 h. Dialysis was carried out to remove unlabeled fluorescent dye. The labeled ODN was concentrated by using Centricon (Amicon). The labeled sense oligonucleotide was added to the micelle solution for labeling the ODN/PLGA micelles. The mixture was heated at 65 $^{\circ}$ C for 5 min and cooled in ice.

Endocytosis of Micelles. NIH 3T3 murine fibroblast was purchased from Korea Cell Line Bank (Seoul, Korea) and were cultured in DMEM supplemented with 10% FBS, streptomycin at 100 μ g/mL, penicillin at 100 IU/mL, and 2 mM L-glutamine. Cells were maintained at 37 $^{\circ}$ C in a humidified 5% CO₂ atmosphere. Ten thousand cells per well were plated in a six-well plate (35 mm diameter, Nunc) containing collagen-coated coverglass in 1.5 mL DMEM with 10% FBS and incubated for 24 h before further experiments. After the replacement of the medium with fresh serum-free medium, the ODN/PLGA micelles hybridized with rhodamine-labeled sense ODN were added to attain a concentration of 80 μ g/mL. After 3 h incubation, medium was discarded and the cells were washed four times with PBS. The cells were fixed by adding a fixing solution (0.2% glutaraldehyde, 0.5% formaldehyde in PBS) followed by incubation at 4 $^{\circ}$ C for 15 min. The cells were then washed once with PBS and visualized by a Zeiss LSM 510 confocal microscopy (Carl Zeiss, Germany). An argon/krypton mixed gas laser with excitation line at 568 nm was used to induce rhodamine fluorescence, which was observed by using 575–640 nm band-pass filter.

Flow Cytometry. The efficiency of endocytosis was determined by using a flow cytometry. Ten thousand cells per well were plated and cultured in a six-well plate as described above. The medium was replaced with fresh serum-free medium prior to the addition of the micelles. The fluorescence-labeled ODN/PLGA micelles were then added to the cells to obtain a final concentration of 80 μ g/mL. After 3 h incubation, the cells were washed four times with PBS, trypsinized, and fixed. The number of cells, which had endocytosed the micelles, was measured in a flow cytometry (FACSCalibur, Becton-Dickinson, Mountain View, CA). The cellular uptake efficiency of ODN/PLGA micelles was quantified by counting the number of cells within an arbitrarily selected gate region (50 < FL2-H < 350).

RESULTS AND DISCUSSION

In our previous reports, various bioactive molecules and drugs, such as peptides, enzymes, and anticancer drugs, were directly conjugated to the terminal group of PLGA for achieving a sustained release from nanoparticles and microspheres (23, 24). Slow degradation of PLGA chain provided the controlled release of conjugated bioactive moieties over the desired period. More recently, doxorubicin was conjugated to the terminal end of PLGA in a block copolymer of PEG-*b*-PLGA to form polymeric micelles, where doxorubicin was chemically entrapped within a hydrophobic micelle core (6). It was found that chemically entrapped doxorubicin within PEG-*b*-PLGA could be transported within cells to a greater extent than doxorubicin alone, possibly due to an endocytosis process. In general, when hydrophilic synthetic polymers were linked to water insoluble PLGA in a diblock polymer structure, the A-B type diblock polymers self-assembled in aqueous solution to form micelles. We selected ODN

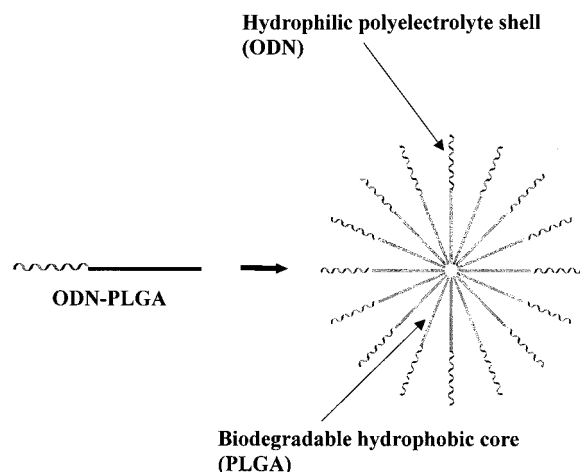


Figure 1. The schematic diagram of micelle formation of amphiphilic conjugate of oligonucleotide and poly(D,L-lactic-co-glycolic acid) in aqueous medium.

as a hydrophilic segment to conjugate to the terminal end of PLGA to form a novel hybrid polymeric micelle structure in aqueous solution (Figure 1). It is postulated that ODN, as the hydrophilic constituent part of ODN/PLGA conjugate, can be more efficiently delivered within cells by an endocytosis mechanism, in contrast to ODN itself which is transported across the cell membrane by passive diffusion. An antisense ODN, *c-myc*, was used as a model ODN in this study. It is a single strand DNA with a molecular weight of 4633 and a nucleotide sequence of CACGTTGAGGGGCAT. The potential application of the antisense *c-myc* ODN for the suppression of smooth muscle cell proliferation was suggested in a previous study (25). Smooth muscle cell proliferation is believed to play a critical role in reoccurring the blockade of coronary artery after a balloon angioplasty treatment (restenosis).

A reaction scheme for the conjugation of oligonucleotide and PLGA is shown in Figure 2. *c-myc* oligonucleotide terminally derivatized with a primary amine group was first dissolved in buffer solution. The solution was then added into DMSO containing preactivated PLGA with DCC and NHS. The volume ratio of aqueous to organic phase was adjusted to 1:10. The ODN/PLGA micelle was obtained and unreacted ODN was removed by dialysis against deionized water. The conjugation efficiency was determined by measuring the amount of ODN in ODN/PLGA micelles via an UV spectrophotometric method and was about 75%. In addition, aqueous gel permeation chromatography was used to confirm the formation of ODN/PLGA micelles (Figure 3). The micelles were eluted in a void volume fraction at ca. 4.3 min, while ODN was eluted at ca. 9.3 min. This GPC result indirectly proves the formation of hybrid DNA/polymer micelles in aqueous solution.

To determine the hydrodynamic volume of ODN/PLGA micelles, DLS was carried out as shown in Figure 4. It showed an effective diameter of 65.2 nm with a narrow size distribution. Atomic force microscopy was also used for the direct visualization of size and morphology of the ODN/PLGA micelles. In Figure 5, it can be seen that the micelles have a spherical shape with an average diameter of 80 nm. The morphological characters of ODN/PLGA micelles are quite similar to those of other reported synthetic polymeric micelles (26, 27). The diameter of micelles in the AFM image is slightly greater relative to that obtained from the DLS measurement. This was possibly caused by the change of micelle size during

drying the spherically shaped hydrated micelles onto the flat surface for the AFM sample preparation.

ODN/PLGA conjugate, consisting of a hydrophilic and negatively charged ODN segment and a hydrophobic PLGA segment, provided an opportunity to form self-assembled micellar structure in an aqueous phase. The conjugate has an amphiphatic structure like other synthetic diblock copolymers. The critical micelle concentration (cmc) of ODN/PLGA micelles was determined by using pyrene as a fluorescence probe, which partitioned into the hydrophobic PLGA core of micelles above the cmc value (21, 22). The cmc is an important physical property of the micelles and also indicates the stability of the micellar structure. Excitation and emission spectra of pyrene are shown at varying concentrations of ODN/PLGA conjugate (Figure 6). In the excitation spectra, with increasing concentration of ODN/PLGA conjugate, greater fluorescence intensity was observed at around 339 nm (Figure 6A). It was reported that the excitation intensity of pyrene in an aqueous phase is very small at 339 nm, but it increases dramatically when pyrene is partitioned into a hydrophobic environment (10, 21). This suggests that the ODN/PLGA conjugate spontaneously formed micelles in aqueous solution that enabled pyrene to partition into the hydrophobic core of micelles. With increasing the concentration of the ODN/PLGA conjugate, a red shift, a characteristic feature of pyrene excitation spectra, was also noticed. The (0,0) band of pyrene shifted from 334 to 339 nm upon the partition of pyrene into the hydrophobic core of the micelles. Fluorescence intensity ratio at the two excitation wavelengths (I_{339}/I_{334}) as a function of ODN/PLGA conjugate concentration was used to determine the cmc value of ODN/PLGA micelles according to the previous reports (10, 28). The cmc value determined by this method was about 5 mg/L as shown in Figure 7A. Figure 7B presents another method of determining the cmc value of micelles based on plotting the intensity ratio of I/III pyrene emission bands as a function of ODN/PLGA conjugate concentration. The cmc value of ODN/PLGA micelles was around 10 mg/L. Thus, an average cmc value of ODN/PLGA micelles calculated from the above two methods was determined as 7.5 mg/L. Although the cmc of ODN/PLGA micelles is higher than that of PEG-PLA diblock copolymer (cmc < 2 mg/L) (28), it is much lower than that of oligo(methyl methacrylate)–poly(acrylic acid) micelles (cmc = 100 mg/L) (10). It is conceivable that negatively charged ODN chains in the shell layer surrounding the PLGA core repel each other by charge-to-charge repulsions, thus increasing the cmc value compared to those of synthetic polymeric micelles containing PEG as a shell layer. This result indicates that the ODN/PLGA micelles are relatively stable compared to conventional diblock polymeric micelles.

ODN/PLGA micelles were subjected to incubation in aqueous solution to examine the release profile of ODN. Since the major constituent of the core region in ODN/PLGA micelles is a biodegradable polymer, it was expected that ODN could be solubilized in an aqueous phase by slow degradation of PLGA chain. Random hydrolytic scission of the PLGA backbone would produce water-soluble oligo(lactic-co-glycolic acid) having a molecular weight of less than 1000. In our previous reports, it was shown that constant release of drugs could be attained from nanoparticles or microspheres prepared by PLGA–drug conjugates (23, 24). It can be seen in Figure 8 that ODN/PLGA micelles exhibited a near zero-order release over 50 days without any burst. The release profile suggests that ODN was released from the polymeric

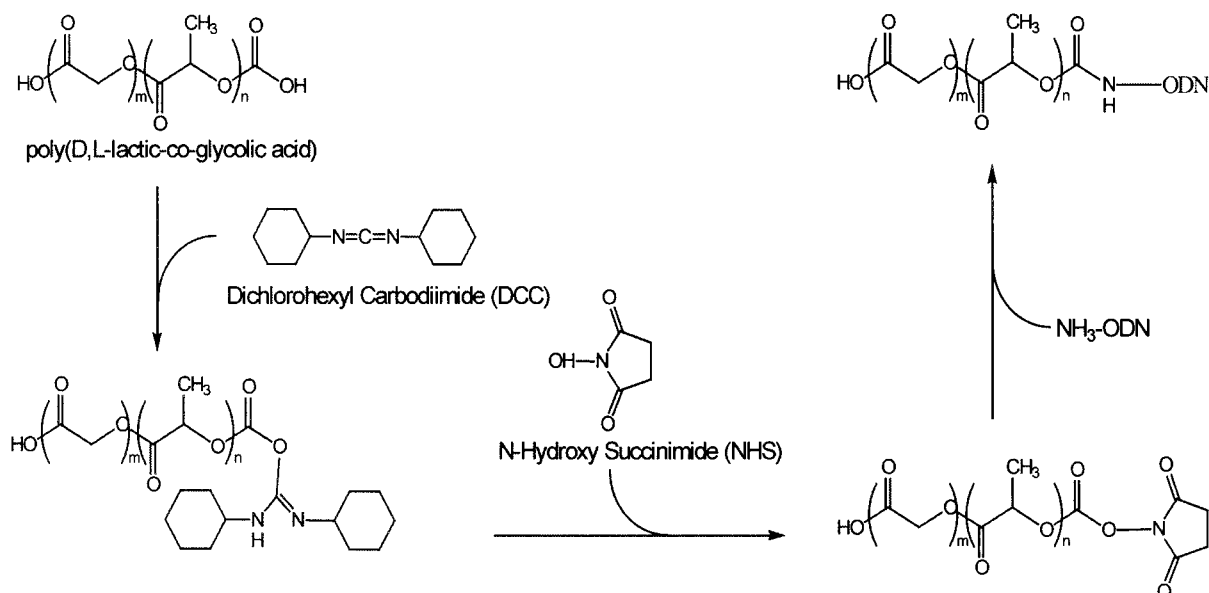


Figure 2. Conjugation scheme of amphiphilic conjugate of oligonucleotide and poly(D,L-lactic-co-glycolic acid).

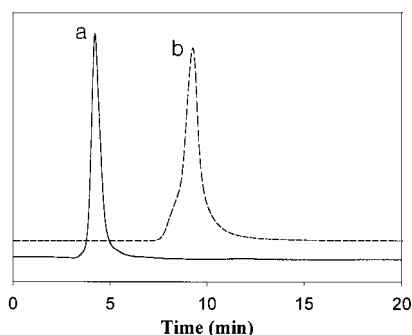


Figure 3. Gel permeation chromatograms of ODN/PLGA micelle (a), and ODN (b).

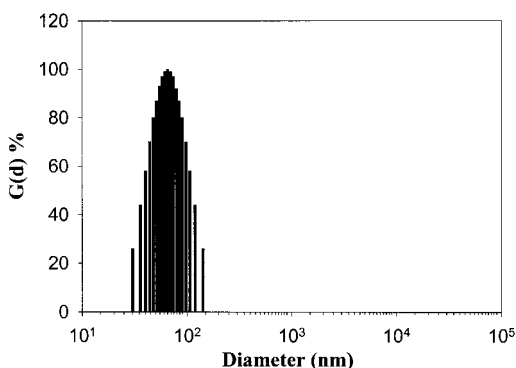


Figure 4. Effective hydrodynamic volume of ODN/PLGA micelles analyzed by dynamic light scattering (DLS).

micelles by a degradation-controlled process of PLGA. Slow degradation of PLGA chains in the micelle core was likely to disintegrate the core structure of micelles, resulting in the release of ODN into the incubation medium. It should be noted that the released ODN fraction was not an intact ODN, but a mixture of ODN-oligo(lactic-co-glycolic acid) conjugates which were soluble in water. The sustained release profile of ODN from ODN/PLGA micelles was not similar to that from PLGA microspheres physically encapsulating ODN molecules (19). The ODN/PLGA micelles exhibited a burst-free zero order release, whereas ODN containing PLGA microspheres showed a nonzero order release pattern with an initial burst release upon incubation. In the case of PLGA

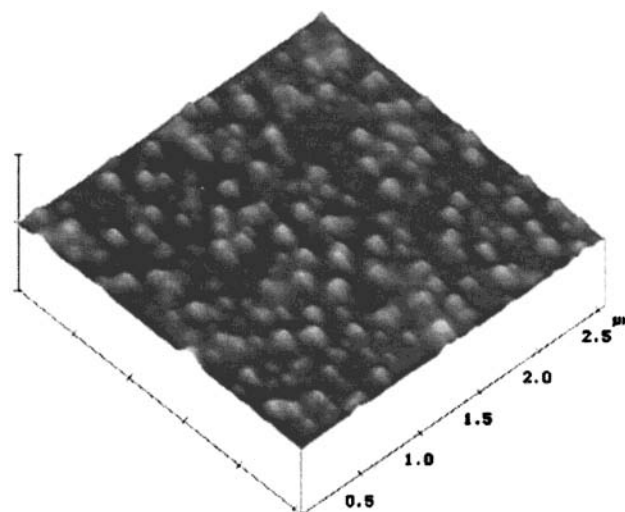


Figure 5. AFM image of ODN/PLGA micelles on glass surface. The concentration of the micelle solution was 100 μg/mL.

microspheres, a depot formulation for subcutaneous and intramuscular tissues, it is difficult to precisely control the release rate of hydrophilic drugs such as ODN over a desired period, because diffusion and erosion processes are combined in a complicated manner to affect the release profile (24). In contrast, ODN/PLGA micelles that can be injected intravenously control the ODN release rate depending on the molecular weight and composition of PLGA to be conjugated.

To visualize the endocytosis process of ODN/PLGA micelles into cells, a rhodamine-labeled sense ODN was hybridized with complementary antisense ODN present in the shell of micelles. This resulted in the micelle structure composed of double stranded ODN in the shell and PLGA in the core. These micelles were readily taken up by NIH3T3 mouse fibroblast cells as shown in Figure 9. It was found that the rhodamine labeled micelles were distributed over the entire intracellular space of the cytoplasm. In contrast, hybridized double stranded ODN labeled with rhodamine exhibited negligible fluorescence intensity within cells due to the limited passive diffusion of the ODN across the cell membrane. The confocal microscopic observation reveals that ODN can be

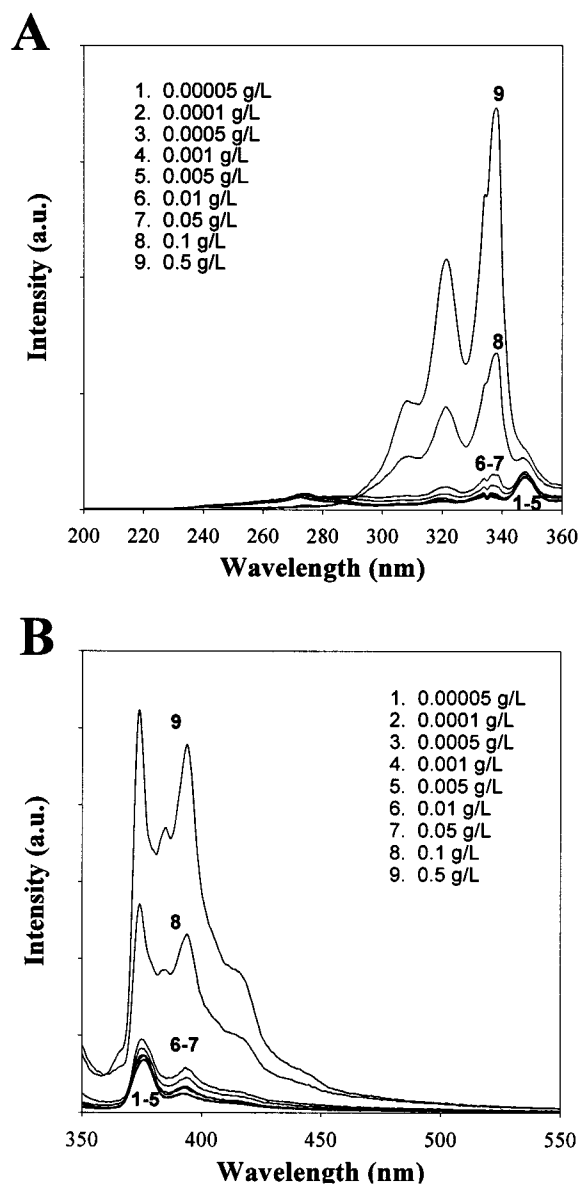


Figure 6. Excitation spectra of pyrene in the presence of ODN/PLGA conjugate at a fixed emission wavelength of 390 nm (A), and fluorescence emission spectra of pyrene in the presence of ODN/PLGA conjugate at a fixed excitation wavelength of 339 nm (B). The concentration of pyrene was 6×10^{-7} M. The concentration of ODN/PLGA conjugate was varied from 0.05 to 500 mg/mL.

effectively transported within cells because cells recognize the self-assembled ODN/PLGA micelles as particulates. Thus, the increased cellular uptake of ODN/PLGA micelles was likely due to an endocytosis process. Presumably, a fluid phase pinocytosis played a pivotal role in the intracellular transport process of ODN/PLGA micelles, because the ODN surface of micelles was highly negatively charged. Due to charge-to-charge repulsion between micelles and cells, adsorptive endocytosis might have a minor role in the transport process. The fact that some micelles were located in the nucleus (dark and round area inside cells) suggested that the micelles can also be delivered into the nucleus. It was reported that PEO–PBLA[poly(β -benzyl L-aspartate)]–FITC micelles could be delivered into the nucleus using endothelial monolayer cells (27). Our results suggest the possibility of ODN fragments intracellularly released from the micelles to enter the nucleus and act as an antisense agent, interacting with specific sequence in mRNA of

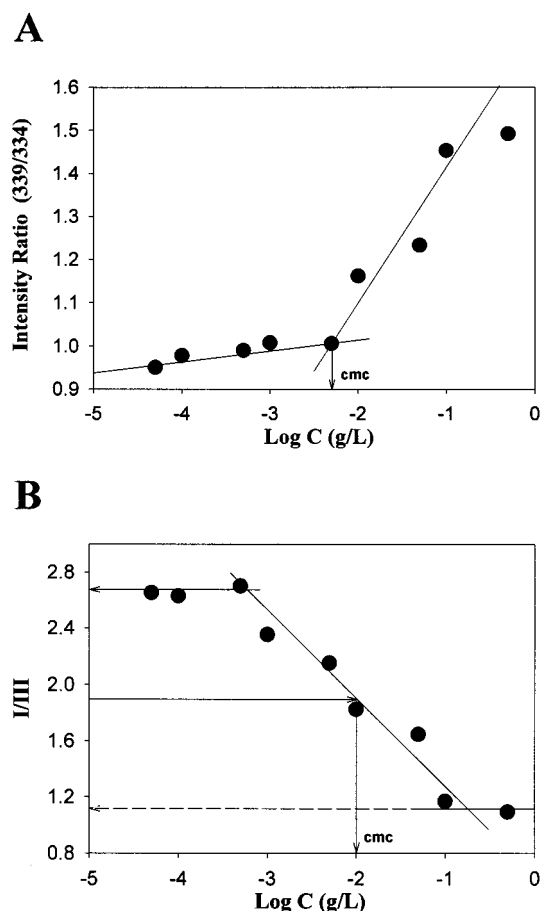


Figure 7. (A) The intensity ratio (339 nm/334 nm) of pyrene in the excitation spectra, (B) I/III band intensity ratio of pyrene as a function of logarithm of the ODN/PLGA conjugate concentration.

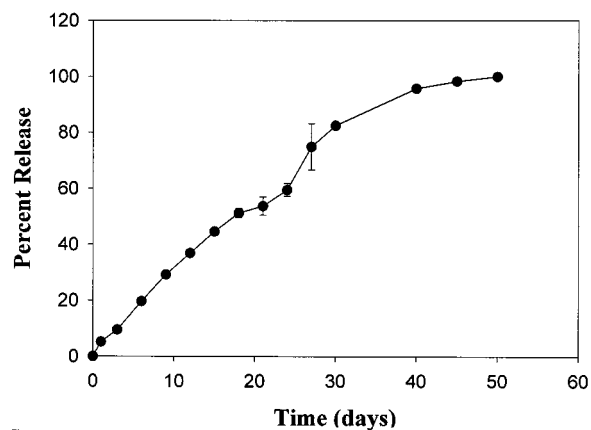


Figure 8. In vitro release profile of ODN from ODN/PLGA micelles in Tris–EDTA buffer, pH 8.0 at 37 °C.

interest to block the expression of a target protein. The cellular uptake efficiency of the rhodamine-labeled ODN/PLGA micelles was measured by a flow cytometry analysis. In an arbitrarily selected gate ($50 < \text{FL2-H} < 350$), the cellular uptake efficiency of ODN/PLGA micelles against fibroblast cells was 68.3% compared to ODN itself that showed a negligible cellular uptake of $< 0.1\%$.

In conclusion, it was shown that a new class of hybrid micelles composed of DNA and biodegradable polymer was synthesized and characterized. The ODN/PLGA micelles were around 65.2 nm in diameter with a very narrow size distribution. ODN and PLGA constituted a

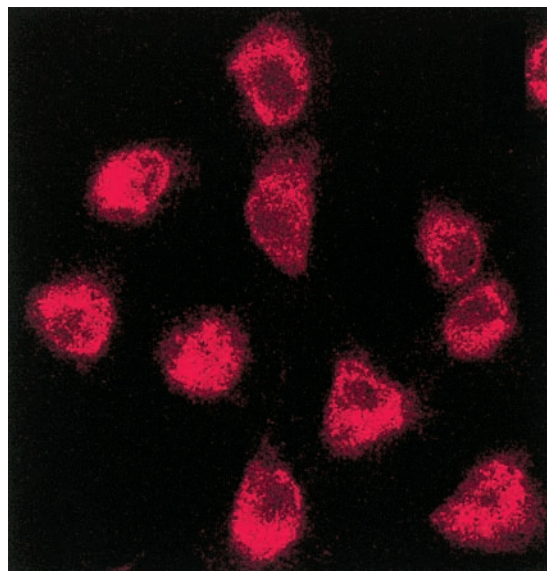


Figure 9. Confocal image of cellular uptake of rhodamine-labeled ODN/PLGA micelles transported within NIH3T3 mouse fibroblast cells. The concentration of the polymeric micelle was 80 $\mu\text{g/mL}$.

hydrophilic shell layer and a hydrophobic core in the micelle structure, respectively. The cmc value, very close to that of other synthetic diblock polymers, indicated that their self-assembling tendency in an aqueous phase was good enough to use as a stable delivery vehicle of ODN. The ODN/PLGA micelles released ODN in a sustained manner by controlled degradation of PLGA. They also exhibited enhanced cellular uptake within cells presumably by an endocytosis mechanism. The ODN/PLGA micelles can be potentially applied in therapeutic uses of inhibiting specific gene expression within cells. Therapeutic effects of ODN delivered in the form of ODN/PLGA micelles are to be evaluated in further studies using *in vitro* and *in vivo* models.

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Construction of a Fusion Enzyme System by Gene Splicing as a New Molecular Recognition Element for a Sequence Biosensor

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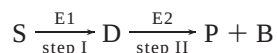
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A bifunctional fusion enzyme system constructed by gene splicing is proposed as a new model to develop sequence biosensors, taking maltose biosensor as an example. The cDNA fragment of *Aspergillus niger* glucoamylase (E.C 3.2.1.3, GA) was fused to the 3' end of *Aspergillus niger* glucose oxidase (E.C 1.1.3.4, GOD) gene with the insertion of a flexible linker peptide [-(Ser-Gly)₅-] coding sequence. The fusion gene was cloned into the vector pPIC9 and expressed in *Pichia pastoris* GS115 under the control of the AOX1 promoter. It was found that a bifunctional hybrid protein with a molecular weight of 430 kDa was secreted after induction with methanol. The fusion enzyme GOD-(Ser-Gly)₅-GA (GLG) was purified using Q Sepharose Fast Flow ion-exchange chromatography. Kinetic analysis demonstrated that GLG retained the typical kinetic properties of both GA and GOD. After being immobilized on an aminosilanized glass slide through covalent bonding by glutaraldehyde, GLG showed much higher sequential catalytic efficiency than the mixture of separately expressed GA and GOD (GA/GOD). Maltose biosensors were fabricated with GLG and GA/GOD, respectively. The performance characteristics of the maltose biosensor with respect to reproducibility, signal level, and linearity were effectively improved by using the fusion enzyme. Our findings offer a basis for the development of other sequence biosensors.

INTRODUCTION

Sequence biosensors, which involve two or more enzymes for sequential catalysis, are widely used in the determination of biochemical substances, such as disaccharides (1–4), starch (5), inosine (6), total cholesterol (7), etc. Upon review of the abstract books of the World Congress on Biosensors of the past decade, it is found that about 30% of the reported biosensors are sequence biosensors. For a two-enzyme sequence biosensor, for example, the reaction in the enzyme membrane could be described as follows:



The substrate S is catalyzed by the first enzyme E1 to produce the intermediate product D in step I. D then serves as the substrate of the second enzyme E2 in step II, giving the final product P and byproduct B, which could be a chemical substance or a physical signal. S is then determined by measuring P or B through an appropriate transducer. However, the performance of the sequence biosensors is normally not as good as the sole enzyme biosensors. The main reason is that the existing

immobilization methods, cross-linking or covalent bonding in most cases, provide no warranty to prepare the high quality multiple enzyme membrane that is the key element of a biosensor. The quality of an enzyme membrane greatly depends on the amount, ratio, and distribution control of E1 and E2 in it. Failure of the control results in poor linearity, sensitivity, and reproducibility (also known as interchangeability); the last one is extremely important in the manufacture of disposable biosensors. Many investigators tried to optimize the enzyme ratio in the membrane simply by adjusting the enzyme loadings (1–7), but the results were not always satisfying because the immobilization efficiency of the enzymes may differ from each other under the same immobilization conditions. On the basis of the studies of protein molecular structure, here we proposed a fusion enzyme method by gene manipulation to solve the problems.

Fusion protein technique has been widely used in the field of molecular biology and biotechnology for a variety of applications. Proteins or peptides which bind to specific molecular structures, such as glutathione S-transferase (8), maltose-binding protein (9), polyhistidine (10), streptag (11), are often employed as fusion partners to facilitate expression, purification, or immobilization of recombinant proteins. Green fluorescent protein (GFP) has been fused to some proteins as a reporter for protein

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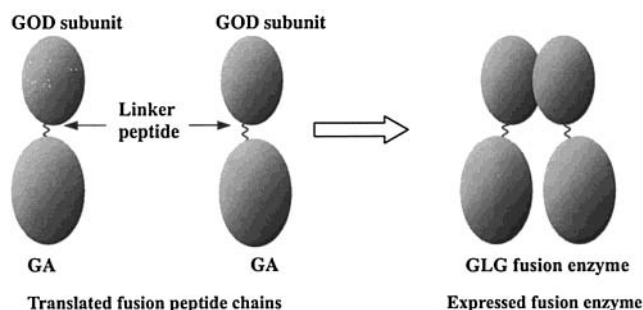


Figure 1. Schematic process of the fusion enzyme formation.

expression and localization (12, 13). Recently, there is a growing interest in a fusion enzyme possessing more than one catalytic function. Though the fragility and complexity of protein structures largely limit the success for accomplishing functionally active fusion enzymes, a few artificial fusion enzymes have been reported to exhibit noticeable performance in a concerned fashion (14–19). To our knowledge, there is no report concerning fusion enzyme application in biosensors yet.

The maltose biosensor, which employs GA and GOD as recognition elements, is one of the typical sequence biosensors (20). GA catalyzes hydrolysis of maltose to produce glucose that serves as specific substrate of GOD. Maltose is thus quantified by electrochemical measurement of oxygen consumption or release of hydrogen peroxide. GA is a monomer with a molecular weight of 99–112 kDa (21), and GOD is a dimeric flavoprotein with a molecular weight ranging from 150 to 186 kDa (22). The fusion enzyme constructed through gene fusion consists of two molecules of GA and one molecule of GOD. Since little information is available for predicting the structure and function change of a fusion enzyme, there is high risk in construction of such a complex hybrid protein. To avoid failure, a flexible linker peptide [-(Ser-Gly)₅-] was inserted between GA and GOD. This was thought to aid the independent folding of the two components (23). Figure 1 shows the schematic formation of GLG. Theoretically, GA and GOD would be adjacently immobilized with constant ratio by the proposed method if the fusion enzyme were successfully expressed. Reaction kinetics and reproducibility of the sequence biosensor should be accordingly improved. Details are reported herein.

MATERIALS AND METHODS

Materials. Restriction enzymes, DNA polymerase, and T4 DNA ligase were obtained from Takara and Promega. Lyticase, bovine serum albumin (BSA), O-dianisidine, soluble starch, PEG3350, glucose oxidase, and glucoamylase were purchased from Sigma. Yeast nitrogen base (YNB) was from Difco. All single strand oligonucleotides used in this study were obtained from Sangon (Shanghai, China). DNA purification columns and Q Sepharose Fast Flow ion exchange chromatography were from Qiagen and Pharmacia, respectively. A stirred cell system used for protein concentration was purchased from Filtron. *Aspergillus niger* 9029 containing GOD encoding region and plasmid pRD112 containing *Aspergillus niger* GA cDNA fragment were from American Type Culture Collection (ATCC). The methylotrophic yeast *Pichia pastoris* GS115 and its expression vector pPIC9 were kindly donated by professor Sheng-Li Yang. *Escherichia coli* DH5 α was used for all bacterial transformations and plasmid propagations. All other reagents used were of analytical-reagent grade available.

Construction of Expression Vectors. All oligonucleotides were designed to incorporate proper restriction sites for cloning. Primers GODN-1 (5'-TACGTAAGCAATGGCATTGAAGCCAGC-3') and GODC-1 (5'-AAGCTTCATGGAAGCATAATCTTCCAAG-3'), spanning the *Aspergillus niger* GOD encoding gene, were synthesized to amplify GOD gene with *Aspergillus niger* genome as template. Restriction sites SnaB I and Hind III were introduced to the N- and C-terminal primers, respectively. The fragment was first cloned into pGEM-T vector, and correct orientation was identified by restriction analysis. The resulting plasmid pGEM-TGOD was digested by SnaB I and Not I, and GOD encoding fragment was recovered and inserted into the SnaB I/Not I site of pPIC9, yielding GOD expression vector pPICGOD. The GA expression vector pPICGA1 was similarly constructed by using primers GAN-1 (5'TACGTAGCGACCTTGGATTCTATGGTTGAG-3') and GAC-1 (5'GCGGCCGCGAAATGGATTGATTGTCACC-3') to amplify GA encoding sequence with plasmid pRD112 as template.

For the construction of GLG fusion gene, GOD gene was first amplified and cloned into pGEM-T vector as described above. The synthesized oligonucleotides containing [-(Ser-Gly)₅-] coding sequence (in bold) and an additional Hind III site (underlined) were annealed at 65 °C for 5 min to form a dsDNA fragment:

5'-AGCTC***AGCGGCTCTGGTTCCGGTAGCGGTTCCGGCAAGCTTAGCGGTGC**-3'
3'-**GTCGCCGAGACCAAGGCCATCGCCAAGGCCGTTCAATCGCCACGCCCGG**-5'

This resulting short fragment was inserted to replace the sequence between Hind III and Not I site in pGEM-TGOD, yielding plasmid pGEM-TGL. Using primers GAN-2 (5'-AAGCTTGCGACCTTGGATTTCATGGTTGAG-3') and GAC-1 (5'-GCGGCCGCGAAATGGATTGATTGTCACC-3'), the GA encoding sequence containing Hind III and Not I site at its N- and C-termini was amplified and cloned into pGEM-T vector. The GA fragment was obtained by digesting the resulting plasmid pGEM-TGA2 with Hind III and Not I and then ligated to pGEM-TGL linearized with the same restriction enzymes, yielding plasmid pGEM-TGLG. Because the Hind III restriction site immediately behind GOD gene had been silenced by a base mutation, which is indicated by an asterisk, in linker peptide coding sequence, the resulting plasmid pGEM-TGLG contains intact GLG encoding sequence. Plasmid pGEM-TGLG was digested with SnaB I and Not I, and the fragment encoding the fusion enzyme was cloned into the SnaB I/Not I site of pPIC9, yielding GLG fusion enzyme expression vector pPICGLG. The vectors, shown in Figure 2, contain expression cassettes of the 5'AOX1 promoter and 3' AOX1 transcription termination fragment for regulated transcription and a-factor signal peptide for secretion.

Preparation of *Aspergillus niger* Genomic DNA.

Aspergillus niger genomic DNA was prepared by a modified method of Zhu et al. (24). *Aspergillus niger* cells were grown in YEP medium (peptone 20 g, yeast extract 10 g, glucose 20 g per liter) for 2 days. The mycelia harvested by centrifugation were washed with 10 mL of extracting solution (100 mM Tris-HCL, 40 mM EDTA, pH 8.5) twice and then mixed with 10 mL of extracting solution, 2 mL of 10% SDS, and 6 mL of benzyl chloride. The mixture was incubated at 50 °C for 1 h to digest the cell walls. The reaction mixture was supplemented with 6 mL of 3 M sodium acetate (pH 5.2) and kept in ice-bath for about 15 min. The mixture was then centrifuged at 10000 rpm for 15 min. After the mixture was deproteinized with PCA (phenol-chloroform-isoamyl alcohol,

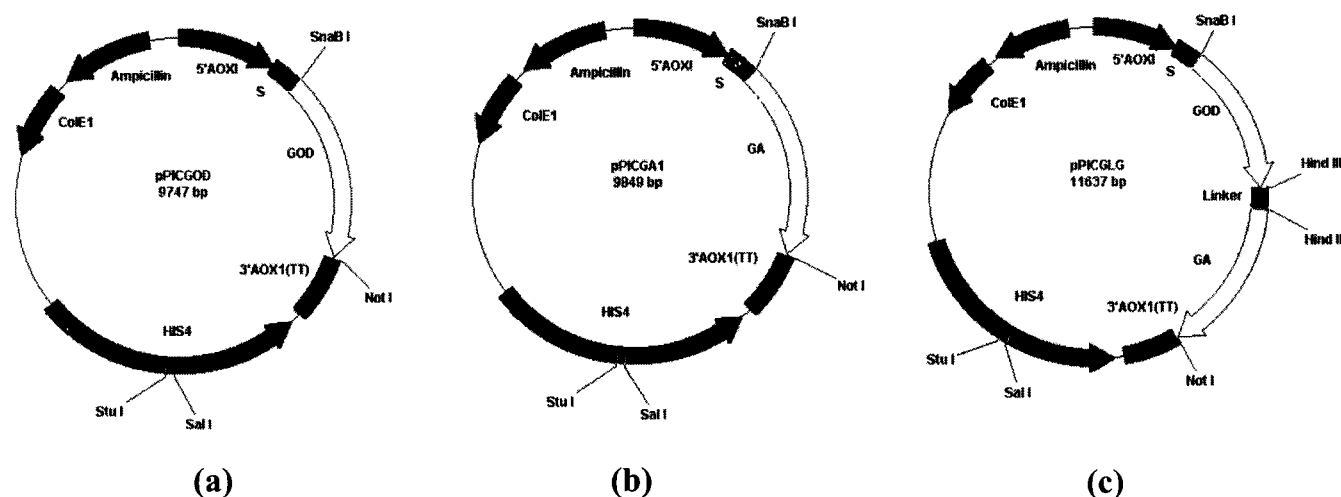


Figure 2. Constructed vectors for expression of (a) GOD, (b) GA, and (c) GLG fusion enzyme in *Pichia pastoris*.

25:24:1, vol/vol/vol), the DNA in the supernatant was precipitated with equal volume of 2-propanol.

Transformation of *Pichia pastoris*. pPICGOD, pPICGA1, and pPICGLG were digested with *Stu* I, and the linearized plasmid fragments containing GOD, GA, and GLG fusion gene, respectively, were introduced into GS115 as described previously (25). Integration of linearized pPICGOD, pPICGA1, and pPICGLG into the genome of GS115 was confirmed by PCR using yeast genomic DNA as templates.

Expression and Purification of the Yeast-Derived Enzymes. GOD, GA, and GLG were expressed respectively in *Pichia pastoris* under the control of AOX1 promoter. Yeast cells were cultured at 30 °C in 400 mL of MD medium (YNB 1.7 g, glucose 20 g, ammonium sulfate 5 g, and biotin 400 µg per liter) until the culture reached an optical density ($\lambda = 600$ nm) of 1.2–1.5. The cells were harvested by centrifugation and resuspended in 200 mL of MM medium (YNB 1.7 g, methanol 12 mL, ammonium sulfate 5 g, biotin 400 µg, and casamino acids 10 g per liter, pH 6.0) to induce the production of recombinant enzymes. After 5-day induction, the culture supernatant was concentrated to 30–40 mL using the FILTRON stirred cell system and dialyzed against 1000 mL of citrate buffer (pH 6.0, 0.02 M) overnight.

The yeast-derived enzymes were purified using Q Sepharose Fast Flow ion exchanger chromatography. Initially, the column was washed with 20% (v/v) ethanol and then equilibrated with citrate buffer (pH 6.0, 0.02 M). The sample solution was applied to the column, and unbound protein was washed out with equilibration buffer. The protein bound to the column was eluted using a salt gradient in the equilibration buffer. Fractions containing enzyme activity were pooled and concentrated using a FILTRON stirred cell system. Homogeneity of the purified enzymes was checked by SDS–PAGE (10%), and a Bio-Rad protein assay kit was used to determine the protein concentration of each sample.

Enzyme Assays. For all enzyme assays one unit of activity was defined as the amount of enzyme that generated 1 µM of product per minute. GOD activity was determined using fresh *Aspergillus niger* GOD (Sigma) as standard. Assays were performed according to the method described by Frederick et al. (26). The steady-state kinetic parameters of purified GOD and GLG to glucose at pH 5.6, 30 °C were calculated by assaying initial velocities over a range of glucose concentrations from 10 mM to 100 mM under oxygen saturation condi-

tion. Lineweaver–Burk plots were used to determine the parameters on the assumption that simple Michaelis–Menten kinetics was followed.

GA was assayed by measuring the amount of glucose released from starch hydrolysis using 3,5-dinitrosalicylic acid (DNS) method (27). When assaying GA activity of the fusion enzyme, nitrogen bubbling was employed to remove oxygen from the reaction system, thereby blocking GOD activity. For determination of kinetic parameters, initial rates of starch hydrolysis catalyzed by yeast-derived GA and GLG were determined using up to 15 substrate concentrations from 0.2 mg/mL to 20 mg/mL.

Enzyme Immobilization. Immobilization of yeast-derived enzymes was carried out by a modified method of Fonseca (28). A glass slide with desired well assay was cleaned thoroughly and then coupled to γ -(aminopropyl)-triethoxysilane in a toluene solution. The glass slide was soaked in 100 mL of 2.5% (v/v) glutaraldehyde solution in phosphate buffer (pH 7.0, 0.05 M) for about 1 h at room temperature. The glutaraldehyde-activated slide was washed five times with phosphate buffer to remove unreacted glutaraldehyde. 5 µL of 50 µg/mL GLG fusion enzyme and a mixture of yeast-derived GOD and GA equivalent to corresponding enzyme activities of GLG (GA/GOD) were applied to the wells of the glass slide and incubated at room temperature for 4 h. Unbound enzymes were removed by washing thoroughly with phosphate buffer containing 0.05% Tween-20 and 0.5 M NaCl. Finally, 5 µL of substrate solution containing 4 mg/mL soluble starch, 0.01 mg/mL horseradish peroxidase, and 0.1 mg/mL *o*-dianisidine were added. Color change of the wells was observed and compared.

Preparation of the Enzyme Electrodes. The enzyme membranes were prepared by cross-linking method using glutaraldehyde as described in our previous report (29). Typically, 3 µL of 20% (w/v) BSA, 4 µL of 2.5% (v/v) glutaraldehyde solution, and 20 µL of enzyme solution were dropped onto a porous Teflon membrane, followed immediately by mixing and spreading on 7 mm diameter circular area with a tiny glass rod. Cross-linking reaction was allowed to perform for 1 h at room temperature. The enzyme membrane was then formed on the Teflon membrane. The enzyme constitutions in the electrodes were 18U of GOD for GOD electrode, 18U of GOD and 10U of GA for both GA/GOD electrode and GLG electrode.

The enzyme membrane was covered with a piece of nylon gauze and fixed on the tip of an oxygen electrode with an 'O' ring. The enzyme electrodes were soaked in

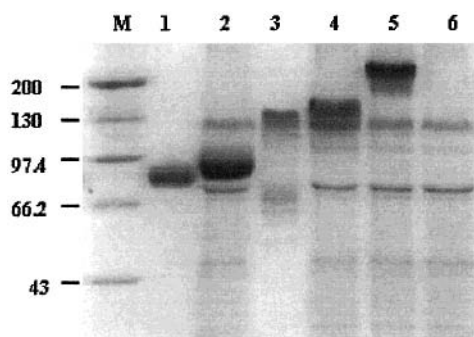


Figure 3. SDS-PAGE analysis of the total secreted proteins of *Pichia pastoris* cells. Lane M, high molecular weight protein marker (kDa); lane 1, *Aspergillus niger* GOD; lane 2, total secreted proteins of the yeast expressing GOD; lane 3, *Aspergillus niger* GA; lane 4, total secreted proteins of the yeast expressing GA; lane 5, total secreted proteins of the yeast expressing GLG; lane 6, total secreted proteins of the yeast carrying pPIC9.

citrate buffer (pH 4.8, 0.02 M), which was also used to prepare maltose solution, overnight to wash out unimmobilized enzymes and activated in 40 mM maltose solution at least 1 h before use.

Maltose Measurement. Maltose was measured using enzyme electrodes in combination with a flow injection analysis system (30). The working system was mainly composed of the enzyme electrode, amplified and digital display, peristaltic pump, injection unit, tubing system with citrate buffer (pH 4.8, 0.02 M) as carrying stream, and a printer. The carrier stream was pumped through the flow line at a flow rate of 2.0 mL/min, and 25 μ L of sample solution was injected into the flow stream when the background current was stabilized. When the sample flowed through the surfaces of the electrodes, consumption of oxygen was measured through current drop.

RESULTS

Expression of GOD, GA, and GLG in *Pichia pastoris*. As a preliminary experiment, the expression vectors were transformed into host yeast separately. Positive transformants were confirmed by PCR analysis of the yeast genome. Expression of the recombinant proteins was induced by methanol. Enzyme assays indicated that pPICGOD, pPICGA1, and pPICGLG directed separately the synthesis and secretion of active GOD, GA, and GLG displaying both GOD and GA activity. The total proteins in the culture supernatant were analyzed by SDS-PAGE (Figure 3). It was found that GOD and GA secreted from *Pichia pastoris* migrated more slowly than the enzymes from *Aspergillus niger*. Yeast-derived GA and GOD monomer were visualized to have molecular weights of about 135 and 85 kDa, respectively. The difference of the molecular weight between proteins expressed in *Aspergillus niger* and *Pichia pastoris* was also observed by other investigators (26, 31). They concluded that this was due to yeast-derived proteins tending to show substantially more N-linked carbohydrate than *Aspergillus niger* proteins. The protein band corresponding to GLG monomer on SDS-PAGE was observed at approximately 215 kDa. The molecular weight of GLG monomer agreed well with the sum of those of GA and GOD monomer. The native GLG which consists of two GA monomer and one GOD dimer has a molecular weight up to 430 kDa.

To optimize the production conditions, effects of pH and methanol concentration on the yield of GLG were studied. Although *Pichia pastoris* cells grew normally in all induction conditions tested, the optimal conditions for

production of GLG were around 1.2% methanol added once a day and initial pH 5–6 (Figure 4). In addition, the activity of GLG did not increase appreciably upon induction for periods longer than 5 days in all shake flask studies. Hence induction in pH 6, MM medium for 5 days with daily addition of 1.2% methanol was chosen to produce GOD, GA, and GLG for further studies.

Purification of the Yeast-Derived Enzymes. After removal of the *Pichia pastoris* cells from the fermentation culture, the supernatant was concentrated by ultrafiltration and dialyzed to remove salt. GOD, GA, and GLG were purified using anion-exchange chromatography on a Q Sepharose Fast Flow column. Elution profile of all yeast-derived enzymes is shown in Figure 5a. It is clear that GOD, GA, and GLG require different elution time and ionic strength of the elution buffer. GOD was eluted from the column by using 0–0.1 M NaCl gradient solution, while GA and GLG were eluted by a NaCl gradient of 0–0.4 M. Among these enzymes, the fusion enzyme required the highest ionic strength. The purity of the proteins was checked by SDS-PAGE (Figure 5b).

Kinetic Properties of the GLG Fusion Enzyme.

To examine the effect of protein fusion on the kinetic properties of both GOD and GA moieties of the fusion enzyme, the kinetic parameters of GLG were compared with those of individual enzymes from either *Pichia pastoris* or *Aspergillus niger*. Table 1 summarizes the kinetic constants of all enzymes studied here. GOD and GA secreted from *Pichia pastoris* had very high catalytic activity and showed about 1.6-fold and 2.0-fold increase of specific activity over that of the *Aspergillus niger* GOD and GA, respectively. Compared with the values of 426.62 U/mg protein and 238.34 U/mg protein measured for yeast-derived GOD and GA, the specific activities of the fusion enzyme toward glucose and soluble starch were determined to be 195.21 U and 116.56 U, respectively, per mg protein, showing that the catalytic activities of the fusion enzyme were successfully maintained. The k_{cat} and K_m of GLG to glucose were similar to the values determined for the yeast-derived GOD. The k_{cat} of GLG to soluble starch was 2.3 times as high as that of the yeast-derived GA due to the fact that 1 mol of GLG consists of 2 mol of GA. The higher turnover number of the fusion enzyme to soluble starch is thought to be advantageous for the sequential catalysis occurred in maltose biosensor. Compared with GOD and GA from *Aspergillus niger*, the yeast-derived GOD, GA, and GLG showed slightly lower affinity for substrates. However, the k_{cat} and k_{cat}/K_m determined for the yeast-derived enzymes were obviously higher than those of *Aspergillus niger* enzymes.

Enzyme Immobilization. Cross-linking and covalent bonding using glutaraldehyde are the most widely used enzyme immobilization methods. Immobilization of the yeast-derived enzymes was performed by depositing GLG and GA/GOD onto the glutaraldehyde-activated glass slide, respectively, with four duplicates. The immobilization profile would be a direct indication of whether the fusion enzyme had benefited the enzyme immobilization or not if so to what extent. After adding substrate solution containing soluble starch, horseradish peroxidase and o-dianisidine, the slide was incubated at 30 $^{\circ}$ C for more than 1 h to develop brown color, which in fact is a measure of the overall reaction efficiency catalyzed sequentially by GA and GOD. Relative recovery activities of GLG and GA/GOD were evaluated according to the darkness of the color spots. It was found that wells with immobilized GLG developed color much faster than those with immobilized GA/GOD. Visible color change appeared

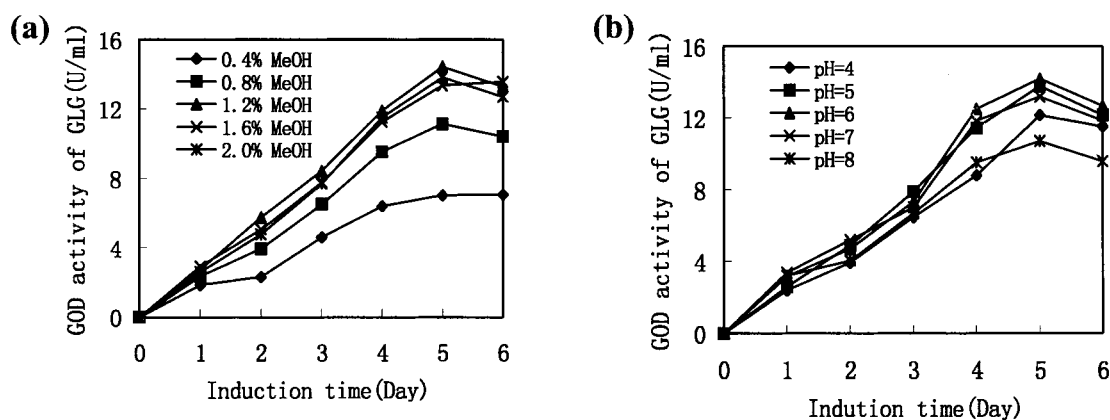


Figure 4. Effects of (a) methanol concentration and (b) pH on the production of GLG.

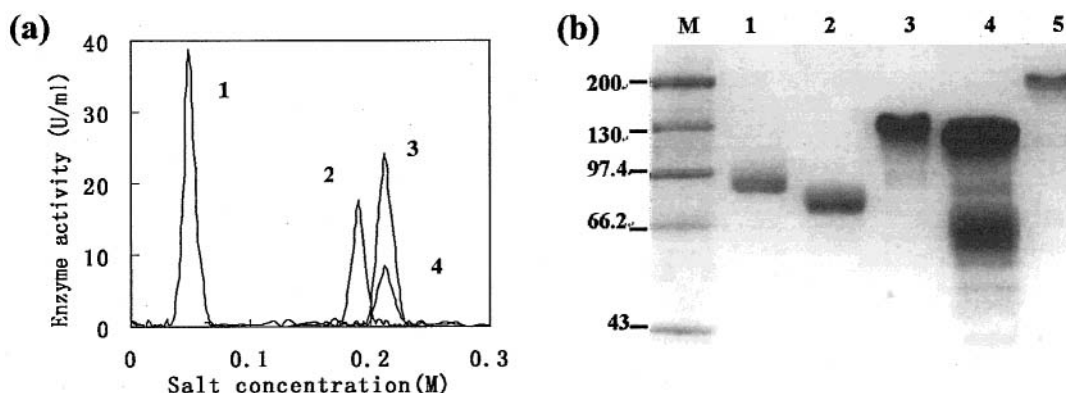


Figure 5. (a) Elution pattern of the yeast-derived enzymes. Peak 1 represents GOD; peak 2 represents GA; peak 3 and peak 4 represent GOD and GA activities of GLG, respectively. (b) SDS-PAGE analysis of the purified enzymes. Lane M, high molecular weight protein marker (kDa); lane 1, purified yeast GOD; lane 2, *Aspergillus niger* GOD; lane 3, purified yeast GA; lane 4, *Aspergillus niger* GA; lane 5, purified GLG.

Table 1. Kinetic Parameters of Native and Yeast-derived Enzymes

enzymes	specific activity (U/mg protein)	K_m (mM for GOD, mg/mL for GA)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} mM^{-1}$ for GOD, $s^{-1} mL \cdot mg^{-1}$ for GA)
<i>A. niger</i> GOD	266.31	33.44	2303.56	68.89
<i>P. pastoris</i> GOD	426.62	38.25	3492.66	91.31
<i>A. niger</i> GA	118.75	1.347	557.33	413.76
<i>P. pastoris</i> GA	238.34	1.703	1123.71	659.84
GOD of GLG	195.21	37.16	3314.13	89.19
GA of GLG	116.56	1.774	2601.33 ^a	1466.36

^a The k_{cat} value was calculated per GLG, which contains two GA active sites.

within 1 h for the fusion enzyme, but only a faint color developed in the spots with immobilized GA/GOD even after 5 h incubation (Figure 6). The result demonstrates that GLG fusion enzyme is obviously superior to GA/GOD in terms of immobilization efficiency. No detectable signal was observed in the spots of negative controls where the yeast-derived GA and GOD were immobilized individually on the glass chip with the same procedure.

Evaluation of the Enzyme Electrodes by Measuring Maltose. The responses of the three kinds of enzyme electrodes to maltose solution were investigated. As shown in Figure 7, the GOD electrode did not response to maltose, while the response values of GLG electrode and GA/GOD electrode were proportional to maltose concentration. To the same maltose solution, the response signal of GLG electrode is higher than that of GA/GOD electrode. A linear response range up to 40 mM was

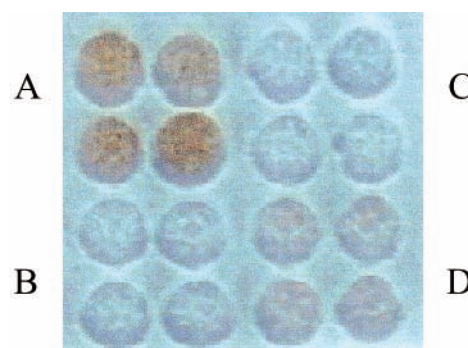


Figure 6. Immobilization of the enzymes on a glass chip. The spots that developed brown show positive reactions, others show negative reactions, and each loaded four duplicates. Experimental detail is in the text. (A) denotes the detection of GLG; (B) denotes the detection of GOD; (C) denotes the detection of GA; (D) denotes the detection of the mixture of GA and GOD (GA/GOD).

obtained for GLG electrode and 30 mM for GA/GOD electrode. The increased signal level and extended measuring linearity of the GLG system should be attributed to the higher immobilization efficiency of GA and GOD as well as their proximity effect in the enzyme membrane.

To investigate the precision and reproducibility of the electrodes, both GLG electrode and GA/GOD electrode were fabricated with six repeats, and each electrode was used to measure 10 mM maltose solution five times in succession. Good precision was obtained for both GLG electrode and GA/GOD electrode. The variation coefficients of the response values varied from 0.6% to 4.2%

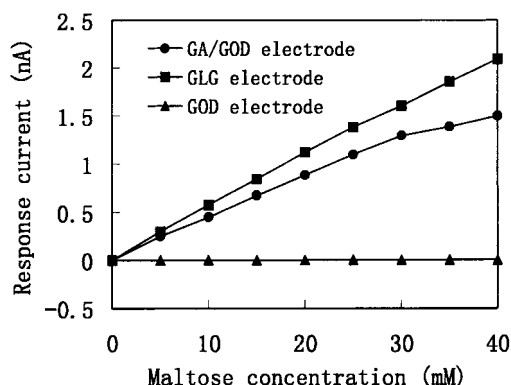


Figure 7. Responses of the enzyme electrodes to maltose.

Table 2. Analysis of Reproducibility of Enzyme Membranes

electrode	the responses of six enzyme membrane ^a (nA)	variation coeff of the enzyme membranes (%)
GA/GOD	0.346, 0.489, 0.401, 0.395, 0.547, 0.528	18.1
GLG	0.527, 0.613, 0.583, 0.645, 0.505, 0.577	9.1

^a Each response value here was the mean of five repeated measurements to 10 mM maltose.

for six GLG electrodes and from 1.3% to 5.4% for six GA/GOD electrodes. However, the reproducibility, which represents the interchangeability between enzyme membranes, of GLG membranes was much better than that of GA/GOD membranes. Compared with the values of 18.1% calculated for GA/GOD membranes, the variation coefficient of GLG membranes was determined to be 9.1% (Table 2).

DISCUSSION

The large hybrid fusion enzyme GLG was successfully constructed in this study. The linker peptide composed of serine and glycine was designed to minimize the steric hindrance between GA and GOD so as to provide enough space for both proteins to remain in native conformation. This was indirectly proved by comparison of its catalytic activities with the native GA and GOD expressed in the same host. The same linker had been successfully used in our previous work where the alkaline phosphate was actively fused to strep-tag through it (32). Our observations suggest that the linker peptide play an important role in the successful expression of the fusion enzyme. The resulting fusion enzyme GLG maintained typical characteristics of both GA and GOD. No any detectable activity of the fusion enzyme was obtained at cellular

level without the linker insertion (data not shown). On the other hand, it is interesting that this heterogeneous hybrid protein up to 430 kDa can still correctly fold in yeast cell and secrete. The mechanism is worth studying.

For process development, GOD and GA have also been separately expressed in the same host. It is presumed that all yeast-derived enzymes here were hyperglycosylated according to their lower electrophoretic mobility (Figures 3, 5b). Yeast is known to hyperglycosylate some secreted foreign protein, especially those using α -Factor signal leader for secretion (26, 31). However, at present the reason for the differential glycosylation of protein between yeast and *Aspergillus niger* is still not clear. A detailed kinetic comparison of the various enzymes demonstrated that the yeast-derived GOD and GA showed increased specific activity and catalytic efficiency than the *Aspergillus niger* enzymes.

In the fabrication of enzyme sensors, the immobilization of enzymes on the electrode surface is a crucially important step, which determines the performance characteristics of the biosensors. Sequence biosensors require coimmobilization of more than one enzyme in the same membrane. The discrepancy of immobilization efficiency between enzymes, without doubt, will affect the performance of the biosensor, especially in case one enzyme having relatively poor immobilization efficiency. The fusion enzyme offers several advantages over individually coimmobilized enzymes. First, it provides fixed molecular ratio of immobilized enzymes, ensuring the desired activity of the enzyme membrane. The compositions and structures of both *Aspergillus niger* GA and GOD were resolved and released (26, 33–36). There are 13 and 30 lysine residues in the structures of GA and GOD, respectively. Most of them are located on the surface of the structures (Figure 8). Lysine provides an amino group for coupling to glutaraldehyde, a bifunctional reagent for cross-linking reaction or covalent bonding. It clearly demonstrates why GA is more difficult to be immobilized than GOD using glutaraldehyde. In case of GLG, neither GA nor GOD could escape whichever reacted with the bifunctional reagent during immobilization process. This assumption has been proved by the evidence shown in Figure 6, where the recovery activity of the sequence reaction catalyzed by GLG was much higher than that of GA/GOD, despite of the same enzyme activity loading in both cases. Second, the ratio control of GA and GOD activity greatly improved the homogeneity of the enzyme membranes from batch to batch of manufacture (Table 2), which is one of the main factors affecting the quality of enzyme sensor. Poor homogeneity results in difficulty of interchangeability between the enzyme membranes. It is, as mentioned above, the major premise to develop

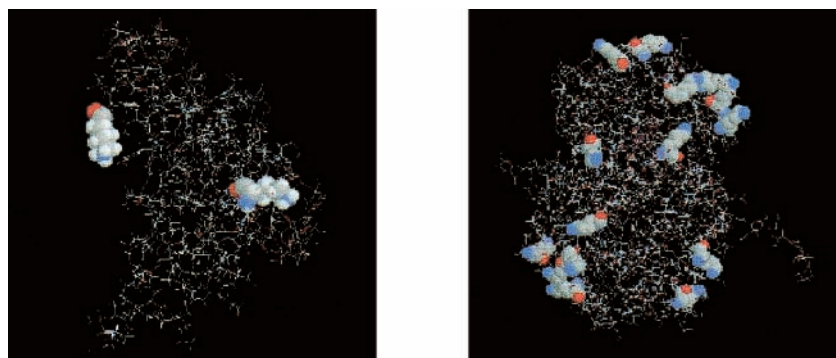


Figure 8. The crystal structures of (a) granular starch binding domain of *Aspergillus niger* GA bound to β -cyclodextrin (Sorimachi et al.) and (b) *Aspergillus niger* GOD subunit (Hecht et al.). Lysines are shown in space-filling models.

the disposable biosensors where no calibration step can be carried out when in use. Similar requirement can be met in construction of other biomolecular devices, such as protein chips. Third, the distance between the active centers of the fused enzymes is shortened and controlled, which makes the intermediate produced by the first enzyme readily available to the second enzyme, leading to a faster reaction rate and thus higher response signal or sensitivity of the biosensor (Figure 7). It is argued that fusion enzymes may not function at their highest active level because the optimal working conditions for the enzymes to be fused may differ from each other. The working condition, however, could be compromised if there is no better choice.

Our findings suggest that GA and GOD can be effectively used as fusion partners to construct a genetically artificial fusion enzyme despite their complex tertiary structure. And, in conclusion, the method proposed in this study offers a basis to develop high quality sequence biosensors as well as other sequential enzyme reaction applications.

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Structural Characteristics of Size-Controlled Self-Aggregates of Deoxycholic Acid-Modified Chitosan and Their Application as a DNA Delivery Carrier

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Precise control of the size and structure is one critical design parameter of micellar systems for drug delivery applications. To control the size of self-aggregates, chitosan was depolymerized with various amounts of sodium nitrite, and hydrophobically modified with deoxycholic acid to form self-aggregates in aqueous media. Formation and physicochemical characteristics of size-controlled self-aggregates were investigated using dynamic light scattering, fluorescence spectroscopy, and computer simulation method. The size of self-aggregates varied in the range of 130–300 nm in diameter, and their structures were found to depend strongly on the molecular weight of chitosan ranging from 5 to 200 kDa. Due to the chain rigidity of chitosan molecule, the structure of self-aggregates was suggested to be a cylindrical bamboolike structure when the molecular weight of chitosan was larger than 40 kDa, which might form a very poor spherical form of a birdnestlike structure. To explore the potential applications of self-aggregates as a gene delivery carrier, complexes between chitosan self-aggregates and plasmid DNA were prepared and confirmed by measuring the fluorescence intensity of ethidium bromide and electrophoresis on agarose gels. The complex formation had strong dependency on the size and structure of chitosan self-aggregates and significantly influenced the transfection efficiency of COS-1 cells (up to a factor of 10). This approach to control the size and structure of chitosan-derived self-aggregates may find a wide range of applications in gene delivery as well as general drug delivery applications.

INTRODUCTION

Colloidal systems have found numerous applications as promising delivery vehicles of drugs, proteins, antigens, and genes due to their reduced toxic side effect and improvement of therapeutic effect (1, 2). Polymeric self-assembly systems, as one of colloidal systems, have been widely investigated in terms of their micellar behavior as well as their application to the fields of biotechnology and pharmaceuticals (3–5). Polymeric self-assemblies are usually formed from block copolymers (6–8) or hydrophobically modified water-soluble polymers (9–11). It may be fascinating to develop polymeric self-aggregates from hydrophobically modified water-soluble polymers rather than block copolymers, as it is generally difficult to synthesize new types of block copolymers.

One attractive utilization of polymeric self-aggregates to date is as a gene delivery vehicle in order to cure many hereditary diseases and treat acquired disease resulting from either multigenetic disorders or foreign viral genes

(12, 13). Viral delivery systems have many advantages for gene delivery due to their high efficiency and wide range of cell targets. However, they have some drawbacks of immune responses and oncogenic effect in vivo. Non-viral delivery systems including cationic liposomes and polymeric systems have therefore been extensively investigated (14–18). Cationic liposomes are widely applicable to many animal cells due to their nonspecific ionic interaction and low toxicity. However, due to the poor stability in vivo of cationic liposomes, many polymeric systems have been studied as alternatives for in vivo and in vitro applications (16–18).

Chitosan is generally considered biocompatible, biodegradable, and of low toxicity (19). In addition, chitosan has cationic characters even in neutral condition to form complexes with negatively charged plasmid DNA, and promotes cell transfection (20–23). We have previously demonstrated that self-aggregated nanoparticles, prepared from hydrophobically modified chitosan, could find a potential application as a gene delivery vehicle, due to the controlled complex formation with plasmid DNA (24). However, the size of the chitosan self-aggregates was not precisely controlled by varying the degree of substitution, pH, and/or ionic strength of the medium, probably due to the rigidity of chitosan backbone chain, and their diameter was in the range of 160–180 nm (10, 24). To be used as a delivery vehicle, the size of self-aggregates should be more precisely controlled, and their structure should be further investigated.

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¹ Abbreviations used: DAMC, deoxycholic acid-modified chitosan; DPH, 1,6-diphenyl-1,3,5-hexatriene; DS, degree of substitution; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EtBr, ethidium bromide; PEO, poly(ethylene glycol).

It is thus the aim of this paper to control the size and its distribution of self-aggregates of deoxycholic acid-modified chitosan (DAMC) and to verify their structure in order to utilize as a gene delivery carrier. In brief, chitosan was depolymerized using sodium nitrite and modified with deoxycholic acid in the presence of carbodiimide. We hypothesized that the chitosan derivatives could form self-aggregates with different sizes and structures, depending on the molecular weight of chitosan. Micellar characteristics were investigated by fluorescence spectroscopy, dynamic light scattering method, and computer simulation. In addition, the chitosan self-aggregates were considered to form complexes with plasmid DNA by ionic interactions. Complexation behavior between plasmid DNA and chitosan self-aggregates, as well as their transfection activity, were investigated and interpreted in relation to their micellar structures.

MATERIALS AND METHODS

Materials. Chitosan ($M_w = 1.0 \times 10^6$, degree of deacetylation = 80%) was purchased from Fluka (Milwaukee, WI). Sodium nitrite, deoxycholic acid, ethidium bromide, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Sigma (St. Louis, MO) and used without further purification. Pyrene and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Aldrich (St. Louis, MO) and used as a fluorescence probe. DNA molecular weight marker II (λ DNA/Hind III digested) was purchased from Boehringer Mannheim GmbH (Germany). The water was purified by distillation, deionization, and reverse osmosis (MilliQ Plus).

Depolymerization of Chitosan. Chitosan was dissolved in a 2% acetic acid solution and treated for 3 h at room temperature with different amounts of sodium nitrite in a range from 0.01 to 0.25 mol/mol glucosamine residues. The resulting solution was neutralized with a 1.0 N sodium hydroxide solution, precipitated with acetone, and dried in a vacuum at room temperature. The molecular weight of the depolymerized chitosan was determined by gel permeation chromatography (GPC) through Ultrahydrogel linear columns (Waters, USA) at a flow rate of 1.0 mL/min. A buffer solution of 0.15 M acetic acid and 0.5 M sodium acetate was used as a mobile phase. Pullulan (Showa, Japan) was used as a molecular weight standard. Degree of deacetylation of chitosan was determined by the titration method as previously reported (25).

Synthesis of Deoxycholic Acid-Modified Chitosan (DAMC). The depolymerized chitosan was hydrophobically modified with deoxycholic acid (0.05–0.34 mol/mol glucosamine residues of chitosan) in the presence of EDC for 24 h at room temperature (24). The reaction mixture was poured into a methanol/ammonia solution (7/3, v/v). The precipitates were filtered off, washed thoroughly with methanol and ether, and then followed by vacuum-drying at room temperature. The degree of substitution, defined as the number of deoxycholic acid groups per 100 anhydroglucose units of chitosan, was determined by calculation of the C/N ratio from an elemental analyzer (LECO). Infrared spectra of chitosan derivatives were monitored on an FT-IR spectrophotometer (resolution, 4 cm^{-1} ; 32 scans; MIDAC) to confirm the conjugation reaction between chitosan and deoxycholic acid (KBr pellet: 1655 (NHC=O) cm^{-1}).

Preparation of DAMC Self-Aggregates. DAMC was suspended in a phosphate-buffered saline (PBS) solution (pH 7.2) under gentle shaking at 37 °C for 48 h, followed by sonication using a probe type sonifier (Sigma Ultra-

sonic Processor, GEX-600) at 30 W for 2 min. The sonication was repeated three times to get an optically clear solution. To inhibit the heat build-up during sonication, the pulse function was used (pulse on, 5.0 s; pulse off, 1.0 s) (10).

Fluorescence Measurements. Fluorescence spectra of DAMC self-aggregate solutions, which contained 6.0×10^{-7} M of pyrene as a probe, were obtained with an ISS K2 fluorometer (ISS, Champaign, IL). For the measurement of the intensity ratio of the first and the third highest energy bands in the pyrene emission spectra, the slit openings were set at 1 mm (excitation) and 0.5 mm (emission). The excitation wavelength (λ_{ex}) was 336 nm, and the spectra were accumulated with an integration time of 5 s/nm.

Assuming simple partition equilibrium, the equilibrium constant (K_v) for partitioning of pyrene between water and micellar phases was calculated according to the method of Wilhelm et al. (26)

$$\frac{F - F_{\min}}{F_{\max} - F} = \frac{K_v x c}{1000 \rho} \quad (1)$$

where F is the intensity ratio ($= I_{336}/I_{333}$) from the fluorescence excitation spectrum at the intermediate concentration range of DAMC ($\lambda_{\text{em}} = 390$ nm), $F_{\min}$ and $F_{\max}$ are the intensity ratios at low concentration and at high concentration, respectively, x is the weight fraction of deoxycholic acid in DAMC, and ρ is the density of the inner core of self-aggregates that is assumed to be the same value of deoxycholic acid in water ($= 1.31$ g/mL) (27).

Fluorescence anisotropy of the DAMC self-aggregate solution containing DPH (2.1×10^{-6} M) was measured using L-format geometry of detection ($\lambda_{\text{ex}} = 360$ nm, $\lambda_{\text{em}} = 430$ nm). Fluorescence anisotropy (r) was calculated from the following relationship.

$$r = \frac{(I_{\text{VV}} - I_{\text{VV}}^s) - G(I_{\text{VH}} - I_{\text{VH}}^s)}{(I_{\text{VV}} - I_{\text{VV}}^s) + 2G(I_{\text{VH}} - I_{\text{VH}}^s)} \quad (2)$$

where I^s is the contribution of scattered light from a sample solution in the absence of DPH, $G (= I_{\text{VH}}/I_{\text{HH}})$ is an instrumental correction factor, and I_{VV} , I_{VH} , I_{HV} , and I_{HH} correspond to the resultant emission intensity polarized in the vertical or the horizontal detection planes (the second subscript) when excited with vertically or horizontally polarized light (the first subscript) (28).

Dynamic Light-Scattering Measurement. An argon ion laser (Lexel Laser Model 95) was operated at a wavelength of 488 nm. The intensity autocorrelation was measured with a Brookhaven BI-9000AT digital autocorrelator ($\theta = 90^\circ$, $T = 25 \pm 0.1$ °C). A nonlinear regularized inverse Laplace transformation technique (CONTIN) was used to obtain the distribution of decay function. Mean diameter (d) was calculated from the Stokes–Einstein equation.

Computer Simulation. Based on the experimentally observed data, the structures of native chitosan, DAMC, and DAMC self-aggregate were simulated using Cerius2 programs that contain the modules such as polymer builder, open force field, energy minimizer, and molecular dynamics (MSI, San Diego, CA). Chitosan and DAMC were initially created on a computer and energy-minimized using the modules of polymer builder and energy minimizer. The micellar structure of DAMC was obtained from the molecular dynamics simulation method

for 20 ps (20000 steps $\times$ 0.001 ps/step) using Dreiding 2.21 force field and Universal 1.02 force field.

Plasmid DNA. The pCMV-Luc+, obtained from the plasmid pGL3 (Promega, Madison, WI) using Xba I and Hind III, consists of a cytosolic form of *Photinus pyralis* luciferase cDNA (29). The plasmid was amplified in the *Escherichia coli* DH5- α strain and purified by a Qiagen kit (Chatsworth, CA) according to the manufacturer's instruction. DNA purity was determined by measuring the optical density (OD), and DNA with $OD_{260}/OD_{280} \geq 1.8$ was used.

Characterization of DAMC Self-Aggregate/DNA Complexes. Complexes were prepared by the addition of DNA solution (0.1 μ g/mL) to DAMC self-aggregate solution (1 mg/mL) at different charge ratios, following incubation for 20 min at room temperature. The charge ratio ($\pm$) was defined as the ratio of the number of amino groups in DAMC self-aggregates to the number of phosphate groups in DNA. Both DNA and DAMC self-aggregate solutions were prepared in PBS solution, and the final volume of the complexes was 25 μ m. Complex formation between plasmid DNA and DAMC self-aggregates was confirmed by electrophoresis using agarose gels as previously reported (24). The DAMC self-aggregate/DNA complexes were further characterized by fluorescence spectroscopy. Briefly, the complex solution containing EtBr (2 nmol) was incubated for 10 min, and the fluorescence intensity was measured using the ISS K2 fluorometer at $\lambda_{ex} = 488$ nm (slit width 2.0 mm) and $\lambda_{em} = 594$ nm (slit width 1.0 mm) ($n = 3$).

Cell Culture and In Vitro Transfection. A simian kidney cell line (COS-1) was cultured in the Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 $^{\circ}$ C in a humidified 5% carbon dioxide incubator. Cells were seeded at 2×10^4 cells per well of a 96-well plate 12 h before transfection. Cells were 70–80% confluent at the time of transfection. The plasmid of pCMV-Luc+ (0.5 μ g) was mixed with the DAMC self-aggregate solution at a charge ratio of 4 in the absence of serum and added to the single well. Naked DNA was also used as a control. After 1 h incubation, the cells were washed with serum-free DMEM to remove the remaining complexes, fed again with fresh media containing FBS, and cultured for 24 h after transfection. The transfected cells were assayed for luciferase activity using a Promega kit (Madison, WI) with a luminometer (Turner Designs Luminometer Model TD-20/20, Promega). The luciferase content was calculated from the relative light units using a standard curve obtained from the purified firefly luciferase (Sigma). The background level of luciferase activity in untreated cell lysates was measured from the organs of mice without any treatment and found to be negligible.

RESULTS AND DISCUSSION

Depolymerization and Hydrophobic Modification of Chitosan. Chitosan was depolymerized using sodium nitrite to obtain samples with various molecular weights. The depolymerization reaction was selective and could be easily controlled by adjusting the amount of sodium nitrite used in the reaction. The weight average molecular weights of the depolymerized chitosans, determined by GPC, were in the range of 5–200 kDa and the polydispersity of about 2. However, their degree of deacetylation was not significantly affected by the depolymerization reaction in this range of molecular weights (Figure 1).

The depolymerized chitosan was hydrophobically modified with deoxycholic acid in the presence of EDC, which

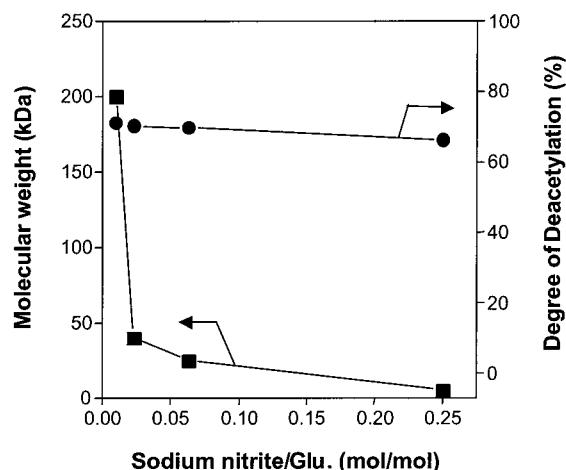


Figure 1. Changes of the molecular weight and the degree of deacetylation of depolymerized chitosans.

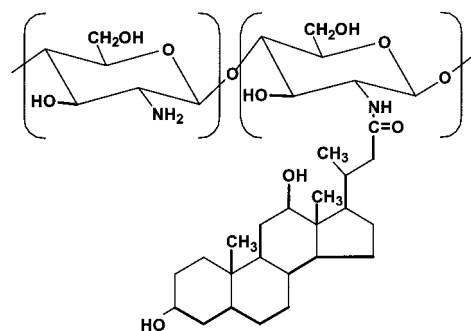


Figure 2. Chemical structure of deoxycholic acid-modified chitosan (DAMC).

Table 1. Characteristics of Chitosan Self-aggregates with Different Molecular Weights

sample ^a	$cac^b \times 10^2$, mg/mL	$K_v^c \times 10^{-5}$	r^d	d^e , nm	d_{real}/d_{cal}^f
DAMC5k	6.7	0.8	0.186	132	40
DAMC25k	4.0	1.2	0.246	170	10
DAMC40k	2.3	2.5	0.302	230	9
DAMC200k	1.2	3.9	0.340	300	2

^a Deoxycholic acid-modified chitosan (DAMC) with DS = 6.0. ^b Critical aggregation concentration. ^c Equilibrium binding constant of pyrene according to eq 1. ^d Anisotropy value of DPH according to eq 2. ^e Mean diameter measured by dynamic light-scattering method. ^f Minimum aggregation number of chitosan molecular chains calculated on the assumption of the formation of a spherical unimer micelle.

is known to promote amide linkage formation between amine and carboxylic acid (30). EDC initially reacts with the carboxyl group of deoxycholic acid to form an active ester intermediate, and the intermediate readily reacts with a primary amine of chitosan as a result of amide linkage formation (Figure 2). The characteristic peak at 1655 cm^{-1} from FT-IR spectra confirmed the amide bond formation between chitosan and deoxycholic acid (data not shown). The degree of substitution (DS) increased with increasing mole ratio of deoxycholic acid and was in the range from 2 to 10. The chitosan derivatives with DS = 6 of various molecular weights were used in this study in order to investigate the effect of the molecular size on self-aggregate formation in aqueous media. The number following the sample code DAMC in Table 1 indicates the molecular weight of chitosan.

Characteristics of DAMC Self-Aggregates. The self-aggregation behavior of DAMC in aqueous media was initially investigated by a fluorometry in the presence of

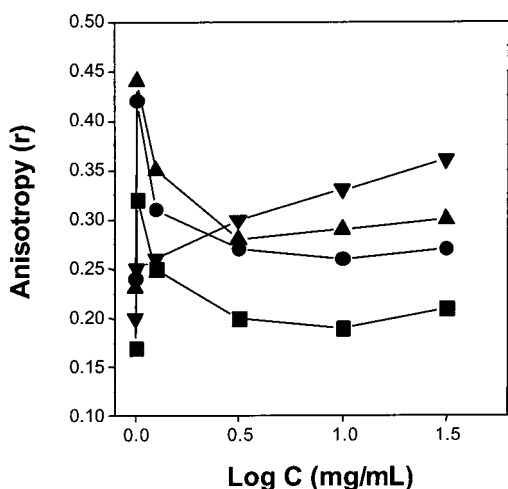


Figure 3. Changes of the anisotropy value (r) for DPH in the presence of DAMC self-aggregates in PBS solution (■, DAMC5k; ●, DAMC25k; ▲, DAMC40k; ▼, DAMC200k).

pyrene as a fluorescent probe. Pyrene is poorly soluble in water, but strongly emits when the self-aggregates or other hydrophobic microdomains are formed in an aqueous solution, as it preferably lies close to (or inside) the microdomains (28). The intensity of the third highest vibrational band as well as the total emission intensity of pyrene started to drastically increase over a certain concentration of DAMC, which was defined as a critical aggregation concentration (cac). The cac was determined from the intensity ratio (I_1/I_3) of the first (372 nm) and the third (383 nm) highest energy band in the emission spectra of pyrene. The cac values of DAMC self-aggregates were in the range of $1.2\text{--}6.7 \times 10^{-2}$ mg/mL, depending on the molecular weight of chitosan (Table 1). Since the unmodified chitosan did not form self-aggregates, it was used as a control.

Polarity of the inner core of self-organized structure is one important factor in its application as a drug delivery vehicle. Polarity of DAMC self-aggregates can be expressed with an equilibrium constant (K_v) of pyrene. In principle, the higher K_v value implies the lower polarity. So, the K_v value increasing with the molecular weight of chitosan indicates the existence of less polar microdomains inside the self-aggregates (Table 1).

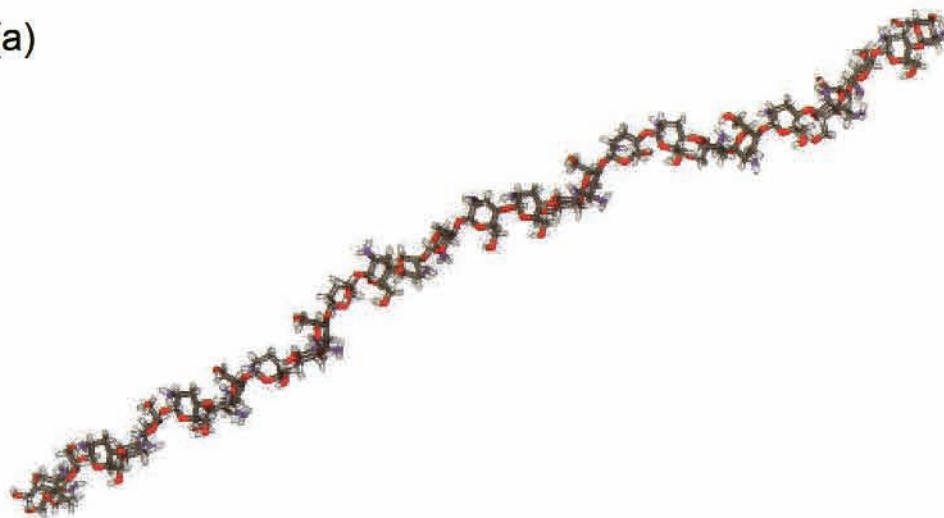
Microviscosity, defined as viscosity of the inner core of self-aggregates, can be determined by measuring the molecular anisotropy (r) resulting from the restricted rotational motion of DPH. The higher r value with increasing molecular weight of chitosan indicates the enhanced rigidity of hydrophobic domain of DAMC self-aggregates (Table 1). The mean diameter of DAMC self-aggregate was mainly dependent on the molecular weight of chitosan and was in the range of 130–300 nm with unimodal size distribution (Table 1).

Structure of DAMC Self-Aggregates. Figure 3 represents the anisotropy value (r) of DPH measured at different concentrations of DAMC self-aggregates with different molecular weights. The r values abruptly increased near cac , probably due to the dye-induced self-aggregate formation, and the values remained almost constant above cac independent of the self-aggregate concentration, except for DAMC200k. One interesting finding in this experiment is the increased r value of DAMC200k with increasing micelle concentrations. This might indicate a transition of self-aggregate shape from bamboo-like cylindrical structure to a poorly organized birdnest-like structure. This could possibly be the case

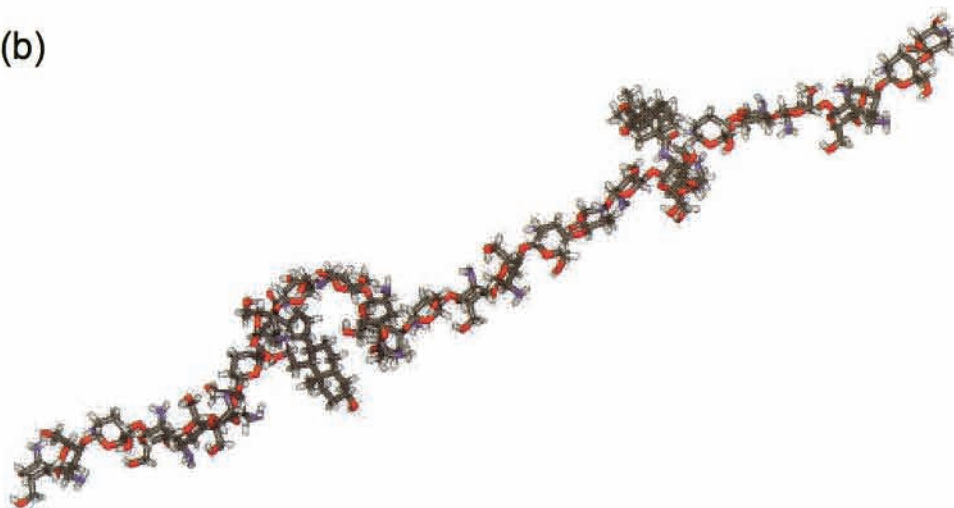
because the DAMC molecules have chain rigidity to a great extent, arising from the rigidity of parent chitosan and bulky deoxycholic acid groups. On the basis of the assumption that a single chain forms a conventional structure of a self-aggregate (i.e., spherical unimer), the diameter of an expected self-aggregate (d_{cal}) can be calculated by considering the length of monomer unit of chitosan (ca. 4 Å) and the molecular weight of chitosan. Therefore, the ratio between the measured and calculated diameter of self-aggregates (d_{real}/d_{cal}) could suggest the minimum aggregation number necessary to assemble a self-aggregate of spherical shape. Table 1 shows the existence of the calculated aggregation number (d_{real}/d_{cal}), which implies the formation of nonspherical self-aggregates above molecular weight of 40 kDa. Computer-simulated structure of chitosan and DAMC also supports the previous results. Indeed, Figure 4 shows that it is very difficult for the chitosan molecule itself to coil due to its chain rigidity (Figure 4a). When chitosan is modified with deoxycholic acid, DAMC chains tend to kink due to some intramolecular interaction at low concentration (Figure 4b). However, when DAMC concentrations increase, DAMC chains tend to be straightened due to the intermolecular interactions with concurrent self-association of deoxycholic acid units of neighboring DAMC chains (Figures 4c and 4d). By this way, the DAMC self-aggregates may form a cylindrical bamboo-like structure, particularly when the molecular weight of chitosan backbone in DAMC is higher than 40 kDa. Even when the molecular weight is lower than 40 kDa, the micellar structure of DAMC may not be in the form of perfect sphere that has been commonly reported for the self-aggregates of poly(ethylene oxide) (PEO) based diblock copolymer that has high chain flexibility. This might be attributed to the rigidity of chitosan chains, likely due to the 1,4- β -glycosidic linkages and/or possible existence of multicore structure (rather than one) as previously reported (10), resulting in the formation of pseudosphere that might be formed very similar to the way of construction of a birdnest with debris of a tree.

Complex Formation between DAMC Self-Aggregates and DNA. One interesting feature of chitosan is its cationic character due to the amino group in each repeating unit. Therefore, DAMC self-aggregates were expected to form complexes with DNA, a representative anionic macromolecule existing in nature. The electrophoretic mobility of DNA, complexed with DAMC self-aggregates, decreased gradually on the agarose gel with increasing the molecular weight of DAMC at the same charge ratio (Figure 5). The addition of low molecular weight of DAMC5k self-aggregates, however, could not result in complete retardation of DNA even at higher charge ratios (data not shown). This could be attributed to the lower stability of DAMC5k self-aggregates (Figure 3). A similar behavior of complex formation was also confirmed by a fluorometer using ethidium bromide (EtBr) as a probe (Figure 6). The fluorescence intensity of EtBr was normalized by the intensity in the presence of DNA only. This loss in the fluorescence intensity indicates the condensation of DNA and the compaction of the complex particle, which could result in the exclusion of EtBr from DNA. The decrease in the fluorescence intensity was dependent on the molecular weight of DAMC. DAMC5k self-aggregates did not show the dramatic reduction in the fluorescence intensity after complexation with DNA, irrespective of charge ratios. However, the complexes from higher molecular weight DAMC were stable, and no significant change in the fluorescence intensity was observed over the charge ratio of 4.

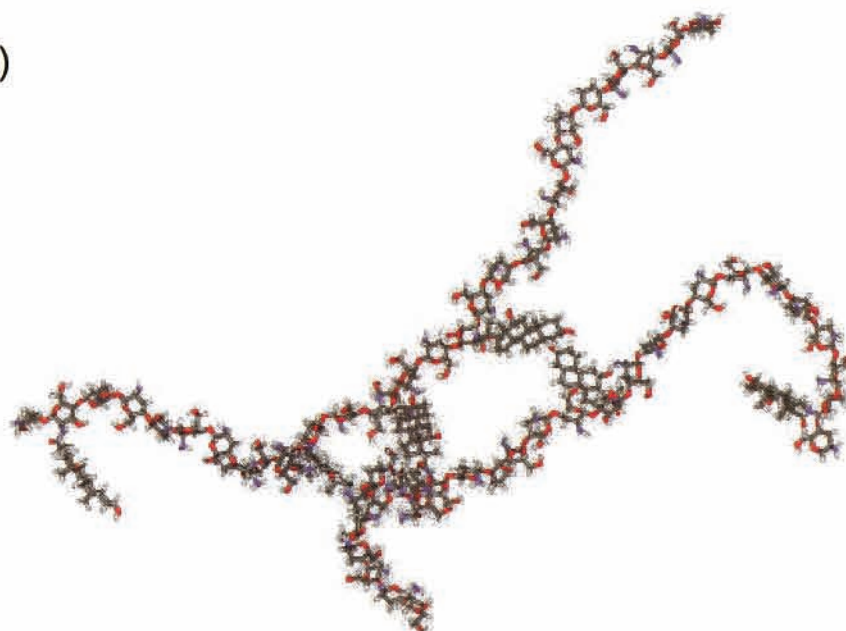
(a)



(b)



(c)



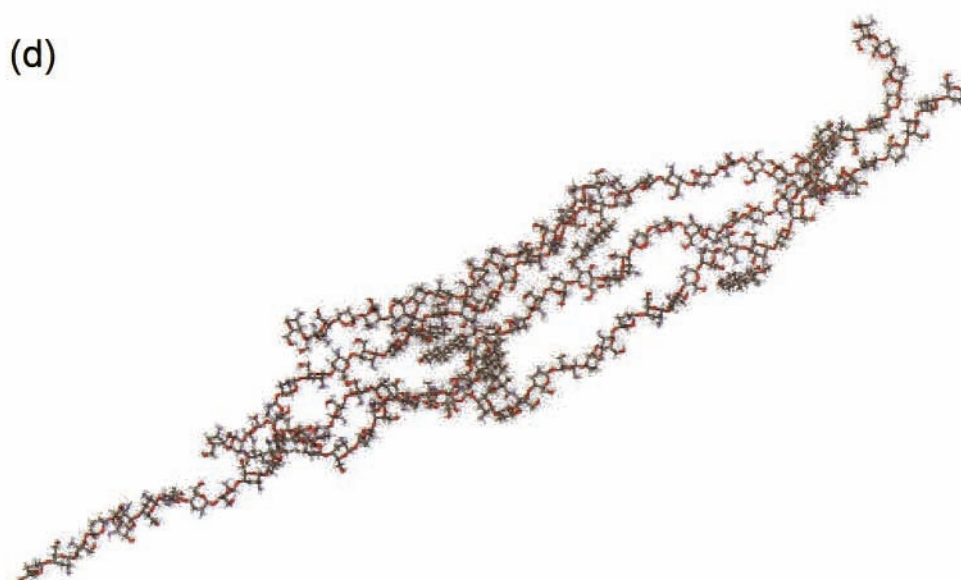


Figure 4. Simulated structure of (a) chitosan, (b) DAMC, (c) and (d) assembled self-aggregates formed by two and four DAMC molecules, respectively.

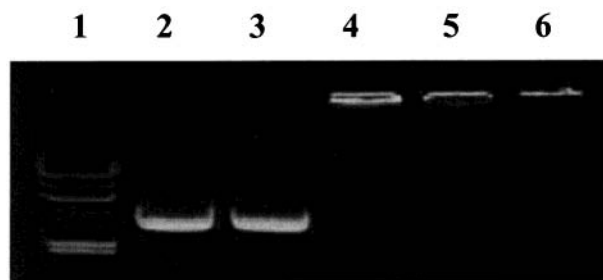


Figure 5. Electrophoresis of DAMC self-aggregate/DNA complexes on an agarose gel. Lane 1, DNA molecular weight marker; lane 2, DNA only; lane 3, DAMC5k/DNA complex; lane 4, DAMC25k/DNA complex; lane 5, DAMC40k/DNA complex; lane 6, DAMC200k/DNA complex at the charge ratio ($\pm$) of 4/1.

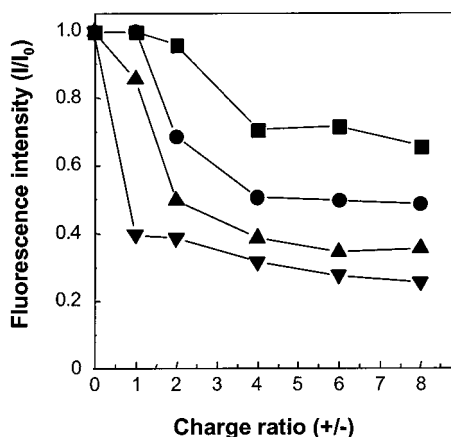


Figure 6. Changes in the fluorescence intensity of ethidium bromide in the presence of DAMC self-aggregate/DNA complexes as a function of charge ratios (■, DAMC5k; ●, DAMC25k; ▲, DAMC40k, ▼, DAMC200k).

In Vitro Transfection Efficiency. The transfection of COS-1 cells with DAMC self-aggregate/DNA complexes were studied next. The complexes at a charge ratio of 4 were chosen, as it was considered stable (Figures 5 and 6). In the absence of serum, it is evident that DAMC40k was the most efficient to deliver DNA to COS-1 cells, whereas DAMC5k self-aggregates showed the lowest

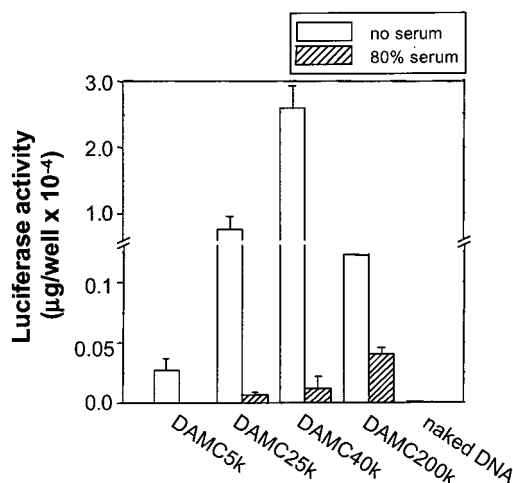


Figure 7. In vitro transfection activity of DAMC self-aggregate/pCMV-Luc+ complexes in COS-1 cells. Gene expression was determined by luciferase activity using a Promega kit.

transfection efficiency, likely due to the low stability of the complexes (Figure 7). Although DAMC5k self-aggregate slightly condenses the plasmid DNA from the measurement of the fluorescence intensity of EtBr (Figure 6), the DAMC5k self-aggregate/DNA complex does not seem to be strong enough at physiological ionic strength. DAMC200k self-aggregates could form highly condensed complexes with DNA. However, the complexes might have been too tight to release DNA. In the presence of serum, the transfection efficiency increased with increasing the molecular weight of DAMC due to the abundant proteins, which may act as an inhibitor of complex formation, resulting in weakening the ionic interaction between DAMC self-aggregates and DNA. Therefore, the optimum condition for cell transfection could be controlled using DAMC self-aggregates with different physicochemical properties including the size and structures.

CONCLUSIONS

Consideration of information generated in this analysis lead us to believe that structure of deoxycholic acid-

modified chitosan self-aggregate is dependent on the molecular weight of chitosan, and the condition of gene transfection is correlated to the structure of self-aggregates. This approach to control the size and structure of self-aggregates may find wide utility in the development of gene carriers, as well as many drug delivery applications.

ACKNOWLEDGMENT

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5'-Sulfhydryl-Modified RNA: Initiator Synthesis, in Vitro Transcription, and Enzymatic Incorporation

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The detailed syntheses of the sulfhydryl-modified guanosine monophosphates 5'-deoxy-5'-thioguanosine-5'-monophosphorothioate (GSMP), *O*-[ω -sulfhydryl-tetra(ethylene glycol)]-*O*-(5'-guanosine) monophosphate (5'-HS-PEG₄-GMP), and *O*-[ω -sulfhydryl-di(ethylene glycol)]-*O*-(5'-guanosine) monophosphate (5'-HS-PEG₂-GMP) are reported. Transcription reactions employing GSMP, 5'-HS-PEG₄-GMP, or 5'-HS-PEG₂-GMP as the initiator nucleotide for T7 RNA polymerase introduce a thiol group at the 5'-end of RNA. The efficiency of thiol incorporation at the 5'-terminus of modified RNA compounds was assayed with three different thiol-reactive biotinylated reagents followed by streptavidin gel-shift methods. The transcription efficiency with various ratios of GTP to 5'-HS-PEG₂-GMP was explored by reaction with a sulfhydryl-reactive maleimide-conjugated protein. This is an efficient method to incorporate enzymatically a thiol group into the 5'-end of RNA.

INTRODUCTION

RNA molecules play important roles in cellular processes including regulation, protein biosynthesis, RNA splicing, and retroviral replication. Site-specific substitution and derivatization provide powerful tools for studying RNA structure and function (Favre, 1990; Uhlmann and Peyman, 1990; Somtheimer and Steitz, 1993; Griffin et al., 1995; Cech and Herschlag, 1996; Dewey et al., 1996; Thomson et al., 1996; Allerson et al., 1997; Sontheimer et al., 1997; Strobel and Shetty, 1997). Although solid phase chemical synthesis can be used to introduce functional groups at any specific position of oligonucleotides shorter than approximately 40 nt (Gait et al., 1998), investigations of larger RNA molecules face a limited number of methodologies for site-specific modification and substitution. Several 5'-modifications of RNA molecules have been shown to have broad applications in studying RNA structures, in mapping RNA–protein interactions, and in the in vitro selection of catalytic RNAs.

In vitro transcription reactions are widely used to synthesize RNA from recombinant DNA templates. RNA generated in vitro has been used for many applications, for example, RNA processing, translation, RNA–protein interactions, and the generation of ribozymes. In vitro selection and in vitro evolution methods (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990) have provided powerful tools for isolating ribozymes that can catalyze a wide range of chemical and biochemical reactions. Molecules have been isolated from pools of random RNA sequences that catalyze polynucleotide kinase activity (Lorsch et al., 1994), alkylation (Wilson et al., 1995), carbon–sulfur bond formation (Wecker et al., 1996), and the Diels–Alder reaction

(Tarasow et al., 1997; Seelig et al., 1999). Aminoacyl transfer reactions can be catalyzed by ribozymes to form 3'-terminal (Illangasekare et al., 1995, 1999; Lee et al., 2000) or 2'-internal aminoacyl esters (Jenne et al., 1998), 5'-terminal esters or amide bonds (Lohse et al., 1996; Wiegand et al., 1997), and peptide bond formation (Zhang and Cech, 1997, 1998). RNA can also catalyze nucleotide synthesis (Unrau et al., 1998). These findings have greatly expanded the catalytic versatility of RNA and provided support for the hypothesis that the first biological catalyst may have been an RNA molecule. Most of these selection methodologies employed the use of 5'-end-modified RNA transcripts. The inclusion of a disulfide bond is a desirable feature of many of these 5'-end modifications. A key functional group required for the selection scheme may be tethered distal to the disulfide bond. Following the isolations of the desired RNA, reduction of the disulfide bond may be used to affect the liberation of the selected RNA in a soluble form suitable for reverse-transcription.

Phosphorothioate modification is one of the most popular methods for functionalizing the 5'-terminus of RNA by a transcription or kinase reaction (Burgin and Pace, 1990; Joseph and Noller, 1996; Zhang and Cech, 1997). Fluorophores are the most attractive probes for RNA structure (Qin and Pyle, 1999), but only a low efficiency of conjugating terminal phosphorothioates with fluorophores has been achieved (Czworkowski et al., 1991). The sulfhydryl group is another special reactive group that can be incorporated into nucleic acids (Fidanaza et al., 1994; Musier-Forsyth and Schimmel, 1994; Sun et al., 1996; Sigurdsson et al., 1996; Cohen and Cech, 1997) as an alternative to the use of phosphorothioates. The thiol-reactive functional groups are primarily alkylating reagents, including haloacetamides, maleimides, benzylic halides, and bromomethyl ketones (Haugland, 1996). The thiol group demonstrates a unique property, that is, the thiol–disulfide exchange reaction. A pyridyl disulfide group is the most popular type of thiol–disulfide exchange functional group used in the construction of cross-linkers or modification reagents. A pyridyl disulfide

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will readily undergo an interchange reaction with a free sulfhydryl to yield a single mixed-disulfide product. Once a disulfide linkage is formed, it may be cleaved subsequently using disulfide reducing agents (DTT etc.). Although 5'-phosphorothioate-RNA (5'-GMPS-RNA) can react with pyridyl disulfide to form a phosphorothioate sulfide compound (R-S-SPO₃-RNA) (Lorsch and Szostak, 1994; Macosko et al., 1999), a limitation of the thiophosphate disulfide product is the relative lability (Goody and Eckstein, 1971; Sengle et al., 2000). A free thiol group can be introduced into the 5'-termini of RNA chemically using carbodiimide and cysteamine, but the phosphoramidate linkage is not very stable (Chu et al., 1986; Chu and Orgel, 1988).

To overcome these limitations, we have developed a method to introduce a 5'-terminal sulfhydryl group into the 5'-termini of RNA molecules by in vitro transcription. We (Zhang et al., 2001) reported that a thiol group can be indirectly introduced into 5'-termini of RNA with an initiator, 5'-deoxy-5'-thioguanosine-5'-monophosphorothioate (GSMP), by T7 RNA polymerase. The method requires an additional step of dephosphorylation of 5'-GSMP-RNA to produce 5'-HS-G-RNA. Herein, we report two new 5'-modified guanoses as initiator for T7 RNA polymerase to directly incorporate a free thiol to 5'-termini of RNA by in vitro transcription. The new initiators are *O*-[ω -sulfhydryl-bis(ethylene glycol)]-*O*-(5'-guanosine) monophosphate (5'-HS-PEG₂-GMP) and *O*-[ω -sulfhydryl-tetra(ethylene glycol)]-*O*-(5'-guanosine) monophosphate (5'-HS-PEG₄-GMP). These initiators are not only to introduce directly a free thiol into 5'-end of RNA, but also to provide a flexible PEG linker between the HS group and RNA, which is important for some bioconjugation applications. We describe the detailed synthesis of 5'-deoxy-5'-thioguanosine-5'-monophosphorothioate, *O*-[ω -sulfhydryl-di(ethylene glycol)]-*O*-(5'-guanosine) monophosphate, and *O*-[ω -sulfhydryl-tetra(ethylene glycol)]-*O*-(5'-guanosine) monophosphate. Three thiol-reactive biotin agents have been tested to couple with the 5'-thiol of RNA molecules. The bioconjugation of maleimide-activated horseradish peroxidase with the 5'-sulfhydryl of RNA is also studied.

EXPERIMENTAL PROCEDURES

General Procedure. (2-Cyanoethyl-*N,N*-diisopropyl)-chlorophosphoramidite was purchased from Peninsula Laboratories. Biotin-PEG₃-iodoacetamide, biotin-HPDP, and biotin-PEG₃-maleimide were purchased from Pierce and Molecular Biosciences. Maleimide-activated horseradish peroxidase was purchased from Pierce, [α -³²P]ATP from NEN Labs, and NTPs and Taq DNA polymerase from New England Biolabs. All other materials were obtained from Aldrich, Sigma, and Acros, and used without additional purification unless otherwise noted. All solvents were distilled before use. Dichloromethane, acetonitrile, and pyridine were dried by refluxing with calcium hydride. ¹H, ¹³C, and ³¹P NMR spectra were obtained on Varian 300 and 400 spectrometers. Corresponding operating frequencies were as follows: 299.95/400.14 MHz (¹H), 75.43/100.61 MHz (¹³C), 121.42/161.98 MHz (³¹P). Internal references used are TMS for ¹H and ¹³C, and 85% H₃PO₄ for ³¹P. Mass spectra were obtained on a Finnigan LCQ^{DUO} spectrometer and high-resolution MS (FAB) spectra on a JMS-700 MStation mass spectrometer.

Di(ethylene glycol) Monotosylate 2b. To a solution of di(ethylene glycol) (**1b**, 95 mL, 1.0 mol) and anhydrous pyridine (40.5 mL, 0.5 mol) in 250 mL of anhydrous

dichloromethane was added dropwise a solution of *p*-toluenesulfonyl chloride (38.1 g, 0.2 mol) in 150 mL of dichloromethane. The mixture was stirred at room temperature overnight. The reaction solution was washed with cold water (2 × 100 mL) and brine (2 × 100 mL). The aqueous solution was extracted with dichloromethane (2 × 100 mL), and the combined organic layers were dried over magnesium sulfate. The solvent was evaporated under reduced pressure to give a slightly yellow oil. The crude product was purified by flash silica gel column chromatography using a gradient of dichloromethane/methanol (0–5%) to yield a colorless oil: 42.3 g; yield = 81.2%; TLC (silica gel, chloroform/methanol = 95:5); *R*_f = 0.42. ¹H NMR (CDCl₃): δ 1.99 (s, 1H), 2.46 (s, 3H), 3.54 (t, *J* = 4.5 Hz, 2H), 3.68 (m, 4H), 4.20 (t, *J* = 4.6 Hz, 2H), 7.35 (d, *J* = 8.4 Hz, 2H), 7.81 (d, *J* = 8.4 Hz, 2H). ¹³C NMR (CDCl₃): δ 21.9, 61.8, 68.8, 69.5, 72.5, 128.2, 130.1, 133.1, 145.2. ESI mass (*m/z*): calcd for C₁₁H₁₆O₅S 260.1, found 283.3 [M+Na]⁺.

Tetra(ethylene glycol) Monotosylate 2c. To a solution of tetra(ethylene glycol) (**1c**, 100 mL, 0.58 mol) and anhydrous pyridine (40 mL, 0.50 mol) in 200 mL of anhydrous dichloromethane was added dropwise a solution of *p*-toluenesulfonyl chloride (19.1 g, 0.10 mol) in 100 mL of dichloromethane. The mixture was stirred at room temperature for 20 h. The reaction solution was washed with cold water (2 × 100 mL) and brine (2 × 100 mL). The aqueous solution was extracted with dichloromethane (2 × 100 mL), and the combined organic layers were dried over magnesium sulfate. Evaporation of solvent under reduced pressure gave a slightly yellow oil. The crude product was purified by flash silica gel column chromatography using a gradient of dichloromethane/methanol (0–5%) to give a colorless oil: 31.3 g; yield = 90%; TLC (silica gel, dichloromethane/methanol = 95:5); *R*_f = 0.43. ¹H NMR (CDCl₃): δ 2.43 (s, 3H), 2.48 (s, 1H), 3.56–3.70 (m, 14H), 4.14 (t, *J* = 4.8 Hz, 2H), 7.32 (d, *J* = 8.4 Hz, 2H), 7.78 (d, *J* = 8.4 Hz, 2H). ¹³C NMR (CDCl₃): δ 21.5, 61.6, 68.6, 69.2, 70.6–70.2 (m), 72.3, 127.9, 129.7, 132.8, 144.7. ESI mass (*m/z*): calcd for C₁₅H₂₄O₇S 348.1, found 371.5 [M+Na]⁺.

2-(Thioacetyl)ethanol 3a. To a suspension of potassium thioacetate (11.4 g, 0.1 mol) in 500 mL of acetone was added dropwise 3.55 mL of bromoethanol (**1a**, 0.05 mol). The mixture was stirred at room temperature for 1 h, producing a white precipitate. The solid was filtered, and the solvent was evaporated under reduced pressure. The residue was stirred in 100 mL of dichloromethane, and refiltered and diluted to 500 mL with dichloromethane. The organic solution was washed with water (2 × 50 mL) and brine (2 × 50 mL). The aqueous wash solutions were reextracted with dichloromethane (2 × 50 mL), and the combined organic layers were dried over magnesium sulfate and evaporated under reduced pressure to give an orange oil in almost quantitative yield and high purity. The crude product was purified through a silica gel column eluted with hexane/ethyl acetate (6:1) to afford 8.4 g of the desired product (70% yield); TLC (silica gel, hexane/ethyl acetate = 1:2); *R*_f = 0.56. ¹H NMR (CDCl₃): δ 2.34 (s, 3H), 2.43 (br, 1H), 3.05 (t, *J* = 6.1 Hz, 2H), 3.72 (t, *J* = 6.1 Hz, 2H). ¹³C NMR (CDCl₃): δ 30.8, 32.2, 61.8, 196.7.

Di(ethylene glycol) Monothioacetate 3b. To a suspension of potassium thioacetate (17.2 g, 0.15 mol) in 650 mL of acetone was added a solution of **2b** (15.6 g, 60.3 mmol) in 100 mL of acetone at room temperature. The mixture was stirred at room temperature for 1 h and then refluxed for 4 h. After cooling to room temperature, the solid was filtered off and the solution was evaporated

under reduced pressure. The residue was dissolved in ethyl acetate (150 mL) and washed with water (2 × 40 mL) and brine (2 × 50 mL). The aqueous wash solutions were reextracted with ethyl acetate (2 × 50 mL), and the combined organic layers were dried over magnesium sulfate and evaporated under reduced pressure to give a yellow oil. The crude product was purified through a flash silica gel column eluted with hexane/ethyl acetate (6:1) to give 8.75 g of desired product, yield = 88.8%. TLC (silica gel, hexane/ethyl acetate = 1:2); R_f = 0.38. ^1H NMR (CDCl_3 , 400 MHz): δ 2.30 (s, 3H), 2.51 (br, 1H), 3.06 (t, J = 6.1 Hz, 2H), 3.59–3.52 (m, 4H), 3.68 (t, J = 4.6 Hz, 2H). ^{13}C NMR (CDCl_3 , 400 MHz): δ 29.0, 30.7, 61.8, 69.7, 72.2, 195.8. ESI MS calcd for $\text{C}_6\text{H}_{12}\text{O}_3\text{S}$ 164.1, found 187.0 $[\text{M}+\text{Na}]^+$.

Tetra(ethylene glycol) Monothioacetate 3c. To a suspension of potassium thioacetate (10.1 g, 88 mmol) in 650 mL of acetone was added a solution of **2c** (15.4 g, 44 mmol) in 100 mL of acetone. The mixture was stirred at room temperature for 1 h and then refluxed for 4 h. After filtration, the solvent was evaporated under reduced pressure. The residue was dissolved in ethyl acetate (150 mL) and washed with water (2 × 50 mL) and brine (2 × 50 mL). The aqueous wash solutions were reextracted with ethyl acetate (2 × 50 mL), and the combined organic layers were dried over magnesium sulfate and evaporated under reduced pressure to give a yellow oil. The crude product was purified through a flash silica gel column eluted with hexane/ethyl acetate (6:1) to afford 10.5 g of the desired product, yield = 95%. TLC (silica gel, hexane/ethyl acetate = 1:4); R_f = 0.24. ^1H NMR (CDCl_3): δ 2.26 (s, 3H), 2.95 (br, 1H), 3.01 (t, J = 6.1 Hz, 2H), 3.56–3.64 (m, 14H). ^{13}C NMR (CDCl_3): δ 28.8, 30.6, 61.7, 69.8, 70.3, 70.4, 70.5, 70.7, 72.6, 195.6. ESI mass (m/z): calcd for $\text{C}_{10}\text{H}_{20}\text{O}_5\text{S}$ 252.1, found 275.3 $[\text{M}+\text{Na}]^+$.

2',3'-O,O-Isopropylidene Guanosine 5. To a suspension of guanosine (8.7 g, 30.7 mmol) in 600 mL of acetone was added 3 mL of 70% perchloric acid. A clear colorless solution was formed after ca. 0.5 h. The mixture was stirred at room temperature for 1 h, and 3 mL of concentrated $\text{NH}_3\cdot\text{H}_2\text{O}$ was added, leading to a white precipitate. The solvent was evaporated under reduced pressure to afford a white solid that was stirred with 40 mL of H_2O for several hours, filtered, and washed with cold water. Drying over phosphorus pentoxide in vacuo gave a white solid (8.3 g, 83.6%). ^1H NMR ($\text{DMSO}-d_6$): δ 1.30 (s, 3H), 1.50 (s, 3H), 3.52 (m, 2H), 4.10 (dt, J = 3.0, 5.4 Hz, 1H), 4.95 (dd, J = 3.0, 6.3 Hz, 1H), 5.05 (t, J = 5.4 Hz, 1H), 5.17 (dd, J = 2.7, 6.3 Hz, 1H), 5.90 (d, J = 2.7 Hz, 1H), 6.50 (br, 2H), 7.90 (s, 1H), 10.66 (s, 1H).

2-N,N-Dimethylaminomethylene-2',3'-O,O-isopropylidene Guanosine 6. A solution of **5** (9.78 g, 30.2 mmol) and dimethylformamide dimethyl acetal (15 mL, 0.11 mol) in anhydrous DMF (100 mL) was stirred under argon at 55 °C for 1 day. The clear light yellow solution was evaporated under reduced pressure. The residue was stirred in 40 mL of methanol, leading to precipitation of a white solid. More product was precipitated by addition of ethyl acetate (100 mL). The mixture was cooled to –20 °C and filtered. The solid was dried over phosphorus pentoxide in vacuo to give 6.6 g of **6**. The mother liquor was concentrated to about 20 mL; white solid was formed again which was filtered and washed with ethyl acetate and dried over phosphorus pentoxide in vacuo to give 2.2 g more product (total 8.8 g, yield = 90%). TLC (silica gel, ethyl acetate/methanol = 3:1); R_f = 0.42. ^1H NMR ($\text{DMSO}-d_6$): δ 1.32 (s, 3H), 1.53 (s, 3H), 3.03 (s, 3H), 3.15 (s, 3H), 3.52 (m, 2H), 4.13 (m, 1H), 4.95 (dd, J = 2.8, 6.4

Hz, 1H), 5.07 (t, J = 5.4 Hz, 1H), 5.26 (dd, J = 3.0, 5.8 Hz, 1H), 6.03 (d, J = 2.8 Hz), 8.02 (s, 1H), 8.57 (s, 1H), 11.37 (s, 1H). ^{13}C NMR ($\text{DMSO}-d_6$): δ 25.2, 27.1, 34.6, 40.8, 61.4, 81.1, 83.5, 86.3, 88.5, 113.1, 119.8, 137.2, 149.5, 157.4, 157.6, 158.2. ESI mass (m/z): calcd for $\text{C}_{16}\text{H}_{22}\text{N}_6\text{O}_5$ 378.2, found 379.2 $[\text{M}+\text{H}]^+$.

2-Cyanoethyl-N,N-diisopropylamino-5'-(2-N-dimethylaminomethylene-2',3'-O,O-isopropylidene guanosine) Phosphoramidite 7. To a suspension of **6** (5.0 g, 13.2 mmol) in anhydrous dichloromethane (50 mL) was added 10 mL of *N,N*-diisopropylethylamine in argon atmosphere. The mixture was cooled to 0 °C, and 2-cyanoethyl-*N,N*-diisopropylaminophosphorus chloride (5 mL, 21.4 mmol) was added dropwise. The reaction was completed after 30 min and diluted with ethyl acetate (500 mL). The solution was washed with cold water (2 × 100 mL) and brine (2 × 100 mL). The combined aqueous layers were reextracted with ethyl acetate (2 × 150 mL), and the combined organic layers were dried over magnesium sulfate and filtered. Evaporation of solvent gave a slightly yellow residue that was applied to flash column chromatography (silica gel) and eluted with ethyl acetate/triethylamine (95:5) to yield a white foam solid (6.37 g, 83.5% yield). TLC (silica gel, ethyl acetate/methanol/triethylamine = 85:10:5); R_f = 0.73. ^1H NMR (CDCl_3): δ 1.14 (m, 12H), 1.38 (s, 3H), 1.61 (s, 3H), 2.68 (m, 2H), 3.10 (s, 2H), 3.18 (s, 3H), 3.55 (m, 2H), 3.78 (m, 4H), 4.41 (m, 1H), 4.97 (m, 1H), 4.97 (m, 1H), 5.12 (m, 1H), 6.11 (dd, J = 2.7, 4.7 Hz, 1H), 7.85, 7.88 (2s, 1H), 8.61 (s, 1H), 9.63 (br, 1H). ^{13}C NMR (CDCl_3): δ 20.6 (dd, J = 2.7, 7.3 Hz), 24.8 (m), 27.5, 35.4, 41.7, 43.3 (dd, J = 6.8, 12.3), 58.8 (dd, J = 18.4, 22.6), 63.4 (dd, J = 16.1, 21.4), 81.7 (d, J = 2.3), 85.2 (d, J = 7.7), 85.9 (t, J = 9.2), 89.8 (d, J = 8.4), 114.4 (d, J = 1.5), 118.0 (d, J = 12.3), 120.8, 136.8, 150.1 (d, J = 1.5), 157.1, 158.3, 158.4. ^{31}P NMR (CDCl_3): δ 150.0, 151.1. ESI mass (m/z): calcd for $\text{C}_{25}\text{H}_{39}\text{N}_8\text{O}_6\text{P}$ 578.3, found 579.1 $[\text{M}+\text{H}]^+$.

2-Cyanoethyl 5'-(2-N-Dimethylformamidino-2',3'-O,O-isopropylidene-guanosine) (ω -Thioacetyethyl) Phosphate 8a. To a solution of 1*H*-tetrazole (1.4 g, 20 mmol) in 40 mL of anhydrous acetonitrile was added a solution of 2-thioacetyethanol **3a** (0.6 g, 5 mmol) in 10 mL of anhydrous acetonitrile under argon atmosphere. A solution of **7** (1.92 g, 3.32 mmol) in 7 mL of acetonitrile was then added dropwise and stirred at room temperature. More 2-thioacetyethanol **3a** (0.6 g, 5 mmol) was added after 0.5 h, leading to complete disappearance of **7** in ca. 0.5 h. An 8.0 mL aliquot of *tert*-butyl hydroperoxide was added, and the mixture was stirred at room temperature for 0.5 h. After evaporation of solvent under reduced pressure, the residue was dissolved in 250 mL of ethyl acetate and washed with cold water (2 × 30 mL) and brine (2 × 50 mL). The combined aqueous layers were back-extracted with ethyl acetate (2 × 50 mL), and the combined organic layers were dried over magnesium sulfate. After removal of solvent under reduced pressure, the residue was applied to a silica gel flash column and eluted with ethyl acetate/methanol (0–20%). Evaporation of solvent gave the desired compounds as a white foam solid (1.51 g, 74.0%). TLC (silica gel, ethyl acetate/methanol = 4:1); R_f = 0.55. ^1H NMR (CDCl_3): δ 1.40 (s, 3H), 1.63 (s, 3H), 2.32, 2.33 (2s, 3H), 2.76 (m, 2H), 3.05 (m, 2H), 3.10 (s, 3H), 3.22 (s, 3H), 4.05–4.35 (m, 6H), 4.42 (m, 1H), 5.05 (dd, J = 3.6, 6.6 Hz, 1H), 5.29 (m, 1H), 6.10 (s, 1H), 7.82, 7.84 (2s, 1H), 8.60 (s, 1H), 9.05 (s, 1H). ^{31}P NMR (CDCl_3): δ –2.3, –2.2. ESI mass (m/z): calcd for $\text{C}_{23}\text{H}_{32}\text{N}_8\text{O}_9\text{P}$ 613.2, found 614.3 $[\text{M}+\text{H}]^+$.

2-Cyanoethyl 5'-(2-N-Dimethylaminomethylene-2'-O,3'-O-isopropylidene-guanosine) [ω -Thioacetyl

Di(ethylene glycol) Phosphate 8b. To a solution of 1*H*-tetrazole (1.4 g, 20 mmol) in 40 mL of anhydrous acetonitrile was added a solution of **3b** (0.82 g, 5 mmol) in 10 mL of anhydrous acetonitrile under argon atmosphere. A solution of phosphoramidite **7** (2.0 g, 3.46 mmol) in 20 mL of acetonitrile was then added dropwise. After the mixture was stirred for 0.5 h at room temperature, more **3b** (0.5 g, 3 mmol) was added, and the mixture was stirred for an additional 0.5 h. An 8.0 mL aliquot of *tert*-butyl hydroperoxide was added, and the mixture was stirred at room temperature for 0.5 h. After evaporation of solvent under reduced pressure, the residue was dissolved in 250 mL of ethyl acetate, and washed with cold water (2 × 30 mL) and brine (2 × 50 mL). The combined aqueous layers were back-extracted with ethyl acetate (2 × 50 mL), and the combined organic layers were dried over magnesium sulfate. After removal of solvent, the residue was applied to a silica gel flash column and eluted with ethyl acetate/methanol (5–15%). The pure product was obtained as a white foam solid (1.86 g, 81.9%). TLC (silica gel, ethyl acetate/methanol = 4:1); R_f = 0.25. ^1H NMR (CDCl_3): δ 1.38 (s, 3H), 1.60 (s, 3H), 2.30, 2.31 (2s, 3H), 2.74 (m, 2H), 3.02 (m, 2H), 3.10 (s, 3H), 3.20 (s, 3H), 3.52–3.64 (m, 4H), 4.11–4.33 (m, 6H), 4.40 (m, 1H), 5.04 (dd, J = 3.5, 6.4 Hz, 1H), 5.27 (dt, J = 2.6, 6.6 Hz, 1H), 6.06 (dd, J = 2.6, 4.0 Hz, 1H), 7.71, 7.72 (2s, 1H), 8.58 (s, 1H), 9.71 (br, 1H). ^{13}C NMR (CDCl_3): δ 19.8, 25.6, 27.4, 28.8, 30.8, 35.5, 41.8, 62.4, 67.1, 67.6, 69.6, 69.9, 81.0, 84.5, 89.8, 114.9, 116.8, 121.0, 136.9, 150.0, 157.2, 158.1, 158.4, 195.6. ^{31}P NMR (CDCl_3): δ -0.7, -0.6. ESI mass (m/z): calcd for $\text{C}_{25}\text{H}_{36}\text{N}_7\text{O}_{10}\text{PS}$ 657.2, found 658.1 $[\text{M}+\text{H}]^+$.

2-Cyanoethyl 5'-(2-*N*-Dimethylaminomethylene-2'-*O*,3'-*O*-isopropylidene-guanosine) [ω -Thioacetyl Di(ethylene glycol)] Phosphate 8c. To a solution of 1*H*-tetrazole (2.9 g, 41.4 mmol) in 80 mL of anhydrous acetonitrile was added a solution of **3c** (2.27 g, 9 mmol) in 20 mL of anhydrous acetonitrile under argon atmosphere. A solution of **7** (4.0 g, 7.0 mmol) in 20 mL of acetonitrile was then added dropwise, and the mixture was stirred at room temperature. More **3c** (0.76 g, 3 mmol) was added after 0.5 h, and the reaction was stirred for an additional 0.5 h. A 10 mL aliquot of *tert*-butyl hydroperoxide was added, and the mixture was stirred at room temperature for 0.5 h. After removing solvent, the residue was dissolved in 400 mL of ethyl acetate and washed with cold water (2 × 50 mL) and brine (2 × 50 mL). The combined aqueous layers were reextracted with ethyl acetate (2 × 50 mL), and the combined organic layers were dried over magnesium sulfate. After evaporation of solvent under reduced pressure, the residue was applied to a silica gel flash column and eluted with ethyl acetate/methanol (5–20%) to yield the desired product as a white foam solid (4.96 g, 95.0%). TLC (silica gel, ethyl acetate/methanol = 4:1); R_f = 0.22. ^1H NMR (CDCl_3): δ 1.37 (s, 3H), 1.59 (s, 3H), 2.30 (s, 3H), 2.72 (m, 2H), 3.05 (m, 2H), 3.09 (s, 3H), 3.19 (s, 3H), 3.52–3.66 (m, 12H), 4.10–4.30 (m, 6H), 4.38 (m, 1H), 5.03 (m, 1H), 5.26 (m, 1H), 6.04 (t, J = 3.0 Hz, 1H), 7.71, 7.72 (2s, 1H), 8.57 (s, 1H), 9.57 (br, 1H). ^{31}P NMR (CDCl_3): δ -1.7. ESI mass (m/z): calcd for $\text{C}_{29}\text{H}_{44}\text{N}_7\text{O}_{12}\text{PS}$ 745.3, found 746.1 $[\text{M}+\text{H}]^+$.

The compound **8c** was also prepared from the reaction of **6** and **11**. To a suspension of **6** (189.2 mg, 0.5 mmol) in anhydrous dichloromethane (15 mL) was added a solution of 1*H*-tetrazole (210.1 mg, 3.0 mmol) in anhydrous acetonitrile (6 mL) and a solution of **11** (226 mg, 0.5 mmol) in anhydrous acetonitrile (5 mL) under argon atmosphere. After stirring the mixture at room temperature for 0.5 h, more 1*H*-tetrazole (210 mg, 3.0 mmol)

and **11** (290 mg, 0.64 mmol) were added. After the disappearance of compound **6**, *tert*-butyl hydroperoxide (2 mL) was added, and the mixture was stirred at room temperature for an additional 0.5 h. After evaporation of solvents under reduced pressure, the oil residue was dissolved in dichloromethane (150 mL) and washed with water (2 × 20 mL) and brine (2 × 20 mL). The combined aqueous solutions were back-extracted with dichloromethane (2 × 20 mL), and the combined organic layers were dried over magnesium sulfate. After removal of solvent, the residue was applied to a flash column (silica gel) eluted with ethyl acetate/methanol (0–20%) to give the desired product as a white foam solid (344 mg, 92.8%).

2-Cyanoethyl 5'-Guanosine (3-Thioacetyl) Phosphate 9a. The fully protected compound **8a** (1.53 g, 2.5 mmol) was dissolved in 40% formic acid (50 mL), and the solution was stirred at room temperature for 3 days to affect a complete deprotection of the 2',3'-acetonide group. After removal of solvent under reduced pressure, the residue was coevaporated with methanol twice to afford a crude product that was used for the next step of the reaction without further purification. An analytical amount of product was obtained by silica gel flash column eluted with ethyl acetate/methanol (3:1). ^1H NMR (D_2O): δ 2.07, 2.09 (2s, 3H), 2.74 (m, 2H), 2.88 (m, 2H), 3.85–4.45 (m, 8H), 4.70 (m, 1H, partially overlapped by H_2O signal), 5.74 (d, J = 5.4 Hz), 7.75 (s, 1H). ^{31}P NMR (D_2O): δ -2.2, -2.1. ESI mass (m/z): calcd for $\text{C}_{17}\text{H}_{23}\text{N}_6\text{O}_9\text{PS}$ 518.1, found 519.5 $[\text{M}+\text{H}]^+$.

2-Cyanoethyl 5'-Guanosine [ω -Thioacetyl Di(ethylene glycol)] Phosphate 9b. The fully protected compound **9b** (1.53 g, 2.5 mmol) was dissolved in 60% formic acid (50 mL), and the solution was stirred at room temperature for 3 days to deprotect the 2',3'-acetonide group. After evaporation of solvent under reduced pressure, the residue was coevaporated with methanol twice to afford a crude product that was used for the next step of the reaction without further purification. ^1H NMR (D_2O): δ 2.12, 2.14 (2s, 3H), 2.72 (m, 2H), 2.80 (dd, J = 6.4 Hz, 12 Hz, 2H), 3.40 (dd, J = 5.6, 12 Hz, 2H), 3.46 (m, 2H), 3.85–4.40 (m, 8H), 4.7 (m, 1H), 5.74 (d, J = 5.4 Hz, 1H), 7.78 (s, 1H). ^{31}P NMR (D_2O): δ -1.9, -1.8. ESI mass (m/z): calcd for $\text{C}_{19}\text{H}_{27}\text{N}_6\text{O}_{10}\text{PS}$ 562.1, found 563.2 $[\text{M}+\text{H}]^+$.

2-Cyanoethyl 5'-Guanosine [ω -Thioacetyl Tetra(ethylene glycol)] Phosphate 9c. The fully protected compound **9c** (1.64 g, 2.5 mmol) was dissolved in 60% formic acid (50 mL), and the solution was stirred at room temperature for 3 days to affect complete deprotection of the 2',3'-acetonide group. After evaporation of solvent under reduced pressure, the residue was coevaporated with methanol twice to yield a crude product that was used for the next step of the reaction without further purification. An analytical amount of product was obtained by silica gel flash column eluted with ethyl acetate/methanol (3:1). ^1H NMR ($\text{DMSO}-d_6$): δ 2.30 (s, 3H), 2.90 (m, 2H), 3.02 (m, 4H), 3.40–4.20 (m, 18 H, overlapped with water signal), 4.44 (m, 1H), 5.72 (d, J = 6.4 Hz, 1H), 6.75 (br, 2H), 7.84 (s, 1H), 8.46 (s, 1H). ^{31}P NMR (D_2O): δ -1.6. ESI mass (m/z): calcd for $\text{C}_{23}\text{H}_{35}\text{N}_6\text{O}_{12}\text{PS}$ 650.2, found 651.1 $[\text{M}+\text{H}]^+$.

O- ω -Mercapto-di(ethylene glycol)-O-(5'-guanosine) Monophosphate 10b. The crude product **9b** (1.4 g, 2.5 mmol) was dissolved in methanol (40 mL) under argon atmosphere, and an excess of 2-mercaptoethanol (2 mL, 28.5 mmol) was added. To the above solution was added ammonia in methanol (7.0 N solution, 20 mL). The mixture was stirred at 55 °C for 1 day. After

removal of solvent, the solid residue was washed with ethyl acetate to remove excess mercaptoethanol. The crude product was applied to a reverse phase column and eluted with water and water/methanol (10–50%). The collected fractions were evaporated under reduced pressure, and the aqueous solution was lyophilized to yield a pure product (1.06 g, 88%). TLC (silica gel, 2-propanol/ammonia/water = 7:1:2); R_f = 0.45. ^1H NMR (D_2O): δ 2.40 (t, J = 6.3 Hz, 2H), 2.51 (s, 1H), 3.33 (t, J = 6.3 Hz, 2H), 3.37 (m, 2H), 3.69 (m, 2H), 3.90 (m, 2H), 4.12 (m, 1H), 4.30 (t, J = 4.7 Hz, 2H), 4.59 (t, J = 5.3 Hz, 1H), 5.71 (d, J = 5.5 Hz, 1H), 7.95 (s, 1H). ^{31}P NMR (D_2O): δ 1.3. HRMS calcd for $\text{C}_{14}\text{H}_{23}\text{O}_9\text{N}_5\text{PS}$ 468.0954, found 468.0927.

O-[ω -Mercapto-tetra(ethylene glycol)]-O-(5'-guanosine) Monophosphate 10c. The compound **9c** (1.5 g, 2.3 mmol) was dissolved in methanol (40 mL) in argon atmosphere, and an excess of 2-mercaptoethanol (2 mL, 28.5 mmol) was added. To the above solution was added ammonia in methanol (7.0 N solution, 20 mL). The mixture was stirred at 55 °C for 1 day. After evaporation of solvent under reduced pressure, the residue was coevaporated with methanol (3×25 mL), and washed with ethyl acetate to remove excess mercaptoethanol. The solid product was dried in vacuo and then was applied to a reverse phase column and eluted with water and water/methanol (10–50%). The desired fractions were collected and evaporated under reduced pressure to remove organic solvent, and the aqueous solution was dried by lyophilization to yield a cotton-like solid (1.22 g, 93%). TLC (silica gel, 2-propanol/ammonia/water = 7:1:2); R_f = 0.45. ^1H NMR (D_2O): δ 2.48 (t, J = 6.3 Hz, 2H), 2.52 (s, 1H), 3.41–3.45 (m, 12H), 3.74 (m, 2H), 3.93 (m, 2H), 4.14 (m, 1H), 4.30 (t, J = 4.5 Hz, 2H), 4.58 (t, J = 5.1 Hz, 1H), 5.75 (d, J = 5.5 Hz, 1H), 8.11 (s, 1H). ^{31}P NMR (D_2O): δ 1.3. HRMS calcd for $\text{C}_{18}\text{H}_{31}\text{N}_5\text{O}_{11}\text{PS}$ 556.1478, found 556.1479.

2-Cyanoethyl *N,N*-Diisopropylamino [ω -Thioacetyl Tetra(ethylene glycol)] Phosphoramidite 11. To a solution of **3c** (1.0 g, 3.96 mmol) in anhydrous dichloromethane (7 mL) was added *N,N*-diisopropylethylamine (2.0 mL, 11.5 mmol). The solution was cooled to 0 °C, and then 2-cyanoethyl diisopropylamino phosphorus chloride was added dropwise. After stirring at 0 °C for 0.5 h, ethyl acetate (100 mL) was added, and the solution was washed with water (2×20 mL) and brine (2×20 mL). The combined aqueous layers were back-extracted with ethyl acetate (2×20 mL), and the combined organic layers were dried over magnesium sulfate. After removal of solvent, the residue was applied to a flash silica gel column eluted with heptane/ethyl acetate/triethylamine (80:15:5) to give a colorless oil (1.2 g, 67.0%). TLC (silica gel, ethyl acetate/heptane/triethylamine = 10:9:1); R_f = 0.57. ^1H NMR (CDCl_3): δ 1.20 (d, J = 6.6 Hz, 6H), 1.21 (d, J = 6.6 Hz, 6H), 2.36 (s, 3H), 2.68 (t, J = 6.5 Hz, 2H), 3.12 (t, J = 6.5 Hz, 2H), 3.60–3.95 (m, 18H). ESI mass (m/z): calcd for $\text{C}_{19}\text{H}_{37}\text{N}_2\text{O}_6\text{PS}$ 452.2, found 475.3 [$\text{M}+\text{Na}$] $^+$.

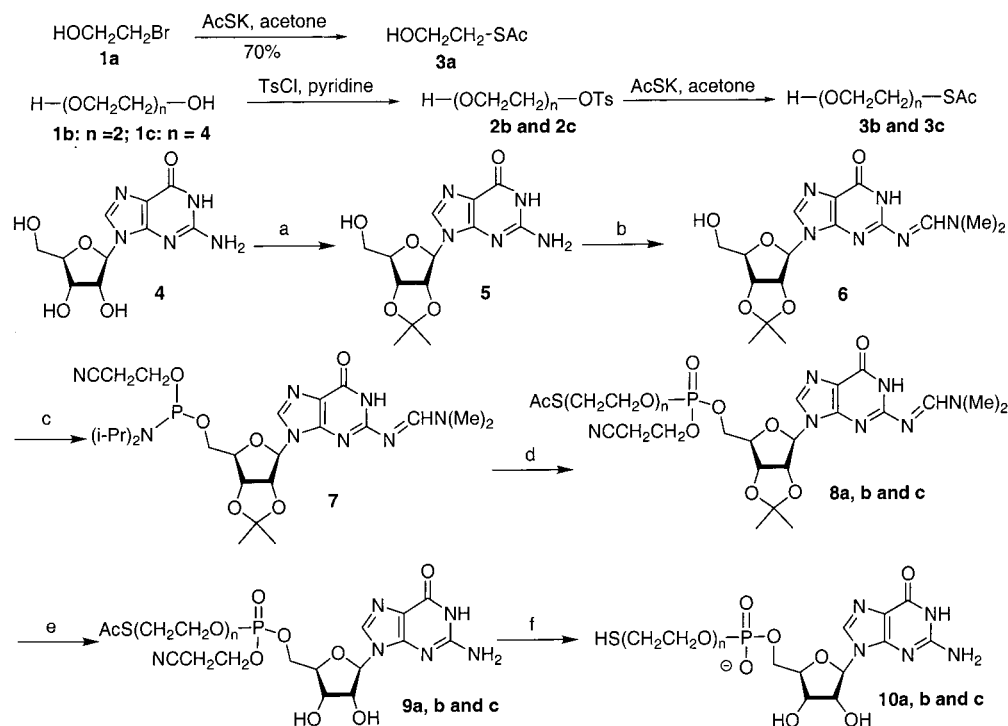
Synthesis of 5'-Deoxy-5'-iodo-2',3'-isopropylidene Guanosine 12. Methyltriphenoxyposphonium iodide (0.86 g, 1.91 mmol) was added to a cooled (−78 °C) suspension of 2',3'-*O*-isopropylidene guanosine (0.41 g, 1.27 mmol) in tetrahydrofuran (20 mL). The mixture was allowed to warm to room temperature after 10 min. After 4 h the excess methyltriphenoxyposphonium iodide was destroyed by addition of 1 mL of methanol, and the solvent was removed under reduced pressure. The residue was suspended in a mixture of ethyl ether and hexane (1:1), and the solid was filtered and washed thoroughly by the addition of ethyl ether and hexane. The

crude product was purified by flash chromatography (gradient of methanol/chloroform) (0.34 g, 61.8%). R_f = 0.53 (chloroform/methanol = 4:1); ^1H NMR ($\text{DMSO}-d_6$): δ 1.31 (s, 3H), 1.50 (s, 3H), 3.35 (m, 2H), 4.25 (m, 1H), 5.04 (dd, J = 4.0 Hz, 8.4 Hz, 1H), 5.30 (dd, J = 2.8 Hz, 8.4 Hz, 1H), 6.01 (d, J = 2.8 Hz, 1H), 6.55 (b, 2H), δ 7.88 (s, 1H).

Synthesis of 5'-Deoxy-5'-thioguanosine-5'-monophosphorothioate 14. A suspension of 5'-deoxy-5'-iodo-2',3'-isopropylidene guanosine **12** (2.88 g, 6.65 mmol) in 50% aqueous formic acid (100 mL) was stirred for 2.5 days, and then the solvent was removed by evaporation. The crude deprotected product **13** (2.83 g) was used without further purification in the next reaction. R_f = 0.78 (*i*-propyl alcohol/ $\text{NH}_3/\text{H}_2\text{O}$ = 6:3:1). To a suspension of 5'-deoxy-5'-iodoguanosine **13** (2.83 g, 7.2 mmol) in 140 mL of water was added trisodium thiophosphate (4.8 g, 26 mmol). The reaction mixture was stirred for 3 days at room temperature under argon atmosphere. After filtration to remove any precipitate, the solvent was evaporated under reduced pressure. The residue was dissolved in 100 mL of water and precipitated by the addition of 200 mL of methanol. After removing the precipitate by filtration, the solvent was evaporated and the residue was dissolved in a small amount of water and subjected to reverse phase chromatography and eluted with water. The desired product was collected and dried by lyophilization (1.9 g, 68% for two steps). R_f = 0.36 (isopropyl alcohol/ $\text{NH}_3/\text{H}_2\text{O}$ = 6:3:1). ^1H NMR ($\text{DMSO}-d_6 + \text{D}_2\text{O}$): δ 2.83 (m, 2H), 4.08 (m, 2H), 4.28 (dd, J = 3.9 Hz, 5.3 Hz, 1H), 5.63 (d, J = 5.9 Hz, 1H), 7.82 (s, 1H). ^{31}P NMR ($\text{DMSO}-d_6 + \text{D}_2\text{O}$): δ 16.4. HRMS: $\text{C}_{10}\text{H}_{15}\text{N}_5\text{O}_7\text{PS}$ calcd 380.0430, found 380.0482.

Preparation of 5'-HS-PEG₂-GMP-RNA, 5'-HS-PEG₄-GMP-RNA, and 5'-HS-G-RNA. The 5'-GTP-RNA, 5'-GSMP-RNA, and 5'-HS-PEG-GMP-RNA were prepared by runoff transcription in the presence of the four ribonucleotides or the four ribonucleotides supplemented with GSMP or **10b** or **10c** (Scheme 4). In general, the 222 bp DNA template for *in vitro* transcription was generated by PCR from pC25 plasmid DNA (Zhang and Cech, 1997). Transcription reactions were carried out with 4 μL of T7 RNA polymerase in the presence of 2 mM each NTP, 7.2 μg of DNA template, 10 μCi of [α - ^{32}P]-ATP, 4 mM spermidine, 0.05% Triton X-100, 12 mM MgCl_2 , 20 mM DTT, and 40 mM Tris buffer (pH 7.5) in a total 200 μL reaction at 37 °C for 3 h. A 4 μL aliquot of 0.5 M EDTA (pH 7.4) was added to dissolve the white Mg^{2+} -pyrophosphate precipitate, and 80 μL of formamide dye was added, and then loaded on an 8% polyacrylamide gel. RNA was purified through an 8% polyacrylamide [29:1 acrylamide:bis(acrylamide)]/8 M urea gel. RNA was visualized by UV-shadowing and excised from the gel. The gel slice was crushed and soaked overnight in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 250 mM NaCl) at 4 °C to elute the RNA. After filtering the soaking solution, RNA was recovered by ethanol precipitation, and the pellet was dissolved in 10–50 μL of ddH₂O. The sequence of the full-length RNA is the following: 5'-GGG AGA GAC CUG CCA UUC ACG CUG GAU AAA ACU UCA CAG CCA UAC GUU GUG UUU GAC UAA GCC AGA AUA UCC AGA UAA GGU AGC UGG AGA GAG CAG CGA CUU ACA UCC CCG GUA GAU ACG AAC AGG ACC CCU GCC AUG CAG UGA CCU UUC GUA GCC GCC AGU UCU UGA CCU CUA AGC AGC GUC AGG AUC CGU G-3'.

To prepare 5'-HS-PEG_{*n*}-GMP-RNA (where *n* = 2 or 4), 5'-HS-PEG_{*n*}-GMP (**10b** or **10c**) was added into the transcription reaction with a ratio of 5'-HS-PEG_{*n*}-GMP

Scheme 1. Synthesis of 5'-HS-PEG_n-GMP 10a, 10b, and 10c^a

^a (a) Acetone, 70% HClO_4 ; (b) $\text{Me}_2\text{NCH(OMe)}_2$, DMF, 55 °C; (c) $\text{CIP(NPr}_2\text{)(OCH}_2\text{CH}_2\text{CN)}$, NPr_2Et , CH_2Cl_2 , 0 °C; (d) (1) $\text{H(OCH}_2\text{CH}_2)_n\text{SCOCH}_3$, 1*H*-tetrazole, MeCN, (2) *t*-BuOOH; (e) 60% $\text{HCOOH/H}_2\text{O}$; (f) NH_3/MeOH , $\text{HS-CH}_2\text{CH}_2\text{OH}$.

to GTP of 1:1, 4:1, 8:1, or 16:1. A 20 μL aliquot of 0.5 M EDTA (pH 7.4) was added to dissolve the white precipitate before adding formamide-loading dye. The RNA transcript was purified as described above.

To prepare 5'-HS-G-RNA, 5'-GSMP-RNA was synthesized by runoff transcription in the presence of GSMP with a ratio of GSMP:GTP:ATP:CTP:UTP = 8:1:1:1:1 mM. The 5'-GSMP-RNA was dephosphorylated by calf intestinal alkaline phosphatase (New England Biolabs) in NEBuffer 3 (50 mM Tris-HCl, 10 mM MgCl_2 , 100 mM NaCl, 1 mM dithiothreitol, pH 7.9) at 37 °C for 3 h to generate 5'-HS-G-RNA. The reaction was stopped by the addition of 10 μL of 200 mM EGTA and incubation at 65 °C for 10 min. The 5'-HS-G-RNA was recovered and resuspended as described above.

Conjugation of Thiol-Reactive Agents with 5'-HS-G-RNA and 5'-HS-PEG_n-RNA. The 5'-GTP-RNA, 5'-HS-G-RNA, and 5'-HS-PEG_n-RNAs were incubated with biotin-PEG₃-iodoacetamide (**15**), biotin-HPDP (**16**), and biotin-PEG₃-maleimide (**17**), in 10 mM HEPES (pH 7.8), 300 mM NaCl, and 1 mM EDTA at room temperature for 2 h. The reaction mixtures were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) (pH 6.7) once and chloroform once, and precipitated with ethanol. The RNA pellets were resuspended in 20 μL of pure water and stored at -20 °C. A 2 μL aliquot of each of the biotinylated RNAs was incubated with 15 μg of streptavidin in the binding buffer (20 mM HEPES, pH 7.4, 5.0 mM EDTA, and 1.0 M NaCl) at room temperature for 20 min prior to mixing with 0.25 volume of formamide loading buffer (90% formamide, 0.01% bromophenol blue, and 0.025% xylene cyanol). The biotinylated RNA products were resolved by electrophoresis through 7.5 M urea/polyacrylamide gels. The biotinylated RNA can complex with streptavidin, and the mobility of the 5'-biotin-RNA/streptavidin complex through the gel will be retarded relative to unbiotinylated RNA. The fraction of product

formation relative to total RNA at each lane was quantitated with a Molecular Dynamics PhosphorImager.

For conjugation with maleimide-activated horseradish peroxidase (HRP), a 1.0 μL aliquot of 5'-GTP-RNA or 5'-HS-PEG_n-GMP-RNA was incubated with 10 μg of HRP in maleimide conjugation buffer (100 mM sodium phosphate, 5 mM EDTA, pH 7.6) at room temperature for 1 h. The HRP-conjugated RNA was then resolved by electrophoresis through an 8% urea/8% polyacrylamide gel. Detection of the HRP-maleimide-RNA conjugate was based on the electrophoretic mobility change of the conjugated RNA which obviated the need to assay for HRP's enzymatic activity. The mobility of HRP-labeled RNA will be slower than unmodified RNA on the 8% urea/8% polyacrylamide gel.

RESULTS AND DISCUSSION

Synthesis and Characterization of 5'-HS-PEG_n-GMP. The *O*-[ω -sulfhydryl-di(ethylene glycol)]-*O*-(5'-guanosine) monophosphate **10b** and *O*-[ω -sulfhydryl-tetra(ethylene glycol)]-*O*-(5'-guanosine) monophosphate **10c** were synthesized by phosphoramidite chemistry. The synthetic strategy was to synthesize 5'-phosphoramidite-2',3'-*O*,*O*-isopropylidene-2-*N*-(*N*',*N*'-dimethylaminomethylene)-guanosine **7** first, and then the free hydroxyl groups of ω -thioacetate-poly(ethylene glycol) compounds (**3a**, **3b**, and **3c**) were coupled with **7** in the presence of 1*H*-tetrazole (Scheme 1). Different lengths of PEG linkers were incorporated at the 5'-phosphate of guanosine depending upon the specific version of **3a-c** chosen.

A large excess of di- or tetra(ethylene glycol) was reacted with *p*-toluenesulfonyl chloride in pyridine and then reacted with potassium thioacetate to afford monothioacetate **3b** or **3c**. Guanosine **4** was treated with acetone and 70% perchloric acid at room temperature to give 2',3'-*O*,*O*-isopropylideneguanosine **5** in 83% yield. Compound **5** was reacted with *N*,*N*-dimethylformamide

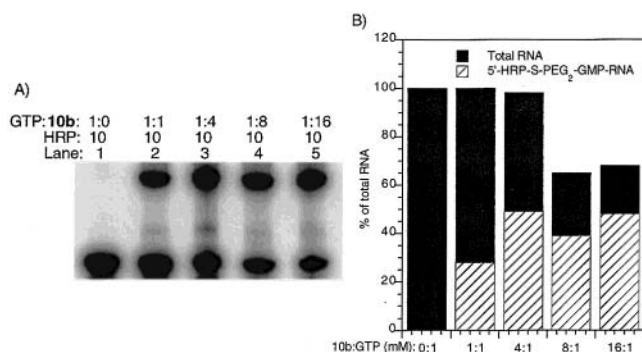
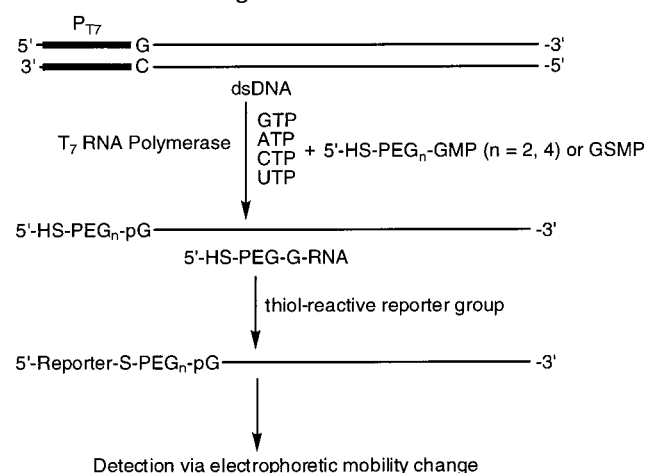


Figure 2. (A) Autoradiogram of RNAs transcribed using various GTP:5'-HS-PEG₂-GMP ratios and incubated with maleimide-activated horseradish peroxidase prior to electrophoresis. (B) Quantitative analysis of transcription yield and incorporation efficiency of **10b** using maleimide-activated horseradish peroxidase to detect 5'-HS-PEG₂-GMP-initiated RNA. The total RNA was quantitated using a Varian UV spectrometer (260 nm); the height of the bars represents the total RNA yield after the gel purification of each RNA transcript. The shaded portions are the fraction of the **10b**-initiated RNA. Percentage values are normalized to an in vitro transcription reaction with each NTP at 1.0 mM without the **10b** initiator nucleotide.

We explored the efficiency of incorporation of 5'-HS-PEG₂-GMP (**10b**) during in vitro transcription reactions performed with varying molar ratios of GTP to 5'-HS-PEG₂-GMP (Figure 2). The molar ratio of GTP to **10b** was adjusted by maintaining a consistent concentration of 1 mM GTP while varying the concentration of **10b** between transcription reactions. The thiol-containing RNAs generated by the transcription reactions were conjugated to maleimide-activated HRP during a subsequent incubation step. Assuming that the thiol-maleimide reaction was quantitative, resolution of the 5'-HRP-S-PEG₂-GMP-RNA from the unconjugated RNA allowed us to determine the percent of RNA transcripts that successfully employed **10b** as the initiator nucleotide in lieu of GTP. No 5'-HRP-RNA was formed when **10b** was absent from the transcription reaction (Figure 2A, lane 1), confirming that the conjugation of the maleimide-activated HRP with the RNA was dependent upon the use of the thiol-containing initiator nucleotide.

The efficiency of incorporation of **10b** may be dissected in terms of both relative and absolute yields (i.e., what fraction of the total transcripts were initiated with **10b**, and how many moles of transcripts were produced). This is an important distinction since the absolute yield from the transcription reactions decreased at the highest concentrations of **10b** tested (Figure 2B). When the ratio of GTP to **10b** was 1:1, approximately 28% of the nascent transcripts were initiated with **10b**. The percent of transcripts initiated with **10b** increased to 51%, 60%, and 72% as the GTP:**10b** ratio was varied from 1 mM:4 mM, 1 mM:8 mM, and 1 mM:16 mM, respectively. Interestingly, the fraction of 5'-HRP-S-PEG₂-GMP-RNA increased significantly over this interval, but the absolute yield of 5'-HRP-S-PEG₂-GMP-RNA remained relatively constant as the absolute total transcription yield (including GTP-initiated transcripts) decreased (Figure 2B). When the concentration of **10b** reached 8 mM, it appeared to slightly inhibit transcription by T7 RNA polymerase. Normalizing the absolute yield of total RNA to 100% when the ratio of GTP to 5'-HS-PEG₂-GMP was 1 mM:0 mM, the yield decreased to 98% for 1 mM:4 mM, 65% for 1 mM:8 mM, and 68% for 1 mM:16 mM transcription reactions.

Scheme 4. General Overview of Enzymatic Incorporation To Yield 5'-Sulfhydryl-Modified RNA and Their Subsequent Detection by Conjugation with Thiol-Reactive Reagents



A comparison of these incorporation efficiencies with published reports using similar initiator nucleotides is complicated by the facts that each laboratory prefers slightly different transcription conditions and that the nucleotide concentrations frequently vary between studies. Certainly, however, our yields compare favorably with the yields reported by Sengle et al. (2000) in which transcription reactions employing a 1 mM:4 mM ratio of GTP to their biotinylated-GMP analogue resulted in one-fourth of their transcripts initiating with their analogue. Under similar GTP:GMP analogue conditions, we observed that 51% of the transcripts initiated with our thiol-containing GMP analogue **10b**. Interestingly, they reported that GMPS at low concentrations appeared to enhance the absolute total transcription yield and then began to decrease the total yield as the GMPS concentration was raised further. In contrast, the addition of AMP and GMP decreased the transcription yields in a concentration-dependent manner. We find that our **10b** nucleotide behaves more similar to the effect reported for AMP and GMP addition, specifically a concentration-dependent decrease in yields from transcription reactions utilizing T7 RNA polymerase.

We chose to use poly(ethylene glycol) (PEG) linkers because the flexibility that they provide enables their future use in applications in which steric hindrance may be an issue. In light of the finding that PEG-containing GMP nucleotides are incorporated less efficiently as initiator nucleotides as the length of the PEG linker increases (Seelig and Jäschke, 1999a,b), we sought to balance the competing demands of linker flexibility with incorporation efficiency. The results from Figure 2 suggest that we have found an acceptable balance; the initiator nucleotide **10b**, containing two PEG subunits, decreased the absolute total transcription yield when present at a GTP:**10b** ratio at 1 mM:8–16 mM but without significantly lowering the absolute yield of the desired **10b**-capped-RNA.

Thiol-Reactive Biotin Conjugation with 5'-HS-PEG₂-GMP-RNA. The streptavidin gel-shift results of 5'-HS-PEG₂-GMP-RNA with the biotinylated thiol-reactive **15**, **16**, and **17** (from Molecular Biosciences, Boulder, CO) (Scheme 5) are shown in Figure 3. When 5'-HS-PEG₂-GMP-RNA was reacted with **15**, **16**, and **17**, the thiol-modified RNA molecules were biotinylated and detected as band-shifts in the presence of streptavidin upon gel electrophoresis that represented streptavidin/

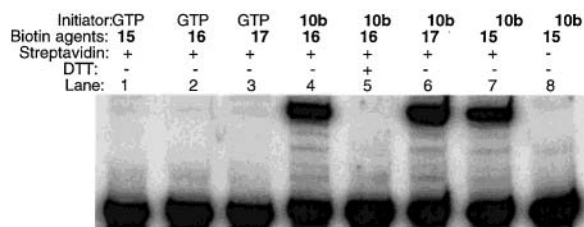


Figure 3. Autoradiogram of the streptavidin gel-shift analysis of transcription products (5'-GTP-RNA and 5'-HS-PEG₂-RNA) following an incubation with **15**, **16**, or **17**. Lanes 1–3, 5'-GTP-RNA; lanes 4–8, 5'-HS-PEG₂-RNA.

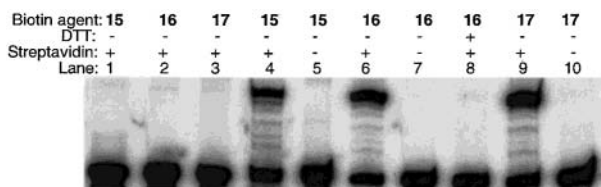
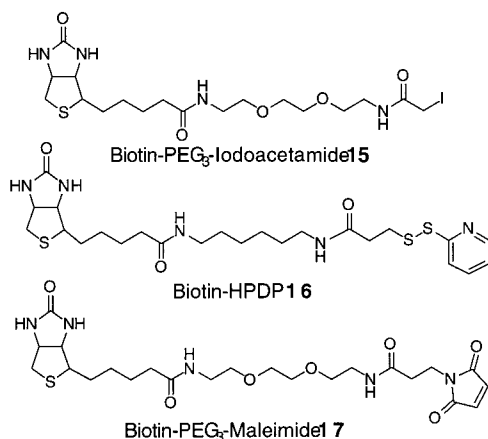


Figure 4. Autoradiogram of the streptavidin gel-shift analysis of transcription products (5'-GTP-RNA and 5'-GSMP-RNA) following an incubation with **15**, **16**, or **17**. Lanes 1–3, 5'-GTP-RNA; lanes 4–10, 5'-HS-G-RNA.

Scheme 5. Chemical Structures of Thiol-Reactive Agents



RNA complexes (lanes 4, 6, and 7); no retarded band was detected without streptavidin (lane 8). When 5'-GTP-capped-RNA was treated with **15**, **16**, and **17**, no biotinylated RNA was detected in the presence of streptavidin (lanes 1, 2, and 3, respectively). The retarded band disappeared after treatment with DTT (lane 5), which reduced the product of the thiol–disulfide exchange reaction between 5'-HS-RNA and biotin-HPDP **16**. The overall fraction of biotinylated RNA was 39% after reaction with **16**, 45% with **17**, and 23% with **15** for 5'-HS-PEG₂-GMP (lanes 4, 6, and 7, respectively).

Thiol-Reactive Biotin Conjugation with 5'-HS-G-RNA. The bridging phosphorothioate 5'-GSMP-RNA was dephosphorylated by alkaline phosphatase to generate 5'-HS-G-RNA (i.e., RNA containing a 5'-thiol instead of a 5'-hydroxyl group). The streptavidin gel-shift results of 5'-HS-G-RNA following reaction with **15**, **16**, and **17** are shown in Figure 4. When 5'-HS-RNA was reacted with **15**, **16**, and **17**, the thiol-modified RNA molecules were biotinylated and detected as band-shifts in the presence of streptavidin (lanes 4, 6, and 9, respectively); no retarded band was detected without streptavidin (lanes 5, 7, and 10) nor when 5'-GTP-capped-RNA was treated with **15**, **16**, and **17** (lanes 1, 2, and 3, respectively). The retarded band disappeared after treatment

with DTT (lane 8), which reduced the product of the thiol–disulfide exchange reaction between 5'-HS-RNA and biotin-HPDP **16**. These results suggest that the biotin group was transferred to the terminal thiol of the 5'-HS-RNA, not to other nucleophilic groups of RNA. The overall yield (three steps) of biotinylated RNA is 57% with **15** and 60% with **16** for GSMP (lanes 6 and 9, respectively). The experiments demonstrated that GSMP **14** can serve as a better initiator nucleotide for transcription by T7 RNA polymerase than **10b** and **10c** for the purpose of introducing a sulfhydryl group at the 5'-end of RNA.

CONCLUSION

We have developed a general method to introduce a single thiol group tethered to the 5'-end of RNA via spacers of various lengths. Our results demonstrate that GSMP and 5'-HS-PEG_n-GMP can serve as initiator nucleotides for T7 RNA polymerase, thus introducing a sulfhydryl group into the 5'-end of RNA. Fluorophores, biotinylated compounds, peptides, proteins, DNAs, RNAs, enzymes, and other chemical entities containing thiol-reactive functional groups can be attached to the thiol-modified RNA molecules using the techniques described here. These methods may have potential applications in analysis and detection of RNA, mapping RNA–protein interactions, in vitro selection of novel catalytic RNAs, and even gene array technologies.

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In Vitro Cationic Lipid-Mediated Gene Delivery with Fluorinated Glycerophosphoethanolamine Helper Lipids

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There is a need for the development of nonviral gene transfer systems with improved and original properties. "Fluorinated" lipoplexes are such candidates, as supported by the remarkably higher in vitro and in vivo transfection potency found for such fluorinated lipoplexes as compared with conventional ones or even with PEI-based polyplexes (Boussif, O., Gaucheron, J., Boulanger, C., Santaella, C., Kolbe, H. V. J., Vierling, P. (2001) Enhanced in vitro and in vivo cationic lipid-mediated gene delivery with a fluorinated glycerophosphoethanolamine helper lipid. *J. Gene Med.* 3, 109–114). Here, we describe the synthesis of fluorinated glycerophosphoethanolamines (*F*-PEs), close analogues of dioleoylphosphatidylethanolamine (DOPE), and report on their lipid helper properties vs that of DOPE, as in vitro gene transfer components of fluorinated lipoplexes based on pcTG90, DOGS (Transfectam), or DOTAP. To evaluate the contribution of the *F*-PEs to in vitro lipoplex-mediated gene transfer, we examined the effect of including the *F*-PEs in lipoplexes formulated with these cationic lipids (CL) for various CL:DOPE:*F*-PE molar ratios [1:(1 - *x*):*x* with *x* = 0, 0.5 and 1; 1:(2 - *y*):*y* with *y* = 0, 1, 1.5, and 2], and various *N/P* ratios (from 10 to 0.8, *N* = number of CL amines, *P* = number of DNA phosphates). Irrespective of the *F*-PE chemical structure, of the colipid *F*-PE:DOPE composition, and of the *N/P* ratio, comparable transfection levels to those of their respective control DOPE lipoplexes were most frequently obtained when using one of the *F*-PEs as colipid of DOGS, pcTG90, or DOTAP in place of part or of all DOPE. However, a large proportion of DOGS-based lipoplexes were found to display a higher transfection efficiency when formulated with the *F*-PEs rather than with DOPE alone while the opposite tendency was evidenced for the DOTAP-based lipoplexes. The present work indicates that "fluorinated" lipoplexes formulated with fluorinated helper lipids and conventional cationic lipids are very attractive candidates for gene delivery. It confirms further that lipophobicity and restricted miscibility of the lipoplex lipids with the endogenous lipids does not preclude efficient gene transfer and expression. Their transfection potency is rather attributable to their unique lipophobic and hydrophobic character (resulting from the formulation of DNA with fluorinated lipids), thus preventing to some extent DNA from interactions with lipophilic and hydrophilic biocompounds, and from degradation.

INTRODUCTION

Gene (or DNA) transfer systems into cells have become powerful tools for gene and cellular therapy that are very promising novel forms of molecular medicine. Successful gene therapy depends on the efficient delivery of genetic material to cells and its effective expression within these cells. Although at present, the in vivo expression levels of synthetic nonviral gene transfer vectors based on (poly)cationic lipids, liposomes, and polymers (e.g., lipoplexes and polyplexes, respectively (1)) are lower than for viral vectors and gene expression is transient, these

systems are likely to present several advantages including low-cost and large-scale production, safety, lower immunogenicity, and capacity to deliver large gene fragments. Such systems have therefore gained wide acceptance over the past decade for in vitro and/or in vivo gene delivery (2–5). However, there is still a need for the development of gene transfer systems with improved and original properties.

Our approach with the development of "fluorinated" lipoplexes based either on highly fluorinated lipospermines (6, 7) or on a fluorinated glycerophosphoethanolamine helper lipid (e.g., [F8E11][C16]OPE in Scheme 1 which is an analogue of dioleoylphosphatidylethanolamine, DOPE) (8) proved to some extent successful as these systems showed a higher in vitro and in vivo transfection potential than conventional lipoplexes (6–8) or PEI polyplexes (8). More particularly, the use of [F8E11][C16]OPE (which on its own is inactive in promoting transfection) as colipid of the cationic lipopolyamine pcTG90 (see structure in Scheme 1) increased the in vitro and in vivo gene transfer capability of pcTG90 to a larger extent than DOPE (8). Although the exact mechanism which governs lipoplex-mediated gene delivery and ex-

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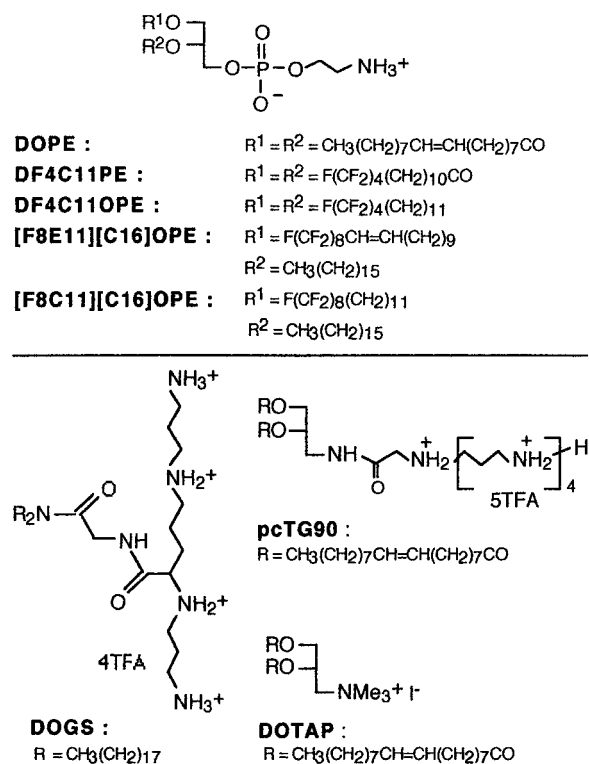
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Scheme 1



pression is unclear, several reasons may account for the gene expression improvement resulting from the use of [F8E11][C16]OPE in place of DOPE. This improvement could be due to the larger ability of the fluorinated colipid to preserve the integrity of complexed DNA in a biological environment. Indeed, owing to its increased hydrophobic and lipophobic character, this colipid is expected to prevent the fluorinated lipoplexes it forms with cationic lipids from interactions with lipophilic and hydrophilic biocompounds, and from degradation to a larger extent than conventional helper lipids, as shown for fluorinated lipoplexes formed from fluorinated lipospermines as compared with conventional ones (7). This improvement could also be related to the larger propensity of the fluorinated colipid to promote fusion with and destabilization of the endosome membrane allowing more efficient DNA release in the cytosol, thus preventing internalized DNA from endosome and lysosome degradation to a larger extent than when DOPE is used as helper lipid (9–12). This fluorinated PE displays also a more pronounced cone-shape geometry than DOPE as a consequence of the larger size of its fluorinated chain when compared with hydrocarbon ones (13). One therefore expects a greater tendency for [F8E11][C16]OPE to promote a lamellar to inverted hexagonal HII phase transition (13) and, thus, a greater effectiveness in disrupting membranes than DOPE (the role in membrane fusion of DOPE was indeed attributed to its capability to initiate such a phase transition (11, 12)).

The goals of the present study was aimed at extending the library of fluorinated glycerophosphoethanolamines (*F*-PEs), and at examining (i) their lipid helper effect on lipofection and (ii) the cationic lipid specificity of their transfection enhancement potential, as compared with that of DOPE. The molecular structure of this second generation of *F*-PEs (listed in Scheme 1) follows a modular design (one or two fluorinated chains, saturated or unsaturated chains, ester or ether bond) aimed at the establishment of structure/properties (transfection helper

potential) relationships. We report here on their synthesis and on their helper lipid properties as in vitro gene transfer components of pcTG90, DOGS (Transfectam), and DOTAP-based lipoplexes (see structures in Scheme 1), as compared with DOPE. These (poly)cationic lipids were selected for their well-documented high transfection efficiency which is significantly improved with DOPE (for pcTG90, see (8, 14, 15), for DOGS, see (16) and for DOTAP, see (17, 18)). They were also chosen for their specific structural and geometrical features (linear or branched polyamino or single quaternary ammonium polar head) which might have quite different effects on the phases these cationic lipids associated to the *F*-PEs are susceptible to form. The thermotropic phase behavior of the *F*-PEs in water was also investigated by differential scanning calorimetry (DSC) in order to highlight its role on lipoplex formulation and lipofection (10, 12). In our initial study (8), the optimized conditions for the in vitro and in vivo transfection tests, which led us to show a significantly higher transfection helper potential of [F8E11][C16]OPE as compared with that of DOPE, were corresponding to pcTG90-based lipoplexes formulated in HEPES and in 5% glucose, respectively. However, in vitro, when the lipoplexes were formulated in 5% glucose, both colipids displayed a comparable transfection helper potential. In anticipation of in vivo testing, lipofection with the *F*-PEs as colipids was investigated here only for lipoplexes formulated in 5% glucose.

EXPERIMENTAL PROCEDURES

General Experimental and Analytical Conditions. Most of the reactions were performed in anhydrous solvents under dry and oxygen-free nitrogen. Anhydrous solvents were prepared by standard methods. The purifications by column chromatography were carried out using silica gel 60 (Merck, 70–230 mesh) and chloroform (CHCl_3), dichloromethane (CH_2Cl_2), methanol (MeOH), or mixtures thereof as indicated. Unless noted otherwise, the ratios describing the composition of solvent mixtures represent relative volume. Advancing of the reaction was followed by thin-layer chromatography (TLC) on silica plates F_{254} (Merck). The following developing systems were used: UV light, KMnO_4 , $\text{H}_2\text{SO}_4/\text{EtOH}$, Dragendorff reagent (Sigma), ninhydrin reagent (Sigma).

The perfluoroalkyl iodides were purified over neutral alumina before use. DF4C11OPE, DOGS (or Transfectam), and DOTAP were prepared as described in refs 19, 6, and 17, respectively. The lipopolyamine pcTG90 was from Transgène (Strasbourg, France) (20). DOPE was purchased from Sigma. All other organic chemicals were from Aldrich, Fluka, or Novabiochem.

^1H , ^{13}C , $^{31}\text{P}\{^1\text{H}\}$, and ^{19}F NMR spectra were recorded on a Bruker AC-200 at 200, 50.3, 81, and 188.3 MHz, respectively. Chemical shifts were measured relative to CHCl_3 (δ 7.27 ppm) or CH_3OD (δ 3.35 ppm) for ^1H , to CDCl_3 (δ 76.9 ppm) for ^{13}C and expressed indirectly in relation to TMS, to external ref 75% H_3PO_4 for ^{31}P , and to CCl_3F as internal reference for ^{19}F . The following abbreviations are used to describe the signal multiplicities: s (singlet), d (doublet), t (triplet), q (quadruplet), and m (multiplet). Chemical shifts are expressed in ppm and listed as follow: shift in ppm (multiplicity, coupling, integration and attribution). Elemental analyses were carried out by the Service Central de Microanalyses du CNRS.

Synthesis of the Glycerol Derivatives 2a–c. *rac*-2,3-Di-[11-(*F*-butyl)undecanoyl]glycerol, 2a. The synthesis of 2a from *rac*-1-*O*-benzylglycerol was performed according to published procedures (21).

rac-3-[11-(F-Octyl)undec-10-enyloxy]-2-(hexadecyloxy)propan-1-ol, 2b. The syntheses of derivatives **4** to **6** from 1,3-*O*-benzylideneglycerol, **3**, were performed according to published procedures (19).

rac-3-(Benzyloxy)-2-(hexadecyloxy)propan-1-ol, 4. The alkylation of **3** with 1-bromohexadecane under phase-transfer catalysis conditions gave 5-hexadecyloxy-2-phenyl-1,3-dioxane [white powder, 73%; TLC (hexane/ethyl acetate 8:2, UV and H₂SO₄): *R*_f = 0.72. ¹H NMR (CDCl₃): δ 0.84 (t, ³*J* = 6.1 Hz, 3H, CH₃); 1.20–1.50 (broad s, 26H, CH₃(CH₂)₁₃); 1.51–1.80 (m, 2H, CH₂CH₂O); 3.10–3.20 (m, part X of a ABX system, 1H, CH₂CHCH₂); 3.68 (t, ³*J* = 6.6 Hz, 2H, CH₂CH₂OCH); 4.05, 4.38 (part AB of a ABX system, ²*J*_{AB} = 12.4 Hz, ³*J*_{AX} = 1.6 Hz, ³*J*_{BX} = 1.3 Hz, 4H, CH₂CHO); 5.60 (s, 1H, CHPh); 7.31–7.49, 7.50–7.65 (m, m, 3H, 2H, Ph). ¹³C NMR (CDCl₃): δ 14.3 (CH₃); 22.8 (CH₃CH₂); 26.3 (CH₂CH₂CH₂O); 29.5, 29.6, 29.7, 29.8, 29.9 (CH₃(CH₂)₂(CH₂)₉, CH₂CH₂O); 32.1 (CH₃CH₂CH₂); 69.2, 69.3 (CH₂O); 70.7 (CH₂OCH); 101.4 (CHPh); 126.2, 128.2, 128.8, 138.3 (Ph)]. The reductive cleavage of the 1,3-*O*-benzylidene group in 5-hexadecyloxy-2-phenyl-1,3-dioxane with BH₃·THF gave **4** (colorless oil, 85%). TLC (CHCl₃; UV and H₂SO₄): *R*_f = 0.25. ¹H NMR (CDCl₃): δ 0.90 (t, ³*J* = 6.1 Hz, 3H, CH₃); 1.10–1.40 (broad s, 26H, CH₃(CH₂)₁₃); 1.50–1.70 (m, 2H, CH₂CH₂O); 2.22 (broad s, 1H, OH); 3.35–3.78 (m, 7H, CH₂O, CHO); 4.56 (s, 2H, CH₂Ph); 7.20–7.35 (m, 5H, Ph). ¹³C NMR (CDCl₃): δ 14.3 (CH₃); 22.8 (CH₃CH₂); 26.3 (CH₂CH₂CH₂O); 29.5, 29.6, 29.7, 29.8, 29.9 (CH₃(CH₂)₂(CH₂)₉, CH₂CH₂O); 32.1 (CH₃CH₂CH₂); 63.0 (CH₂OH); 70.2, 70.6 (CH₂O); 73.7 (CH₂Ph); 78.7 (OCH); 127.6, 127.7, 128.4, 138.1 (Ph).

rac-1-[3-(Undec-10-enyloxy)-2-(hexadecyloxy)propyloxy]benzene, 5. The alkylation of **4** via its anion with 11-bromoundecene gave **5** (colorless oil, 84%). TLC (CHCl₃; UV and H₂SO₄): *R*_f = 0.47. ¹H NMR (CDCl₃): δ 0.90 (t, ³*J* = 6.1 Hz, 3H, CH₃); 1.10–1.40 (broad s, 38H, CH₃(CH₂)₁₃, (CH₂)₆(CH₂)₂O); 1.41–1.70 (m, 4H, CH₂CH₂O); 2.00–2.20 (m, 2H, CH₂=CHCH₂); 3.35–3.78 (m, 9H, CH₂O, CHO); 4.56 (s, 2H, CH₂Ph); 4.80–5.10 (m, 2H, CH₂=CH); 5.65–5.95 (m, 1H, CH₂=CH); 7.20–7.35 (m, 5H, Ph).

rac-1-[3-(11-(F-Octyl)undec-10-enyloxy)-2-(hexadecyloxy)propyloxy]benzene, 6. The addition of CF₃(CF₂)₇I on the terminal CH₂=CH bond of **5** gave **6** (colorless oil, 73%). TLC (CH₂Cl₂; UV and H₂SO₄): *R*_f = 0.40. ¹H NMR (CDCl₃): δ 0.92 (t, ³*J* = 6.1 Hz, 3H, CH₃); 1.10–1.40 (broad s, 38H, CH₃(CH₂)₁₃, (CH₂)₆(CH₂)₂O); 1.41–1.70 (m, 4H, CH₂CH₂O); 2.10–2.30 (m, 2H, CH=CHCH₂); 3.35–3.78 (m, 9H, CH₂O, CHO); 4.56 (s, 2H, CH₂Ph); 5.30–5.80 (m, 1H, CF₂CH=CH); 6.30–6.55 (m, 1H, CF₂CH=CH); 7.20–7.35 (m, 5H, Ph). ¹³C NMR (CDCl₃): δ 14.2 (CH₃); 22.8 (CH₃CH₂); 26.2, 26.3 (CH₂CH₂CH₂O); 28.1, 29.1, 29.2, 29.4, 29.5, 29.6, 29.7, 29.8, 29.9, 30.3 (CH₂CH₂O, CH₃(CH₂)₂(CH₂)₁₀, CH=CHCH₂(CH₂)₅); 32.1 (CH₃CH₂CH₂, =CHCH₂); 70.5, 70.8, 70.9 (CH₂O); 73.5 (CH₂Ph); 78.6 (CHO); 116.9 (t, ²*J*_{CF} = 23 Hz, CF₂CH=CH); 126.2, 128.2, 128.8, 138.3 (Ph); 143.4 (t, ³*J*_{CF} = 9 Hz, CF₂CH=CH trans); 144.0 (t, ³*J*_{CF} = 5 Hz, CF₂CH=CH, cis). ¹⁹F NMR (CDCl₃): δ -81.3 (3F, CF₃); -107.2 (0.2F, CF₂CH cis); -112.7 (1.8F, CF₂CH trans); -122.0, -122.5, -123.2, -124.0 (2F, 4F, 2F, 2F, CF₃CF₂(CF₂)₅); -126.6 (2F, CF₃CF₂).

rac-3-[11-(F-Octyl)undec-10-enyloxy]-2-(hexadecyloxy)propan-1-ol, 2b. A suspension of FeCl₃ and 4.4 g of **6** in 15 mL CH₂Cl₂ was stirred for 90 min at room temperature. The organic phase was then washed, dried over Na₂SO₄ and evaporated, and the residue was purified by silica gel chromatography (CH₂Cl₂:MeOH 100:0

to 99:1) to afford 2.9 g of **2b** (white powder, 73%). TLC (CHCl₃; H₂SO₄): *R*_f = 0.20. ¹H NMR (CDCl₃): δ 0.85 (t, 3H, ³*J* = 6.5 Hz, CH₃); 1.05–1.45 (broad s, 38H, (CH₂)₆-CH₂CH₂O, CH₃(CH₂)₁₃); 1.45–1.70 (m, 4H, CH₂CH₂O); 2.07–2.28 (m, 2H, CH=CHCH₂); 2.48 (broad s, 2H, CH₂OH); 3.30–3.80 (m, 9H, CH₂O, CHO); 5.40–5.74 (m, 1H, CF₂CH=CH); 6.35–6.50 (m, 1H, CF₂CH=CH). ¹³C NMR (CDCl₃): δ 13.8 (CH₃); 22.6 (CH₃CH₂); 26.0 (CH₂CH₂CH₂O); 27.9, 28.9, 29.2, 29.3, 29.6, 30.0 (CH₂CH₂O, CH₃(CH₂)₂(CH₂)₉, =CHCH₂(CH₂)₅); 31.8 (CH₃CH₂CH₂, =CHCH₂); 62.8 (CH₂OH); 70.3, 70.7, 71.6 (CH₂O); 78.3 (CHO); 116.7 (t, ²*J*_{CF} = 23 Hz, CF₂CH=CH); 143.0 (t, ³*J*_{CF} = 9 Hz, CF₂CH=CH trans); 145.0 (CF₂CH=CH cis). ¹⁹F NMR (CDCl₃) identical to that of **6**.

rac-3-[11-(F-Octyl)undecyloxy]-2-(hexadecyloxy)propan-1-ol, 2c. The simultaneous benzyl deprotection and C=C hydrogenation of **4** (1.8 g; 1.8 mmol) in 15 mL of EtOH and 0.2 mL of AcOH was performed at room temperature under 40 atm H₂ pressure with Pd/C (10%) as catalyst. Usual workup and silica gel chromatography (CH₂Cl₂) afforded 1.0 g (60%) of **2c** as a white powder (60%). TLC (CH₂Cl₂; H₂SO₄): *R*_f = 0.55. ¹H NMR (CDCl₃): δ 0.88 (t, 3H, ³*J* = 6.5 Hz, CH₃); 1.15–1.48 (broad s, 40H, (CH₂)₇CH₂CH₂O, CH₃(CH₂)₁₃); 1.48–1.72 (m, 4H, CH₂CH₂O, CF₂CH₂CH₂); 2.06 (t, ³*J* = 7.5 Hz, ³*J*_{HF} = 19.0 Hz, 2H, CF₂CH₂); 2.22–2.34 (broad s, 1H, CH₂OH); 3.38–3.83 (m, 9H, CH₂O, CHO). ¹³C NMR (CDCl₃): δ 14.0 (CH₃); 20.6 (t, ³*J*_{CF} = 4 Hz, CF₂CH₂CH₂); 22.6 (CH₃CH₂); 26.1 (CH₂CH₂CH₂O); 29.2, 29.3, 29.6, 30.0 (CH₂CH₂O, CH₃(CH₂)₂(CH₂)₁₀, CF₂(CH₂)₂(CH₂)₆); 30.9 (t, ²*J*_{CF} = 22 Hz, CF₂CH₂); 31.8 (CH₃CH₂CH₂); 63.1 (CH₂OH); 70.4, 70.9, 71.8 (CH₂O); 78.3 (CHO). ¹⁹F NMR (CDCl₃): δ -79.6 (3F, CF₃); -113.3 (2F, CF₂CH₂); -120.7 (6F, (CF₂)₃CF₂-CH₂); -121.6 (2F, CF₃(CF₂)₂CF₂); -122.4 (2F, CF₃-CF₂CF₂); -125.0 (2F, CF₃CF₂).

Synthesis of the F-PE Derivatives. The phosphorylation of the glycerol derivatives **2a–c** and the deprotection step of the BOC-protected intermediates **7a–c** with TFA were performed according to the procedures described for the synthesis of DF4C11OPE (19). The F-PE derivatives were isolated as TFA salts.

rac-2,3-Di[11-(F-butyl)undecanoyl]glycero-1-phosphoethanolamine, DF4C11PE. **rac-1,2-Di[10-(F-butyl)undecanoyl]glycero-3-phospho(tert-butoxy-carbonyl)ethanolamine, 7a** (30% yield): ¹H NMR (CDCl₃:CD₃OD): δ 1.02–1.37 (m, 37H, (CH₂)₇(CH₂)₂C(O), CH₃ (BOC); 1.37–1.57 (m, 4H, CH₂CH₂C(O)); 1.92 (tt, ³*J* = 7.5 Hz, ³*J*_{HF} = 19.0 Hz, 4H, CF₂CH₂); 2.09–2.26 (m, 4H, CH₂C(O)); 3.07–3.26 (m, 2H, CH₂N); 3.66–3.90 (m, 4H, CH₂OPOCH₂); 3.95–4.32 (m, 2H, C(O)OCH₂); 5.03–5.16 (m, 1H, CH). ¹³C NMR (CDCl₃/CD₃OD): δ 20.3 (t, ³*J*_{CF} = 4 Hz, CF₂CH₂CH₂); 25.0, 25.1 (CH₂CH₂C(O)); 28.4 (C(CH₃)₃); 29.5, 29.6 ((CH₂)₆(CH₂)₂C(O)); 30.9 (t, ²*J*_{CF} = 22 Hz, CF₂CH₂); 34.2, 34.4 (CH₂C(O)); 41.1 (d, ³*J*_{CP} = 6 Hz, CH₂N); 62.9 (OCH₂); 64.1 (d, ²*J*_{CP} = 5 Hz, POCH₂-CH₂N); 65.1 (d, ²*J*_{CP} = 5 Hz, CHCH₂OP); 71.1 (d, ³*J*_{CP} = 8 Hz, CH); 80.1 (C(CH₃)₃); 157.1 (NC(O)); 173.6, 174.6 (CH₂C(O)). ¹⁹F NMR (CDCl₃/CD₃OD): δ -80.0 (3F, CF₃); -113.5 (2F, CF₂CH₂); -123.3, -124.9 (2F, 2F, CF₃(CF₂)₂). ³¹P{¹H} NMR (CDCl₃/CD₃OD): δ -8.1 (s).

DF4C11PE (15% yield from **7a**): TLC (CH₂Cl₂:MeOH 7:3; ninhydrine): *R*_f = 0.70. ¹H NMR (CDCl₃/CD₃OD): δ 0.80–1.18 (m, 28H, (CH₂)₇(CH₂)₂C(O)); 1.18–1.50 (m, 4H, CH₂CH₂C(O)); 1.75 (tt, ³*J* = 7.5 Hz, ³*J*_{HF} = 19.0 Hz, 4H, CF₂CH₂); 1.90–2.12 (m, 4H, CH₂C(O)); 2.75–2.96 (m, 2H, CH₂N); 3.48–4.18 (m, 6H, CH₂OPOCH₂, C(O)OCH₂); 5.06–5.21 (m, 1H, CH). ¹³C NMR (CDCl₃/CD₃OD): δ 19.6 (t, ³*J*_{CF} = 4 Hz, CF₂CH₂CH₂); 24.5 (CH₂CH₂CO); 28.7, 28.8, 30.0 ((CH₂)₆(CH₂)₂CO); 30.4 (t, ²*J*_{CF} = 22 Hz,

CF₂CH₂); 33.6, 33.7 (CH₂C(O)); 40.1 (d, ³J_{CP} = 6 Hz, CH₂N); 61.3 (d, ²J_{CP} = 5 Hz, POCH₂CH₂N); 62.2 (C(O)-OCH₂); 63.5 (d, ²J_{CP} = 5 Hz, CHCH₂OP); 70.1 (d, ³J_{CP} = 8 Hz, CH); 173.5, 173.8 (CO). ¹⁹F NMR (CDCl₃/CD₃OD): δ -82.5 (3F, CF₃); -115.9 (2F, CF₂CH₂); -125.7, -127.4 (4F, CF₃(CF₂)₂). ³¹P NMR {¹H} (CDCl₃/CD₃OD): δ -0.2 (s). Anal. (C₃₅H₅₂F₁₈NO₈P + 1H₂O) Calcd: C, 41.80; H, 5.21; N, 1.39. Found: C, 41.78; H, 5.17; N, 1.38.

rac-3-[11-(F-Octyl)undec-10-enyl]-2-(hexadecyl)-glycero-1-phosphoethanolamine, [F8E11][C16]OPE. rac-1-[11-(F-Octyl)undec-10-enyl]-2-(hexadecyl)-glycero-3-phospho(tert-butoxycarbonyl)ethanolamine, 7b (70% yield): TLC (CHCl₃/MeOH: 7:3, v/v; molybdenum blue): R_f = 0.60. ¹H NMR (CDCl₃/CD₃OD): δ 0.85 (t, ³J = 6.1 Hz, 3H, CH₃); 1.05–1.35 (s large, 38H, CH₃(CH₂)₁₃, (CH₂)₆(CH₂)₂O); 1.35–1.63 (m, 13H, CH₂-CH₂O, CH₃(BOC)); 2.10–2.28 (m, 2H, CH=CHCH₂); 3.17–3.32 (m, 2H, CH₂N); 3.32–3.65 (m, 7H, CH₂OCH₂, CH₂OCH); 3.73–3.95 (m, 4H, CHCH₂OP, POCH₂CH₂N); 5.42–5.72 (m, 1H, CF₂CH=CH); 6.38–6.50 (m, 1H, CF₂CH=CH). ¹³C NMR (CDCl₃/CD₃OD): δ 13.6 (CH₃); 22.4 (CH₃CH₂); 25.9 (CH₂CH₂CH₂O); 28.0 (CH₃(BOC)); 27.8, 28.8, 29.2, 29.4, 29.6, 30.0 (CH₂CH₂O, CH₃(CH₂)₂-(CH₂)₁₀, CH=CHCH₂(CH₂)₅); 31.8, 31.9 (CH₃CH₂CH₂, =CHCH₂); 40.8 (d, ³J_{CP} = 6 Hz, CH₂N); 64.3 (d, ²J_{CP} = 5 Hz, POCH₂CH₂N); 65.2 (d, ²J_{CP} = 5 Hz, CHCH₂OP); 70.1, 70.5, 71.6 (CH₂OCH₂, CH₂OCH); 77.7 (CH); 79.2 (C(CH₃)₃); 116.7 (t, ²J_{CF} = 23 Hz, CF₂CH=CH); 143.0 (t, ³J_{CF} = 9 Hz, CF₂CH=CH trans); 145.0 (CF₂CH=CH cis); 157.2 (C(O)). ¹⁹F NMR (CDCl₃/CD₃OD): identical to that of **6**. ³¹P {¹H} NMR (CDCl₃/CD₃OD): δ 2.1 (s).

[F8E11][C16]OPE, 2.3 TFA (white powder; 75% yield from **7b**): TLC (CHCl₃/MeOH/NH₄OH 8:2:0.2, molybdenum blue and H₂SO₄): R_f = 0.30. ¹H NMR (CDCl₃/CD₃OD): δ 0.85 (t, ³J = 6.1 Hz, 3H, CH₃); 1.10–1.45 (broad s, 38H, CH₃(CH₂)₁₃, (CH₂)₆(CH₂)₂O); 1.45–1.67 (m, 4H, CH₂CH₂O); 2.10–2.30 (m, 2H, CH=CHCH₂); 3.05–3.27 (m, 2H, CH₂N); 3.35–3.70 (m, 7H, CH₂OCH₂, CH₂OCH); 3.80–4.01 (m, 2H, CHCH₂OP); 4.01–4.21 (m, 2H, POCH₂CH₂N); 5.40–5.74 (m, 1H, CF₂CH=CH); 6.35–6.50 (m, 1H, CF₂CH=CH). ¹³C NMR (CDCl₃/CD₃OD): δ 13.6 (CH₃); 22.4 (CH₃CH₂); 25.9 (CH₂CH₂-CH₂O); 27.8, 28.8, 29.2, 29.4, 29.6, 30.0 (CH₂CH₂O, CH₃(CH₂)₂(CH₂)₁₀, CH=CHCH₂(CH₂)₅); 31.8 (CH₃CH₂CH₂, =CHCH₂); 40.2 (d, ³J_{CP} = 6 Hz, CH₂N); 61.5 (d, ²J_{CP} = 5 Hz, POCH₂CH₂N); 65.2 (d, ²J_{CP} = 5 Hz, CHCH₂OP); 70.1, 70.5, 71.6 (CH₂OCH₂, CH₂OCH); 77.7 (d, ³J_{CP} = 8 Hz, CH); 116.7 (t, ²J_{CF} = 23 Hz, CF₂CH=CH); 143.0 (t, ³J_{CF} = 9 Hz, CF₂CH=CH trans); 145.0 (CF₂CH=CH cis). ¹⁹F NMR (CDCl₃/CD₃OD): δ -72.6 (1.3F, CF₃COOH); -81.3 (3F, CF₃); -107.2 (0.2F, CF₂CH cis); -112.7 (1.8F, CF₂CH trans); -122.0, -122.5, -123.2, -124.0 (2F, 4F, 2F, 2F, CF₃CF₂(CF₂)₅); -126.6 (2F, CF₃CF₂). ³¹P NMR (CDCl₃/CD₃OD): δ 1.0 (s). Anal. (C₄₀H₆₅F₁₇NO₆P + 1.3 CF₃COOH + 2H₂O) Calcd: C, 42.64; H, 5.55; N, 1.16. Found: C, 42.56; H, 5.45; N, 1.31.

rac-3-[11-(F-Octyl)undecyl]-2-(hexadecyl)glycero-1-phosphoethanolamine, [F8C11][C16]OPE. rac-1-[11-(F-Octyl)-10-undecyl]-2-(hexadecyl)glycero-3-phospho(tert-butoxycarbonyl)ethanolamine, 7c: (40% yield) TLC (CHCl₃/MeOH: 7:3 v/v; molybdenum blue, ninhydrine): R_f = 0.65. ¹H NMR (CDCl₃/CD₃OD): δ 0.85 (t, ³J = 6.1 Hz, 3H, CH₃); 1.04–1.37 (s large, 40H, (CH₂)₇-(CH₂)₂O, CH₃(CH₂)₁₃); 1.37–1.70 (m, 15H, CH₂CH₂O, CF₂CH₂CH₂, CH₃(BOC)); 2.04 (tt, ³J = 7.5 Hz, ³J_{HF} = 19.0 Hz, 2H, CF₂CH₂); 3.12–3.35 (m, 2H, CH₂N); 3.35–3.69 (m, 7H, CH₂OCH₂, CH₂OCH); 3.73–4.03 (m, 4H, CH₂-OPOCH₂). ¹³C NMR (CDCl₃/CD₃OD): δ 13.9 (CH₃); 20.1 (t, ³J_{CF} = 4 Hz, CF₂CH₂CH₂); 22.6 (CH₃CH₂); 26.1

(CH₂CH₂CH₂O); 28.3 (CH₃(BOC)); 29.1, 29.3, 29.4, 29.5, 29.6, 29.7, (CH₂CH₂O, CH₃(CH₂)₂(CH₂)₁₀, CF₂(CH₂)₂-(CH₂)₆); 30.9 (t, ²J_{CF} = 22 Hz, CF₂CH₂); 31.9 (CH₃-CH₂CH₂); 40.9 (d, ³J_{CP} = 6 Hz, CH₂N); 64.8 (d, ²J_{CP} = 5 Hz, POCH₂CH₂N); 65.0 (d, ²J_{CP} = 5 Hz, CHCH₂OP); 70.6, 70.7, 71.7 (CH₂OCH₂, CH₂OCH); 77.1 (CH); 79.0 (C(CH₃)₃); 157.2 (C(O)). ¹⁹F NMR (CDCl₃/CD₃OD): identical to that of **2c**. ³¹P {¹H} NMR (CDCl₃/CD₃OD): δ -2.5 (s).

[F8C11][C16]OPE, 2.4 TFA (white powder; 80% yield from **7c**): TLC (CHCl₃/MeOH/NH₄OH 8:2:0.2, molybdenum blue and H₂SO₄): R_f = 0.36. ¹H NMR (CDCl₃/CD₃OD): δ 0.80 (t, ³J = 6.1 Hz, 3H, CH₃); 1.02–1.40 (broad s, 40H, (CH₂)₇CH₂CH₂O, CH₃(CH₂)₁₃); 1.40–1.67 (m, 6H, CH₂CH₂O, CF₂CH₂CH₂); 1.97 (tt, ³J = 7.5 Hz, ³J_{HF} = 19.0 Hz, 2H, CF₂CH₂); 2.99–3.19 (m, 2H, CH₂N); 3.38–3.64 (m, 7H, CH₂OCH₂, CH₂OCH); 3.70–3.91 (m, 2H, CHCH₂OP); 3.91–4.12 (m, 2H, POCH₂CH₂N). ¹³C NMR (CDCl₃/CD₃OD): δ 13.8 (CH₃); 20.1 (t, ³J_{CF} = 4 Hz, CF₂CH₂CH₂); 22.5 (CH₃CH₂); 25.9 (CH₂CH₂CH₂O); 29.0, 29.2, 29.3, 29.6, 29.9 (CH₂CH₂O, CH₃(CH₂)₂(CH₂)₁₀, CF₂(CH₂)₂(CH₂)₆); 30.9 (t, ²J_{CF} = 22 Hz, CF₂CH₂); 31.8 (CH₃CH₂CH₂); 40.2 (d, ³J_{CP} = 6 Hz, CH₂N); 61.7 (d, ²J_{CP} = 5 Hz, POCH₂CH₂N); 65.2 (d, ²J_{CP} = 5 Hz, CHCH₂OP); 70.1, 70.6, 71.76 (CH₂OCH₂, CH₂OCH); 77.7 (d, ³J_{CP} = 8 Hz, CH). ¹⁹F NMR (CDCl₃/CD₃OD): δ -74.6 (0.2F, CF₃COOH); -79.6 (3F, CF₃); -113.3 (2F, CF₂CH₂); -120.7 (6F, (CF₂)₃CF₂CH₂); -121.6 (2F, CF₃(CF₂)₂CF₂); -122.4 (2F, CF₃CF₂CF₂); -125.0 (2F, CF₃CF₂). ³¹P NMR (CDCl₃/CD₃OD): δ -3.6 (s). Anal. (C₃₅H₆₇F₁₈NO₈P + 0.2 CF₃COOH + 1H₂O) Calcd: C, 46.07; H, 6.43; N, 1.33. Found: C, 46.09; H, 6.58; N, 1.41.

Differential Scanning Calorimetry (DSC). For DSC measurements in the +4 to 95 °C temperature range, the samples were prepared by weighing the powdered phospholipid (~10 mg) into pierced aluminum pans and adding a weighed amount of deionized water to obtain a water concentration of 60% (w/w). DSC measurements were carried out at a rate of 10 °C/min with a Mettler-Toledo DSC 821e apparatus which was previously calibrated using indium, zinc, and lead standards. The samples were sealed and heated in a static atmosphere. Transition enthalpies and temperatures were determined after several heating/cooling cycles. The reported values of the T_c represent the temperature at maximum excess heat capacity.

Preparation of Complexes Composed of the Cationic Lipids, the Helper Lipids and Plasmid pTG11033. The plasmid pTG11033 was produced by Transgène. The endotoxin content of the plasmid preparation was checked using a Limulus Amebocyte Lysate kit (Biogenic, Maurin, France). This value was below 1 endotoxin unit/mg of plasmid, hence below the 5 e.u./mg of DNA recommended for in vivo protocols. The quantities of compounds used were calculated according to the desired DNA concentration of 0.1 mg/mL, the N/P ratio, the molar weight, and the number of positive charges in the selected cationic lipid (CL). The N/P ratio of 10, for example, corresponds to the molar amount of CL necessary to have a ratio of 10 amino group nitrogens (for 1 mol of CL) per one phosphate in the DNA (330 Da mean Mw), as described elsewhere (1, 22, 27). The DNA/CL: colipid(s) complex is formulated by adding a desired volume of the liposomal CL:colipid(s) preparation at a CL concentration of 10 mg/mL in 5% glucose to the desired volume of DNA solution to reach a DNA concentration of 0.1 mg/mL. Thus for the preparation of the DNA/DOGS:DOPE (1:1 mol) complex at N/P ratio 5 and 0.1 mg/mL DNA, the desired volume of DOPE (10 mg/mL chloroform solution) to get mol:mol DOGS:DOPE was

added to 50 μL of DOGS solution (10 mg/mL in EtOH), and then the mixture was transferred to a borosilicate glass tube (16 $\times$ 100 mm). The solvent was evaporated in Rotavap evaporation system (45 $^{\circ}\text{C}$, 30 pm, 0.2 bar, 40 min). 50 μL of 5% glucose were added to the film obtained. The preparation was vortexed for 2 h and then sonicated for 5–10 min to yield a liposomal preparation of 50–100 nm mean size as measured by photon correlation spectroscopy (see below). Then, 47.9 μL of this preparation was added to 952.1 μL of DNA solution [(100 μL DNA (1 mg/mL) diluted with 852.1 μL of 5% glucose)]. This preparation was vortexed for 10 s and was used within 1 h for the particle size measurements and the in vitro transfection experiments.

Measurement of the Size of the Lipoplexes. The sample was diluted with 5% glucose in the measurement tube and homogenized, and the average sizes were measured by photon correlation spectroscopy using a Coulter N4Plus particle size analyzer, as described in ref 5. The formulations and analyses were reproduced twice.

Agarose Gel Electrophoresis. Each sample was analyzed and plasmid integrity in each sample was confirmed by electrophoresis after decomplexing the lipoplex with sodium dodecyl sulfate, following the procedures described in ref 5.

In Vitro Transfection of A549 Cells. Twenty-four hours before transfection, A549 cells (epithelial cells derived from human pulmonary carcinoma) were grown in Dulbecco-modified Eagle culture medium (DMEM) (GIBCO-BRL, Life Technologies, Cergy Pontoise, France), containing 10% fetal calf serum, FCS (SIGMA, Saint Quentin Fallavier, France), in 96-well plates (2 $\times$ 10⁴ cells per well), in a wet (37 $^{\circ}\text{C}$) and 5% CO₂/95% air atmosphere. Volume of DNA/CL:colipid(s) (1:1 mol) or (1:2 mol) lipoplex (5 and/or 1 μL) was diluted to 100 μL in DMEM supplemented with 10% FCS in order to obtain various amounts of DNA (0.5 and/or 0.1 μg , respectively) in the preparation. The culture medium was removed and replaced by 100 μL of DMEM supplemented with 10% FCS and containing the desired amount of DNA. After 4 and 24 h, 50 μL and 100 μL of DMEM supplemented with 30 and 10% FCS, respectively, were added. Forty-eight hours after transfection, the culture medium was discarded, and the cells were washed twice with 100 μL of PBS and then lysed with 50 μL of lysis buffer (Promega, Charbonnières, France). The lysates were frozen at -40°C awaiting analysis of luciferase activity. These measurements were done for 10 s on 10 μL of the lysis mixture in a Berthold LB96P luminometer in dynamic mode, using the "Luciferase" determination system (Promega) in 96-well plates. The total protein concentration per well was determined using conventional techniques (BCA test, Pierce, Montluçon, France). For cells grown in the absence of lipoplexes, a well contains around 30 to 50 μg of proteins. The percentage of cell viability of the lipoplexes was calculated as the ratio of the total protein amount per well of the transfected cells relative to that measured for untreated cells $\times$ 100%. The given means $\pm$ SEM were calculated from four independent experiments.

Statistical Analyses. Statistical analyses were performed using a Student *t* test. The difference between two means was considered as statistically significant when *p* is $\leq$ 0.05.

RESULTS AND DISCUSSION

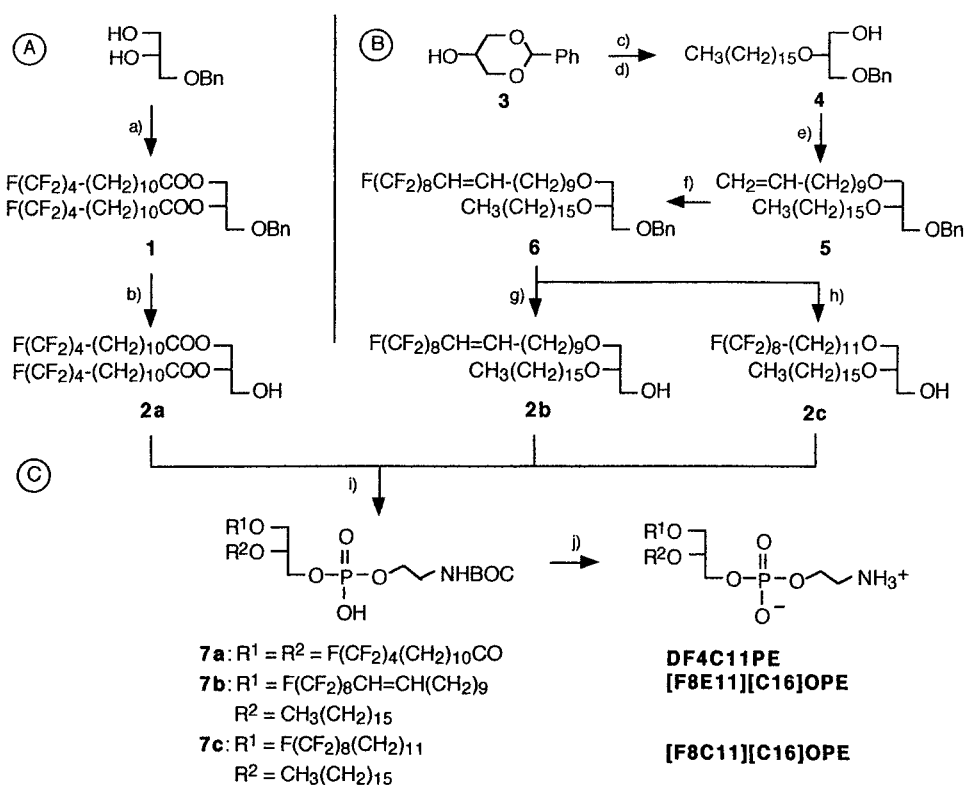
Synthesis. The synthesis of the fluorocarbon DF4C11PE, [F8E11][C16]OPE, and [F8C11][C16]OPE

phosphoethanolamines from their respective glycerol precursors **2a–c** was performed according to well documented procedures that have been published for the preparation of DF4C11OPE (19). These procedures, illustrated in Scheme 2 part C, involved phosphorylation of these **2a–c** synthons with POCl₃, condensation with *N*-BOC-ethanolamine, hydrolysis, and then BOC-deprotection using trifluoroacetic acid (TFA), affording the expected *F*-PEs as TFA salts (15–50% yields from **2a–c**).

The ester-linked glycerol derivative **2a** was obtained in two steps from benzylglycerol which included conventional acylation and benzyl deprotection by hydrogenolysis (Scheme 2, part A). The more demanding preparation of the mixed fluorocarbon/hydrocarbon 1,2-di-ether linked glycerol derivatives **1b,c** (Scheme 2, part B) was best performed from 1,3-*O*-benzylideneglycerol **3** using a strategy developed for close analogues (19). In this cyclic-strained protected glycerol, the secondary hydroxyl function has been shown to be more accessible and easier to alkylate than in acyclic protected analogues (23). Alkylation of **3** performed under phase catalysis conditions using hexadecyl bromide, followed by the reductive cleavage of the 1,3-benzylidene-protecting group with BH₃/THF (23) afforded derivative **4** (70% yield). Subsequent alkylation of the hydroxyl thus liberated in **4** using 11-undecenyl bromide yielded the benzyl-protected derivative **5** (70% yield). The addition of the linear perfluorooctyl iodide (C₈F₁₇I) on the terminal CH₂=CH—double bond of **5** was performed using the modified method of Burton and Kehoe (24): this two-step one-pot reaction led to the perfluorooctyl unsaturated derivative **6**, in 75% yield. According to ¹⁹F NMR which showed more particularly the presence of two resonances for the CF₂ in α to the double bond, the isolated compound consisted in a mixture of *E*- and *Z*-isomers. The most abundant one was the *E*-isomer (at least 85%). These products were also contaminated by a compound (less than 5%) which possesses a CF₂—CH₂—CH=CH— sequence, as a result of a β,γ -HI elimination during the perfluoroalkylation step (19, 24). This was evident from the presence of, respectively, a doublet of triplets (³*J*_{HH} = 7 Hz, ³*J*_{HF} = 18 Hz) at 2.7 ppm, a triplet (²*J*_{CF} = 23 Hz) at 34.7 ppm for the CH₂ group and a CF₂ resonance at -115.8 ppm, in the ¹H, ¹³C, and ¹⁹F NMR spectra of **6**.

Selective benzyl-deprotection was performed by action of excess FeCl₃ in anhydrous CH₂Cl₂ followed by hydrolysis (25). This procedure afforded the mixed fluorocarbon/hydrocarbon unsaturated 1,2-glycerol derivative **2b** in 75% yield. The saturated and benzyl-deprotected analogue **2c** was obtained in nearly 60% yield by simultaneous benzyl-deprotection and double-bond hydrogenation using high-pressure hydrogenolysis conditions (40 atm H₂ in the presence of 10% Pd/C). Under milder hydrogenolysis conditions (1 atm H₂), double-bond hydrogenation but only partial debenzylation was observed.

Thermotropic Phase Behavior. The thermotropic phase behavior of samples consisting of hydrated powders of the *F*-PEs was investigated by DSC. Table 1 collects the thermodynamic parameters (*T*_c, gel (crystalline) to liquid-crystalline main phase transition and/or lamellar to inverted hexagonal phase transition, see hereafter, and their associated ΔH and ΔS) determined on these dispersions by DSC. The phase behavior of hydrated DF4C11OPE has been investigated in detail by X-ray diffraction, DSC, and optical microscopy with polarized light (26). This latter study, performed as a function of

Scheme 2^{a–j}

^a (a) $F(CF_2)_4(CH_2)_{10}COCl$ /pyridine/ Et_2O ; (b) H_2 /Pd/C/THF; (c) $CH_3(CH_2)_{15}Br$ /phase transfer catalysis; (d) BH_3 /THF; (e) NaH/toluene, then $CH_2=CH(CH_2)_9Br$; (f) $C_8F_{17}I/CuCl/NH_2(CH_2)_2OH/t-BuOH$; (g) anhydrous $FeCl_3/CH_2Cl_2$ then H_2O ; (h) H_2 (40 atm)/Pd/C/ $EtOH/AcOH$; (i) 1. $POCl_3/NEt_3/THF$; 2. $HO(CH_2)_2NHBoc/CHCl_3$ /pyridine; 3. H_2O ; (j) TFA/ CH_2Cl_2 .

Table 1. Thermodynamic Parameters of the Lamellar to Inverted Hexagonal HII Phase Transition for the Fluorinated Glycerophosphoethanolamines (F-PEs). DSC Experiments Were Performed in Deionized Water (60% w:w)

F-PE compound	$T_c (\pm 1^\circ C)$ ($\Delta T_{1/2}$) ^a	$\Delta H (\pm 5\%)$ (kJ/mol)	ΔS (J/mol·K)
DF4C11PE	25 (3)	36.4	122
[F8E11][C16]OPE	38 (4)	2.9	9.3
[F8C11][C16]OPE	45 (5)	27.7	87
DF4C11OPE ^b	32 (2)	20.2	66.2

^a $\Delta T_{1/2}$ is the transition width at half-maximal excess specific heat capacity. ^b Data from ref 26.

temperature, showed that DF4C11OPE, in excess water, forms a hexagonal phase above 28 °C, (i.e., below but close to the phase transition temperature measured by DSC). It showed also that the unique and broad phase transition detected by DSC consisted in a superposition of transitions, which included the lamellar gel to liquid-crystalline phase transition followed, almost simultaneously, by a transition to a hexagonal phase. Owing to their very close molecular structures, one can therefore assume that the unique and broad phase transition detected by DSC for the other fluorinated PEs is also most likely a transition to a hexagonal phase. The presence of a HII phase at a temperature above the phase transition temperature detected by DSC was further attested by optical microscopy with polarized light.

Lipoplex Formation and Characterization. The capability of the various F-PEs as colipid of cationic lipids, CL (= DOGS, pcTG90 or DOTAP) to form lipoplexes was analyzed for various CL:DOPE:F-PE molar ratios $[1:(1-x):x]$ with $x = 0.5, 0.75$, or 1, and $[1:(2-y):y]$ with $y = 1, 1.5$, or 2, and for various N/P ratios (10, 5, 2.5, 1.25, and 0.8), as compared to CL:DOPE (1:1) or (1:2

mol) control lipoplexes. These studies were performed with pTG11033 plasmid, also used for the in vitro transfection assays (see next section). The procedure applied for the lipoplex preparation relies on the dilution of a liposomal solution obtained from CL and the colipid(s) in 5% glucose with the DNA solution in 5% glucose, using N/P ratios of 10, 5, 2.5, 1.25, and 0.8 (N = number of lipid cations; P = number of DNA phosphates (22, 27)). These formulations were analyzed by agarose gel electrophoresis which showed the absence of « free » plasmid for N/P ratios from 10 to 1.25, DNA being totally protected from ethidium bromide interaction. By contrast, the plasmid was accessible to ethidium bromide in the case of N/P 0.8 formulations. These formulations were also analyzed by light scattering spectroscopy (LSS) for size determination (which is an important parameter to control when in vivo uses are contemplated or when their in vitro transfection efficiency is evaluated (16, 22)). For the CL:colipid(s) (1:1 mol) formulations, and irrespective of the nature of CL and colipid(s) and of colipid composition, one observed most often lipoplexes of mean size in the 50–180 nm range for an excess of cationic charges ($N/P \geq 2.5$) and in the 150–400 nm range for $N/P = 0.8$ while precipitates were systematically detected for $N/P = 1.25$ (data not shown). Concerning the pcTG90:colipid(s) (1:2 mol) formulations, a very similar behavior was observed, i.e., lipoplexes of mean size in the 50–150 nm range for $N/P \geq 2.5$, in the 150–300 nm range for $N/P = 0.8$, and precipitates for $N/P = 1.25$ except when co-formulated with DF4C11OPE or DF4C11PE (data not shown). This latter result is noteworthy as most often precipitates for a N/P ratio of 1.25 (which corresponds to almost neutrally charged lipoplexes) were reported in the literature (4, 5, 28). Concerning the N/P 0.8, 2.5, 5, and 10 formulations, our results are also close to those

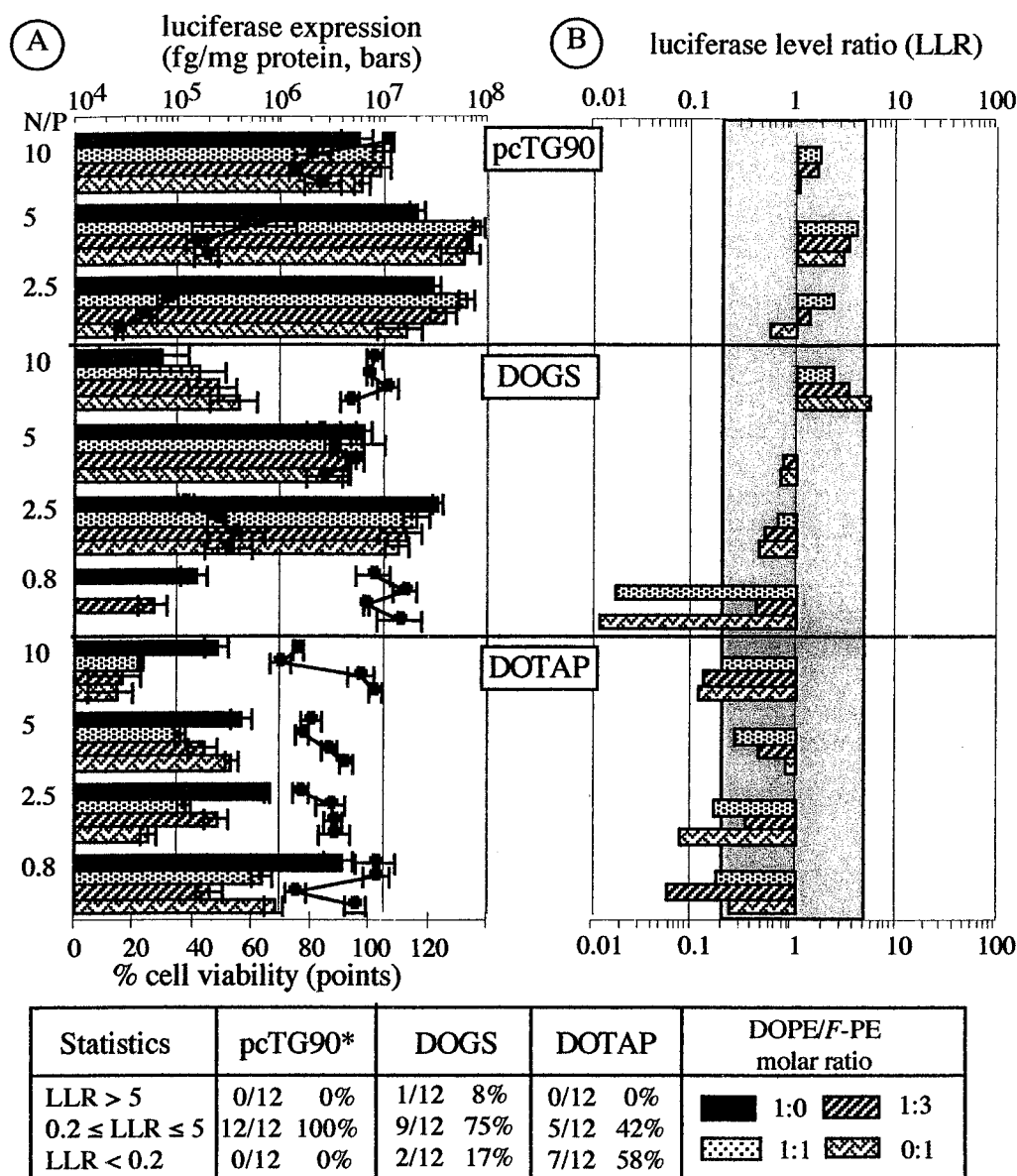


Figure 1. A: Luciferase expression (bars) and cell viability (points) in A549 cells of the lipoplexes prepared in 5% glucose and made of CL(= pcTG90, DOGS, or DOTAP):DOPE:[F8E11][C16]OPE (1:1 - $x:x$ with $x = 0.5, 0.75$, or 1) and plasmid pTG11033 (DNA) for various N/P ratios, as compared to control DOPE lipoplexes ($x = 0$). The given means $\pm$ SEM were calculated from four independent experiments. B: Transfection efficiency of the [F8E11][C16]OPE-based lipoplexes vs that of their corresponding DOPE control lipoplexes. The luciferase level LLR is the ratio of luciferase amount measured for the fluorinated formulation vs that measured for its corresponding DOPE control. The efficiency is significantly higher if LLR is ≥ 5 , significantly lower if LLR ≤ 0.2 , or comparable if $0.2 < \text{LLR} < 5$. When outside this range, $p < 0.01$ were carried out in a pairwise comparison. The values given as statistics correspond to the number of formulations that satisfy the given LLR condition vs the total number of formulations investigated, and to the respective percentages. *The luciferase expression levels (in the 2×10^3 to 7×10^3 fg luciferase per mg protein range) corresponding to the F-PE and control N/P 0.8 pcTG90-based formulations are not shown.

reported for other lipoplexes, i.e., stable dispersions for "negatively" ($N/P = 0.8$) and positively charged lipoplexes ($N/P \geq 2.5$) (4, 5, 28).

As compared with DOPE alone, no impact of the F-PE's nature or of their molecular structure on lipoplex size could be evidenced. Our results indicate that the fluorinated PEs are as effective colipids as DOPE in terms of compacting DNA with DOGS, pcTG90 or DOTAP into small-sized particles. These data suggest further that the lamellar to hexagonal phase transition temperature of the F-PEs, and the expected lower miscibility between the fluorinated colipids and the cationic lipids as compared with DOPE (fluorinated amphiphiles are usually not miscible with hydrocarbon ones (29)) are not determinant for the DNA compaction process when using cationic lipids in combination with such colipids.

In Vitro Transfection. To evaluate the contribution of the various F-PEs to in vitro lipoplex-mediated gene transfer, we examined the transfection potential of the above-described lipoplexes formulated with these F-PEs (alone or in combination with DOPE) as colipids of pcTG90, DOGS, or DOTAP. Both CL:colipid(s) (1:1 mol) and (1:2 mol) formulations were investigated as the pcTG90:colipid(s) (1:1 mol) and (1:2 mol) lipoplexes proved to be more efficient in vitro and in vivo transfection assays, respectively (8, 14, 15). Lipofection was assayed in vitro on lung epithelial A549 cells, from human pulmonary carcinoma. These assays were performed using the luciferase reporter plasmid pTG11033 (pCMV-intronHMG-luciferase-SV40pA, 9572 bps) in the presence of 10% fetal calf serum for 48 h. All the lipoplex formulations described in the precedent section were

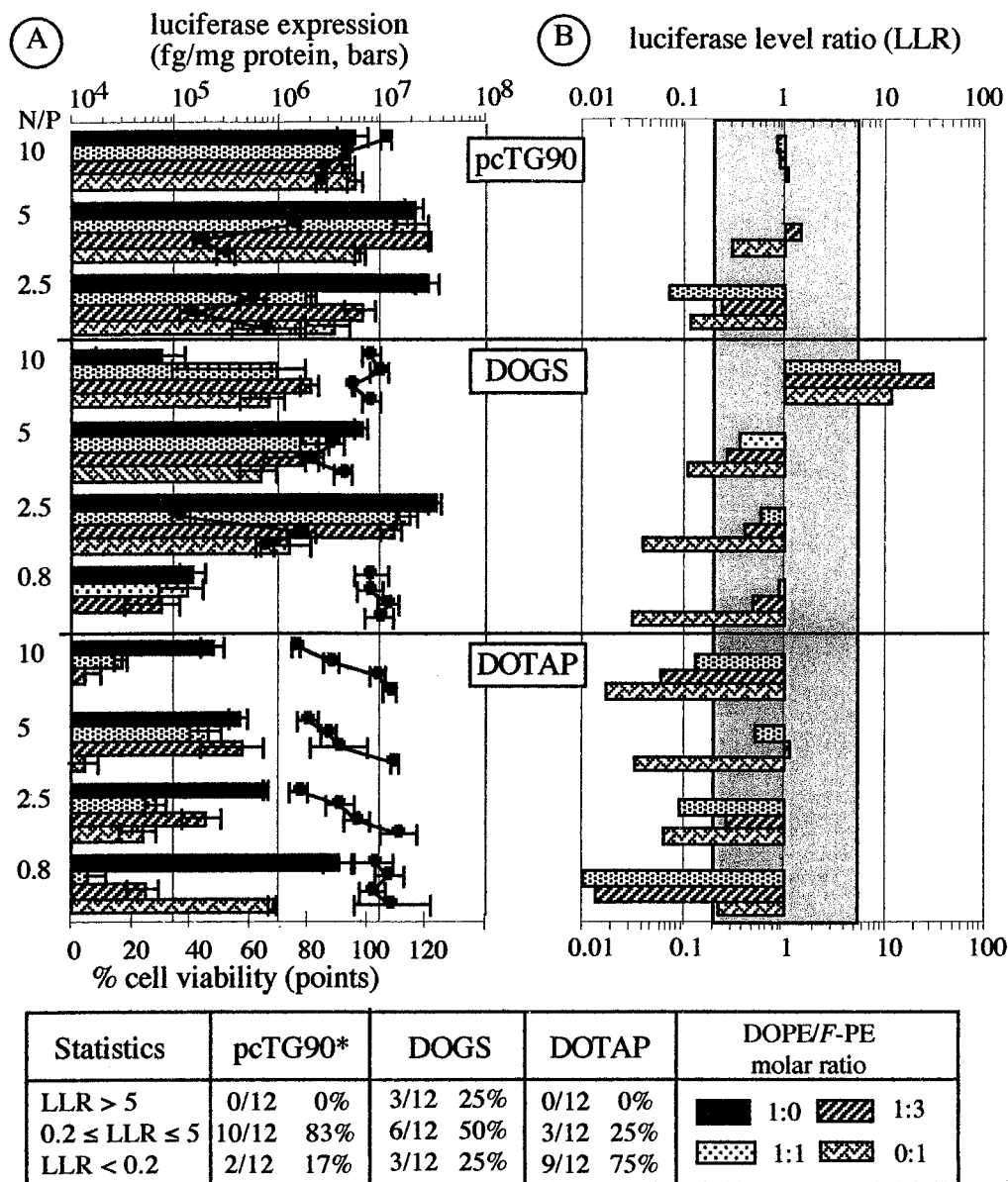


Figure 2. A: Luciferase expression (bars) and cell viability (points) in A549 cells of the lipoplexes prepared in 5% glucose and made of CL(= pcTG90, DOGS, or DOTAP):DOPE:[F8C11][C16]OPE (1:1 - x : x with $x = 0.5, 0.75$, or 1) and plasmid pTG11033 (DNA) for various N/P ratios, as compared to control DOPE lipoplexes ($x = 0$). The given means $\pm$ SEM were calculated from four independent experiments. B: Transfection efficiency of the [F8E11][C16]OPE-based lipoplexes vs that of their corresponding DOPE control lipoplexes. *The luciferase expression levels (in the 2×10^3 to 7×10^3 fg luciferase per mg protein range) corresponding to the F-PE and control N/P 0.8 pcTG90-based formulations are not shown. For more details concerning LLR, and the statistics, see caption of Figure 1. When LLR is outside the 0.2–5 range, $p < 0.01$ in a pairwise comparison.

tested, except those that precipitated. The transfection efficiency of the lipoplexes, expressed in femtograms (fg) of luciferase/mg of protein, was evaluated for a DNA amount of 0.5 μ g/well: in our transfection protocol, a plateau of luciferase expression was generally obtained for such a DNA amount. For comparison, naked DNA and the control lipoplexes based on DOPE as sole colipid were also tested. Cells treated with naked DNA under equivalent conditions showed expression levels of about 10^{2-3} fg of luciferase/mg of protein. The cell viability of the lipoplexes was also checked by determining the total protein amount per well of the transfected cells relative to that measured for untreated cells (for which the total protein amount per well is in a 30–50 μ g/well range).

The transfection and cell viability results for the lipoplexes based on [F8E11][C16]OPE, [F8C11][C16]OPE, DF4C11OPE or DF4C11PE are illustrated in Figures 1A

to 4A for the CL:colipid(s) (1:1 mol) formulations and in Figure 5A for the pcTG90:colipid(s) (1:2 mol) ones. These results indicate that the “fluorinated” lipoplexes formed from any of these F-PEs (used as sole colipid or in combination with DOPE) and any of the cationic lipids (pcTG90, DOGS, or DOTAP) do transfect cells to a larger extent than naked DNA.

Concerning the CL:colipid(s) (1:1 mol) formulations (Figures 1A to 4A), the highest luciferase expression levels (LEL) were most frequently observed for N/P ratios of 2.5, 5, and 10 in the case of pcTG90, of 2.5 and 5 in the case of DOGS (LEL $\geq 10^6$ fg per mg protein), and of 0.8, 2.5, and 5 in the case of DOTAP (LEL $\geq 10^5$ fg per mg protein). A much lower plasmid expression was evidenced for the N/P 0.8 lipoplexes formulated with pcTG90 ($2 \times 10^3 < \text{LEL} < 7 \times 10^3$ fg per mg protein, data not shown) or DOGS ($10^4 < \text{LEL} \leq 10^6$ fg per mg

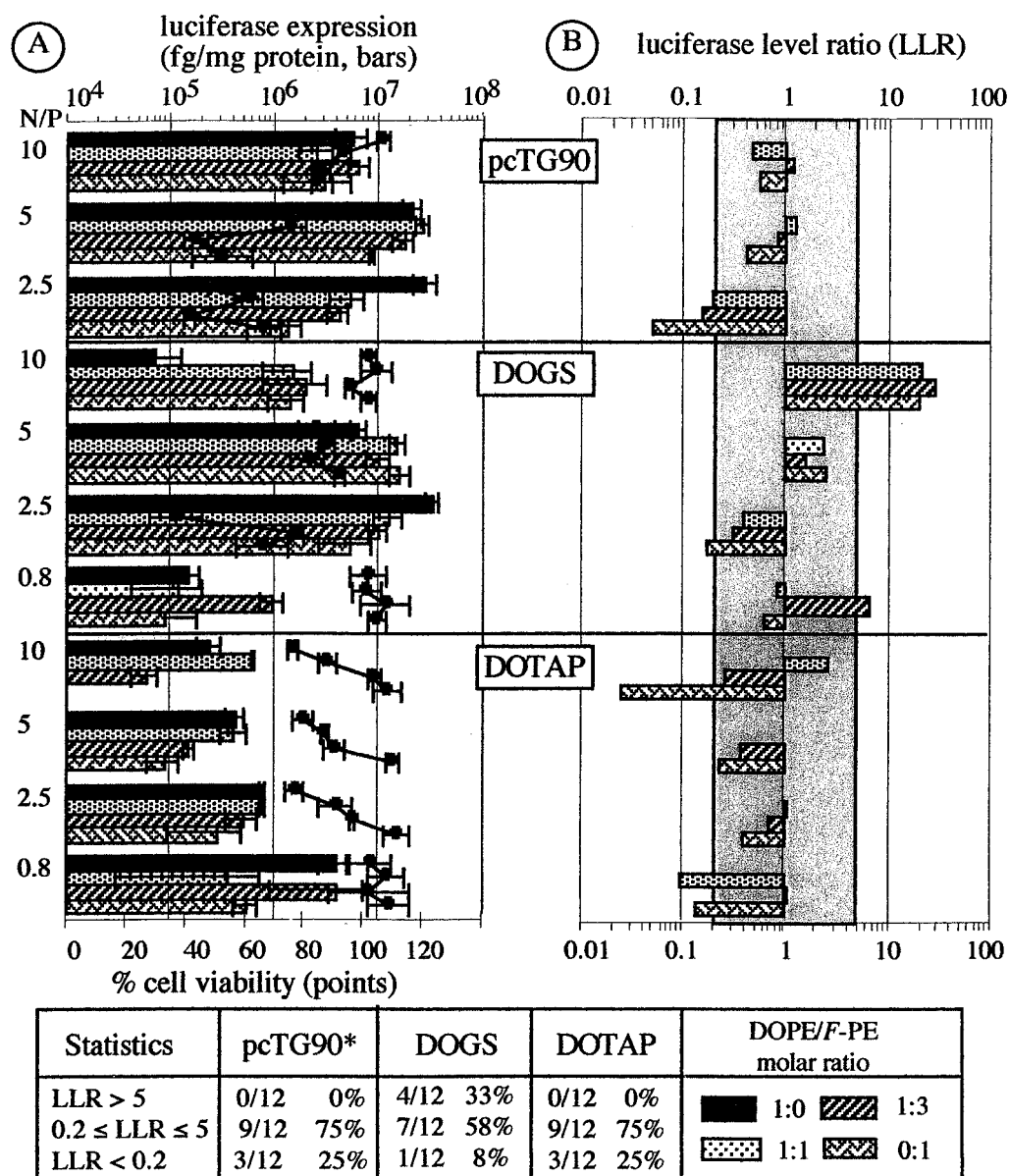


Figure 3. A: Luciferase expression (bars) and cell viability (points) in A549 cells of the lipoplexes prepared in 5% glucose and made of CL(= pcTG90, DOGS, or DOTAP):DOPE:DF4C11OPE (1:1 - $x:x$ with $x = 0.5, 0.75$, or 1) and plasmid pTG11033 (DNA) for various N/P ratios, as compared to control DOPE lipoplexes ($x = 0$). The given means $\pm$ SEM were calculated from four independent experiments. B: Transfection efficiency of the DF4C11OPE-based lipoplexes vs that of their corresponding DOPE control lipoplexes. *The luciferase expression levels (in the 2×10^3 to 7×10^3 fg luciferase per mg protein range) corresponding to the F-PE and control N/P 0.8 pcTG90-based formulations are not shown. For more details concerning LLR, and the statistics, see caption of Figure 1. When LLR is outside the 0.2–5 range, $p < 0.01$ in a pairwise comparison.

protein). Concerning the pcTG90:colipid(s) (1:2 mol formulations (Figure 5A), the highest LEL ($\geq 10^5$ fg per mg protein) were observed for N/P 5 and 2.5 while for N/P 1.25 and 0.8, LELs remained below 10^4 fg per mg protein (results not shown).

The lower transfection efficiency of the N/P 0.8 and 1.25 lipoplexes is most probably related to a lower cellular uptake of these “anionic” and “neutral” formulations as compared with that of the more cationic lipoplexes owing to expected lower lipoplex-cell electrostatic interactions. The mechanism of cellular endocytic uptake of the lipoplexes is indeed mainly governed by electrostatic interactions between the DNA complexes and the anionic proteoglycans expressed at the surface of these adherent cells (30, 31), this uptake increasing further with raising the N/P ratio of the lipoplexes (32). However, lower luciferase expression was evidenced for some of the N/P

10 lipoplexes as compared with that of their corresponding N/P 5 and 2.5 ones (and even of the N/P 0.8 ones in the case of DOTAP). Concomitantly, cell viability of the N/P 10 lipoplexes (and more particularly for pcTG90 or DOGS) was improved, indicating that cytotoxicity induced by these lipoplexes was not responsible for the lower gene expression. That the transfection efficiency of the lipoplexes does not necessarily increase with a N/P ratio increase was recently shown to be related to significant differences in intracellular distribution and trafficking (probably release from the endosomes or lysosomes) of the lipoplexes between these N/P ratios (32).

As illustrated in Figure 6, it appears, for $N/P \geq 2.5$ and irrespective of the fluorinated colipid and of the F-PE:DOPE molar ratio, that most of the “fluorinated” pcTG90- and DOGS-based formulations (90 and 79% of

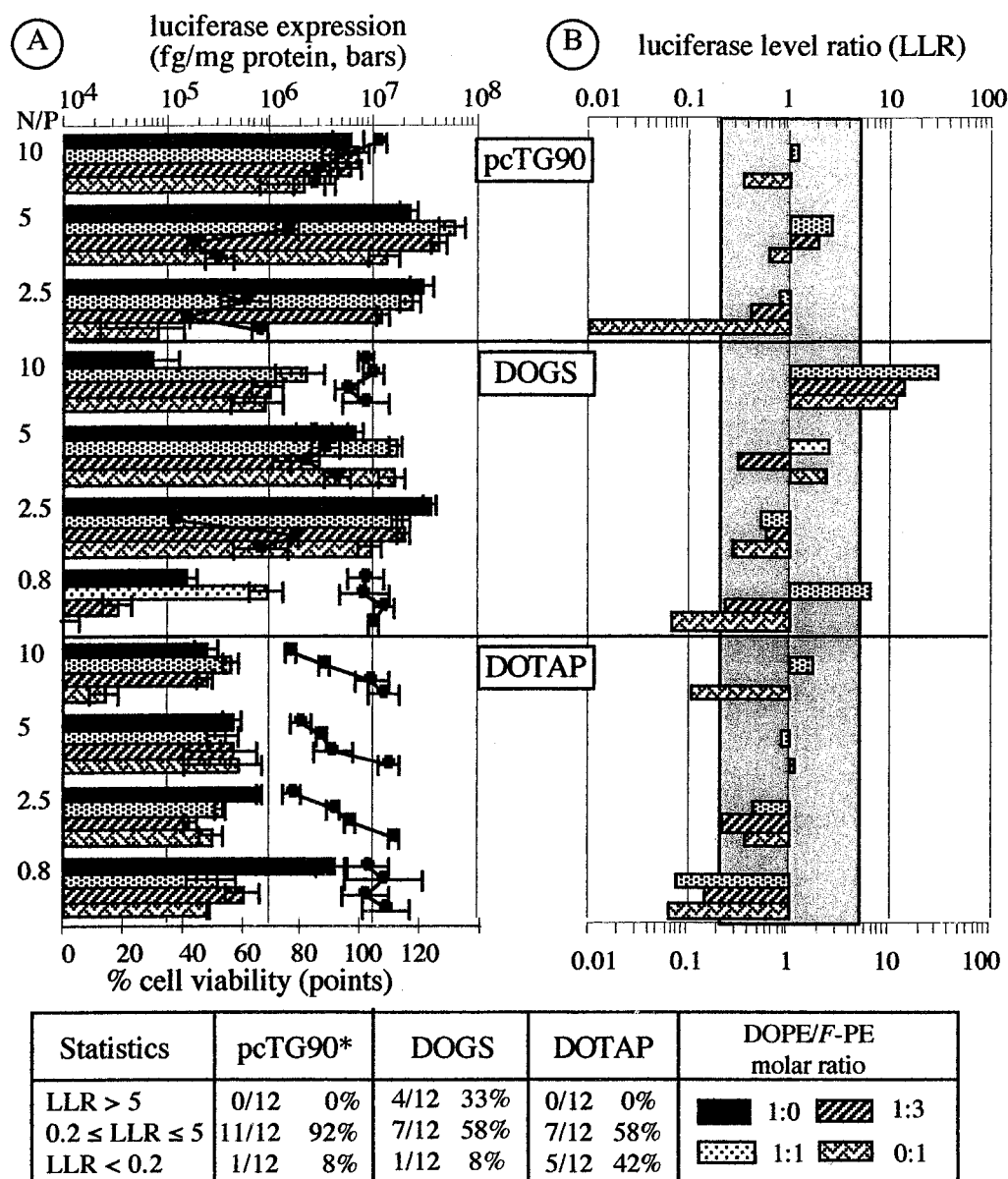


Figure 4. A: Luciferase expression (bars) and cell viability (points) in A549 cells of the lipoplexes prepared in 5% glucose and made of CL (= pcTG90, DOGS, or DOTAP):DOPE:DF4C11PE(1:1 - $x:x$ with $x = 0.5, 0.75$, or 1) and plasmid pTG11033 (DNA) for various N/P ratios, as compared to control DOPE lipoplexes ($x = 0$). The given means $\pm$ SEM were calculated from four independent experiments. B: Transfection efficiency of the DF4C11PE-based lipoplexes vs that of their corresponding DOPE control lipoplexes. *The luciferase expression levels (in the 2.10^3 to 7.10^3 fg luciferase per mg protein range) corresponding to the F-PE and control N/P 0.8 pcTG90-based formulations are not shown. For more details concerning LLR, and the statistics, see caption of Figure 1. When LLR is outside the 0.2–5 range, $p < 0.01$ in a pair-wise comparison.

48 formulations, respectively) were at least 10-fold more efficient ($p < 0.01$) for transfecting A549 cells than their corresponding DOTAP lipoplexes. In a pairwise comparison, two formulations were considered to have comparable transfection efficiency when the LLR ratio of their respective LEL was within the 0.2–5 range, and of significantly different efficiency if this ratio is outside this range, providing its associated p value is ≤ 0.05 . One should further underscore the higher transfection potential of the pcTG90-based lipoplexes when compared with the DOGS ones: if 60% of the 48 pcTG90 formulations investigated were found to lead to comparable LELs to those of their corresponding DOGS complexes, 35% of the 48 pcTG90 formulations led however to LELs that were at least 5-fold higher ($p < 0.05$). The transfection efficacy sequence evidenced for the F-PE-based lipoplexes (i.e., pcTG90 $\geq$ DOGS $>$ DOTAP) for $N/P \geq 2.5$ is further

identical to that observed for the lipoplexes based on DOPE as sole colipid. The higher in vitro transfection efficiency of the pcTG90 and DOGS lipopolyamines as compared with that of DOTAP can be attributed to their endosomolytic activity related to the presence of protonable amine functions of low pK_a ("proton sponge" effect) (2, 14, 15). However, for the "negatively charged" lipoplexes (N/P 0.8), the opposite tendency was found for both the F-PEs and DOPE, as shown in Figure 6. Their transfection efficiency was indeed seen to decrease along the sequence DOTAP $>$ DOGS $>$ pcTG90. Concerning the fluorinated lipoplexes, 63 and 100% of the 16 DOTAP-based formulations were significantly more efficient than their corresponding DOGS- (at least 10-fold, $p < 0.01$) or pcTG90-based lipoplexes (at least 10- to 100-fold, $p < 0.01$), respectively, and 69% of the 16 DOGS-based lipoplexes were significantly more efficient (at least 10-

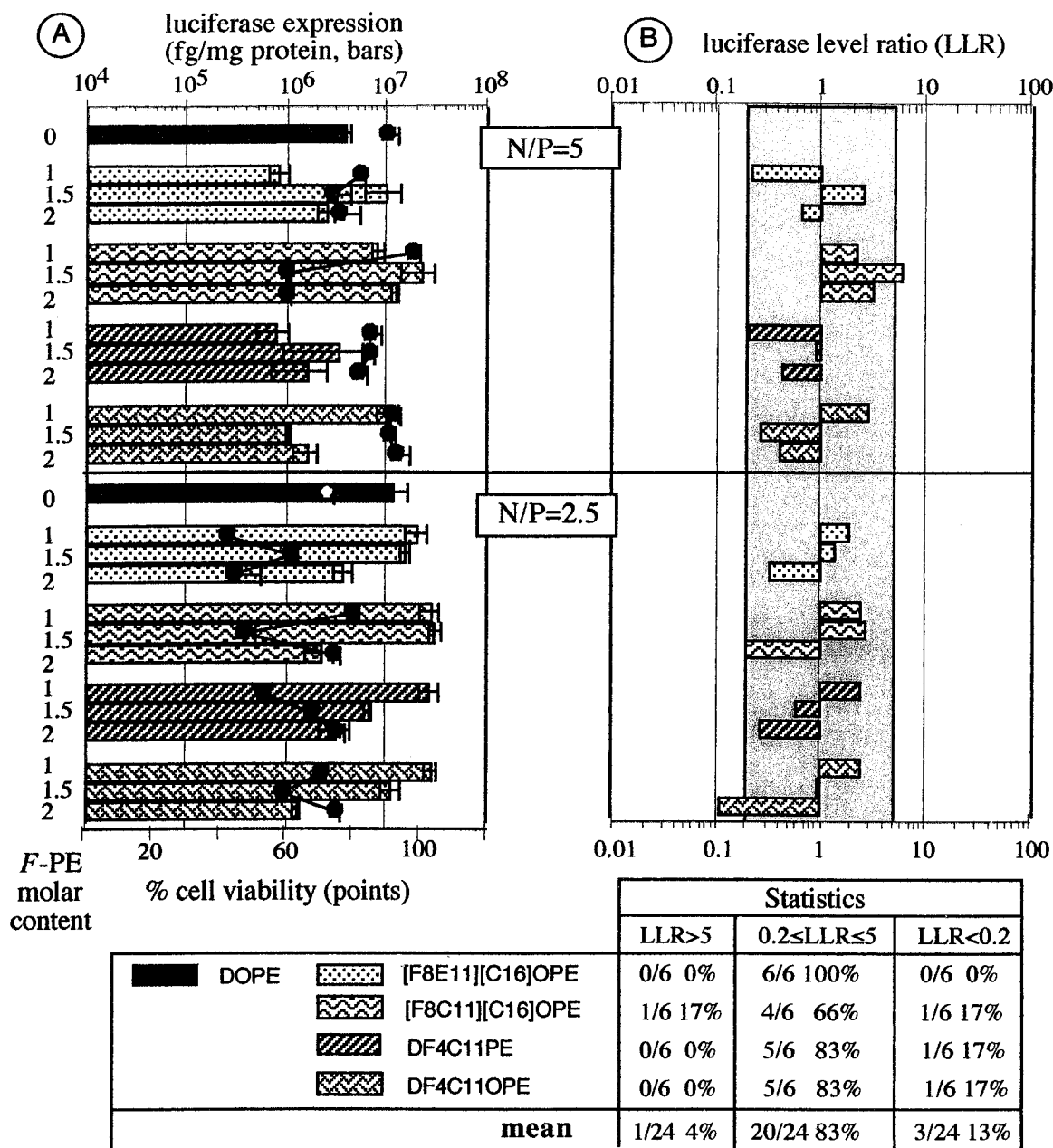


Figure 5. A: Luciferase expression (bars) and cell viability (points) in A549 cells of the lipoplexes prepared in 5% glucose and made of pcTG90:DOPE:F-PE (1:2 - y:y with $y = 1, 1.5$, or 2) and plasmid pTG11033 (DNA) for N/P 2.5 and 5, as compared to control DOPE lipoplexes ($y = 0$). The given means $\pm$ SEM were calculated from four independent experiments. B: Transfection efficiency of the F-PE-based lipoplexes vs that of their corresponding DOPE control lipoplexes. For more details concerning LLR, and the statistics, see caption of Figure 1. When LLR is outside the 0.2–5 range, $p < 0.01$ in a pair-wise comparison.

fold, $p < 0.01$) than the pcTG90-based ones. As for the cationic lipids, the different tendency observed for their N/P 0.8 lipoplexes as compared with that found for their more cationic ($N/P \geq 2.5$) ones is most likely attributable to significant differences in the mechanism of cellular uptake and of intracellular traffic of the lipoplexes between these cationic lipids and N/P values.

Irrespective of the nature of the F-PE and of the DOPE: F-PE molar ratio, increasing the colipid content from 50 to 66.7% mol (1:1 to 1:2 formulations) led, as shown in Figure 7, most often for the N/P 5 lipoplexes to a significant decrease in transfection (67% of the 12 F-PE formulations) while comparable and higher LELs were observed for 67 and 33% of the 12 N/P 2.5 F-PE formulations, respectively. These differences can tentatively be attributed to differences in intracellular distribution and

trafficking of the lipoplexes between these N/P and pcTG90:colipid molar ratios (32). One can further observe that, whatever the N/P ratio, [F8C11][C16]OPE constituted the sole colipid (among the F-PEs and DOPE) for which transfection was not affected by or increased with this CL:colipid molar ratio increase.

The main objectives of this study were aimed at examining the lipid helper effect on lipofection of the F-PEs as compared with that of DOPE and at determining whether their lipid helper potential was cationic lipid specific or not. Our results indicate that the F-PEs display such a lipid helper effect that is most often comparable with that of DOPE. However, their helper effect shows some dependency on the cationic lipid. This is strongly supported by the statistics deduced from panels B of Figures 1 to 4 and of Figure 5 for the (1:1)

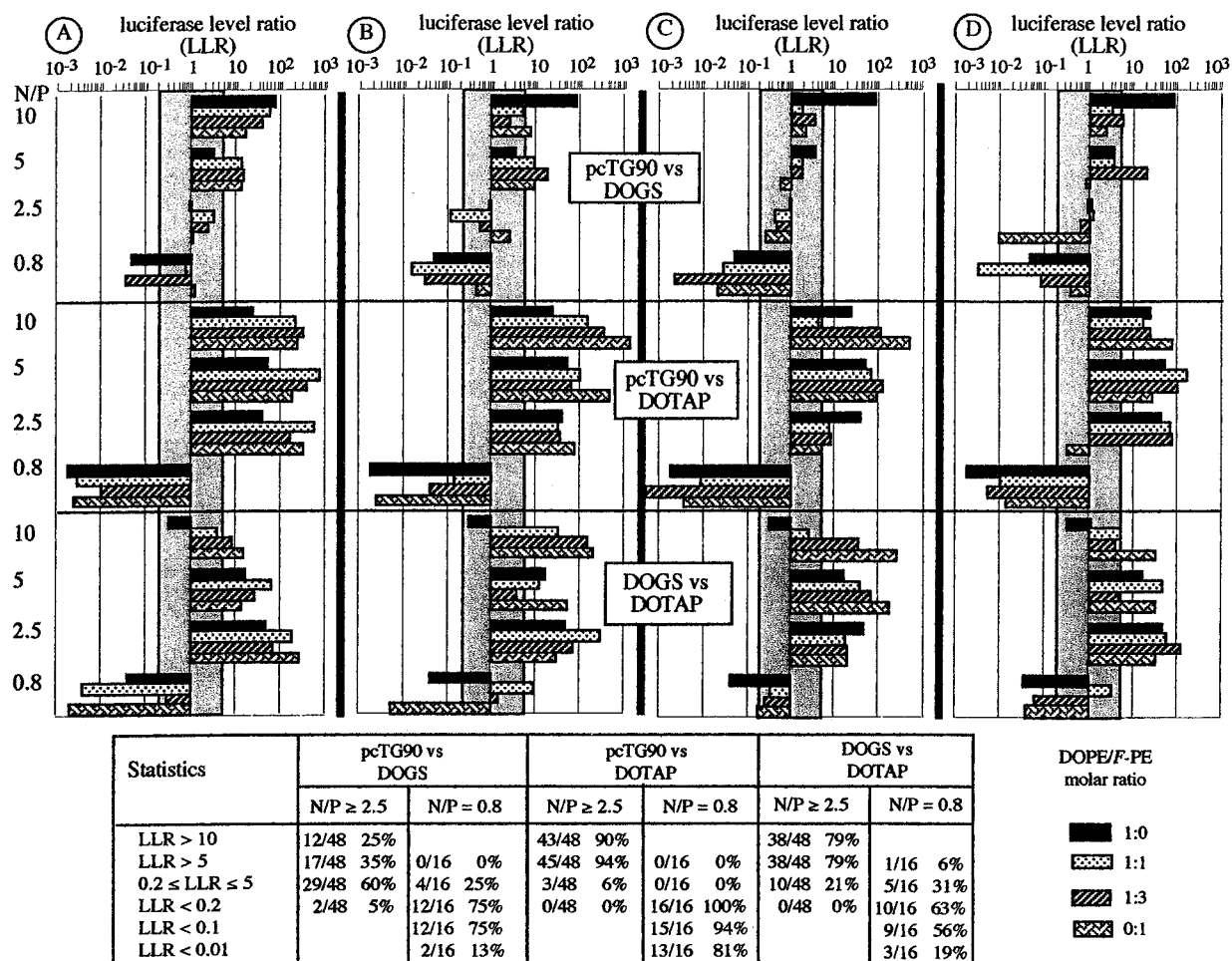


Figure 6. Dependence of the *F*-PE lipid helper effect with the cationic lipid. The luciferase level ratios, LLR, correspond to the ratios of luciferase amount measured for the formulations based on [F8E11][C16]OPE (A), [F8C11][C16]OPE (B), DF4C11OPE (C), or DF4C11PE (D) and (i) on pcTG90 vs that measured for their corresponding DOGS (upper section) or DOTAP (middle section) lipoplexes, or (ii) on DOGS vs that measured for their corresponding DOTAP (lower section) lipoplexes. For more details concerning LLR, and the statistics, see caption of Figure 1. When LLR is outside the 0.2–5 range, $p < 0.01$ in a pair-wise comparison.

and (1:2 mol) formulations, respectively. These panels display the luciferase level ratios (LLR) measured for the formulations based on-[F8E11][C16]OPE, [F8C11][C16]OPE, DF4C11OPE, or DF4C11PE, respectively, vs that measured for their corresponding control lipoplexes (which contain DOPE as sole colipid). The “helper” effect related to the presence of *F*-PE (in place of part of or of all DOPE) is significantly higher or lower to that of DOPE if this ratio is higher or lower than 5 or 0.2, respectively, providing its associated p value is ≤ 0.05 , and this effect is comparable with that of DOPE if this ratio is within the 0.2–5 range. Irrespective of the *F*-PE chemical structure, of the colipid mixture composition, and of the *N/P* ratio, we found indeed that, when using one of the *F*-PEs as colipid in place of part of or of all DOPE, comparable transfection levels were most frequently measured for the fluorinated lipoplexes and their respective control DOPE lipoplexes. This is the case for 75–100% of the 12 pcTG90-based (1:1 mol) formulations investigated for each *F*-PE, for 83% of the 24 pcTG90-based (1:2 mol) formulations, and for 75–50% of the 12 DOGS-based formulations investigated for each *F*-PE. However, a significant proportion of fluorinated DOGS-based lipoplexes (8–33% of the 12 formulations investigated for each *F*-PE) were found to display a higher transfection efficiency than their DOPE control. By contrast for the DOTAP-based lipoplexes, the opposite tendency was evidenced: if statistically comparable

transfection levels were obtained for 75–25% of the 12 formulations investigated for each *F*-PE, the complementary 25–75% ones displayed significantly lower transfection levels than their respective DOPE control lipoplexes.

The dependence of the lipid helper potential of the *F*-PEs on the cationic lipid can be tentatively explained by considering the geometrical complementarity between the cationic lipids and the colipids, which might have an effect on the packing within the lamellar phases formed between each of the CLs and each of the *F*-PEs or DOPE, and, consequently, on the physicochemical and biological properties of the DNA lipoplexes they form, and on transfection. Of the three cationic lipids investigated, it is DOGS with its branched polar head which displays the most-pronounced “direct” cone-shaped geometry (pure DOGS was shown to form mainly direct micelles when dispersed in water (33)). The *F*-PEs displaying a more-pronounced “inverted” cone-shaped geometry than DOPE, one therefore expects a higher degree of geometrical complementarity between the *F*-PEs and DOGS, which should result in more tightly packed (or more stable) lamellar phases than between DOPE and DOGS or than between any of the *F*-PEs and any of the two other cationic lipids. Consequently, one expects that, among the different combinations, more stable lipoplexes are formed from the *F*-PEs and DOGS, and thus a more pronounced helper effect for the *F*-PEs when used with

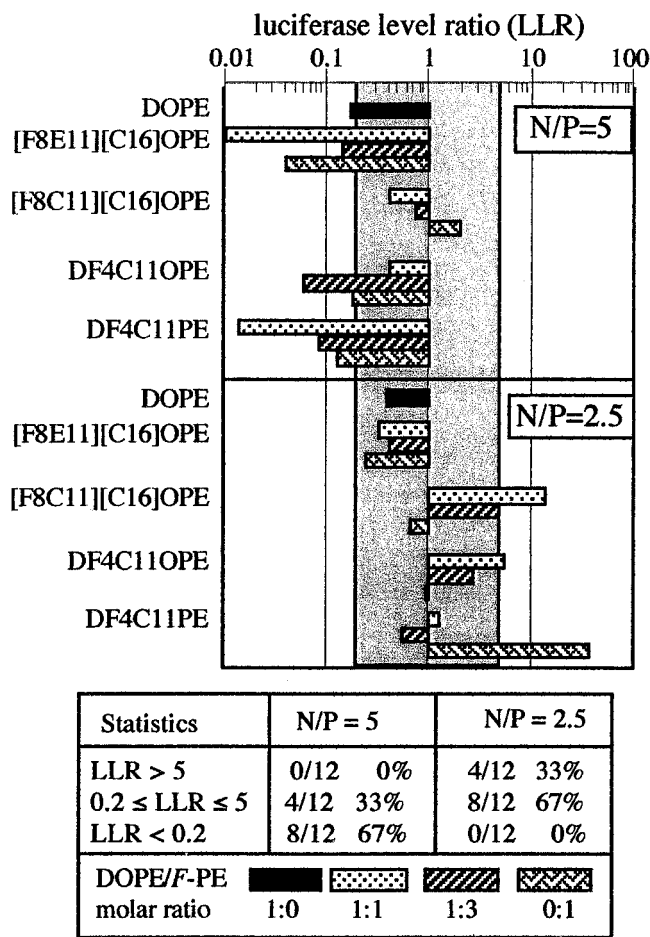


Figure 7. Effect of increasing the colipid content on luciferase expression in A549 cells of the pcTG90:colipid(s) lipoplexes (from 1:1 to 1:2). The luciferase level ratio LLR is the ratio of luciferase amount measured for the 1:2 mol formulation vs that measured for its corresponding 1:1 mol formulation. For more details concerning LLR, and the statistics, see caption for Figure 1. When LLR is outside the 0.2–5 range, $p < 0.01$ in a pair-wise comparison.

DOGS. Conversely, DOTAP which, of the three cationic lipids, displays a geometry that is the closest to a cylinder, forms lamellar phases which when combined with the *F*-PEs are expected to be substantially less tightly packed (or more destabilized) than those formed between DOTAP and DOPE. Owing to the lower degree of geometrical complementarity between DOTAP and the *F*-PEs, one expects a helper effect of the *F*-PEs that is below that of DOPE, as observed.

Where the helper effect on transfection resulting from the composition of the colipid mixture (DOPE:*F*-PE molar ratio) is concerned, it seems most preferable to use a DOPE:*F*-PE (1:3 mol) colipid combination than *F*-PE alone or than a *F*-PE:DOPE (1:1 mol) mixture, whatever the cationic lipid and the *F*-PE. If a preference for a specific DOPE:*F*-PE combination could not be evidenced among the few (eleven) CL:colipid(s) (1:1 mol) formulations that led to significantly higher transfection levels than their respective DOPE controls (see Figure 1B to 4B), this is however not the case for the 36 formulations that displayed a significantly lower transfection level than the DOPE controls. Indeed, of these 36 less efficient formulations, only 7 (19%) concerned the DOPE:*F*-PE (1:3 mol) colipid combination while 12 (33%) and 17 (48%) concerned lipoplexes based on the (1:1 mol) colipid mixture and on *F*-PE as sole colipid, respectively, indicating

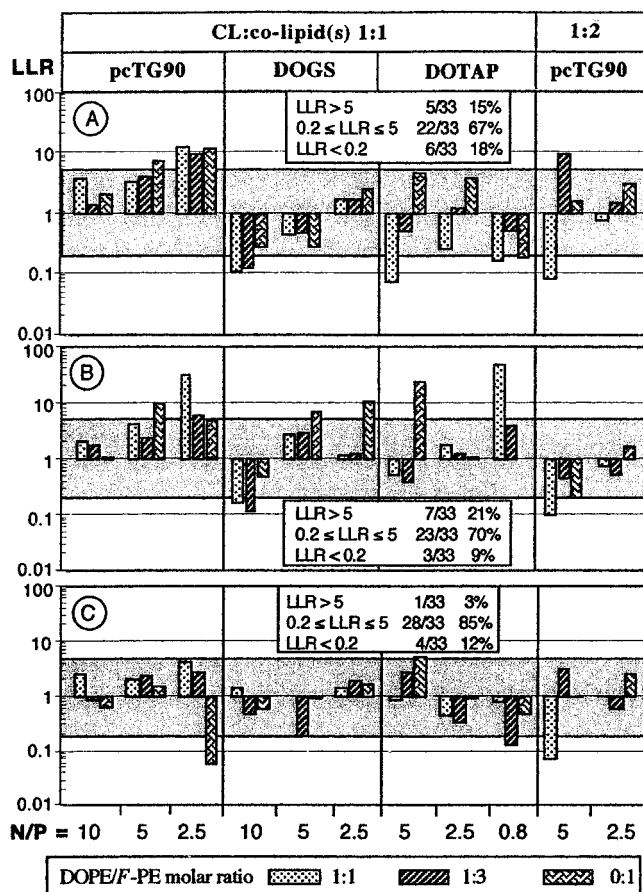


Figure 8. Panel A: Effect of the presence of a fluorinated tail on one or on both hydrophobic chains on lipofection. LLR represents the ratio of luciferase amount measured for the [F8E11][C16]OPE-based formulation vs that measured for its corresponding DF4C11OPE-based one. Panel B: Effect of the presence of a double bond in the hydrophobic chain on lipofection. LLR represents the ratio of luciferase amount measured for the unsaturated [F8E11][C16]OPE-based formulation vs that measured for its corresponding saturated [F8C11][C16]OPE-based one. Panel C: Impact of the chemical connexion (ester vs ether) on lipofection. LLR represents the ratio of luciferase amount measured for the ester DF4C11PE-based formulation vs that measured for its corresponding ether DF4C11OPE-based one. For more details concerning LLR, and the statistics, see caption of Figure 1. When LLR is outside the 0.2–5 range, $p < 0.01$ in a pair-wise comparison.

that transfection was likely optimal for a DOPE:*F*-PE (1:3 mol) colipid combination.

The aim of this study was also to highlight the impact of the *F*-PE structural elements (one fluorinated tail on one or on both hydrophobic chains, saturated or unsaturated hydrophobic chains, ester or ether-linkage) on their helper potential. Concerning the effect of the presence of a fluorinated tail on one or on both hydrophobic chains (as in [F8E11][C16]OPE or DF4C11OPE, respectively), a statistical analysis performed on the 33 [F8E11][C16]OPE-based formulations as compared with their respective DF4C11OPE ones did not allow to distinguish between these two *F*-PEs (Figure 8, panel A). This analysis demonstrated comparable, higher and lower transfection levels for 65, 15, and 20% of the formulations, respectively. Although of very different structures, these two *F*-PEs contain almost the same number of fluorine atoms (17 vs 18). Both compounds display furthermore a phase transition temperature which is below or close to the incubation temperature. One expects therefore a similar hydrophobic and lipophobic character,

and comparable interactions with the cationic lipids, serum proteins, and cellular constituents that have no consequences on transfection.

The presence of a double bond in the hydrophobic chain, although not determinant, was found to favor transfection, as supported by the statistical analysis performed on the 33 formulations containing [F8E11]-[C16]OPE as compared with their respective [F8C11]-[C16]OPE ones. This analysis (panel B of Figure 8) showed that, irrespective of the cationic lipid, the composition of the colipid mixture, the CL:colipid(s) molar ratio, and the *N/P* ratio, higher and comparable transfection levels were measured for 21 and 70% of these formulations, respectively. This result is in line with literature which showed that optimal transfection activity was preferably achieved when using cationic lipids and/or colipids containing hydrophobic chains that organize as "fluid" lamellar phases (e.g., lipids with olefinic chains such as [F8E11][C16]OPE which displays a phase transition temperature of 38 °C) rather than "gel" lamellar ones (e.g. lipids with long saturated chains such as [F8C11]-[C16]OPE which displays a phase transition temperature of 45 °C) (3, 10, 11, 34–37).

Finally, a comparable helper potential was evidenced for the ester DF4C11PE and ether DF4C11OPE: indeed 85% of the 33 formulations containing DF4C11PE displayed similar transfection levels than their corresponding ones based on DF4C11OPE (panel C of Figure 8). This result is not surprising as both colipids possess a very close thermotropic phase behavior and is in line with data from literature concerning DOTAP and its ether analogue DOTMA (17). One should underscore that, under the conditions of transfection used, the chemical nature of the linkage had no impact on lipoplex cell viability.

CONCLUSION

The present work indicates that "fluorinated" lipoplexes formulated with fluorinated helper lipids and conventional cationic lipids are definitely very attractive candidates for gene delivery, highlighting the diversity of lipids leading to efficient transfection. The remarkable in vitro and in vivo transfection potency found for such fluorinated lipoplexes (8) and that reported for fluorinated lipoplexes formulated with fluorinated lipospermines (6, 7) confirms further that lipophobicity and restricted miscibility of the lipoplex lipids with the endogenous lipids does not preclude efficient gene transfer and expression. Their transfection potency is rather attributable to their unique lipophobic and hydrophobic character (resulting from the formulation of DNA with fluorinated lipids), thus preventing DNA to some extent from interactions with lipophilic and hydrophilic biocompounds, and from degradation (7). Further experiments aimed at evaluating the in vivo helper transfection potential of the fluorinated PEs are currently underway.

ACKNOWLEDGMENT

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Supporting Information Available: Two tables listing the mean sizes of the CL(= pcTG90, DOGS, DOTAP:DOPE:F-PE (1:1 – *x*:*x* mol) and pcT90:DOPE:F-PE (1:2 – *y*:*y* mol) lipoplexes formed with plasmid pTG11033, respectively, as determined by light scattering spectroscopy.

This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Synthesis and Characterization of ^{111}In -DTPA-N-TIMP-2: A Radiopharmaceutical for Imaging Matrix Metalloproteinase Expression

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The matrix metalloproteinases (MMPs) are enzymes involved in the turnover of the extracellular matrix. Their overexpression in tumors is implicated in the metastatic process and may provide a target for diagnostic tumor imaging by using a radiolabeled inhibitor. MMPs are inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs). Thus, TIMPs are potential targeting molecules which could be used as vehicles for selective radionuclide delivery by virtue of their binding to MMPs. The aim of this work was to produce a radiopharmaceutical with which to evaluate this potential. The 127 amino acid N-terminal domain of recombinant human TIMP-2 (N-TIMP-2) was conjugated with the bifunctional chelator diethylenetriamine pentaacetic acid (DTPA). Singly modified DTPA-N-TIMP-2 conjugate (identified by electrospray ionization mass spectrometry) was isolated by anion-exchange chromatography. The primary site of DTPA modification on N-TIMP-2 was mapped to lysine-116, which is distant from the site of MMP interaction. The conjugate was radiolabeled with indium-111 to give ^{111}In -DTPA-N-TIMP-2 with a specific activity of at least 4 MBq/ μg and a radiochemical yield and purity of >95%, by incubation with $^{111}\text{InCl}_3$, without need for postlabeling purification. The product was sterile, pyrogen-free, and stable in serum over 48 h and retained full inhibitory activity in a fluorimetric binding assay. With these attributes, ^{111}In -DTPA-N-TIMP-2 is a suitable radiopharmaceutical for in vivo biological and clinical investigation of the potential benefits of imaging MMP expression.

INTRODUCTION

MMPs are a family of matrix-degrading enzymes, consisting of 18 currently known members, which are involved in the turnover of the extracellular matrix (ECM). They have a broad substrate specificity, and their synergetic action enables them to degrade all components of the ECM and basement membrane. To control tissue remodeling, MMP activity is tightly regulated at several levels, including enzyme expression, enzyme activation, enzyme autodegradation, and enzyme inhibition. Malfunction of these regulatory mechanisms results in uncontrolled MMP activity and the potential development of pathological conditions including rheumatoid arthritis, arteriosclerosis, and tumor invasion and metastasis (White et al., 1997).

Initial evidence for the involvement of MMPs in tumor progression arose from several in vivo studies correlating the level of MMPs with tumor appearance and progression (Stetler-Stevenson et al., 1996). The level of MMP expression appears to correlate with tumor stage, with metastatic tumors having the highest levels. Consequently, MMPs are increasingly considered as biochemical markers of metastatic potential and prognosis in cancer (Duffy and McCarthy, 1998). We propose that, in addition to serving as a useful biochemical marker, MMP expression may also provide a target for the diagnostic

in vivo imaging of tumors using a radiolabeled inhibitor. Imaging MMP expression in this way might be a useful noninvasive method of localizing tumors and evaluating MMP activity within them.

MMPs are inhibited in vivo by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs). TIMPs are proteins that bind to MMPs noncovalently with high affinity (10^{-10} to 10^{-16} M, Murphy and Willenbrock, 1995; Hutton et al., 1998). To date four TIMPs have been identified: TIMP-1 (Sellers et al., 1979), TIMP-2 (Stetler-Stevenson et al., 1989), TIMP-3 (Uria et al., 1994), and TIMP-4 (Greene et al., 1996). All consist of two domains (the N- and C-terminal), each of which is defined by three disulfide bonds (Williamson et al., 1990). The N-terminal of TIMP-2 (N-TIMP-2) has been shown to fold independently of the C-terminal (Murphy et al., 1991; Williamson et al., 1994), forming an autonomous protein domain that retains inhibitory activity (Murphy et al., 1991). N-TIMP-2 has been structurally characterized (Williamson et al., 1994; Muskett et al., 1998; Williamson et al., 1999), and its interaction with MMPs has been elucidated (Williamson et al., 1997; Fernandez-Catalan et al., 1998).

N-TIMP-2 has several attributes that render it attractive as a carrier molecule for radionuclides to investigate the feasibility of in vivo MMP imaging. It has a higher MMP binding affinity (K_d range 10^{-12} to 10^{-9} M) than any of the synthetic peptide-based inhibitors (typically 10^{-9} M) that have been devised as drugs to reduce the rate of tumor proliferation (for a review of these see, Brown, 1999). It should be rapidly cleared from circulation due to its modest molecular weight (14084 Da), and it has several exposed lysine residues that could be

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conjugated with a bifunctional chelator. It is a human protein and thus should not elicit an immune response. Finally, recombinant N-TIMP-2 can be expressed and purified from *E. coli* in a pure, pyrogen-free form (Williamson et al., 1996).

Indium-111 rather than technetium-99m was chosen as the gamma-emitting radionuclide with which to radiolabel N-TIMP-2. Technetium-labeling entails the use of reducing agents that might cleave the intramolecular disulfide bonds, which are critical for the inhibitory activity of N-TIMP-2.

This paper describes the synthesis and full in vitro characterization of an indium-111 labeled conjugate of N-TIMP-2 designed to assess the clinical value of MMP imaging in vivo. Although the labeling of biomolecules with indium-111 using the diethylenetriamine pentaacetic acid (DTPA) bifunctional chelator is well-established, the analytical methods used in this work provide a more detailed characterization of the In-DTPA-conjugated protein than has been reported hitherto for any other protein of comparable size.

MATERIALS AND METHODS

Preparation and Purification of DTPA-N-TIMP-2 and DTPA-Myoglobin. Human N-TIMP-2 was expressed in *E. coli* BL21 (DE3) and purified from inclusion bodies as described by Williamson et al. (1996). To produce a pyrogen-free product, all buffers were prepared using pyrogen-free water, and the chromatography columns were sanitized before use by washing with several column volumes of 0.5 M NaOH followed by thorough rinsing with pyrogen-free water. Similarly, all glassware, nonsterile plastic ware, and the chromatography system were sanitized with 0.5 M NaOH and rinsed with pyrogen-free water. Sterile plastic ware was used wherever possible for the collection and handling of the protein. All batches of DTPA-N-TIMP-2 produced using these measures were certified as pyrogen-free by in vivo testing in rabbits by a commercial laboratory.

N-TIMP-2 (5 mg, 1.0 mg/mL) was conjugated with DTPA in 50 mM sodium bicarbonate, pH 8.2. A 3-fold molar excess of DTPA cyclic anhydride (dissolved in anhydrous DMSO at a concentration of 7.5 μ M under N₂ gas) was added to the protein solution and the mixture incubated for 30 min at room temperature (final DMSO concentration of 1% v/v). To determine the degree of DTPA conjugation, the reaction mixture was analyzed by SDS-PAGE (Novex NuPage), isoelectric focusing (IEF; Novex pH 3–10 precast gels), and electrospray mass spectrometry (ES-MS). ES-MS was carried out on a Finnigan Mat LCQ ion trap mass spectrometer with in-line desalting by reverse-phase HPLC. Spectra were acquired in the high mass mode and deconvoluted using Bioexplore software.

DTPA-N-TIMP-2 was purified from the conjugation mixture by anion-exchange chromatography. The reaction mixture (2 mL) was loaded onto a 1 mL Resource-Q column (Pharmacia) equilibrated in buffer A (25 mM Tris-HCl, pH 8.0). The column was eluted with a linear gradient of 0–0.25 M NaCl in buffer A over 25 min at a flow rate of 1 mL/min. Those fractions (1 mL each) containing protein (detected by absorbance at 280 nm) were analyzed by IEF and ES-MS as described above.

Myoglobin was conjugated with DTPA as described for N-TIMP-2. Excess DTPA was removed from the crude conjugation mixture by gel filtration on Sephacryl S-100 (2.6 (diam) $\times$ 38 cm column, Pharmacia). The column was run in 50 mM citrate buffer, pH 5.5, at a flow rate of 1.4

mL/min and 2 mL fractions were collected. Absorbance was measured at 280 nm (to detect elution of protein) and 230 nm (to detect elution of excess unconjugated DTPA). Fractions containing DTPA-myoglobin were pooled and concentrated to 1 mg/mL by ultrafiltration in a 50 mL Amicon stirred cell with a YM10 membrane.

Peptide Mapping of DTPA-N-TIMP-2. N-TIMP-2 and the purified singly modified conjugate DTPA-N-TIMP-2 (200 μ g, 1 mg/mL), were incubated with dithiothreitol (DTT; 0.3 mM) in 50 mM MOPS buffer, pH 7, for 10 min at 30 °C. Trypsin was added to a final concentration of 0.05 mg/mL, and the samples were incubated for 4 h at 30 °C. Digestion was terminated by freezing at –20 °C. Additional DTT (to a final concentration of 2.2 mM) was added to each sample to ensure complete disulfide bond reduction before analysis by reverse-phase HPLC (Vydac 218TP54 C₁₈ column) at room temperature. The column was equilibrated with solvent A (0.1% v/v TFA in water) prior to loading the sample (100 μ g) and eluted with a linear gradient of 5–70% solvent B (0.1% v/v TFA, 70% v/v acetonitrile in water) over 120 min at a flow rate of 1 mL/min. Fractions were collected manually by following their absorbance at 215 nm. MALDI-TOF (matrix assisted laser desorption ionization time-of-flight) mass spectra were recorded for each fraction on a VG ToF spectrometer with an α -cyano-4-hydroxycinnamic acid (97%) matrix. The mass spectrometer was calibrated with bradykinin (572 Da) and insulin (5733.5 Da). For all samples, the detector was set in linear mode with a laser voltage of 1750 V, and spectra were measured using the lowest possible laser energy necessary for ionization of the sample. N-Terminal amino acid sequencing of selected peptides was carried out at the Wellcome Trust Protein Science Facility, University of Kent, on an Applied Biosystems 492 Protein Sequencer.

Radiolabeling of DTPA-N-TIMP-2 and Evaluation of the Radiolabeling Reaction. Samples of DTPA-N-TIMP-2 (1 mg/mL) were dialyzed against 2 L of 100 mM sodium citrate buffer, pH 5.5, overnight at room temperature in order to remove adventitious metal ions bound to the conjugate prior to radiolabeling. ¹¹¹InCl₃ (Mallinckrodt Medical b.v., Petten, Netherlands) was diluted 8-fold to a volume of 1.5–2.5 mL with 100 mM citrate buffer, pH 5.5, prior to use in the radiolabeling procedure to increase the pH of the acidic ¹¹¹InCl₃ solution to pH 5.5. ¹¹¹InCl₃ and ¹¹¹In-citrate were manipulated using Hamilton glass syringes and stainless steel needles, and radiolabeling reactions were carried out in 3 mL glass vials. The desired amount of conjugate (30 μ L of 1 mg/mL solution) was added to the ¹¹¹In-citrate solution (4 MBq/ μ g protein) and incubated at 37 °C for 1 h. To check for ¹¹¹In complexed to any contaminating unconjugated DTPA, a TLC system was used comprising Merck 60F₂₄₅ silica gel plates with a mobile phase of 40% aqueous ammonium formate (1.56 M)/40% methanol/20% citric acid (0.5 M). Nonspecific binding of ¹¹¹In to protein was checked by ITLC (Gelman ITLC-SG) using 100 mM citrate buffer, pH 5.5, as the mobile phase. The percentage of radioactivity remaining at the origin (¹¹¹In-DTPA-N-TIMP-2) was taken as the radiochemical yield. Samples were spotted using glass capillaries, and the TLC plates were scanned on a Bioscan TLC plate scanner at 2.5 cm/min.

Serum Stability of ¹¹¹In-DTPA-N-TIMP-2. Fresh human serum was obtained by the centrifugation (5000 g for 10 min) of clotted blood (20 mL). ¹¹¹In-DTPA-N-TIMP-2, ¹¹¹In-DTPA and ¹¹¹InCl₃ (1 MBq of each) were incubated in 6 mL of serum under 5% CO₂, and samples

(50 kBq) were taken at 4, 24, and 48 h. Each sample (diluted to 1 mL in running buffer) was loaded directly on to the Sephacryl S-100 gel filtration column (2.6 cm (diameter) $\times$ 38 cm, Pharmacia) run in 100 mM sodium citrate buffer, pH 5.5, at 1.4 mL/min. Fractions (2 mL) were collected and counted for 60 s. The activity of each fraction was recorded as a percentage of the total eluted activity. A standard chromatogram for each protein was also determined using unlabeled material by measuring the absorbance of each fraction at 280 nm (transferrin and N-TIMP-2) and 230 nm (In-DTPA). In a separate stability study, purified ^{111}In -DTPA-N-TIMP-2, ^{111}In -DTPA, and $^{111}\text{InCl}_3$ (10 MBq of each) were diluted to 20 μL with 100 mM sodium citrate buffer, pH 5.5, and incubated with serum (200 μL) at 37 $^\circ\text{C}$ as described above. Samples (10 μL) were taken at several time intervals between 5 min and 48 h and analyzed by nonreducing SDS-PAGE. Nonreducing conditions were used to avoid boiling with dithiothreitol, which causes indium to be transchelated from the DTPA conjugate to form a low molecular weight indium thiolate complex. The gels were developed by Coomassie-blue staining and autoradiography.

MMP Activity Assay. The inhibitory activity of In-DTPA-N-TIMP-2, (In-DTPA) $_2$ -TIMP-2, and In-DTPA-myoglobin was compared to that of N-TIMP-2 using the fluorimetric peptide assay described by Murphy et al. (1995). A 10-fold molar excess of nonradioactive InCl_3 (1 mg/mL) was added to 5 nmol of N-TIMP-2, DTPA-N-TIMP-2, (DTPA) $_2$ -N-TIMP-2, and DTPA-myoglobin at a protein concentration of 1 mg/mL. Chelation of indium was confirmed by ES-MS. The solutions were diluted to 200 μL with incubation buffer (50 mM Tris-HCl, 0.1 M NaCl, 10 mM CaCl_2 , 0.05% Brij 35, pH 7.5) and excess InCl_3 removed by ultrafiltration (microconcentrator devices, Amicon). All four proteins were diluted to a stock solution of 1 μM in incubation buffer using the A_{280} extinction coefficient of $12450 \text{ M}^{-1}\text{cm}^{-1}$ for N-TIMP-2, DTPA-N-TIMP-2, and (DTPA) $_2$ -N-TIMP-2, and the A_{556} extinction coefficient of $12900 \text{ M}^{-1}\text{cm}^{-1}$ for DTPA-myoglobin. A 1 μM stock solution of the catalytic N-terminal domain of stromelysin (N-MMP-3) was also prepared (Marcy et al., 1991), and 50 μL of this was added to each of a series of tubes containing 0–100 μL of the inhibitor stock solution diluted with incubation buffer to bring the total incubation volume to 150 μL . Thus each incubation mixture contained enzyme at a concentration of 0.33 μM and a putative inhibitor at 0–0.67 μM . These inhibitor/enzyme mixtures were incubated for 30 min before adding 50 μL of each to 445 μL assay buffer and 5 μL substrate peptide solution, as described by Knight et al. (1992). The fluorescence (excitation 328 nm, emission 393 nm) was monitored over 4 min at 25 $^\circ\text{C}$, corrected for background (nonenzymatic) peptide cleavage, and plotted against inhibitor concentration.

RESULTS

Preparation and Characterization of DTPA-N-TIMP-2. Production, refolding, and purification of N-TIMP-2 was performed according to the methods of Williamson et al. (1996). The final protein product was analyzed by electrospray mass spectrometry (ES-MS) and was of the correct molecular weight (found: 14085 Da; expected: 14084) for the N-TIMP-2 sequence containing three disulfide bonds (Figure 1, top panel, peak a). A trace amount of the N-terminally acetylated form seen on recombinant expression in *E. coli* was also detected (peak b). N-TIMP-2 incubated with a 3-fold molar excess of DTPA produced the highest yield of singly modified

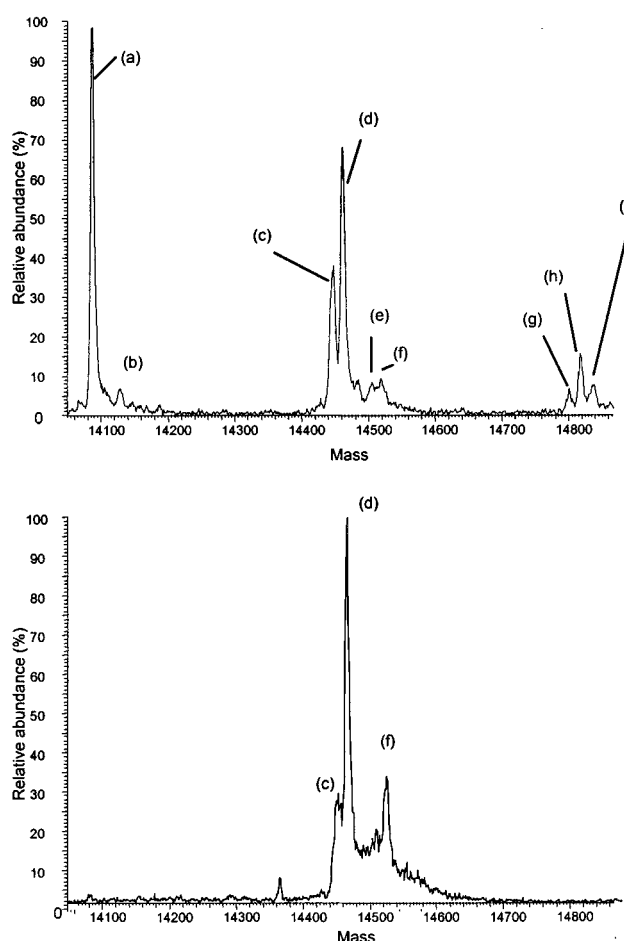


Figure 1. Deconvoluted electrospray mass spectra of N-TIMP-2 conjugates with DTPA. Top: crude reaction mixture; bottom: fraction 25 isolated from the crude reaction mixture by ion-exchange chromatography. Assignments: (a) 14085 (calcd for unmodified N-TIMP-2: 14084); (b) 14127 (calcd for acetylated N-TIMP-2: 14126); (c) 14441 (calcd for internally cross-linked DTPA-N-TIMP-2: 14441); (d) 14459 (calcd for DTPA-N-TIMP-2: 14459); (e) 14501 (calcd for peak (c) + Cu^{2+} - 2H^+ : 14502); (f) 14517 (calcd for DTPA-N-TIMP-2 + Cu^{2+} - 2H^+ : 14520); (g) 14799 (calcd for (DTPA) $_2$ -N-TIMP-2 with two internal cross-links: 14798); (h) 14816 (calcd for (DTPA) $_2$ -N-TIMP-2 with one internal cross-link: 14816); (i) 14837 (calcd for (DTPA) $_2$ -N-TIMP-2: 14834).

DTPA-N-TIMP-2 conjugate (peak d, estimated at 25% of total protein from relative abundance by ES-MS), while minimizing the amount of doubly modified conjugate (peak i). A secondary peak (peak c, 14% of total protein) of 18 mass units below the singly modified conjugate was consistently observed. As detailed below, this was assigned as an internally cross-linked species in which the DTPA bridges between two lysine residues. The doubly conjugated protein peak i was associated with two such peaks (g and h, respectively) of 18 and 36 Da less than the mass of the conjugate. Peaks e and f correspond to the addition of a metal ion (adventitious copper originating both from buffer salts and as an artifact from within the ES-MS instrument) to peaks c and d. Much of the metal was found to be readily removable by dialysis against citrate buffer. SDS-PAGE showed the presence of an additional minor species with twice the molecular weight of N-TIMP-2. This was assigned to protein dimers cross-linked by DTPA.

IEF resolved the proteins into three distinct groups of bands with pI 6.4–6.5, 5.1–6.2, and 4.5–4.9 (Figure 2). The results suggested that these protein species could

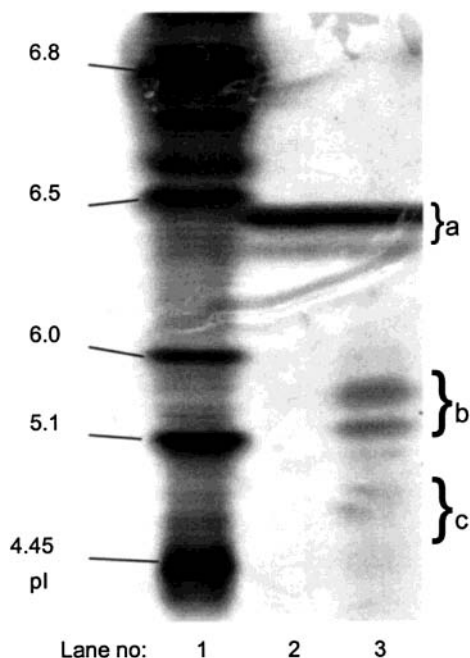


Figure 2. Isoelectric focusing of N-TIMP-2 conjugates with DTPA. Lane 1: pI markers; lane 2: N-TIMP-2 standard. The band with pI slightly lower than that of N-TIMP-2 is assigned to the N-terminally acetylated form. Lane 3: Crude conjugation mixture after incubation of N-TIMP-2 with DTPAA. Band group a is unmodified N-TIMP-2 and its N-terminally acetylated form; group b includes forms of DTPA–N-TIMP-2; group c includes forms of (DTPA)₂–N-TIMP-2.

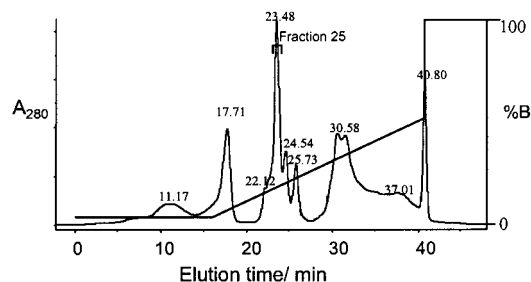


Figure 3. Ion-exchange chromatography of the crude conjugation mixture produced by incubation of N-TIMP-2 with DTPAA. ES-MS and IEF analysis of the fractions show that unmodified N-TIMP-2 elutes at 17.71 min, DTPA–N-TIMP-2 at 23.48 min (referred to in the text as fraction 25), and (DTPA)₂–N-TIMP-2 at 30.58 min.

be separated by elution with increasing salt concentration at basic pH on an anion-exchange column. Indeed, three groups of peaks were well-resolved by anion exchange chromatography at pH 8 (Figure 3), with elution times of 18–20 min, 23–27 min, and 31–33 min. IEF of these fractions showed that they corresponded to the groups with pI 6.4–6.5, 5.1–6.0, and 4.5–4.9, respectively. ES-MS of the fractions (data not shown) showed that the pI 6.4–6.5 group consisted of unmodified N-TIMP-2 and its N-terminally acetylated form, the pI 5.1–6.2 group consisted of various forms of DTPA–N-TIMP-2, and the pI 4.5–4.9 groups consisted of forms of (DTPA)₂–N-TIMP-2. ES-MS also showed that the multiplicity of bands within each group was due to combinations of both internal cross-linking and metal ion scavenging. Fraction 25 from the ion-exchange column, containing the major peak in the DTPA–N-TIMP-2 group (elution time 23.48 min) and having a major IEF band at a pI of approximately 5.4, was selected for further development as a radiopharmaceutical and subjected to tryptic peptide

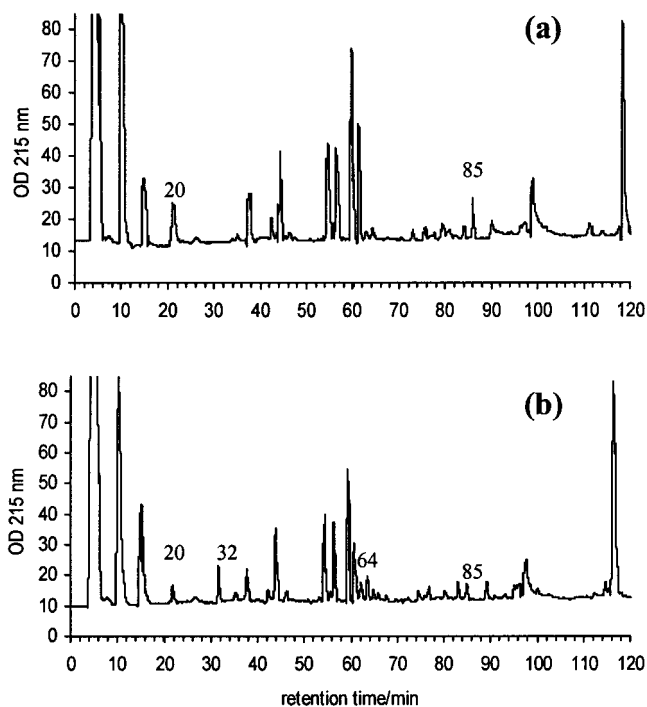


Figure 4. Reverse-phase HPLC of tryptic digests of (a) N-TIMP-2 and (b) ion-exchange purified DTPA–N-TIMP-2 (fraction 25 in Figure 3). Peak 32 contains a DTPA conjugated to lysine-116, while peak 64 contains two peptide sequences cross-linked by DTPA between lysine-116 and lysine-51.

mapping to determine the site of DTPA modification (see below). ES-MS of this fraction showed that it contained DTPA–N-TIMP-2 together with up to 20% of its internally cross-linked form, but no unmodified N-TIMP-2 and no (DTPA)₂–N-TIMP-2 (see Figure 1, bottom panel).

Peptide Mapping of DTPA–N-TIMP-2. The primary site of DTPA modification on N-TIMP-2 was determined by tryptic peptide mapping of fraction 25 isolated by ion-exchange chromatography (see Figure 3). Reverse-phase HPLC chromatograms of tryptic digests of unmodified N-TIMP-2 and DTPA–N-TIMP-2 are shown in Figure 4. Two new peaks numbered 32 (major) and 64 (minor), appear following conjugation, with a concomitant decrease in the heights of peaks 20 and 85 compared to unmodified N-TIMP-2. Peaks 32 and 64 from the DTPA–N-TIMP-2 digest were collected and analyzed by MALDI-TOF-MS. The masses obtained for the peptides in these peaks were compared to the theoretical masses of the peptides predicted from complete and incomplete (i.e., with cleavage blocked at modified lysine residues) N-TIMP-2 tryptic digestion (Table 1).

The mass of peptide 32 corresponded to residues 116–121, which has the sequence KSLNHR. This peptide contains only one lysine residue, lysine-116 (at which trypsin cleavage did not occur); therefore, the position of the DTPA-modification in this sequence was unambiguous and sequencing was considered unnecessary. The mass of the peptide contained in peak 64, however, did not correspond to the theoretical mass of any simple linear sequence from N-TIMP-2 or DTPA-conjugate thereof. MALDI-TOF analysis after the addition of indium chloride to peaks 32 and 64 confirmed that each peptide contained a single DTPA moiety, as the mass of both peptides increased by approximately 110 corresponding to replacement of four protons by a single indium ion (results not shown).

The first 12 amino acids of peptide 64 were sequenced in order to identify the modified lysine residue. Three

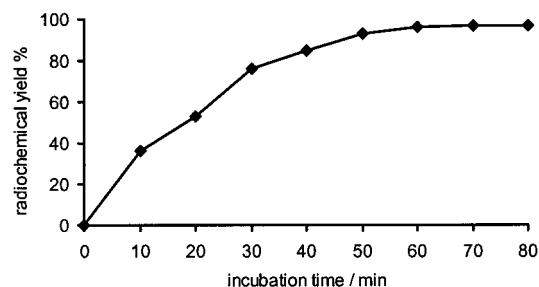
Table 1. Mass Spectrometry (MALDI-TOF MS) and Amino Acid Sequence Analysis for Peptides 32 and 64

	Peptide 32	Peptide 64 (crosslinked)
Experimentally observed mass (Da)	1132	1906
Sequence of "best match" peptide	K ¹¹⁶ SLNHR DTPA	QIK ⁵¹ MFK DTPA K ¹¹⁶ SLNHR
Calculated mass of "best match" peptide	1129	1905
Residue numbers of sequence	116-121	49-54 & 116-121
Number of missed cleavages due to DTPA modification	1 (K ¹¹⁶)	2 (K ⁵¹ & K ¹¹⁶)

sequences were detected, one of which was a minor impurity present at very low concentration and corresponded to the first 12 residues of the N-TIMP-2 sequence (C¹–R²⁰). The remaining two peptides produced amino acid residues expected for sequences Q⁴⁹–K⁵⁴ and K¹¹⁶–R¹²¹ except that one lysine residue was missing from each sequence (lysine-51 and lysine-116, respectively). The observation that this peptide binds only one indium ion, despite the presence of two modified lysine residues, suggests that the same DTPA moiety was bound simultaneously to both lysine-51 and lysine-116, i.e., the sequences were cross-linked to give a single peptide species Q⁴⁹–K⁵⁴ and K¹¹⁶–R¹²¹ with a DTPA linker between K⁵¹ and K¹¹⁶. This is supported by the ES-MS of the intact conjugate in which a cross-linked species was identified as a peak with a mass 18 units less than expected (Figure 1), and the fact that the calculated mass of the suggested cross-linked species (1905 Da) is close to the experimental mass of peptide 64 determined by MALDI-TOF MS (1906 Da, Table 1).

Radiolabeling of DTPA–N-TIMP-2 with Indium-111. Two chromatographic assays were used to evaluate the purity and yield of the radiolabeled product. ¹¹¹In–DTPA has an *R_f* of 0.6 in the silica gel thin-layer chromatography (TLC) assay, while uncomplexed ¹¹¹InCl₃ and ¹¹¹In–protein have an *R_f* of zero, permitting this system to be used as a check for unconjugated DTPA in the sample. Instant thin-layer chromatography (ITLC) in 100 mM citrate buffer, pH 5.5, was used to determine incorporation of ¹¹¹In into DTPA–N-TIMP-2. In this system radiolabeled protein has an *R_f* of zero while uncomplexed ¹¹¹InCl₃ and ¹¹¹In–DTPA have an *R_f* close to 1. A control ITLC after incubation of unconjugated N-TIMP-2 with ¹¹¹InCl₃ showed no radioactivity at an *R_f* of zero (all radioactivity being close to an *R_f* value of 1) showing that ¹¹¹InCl₃ does not bind nonspecifically to the protein under the conditions of the chromatography. Purified DTPA–N-TIMP-2 that had been freed from adventitious metal ions by dialysis against citrate buffer was consistently radiolabeled with ¹¹¹In to a specific activity of 4.5 MBq/μg and a radiochemical yield and purity of >95% after 1 h of incubation (Figure 5). Undialyzed samples of DTPA–N-TIMP-2 containing significant amounts of adventitious metal ion chelated by the DTPA conjugate, as detected by ES-MS, consistently failed to radiolabel satisfactorily.

Serum Stability of ¹¹¹In–DTPA–N-TIMP-2. The stability of ¹¹¹In–DTPA–N-TIMP-2 in serum was determined by size exclusion chromatography and SDS–PAGE electrophoresis. Nonradioactive standards transferrin, N-TIMP-2, and In–DTPA were analyzed beforehand in order to determine their elution profiles by UV absorbance measurement (results not shown). Transferrin eluted in fractions 1–10, N-TIMP-2 in fractions 18–28, and In–DTPA in fractions 43–50. The elution profiles of ¹¹¹In–DTPA–N-TIMP-2 and the controls ¹¹¹InCl₃ (which in serum rapidly incorporates ¹¹¹In into transferrin) and ¹¹¹In–DTPA after incubation in serum for up to 48 h were also determined (Figure 6). No radioactive peaks corresponding to ¹¹¹In–DTPA or low molecular weight protein fragments appeared, showing that ¹¹¹In–DTPA–N-TIMP-2 is stable to degradation by serum proteases. However, approximately 5% of the radioactivity is lost from the conjugate to a species coeluting with transferrin over 48 h. Approximately 10% of the radioactivity in the incubation mixture eluted close to ¹¹¹In–DTPA. This is also present in the ¹¹¹In–serum control and is due to a contaminant introduced during labeling of the conjugate prior to addition to serum. It arises from plasticware used to handle the ¹¹¹InCl₃ and is not a DTPA complex. [We found that undiluted ¹¹¹InCl₃ reacted with certain types of plasticware, including pipet tips and microcentrifuge tubes, to form an unidentified complex that was stable to competition by excess DTPA and had an *R_f* value of 0.7 on the silica gel TLC assay. Excessive manipulation of ¹¹¹InCl₃ caused this contamination, and consequently ¹¹¹InCl₃ was handled exclusively with glassware, i.e., glass vials, glass syringes, and capillaries. With

**Figure 5.** Time-dependent incorporation of ¹¹¹In into purified DTPA–N-TIMP-2 at 37 °C. From these results, an incubation time of 60 min was adopted as standard for preparation of ¹¹¹In–DTPA–N-TIMP-2.

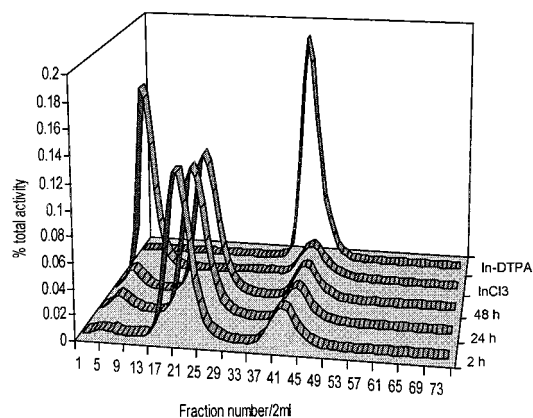


Figure 6. Serum stability, determined by size exclusion chromatography after 2, 24, and 48 h of incubation, of ^{111}In -DTPA-N-TIMP-2 and control species $^{111}\text{InCl}_3$ and ^{111}In -DTPA. ^{111}In -DTPA-N-TIMP-2 elutes in fraction 20, and this peak is only slightly depleted by 48 h while a small new peak corresponding to ^{111}In -transferrin (fraction 5) appears. Free ^{111}In in the control experiment binds to transferrin and elutes at fraction 5 (labeled " InCl_3 "), and ^{111}In -DTPA elutes at fraction 40 (labeled " In -DTPA"). The constant peak at fraction 42 is an ^{111}In -labeled impurity arising prior to incubation in serum (see text) and is not attributed to instability of the labeled conjugate in serum.

these precautions the contaminant did not appear. The contaminant was responsible for the appearance of the peak seen in fractions 43–50 of the ^{111}In -DTPA-N-TIMP-2 samples in the serum stability determination. It was therefore considered to be an artifact of the radiolabeling procedure rather than an indication of instability of ^{111}In -DTPA-N-TIMP-2. It does not increase during incubation in serum and its presence does not indicate any instability of the labeled conjugate.

The stability of ^{111}In -DTPA-N-TIMP-2 in serum was further supported by nonreducing SDS-PAGE of the radiolabeled conjugate following incubation in serum. After a 48 h incubation, no changes in the intensity of the radioactive ^{111}In -DTPA-N-TIMP-2 band were seen, and no new radioactive bands were evident, indicating that In -DTPA-N-TIMP-2 was not degraded over this period of time (data not shown).

MMP-Binding Activity of In -DTPA-N-TIMP-2. Nonradioactive In -DTPA-N-TIMP-2 and $(\text{In-DTPA})_2$ -N-TIMP-2 (separated from one another by ion exchange chromatography), and a control protein DTPA-myoglobin, were subjected to the fluorimetric binding assay described by Murphy et al. (1995). In this experiment, increasing amounts of inhibitor are incubated with the catalytic domain of MMP-3 (N-MMP-3) and the resulting proteinase activity measured subsequently by the addition of an internally quenched substrate. The rate of fluorescence increase is proportional to the rate of substrate cleavage and hence MMP activity. Figure 7 shows that In -DTPA-N-TIMP-2 inhibited N-MMP-3 to the same extent as unmodified N-TIMP-2. Hence incorporation of a single DTPA into N-TIMP-2, and subsequent reaction with InCl_3 did not affect inhibitory activity under the conditions used. In contrast, $(\text{In-DTPA})_2$ -N-TIMP-2 had no inhibitory activity under these conditions, and its binding to N-MMP-3 was comparable to DTPA-myoglobin, the negative control. Thus the incorporation of a second DTPA severely compromised the inhibitory activity.

DISCUSSION

Conjugation of N-TIMP-2 with DTPA resulted in a yield of approximately 25% of singly modified protein and

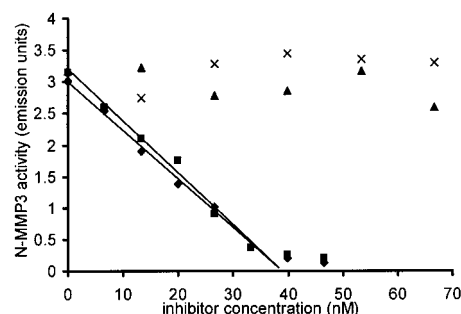


Figure 7. N-MMP-3 inhibition assay comparing the activity of In -DTPA-N-TIMP-2 and $(\text{In-DTPA})_2$ -N-TIMP-2, with positive (unmodified N-TIMP-2) and negative (In -DTPA-myoglobin) controls. N-MMP-3 was present at 33 nM in all incubations with substrate peptide. N-TIMP-2 ($\blacklozenge$) gave almost full inhibition at a concentration of 33 nM, as expected for the formation of a 1:1 complex. In -DTPA-N-TIMP-2 ($\square$) retained full inhibitory activity identical to unmodified N-TIMP-2. $(\text{In-DTPA})_2$ -N-TIMP-2 ($\square$) and In -DTPA-myoglobin ($\times$) showed no N-MMP-3 inhibitory activity.

an additional 14% of internally cross-linked singly modified protein. The latter was identified on the ES-MS spectrum as a peak of 18 mass units less than the conjugate peak. Also present, identified by SDS-PAGE, ES-MS, and IEF, were unmodified N-TIMP-2 and small amounts of intermolecularly cross-linked dimers, multiply conjugated N-TIMP-2 species and their intramolecularly cross-linked derivatives, and complexes of the conjugates with metal ions.

Product heterogeneity resulting from DTPA conjugation is well documented (Maisano et al., 1992; Ogan et al., 1987). Heterogeneity can result from conjugation at more than one lysine residue, conjugation at tyrosine residues, DTPA cross-linking, and metal ion contamination of the conjugate (Hnatowich, 1985). Most imaging studies with ^{111}In -DTPA conjugates utilize the crude conjugate, which may have a comparatively low specific radioactivity and may contain some inactivated material as a result of multiple conjugation. Separation of these various conjugation products is desirable as it allows thorough characterization of the various species leading to the development of a homogeneous product with optimal biological activity and specific radioactivity. Isolation of the desired species DTPA-N-TIMP-2 was shown to be straightforward using ion-exchange chromatography, as a result of the significant change in isoelectric point caused by the DTPA conjugation. This method also removes excess DTPA, offering a convenient single-step procedure for purification of the required conjugate.

Tryptic peptide mapping of the purified DTPA-N-TIMP-2 isolated two HPLC fractions containing DTPA-modified peptides. The major peak, peak 32, contained a peptide whose mass corresponded to that expected for the cleaved sequence $\text{K}^{116}\text{SLNHR}^{121}$. The primary site of DTPA modification in singly conjugated N-TIMP-2 is thus lysine-116. This is located on helix 2 of N-TIMP-2 and is spatially distant from the site of MMP interaction (Fernandez-Catalan et al., 1998; Muskett et al., 1998). Lysine-116 was also identified as the primary conjugation site following the reaction of N-TIMP-2 with Bolton-Hunter reagent (Giersing et al., 2000). The principal peptide of peak 64 consisted of two sequences present in equal concentrations corresponding to the cleavage sequences $\text{Q}^{49}\text{-K}^{54}$ and $\text{K}^{116}\text{-R}^{121}$. The combination of amino acid sequencing and MALDI-TOF MS suggests that peptide 64 consists of two sequences bridged by a DTPA linker between lysine-116 and lysine-51.

Examination of the three-dimensional structure of N-TIMP-2 (Muskett et al., 1998) and the dimensions of DTPA suggests that lysine-116 and lysine-51 are close enough together on the surface of N-TIMP-2 for a molecule of DTPA to link simultaneously with both ϵ -amino groups. Thus, a plausible model for the conjugation reaction is that the primary site of modification is lysine-116, and a fraction of the DTPA linked at this site then reacts with nearby lysine-51. In contrast, analysis of myoglobin by ES-MS shows no signs of formation of intramolecular cross-linked species on reaction with DTPAA. Intramolecular cross-linking does not prevent the chelator from binding In^{3+} , as shown by ES-MS. However, we have not determined whether the cross-linked species becomes labeled with ^{111}In under the labeling conditions used, in which DTPA–N-TIMP-2 is present in a large molar excess over indium. The fluorescence binding assay showed that no inhibitory activity was lost on conjugation with a single DTPA, even in the cross-linked form (which constituted approximately 20% of the conjugate used in this assay). On the other hand, the fact that the inhibitory activity of $(\text{DTPA})_2$ –N-TIMP-2 is abolished upon conjugation and subsequent reaction with indium suggests that the second site of DTPA modification is important for the correct functioning of the MMP interaction site. The second site of modification has yet to be identified, but it is unlikely to be lysine-51 since this is spatially distant from the MMP-binding site, and modification at lysine-51 (as seen in the internally cross-linked form of the conjugate) retains MMP-binding activity. A likely candidate for the second modification is the amino-terminus. Higashi and Miyazaki (1999) showed that this was the primary site of modification of full-length TIMP-2 with cyanate, and that this modification abolished MMP-inhibitory activity. This would be consistent with our observation that the second modification inactivates N-TIMP-2. It is possible, in light of the proven accessibility of the N-terminus, that one of the discarded singly modified fractions immediately following fraction 25 in the ion-exchange purification is conjugated at the N-terminus.

The ^{111}In –DTPA–N-TIMP-2 used here is sufficiently stable in serum to undergo evaluation as a diagnostic imaging agent. Approximately 5% of indium-111 is lost to transferrin over a 48 h period. This is consistent with the findings of Gobuty et al. (1985).

In summary, DTPA–N-TIMP-2 can be synthesized in high yield and isolated free from unmodified N-TIMP-2 (so that it can be labeled to high specific activity), free from $(\text{DTPA})_2$ –N-TIMP-2 (so that it retains full activity), and free from pyrogens. It can be radiolabeled in >95% radiochemical yield to a specific activity of at least 4 MBq/ μg (58 MBq/nmol). The conjugate has been produced in a kit formulation which can be stored at -20°C until needed for radiolabeling. The radiolabeling reaction and the appropriate TLC and ITLC quality controls can be completed within 2 h. These attributes render DTPA–N-TIMP-2 suitable for in vivo evaluation.

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Mechanisms Leading to an Oriented Immobilization of Recombinant Proteins Derived from the P24 Capsid of HIV-1 onto Copolymers

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To investigate the mechanism leading to an oriented immobilization of recombinant proteins onto synthetic copolymers, five genetically modified HIV-1 p24 capsid proteins (RH24, RH24A₄K₂, RH24R₆, RH24R₄K₂, and RH24K₆) were tested for their efficiency to covalently bind to maleic anhydride-*alt*-methyl vinyl ether (MAMVE) and *N*-vinyl pyrrolidone-*alt*-maleic anhydride (NVPMA) copolymers. These proteins contain, at their C-termini, tags differing in cationic and/or reactive amino acids density. We demonstrated that an increase of the charge and amine density in the tag enhances the coupling yield, the most efficient tag being a six lysine one. The reactivity of the proteins depends directly on the reactivity of the tag, and this led us to conclude that the tag was the site where the covalent grafting with the polymer occurred. Thus, design of such tags provides a new efficient and versatile method allowing oriented immobilization of recombinant proteins onto copolymers.

INTRODUCTION

Immobilization of proteins has been widely studied and has found numerous applications in purification processes (affinity chromatography), diagnostic field (immunoassays, biosensors), and large-scale industrial production (bioreactors). According to the expected application, many immobilization processes of proteins are commonly used ranging from ionic interactions, adsorption, complexation, or covalent binding, but covalent immobilization is often required to obtain very stable preparations with extended active life (1). However, binding often occurs via a random attachment of the proteins to the support through several amino acid residues, leading to a partial loss of biological properties, i.e., loss of enzymatic activity for an enzyme or loss of biological recognition for immobilized antibodies or antigens (2). The advantage of oriented grafting of biologically active proteins is mainly good steric accessibility of the active binding site. Regarding protein covalent immobilization as a chemical alteration of the biomolecule, it is of great interest to make this reaction occur at a specific site of the protein that is not involved in the biorecognition process, which is called oriented binding (3). This can be achieved using specific chemistry, for instance, the chemistry of thio groups of antibody Fab fragments or aldehyde chemistry after mild oxidation of carbohydrate moieties (3). With recombinant proteins, site-directed mutagenesis allows introduction of reactive groups, such as cystein residues, at virtually any position of a protein to achieve site-specific conjugation with functional polymers (4, 5).

The use of amine groups for the conjugation of a protein is a more generic method than using specific chemistry since every protein has primary amines avail-

able for conjugation. Furthermore, it is easier to use than thiol chemistry, as this chemistry does not suffer from the unwanted oxidation of the free cysteine groups, leading to unreactive protein dimers. Finally, it is a very flexible approach, as many different reaction conditions in terms of pH, ionic strength, and temperature can lead to the formation of a stable amide bond, linking the protein to the polymer. Hershfield et al. used site-directed mutagenesis to enhance protein modification with poly(ethylene glycol) by mutating arginine to lysine (6). Controlling the site of conjugation using amine chemistry is not a trivial issue, as lysine residues are randomly distributed along the protein. Our idea was to use electrostatic interactions with a polyanionic polymer to favor and direct the chemical immobilization of lysine-tagged protein onto poly(maleic anhydride-*alt*-methyl vinyl ether), the hexalysine tag being both positively charged and reactive toward the anhydride moieties of the copolymer. Preliminary investigations demonstrated the feasibility of this approach, as the coupling yields of the tagged protein were higher than its nontagged counterpart. Furthermore, we showed that the reaction was enhanced by electrostatic interactions between the polymer and the biomolecule (7).

This paper reports on a more thorough investigation on this approach, willing to address the effects of the reactivities and of the natures of both the polymer and the tag introduced on a HIV-1 modified p24 capsid protein. To assess the impact on the efficiency of the coupling reaction of (i) the primary amines density and (ii) the charge density composing the tag, competitive experiments using peptides have been performed. Investigation of the coupling isotherms and the corresponding Scatchard plots demonstrated a positive cooperative effect of the presence of the tag, provided it bore positively charged amino acids.

EXPERIMENTAL PROCEDURES

Proteins. Five HIV-1 p24 recombinant proteins were obtained from *Escherichia coli* from a procedure derived

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RH24	MRGSHHHHHHGSVDESMVQNIQGQMVHQAI ~~SP~~ RTLNAWVKVVEEKAFSP ~~EV~~ IPMF ~~S~~ AL ~~S~~ E	60
RH24A ₄ K ₂	MRGSHHHHHHGSVDESM.....	60
RH24R ₆	MRGSHHHHHHGSVDESM.....	60
RH24R ₄ K ₂	MRGSHHHHHHGSVDESM.....	60
RH24K ₆	MRGSHHHHHHGSVDESM.....	60
RH24	GATPQDLNTMLNTVGGHQAAMQMLKETINEEAAEWDRVHPVHAGPIAPGQMREPRGSDIA	120
RH24A ₄ K ₂		120
RH24R ₆		120
RH24R ₄ K ₂		120
RH24K ₆		120
RH24	GTTSTLQEQIGWMTNNPPIPVGEIYKRWIIILGLNKIVRMYSPTSILDIRQGPK ~~EP~~ FRDYV	180
RH24A ₄ K ₂		180
RH24R ₆		180
RH24R ₄ K ₂		180
RH24K ₆		180
RH24	DRFYKTLRAEQASQEVKNWMTETLLVQNANPDCKTILKALGPAATLEEMMTACQGVGGPG	240
RH24A ₄ K ₂		240
RH24R ₆		240
RH24R ₄ K ₂		240
RH24K ₆		240
RH24	DLV	243
RH24A ₄ K ₂	AAAA KK SVDESL	252
RH24R ₆	RRRRRR SVDESL	252
RH24R ₄ K ₂	RRRRKK SVDESL	252
RH24K ₆	KKKKKK SVDESL	252

Figure 1. Sequence alignment of recombinant RH24, RH24A₄K₂, RH24R₆, RH24R₄K₂, and RH24K₆ proteins (derived from HIV-1 p24 strain HXB2). The polyhistidine tag (H₆) used for metal chelate affinity chromatography is indicated in italic, arginine (R) and lysine (K) residues are indicated in bold letters.

from Cheynet et al. (8). They all present a six-histidine tag (H) at their N-terminal end, allowing a one-step purification by ion metal affinity chromatography (IMAC). They were termed RH24, RH24A₄K₂, RH24R₆, RH24R₄K₂, and RH24K₆ for recombinant protein with respectively no tag at their C-terminal end, a tag containing four alanines and two lysines (A₄K₂), six arginines (R₆), four arginines and two lysines (R₄K₂), and six lysines (K₆) for the last one. *E. coli* strain XL1 was transformed with the expression plasmids. The extracts of soluble proteins were purified on an FPLC Äkta system using a Zn-NTA activated gel. Purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and by size exclusion chromatography on a Shodex Protein Pack column on a Kontron HPLC (detection 280 nm).

Theoretical molar masses and isoelectric points were determined with the Mac Vector software (version 6.5.3) and compared with the experimental values obtained by mass spectrometry and isoelectric focusing. The amino acid sequence for each protein is reported in Figure 1. Ten lysines and 11 arginines groups are randomly dispersed along the nontagged RH24 protein macromolecule and conserved within all five recombinant proteins. NMR (9) and crystal structure data (10, 11, 12) demonstrated that, in the 3D structure, the 10 lysine groups were accessible for grafting though displaying different reactivities (13).

Protein Characterization. Mass Spectrometry (LC-ESI-MS). Analyses were performed with a single quadrupole API 100 mass spectrometer, 140B pumps, and a

785A detector (Perkin-Elmer). Reversed-phase liquid chromatography was performed on a C4 column (Vydac ref 214PT5115, 5 µm particle size). Solvent A was 0.1% (v/v) formic acid in water and solvent B was 0.1% (v/v) formic acid in water/acetonitrile mixture (5:95 v/v). A gradient of 40 to 60% of B was used.

Isoelectric Focusing (IEF). IEF were performed with a PhastSystem and PhastGel IEF 5-8 from Pharmacia. The separation method used for that IEF required three steps: a prefocusing step (2000 V, 2 mA, 3.5 W, 15 °C, 75 Vh), the sample application (200 V, 2 mA, 3.5 W, 15 °C, 15 Vh) and the focusing step (2000 V, 5 mA, 3.5 W, 15 °C, 510 Vh). After separation, PhastGel was colored using PhastGel Blue R, a coomassie R350 dye on the Phast-System from Pharmacia. The different steps of the fast coomassie staining were (i) fix: 20% trichloroacetic acid, (ii) wash/destain: 30% methanol and 10% acetic acid in distilled water (3:1:6), and (iii) stain: 0.02% PhastGel Blue R solution in approximately 30% methanol and 10% acetic acid in distilled water and 0.1% (w/v) CuSO₄. After development, gels were dried.

Copolymers. Four polymers were used, differing in molar masses and in nature of the comonomer used with maleic anhydride (MA). Alternate copolymers were obtained with either methyl vinyl ether (MVE) [sample MAMVE67 = 67000 g/mol and sample MAMVE20 = 20000 g/mol] or *N*-vinylpyrrolidone (NVP) [sample NVP-MA58 = 58400 g/mol and sample NVPMA29 = 29100 g/mol]. MAMVE copolymers were obtained from Poly-science Inc. (Warrington, PA). NVPMA were synthesized in the laboratory according to Veron et al (14).

Table 1. Design of the C-Terminal Tag of the Different Recombinant p24^a

	design of the C-terminal tag	amount of cationic amino acid per tag	amount of amino groups per tag
RH24	DLV	0	0
RH24A ₄ K ₂	AAAAKKSVDL	2	2 (16.6% of total K)
RH24R ₆	RRRRRSVDL	6 (22% of total R + K)	0
RH24R ₄ K ₂	RRRRKKSVDL	6 (22% of total R + K)	2 (16.6% of total K)
RH24K ₆	KKKKKSVDL	6	6 (37.5% of total K)

^a The conserved region of all the five recombinant p24 proteins contains 10 lysines and 11 arginines.

Table 2. Theoretical and Experimental Molar Masses and Isoelectric Points of HIV-1 Recombinant P24 Capsid Proteins

	amino acids	theoretical MM ^a	LC/ESI/MS determined MM ^a	theoretical Ip ^b	IEF determined Ip ^b
RH24	243	26950	26961	5.90	5.9
RH24A ₄ K ₂	252	27794	27803	6.06	6.06 and 6.5
RH24R ₆	252	28190	28199 and 27477	6.93	6.9 and 6.5
RH24R ₄ K ₂	252	28134	28144	6.93	6.9 and 6.7
RH24K ₆	252	28022	28032	6.93	6.9

^a Molar mass. ^b Isoelectric point.

Coupling of Proteins to Copolymers. The appropriate amount of copolymers was dissolved in anhydrous dimethyl sulfoxide (DMSO from Aldrich) at 37 °C. To a protein buffer solution at the appropriate concentration was added the required volume of polymer solution in DMSO. A standard reaction consisted of 100 µg of protein and 5 µg of polymer in 100 µL of buffer. The final concentration of DMSO in the mixture was 5%. The reaction mixture was stirred for 3 h at 37 °C. Bioconjugates were stored at 4 °C. For the bioconjugates obtained with partially hydrolyzed polymers, the polymers were dissolved in DMSO containing 10% (volume percent) H₂O and incubated 48 h at 37 °C before conjugation with proteins.

Bioconjugate Characterization. Bioconjugates were analyzed by size exclusion chromatography using a Shodex Protein KW-803, a Kontron HPLC 422 pump, a Kontron HPLC autosampler 465, and a Kontron UV diode array detector. Purification was run in a 0.1 M phosphate buffer pH 6.8 0.5% (w/v) of sodium dodecyl sulfate (SDS) with a flow rate of 0.5 mL/min. Detection was achieved by measuring the protein absorbance at 280 nm (the polymer did not generate any signal under our experimental conditions). The ratio of the peak area corresponding to the polymer bound protein versus the sum of the two peaks corresponding to the unbound and bound protein (i.e., the total amount of protein involved in the reaction) gave the coupling yield (Y). To assess the average amount of protein molecules per polymer chain: $N = nY/n'$ where n and n' , respectively, correspond to the number of protein molecules and the number of polymer chains in the reaction mixture.

RESULTS

Protein Characterization. The five recombinant proteins described above differ at their C-terminal end by the introduction of a tag containing variable density of primary amines and/or cationic charges (Table 1). The nontagged recombinant RH24 protein contains 11 arginines and 10 lysines representing respectively 4.5 and 4.1% of the total amount of amino acids. After purification by IMAC on a Zn-NTA activated gel, the purity of the proteins was greater than 95% as determined on a Coomassie Blue stained SDS polyacrylamide gel.

Every protein displayed the same behavior by size exclusion chromatography (HPLC) as well as in SDS polyacrylamide gel electrophoresis. Experimental determination of the molar masses by electrospray mass spectrometry was in accordance with the expected theoretical value for all recombinant proteins (Table 2). RH24R₆ protein exhibited an additional peak correspond-

ing to the expected value minus 722 mass unit. Such a deletion may occur at the N-terminal of the protein and correspond to the MRGSHH sequence, representing a 723 Da sequence. Moreover, MRGSHH-deleted RH24R₆ protein would exhibit a theoretical isoelectric point of 6.5 which is compatible with the experimental IEF gel pattern obtained (see below). Although such a deletion has never been observed for any six-histidine-tagged proteins manufactured in our laboratory (8, 15,16), the four remaining histidines are enough for an efficient purification (17). As the six-arginine tag remains nonaltered in the cleaved RH24R₆ protein, even if a C-terminal deletion might occur (SVDES- COOH and RSVDES- COOH deletion would represent 649 and 805 Da, respectively), this protein preparation was used in further experiments.

Theoretical isoelectric points were determined using Mac Vector software (version 6.5.3) and were compared to the experimental values. After isoelectric focusing, it appears on a Coomassie Blue stained gel that all the five proteins displayed a band corresponding to the expected isoelectric point (Table 2). Nevertheless, RH24A₄K₂, RH24R₄K₂, and RH24R₆ proteins displayed one or more unexpected bands which may be due to impurities, oxidized forms of p24 (data not shown) as previously described (18), or cleaved recombinant proteins as observed for RH24R₆ by MS analysis.

Protein Immobilization Conditions. In our previous paper (7), we demonstrated that the six-lysine-tagged protein reacted under a broader range of experimental conditions than the nontagged RH24 protein. Furthermore, it came out from this previous investigation that ionic strength was the physicochemical factor that had the most important impact on the course of the reaction, a reduction of the ionic strength of the coupling medium leading to an increase in coupling efficiency. Interestingly, the improvement was more important with the lysine-tagged protein. The present work aims at getting a better insight of the mechanisms involved in the formation of covalent conjugates between tagged proteins and reactive copolymers. Therefore, we investigated the effects of the natures and reactivities of both the polymer and the tag on the course of the immobilization reaction. Two different polymers were investigated whose reactivities were modified by controlling the level of hydrolysis of the functional groups prior to running the reaction. Tags of various compositions were genetically introduced in the proteins, and different reaction buffers were investigated. The results will be discussed first in term of immobilization efficiencies and, then, more in a fundamental viewpoint on the role of the tag in the covalent process.

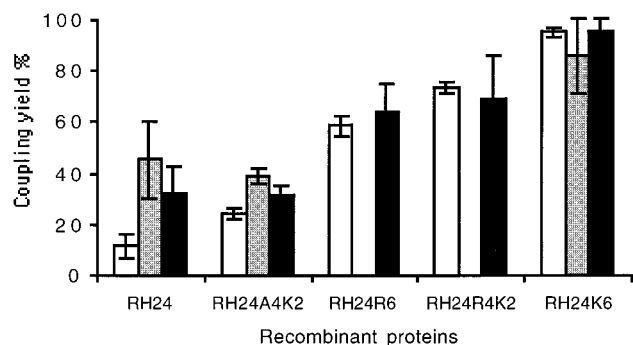


Figure 2. Coupling yield of recombinant proteins RH24, RH24A₄K₂, RH24R₆, RH24R₄K₂, and RH24K₆ to MAMVE67 copolymer, 3 h at 37 °C, in 20 mM carbonate buffer pH 8.2 (empty bar), 50 mM tris pH 7.0 (grey bar), and 50 mM phosphate pH 7.8 (black bar). [protein] = 0.95 g/L (3.56×10^{-9} mol) and [MAMVE67] = 0.048 g/L (7.46×10^{-11} mol). Experiments were performed in triplicate. The indicated data is the mean value $\pm$ two standard deviations.

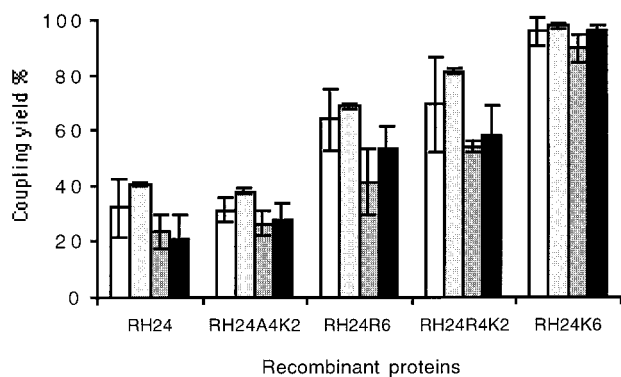


Figure 3. Coupling yield of recombinant proteins RH24, RH24A₄K₂, RH24R₆, RH24R₄K₂, and RH24K₆ to MAMVE67 (empty bar), MAMVE20 (clear grey bar), NVPMA58 (dark grey bar), and NVPMA29 (black bar) copolymers at 37 °C during 3 h in 50 mM phosphate buffer pH 7.8. [protein] = 0.95 g/L (3.56×10^{-9} mol), [MAMVE67] = 0.048 g/L (7.46×10^{-11} mol), [MAMVE20] = 0.048 g/L (2.5×10^{-10} mol), [NVPMA58] = 0.048 g/L (8.56×10^{-11} mol), and [NVPMA29] = 0.048 g/L (1.71×10^{-10} mol). Experiments were performed in triplicate. The indicated data is the mean value $\pm$ two standard deviations.

The effect of the buffer on the course of the reaction is reported in Figure 2 for each of the proteins. Carbonate and phosphate buffers presented similar patterns for the five proteins, excepted for RH24 whose coupling yield was quite poor in carbonate buffer as previously obtained (7). Conversely, the Tris buffer exhibited a quite different behavior. The coupling yields obtained with the less-reactive RH24 and RH24A₄K₂ proteins were higher in Tris buffer than in phosphate or in carbonate buffers.

Unexpectedly, in the Tris buffer, the two proteins containing arginine in the tags (RH24R₆ and RH24R₄K₂) precipitated on addition of the polymer solution in DMSO, leading to coupling yields close to 0%. Finally, the most reactive RH24K₆ protein exhibited the best coupling yields close to 100% whatever the buffer. This result led us to conclude that the more reactive the tag on the protein, the less important the influence of the coupling medium on the coupling yield. The phosphate buffer was retained for extensive investigations of the parameters involved in protein–copolymer coupling reaction. This is, in addition, convenient as it is the protein storage buffer.

Using the phosphate buffer, the effect of the nature and the molar mass of the polymer were investigated in terms of coupling yield for each of the five recombinant proteins used. In most cases, the lower molar mass polymers allowed slightly more efficient grafting reactions than the higher molar mass ones (Figure 3). As a general trend, the *N*-vinylpyrrolidone copolymers were slightly less reactive than the methyl vinyl ether based ones, reflecting their higher susceptibility to hydrolysis as reported by Véron et al (14). Nevertheless, as already noticed for the buffer, no significant difference was obtained when coupling the most reactive protein RH24K₆ to the four copolymers.

The level to which their functional groups were hydrolyzed by water (Figure 4) altered the reactivities of the polymers. Preliminary experiments showed that after storing the MAMVE polymer in a 10% (v/v) water DMSO mixture for 24 h, no alteration was observed in coupling efficiency. After 96 h of storage, the coupling yields were lower than 70% for RH24K₆, illustrating the loss of reactivity of the polymer due to hydrolysis of the functional groups. In Table 3, the effect of prehydrolysis of the polymers for 48 h prior to running the coupling reaction is shown. For RH24K₆, prehydrolysis of the MAMVE copolymer resulted in a slight decrease in the immobilization efficiency. The observed decrease was more important for the NVPMA copolymer, as its hydrolysis rate is higher than MAMVE (14). For RH24 and RH24R₆, which are less reactive than RH24K₆, the effect of prehydrolysis on the course of the reaction is more strongly marked. In addition, the reproducibility of the reaction under these conditions seems poor; the more sensitive to hydrolysis the polymer, the more variations in the results were observed. For ease of comparison, these results can be reported in percent of decrease of the coupling yield using the nonhydrolyzed polymer as a reference (Table 3). For the nontagged RH24 protein, the drop in binding efficiency is quite dramatic ranging from 80 to 98%. Conversely, the binding efficiency is moderately affected for the lysine-tagged RH24K₆ pro-

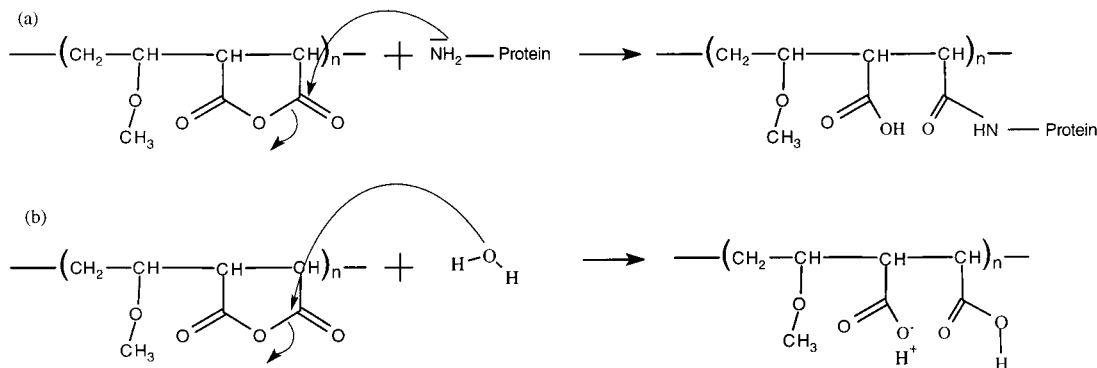


Figure 4. (a) Coupling reaction between recombinant protein and copolymer MAMVE and (b) hydrolysis competitive reaction.

Table 3. Coupling Yield of Recombinant Proteins RH24, RH24R₆, and RH24K₆ to Non Hydrolyzed or Partially Hydrolyzed Copolymers^a

proteins ^b	RH24				RH24R ₆				RH24K ₆			
polymers ^c	A67	A20	N58	N29	A67	A20	N58	N29	A67	A20	N58	N29
nonhydrolyzed ^d	22 ± 3	37 ± 7	24 ± 6	13 ± 3	64 ± 3	68 ± 4	42 ± 21	52 ± 3.5	98 ± 2	99 ± 2	94 ± 8	97 ± 5
hydrolyzed ^d	4 ± 1.5	2 ± 0.7	0.5 ± 0.6	0.4 ± 0.7	10 ± 17	19 ± 19	13 ± 22	9 ± 7.4	81 ± 6	82 ± 5	74 ± 19	62 ± 20
decrease ^e	82	94	98	97	84	72	68	82	17	17	22	37

^a Conjugation reaction 3 h at 37 °C, in 50 mM phosphate buffer pH 7.8. ^b [proteins] = 0.95 g/L (3.56×10^{-9} mol). ^c A67: [MAMVE67] = 0.048 g/L (7.46×10^{-11} mol), A20: [MAMVE20] = 0.048 g/L (2.5×10^{-10} mol), N58: [NVPMA58] = 0.048 g/L (8.56×10^{-11} mol) and N29: [NVPMA29] = 0.048 g/L (1.71×10^{-10} mol). Experiments were performed in triplicate. ^d Coupling yield of recombinant proteins to nonhydrolyzed and hydrolyzed copolymers; the indicated data are the mean value ± two standard deviations. ^e Decrease of the coupling yields obtained with hydrolyzed polymers referring to their nonhydrolyzed counterparts expressed in percentage.

tein, ranging from 17 to 37%. An intermediate situation is obtained for the six-arginine-tagged RH24R₆ protein, which exhibits decrease binding efficiency ranging from 68 to 84%. As already noticed for the previous parameters, the overall reaction efficiency is moderately affected by the amount of copolymer reactive functions when the protein counterpart is highly reactive, such as RH24K₆.

Finally, the nature of the tag was investigated as shown in Table 1. In the six-lysine tag, the amino groups were both positively charged and potentially reactive with the anhydride groups of the polymer. Therefore, in the following experiments, the chemical reactivity and the presence of positive charges in the tag ('physicochemical reactivity') were investigated. Recombinant RH24A₄K₂ protein bears a tag in which four out of six lysines were substituted by alanine, a neutral amino acid, reducing therefore the amount of charged/reactive amino acids in the tag. In recombinant protein RH24R₆, the tag is only composed of six arginine residues, still positively charged but barely capable of reacting with anhydride groups. The tag in protein RH24R₆K₂ is fully positively charged and bears two lysines that are the reactive amino acids of the tag. From Figures 2 and 3, it appears that the reactivity of the proteins depends on the presence in the tag of both positive charges and reactive amino groups. The least reactive tagged protein is RH24A₄K₂ featuring only two charged lysines in the tag whereas the most reactive one is the six-lysine-tagged RH24K₆ (higher coupling yields). Between these two extreme examples, RH24R₆ binds more efficiently to the polymer than RH24A₄K₂. Substituting two unreactive arginines of the R₆ tag by lysines (RH24R₄K₂) allowed an increase in the reactivity of the protein toward coupling onto the polymer. These results clearly demonstrate that the reactivities of the proteins are directly related to the reactivities of the tag.

Role of the Tag in the Immobilization Process.

The reactive amino acids of the tag which so drastically affect the course of the reaction as demonstrated above, correspond to ca. 2% of modification in the amino acid composition of the proteins. So, to better understand how such low amino acid variations in protein composition can impact so drastically on the reactivity of the protein, we investigated both the intrinsic reactivity of the tag toward the polymer and their behaviors in competition experiments with the proteins. In Table 4 is reported the composition of the peptides used in further experiments. The peptides contain 18 amino acid residues. The 12 carboxyl-terminal amino acids perfectly mimic the C-terminal end of a protein. The amino-terminal sequence consists of a stretch of six amino acids different from the flanking sequences of the recombinant proteins. The two N-terminal acetylated (Ac-K₆ and Ac-R₆) peptides mimic a situation where a protein does not contain, in a close vicinity of the tag, any primary amino group available for grafting. Conversely, NH₂-K₆ and NH₂-R₆ peptides

Table 4. Amino Acid Sequences of the Peptides Used to Mimic the Tags and Their Coupling Yield to MAMVE67 Copolymer

name	amino acid sequence	coupling yield %
NH ₂ -Ct	H ₂ N-SFADTPYPWGWLDEGYDAE-COOH	n.d.
NH ₂ -K ₆	NH ₂ -IEGRPGKKKKKKSVDES-COOH	80
Ac-K ₆	AcNH-IEGRPGKKKKKKSVDES-COOH	68
NH ₂ -R ₆	NH ₂ -IEGRPGRRRRRRSVDES-COOH	94
Ac-R ₆	AcNH-IEGRPGRRRRRRSVDES-COOH	32

exhibit a free terminal amine accessible for grafting. A non K non R containing peptide was used as control (NH₂-Ct).

The intrinsic reactivities of the tags were assessed in conjugation reactions of the peptides with copolymer MAMVE67 under identical experimental conditions used for the proteins (50 mM phosphate buffer, pH 7.8). The following gradation in coupling efficiency was observed, NH₂-R₆ > NH₂-K₆ > Ac-K₆ >> Ac-R₆, from the most to the least reactive peptide (Table 4). Comparison from acetylated versus nonacetylated peptides showed that, at least in such short peptides, the amino terminal group is a major site involved in grafting. In an NH₂-terminal free reactive group context, the R₆-containing peptide proves more valuable in grafting than the K₆ one. Taken together, these two last results confirm our preliminary study on a model peptide reported earlier (19). From these data, it can be stated that arginine groups are as efficient as lysine groups at generating attractive electrostatic interactions with the copolymer. The lower grafting yield of the NH₂-K₆ peptide may be due to multiple reaction sites on the polymer because of the presence of seven potentially reactive primary amine. Conversely, NH₂-R₆ can only react via the terminal amino group and is so bound to the polymer via a single attachment point. Quite logically, Ac-K₆ peptide was much more reactive than the Ac-R₆ one, whose residual reactivity may be explained by the presence of the weak nucleophilic hydroxyl of serine, or/and by a disfavored, but still existing, reaction of arginine groups onto reactive anhydrides.

The properties of these peptides were further evaluated in competition assays with the most reactive RH24K₆ protein in grafting reactions with MAMVE67 copolymer. The deduced percentages of inhibition, resulting from the addition of increasing amounts of peptides to the coupling mixture, are reported in Figure 5. All curves displayed a characteristic shape with a more or less steep slope and a variable plateau value. At a peptide/protein molar ratio lower than 25, the percentage of inhibition increased with increasing amount of peptides in the coupling mixture, except for the control peptide NH₂-Ct, which being uncharged, had no interference with the immobilization of RH24K₆. The following gradation in competition efficiency was observed NH₂-K₆ > NH₂-R₆ > Ac-K₆ >> Ac-

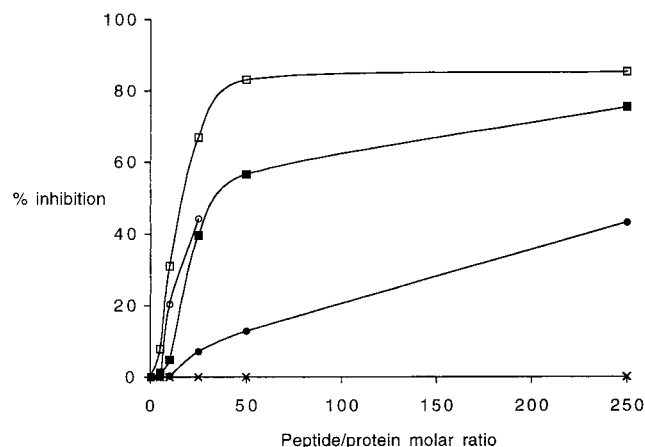


Figure 5. Percent inhibition of the formation of bioconjugates RH24K₆ to MAMVE67 as a function of the nature of the peptide during a competitive coupling reaction. [protein] = 0.34 g/L (1.28×10^{-9} mol), [MAMVE67] = 0.048 g/L (7.46×10^{-11} mol). Peptides: NH₂-K₆ (□), Ac-K₆ (■), NH₂-R₆ (○), Ac-R₆ (●), NH₂-Ct (×). Coupling reaction in 50 mM phosphate buffer pH 7.8, 3 h at 37 °C. Because samples precipitated when the peptide/protein molar ratio was greater than 25/1, a plateau value was not obtained for curve NH₂-R₆ (○).

R₆ in descending order. The same gradation is observed at molar ratio higher than 25. All curves tend toward a plateau, which was reached as early as a ratio of 50 by NH₂-K₆ peptide. No plateau could be obtained for the NH₂-R₆ peptide due to a precipitation of the conjugates when peptide/protein molar ratio was greater than 25.

These data clearly show that in this reaction, the reactivity of the peptide is related to both its amine and charge density.

Finally, the most reactive NH₂-K₆ peptide and its acetylated counterpart were used in competition assays, in the grafting reaction, with the five recombinant proteins bearing tags of variable reactivities. The deduced percentage of inhibition are reported in Figure 6. The curves displayed patterns similar to those described above. It appears that the inhibition potential of the peptide strongly depended on the nature of the tag on the protein (Figure 6). Ac-K₆ peptide competed more efficiently with the least reactive proteins (RH24 and RH24A₄K₂) than with the most reactive ones (RH24R₆, RH24R₄K₂, or RH24K₆), as shown by the steepest slope and highest plateau value of the inhibition curves. The same inhibition pattern was obtained with NH₂-K₆ peptide, although with a more pronounced effect than its acetylated counterpart, due to greater reactivity of NH₂-K₆ peptide as discussed above. These results show evidence that recombinant protein reactivity in the grafting reaction is directly correlated with the density of charges (R and K) and primary amines (K) within the C-terminal-fused tag.

DISCUSSION

The molar masses and compositions of the polymers, as well as their reactivities, have moderate effects on the course of the reaction compared to the drastic ones observed with tags of differing compositions. The reactivities of the proteins were increased if the tag bore six positively charged amino acids, and to a better extent if it comprised functional groups susceptible to react with the anhydride moieties of the polymer (Figure 2, Figure 3, and Table 3). These results prompted us to investigate the effects of the reactivities of the tag on the mechanism of binding of the proteins. By definition, a reactive tag is

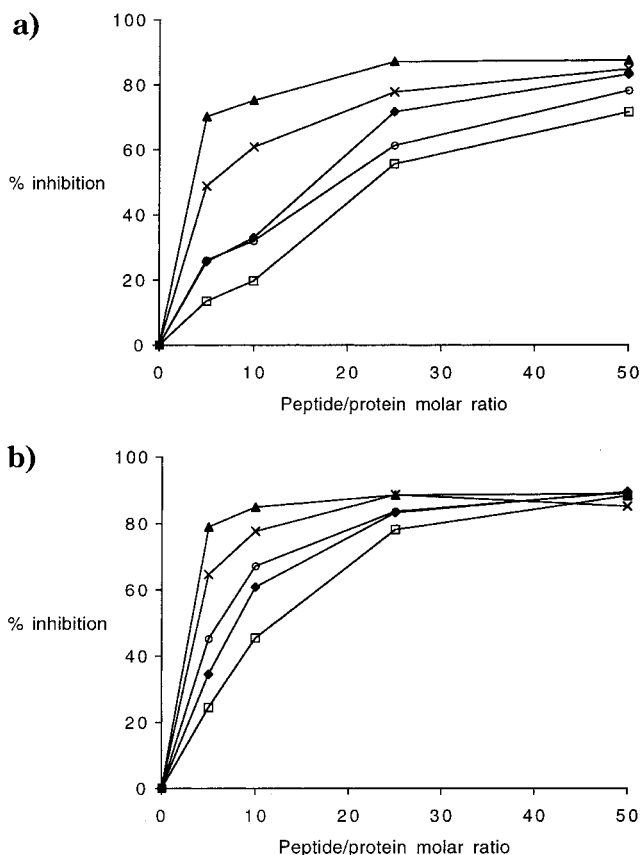


Figure 6. Percent inhibition of the five recombinant proteins RH24 (×), RH24A₄K₂ (▲), RH24R₆ (○), RH24R₄K₂ (◆), and RH24K₆ (□) in competitive reaction with (a) peptide Ac-K₆ and (b) NH₂-K₆. [protein] = 0.34 g/L (6.4×10^{-10} mol), [MAMVE67] = 0.048 g/L (3.73×10^{-11} mol). Coupling reaction in 50 mM phosphate buffer pH 7.8, 3 h at 37 °C.

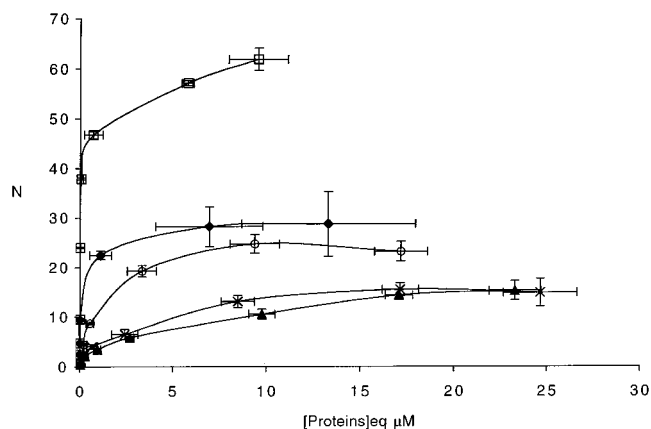


Figure 7. Immobilization isotherms of RH24 (×), RH24A₄K₂ (▲), RH24R₆ (○), RH24R₄K₂ (◆), and RH24K₆ (□) to MAMVE67 copolymer in 50 mM phosphate buffer pH 7.8, 3 h at 37 °C. Experiments were performed five times. The indicated data is the mean value $\pm$ one standard deviation. N corresponds to the number of proteins per polymer chain.

positively charged and its overall reactivity increased by increasing the amount of lysine residues in its composition. Immobilization isotherms presented in Figure 7 show that both the maximal amount of covalently bound protein (plateau value) and the affinity (slope) of the protein for the polymer are directly related to the amino acid composition of the tags (Table 5). The more reactive the tag, the steeper the slope of the isotherms and the more proteins were bound per polymer chain. The Scatchard plots presented in Figure 8, corresponding to the

Table 5. Correlation between Physicochemical Features of Recombinant Proteins and Isotherms Curves Characteristics

	RH24		RH24A ₄ K ₂		RH24R ₆		RH24R ₄ K ₂		RH24K ₆	
	protein	tag	protein	tag	protein	tag	protein	tag	protein	tag
cations ^a	21	0	23	2	27	6	27	6	27	6
amines ^a	10	0	12	2	10	0	12	2	16	6
slope ^{b,c}	6.7 ± 3.4		3.3 ± 0.8		18 ± 4.4		437 ± 81		1743 ± 96	
plateau ^{b,c}	18 ± 4.8		19 ± 3.5		27 ± 2		28 ± 4.5		54 ± 6	

^a Number of cationic and amine residues in the recombinant protein. ^b Slope and plateau value of the isotherms curves determined with GraFit Software version 3.0. ^c Experiments were performed five times. The indicated data is the mean value plus one standard deviation.

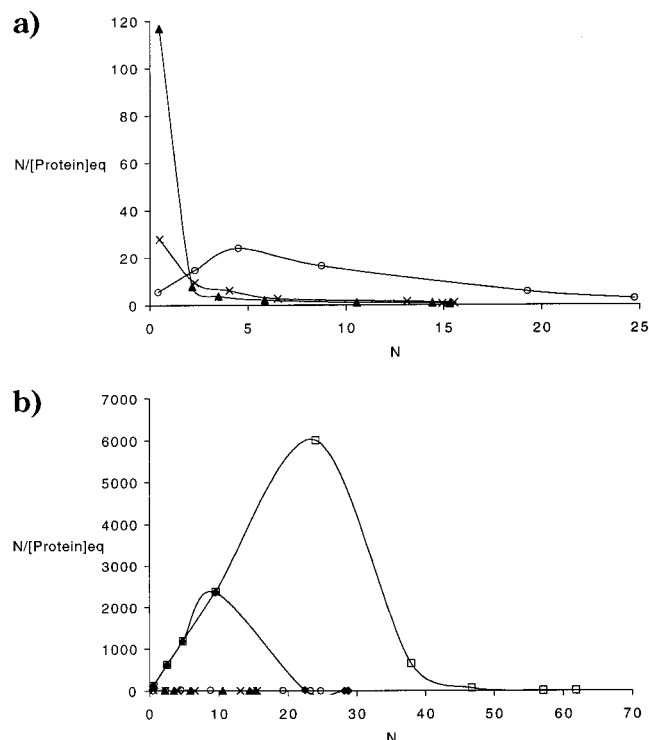


Figure 8. Scatchard plots of (a) RH24 (×), RH24A₄K₂ (▲), RH24R₆ (○); (b) RH24R₄K₂ (◆) and RH24K₆ (□). Coupling reaction in 50 mM phosphate buffer pH 7.8, 3 h at 37 °C. N corresponds to the number of proteins per polymer chain.

isotherms, can be divided into two groups. The first group corresponds to the exponential decrease observed for proteins RH24 and RH24A₄K₂, respectively bearing no tag or a poorly reactive one. For these two proteins, the curve is concave up, corresponding to a binding process taking place with negative cooperativity (20), i.e. the already bound proteins preventing the binding of others via steric hindrance and/or electrostatic repulsive forces. Conversely, for proteins with a reactive tag, the Scatchard plots displayed a bell-shaped curve, revealing that the binding process occurred with positive cooperativity (21). The maxima of the bell-shaped curves were directly dependent on the reactivities of the tags; the more reactive, the higher the ratio and, thus, the more efficient the binding reaction. Similarly, the positions of the maxima along the X-axis respected the order of reactivities of the tags: situated at five proteins per chain for the least charged tag, the maximum is found at 30 for the most reactive one.

The strong dependency of the performance of the reaction with the reactivities of the tags suggests the reaction to be mainly mediated by the tag, in a regioselective manner. In other words, the binding reaction preferentially takes place at the site of the tag, or in its close vicinity. Supposing saturation of the polymer is

obtained for 60 protein molecules bound per polymer chain ($N_{\max} = 60$) as observed with the most reactive RH24K₆, then the fractional saturation can be determined as $\theta = N/N_{\max}$. Hill's plots ($\log(\theta/(1 - \theta))$ vs C_{eq}) of grafting data for RH24 and RH24A₄K₂ featured the same linearity, and the slope (Hill coefficient) was 0.49. This low value confirms the negative cooperativity observed in the Scatchard plots. Conversely, for the data obtained with the positively charged tags, no existing model could fit our experimental data despite that Langmuir, multi-equilibrium, or Freundlich models were taken into account. Even 'the multibinding sites ligand' model, developed by Mc Ghee and von Hippel (22), was not successfully fitted with our experimental data though it actually predicts bell-shaped Scatchard plots for positive cooperativity. In fact, our data feature a deformed-bell shape with a linear increase of the ratio values up to the maximum. This linear increase can be explained by a high affinity of the protein for the polymer due to the existence of electrostatic interactions that exhausts the protein content of the medium, up to a level of pseudo-saturation (the decreasing and plateau sections). After this saturation level, the binding process gets less efficient and becomes dependent on the protein concentration. In addition, the chemical reactivity is an important factor as, when the amino acids are susceptible to react with the polymer, the protein/polymer affinity is higher and more protein can be accommodated onto the macromolecular chain, because the grafting reaction takes place at the site of the tag. Interestingly, the shapes of the Scatchard plots obtained for the proteins proved similar to those reported earlier for peptides, the chemical reactivities of which were directly related to the amino acid composition of the tags.

CONCLUSION

This paper points out the importance of the reactivity of the tag introduced in the protein on the efficiency of the covalent grafting. We demonstrated that the more reactive the tag, the less important the other parameters (i.e., the nature of the buffer, the nature and size of the polymer, and then the reactivity of the polymer) on the control of the covalent immobilization. This allows the use of a great variety of many experimental conditions to perform the covalent grafting, provided the tag be reactive enough.

In a previous work, we had demonstrated that 50 molecules of a lysine-tagged protein could be immobilized per polymer chain. The accommodation of so many molecules on the polymer required an end-on conformation from the protein, taking into account its dimensions, the link between the polymer and the protein was supposed to take place via the tag. In the present work, we demonstrated that the reactivity of a protein depended directly on the reactivity of its tag, which confirmed our previous hypothesis that the tag was the

site where the interactions with the polymer occurred. The coupling isotherms and the Scatchard plots of the immobilization process of each of the tagged proteins pointed out that the binding of proteins bearing highly reactive tags took place with a positive cooperativity. Conversely, in absence of tag or with a poorly reactive one, the binding occurred with a negative cooperativity.

Our investigations demonstrated that the immobilization of tagged recombinant proteins can occur in a regioselective manner, by taking profit of attractive electrostatic forces between the polymer and the natural macromolecule. This concept will be used in further developments for various applications such as diagnostics or for the realization of macromolecular assemblies for the delivery of bioactive substances.

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Synthesis and Biological Studies of 5-Aminolevulinic Acid-Containing Dendrimers for Photodynamic Therapy

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Using a convergent growth approach, a series of novel 5-aminolevulinic acid (ALA)-containing dendrimers have been synthesized. In these molecules, ALA residues are attached to the periphery by ester linkages, with amide bonds connecting the dendrons. Three first-generation dendrimers, bearing either 6 or 9 ALA residues, were synthesized by attachment of a tris(Boc-protected ALA)-containing wedge (**1**) to a di- or tripodent aromatic, or tripodent aliphatic core. Two second generation 18-ALA-containing dendrimers were also synthesized using a 3,3'-iminodipropionic acid spacer unit between wedge **1** and the aromatic core. These compounds differed only in the distance between the core and the linker unit. The Boc-protected dendrimers were deprotected using trifluoroacetic acid and isolated as their TFA salts. The potential of these ALA ester dendrimers as macromolecular prodrugs for photodynamic therapy has been demonstrated in the tumorigenic keratinocyte PAM 212 cell line.

INTRODUCTION

Photodynamic therapy (PDT) is a nonthermal technique for inducing tissue damage with light following administration of a light-activated photosensitizing drug which can be selectively retained in malignant or diseased lesions relative to normal adjacent tissue (1). Activation of the photosensitizer results in the generation of reactive oxygen species (ROS), mainly singlet oxygen via interaction of the sensitizer triplet state with molecular oxygen. The ROS oxidize intracellular substrates such as lipids and amino acid residues leading to cell death.

The most widely studied application of PDT to date is in cancer therapy. However, using conventional exogenous sensitizers, skin photosensitivity can be prolonged, and damage to normal tissue is rarely avoidable due to poor tumor selectivity. The use of 5-aminolevulinic acid (ALA) is a relatively new approach in PDT. ALA is a natural precursor of protoporphyrin IX (PpIX), an effective photosensitizer, and cellular concentrations of PpIX can be increased by the administration of 5-aminolevulinic acid (ALA) (2). A certain amount of tumor selectivity can be achieved since porphobilinogen deaminase (a rate-limiting enzyme in heme biosynthesis) activity is increased (3) and ferrochelatase (the enzyme which catalyzes the conversion of photoactive PpIX into inactive heme) levels are decreased in tumor cells (4). For therapeutic applications, PpIX is activated using red light (typically at 635 nm), with mitochondria (5) believed to be key targets of singlet oxygen oxidative damage resulting in cell death mainly via apoptosis (6). PpIX levels return to normal after 24 h, following administra-

tion so skin photosensitization is relatively short-lived compared to many exogenous sensitizers (7).

A major limitation of ALA-PDT is the low intracellular availability of ALA. Cellular uptake of ALA occurs via a relatively slow active transport mechanism (8, 9). Recently it has been shown that ALA esters, being more lipophilic than ALA (e.g., ALA hexyl ester), can quickly cross the cell membrane by diffusion and (10, 11), once inside the cell, are cleaved by nonspecific esterases (11–18). Since higher intracellular ALA levels lead to higher PpIX concentrations, lipophilic ALA prodrug derivatives are more efficient at inducing PpIX photosensitization. A further improvement based on this strategy would be to design a prodrug that can deliver several ALA molecules into the cell, and selectivity of the prodrug for target cells would be an obvious advantage.

Polymeric drug delivery systems present an attractive method for drug targeting, particularly in cancer chemotherapy (19). In the past, most of the polymers investigated as drug carriers have been polydisperse and either linear or highly branched. Recent advances in polymer chemistry now allow the synthesis of structurally defined, hyperbranched polymers (or dendrimers) which can be conjugated with drug molecules (20). The drug can either be reacted with a preformed dendrimer (21–23), in which case the exact loading of the drug cannot easily be controlled, or can be incorporated into the structure of the dendrimer during synthesis. The latter offers the opportunity for a polymeric prodrug which is structurally defined with a known size and drug loading. Here we describe the synthesis of a novel series of dendritic ALA ester prodrugs for use in PDT applications.

MATERIALS AND METHODS

General Methods. ¹H and ¹³C NMR spectra were recorded on a JEOL EX270 MHz spectrometer. Chemical shifts are quoted in ppm measured downfield relative to

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TMS. Ultraviolet–visible spectra were recorded on a Unicam UV2 spectrophotometer in dichloromethane solution. Infrared spectra were recorded on a Nicolet FTIR spectrophotometer as thin films in dichloromethane. Mass spectra were obtained using either an EI, CI, FAB or electrospray technique with a NOBA matrix. Melting points were measured using a Kofler hot-plate microscope. Analytical thin-layer chromatography was carried out using Merck silica gel 60, aluminum-backed silica-coated plates which were visualized using ultraviolet light (254 nm). Column chromatography was carried out using Prolabo silica gel (220–400 mesh flash grade). All compounds were purified to one spot by TLC and R_f values are quoted using the same solvent system as used for column chromatography unless otherwise stated. All reagents were purchased from Aldrich, Avocado, or Acros chemical companies and used without further purification.

Dendrimer Synthesis. ALA was prepared by the method of Hart et al. (24). Compounds **2**, **3**, **4**, **7**, and **11** were prepared according to Ashton et al. (25).

N-tert-Butyloxycarbonyl-5-aminolevulinic Acid (8, Boc-ALA). A solution of 5-aminolevulinic acid (0.42 g, 2.50 mmol) in water (5 mL) was adjusted to pH 8–10 with aqueous sodium hydroxide (0.1 N). Di-tert-butyl dicarbonate (1.16 g, 5.33 mmol) was dissolved in 1,4-dioxane (5 mL) and added to the mixture which was stirred at room temperature for 18 h. The excess of di-tert-butyl dicarbonate was removed by washing the mixture with diethyl ether (2 × 50 mL). Ethyl acetate (50 mL) was added, and the aqueous layer was acidified with dilute hydrochloric acid (1 N). Separation of the organic layer, followed by removal of the solvent, gave 0.35 g (60%) of Boc-ALA as a colorless oil; IR (film) 3445, 2765, 2242, 1721, 1389, 1364 cm^{-1} ; ^1H NMR (270 MHz, CDCl_3) δ 10.42 (br s, 1H), 5.25 (br s, 1H), 4.00 (s, 2H), 3.26 (m, 4H), 1.42 (s, 9H); ^{13}C NMR (67.5 MHz, CDCl_3) δ 204.47, 176.75, 155.89, 128.58; 80.10, 49.61, 33.88, 27.34; CI-MS m/z 232 (M^+ + H).

N-(Benzyloxycarbonyl)[tris(N-tert-butyloxycarbonyl-5-aminolevulinylloxymethyl)methyl]methylamine (9). Compound **7** (0.31 g, 1.22 mmol) in DMF (10 mL) was added dropwise, over 50 min, to a mixture of Boc-ALA (**8**, 1.0 g, 4.30 mmol), DCC (0.82 g, 4.10 mmol), and DMAP (0.05 g, 0.42 mmol) in dichloromethane and DMF (15 mL, 2:1 v/v). The mixture was stirred at room temperature for 48 h, after which time the heavy precipitate was filtered off, and the solvents were evaporated in vacuo. The residue was purified by column chromatography on silica gel (EtOAc/hexane 7:3, R_f = 0.46) to obtain 0.52 g (75%) of compound **9** as a colorless oil; IR (film) 3372, 2680, 2352, 1755, 1718, 1513, 1457, 1370 cm^{-1} ; ^1H NMR (270 MHz, CDCl_3) δ 7.41 (m, 5H), 5.86 (br s, 1H), 5.58 (br s, 3H), 5.13 (s, 2H), 4.47 (s, 6H), 4.05 (d, J = 5.2 Hz, 6H), 2.76 (m, 6H), 2.09 (m, 6H), 1.37 (s, 27H); ^{13}C NMR (67.5 MHz, CDCl_3) δ 127.68, 127.86, 153.23, 155.17, 171.17, 172.33, 203.44; 127.54, 79.11, 61.85, 56.50, 48.92, 33.48, 27.64, 27.35, 26.90; FAB-MS m/z 917 (M^+ + Na); FAB-HRMS calcd for $\text{C}_{42}\text{H}_{62}\text{N}_4\text{O}_{17}\text{Na}$ (M^+) 917.4008, found 917.3998.

Tris(tert-butyloxycarbonyl-5-aminolevulinylloxymethyl)methylamine (1). The Z-protected amine **9** (0.53 g, 0.59 mmol) was dissolved in ethyl acetate and methanol (15 mL, 2:1 v/v). Palladium on carbon (0.17 g, 10%) was added, and the mixture was stirred under an atmosphere of hydrogen for 8 h at room temperature. The reaction mixture was filtered through Celite which was washed with a further amount of ethyl acetate. The solvents were evaporated in vacuo, and the residue was

dried to obtain 0.44 g (97%) of compound **1** as a colorless oil; IR (film) 3375, 2654, 1760, 1718, 1370 cm^{-1} ; ^1H NMR (270 MHz, CDCl_3) δ 8.95 (br s, 2H), 5.54 (br s, 3H), 4.32 (s, 6H), 3.98 (d, J = 4.3 Hz, 6H), 2.75 (s, 6H), 2.69 (m, 6H), 1.38 (s, 27H); ^{13}C NMR (67.5 MHz, CDCl_3) δ 205.56, 171.90, 155.62, 79.64, 65.36, 54.05, 49.99, 34.07, 28.15, 27.45; ES-MS m/z 761 (M^+ + H); ES-HRMS calcd for $\text{C}_{34}\text{H}_{57}\text{N}_4\text{O}_{15}$ (M^+ + H) 761.3820, found 761.3821.

1,3,5-Benzenetricarbamido-N,N',N''-tris(ethylpropionate) (10). A solution of 1,3,5-benzenetricarbonyl trichloride (3.10 g, 11.34 mmol) in dichloromethane and DMF (15 mL, 2:1 v/v) was slowly added, over a period of 2 h, to a stirred mixture of β -alanine ethyl ester hydrochloride (6.90 g, 45.0 mmol) and triethylamine (4.20 mL, 30.0 mmol) in dichloromethane and DMF (15 mL, 2:1 v/v) at 0 °C. Stirring was continued at the same temperature for 4 h and at room temperature for 16 h. The reaction mixture was then filtered, and the solvents were evaporated in vacuo. The resulting residue was dissolved in dichloromethane (50 mL) and washed successively with aqueous hydrochloric acid (50 mL, 2 N), saturated aqueous sodium hydrogen carbonate (50 mL), and water (20 mL), dried (MgSO_4), and evaporated to obtain 6.20 g (61%) of the title compound as a white powder, mp 181–183 °C; IR (film) 3419, 2276, 2340, 1710, 1650 cm^{-1} ; ^1H NMR (270 MHz, CDCl_3) δ 8.10 (s, 3H), 7.49 (s, 3H), 4.18 (q, J = 4.0 Hz, 6H), 3.71 (m, 6H), 2.69 (m, 6H), 1.27 (t, J = 4.0 Hz, 9H); ^{13}C NMR (67.5 MHz, CDCl_3) δ 173.00, 166.42, 135.36, 128.56, 61.30, 36.28, 34.26, 14.55; CI-MS m/z 508 (M^+ + H); EI-HRMS calcd for $\text{C}_{24}\text{H}_{33}\text{N}_3\text{O}_9$ (M^+) 507.2217, found 507.2228.

1,3,5-Benzenetricarbamido-N,N',N''-tripropionic Acid (5). Compound **10** (4.00 g, 7.92 mmol) was dissolved in methanol (15 mL), and the solution was cooled to 0 °C. Aqueous sodium hydroxide (70 mL, 2 N) was added, and the solution was left to stir for 3 h. The resultant precipitate was dissolved by the addition of water (7 mL), and the solution was neutralized with Amberlite IR-120 (H^+ form) ion-exchange resin, filtered, evaporated, and dried thoroughly to afford 0.61 g (93%) of **5** as a white powder, mp 251–252 °C; IR (Nujol) 3455, 2237, 1689, 1631 cm^{-1} ; ^1H NMR (270 MHz, d_6 -DMSO) δ 12.42 (br s, 3H), 8.91 (t, J = 5 Hz, 3H), 8.51 (s, 3H), 3.68 (m, 6H), 2.63 (m, 6H); ^{13}C NMR (67.5 MHz, d_6 -DMSO) δ 172.88, 165.49, 134.80, 128.51, 35.72, 33.61; CI-MS m/z 424 (M^+ + H); CI-HRMS calcd for $\text{C}_{18}\text{H}_{22}\text{N}_3\text{O}_9$ (M^+ + H) 424.1356, found 424.1368.

N,N'-[N-[Tris(N-tert-butyloxycarbonyl-5-aminolevulinylloxymethyl)methyl]acetamido]terephthalamide (11). A solution of **1** (0.42 g, 0.58 mmol) in dichloromethane (10 mL) was added dropwise to a mixture of **3** (70 mg, 0.22 mmol), EDC (0.12 g, 0.50 mmol), and HOBt (74 mg, 0.50 mmol) in dichloromethane and DMF (15 mL, 2:1 v/v) at 0 °C under argon. The reaction mixture was allowed to stir for 48 h at room temperature. The solvents were then evaporated, and the residue was dissolved in ethyl acetate (30 mL) and washed with hydrochloric acid (2 × 20 mL, 2 N), saturated aqueous sodium hydrogen carbonate (2 × 20 mL), and water (2 × 20 mL). The solvent was evaporated to give a crude oily product which was subjected to column chromatography on silica gel (EtOAc/MeOH 9:1, R_f = 0.55) to give 0.19 g (46%) of compound **11** as a white solid, mp 106 °C; IR (film) 3397, 2359, 2229, 2130, 1695, 1598, 1394 cm^{-1} ; UV (MeOH) 202, 241 nm; ^1H NMR (270 MHz, CDCl_3) δ 7.95 (s, 4H), 7.82 (br s, 2H), 6.93 (br s, 2H), 5.49 (br s, 6H), 4.44 (s, 12H), 4.07 (d, J = 6.1 Hz, 4H), 4.01 (d, J = 7.5 Hz, 12H), 2.88 (m, 12H), 2.61 (m, 12H), 1.43 (s, 54H); ^{13}C NMR (67.5 MHz, CDCl_3) δ 206.17,

171.51, 166.60, 155.22, 135.86, 127.08, 79.45, 62.33, 59.88, 49.64, 43.45, 33.70, 28.84, 27.81, 27.53; FAB-MS m/z 1787 ($M^+ + Na$). Anal. Calcd for $C_{80}H_{120}N_{10}O_{34}$: C, 54.4; H, 6.9; N, 7.9. Found: C, 53.9; H, 6.9; N, 7.7.

***N,N'*-[*N*-[Tris(5-aminolevulinylloxymethyl)methyl]acetamido]terephthalamide-6Trifluoroacetic Acid (12).** Compound **11** (30 mg, 20 μ mol) was dissolved in dichloromethane (10 mL), trifluoroacetic acid TFA (2 mL) was added, and the mixture was stirred at room temperature for 2 h. The solvents were evaporated under reduced pressure to yield an oily product which was crystallized from a mixture of methanol, ethyl acetate, and diethyl ether. 20 mg (63%) of compound **12** was obtained as a pale yellow solid; ^1H NMR (270 MHz, CD_3OD) δ 7.98 (s, 4H), 4.43 (s, 12H), 4.40 (s, 4H), 4.01 (s, 12H), 2.82 (m, 12H), 2.68 (m, 12H); ^{13}C NMR (67.5 MHz, CD_3OD) δ 200.89, 171.57, 169.66, 160.62, 135.76, 126.63, 76.51, 61.27, 58.81, 45.99, 42.25, 33.12, 29.17, 26.31.

1,3,5-Tris(*N*-[*N*-[tris(*N*-*tert*-butyloxycarbonyl-5-aminolevulinylloxymethyl)methyl]acetamido]carbamido)benzene (13). A solution of **1** (0.50 g, 2.16 mmol) in dry dichloromethane (10 mL) was added dropwise to a stirred solution of **4** (0.23 g, 0.60 mmol), DCC (0.43 g, 2.14 mmol), and HOBt (0.28 g, 2.14 mmol) in dichloromethane and DMF (15 mL, 2:1 v/v) at 0 °C under argon. The reaction mixture was left to stir at room temperature for 4 days and then filtered, and the solvents were evaporated. The resulting residue was dissolved in ethyl acetate and washed with 5% hydrochloric acid (2 $\times$ 30 mL), saturated sodium hydrogen carbonate (30 mL), and water (2 $\times$ 30 mL). The organic layer was evaporated and the residue purified by column chromatography (EtOAc/MeOH 8:2, R_f = 0.45) to obtain 0.61 g (40%) of **13** as a foamy solid, mp 115–116 °C; IR (film) 3366, 2955, 2365, 2137, 1693, 1678, 1519, 1364 cm^{-1} ; UV (MeOH) 209, 240 nm; ^1H NMR (270 MHz, $CDCl_3$) δ 8.81 (s, 3H), 8.22 (br s, 3H), 6.17 (br s, 3H), 6.12 (br s, 9H), 4.36 (s, 6H), 3.97 (s, 18H), 3.94 (d, J = 6.5 Hz, 18H), 2.65 (m, 18H), 2.45 (m, 18H), 1.31 (s, 81H); ^{13}C NMR (67.5 MHz, d_6 -acetone) δ 206.22, 172.74, 170.32, 156.88, 135.63, 129.98, 79.36, 63.16, 62.97, 59.10, 50.57, 44.39, 34.67, 28.96, 28.56; FAB-MS m/z 2632 ($M^+ + Na$). Anal. Calcd for $C_{117}H_{177}N_{15}O_{51}$: C, 53.8; H, 6.8; N, 8.1. Found: C, 54.6; H, 7.4; N, 7.7.

1,3,5-Tris[*N*-[*N*-[tris(5-aminolevulinylloxymethyl)methyl]acetamido]carbamido]benzene-9Trifluoroacetic Acid (14). Compound **13** (30 mg, 10 μ mol) was dissolved in dichloromethane (10 mL). TFA (2 mL) was added to the solution, and the mixture was stirred at room temperature for 2 h. The solvents were evaporated under reduced pressure to yield an oily product which was crystallized from a mixture of methanol, ethyl acetate, and diethyl ether to obtain 2 mg (66%) of the TFA salt as a pale yellow solid; ^1H NMR (270 MHz, CD_3OD) δ 8.58 (s, 3H), 4.45 (s, 18H), 4.35 (s, 6H), 4.04 (d, J = 4.5 Hz, 18H), 2.87 (m, 18H), 2.70 (m, 18H); ^{13}C NMR (67.5 MHz, CD_3OD) δ 206.22, 205.92, 172.74, 170.32, 156.88, 135.63, 129.98, 79.36, 63.16, 62.97, 59.10, 50.57, 44.39, 34.67.

Tris[*N*-[tris(*N*-*tert*-butyloxycarbonyl-5-aminolevulinylloxymethyl)methyl]acetamido]amine (15). Nitrotri-acetic acid (**6**, 60 mg, 0.32 mmol) was dissolved in dichloromethane and DMF (20 mL, 2:1 v/v), and DCC (0.23 g, 1.10 mmol) and HOBt (0.15 g, 1.10 mmol) were added. The mixture was stirred under argon for 30 min at 0 °C, and then compound **1** (0.85 g, 1.14 mmol) was added dropwise over 50 min. The mixture was allowed to stir at room temperature for 3 days, the precipitate was filtered off, and the solvents were evaporated. The

residue was dissolved in ethyl acetate (30 mL) and washed with hydrochloric acid (2 $\times$ 30 mL, 2 N), saturated sodium hydrogen carbonate (30 mL), and water (2 $\times$ 30 mL). The organic layer was evaporated to give an oily product which was subjected to flash chromatography (EtOAc/MeOH 49:1, R_f 0.6). The product was crystallized from a mixture of methanol, diethyl ether, and ethyl acetate giving 0.45 g (60%) of compound **15** as a foamy solid, mp 102–104 °C; IR (film) 3372, 2980, 2365, 1699, 1640, 1364 cm^{-1} ; UV (MeOH) 214 nm; ^1H NMR (270 MHz, $CDCl_3$) δ 6.39 (br s, 3H), 5.41 (br s, 9H), 4.37 (s, 18H), 4.04 (s, 18H), 2.75 (m, 18H), 2.65 (m, 18H), 2.51 (s, 6H), 1.44 (s, 81H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 204.28, 171.51, 171.38, 155.37, 61.78, 57.64, 49.64, 33.91, 33.72, 29.41, 27.01, 27.16; FAB-MS m/z 2419 (M^+). Anal. Calcd for $C_{108}H_{171}N_{13}O_{48}$: C, 53.6; H, 7.1; N, 7.5. Found: C, 53.9; H, 7.0; N, 7.6.

Tris[*N*-[tris(5-aminolevulinylloxymethyl)methyl]acetamido]amine-9Trifluoroacetic Acid (16). Compound **15** (0.34 g, 0.22 mmol) was dissolved in dichloromethane (15 mL), TFA (3 mL) was added, and the solution was stirred at room temperature for 2 h. The solvents were evaporated under reduced pressure to yield an oily product which was crystallized from a mixture of methanol, ethyl acetate, and diethyl ether to obtain 0.24 g (69%) of the TFA salt as a pale yellow solid; ^1H NMR (270 MHz, CD_3OD) δ 4.31 (s, 18H), 3.97 (s, 18H), 2.80 (m, 18H), 2.63 (m, 18H), 2.54 (s, 6H); ^{13}C NMR (67.5 MHz, CD_3OD) δ 203.78, 203.32, 174.72, 173.66, 163.07, 162.54, 63.31, 59.05, 35.32, 30.37, 28.31.

***N*-(Benzyloxycarbonyl)imino-3,3'-bis[*N*-[tris(*N*-*tert*-butyloxycarbonyl-5-aminolevulinylloxymethyl)methyl]propionamide] (18).** Compound **2** (0.12 g, 0.43 mmol) was dissolved in dichloromethane and DMF (20 mL, 2:1 v/v). DCC (0.21 g, 0.96 mmol) and HOBt (0.14 g, 0.96 mmol) were added, and the mixture was allowed to stir for 30 min at 0 °C under argon. Compound **1** (0.80 g, 1.11 mmol) was added dropwise over 50 min. The reaction mixture was stirred at room temperature for 3 days, and then the solvents were evaporated. The residue was dissolved in ethyl acetate (20 mL) and washed with hydrochloric acid (2 $\times$ 25 mL, 2 N), saturated aqueous sodium hydrogen carbonate (25 mL), and water (2 $\times$ 25 mL). The organic layer was evaporated to give an oily product, which was subjected to flash chromatography on silica gel (EtOAc/MeOH 19:1, R_f 0.6) to yield 0.51 g (66%) of compound **18** as a colorless oil; IR (film) 3347, 2974, 2924, 1712, 1709, 1513, 1370 cm^{-1} ; UV (MeOH) 209, 240 nm; ^1H NMR (270 MHz, $CDCl_3$) δ 7.48 (m, 5H), 7.45 (br s, 2H), 6.34 (br s, 6H), 5.21 (s, 2H), 4.48 (s, 12H), 4.05 (s, 12H), 2.88 (m, 12H), 2.79 (m, 12H), 2.69 (m, 12H), 2.59 (m, 12H), 1.49 (s, 54H); ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 203.740, 173.97, 170.79, 157.96, 134.44, 130.45, 129.89, 80.50, 74.58, 73.63, 67.76, 52.14, 51.82, 36.82, 35.87, 31.15, 30.86, 29.29, 23.69; ES-MS m/z 1803 ($M^+ + Na$).

Imino-3,3'-bis[*N*-[tris(*N*-*tert*-butyloxycarbonyl-5-aminolevulinylloxymethyl)methyl]propionamide] (19). Compound **18** (0.50 g, 0.32 mmol) was dissolved in ethyl acetate and methanol (30 mL, 2:1 v/v). Palladium on carbon (0.17 g, 10%) was added, and the mixture was stirred under an atmosphere of hydrogen gas for 8 h at room temperature. The reaction mixture was filtered through Celite and washed with ethyl acetate. The solvents were evaporated, and the residue was dried to obtain 0.38 g (82%) of **19** as a colorless oil; IR (film) 3353, 2980, 2930, 2365, 1712, 1513, 1364, 1258, 1159, 1046, 860 cm^{-1} ; UV (MeOH) 208 nm; ^1H NMR (270 MHz, $CDCl_3$) δ 7.43 (br s, 2H), 6.30 (m, 7H), 4.09 (s, 12H), 4.05 (s, 12H), 2.89 (m, 12H), 2.72 (m, 12H), 2.70 (m, 12H), 2.67

(m, 12H), 1.49 (s, 45H); ^{13}C NMR (67.5 MHz, CDCl_3) δ 202.32, 176.84, 155.91, 80.06, 77.50, 60.42, 50.06, 34.03, 28.19, 27.58, 20.95, 14.07, 9.16; FAB-MS m/z 1647 (M^+).

1,3,5-Tris[*N*-(*N*-bis[*N*-[tris(*N*-*tert*-butyloxycarbonyl-5-aminolevulinylloxymethyl)methyl]propionamido}acetamido)carbamido]benzene (20). Compound **19** (0.05 g, 0.11 mmol) was dissolved in dichloromethane and DMF (20 mL, 2:1 v/v), EDC (90 mg, 0.48 mmol) and HOBt (0.06 g, 0.48 mmol) were added, and the mixture was stirred for 30 min under argon at 0 °C. Compound **4** (0.77 g, 0.55 mmol) was dissolved in dichloromethane (5 mL) and added dropwise over 50 min. The mixture was stirred at room temperature for 3 days, and then the solvents were evaporated. The residue was dissolved in ethyl acetate and washed with hydrochloric acid (2 $\times$ 25 mL, 2 N), saturated aqueous sodium hydrogen carbonate (25 mL), and water (2 $\times$ 20 mL). The organic layer was evaporated to give an oily product which was purified by flash chromatography on silica gel (EtOAc/MeOH 3:1, R_f 0.35) yielding 0.37 g (54%) of compound **20** as a foamy white solid, mp 126–128 °C; IR (film) 3384, 2974, 2918, 2359, 1711, 1699, 1513, 1364 cm^{-1} ; ^1H NMR (270 MHz, CDCl_3) δ 7.28 (s, 3H), 6.39 (br s, 3H), 5.34 (br s, 6H), 5.06 (br s, 18H), 4.31 (m, 6H), 4.23 (s, 36H), 3.97 (s, 36H), 3.68 (m, 12H), 2.70 (m, 36H), 2.57 (m, 36H), 2.45 (m, 12H), 1.37 (s, 162H); ^{13}C NMR (67.5 MHz, CDCl_3) δ 203.56, 172.49, 171.61, 155.39, 134.31, 128.01, 79.46, 65.32, 62.30, 61.74, 59.66, 57.52, 49.61, 33.78, 33.72, 29.50, 27.80, 14.74. Anal. Calcd for $\text{C}_{237}\text{H}_{366}\text{N}_{30}\text{O}_{102}$: C, 54.0; H, 7.0; N, 8.0. Found: C, 54.1; H, 7.4; N, 8.1.

1,3,5-Tris[*N*-(*N*-bis[*N*-[tris(5-aminolevulinylloxymethyl)methyl]propionamido}acetamido)carbamido]benzene-18Trifluoroacetic Acid (21). Compound **20** (0.28 g, 50 μmol) was dissolved in dichloromethane (20 mL), TFA (4 mL) was added, and the solution was stirred at room temperature for 2 h. The solvents were evaporated under reduced pressure to yield an oily product which was crystallized from a mixture of methanol, ethyl acetate, and diethyl ether to obtain 0.18 g (62%) of the TFA salt as a pale yellow solid; ^1H NMR (270 MHz, CD_3OD) δ 7.36 (s, 3 H), 4.40 (s, 36 H), 4.35 (m, 6 H), 4.03 (s, 36 H), 3.76 (m, 12 H), 2.89 (m, 36 H), 2.85 (m, 36 H), 2.69 (m, 12 H); ^{13}C NMR (67.5 MHz, CD_3OD) δ 204.22, 172.00, 155.70, 130.60, 128.00, 79.87, 65.51, 62.84, 58.05, 54.22, 50.17, 41.78, 34.59, 34.24, 28.30, 27.73, 27.60.

1,3,5-Tris[*N*-(*N*-bis[*N*-[tris(*N*-*tert*-butyloxycarbonyl-5-aminolevulinylloxymethyl)methyl]propionamido}propionamido)carbamido]benzene (22). Compound **5** (30 mg, 60 μmol) was dissolved in dichloromethane and DMF (15 mL, 2:1 v/v), EDC (40 mg, 0.20 mmol) and HOBt (30 mg, 0.20 mmol) were added, and the mixture was stirred under argon for 30 min at 0 °C. Compound **19** was dissolved in dichloromethane (0.39 g, 0.21 mmol) and added dropwise to the mixture over 50 min. The mixture was stirred at room temperature for 3 days, the heavy precipitate was filtered off, and the solvents were evaporated. The residue was dissolved in ethyl acetate (25 mL) and washed with hydrochloric acid (2 $\times$ 25 mL, 2 N), saturated aqueous hydrogen carbonate (25 mL), and water (2 $\times$ 25 mL). The organic layer was evaporated to give an oily product which was subjected to flash chromatography on silica gel (EtOAc/MeOH 3:1, R_f 0.35). Crystallization of the product using a mixture of dichloromethane, diethyl ether, and ethyl acetate gave 0.22 g (63%) of compound **22** as a foamy solid, mp 129–128 °C; IR (film) 3384, 2980, 2930, 2359, 2253, 1706, 1699, 1507, 1370 cm^{-1} ; ^1H NMR (270 MHz, d_6 -acetone) δ 8.46 (br s, 3H), 8.30 (s, 3H), 6.17 (br s, 6H), 5.62 (br s, 18H), 4.41 (s, 36H), 4.11 (m, 6H),

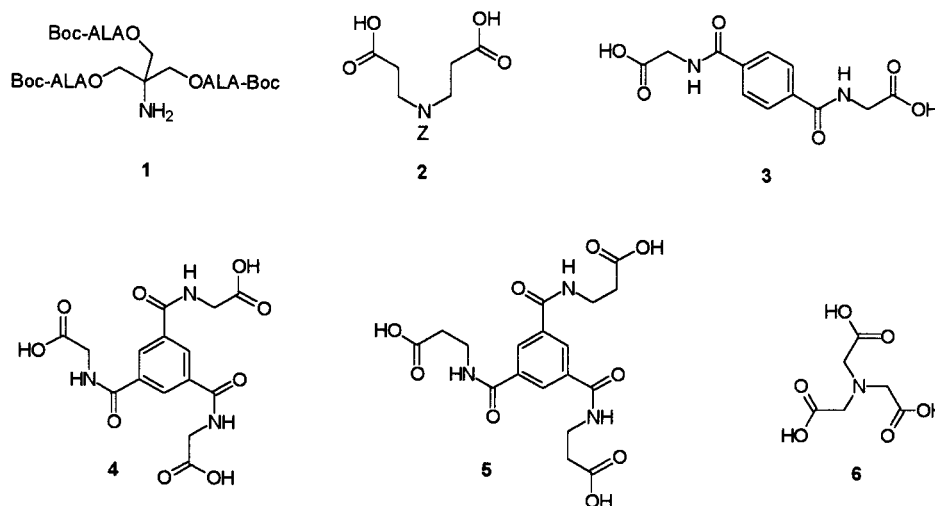
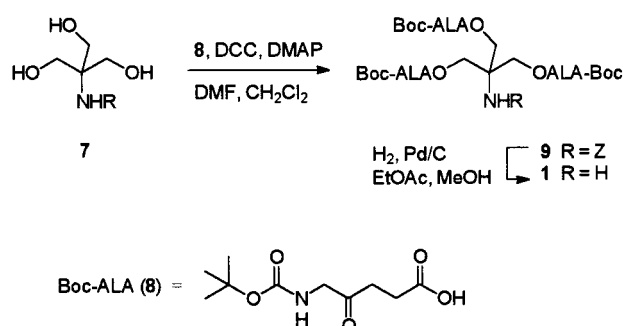
4.03 (s, 36H), 3.73 (m, 12H), 2.73 (m, 36H), 2.70 (m, 6H), 2.66 (m, 36H), 2.63 (m, 12H), 1.44 (s, 162H); ^{13}C NMR (67.5 MHz, CDCl_3) δ 204.48, 172.06, 171.98, 168.92, 155.69, 128.46, 72.26, 65.79, 61.97, 60.06, 54.16, 50.12, 48.98, 34.18, 33.84, 28.24, 27.54, 25.54, 24.86. Anal. Calcd for $\text{C}_{240}\text{H}_{372}\text{N}_{30}\text{O}_{102}$: C, 54.3; H, 7.1; N, 7.9. Found: C, 54.2; H, 7.2; N, 7.6.

1,3,5-Tris[*N*-(*N*-bis[*N*-[tris(5-aminolevulinylloxymethyl)methyl]propionamido}propionamido)carbamido]benzene-18Trifluoroacetic Acid (23). Compound **22** (0.31 g, 60 μmol) was dissolved in dichloromethane (20 mL), TFA (4 mL) was added, and the solution was stirred at room temperature for 2 h. The solvents were evaporated under reduced pressure to yield an oily product that was crystallized from a mixture of methanol, diethyl ether, and ethyl acetate to obtain 0.12 g (41%) of the TFA salt as a pale yellow solid; ^1H NMR (270 MHz, CD_3OD) δ 8.40 (s, 3H), 4.40 (s, 36H), 4.17 (m, 6H), 4.14 (s, 36H), 4.01 (m, 12H), 2.95 (m, 36H), 2.93 (m, 6H), 2.84 (m, 36H), 2.82 (m, 12H); ^{13}C NMR (67.5 MHz, CDCl_3) δ 203.71, 203.03, 173.76, 173.14, 163.02, 162.38, 132.67, 127.23, 63.12, 58.36, 51.80, 36.14, 35.27, 35.14, 34.67, 29.02, 28.34, 28.01.

Cell Culture. PAM 212 cells (naturally transformed murine keratinocytes)(26) were grown monolayered in sterile, vented-capped, angled-necked cell culture flasks. The culture medium was RPMI 1640 medium containing L-glutamine (0.02 mM) and phenol red, supplemented with 10% foetal calf serum (FCS) (First Link), penicillin (500 units/mL), streptomycin (0.5 mg/mL), and HEPES buffer (0.01 M). Cells were maintained at 37 °C in a humidified 5% CO_2 incubator until confluent. Approximately twice weekly, the cells were passaged. The cells were detached from the flasks with a solution of 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS), spun down in a centrifuge at 1500 rpm for 5 min, resuspended in fresh medium, and subcultured in new flasks.

Chemicals and Solutions. ALA was purchased from Photocure (Norway) as the hydrochloride salt. ALA and dendrimers **16** and **21** were dissolved in FCS- and phenol red-free RPMI medium immediately prior to incubation (serum is known to cause release of PpIX from the cells, thus resulting in loss of the fluorescence signal(27) and phenol red would optically interfere with the detection of PpIX). Solutions were sterile filtered using a 0.2 μm syringe-tip filter before incubation. Incubation in the presence of ALA or an ALA-containing dendrimer was performed under subdued lighting.

Fluorescence Spectroscopy. Fluorescence spectroscopy allows quantification of porphyrin yields over a period of time. Cells were seeded in gamma-sterilized 96-well plates at a density of 5×10^4 cells per well, 48 h prior to constant drug exposure, and solutions of ALA and ALA-containing dendrimers were freshly prepared and sterile-filtered. PpIX fluorescence was recorded at 2, 4, and 6 h after drug exposure. PpIX fluorescence was measured with a well plate fluorescence reader (Perkin-Elmer, UK) connected to a Perkin-Elmer LS 50B fluorescence spectrophotometer (Perkin-Elmer, UK) using 410 nm excitation and 635 nm emission with slit widths set to 10 nm both on the excitation and emission monochromators, and an internal 530 nm highpass filter used on the emission side; spectral scans were made between 550 and 750 nm to check for the presence of other porphyrins. Intensity calibrations were performed using rhodamine B embedded in a Perspex disk as a standard. Control wells containing RPMI alone were included. The experiments were performed in quadruplicate, and stan-

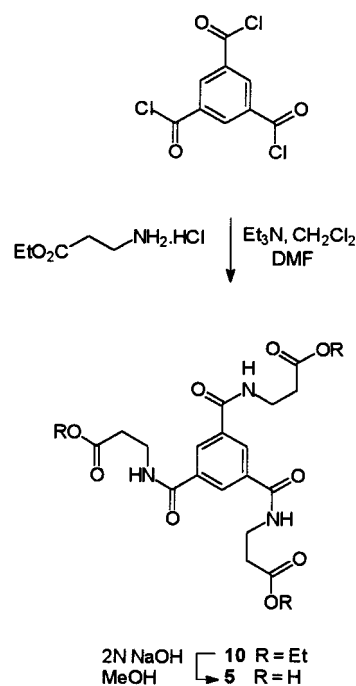
**Figure 1.** Dendrimer building blocks.**Figure 2.** Synthesis of ALA-containing dendrimer, 1.

dard deviations expressed as error bars. The respective controls were used for background subtraction of the spectrum, and equalization of the number of cells in the solution to reduce the error caused by scattering effects.

Photocytotoxicity. Forty-eight hours prior to the experiment, 5×10^4 cells were seeded in 16 wells located in the middle of each 96-well plate—the irradiation zone produced by the laser is up to 6 cm in diameter and covers 16 wells (4 wells $\times$ 4 wells) on the well plate. Three different controls were prepared. Two controls consisted of cells incubated with RPMI, and only one of them was irradiated with light. In the third control, cells were incubated with the maximum concentration of each compound (2 mM) with no light irradiation to determine dark cell toxicity.

The cells were incubated with ALA or an ALA-containing dendrimer for 4 h at 37 °C in a humidified 5% CO₂ atmosphere. After 4 h, the compounds were removed and replaced with 0.1 mL of RPMI containing 10% FCS (warmed at 37 °C). For illumination a diode laser was used (Diomed) emitting at a wavelength of 630 nm, for which the power output at the fiber tip was set at 1.13 W using a power meter (Gentech). The cells were irradiated in a 6 cm diameter area within a single field, providing simultaneous illumination of all wells. Exposure time was 125 s, providing an energy density of 5 J/cm² (fluence rate: 40 mW/cm²). Cells were not rinsed before irradiation; loss of PpIX in the medium has been neglected since it does not affect cell survival (28). Once irradiated, the cells were placed into the incubator (37 °C in a humidified 5% CO₂ atmosphere) and cell cytotoxicity was determined 18 h later using the MTT assay.

MTT Assay. Cell viability was tested by means of an MTT assay. This technique allows quantification of cell survival after cytotoxic insult by testing the enzymatic activity of the mitochondria. It is based on the reduction of a water-soluble tetrazolium salt to a purple, insoluble formazan derivative by mitochondrial enzyme dehydrogenases. This enzymatic function is only present in living, metabolically active cells. The optical density of the product was quantified by its absorption at 570 nm using a 96-well microplate reader (MR 700 Dynatech). A 0.1 mL volume of a solution of 3-[4,5-dimethylthiazolyl]-2,5-diphenyltetrazolium bromide (MTT) in PBS (1 mg/mL) was added to each well, and the cells were incubated for 3 h at 37 °C in a humidified 5% CO₂ atmosphere (29). Control studies showed no cytotoxicity induced by the dendrimers in the absence of light. Phototoxicity results are expressed as percentage of cell viability against control cells under the same conditions but in the absence of test compound.

**Figure 3.** Synthesis of core molecule, 5.

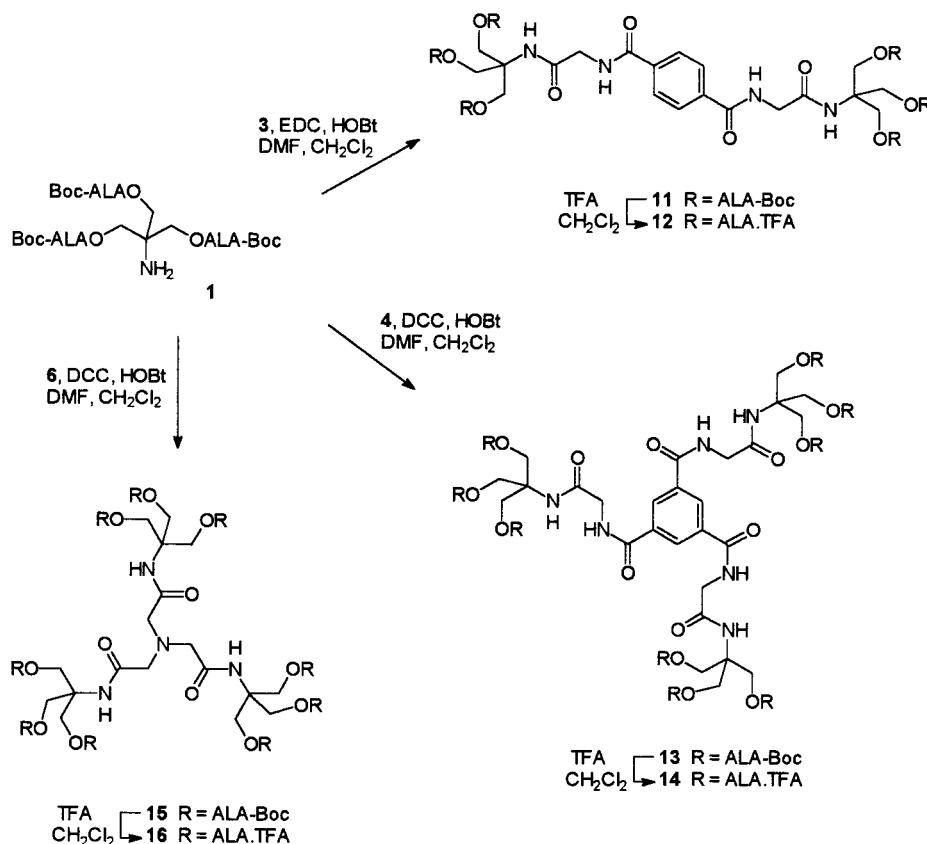


Figure 4. Synthesis of the first-generation dendrimers.

RESULTS AND DISCUSSION

Dendrimers are constructed by stepwise methods yielding structurally and topologically defined, monodisperse, unimolecular entities. There are two approaches to the synthesis of dendritic polymers based on either convergent or divergent synthesis. In the divergent approach, growth of the polymer begins at the center and proceeds radially outward in a series of coupling reactions, whereas in the convergent approach, growth begins at the chain ends. A disadvantage of using the divergent method is that, because the number of polymer chain ends increases geometrically at each coupling or activation process, it eventually becomes difficult to ensure that all chain ends have reacted at each stage of growth. A convergent growth strategy, similar to that of Ashton et al. (25), has therefore been adopted for the synthesis of the ALA-containing dendrimers. The dendrimers were assembled in segments: first the individual building blocks were synthesized, and then these dendrons were attached to a multipodent core unit in the final steps of dendrimer construction. Classical coupling chemistry using either DCC or EDC with HOBt was used to join the individual dendrons by amide bonds.

The synthetic strategy adopted makes use of easily accessible building blocks (Figure 1). Dendron **1**, which bears three Boc-protected ALA molecules, is the terminal dendron in all five dendrimers. Four different core molecules were used: the diglycyl amide of 1,4-benzenedicarboxylic acid (**3**), the triglycyl amide of 1,3,5-benzenetricarboxylic acid (**4**), the tri- β -alanyl amide of 1,3,5-benzenetricarboxylic acid (**5**), and nitrilotriacetic acid (**6**). In the larger dendrimers, Z-protected 3,3'-iminodipropionic acid (**2**) was used as a branching unit. These building blocks, when combined in a suitable synthetic

sequence, gave rise to a series of dendrimers varying in size and ALA loading.

The three primary hydroxyl groups of tris(hydroxymethyl)aminomethane (TRIS) allowed attachment of three ALA molecules through ester linkages, and the free amino group enabled further elaboration through the formation of an amide bond with either a branching unit or a core molecule. Therefore, Z-protection of TRIS using benzyl chloroformate gave **7** (25), which was then coupled with *N*-*tert*-butoxycarbonyl-5-aminolevulinic acid (Boc-ALA, **8**), giving rise to compound **9**. Removal of the Z-protecting group by hydrogenation gave the terminal dendron **1** (Figure 2).

The core molecules **3** and **4** were prepared by reacting the corresponding di- and tribenzoic acid chlorides and glycine methyl ester, followed by basic hydrolysis of the di- and triester products (25). Core molecule **5** was synthesized in an analogous fashion: 1,3,5-benzenetricarbonyl trichloride reacted with β -alanine ethyl ester to give the triester **10** and base-induced hydrolysis gave **5**. Compound **6** is commercially available (Figure 3).

Attempts to directly couple dendron **1** with 1,3,5-benzenetricarboxylic acid failed due to steric congestion around the core. However, when **1** was reacted with the extended core **4**, using DCC as the coupling reagent, the reaction proceeded smoothly to give dendrimer **13**. Dendron **1** also reacted with core **3** to give dendrimer **11** which contains six ALA residues. The 9-ALA dendrimer **15**, which has an aliphatic core, was synthesized by the reaction between dendron **1** and nitrilotriacetic acid in the presence of DCC and HOBt (Figure 4).

Branching unit **2** was prepared by hydrolysis of the nitrile groups in 3,3'-iminodipropionitrile using barium hydroxide to give the corresponding dicarboxylic acid as

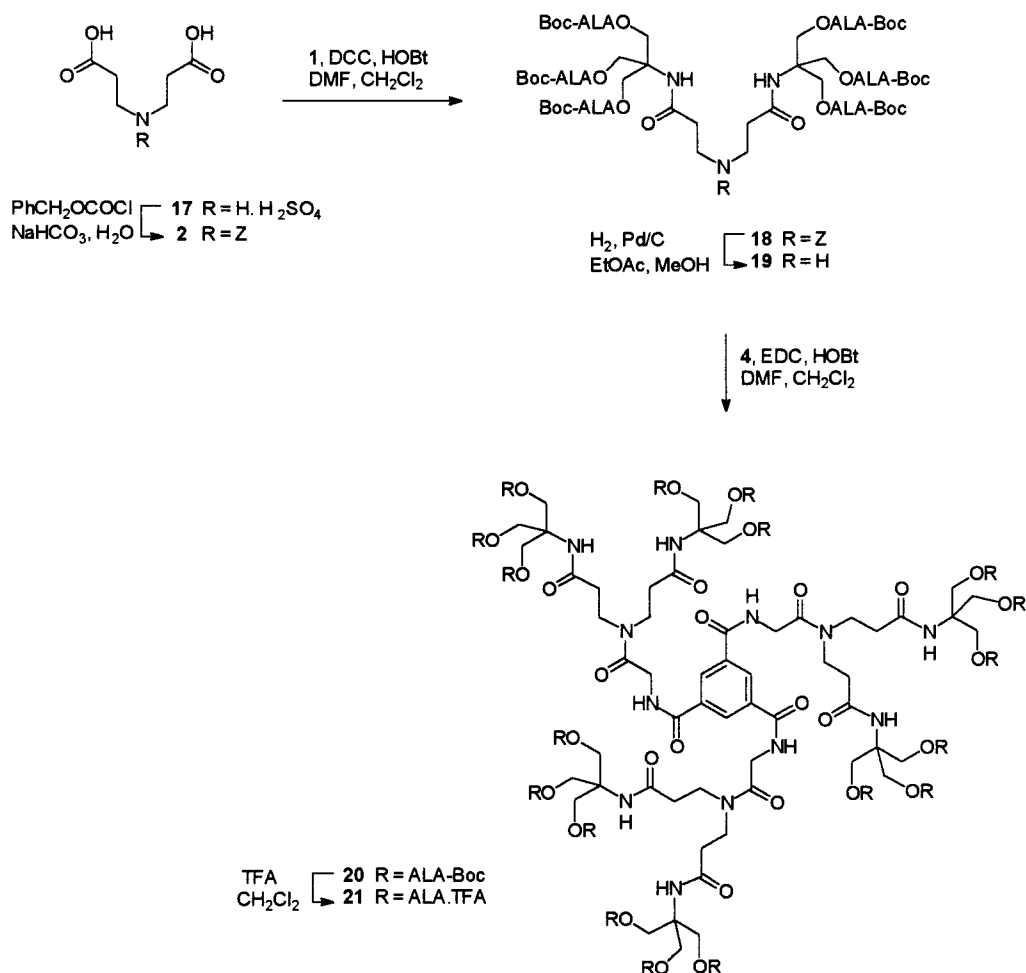


Figure 5. Synthesis of the second-generation dendrimer, **21**.

its hydrogen sulfate salt (**17**) (25). Z-Protection using benzyl chloroformate gave compound **2** (25). DCC coupling between dendron **1** and branching unit **2** gave dendron **18**. Hydrogenation gave amine **19**, however, using DCC as the coupling reagent, the reaction between this new 6-ALA dendron and core molecule **4** did not give the desired second generation dendrimer **20**. When EDC was used for the amide coupling, **20** was obtained in good yield (Figure 5). The homologous dendrimer **22** was prepared by coupling dendron **20** with core molecule **5** (Figure 6).

The Boc protecting groups were removed from dendrimers **11**, **13**, **15**, **20**, and **22** by treatment with trifluoroacetic acid, and the cationic dendrimers **12**, **14**, **16**, **21**, and **23**, respectively, were isolated as their trifluoroacetate salts (Figures 5 and 6).

The simplicity and reliability of this approach should permit an extension of this synthetic methodology to the synthesis of higher-generation dendrimers by the incorporation of additional branching units. Although higher molecular weight dendrimers would be required for enhanced permeability and retention in tumor tissue, we have evaluated the concept of using dendrimers as macromolecular carriers for the delivery of ALA to the tumorigenic cell line, PAM 212, in vitro. Figure 7(a) shows the PpIX fluorescence induced by ALA, the 9-ALA dendrimer **16**, and the 18-ALA dendrimer **21** at 0.1 mM concentration in PAM 212 cells. The ALA-containing dendrimers are clearly able to pass through the cell membrane and release ALA intracellularly; however,

there is no direct correlation between the number of ALA molecules attached to the dendrimer and PpIX accumulation. The more than 2-fold greater efficacy of **21**

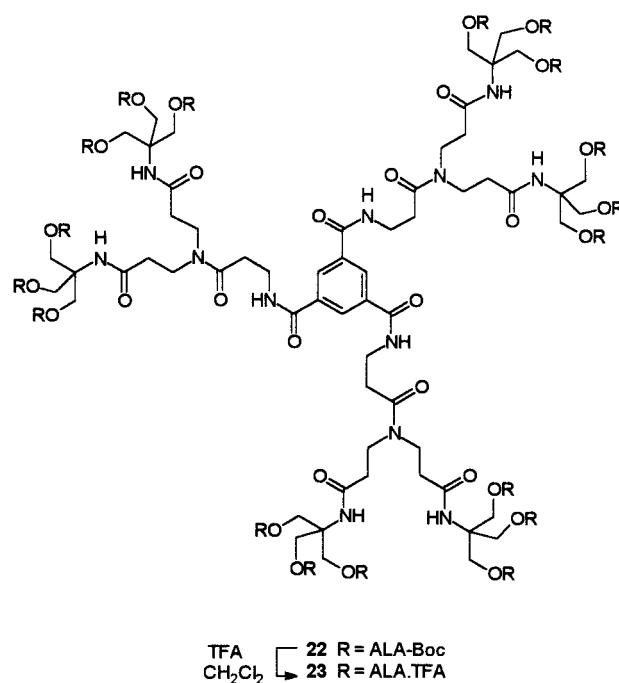


Figure 6. The second-generation dendrimer, **23**.

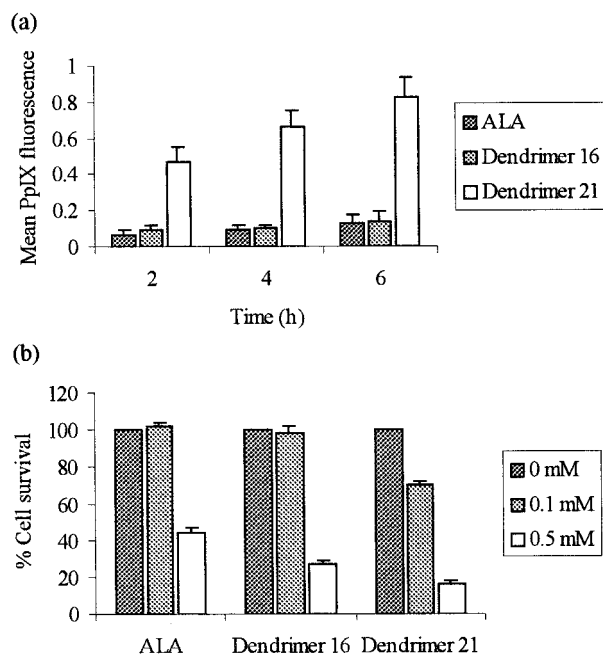


Figure 7. (a) Mean PpIX fluorescence induced by 0.1 mM ALA, dendrimer **16** or dendrimer **21**. (b) Cell survival using 4 h incubation with 0.1 mM and 0.5 mM ALA, dendrimer **16** or dendrimer **21**, after illumination with 5 J cm⁻².

over **16** may be due to increased uptake of the larger dendrimer by endocytosis. Figure 7b shows the results of a PDT experiment in which the cells were illuminated 4 h after incubation with either ALA, dendrimer **16**, or dendrimer **21**. At the concentrations used, neither of the dendrimers induced cytotoxicity in the absence of light, and dendrimer **21** again shows greatest potency. These results demonstrate the potential of this type of multi-valent ALA carrier for the delivery of ALA in PDT applications.

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Efficient Gene Transfer Using Reversibly Cross-Linked Low Molecular Weight Polyethylenimine

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Polyethylenimine (PEI) is a polycation with potential application as a nonviral vector for gene delivery. Here we show that after conjugation with homobifunctional amine reactive reducible cross-linking reagents, low molecular weight polyethylenimine efficiently mediates *in vitro* gene delivery to Chinese hamster ovary (CHO) cells. Two cross-linking reagents, dithiobis(succinimidylpropionate) (DSP) and dimethyl-3,3'-dithiobispropionimidate·2HCl (DTBP), were utilized based on their reactivity and chemical properties. Both reagents react with primary amines to form reducible cross-links; however, unlike DSP, the DTBP cross-linker maintains net polymer charge through amidine bond formation. PEI with a reported weight-average molecular weight (M_w) of 800 Da was reacted with either DSP or DTBP at PEI primary amine:cross-link reactive group ratios of 1:1 and 2:1. The transfection efficiencies of the resulting cross-linked products were evaluated in CHO cells using a luciferase reporter gene under a cytomegalovirus (CMV) promoter. Our results show that cross-linked polymers mediate variable levels of transfection depending on the cross-linking reagent, the extent of conjugation, and the N/P ratio. In general, we found conjugate size to be proportional to gene transfer efficiency. Using gel retardation analysis, we also evaluate the capacity of the cross-linked polymers to condense plasmid DNA before and after reduction with 45 mM dithiothreitol (DTT). DTT mediated reduction of intra-cross-link disulfide bonds and inhibited condensation of DNA by conjugates cross-linked with DSP at a ratio of 1:1, but had little effect on the remaining polymers. Analogous intracellular reduction of transfection complexes by reduced glutathione could facilitate uncoupling of PEI from DNA to enhance gene expression.

INTRODUCTION

The primary obstacle toward implementing an effective gene therapy using nonviral vectors remains their relatively inefficient gene delivery *in vivo*. Although viral vectors are able to overcome this problem, and generally yield higher gene expression, there are concerns over the immunogenic, cytotoxic, and recombinogenic potential of viral gene transfer. Due to the improved safety profile and ease of preparation and manipulation, nonviral techniques for gene delivery continue to be explored and optimized.

Polyethylenimine (PEI) is a cationic polymer which has proven to be an effective transfection agent both *in vitro* (1–3) and *in vivo* (4–8). Due to the chemical properties of PEI, primarily the high cationic charge density, it is able to effectively condense DNA and form nanometric particles capable of being endocytosed (9). In addition, since every third atom in the polymer is an amino nitrogen capable of being protonated, PEI effectively buffers the endosomal environment, thus protecting DNA from lysosomal nuclease degradation and facilitating endosomal escape to the cytoplasm. Cellular uptake of PEI–DNA complexes likely results from nonspecific electrostatic interactions between positively charged polymer–DNA complexes and negatively charged residues on the cell surface (10). Several groups have

demonstrated improved transfection efficiencies with PEI by incorporating targeting ligands such as galactose (11), mannose (12), transferrin (13), or antibodies (14, 15).

Dithiobis(succinimidylpropionate) (DSP) is a homobifunctional NHS (*N*-hydroxysuccinimide) ester-based electrophilic cross-linking reagent containing an 8 atom spacer 12 Å in length (16). Dimethyl-3,3'-dithiobispropionimidate·2HCl (DTBP) is a similar molecule containing an 8 atom spacer 11.9 Å in length, but utilizes an imidoester reactive group to form amidine bonds, resulting in the retention of net positive charge (16). Both cross-linking reagents react with primary amines to form stable covalent linkages, and are constructed around a centrally located disulfide bond, which is cleavable with common reducing agents after conjugation (Figure 1).

It is well established that branched high molecular weight polyethylenimines (>25 kDa) are effective transfection agents, and recent reports indicate that relatively low molecular weight PEI (~10 kDa) can also be used to deliver DNA efficiently with lower toxicity (17, 18). With this in mind, we hypothesized that high molecular weight conjugates composed of cross-linked 800 Da PEI would yield transfection efficiencies comparable to those obtained with high molecular weight PEI. Furthermore, we expected that when the transfection complexes were exposed to the reducing environment of the cytoplasm that the disulfide bonds introduced by the cross-linking reagents would be cleaved. This reduction would promote reversion of the high molecular weight complexes back to their low molecular weight counterparts, presumably leading to lower toxicity and potentially easier access by the transcription machinery (Figure 2).

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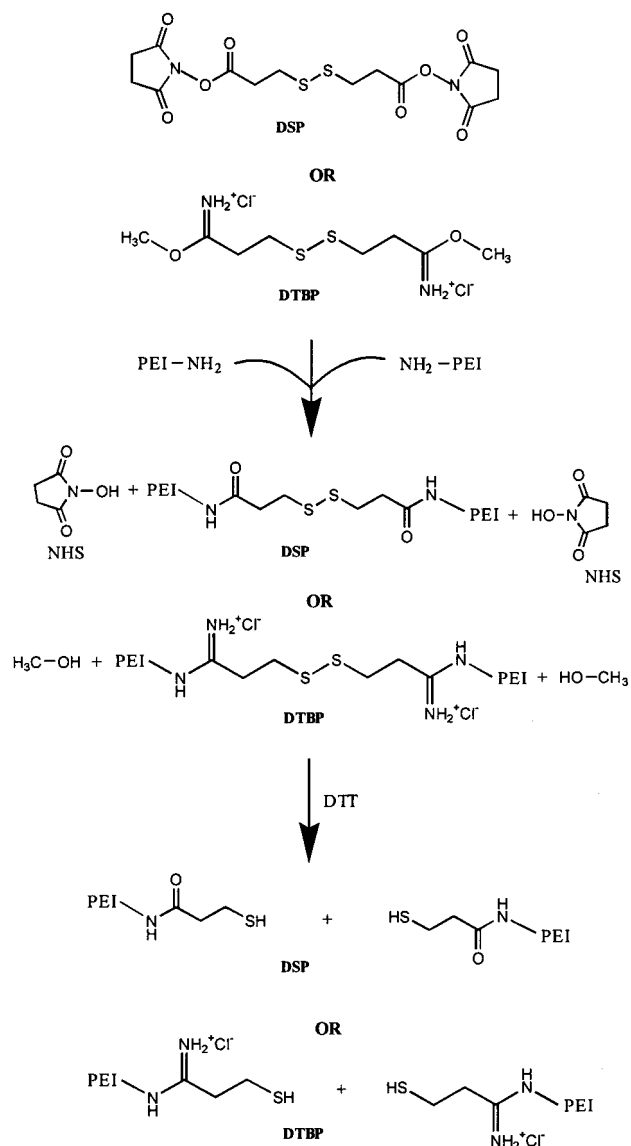


Figure 1. Proposed reaction scheme for conjugating polyethylenimine with cross-linking reagents DSP and DTBP. Also shown is the effect of disulfide bond reduction by DTT.

In this report, we examine the transfection properties of low molecular weight PEI cross-linked with either DSP or DTBP in CHO cells. We also evaluate the DNA condensing properties of the cross-linked conjugates before and after reduction with 45 mM DTT.

MATERIALS AND METHODS

Polymers, Plasmid, and Cross-Linking Reagents.

PEI with M_w of 800 Da and 25 kDa were purchased from Aldrich, and used as 0.16 mM aqueous solutions (3 nmol of N/ μ L) in all transfection experiments. Plasmid DNA containing the luciferase reporter gene, pcDNA3-CMV-Luc (7.1 kb), was kindly provided by Dr. Leaf Huang (University of Pittsburgh Medical Center). Cloning and preparation of plasmid DNA were performed by propagating transformed *E. coli* in LB medium containing ampicillin (50 μ g/mL), followed by isolation and purification with a commercially available plasmid purification kit (Qiagen). Concentration and purity of DNA were assessed spectrophotometrically by measuring absorbance at wavelengths of 260 and 280 nm ($OD_{260}/OD_{280} \sim 1.7$). Plasmid size and integrity were confirmed using agarose gel electrophoresis (0.9% agarose gel containing

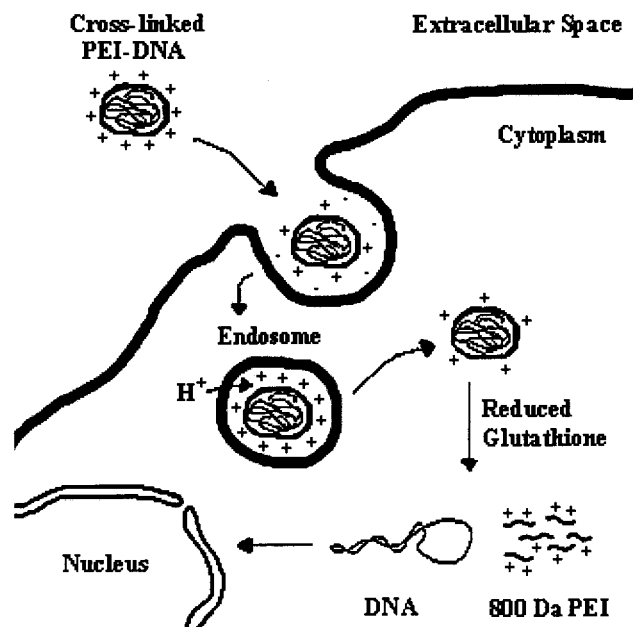


Figure 2. Proposed transfection scheme using reversibly cross-linked low molecular weight polyethylenimine. Following endocytosis and subsequent endosome release, intracellular reduction of polymer disulfide bonds leads to the release of DNA for nuclear uptake and transcription.

0.5 μ g/mL ethidium bromide) and cleavage with the restriction endonuclease *Hind*III (Gibco BRL). Cross-linking reagents, dithiobis(succinimidylpropionate) (DSP) (MW 404.42) and dimethyl-3,3'-dithiobispropionimidate·2HCl (DTBP) (MW 309.28), were purchased from Pierce.

PEI Cross-Linking with DSP and DTBP. Prior to cross-linking, PEI (800 Da) was labeled with fluorescein isothiocyanate (FITC) (Sigma) at a molar ratio of 100:1 (PEI:FITC) to aid in concentration determination of the cross-linked polymers. Briefly, 800 mg of PEI was dissolved in 4 mL of DMSO for a final concentration of 200 mg/mL. Next, 389.4 μ L of FITC dissolved in DMSO (10 mg/mL) was added dropwise to the PEI solution while vortexing. The reaction was allowed to proceed in the dark at room temperature (RT) for 30 min, and was then stored at 4 $^{\circ}$ C protected from light. Precipitation of the labeled polymer confirmed that the reaction between PEI and FITC proceeds to completion, shown by the absence of free FITC in the supernatant. PEI-FITC was then cross-linked with either DSP or DTBP at PEI primary amine:cross-link reactive group ratios of 1:1 and 2:1. Based on reports in the literature (17,19), we estimated the primary amine content of PEI (800 Da) to be 25% of the total amines. To achieve PEI primary amine:cross-link reactive group molar ratios of 1:1 or 2:1, 117 or 58.5 mg of the appropriate cross-linking reagent was dissolved in 1 or 0.5 mL of DMSO, respectively. The DSP or DTBP solutions were then added dropwise to PEI-FITC solutions containing 100 mg of PEI-FITC in approximately 550 μ L of DMSO. The reactions were then allowed to proceed for 1 h at RT. Prior to use, aliquots of cross-linked polymer samples were diluted 1:4 with ddH₂O to a volume of 0.5 mL. The diluted samples were then purified on a 1.5 $\times$ 8 cm Sephadex G-25 fine column using ddH₂O as the eluent under atmospheric pressure. One milliliter fractions were collected, and the concentration of each fraction was determined spectrophotometrically by measuring the absorbance at 495 nm and extrapolating concentration from a standard curve obtained with uncross-linked PEI-FITC of known concentrations. Finally,

each sample was diluted to 0.16 mM (3 nmol of N/ μ L) for use in transfection experiments.

Transfection of CHO Cells. CHO cells were cultured in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), 100 units/mL penicillin, 100 μ g/mL streptomycin, 300 μ g/mL L-glutamine, and 100 μ M sodium citrate (Gibco BRL). For transfection experiments, 1.5×10^5 cells/well were seeded on 24-well plates (Falcon) and allowed to incubate for approximately 20 h in normal growth media at 37 °C in a humidified atmosphere containing 5% CO₂ (~70% confluent). Prior to transfection, the medium was replaced with 0.75 mL of serum-free growth medium. Polymer–DNA complexes were prepared in 37.5 mM NaCl solutions by addition of 150 μ L of PEI or cross-linked PEI-FITC to 7.5 μ g of pcDNA3-CMV-Luc plasmid DNA to achieve the desired N/P ratios in a final volume of 300 μ L (experiments performed in triplicate for 100 μ L/well or 2.5 μ g of DNA/well). Following a 20 min incubation at RT, the transfection complexes were applied to CHO cells and allowed to incubate for 4 h. The medium was then replaced with normal growth medium supplemented with 10% FBS, and luciferase gene expression was measured 24 h later using a commercially available kit (Promega). Protein concentrations were determined using the bicinchoninic acid (BCA) protein determination assay (Pierce), using bovine serum albumin (BSA) standards (Sigma).

Gel Retardation Assays. One microgram of pcDNA3-CMV-Luc plasmid DNA was condensed with PEI or cross-linked PEI at selected N/P ratios by addition of polymer to DNA in 37.5 mM NaCl solution. Prior to electrophoresis (0.9% agarose gel containing 0.5 μ g/mL ethidium bromide), the complexes were allowed to incubate at RT for 20 min. To evaluate the effect of a reducing agent on DNA condensation, some complexes were incubated further for 30 min in the presence of 45 mM DTT (Sigma); 0.5 μ g of DNA was loaded in each well.

Viscosity Measurements. One milliliter of PEI or cross-linked PEI was prepared at a concentration (*c*) of 50 mg/mL in ddH₂O. The time required for each sample to flow through a 1 mm (outside diameter) capillary tube under atmospheric pressure was recorded, and these values were used to calculate the inherent viscosity (η_{inh}) of the polymer solutions. The $\eta_{inh} = \ln \eta_{rel}/c$, where η_{rel} is the relative viscosity which equals $t_{solution}/t_{solvent}$, or the time required for the polymer–solvent solution to flow through the capillary tube divided by the time required for the solvent alone. The molecular weight of cross-linked polymers was estimated by comparison with unconjugated polyethylenimine standards.

RESULTS

CHO cells were transfected in vitro with 2.5 μ g of pcDNA3-CMV-Luc plasmid DNA condensed with various polyethylenimines. Luciferase gene expression is quantified as relative light units (RLU) and femtomoles of luciferase standardized for protein concentration and corrected for background (Figure 3). The data show that among the different PEI polymers tested, 800 Da PEI was the least effective transfection agent. In fact, gene expression using this vector was immeasurable below a N/P ratio of 18. This finding is consistent with previous reports, which show negligible gene expression for PEI with nominal molecular weights below 1800 Da (1). In contrast, cells transfected with 25 kDa PEI produced high levels of luciferase activity, yielding optimal gene expression of 2×10^9 RLU/mg of protein at a N/P ratio of 9.

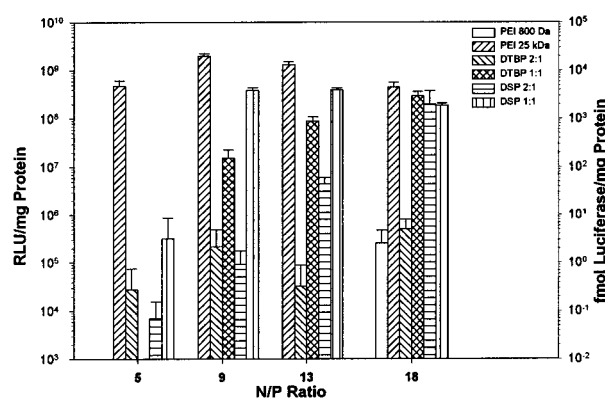


Figure 3. Transfection efficiency of various polyethylenimines in CHO cells at selected N/P ratios. Results are shown as relative light units (RLU)/mg of protein on the left ordinate, and as fmol of luciferase/mg of protein on the right ordinate. Transfection agents are identified in the figure legend.

Transfection with cross-linked 800 Da PEI-FITC yielded variable results depending on the cross-linking reagent, the extent of conjugation, and the N/P ratio. The DTBP conjugate prepared at a PEI primary amine:imidoester reactive group ratio of 2:1 (DTBP 2:1) was relatively ineffective, yielding gene expression on the order of 10^4 – 10^6 RLU/mg of protein. Changing the cross-linking ratio to 1:1 (DTBP 1:1) increased gene expression by several orders of magnitude at N/P ratios ≥ 9 . Interestingly, no luciferase activity was detected using this conjugate at a N/P ratio of 5. This result is surprising given the fact that the DTBP 2:1 conjugate was able to mediate gene transfer at this ratio, albeit at relatively low levels. The DSP conjugate prepared at a PEI primary amine:NHS reactive group ratio of 2:1 (DSP 2:1) yielded gene expression over a wide range, 10^3 – 10^8 RLU/mg of protein, facilitating higher gene expression at larger N/P ratios. Analogous to the DTBP conjugates, we found that increasing the cross-linking ratio to 1:1 (DSP 1:1) led to increased luciferase activity. In fact, the DSP 1:1 conjugate was able to mediate the highest levels of transgene expression among the cross-linked polymers tested, 4×10^8 RLU/mg of protein at N/P ratios of 9 and 13.

By measuring protein concentrations of cellular lysates from adherent cells in each well after transfection, we were able to infer the relative toxicities of cross-linked polymers in relation to unmodified PEI (Figure 4). We assumed an inverse relationship between the protein concentration and cytotoxicity, concluding that lower protein concentrations were indicative of fewer adherent cells and therefore greater toxicity. Wells transfected with 25 kDa PEI consistently produced the lowest protein concentrations, yielding less protein as the charge ratio increased. This finding corroborates earlier reports of toxicity observed after transfection with high molecular weight PEI at large N/P ratios (3). Conversely, protein concentrations measured from cells transfected with 800 Da PEI remained steady at approximately 175 μ g/mL at all charge ratios, and yielded the highest protein concentrations among the transfection agents studied.

Similar to the gene expression data, conjugated PEI-FITC yielded intermediate protein concentrations depending on the cross-linking reagent, the degree of conjugation, and the N/P ratio. PEI-FITC conjugated with DSP at a ratio of 1:1 produced the lowest protein concentrations among cross-linked polymers. Analogous to the trend observed with 25 kDa PEI, concentrations decreased with increasing charge ratio. The DTBP 1:1 conjugate yielded a similar profile, but protein values

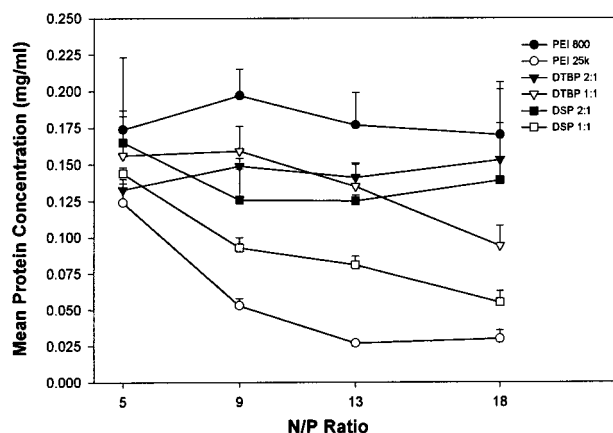


Figure 4. Protein concentrations from cellular lysates after transfection with various polyethylenimines. Solid circles, 800 Da PEI; open circles, 25 kDa PEI; solid triangles, PEI-FITC cross-linked with DTBP at a primary amine:imidoester reactive group ratio of 2:1; open triangles, PEI-FITC cross-linked with DTBP at a primary amine:imidoester reactive group ratio of 1:1; solid squares, PEI-FITC cross-linked with DSP at a primary amine:NHS ester reactive group ratio of 2:1; open squares, PEI-FITC cross-linked with DSP at a primary amine:NHS ester reactive group ratio of 1:1.

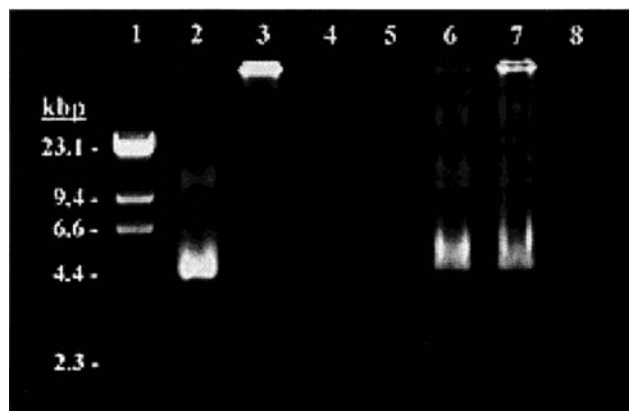


Figure 5. Gel retardation analysis of PEI-FITC cross-linked with DSP at a primary amine:NHS ester reactive group ratio of 1:1. Lane 1 is phage λ DNA digested with *Hind*III used as a size marker; lane 2 is pcDNA3-CMV-Luc plasmid DNA only; lanes 3–5 are plasmid DNA condensed with the DSP 1:1 conjugate at N/P ratios of 5, 9, and 13, respectively; lanes 6–8 are identical to lanes 3–5 except the polymer–DNA complexes were treated with 45 mM DTT.

were higher than those found with DSP 1:1 and 25 kDa PEI. Conjugates prepared at a ratio of 2:1 yielded concentrations $\geq 125 \mu\text{g/mL}$, and did not display the characteristic decrease at higher charge ratios observed with the 1:1 conjugates.

Using gel retardation analysis, we evaluated the DNA condensing properties of the various polyethylenimines used for transfection before and after reduction with 45 mM DTT. We hypothesized that reduction of intramolecular disulfide bonds present in the cross-linked PEI would inhibit DNA condensation by disrupting the structure of the high molecular weight conjugates. However, we found this to be true only in the case of DSP conjugates formed at a ratio of 1:1 (Figure 5). Our results show that at a N/P ratio of 5, the DSP 1:1 conjugate is unable to completely condense DNA as shown by the lack of ethidium bromide exclusion. However, after DTT reduction, this effect is even more pronounced and very little DNA condensation is observed. In contrast, at a N/P ratio of 9, DNA can be visualized only after cleavage with

DTT. At higher charge ratios, condensation is complete regardless of disulfide bond integrity. Unmodified PEI, both the 800 Da and 25 kDa forms, was able to effectively condense DNA at all charge ratios examined (data not shown). This result is in agreement with previous work, which shows complete DNA condensation with PEI above N/P ratios of 2 (11).

Finally, using inherent viscosity calculations we were able to approximate the weight-average molecular weights of the cross-linked PEI conjugates. We found that 800 Da PEI cross-linked with DTBP at a PEI primary amine:imidoester reactive group ratio of 2:1 yielded conjugates with an average molecular weight of nearly 8 kDa. Increasing the cross-linking ratio to 1:1 led to an increase in the molecular weight, yielding conjugates around 23 kDa. Similarly, PEI cross-linked with DSP at a PEI primary amine:NHS ester reactive group ratio of 2:1 also produced conjugates with an average molecular weight of about 23 kDa. The DSP 1:1 conjugate yielded the largest molecular weight, with a value of approximately 75 kDa. These results show that DSP mediated more extensive cross-linking at equal cross-linking ratios.

DISCUSSION

In order for nonviral vectors to be clinically useful, it will be necessary to develop formulations that mediate high levels of cell-specific gene expression with favorable toxicity profiles. Here we build on the foundation of successful gene delivery using polyethylenimine by evaluating a new strategy to enhance gene expression. Previous research has shown that several parameters affect the performance of polyethylenimine vectors for gene delivery (3, 20–23). Our findings verify the importance of polymer molecular weight on transfection efficiency. We show that cross-linking low molecular weight PEI with homobifunctional amine reactive cross-linking reagents to form high molecular weight conjugates leads to efficient transfection of CHO cells *in vitro*. In general, transfection results show that DSP conjugates outperformed DTBP conjugates at similar cross-linking ratios. Furthermore, although 25 kDa PEI proved to be the most effective transfection agent, cross-linked PEI-FITC was able to achieve gene expression of similar magnitude at higher N/P ratios.

Based on relative viscosity measurements, we obtained approximate weight-average molecular weights for PEI-FITC conjugated with DSP and DTBP. Cross-linking 800 Da PEI-FITC with DTBP at a ratio of 2:1 produced conjugates with an average molecular weight of nearly 8 kDa. Therefore, it is not surprising to find that this conjugate consistently yielded relatively low gene expression. The DTBP 1:1 and DSP 2:1 conjugates had similar molecular weights around 23 kDa. However, transfection experiments show that these polymers mediated variable degrees of gene expression despite their similar size. Specifically, the DSP 2:1 conjugate proved less effective at lower N/P ratios. Finally, cross-linking PEI with DSP at a ratio of 1:1 led to the formation of conjugates having an approximate molecular weight of 75 kDa. As expected, this conjugate yielded the highest luciferase activity among the cross-linked polymers tested.

The success of the cross-linking reactions, characteristics of the resulting conjugates and subsequent transfection performance, can be explained, in part, by the chemical nature of the cross-linking reagents utilized for conjugation. At identical cross-linking ratios, we found DTBP conjugates to have lower molecular weights than DSP conjugates. We suspect that this difference is due

to electrostatic repulsion between the positive charge carried on the imidoester reactive group of DTBP and the highly cationic polyethylenimine polymer. This repulsion likely led to less extensive cross-linking, and ultimately smaller conjugates. Differences in the inherent reactivities of the cross-linking reagents toward primary amines could also play a role. Although DSP was able to mediate more extensive cross-linking, reaction of the DSP NHS esters with PEI primary amines led to the elimination of positive charges carried by the polymer, whereas amidine bonds introduced by DTBP compensated for this loss. In effect, DTBP conjugates were able to achieve higher gene expression than DSP conjugates of similar molecular weight at equal N/P ratios because even though the N/P ratios were the same, the charge ratios were different. The increase in net charge for DTBP conjugates would promote greater interaction between positively charged transfection complexes and negatively charged cell membranes, potentially leading to increased gene transfer.

Measurements of protein concentration allowed us to infer the relative toxicities of the various transfection complexes. In general, wells transfected with DSP conjugates yielded lower protein concentrations (higher toxicity) than DTBP conjugates formed at analogous cross-linking ratios, although this effect was more pronounced with conjugates prepared at a ratio of 1:1. Furthermore, protein levels decreased to a greater extent at high N/P ratios with conjugates cross-linked at a ratio of 1:1. The lack of increased toxicity at higher charge ratios is particularly encouraging for the DSP 2:1 conjugate, which showed relatively high gene expression at a N/P ratio of 18. Furthermore, although 25 kDa PEI yielded higher transfection, it also produced the greatest toxicity among the polymers tested.

To our knowledge, this is the first report describing the transfection properties of cross-linked low molecular weight polyethylenimine. However, cross-linking strategies have been used previously to increase the transfection performance and stability of nonviral vectors. Trubetskoy et al. utilized DTBP to cross-link the amine groups of polylysine and polyallylamine after condensation of DNA to form a caged DNA complex that increased their stability in salt solution (24). McKenzie et al. hypothesized that incorporating cysteine residues into low molecular weight peptides would result in the formation of reducible disulfide bridges between the cysteine residues after DNA condensation. They found they were able to create small, stable complexes with enhanced transfection properties in several cell lines (25). Our work shows that 800 Da PEI-FITC cross-linked with DSP or DTBP can be used to efficiently transfect CHO cells in vitro with lower overall toxicity. Furthermore, in the case of conjugates cross-linked with DSP at a ratio of 1:1, reduction of disulfide bonds leads to a decrease in the DNA condensing properties of this polymer. Since the cytoplasmic environment is markedly reducing, it is reasonable to expect that disulfide bonds introduced via cross-linking reagents will be reduced intracellularly by reduced glutathione, leading to the breakdown of PEI conjugates. In fact, glutathione reductase acts essentially in one direction so that the ratio of reduced glutathione to oxidized glutathione is approximately 500:1 in most cells (26). Although the transfection efficiency using conjugated polymers does not appear to be superior to 25 kDa PEI, there is the apparent advantage of reduced toxicity. Transfection with polyethylenimine could lead to an accumulation of PEI localized in the nucleus. This PEI would be able to interact with endogenous DNA,

potentially leading to undesirable side effects. Using cross-linked polymers at the appropriate conjugation ratio would alleviate these concerns due to a decrease in the affinity for DNA observed after reduction of intracellular disulfide bonds.

In closing, cross-linking low molecular weight PEI with DSP or DTBP may confer a pharmacokinetic advantage to polyethylenimine vectors in vivo. PEI is an organic polymer that cannot be metabolized or broken down by cellular enzymes. As a result, total body clearance of high molecular weight PEI will likely be a slow process potentially leading to an accumulation of the polymer in vivo. The introduction of disulfide bonds through DSP and DTBP should promote reversion of the high molecular weight transfection complexes back to their low molecular weight counterparts. These low molecular weight fragments should be cleared more easily from the body due to enhanced diffusion and potentially easier filtration through glomerular capillaries.

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Pharmacokinetics and Delivery of Tat and Tat-Protein Conjugates to Tissues in Vivo

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The membrane permeation in vivo of therapeutic proteins may be enhanced by conjugation of the protein to cationic import peptides, such as the tat protein of the human immune deficiency virus. The organ uptake, expressed as a percent of injected dose (ID) per gram of tissue, is a function of both membrane permeability and the area under the plasma concentration curve (AUC), which is a function of the plasma pharmacokinetics. The purpose of the present studies was to examine the effect of the tat peptide on the plasma AUC of a model exogenous protein, streptavidin, and to examine the extent to which changes in the plasma AUC influence organ uptake (%ID/g) of the protein. The cationic portion of the tat protein is comprised of a lysine/arginine-rich sequence, designated tat^{48–58}. A biotin analogue of this cationic peptide, tat-biotin, was radioiodinated and injected intravenously into rats with or without conjugation to streptavidin. The unconjugated tat-biotin peptide was nearly instantaneously cleared from plasma by all tissues with a very high systemic clearance of 29 ± 4 mL/min/kg and a high systemic volume of distribution of 4160 ± 450 mL/kg. The plasma clearance of the tat-biotin/streptavidin conjugate, 1.37 ± 0.01 mL/min/kg, was reduced relative to the clearance of unconjugated tat peptide, but was higher than the plasma clearance of the unconjugated streptavidin, 0.058 ± 0.005 mL/min/kg. Conjugation of cationic import peptides such as tat^{48–58} to higher molecular weight proteins results in a marked increase in the rate of removal of the protein from the circulation, which is reflected in the reduced plasma AUC. In summary, tat conjugation of a protein has opposing effects on membrane permeation and the plasma AUC. Therefore, the organ %ID/g is not increased in proportion to the increase in membrane permeation caused by tat conjugation of proteins.

INTRODUCTION

The application of genomics and gene microarray is predicted to lead to the discovery of many new therapeutic proteins (1). However, the delivery of large-molecule pharmaceuticals into tissues in vivo is restricted by transport across the capillary microvascular endothelial barrier. Transport of therapeutic proteins to the brain is particularly restricted because of the presence of the blood–brain barrier (BBB).¹ One strategy for mediating the uptake of proteins by cells in culture and by tissues in vivo is cationization of the protein (2). The cationic charge forms electrostatic interactions with anionic charges on the cellular surface, and this triggers absorptive-mediated endocytosis and absorptive-mediated transcytosis across endothelial barriers (3, 4). Proteins may be cationized directly by conversion of surface carboxyl moieties into extended primary amino groups, and this raises the isoelectric point (pI) of the protein from the neutral/acidic range to the cationic range (5). Alternatively, a cationic peptide or protein, such as

polylysine, may be conjugated to the therapeutic protein (6). Polycations that have been used in the past include polylysine, protamine, histone, or import peptides. Import peptides are oligopeptide-containing sequences derived from larger proteins and are arginine (R)- or lysine (K)-rich peptides of strong cationic charge. Import peptides include the R-rich sequence from the third helix of the Antennapedia (Antp) protein (7, 8), which is a *Drosophila* transcription factor, the K-rich transportan peptide (9), the R/K-rich D-penetrin or the R-rich SynB1 (10), and the R-rich sequence from the tat protein of the human immune deficiency virus (HIV)-1 (11, 12). When arginine and/or lysine residues are clustered in a particular conformation, there is high-affinity binding to anionic proteoglycans such as heparin on cellular surfaces (13).

The R-rich sequence of the tat peptide encompasses the amino acids from positions 48–58 of the tat protein, which has a sequence of GRKKRRQRRP (14), and the cationic region of the tat peptide was observed to mediate uptake by cells in tissue culture (15). The conjugation of synthetic peptides containing the R/K-rich tat sequence to proteins, such as β -galactosidase, results in enhanced tissue uptake in vivo based on histochemical assays (11, 12). Histochemistry is a qualitative assay of protein uptake, and does not evaluate the plasma pharmacokinetics of the conjugate. The extent to which tissue uptake of an exogenous protein, which is expressed as percent injected dose (%ID) per gram of tissue, is increased by conjugation of cationic peptides to the protein is a dual function of (a) the increased membrane permeability of the conjugate and (b) the area under the plasma concentration curve (AUC), which is determined by the plasma

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¹ Abbreviations: BBB, blood–brain barrier; pI, isoelectric point; Antp, Antennapedia; HIV, human immune deficiency virus; R, arginine; K, lysine; G, glycine; Q, glutamine; P, proline; Y, tyrosine; ID, injected dose; SA, streptavidin; TCA, trichloroacetic acid; Cl, systemic clearance; V_{ss}, systemic volume of distribution; AUC, plasma area under the concentration curve; V_d, organ volume of distribution; V_o, plasma organ volume of distribution.

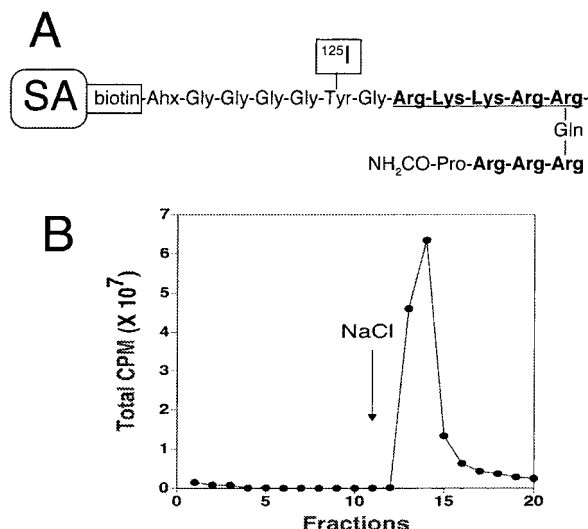


Figure 1. (A) Structure of [^{125}I]tat-biotin/streptavidin conjugate. (B) Elution of [^{125}I]tat $^{48-59}$ -biotin with high salt from a cationic exchange column results in a single homogeneous peak. There is no elution of noncationic components from the column prior to the high-salt wash, indicating the absence of radiochemical impurities from the iodinated tat $^{48-58}$ peptide.

pharmacokinetics of the tat-protein conjugate. Conjugation of cationic peptides to protein therapeutics would be expected both to increase membrane permeability and to decrease the plasma AUC, and these could have offsetting effects on the organ %ID/g. The organ %ID/g, the plasma AUC, and membrane permeation were examined in the present study. A biotinylated tat $^{48-58}$ synthetic peptide was prepared and conjugated to streptavidin (SA). The tat peptide contained a tyrosine residue, which allowed for radiolabeling with ^{125}I . The conjugate of the [^{125}I]tat-biotin and SA is designated [^{125}I]tat-biotin/SA, and is shown in Figure 1A. The organ uptake in vivo of [^{125}I]tat-biotin/SA was compared to the organ uptake of native SA, which was labeled with [^3H]biotin, and designated [^3H]biotin/SA.

EXPERIMENTAL PROCEDURES

Materials. Na ^{125}I was purchased from Amersham-Pharmacia Biotech (Arlington Heights, IL), and [^3H]biotin was obtained from NEN Life Sciences (Boston, MA). Sep-Pak C $_{18}$ cartridge and CM Bio-Gel were obtained from Waters (Milford, MA) and Bio-Rad (Hercules, CA), respectively. Acetonitrile was purchased from Fisher Scientific (Pasadena, CA), and trifluoroacetic acid was supplied by Pierce (Rockford, IL). Recombinant streptavidin (SA) and all other chemicals were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). Male Sprague Dawley rats were obtained from Harlan Sprague Dawley (San Diego, CA). The tat-biotin peptide with the sequence biotin-Ahx-GGGGYGRKKRRQRRP-NH $_2$, where Ahx = 6-aminoheptanoic acid, was synthesized and purified to >95% homogeneity on HPLC by Multiple Peptide Systems (San Diego, CA). The molecular mass, 2224 daltons, determined by mass spectrometry, equaled the expected molecular mass. This peptide contains an amino-terminal biotin, an Ahx-(G) $_4$ linker, and the Y ^{47} -P ^{58} sequence of the tat protein of HIV-1 (14). The tyrosine (Y) at position 47 enables radio-iodination (Figure 1A).

Iodination of Tat-Biotin Peptide. The tat-biotin peptide (29 μg , 13 nmol) was iodinated with [^{125}I]iodine (2 mCi, 1 nmol) and chloramine T (10 nmol), and the reaction was quenched by the addition of sodium metabisulfite (62 nmol). The mixture was then applied to an

activated C $_{18}$ Sep-Pak cartridge, followed by elution in 40% acetonitrile/0.1% trifluoroacetic acid. The radiochemical purity of this preparation was assayed by measurement of the fractional precipitation of the peptide by cold 10% trichloroacetic acid (TCA) in the presence of 5% carrier albumin, and the TCA precipitation of the peptide was only 65%. A TCA precipitation of <95% is indicative of either radiochemical impurities or an incomplete acid precipitation of the peptide owing to the small size of the peptide. Therefore, the radiochemical purity was assayed further with cation exchange chromatography. The fraction of [^{125}I]tat-biotin eluted from the C $_{18}$ Sep-Pak cartridge was evaporated to 0.5 mL and then applied to a 0.7 $\times$ 6 cm Biogel CM column. The column was eluted with 10 mL of 0.01 M Na $_2\text{HPO}_4$, pH 7.4, followed by 10 mL of 0.01 M Na $_2\text{HPO}_4$, pH 7.4, containing 0.5 M NaCl. No radiolabeled impurities eluted in the low-salt fraction, and the [^{125}I]tat-biotin peptide eluted as a single peak with the high-salt fraction, indicating a high degree of radiochemical purity of the iodinated tat-biotin peptide (Figure 1B). The [^{125}I]tat-biotin was added to a molar excess of recombinant SA 30 min prior to injection in rats. Owing to the very high affinity of SA binding of biotin ($K_D = 10^{-15}$ M, dissociation $t_{1/2} = 89$ days) (16), there is nearly instantaneous formation of the [^{125}I]tat-biotin/SA complex.

Pharmacokinetics and in Vivo Biodistribution.

Rats (320–350 g) were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2 mg/kg) intraperitoneally, and a femoral vein and artery were cannulated with PE50 tubing. A 0.25 mL aliquot of 0.005 M Na $_2\text{HPO}_4$ /0.25 M NaCl, pH 7.4, containing either 4 μCi of unconjugated [^{125}I]tat-biotin peptide or 4 μCi of [^{125}I]tat-biotin peptide conjugated to 20 μg of SA was injected into the femoral vein. In addition, SA bound to [^3H]biotin was injected in a separate group of rats. Blood was removed from the femoral artery at 0.25, 1, 2, 5, 15, 30, and 60 min after injection of the compound. The volume of blood removed was replaced by an equal volume of 0.9% saline via the femoral artery catheter. The blood samples were centrifuged, serum was counted for total radioactivity, and samples containing ^{125}I were precipitated by 10% TCA. The animals were decapitated at 60 min after the isotope administration, and radioactivity (cpm/g) was measured in brain, liver, kidney, heart, lung, and spleen.

Pharmacokinetic parameters were calculated by fitting serum radioactivity data to either a mono- or a biexponential equation using a derivative-free nonlinear regression analysis (PARBMDP, Biomedical computer P-Series, developed at UCLA Health Science Computing Facilities). For the studies with the tat-peptide, the TCA-precipitable serum radioactivity was used. The data were either unweighted or weighted with weight = $1/(\text{concentration})^2$, where concentration = %ID/mL of serum and ID = injected dose, so as to obtain the lowest residual sum of squares. The systemic clearance (Cl), the systemic volume of distribution (V_{ss}), and the area under the plasma concentration curve (AUC) were determined as described previously (17). The organ clearance was determined as follows:

$$\text{clearance} = (V_d - V_o) \cdot C_p(60 \text{ min}) / \text{AUC}_{0-60 \text{ min}}$$

where V_d is the organ volume of distribution ($\mu\text{L/g}$), $C_p(60 \text{ min})$ is the terminal serum concentration, and V_o are the plasma volumes for the respective organs, which have been reported previously (17). The organ uptake, %ID/g, was computed from the product of organ clearance and $\text{AUC}_{0-60 \text{ min}}$. The organ V_d was determined from the

Table 1. Pharmacokinetic Parameters

parameter (units)	[¹²⁵ I]tat-biotin	[¹²⁵ I]tat-biotin/SA ^a	[³ H]biotin/SA
A ₁ (%ID/mL)	0.39 ± 0.02	3.0 ± 0.2	9.7 ± 0.4
A ₂ (%ID/mL)	0.063 ± 0.005		
K ₁ (min ⁻¹)	0.88 ± 0.20	0.014 ± 0.001	0.0056 ± 0.0006
K ₂ (min ⁻¹)	0.0069 ± 0.0012		
t _{1/2} ¹ (min)	0.87 ± 0.17	49 ± 3	124 ± 12
t _{1/2} ² (min)	107 ± 19		
AUC(60) (%ID·min/mL)	3.6 ± 0.3	123 ± 5	494 ± 50
AUC(∞) (%ID·min/mL)	10 ± 1	215 ± 8	1734 ± 136
V _{ss} (mL/kg)	4160 ± 450	97 ± 5	36 ± 1
Cl (mL/min/kg)	29 ± 4	1.37 ± 0.01	0.058 ± 0.005

^a SA = streptavidin.

ratio of total dpm/g in the tissue divided by the dpm/μL of TCA-precipitable serum radioactivity. The plasma TCA precipitation data were normalized by dividing by the control TCA precipitation of the uninjected peptide, which was 63% and 85% for the unconjugated tat-biotin peptide and for the tat-biotin/SA conjugate, respectively. This approximation was necessary owing to the incomplete precipitation of the tat^{48–58} peptide by TCA (Figure 1). However, the differences in TCA precipitation of [¹²⁵I]tat-biotin and the [¹²⁵I]tat-biotin/SA complex are small compared to the large differences in systemic clearance of these conjugates.

The organ clearance, also called a membrane permeability–surface area (PS) product, is a measure of membrane permeation of the tat-SA conjugate or the unconjugated SA protein. Since organ radioactivity was measured at 60 min after injection, the organ clearance may underestimate the actual PS product if there is significant efflux of radioactivity from the organ after initial uptake.

RESULTS

The serum concentration following intravenous injection of [³H]biotin/SA, of unconjugated [¹²⁵I]tat-biotin, and of [¹²⁵I]tat-biotin/SA is shown in Figure 2 (left panel). These data demonstrate marked differences in the rate of removal of the proteins from blood in the rat in vivo. The serum radioactivity data were subjected to pharmacokinetic analysis (Experimental Procedures), and the pharmacokinetic parameters are listed in Table 1. The unconjugated [¹²⁵I]tat-biotin peptide is rapidly removed from blood following intravenous injection as demonstrated by the extremely high rate of plasma clearance, 29 ± 4 mL/min/kg, and the high systemic volume of distribution, 4160 ± 450 mL/kg (Table 1). The plasma clearance of the [¹²⁵I]tat-biotin peptide was reduced 21-fold following conjugation to SA (Table 1). The plasma clearance of the tat-biotin/SA conjugate was 24-fold greater than the plasma clearance of biotin/SA (Table 1). In parallel with the rapid rate of removal of the tat peptide from plasma, there was rapid degradation of the unconjugated tat peptide in vivo as demonstrated by the rapid decline in the serum radioactivity that was precipitable by TCA (Figure 2, right panel).

The individual organ clearance for the unconjugated tat peptide was high in all tissues (Figure 3). There was a reduction in the organ clearance of the tat peptide following conjugation to SA (Figure 3). However, the organ clearance of the tat-SA conjugate was still greater than the organ clearance of the unconjugated SA, as shown in Figure 4 (top panel). These data demonstrate that conjugation of the tat peptide to SA increases protein uptake in all tissues, particularly kidney. However, when organ uptake was measured as percent injected dose (%ID) per gram of tissue, the increased uptake of SA

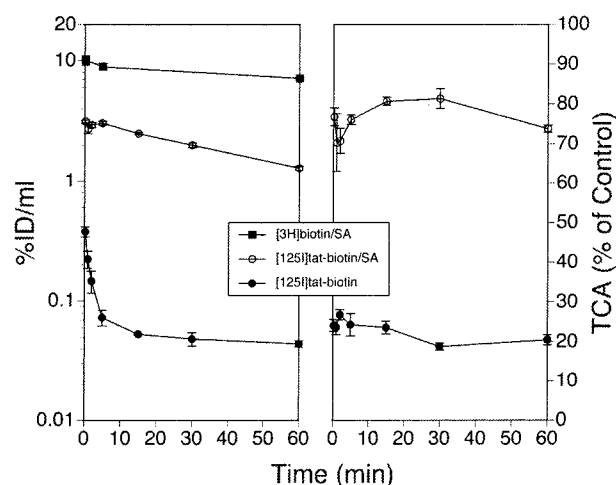


Figure 2. (Left panel) The percent injected dose (%ID) per milliliter of serum is plotted versus time for [³H]biotin/SA, [¹²⁵I]tat-biotin/SA, and unconjugated [¹²⁵I]tat-biotin. Data are mean ± SE (*n* = 3 rats per point). (Right panel) Percent of serum radioactivity that is precipitable by trichloroacetic acid (TCA) is plotted versus time after intravenous injection.

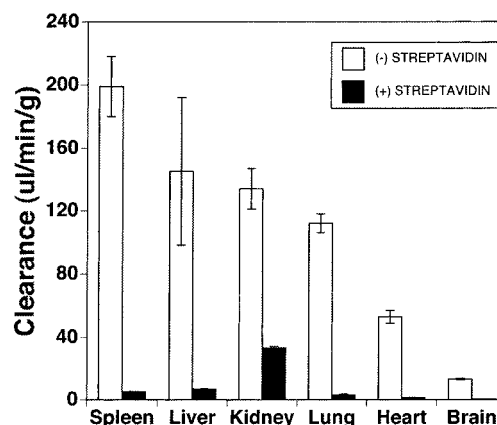


Figure 3. Organ clearance values for [¹²⁵I]tat-biotin without (–) or with (+) conjugation to streptavidin. These organ clearances are related to membrane permeation of the conjugates. Values were determined at 60 min after a single intravenous injection. Data are mean ± SE (*n* = 3 rats).

following conjugation of the tat peptide was more modest, as demonstrated in Figure 4 (bottom panel). The differences between the organ clearance and the organ %ID/g values in Figure 4 are due to the difference in the plasma AUC values. The plasma AUC at 60 min for [³H]biotin/SA is decreased 4-fold compared to the plasma AUC of [¹²⁵I]tat-biotin/SA, and the steady-state plasma AUC of SA is decreased 8-fold following conjugation with the tat peptide (Table 1).

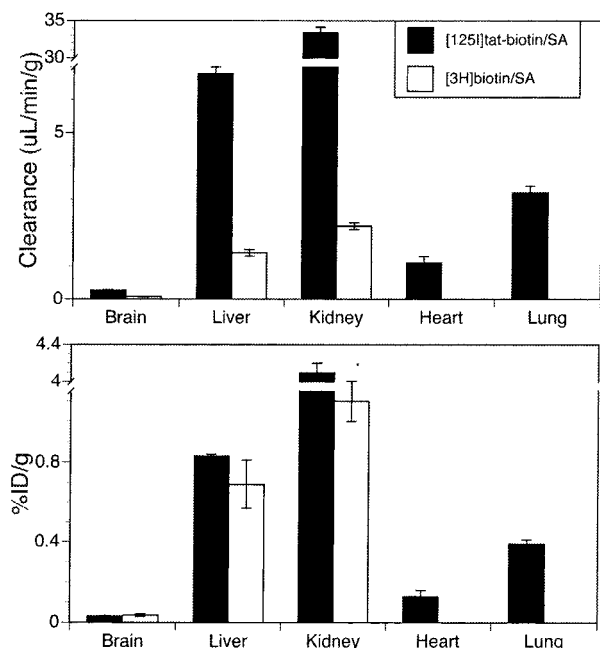


Figure 4. (Top panel) Organ clearance of $[^3\text{H}]$ biotin/SA (white columns) or $[^{125}\text{I}]$ tat-biotin/SA (black columns) at 60 min after intravenous injection. Data are mean $\pm$ SE ($n = 3$ rats). (Bottom panel) Organ uptake is expressed as percent injected dose (%ID) per gram of tissue. %ID/g = (clearance) $\times$ (60 min plasma AUC). The 60 min AUC values are shown in Table 1. The heart and lung values for $[^3\text{H}]$ biotin/SA are not visible as these are zero.

DISCUSSION

The results of these studies are consistent with the following conclusions. First, cationic oligopeptides such as tat^{48–58} are removed from blood nearly instantaneously owing to rapid uptake by virtually all tissues. Second, the organ uptake of a model protein, SA, is increased following conjugation with a cationic oligopeptide such as tat^{48–58}. Third, conjugation of proteins with cationic peptides such as tat^{48–58} has opposite effects on (i) the membrane permeation, as reflected in the estimate of organ clearance, and (ii) the plasma AUC. These opposing effects on membrane permeation and plasma AUC result in a more modest change in the organ uptake of the tat-protein conjugate, as reflected in the %ID/g (Figure 4). The %ID/g is directly proportional to both membrane permeation and the plasma AUC (Experimental Procedures).

Conjugation of cationic import peptides such as polylysine or tat oligopeptides triggers electrostatic interactions with anionic sites on cellular membranes, and this causes absorptive-mediated endocytosis (2). The mechanism of cellular uptake of cationic peptides is said not to involve endocytosis, because the uptake is not suppressed by incubations at 4 °C in tissue culture (18). However, confocal microscopy investigations of the uptake of tat or Antp import peptides uniformly show sequestration of the peptide in intracellular endosomes (8, 18, 19), which suggests the cationic peptides enter cells via absorptive-mediated endocytosis (2).

Cationic import peptides such as tat^{48–58} are rapidly cleared from blood following intravenous injection, and more than 90% of the injected dose is removed from blood within the first 60 s after intravenous administration (Figure 2, left panel). The V_{ss} of the $[^{125}\text{I}]$ tat-biotin peptide, 4160 ± 450 mL/kg (Table 1), is 50-fold higher than the blood volume, which is indicative of rapid sequestration of the peptide in peripheral tissues follow-

ing the initial extravascular distribution of the $[^{125}\text{I}]$ tat-biotin peptide. In the present studies, a radio-iodinated form of the tat peptide was used. It is possible that the organ transport of the tat peptide is altered by the addition of the ^{125}I -labeled nuclide. However, the ^{125}I moiety comprises only 5% of the mass of the iodinated tat peptide. In addition, prior work has shown that the pharmacokinetics of peptide clearance from blood are identical whether the peptide is labeled with ^{125}I versus another radionuclide, such as ^{111}In (20). The identical clearance of a peptide labeled with either ^{125}I or ^{111}In indicates that surface deiodination of the peptide in vivo is not significant. In the case of the tat peptide, surface deiodination of the peptide in vivo would have to be nearly instantaneous to explain the rapid removal of the radioactive peptide from blood (Figure 2), and this has not been described for iodinated peptides in vivo.

The tat^{48–58} is an oligopeptide with a molecular mass of 2200 daltons, and this oligopeptide rapidly traverses the microvascular endothelial barrier in tissues in vivo. The rapid extravascular distribution of the unconjugated tat peptide is exemplified by the very high systemic volume of distribution, 4160 ± 450 mL/kg (Table 1). There is a marked reduction in the systemic volume of distribution of the tat peptide following conjugation to SA, but the V_{ss} of the tat-biotin/SA conjugate is still nearly 3-fold greater than the V_{ss} of the unconjugated SA (Table 1). The increased V_{ss} of the tat-biotin/SA conjugate relative to biotin/SA is paralleled by the organ clearance data shown in Figure 4 (top panel). These data indicate that conjugation of the tat^{48–58} import peptide enhances the organ clearance of streptavidin, a 50 000 dalton protein.

The dose of the streptavidin delivered to the tissue, as reflected in the %ID/g parameter, is not increased in proportion to changes in the organ clearance parameter (Figure 4). This is because the %ID/g is an equal function of the organ clearance and the 60 min plasma AUC. Owing to the rapid uptake of the tat-biotin/SA conjugate in all tissues, there is a substantial decrease in the 60 min plasma AUC of the SA protein following tat conjugation (Table 1). Therefore, conjugation of SA with the tat peptide increases the organ permeability (clearance) for the exogenous protein, but the conjugation of the tat peptide causes a parallel decrease in the plasma AUC, owing to enhanced uptake in nearly all tissues.

The rapid rate of systemic clearance of conjugates of proteins and cationic peptides such as tat may require the systemic administration of relatively large amounts of the conjugate in order to exert pharmacologic effects in vivo. For example, to detect β -galactosidase enzyme activity in tissues in vivo in mice, it was necessary to inject 7–25 mg/kg doses of the conjugate of β -galactosidase and either tat^{37–72} or tat^{48–58} (11, 12). The injection of mg/kg doses of cationic peptide, which are required because of the low plasma AUC of the cationic peptide conjugate, may have toxic effects in vivo. Comparable doses of cationic peptides increase membrane permeability in vivo (21, 22).

In summary, cationization of a protein therapeutic has effects on the plasma pharmacokinetics and tissue uptake that are similar to the effects of lipidization of small-molecule drugs. Assuming no significant plasma protein binding, increasing the lipid solubility of a small molecule, or increasing the cationic charge of a large molecule, has two parallel effects: (i) the membrane permeation (organ clearance) parameter is increased, and (ii) the plasma AUC is decreased. The increase in the membrane permeation and the decrease in the plasma

AUC have opposing effects on the tissue %ID/g. Therefore, the organ %ID/g is not increased in proportion to changes in membrane permeation following drug lipidization (23). Similarly, attachment of a cationic import peptide, such as tat^{48–58}, results in an increase in membrane permeation, but also causes a parallel decrease in the plasma AUC, and these have offsetting effects on the organ %ID/g. Pharmacological effects in vivo are determined not by the organ clearance parameter, but by the %ID/g.

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A Streptavidin Mutant Useful for Directed Immobilization on Solid Surfaces

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A streptavidin mutant has been designed and produced that allows the specific, covalent immobilization of streptavidin on solid surfaces. This streptavidin mutant was constructed by fusing a six-residue sequence, containing a single cysteine, to the carboxyl terminus of streptavidin. Because this mutant has no other cysteine residues, the reactive sulfhydryl group of the cysteine residue serves as a unique immobilization site for conjugation using sulfhydryl chemistry. This streptavidin mutant was efficiently immobilized on maleimide-coated solid surfaces via its unique immobilization site. Characterization of the immobilized streptavidin mutant for the ability to bind to biotinylated macromolecules and the dissociation rates of bound biotin showed that the biotin-binding properties of this mutant were minimally affected by immobilization on solid surfaces. This streptavidin could be readily incorporated into a wide variety of solid-phase diagnostic tests and biomedical assays. This could enhance the performance of streptavidin-based solid-phase assay systems.

INTRODUCTION

Streptavidin is a tetrameric protein produced by *Streptomyces avidinii*. This protein binds the vitamin D-biotin with an affinity higher than any other known biological binding site ($K_d \sim 10^{-15}$ M) (1–4). Streptavidin is a highly stable protein that can maintain its functional structure even under a variety of harsh conditions, such as high temperatures and the presence of high concentrations of denaturants and organic solvents. These unique properties, along with the ability of biotin to be incorporated easily into various biological materials without disturbing their properties and structures, allow streptavidin to serve as a powerful, versatile tool in biomedical science and clinical diagnostics.

One group of applications of streptavidin involves solid-phase assays, in which streptavidin is immobilized covalently on a solid surface. The extremely high biotin-binding affinity of streptavidin allows tight, efficient immobilization or capture of biotinylated materials on streptavidin-coated solid surfaces. The properties of such immobilized streptavidin were recently analyzed (5). The streptavidin-coated solid surface was produced by using natural core streptavidin, lacking both the N- and C-terminal sequences present in the parental full-length streptavidin, which was immobilized covalently through its amino groups on a polystyrene surface. This study indicated that the properties of the immobilized streptavidin are heavily altered as compared to those in solution. In particular, its binding affinity for biotin is much lower than that in solution, and its accessibility to biotinylated materials is significantly reduced. These and other alterations severely reduce the efficacy of immobilized streptavidin as a solid support for biomedical assays. This suggests that, if streptavidin could be

immobilized specifically on solid surfaces without affecting its properties, various solid-phase assay systems with immobilized streptavidin could be considerably enhanced. In this work, we designed and produced a streptavidin mutant that can be immobilized efficiently on a solid surface with minimal effects on the fundamental properties of streptavidin.

MATERIALS AND METHODS

Construction of an Expression Vector. An expression vector was constructed by using pTSA-13 carrying a cloned streptavidin gene (6) as a starting material. A PCR (polymerase chain reaction)-based site-directed mutagenesis method was used to modify the coding sequence for a C-terminal region of streptavidin. A DNA sequence encoding six amino acid residues with a translation termination codon, GGT GGT TCT GGT TGC CCG TAG (Gly-Gly-Ser-Gly-Cys-Pro-Stop), was fused to the codon for Val-133 (GTG) (7). The entire coding sequence was cloned between the *Nde* I and *Bam* H I sites of the plasmid pET-3a under the control of the $\phi 10$ promoter of bacteriophage T7 (8). The resulting expression vector pTSA-C encodes a streptavidin mutant, Stv-C, which consists of the core region of streptavidin encoded in pTSA-13 (6), with the six-residue sequence Gly-Gly-Ser-Gly-Cys-Pro at the C-terminus.

Expression and Purification of a Streptavidin Mutant. Expression and purification of Stv-C was carried out as previously described (6, 9, 10) by using the *Escherichia coli* strain BL21(DE3)(pLysE) (8) carrying pTSA-C. During renaturation of Stv-C, 2-mercaptoethanol was included in all solutions at a concentration of 10 mM to prevent the formation of disulfide bonds (9, 10).

Immobilization of Stv-C on a Maleimide-Coated Solid Surface. Stv-C was immobilized on microtiter wells coated with maleimide groups (Reacti-Bind Male-

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imide Activated Plates; Pierce Chemical). Stv-C was added to maleimide-coated microtiter wells at various concentrations (0.4–4 μ M subunits) in phosphate-buffered saline (PBS; 0.137 M sodium chloride, 2.7 mM potassium chloride, 10 mM phosphate buffer, pH 7.4). TCEP-HCl [tris(2-carboxyethyl)phosphine hydrochloride] was included in the reaction mixture at a 10-fold molar excess over sulfhydryl groups present to avoid the formation of disulfide bonds. The immobilization reaction was carried out with shaking at room temperature ($\sim 22^\circ\text{C}$) overnight. Unbound Stv-C was removed by washing microtiter wells three times with PBS containing 1 mM TCEP-HCl. To each microtiter well, 200 μ L of 5 mM iodoacetamide in 0.2 M Tris-HCl pH 8.5 was added and incubated for 2 h in the dark to block unreacted sulfhydryl groups of Stv-C, followed by washing the microtiter wells with PBS.

The biotin-binding capacity of immobilized Stv-C was estimated by using D-[carbonyl- ^{14}C]biotin (54 mCi/mmol; Amersham Pharmacia). A known amount of D-[carbonyl- ^{14}C]biotin in PBS was added to each microtiter well and incubated for 30 min. Then, unbound D-[carbonyl- ^{14}C]biotin was collected, and its radioactivity was determined by liquid scintillation counting. The amount of bound D-[carbonyl- ^{14}C]biotin was estimated from the difference in the amount between the total D-[carbonyl- ^{14}C]biotin added and unbound D-[carbonyl- ^{14}C]biotin.

Preparation of a Biotinylated, Radiolabeled DNA.

A biotinylated 500-bp double-stranded DNA was generated by PCR using the plasmid pUC19 as a template with the oligonucleotide primers 5'-GGCGTATCACGAGG-3' and 5'-biotin-AACAGCTATGACCATG-3'. The 5'-terminus of the nonbiotinylated oligonucleotide primer had been labeled with ^{32}P by phosphorylation using T4 polynucleotide kinase (New England Biolabs) and [γ - ^{32}P]-adenosine 5'-triphosphate (3000 Ci/mmol; Amersham Pharmacia). The resulting biotinylated, ^{32}P -labeled 500-bp PCR product (4.88 Ci/mmol), which contained one biotin moiety per molecule, was purified by using a PCR purification kit (Qiagen) and stored at -20°C until used.

Preparation of Biotinylated, Radiolabeled IgG and F(ab')₂ Fragment. Approximately 350 μ g of goat immunoglobulin G (IgG) against rabbit IgG (150 kDa; Roche Molecular Biochemicals) or 350 μ g of a goat F(ab')₂ fragment against rabbit IgG (110 kDa; Sigma) was incubated on ice for 90 min with 3.2 pmol of a [^{35}S]sulfur labeling reagent (>800 Ci/mmol; Amersham Pharmacia) in 100 mM boric acid pH 8.0 (reaction volume, 175 μ L). Then, 1 mg (1.8 μ mol) of sulfo-NHS-LC-biotin (Pierce Chemical) in 25 μ L of 100 mM boric acid pH 8.0 was added to each mixture, which was incubated for an additional 90 min. Each reaction mixture was subjected to gel filtration chromatography on a Superdex 75 HR column (1 cm $\times$ 30 cm; Amersham Pharmacia) using 150 mM NaCl, 20 mM Tris-HCl pH 7.4 as the eluent to remove unreacted ^{35}S -sulfur labeling reagents and sulfo-NHS-LC-biotin. The eluted protein samples [biotinylated ^{35}S -labeled IgG (3.25 Ci/mmol) and a biotinylated ^{35}S -labeled F(ab')₂ fragment (2.08 Ci/mmol)] were stored at 4°C until used. By a modified 2-[4'-hydroxyazobenzene]-benzoic acid (HABA) displacement assay (11) using the protocol provided with ImmunoPure HABA (Pierce Chemical), the number of biotin moieties per molecule was estimated to be 18 and 10 for the biotinylated ^{35}S -labeled IgG and the biotinylated ^{35}S -labeled F(ab')₂ fragment, respectively.

Binding Capacities of Immobilized Stv-C for Biotinylated Macromolecules. The binding capacity of immobilized Stv-C for biotinylated DNA was analyzed

by adding 10 pmol of the biotinylated, ^{32}P -labeled 500-bp double-stranded DNA, prepared above, to each Stv-C-coated microtiter well (biotin-binding capacity, 10.1 pmol/well) in 1 M NaCl, 10 mM Tris-HCl pH 7.4 containing 0.02% Nonident P-40. The mixtures were incubated for 1 h, after which time unbound DNA was collected and quantitated by liquid scintillation counting. The difference between the total DNA added and unbound DNA was used to estimate the amount of bound DNA. Non-specific binding of DNA to Stv-C-coated microtiter wells was determined in the same manner, except that target ^{32}P -labeled DNA contained no biotin.

The binding capacities of immobilized Stv-C for the biotinylated ^{35}S -labeled IgG and the biotinylated ^{35}S -labeled F(ab')₂ fragment, prepared above, were analyzed by adding 25 pmol of each protein target to each Stv-C-coated microtiter well (biotin-binding capacity, 25 pmol/well) in PBS containing 0.02% Nonident P-40. The mixtures were incubated for 1 h, after which time unbound materials were collected and quantitated by liquid scintillation counting. The difference between the total protein added and unbound protein was used to estimate the amount of bound protein. Nonspecific binding of these protein targets to Stv-C-coated microtiter wells was measured by using the same procedure, except that ^{35}S -labeled protein targets contained no biotin.

Determination of Dissociation Rate Constants of Bound Biotin from Immobilized Stv-C.

Rate constants of dissociation of bound biotin from immobilized Stv-C were determined by using the following procedure. Each Stv-C-coated microtiter well (biotin-binding capacity, 64 pmol/well) was incubated with approximately 64 pmol of D-[8,9- ^3H]biotin (0.875 Ci/mmol; Amersham Pharmacia) for 30 min in 300 μ L of 20 mM Tris-HCl pH 8.0. Stv-C-coated microtiter wells were washed three times with 20 mM Tris-HCl pH 8.0 to remove unbound D-[8,9- ^3H]biotin. Then, 64 nmol of biotin in 300 μ L of 20 mM Tris-HCl pH 8.0 was added to each microtiter well, which was incubated with shaking at room temperature. At various incubation times over a period of 5 days, 100- μ L aliquots were collected from three wells at a time. The radioactivity of the collected aliquots, derived from D-[8,9- ^3H]biotin released from immobilized Stv-C, was measured by liquid scintillation counting and used to calculate the dissociation rate constant of bound biotin from immobilized Stv-C.

RESULTS AND DISCUSSION

Streptavidin-coated solid surfaces, on which streptavidin is covalently immobilized, have proven very useful for a variety of biomedical assays, including clinical diagnostics (12–14). The properties of one such streptavidin-coated solid surface (5) were recently investigated. This study showed that the properties of natural core streptavidin are heavily altered by immobilization on a solid surface. In particular, several different classes of biotin-binding sites are present, each of which has a weaker biotin-binding affinity by, at least, a few orders of magnitude than in solution. Molecular simulation analysis using the known three-dimensional structure of streptavidin (15, 16) suggested that two of the four biotin-binding sites of each immobilized natural core streptavidin molecule are very unlikely to be available for binding to biotin due to their close proximity to the solid surface. These alterations are likely to result from the direct interaction of streptavidin molecules with the solid surface, which may cause a structural distortion of streptavidin. This suggests that, if an appropriate dis-

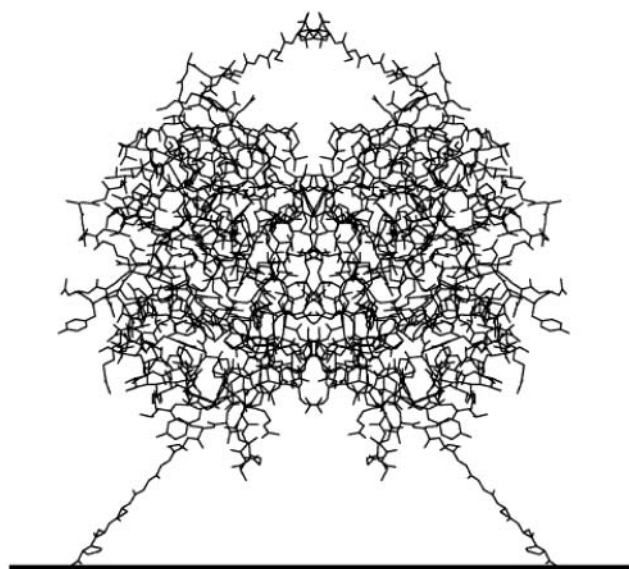


Figure 1. Schematic illustration of a streptavidin mutant with a six-residue immobilization sequence, Stv-C, immobilized on a solid surface. The immobilization sequence (Gly-Gly-Ser-Gly-Cys-Pro) is shown in a fully extended conformation.

tance could be introduced between an immobilized streptavidin molecule and a solid surface, the properties of streptavidin could be maximally maintained upon immobilization. To test this, we attempted to design a specific immobilization sequence, which can be fused to a terminus of streptavidin recombinantly. The immobilization sequence has a unique immobilization site, which serves as the point of attachment to a solid surface. The sequence between the immobilization site and the core region of streptavidin should serve as a linker that can be used to adjust the distance between the streptavidin molecule and the solid surface.

Cysteine was chosen as an immobilization site because of the high reactivity of its sulfhydryl group. Due to the absence of cysteines in natural streptavidin (9, 10), a cysteine residue in a recombinant streptavidin should be useful for specific, directed immobilization of streptavidin on a solid surface using sulfhydryl chemistry. The appropriate length of the immobilization sequence was estimated by using the known three-dimensional structure of streptavidin (15, 16) with the protein-modeling program QUANTA (Pharmacopeia, Princeton, NJ). This analysis suggested that four amino acid residues between the immobilization site (cysteine residue) and the C-terminus of the core region of streptavidin should be optimal to maintain the properties of streptavidin upon immobilization. Shorter sequences between the immobilization site and streptavidin may reduce the capacity of immobilized streptavidin for biotinylated macromolecules due to the close proximity of biotin-binding sites to the solid surface. Longer immobilization sequences seem to be unnecessary and even disadvantageous since they might sterically prevent biotinylated macromolecules from docking into the biotin-binding sites of streptavidin. With a distance of four residues between the immobilization site and streptavidin, only two covalent linkages should be formed between each streptavidin molecule and the solid surface, thereby fully exposing two of the biotin-binding sites (Figure 1). On the basis of these analyses, the immobilization sequence designed is Gly-Gly-Ser-Gly-Cys-Pro. This six-residue sequence is flexible and has four residues between the C-terminus of the core region of

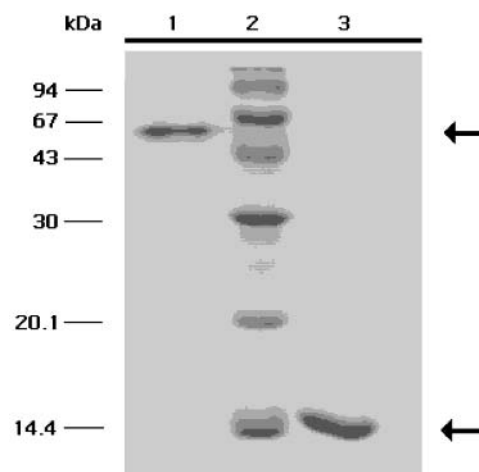


Figure 2. SDS-PAGE analysis of purified Stv-C. Stv-C (approximately 2.5 μ g) in the presence of 1.5% SDS and 10 mM TCEP, with and without heat treatment in boiling water for 3 min, was analyzed by SDS-PAGE (18) using a 15% polyacrylamide gel. Streptavidin is known to retain its tetrameric structure in the presence of SDS without heat treatment (19). Proteins were stained with Coomassie brilliant blue R. Lane 1, Stv-C without heat treatment; lane 2, molecular mass standard proteins; and lane 3, Stv-C with heat treatment. The positions of subunit tetramers (top) and monomers (bottom) are shown by arrows.

streptavidin and the immobilization site (cysteine). A proline residue was placed at the C-terminus to enhance the stability of the C-terminal sequence against exopeptidases. The design of the linker sequence took into account the absence of highly charged or hydrophobic residues to avoid electrostatic or hydrophobic interactions, respectively, with the core region of streptavidin or other molecules present in the sample.

We constructed an expression vector for a streptavidin mutant, Stv-C, which consists of the core region (from Gly-16 to Val-133) of streptavidin (7) with a methionine at the N-terminus and the six-residue immobilization sequence, described above, at the C-terminus. The encoded Stv-C was expressed efficiently in *E. coli* by using the bacteriophage T7 expression system (8). Expressed Stv-C was purified to homogeneity (Figure 2) by a procedure that includes 2-iminobiotin affinity chromatography (9, 10). The average yield was 1.2 mg of purified Stv-C from 100 mL of culture. Purified Stv-C formed a tetrameric molecule and had full biotin-binding ability, indicating that the fusion of the six-residue immobilization sequence to the C-terminus has little, if any, effect on the fundamental properties of streptavidin.

Purified Stv-C was used to test if it can be immobilized efficiently on a solid surface. A maleimide-coated microtiter plate (Pierce Chemical) was used as the solid surface because of the specific reactivity of maleimide for sulfhydryl groups. Biotin-binding capacities of the resulting Stv-C-coated microtiter wells, determined by using D-[carboxyl- ^{14} C]biotin, showed that approximately 30 pmol of Stv-C subunits were immobilized per well, when Stv-C was used at a subunit concentration of 4 μ M during immobilization. This corresponds to a Stv-C density of 420 fmol subunits/mm².

The binding capacities of immobilized Stv-C, prepared as above, for biotinylated macromolecules were determined by using a biotinylated 500-bp double-stranded DNA (one biotin moiety per molecule), a biotinylated F(ab')₂ fragment (10 biotin moieties per molecule), and a biotinylated IgG (18 biotin moieties per molecule) as targets (see Materials and Methods). Stv-C-coated mi-

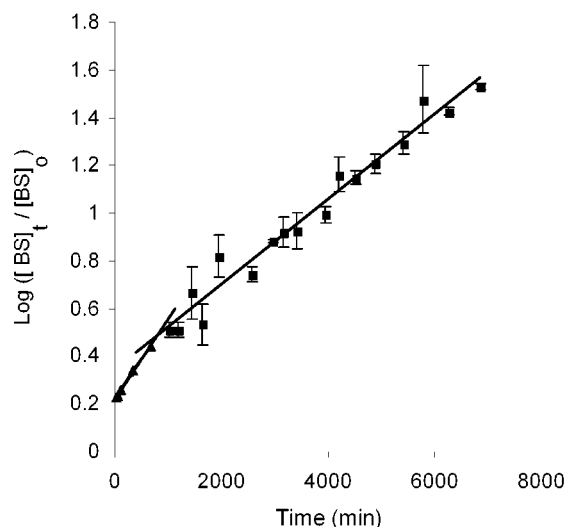


Figure 3. Dissociation of bound biotin from Stv-C immobilized on maleimide-coated microtiter wells. The logarithm of the ratio of the initial concentration of Stv-C-biotin complexes (time 0) ($[BS]_0$) to that at time t ($[BS]_t$) is plotted as a function of time. Each data point represents the mean $\pm$ standard deviation ($n = 3$). Best-fit lines are shown, which indicate the biphasic nature of the dissociation of bound biotin from immobilized Stv-C.

cro-titer wells with a biotin-binding capacity of 10.1 pmol/well bound 1.21 pmol of the biotinylated 500-bp DNA. Stv-C-coated microtiter wells with a biotin-binding capacity of 20.8 pmol per well bound 2.73 pmol of the biotinylated F(ab')₂ fragment or 1.77 pmol of the biotinylated IgG. The reduced binding capacities of immobilized Stv-C for these biotinylated macromolecules, as compared to that for biotin, are very likely caused by steric hindrance, generated by the binding of the biotinylated macromolecules to Stv-C that was immobilized in a high density on a solid surface. The Stv-C-coated microtiter wells used showed no detectable nonspecific binding by the equivalent target molecules lacking a biotin moiety. In a previous study (5), a streptavidin-coated solid surface, which was produced by the immobilization of natural core streptavidin on a polystyrene surface through its amino groups, bound approximately 22 pmol of a biotinylated 526-bp double-stranded DNA per 300 pmol of biotin-binding sites. Thus, approximately 7.3% of the biotin-binding sites can be bound by the biotinylated DNA (17). In contrast, approximately 12% of the biotin-binding sites of the Stv-C-coated microtiter wells can be bound by the equivalent biotinylated DNA. This implies that the accessibility of the biotin-binding sites of immobilized Stv-C by biotinylated macromolecules is higher than that of immobilized streptavidins prepared by using the natural protein.

The rate constant of dissociation of bound biotin from immobilized Stv-C was determined to estimate the homogeneity of the biotin-binding sites. The dissociation of streptavidin-biotin complexes should be a first-order process. Thus, the logarithm of the ratio of the initial concentration of Stv-C-biotin complexes (time 0) to that at time t is plotted as a function of time (Figure 3). This plot shows a biphasic curve, suggesting that there are, at least, two types of biotin-binding sites. Their dissociation rate constants are estimated at $5.0 \times 10^{-6} \text{ sec}^{-1}$ and $3.3 \times 10^{-6} \text{ s}^{-1}$ at pH 7.4. These dissociation rate constants appear to be slightly faster than that of streptavidin in solution ($2.8 \times 10^{-6} \text{ sec}^{-1}$ at pH 7.0) (3). In contrast, natural streptavidin, immobilized on a polystyrene surface through its amino groups, showed three types of

biotin-binding sites with dissociation rates of $4.9 \times 10^{-5} \text{ s}^{-1}$, $8.3 \times 10^{-6} \text{ s}^{-1}$, and $2.5 \times 10^{-6} \text{ s}^{-1}$ (5).

In conclusion, the fusion of the six-residue immobilization sequence containing a unique cysteine as the immobilization site to the carboxyl terminus of streptavidin has made the resulting streptavidin (Stv-C) immobilizable on a solid surface with minimal effect on the biotin-binding characteristics upon immobilization. In particular, immobilized Stv-C appears to have a higher binding capacity for biotinylated macromolecules than streptavidin-coated solid surfaces produced by using the natural protein. In addition, the biotin-binding characteristics of immobilized Stv-C are only slightly different from those of streptavidin in solution, suggesting that the six-residue immobilization sequence allows the maintenance of the biotin-binding properties of streptavidin upon immobilization. This streptavidin construct should be readily adapted to solid surfaces currently used for a variety of solid-phase assays. This could enhance the performance of current streptavidin-based solid-phase assay systems.

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Stearyl-Arginine-Rich Peptides: A New Class of Transfection Systems

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Membrane-permeable arginine-rich peptides, such as HIV-1 Tat-(48–60), HIV-1 Rev-(34–50), and flock house virus (FHV) coat-(35–49), have been shown to possess the ability to transfect COS-7 cells with luciferase-coding plasmid as efficiently as polyarginine (MW 5000–15 000) and polylysine (MW 9800). Not only these virus-derived cationic peptides but also oligoarginines of 4–16 residues were found to be able to transfect cells. In the case of the Tat, FHV, and octaarginine peptides, N-terminal stearylation of the peptides increases the transfection efficiency by approximately 100 times to reach the same order of magnitude as that of LipofectAMINE, one of the most efficient commercially available transfection agents. Also, a certain correlation was observed between the transfection efficiency of stearyl-(Arg)_n peptides (stearyl-R_n: *n* = 4, 8, 12, 16) and the membrane permeability of the corresponding (Arg)_n peptides (R_n).

INTRODUCTION

Arginine-rich peptides derived from human immunodeficiency virus (HIV)-1 Tat and Antennapedia homeo-domain have been reported to translocate through cell membranes quite easily and to be utilized as carriers of macromolecules such as proteins and oligoDNAs by conjugating these basic peptides with the cargo macromolecules (1–10). Intracellular delivery of β -galactosidase (120 kDa) was achieved by the covalent conjugation with these carrier peptides (4). OligoDNAs up to ~30 bases were also introduced to cells with the help of these carrier peptides aiming at antisense or triple-helix therapy (9, 10). Recently, we reported that not only the Tat-derived peptide but also various arginine-rich basic peptides derived from RNA-binding proteins, such as HIV-1 Rev and flock house virus (FHV) coat proteins, can penetrate through the cell membranes and bring exogenous proteins into the cells (11). The peptides composed of only arginine residues were also found to have the above-mentioned property (11, 12). The fluorescence microscopic observation indicated that the peptide comprising eight residues of arginine translocated through cell membranes quite easily and accumulated in the nucleus. There was a certain dependence on the chain length for the cellular localization as well as the membrane permeability among these oligoarginine peptides. Quite interestingly, not only the smaller but also the larger the number of arginine residues became, the lower the membrane permeability and the less evident nuclear accumulation of the peptides became. In the case of the peptide comprising 16 arginine residues, the peptide was prominently observed on the membrane, and the accumulation in the nucleus was not significant (11). The results contradicted our expectation that, if only the

positive charge was the driving force for the translocation, the efficiency should become higher as the peptide charge increased.

On the other hand, polyarginine and polylysine have been employed for the delivery of plasmid DNAs and antisense oligonucleotides into mammalian cells (13). In the delivery using the above membrane-permeable arginine-rich peptides, the cargo proteins or oligoDNAs were covalently attached to the arginine-rich carrier peptides and applied to the cells. In contrast, transfection of the plasmid DNA and the antisense nucleic acid using these polypeptides was usually conducted simply by mixing them and forming a peptide/nucleic acid complex. In such a case, peptides of high molecular weight (5000 or the higher) have often been employed with the hope of forming a tight and compact complex with plasmid DNAs (14). The transfection efficiency of small peptides of a distinctive structure, such as octaarginine, has been studied less extensively compared with other cationic polymers. In relationship to the potential of the arginine-rich peptides as a protein carrier, we were interested in the applicability of these “short” arginine-rich peptides to the transfection for plasmid DNA of a few kilobase pairs. In this report, the transfection efficiency of arginine-rich membrane-permeable peptides (Figure 1) was assessed for the first time on the basis of the luciferase assay, using a strategy of simply mixing the peptides with the plasmid DNA. The effect of peptide modification by hydrophobic moieties was also examined.

EXPERIMENTAL PROCEDURES

Materials. Fmoc-amino acid derivatives were purchased from Calbiochem-Novabiochem (Läufelfingen, Switzerland). Rink amide resin (TGS-RAM) was purchased from Shimadzu (Kyoto, Japan). Poly-L-lysine (MW 9800) and poly-L-arginine (MW 5000–15 000) were from Sigma (St. Louis, MO). LipofectAMINE and fetal bovine serum (FBS) were obtained from Life Technologies (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM) was from Nissui Pharmaceutical (Tokyo, Ja-

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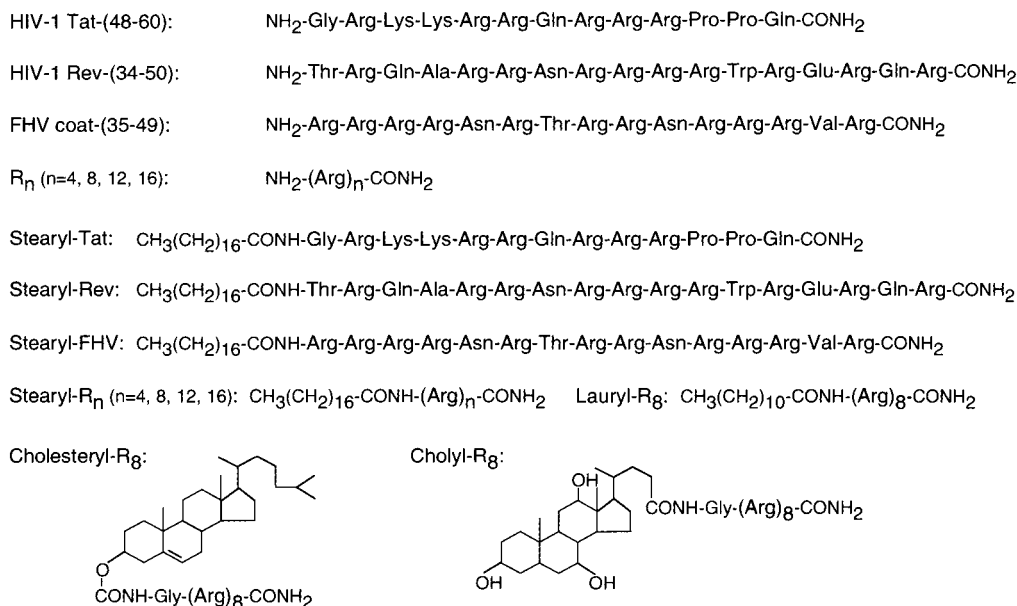


Figure 1. Structures of peptides used in this study.

pan). Plasmid DNA containing a firefly luciferase gene together with a simian virus 40 (SV40) promoter (PicaGene control vector2, PGV-C2) was from Toyo Ink (Tokyo, Japan). Other reagents were purchased from Aldrich (Milwaukee, MI) and Wako Chemical (Osaka, Japan).

Peptide Synthesis. All the peptides used in this report were synthesized by Fmoc-solid-phase peptide synthesis on Rink amide resin using a Shimadzu PSSM-8 synthesizer as reported (15). Stearyl-peptides were prepared by treatment of the corresponding peptide resins with stearic acid and diisopropylcarbodiimide in the presence of *N*-hydroxybenzotriazole followed by deprotection using trifluoroacetic acid (TFA)/ethanedithiol (EDT) (95:5). Lauryl- R_8 and cholyl- R_8 were similarly prepared using lauric acid and cholic acid in place of stearic acid, respectively. Cholesteryl- R_8 was prepared by treatment of the peptide resin with cholesteryl chloroformate in the presence of diisopropylethylamine followed by deprotection using TFA/EDT (95:5). All of the peptides were purified by reverse-phase HPLC. Fidelity of the products was ascertained by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS): HIV-1 Tat-(48–60), 1719.0/1719.1; HIV-1 Rev-(34–50), 2437.5/2437.8; FHV coat-(35–49), 2665.0/2664.5; R_4 , 642.7/642.8; R_8 , 1267.5/1267.5; R_{12} , 1891.5/1892.3; R_{16} , 2517.6/2517.0; stearyl-Tat, 1984.8/1985.2; stearyl-Rev, 2702.1/2702.8; stearyl-FHV, 2430.4/2430.6; stearyl- R_4 , 909.2/909.2; stearyl- R_8 , 1534.0/1533.9; stearyl- R_{12} , 2158.0/2158.7; stearyl- R_{16} , 2783.4/2783.5; stearyl-D- R_8 , 1533.7/1533.9; lauryl- R_8 , 1449.2/1449.9; cholesteryl- R_8 , 1737.5/1737.3; cholyl- R_8 , 1714.8/1715.2 [found/expected for $(\text{M}+\text{H})^+$].

In Vitro Gene Transfection and Luciferase Assay. COS-7 cells, a simian kidney cell line transformed with SV40 (5×10^5 cells/well), were grown to just before confluence in a 24-well plate in DMEM containing 10% FBS under 5% CO_2 at 37 °C. Plasmid DNA coding a luciferase (PGV-C2, 5.2 kbp) (2.5 μg) in serum-free DMEM (25 μL) was added to the solution of a peptide or an N-terminal modified peptide in serum-free DMEM (25 μL), allowed to stand for 30 min, and diluted with serum-free DMEM (200 μL). The cells were washed 3 times with 500 μL of serum-free DMEM. The above peptide/DNA mixture was then gently added to the cells. After incuba-

tion for 12 h at 37 °C, DMEM (250 μL) containing 20% FBS was added. After 12 h, the medium was replaced with fresh DMEM (500 μL) containing 10% FBS. The cells were further incubated for 36 h. Harvest of cells and luciferase assay were performed following the protocol of a Pica Gene luminescence kit (Toyo Ink, Tokyo, Japan) as reported by Niidome et al. (16). The light units were analyzed by a BLR-201 luminescence reader (Aloka, Japan). The light unit values shown in the figures represent the specific luciferase activity (RLU/mg of protein) which is standardized for the total protein content of the cell lysate [10^6 RLU corresponds to 0.37 ng (6 fmol) of luciferase]. The measurement of gene transfer efficiency was performed in triplicate.

Transfection using LipofectAMINE was similarly conducted as stated above. Following the supplier's protocol, LipofectAMINE (2.5 μL) was employed for 2.5 μg of the plasmid DNA in place of oligoarginine in serum-free DMEM (25 μL).

Particle Size Determination. Particle size determination was carried out using a DLS-700 dynamic light scattering spectrophotometer equipped with a vertically polarized 5-mW He–Ne laser (633 nm) (Otsuka Electronics, Hirakata, Japan). Plasmid DNA (PGV-C2) (25 μg) in HEPES-buffered saline (HBS) (21 mM HEPES containing 135 mM NaCl, 5.0 mM KCl, and 0.76 mM Na_2HPO_4 , pH 7.4) (250 μL) was mixed with the appropriate amount of a peptide or liposomes in HBS (250 μL) to give a cation:anion charge ratio of 2:1 and subjected to size determination.

Ethidium Bromide Displacement Assay (17). To a solution of plasmid DNA (PGV-C2) (3.5 μg) in HBS (600 μL) was added ethidium bromide (EtBr) (21 ng) in HBS (0.4 μL) and vortexed. An appropriate amount of stearyl- R_8 in HBS (2 μL) was added to the solution to give the desired cation:anion charge ratio. The fluorescence from the intercalated EtBr was monitored using a Hitachi fluorescence spectrophotometer F-3010 by the excitation and emission wavelengths at 510 and 595 nm, respectively.

MTT Assay (18). Cytotoxicity on transfection using stearyl- R_8 and LipofectAMINE was evaluated by MTT assay. COS-7 cells were placed in a 96-well plate (1×10^4 cells/well) in serum-free DMEM (50 μL) in the presence of the plasmid DNA (PGV-C2) (0.5 μg) and

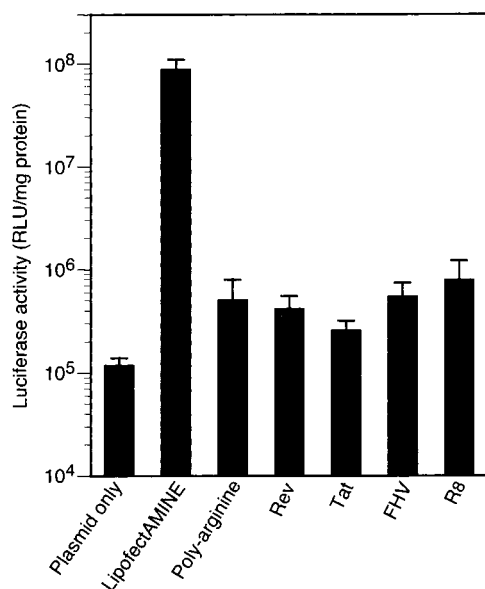


Figure 2. In vitro luciferase activity for PGV-C2 plasmid DNA after transfection of COS-7 cells using membrane-permeable arginine-rich peptides. Peptides were mixed with plasmid DNA at a cation:anion charge ratio of 2:1, where 15 nmol (as cation charge) of each peptide was used for 2.5 μ g of plasmid DNA, respectively. Each bar represents the mean and standard deviation of three determinations.

stearyl- R_8 (0.6 μ g) [or LipofectAMINE (0.5 μ L)] and incubated at 37 $^{\circ}$ C under 5% CO_2 for 12 h. DMEM containing 20% FBS (50 μ L) was then added, and incubation was continued for another 12 h. The medium was removed, and the cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (50 μ g) in DMEM containing 10% FBS (100 μ L) for 4 h. Cell viability was expressed as the ratio of the A_{570} of cells treated with a transfection agent together with plasmid to that of the control samples.

RESULTS

Transfection Efficiency of Membrane-Permeable Arginine-Rich Peptides. The efficiency of HIV-1 Tat-(48–60), HIV-1 Rev-(34–50), FHV coat-(35–49), and octaarginine (R_8) in the transfection of COS-7 cells was examined using a plasmid DNA containing a firefly luciferase reporter gene (Figure 2). All of the peptides have already been proved to be highly membrane-permeable. As references, the transfection efficiency of conventional transfection agents, polylysine (molecular weight ~9800) and polyarginine (molecular weight 5000–15 000), was examined. In these experiments, a peptide and the plasmid were mixed in a cation:anion charge ratio of 2:1. Comparison of the transfection efficiency with that of LipofectAMINE, which is often employed as one of the most efficient cationic liposomes for transfection, was also conducted. The dose of LipofectAMINE for the plasmid DNA was selected following the manufacturer's protocol. The cation:anion charge ratio in this case was calculated to be 1.6:1. After mixing the plasmid with a peptide or LipofectAMINE, the mixture was applied to COS-7 cells, a simian kidney cell line transformed with SV40. Transfection was conducted for 12 h in the absence of serum, and the cells were incubated for 48 h in the presence of serum. Luciferase activity from the cells transfected with the peptides corresponding to HIV-1 Tat-(48–60), HIV-1 Rev-(34–50), and FHV coat-(35–49) was found to be comparable with that from the cells transfected with polyarginine. Transfection efficiency of the

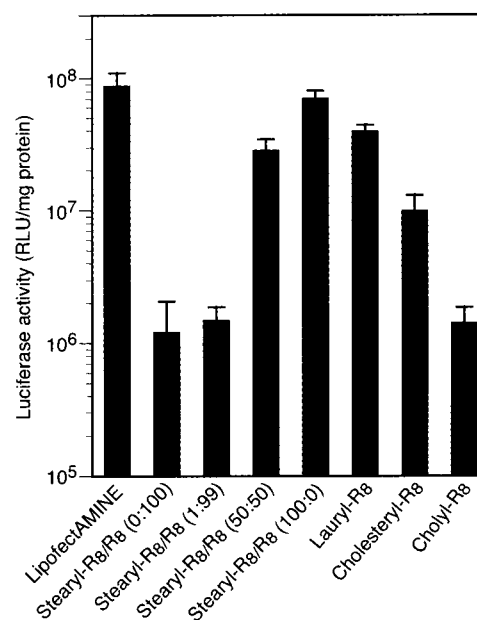


Figure 3. Effect of N-terminal modification of R_8 peptide on transfection efficiency. Peptides were mixed with plasmid DNA at a cation:anion charge ratio of 2:1, where 15 nmol (as cation charge) of each peptide was used for 2.5 μ g of plasmid DNA, respectively. Each bar represents the mean and standard deviation of three determinations.

R_8 peptide was also determined to be comparable to those for the above peptides. Therefore, the arginine-rich peptides as short as ~10 residues have the ability to transfect the cells in almost the same efficiency as those for polylysine and polyarginine. In another words, a long peptide chain length is not necessarily needed for cationic peptide-mediated transfection. However, the transfection efficiency of these peptides was almost 2 orders of magnitude lower than that of LipofectAMINE.

Effect of N-Terminal Modification of R_8 . To improve the transfection efficiency, efforts have been focused on the compaction of DNA-carrier complexes, with the hope of facilitating the cellular uptake of DNA through endocytosis or pinocytosis. For example, the application of polylysine bearing cysteine cross-linking (19, 20), branched cationic peptides (17), cationic polymers (21), and cationic dendrimers (22) has been reported. On the other hand, Nishikawa et al. successfully introduced hydrophobic cores such as aliphatic and cholesteryl moieties in the hydrophilic polysaccharides to obtain a complex of well-defined particle size (23). It has also been reported that the use of partially stearylated polylysine in the presence of low-density lipoprotein increased the transfection efficiency compared with the usage of polylysine (24). If arginine residues attach to the DNA phosphates, partial introduction of a hydrophobic moiety might result in the aggregation of these hydrophobic moieties to produce a compact plasmid/ R_8 complex. Utilization of amino acid-based cationic lipid for the compaction of DNA has also been reported (25, 26). We thus prepared stearyl-octaarginine (stearyl- R_8) as a model of the 'short' membrane-permeable arginyl peptides and examined the transfection efficiency of R_8 in the presence of stearyl- R_8 (Figure 3). The results were, however, not as intended. We did transfection using mixtures comprising stearyl- R_8 : R_8 molar ratios of 0:100, 1:99, 50:50, and 100:0 (Figure 3). The transfection efficiency of these mixtures increased as the ratios of stearyl- R_8 increased. Maximum efficiency was obtained when 100% of stearyl- R_8 was employed for transfection.

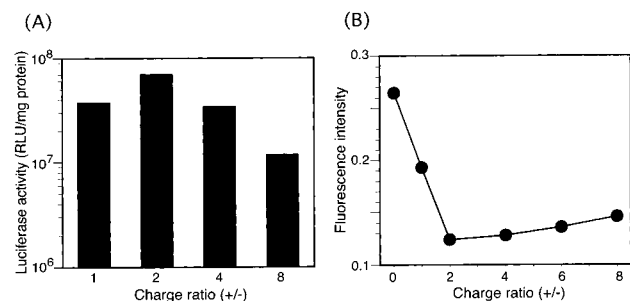


Figure 4. (A) Effect of cation:anion charge ratios on the luciferase activity from COS-7 cells transfected with plasmid DNA (PGV-C2, 2.5 μ g) using stearyl- R_8 . Each bar represents the mean of three determinations. The standard deviation in these cases was so small that the error bars do not appear on the figure. (B) Ethidium bromide (EtBr) displacement assay (17) for stearyl- R_8 . Aliquots of stearyl- R_8 in HEPES-buffered saline (HBS) were added to a complex of plasmid DNA (PGV-C2) and EtBr in HBS. Decreases in fluorescence intensity, representing binding of the peptide to DNA and subsequent dissociation of EtBr, were recorded using excitation and emission at 510 and 595 nm, respectively.

In this case, transfection efficiency was improved by almost 2 orders of magnitude over the unmodified R_8 ; the efficiency became comparable to that of LipofectAMINE. The N-terminal stearylation of the R_8 peptide thus turned out to produce considerable improvement in the transfection efficiency.

The contribution of hydrophobic moieties to transfection efficiency has also been reported in other peptide carriers (27). Therefore, we prepared three more R_8 peptides bearing a hydrophobic moiety. These included lauryl- R_8 , cholesteryl- R_8 , and cholyl- R_8 peptides (Figure 1). Again, stearyl- R_8 gave the best transfection efficiency (Figure 3), followed by lauryl- R_8 and cholesteryl- R_8 . A significant effect on modification was not recognized for cholyl- R_8 .

Effect of Charge Ratio. The above results suggested that stearyl- R_8 is promising as a new type of transfection agent. To establish the peptide/DNA stoichiometry to achieve maximum transfection efficiency, the effect of the charge ratio of stearyl- R_8 and plasmid DNA was next examined. Transfection was carried out using different charge ratios of stearyl- R_8 /DNA mixtures from 1:1 to 8:1. The highest luciferase activity was obtained from the cells treated with a cation:anion charge ratio of 2:1 (Figure 4A). The ethidium bromide displacement assay (17) showed a plateau in the fluorescence intensity at a charge ratio of 2:1 or greater, suggesting that stearyl- R_8 effectively complexed with DNA at a charge ratio of 2:1 and higher (Figure 4B).

Sequence Effect of the Arginyl Peptides. In our preceding paper on the membrane permeability of the arginine-rich peptides, we observed that a subtle difference in the number of arginine residues dramatically influenced the mode of membrane permeability and cellular localization of the peptides (11). A peptide comprising four arginine residues rarely crossed the membrane. In contrast, a highly efficient translocation and accumulation in the nucleus was observed for a peptide comprising six or eight arginine residues. However, as the number of arginine residues in the peptide further increased, membrane permeability again decreased. Internalization and nuclear localization of the peptide bearing 16 arginine residues were not significant; the peptide was observed mostly to stay on the membrane. A similar tendency was observed in the intracellular protein delivery using arginine-rich peptides as

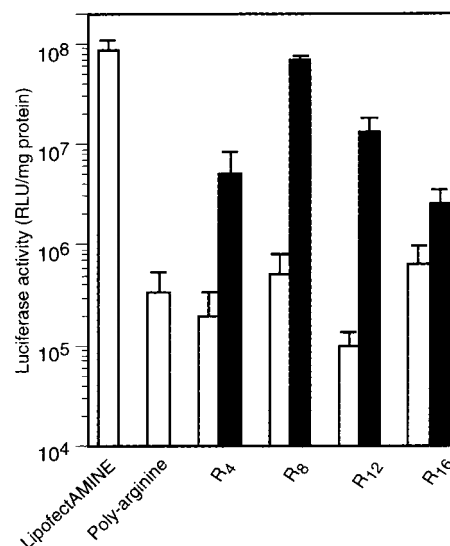


Figure 5. Comparison of transfection efficiency of stearyl-oligoarginine (stearyl- R_n) and oligoarginine (R_n) having a different number of arginine residues ($n = 4, 8, 12, 16$). Peptides (15 nmol as cation charge) were respectively mixed with plasmid DNA (PGV-C2, 2.5 μ g) at a cation:anion charge ratio of 2:1 and used for transfection of COS-7 cells. Open bars indicate the transfection efficiency for R_n peptides. Closed bars indicate that for stearyl- R_n peptides.

a carrier molecule (11). Carbonic anhydrase (MW 29 000) covalently conjugated with a peptide comprising eight arginine residues was quite efficiently delivered into the cells. On the other hand, the protein conjugated with a peptide comprising 16 arginine residues was not able to translocate through the cell membrane as efficiently. Therefore, we examined whether a similar tendency was recognized in the transfection using stearyl-oligoarginine. In this experiment, DNA was simply mixed with stearyl-arginine peptides but not covalently cross-linked. We thus synthesized stearyl-oligoarginine of different chain lengths [stearyl- R_n ($n = 4, 8, 12, 16$)] (Figure 1). For comparison, oligoarginine peptides which lack the stearyl moiety [R_n ($n = 4, 8, 12, 16$)] were also prepared.

Without the stearyl moiety, slight differences were observed in transfection efficiency (Figure 5). However, the efficiency of these peptides was almost of the same order as that for polyarginine. On the other hand, considerable improvement in the transfection efficiency was observed by the N-terminal stearylation, especially in the case of stearyl- R_8 and stearyl- R_{12} . Almost 2 orders of magnitude increase in the transfection efficiency were obtained by stearylation of these peptides. Significant influence of the chain length on translocation efficiency was also observed. Stearyl- R_8 gave the best result in transfection efficiency. The smaller or the larger the number of arginine residues became, the less efficient was the transfection using the peptide. In the case of stearyl- R_{16} , improvement in translocation efficiency was not as obvious as in the case of other stearyl- R_n peptides. These tendencies were quite parallel to those observed in the above-mentioned internalization of the arginine peptides themselves and the peptide-mediated intracellular protein delivery (11). Transfection efficiency of the D-amino acid derivative of stearyl- R_8 (stearyl-D- R_8), where all the arginine residues were D-form, was 2.3×10^8 RLU/mg of protein, almost 3 times that of the L-form, stearyl- R_8 . This result is also parallel to that observed in the quantification of internalized peptides where the (D-Arg) $_8$ peptide internalized more efficiently than the (Arg) $_8$ peptide (12).

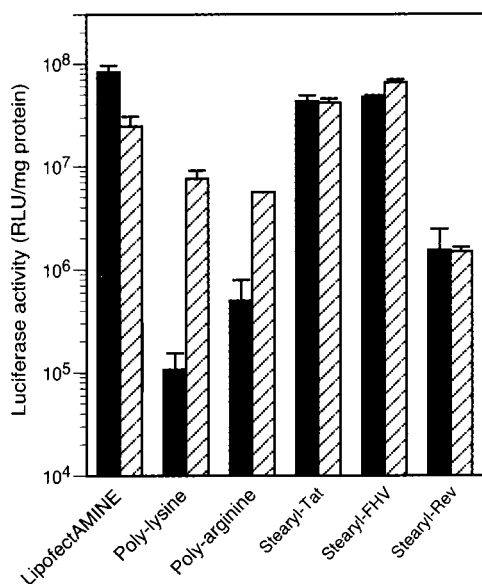


Figure 6. Transfection efficiency of stearylated arginine-rich membrane-permeable peptides and the effect of chloroquine. Peptides were mixed with plasmid DNA at a cation:anion charge ratio of 2:1, where 15 nmol (as cation charge) of each peptide was used for 2.5 μ g of plasmid DNA, respectively. Cells were treated with 50 μ M chloroquine during the transfection. Each bar represents the mean and standard deviation of three determinations. Closed bars indicate the efficiency in the absence of chloroquine. Hatched bars indicate that with chloroquine treatment.

The effect of stearylation on the transfection efficiency was also examined for the HIV-1 Tat-(48–60), HIV-1 Rev-(34–50), and FHV coat-(35–49) peptides (Figure 6). Two orders of magnitude of improvement were also observed by stearylation of the HIV-1 Tat-(48–60) and FHV coat-(35–49) peptides, and a transfection efficiency close to that of LipofectAMINE was achieved. Of interest is the result for the stearyl-Rev peptide. The ability for translocation as well as protein delivery was similarly observed for all three of these peptides. However, little improvement in transfection efficiency by stearylation was observed only for the Rev peptide.

Addition of chloroquine has often been reported to be effective in improving the transfection efficiency using polyarginine and other cationic peptides (13, 27). A considerable increase was actually observed for the transfection efficiency using polylysine and polyarginine in the presence of chloroquine. For transfection using the stearyl-Tat and stearyl-FHV peptides, little improvement with the addition of chloroquine was observed (Figure 6). In the case of stearyl- R_n peptides ($n = 4, 8, 12, 16$), a significant effect of chloroquine on the transfection efficiency was not recognized (Figure 7).

Particle sizes of peptide/DNA complex were estimated using direct light scattering in HBS 3 min after mixing the peptides with DNA and then 30 min later. The latter incubation period is identical with that of the peptide/DNA mixture before the addition to cells (Table 1). Compared to polyarginine and LipofectAMINE, stearyl- R_8 formed a larger complex with DNA. The average particle size of the complex was almost 3 times larger than the lipofectAMINE/DNA complex 3 min after mixing, and 8 times larger after 30 min. The large standard deviation in the particle size also suggested that stearyl- R_8 formed complexes with DNA that easily aggregated into particles with a wide size distribution. While stearyl-Rev also formed large complexes with DNA, the average particle size and the standard deviation after 30 min

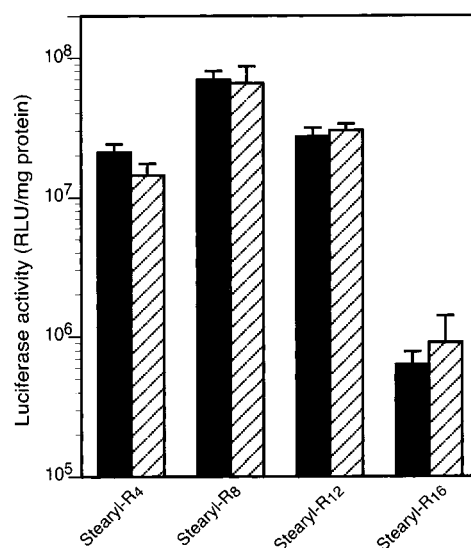


Figure 7. Effect of chloroquine on the transfection efficiency of stearyl- R_n peptides ($n = 4, 8, 12, 16$). Conditions are the same as mentioned in Figure 6. Closed bars indicate the efficiency in the absence of chloroquine (same as in Figure 5). Hatched bars indicate that with chloroquine treatment.

Table 1. Particle Size Determination of Peptide–Plasmid Complexes Using Dynamic Light Scattering^a

	particle size (nm)	
	3 min	30 min
stearyl- R_8	1437 $\pm$ 1273	5148 $\pm$ 3377
stearyl-Rev	2198 $\pm$ 769	3692 $\pm$ 1120
R_8	1667 $\pm$ 537	2917 $\pm$ 738
poly-arginine	317 $\pm$ 43	663 $\pm$ 341
LipofectAMINE	462 $\pm$ 92	752 $\pm$ 398

^a Each peptide was mixed with plasmid DNA (PGV-C2) to give a cation:anion charge ratio of 2:1. The amount of LipofectAMINE mixed with the plasmid was adjusted following the manufacturer's protocol. The particle size of each complex was determined 3 min after mixing and again 30 min later. The mean and standard deviation of three determinants are shown.

were smaller than those for stearyl- R_8 . The effect of R_8 on the compaction of DNA was not so large as that of polyarginine.

Cytotoxicity of the administration was assessed by the MTT assay (18) after incubation of cells with transfection reagents in serum-free medium for 12 h followed by incubation in serum for another 12 h. These incubation periods correspond to that where the peptide/DNA mixture was actually exposed to the cells. The cells were then harvested and subjected to the assay (Figure 8). The cytotoxicity of stearyl- R_8 , polyarginine, and LipofectAMINE in the presence of plasmid DNA was judged to be comparable.

DISCUSSION

In this paper, we have exemplified that membrane-permeable arginine-rich peptides, such as HIV-1 Tat-(48–60), HIV-1 Rev-(34–50), and FHV coat-(35–49), have the ability to transfect COS-7 cells with luciferase-coding plasmid as efficiently as polyarginine (MW 5000–15 000) and polylysine (MW 9800). Not only these virus-derived cationic peptides, but also R_n peptides ($n = 4, 8, 12, 16$) were found to be able to transfect cells. In the case of the Tat, FHV, and R_8 peptides, stearylation on the N-terminus of the peptides dramatically increased the efficiency of transfection to reach the order of

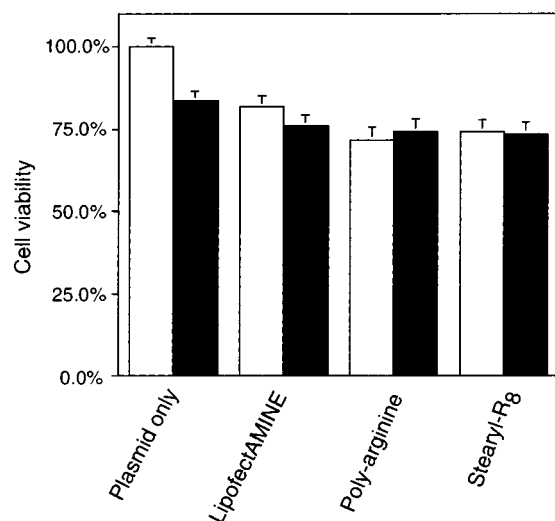


Figure 8. Cytotoxicity of the peptides and those complexed with the plasmid DNA. Cytotoxicity on transfection was evaluated by MTT assay (18). COS-7 cells were placed in a 96-well plate (1×10^4 cells/well) in serum-free DMEM and treated with a peptide or LipofectAMINE in the presence and absence of plasmid DNA (PGV-C2) for 12 h. An equal amount of DMEM containing 20% FBS was then added, and incubation was continued for another 12 h. The medium was removed, and the cells were subjected to MTT assay. Open bars, cell viability in the absence of plasmid DNA; closed bars, cell viability in the presence of plasmid DNA.

magnitude of that of LipofectAMINE, one of the most efficient commercially available transfection agents.

Polyarginine and polylysine of molecular weights more than 5000 have often been used for transfection. Presumably because of the availability of peptides, the transfection efficiency of 'short' arginine-rich peptides of distinctive molecular structure as used in this study has not been as extensively studied as those using cationic polypeptides and polymers. In relationship with the membrane permeability of these 'short' arginine-rich peptides, it is quite interesting that these peptides have the ability to deliver plasmid DNA into cells even without covalent conjugation with the plasmid.

N-terminal stearylation of the R₈ peptide showed a dramatic effect. Improvement of the transfection efficiency by almost 2 orders of magnitude was achieved. The effect of N-terminal modification by lauryl, cholesteryl, and cholyl moieties was studied. Modification by a straight chain lauryl moiety also gave about a 30-times increase in transfection efficiency. The cholyl moiety, which has an amphiphilic structure, contributed little to the improvement of transfection efficiency.

The average particle size of the stearyl-R₈/DNA complex 3 min after their mixing (1437 nm) was significantly larger than for the polyarginine/DNA complex (317 nm) and LipofectAMINE/DNA complex (462 nm). The average particle size of the stearyl-R₈/DNA complex increased faster than the other particles to exceed 5000 nm after 30 min. Because the 30-min incubation period was identical with that before transfection, it could be deduced that stearyl-R₈ forms a considerably larger complex than polyarginine and LipofectAMINE when added to the cells. Considering that stearyl-R₈ was added to plasmid DNA at a charge ratio of 2:1, it is expected that many stearyl moieties are exposed to the surface of the complex. One possible explanation for the high transfection efficiency of the stearyl-R₈/DNA complex could, therefore, be that the hydrophobic moiety contributes to absorbing of the complex on the membranes. A detergent-like effect

could also be expected for stearyl-R₈ to destabilize the membranes to facilitate cellular uptake of the peptide/DNA complex.

One of the mechanisms for chloroquine to improve transfection efficiency has been attributed to destabilization of endosomal particles (13). Though a significant improvement was observed in the transfection efficiency for polyarginine and a slight decrease for LipofectAMINE with the addition of chloroquine, it did not produce a certain effect on the cellular uptake of the stearyl-R_n/DNA complex. The ineffectiveness of chloroquine on the transfection using the stearyl-R₈/DNA complex suggests that stearyl-R₈ would disrupt endosomes by itself. Also, the uptake mechanism of the stearyl-R₈/DNA complex by the cells may not be exactly the same as that of polyarginine and LipofectAMINE.

Dependence on the number of arginine residues for the transfection efficiency of stearyl-R_n peptides ($n = 4, 8, 12, 16$) correlated well with that observed for the internalization of the R_n peptides. Superiority of stearyl-D-R₈ over the L-peptide (stearyl-R₈) in transfection efficiency also coincided with that of the (D-Arg)₈ peptide over the (Arg)₈ peptide in membrane permeability reported by Wender et al. (12). The R₈ peptides showed the highest degree of nuclear localization among the R_n peptides in our previous observations (11). It would be quite interesting if the tendency in nuclear localization of R_n peptides could have something to do with that in transfection efficiency of the corresponding stearyl peptides. The Tat and FHV peptides also accumulate in the nucleus (2, 11), and the corresponding stearyl peptides gave high efficiency of transfection. Inefficiency of stearyl-Rev for transfection may have some relation to the peptide conformation. Among the arginine-rich peptides examined in this study, only Rev peptide takes on a helical structure (11). As a result, stearyl-Rev may form a complex with DNA in a different manner from those in the case of other stearyl peptides.

Taken together, the combination of the stearyl moiety and membrane-permeable basic peptides would provide a novel framework for the development of an efficient transfection system. Among other advantages expected for the stearyl-arginine peptides, low immunogenicity can be pointed out. Arginyl peptides delivered into the cells are expected to be easily degraded by cellular trypsin-like proteases as we observed in the translocation of arginine-rich peptides (11). Also, in accordance with degradation or dissociation of stearyl-arginine peptides in the cell, free plasmid DNA would be released to the cytosol followed by reaching the nucleus. It has been suggested that cationic polymers forming a very tight complex with DNA sometimes face difficulty in unwinding the complex in the cell, causing a reduction of transfection efficiency (17). COS-7 cells express the SV40 large T-antigen. This protein binds the SV40 promoter of the plasmid and may influence intracellular trafficking. However, the effect of the T-antigen on the delivery of the plasmid in the presence of stearyl-peptides is unclear at this stage.

Further study is necessary to understand the precise mechanism of transfection using stearyl-arginine-rich peptides. However, the above data as well as our previous report on peptide translocation (11) indicated that 'short' arginyl peptides are an abundant source of peptides having therapeutic potential. Further refinement of the peptide structure will result in more sophisticated molecules for intracellular gene delivery.

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Synthesis of LJP 993, a Multivalent Conjugate of the N-Terminal Domain of β_2 GPI and Suppression of an Anti- β_2 GPI Immune Response

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LJP 993, a tetravalent conjugate of the amino-terminal domain (domain 1) of β_2 GPI, was synthesized, and studies were carried out to explore the ability of LJP 993 to bind anti- β_2 GPI antibodies and to function as a B cell toleragen. Domain 1 was expressed in *Pichia pastoris*, and the N-terminus was site-specifically modified by a transamination reaction converting the N-terminal glycine to a glyoxyl group. A tetravalent platform was synthesized with linkers that terminate in aminoxy groups. This was accomplished by preparing an ethylene glycol-based heterobifunctional linker that contains both a Boc-protected aminoxy group and a free primary amine. The linker was used to modify a tetravalent platform molecule by reacting the amino groups on the linker with 4-nitrophenyl carbonate esters on the platform to provide a linker-modified platform, and the Boc protecting groups were removed to provide a tetravalent aminoxy platform. Glyoxylated domain 1 was attached to the platform to provide LJP 993 by formation of oxime bonds. The protein domains of LJP 993 retain activity as evidenced by the ability of LJP 993 to bind to anti- β_2 GPI antibodies. Dissociation constants (K_d) for domain 1 and LJP 993 bound to immobilized affinity-purified anti- β_2 GPI antibodies from autoimmune thrombosis patients were determined using surface plasmon resonance. An immunized mouse model was developed to test the ability of LJP 993 to act as a toleragen. A thiol containing domain 1 analogue was expressed in insect cells using the baculovirus expression system, and it was used to prepare an immunogenic conjugate of domain 1 and maleimide-derivatized keyhole limpet hemocyanin (KLH). Mice were immunized with the KLH conjugate, and spleen cells were harvested from the immunized mice. The cells were incubated with various concentrations of LJP 993 and transferred to mice whose immune systems had been compromised by irradiation. The hosts were then boosted with the KLH-domain 1 conjugate, and after 7 days their antibody levels were measured. Host mice receiving cells that were treated with LJP 993 produced significantly lower amounts of anti-domain 1 antibodies than controls which received untreated cells, indicative of B cell tolerance.

INTRODUCTION

Multivalent presentation of a B cell epitope, in the absence of T cell epitopes, can specifically tolerize B cells (1). The B cells become nonresponsive to immunogenic forms of the epitope resulting in an antigen-specific decrease in antibody production (2, 3). Molecules that present B cell epitopes in a multivalent fashion, and that immunospecifically suppress antibody production, are called B cell toleragens; likewise, antigen-specific suppression of antibody production in response to treatment with a toleragen is termed B cell tolerance. An early example of B cell tolerance was the demonstration that 2,4-dinitrophenyl (DNP) groups attached to the D-lysines in polymers consisting of D-amino acids could suppress IgG production in animals that had been immunized with DNP-derivatized proteins (2).

The ability to specifically suppress antibody production provides a basis for a therapeutic approach to the treatment of autoimmune disorders by specifically tolerizing the B cells responsible for producing pathogenic

autoantibodies. B cell tolerance was demonstrated in mice that were immunized with synthetic double-stranded oligonucleotides. Treatment with a tetravalent oligonucleotide conjugate on a poly(ethylene glycol) based platform suppressed anti-oligonucleotide antibody formation (4). Further refinement of that molecule led to LJP 394, which consists of four double-stranded oligonucleotides connected to a defined, nonpolymeric platform. LJP 394 was designed to treat lupus nephritis and has been shown to reduce anti-dsDNA antibodies in human clinical trials (5, 6).

The prospect of using this approach to develop treatments for other autoimmune diseases led us to search for peptide mimotopes of naturally occurring proteinaceous antigens. β_2 -Glycoprotein I (β_2 GPI) has been implicated as the natural antigen of what have been historically referred to as anticardiolipin antibodies (ACA) which are associated with arterial and venous thrombosis, recurrent fetal loss, and thrombocytopenia (7). Anti- β_2 GPI binding peptides were discovered by screening random-peptide phage libraries, and multivalent conjugates of the peptides were prepared. The peptide conjugates could tolerize mice that had been immunized with the peptides resulting in down-regulation of anti-peptide antibodies (8). Although toleragenic,

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peptide conjugates lacked cross-reactivity between patient antibodies. The effectiveness of a peptide was restricted to the specificity of the antibody used to screen the phage libraries. (9).

The discovery that the first domain of β_2 GPI (domain 1) was broadly cross-reactive with patient anti- β_2 GPI antibodies has led to efforts aimed at developing a multivalent construct of this native polypeptide sequence as a toleragen for autoimmune thrombosis (10). We describe a prototypical synthesis of a tetravalent conjugate of domain 1 and experiments that illustrate the potential of using such a conjugate as a therapeutic toleragen for anti- β_2 GPI mediated pathologies. Studies are presented to show that the conjugate binds anti- β_2 -GPI antibodies and acts as a toleragen by suppressing antibody formation in the spleen cells of mice that had been immunized and boosted with a conjugate of domain 1 on KLH.

EXPERIMENTAL PROCEDURES

General. All chemicals were used as purchased unless otherwise specified. *N*-(*tert*-Butyloxycarbonyl)hydroxylamine, 1,2-bis(2-chloroethoxy)ethane, and glyoxylic acid were purchased from Aldrich. Carboxypeptidase A-agarose was purchased from Sigma. Reductacryl beads were purchased from Calbiochem. Nestle Carnation nonfat dry milk was purchased at a supermarket. Optical densities of microtiter plate wells were read using an EL310 Microplate autoreader (Bio-Tek Industries, Inc.). Maleimidyl-activated KLH was purchased from Pierce Chemical Company (cat. no. 77105). *Bordetella pertussis* organisms were obtained from the Massachusetts Department of Public Health.

Silica gel chromatography was performed on silica gel (230–400 mesh ASTM) purchased from Baxter. TLC was performed on silica gel TLC plates (5554) manufactured by EM Separations. Phosphate buffered saline (PBS) was prepared by dissolving 175 g of NaCl, 6.5 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, and 40.9 g of $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ in H_2O and diluting to a final volume of 20 L. Tris-buffered saline (TBS) was prepared by dissolving 219.9 g of NaCl and 151.4 g of tris(hydroxymethyl)aminomethane in H_2O , adjusting the pH to 8.0, at a final volume of 25 L, with HCl. Melting points are reported uncorrected. NMR spectra were recorded on a Bruker AC-300 spectrometer with broad band probe. Elemental analyses were performed by Desert Analytics of Tucson, Arizona. Mass spectra were recorded on a Finnigan LCQ electrospray mass spectrometer or obtained from the Mass Spectrometry Lab at the Scripps Research Institute, San Diego, CA. High-resolution MALDI mass spectra and high-resolution FAB mass spectra were obtained from the Mass Spectrometry Lab at the Scripps Research Institute, San Diego, CA.

Protein concentrations were determined by UV absorbance at 280 nm using a molar extinction coefficient of $9531 \text{ L mol}^{-1} \text{ cm}^{-1}$ ($1.35 \text{ mL mg}^{-1} \text{ cm}^{-1}$) for the domain 1 polypeptide.

Surface plasmon resonance experiments were performed on a Biacore 2000 using a CM5 chip, *N*-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC), and HBS-EP buffer [0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% (v/v) surfactant P20], all obtained from Biacore (Piscataway, NJ). Pooled normal human IgG was obtained from Zymed (South San Francisco, CA).

1,2-Bis(2-iodoethoxy)ethane, 1. This compound was prepared in a manner similar to that previously described (11). A solution of 10.0 g (5.3 mmol) of 1,2-bis(2-chloro-

ethoxy)ethane and 16.0 g (107 mmol) of sodium iodide in 110 mL of acetone was heated at reflux for 18 h. The mixture was concentrated, and the residue was triturated with CHCl_3 , which dissolved the product. The undissolved salts were removed by filtration, and the filtrate was concentrated to give an orange oil. Purification by silica gel chromatography (step gradient, 10/90 EtOAc/hexanes to 15/85 EtOAc/hexanes) provided 17.8 g (90%) of compound **1** as an orange oil: ^1H NMR (CDCl_3) δ 3.28 (t, 4H), 3.67 (s, 4H), 3.78 (t, 4H); ^{13}C NMR (CDCl_3) δ 3.6, 70.5, 72.2.

2-[2-(2-*N*-(*tert*-Butyloxycarbonyl)aminoxyethoxy)-ethoxy]ethyl iodide, 2. DBU (284 μL , 290 mg, 1.90 mmol) was added to a suspension of 266 mg (2.0 mmol) of *N*-(*tert*-butyloxycarbonyl)hydroxylamine in 2.96 g (8.0 mmol) of compound **1**, and the mixture was stirred to obtain a viscous homogeneous solution, which solidified within 15 min. The mixture was allowed to stand for an additional 45 min, and it was dissolved in 5 mL of CH_2Cl_2 . The resulting solution was added to 50 mL of EtOAc resulting in a white precipitate that was removed by filtration. The filtrate was concentrated to a viscous oil that was partitioned between 100 mL of EtOAc and $3 \times 50 \text{ mL}$ of 1 N HCl solution. The EtOAc layer was washed with 5% sodium bisulfite solution and concentrated to provide 2.6 g of yellow oil. Purification by silica gel chromatography (step gradient, 20/80 to 45/55 EtOAc/hexanes) gave 515 mg (69%) of compound **2** as a yellow oil: ^1H NMR (CDCl_3) δ 1.50 (s, 9H), 3.28 (t, 2H), 3.68 (s, 4H), 3.72 (m, 4H), 4.02 (t, 2H), 7.72 (s, 1H); ^{13}C NMR (CDCl_3) δ 2.9, 28.3, 68.9, 69.4, 70.2, 70.6, 72.0, 75.4, 81.6, 156.9; HRMS (MALDI) calculated for $\text{C}_{11}\text{H}_{22}\text{INaO}_5$ ($\text{M}+\text{Na}$): 398.0435. Found: 398.0435.

2-[2-(2-*N*-(*tert*-Butyloxycarbonyl)aminoxyethoxy)-ethoxy]ethyl azide, 3. To a solution of 258 mg (0.69 mmol) of compound **2** in 5 mL of DMF under nitrogen atmosphere was added 358 mg (5.50 mmol) of sodium azide. The mixture was stirred for 18 h at room temperature, 100 mL of water was added, and the mixture was extracted with $3 \times 50 \text{ mL}$ of EtOAc. The EtOAc layers were combined and washed with 50 mL of water, dried (Na_2SO_4), filtered, and concentrated to provide 294 mg of a colorless oil. Purification by silica gel chromatography (30/70 acetone/hexanes) provided 187 mg (93%) of compound **3** as a colorless oil: ^1H NMR (CDCl_3) δ 1.47 (s, 9H), 3.41 (t, 2H), 3.69 (brd s, 4H), 3.73 (m, 4H), 4.03 (t, 2H), 7.50 (s, 1H); ^{13}C NMR (CDCl_3) δ 28.1, 50.5, 69.1, 70.1, 70.4 (two signals), 75.2, 81.3, 156.7; HRMS (MALDI) calculated for $\text{C}_{11}\text{H}_{22}\text{NaN}_4\text{O}_5$ ($\text{M}+\text{Na}$): 313.1482. Found: 313.1496.

2-[2-(2-*N*-(*tert*-Butyloxycarbonyl)aminoxyethoxy)-ethoxy]ethylamine, 4. Compound **3** (1.36 g, 4.70 mmol) and triphenylphosphine (1.48 g, 5.64 mmol) were dissolved in 24 mL of THF and 8 mL of H_2O , and the resulting solution was stirred at room temperature for 2 h. Approximately 160 μL (eight drops) of 1 N NaOH was added, and the mixture was stirred for 18 h. The mixture was concentrated under vacuum, and the concentrate was purified by silica gel chromatography (80/8/2 $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{con NH}_4\text{OH}$) to give 1.16 g (94%) of **4** as a yellow oil: ^1H NMR (CDCl_3) δ 1.50 (s, 9H), 1.90 (brd, 2H), 2.88 (t, 2H), 3.56 (t, 2H), 3.65 (m, 4H), 3.71 (m, 2H), 4.01 (m, 2H); HRMS (MALDI) calculated for $\text{C}_{11}\text{H}_{25}\text{N}_2\text{O}_5$ ($\text{M}+\text{H}$): 265.1758. Found: 265.1758.

Boc-protected aminoxy platform, 6. Triethylamine (157 μL , 114 mg, 1.13 mmol) was added to a stirred solution of 193 mg (0.188 mmol) of compound **5** (obtained as described in ref 12) in 1 mL of CH_2Cl_2 . The mixture was stirred at 0°C , and a solution of 298 mg (1.13

mmol) of compound **4** in 2 mL of CH_2Cl_2 was added. The mixture was allowed to come to room temperature and was stirred overnight. The mixture was cooled to 0°C , acidified with 1 N HCl, and partitioned between 20 mL of 1 N HCl and 4×20 mL of CH_2Cl_2 . The combined organic layers were washed with saturated NaHCO_3 solution, dried (MgSO_4), filtered, and concentrated to give 279 mg of yellow oil. Purification by silica gel chromatography (97/3 $\text{CH}_2\text{Cl}_2/\text{MeOH}$) provided 210 mg (73%) of **6** as an oil: ^1H NMR (CDCl_3) δ 1.49 (s, 36H), 3.35 (m, 8H), 3.46–3.78 (m, 44H), 4.04 (t, 8H), 4.21 (m, 12H), 5.80 (m, 4H), 7.91 (s, 4H); ^{13}C NMR (CDCl_3) δ 28.4, 41.0, 47.7, 48.2, 63.0, 64.8, 69.3, 69.5, 70.2, 70.3, 70.6, 75.4, 81.7, 156.0, 156.6, 157.1; mass spectrum (ESI) m/z calculated for $\text{C}_{62}\text{H}_{117}\text{N}_{10}\text{O}_{33}(\text{M}+\text{H})^+$: 1528.8. Found: 1528.5; HRMS (FAB) calculated for $\text{C}_{62}\text{C}_s\text{H}_{116}\text{N}_{10}\text{O}_{33}(\text{M}+\text{Cs})^+$: 1661.6761. Found: 1661.6672.

Aminoxy Dissolved in 10 mL of 1/9 trifluoroacetic acid/ CH_2Cl_2 , and the mixture was kept at room temperature for 3 h. A gentle stream of nitrogen was used to evaporate the solvent, and the residue was dissolved in a minimal amount of chromatography solvent (5/7.5/87.5 concentrated $\text{NH}_4\text{OH}/\text{H}_2\text{O}/\text{CH}_3\text{CN}$), which was used to load the mixture onto a silica gel column. Purification by silica gel chromatography (step gradient, 5/7.5/87.5 to 5/10/85 conc $\text{NH}_4\text{OH}/\text{H}_2\text{O}/\text{CH}_3\text{CN}$) provided 36 mg (82%) of **7 as a colorless oil: ^1H NMR (CDCl_3) δ 3.37 (m, 8H), 3.58 (m, 16H), 3.67 (s, 16H), 3.71 (m, 12H), 3.86 (m, 8H), 4.17–4.29 (m, 12H), 4.93 (brd, 8H), 5.91 (m, 4H); ^{13}C NMR (CDCl_3) δ 40.9, 47.7, 48.2, 62.9, 64.7, 69.4, 69.6, 70.2, 70.3, 70.5, 74.8, 156.1, 156.6; mass spectrum (ESI) m/z calculated for $\text{C}_{42}\text{H}_{85}\text{N}_{10}\text{O}_{25}(\text{M}+\text{H})^+$: 1129. Found: 1129; HRMS (MALDI) calculated for $\text{C}_{42}\text{H}_{85}\text{N}_{10}\text{O}_{25}(\text{M}+\text{H})^+$: 1129.5682. Found: 1129.5669.**

For the purpose of checking the purity of compound **7** by analytical HPLC, the tetraacetone oxime derivative was prepared as follows. Compound **7** (0.38 mg, 0.34 μmol) was dissolved in 240 μL of 0.1 M NaOAc pH 4.6 buffer in an HPLC sample vial. To the solution was added 10 μL of a solution of 49 μL of acetone in 2.0 mL of 0.1 M NaOAc buffer. The mixture was allowed to stand for 1 h, and an aliquot was analyzed by HPLC (4.6 mm $\times$ 250 mm C_{18} column, 1 mL/min, 210 nm detection, gradient, 10–60% B over 20 min, A = 0.1% TFA/ H_2O , B = 0.1% TFA/ CH_3CN , t_R = 19 min); mass spectrum of collected eluent (ESI) m/z calculated for $\text{C}_{54}\text{H}_{101}\text{N}_{10}\text{O}_{25}(\text{M}+\text{H})^+$: 1289. Found: 1289.

Recombinant Synthesis of the First Domain of $\beta_2\text{GPI}$, **9. Expression of Domain 1 Polypeptide with Histidine Expression Tag, **8**, in Insect Cells.** Compound **8** was prepared using the baculovirus expression vector system and isolated using nickel affinity chromatography as previously described (10).

Preparation of Compound **9 by Digestive Removal of Expression Tag.** Compound **8** was dialyzed against 1/9 glycerol/TBS to provide a solution of the polypeptide at a concentration of 2 mg/mL. A 16 mL aliquot of the solution (32 mg, 4.5 μmol) of compound **8** was added to 15 mL of carboxypeptidase A derivatized agarose beads (Sigma) that had been washed with six 30 mL portions of TBS. The mixture was incubated at 37°C for 2 days at which time the digestion was complete as evidenced by analytical mass spectrometry (ESI, conversion of m/z 8043 to 7060). The supernatant was recovered using TBS to wash the beads. The washes were combined to provide a solution of approximately 28 mg of **9** in 73 mL of TBS. Purification was accomplished by preparative HPLC (10 mm diphenyl column (Vydac); gradient 25% B to 40% B

over 40 min, 3 mL/min, A = 0.1% TFA/ H_2O , B = 0.1% TFA/ CH_3CN) to provide 23 mg (72%) of **9**.

Expression of Compound **9 in Yeast.** Compound **9** was expressed as a secreted product in *Pichia pastoris*. (13) Standard molecular biological techniques were used to create a fusion of domain 1 with the yeast alpha factor signal peptide in the vector pPICZalpha (Invitrogen Corporation, Carlsbad, CA). *Pichia* transformants were screened for secreted product using dot blots with an antibody to domain 1. Fermentation of selected clones yielded recombinant domain 1 that was correctly cleaved from the signal peptide as confirmed by mass spectrometry. The protein was isolated from the supernatant using a strong cation-exchange resin. Approximately 6 L of supernatant at pH 3 was loaded onto a 2-in column packed with strong cation-exchange resin (Toso Haas, Toyopearl SP-650M) using 0.2 M NaCl adjusted to pH 3 prepared by addition of HCl. The column was eluted with a step gradient (0.2 M NaCl, pH 3 to 0.4 M NaCl, pH 3) to provide approximately 400 mg of protein. Fractions containing the domain 1 polypeptide were pooled and purified by reversed phase HPLC (2-in C_4 column (Vydac); gradient 25% B to 40% B over 1 h, 50 mL/min, A = 0.1% TFA/ H_2O , B = 0.1% TFA/ CH_3CN) to provide 262 mg of compound **9**. An additional 109 mg of protein was isolated in which the methionine was oxidized to a sulfoxide. An additional 99 mg of partially purified compound **9** was isolated. Compound **9** was characterized using mass spectroscopy: mass spectrum (ESI, average m/z) calculated for $\text{C}_{320}\text{H}_{496}\text{N}_{82}\text{O}_{88}\text{S}_5$ 7060. Found: 7060.

Transamination of Domain 1; Synthesis of Compound **10.** Water and sodium acetate buffer were sparged with helium before use. Compound **9** (10.55 mg, 1.49 μmol) was dissolved in 0.5 mL of H_2O in a polypropylene tube, and 4.0 mL of 2 M pH 5.5 NaOAc buffer was added. A solution of 3.73 mg (14.9 μmol) of CuSO_4 in 0.5 mL of H_2O was added to the mixture, followed by a solution of 2.75 mg (29.9 μmol) of glyoxylic acid in 0.5 mL of 2 M pH 5.5 NaOAc buffer. The mixture was kept under nitrogen atmosphere and agitated gently for 18 h at which time the reaction appeared complete by analytical HPLC using a 4.6 $\times$ 250 mm, 300 $\text{\AA}$, 5 μm , diphenyl column (Vydac) with detection at 280 nm (1 mL/min; gradient 25–45% B, 0–20 min, A = 0.1% TFA/ H_2O , B = 0.1% TFA/ CH_3CN). Approximate retention times are as follows: domain 1, 13.2 min; transaminated domain 1, 13.7 min; oxidized transaminated domain 1, 13.4 min). The mixture was diluted to a volume of 20 mL with H_2O , filtered, and purified by HPLC (22.4 $\times$ 250 mm, 300 $\text{\AA}$, 10 μm , diphenyl column (Vydac) (12 mL/min; gradient 25%–40% B, 0–40 min, A = 0.1% TFA/ H_2O , B = 0.1% TFA/ CH_3CN). Fractions containing pure transaminated domain 1 (compound **10**), as evidenced by analytical HPLC, were pooled and lyophilized to provide 5.0 mg (48%) of compound **10**: mass spectrum (ESI, average m/z) calculated for $\text{C}_{320}\text{H}_{493}\text{N}_{81}\text{O}_{89}\text{S}_5$: 7059. Found: 7041 (loss of H_2O).

Synthesis of Tetravalent Domain 1 Conjugate, LJP 993. Compound **10** (5.20 mg, 7.37×10^{-7} mol) was dissolved in 2.0 mL of He sparged 0.1 M sodium acetate pH 4.60 buffer in a polypropylene tube. To the mixture was added 15.07 μL (139 μg , 1.23×10^{-7} mol) of a 8.147 $\mu\text{mol}/\text{mL}$ solution of compound **7** in 0.1 M sodium acetate pH 4.60 buffer. The mixture was agitated gently under nitrogen for 23 h at which time the reaction appeared to be complete by analytical HPLC using a 4.6 mm $\times$ 250 mm, 300 $\text{\AA}$, 5 μm , diphenyl column (Vydac) with detection at 280 nm (1 mL/min; gradient 25–45% B, 0–20 min, A = 0.1% TFA/ H_2O , B = 0.1% TFA/ CH_3CN). Approximate

retention times are as follows: compound **10**, 13.7 min; LJP 993, 17.2 min). The mixture was diluted with water to a volume of 5 mL and purified by HPLC (10 $\times$ 250 mm, 300 Å, 5 μ m, diphenyl column (Vydac) (3 mL/min; gradient 25–45% B, 0–40 min, A = 0.1% TFA/H₂O, B = 0.1% TFA/CH₃CN). Fractions containing pure LJP 993, as evidenced by analytical HPLC, were pooled and lyophilized to provide 1.73 mg (48%) of LJP 993: mass spectrum (ESI, average *m/z*) calculated for C₁₃₂₂H₂₀₄₈N₃₃₄O₃₇₇S₂₀: 29 294. Found: 29 294.

Domain 1 Polypeptide Analogue with C-Terminal Cysteine-Glutathione Disulfide, Compound 11. Compound **11** was prepared recombinantly using the baculovirus expression vector system and isolated using nickel affinity chromatography in a manner essentially similar to that previously described for domain 1 with a histidine expression tag (10). The purified fractions were pooled and dialyzed against pH 6.8 phosphate buffered saline containing 2 mM EDTA (PBS/EDTA/6.8) to provide approximately 100 mL of a solution of compound **11** at a concentration of 1 mg/mL: mass spectrum (ESI, average *m/z*) calculated for C₃₇₉H₅₇₈N₁₀₄O₁₀₃S₇: 8463. Found: 8463.

Reduction of Compound 11 to Provide Compound 12. The solution of compound **11** from the previous step was sparged with helium, and 1.2 g of Reductacryl beads were washed with helium-sparged PBS/EDTA/6.8 until the wash solutions tested negative for sulphydryl groups using Ellman's reagent (14). The freshly washed Reductacryl beads were added to the solution, and the mixture was gently mixed for 1 h and centrifuged at 150 g. The supernatant was collected and dialyzed against PBS/EDTA/6.8 at 4°C for 18 h using a 1000 MW cutoff membrane to provide a solution of compound **12**: mass spectrum (ESI, average *m/z*) calculated for C₃₆₉H₅₆₃N₁₀₁O₉₇S₆: 8158. Found: 8158.

Preparation of Domain 1 Immunogen, Compound 14. To the solution of compound **12** was added 12.9 mL of a 10 mg/mL solution of maleimidyl-activated KLH, compound **13**, and the mixture was mixed by rotation for 2 h at room temperature. The mixture was dialyzed against cold PBS at 4°C using a 25 000 MW cutoff membrane to remove unconjugated polypeptide. The resulting solution contained approximately 130 mg of protein at a concentration of approximately 0.8 mg/mL. An aliquot of the dialyzed sample was removed and shown to contain immunoreactive domain 1 as evidenced by binding antiphospholipid antibodies in an ELISA using patient-derived affinity purified antiphospholipid antibodies. A solution of unreacted maleimidyl-activated KLH, used as a negative control, did not bind antiphospholipid antibodies.

Determination *K_d* for Compounds 9 and LJP 993. Anti- β_2 GPI antibodies 6501 and 6626 were obtained from autoimmune thrombosis patients and affinity-purified using cardiolipin-containing multilamellar dispersions with bound β_2 GPI (15). Dissociation constants (*K_d*) were measured using surface plasmon resonance at 25°C with a flow rate of 10 μ L/min. Chip equilibration and binding studies were done with degassed HBS-EP buffer. Covalent coupling of antibodies to the CM5 chip was accomplished as follows. The carboxyl groups on the chip were activated by flowing 40 μ L of a solution of 0.05 M NHS and 0.2 M EDC in water over the chip. Affinity purified antibodies and normal IgG, used as a negative control, were immobilized by flowing 100 μ L of a 100 μ g/mL solution of the antibody in 10 mM acetate, pH 4.8, over the activated chip. Excess reactive groups on the

Table 1. *K_d* Values for Compound 9 and LJP 993 Binding to Surface Plasmon Resonance Sensor Chips Coated with Antibodies from Three Patients^a

	patient 6501	patient 6626	patient 6701
9	533 nM	456 nM, 363 nM	571 nM, 534 nM
LJP 993	73 nM, 96 nM	98 nM, 123 nM	73 nM

^a Concentrations determined by absorbance at 280 nm using a molar extinction coefficient of 9531 L mol⁻¹ cm⁻¹ (1.35 mL mg⁻¹ cm⁻¹) for the domain 1 polypeptide (20).

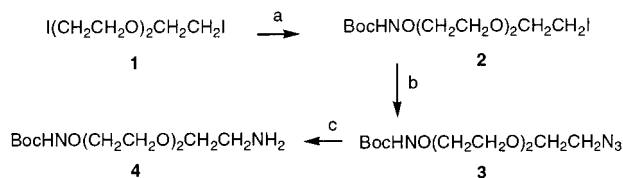
chip surface were quenched with 40 μ L of 1 M ethanolamine, pH 8.5.

Solutions of **9** and LJP 993 at concentrations of 3.73, 1.24, 0.42, 0.14, 0.05, and 0.00 μ M in HBS-EP buffer were flowed over the chip, and response values were collected for 780 s. The chips were regenerated between sample exposures with 80 μ L of 0.1 M glycine-HCl (pH 2.1), 0.1 M NaCl. Since the approach to binding equilibrium was incomplete during the measurement period, the equilibrium binding value (*R_{eq}*) was determined by fitting the association curves to the equation [*R_t* = *R_{eq}*(1 - e^{-*k_s*(*t* - *t*₀)}) + *R*₀] using the manufacturer software (BiaEvaluation version 2.2, Uppsala, Sweden) where *R_t* is the measured response at time *t*, *R_{eq}* is the equilibrium plateau response, *t* is time, *t*₀ is initial time, *k_s* is an apparent association constant (*k_s* = *k_a**C* + *k_{dis}*, where *k_a* is the association constant, *C* is the analyte concentration, and *k_{dis}* is the dissociation constant), and *R*₀ is a response offset (Marquart-Levenberg algorithm).

Each titration association curve was corrected by subtracting the signal from a negative control (normal IgG) flow cell. The calculated *R_{eq}* were plotted versus concentration, and molar *K_d* values were calculated by fitting the data to a one site binding equation using GraphPad Prism software version 2.01. The values are reported in Table 1.

Ex Vivo Tolerance in Mice Immunized with Compound 14. Female C57Bl/6 mice 6–8 weeks of age (Jackson Laboratories, Bar Harbor, ME) were immunized with compound **14**, a conjugate of domain 1 polypeptide covalently attached to KLH. Five mice were used for each dosing group. The immunizations were accomplished by intraperitoneal (IP) injection of 0.5 mL of a solution containing 10 μ g of compound **14** precipitated on 2 mg of alum and 2 $\times$ 10⁹ *B. pertussis* organisms as an adjuvant (16). Three weeks after immunization, spleens were harvested and made into a single cell suspension. The cells were incubated in complete RPMI-1640 medium (Gibco/BRL) at 37°C for 1 h with the indicated concentrations of LJP 993. The cells were washed three times and suspended at a concentration of approximately 1 spleen per 1.5 mL with Hanks' balanced salt solution (Gibco). The washed cells from one-third of a spleen (0.5 mL) were transferred IP into irradiated (650R, ⁶⁰Co) syngeneic recipient mice (C57Bl/6). One day later the recipient mice were boosted with an IP injection of a solution of 10 μ g of conjugate **14** in 0.5 mL of PBS. Seven days after boosting, the mice were bled, and their sera were analyzed for anti-KLH antibodies and anti- β_2 GPI antibodies.

ELISA Assay for Measurement of Anti-KLH Antibodies. The amount of anti-KLH antibodies was determined relative to a standard pool of plasma. Each test sample was run in duplicate at three dilutions. The standard pool was obtained from mice that had been primed and boosted with compound **14**, as described for the ex vivo tolerance experiments. Standard curves were generated by testing eight dilutions of the standard pool in duplicate. Wells of 96-well Falcon plates (Becton

Scheme 1. Synthesis of Linker, 4

(a) Boc-NHOH, DBU, CH_2Cl_2 ; (b) NaN_3 , DMF; (c) triphenylphosphine, THF, H_2O .

Dickinson and Co., NJ) were coated with KLH (Pacific Biomarine) by adding 100 μL of a 4 $\mu\text{g}/\text{mL}$ solution of KLH in 0.1 M sodium carbonate buffer pH 9.5. The plates were incubated at 4 $^\circ\text{C}$, overnight, and washed three times with PBS. The wells were then treated with 0.5% bovine serum albumin (BSA) in PBS for 1 h at room temperature. The plates were again washed three times with PBS and treated with 100 μL of serum sample in 0.5% BSA-PBS for 1 h at 37 $^\circ\text{C}$. The plates were again washed three times with PBS, and all wells were treated with 100 μL of alkaline phosphatase conjugated rabbit-anti-mouse IgG (gamma chain specific) (AP-anti-MIgG, # 61-022, Zymed) which had been diluted 1/1000, for 1 h at 37 $^\circ\text{C}$. The plates were washed three times with PBS, treated with chromogenic substrate, and incubated for 30 min at room temperature.

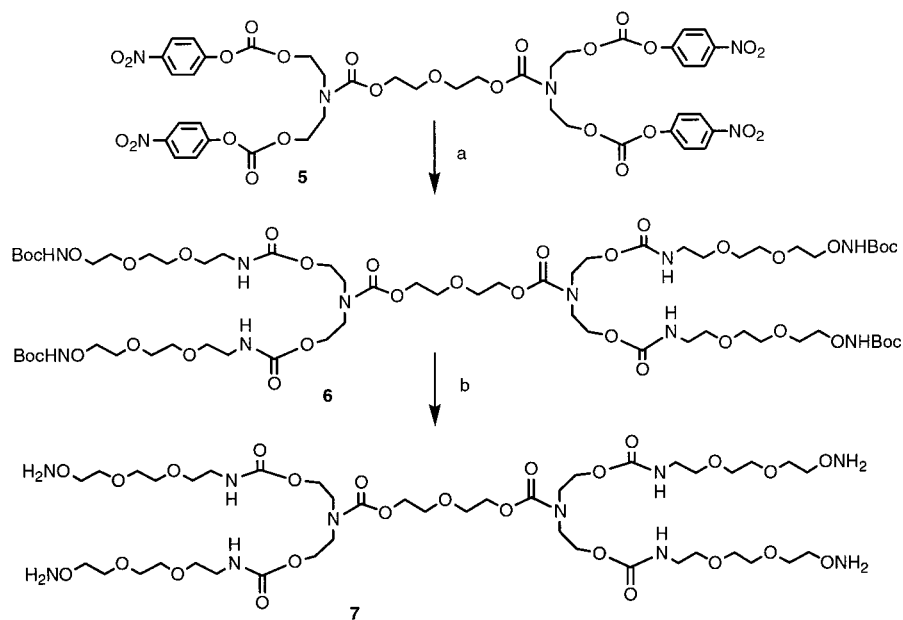
ELISA Assay for Measurement of anti- $\beta_2\text{GPI}$ Antibodies. The amount of anti- $\beta_2\text{GPI}$ antibodies was determined relative to the same standard pool of plasma used to determine the amount of anti-KLH antibodies. Each test sample was run in duplicate at three dilutions. Standard curves were generated by testing eight dilutions of the standard pool in duplicate. Wells of 96-well Maxisorp plates (Nunc-Nalgene) were coated by treating overnight with 50 μL of a 5 $\mu\text{g}/\text{mL}$ solution of $\beta_2\text{GPI}(\text{His})_6$ (10) in pH 9.5 0.1 M sodium carbonate. The wells were washed with PBS and then blocked for 1 h with reconstituted 2% nonfat dry milk (NFDm). The wells were washed with PBS and treated with 50 μL of serial dilutions of individual mouse serum, in 2% NFDm, for 1 h at room temperature. The wells were washed with PBS

and treated for 1 h at room temperature with 50 μL of alkaline phosphatase conjugated rabbit-anti-mouse IgG (gamma chain specific) (AP-anti-MIgG, # 61-022, Zymed) which had been diluted 1/1000, for 1 h at 37 $^\circ\text{C}$. The plates were washed three times with PBS, treated with chromogenic substrate, and incubated for 30 min at room temperature.

RESULTS

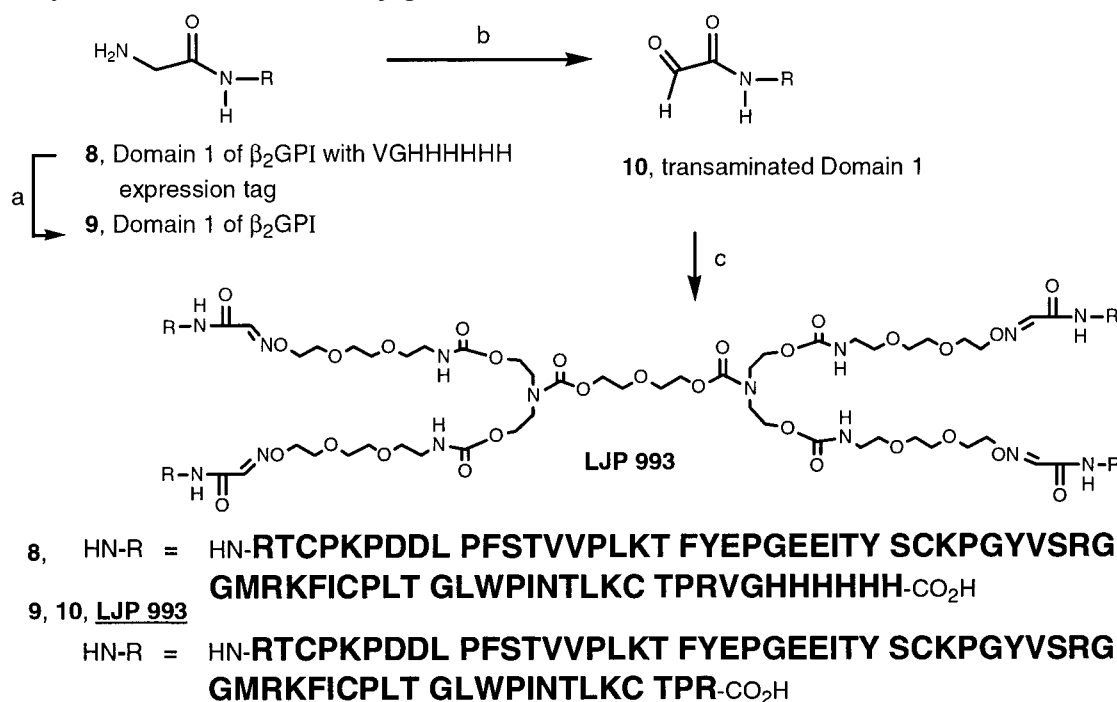
A tetravalent aminoxy platform was developed to which glyoxylated domain 1 could be attached. The approach taken is described as follows. Heterobifunctional linker 4 was prepared as described in Scheme 1. Thus 1,2-bis(2-iodoethoxy)ethane, compound 1 (obtained as described in ref11), was treated with 0.25 equiv of *N*-(*tert*-butoxycarbonyl)hydroxylamine and DBU to provide compound 2 (17). Compound 2 was converted to azide 3 by treatment with sodium azide in DMF. The azide of compound 3 was subsequently reduced with triphenylphosphine in THF/water to provide the amine-containing linker, compound 4.

The linkers were attached to a tetrameric platform intermediate to provide the tetrameric platform, compound 7, as described in Scheme 2. Compound 5 was obtained using a one-pot two-step procedure that is a convenient approach to multivalent platforms comprised of branched carbamates (12). Compound 5 has four 4-nitrophenyl carbonate esters that react with compound 4 in the presence of triethylamine to provide the tetra-carbamate ester, 6. Removal of the Boc protective groups from compound 6 was accomplished using 10% trifluoroacetic acid in CH_2Cl_2 to yield the tetra-aminoxy platform 7. Purification of 7 was accomplished using silica gel chromatography under basic conditions. Under acidic conditions, compound 7 is particularly reactive with trace aldehydes and ketones; however, at alkaline pH compound 7 is reasonably stable and can be stored in solution (1/1 100 mM pH 8.0 tris acetate/acetonitrile) for extended periods of time. For analytical purposes, it was convenient to derivatize compound 7 as an acetone oxime which was amenable to HPLC analysis.

Scheme 2. Synthesis of Platform, 7

a) Compound 4, Et_3N , CH_2Cl_2 ; b) 1/9 trifluoroacetic acid/ CH_2Cl_2 .

Scheme 3. Synthesis of Tetravalent Conjugate, LJP 993



(a) carboxypeptidase A; (b) pH 5.5, 2 M NaOAc, CuSO₄, glyoxylic acid; (c) compound 7, pH 4.6, 100mM NaOAc.

A tetravalent conjugate of domain 1, LJP 993, was prepared in a two-step process involving site-specific introduction of an aldehyde functionality at the N-terminus of compound 9 followed by oxime formation with the tetravalent aminooxy platform, compound 7, as described in Scheme 3. Recombinant human domain 1, compound 9, was produced in two different expression systems. Initially, it was produced in insect cells using the baculovirus expression system using a histidine affinity tag at the carboxy-terminus which is comprised of the additional amino acid sequence VGHHHHHHH at the carboxy terminus (10). The protein was isolated by affinity chromatography on a nickel column providing compound 8. The expression tag was removed by digestion with an immobilized form of carboxypeptidase A, which cannot digest the arginine, providing the domain 1 polypeptide 9. Compound 9 could also be expressed as a secreted product in *P. pastoris* using standard techniques (13, 18). Purification of compound 9 produced from *Pichia* was accomplished using cation exchange chromatography. Compound 9 produced by either method could be further purified using reversed phase preparative HPLC.

The N-terminal glycine of domain 1 was converted to a glyoxyl group by treatment of the polypeptide with 10 equiv of CuSO₄ and 20 equiv of glyoxylic acid in 2.0 M sodium acetate buffer at pH 5.5. Domain 1 contains one methionine which was prone to oxidation. It was important to exclude oxygen in the reaction to avoid converting the methionine thioether to a sulfoxide. Methionine oxidation could be minimized by using He-sparged buffer and performing additions of reagents under a nitrogen atmosphere, however, even with those precautions some oxidation always took place. Purification of the transaminated polypeptide, compound 10, was accomplished by reverse phase preparative HPLC.

LJP 993 was prepared by treating compound 7 with an excess of compound 10 in 0.1 M sodium acetate buffer at pH 4.6. The reaction appeared to be complete after approximately 1 day as evidenced by analytical HPLC.

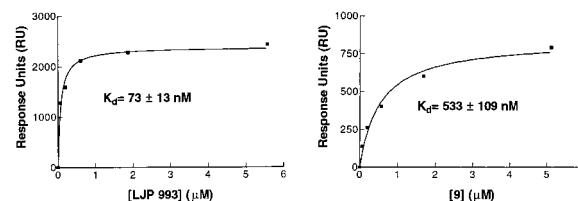
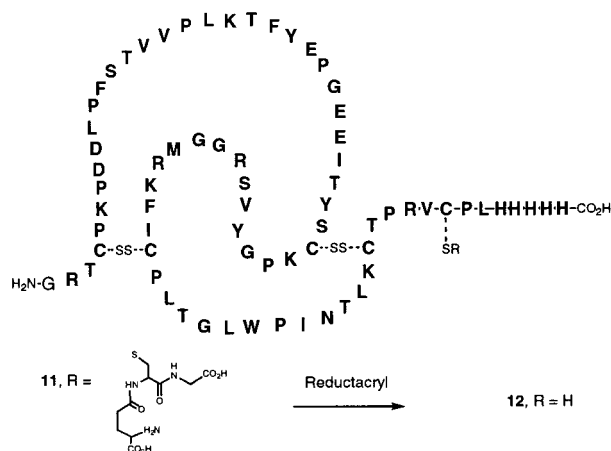
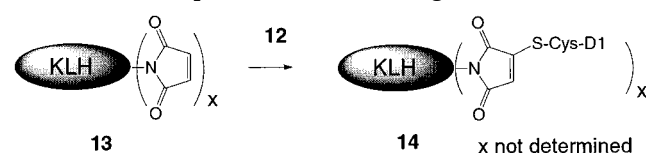


Figure 1. Surface plasmon resonance response plots for LJP 993 and 9 flowing over patient 6501 antibody covalently bound to sensor chip.

Purification was accomplished by preparative reverse phase HPLC.

The ability of LJP 993 and domain 1 to bind to affinity purified antibody from three patients with elevated anticardiolipin antibodies (anti- β_2 GPI) was measured using surface plasmon resonance (Biacore). Affinity purified anti- β_2 GPI antibodies from three autoimmune thrombosis patients were immobilized on sensor chips following the manufacturer's protocol. Solutions of compound 9 or LJP 993 were passed over the sensor surface, and responses were measured. No loss of antibody binding activity was observed in the sensorgram for compound LJP 993 as compared with compound 9; rather, an apparent increase in affinity was observed which roughly corresponds to the 4-fold increase in valence. Table 1 presents dissociation constants (K_d) which were calculated from the equilibrium response plateaus (Req) (15, 19). Typical response plots are described in Figure 1 for the binding of 9 and LJP 993 to the patient antibody 6501 bound to a sensor chip.

The ability of LJP 993 to tolerize B cells was tested in mice immunized with a conjugate of domain 1 and keyhole limpet hemocyanin (KLH). Spleen cells were treated in vitro to avoid having to deal with drug clearance and distribution. Recombinant human domain 1 with an additional cysteine and a histidine expression tag was expressed in insect cells using the baculovirus expression vector system (10). Compound 11 was isolated as a glutathione mixed disulfide using nickel affinity

Scheme 4. Preparation of the Domain 1 Polypeptide with an N-Terminal Cysteine**Scheme 5. Preparation of Immunogen**

chromatography. The glutathione disulfide was removed by selective reduction using immobilized dithiothreitol to provide compound **12**, a form of domain 1 with a free thiol (Scheme 4).

Compound **12** was reacted with maleimidyl-KLH, compound **13** (Pierce), to provide compound **14**, a conjugate of domain 1 on KLH as shown in Scheme 5. Small molecules including unreacted **12** were removed by dialysis. KLH is an approximately 4 MDa multidomain protein complex (21), so chemical characterization of compound **14** was not practical. When coated on plastic microtiter plates, however, compound **14** was able to bind anti- β_2 GPI antibodies as demonstrated in a direct binding ELISA. Most importantly, conjugate **14** elicited an immune response against domain 1 when used to immunize mice (data not shown).

Spleen cells of immunized mice were treated with LJP 993 and transferred to naive syngeneic mice whose immune systems had been compromised by irradiation. One day later, the mice were boosted with compound **14**. Seven days after boosting the mice were bled, and their sera were analyzed for anti-KLH antibodies and anti-domain 1 antibodies using ELISA assays with either KLH or β_2 GPI coated on plastic microtiter plates. Mice treated with compound LJP 993 showed a dose-dependent reduction of anti-domain 1 antibodies, whereas the anti-KLH response was the same in all groups, demonstrating that the effect was antigen specific. These results, presented in Table 2 and graphically in Figure 2, show that treatment of primed cells with LJP 993 suppresses antibody formation.

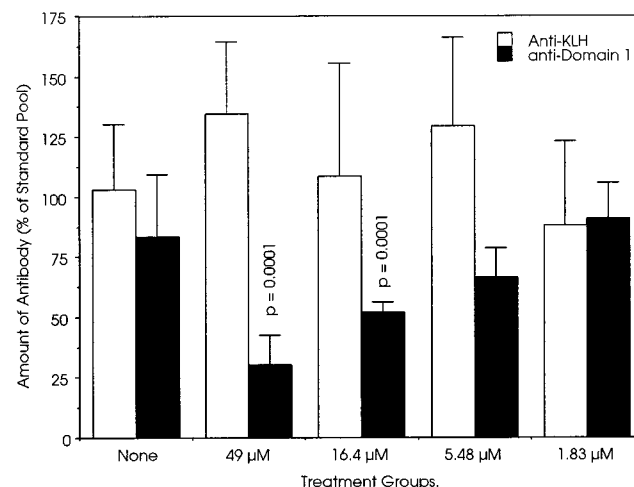
DISCUSSION

The objective was to prepare a tetravalent domain 1 conjugate with the following properties. Each domain 1 polypeptide should retain the ability to bind anti- β_2 GPI antibodies, and the conjugate should be biologically stable, nontoxic, and nonimmunogenic. At the outset, it was recognized that it would be preferable that the conjugate comprises a site-specific attachment of the polypeptide to a tetravalent platform and that the polypeptide retain both affinity and specificity of antibody binding.

Table 2. Relative Amounts of Anti-Domain 1 Antibody Measured in an ELISA Assay

conc of LJP 993 (μ M)	anti-domain 1 antibodies		anti-KLH antibodies	
	amounts ^a	% reduct	amounts ^a	% reduction
0	83.3 $\pm$ 26.2		103.0 $\pm$ 27.2	
1.8	90.8 $\pm$ 14.8	0	87.9 $\pm$ 35.2	15
5.5	66.3 $\pm$ 12.2	20	108.5 $\pm$ 46.7	0
16.4	52.0 ^a $\pm$ 4.1	38	129.5 $\pm$ 36.3	0
49.0	30.1 ^a $\pm$ 12.5	64	134.5 $\pm$ 29.9	0

^a Statistically significant, $p = 0.0001$. ^b Amount of antibodies as a percentage of antibodies in the standard pool.

**Figure 2.** Graphical presentation of the data in Table 2.

For a multivalent B cell epitope to function as a toleragen, it is necessary that it does not contain T cell epitopes which would make it immunogenic. The domain 1 polypeptide did not stimulate T cells harvested from mice immunized with **14**, as evidenced by lack of thymidine uptake by those cells after treatment with compound **9** (data not shown).

The chemical modification of proteins has been an area of intense interest over the past decades, and a number of excellent reviews have been published (22–25). Site-specific modifications of proteins can provide well-defined derivatives that retain native biological activity; whereas, other less specific modifications tend to provide heterogeneous derivatives with possible loss of activity. Oxime bond formation has been used extensively to form bioconjugates by the reaction of one molecular partner, containing an aminoxy group, with another partner containing an aldehyde or ketone. Oxime bond formation involving polypeptides is generally a very selective and predictable reaction. The reaction yields are good because the functional groups present in native polypeptides are not reactive with aldehyde or aminoxy groups, neither of which is present in native polypeptides.

Formation of oximes from aminoxy compounds is chemically analogous to hydrazone formation using hydrazides (26); however, the oxime bond is reported to be hydrolytically more stable than the hydrazone bond (27). Oxime bond formation has been used to chemically ligate aminoxy-containing synthetic polypeptide segments with ketone-containing polypeptide segments (28). Recently, aminoxy containing saccharides have been chemically attached to ketones generated on the surfaces of cells (29). Aminoxy-derivatized PEG has been attached site-specifically to the N-terminus of polypeptides which have an N-terminal glyoxyl group (27). The strategy described in this report, for attaching the domain 1 polypeptide to

a multivalent platform, was to generate a glyoxyl group at the N-terminus of the polypeptide. The glyoxyl group would provide an effective handle for a selective reaction with aminoxy groups on a multivalent platform and provide a well-defined multivalent domain 1 conjugate.

There are two ways to generate a glyoxyl group at the N-terminus of a polypeptide. A process, called transamination, converts the N-terminus to an aldehyde or ketone (30). Transamination of a polypeptide with a glycine at the N-terminus converts the glycine to a glyoxyl group. N-terminal glyoxyl groups can also be generated from N-terminal serine or threonine containing polypeptides by periodate oxidation (27, 31). There are a number of precedent bioconjugates of transaminated polypeptides. For instance, transaminated staphylococcal nuclease has been conjugated to dinitrophenylhydrazine, via hydrazone formation (32). Transaminated IL-1 receptor antagonist has been conjugated to aminoxy modified poly(ethylene glycol) by forming an oxime bond (27). Since recombinantly produced domain 1 polypeptide has an N-terminal glycine, a logical approach toward creating a glyoxylated derivative was to convert the glycine to a glyoxyl group through a transamination reaction.

Transamination of the domain 1 polypeptide provided a site for attachment to a multivalent aminoxy platform. The resulting oxime conjugate, LJP 993, retained biological activity as determined using surface plasmon resonance to measure affinity for anti- β_2 GPI antibody from autoimmune thrombosis patients, and it suppressed anti-domain 1 antibody production in animals immunized with domain 1. These results indicate that multivalent domain 1 conjugates can act as B cell toleragens in vitro in a dose-dependent manner when the cells are exposed to drug for 1 h. In addition to dose, the efficacy of a toleragen may be related to the time of exposure of drug to the B cell, the affinity of the epitopes for the B cell receptors, and the physical properties of the toleragenic molecule which affect its ability to aggregate B cell receptors.

Future studies are aimed at developing a therapeutic for autoimmune thrombosis based on the multivalent presentation of domain 1 with pharmacokinetics, affinity, and dimensions and flexibility of platform all expected to influence the potency of the drug.

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Synthesis of a Hoechst 33258 Analogue Amino Acid Building Block for Direct Incorporation of a Fluorescent, High-Affinity DNA Binding Motif into Peptides

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The synthesis of a new versatile “Hoechst 33258-like” Boc-protected amino acid building block for peptide synthesis is described. It is demonstrated that this new ligand is an effective mimic of Hoechst 33258 in terms of DNA affinity and sequence specificity. Furthermore, this minor groove binder was conjugated to a DNA condensing peptide (KSPKKAKK) by continuous solid-phase peptide synthesis, and the conjugate exhibited increased DNA affinity (ca. 10-fold), but similar sequence preference compared to Hoechst 33258 as analyzed by DNase I footprinting. Finally, the fluorescence quantum yield of the new chromophore is found to increase 30% upon binding to double stranded DNA.

INTRODUCTION

Sequence-specific recognition of DNA by proteins such as transcription factors and repressors play a central role in cellular gene regulation at the transcriptional level and is also crucial in DNA replication and recombination. One long-term objective of structural and biophysical studies of protein DNA interactions is to understand the molecular basis for these processes (1, 2). Such understanding can provide valuable input to the development of new principles for design of DNA recognizing drugs for direct modulation of gene expression, e.g., for use as gene therapeutic drugs (3).

Protein DNA recognition relies on hydrogen bonds, electrostatic interactions, and van der Waals contacts between individual amino acid residues of the protein and nucleotides in the cognate DNA target. However, no simple “amino acid–nucleotide” recognition code has been identified, and the sequence specificity of protein binding to DNA is therefore not a “digital readout” but rather an “analogue readout” of the DNA base sequence in which the entire binding region of the protein exhibit “molecular complementarity” to the major (or more rarely the minor) groove of the DNA target (1, 2, 4).

Most success so far in developing small synthetic ligands for sequence specific recognition of double stranded DNA has come from using major groove binding, triple helix forming oligonucleotides (5), helix-invading peptide nucleic acids (PNA) (6), and minor groove binding, hairpin polyamides (7). Furthermore, it has been possible to use “zink-finger” peptide modules (each recognizing three base pairs) to construct sequence-targeted proteins (8). Finally, several attempts without major breakthroughs have been described to select small peptides from chemical libraries that sequence selectively bind

double-stranded DNA, to discover novel recognition motifs and/or principles (9–10).

We recently described such a combinatorial method for probing small peptides for DNA minor groove recognition potential (9). Bead-supported tripeptides, each placed between *N*-methylpyrrole amino acid containing polyamides operating as DNA minor groove binding anchoring points, were synthesized in a library format and subsequently screened against double-stranded DNA probes (9). Although reliable procedures are available for synthesizing polyamides of *N*-methylpyrrole amino acids by peptide coupling of protected *N*-methyl-4-aminopyrrole-2-carboxylate units (11), their synthesis is resourceful as three or more interlinked units are required for obtaining strong DNA minor groove binding. To improve our methodology, we aimed to minimize the number of synthetic operations needed for library production and therefore required a monomer building block that in a single synthesis step introduces a DNA minor groove binding anchoring point into peptides.

The fluorescent chromosomal stain Hoechst 33258 is a well-known DNA binding agent made of two linked benzimidazoles, with a phenol and a *N*-methylpiperazine substituent in either end. The interaction between Hoechst 33258 and double-stranded DNA has been extensively studied using DNase I footprinting (12), electric linear dichroism, and solution-phase NMR techniques (13) as well as X-ray crystallography (14–16). Studies have shown that Hoechst 33258 binds with high affinity to the minor groove of double stranded B-DNA with a strong preference for AT-rich regions.

As a part of our continuous studies on peptide DNA interactions, we now report the synthesis of a novel protected amino acid based on Hoechst 33258. The building block is suitable for use in solid-phase peptide synthesis following Boc strategy and designed to allow for direct incorporation of one or multiple DNA minor groove-binding motifs into any arbitrary position of a peptide sequence. We illustrate the potential of this novel Hoechst 33258 amino acid analogue by preparing a small series of peptides and 9-amino acridine conjugates and

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analyzing their DNA binding properties by DNaseI footprinting.

EXPERIMENTAL SECTION

General Comments. All reagents (Aldrich, Fluka, or NovaBiochem) and solvents (LabScan) were of standard quality and used without further purification unless indicated. NMR spectra were recorded on either a Bruker 250 MHz or a Varian 400 MHz spectrometer with tetramethylsilane (^1H and ^{13}C) as internal standard. Melting points were recorded on a Büchi melting point apparatus and are uncorrected. Microanalyses were performed at The Microanalytical Laboratory, Department of Chemistry, University of Copenhagen.

2-(4-Cyanophenyl)benzimidazole-5-carboxylic Acid (3). 3,4-Diaminobenzoic acid (4.00 g, 26 mmol) and *p*-cyanobenzaldehyde (3.45 g, 26 mmol) were dissolved in nitrobenzene (120 mL) and heated to 150 °C. After 1 h, a turbid solution was obtained. DMF (20 mL) was added to dissolve the precipitate, and heating was resumed for an additional 5 h (TLC¹ monitoring in $\text{CHCl}_3/\text{NEt}_3/\text{MeOH}$ (7:1:2 v/v/v)). The mixture was cooled on an ice bath, and the crystals that separated out were collected by filtration and washed several times with cold petroleum ether to remove traces of nitrobenzene. Recrystallization from ethanol/water afforded 5.02 g (73%) of the title material. $R_f = 0.45$ in $\text{CHCl}_3/\text{NEt}_3/\text{MeOH}$ (7:1:2 v/v/v). Mp > 300 °C. ^1H NMR (DMSO- d_6) δ : 7.70 (d, 1H); 7.86 (dd, 1H); 8.04 (d, 2H); 8.22 (d, 1H); 8.37 (d, 2H), 12.75 (bs, 1H). ^{13}C NMR (DMSO- d_6) δ : 112.78; 115.17; 117.06; 117.82; 124.33; 125.94; 127.49; 133.19; 133.90; 139.59; 142.23; 151.82; 168.13. MS-FAB+: 264.09 [M + H]⁺. Anal. Calcd for $\text{C}_{15}\text{H}_9\text{N}_3\text{O}_2 \cdot \text{H}_2\text{O}$: C, 64.05; H, 3.94; N, 14.93. Found: C, 64.11; H, 3.96; N, 14.78.

Ethyl *N*³-(2-(4-Cyanophenyl)benzimidazole-5-carboxyl)-3,4-diaminobenzoate (4). 2-(4-Cyanophenyl)benzimidazole-5-carboxylic acid (**3**, 5.02 g, 19 mmol) was suspended in DMF (120 mL). HBTU (7.22 g, 19.0 mmol) was added followed by DIEA (5.0 mL). The mixture was stirred at room temperature for 10 min before the addition of ethyl 3,4-diaminobenzoate (3.43 g, 19 mmol). After the mixture was stirred for 24 h at room temperature, water (400 mL) was added. The precipitated product was collected by filtration, washed with water three times, and dried in a vacuum oven overnight. Yield: 5.75 g (71%). $R_f = 0.4$ in $\text{MeOH}/\text{CH}_2\text{Cl}_2$ (1:9, v/v). MS-FAB+: 426.16 [M + H]⁺. Mp = 222–225 °C. ^1H NMR (DMSO- d_6) δ : 1.28 (t, 3H); 4.22 (q, 2H); 5.83 (bs, 2H); 6.81 (d, 1H); 7.58 (d, 1H); 7.70 (m, 1H); 7.83 (d, 1H); 7.96 (d, 1H); 8.06 (d, 2H); 8.36 (m, 1H); 8.40 (d, 2H); 9.73 (bs, 1H); 13.46 (bs, 1H). ^{13}C NMR (DMSO- d_6) δ : 14.45; 59.82; 111.84; 112.38; 114.72; 116.62; 118.63; 119.21; 119.46; 122.21; 123.57; 127.33; 128.38; 129.00; 133.12; 133.96; 144.98; 146.29; 148.47; 151.78; 165.76; 166.12. Anal. Calcd for $\text{C}_{24}\text{H}_{19}\text{N}_5\text{O}_3$: C, 67.76; H, 4.50; N, 16.46. Found: C, 66.57; H, 4.31; N, 16.56.

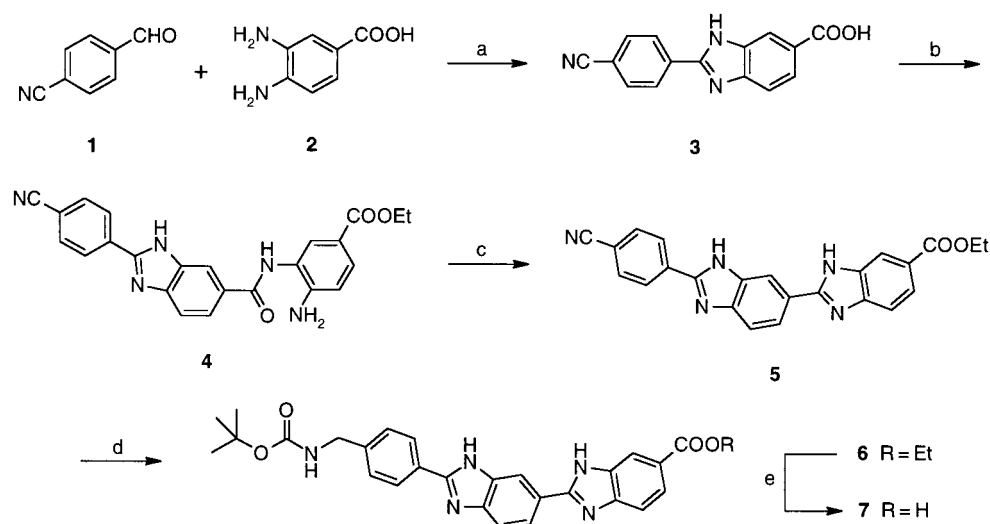
Ethyl 2-(2-[4-Cyanophenyl]benzimidazol-5-yl)benzimidazole-5-carboxylate (5). Ethyl *N*³-(2-(4-cyanophenyl)benzimidazole-5-carboxyl)-3,4-diaminobenzoate (**4**, 5.75 g, 13.5 mmol) was suspended in nitrobenzene (120 mL), and concentrated H_2SO_4 (0.5 mL) was added. The mixture was heated to 190 °C for 12 h during which time complete ring closure took place according to TLC

($\text{MeOH}/\text{CH}_2\text{Cl}_2$ (1:9, v/v)). After being cooled to room temperature, the product was precipitated as an oil by addition of petroleum ether. The adhesive oil turned into a fine powder after repeated addition and decanting of petroleum ether. The powder, which contained traces of hydro sulfate salts, was dissolved in a mixture of DMF (120 mL), saturated Na_2CO_3 (50 mL), and water (150 mL) by heating. The clear dark solution thus obtained was allowed to cool slowly, and the precipitating product was collected by filtration. $R_f = 0.45$ in $\text{MeOH}/\text{CH}_2\text{Cl}_2$ (1:9, v/v). The yield after oven-drying overnight was 4.15 g (75%). Mp = 257–259 °C. ^1H NMR (DMSO- d_6) δ : 1.35 ppm (t, 3H); 4.33 (q, 2H); 7.67 (d, 1H); 7.80 (d, 1H); 7.83 (d, 1H); 8.04 (d, 2H); 8.14 (d, 1H); 8.19 (s, 1H); 8.37 (d, 2H); 8.45 (s, 1H). ^{13}C NMR (DMSO- d_6) δ : 14.28 ppm.; 60.92; 112.52; 114.24; 115.45; 118.52; 119.84; 122.33; 123.39; 124.86; 125.41; 127.34; 129.95; 132.89; 135.08; 135.39; 138.40; 139.72; 141.21; 151.75; 152.83; 165.55. MS-FAB+: 408.09 [M + H]⁺. Anal. Calcd for $\text{C}_{24}\text{H}_{17}\text{N}_5\text{O}_2 \cdot \text{H}_2\text{O}$: C, 67.75; H, 4.50; N, 16.46. Found: C, 67.49; H, 4.32; N, 16.40.

Ethyl 2-[2-[4-(*tert*-Boc-aminomethyl)phenyl]benzimidazol-5-yl]benzimidazole-5-carboxylate (6). Ethyl 2-(2-[4-cyanophenyl]benzimidazol-5-yl)benzimidazole-5-carboxylate (**5**, 4.00 g, 9.8 mmol) was dissolved in DMF (120 mL) by heating. After the solution was cooled to room temperature, di-*tert*-butyl pyrocarbonate (2.50 mL, 10.8 mmol) and DIEA (6.0 mL, mmol) were added, followed by palladium catalyst (10% on charcoal, 520 mg). The solution was then hydrogenated at room temperature for 48 h (1 atm hydrogen pressure). The catalyst was removed by filtration and the solvent removed by rotary evaporation, in vacuo, to leave a dark tar. This was redissolved in ethanol (100 mL), and the product was precipitated as a solid by addition of water (200 mL). Pure title material was obtained after recrystallization from ethanol/water. Yield: 4.20 g (83%). $R_f = 0.4$ in $\text{MeOH}/\text{CH}_2\text{Cl}_2$ (1:9, v/v). Mp: 195–200 °C dec. ^1H NMR (DMSO- d_6) δ : 1.36 ppm (t, 3H); 1.41 (s, 9H); 4.22 (d, 2H); 4.34 (q, 2H); 7.44 (d, 2H); 7.51 (t, 1H); 7.68 (d, 1H); 7.75 (d, 1H); 7.85 (d, 1H); 8.10 (d, 1H); 8.17 (d, 2H); 8.20 (s, 1H); 8.42 (s, 1H). ^{13}C NMR (DMSO- d_6) δ : 14.36; 28.34; 43.34; 60.58; 78.06; 121.62; 123.31; 123.54; 123.65; 126.82; 127.60; 128.11; 142.78; 153.15; 154.83; 155.96; 166.35; 197.16 (only the strong signals are reported). MS-FAB+: 512.26 [M + H]⁺. Anal. Calcd for $\text{C}_{29}\text{H}_{29}\text{N}_5\text{O}_4 \cdot 3 \text{H}_2\text{O}$: C, 61.58; H, 6.23; N, 12.38; found: C, 61.49; H, 6.01; N, 12.52.

2-[2-[4-(*tert*-Butoxycarbonylaminomethyl)phenyl]benzimidazol-5-yl]benzimidazole-5-carboxylic Acid (7). Ethyl 2-[2-[4-(*tert*-Boc-aminomethyl)phenyl]benzimidazol-5-yl]benzimidazole-5-carboxylate (**6**, 2.98 g, 5.83 mmol) was dissolved in ethanol (80 mL), and aqueous sodium hydroxide solution (1 N, 50 mL) was added. The clear solution was heated to reflux for 2 h. The solution was cooled to room temperature and the reaction volume reduced to one-third by rotary evaporation, in vacuo. Water was added to a total volume of 150 mL and the clear solution extracted with diethyl ether (2 $\times$ 100 mL). The solution was acidified to pH 3.5 with 4 N HCl and extracted twice with *n*-butanol. The organic phase was dried with Na_2SO_4 , filtered, and taken to dryness. The oil thus obtained was dissolved in a 5% ethanolic water solution by gentle heating, and after lyophilization of this solution, a fine white powder was obtained. Yield: 1.98 g (70%). $R_f = 0.3$ in $\text{CHCl}_3/\text{NEt}_3/\text{MeOH}$ (7:1:2, v/v/v). Mp > 300 °C. ^1H NMR (DMSO- d_6) δ : 1.28 (t, 3H); 4.07 (d, 2H); 7.41 (d, 2H); 7.48 (d, 1H); 7.70 (d, 1H); 7.84 (d, 1H); 8.08 (d, 1H); 8.15 (s, 1H); 8.23 (d, 2H); 8.42 (s, 1H). ^{13}C

¹ Abbreviations: CT-DNA (calf thymus DNA), DIEA (diisopropylethylamine), DMF (dimethylformamide), DMSO (dimethyl sulfoxide), HBTU (benzotriazol-1-yl 1,1,3,3-tetramethyluronium hexafluorophosphate), TFMSA (trifluoromethanesulfonic acid), TLC (thin-layer chromatography).

Scheme 1^a

^a Key: (a) nitrobenzene/DMF, 190 °C, 6 h (73%); (b) HBTU, DIEA in DMF, 10 min, rt., then ethyl 3,4-diaminobenzoate in DMF, 24 h, rt (71%); (c) cat. H₂SO₄, nitrobenzene, 150 °C, 24 h (75%); (d) H₂/Pd-C, Boc₂O, DIEA, DMF rt, 24 h (83%); (e) NaOH in water, then HCl (70%).

NMR (DMSO-*d*₆) δ : 28.34; 43.33; 77.99; 120.97; 123.74; 124.21; 126.78; 127.44; 128.97; 134.41; 142.15; 153.01; 153.41; 155.94; 171.36 (only the strong signals are reported). HRMS: calcd for C₂₇H₂₅N₅O₄ 484.1985, found 484.1978.

3-Dimethylaminopropyl 2-[2-[4-(Aminomethyl)-phenyl]benzimidazol-5-yl]benzimidazole-5-carboxamide (8). 2-[2-[4-(*tert*-Butoxycarbonylaminoethyl)-phenyl]benzimidazol-5-yl]benzimidazole-5-carboxylic acid (**7**, 250 mg; 0.51 mmol) was dissolved in dimethylformamide (8 mL), and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 195 mg; 0.51 mmol) and diisopropylethylamine (DIEA, 500 μ L; 2.87 mmol) were added. The mixture was stirred at room temperature for 15 min, and then 3-(dimethylamino)propylamine (500 μ L; 3.97 mmol) was added. After being stirred for a further 2 h at room temperature, the solvent was removed by rotary evaporation to leave an oil. The oil was dissolved in saturated aqueous sodium hydrogen carbonate (20 mL). Aqueous sodium hydroxide (1 M, 4 mL) was added, and the solution was extracted twice with *n*-butanol (20 mL). The combined organic phases were dried with anhydrous sodium sulfate and taken to dryness to leave a colorless foam. The foam was suspended in 2 N aqueous hydrochloric acid (40 mL), and the suspension was refluxed until a clear solution was obtained. Solvent was removed, in vacuo, and the crystalline residue recrystallized from ethanol to leave the title material as a crystalline slightly hygroscopic powder. Yield: 130 mg (68%). Mp = 283–286 °C dec. ¹H NMR (D₂O, 250 MHz) δ : 1.97 ppm (d, 2H); 2.88 (s, 6H); 3.14 (d, 2H); 3.26 (t, 2H); 3.95 (s, 2H); 7.36–7.40 (m, 4H); 7.52 (s, 2H); 7.59 (s, 2H); 7.61 (s, 1H); 7.67 (s, 1H). Anal. Found: C, 51.26; H, 6.62; N, 15.48 (agree with the dihydrochloride pentahydrate). MS found 468.29; calcd for [C₂₇H₂₉N₇O + H]⁺ 468.58.

Spectroscopic Methods. Absorption spectra were recorded on a Perkin-Elmer UV-vis Lambda 16 spectrophotometer in 1 cm cells. Fluorescence spectra were recorded on an LS 50B Perkin-Elmer luminescence spectrometer in perpendicular geometry. In all excitation and fluorescence spectra shown, excitation and emission band-pass were 1 nm. All excitation spectra are corrected. However, no correction was performed for the wavelength-

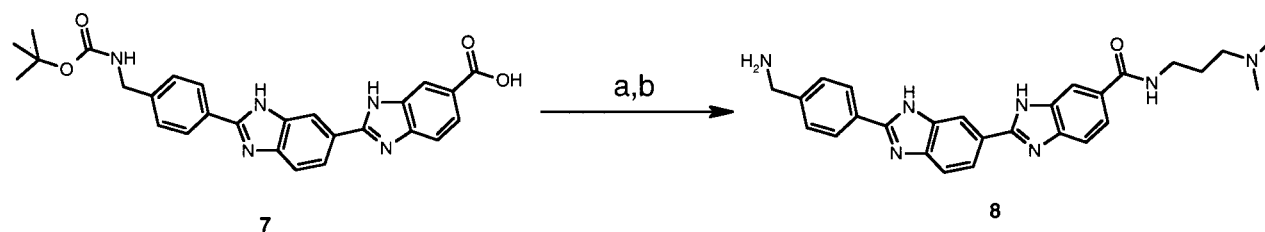
dependent response function of the detection system. Concentration of CT-DNA was calculated using $\epsilon_{258} = 16\,800\text{ M}^{-1}\text{ cm}^{-1}$ pr. base-pair from the absorption at 256 nm (Figure 3, trace b) corrected for absorption of **8** (using the unbound absorption coefficient).

Oligomer Synthesis. Peptides and peptide conjugates were synthesized by standard Boc protocol on an MBHA resin at a loading of 0.1 mmol/g. Oligomers were cleaved off the resin using the low/high TFMSA procedure, purified by reversed-phase HPLC, and were characterized by MALDI-TOF mass spectrometry recorded on a Kratos Compact MALDI II instrument operating in the positive-ion mode, using 3,5-dimethoxy-4-hydroxycinnamic acid as the matrix (17).

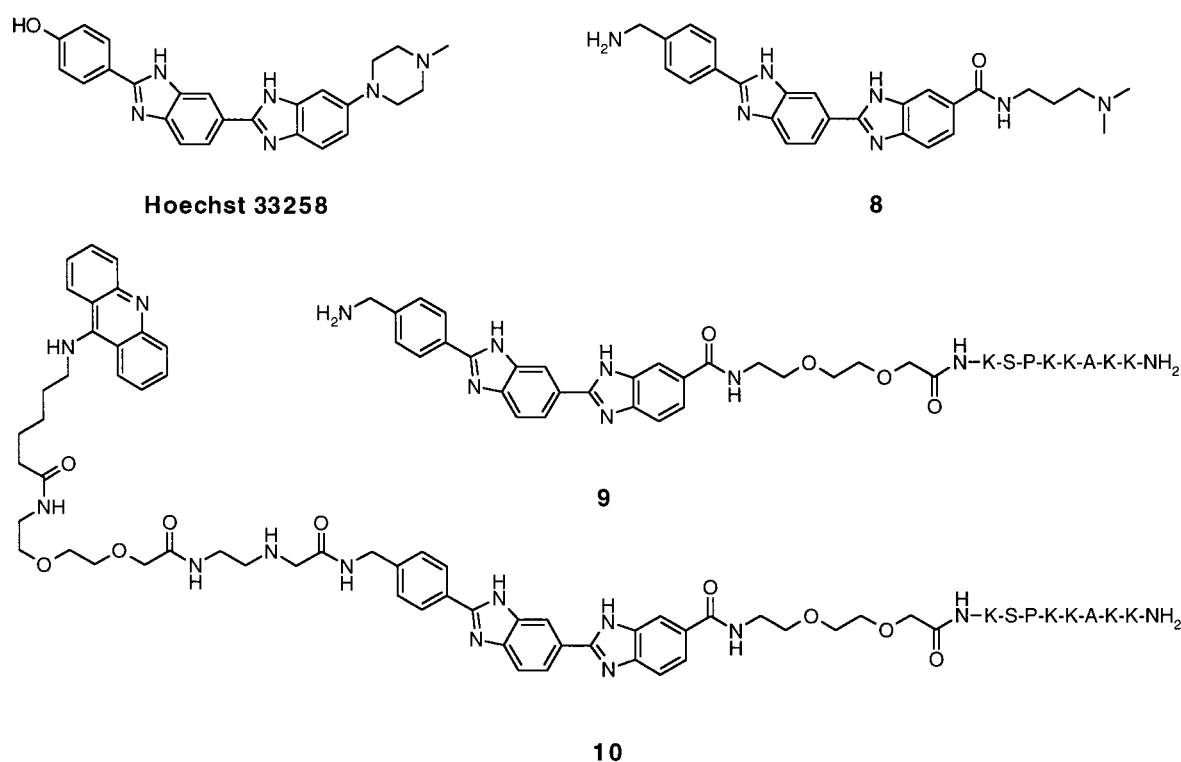
DNA Footprinting. Footprinting with DNaseI was performed in 100 μ L of buffer (50 mM Tris-HCl, pH 7.4, 1 mM MgCl₂) using a 3'-³²P-end labeled *Eco*RI-*Pvu*II fragment of plasmid pUC19. The DNaseI was titrated to yield ca. 20% cleavage after 30 min incubation at room temperature (20 °C). The DNA was precipitated with 66% ethanol, 0.1 M K-acetate, washed, dried, and analyzed by electrophoresis in 10% polyacrylamide sequencing gels run in standard Tris-Borate-EDTA buffer, pH 8.3. Radioactive DNA bands were visualized by autoradiography (18).

RESULTS AND DISCUSSION

Chemistry. The construction of the 2-(2-arylbenzimidazol-5-yl)benzimidazole framework is outlined in Scheme 1 and essentially followed previously described strategies (19). Initially, 4-cyanobenzaldehyde (**1**) was condensed with 3,4-diaminobenzoic acid (**2**) under oxidative conditions, leaving 2-(4-cyanophenyl)benzimidazol-5-carboxylic acid (**3**) in 73% yield. Installation of the second benzimidazole moiety was accomplished in three steps: (1) conversion of **3** to its 1,2,3-hydroxybenzotriazole ester (HOBt ester) by reaction with HBTU, (2) condensation with ethyl 3,4-diaminobenzoate to form amide **4**, and finally, (3) acid-catalyzed ring closure using sulfuric acid in nitrobenzene (53% overall for the three steps). Reduction of the cyano group in **5**, in the presence of di-*tert*-butyl pyrocarbonate and DIEA, directly afforded the Boc-protected benzylic amine (**6**), which after saponification provided the target building block **7** in 70% yield.

Scheme 2^a

^a Key: (a) HBTU, DIEA in DMF, 10 min, rt, then 3-dimethylaminopropylamine in DMF, 2 h, rt; (b) 2 N HCl, reflux 10 min (68% overall).

Scheme 3

DNA Recognition of Conjugates. To evaluate the potential of this new amino acid, we used it to prepare a simple Hoechst 33258 analogue (**8**, Scheme 2), a conjugate to a DNA binding (condensing) peptide motif, that is found in histone H1 (20) (**9**), and a further conjugation of **9** to the DNA intercalator 9-amino acridine (**10**) (Scheme 3). All conjugates were synthesized on solid support starting with the peptide, and were characterized by MALDI-TOF mass spectrometry.

The DNA recognition properties of these compounds were evaluated by DNaseI footprinting (Figure 1) using a ³²P-end labeled plasmid DNA restriction fragment (Figure 2). Comparing the footprint produced by **8** with that of Hoechst 33258 shows close to identical (AT target) sequence preference of the two compounds. Concordant with this conclusion, an NMR study of a DNA–**8** complex shows AT-minor groove binding (21) fully analogous to that typically observed for Hoechst 33258. The present results (Figure 1a, e.g., compare lanes 4 and 10–11) also indicate a 4–8-fold higher affinity of **8** as compared to Hoechst 33258.

In terms of sequence specificity, the binding of the conjugates **9** and **10** is almost identical to that of Hoechst 33258 (and thus also identical to that of **8**). All major binding sites contain (A/T)₄ motifs (cf. Figure 2), exhibiting the following order of potency (5′-3′-orientation) as

judged from the concentration resulting in ca. 50% protection from DNaseI cleavage: AATTAAT ~ AATTT > AATT > ATTA > TTTAT > ATAA > TTAA. Furthermore, as estimated from the binding to the most 3′ ATTA target (and also the TTAA target), similar protection by **9** is achieved at 60-fold lower concentration than that required for Hoechst 33258 (compare, e.g., lanes 3 and 7 of Figure 1). Therefore, **9** binds to the DNA targets with ~60-fold higher affinity than Hoechst 33258, and thus with ca. 10-fold higher affinity than the nonconjugated **8**. Finally, no sequence-specific binding of the peptide alone (**11**, H-KSPKKAKK-NH₂) was detected by DNaseI footprinting. Therefore, conjugation of the cationic peptide, KSPKKAKK increases the DNA affinity of the minor groove binder by approximately 10-fold without affecting the sequence preference. Attachment of a DNA intercalating acridine to **9** yielding **10**, surprisingly only seem to contribute little additional affinity. As would be expected from the very limited sequence preference found for DNA binding of 9-aminoacridines (22), the sequence preference of **10** was not changed either as compared to **9**.

Fluorescence Properties. Hoechst 33258 is widely used as a DNA stain in molecular biology and in cytology based on the great increase in quantum yield from 0.01 to 0.6 of its bright blue fluorescence upon binding to DNA

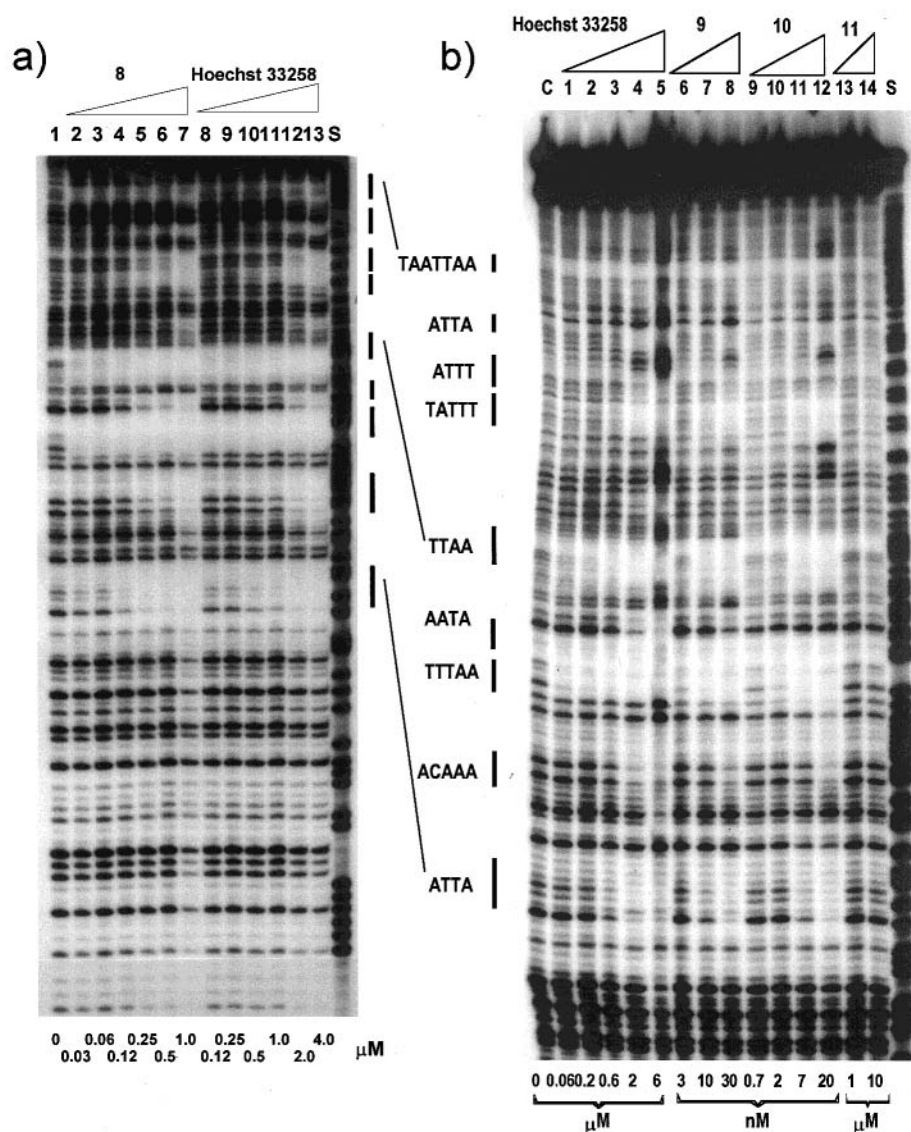


Figure 1. Autoradiograms of DNaseI footprinting experiments. The compound used is indicated above the lanes, while the concentrations are indicated below the lanes. The putative binding sites are indicated by bars between the autoradiograms. S is an A/G sequence reaction, while C is a control without reagent.

3' AGCTCGAGCCATGGGCCCTAGGAGATCTCAGC
 TGGACGTCCGTACGTTTGAACCGCATAGTACCAG
 TATCGACAAAGACACACTTTAACAATAGGCGAGT
 GTTAAGGTGTGTGTATGCTCGGCCTTCGTATTTT
 ACATTTTCGGACCCACGGATTACTCACTCGATTGA
 GTGTAATTAACGCAACGCGAGTGACGGCGAAAGG
 TCAGCCCTTTGCACAGCACGGTC-5'

Figure 2. Base sequence of the *EcoRI*-*PvuII* restriction fragment used in the DNaseI footprinting experiments. The binding sites detected by the footprinting are indicated.

(23). The dye shows pronounced preference for A/T-rich regions, but also exhibits multiple binding modes and distinct fluorescence emission spectra depending on dye/base pair ratios (24). As the benzimidazole derivative **8** is closely related to the Hoechst chromophore, we decided to investigate the fluorescence properties of the compound.

The electronic spectra of **8** and its complex with CT-DNA are displayed in Figure 3. The absorption spectrum (trace a) displays two absorption maxima in the range 250–400 nm. The long-wavelength absorption undergoes

a blue shift from 332 to 340 nm upon complexation with DNA (traces a and b). Upon excitation into the long wavelength band, unbound **8** fluoresces with a maximum at 390 nm (trace e). The spectral profile features an unresolved vibronic structure. However, the fluorescence from the **8**-DNA complex (trace f) is more intense (integrated intensity is up by 31% relative to unbound) and appears with three clearly resolved vibronic bands at 372, 392 (max), and 412 nm, corresponding to an average progression of 1300 cm^{-1} .

The excitation spectra derived from the fluorescence of compound **8** are seen to comply completely with the long wavelength absorption bands, whether in the free (traces a and c) or in the DNA-bound state (traces b and d). Furthermore, excitation spectra recorded with emission wavelengths set at the three vibronic bands at 372, 392 and 412 nm of trace f were found to be completely identical (spectra not shown), which implies that only one fluorescing species is present when **8** binds to DNA. The maximum observed at 280 nm in the excitation spectrum of DNA-bound **8** (trace d) indicates a small contribution of resonance energy transfer from DNA, the DNA absorption itself is seen going off-scale in trace b.

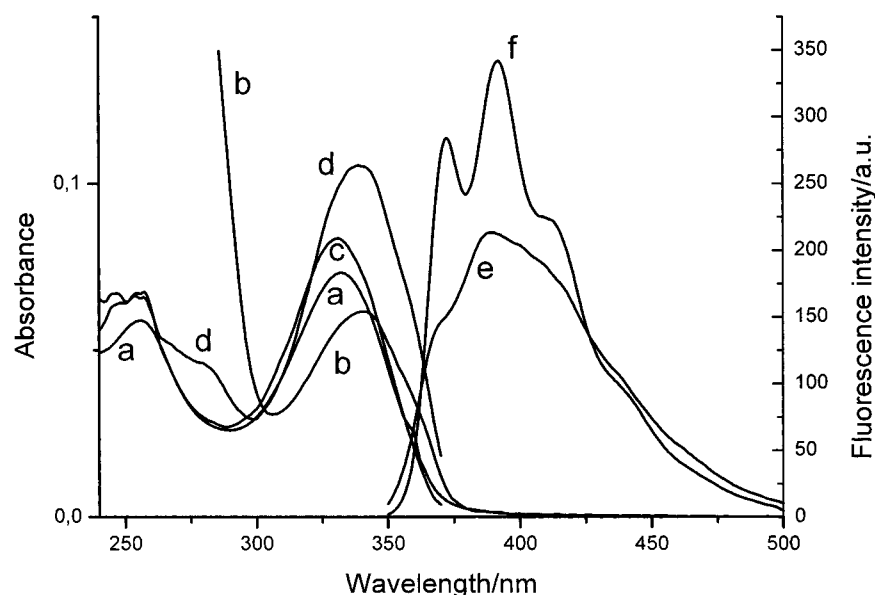


Figure 3. Electronic spectra of compound **8** and its complex with CT-DNA in water containing NaCl (0.1 M), KH_2PO_4 (10 mM), and EDTA (0.1 mM). In all spectra $[\mathbf{8}] = 2.2 \mu\text{M}$. In traces b, d, and f [basepair CT-DNA] = $22 \mu\text{M}$ (see the Experimental Section): (a) absorption, compound **8** ($\epsilon_{256} = 2.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{332} = 3.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$); (b) absorption, compound **8** ($\lambda_{\text{max}} = 340 \text{ nm}$) bound to CT-DNA; (c) excitation ($\lambda_{\text{em}} = 400 \text{ nm}$) of compound **8** ($\lambda_{\text{max}} = 331 \text{ nm}$); (d) excitation ($\lambda_{\text{em}} = 400 \text{ nm}$) of compound **8** ($\lambda_{\text{max}} = 339 \text{ nm}$) bound to CT-DNA; (e) fluorescence ($\lambda_{\text{ex}} = 338 \text{ nm}$, same intensity scale as spectrum f) of compound **8** ($\lambda_{\text{max}} = 390 \text{ nm}$); (f) fluorescence ($\lambda_{\text{ex}} = 338 \text{ nm}$, same intensity scale as spectrum e) of compound **8** bound to CT-DNA.

In general, when comparing corresponding absorption and fluorescence bands, it is fairly common to see a vibrational fine structure in the absorption spectrum being lost in fluorescence (25). Far more unusual is the observation of fine structure in a fluorescence band that corresponds to a structureless absorption, as it can be seen for the **8**-DNA complex by comparing traces b and f of Figure 3, respectively. To a lesser extent the same feature is found in the spectra of unbound **8** (traces a and e).

The default example of this phenomenon is biphenyl for which a range of torsional conformers is present among ground-state molecules while the distribution narrows in on a more planar conformation in the excited singlet state (26). The same process seems to occur in case of **8** and is especially pronounced when it is bound to DNA. This conclusion is supported by the full-width-at-half-maximum (fwhm) values of the fluorescence spectra, being 4660 cm^{-1} for unbound **8** (trace e) and 3600 cm^{-1} for the **8**-DNA complex (trace f). The conformational distribution is narrower among the molecules of **8** when it is bound to DNA. Furthermore, the fluorescence appear at virtually the same wavelength whether **8** is free (trace e) or DNA bound (trace f). Most likely, DNA-bound **8** is already forced into planar configuration in the ground state. Thus, the Stoke shift is smaller (3900 cm^{-1} , traces b and f) when **8** is bound than in the unbound form (4490 cm^{-1} , traces a and e) indicating that less reorientation occurs in the excited state of **8** in the complex relative to the unbound molecule. This conclusion is also consistent with minor groove binding of **8**, which indeed requires (enforces) coplanarity of the two aromatic systems, as is also consistent with the NMR structure determination of the complex (21).

CONCLUSION

The presently described Hoechst analogue represents a versatile and convenient building block for introducing a high affinity minor groove binder into peptides or other

oligoamides via Boc solid-phase chemistry. Thus the monomer should also be useful in combinatorial library approaches. Furthermore, the fluorescence properties of the ligand may be exploited to study DNA binding.

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^{99m}Tc-Labeled Neuropeptide Y Analogues as Potential Tumor Imaging Agents

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The possible use of neuropeptide Y (NPY) as a novel radiopeptide has been investigated. NPY is a 36-amino acid peptide of the pancreatic polypeptide family, which is expressed in the peripheral and central nervous system, and is one of the most abundant neuropeptides in the brain. Its receptors are produced in a number of neuroblastoma and the thereof derived cell lines. As structure–activity relationships of NPY are well-known, we could assume where a radionuclide might be introduced without affecting receptor affinity. We applied the novel [^{99m}Tc(OH₂)₃(CO)₃]⁺ aqua complex and PADA (2-picolylamine-*N,N*-diacetic acid) as bifunctional chelating agent. The peptides were synthesized by solid-phase peptide synthesis, and PADA was coupled to the side chain of Lys⁴ of the resin-bound peptide. Upon postlabeling of [K⁴(PADA)]-NPY, ^{99m}Tc(CO)₃ did not only bind to the desired PADA, but presumably as well to the His in position 26. Since the replacement of His²⁶ by Ala only slightly decreased binding affinity, [K⁴(PADA),A²⁶]-NPY was specifically postlabeled, and the ¹⁸⁵Re surrogate maintained high binding affinity. Furthermore, the prelabeling approach has been applied for the centrally truncated analogue [Ahx^{5–24}]-NPY, which is highly selective for the Y₂ receptor. The resulting Ac-[Ahx^{5–24},K⁴(^{99m}Tc(CO)₃-PADA)]-NPY was produced with a yield of only 16%. Therefore, postlabeling was applied for the short analogue as well, again substituting His²⁶ by Ala. Competitive binding assays using ¹⁸⁵Re as a surrogate for ^{99m}Tc showed high binding affinity of Ac-[Ahx^{5–24},K⁴(¹⁸⁵Re(CO)₃-PADA),A²⁶]-NPY. Internalization studies with the corresponding ^{99m}Tc-labeled analogue revealed receptor-mediated internalization. Furthermore, biodistribution studies were performed in mice, and stability was tested in human plasma. Our centrally truncated analogue revealed a 6-fold increased stability compared to the natural peptide NPY. We conclude that Ac-[Ahx^{5–24},K⁴(^{99m}Tc(CO)₃-PADA),A²⁶]-NPY has promising characteristics for future applications in nuclear medicine.

INTRODUCTION

Peptides can be considered as ideal agents for diagnostic and therapeutic applications, adopting Paul Ehrlich's concept of the "magic bullet" (1). Tumor tissues expressing receptors for a peptide can be addressed specifically by the peptide or differently labeled analogues (2). To date, radiolabeled receptor-binding peptides have emerged as a new class of radiopharmaceuticals (3, 4).

However, there are several prerequisites for peptides used for scintigraphy. The corresponding receptors have to be expressed on the target in suitable amounts, and it is crucial that the radiolabeled analogue retains a high affinity. Specificity is to be considered as well. Whereas the natural ligand usually binds with similar affinities to several receptor subtypes, chemically modified synthetic analogues often display pronounced subtype selectivity for only one or few receptor subtypes (3). Peptides that are taken up by receptor-mediated internalization may be preferred, as this results in accumulation of the radioligand in the target, providing an

increased signal for scintigraphy (5). A main concern of radiolabeled peptides is their stability under physiological conditions, i.e., peptide fragmentation by peptidases and the stability of the radiometal–chelator complex (6). As the natural peptides usually exhibit a short half-life in plasma, metabolically more stable analogues had to be developed. Somatostatin (SST) receptor scintigraphy using different radiolabeled SST analogues has been extensively clinically applied for tumor diagnosis (7, 8). Furthermore, bombesin and VIP have been evaluated as radiopharmaceuticals (9–11), and many further peptides are promising candidates, for instance neurotensin, α-MSH, substance P, and CCK (12).

Here, we investigate the possibility to use neuropeptide Y (NPY) as a novel radiopeptide, while taking into account the above-mentioned prerequisites. NPY is a 36-amino acid peptide of the pancreatic polypeptide family. It is expressed in the peripheral and central nervous system and is one of the most abundant neuropeptides in the brain (13). Several physiological activities such as induction of food intake, inhibition of anxiety, increase in memory retention, presynaptic retention of neurotransmitter release, and vasoconstriction have been attributed to NPY (14). Its receptors are produced in a number of neuroblastoma and the thereof derived cell lines, making them optimal targets for tumor scintigraphy. Five distinct NPY receptors have been cloned, which have been named Y₁, Y₂, Y₄, Y₅, and y₆ receptor subtype

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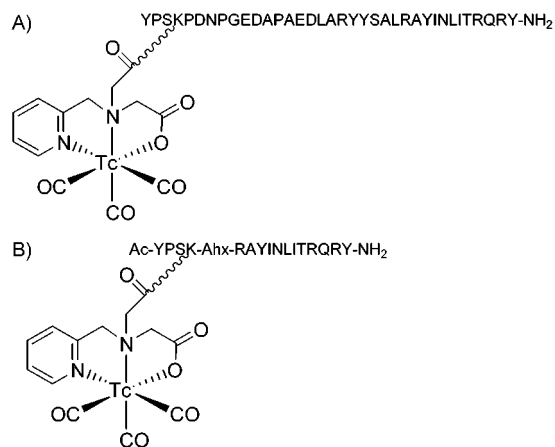


Figure 1. Structures of ^{99m}Tc -labeled NPY analogues. (A) Full length NPY analogue $[\text{K}^4(^{99m}\text{Tc}(\text{CO})_3\text{-PADA}),\text{A}^{26}\text{]-NPY}$ and (B) Centrally truncated analogue $\text{Ac-}[\text{Ahx}^{5-24},\text{K}^4(^{99m}\text{Tc}(\text{CO})_3\text{-PADA}),\text{A}^{26}\text{]-NPY}$. Ahx stands for 6-aminohexanoic acid, and PADA for 2-picolylamine-*N,N*-diacetic acid.

(15). Because structure–activity relationships of NPY are well-known (16, 17), we could assume where the radionuclide ^{99m}Tc might be introduced without affecting receptor affinity. The nuclear properties of ^{99m}Tc (monoenergetic γ -radiation of 140 keV, $t_{1/2}$ of 6 h) are virtually ideal for diagnostic imaging (18). We applied the method of Alberto (19) by using the $[\text{K}^4(^{99m}\text{Tc}(\text{OH})_2)_3(\text{CO})_3]^+$ aqua complex and PADA (2-picolylamine-*N,N*-diacetic acid) as bifunctional chelating agent (BFCA). Pre- and postlabeling approaches with attachment of PADA on the Lys⁴ side chain of NPY were investigated, and the full length analogue $[\text{K}^4(^{99m}\text{Tc}(\text{CO})_3\text{-PADA}),\text{A}^{26}\text{]-NPY}$ as well as centrally truncated ones such as $\text{Ac-}[\text{Ahx}^{5-24},\text{K}^4(^{99m}\text{Tc}(\text{CO})_3\text{-PADA}),\text{A}^{26}\text{]-NPY}$ (Ahx = 6-aminohexanoic acid) were produced (Figure 1). Synthesis, labeling, stability, binding affinities, internalization, and biodistribution studies are presented.

MATERIALS AND METHODS

Materials. Fmoc-protected amino acids, 4-(2',4'-dimethoxyphenyl)-Fmoc-aminomethyl)phenoxy resin, and 1*H*-benzotriazolium,1-[bis(dimethylamino)methylene]-, tetrafluoroborate(1-),3-oxide (TBTU) were obtained from NovaBiochem (Läufelfingen, Switzerland), diisopropylcarbodiimide (DIC) and thioresol from Aldrich, 1-hydroxybenzotriazole (HOBt), *N*-ethyl-diisopropylamine (DIPEA), trifluoroacetic acid, thioanisole, acetic anhydride, hydrazine, and piperidine from Fluka, and *tert*-butyl alcohol from Merck. Dimethylformamide and diethyl ether were purchased from Scharlau (La Jota, Barcelona, Spain). Acetonitrile was obtained from Romil (Cambridge, England). Bovine serum albumin and bacitracin were purchased from Sigma, Minimum Essential Medium with Earle's salts (MEM), Dulbecco's MEM/Nut-Mix F12 medium, fetal calf serum, trypsin-EDTA, Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium, glutamine, sodium pyruvate, and nonessential amino acids from Gibco. EDTA, Pefabloc SC, and all other chemicals were from Fluka. ^3H -propionyl-NPY (specific activity of 3.63 TBq/mmol) was purchased from Amersham. $\text{Na}[^{99m}\text{TcO}_4]$ was eluted from a Mallinckrodt $^{99}\text{Mo}/^{99m}\text{Tc}$ generator (Petten, The Netherlands) using 0.9% saline.

Peptide Synthesis. The peptides were synthesized by automated multiple solid-phase peptide synthesis using a robot system (Syro, MultiSynTech, Bochum, Germany). To obtain the peptide amide, 4-(2',4'-dimethoxyphenyl)-

Fmoc-aminomethyl)phenoxy resin was used. The polymer matrix was polystyrene–1%-divinylbenzene (30 mg; 15 μmol). The side-chain protection was chosen as follows: Tyr(*tert*-butyl), Glu(*tert*-butyl), Arg(2,2,3,5,5-pentamethylchromansulfonyl), His(trityl), Gln(trityl), Asn(trityl), Thr(*tert*-butyl), Lys(*tert*-butoxy-carbonyl), or Lys(4,4-dimethyl-2,6-dioxocyclohex-1-ylideneethyl) (Lys(Dde)). Double coupling procedures were performed with diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) activation, 10-fold excess and a coupling time of 40 min (20). Upon completion of the peptide sequence, the coupling of $\text{Re}(\text{CO})_3\text{-PADA}$ was performed on the resin (after hydrazine-based deprotection of Lys⁴(Dde)) (21) by DIC/HOBt activation, 10-fold excess, and a coupling time of 24 h. For the PADA-peptides, PADA was activated by diisopropylcarbodiimide for 45 min and subsequently coupled with a 10-fold excess to the selectively deprotected Lys⁴ side chain. The N-termini were either Boc-protected or had been acetylated.

The peptide amides were cleaved with 1 mL of trifluoroacetic acid/thioanisole/thiocresol (90:5:5) within 3 h. The peptide was precipitated from cold diethyl ether, collected by centrifugation, and lyophilized from water/*tert*-butyl alcohol (1:1). Analysis of the product was performed by analytical reversed-phase HPLC at 220 nm on nucleosil C-18 columns from Merck-Hitachi (5 μm , 3×125 mm). Acetonitrile (25% to 75%) with 0.1% trifluoroacetic acid within 30 min was used as an eluent for the full length analogues, whereas for the centrally truncated ones 5% to 60% within 30 min was applied. Correct mass was identified by ion-spray mass spectrometry (SSQ 710, Finnigan MAT, Bremen, Germany).

Radiolabeling. The preparation of $[\text{K}^4(^{99m}\text{Tc}(\text{OH})_2)_3(\text{CO})_3]^+$ was done according to earlier description (19). A mixture of 4 mg disodium carbonate (0.04 mmol), 5.5 mg sodium borohydride (0.15 mmol), and 20 mg potassium sodium tartrate tetrahydrate (0.07 mmol), inclosed in a Wheaton sample vial, were flushed with 1 atm CO for 15 min. One milliliter of a $[\text{K}^4(^{99m}\text{TcO}_4)]^-$ generator eluate in saline (about 1 GBq) was added, and the solution was heated for 30 min at 75°C. The solution was adjusted with approximately 100 μL of a mixture of 1 M phosphate buffer pH 7.4 and 1 N HCl (1:2) to pH 7. For postlabeling, 10 μL of 0.001 M solutions of the different PADA-NPY analogues were added to 90 μL of the neutralized ^{99m}Tc -(I)-tricarbonyl solution and heated for other 60 min at 75°C. Prelabeling was done as described in (10). The labeling procedures and conjugates have been analyzed by RP-HPLC on a Macherey-Nagel CC Nucleosil 100–5 C18 reversed-phase column (10 mm, 250×4.6 mm). A gradient system from 5% to 60% acetonitrile with 0.1% trifluoroacetic acid in 30 min and a flow rate of 1.5 mL/min were applied.

Stability. To analyze the chemical stability of the radiolabeled NPY conjugates, challenging assays were done according to the method of Stalteri et al. (22) with some modifications. A 10 μL amount of the ^{99m}Tc labeled peptide was added to 90 μL of 0.1 M solution of histidine or cysteine (1000-fold molar excess of challenging agents). The resulting solutions were incubated at 37 °C, and progress of the reaction was followed by RP-HPLC with radiometric detection. The proteolytic degradation in plasma of the NPY analogues was determined in vitro in freshly prepared human plasma. Plasma from healthy donors was incubated with the ^{99m}Tc -labeled NPY analogues at a concentration of 0.2 pmol/mL for different time periods at 37 °C. After incubation, samples were precipitated with acetonitrile/ethanol and centrifuged at

4 °C. The supernatant was analyzed by reverse-phase HPLC using a Superdex peptide HR 10/30 (300 mm, 13–15 μ m).

Cell Culture. SK-N-MC cells were grown in Minimum Essential Medium (MEM) with Earl's salts containing 10% fetal calf serum, 4 mM glutamine, 1 mM sodium pyruvate, and 1% nonessential amino acids. SMS-KAN cells were grown under the same conditions using Dulbecco's MEM/Nutrient mix F12 1:1 with 15% fetal calf serum. Cells were grown to confluency at 37°C and 5% CO₂. The growth medium was removed, and the cells were washed with Dulbecco's PBS. After incubation at room temperature for 3 min with PBS containing 0.02% EDTA, the cells were detached by mechanical agitation and suspended in new medium or incubation buffer.

Receptor Binding Studies. Binding assays on SK-N-MC and SMS-KAN cells were performed as previously described (23). In brief, cells were washed with PBS and resuspended in incubation buffer (minimum essential medium with Earl's salts containing 0.1% bacitracin, 50 μ M Pefabloc SC, and 1% bovine serum albumin). A 200 μ L volume of the suspension containing 400000 cells was incubated with 25 μ L of 10 nM ³H-propionyl-NPY and 25 μ L 10 pM to 10 μ M of NPY-analogues. Nonspecific binding was defined in the presence of 1 μ M cold NPY. After 1.5 h at room temperature, the incubation was terminated by centrifugation at 2000g and 4 °C for 5 min. The pellets were then washed once with PBS by centrifugation, resuspended in PBS, transferred to scintillation vials, and mixed with scintillation cocktail. Radioactivity was determined using a β -counter.

Internalization Studies. Studies of internalization of receptor-bound ^{99m}Tc labeled NPY analogues were performed as previously described (24, 25) with some modifications. Briefly, SK-N-MC or SMS-KAN cells were incubated at 37 °C in 6-well plates with about 300000 cpm [$K^4(^{99m}\text{Tc}(\text{CO})_3\text{-PADA})$,A²⁶]-NPY or Ac-[Ahx⁵⁻²⁴, $K^4(^{99m}\text{Tc}(\text{CO})_3\text{-PADA})$,A²⁶]-NPY, respectively. To determine nonspecific binding and internalization, the incubation was also performed in the presence of 1 mM NPY or [Ahx⁵⁻²⁴]-NPY. Incubation was terminated after different time periods (5, 30, 60, 120, and 180 min), and the cells were washed with ice-cold medium containing 1% bovine serum albumin. Cell-surface bound radioligand was removed by two steps of acid wash (50 mM glycine·HCl/100 mM NaCl, pH 2.8) at room temperature for 5 min. The pH were neutralized with PBS, and subsequently the cells were solubilized by incubation with 1 N NaOH at 37°C to determine internalized radioligand.

Biodistribution. Biodistribution of the radioconjugate Ac-[Ahx⁵⁻²⁴, $K^4(^{99m}\text{Tc}(\text{CO})_3\text{-PADA})$,A²⁶]-NPY was estimated in BALB/c mice. The analogue was injected intravenously into the tail vein (3.7 MBq/mouse). For blocking studies, coinjections with [Ahx⁵⁻²⁴]-NPY (0.3 mg/mouse) and NPY (0.25 mg/mouse) were performed. Organs and tissues (blood, heart, lung, spleen, kidneys, stomach, pancreas, duodenum, ileum, colon, liver, muscle, bone, brain) were excised from the sacrificed animals 1.5 and 24 h postinjection. The tissues were weighed, and the radioactivity was determined by γ -counting. Results were expressed as percentage of injected dose per gram of tissue (% ID/g).

RESULTS

Peptide Synthesis. Upon completion of the peptide sequence on the robot, the coupling of PADA or Re(CO)₃-PADA was performed manually to the resin-bound peptide. For the conjugation of Re(CO)₃-PADA, activation by

TBTU/HOBT/DIPEA was tested, but resulted in an incomplete coupling, even after several repetitive coupling steps. Therefore, the activation with DIC/HOBT was used, where a 10-fold excess of DIC/HOBT and Re(CO)₃-PADA and a coupling time of 24 h was applied. For the PADA-peptides, a preactivation of PADA with DIC for 45 min and subsequently coupling with a 10-fold excess to the peptide was suitable. As the BFCA PADA was coupled to the selectively deprotected Lys⁴ side chain, the N-termini had to be protected, which was achieved by either Boc-protection or acetylation. The PADA-peptides and Re(CO)₃-PADA conjugates yielded in high purity (>95%), as determined by analytical HPLC. A synthesis scheme is shown in Figure 2, and the analytical data are summarized in Table 1.

Radiolabeling. Upon postlabeling of [$K^4(\text{PADA})$]-NPY, the aquaion ^{99m}Tc(CO)₃ did not only bind to the desired PADA, but as well to a secondary labeling site, as indicated by two peaks in the HPLC chromatogram. As His is known to be a strong binding chelator for the aquaion as well (26), we assumed that the His in position 26 of NPY is the second labeling site. Therefore, His²⁶ was substituted by Ala, and this [$K^4(\text{PADA})$,A²⁶]-NPY was selected for postlabeling. Specific labeling could be achieved, as shown by a single peak in the HPLC chromatogram. By the comigration of the resulting [$K^4(^{99m}\text{Tc}(\text{CO})_3\text{-PADA})$,A²⁶]-NPY with its cold ¹⁸⁵Re surrogate on HPLC (retention times 23.5 min and 23.7, respectively), identity was confirmed.

The prelabeling approach has been applied for labeling of the centrally truncated analogue [Ahx⁵⁻²⁴]-NPY. The resulting Ac-[Ahx⁵⁻²⁴, $K^4(^{99m}\text{Tc}(\text{CO})_3\text{-PADA})$]-NPY was produced with a yield of about 16%. As subsequent purification steps make the prelabeling approach very time-consuming, we applied postlabeling for the short analogue as well, again substituting His²⁶ by Ala. The resulting Ac-[Ahx⁵⁻²⁴, $K^4(^{99m}\text{Tc}(\text{CO})_3\text{-PADA})$,A²⁶]-NPY nicely comigrated with its cold analogue on RP-HPLC (retention times 20.5 min and 20.8, respectively).

Stability. Neither histidine nor cysteine led to significant ligand exchange and/or decomposition after 24 h incubation at 37 °C. The results of these challenge experiments are shown in Table 2. In addition, the degradation of [$K^4(^{99m}\text{Tc}(\text{CO})_3\text{-PADA})$,A²⁶]-NPY and Ac-[Ahx⁵⁻²⁴, $K^4(^{99m}\text{Tc}(\text{CO})_3\text{-PADA})$,A²⁶]-NPY was determined in human plasma. The former full length NPY analogue showed almost no degradation after 1 h. In contrast, the centrally truncated analogue Ac-[Ahx⁵⁻²⁴, $K^4(^{99m}\text{Tc}(\text{CO})_3\text{-PADA})$,A²⁶]-NPY was metabolized as shown in Figure 3, and a half-life time of 24.7 $\pm$ 2.0 min was calculated.

Receptor Binding Studies. To determine the affinity of the NPY analogues, competition binding assays at two neuroblastoma cell lines were performed ($n = 3$). The SK-N-MC cell line has been shown to express the NPY Y₁ receptor, whereas the SMS-KAN line is known to express the Y₂ receptor (27, 28). ¹⁸⁵Re was used as a cold surrogate for ^{99m}Tc. As shown in Table 3, the peptides were still able to bind to the corresponding receptors. The natural ligand pNPY has a high affinity to the Y₁ and Y₂ receptor subtype (IC₅₀ 1 and 0.5 nM, respectively). By replacing His²⁶ by Ala, this affinity decreased to 8.5 and 5.3 nM, respectively. The introduction of the BFCA PADA on the Lys⁴ side chain resulted only in a slight affinity reduction, and finally the cold radiopeptide analogue [$K^4(^{185}\text{Re}(\text{CO})_3\text{-PADA})$,A²⁶]-NPY exhibited an IC₅₀ value of 16.0 nM for the Y₁ and 8.5 nM for the Y₂ receptor. A similar stepwise reduction of receptor binding upon acetylation, attachment of PADA and Re(CO)₃-PADA was also seen for the short NPY analogue on the Y₂ receptor.

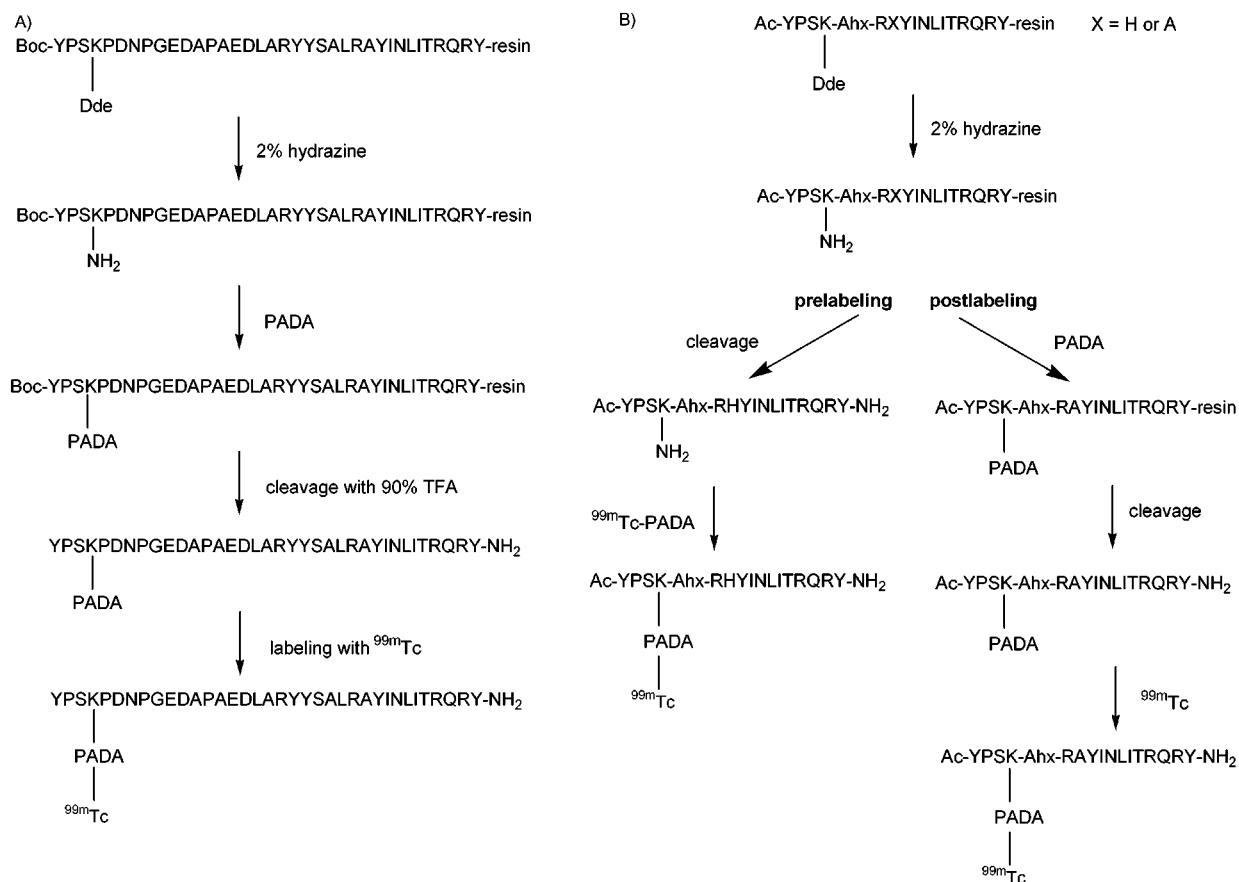


Figure 2. Synthesis scheme for ^{99m}Tc -labeled NPY analogues. (A) Synthesis and postlabeling of the full length NPY analogue. (B) Synthesis and pre- or postlabeling of the centrally truncated analogue.

Table 1. Analytical Data of the Prepared NPY Analogs

compound	molecular weight		retention time
	calcd	exptl	
[K ⁴ (PADA),A ²⁶]-NPY	4393.7	4394.3	21.1 ^a
[K ⁴ (¹⁸⁵ Re(CO) ₃ -PADA),A ²⁶]-NPY	4662.2	4663.5	21.7 ^a
Ac-[Ahx ⁵⁻²⁴ ,K ⁴ (PADA),A ²⁶]-NPY	2402.6	2403.0	26.2 ^b
Ac-[Ahx ⁵⁻²⁴ ,K ⁴ (¹⁸⁵ Re(CO) ₃ -PADA)]-NPY	2737.8	2739.2	27.2 ^b
Ac-[Ahx ⁵⁻²⁴ ,K ⁴ (¹⁸⁵ Re(CO) ₃ -PADA),A ²⁶]-NPY	2670.5	2672.1	28.5 ^b

^a 25% to 75% acetonitrile with 0.1% trifluoroacetic acid within 30 min. ^b 5% to 60% acetonitrile with 0.1% trifluoroacetic acid within 30 min.

Table 2. Chemical Stability [%] of the ^{99m}Tc -labeled NPY Derivatives against Ligand Exchange in 10 mM Histidine and Cysteine at 37 °C

	histidine	cysteine
[K ⁴ (^{99m}Tc (CO) ₃ -PADA),A ²⁶]-NPY		
1 h	98	99
4 h	98	98
24 h	96	97
Ac-[Ahx ⁵⁻²⁴ ,K ⁴ (^{99m}Tc (CO) ₃ -PADA),A ²⁶]-NPY		
1 h	98	99
4 h	97	98
24 h	95	96

[Ahx⁵⁻²⁴]-NPY has an IC₅₀ value of 0.2 nM, whereas the cold surrogate Ac-[Ahx⁵⁻²⁴,K⁴(¹⁸⁵Re(CO)₃-PADA),A²⁶]-NPY binds with 2.5 nM affinity. As the replacement of the amino acid sequence 5–24 of NPY by the spacer Ahx leads to subtype-specific high affinity analogue for the Y₂ receptor and a complete loss of affinity at all other receptors (29), these short analogues were not tested at the Y₁ receptor.

Internalization Studies. Internalization of the ^{99m}Tc -labeled peptides was investigated in vitro by incubating

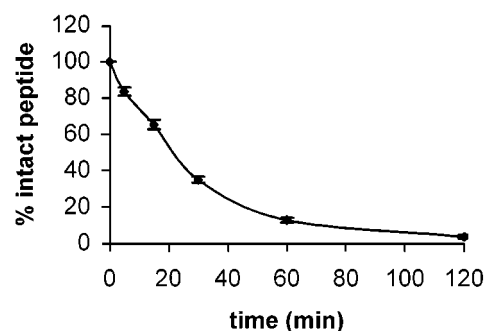
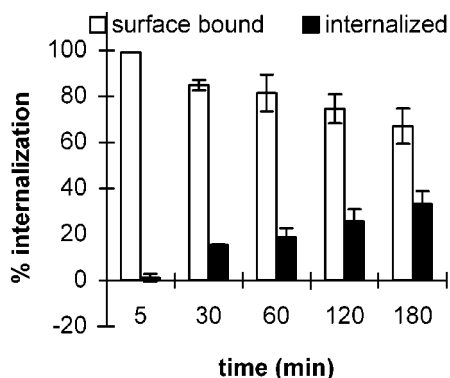


Figure 3. Proteolytic degradation of Ac-[Ahx⁵⁻²⁴,K⁴(^{99m}Tc (CO)₃-PADA),A²⁶]-NPY after incubation in human plasma at 37 °C.

[K⁴(^{99m}Tc (CO)₃-PADA),A²⁶]-NPY on SK-N-MC and Ac-[Ahx⁵⁻²⁴,K⁴(^{99m}Tc (CO)₃-PADA),A²⁶]-NPY on SMS-KAN cells at 37 °C. Almost no specific binding and internalization was observed for [K⁴(^{99m}Tc (CO)₃-PADA),A²⁶]-NPY. In contrast, Ac-[Ahx⁵⁻²⁴,K⁴(^{99m}Tc (CO)₃-PADA),A²⁶]-NPY was specifically internalized in SMS-KAN cells. The uptake was time-dependent, as shown in Figure 4. After 120 min, 25%, and after 180 min, 33% of the radiolabeled ligand

Table 3. Binding Affinities of PNPY Analogs at SK-N-MC (Y₁ Receptor) and SMS-kan (Y₂ Receptor) Cells

compound	IC ₅₀ [nM] at Y ₁	IC ₅₀ [nM] at Y ₂
NPY	1.0 ± 0.3	0.5 ± 0.1
[A ²⁶]-NPY	8.5 ± 0.2	5.3 ± 0.1
[K ⁴ (PADA),A ²⁶]-NPY	9.6 ± 0.3	6.2 ± 0.1
[K ⁴ (¹⁸⁵ Re(CO) ₃ -PADA),A ²⁶]-NPY	16.0 ± 0.1	8.5 ± 0.3
[Ahx ⁵⁻²⁴]-NPY	>4000	0.2 ± 0.1
Ac-[Ahx ⁵⁻²⁴]-NPY	n.d. ^a	0.7 ± 0.3
Ac-[Ahx ⁵⁻²⁴ ,K ⁴ (PADA),A ²⁶]-NPY	n.d.	1.3 ± 0.8
Ac-[Ahx ⁵⁻²⁴ ,K ⁴ (¹⁸⁵ Re(CO) ₃ -PADA),A ²⁶]-NPY	n.d.	1.4 ± 0.5
Ac-[Ahx ⁵⁻²⁴ ,K ⁴ (¹⁸⁵ Re(CO) ₃ -PADA),A ²⁶]-NPY	n.d.	2.5 ± 0.1

^a n.d. = not determined.**Figure 4.** Internalization studies. Specific internalization of Ac-[Ahx⁵⁻²⁴,K⁴(^{99m}Tc(CO)₃-PADA),A²⁶]-NPY on SMS-KAN cells at 37 °C over the time. Surface bound and internalized fractions of the radiolabeled ligand are shown.**Table 4. Biodistribution of Ac-[Ahx⁵⁻²⁴,K⁴(^{99m}Tc(CO)₃-PADA),A²⁶]-NPY in BALB/c Mice after iv Injection. Data Are Expressed as % ID/g of Tissue ± SD**

tissue	1.5 h			24 h
	A ^a	B ^b	C ^c	D ^d
blood	1.02 ± 0.30	1.17 ± 0.33	1.52 ± 0.28	0.17 ± 0.03
heart	0.65 ± 0.13	0.82 ± 0.28	1.06 ± 0.19	0.25 ± 0.05
lung	1.44 ± 0.29	3.61 ± 1.61	3.74 ± 0.94	1.02 ± 0.68
spleen	0.83 ± 0.20	1.06 ± 0.25	1.15 ± 0.06	0.36 ± 0.08
kidney	8.21 ± 1.69	11.05 ± 2.19	8.74 ± 1.92	4.08 ± 0.59
pancreas	1.01 ± 0.34	2.79 ± 3.97	4.50 ± 2.27	0.32 ± 0.05
stomach	3.11 ± 1.34	4.19 ± 1.78	8.27 ± 0.20	0.36 ± 0.07
duodenum	4.10 ± 2.53	6.67 ± 3.34	28.29 ± 10.43	0.24 ± 0.06
ileum	8.37 ± 7.06	11.83 ± 9.79	2.59 ± 1.05	0.22 ± 0.06
colon	1.89 ± 1.67	1.36 ± 0.67	3.54 ± 0.47	0.24 ± 0.07
liver	8.24 ± 1.64	11.79 ± 2.73	15.40 ± 1.60	4.19 ± 0.91
muscle	0.32 ± 0.08	0.35 ± 0.12	0.53 ± 0.12	0.10 ± 0.01
bone	0.41 ± 0.103	0.50 ± 0.16	0.60 ± 0.07	0.10 ± 0.02
brain	0.05 ± 0.01	0.08 ± 0.04	0.11 ± 0.01	0.01 ± 0.00

^a Group A (*n* = 7) received 3.7 MBq of Ac-[Ahx⁵⁻²⁴,K⁴(^{99m}Tc(CO)₃-PADA),A²⁶]-NPY; ^b Group B (*n* = 7) received 3.7 MBq of Ac-[Ahx⁵⁻²⁴,K⁴(^{99m}Tc(CO)₃-PADA),A²⁶]-NPY coinjected with cold [Ahx⁵⁻²⁴]-NPY (0.3 mg/mouse); ^c Group C (*n* = 3) received 3.7 MBq of Ac-[Ahx⁵⁻²⁴,K⁴(^{99m}Tc(CO)₃-PADA),A²⁶]-NPY coinjected with cold NPY (0.25 mg/mouse); ^d Group D (*n* = 4) received 3.7 MBq of Ac-[Ahx⁵⁻²⁴,K⁴(^{99m}Tc(CO)₃-PADA),A²⁶]-NPY.

were internalized, whereas the surface-bound fraction accordingly decreased.

Biodistribution. Table 4 shows the tissue distribution of Ac-[Ahx⁵⁻²⁴,K⁴(^{99m}Tc(CO)₃-PADA),A²⁶]-NPY in BALB/c mice after intravenous injection. Time points at 1.5 (row A) and 24 h p.i. (row D) were chosen in order to follow the time course of disappearance from the blood, kidney, and liver. The peptide exhibited a rapid clearance from the blood pool. The levels after 24 h in both kidneys and liver might indicate urinary and hepatobiliary excretion. Blocking has been performed with cold [Ahx⁵⁻²⁴]-NPY and full length NPY. Blood, heart, spleen, brain, muscle, and bone had small activities and no difference

in the uptake with or without coinjection. Differences were observed after coinjection with cold NPY, especially at the gastrointestinal tract, where the highest uptake was found in the duodenum. However, specific blocking by a cold analogue could usually not be observed, and the interindividual variations in the mice were considerably high.

DISCUSSION

In the postlabeling approach, a BFCA is first attached to the peptide, and the radionuclide is subsequently coupled to the free chelating group of the BFCA (30). This method can be combined with the advantages of solid-phase peptide synthesis (SPPS). Applying a molar excess of BFCA, complete coupling to the resin-bound peptide can be achieved, and unreacted BFCA is easily washed away. Subsequent cleavage from the resin yields the highly pure BFCA-peptide conjugate. Using the organometallic aquaion [^{99m}Tc(OH)₂(CO)₃]⁺ has been shown to be very suitable for labeling (31). Efficient postlabeling of histidine derivatives of neurotensin has been performed (32, 33), and a bombesin analogue was successfully prelabeled applying PADA as BFCA (10).

Accordingly, we synthesized NPY derivatives on a rinkamide resin, selectively deprotected Lys⁴ by hydrazine and coupled the BFCA PADA to this side chain. Upon postlabeling of [K⁴(PADA)]-NPY, the ^{99m}Tc did not only bind to the desired PADA, but presumably as well to the His in position 26, as His is a preferred binding chelator (26). Therefore, we tried to replace the disturbing amino acid, which is not an essential position for binding (16, 34). The replacement of His²⁶ by Ala resulted in a specific ^{99m}Tc-labeling of PADA. As assumed, high binding affinity at the Y₁ and Y₂ receptor was maintained, as tested by the cold ¹⁸⁵Re surrogate.

Another way how to circumvent the attachment of ^{99m}Tc to His²⁶ is the prelabeling approach, where the radionuclide-BFCA complex is formed prior to its attachment to the peptide (30). To avoid polymerization due to the activation in solution, we applied the shortened analogue [Ahx⁵⁻²⁴]-NPY, in which all the reactive aspartate amino acids are omitted. By replacing the sequence 5–24 in NPY by Ahx, receptor subtype selectivity for the Y₂ receptor is achieved (29). The resulting Ac-[Ahx⁵⁻²⁴,K⁴(^{99m}Tc(CO)₃-PADA)]-NPY was produced with a yield of only 16%. Therefore, we again substituted His²⁶ by Ala and attached PADA on the Lys⁴ side chain. Competitive binding assays showed surprisingly high affinity of the so produced Ac-[Ahx⁵⁻²⁴,K⁴(¹⁸⁵Re(CO)₃-PADA),A²⁶]-NPY, considering the bulky chelator on the short peptide sequence. Thus, although substitution of a single amino acid, introduction of spacer, N-terminal protection and introduction of an organometallic moiety was performed, the NPY analogues maintained high binding to their corresponding receptor.

Internalization studies with the two ^{99m}Tc labeled analogues revealed receptor-mediated internalization only for the short analogue Ac-[Ahx⁵⁻²⁴,K⁴($^{99m}\text{Tc}(\text{CO})_3\text{-PADA,A}^{26}$)]-NPY. We have previously shown that fluorescent and ^3H -marked NPY internalizes in a time- and temperature-dependent manner (25), determined to be 20–30%, which corresponds to our current measurements. As internalization may lead to higher uptake and retention in the target tissue, the short analogue seems to be more appropriate for future application as a tumor imaging agent. Moreover, it revealed to be stable to enzymatic degradation. Most peptides undergo rapid proteolysis in plasma by endogenous peptidases (35) and have to be molecularly engineered to prevent their enzymatic destruction (12). The N-terminal acetylation and introduction of an unnatural spacer Ahx within the sequence made our analogue a stable candidate, as the natural ligand NPY exhibits a half-life time of only 4 min in humans (36), whereas we were able to increase the stability to a half-life time of about 24 min. This is a crucial step in the development of a novel peptide radiopharmaceutical, since the normally rapid enzymatic degradation is one of the major disadvantages of peptides. As an example, only the discovery of the stable somatostatin analogue octreotide ($t_{1/2}$ 90 min vs the $t_{1/2}$ 3 min of somatostatin) enabled the use and success of OctreoScan, the most commonly used radiopharmaceutical to date.

The full length NPY analogue [K⁴($^{99m}\text{Tc}(\text{CO})_3\text{-PADA,A}^{26}$)]-NPY seems to have lost its biological activity upon postlabeling, as no specific binding to the Y₁ receptor or receptor-mediated internalization could be detected. This might explain as well its too-good stability in plasma: the enzymes involved in the metabolism of NPY might be not capable of degrading this biologically inactive form.

In the biodistribution studies, the short peptide exhibited a rapid clearance from the blood pool. As seen by the low activity in the brain, the peptide is not crossing the intact blood–brain barrier. Blocking studies have been performed with cold [Ahx⁵⁻²⁴]-NPY (Y₂ selective) and full length NPY (binds to all NPY subtype receptors). Blood, heart, spleen, muscle, and bone had small activities and no difference in the uptake with or without coinjection, indicating either no receptors or at least no Y₂ receptors in these tissues. Differences were observed after coinjection with cold NPY, especially at the gastrointestinal tract, an area which expresses NPY receptors. An explanation for this fact could be the inhibitory effect of the cold NPY on contractility and secretion in the intestine (37). Upon injection of the labeled short analogue alone, the highest activity was found at the ileal level, whereas upon coinjection with cold NPY, the highest activity corresponded to the duodenal level. This might be provoked by the high NPY dosage inducing slower transit and inhibition of intestinal fluid secretion. However, this high accumulation at intestinal level could hide a possible specific binding or blocking of the labeled analogue to its receptor. Generally, there is little known about the Y₂ receptor distribution in mice, and distribution varies from one species to another, as shown in autoradiographic studies performed in rabbit and rat (38, 39).

In conclusion, we here demonstrate for the first time the novel application of modified NPY analogues as radiopharmaceuticals. Using our knowledge of structure–activity relationships of NPY, and combining the advantages of SPPS and a stable, well-defined BFCA such as PADA for complexing [$^{99m}\text{Tc}(\text{OH}_2)_3(\text{CO})_3$]⁺, we could produce a chemically and metabolically stable candidate,

which maintains high binding affinity to its receptor and even exhibits receptor-mediated internalization. Therefore, Ac-[Ahx⁵⁻²⁴,K⁴($^{99m}\text{Tc}(\text{CO})_3\text{-PADA,A}^{26}$)]-NPY has promising characteristics for future applications in tumor diagnosis or even therapy, when using a suitable radionuclide such as ^{188}Re .

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A Novel Approach to the High-Specific-Activity Labeling of Small Peptides with the Technetium-99m Fragment [$^{99m}\text{Tc}(\text{N})(\text{PXP})$] $^{2+}$ (PXP = Diphosphine Ligand)

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A new labeling approach for incorporating bioactive peptides into a technetium-99m coordination complex is described. This method exploits the chemical properties of the novel metal-nitrido fragment [$^{99m}\text{Tc}(\text{N})(\text{PXP})$] $^{2+}$, composed of a terminal $\text{Tc}\equiv\text{N}$ multiple bond bound to an ancillary diphosphine ligand (PXP). It will be shown that this basic, molecular building block easily forms in solution as the dichloride derivative [$^{99m}\text{Tc}(\text{N})(\text{PXP})\text{Cl}_2$], and that this latter complex selectively reacts with monoanionic and dianionic, bidentate ligands (YZ) having soft, π -donor coordinating atoms to afford asymmetrical nitrido heterocomplexes of the type [$^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{YZ})$] $^{0/+}$ without removal of the basic motif [$^{99m}\text{Tc}(\text{N})(\text{PXP})$] $^{2+}$. The reactions of the amino acid cysteine was studied in detail. It was found that cysteine readily coordinates to the metal fragment [$^{99m}\text{Tc}(\text{N})(\text{PXP})$] $^{2+}$ either through the [NH_2 , S^-] pair of donor atoms or, alternatively, through the [O^- , S^-] pair, to yield the corresponding asymmetrical complexes in very high specific activity. Thus, these results were conveniently employed to devise a new, efficient procedure for labeling short peptide sequences having a terminal cysteine group available for coordination to the [$^{99m}\text{Tc}(\text{N})(\text{PXP})$] $^{2+}$ fragment. Examples of the application of this novel approach to the labeling of the short peptide ligand H-Arg-Gly-Asp-Cys-OH ($\text{H}_2\text{1}$) and of the peptidomimetic derivative H-Cys-Val-2-Nal-Met-OH (H_2) will be discussed.

INTRODUCTION

The labeling of biologically relevant molecules with γ - and positron-emitting radionuclides is currently an active field of research in diagnostic nuclear medicine, as it is mostly focused on the production and development of new categories of imaging agents for in vivo monitoring of metabolic processes and receptor distributions (1–12). A central issue of this effort comes from the requirement that the labeling procedure should not dramatically change the biological properties of the selected active molecule. Generally, the introduction of a positron-emitting radionuclide such as ^{11}C , ^{18}F , and ^{13}N requires only a slight modification of the chemical structure of the starting biological substrate due to the natural occurrence of these elements in biological compounds. However, these positron-emitting nuclides can be obtained only through the use of a cyclotron, and their radioactive decays are, usually, characterized by short half-lives. This fact strongly limits the application of positron-emitting tracers for routine clinical studies. In contrast, the γ -emitting radionuclide technetium-99m exhibits almost ideal nuclear properties for nuclear imaging ($E_\gamma = 140.5$ keV, $t_{1/2} = 6.01$ h) and is easily available through a $^{99}\text{Mo}/^{99m}\text{Tc}$ generator (13). Nonetheless, technetium is a transition metal having almost no natural occurrence, and its chemical behavior is far from being similar to that of any biologically relevant element. Thus, it has to be expected that, in some situations, the labeling of a

biomolecule with ^{99m}Tc might give rise to a strong perturbation of its chemical structure and, consequently, to the loss of its biological properties. To avoid this problem, a number of chemical strategies have been proposed. At present, the most successful procedure, known as 'bifunctional approach', consists of tethering a strong chelating group for the metal to a point of a biomolecule that is irrelevant for preserving its biological properties (1, 8, 12). The resulting bifunctional ligand would act as a bridging backbone between the metal center and the bioactive group. This method has been successfully applied to the production of a number of new Tc-99m radiopharmaceuticals with different bioactive compounds and was mostly exploited using the chemistry of oxo complexes containing the [$\text{Tc}=\text{O}$] $^{3+}$ basic motif (1, 3, 8–11). However, it suffers from many drawbacks, as it usually requires a large excess of ligand to achieve a satisfactory labeling yield (>90%) and, in addition, the resulting complexes are not always sufficiently stable.

It is apparent that the bifunctional approach puts its main concern on the design of the bifunctional ligand itself. Recently, an alternative strategy for the labeling of biomolecules has emerged. The central idea behind this new radiopharmaceutical design is to set up a specific arrangement of ligands bound to a technetium center, which should exhibit a remarkable inertness toward oxidation–reduction reactions. An additional characteristic of this hypothetical precursor metal complex should be also in possessing a few substitution-labile coordination positions where some transient ligand could be easily replaced by a stronger coordinating group without affecting that part of the complex corresponding to the 'robust' metal-containing fragment. In this new context,

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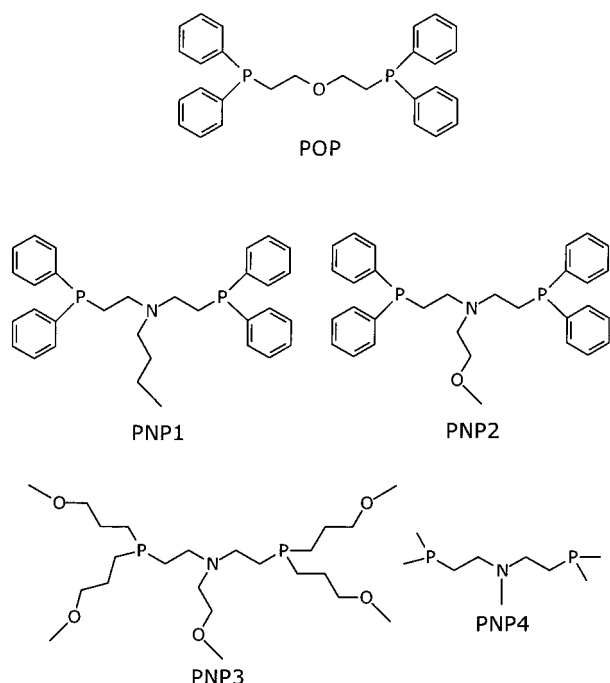


Figure 1. The diphosphine ligands (PXP) utilized in this study.

therefore, the main focus becomes shifted from the ligand to the metal-containing block. When the incoming ligand has been made to carry on a bioactive side-chain, its selective interaction with the metal fragment would result in the prompt formation of a final complex incorporating the biological substrate. The first, definite example of this novel approach has been reported by Alberto and co-workers (14–17). These authors were able to devise an efficient preparation, at tracer level, of the precursor aquo-carbonyl Tc-99m complex $[^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$. In this species, the technetium ion has a +1 oxidation state and appears highly stable toward changes of its oxidation number due to the inertness of the d^6 electronic configuration. In addition, the three water molecules are only weakly bound to the metal center and can be easily displaced by some incoming bidentate or tridentate ligand to form new complexes. In contrast, the carbonyl fragment $[^{99m}\text{Tc}(\text{CO})_3]^+$ remains untouched in the course of the substitution reaction. Thus, the metal fragment $[^{99m}\text{Tc}(\text{CO})_3]^+$ could be viewed as an efficient synthon for the preparation of a wide class of carbonyl Tc(I) complexes.

A further example of this type of radiopharmaceutical design is described here. It is based on the chemical properties of the new metal nitrido fragment $[^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$, where PXP represents an ancillary diphosphine ligand having some heteroatom ($X = \text{N}, \text{O}$) within the backbone connecting the two P atoms (Figure 1). The fragment $[^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$ is easily produced in aqueous physiological solution as the dichloride derivative $[^{99m}\text{Tc}(\text{N})(\text{PXP})\text{Cl}_2]$. It will be shown that this precursor complex selectively reacts with ligands having soft π -donor coordinating atoms such as S, O, and N without removal of both the $\text{Tc}\equiv\text{N}$ multiple bond and the ancillary PXP ligand. In particular, it was found that, at tracer level, cysteine readily reacts with $[^{99m}\text{Tc}(\text{N})(\text{PXP})\text{Cl}_2]$ as a bidentate ligand through the $[\text{NH}_2, \text{S}^-]$ or $[\text{O}^-, \text{S}^-]$ pair of chelating atoms in nearly stoichiometric ratio with the radiometal. In principle, therefore, biologically active molecules having a terminal cysteine group could be efficiently labeled with Tc-99m using this alternative

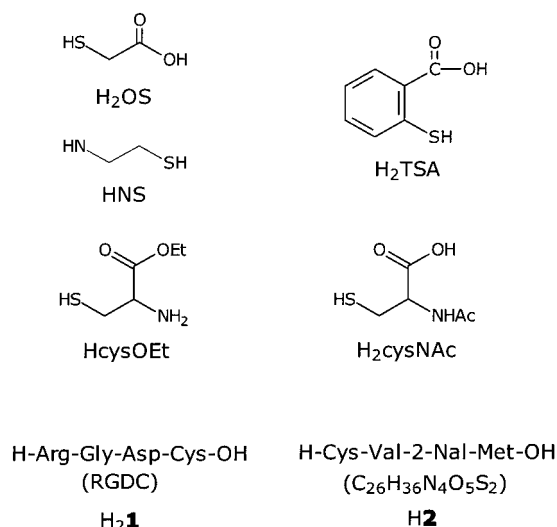


Figure 2. Ligands and peptide-derived ligands utilized in this study.

route. The application of these findings to the labeling of short peptide sequences will be discussed.

EXPERIMENTAL PROCEDURES

General. Reagents employed in the preparations were purchased from Aldrich Chimica (Milan, Italy) or Fluka (Milan, Italy) and used without further purification. Succinic dihydrazide [SDH = $\text{H}_2\text{N}-\text{NH}-(\text{O}=\text{C})-(\text{CH}_2)_2-\text{C}(=\text{O})-\text{NH}-\text{NH}_2$], L-cysteine ethyl ester hydrochloride (HcysOEt·HCl), N-acetyl-L-cysteine (H₂cysNAC), glutathione (GSH), and the bidentate ligands 2-aminoethanethiol hydrochloride (HNS·HCl), mercaptoacetic acid (H₂OS), and thiosalicylic acid (H₂TSA) (Figure 2) were obtained from Aldrich Chimica. The peptide ligand H₂1 and the peptidomimetic derivative H₂2 (Figure 2) were obtained from Bachem AG (Bubendorf, Switzerland). The diphosphine ligands bis(diphenylphosphino)ether [POP = $(\text{C}_6\text{H}_5)_2\text{P}(\text{CH}_2)_2\text{O}(\text{CH}_2)_2\text{P}(\text{C}_6\text{H}_5)_2$], bis(diphenylphosphino)ethyl n-butylamine [PNP1 = $(\text{C}_6\text{H}_5)_2\text{P}(\text{CH}_2)_2-\text{N}(\text{C}_4\text{H}_9)-(\text{CH}_2)_2\text{P}(\text{C}_6\text{H}_5)_2$], bis(diphenylphosphino)ethyl methoxyethylamine [PNP2 = $(\text{C}_6\text{H}_5)_2\text{P}(\text{CH}_2)_2-\text{N}(\text{C}_2\text{H}_4\text{OCH}_3)-(\text{CH}_2)_2\text{P}(\text{C}_6\text{H}_5)_2$], bis(dimethoxypropylphosphino)ethyl methoxyethylamine [PNP3 = $(\text{CH}_3\text{OC}_3\text{H}_6)_2\text{P}(\text{CH}_2)_2-\text{N}(\text{C}_2\text{H}_4\text{OCH}_3)-(\text{CH}_2)_2\text{P}(\text{C}_3\text{H}_6\text{OCH}_3)_2$], and bis(dimethylphosphino)ethyl methylamine [PNP4 = $(\text{CH}_3)_2\text{P}-\text{N}(\text{CH}_3)-\text{P}(\text{CH}_3)_2$] (Figure 1) were prepared using literature procedures (18–20) or purchased from Argus Chemical (Florence, Italy). High performance liquid chromatography (HPLC) was performed on a System Gold Instrument equipped with a programmable solvent Module 126, a scanning detector Module 166, and a radioisotope detector Module 170 (Beckman Instruments, Fullerton, CA). Chromatographic analyses were carried out using a reversed-phase C₁₈ Beckman ODS precolumn (25 × 4.6 mm) and a reversed-phase C₁₈ Beckman ODS column (250 × 4.6 mm, $\phi = 10 \mu\text{m}$). Thin-layer chromatography (TLC) was carried out on silica gel plates and reversed-phase C18 plates (Merck). TLC chromatograms were analyzed with a Cyclone instrument equipped with a phosphor imaging screen and an OptiQuant image analysis software (Packard, Meriden, CT). The radiochemical purity (RCP) of Tc-99m complexes was expressed as the ratio between the activity associated with a single radiocompound, revealed as a single spot on a TLC strip, and the total activity on the strip or, alternatively, as the percent of the area under the corresponding HPLC peak.

Table 1. Relevant Chromatographic Data for Tc-99m Complexes

complex	R_f (SiO_2)	R_f (C_{18})	retention time (min)
$^{99m}\text{Tc}(\text{N})(\text{POP})\text{Cl}_2$	0.66 ^a		
$^{99m}\text{Tc}(\text{N})(\text{PNP1})\text{Cl}_2$	0.82 ^a		
$^{99m}\text{Tc}(\text{N})(\text{PNP2})\text{Cl}_2$	0.90 ^a		
$^{99m}\text{Tc}(\text{N})(\text{PNP3})\text{Cl}_2$	0.86 ^a		20.31 ^e
$^{99m}\text{Tc}(\text{N})(\text{PNP4})\text{Cl}_2$	0.13 ^a		19.68 ^f
$^{99m}\text{Tc}(\text{N})(\text{POP})(\text{TSA})$	0.88 ^a		
$^{99m}\text{Tc}(\text{N})(\text{PNP2})(\text{NS})^+$	0.38 ^a		
$^{99m}\text{Tc}(\text{N})(\text{PNP2})(\text{TSA})$		0.61 ^c	
$^{99m}\text{Tc}(\text{N})(\text{PNP3})(\text{NS})^+$	0.16 ^a	0.48 ^c	6.20 ^f
$^{99m}\text{Tc}(\text{N})(\text{PNP3})(\text{OS})$	0.76 ^a	0.26 ^c	6.40 ^f
$^{99m}\text{Tc}(\text{N})(\text{PNP3})(\text{cysNac})$		0.24, ^d	52.50, ^g
		0.17 ^d	56.20 ^g
<i>syn, anti</i> - $^{99m}\text{Tc}(\text{N})(\text{PNP3})(\text{cysOEt})^+$		0.16, ^d	24.78, ^e
		0.10 ^d	26.57 ^e
$^{99m}\text{Tc}(\text{N})(\text{PNP4})(\text{OS})$		0.41 ^c	
$^{99m}\text{Tc}(\text{N})(\text{PNP4})(\text{TSA})$		0.35 ^c	
<i>syn, anti</i> - $^{99m}\text{Tc}(\text{N})(\text{PNP4})(\text{cysNac})$	0.30, ^a	0.36, ^c	
	0.18 ^a	0.18 ^c	
<i>syn, anti</i> - $^{99m}\text{Tc}(\text{N})(\text{PNP4})(\text{cysOEt})^+$	0.16, ^a	0.70, ^c	
	0.15 ^a	0.68 ^c	
<i>syn, anti</i> - $^{99m}\text{Tc}(\text{N})(\text{PNP3})(1)$	0.12 ^b		5.38, ^h
			7.12 ^h
<i>syn, anti</i> - $^{99m}\text{Tc}(\text{N})(\text{PNP3})(2)^+$	0.47 ^b		22.58 ⁱ

^a Ethanol/ $\text{CHCl}_3/\text{C}_6\text{H}_6$ (1.5:2:1.5). ^b Ethanol/ CHCl_3 /Toluene/ NH_4Ac (0.5 M) (6:2:0.5:1.5). ^c $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (90:10). ^d $\text{CH}_3\text{CN}/\text{Et}_3\text{N}$ (0.1 M in 1.0 M H_3PO_4 , pH = 3.0) (50:50). ^e Reversed-phase C_{18} column; A, Et_3N (0.1 M in 1.0 M- H_3PO_4 , pH = 3.0), B, CH_3CN . Gradient: 0–5 min, B = 0%; 5–15 min, B = 30%; 15–20 min, B = 50%; 20–30 min, B = 50%; 30–35 min, B = 0%. Flow rate: 1 mL/min. ^f Reversed-phase C_{18} column; A, Et_3N (0.1 M in 1.0 M- H_3PO_4 , pH = 3.0), B, CH_3CN . Gradient: 0 min, B = 40%; 1–10 min, B = 60%; 10–28 min, B = 40%. Flow rate: 1 mL/min. ^g Isocratic: $\text{CH}_3\text{CN}/\text{PBS}$ buffer (0.02 M, pH = 7.0) (20:80). Flow rate: 1 mL/min. ^h Reversed-phase C_{18} column; A, H_2O [containing trifluoroacetic acid (TFA) 0.1% v/v], B, CH_3CN [containing TFA 0.1% v/v]. Gradient: 0–5 min, B = 30% (isocratic); 5–30 min, B = 60%; 30–40 min, B = 70%; 40–45 min, B = 30%. Flow rate: 1 mL/min. ⁱ Reversed-phase C_{18} column; A, H_2O [containing trifluoroacetic acid (TFA) 0.1% v/v], B, CH_3CN [containing TFA 0.1% v/v]. Gradient: 0–5 min, B = 40% (isocratic); 5–30 min, B = 60%; 30–40 min, B = 70%; 40–45 min, B = 40%. Flow rate: 1 mL/min.

The chemical nature of all Tc-99m compounds described in this study was investigated by chromatographic analysis. For selected complexes, a further comparison was performed between their chromatographic behavior and that of the corresponding complexes prepared at the macroscopic level with the long-lived isotope Tc-99 g as described elsewhere (21–24). Chromatographic data for the resulting products are showed in Table 1.

Preparation of the Complexes $^{99m}\text{Tc}(\text{N})(\text{PXP})\text{Cl}_2$ (PXP = POP, PNP1, PNP2, PNP3, PNP4). A 5.0 mg amount of SDH, 0.05 mg of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ (suspended in 0.1 mL of saline), and 1.0 mL of ethanol were placed in a vial purged with argon, followed by addition of 0.250 mL of generator-eluted $\text{Na}^{99m}\text{TcO}_4$ (activity range 50 MBq–2 GBq). The mixture was kept at room temperature for 30 min. Then, 3.0 mg of the appropriate diphosphine ligand PXP (dissolved in 0.25 mL of ethanol) were added, and the reaction solution was further kept at room temperature for 30 min (yield > 98%).

Preparation of the Complexes $^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{YZ})^{0/+}$ (PXP = PNP2, PNP3, PNP4; YZ = H_2TSA , NSH , H_2OS). *Method a.* After completing the procedure described above for the preparation of the precursor complex $^{99m}\text{Tc}(\text{N})(\text{PXP})\text{Cl}_2$, 5.0 mg of the appropriate YZ ligand was added, and the reaction mixture was heated at 100 °C for 30 min (yield > 95%).

Method b. A 5.0 mg amount of SDH, 0.05 mg of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ (suspended in 0.1 mL of saline) and 1.0 mL of ethanol were placed in a vial purged with argon, followed by 0.250 mL of generator-eluted $\text{Na}^{99m}\text{TcO}_4$ (activity range 50 MBq–2 GBq). The solution was kept at room temperature for 30 min. Then, 5.0 mg of the appropriate YZ ligand was added to the reaction mixture that was further kept at room temperature for 30 min or, alternatively, heated at 100 °C for 15–20 min. Finally, after addition of 3.0 mg of the appropriate diphosphine ligand PXP (dissolved in 0.20 mL of ethanol), the reaction solution was heated at 100 °C for 30 min (yield > 90%).

Method c. A 5.0 mg amount of SDH, 0.05 mg of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ (suspended in 0.1 mL of saline) and 1.0 mL of ethanol were placed in a vial purged with argon. Then, 0.250 mL of generator-eluted $\text{Na}^{99m}\text{TcO}_4$ (activity range 50 MBq–2 GBq) were added, and the mixture was kept at room temperature for 30 min. Finally, 1.0 mg of PXP (dissolved in 0.20 mL of ethanol) and 3.0 mg of the appropriate YZ ligand were added to the resulting solution, and the reaction was heated at 100 °C for 30 min (yield > 95%).

Preparation of the Complexes $^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{cysOEt})^+$ and $^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{cysNac})$ (PXP = PNP3, PNP4). These complexes were prepared following both methods a and c as described before. Briefly, using method a, 5.0 mg of cysOEt (or cysNac) was added to the vial containing the intermediate complex $^{99m}\text{Tc}(\text{N})(\text{PXP})\text{Cl}_2$. Alternatively, using method c, 3.0 mg of the appropriate PXP ligand and 5.0 mg of cysOEt (or cysNac) were mixed in the same vial containing the mixture of Tc-99m precursor nitrido complexes first produced by reaction of $\text{Na}^{99m}\text{TcO}_4$ with SDH and Sn^{2+} ions. In both preparations, the resulting solutions were heated at 100 °C for 30 min (yield > 95% for both preparations).

High-Specific-Activity Labeling of Cysteine-Derived Ligands. In all preparations described below, freshly generator-eluted $\text{Na}^{99m}\text{TcO}_4$ (4.0 GBq), contained in a volume of saline ≤ 0.25 mL, was used. The final volume of the reaction solution was always kept within the range 1.5–2.0 mL by addition of saline. Solutions of the cysteine-derived ligands were always stored under an argon atmosphere and checked before use with Ellman's test (25) to ensure the absence of disulfide bridges.

In a vial purged with argon, $\text{Na}^{99m}\text{TcO}_4$ was mixed with 5.0 mg of SDH, 0.05 mg of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ (suspended in 0.1 mL of saline), and 1.0 mL of ethanol. The resulting solution was kept at room temperature for 30 min. Then, the ligands PNP3 (dissolved in 0.2 mL of ethanol) and cysOEt (or cysNac) (dissolved in 0.2 mL of water) were added together to the same vial, and the reaction solution was heated at 100 °C for 1 h. More precisely, in a series of preparations, the amount of the diphosphine ligand was fixed and that of the cysteine-derived ligand was progressively decreased. The final labeling yield was determined by HPLC analysis. Results were analyzed by plotting the percent radiochemical yield as a function of the amount of the cysteine-derived ligand used in each preparations (see Results).

Stability. The in vitro stability of the heterocomplexes described here was evaluated by monitoring RCP at different time points (15, 30, 60, 120, 360 min) using the following procedure. After preparation, the selected complex was purified by HPLC. Then, 100 μL of the resulting eluate were incubated at 37 °C with 900 μL of (a) saline, (b) PBS, (c) rat serum, and (d) human serum. RCP was measured by TLC analysis.

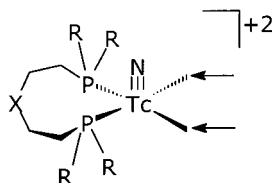


Figure 3. Schematic drawing of the structure of the metal-containing fragment $[^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$.

Cysteine and Glutathione (GSH) Challenge. A 250 μL volume of PBS (0.2 M, pH = 7.4), 150 μL of water, 50 μL of a freshly prepared aqueous solution of L-cysteine or, alternatively, of GSH (10.0 mM), and 50 μL of the appropriate HPLC-purified complex were placed in a test tube and incubated in a water bath at 37 $^{\circ}\text{C}$. A blank experiment was carried out using an equal volume of H_2O in place of the cysteine (GSH) solution. Aliquots of the resulting solutions were withdrawn at 1, 15, 30, 60, and 120 min, and analyzed by TLC.

RESULTS

Preparation of the Complexes $[^{99m}\text{Tc}(\text{N})(\text{PXP})\text{Cl}_2]$ (PXP = POP, PNP1, PNP2, PNP3, PNP4). The precursor metal fragment $[^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$ ($\text{X} = \text{O}, \text{N}$) easily forms, at tracer level (μM concentration), by simple mixing of the ligand PXP with a solution containing a starting nitrido Tc(V) complex. Essentially, this two-step preparation requires the preliminary action on Tc-99m pertechnetate of different reagents such as a donor of nitrido nitrogen atoms (N^{3-}) (SDH) and a reducing agent (SnCl_2) to obtain a mixture of intermediate complexes all containing the $\text{Tc}\equiv\text{N}$ group. The detailed description of this latter procedure for preparing the $\text{Tc}\equiv\text{N}$ bond at tracer level has been reported previously (26–28). In turn, the resulting mixture of nitrido intermediates is quantitatively converted into the precursor metal fragment by reaction with PXP. The structure of the fragment $[^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$ is schematically drawn in Figure 3. It is formed by a terminal $\text{Tc}\equiv\text{N}$ multiple bond coordinated to a diphosphine ligand PXP through the two neutral phosphorus atoms. It is easy to note that the molecular arrangement $[^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$ is coordinatively unsaturated and thus it cannot exist in solution as an independent species. In fact, two residual positions of the coordination geometry are available for the bonding of two monodentate ligands or of one bidentate ligand (arrows in Figure 3). This suggests that, in aqueous physiological solution, this fragment should appear under different chemical forms depending on the pH. In particular, the formation of the aqua complex $[^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{H}_2\text{O})_2]^{2+}$, and of the hydroxo complexes $[^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{H}_2\text{O})(\text{OH})]^+$ and $[^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{OH})_2]$, could be envisaged as well as that of a series of chloro complexes of the type $[^{99m}\text{Tc}(\text{N})(\text{PXP})\text{Cl}_2]$, $[^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{H}_2\text{O})\text{Cl}]$, and $[^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{OH})\text{Cl}]$. This prediction was nicely confirmed by HPLC analysis of aqueous solutions of this fragment. Actually, though it was commonly observed that the dichloro complex was always the predominant species in solution (Figure 4), a mixture of different compounds was produced after addition of a buffer. In particular, when the preparation was conducted at pH = 7.4 by addition of a phosphate buffer, only 60% of the activity in solution was associated with the mixed halogeno-diphosphine complex $[^{99m}\text{Tc}(\text{N})(\text{PXP})\text{Cl}_2]$. Furthermore, a mixture of different compounds, always including the dichloro-diphosphine complex, was obtained both at lower pH (acetate buffer, pH = 4.5) and at higher pH (carbonate buffer, pH = 8.5). The distribution of the activity between the various species was also found to

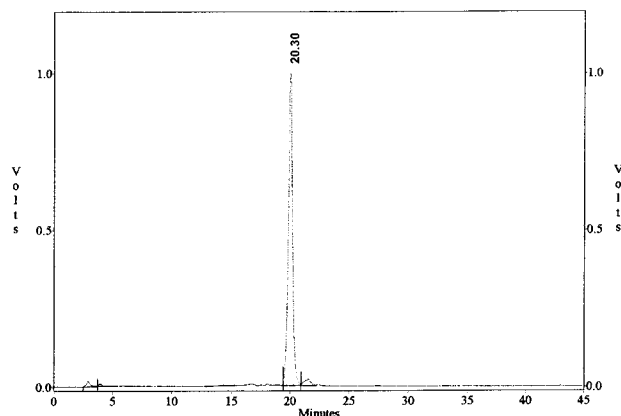


Figure 4. HPLC chromatogram of the complex $[^{99m}\text{Tc}(\text{N})(\text{PNP3})\text{Cl}_2]$. It elutes at a retention time of 20.30 min.

be dependent on temperature. Though it was difficult to determine exactly the chemical nature of all complexes in the mixture, the finding that these compounds were easily and quantitatively converted into a single product of the type $[^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{YZ})]^{0/+}$ by reaction with a bidentate chelating ligand (YZ) (see below) strongly indicates that they should possess the same basic structural framework corresponding to the motif $[^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$.

Despite this, an accurate characterization of one of the compounds in the mixture, namely the dichloro complex $[^{99m}\text{Tc}(\text{N})(\text{PXP})\text{Cl}_2]$ (PXP = POP, PNP1, PNP2, PNP3, PNP4), has been possible by comparison of its HPLC profile with that recorded for the analogous complex prepared at macroscopic level (mM concentration range) with the long-lived isotope Tc-99g or, alternatively, with the seventh-group transition-metal congener rhenium, an element exhibiting a chemical behavior closely similar to that of technetium. Specifically, the complexes $[^{99g}\text{Tc}(\text{N})(\text{PXP})\text{Cl}_2]$ (PXP = PNP3, PNP4) have been prepared by reacting the precursor complex $[^{99g}\text{Tc}(\text{N})(\text{PPh}_3)_2\text{Cl}_2]$ with PXP in dichloromethane, and characterized by elemental analysis and spectroscopic methods (29). In addition, the synthesis of similar complexes with the diphosphine ligands POP, PNP1, and PNP2 have been reported previously (20–24). Results showed that the compound $[^{99g}\text{Tc}(\text{N})(\text{POP})\text{Cl}_2]$ can exist in two different isomeric forms depending on the relative cis or trans position of the two chloride atoms or, equivalently, of the cis or trans position of the two phosphorus atoms showing an identical geometrical behavior. In solution, the cis isomer was irreversibly converted into the trans isomer. This fact suggests that this latter arrangement should be the most stable form of this complex. The crystal structures of the trans isomer of the complex $[^{99g}\text{Tc}(\text{N})(\text{POP})\text{Cl}_2]$ (21) and that of the cis isomer of the rhenium analogue $[\text{Re}(\text{N})(\text{PNP2})\text{Cl}_2]$ (24, 29) have been determined. No evidence of the existence in solution of a cis–trans isomerism was found for all complexes with PNP-type ligands, a fact that was attributed to the steric hindrance determined by the alkyl substituent on the nitrogen atom of PNP. HPLC characterization of Tc-99g complexes clearly showed that they have the same retention time of the corresponding Tc-99m counterparts, thus suggesting that these complexes possess an identical chemical structure.

Preparation of the Complexes $[^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{YZ})]^{0/+}$ (PXP = PNP2, PNP3, PNP4; YZ = H_2TSA , NSH , H_2OS). It was further observed that $[\text{Tc}(\text{N})(\text{PXP})]^{2+}$ selectively reacted with bidentate chelating ligands (YZ) possessing a pair of soft π -donor coordinating atoms.

These reactions afforded the asymmetrical heterocomplexes $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{YZ})]^{0/+}$ composed by two different bidentate chelating ligands bound to the same $\text{Tc}\equiv\text{N}$ group. Figure 2 illustrates the bidentate ligands employed here to afford various examples of asymmetrical heterocomplexes. Using dianionic bidentate ligands, having $[\text{S}^-, \text{O}^-]$ as a pair of coordinating atoms, the neutral heterocomplexes $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{YZ})]$ were obtained. In contrast, with monoanionic bidentate ligands having $[\text{S}^-, \text{NH}_2]$ as coordinating pair, monocationic species of the type $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{YZ})]^+$ were isolated. It is interesting to note that ligands having $[\text{N}, \text{O}^-]$, $[\text{N}, \text{N}]$, or $[\text{O}, \text{O}^-]$ as chelating system exhibited a low reactivity toward the fragment $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$. In these preparations, negligible yields ($<10\%$) of formation of the corresponding asymmetrical complexes were obtained. Table 1 reports TLC and HPLC characterization of the various Tc-99m heterocomplexes described here. As before, the chemical nature of selected, asymmetrical complexes was investigated by comparison of their HPLC behavior with that of the corresponding Tc-99g complexes, synthesized previously and fully characterized by elemental analysis, mass spectroscopy, and ^{31}P and proton NMR spectra (21–24, 29).

The reactions of the fragments comprising PNP-type diphosphine ligands were investigated in much detail. Basically, these preparations have been conducted in two different ways. A three-step procedure involved the preliminary production of the starting mixture of intermediate nitrido complexes, generated by reacting $[\text{}^{99m}\text{TcO}_4]^-$ with SDH and SnCl_2 . This was followed by the formation of the metal fragment $[\text{}^{99m}\text{Tc}(\text{N})(\text{PNP})]^{2+}$ (PNP = PNP2, PNP3, PNP4) obtained through the reaction with PNP. The preparation was, then, completed by the addition of the appropriate bidentate chelating ligand YZ to yield the final asymmetrical heterocomplex. Alternatively, a two-step preparation could be also used. This procedure was carried out through the simultaneous addition of both ligands PNP and YZ to the same reaction vial containing the intermediate nitrido complexes. Interestingly, both procedures gave almost the same final radiochemical yields of formation of the asymmetrical products, and conversion to the corresponding symmetrical complexes comprising two identical bidentate ligands was never detected.

Preparation of the Complexes $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{cysOEt})]^+$ and $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{cysNAc})]$ (PXP = PNP3, PNP4). Due to its potential relevance in setting up a convenient new route for labeling peptides as schematically represented in Figure 5 (see Discussion), the reactions of some cysteine derivatives with the metal fragment $[\text{}^{99m}\text{Tc}(\text{N})(\text{PNP3})]^{2+}$ were studied extensively. Figure 2 shows the drawings of the two ligands *cysNAc* and *cysOEt* utilized here. They differ mainly in the set of two-donor atoms used for coordination to the $[\text{}^{99m}\text{Tc}(\text{N})(\text{PNP3})]^{2+}$ fragment. Specifically, the ligand *cysOEt*, bearing an ethoxy protecting group on the carboxylic function of cysteine, was designed to bind the metal ion through the $[\text{S}^-, \text{NH}_2]$ pair, thus affording the monocationic complex $[\text{}^{99m}\text{Tc}(\text{N})(\text{PNP3})(\text{cysOEt})]^+$. The HPLC profile of this complex is reported in Figure 6. On the contrary, the ligand *cysNAc*, having an acetyl protecting group on the cysteine amine nitrogen, bound through the $[\text{S}^-, \text{O}^-]$ pair to give rise to the neutral complex $[\text{}^{99m}\text{Tc}(\text{N})(\text{PNP3})(\text{cysNAc})]$. Both complexes were formed as a mixture of two isomers arising from the relative syn or anti position of the lateral (ethoxy or acetyl) substituents with respect to the $\text{Tc}\equiv\text{N}$ multiple bond. This mixture was clearly detected by HPLC, which always showed the

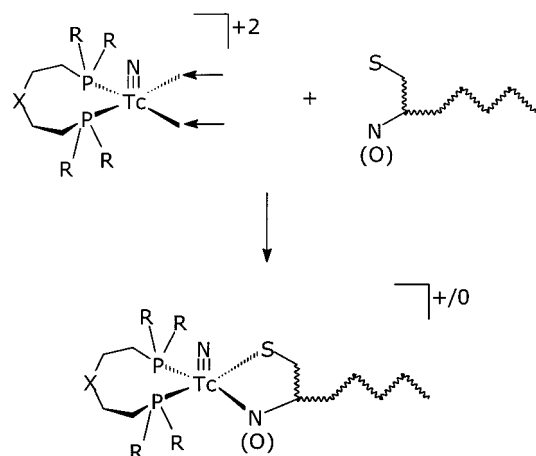


Figure 5. Schematic representation of the new labeling approach described in this study.

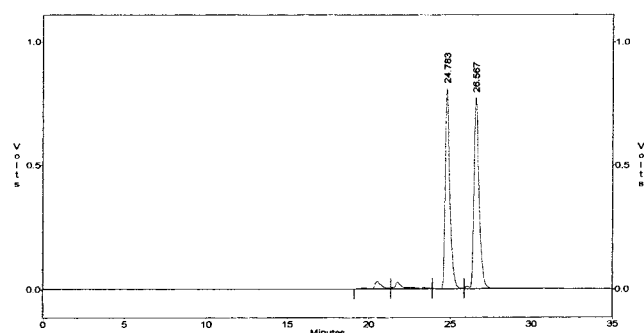


Figure 6. HPLC chromatogram of the complex $[\text{}^{99m}\text{Tc}(\text{N})(\text{PNP3})(\text{cysOEt})]^+$. Retention times are 24.783 and 26.567 min.

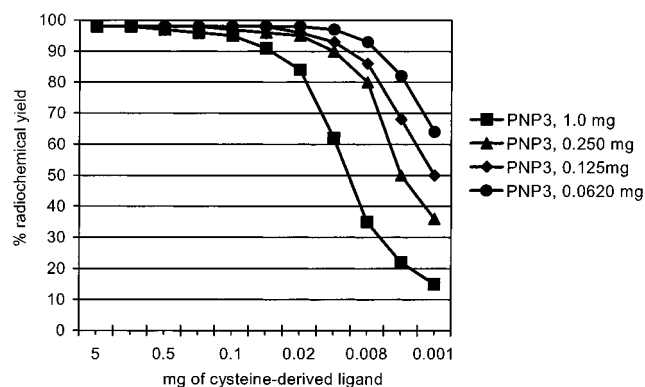


Figure 7. Variation of the labeling yield (%) of the complex $[\text{}^{99m}\text{Tc}(\text{N})(\text{PNP3})(\text{cysOEt})]^+$ as a function of the amount (mg) of the ligand *cysOEt* utilized in the preparation (see Experimental Section). In each curve, the amount of PNP3 is kept constant. A similar behavior was found for the complex $[\text{}^{99m}\text{Tc}(\text{N})(\text{PNP3})(\text{cysNAc})]$ (not shown here).

presence of two peaks of different intensity associated with this syn and anti isomeric pair. Though the overall yield of formation was higher than 95%, the relative labeling yields of the two isomers were not equal and one isomer was always formed in a greater amount (relative yield of the main isomer, approximately 60–70%). It should be noted that the existence of this isomeric pair was not always detected by TLC chromatography.

High-Specific-Activity Labeling of Cysteine-Derived Ligands. In studying the preparation of the complexes with cysteine-derived ligands, the influence of the molar stoichiometric ratio between PNP3 and each of these two ligands on the radiochemical yield of the resulting heterocomplexes was also investigated. Figure 7 illustrates the dependence of the radiochemical yield

of formation of the complex $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\text{cysOEt})]^+$ as a function of the concentration of cysOEt. The diagram was drawn by plotting the labeling yield of the radioactive complex (measured as percent of the total starting activity) as a function of the weighted amount of cysOEt added to the various preparations. A fixed amount of the initial activity (approximately 4.0 GBq) of freshly eluted $[^{99m}\text{TcO}_4]^-$ was used in each preparation, and the final volume of the reaction mixture was always kept in the interval 1.5–2.0 mL. The amount of cysOEt was decreased from 5.0 mg to 1.0 μg . A series of sigmoid curves were obtained showing the variation of the yield of formation upon decreasing the concentration of cysOEt. Inspection of this diagram revealed that, for a given amount of PNP3, the highest labeling yield was always achieved when the molar ratio PNP3/cysOEt did not exceed a specific value. An almost identical behavior was observed with the ligand cysNAC (data not showed). In particular, for all curves, the maximum radiochemical yield (>95%) obtained using the lowest amount of L (L = cysOEt, cysNAC) matched approximately the molar ratio $\text{PNP3/L} = 3.5$. A final specific activity of approximately 70 $\text{GBq}/\mu\text{mol}$ (2 $\text{Ci}/\mu\text{mol}$) was attained for both ligands cysOEt and cysNAC.

The results reported above with the two cysteine-derived ligands demonstrated that a terminal cysteine group was an efficient chelating system for the fragment $[^{99m}\text{Tc}(\text{N})(\text{PNP3})]^{2+}$. Thus, the labeling of the short peptide ligand **H₂1** and of the peptidomimetic derivative **H2** (Figure 2) was investigated in detail. These peptidic ligands were selected because they possess a cysteine residue at one terminus of the amino acidic chain. In **H2**, cysteine is joined to the remaining sequence through its carboxylic group, thus yielding a $[\text{NH}_2, \text{S}^-]$ pair of coordinating atoms. On the contrary, in **H₂1** the linkage of cysteine is through its amino nitrogen atom, and the resulting chelating system is composed by a $[\text{O}^-, \text{S}^-]$ pair of atoms. The most suitable reaction conditions were determined through a careful balance of the relative amounts of PNP3 and **H₂1**, and of PNP3 and **H2**, used in the two preparations, respectively. The optimal molar ratio $\text{PNP3/H}_2\mathbf{1}$ was found to be 3.5, and the resulting asymmetrical complex $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\mathbf{1})]$ was obtained with a radiochemical yield > 90%. The final specific activity for the labeling of **H₂1** was 180 $\text{GBq}/\mu\text{mol}$ (5 $\text{Ci}/\mu\text{mol}$). Similarly, the optimal molar ratio PNP3/H2 was 3.3, and the complex $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\mathbf{2})]^+$ was obtained with a radiochemical yield > 90% and a specific activity equal to 220 $\text{GBq}/\mu\text{mol}$ (6 $\text{Ci}/\mu\text{mol}$). The two complexes $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\mathbf{1})]$ and $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\mathbf{2})]^+$ were characterized by chromatographic methods (Table 1). In $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\mathbf{1})]$, the ligand **1** is coordinated to the metal through the $[\text{O}^-, \text{S}^-]$ pair of donor atoms of the terminal cysteine group, and the resulting complex is neutral. In $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\mathbf{2})]^+$, the terminal cysteine group is bound to the central ion through the $[\text{NH}_2, \text{S}^-]$ pair of atoms, thus giving rise to a monocationic compound. As observed with the ligands cysOEt and cysNAC, HPLC analysis showed that the complex $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\mathbf{1})]$ can exist in two distinct isomeric forms corresponding to the syn or anti orientations of the peptide side chains with respect to the apical $\text{Tc}\equiv\text{N}$ bond. However, in this situation, the final yield of one isomer (84%) was significantly higher than that of the other (12%). Apparently, this effect was even more pronounced with the complex $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\mathbf{2})]^+$ where only one peak was detected also under different chromatographic condi-

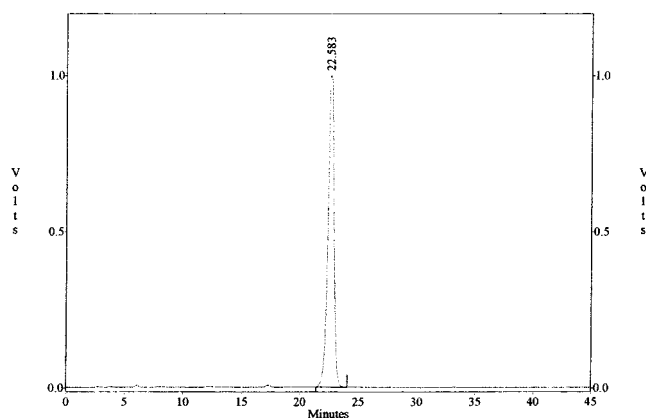


Figure 8. HPLC chromatogram of the complex $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\mathbf{2})]^+$. It elutes at a retention time of 22.583 min.

tions (Table 1). A representative HPLC chromatogram for the complex $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\mathbf{2})]^+$ is reported in Figure 8.

Stability. It was observed that all the heterocomplexes with two different bidentate ligands described in this study were highly stable both in solution and in blood. A remarkable result was obtained by studying the in vitro stability of the complexes $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\text{cysOEt})]^+$, $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\text{cysNAC})]$, $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\mathbf{1})]$, and $[^{99m}\text{Tc}(\text{N})(\text{PNP3})(\mathbf{2})]^+$. Data showed that these compounds were completely stable in solution and in blood, and highly inert toward transchelation by free cysteine and glutathione, a fact which is of fundamental importance in view of the potential, in vivo application of this novel class of radiopharmaceuticals.

DISCUSSION

In this study, a novel route to the labeling of short peptide sequences with Tc-99m was investigated. This new procedure is driven by the chemical properties exhibited by the nitrido metal fragment $[^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$ (Figure 3). A salient property of this arrangement of atoms around the Tc(V) center is that the overall structure can switch between square pyramidal (sp) and trigonal bipyramidal (tbp) geometries depending on the relative cis or trans position of the two π -acceptor phosphorus substituents. A tbp arrangement is much more favored as a result of the strong tendency of the two P atoms to span a reciprocal trans position (24). This apparently less remarkable feature is the key factor controlling the selective reactivity of this fragment toward nucleophilic ligands. In fact, the transition from sp to tbp structure brings also a complete rearrangement of the electronic density, which appears to concentrate on the two π -acceptor atoms. This leaves the other two coordination positions depleted of electrons and, thus, activated toward reaction with electron-rich, incoming ligands surrounded by a set of filled atomic orbitals having the appropriate symmetry to overlap with the empty antibonding π -orbitals of the metal fragment (29). Experimental findings completely agreed with this prediction. Indeed, the fragment $[^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$ was found to react only with ligands carrying soft π -donors as coordinating atoms. Such a behavior is highly specific and is particularly interesting in reactions where bidentate chelating ligands (YZ) are employed. When mono- or dianionic bidentate ligands, YZ, react with the fragment $[^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$, only the asymmetrical mixed heterocomplexes, $[^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{YZ})]^{0/+}$, are formed, and no concomitant production of the symmetrical species

$[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})_2]^{2+}$ or $[\text{}^{99m}\text{Tc}(\text{N})(\text{YZ})_2]^{0/2-}$ does occur. Furthermore, the fragment $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$ appears to be almost inert toward coordination by electrophilic or hard π - and σ -donor ligands. The reason of the high stability of the coordination arrangement composed by two P atoms and two Y, Z atoms bound to a $\text{Tc}\equiv\text{N}$ group originates from the perfect balance between the π -acceptor properties of the diphosphine ligand and the π -donor properties of the YZ ligand.

The peculiar chemical behavior of the fragment $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$ was experimentally evidenced here by using, in particular, PNP-type diphosphine ligands (Figure 1) and the bidentate YZ ligands illustrated in Figure 2. A key advantage of these reactions was that all the asymmetrical complexes $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{YZ})]^{0/+}$ could be also efficiently prepared by simply mixing in the same vial both the ligands PXP and YZ with the starting mixture of nitrido precursors. This procedure, therefore, did not require separate formation of the fragment $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$ and then letting it react with the bidentate ligand YZ. As predicted, during these preparations, no detectable formation of the symmetrical nitrido complexes, comprising two identical bidentate ligands, was observed.

The finding that the amino acid cysteine exhibited excellent coordinating properties toward the fragment $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$ prompted us to make use of this chelating system for incorporating short peptide chains into a Tc-99m asymmetrical complex. Cysteine was able to coordinate the fragment $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$ both through the $[\text{NH}_2, \text{S}^-]$ pair of coordinating atoms or, alternatively, through the $[\text{O}^-, \text{S}^-]$ pair. This reaction was highly specific and quantitative, due to the peculiar properties of the fragment $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})_2]^{2+}$. Thus, it was almost straightforward to exploit these coordination properties for labeling short peptide sequences having a cysteine group sufficiently accessible to bind the technetium center of the basic motif $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$. A schematic drawing of the novel labeling method proposed here is shown in Figure 5. The main approach can be outlined as follows. A selected peptide is combined with a bidentate cysteine chelating group either through the N-terminus or through the COO-terminus. Then, the resulting bifunctional ligand, $\text{cys}\sim$, is reacted with the metal fragment $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})_2]^{2+}$ to afford the mixed, asymmetrical complex $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})(\text{cys}\sim)]^{0/+}$. As a result, the peptide sequence remains stably incorporated into the structure of the final complex. The overall charge of the resulting asymmetrical complex is dependent on the nature of the π -donating atoms of cysteine. In fact, when the metal chelating system is composed by the $[\text{NH}_2, \text{S}^-]$ pair, the resulting complex will be monocationic. On the other hand, neutral complexes will arise when cysteine coordinates through the $[\text{O}^-, \text{S}^-]$ pair. It should be noted that cysteine has been already used as a coordinating moiety for technetium (30, 31), in particular in conjunction with other amino acids to form tetradentate chelating systems in oxo-Tc(V) complexes (1, 3). Generally, however, a large excess of the tetradentate ligand has to be employed to achieve satisfactory radiochemical yields owing to the lack of bonding specificity of these chelating groups toward the $[\text{Tc}=\text{O}]^{3+}$ core.

A successful application of the novel labeling approach was carried out using the two simple peptidic ligands showed in Figure 2. These two ligands efficiently coordinated to the $[\text{}^{99m}\text{Tc}(\text{N})(\text{PNP3})]^{2+}$ fragment through their terminal cysteine residues, which were oriented in an opposite manner in the two sequences. It was found that these reactions were highly selective, and only the

formation of the asymmetrical complexes were detected. Moreover, a quite surprising result was obtained by studying the stability of these two complexes in solution. Actually, results demonstrated that they were completely stable in solutions containing excess of free cysteine or glutathione, which are known to behave as excellent competing ligands toward metal transchelation in physiological conditions. This fact appears to further support the view that the arrangement composed by two π -acceptor and two π -donor coordinating atoms around a $[\text{Tc}\equiv\text{N}]^{2+}$ core should possess a rather considerable thermodynamic and kinetic stability.

CONCLUSIONS

A new approach to the labeling of peptides with Tc-99m was proposed here. This procedure makes use of the selective reactivity of cysteine toward the $[\text{}^{99m}\text{Tc}(\text{N})(\text{PXP})_2]^{2+}$ metal fragment, which exclusively coordinates to this amino acid residue through both the $[\text{NH}_2, \text{S}^-]$ and $[\text{O}^-, \text{S}^-]$ chelating systems. Other amino acids possessing different chelating systems were found to be less reactive. These properties could be conveniently exploited to set up a general procedure for the labeling of short peptides simply by selecting those sequences having a terminal cysteine group or, alternatively, by placing a cysteine residue at either the N- or COO-terminus of a specific peptide chain. It is expected that this almost 'natural' way of introducing a strong chelating group for the metal into a peptide would not dramatically alter its biological properties as it occurred with other labeling methods. Obviously, to fully evaluate the usefulness of the proposed approach, future work should be devoted to investigate both the influence of the peptide length and conformation on the efficiency of the labeling procedure and, ultimately, the possibility to preserve the biological properties of the native peptide sequence.

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Quadruplex and Pentaplex Self-Assemblies of Oligonucleotides Containing Short Runs of 8-Aza-7-deaza-2'-deoxyisoguanosine or 2'-Deoxyisoguanosine

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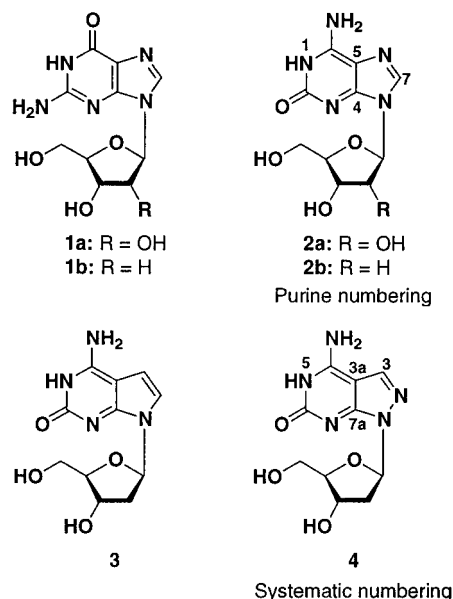
Oligonucleotides containing 8-aza-7-deaza-2'-deoxyisoguanosine (**4**) were investigated regarding their self-assembly in aqueous solution. The aggregation of **4** was compared with that of oligonucleotides containing 2'-deoxyisoguanosine (**2b**) and 2'-deoxyguanosine (**1b**). For this purpose the phosphoramidite of **4** was synthesized which was protected by a dibutylaminomethylidene residue at the amino group and a diphenylcarbamoyle residue at the 2-oxo function. Solid-phase synthesis furnished oligonucleotide containing short runs of the nucleoside **4**. The self-assembly of the oligonucleotide 5'-d(T₄**4**T₂) was studied by ion-exchange chromatography. The formation of a pentaplex was observed in the presence of Cs⁺, while a tetraplex is formed when the counter ion is Na⁺ or Rb⁺. The cation selectivity of the oligonucleotide 5'-d(T₄**4**T₂) was found to be different from the parent 5'-d(T₄isoG₄T₂) which was forming the tetraplex as well as a pentaplex in aq RbCl solution.

INTRODUCTION

Guanosine (**1a**) as well as guanosine derivatives such as 2'-deoxyguanosine (**1b**) and their oligonucleotides form self-assemblies in the presence of metal ions (1–3). These aggregates can be considered as nucleoside conjugates held together by hydrogen bonds and coordination forces (metal ions) and not by covalent linkages. Oligonucleotides with short runs of guanine, such as d(T₄G₄), d(T₄G₄T), or d(T₄G₄T₄), were shown to form tetrameric complexes, and the G-quartet binds cations with a selectivity of K⁺ (1.33 Å) > Rb⁺ (1.49 Å) > Na⁺ (0.98 Å) >> Cs⁺ (1.65 Å), Li⁺ (0.78 Å) (4–7) following the Hoogsteen base-pair motif. The ions are surrounded by the 6-oxo groups of the hydrogen-bonded guanine quartet. Investigations on the structure of self-assembled nucleosides as well as oligonucleotides have been performed using various techniques such as NMR spectroscopy (8–11), X-ray analyses (12, 13), electrospray ionization mass spectroscopy (14), electrophoresis (5), and energy perturbation studies (15). The X-ray analyses gave a detailed picture of the G-quartet structure showing that the central cation of the complexes is located between two layers of the guanine tetrads.

The guanosine-related nucleoside isoguanosine (**2a**) was found to form also aggregates in aqueous solution. The phenomenon was already observed in 1976 by Golas et al. on poly(isoguanic acid) (16). Several structures of the aggregated homopolymer have been suggested. The first defined complex formed by an 2'-deoxyisoguanosine (**2b**) containing oligonucleotide has been reported by our laboratory. The dodecamer d(T₄isoG₄T₄) was found to form a tetrameric assembly in the presence of sodium ions which is structurally different from that formed by 2'-deoxyguanosine (17, 18). Nitrogen-7 is not participat-

ing in the hydrogen bonding pattern. Thus, 7-deaza-2'-deoxyisoguanosine (**3**) forms an aggregate structure (19) while 7-deaza-2'-deoxyguanosine prevents guanine-rich oligonucleotides from aggregation (20). Recently pentameric complexes have been reported for an isoguanosine derivative (21) as well as for 2'-deoxyisoguanosine containing oligonucleotides both, in the presence of cesium ions (22).



This manuscript reports on the self-assembly of oligonucleotides containing 8-aza-7-deaza-2'-deoxyisoguanosine (**4**). Corresponding oligonucleotides are prepared by solid-phase synthesis using the protocol of phosphoramidite chemistry. The self-assembly of those oligonucleotides is investigated for the dependence on the size of the alkali cation, and the aggregate formation of 8-aza-

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7-deaza-2'-deoxyisoguanosine oligonucleotides is compared to those containing 2'-deoxyisoguanosine (**2b**) and 2'-deoxyguanosine (**1b**).

EXPERIMENTAL PROCEDURES

General Remarks. Thin-layer chromatography (TLC) was performed on TLC aluminum sheets silica gel 60 F₂₅₄ (0.2 mm, Merck, Germany). Solvent systems for FC and TLC: CH₂Cl₂/MeOH 9:1 (A), CH₂Cl₂/MeOH 95:5 (B), CH₂Cl₂/MeOH 98:2 (C), CH₂Cl₂/acetone 9:1 (D), CH₂Cl₂/acetone 98:2 (E), CH₂Cl₂/EtOAc 85:15 (F). Reverse phase HPLC was carried out on a 4 × 250 mm RP-18 (10 μm) LiChrosorb column (Merck) with a Merck-Hitachi HPLC pump (model 655 A-12) connected with a variable wavelength monitor (model 655-A), a controller (model L-5000), and an integrator (model D-2000). UV-spectra were recorded on a U-3200 spectrophotometer (Hitachi, Japan), λ_{max} in nm, ε in dm³ mol⁻¹ cm⁻¹. Half-life values (τ) were measured on a U-3200 spectrophotometer (Hitachi, Japan) connected with a temperature controller (Lauda, Germany). NMR spectra were measured on an Avance DPX 250 and an AMX 500 spectrometer (Bruker, Germany); chemical shifts (δ) are in ppm downfield from internal TMS (¹H, ¹³C) or external 85% H₃PO₄ (³¹P). The *J*-values are given in Hz. The solvents were purified and dried according to standard procedures.

Ion-Exchange HPLC. The ion-exchange chromatography was performed on a 4.6 × 100 mm Gen-Pak Fax Column (Waters, WAT015490) using a Merck-Hitachi HPLC apparatus with a pump (model L-7100), and a UV-detector (model L-4250) connected with an integrator (model D-2000). A column oven (model L-7350, Merck, Germany) was used to control the temperature of the ion-exchange column. The oligonucleotides were prepared as follows. A sample of 0.1 A₂₆₀-units was dissolved in 20 μL of 1.0 M MCl-buffer (M: Na, K, Rb, Cs). The solution was heated to 60 °C for 5 min, brought to room temperature and kept in a refrigerator (−23 °C) for 15 min up to 16 h (depending on the oligonucleotide). Then, the sample was brought to room temperature, diluted with 100 μL of buffer A, and injected into the system, which had been preheated to 30 °C. The column was eluted using the following systems: 0–40 min with 0–80% B in A with a flow rate of 0.5 mL min⁻¹ (A: 25 mM Tris-HCl, 1.0 mM EDTA, pH 8.0; B: 1.0 M MCl (M: Na, K, Rb, Cs) 25 mM Tris-HCl, 1.0 mM EDTA, pH 8.0).

Oligonucleotides. The oligonucleotide syntheses were carried out on an ABI 392-08 DNA synthesizer (Applied Biosystems, Weiterstadt, Germany) in a 1 μmol scale using the phosphoramidite **10**, those of the regular 2'-deoxynucleosides (Applied Biosystems, Weiterstadt, Germany) together with the DPC- and acetamidide-protected phosphoramidite of 2'-deoxyisoguanosine following the synthesis protocol for 3'-β-cyanoethylphosphoramidites. After cleavage from the solid support, the oligonucleotides were deprotected in 25% aqueous ammonia solution for 12–16 h at 60 °C. Purification of the 5'-dimethoxytrityl-oligomers was performed by reversed-phase HPLC (RP-18) with the following solvent gradient system (A: 0.1M (Et₃NH)OAc (pH 7.0)/MeCN 95:5; B: MeCN): 3 min 20% B in A with a flow rate of 1.0 mL min⁻¹, 12 min 20–40% B in A with a flow rate of 1 mL min⁻¹. Treatment with 2.5% CHCl₂COOH/CH₂Cl₂ for 5 min at room temperature to remove the 4,4'-dimethoxytrityl residues. The detritylated oligomers were purified by reversed-phase HPLC with the gradient: 20 min 0–20% B in A with a flow rate of 1 mL min⁻¹. The oligomers were desalted and lyophilized on a Speed-Vac evaporator to yield colorless solids.

4-Amino-1-(2-deoxy-β-D-erythro-pentofuranosyl)-1H-pyrazolo[3,4-d]pyrimidin-6-one (4). To a solution of 4,6-diamino-1-(2-deoxy-β-D-erythro-pentofuranosyl)-1H-pyrazolo[3,4-d]pyrimidine (**5**) (200 mg, 0.75 mmol) and sodium nitrite (200 mg, 2.9 mmol) in water (6 mL) was added acetic acid (300 μL) dropwise at 60 °C under stirring. Stirring was continued for 8 min, and the pH of the yellowish solution was adjusted to 8.0 with 25% aqueous NH₃ solution. The precipitated crude material was filtered, and the residue dissolved in water and applied to a Serdolit AD-4 column (4 × 20 cm, resin 0.1–0.2 mm, Serva, Germany). The column was washed with water (500 mL), and the product was eluted with H₂O–PrⁱOH (1:1). Compound **4** was obtained as a yellowish powder (98 mg, 49%). UV, ¹H and ¹³C NMR spectra are identical with the published data (23).

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-4-(N,N-dibutylaminomethylidene)-1H-pyrazolo[3,4-d]pyrimidin-6-one (6). To a stirred suspension of **4** (440 mg, 1.65 mmol) in MeOH (15 mL) was added *N,N*-dibutylformamide dimethylacetal (800 μL) (24). The mixture was stirred at 40 °C for 2 h and evaporated to dryness. The residue was applied to flash chromatography (FC; silica gel, column 2 × 10 cm, CH₂Cl₂–MeOH 98:2 to 95:5) and eluted stepwise. Colorless foam of **6** (450 mg, 67%). TLC (A): *R*_f 0.32; UV (MeOH): 233 (24300); 278 (13000); 338 (15500). ¹H NMR (*d*₆-DMSO): 2.20 (1H, m, 2'-H_α); 2.65 (1H, m, 2'-H_β); 3.36 (2H, m, 5'-H₂); 3.80 (1H, m, 4'-H); 4.37 (1H, s, 3'-H); 5.21 (2H, m, 3'-OH, 5'-OH); 6.30 (1H, "t", *J* = 6.5, 1'-H); 7.93 (1H, s, 3-H); 8.56 (1H, s, H–C=N). Anal. Calcd for C₁₉H₃₀N₆O₄ (406.5): C, 56.14; H, 7.44; N, 20.68. Found: C, 56.08; H, 7.50; N, 20.59.

4-Amino-1-(2-deoxy-β-D-erythro-pentofuranosyl)-6-[(diphenylcarbamoyl)oxyl]-1H-pyrazolo[3,4-d]pyrimidine (7). Compound **4** (100 mg, 0.37 mmol) was dried by repeated coevaporation with anhydrous pyridine and then suspended in anhydrous pyridine (2 mL). Diphenylcarbamoyl chloride (DPC-Cl) (150 mg, 0.65 mmol) and *N,N*-diisopropylethylamine (100 μL, 0.57 mmol) were added, and the reaction mixture was stirred for 2 h at room temperature. The excess of DPC-Cl was hydrolyzed with crushed ice. Then, the mixture was poured into a 5% aqueous NaHCO₃ (4 mL) and extracted with CH₂Cl₂ (3 × 5 mL). The combined CH₂Cl₂ layers were dried (Na₂SO₄) and filtered. After evaporation of the solvent, the residue was applied to FC (silica gel, column 2 × 15 cm). Elution with CH₂Cl₂–MeOH (98:2) followed by CH₂Cl₂–MeOH (9:1) furnished as a colorless foam (105 mg, 61%). TLC (A): *R*_f 0.41. ¹H NMR (*d*₆-DMSO): 2.27 (1H, m, 2'-H_α); 2.80 (1H, m, 2'-H_β); 3.35–3.57 (2H, m, 5'-H₂); 3.81 (1H, m, 4'-H); 4.42 (1H, m, 3'-H); 4.71 (1H, "t", *J* = 5.9, 5'-OH); 5.23 (1H, d, *J* = 4.6, 3'-OH); 6.43 (1H, "t", *J* = 6.3, 1'-H); 7.26–7.50 (10H, m, arom. H), 7.80–8.24 (3H, m, NH₂, 3-H). Anal. Calcd. for C₂₃H₂₂N₆O₅ (462.5): C, 59.73; H, 4.79; N, 18.17. Found: C, 59.65; H, 4.79; N, 18.11.

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-4-(N,N-dibutylaminomethylidene)-6-[(diphenylcarbamoyl)oxyl]-1H-pyrazolo[3,4-d]pyrimidine (8). To a suspension of compound **6** (290 mg, 0.71 mmol) in dry pyridine (5 mL) were added diphenylcarbamoyl chloride (300 mg, 1.29 mmol) and *N,N*-diisopropylethylamine (220 μL, 1.26 mmol), and the reaction mixture was stirred for 1 h at room temperature. The mixture was poured into 5% aqueous NaHCO₃ (7 mL) and extracted with CH₂Cl₂ (3 × 5 mL). The combined CH₂Cl₂ layers were dried (Na₂SO₄) and filtered. After evaporation of the solvent, the

Table 1. $^3J_{\text{H,H}}$ Coupling Constants of the Sugar Moieties and Conformer Populations of 2'-Deoxynucleosides^a

	$^3J_{1',2'}$	$^3J_{1',2''}$	$^3J_{2',3'}$	$^3J_{2'',3'}$	$^3J_{3',4'}$	$^3J_{4',5'}$	$^3J_{4'',5'}$	%N	%S	% $\gamma^{\text{g}+}$	% γ^{t}	% $\gamma^{\text{g}-}$
4	6.70	6.70	6.35	3.80	3.45	3.90	5.60	34	66	39	40	21
2b (31)	6.95	6.65	6.45	3.55	3.45	3.60	4.70	32	68	53	17	30
1b (32)	7.30	6.50	6.30	3.60	3.20	3.60	4.70	29	71	53	30	17
3b (31)	7.00	6.60	6.70	3.40	3.60	3.90	5.00	31	69	46	21	34

^a RMS ≤ 0.4 for all calculations; $|\Delta J_{\text{max}}| \leq 0.5$ Hz.

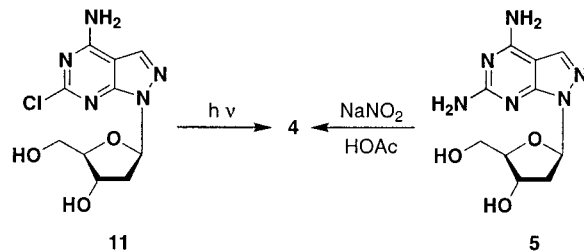
residue was applied to FC (silica gel, column 2×15 cm). Elution with CH_2Cl_2 followed by CH_2Cl_2 -MeOH (stepwise from 98:2 to 9:1) furnished a colorless foam (304 mg, 71%). TLC (A): R_f 0.46. UV (MeOH): 238 (20700), 322 (32000). ^1H NMR (d_6 -DMSO): 0.92 (6H, m, CH_3); 1.33, 1.61 (8H, m, CH_2); 2.26 (1H, m, $2'\text{-H}_\alpha$); 2.80 (1H, m, $2'\text{-H}_\beta$); 3.27–3.67 (6H, m, NCH_2 , $5'\text{-H}_2$); 3.82 (1H, m, $4'\text{-H}$); 4.44 (1H, m, $3'\text{-H}$); 4.76 (1H, "t", $J = 5.8$, $5'\text{-OH}$); 5.29 (1H, d, $J = 4.6$, $3'\text{-H}$); 6.51 (1H, "t", $J = 6.3$, $1'\text{-H}$); 7.27–7.45 (10H, m, arom. H); 8.13 (1H, s, 3-H); 8.85 (1H, s, $\text{HC}=\text{N}$). Anal. Calcd for $\text{C}_{32}\text{H}_{39}\text{N}_7\text{O}_5$ (601.7): C, 63.88; H, 6.53; N, 16.30. Found: C, 64.00; H, 6.78; N, 15.88.

1-[2-Deoxy-5-O-(4,4'-dimethoxytrityl)- β -D-erythro-pentofuranosyl]-4-(N,N-dibutylaminomethylidene)-6-[(diphenylcarbamoyl)oxyl]-1H-pyrazolo[3,4-d]pyrimidine (9). Compound **8** (200 mg, 0.33 mmol) was dried by repeated coevaporation with anhydrous pyridine and dissolved in anhydrous pyridine (1 mL). The solution was treated with dimethoxytrityl chloride (163 mg, 0.48 mmol) at 30 °C under stirring (45 min). MeOH (1 mL) was added, and the stirring was continued for 5 min. The mixture was poured into 5% aqueous NaHCO_3 solution (5 mL) and extracted with CH_2Cl_2 (3×5 mL). The combined organic layers were dried (Na_2SO_4). After evaporation of the solvent, the residue was applied to FC (silica gel, column 2×15 cm), which was prewashed with CH_2Cl_2 and eluted with CH_2Cl_2 -acetone (stepwise elution from 98:2 to 9:1) to give a colorless foam (195 mg, 65%). TLC (D): R_f 0.60. UV (MeOH): 236 (31200), 319 (44800). ^1H NMR (d_6 -DMSO): 0.94 (6H, m, CH_3); 1.28, 1.99 (8H, m, 4 CH_2); 2.31 (1H, m, $2'\text{-H}_\alpha$); 2.77 (1H, m, $2'\text{-H}_\beta$); 3.01, 3.53 (4H, m, NCH_2); 3.65–3.67 (8H, m, $5'\text{-H}_2$, OCH_3); 3.94 (1H, m, $3'\text{-H}$); 5.32 (1H, d, $J = 5.1$, $3'\text{-OH}$); 6.56 (1H, m, $1'\text{-H}$); 6.68–7.44 (23H, m, arom. H); 8.05 (1H, s, 3-H); 8.85 (1H, s, $\text{HC}=\text{N}$). Anal. Calcd. for $\text{C}_{53}\text{H}_{57}\text{N}_7\text{O}_7$ (904.1): C, 70.41; H, 6.35; N, 10.85. Found: C, 70.08; H, 6.41; N, 10.77.

1-[2-Deoxy-5-O-(4,4'-dimethoxytriphenylmethyl)- β -D-erythro-pentofuranosyl]-4-(N,N-dibutylaminomethylidene)-6-[(diphenylcarbamoyl)oxyl]-1H-pyrazolo[3,4-d]pyrimidine 3'-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (10). A solution of compound **9** (200 mg, 0.22 mmol) in CH_2Cl_2 (2 mL) was preflushed with argon and kept under argon atmosphere. Then, 2-cyanoethyldiisopropylphosphoramidochloridite (233 μL , 1.04 mmol) and N,N -diisopropylethylamine (88 μL , 0.51 mmol) were added at room temperature. Stirring was continued for 45 min. An aqueous solution of 5% NaHCO_3 (10 mL) was added. The mixture was shaken, the layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (3×15 mL). The combined organic extracts were dried (Na_2SO_4), filtered, evaporated, and applied to FC (silica gel, column 2×10 cm, CH_2Cl_2 -EtOAc (85:15) containing a few drops of Et_3N) yielding a colorless foam (196 mg, 80%). TLC (F): R_f 0.95. ^{31}P NMR (CDCl_3): 149.6, 149.9.

RESULTS AND DISCUSSION

Synthesis of Monomers. Earlier, 8-aza-7-deaza-2'-deoxyisoguanosine (**4**) has been synthesized from 8-aza-

Scheme 1


2-chloro-7-deaza-2'-deoxyadenosine (**11**) by photochemical substitution of the 2-chloro substituent (23). As this synthetic route is limited to small scale preparation, the nucleoside **4** was now prepared by deamination of the 4,6-diamino-1-(2-deoxy- β -D-erythro-pentofuranosyl)-1H-pyrazolo[3,4-d]pyrimidine **5** (25) as it was reported for the ribonucleoside **2a** (26). Selective deamination of compound **5** at position-2 with sodium nitrite and glacial acetic acid furnished the nucleoside **4** (Scheme 1).

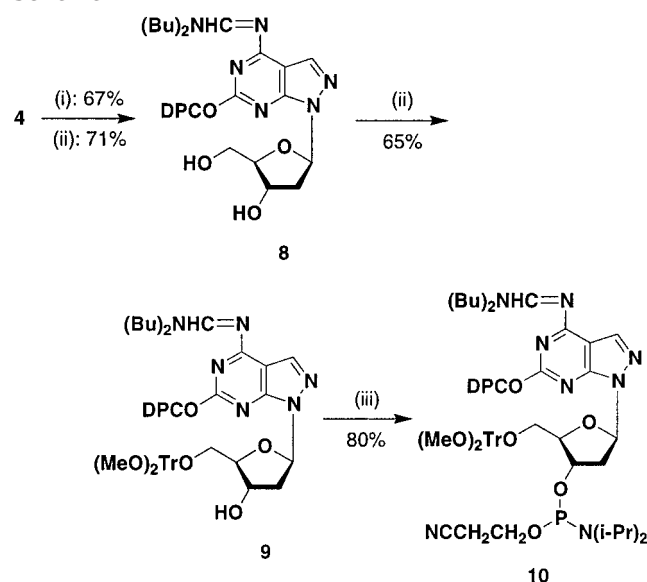
To investigate the stability of the glycosylic bond of compound **4**, it was hydrolyzed in 0.5 M HCl at room temperature, and the kinetic of hydrolysis was followed UV-spectrophotometrically at 268 nm leading to a half-life (τ) of 12.5 min. Compared to the acid-labile purine nucleoside **2b** ($\tau = 14$ min, 0.1 M HCl, 25 °C) (27) it possesses a significantly more stable glycosylic bond. This favorable property makes this compound to a useful substitute for 2'-deoxyisoguanosine as depurination can be avoided during oligonucleotide synthesis or longer storage of oligonucleotides.

Next, the conformational analysis of the 2'-deoxyribose moiety of **4** was performed using the program PSEUROT 6.2 (28, 29) which calculates the conformational parameters from ^1H , ^1H coupling constants and relates them to the corresponding mole fractions of the conformers. The populations of the staggered rotamers across the $\text{C}(4')\text{—C}(5')$ bond were calculated according to Westhof et al. (30). Table 1 summarizes the coupling constants and the conformational data. For comparison, the data of 2'-deoxyisoguanosine (**2b**) and 2'-deoxyguanosine (**1b**) as well as of 7-deaza-2'-deoxyisoguanosine (**3**) are listed in Table 1. While the population of N vs S conformers is similar in all cases, the nucleosides **1b**, **2b**, and **3** prefer the $\gamma^{\text{g}+}$ (+sc) conformation around the $\text{C}(4')\text{—C}(5')$ bond and for the base-modified compound **4** the +sc-population is reduced to 39%.

Earlier, the amino group of 2'-deoxyisoguanosine (**2b**) was protected with an amidine function, and the 2-oxo group with a diphenylcarbamoyl residue (27). The same protocol was now applied to compound **4**. Three different amidine residues were employed which lead on one hand to an increase of the stability of the glycosylic bond and on the other hand to a greater lipophilicity, thus facilitating chromatographic separation (24). Treatment of compound **4** with N,N -dimethylformamide dimethylacetal furnished **12**; the isomeric dibutylformamide dimethylacetates gave compounds **6** and **13** while the reaction

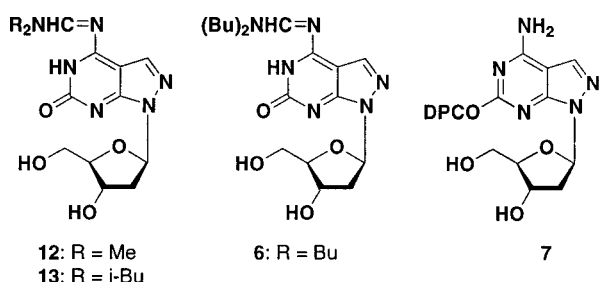
Table 2. Half-Life Values (τ) of Deprotection of 8-Aza-7-deaza-2'-deoxyisoguanosine Derivatives in 25% Aqueous Ammonia at 40 °C^a

compound	τ [min]	compound	τ [min]
12	1	6	38
13	82	7	29
		8	45

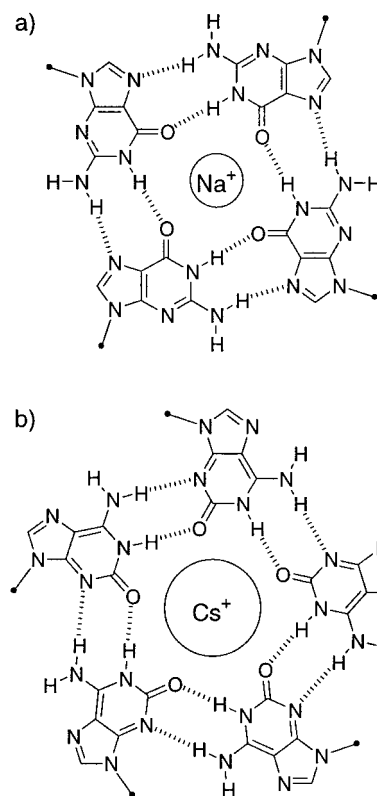
^a Data observed UV-spectrophotometrically.**Scheme 2^a**

^a (i) *N,N*-Dibutylformamide dimethylacetal/methanol; 40 °C; 2 h; (ii) DPC-Cl/pyridine/ Pr_2EtN ; rt; 1 h; (iii) DMT-Cl/pyridine; 30 °C; 45 min; (iv) 2-cyanoethyl-diisopropylphosphoramidochloridite/ Pr_2EtN ; rt; 45 min.

with diphenylcarbamoyl chloride in pyridine furnished compound 7.



To test the applicability of the protecting groups for oligonucleotide synthesis, their stability was studied under alkaline conditions. The kinetics of deprotection were followed UV-spectrophotometrically in 25% aqueous ammonia (Table 2). As the formamidine 12 was found to be rather labile and the isobutylamidine 13 was too stable to be used, thus the butylamidine 6 was chosen for further reactions.

**Figure 1.** Structures of (a) G₄-quartet, and (b) isoG₄-pentaplex

The resulting amidine 6 was then blocked at the 2-oxo group with the diphenylcarbamoyl residue (33). This protecting group has already been used for the 2-oxo-protection of related purine nucleosides (27, 34–36). From the NMR spectra it was concluded that the DPC-group is protecting the 2-oxo function as the chemical shift (δ) of C(3a) is remarkably shifted (downfield) about 7 ppm by introduction of a DPC group. An additional change of the C(4)-signal can also be observed. Nevertheless, this is not an unambiguous proof since protection at one of the pyrimidine nitrogens formed by tautomerization cannot be completely ruled out as such reactions have been observed in the case of 2-aza-2'-deoxyinosine by Fernandez-Forner et al. (37). Compound 8 was then converted into the 5'-O-(dimethoxytriphenylmethyl)-protected ((MeO)₂Tr) derivative 9 under standard conditions (38) (Scheme 2). Finally, the phosphoramidite 10 was prepared from 9 by treatment with 2-cyanoethyl-diisopropylphosphoramidochloridite in the presence of *N,N*-diisopropylethylamine under argon atmosphere. All compounds were characterized by ¹H and ¹³C NMR spectroscopy (see Table 3 and Experimental Procedures) as well as by elemental analysis.

The Self-Assembly of Oligonucleotides Containing Short Runs of 8-Aza-7-deaza-2'-deoxyisoguanosine (4). The formation of oligonucleotide aggregates containing 2'-deoxyisoguanosine is well established

Table 3. ¹³C NMR Chemical Shifts of 8-Aza-7-deaza-2'-deoxyisoguanosine Derivatives (*d*₆-DMSO, 25 °C)

	C(3) ^a C(7) ^b	C(3a) ^a C(5) ^b	C(4) ^a C(6) ^b	C(6) ^a C(2) ^b	C(7a) ^a C(4) ^b	C(1')	C(2')	C(3')	C(4')	C(5')	HC=N	CO
4 (23)	134.7	92.6	157.0 ^d	155.2 ^d	<i>e</i>	84.3	<i>c</i>	71.2	87.6	62.5		
6	136.2	98.2	159.2 ^d	157.8 ^d	<i>e</i>	83.7	<i>c</i>	71.2	87.4	62.6	159.8	
7	133.4	99.1	155.3	159.3	141.8	83.7	<i>c</i>	71.0	87.6	62.4		151.0
8	141.5	106.1	158.5 ^d	157.7 ^d	155.6	83.5	<i>c</i>	70.7	87.3	62.2	163.6	
9	141.8	106.4	158.9 ^d	157.9 ^d	155.9	83.2	<i>c</i>	74.2	85.1	64.4	163.8	151.2

^a Systematic numbering. ^b Purine numbering. ^c Superimposed by DMSO. ^d Tentative assignment. ^e Not detected.

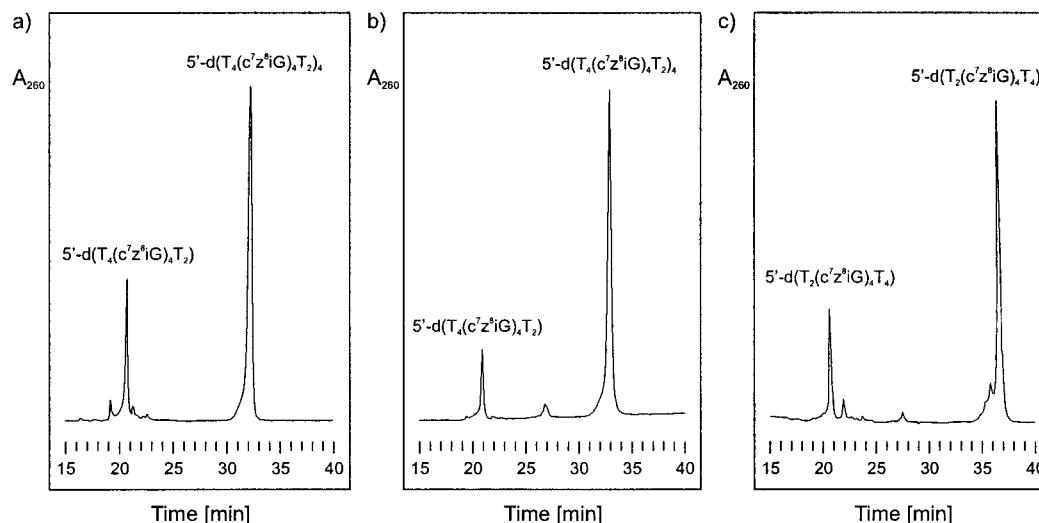


Figure 2. Ion-exchange HPLC elution profile of 5'-d(T₄(c'z⁸iG)₄T₂) (**14**) at 30 °C in the presence of Na⁺ (a), Rb⁺ (b), and Cs⁺ (c); conditions: see Experimental Procedures.

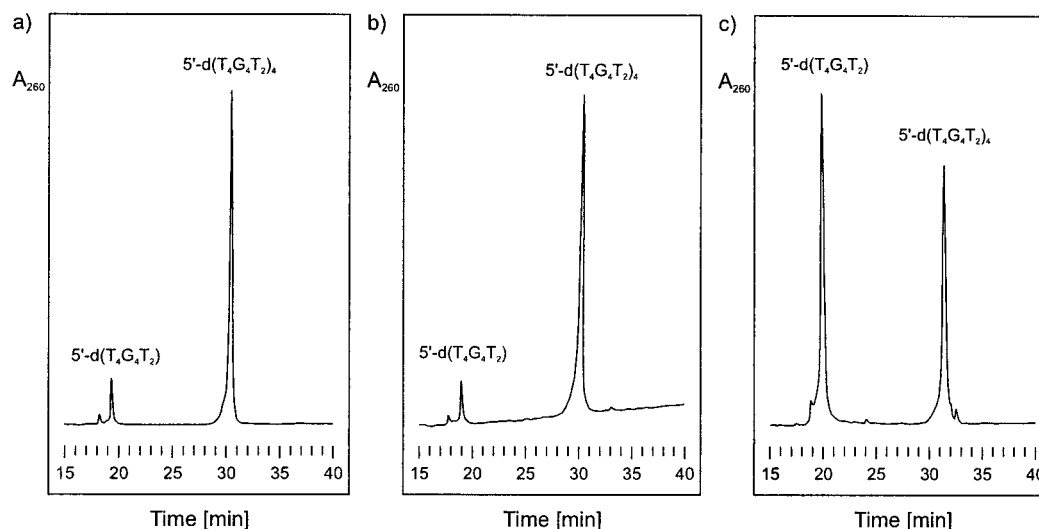


Figure 3. Ion-exchange HPLC elution profiles of 5'-d(T₄G₄T₂) (**16**) at 30 °C in the presence of Na⁺ (a), Rb⁺ (b), and Cs⁺ (c); conditions: see Experimental Procedures.

(16, 17, 22). The 12-mer d(T₄isoG₄T₄) forms two well-separated peaks when applied to an ion-exchange resin (Dionex NucleoPac PA-100) in the presence of sodium ions (18). The fast migrating peak refers to the single strand, while the slow-migrating one represents the aggregate. When the oligomer d(T₄G₄T₄) is analyzed under identical conditions, two peaks are also detected showing similar retention times (7). Again, the first one is the monomer while the second one was assigned to be the tetramer. So far oligonucleotides containing 2'-deoxyisoguanosine show a similar tendency to form aggregates as those containing 2'-deoxyguanosine. An equivalent study as performed by our laboratory applying ion-exchange chromatography and using the mobility differences of the monomeric and tetrameric assemblies (17) was performed later by gel electrophoresis (39). The complex formation of d(T₄isoG₄T₄) was also studied in the presence of other monovalent cations indicating that rather stable isoguanine conjugates are formed in the presence of cesium ions, while in the case of the guanine-containing oligomer d(T₄G₄T₄) fairly unstable complexes did occur. The stabilizing ability of dG-tetrads by Cs⁺ is ranking in the range of Li⁺ (40), and in this case K⁺ has the optimum ionic radius for the formation of the tetraplex (41). The aggregate stability of oligonucleotides

containing 2'-deoxyisoguanosine (**2b**) was also investigated in dependence of the ion radii of the alkali cations. However, little attention was given by our laboratory to a discontinuity of the complex stabilities between the potassium-, rubidium-, and cesium-ions (7). Chaput and Switzer found that oligonucleotides containing short runs of 2'-deoxyisoguanosine residues such as d(T₄iG₄T) or d(T₈iG₄T) form pentameric assemblies in the presence of cesium ions while in the presence of potassium ions tetrameric complexes or mixtures of tetrameric and pentameric structures are observed (22) (Figure 1).

As nitrogen-7 is not participating in the hydrogen-bonding pattern of the 2'-deoxyisoguanosine assembly (19) the 8-aza-7-deaza-2'-deoxyisoguanosine, which usually forms stronger hydrogen bonds upon base pairing than 2'-deoxyisoguanosine (**2b**) does, was incorporated in an oligonucleotide containing a short run of compound **4** residues. A decanucleotide 5'-d(T₄(c'z⁸iG)₄T₂) (**14**), containing four consecutive nucleoside **4** residues as well as tails of 4 dT's at the 5'-terminus and two dT's at the 3'-end, was synthesized. The oligonucleotides 5'-d(T₄isoG₄T₂) (**15**) and 5'-d(T₄G₄T₂) (**16**) having a similar structure, but containing either four 2'-deoxyisoguanosine or four 2'-deoxyguanosine residues, were synthesized for comparison.

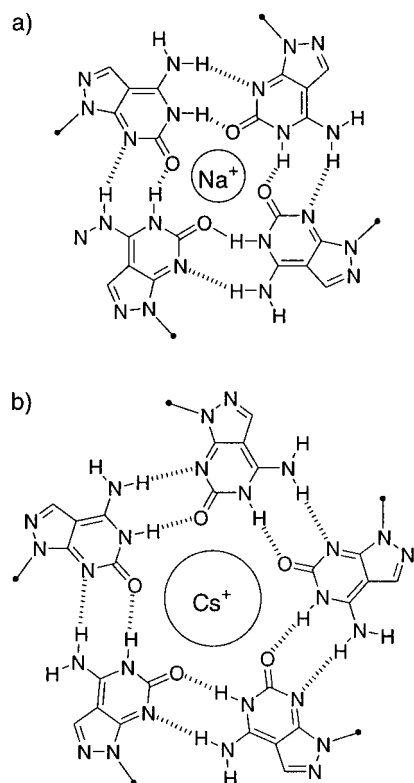


Figure 4. Structures of (a) $c^7z^8iG_d$ -tetraplex, and (b) $c^7z^8iG_d$ -pentaplex.

At first, the self-assembly of $5'-d(T_4(c^7z^8iG)_4T_2)$ (**14**) was studied. The oligonucleotide was stored in 1.0 M MCl solution (M: Na^+ , Rb^+ , Cs^+) at $-23^\circ C$ for 16 h. Subsequently, the solution was injected onto an ion-exchange Gen-Pak Fax column thermostated at $30^\circ C$. The ion-exchange chromatography of the sodium complex performed in the presence of NaCl is shown in Figure 2a. The peak with the short retention time contains the single-stranded oligomer while the slower migrating peak represents the conjugate. The same experiment (storage and ion exchange chromatography) was then performed with the 2'-deoxyguanosine-containing oligonucleotide $5'-d(T_4G_4T_2)$ (**16**) showing the same sequence, but $c^7z^8iG_d$ was replaced by dG. This HPLC profile (Figure 3a) shows also two peaks when Na^+ is the counterion. Earlier

studies have shown that the faster migrating peak results from the single-stranded oligonucleotide while the slower one represents the tetramer. As the peak of the aggregate formed by oligomer **14** shows a similar retention time as that of **16** (33 min vs 30 min), the complex of the aggregate was also assigned to be a tetraplex. The same protocol was performed with Rb^+ as counterion (see Figure 2b); according to this figure a tetraplex is also formed in this case. The chromatographic mobility changed when Cs^+ was used. Incubation of **14** in 1.0 M CsCl and chromatography in a running buffer containing CsCl leads to a chromatographic profile shown in Figure 2c. The mobility of the second peak of oligonucleotide **14** shows a significantly increased retention time over that of the sodium complex (37 min vs 33 min) while that of **16** remains unaffected. From this observation it is concluded that the aggregate containing 8-aza-7-deaza-2'-deoxyisoguanosine is a tetraplex in the presence of Na^+ , K^+ , and Rb^+ while a pentaplex is formed in Cs^+ . This observation is in line with findings on oligonucleotide assemblies containing 2'-deoxyisoguanosine (22). From the series of experiments shown in Figure 2a–c and 3a–c, it is apparent that the Cs^+ complex of **14** has a similar stability as the sodium complex while the complex of the oligomer **16** containing 2'-deoxyguanosine becomes less stable in an aqueous CsCl solution.

As there was no unambiguous proof whether the lower mobility of this complex formed in the presence of Cs^+ results from the formation of a pentameric structure or other phenomena induced by the pyrazolo[3,4-*d*]pyrimidine system we searched for experimental conditions which allow the detection of both types of complexes in the same solution. The experiments were performed in the presence of a counterion which is smaller than Cs^+ (1.65 Å) but significantly larger than sodium (0.98 Å) (Figure 4). For this purpose Rb^+ was chosen exhibiting an ion radius of 1.49 Å. However, as shown in Figure 2b, both complexes are not formed in the case of the oligonucleotide **14**. Therefore, we went back to the oligonucleotide $5'-d(T_4isoG_4T_2)$ (**15**) containing the purine nucleoside 2'-deoxyisoguanosine. This oligomer forms a tetraplex in the presence of Na^+ and a pentaplex in the presence of Cs^+ (Figure 5a,b). Then, the experiments were performed in the presence of RbCl which was used for incubation and in the chromatographical runs (Figure 5c). In this case two well-separated slow-migrating peaks

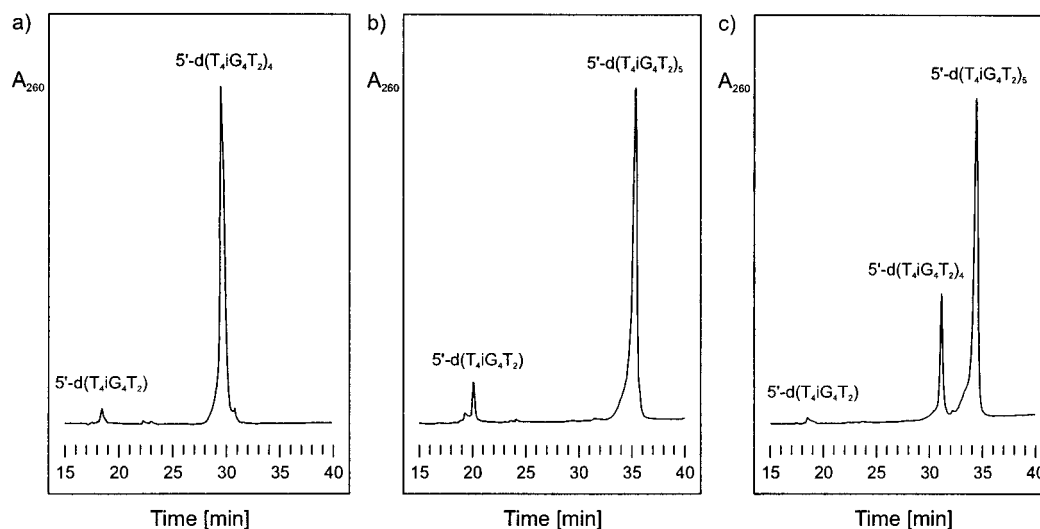


Figure 5. Ion-exchange HPLC elution profiles of $5'-d(T_4isoG_4T_2)$ (**15**) at $30^\circ C$ in the presence of Na^+ (a), Cs^+ (b), and Rb^+ (c); conditions: see Experimental Procedures.

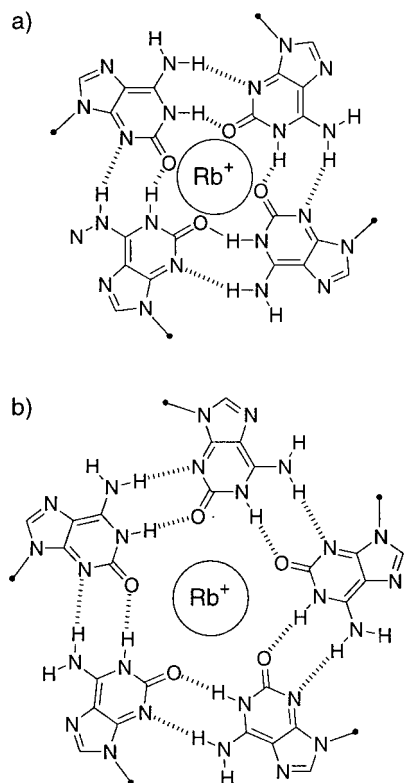


Figure 6. Structure of (a) isoG₄-tetraplex, and (b) pentaplex.

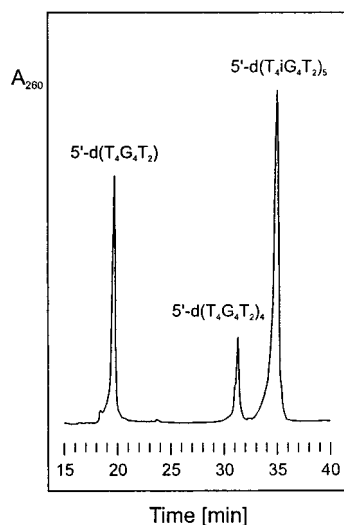


Figure 7. Ion-exchange HPLC elution profiles of 5'-d(T₄-isoG₄T₂) (**15**) and 5'-d(T₄G₄T₂) (**16**) in the presence of Cs⁺; conditions: see Experimental Procedures.

appeared. The faster migrating one was considered to be the tetraplex while the slower one represents the pentameric structure. The mobility change of the conjugates arises from the increased number of negative charges of the pentameric complex resulting in a stronger binding to the ion-exchange resin than the less charged tetrameric complex.

To show that the tetrameric complex can be distinguished from the pentamer another experiment was performed. It is now well established that 2'-deoxyisoguanosine-containing oligomers form pentameric assemblies in the presence of Cs⁺ ions while 2'-deoxyguanosine forms exclusively tetraplexes even in aqueous CsCl solution. Thus, a chromatographical run of a mixture of the oligonucleotides 5'-d(T₄G₄T₂) (**16**) and 5'-d(T₄-isoG₄T₂) (**15**) in the presence of CsCl should lead to two

aggregate peaks—the tetramer of **16** and the pentamer of **15**. This is actually the case. Apart from the rather high amount of the monomeric oligonucleotide of 5'-d(T₄G₄T₂) (**16**) and its tetrameric assembly which is present in aqueous CsCl solution the pentameric assembly of **15** is observed. As the mobilities of the self-assembled aggregates of **15** and **16** (Figure 7) look similar to those of Figure 5c it is obvious that the peaks of the tetraplexes migrate significantly faster than those of the pentaplexes, no matter whether the tetraplex is formed by dG or an isoG₄ quartet. It also confirms the presence of two complexes, the tetraplex and the pentaplex of **15**, in the presence of Rb⁺.

The difference of the complex stability observed for the various cations in the case of the oligonucleotide **14** containing 8-aza-7-deaza-2'-deoxyisoguanosine and **15** containing 2'-deoxyisoguanosine was unexpected. It might be traced back to conformational differences of the monomeric pyrazolo[3,4-*d*]pyrimidine vs the purine nucleoside residues (high-anti vs anti conformation) (42). This can affect the thermodynamic stability of the aggregates and/or the kinetics of complex formation and decay. In this regard it is worth mentioning that the self-aggregation of 2'-deoxyisoguanosine containing oligonucleotides occurs much faster than that of containing 2'-deoxyguanosine or 8-aza-7-deaza-2'-deoxyisoguanosine.

CONCLUSION

Oligonucleotides containing short runs of 8-aza-7-deaza-2'-deoxyisoguanosine (**4**) form tetrameric as well as pentameric self-assemblies, which can be considered as oligonucleotide conjugates held together by hydrogen bonds and coordination forces (metal ions) and not by covalent linkages. Those conjugates require the presence of a central cation, as it has been observed for oligomers containing 2'-deoxyisoguanosine (**2b**) and 2'-deoxyguanosine (**1b**). In the latter cases it was shown that the metal ion is surrounded by the tetrameric or pentameric assembly of the nucleobases in a sandwich type structure (X-ray) (43). Such a structure is also expected for the self-assemblies of 8-aza-7-deaza-2'-deoxyisoguanosine and its oligonucleotides. The formation of the various complexes (tetraplexes or pentaplexes) depends on the size of the cation. Cs⁺ generates a pentaplex while the smaller cations (Na⁺: 0.98 Å, Rb⁺: 1.49 Å, Cs⁺: 1.65 Å) can induce a tetraplex structure. In the case of the 2'-deoxyisoguanosine containing oligonucleotide **15** a tetraplex as well as a pentaplex can be observed in the same solution when Rb⁺ is the counterion, while the self-assembly formed by the 8-aza-7-deaza-2'-deoxyisoguanosine containing oligonucleotide 5'-d(T₄G₄T₂) yields only one species which is expected to be the tetraplex.

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Systematic Modulation of Michael-Type Reactivity of Thiols through the Use of Charged Amino Acids

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A quantitative structure–reactivity relationship for the Michael-type addition of thiols onto acrylates was determined. Several thiol-containing peptides were investigated by examining the correlation between the second-order rate constant of their addition onto PEG-diacrylate and the pK_a of the thiols within a peptide. By introducing charged amino acids in close proximity to a cysteine, the pK_a of the thiol was systematically modulated by electrostatic interactions. Positive charges from the amino acid arginine decreased the pK_a of the thiol and accelerated the reaction with acrylates while negative charges from aspartic acids showed the opposite effect. A linear correlation between thiolate concentrations and kinetic constants was found, confirming the role of thiolates as the reactive species in this Michael-type reaction. The relevant factors influencing the reactivity were the sign and the number of the neighboring charges, while the position of these charges had little effect on reactivity. These results provide a basis for the rational design of peptides, where the kinetics and thus selectivity of protein/peptide conjugation with polymeric structures via Michael-type addition reactions can be controlled.

INTRODUCTION

Conjugation with poly(ethylene glycol) (PEG), also termed PEGylation, is a common method for the modification of proteins and peptides, as well as other drugs, with synthetic polymers. As a result of the favorable properties of PEG, such as protein repellence, high hydrophilicity, and low degradability by mammalian enzymes, PEGylation can dramatically extend the stability and improve the pharmacology of a biomolecule in vivo (1, 2). One common coupling strategy involves the modification of terminal hydroxyl groups with electrophilic functionalities, such as the *N*-hydroxysuccinimide esters of PEG carboxylic acids (PEG-NHS) (3–5), PEG-oxycarbonylimidazole (CDI-PEG) (6), or PEG nitrophenyl carbonate (7, 8). However, since these functionalized PEGs react unselectively with both thiols and amines, a distribution of products can be obtained, resulting in grafting at multiple sites of the biomacromolecule, additionally with an uncontrolled PEGylation degree (due to the limited stability of thioester linkages). To achieve a better coupling selectivity, two classes of functional groups have been utilized. The first one is based on substitution onto groups such as iodo-, bromo-, and chloroacetamides and -acetates (9, 10); the second class on the conjugate addition (Michael-type) onto unsaturated groups such as maleimides (10), vinyl sulfones (11, 12), acrylamides (13), and acrylates (14).

Our recent interests have focused on the development of PEG-based synthetic hydrogels in contact with pro-

teins (15), isolated cells, and tissues via the use of in situ cross-linking reactions, e.g., by reacting a multifunctional PEG with a correspondingly reactive multifunctional PEG or peptide. A satisfactory exploitation of this scheme depends on the possibility of obtaining quantitative conversions in biologically or surgically relevant time scales and with adequate selectivity. Furthermore, the same scheme could be applied to PEGylate biologically active molecules, so as to graft them into the above matrixes. Among the other reactions, the Michael-type addition of thiols onto unsaturated esters is an ideal candidate for such processes, due to its high speed, the ease of preparation of the unsaturated compound, the negligible influence of competing reactions with other nucleophiles (at physiological pH, amines react generally at least 1 order of magnitude slower than thiols (16)), and the absence of any byproducts. Preliminary experiments (17) have shown acrylates to react considerably faster than other unsaturated esters (methacrylates, crotonates, 2,2-dimethylacrylates, cinnamates, cyclopropanecarbonylates) and were therefore chosen as standard conjugated unsaturated partner.

To better meet the requirements for bioconjugation and gel formation and to optimally exploit the selectivity of the Michael-type addition reaction, knowledge of the thiol structure–reactivity relationship is crucial. Thiols are key players in determining the structure and function of many proteins, for example, as active sites of enzymes. The protonation state of the active site of many enzymes is determined by its pK_a which in turn strongly influences protein catalytic activity (18, 19). For example, thioredoxin (20) and thioltransferase (21) have highly reactive thiols with very low pK_a 's, which increases their activity toward reducing disulfide bonds. Many examples in the literature reveal that pK_a shifts of thiols can be induced by electrostatic interactions with neighboring ionizable amino acids (22) and/or polar residues (e.g. amides

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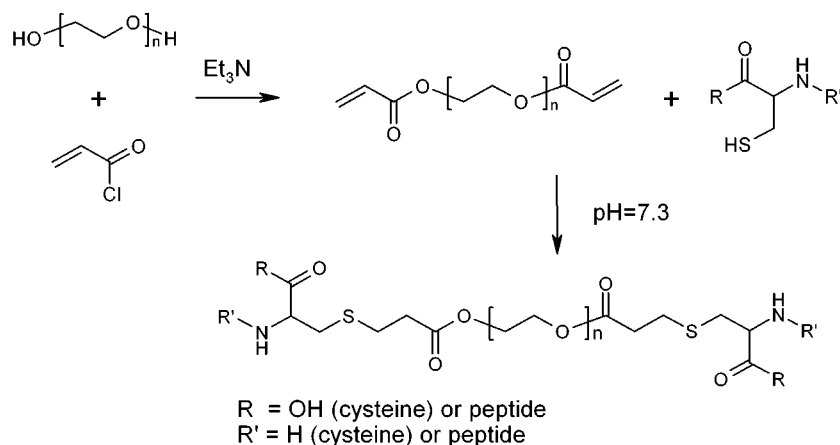


Figure 1. Synthesis of and Michael-type reaction on PEG diacrylate.

Table 1. Series of Peptides with Ionizable and/or Neutral Amino Acids in Close Proximity to a Cysteine Residue to Alter Its Protonation State (pK_a) by Electrostatic Interaction

peptide	GCRRG	GRCRG	GCRG	GRCDG	GCRDG	GCDRG	GCDG	GDCDG	GCDDG
abbreviation	crr	rcr	cr	rcd	crd	cdr	cd	dcd	cdd
net charge	+2	+2	+1	0	0	0	-1	-2	-2

dipoles (23)) or surfactants (19). For instance, changes in enzymatic activity have been observed for thioredoxin (22) and 4-oxalocrotonate tautomerase (24) as a result of pK_a shifts in the active site due to mutations on charged residues close to the active thiol. It was stated that charge not only influences pK_a shifts, but can also change the transition state energy (19) influencing the reaction kinetics. Since thiolates rather than thiols are reported to be the reactive species also in Michael-type reactions (9,16,25), the pK_a value of the sulfhydryl-group is the design parameter of choice for this study.

The present work outlines a quantitative relationship between the chemical structure and the Michael-type reactivity for a model system constituted of PEG-diacrylate and peptide thiols (see Figure 1). A homologous series of peptides with charged and/or neutral amino acids in close proximity to a cysteine residue was designed in order to confirm the role of thiolates in the Michael addition and quantitatively show how the charge of the neighboring groups of a thiol can influence its pK_a . In this system, pK_a -shifts— independent of any other structural factors—linearly correlate with a change in reaction kinetics. This knowledge will aid in the rational design of peptides and proteins conjugated to polymeric structures with controlled kinetics and selectivity.

EXPERIMENTAL SECTION

Materials. Poly(ethylene glycol) mol wt 3400 (PEG3400) and triethylamine were purchased from Aldrich (Buchs, Switzerland) and were used without purification. Acryloyl chloride was purchased from Aldrich (Buchs, Switzerland) and was used freshly after distillation over quinoline.

All standard chemicals used in peptide synthesis were analytical grade or better and were purchased from Novabiochem (Läufelfingen, Switzerland).

^1H NMR spectra were recorded on a 300 MHz Bruker spectrometer. FT-IR spectra were recorded in ATR mode on a Spectrum One Perkin-Elmer spectrometer. UV-Vis spectra were recorded on Perkin-Elmer Lambda 20 spectrometer

Synthesis of PEG Diacrylate. PEG diol with 3400 MW (10 g, 2.94 mmol) was dissolved under argon in 300 mL of toluene and dried azeotropically. After cooling to

5 °C, 50 mL of dichloromethane was added. After the addition of triethylamine (1.64 mL, 2.0 equiv), acryloyl chloride (0.72 mL, 1.5 equiv) was added dropwise, and the reaction proceeded overnight, in the dark, at room temperature. The resulting solution was filtered through a neutral alumina bed. Sodium carbonate was added to the toluene solution and removed by filtration, and the volume of the solution was reduced by rotary evaporation. The polymeric material was precipitated twice in with diethyl ether and dried in vacuo to yield a white powder. Yield: 88%; conversion of OH to acrylate: 100% (from ^1H NMR analysis). ^1H NMR (CDCl_3): 3.6 (156H, PEG chain protons), 4.3 (t, 2H, $\text{CH}_2\text{CH}_2\text{OCOCH}=\text{CH}_2$), 5.8 (dd, 1H, $\text{CH}_2=\text{CHCOO}$), 6.1 and 6.4 (dd, 1H, $\text{CH}_2=\text{CHCOO}$) ppm. FT-IR (film on KBr plate): 2990–2790 (ν C–H), 1724 (ν C=O), 1460 (δ_s CH_2), 1344, 1281, 1242, 1097 (ν_{as} C–O–C), 952, 842 (ν_s C–O–C) cm^{-1} .

Peptide Synthesis and Purification. A series of cysteine-containing peptides was synthesized, derived from four amino acid building blocks: a positively charged (at neutral pH) (arginine, R), a negatively charged (aspartic acid, D), a neutral (glycine, G), and a thiol-bearing amino acid (cysteine, C). The peptide sequences and their abbreviations used throughout the paper are listed in Table 1.

Synthesis was performed by solid-state chemistry on resin using an automated peptide synthesizer (Perceptive Biosystems, Farmington, MA), with standard 9-fluorenylmethyloxycarbonyl chemistry (26). Peptides were purified by C18 chromatography (Perceptive Biosystems Biocad 700E) and analyzed by MALDI-TOF mass spectrometry. All peptides were synthesized bearing a *N*-acetylglycine at the amino terminus, to suppress reaction of acrylate-groups with the α -amine.

Validation of an Analytical Method To Determine Reaction Kinetics. The Michael-type reactions on two model compounds, namely cysteine and PEGDA, was followed monitoring the absorbance at 233 nm, where a pH-dependent absorption peak is shown by thiols (associated to the thiolate form) (22,27) and where unsaturated esters absorb too. The method was validated using reaction mixtures prepared by keeping the overall number of reacting groups (the sum of acrylates and thiols) constant, but varying their relative quantities. In this

way, the absorption could be divided by the number of reacting groups, yielding an averaged extinction coefficient, ϵ , which is a linear combination of the extinction coefficients of the absorbing species.

Determination of Michael Addition Kinetics for Thiol-Containing Model Peptides on PEGDA. Peptides (Table 1) were dissolved in 100 mM HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid] buffered saline and reacted with PEGDA (pH 7.4 and 37 °C) at different molar ratios. The overall concentration of both reacting groups was kept constant at 2.5 mM. The absorption data at 233 nm were fitted with single exponential decays of the form $y(t) = y_0 + A_1(\exp - t/\tau)$. For a second-order reaction, the equation

$$\ln([PEGDA]/[peptide]) = \ln([PEGDA]_0/[peptide]_0) + k_{\text{eff}}t \quad (1)$$

with $k_{\text{eff}} = k([PEGDA]_0 - [peptide]_0)$, implies a linear time dependence for $\ln([PEGDA]/[peptide])$.

pK_a Determination. The pK_a of all thiols was determined spectrophotometrically using a method related to the one described above for measurement of reaction kinetics. The absorption at 233 nm was measured for each peptide at various pH for solutions having identical concentrations. Solutions of peptides (0.1 mM) were prepared in a buffer system (depending on the pH regime), composed of two components: a 0.01 M acetate-, phosphate-, tris- or borate-buffer (depending on the pH), and 0.1 M NaCl (stable ionic strength). Freshly prepared solutions were immediately measured in the UV region with a scan from 190 to 300 nm.

Statistics. Kinetic studies were conducted in triplicate for every peptide. Mean values and standard error of the mean are shown.

RESULTS AND DISCUSSION

Validation of an Analytical Method To Determine Reaction Kinetics. In a buffered environment, the intensity of the 233 nm peak is proportional to the concentration of unreacted acrylates and thiolates; compared to the Michael-type addition, the proton transfer is instantaneous, and from the equilibrium $[S^-] = [SH]/K_a/[H^+]$ one can assume $[S^-]$ to be constantly proportional to the overall thiol concentration $C_{\text{thiol}} = [S^-] + [SH] = [S^-](1 + [H^+]/K_a)$. Thus, whether the active group is the thiol, the thiolate, or both of them, the time evolution of this peak allows one to monitor on-line the extent of every thiol-consuming reaction. Saturated esters and thioethers (in the reaction product) have a negligible absorption at that wavelength.

If the change of the 233 nm absorption depends only on the reaction extent, this is indicative of a 1:1 reaction, and if disulfide formation plays a negligible role during the reaction (at least for cysteine), then a graph of ϵ vs the equivalent fraction of PEGDA (number of acrylates divided by the number of reacting groups; the same applies to cysteine) at time ∞ (complete reaction) should show a minimum at 0.5, corresponding to ϵ of the pure product. Moreover, the time dependence of ϵ should follow a single-exponential decay, because thiols and acrylates would be consumed at the same rate. These assumptions were all indeed confirmed, as shown by the reaction diagram in Figure 2A and by the time dependence of the absorption in Figure 2B. Thus, this method was used for all further kinetic experiments.

Influence of the Primary Structure of the Peptide on the Michael Addition Reactivity. According

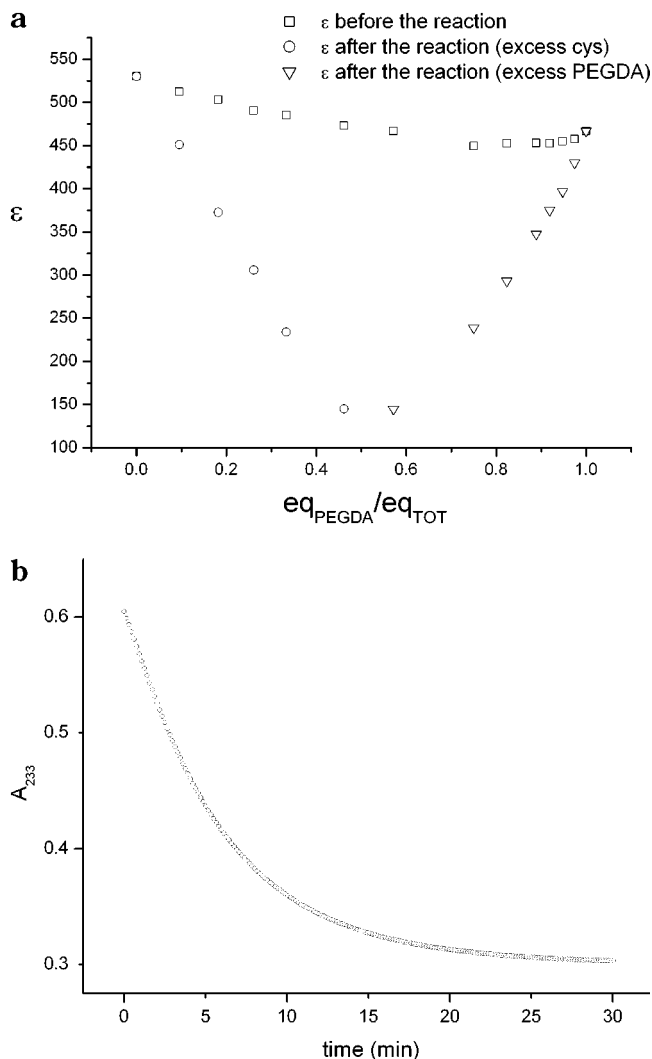


Figure 2. (A) Reaction diagram for PEGDA and cysteine as model compounds obtained by measuring absorbance at 233 nm for various PEGDA/Cys molar ratios. The overall concentration c of both reacting groups was kept constant at 2.5 mM. The absorbance divided by c is depicted on the ordinate as ϵ . The ratio between the concentrations of reacting groups was drawn on the abscissa as fraction of acrylic groups. (B) Absorbance of a PEGDA-cysteine solution over time ($c = 2.5$ mM). The reaction is complete after approximately 30 min under these conditions (pH 7.4, 37 °C), yielding a minimum of ϵ corresponding to the pure product.

to eq 1, true second order kinetics would imply a linear time dependence for $\ln([PEGDA]/[peptide])$. This was indeed shown by all peptides investigated. In Figure 3, the kinetic data for some peptides upon reaction with PEGDA at 80:20 acrylate over thiol ratio are shown.

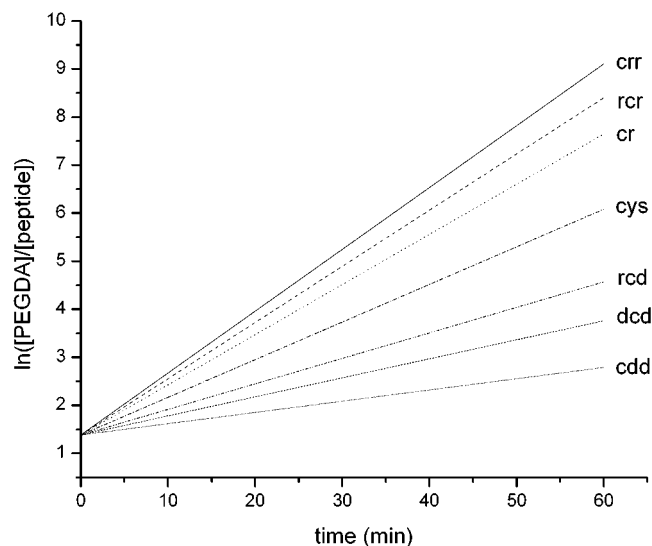
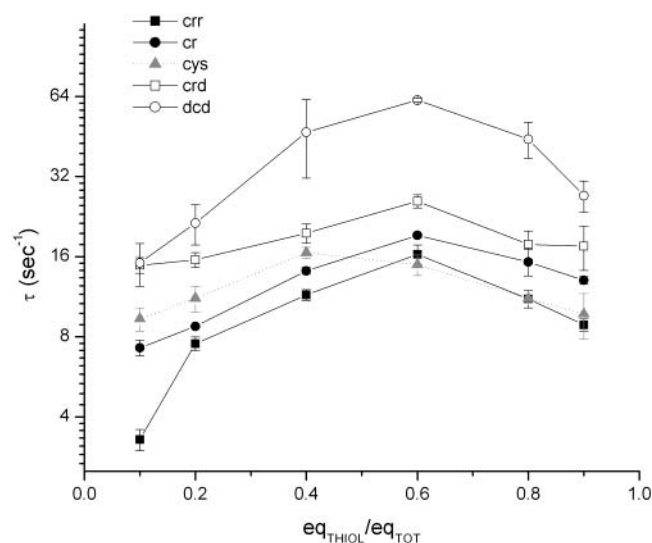
Positive charges close to the SH-groups were observed to speed the reaction considerably, while the opposite effect was shown by nearby groups of negative charge. The effect did not depend on the precise location of the charges: the rate constant for **crr** and **rcr** or **rcd**, **crd**, and **cdr** were not significantly different from each other. The sequence **cdd** possessed a considerably higher k -value than **dcd**, but this may be due to the low precision in the **dcd** measurement.

Michael-type addition was generally faster in the presence of excess of acrylic groups, particularly when positive charges were close to the thiol, as depicted in Figure 4 for some peptides and cysteine. In our interpretation, however, this difference derives from an incorrect estimation of the concentration of initial thiols in

Table 2. k and pK_a Values of the Cysteine-Containing Peptides. Both pK_a and k Respond to Changes in the Primary Peptide Structure

peptide	crr	rcr	cr	cys	rcd	crd	cdr	cd	dcd	cdd
k^a [L/mol·min]	136.1	124.0	107.1	88.5	58.3	58.9	58.9	43.9	40.4	21.9
SD ^b	20.49	7.82	11.09	9.11	10.11	7.91	5.70	13.01	17.61	6.14
pK_a^c	8.12	8.22	8.31	8.49	8.50	8.51	8.43	8.57	8.85	8.93

^a Averaged over three to four measurements. ^b SD: standard deviation. ^c Averaged over two or three measurements, standard deviation ± 0.05 (estimated max.).

**Figure 3.** $\ln([PEGDA]/[peptide])$ over time for several peptides (as examples) reacted with PEGDA (here at 80:20 acrylate over thiol ratio). The linearity indicates true second-order kinetics according to eq 1.**Figure 4.** τ values from single-exponential fit from second-order reaction for various PEGDA/peptide ratios. Error bars are standard error of the mean, $n = 3$.

reduced form. We assume the disulfide concentration to be roughly quadratically dependent on the peptide concentration. Thus, in high excess of peptide, disulfide formation could reduce the effective thiol concentration and hence also the calculated reaction rate: e.g., changing a theoretical 80:20 to an actual 75:25 molar ratio. This assumption can be confirmed by the asymmetry in the τ plot of Figure 4. The slowest reacting mixture should be the one having acrylic and thiol groups in stoichiometric amounts. However, an interpolation of the graphic revealed that this was exhibited with samples having a peptide excess of ca. 5–10%. Furthermore,

cysteine, generally assumed to dimerize at a rate 2–4 times lower than other peptides (10), did not show this asymmetry.

Relationship between Primary Structure of the Thiol and Its pK_a . A typical titration curve followed spectrophotometrically at 233 nm is shown in Figure 5A for the peptide GCDDG (**cdd**). Since at high pH, thiols are completely deprotonated, the absorbance $A_{\max}$ was assumed equal to $\epsilon_{S^-}C_{\text{thiol}}$, where ϵ_{S^-} is the absorption coefficient of the thiolate, and C_{thiol} is the peptide concentration.

The pK_a was determined by observing, that for a measurement i , the following holds:

$$-\log([SH]_i/[S^-]_i) = pH_i - pK_a \quad (2)$$

and

$$[SH]_i/[S^-]_i = (C_{\text{thiol}} - [S^-]_i)/[S^-]_i = (A_{\max} - A_i)/A_i \quad (3)$$

As required by eq 3, the intercept with the abscissa in a graphical representation of $-\ln[(A_{\max} - A_i)/A_i]$ vs pH yielded the pK_a value (Figure 5B).

The pK_a of cysteine agrees with the commonly accepted value of 8.4–8.5. The presence of charges close to the thiols strongly influenced the pK_a (Table 2), hindering the deprotonation in case of negative charges and facilitating it for positive ones. In contrast to the results obtained for the second-order rate constants, pK_a values generally responded to the charge location. The pK_a of **crr** and **cdd** could be distinguished from **rcr** and **dcd**, respectively. A stronger charge effect was found for peptides bearing vicinal charges (**crr**, **cdd**). For the zwitterionic peptides **rcd**, **crd**, and **cdr**, as well as for cysteine (which is zwitterionic itself), similar values were measured.

Quantitative Relationship between Thiol pK_a and Michael Addition Reactivity. Positive charges were shown to decrease the pK_a of thiols, that is, at constant pH, to increase the concentration of thiolates. Along with the protonation state, an increase in Michael-type reactivity was observed (as described above). The parallel change of both parameters would confirm the observation of other groups (25), that thiolates are the reactive moiety in this reaction. Under this assumption, the second-order kinetic equation $v = kC_{\text{thiol}}C_{\text{acrylate}}$ can be expressed as

$$v = k_{\text{real}}[S^-]C_{\text{acrylate}} \quad (4)$$

$$\text{thus, } k = k_{\text{real}}[1/(1 + [H^+]/K_a)] = k_{\text{real}}K_a/(K_a + [H^+]) \quad (5)$$

According to eq 5, a linear relationship should be seen between $K_a/(K_a + [H^+])$ and k . Whereas cysteine did not precisely follow this correlation, a good agreement was shown for all peptides (Figure 6). The behavior of cysteine is likely due to the shorter distance of the ionic groups from the thiol and to the different structure of the molecule (no peptide bond).

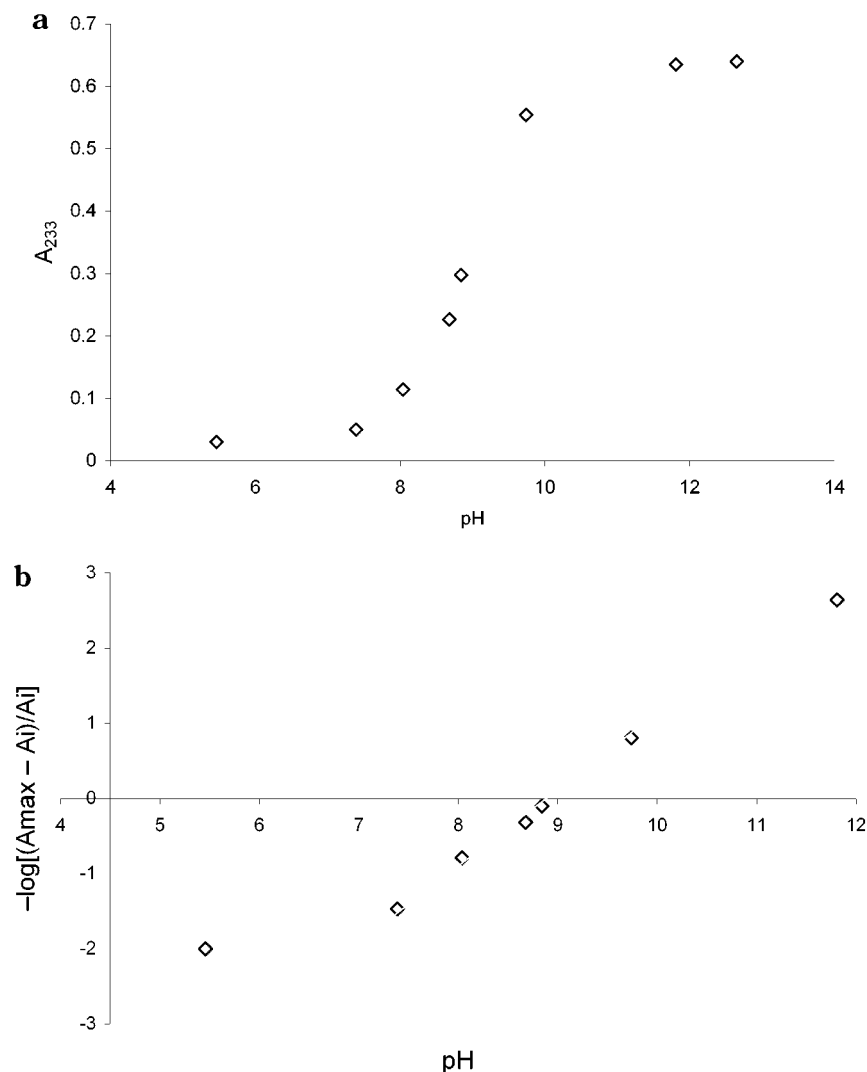


Figure 5. (A) Absorbance at 233 nm as a function of pH for the peptide GCDDG. (B) $\log[(A_{\max} - A_i)/A_i]$ vs pH. The pK_a value corresponds to the intercept with the abscissa. The linear approximation was only used in the buffer region (in this case, the five central points).

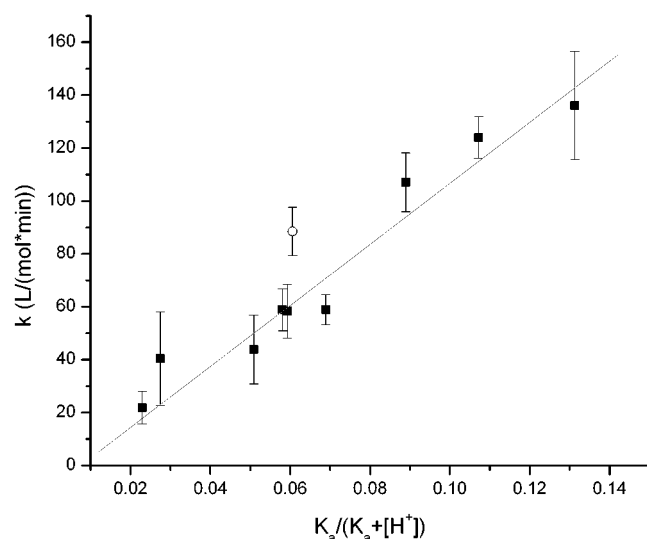


Figure 6. Correlation of $K_a/(K_a + [H^+])$ and k . Peptides (○), cysteine (■). Error bars indicate standard error of the mean, $n = 3$.

A linear fit of the k values for the all investigated peptides gave a k_{real} value of $1040 \pm 40 L/(mol \times e1 \cdot min)$.

CONCLUSION

Due to its selectivity and favorable kinetics in physiological conditions, the Michael-type addition of sulfhydryl-containing biomolecules onto unsaturated groups is an ideal method for many bioconjugation purposes, both in the formation of cross-linked gels and in the PEGylation of therapeutic proteins. Obtaining high self-selectivity in the coupling and tailoring the reaction kinetics to the needs of an application can be crucial in many applications, e.g., in the preparation of polymer-peptide conjugates for in vivo cross-linking materials. In the present work, this issue was approached by designing a homologous series of peptides with charged amino acids flanking a cysteine, to elaborate the relationship between structure of the reaction partners and Michael-type reactivity. The influence of the peptide primary structure on thiol reactivity was significant: charged amino acids close to the SH groups considerably altered the second-order reaction kinetics, positive charges increasing and negative charges decreasing the reaction rate. In terms of changes in pK_a , a decrease of approximately 0.2 or an increase of approximately 0.2 units per positive or negative charge, respectively, was observed. The good correlation between the variations in pK_a and reaction rate confirmed the thiolates to be the only active group

and that the effect of thiolate concentration on reaction kinetics most likely overwhelms any other structural factor.

These results provide the basis for the rational design of peptides or proteins for optimal use in conjugation schemes with polymeric structures, where selectivity and controlled reaction kinetics are desired.

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Evaluation of Radiolabeled Type IV Collagen Fragments as Potential Tumor Imaging Agents

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The objective of this study was to examine radiopharmaceuticals that target the $\alpha\beta 1$ integrin to determine if these agents target tumors for diagnostic imaging and/or targeted radiotherapy of cancer. Prior studies had shown that residues 531–542 from the $\alpha 1$ chain of type IV collagen bind a variety of tumor cell $\alpha\beta 1$ integrins. A peptide mimic of this sequence containing all D-amino acids (designated D-Hep-III) was synthesized by solid-phase methods. The tetraazamacrocyclic chelator, TETA, was conjugated to the peptide while it was resin-bound. TETA-D-Hep-III and D-Hep-III were radiolabeled with ^{64}Cu and ^{125}I , respectively, in high specific activity and radiochemical purity. Heterologous competitive binding assays between D-Hep-III and either ^{125}I -D-Hep-III or ^{64}Cu -TETA-D-Hep-III indicated low micromolar affinity of D-Hep-III. The biodistribution of each radiolabeled analogue of D-Hep-III was carried out in rats and tumor-bearing mice. Both analogues were rapidly cleared from the blood in normal rats, with the kidneys receiving the highest accumulation of each. SKOV3 human ovarian tumor cells, known to strongly express $\alpha\beta 1$, were xenografted in SCID mice. Localization of ^{125}I -D-Hep III and ^{64}Cu -TETA-D-Hep III in the xenografts were low ($<2\%$ ID/g), and in the case of ^{125}I -D-Hep III, not inhibited by a competitive dose of D-Hep III. The low tumor accumulation is likely not due to receptor down-regulation, but rather due to the weak affinity of the radioligands for the $\alpha\beta 1$ integrin.

INTRODUCTION

Radiolabeled analogues of peptide hormones are under current investigation as diagnostic imaging agents and radiotherapeutics for the diagnosis or treatment of cancer (for reviews see refs 1–3)). These radiolabeled peptide receptor ligands are targeted to tumors that have up-regulated receptors on the tumor cell surface. For example, ^{111}In -DTPA–octreotide is currently in routine clinical use for imaging neuroendocrine tumors overexpressing the somatostatin receptor (4).

Integrins are a family of receptors that bind various components of the ECM¹. They are heterodimeric glycoproteins comprised of two subunits, α and β , that each span the cell membrane once. A β -subunit can pair with a number of different α -subunits, and this combination confers the unique binding properties of that integrin. Also, cell type-specific factors appear to play a role in determining specificities of integrins. For example, $\alpha 2 \beta 1$ is a receptor for collagen but not laminin on platelets (5); however, $\alpha 2 \beta 1$ is a receptor for both ligands on other cell types (6). In addition to promoting adhesion, integrins

provide a linkage between the ligand and intracellular proteins and can promote signal transduction (7). The cells of most normal human tissues express a variety of integrins including $\alpha 1 \beta 1$, $\alpha 2 \beta 1$, $\alpha 3 \beta 1$, and $\alpha 6 \beta 1$, which are primarily required for adhesion to collagen and laminin (for reviews, see refs 8, 9, 10). Radiolabeled ECM fragments have been investigated as imaging agents (11–14) since their integrins are up-regulated in certain types of tumors and can therefore be a target for diagnosis or therapy.

Immunohistochemical analysis of human tissue showed expression of $\alpha 3 \beta 1$ in dermal and lung epithelium, in large vessel and capillary endothelium (15), and in certain cell types within rat and human kidney (16, 17). Immunohistochemical methods on surgically excised specimens from human tumors showed strong expression of $\alpha 3 \beta 1$ on primary tumors from the ovary, colon–rectum, stomach, and skin as well as on tumors from their metastatic sites (18). Moreover, $\alpha 3 \beta 1$ is frequently expressed on cultured human tumor cell lines such as ovarian carcinoma (19), breast carcinoma (20), and melanoma (21, 22). A noninvasive means of assessing $\alpha 3 \beta 1$ expression could provide useful information about the extent of metastasis prior to therapy or surgery by possibly revealing metastatic sites too small for conventional imaging techniques.

Since the natural ligands for several integrins are collagens, radiolabeled fragments of collagen have the potential to serve as imaging agents. Recently, peptides incorporating segments of type IV collagen have demonstrated specific binding to the $\alpha 3 \beta 1$ integrin. One of these peptides, Hep-III (GEFYFDLRLKGDK), consisting of residues 531–543 from the $\alpha 1$ chain of type IV collagen, promoted 40–50% adhesion of human ovarian, breast

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¹ ECM, extracellular matrix; PET, positron emission tomography; TETA, 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; HBTU, O-(1H-benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate; BSA, bovine serum albumin; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; Mab, monoclonal antibody; ATCC, American Type Culture Collection).

carcinoma, and melanoma cell lines at concentrations of 7–11 μM (23). An all D-isomer of Hep-III (D-Hep-III) was as effective as the L-isomer in promoting cell adhesion of these cell lines, indicating that the receptor does not discriminate between the two enantiomers (23, 24). Furthermore, D-Hep-III was found to bind $\alpha 3\beta 1$ integrins on cultured human tumor cells in a fashion similar to Hep-III (19, 24). Radiopharmaceuticals derived from all D-peptides are particularly attractive due to their anticipated resistance to proteolysis (24, 25).

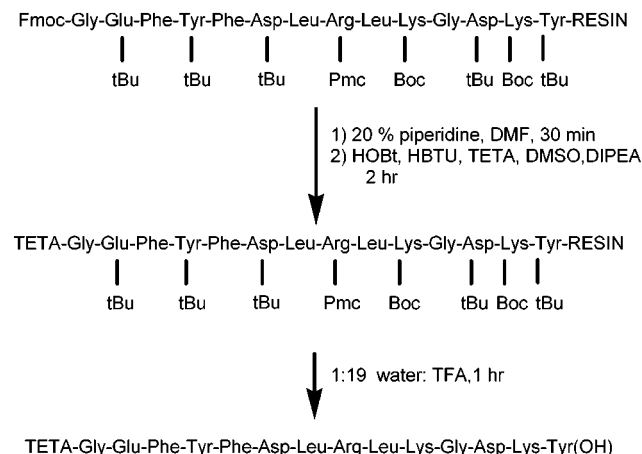
Copper-64 ($t_{1/2} = 12.7$ h, $\beta^+ = 0.655$ MeV [19.3%], $\beta^- = 0.573$ MeV [39.6%]) has emissions suitable for PET imaging (β^+) as well as radiotherapy (β^-) (3, 26). A commercially available chelator, TETA, has been conjugated to both peptides and monoclonal antibodies (Mabs) for subsequent labeling with radiometals such as ^{64}Cu . The resulting complex has demonstrated relatively high *in vivo* stability (27–29). The N-terminal amine of D-Hep III was chosen as the site for conjugation of one of the carboxylic arms of TETA because the N-terminus did not appear important in binding (23). Iodine radionuclides are also of interest due to the availability of cyclotron-produced ^{124}I ($t_{1/2} = 4.2$ d, $\beta^+ = 2.14$ MeV [25%]) for PET imaging (30–32) and ^{131}I for therapy. Because $\alpha 3\beta 1$ is highly expressed in several types of human neoplasms, it was hypothesized that ^{64}Cu - or ^{125}I -labeled D-Hep III would localize to tumors in a receptor specific manner. The aim of this investigation was to demonstrate that ^{64}Cu - and ^{125}I -labeled D-Hep III peptides were specifically bound to receptor positive cells or tissues, estimate receptor affinities *in vitro*, and determine whether they would localize within tumor xenografts in a receptor specific manner *in vivo*.

EXPERIMENTAL SECTION

Reagents. All standard peptide synthesis chemicals were analytical reagent grade or better and obtained from Applied Biosystems (Foster City, CA) or Fisher Scientific (St. Louis, MO). DBU and TETA·4HCl were obtained from Aldrich (Milwaukee, WI). Fmoc-Tyr(t-Bu)-HMP resin (substitution level = 0.31 mmol/g) was from Millipore Corporation (Bedford, MA). HBTU was from Richlieu Biotechnologies (St. Hyacinthe, Quebec). Glass test tubes precoated with Iodogen were obtained from Pierce (Rockford, IL). The mouse anti-human integrin, $\alpha 3$ monoclonal antibody (Mab anti- $\alpha 3$) was obtained from Chemicon International (Temecula, CA). BSA and 0.5 M EDTA were from Sigma (St. Louis, MO), and ammonium acetate was from Fluka (Ronkonkoma, NY). C-18 Sep-pak Light cartridges were from Waters (Milford, MA). Sprague–Dawley rats were obtained from either Charles River (Wilmington, MA) or Harlan Bioproducts (Indianapolis, IN), and SCID mice were from Taconic (Germantown, NY). Copper-64 was produced as previously described (33). ^{125}I was obtained from Amersham (Piscataway, NJ). Radio-TLC detection was accomplished using a BIOSCAN System 200 Imaging Scanner (Washington, DC). Radioactive samples were counted using a Beckman 8000 automated well-type counter (Fullerton, CA).

High Performance Liquid Chromatography. HPLC analysis and purification was performed on one of two systems: Spectra Physics 8700 ternary pump and controller with a Waters 486 UV detector, or a Waters 600E quaternary pump and controller with a Waters 991 photodiode array UV–Vis detector and Waters 771 autosampler. Both systems were fitted with Rheodyne 7125 injectors and radioactivity was detected by NaI scintillation.

Scheme 1. Solid-Phase Synthesis of TETA-D-Hep-III. All Residues Are in the D-Configuration Except for Glycine



Peptide Synthesis, Purification, and Analysis.

The peptides were assembled on the Fmoc-Tyr(t-Bu)-HMP resin by Fmoc chemistry as previously described (34) on an Applied Biosystems 431A Peptide Synthesizer. Purity was assessed on an analytical Vydac C-18 RP-HPLC column (300 Å, 5 μm , 4.6 $\times$ 250 mm) using a solvent system of A, consisting of H₂O (0.1%TFA) and B, consisting of acetonitrile (0.1%TFA):H₂O (0.1%TFA), 9:1, v:v. The peptides were purified on a Vydac C-18 semipreparative column (300 Å, 15–20 μm , 22 $\times$ 250 mm) unless noted. The flow rates were 1.0 and 12.0 mL/min for the analytical and semipreparative column, respectively. Edman degradation analysis was performed on an Applied Biosystems 477A Protein Sequencer/120A Analyzer. Peptide molecular weights were determined on a Finnigan (San Jose, CA) LCQ ion-trap electrospray mass spectrometer by the Protein and Nucleic Acid Chemistry Laboratories in the Departments of Medicine and Molecular Biology and Pharmacology at the Washington University School of Medicine. Data were collected and analyzed using the Finnigan LCQ Bioworks software (version 1.1). Size-exclusion chromatography was performed on a Superdex HR 10/30, 10 $\times$ 100 mm, using a solvent system consisting of 20 mM NH₄OAc pH 7.5 at a flow rate of 0.8 mL/min. The column was calibrated against insulin, D-Hep-III and 3-iodotyrosine, which eluted at 13.6, 18.5, and 38.2 min, respectively (BSA eluted at 10.4 min in the void volume). A plot of the elution volume vs log molecular weight was linear ($r = 0.98$). Peptide concentrations were determined by UV absorbance at 280 nm utilizing the extinction coefficient for tyrosine in water ($\epsilon = 1200$ L M⁻¹ cm⁻¹) (35). Because D-Hep-III contains two tyrosine residues, the molar extinction coefficient (ϵ) was for D-Hep-III was assumed to be 2400 L M⁻¹ cm⁻¹.

Solid-Phase Synthesis of TETA-D-Hep III. TETA-D-Hep III was synthesized by coupling TETA to the N-terminal amine of the resin-bound peptide (Scheme 1). LiOH·H₂O (34.6 mg, 0.82 mmol) was added to TETA·4HCl (106 mg, 0.18 mmol) and dissolved in 1.5 mL of DMSO. HBTU (63 mg, 0.16 mmol) was added to 208 mg (0.046 mmol) of resin containing the D-Hep-III sequence. The TETA solution was added, immediately followed by 64 μL (0.37 mmol) of DIEA. After 4 h, the mixture was filtered over a fine, fritted, glass funnel, and the resin was rinsed with 3 $\times$ 5 mL DMSO, 3 $\times$ 5 mL DMF, 2 $\times$ 5 mL methanol:H₂O, 1:1, 2 $\times$ 5 mL H₂O, and 3 $\times$ 5 mL methanol and was then dried *in vacuo*. The peptide was released from the resin and side-chain protecting groups

were simultaneously removed in 3.5 h with 3.5 mL of TFA: H₂O, 19:1. The resin was filtered through a fine, fritted glass funnel and rinsed with 2 mL of TFA. Greater than 90% of the peptide was cleaved from the resin. The filtrate was concentrated to 1.5 mL in vacuo and added to 35 mL of *tert*-butyl-methyl ether (4 °C). The white precipitate was collected by centrifugation at 3000 rpm for 10 min at 4 °C. The pellet was rinsed twice with 35 mL of *tert*-butyl-methyl ether (4 °C). RP-HPLC analysis showed about 40% coupling of TETA to the *N*-terminal amine. The crude mixture was dissolved in TFA (30 mg/mL) and 4.9 mg was loaded onto a semipreparative Vydac C-18 RP-HPLC column and purified with a gradient of 24 to 34% B in 45 min. TETA-D-Hep III (1.0 mg) was recovered in 20% yield and D-Hep III (0.8 mg) in 16% yield. Both were greater than 98% pure as determined by RP-HPLC. The peptides were identified by ES-MS (D-Hep III: calcd 1751, obsvd 1751; TETA-D-Hep-III: calcd 2165, obsvd 2165).

Radiolabeling of TETA-D-Hep III with ⁶⁴Cu. TETA-D-Hep III (1.5 μg) was labeled in ammonium acetate (100 mM, pH 5.5, 0.1% BSA) with 200–1000 μCi of ⁶⁴Cu-acetate at room temperature. After 15–30 min, 0.5 M EDTA was added to the reaction mixture (for a final concentration of 1 mM EDTA). By HPLC, ⁶⁴Cu-TETA-D-Hep III eluted at 20.26 min (22 to 32% B in 20 min, analytical) while unbound ⁶⁴Cu or ⁶⁴Cu-EDTA both eluted in the void volume. By radio-TLC (C-18, Whatman, 3:2 methanol: 10% ammonium acetate), ⁶⁴Cu-TETA-D-Hep III had a *R_f* of 0.6, compared to ⁶⁴Cu-acetate, which remained at the origin, and ⁶⁴Cu-EDTA, which migrated to the solvent front. The mixture was purified on a C-18 Sep-Pak preconditioned with ethanol and water and eluted with labeling buffer to remove ⁶⁴Cu-EDTA. Elution with ethanol produced ⁶⁴Cu-TETA-D-Hep III in ≥ 95% purity as demonstrated by HPLC and TLC.

Radiolabeling of D-Hep III with ¹²⁵I. D-Hep-III (10 μg, 1.0 mg/mL, 1:9 DMSO:H₂O) was added to 20 μL of sodium phosphate (200 mM, pH 7.5) in a 1.5 mL polypropylene vial. Sodium (¹²⁵I)-iodide (1.3 mCi) was added to the vial followed by 100 μg of chloramine-T (10 mg/mL in sodium phosphate, 200 mM, pH 7.5). After 2 min, the iodination was quenched with 500 μg of Na₂S₂O₅ (10 mg/mL in sodium phosphate, 200 mM, pH 7.5). The mixture was diluted with 200 μL of TFA and purified on an analytical Vydac C-18 column eluted with 25 to 35% B in 45 min. D-Hep-III eluted at 15.0 min, and the major radioactive product, ¹²⁵I-D-Hep-III, eluted at 21.0 min. Radiochemical yield was 24% and purity was ≥95%. Purity was assessed on an analytical Vydac C-18 RP-HPLC column. As a control to test for degradation of D-Hep-III by chloramine-T, 40 μg of D-Hep-III in 1:9 DMSO:H₂O was added to 40 μL of chloramine-T (10 μg/μL in sodium phosphate, 200 mM, pH 7.5). After 2 min, the mixture was added to 500 μg of Na₂S₂O₅ (10 mg/mL in sodium phosphate, 200 mM, pH 7.5) to quench the chloramine-T.

Iodination of Mab anti-α3. A precoated Iodogen tube was rinsed with 1 mL of Tris-HCl (200 mM, pH = 7.5). Tris-HCl (40 μL, 200 mM, pH = 7.5) was added to the glass tube followed by Na(¹²⁵I)I (181 μCi) and then Mab anti α3 (5 μg). The mixture was allowed to react for 20 min. The iodination mixture was transferred to a 1.5 mL polypropylene tube and allowed to stand for 5 min before addition of Tris-HCl (200 mM, 1 mg/mL BSA, pH = 7.5) to a final volume of 200 μL. Incorporation of ¹²⁵I into the Mab was determined by precipitation of total protein with 10% trichloroacetic acid. Incorporation was 92% and the

¹²⁵I-Mab-anti-α3 was not further purified. Specific activity of ¹²⁵I-Mab-anti-α3 was determined to be 33 μCi/μg.

Serum Stability of Radiolabeled D-Hep III Analogues. ¹²⁵I-D-Hep-III (50 μL) in ethanol was added to 500 μL of rat serum and incubated at 37 °C for 3 h. The incubation mixture was monitored on an analytical Vydac C-18 RP-HPLC column eluted with a gradient of 20 to 90% B in 30 min (1 mL/min, A = H₂O, 0.1% TFA; B = acetonitrile, 0.1% TFA). Stability was monitored for 3 h by RP-HPLC. To estimate serum protein binding of ¹²⁵I-D-Hep-III, it was concentrated from RP-HPLC eluant in vacuo with 20 μL of PBS containing 1 mg/mL BSA and incubated for 40 min at 37 °C and then analyzed by SEC. To investigate whether higher concentrations of D-Hep-III caused aggregation of ¹²⁵I-D-Hep-III, 280 μM D-Hep III was incubated with ¹²⁵I-D-Hep-III prepared as described above (serum protein binding experiment) in both rat serum (75 μL) and PBS (75 μL) at 37 °C for 30 min followed by analysis by SEC.

Copper-64-TETA-D-Hep-III in ethanol was concentrated in vacuo with 10 μL of NH₄OAc (100 mM, pH 5.5, 10 mg/mL BSA). Rat serum (75 μL) was added, and the mixture was incubated at 37 °C for 1 h. Stability was monitored for 1 h by RP-HPLC.

Cell Maintenance. The SKOV3 human ovarian tumor cell line was obtained from ATCC (Rockville, MD) and maintained in McCoy's 5A media (Iwakata and Grace modification with glutamine) from Mediatech (Herndon, VA) with 10% fetal bovine serum supplemented with 50 units/mL penicillin and 0.05 mg/mL streptomycin. Cells were passaged for 4–5 weeks and then replaced from frozen stocks of earlier passages to minimize phenotypic drift. SKOV3 cells were detached with either 0.05% trypsin with 0.53 mM EDTA in HBSS or 5 mM EDTA in PBS each without calcium or magnesium salts.

Preparation of Membranes from Harvested Xenografts and SKOV3 Cultured Cells. SKOV3 cells were split 1:2 24 h prior to harvesting to ensure they were in the growth phase. Cells were detached at about 80% confluency with 5 mM EDTA in PBS and concentrated by centrifugation at 200g for 10 min at 4 °C. The supernatant was discarded, and the cells were suspended in a homogenizing buffer consisting of 50 mM Tris·HCl, 5 mM MgCl₂, pH 7.5, containing aprotinin (0.5 μg/mL), leupeptin (10 μg/mL), pepstatin A (10 μg/mL), phenylmethylsulfonyl fluoride (2 μg/mL), and *N*-ethylmaleimide (2 μg/mL). The cells were lysed by sonication and centrifuged at 34000g for 30 min at 4 °C. After Dounce homogenization of the pellet in the homogenizing buffer, the membranes were quantified with the BCA assay (Pierce, Rockford, IL) relative to BSA. The membranes were centrifuged at 34000g for 30 min at 4 °C and then suspended at a concentration of 1.5 mg/mL in PBS, 1 mM MgCl₂, 1 mg/mL BSA, and stored in aliquots at –80 °C.

Membranes from xenografted tumor were prepared by homogenization of 0.5 g tumor/10 mL homogenizing buffer at 4 °C. After centrifugation at 2000g, the supernatant was stored on ice while the pellet was rehomogenized and recentrifuged twice. The combined supernatants were then recentrifuged at 34000g for 30 min at 4 °C. The pellet was resuspended in 15 mL of homogenizing buffer, and protein content quantified by the BCA method relative to BSA. The membranes were centrifuged at 34000g for 30 min at 4 °C and then suspended at a concentration of 1.5 mg/mL in PBS, 1 mM MgCl₂, and 1 mg/mL BSA, and stored in aliquots at –80 °C.

Binding of ¹²⁵I-Mab-anti-α3 to Membranes Prepared from Xenografted SKOV3 tumor. The specific binding of ¹²⁵I-Mab-anti-α3 was measured at infinite

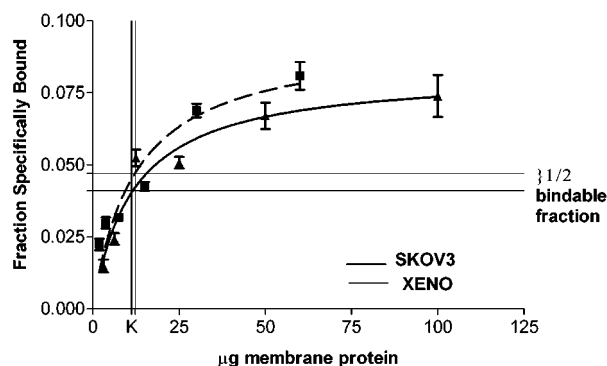


Figure 1. Binding of ^{125}I -Mab-anti- $\alpha 3$ to membranes from SKOV3 cells (▲) and SKOV3 xenografts (■). K represents the amount of membrane protein required to bind one-half of the bindable fraction of ^{125}I -Mab-anti- $\alpha 3$. K for xenografted SKOV3 membranes = $12.3 \pm 4.7 \mu\text{g}$ and K for SKOV 3 membranes from cell culture = $11.7 \pm 3.0 \mu\text{g}$.

antigen excess (36) with membranes from SKOV3 cells and SKOV3 xenografted tumor (passage 10). Seven 1:2 serial dilutions of $833 \mu\text{g/mL}$ membrane protein were made in PBS containing MgCl_2 (1 mM) and BSA (1 mg/mL). Iodine-125-labeled Mab-anti- $\alpha 3$ was added to the membranes for a final concentration of 466000 to 497000 cpm/mL. To assess nonspecific binding, Mab-anti- $\alpha 3$ was added to membranes containing the radioiodinated Mab for a final concentration of $33 \mu\text{g/mL}$. Nonspecific binding of ^{125}I -Mab-anti- $\alpha 3$ to the glass fiber filters was assessed by adding the tracer to the binding buffer alone. The samples were allowed to bind at 24°C for 2 h, and then five 125 mL aliquots were filtered in a 96-well plate with $1.0 \mu\text{m}$ glass fiber filters. The filters were rinsed with $3 \times 50 \mu\text{L}$ of cold (4°C) binding buffer, dried at $\sim 40^\circ\text{C}$, and removed to 1.5 mL polypropylene centrifuge tubes by the Millipore Multiscreen system (Bedford, MA) and counted in a gamma counter. The data (Figure 1) were fit by nonlinear regression according to a one-site binding hyperbola model with the following equation: $Y = (R_f^{\text{max}}) \cdot (X) / (K + X)$ with Graphpad Prism, where $X = \mu\text{g}$ membrane protein; $Y = \text{reactive fraction}$; R_f^{max} = maximum reactive fraction; K = amount of membranes (μg) required to bind one-half of the ^{125}I -Mab-anti- $\alpha 3$.

To establish $\alpha 3\beta 1$ density, a homologous competitive binding assay was carried out between the ^{125}I -Mab-anti- $\alpha 3$ and Mab-anti- $\alpha 3$. Briefly, membranes from SKOV3 xenografted tumor ($200 \mu\text{g}$, passage 10) were suspended in binding buffer (PBS, 1 mg/mL BSA, 1 mM MgCl_2) along with ^{125}I -Mab-anti- $\alpha 3$ (0.0144 nM , $\sim 80000 \text{ cpm}$) and either 0.02, 0.1, 0.6, 2.9, 14, 72, or 177 nM Mab-anti- $\alpha 3$. One preparation contained only ^{125}I -Mab-anti- $\alpha 3$. The total volume was $370 \mu\text{L}$. The samples were allowed to bind at 24°C for 2 h, and then four $40 \mu\text{L}$ aliquots were filtered in a 96-well plate with $1.0 \mu\text{m}$ glass fiber filters. The filters were rinsed with $3 \times 50 \mu\text{L}$ cold (4°C) binding buffer, dried at $\sim 40^\circ\text{C}$, removed to 1.5 mL polypropylene centrifuge tubes by the Millipore Multiscreen system (Bedford, MA), and counted in a gamma counter. Specifically bound cpm were plotted against $\log [\text{Mab-anti-}\alpha 3]$ concentration, and the data was fit by nonlinear regression to a one-site competition curve in Prism (Graphpad, San Diego, CA) (Figure 2).

Heterologous Competitive Binding Assays. Membranes from SKOV3 xenografts (passage 10) were thawed, Dounce homogenized, and diluted in PBS containing MgCl_2 (1 mM), BSA (1 mg/mL) (binding buffer) to prepare a stock solution of membranes. Either ^{125}I -D-Hep-III or ^{64}Cu -TETA-D-Hep-III was diluted to prepare a stock

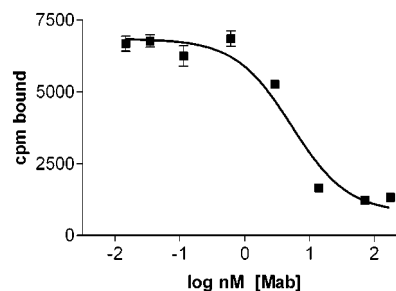


Figure 2. Homologous competitive binding between ^{125}I -Mab-anti- $\alpha 3$ and Mab anti $\alpha 3$ to membranes from SKOV3 xenografts, passage 10 ($\text{IC}_{50} = 5.3 \text{ nM}$).

solution of tracer in the binding buffer. D-Hep-III (1 mg/mL in 1:9 DMSO:water) was serially diluted in the same vehicle at a volume of $25 \mu\text{L}$ in 1.5 mL polypropylene centrifuge tubes. Membranes ($500 \mu\text{L}$) and tracer ($250 \mu\text{L}$) were added to the D-Hep-III for a final concentration of 400 to $600 \mu\text{g/mL}$ membrane protein and 200 to 300 kcpm/mL tracer, respectively. The mixtures were allowed to mix at room temperature for 2 h, and then four to five aliquots of $100\text{--}125 \mu\text{L}$ were layered over $250 \mu\text{L}$ of dioctylphthalate:dibutylphthalate (55:45) in 1.5 mL polypropylene centrifuge tubes. To separate tracer bound to membrane protein from unbound, the aliquots were centrifuged at 14000 rpm in an Eppendorf 5415 benchtop centrifuge. After removing the supernatant, the pellets were counted in a gamma counter. Specifically bound cpm were plotted against $\log [\text{D-Hep-III}]$ concentration, and the data was fit by nonlinear regression to a one-site competition curve using Prism.

Biodistribution Studies: General. The laboratory rats and mice were maintained in the animal facilities operated by the Division of Comparative Medicine at Washington University School of Medicine in accordance with the American Association for Accreditation of Laboratory Animal Care (AAALAC) requirements. The radiolabeled D-Hep-III analogues and blocking doses were injected into the tail vein of anesthetized rats and mice. The vehicle was either 0.9% NaCl or 10 mM NH_4OAc , pH 6, each containing 0.1% BSA. Animals were sacrificed by cervical dislocation while under Metofane anesthesia at specific time points postinjection. Organs of interest were excised, counted in a gamma counter, and then weighed to generate the percent injected dose/organ (% ID/organ) and percent injected dose/gram tissue (% ID/g).

Biodistribution of ^{64}Cu -TETA-D-Hep-III and ^{125}I -D-Hep-III in Male Sprague–Dawley Rats. Copper-64-TETA-D-Hep-III ($5.0 \mu\text{Ci}/100 \mu\text{L}/\text{rat}$ 0.1 M ammonium acetate, 1 mg/mL BSA $\geq 95\%$ radiochemical purity, specific activity $798 \text{ Ci}/\text{mmol}$) and ^{125}I -D-Hep-III ($5.0 \mu\text{Ci}/100 \mu\text{L}/\text{rat}$; water, 1 mg/mL BSA, $\geq 98\%$ radiochemical purity, specific activity $2000 \text{ Ci}/\text{mmol}$) were coinjected into male-Sprague–Dawley rats ($\sim 500 \text{ g}$). The gamma counter windows were adjusted so that none of the cpm from the ^{125}I contributed to the cpm of ^{64}Cu . After 6 days (> 10 half-lives, ^{64}Cu) the tissues were recounted for ^{125}I .

SKOV3 Xenografts in SCID Mice. One hundred microliter aliquots (7.5×10^6 cells) were implanted sc in the right and left flank of ≥ 5 week old female SCID mice. Tumor growth was measured every 3–5 days with calipers. The tumors grew to 500 to 1500 mm^3 within 3–4 weeks. This tumor model was maintained by serial passages of solid tumor out to passage 12 by implantation of two tumor pieces ($\sim 3 \text{ mm}^3$) in the right and left flank of female SCID mice.

Biodistribution of ^{64}Cu -TETA-D-Hep-III in SCID Mice Bearing SKOV3 Tumors. Nine mice bearing xenografted SKOV3 tumors (passage 3) implanted bilaterally on the flank were used at 21 days post-implantation. Five mice received ^{64}Cu -TETA-D-Hep-III ($5.8 \mu\text{Ci}/100 \mu\text{L}/\text{mouse}$, 0.1 M ammonium acetate, 1 mg/mL BSA, 540 Ci/mmol). The animals were sacrificed at 1 h post-injection, and tissues were removed and counted in a gamma counter for biodistribution as described above.

Biodistribution of ^{125}I -D-Hep-III in SCID Mice Bearing SKOV3 Tumors. Four SCID mice bearing bilateral SKOV3 xenografts (passage 5) received ^{125}I -D-Hep-III ($1 \mu\text{Ci}/100 \mu\text{L}/\text{mouse}$, 0.9% NaCl, 1 mg/mL BSA, $\geq 2000 \text{ Ci/mmol}$). Four tumor-bearing mice were coinjected with $1 \mu\text{Ci}$ ^{125}I -D-Hep-III ($1 \mu\text{Ci}/100 \mu\text{L}/\text{mouse}$, 0.9% NaCl, 1 mg/mL BSA, $\geq 2000 \text{ Ci/mmol}$) and $9 \mu\text{g}$ D-Hep-III. Three mice were coinjected with $1 \mu\text{Ci}$ ^{125}I -D-Hep-III ($1 \mu\text{Ci}/100 \mu\text{L}/\text{mouse}$, 0.9% NaCl, 1 mg/mL BSA, $\geq 2000 \text{ Ci/mmol}$) and $1.7 \mu\text{g}$ D-Hep-III. The animals were sacrificed at 1 h postinjection and biodistribution was determined as described above.

RESULTS

Peptide Synthesis. Following Fmoc solid-phase synthesis of D-Hep-III, the peptide was found to be compositionally correct by Edman degradation sequence analysis. D-Hep-III was isolated by RP-HPLC and further characterized by mass spectrometry. Purities were determined by analytical RP-HPLC, with monitoring both at $\lambda = 222$ and 280 nm , and were greater than 98% .

TETA was coupled to D-Hep-III while it was resin-bound. TETA-4HCl had low solubility in DMF or DMSO; however, neutralization with lithium hydroxide improved its solubility in DMSO. Coupling yields were in the range of 40 to 50% and could not be improved by further coupling reactions. RP-HPLC analysis of the unpurified cleavage mixtures showed that D-Hep-III and TETA-D-Hep-III eluted closely, even with a relatively shallow gradient, which resulted in numerous fractions from the semipreparative purification to be mixtures of both peptides in varying amounts. TETA-D-Hep III was recovered in 20% yield and D-Hep III in 16% yield.

Radiochemistry. Radiochemical yields of ^{64}Cu -TETA-D-Hep-III ranged from 40 to 90% and radiochemical purity $\geq 95\%$ and specific activities ranged from 500 to 2000 Ci/mmol . D-Hep-III contains two tyrosine residues, each of which has the potential for radioiodination. RP-HPLC of the purified ^{125}I -D-Hep-III utilizing a shallow gradient showed a small shoulder in the trace for the major component, suggesting a mixture of two mono-radioiodinated species. Therefore, the ^{125}I -D-Hep-III used in vitro assays and in vivo studies was a probable mixture of two mono-radioiodinated peptides. Whether or not this affected the binding affinity of the peptide is unknown. RP-HPLC investigation of a mixture of D-Hep-III and chloramine-T showed no degradation of the peptide. Separation of the ^{125}I -D-Hep-III from D-Hep-III yielded specific activities of $\geq 2000 \text{ Ci/mmol}$.

Serum Stability. The stability of ^{125}I -D-Hep-III and ^{64}Cu -TETA-D-Hep-III were monitored by RP-HPLC and no changes in retention time or the appearance of new radiolabeled components were observed within 3 h which is indicative of the stability of the radiolabeled D-Hep III analogues. Although RP-HPLC is sensitive to changes in structure, such as cleavage of the peptide bonds, it may not have revealed whether either of the radiolabeled D-Hep-III analogues bound serum proteins. Furthermore, in cases where there were mixtures of the radiolabeled

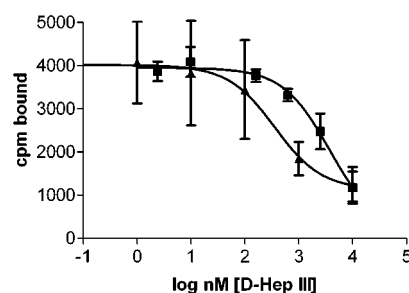


Figure 3. Heterologous competitive binding of ^{125}I -D-Hep-III and D-Hep-III to membranes from SKOV3 cultured cells ($\blacktriangle$, $\text{IC}_{50} = 360 \text{ nM}$) and SKOV3 xenografts, passage 10 ($\blacksquare$, $\text{IC}_{50} = 3700 \text{ nM}$).

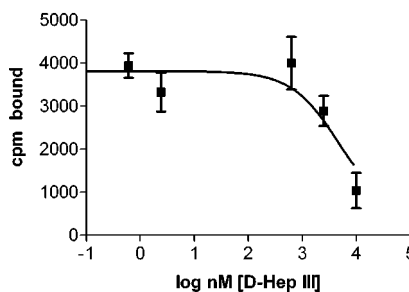


Figure 4. Heterologous competitive binding between ^{64}Cu -TETA-D-Hep-III and D-Hep-III on membranes from SKOV3 cultured cells ($\text{IC}_{50} = 4700 \text{ nM}$).

Table 1. Biodistribution of Radiolabeled D-Hep-III Analogues in Normal Male Sprague-Dawley Rats at 4 h Postinjection. The Data Are Reported as %ID/Gram $\pm$ Standard Deviation

organ	^{64}Cu -TETA-D-Hep-III ($n = 5$)	^{125}I -D-Hep-III ($n = 5$)
blood	0.03 ± 0.002	0.06 ± 0.01
lung	0.06 ± 0.04	0.13 ± 0.02
liver	0.26 ± 0.03	0.61 ± 0.15
kidney ^a	2.98 ± 0.48	27.3 ± 2.61
muscle	0.01 ± 0.001	0.02 ± 0.002
fat	0.01 ± 0.001	0.01 ± 0.003
heart	0.02 ± 0.003	0.04 ± 0.006
bone	0.03 ± 0.004	0.10 ± 0.02
thyroid	0.05 ± 0.02	3.67 ± 1.33
pancreas	0.03 ± 0.01	0.03 ± 0.01
stomach	0.08 ± 0.05	0.13 ± 0.01
intestines	0.55 ± 0.26	0.22 ± 0.04

^a Single kidney.

D-Hep-III analogues and D-Hep-III at a concentration equal to or in excess of $10 \mu\text{M}$, a SEC HPLC column especially designed for low molecular weight peptides showed there was no aggregation of D-Hep-III and its radiolabeled analogues. This is an important control for biodistributions where ^{125}I -D-Hep-III was coinjected with D-Hep-III ($25 \mu\text{M}$) as well as for the competitive binding assays where ^{125}I -D-Hep-III analogues were combined with D-Hep-III ($10 \mu\text{M}$). D-Hep-III at $280 \mu\text{M}$ did not cause the aggregation of ^{125}I -D-Hep-III either in aqueous buffer or rat serum.

Heterologous Competitive Binding Assays between Radiolabeled D-Hep III Analogues and D-Hep III. The heterologous competitive binding assay between ^{125}I -D-Hep-III or ^{64}Cu -TETA-D-Hep-III and D-Hep-III on membranes from SKOV3 cultured cells and xenografts (Figures 3 and 4, Table 3) show that both the D-Hep-III and the radiotracers are specifically bound to cell surface receptors. The 95% confidence intervals ($95\% \text{ CI}$) for the IC_{50} values are large in cases where the concentration of D-Hep-III was not great enough to completely block tracer

Table 2. Biodistribution of ^{125}I -D-Hep-III in SCID Mice Bearing sc Bilateral SKOV3 Tumors at 1 h Postinjection. The Data Are Reported as %ID/Gram $\pm$ Standard Deviation

organ	^{64}Cu -TETA-D-Hep-III (<i>n</i> = 5)	^{125}I -D-Hep-III (<i>n</i> = 4)	^{125}I -D-Hep-III + 1.7 μg D-Hep-III (<i>n</i> = 4)	^{125}I -D-Hep-III + 9 μg D-Hep-III (<i>n</i> = 3)
blood	0.53 $\pm$ 0.14	1.44 $\pm$ 0.526	1.64 $\pm$ 0.478	1.95 $\pm$ 0.255
lung	1.13 $\pm$ 0.10	2.10 $\pm$ 0.199	2.55 $\pm$ 0.338	3.30 $\pm$ 0.528
liver	3.37 $\pm$ 0.97	2.87 $\pm$ 0.340	5.12 $\pm$ 1.36	9.79 $\pm$ 2.63
spleen	1.82 $\pm$ 1.07	1.59 $\pm$ 0.251	2.59 $\pm$ 0.884	4.86 $\pm$ 1.62
kidney ^a	29.7 $\pm$ 2.82	244 $\pm$ 17	224 $\pm$ 54	237 $\pm$ 45
muscle	0.19 $\pm$ 0.03	0.66 $\pm$ 0.25	0.63 $\pm$ 0.089	0.69 $\pm$ 0.13
fat	0.15 $\pm$ 0.07	0.20 $\pm$ 0.06	0.50 $\pm$ 0.25	0.31 $\pm$ 0.19
bone	0.43 $\pm$ 0.13	0.72 $\pm$ 0.02	0.81 $\pm$ 0.06	0.91 $\pm$ 0.094
ovaries	0.33 $\pm$ 0.18	0.75 $\pm$ 0.19	1.11 $\pm$ 0.41	0.83 $\pm$ 0.25
intestines	0.46 $\pm$ 0.10	1.11 $\pm$ 0.10	1.18 $\pm$ 0.17	2.14 $\pm$ 0.36
tumor ^b	0.55 $\pm$ 0.05	1.25 $\pm$ 0.12	1.93 $\pm$ 0.53	1.72 $\pm$ 0.20

^a Single kidney. ^b Average of right and left tumors.

Table 3. IC₅₀ Values for D-Hep-III in Heterologous Binding Assays

radioligand	membrane source	IC ₅₀ , nM	95% CI, nM
^{125}I -D-Hep-III	SKOV3 cultured cells	360	210–614
^{125}I -D-Hep-III	SKOV3 xenograft passage 10	3700	1682–8061
^{64}Cu -TETA-D-Hep-III	SKOV3 cultured cells	4700	1402–15620

binding. The concentrations of the radioligands in the binding assays did not exceed 100 pM.

Biodistributions of ^{64}Cu -TETA-D-Hep-III and ^{125}I -D-Hep III in Normal Rats. A biodistribution of ^{64}Cu -TETA-D-Hep-III and ^{125}I -D-Hep-III in male Sprague–Dawley rats showed that both radiolabeled analogues cleared from the circulation by 4 h ($\leq 0.06 \pm 0.01\%$ ID/g) with the highest accumulation for each in the kidney (Table 1). ^{125}I -D-Hep-III ($27.3 \pm 2.61\%$ ID/g) accumulated 9-fold more ($P < 0.01$) in a single kidney than did ^{64}Cu -TETA-D-Hep-III ($2.98 \pm 0.48\%$ ID/g). Accumulation in the other organs was low except for the thyroid accumulation in the case of ^{125}I -D-Hep-III ($3.67 \pm 1.33\%$ ID/g).

Biodistribution of ^{64}Cu -TETA-D-Hep-III and ^{125}I -D-Hep-III in Tumor Bearing SCID Mice. ^{64}Cu -TETA-D-Hep-III was evaluated in SCID mice bearing bilateral SKOV3 human ovarian tumors (Table 2). ^{64}Cu -TETA-D-Hep-III cleared rapidly from the blood ($0.53 \pm 0.14\%$ ID/g) but demonstrated low tumor accumulation ($0.55\% \pm 0.055\%$ ID/g) at 1 h. High accumulation of ^{64}Cu -TETA-D-Hep III in the kidney was observed ($29.7 \pm 2.82\%$ ID/g).

^{125}I -D-Hep-III was evaluated in SCID mice bearing bilateral sc SKOV3 xenografts with and without competitive doses of 1.7 and 9 μg of D-Hep III. Tumor accumulation was low ($1.25 \pm 0.12\%$) and no dose dependent blocking of tumor accumulation was observed ($\geq 1.72 \pm 0.20\%$ ID/g) at the doses administered ($P > 0.05$). Kidney accumulation after 1 h was very high ($244 \pm 17\%$ ID/g) but dose-dependent blocking was also not observed ($\geq 237 \pm 45\%$ ID/g, $P > 0.05$). However, increases in liver (3.4-fold, $P < 0.05$) and spleen (3.1-fold, $P < 0.01$) accumulation of the radioiodinated peptide were observed with coinjection of D-Hep-III (9 μg), but concomitant decreases in accumulation in other tissues were not apparent.

Binding of ^{125}I -Mab-anti- $\alpha 3$ to Membranes from SKOV3 Cells and Xenografted Tumor. One possible explanation for the low accumulation of either of the radiolabeled D-Hep-III analogues in tumors may be down-regulation of $\alpha 3\beta 1$ expression. Therefore, binding assays were carried out with a radioiodinated monoclonal antibody specific for the $\alpha 3$ subunit of the targeted integrin. Equal amounts of ^{125}I -Mab-anti- $\alpha 3$ at the same specific activity were added to varying amounts of membrane protein. The reactive fraction of ^{125}I -Mab-anti- $\alpha 3$ was

relatively low, $9.4 \pm 1.3\%$, for xenografted membranes and $8.2 \pm 0.7\%$ for SKOV3 cells grown in culture. Moreover, the amount of membrane protein required to bind one-half the tracer was $12.3 \pm 4.7 \mu\text{g}$ for xenografted membranes and $11.7 \pm 3.0 \mu\text{g}$ for SKOV3 membranes (Figure 1). This indicates that $\alpha 3\beta 1$ is present in membranes from SKOV3 xenografts, as well as from SKOV3 membranes of cells grown in culture. Furthermore, since $\alpha 3\beta 1$ is proportional to the amount of membrane protein, and since the same amount of membrane protein from each receptor source will bind one-half the ^{125}I -Mab-anti- $\alpha 3$, it is concluded that the density of $\alpha 3\beta 1$ is equivalent between the two preparations.

In the homologous competitive binding assay between the ^{125}I -Mab-anti- $\alpha 3$ and Mab anti- $\alpha 3$, all of the radioligand bound at concentrations of competitive Mab of 0.01, 0.02, 0.1, and 0.6 nM because of the low immunoreactivity of the ^{125}I -Mab (Figure 2). Insufficient data points remained for Scatchard analysis. Therefore, the minimum density of $\alpha 3\beta 1$ integrin can be estimated from the amount of ^{125}I -Mab specifically bound in the preparations containing 14 and 72 nM Mab, where 1400 and 970 cpm bound, respectively. Using the following conversion factors, values of 2700 and 9800 pM total bound were obtained: 33 $\mu\text{Ci}/\mu\text{g}$ Mab, 150 KDa, 0.6 dpm/cpm, $2.22 \times 10(6)$ dpm/ μCi , $1 \times 10(6)$ pmol/ μmol . With 75 μL aliquot containing 40 μg of protein, a log-dose dependent displacement of ^{125}I -Mab-anti- $\alpha 3$ by Mab anti $\alpha 3$ was observed (Figure 2; $\text{IC}_{50} = 5.3$ nM, 95% confidence interval 3.2 to 8.7 nM).

DISCUSSION

Radiolabeled derivatives of peptide hormones targeted to cell surface receptors, such as vasoactive intestinal peptide (VIP) (38), somatostatin (39), and melanotropin stimulating hormone (αMSH) (40), have demonstrated receptor mediated accumulation of the radiotracer at tumor sites in animal models or humans and therefore have potential as tumor imaging agents. Besides peptide hormones, another class of peptides derived from ECM proteins has been investigated as tumor imaging agents (11–14). D-Hep-III, derived from the ECM protein, type IV collagen, was investigated as an imaging agent for the $\alpha 3\beta 1$ integrin.

The tetraazamacrocyclic TETA was coupled to the N-terminal amine of D-Hep-III while it was resin bound,

as structure–activity studies indicated that the *N*-terminal region of D-Hep-III does not significantly impact receptor binding (23). TETA-D-Hep-III and D-Hep-III were radiolabeled with ^{64}Cu and ^{125}I , respectively, for evaluation as a receptor specific imaging agents.

To establish that both ^{64}Cu -TETA-D-Hep-III and ^{125}I -D-Hep-III bound specifically to $\alpha 3\beta 1$ positive tissues, heterologous competitive binding assays between D-Hep-III and ^{125}I -D-Hep-III or ^{64}Cu -TETA-D-Hep-III were carried out on membranes isolated from SKOV3 cultured cells and SKOV3 xenografts. Both ^{125}I -D-Hep-III and ^{64}Cu -TETA-D-Hep-III were specifically bound to cell surface receptors on membranes originating from the SKOV3 ovarian tumor cell line. However, the IC_{50} value for D-Hep-III was 10-fold higher on membranes isolated from SKOV3 xenografts ($\text{IC}_{50} = 3700 \text{ nM}$) than from those isolated from cultured cells ($\text{IC}_{50} = 360 \text{ nM}$). The concentration of ^{125}I -D-Hep-III was 50 and 26 pM in the experiments with SKOV3 membranes and xenografts, respectively. According to the Cheng-Prusoff equation [$K_D = \text{IC}_{50} / (1 + [\text{radioligand}] / K_D)$], where K_D = affinity of the competitor for the receptor; K_D = affinity of the radioligand for the receptor; IC_{50} = concentration of competitive ligand required to displace one-half the radioligand], a 2-fold decrease in tracer concentration cannot account for a 10-fold increase in IC_{50} unless either the K_D of D-Hep-III or ^{125}I -D-Hep-III or both were different on membranes isolated from SKOV3 cells or xenografts. This indicates that either or both of the ligands were interacting with different receptors between the two preparations. The higher affinity interaction observed on membranes from SKOV3 cells may have been with an integrin other than $\alpha 3\beta 1$ since the Hep III sequence is contained within a region of type IV collagen that is known to bind both the $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins (ref 7 and references therein). Furthermore, L-Hep-III (i.e., all residues except glycine are in the *L*-configuration) binds both the $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins from human mesangial (kidney) cells (41).

The radioligands were also evaluated in normal and tumor-bearing animal models. The biodistribution of ^{64}Cu -TETA-D-Hep-III and ^{125}I -D-Hep-III in normal rats resulted in rapid blood clearance and low accumulation in most of the tissues examined. The highest accumulation was in the kidney, which is known to highly express $\alpha 3\beta 1$ (16, 17, 37). While kidney uptake could have been receptor-mediated, it is important to note that renal clearance is also the major excretory pathway of the radiotracers. However, coadministration of up to 9 μg doses of D-Hep-III in SCID mice resulted in no observed decreases in kidney accumulation. The observed accumulation of the tracers in the kidneys could simply be a result of clearance although other radiolabeled peptides, such as ^{64}Cu - and ^{125}I -labeled somatostatin analogues, do not accumulate in the kidneys to such a great extent (42, 43). Alternatively, the 9 μg blocking dose may not have been sufficient to block the potentially large number of integrin receptors present in the kidneys.

To determine if ^{64}Cu -TETA-D-Hep-III would accumulate preferentially in $\alpha 3\beta 1$ rich tumor tissues, a biodistribution of ^{64}Cu -TETA-D-Hep-III was obtained in SCID mice bearing bilateral SKOV3 xenografts. SKOV3 cells strongly express $\alpha 3\beta 1$ in culture (44) but $\alpha 3\beta 1$ density of SKOV3 or any other xenografts of cultured cells known to express $\alpha 3\beta 1$ has not been reported. To determine if SKOV3 xenograft accumulation was receptor mediated for ^{125}I -D-Hep-III, a blocking dose of D-Hep-III (1.7 and 9 μg) was coinjected with ^{125}I -D-Hep-III. Accumulation of the radioactivity in the liver and spleen increased with

increasing doses of D-Hep-III. However, the accumulation of the radioactivity in the tumor was low and not significantly different than that of the blood or tumors from animals that received the blocking doses. The accumulation of radiolabeled D-Hep-III analogues in the tumor may not be receptor-mediated; however, if the $\alpha 3\beta 1$ expression is dense, higher blocking doses may be needed to displace the tracers.

Low tumor accumulation of ECM-derived peptides had been previously observed with peptides derived from other ECM proteins. Peptides incorporating the sequence YIGSR, a fragment proposed to bind to laminin receptors, were radiolabeled with ^{131}I and investigated in a tumor bearing animal model (12). In mice bearing Lewis lung carcinoma tumors, ^{131}I -YIGSR uptake in the tumor, while low ($1.15 \pm 0.28\% \text{ ID/gram}$, 24 h), was much higher than blood ($0.014 \pm 0.007\% \text{ ID/gram}$, 24 h). However, this ratio was achieved at 24 h postinjection. In contrast, previous work in mice bearing xenografted melanoma showed that cyclic peptides incorporating the RGD sequence [cyclo-(RGD-D-YV)] and radioiodinated with ^{125}I displayed low tumor accumulation ($1.30 \pm 0.13\% \text{ ID/gram}$, 1 h) (11). In these studies, clearance from the blood was rapid ($0.17 \pm 0.02\% \text{ ID/gram}$, 1 h), and a tumor-to-blood ratio of ~ 8 was achieved. In our study, the evaluation of the radiolabeled D-Hep-III analogues in tumor-bearing animals extended to only 1 h, and this time interval may not have been long enough to reach optimal tumor-to-blood ratios indicative of receptor-mediated accumulation.

The primary goal of this study was to determine whether ^{125}I -D-Hep-III or ^{64}Cu -TETA-D-Hep-III would localize in a receptor specific manner in $\alpha 3\beta 1$ rich tissues, and the results suggest that the observed accumulation was not receptor-mediated. Receptor down-regulation or degradation of the radiotracers are two factors that could have adversely affected receptor-mediated tumor accumulation. However, the results from the binding assay at infinite $\alpha 3\beta 1$ excess to ^{125}I -Mab-anti- $\alpha 3$ indicate that $\alpha 3\beta 1$ density is equivalent between SKOV3 cultured cells and SKOV3 xenografts. Furthermore, the minimum density of $\alpha 3\beta 1$ of 5 to 18 pmol/mg protein is a relatively high value since xenografted somatostatin-positive tumors routinely express 300 fmol/mg protein (26). Therefore, $\alpha 3\beta 1$ down-regulation or sparse density cannot explain the lack of receptor mediated accumulation. Moreover, both ^{64}Cu -TETA-D-Hep-III and ^{125}I -D-Hep-III are stable in serum long enough to reach their targets.

It was previously shown that a monoclonal antibody, GA17 ($K_D = \sim 2 \text{ nM}$), directed toward $\alpha 3\beta 1$ and radiolabeled with either ^{125}I or ^{111}In , selectively localized to human tumor xenografts which expressed $\alpha 3\beta 1$ in vitro, thus demonstrating $\alpha 3\beta 1$ as a viable target for high affinity radiolabeled ligands (45). Although the epitope of GA17 is unknown, Mab binding is not necessarily dependent on $\alpha 3\beta 1$ conformation or competition from endogenous ligand, two possibilities that would explain the low tumor accumulation of either ^{125}I -D-Hep-III or ^{64}Cu -TETA-D-Hep-III in the xenograft.

Another possible explanation for the low accumulation of the ^{64}Cu -TETA-D-Hep-III and ^{125}I -D-Hep-III is low affinity for the receptor. The K_D of radiolabeled D-Hep-III analogues would have to approach the concentration of the tracer in the competitive binding experiments, which ranged from 26 to 100 pM, for IC_{50} values of D-Hep-III to differ greatly from the K_D of D-Hep-III. The $\text{IC}_{50} = 3700 \text{ nM}$ for D-Hep-III on membranes from SKOV3 xenografts suggests a weak affinity for D-Hep-III. Furthermore, $\alpha 3\beta 1$ is known to be recruited to focal contacts of cells adhering to type IV collagen after the collagen is

bound by integrins ($\alpha 2\beta 1$ and $\alpha 1\beta 1$) with demonstrated high affinity for type IV collagen, suggesting that $\alpha 3\beta 1$ has a weak affinity for type IV collagen (7). Successful radiotracers that have accumulated in their target tissue by a receptor-mediated process have demonstrated affinity in the low nanomolar region. Micromolar affinity is likely to be insufficient for receptor-mediated uptake in target tissue (46) and would explain the low accumulation of ^{64}Cu -TETA-D-Hep-III and ^{125}I -D-Hep-III.

CONCLUSIONS

Radiolabeled TETA-D-Hep-III and D-Hep-III were evaluated as imaging agents for human neoplasms where the $\alpha 3\beta 1$ integrin is strongly expressed. On tissues known to express $\alpha 3\beta 1$ integrin, binding of both of ^{64}Cu -TETA-D-Hep-III and ^{125}I -D-Hep-III was inhibited in a log-dose dependent manner on membranes from cultured SKOV3 cells and SKOV3 xenografts, demonstrating that these ligands are bound to cell surface receptors. IC_{50} values of D-Hep-III in the low micromolar region suggest a weak affinity of D-Hep-III and radiolabeled analogues of D-Hep-III for $\alpha 3\beta 1$. In the in vivo tumor model used in this study low accumulation of both radiotracers was observed within the xenograft at the time points investigated despite the relatively dense expression of $\alpha 3\beta 1$. The combination of the results obtained from the in vitro and in vivo data strongly suggest that peptides of this class targeted to the $\alpha 3\beta 1$ would not be suitable as in vivo imaging agents in humans.

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Targeting Endothelial Cells Overexpressing VEGFR-2: Selective Toxicity of Shiga-like Toxin–VEGF Fusion Proteins

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Growing endothelial cells at the sites of angiogenesis express high numbers of VEGF receptors and therefore may be particularly sensitive to VEGF-mediated drug delivery. To test this hypothesis we have constructed a protein containing the catalytic A-subunit of Shiga-like toxin I fused to VEGF₁₂₁ (SLT-VEGF/L). Wild-type A-subunit is a site-specific N-glycosidase of 28S rRNA that inhibits protein synthesis after being delivered into cells by separate cell-binding B-subunits. SLT-VEGF/L retains functional activities of both SLT and VEGF₁₂₁ moieties, since it inhibits protein synthesis in a cell-free translation system and induces VEGFR-2 tyrosine autophosphorylation. SLT-VEGF/L selectively inhibits growth of porcine endothelial cells expressing 2.5×10^5 VEGFR-2/cell with an IC₅₀ of 0.2 nM and rapidly induces apoptosis at concentrations >1 nM. We found that sensitivity of VEGFR-2 transfected PAE cells to SLT-VEGF/L declined as the cellular VEGFR-2 density decreased; PAE cells expressing 25000 VEGFR-2/cell were as sensitive as parental cells lacking the receptor. Growth inhibition and induction of apoptosis by SLT-VEGF/L require intrinsic N-glycosidase activity of the SLT moiety, but take place without significant inhibition of protein synthesis. Selective cytotoxicity of SLT-VEGF/L against growing endothelial cells overexpressing VEGFR-2 suggests that it may be useful in targeting similar cells at the sites of angiogenesis.

INTRODUCTION

Angiogenesis is a tightly controlled process of growing new blood vessels that in the adult organism under normal circumstances takes place only during muscle or wound healing (reviewed in refs 1, 2). However, angiogenesis occurs in a large number of pathologies, such as solid tumor growth, various eye diseases, chronic inflammatory states, and ischemic injuries (reviewed in ref 3). Growing endothelial cells at the sites of angiogenesis are unique targets for treatment of several major pathologies, and a number of compounds targeting angiogenesis are currently at various stages of development (reviewed in ref 4). Several positive and negative regulators control angiogenesis, and the shift in equilibrium between these regulators is presumably responsible for angiogenesis in pathological situations (reviewed in refs 5, 6). The crucial positive regulator of angiogenesis is vascular endothelial growth factor (VEGF) also known as vascular permeability factor (reviewed in ref 7). VEGF is a secreted homodimeric glycoprotein that, as a result of alternative mRNA splicing, may consist of polypeptides with 121, 145, 165, 189, or 206 amino acid residues. VEGF is expressed by normal and tumor cells in response to hypoxia and nutritional deprivation, and its expression appears to be regulated on several levels (reviewed in refs 8, 9). The action of VEGF on endothelial cells is mediated by tyrosine kinase Flt-1 and KDR/Flk-1 receptors, also known as VEGFR-1 and VEGFR-2, with the latter playing the dominant role in angiogenesis (reviewed in refs 9, 10). These receptors are preferentially expressed on endothelial cells, and there is evidence that

endothelial cells at the sites of angiogenesis express significantly higher numbers of VEGFR-2 than quiescent endothelial cells (11–15). VEGF binding to VEGFR-2 induces receptor dimerization followed by tyrosine phosphorylation of the SH2 and SH3 domains and internalization of the complexes (reviewed in refs 9, 10). Since VEGF binds specifically to endothelial cells, this growth factor provides a unique opportunity for targeted drug delivery to the sites of angiogenesis. Indeed, it has been shown that conjugation or fusion of the catalytically active fragments of diphtheria toxin (DT) to VEGF yielded proteins that inhibited growth of endothelial cells and displayed anti-angiogenic activity in in vivo models of angiogenesis (16–18).

To inhibit angiogenesis without damaging quiescent endothelium, the toxin–VEGF fusion proteins should selectively target endothelial cells at the sites of angiogenesis that overexpress VEGFR-2. We hypothesized that overexpression of VEGFR-2 receptor in the growing endothelial cells may endowed them with enhanced sensitivity to some toxin–VEGF fusion proteins. To test this hypothesis we have constructed a protein containing the catalytic A-subunit of Shiga-like toxin I fused to VEGF₁₂₁ (SLT-VEGF/L). SLT-1 produced by *E. coli* O157:H7 is a “natural killer” for endothelial cells. Damage to endothelial cells caused by SLT-1 plays a causative role in the pathogenesis of hemorrhagic colitis and hemolytic uremic syndrome induced by *E. coli* O157:H7 (19–22). SLT-1 is composed of a single copy of a 32 kDa A-subunit associated with a ring shaped pentamer of 7 kDa B-subunits that bind to the cellular receptor globotriaosylceramide known as Gb₃/CD77. SLT-1 enters cells via CD77-mediated endocytosis, whereby the A-subunit is cleaved into disulfide bond linked 27.5 kDa A₁ and 4.5 kDa A₂ fragments (22, 23). The processed A-subunit is a site-specific N-glycosidase that cleaves off A₄₃₂₄ in the 5' terminus of 28S rRNA (24). Depurination of A₄₃₂₄ inhibits

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binding of the elongation factor EF-1/aminocyl-tRNA complex to ribosomes, resulting in the inhibition of the protein synthesis and initiation of apoptosis. Proteolytic processing of the A-subunit is not required for its enzymatic activity, since the full length A-subunit, and truncated A-subunits missing most of the A₂ region, retain significant N-glycosidase activity in vitro (25–27). Furthermore, fusion proteins containing the full length A-subunit as well as various truncated A-subunits fused to the N-terminus of CD4 retain N-glycosidase activity and are cytotoxic for cells expressing the HIV-1 gp120/gp41 complex (26, 27).

Our preliminary data indicated that SLT-VEGF/L inhibits growing cells overexpressing VEGFR-2 (28). We report here that the number of VEGFR-2 receptors on endothelial cells determines their sensitivity to SLT-VEGF/L. Furthermore, contrary to a commonly held view that plant and bacterial toxin act via inhibition of protein synthesis we found that SLT-VEGF/L induces apoptosis without significant inhibition of protein synthesis.

MATERIALS AND METHODS

Plasmids. The pJB144 plasmid encoding VT1/SLT-1 holotoxin was kindly provided by Dr. A. Solyk (Samuel Lunfield Research Institute, Toronto, Canada). The pBalPst/KDR mammalian expression plasmid containing the full-length human VEGFR-2 was kindly provided by Dr. B. Terman (Albert Einstein School of Medicine, NY).

Construction of Expression Plasmids. Primers for cloning and mutagenesis of SLT-1 fragments (GenBank accession: AB015056) were synthesized by CeneLink (Thornwood, NY). The full-length (1–293 aa) and truncated (62–264 aa) fragments of the SLT-1 A-subunit were amplified by PCR using the pJB144 plasmid as a template. The “sense” primers for the full-length (5′-CCG-AGATCTGAAGGAATTTACCTTAGAC) and for the truncated (5′-CCCAGATCTGCTACGGCTTATTGTTGAACG) SLT-1 fragments included *Bgl*II sites (underlined). The “antisense” primers for the full-length (5′-ATAGGTACCACTGCTAATAGTTCTGCG) and for the truncated (5′-ATAGGTACCATCTGCCGACACATAGAAG) SLT-1 fragments introduced *Kpn*I sites (underlined). PCR-amplified full-length (L) and truncated (S) SLT-1 fragments were cloned separately into VEGF₁₂₁ encoding pET32-VEGF₁₂₁ plasmid (constructed as described in 29) in-frame with VEGF₁₂₁. The resulting plasmids, pET32/SLT-VEGF/L and pET32/SLT-VEGF/S, were confirmed by sequencing (CSU-Macromolecular Resources, Ft. Collins, CO). Site-specific mutagenesis of SLT-1 A-subunit encoded by pET32/SLT-VEGF/L was done using GeneEditor in vitro Site-Directed Mutagenesis System (Promega). Two mutagenic primers were designed to introduce three point mutations (underlined): Y114S (5′-ACGTGGTAGAGCTACTGTCACC), and E167Q and R170L (5′-TTGCCGAAAAAGTAAAGCTTGAGCTGTACAG). The Y114S and R170L mutations were confirmed by sequencing of mutated DNA isolated from two clones. The E167Q mutation was not detected in either clone.

Expression of Fusion Proteins. VEGF₁₂₁ and SLT-VEGF fusion proteins were expressed in *E. coli* strain Origami(DE3)pLysS (Novagen). After induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside (Life Technologies), bacteria were grown for 4 h at 30 °C, harvested, and lysed by freezing-thawing in 50 mM Tris-HCl pH 7.5, 0.1 mM MgCl₂, 1% NP-40, and all protease inhibitors. After DNase treatment inclusion bodies were pelleted at 5000g for 30 min, washed twice with 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 5 mM imidazole, and solubilized in

8 M urea by sonication. The proteins were refolded by dialysis against 10 mM Tris-HCl pH 8.0, 150 mM NaCl. For dialysis of truncated form of SLT-VEGF, the buffer was supplemented with 0.01% Brij-35. All fusion proteins contained S-tags on the N-termini, making it possible to determine their molar concentrations by S-Tag Assay (Novagen).

Cell Cultures. Porcine aortic endothelial (PAE) cells and 293 human primary embryonic kidney cells were obtained from American Type Culture Collection (Rockville, MD). PAE cells expressing 2×10^5 VEGFR-2 per cell (PAE/KDR) and PAE cells transfected with the empty vector (PAE/V) were kindly provided by Dr. B. Terman (Albert Einstein School of Medicine, NY). 293 cells expressing 2.5×10^6 VEGFR-2 per cell (293/KDR) were described elsewhere (29). PAE cells expressing different numbers of VEGFR-2 (PAE/0.25-KDR and PAE/0.10-KDR clones) were constructed by transfection of PAE cells with the pBalPst/KDR plasmid using TransIT-LT1 reagent (PanVera Corporation), followed by selection in the presence of 0.375 μg/mL puromycin. All cells and their derivatives were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Life Technologies), 2 mM L-glutamine, and antibiotics at 37 °C, 5% CO₂.

VEGFR-2 Autophosphorylation. VEGFR-2 autophosphorylation was assayed as described (28). Briefly, subconfluent cells were incubated in serum-free DMEM at 37 °C for 4 h and then shifted to serum-free DMEM containing 0.1 mM orthovanadate, 100 ng/mL bovine serum albumin, 25 mM HEPES pH 7.2, for 20 min at 37 °C, followed by a 20-min incubation at 4 °C. After addition of SLT-VEGF cells were incubated for 1 h at 4 °C and then for 10 min at 37 °C, lysed, and analyzed by SDS-PAGE.

Western Blot Analysis. Cell lysates were separated by SDS-PAGE on 7.5% gels and transferred to nitrocellulose membranes (Life Technologies). After incubation with primary antibodies, phosphotyrosine RC20H (PharMingen), α-fodrin (Chemicon), or rabbit polyclonal anti-VEGFR-2 serum (kindly provided by Dr. B. Terman, Albert Einstein School of Medicine, NY), immune complexes were visualized by the *femto*Lucent Chemiluminescence System (Genotechnology).

Protein Synthesis Inhibition in Vitro. The protein inhibition activities of SLT-VEGF fusion proteins were measured in an in vitro translation system containing a rabbit reticulocyte lysate programmed with luciferase mRNA (Promega) according to the manufacturer's instructions. Protein synthesis in the presence of 1 μM recombinant VEGF₁₂₁ was taken as 100%.

³⁵S-Incorporation. PAE/KDR were plated onto 24-well plates, $(6-8) \times 10^3$ cells/well. Twenty hours later cells were shifted to fresh culture medium containing 2.5 nM SLT-VEGF/L, 1 mL/well. After 1–52 h of incubation at 37 °C cells from duplicate wells were counted in a Coulter Counter. Protein synthesis was measured at each time point by replacing media with methionine-free DMEM containing 0.45 μCi/mL [³⁵S]-Methionine (1000 Ci/mmol, Amersham Pharmacia Biotech). After a 30-min incubation at 37 °C cells were washed twice with ice-cold 10% TCA, lysed in 0.1 M KOH, and counted in a beta-counter. ³⁵S-incorporation assays were performed in triplicate.

DNA Laddering. PAE/KDR cells were plated onto six-well plates, 2×10^5 cells/well. Twenty-four hours later cells were shifted to fresh culture medium containing 2.5 nM SLT-VEGF/L, 1.5 mL/well. After 3–72 h of incubation at 37 °C, DNA was extracted and purified with Suicide-

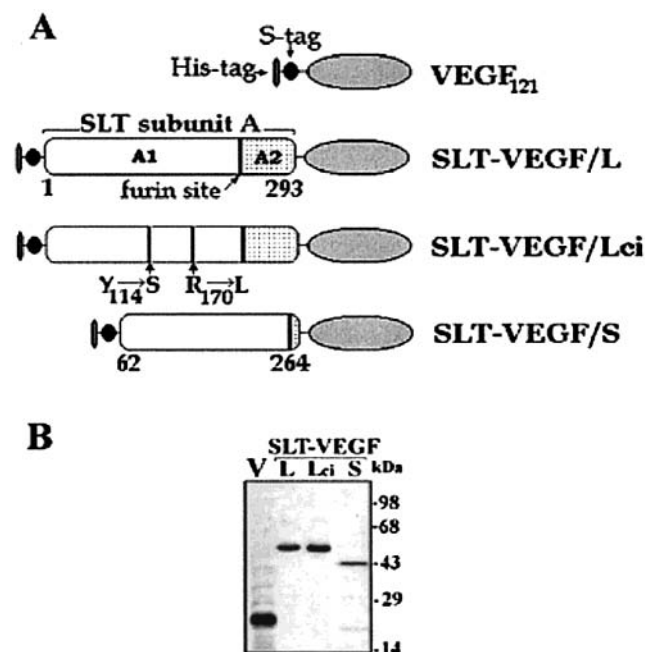


Figure 1. SLT-VEGF fusion proteins. All proteins were expressed in *E. coli* strain Origami(DE3)pLysS, solubilized from inclusion bodies, and refolded as described in Materials and Methods. A, Schematic representation of the proteins. B, SDS-PAGE analysis of the purified proteins (15% PAAG gel, Coomassie Blue staining). Lane V, VEGF₁₂₁.

Track DNA Ladder Isolation kit (Oncogene) and fractionated on 1.5% agarose gels.

Cytotoxicity Assay. Cells were plated in 24-well plates, $5-10 \times 10^3$ cells/well. Twenty hours later 2.5 nM SLT-VEGF/L (unless otherwise indicated) in fresh culture medium was added to duplicate wells, 1 mL/well. After 72-h, treatments cells were detached with trypsin-EDTA and counted in a Coulter Counter. Cytotoxicity assays were performed in triplicate.

RESULTS

Expression of SLT-VEGF Proteins. SLT-VEGF fusion proteins (Figure 1A) were constructed, expressed, and purified as described in Materials and Methods. The VEGF₁₂₁ isoform was chosen for construction of these proteins because, unlike other VEGF isoforms, (1) it does not contain heparin-binding domain(s) that are involved in nonspecific electrostatic interactions with the cell surface and extracellular matrix components, and (2) it has a selective affinity to VEGFR-2 (30). Full-length and truncated SLT-1 A-subunits were fused to VEGF₁₂₁ with the expectation that the resulting SLT-VEGF/L and SLT-VEGF/S fusion proteins would display different N-glycosidase activities (25–27). To separate the effects of ribosome inactivation from other effects that might be induced by recombinant SLT-VEGF proteins we have constructed SLT-VEGF/Lci protein containing a double mutant A-subunit with the Y114S and R170L amino acid substitutions. Each substitution significantly decreases the enzymatic activity of SLT-1 A-subunit, while not affecting its antigenic properties (31, 32). We therefore reasoned that simultaneous mutation of these residues might yield an SLT-1 moiety that lacks N-glycosidase activity. VEGF₁₂₁ and SLT-VEGF proteins were expressed in Origami(DE3)pLysS *E. coli*, recovered from inclusion bodies as 90–95% pure proteins (Figure 1B), and used in the following experiments without additional purification.

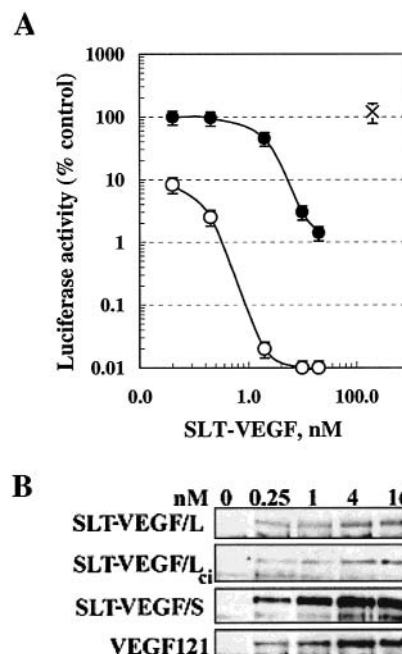


Figure 2. Functional activities of SLT-VEGF fusion proteins. A, Effects of SLT-VEGF/L (○), SLT-VEGF/S (●), or SLT-VEGF/Lci (X) on luciferase production in an in vitro translation system containing a rabbit reticulocyte lysate programmed with luciferase mRNA. Activity of luciferase translated in the presence of 1 μ M recombinant VEGF₁₂₁ was taken as 100%. B, Dose-dependence of VEGFR-2 tyrosine autophosphorylation in 293/KDR cells induced by SLT-VEGF fusion proteins and VEGF₁₂₁. Arrows indicate positions of 205 kDa markers.

SLT and VEGF Moieties Are Active in SLT-VEGF.

The fully activated A-subunit isolated from the AB₅ holotoxin inhibits protein synthesis in cell-free system with the IC₅₀ value of 0.1 nM (33). We therefore tested catalytic activity of SLT-VEGF in a cell-free translation system programmed with luciferase mRNA. We found that SLT-VEGF/L and SLT-VEGF/S inhibited protein synthesis by 90% at concentrations 0.04 nM and 2 nM, respectively (Figure 2A). Thus, SLT-VEGF/L may be even more effective inhibitor of protein synthesis than the wild type A-subunit. A 50-fold difference in activities of SLT-VEGF/L and SLT-VEGF/S suggested that truncation affected either the intrinsic catalytic activity of SLT-1 moiety, or its ability to fold properly within the fusion protein (Figure 2A). SLT-VEGF/Lci did not inhibit protein synthesis (Figure 2A).

The ability of SLT-VEGF fusion proteins to interact with VEGFR-2 was tested in a VEGFR-2 tyrosine autophosphorylation assay with 293/KDR cells. All SLT-VEGF fusion proteins induced VEGFR-2 tyrosine autophosphorylation in a dose-dependent manner in the same concentration range as parental VEGF₁₂₁ (Figure 2B). Different plateau levels observed in this assay suggested that complexes of VEGF fusion proteins with VEGFR-2 possess different intrinsic efficacies in tyrosine autophosphorylation. SLT-VEGF/L and SLT-VEGF/Lci were similarly efficacious (Figure 2B), indicating that mutations in the A-subunit, which eliminated N-glycosidase activity, did not affect interactions with the VEGFR-2. SLT-VEGF/S was more efficacious than SLT-VEGF/L and SLT-VEGF/Lci (Figure 2B), suggesting that SLT-VEGF/S may be better suited for induction of VEGFR-2 mediated processes. The ability of SLT-VEGF/L and SLT-VEGF/S to induce VEGFR-2 tyrosine autophosphorylation in PAE/KDR cells was reported previously (28).

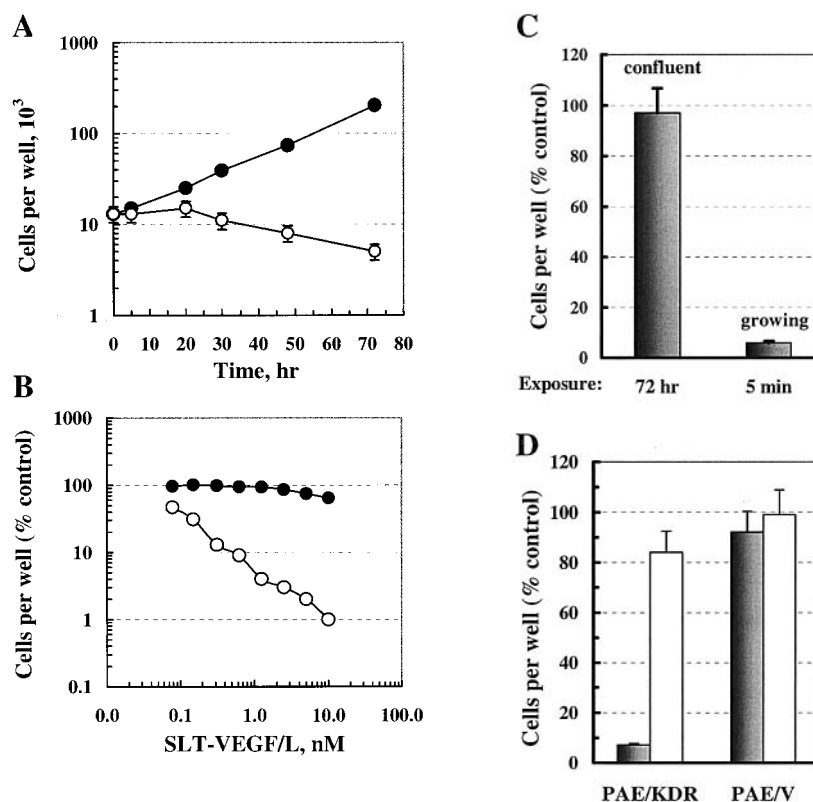


Figure 3. SLT-VEGF/L is selectively cytotoxic against growing endothelial cells overexpressing VEGFR-2. PAE/KDR cells were plated onto 24-well plates at densities of $5\text{--}10 \times 10^3$ cells/well and exposed to SLT-VEGF/L 20 h later. After a 72-h exposure cells were counted in a Coulter Counter. A, PAE/KDR cells treated with 2.5 nM SLT-VEGF/L (○) and untreated cells (●). B, PAE/KDR cells (○) and PAE cells lacking VEGFR-2 (●) exposed to varying concentrations of SLT-VEGF/L. C, Confluent PAE/KDR were maintained at 100% confluence for 3 days; then treated with 20 nM SLT-VEGF/L for 72 h. Growing PAE/KDR were exposed to 20 nM SLT-VEGF/L for 5 min and then shifted to fresh culture medium and counted after 72 h. D, effect of depletion of SLT-VEGF preparation. SLT-VEGF/L (0.5 mL of 25 nM solution in DMEM serum-free) was combined with 0.1 mL S-protein agarose (Novagen). After a 30-min incubation at 4 °C, the solution was cleared at 500g for 5 min, diluted 10-fold with complete culture medium, and added to cells (open bars). Control cells were treated with 2.5 nM SLT-VEGF/L (filled bars).

SLT-VEGF/L Is Selectively Cytotoxic for Growing Cells Overexpressing VEGFR-2.

To test VEGFR-2-mediated effects of SLT-VEGF fusion proteins on endothelial cells we have used PAE/KDR cells engineered to express 2.5×10^5 VEGFR-2/cell (29). PAE/V or wild type PAE cells were used as controls. After an exposure to 2.5 nM SLT-VEGF/L, PAE/KDR, but not control cells, ceased to grow, and their number progressively decreased below the number of plated cells because of widespread death (Figure 3A). SLT-VEGF/L inhibited growth of PAE/KDR cells in a dose-dependent manner with an IC_{50} of 0.19 ± 0.13 nM (average $\pm$ STD for five independent preparations of SLT-VEGF/L) and was highly cytotoxic to PAE/KDR cells at concentrations ≥ 1 nM (Figure 3B for a typical SLT-VEGF/L preparation). These effects were mediated by VEGFR-2, since parental PAE cells lacking VEGFR-2 were inhibited by SLT-VEGF/L with an IC_{50} of 3.95 ± 0.95 (Figure 3B). This sensitivity of nontransfected PAE cells might be mediated by VEGFR-1 present on parental PAE cells (34), since several other cell lines without known VEGF receptors were not affected by SLT-VEGF/L at concentrations as high as 20 nM (28). Presence of VEGFR-2 appears to be necessary, but not sufficient for induction of endothelial cell death by SLT-VEGF/L. We found that quiescent PAE/KDR cells were not affected by a 72-h exposure to SLT-VEGF/L even at concentrations as high as 20 nM (Figure 3C). In contrast, growing PAE/KDR cells exposed to 20 nM SLT-VEGF/L for 5 min, and then shifted to fresh culture medium and counted after 72 h, were dramatically affected (Figure 3C). To confirm a causative role of SLT-VEGF/L in the

observed effects, a preparation of SLT-VEGF/L was depleted of recombinant proteins by capturing them with S-protein agarose. The depleted preparation was ineffective against PAE/KDR and PAE/V cells (Figure 3D).

Effect of SLT-VEGF/L on Endothelial Cells Depends on the VEGFR-2 Density. To test effects of VEGFR-2 density on cytotoxicity of SLT-VEGF/L, we have constructed two clones of PAE cells expressing different numbers of VEGFR-2. The levels of VEGFR-2 expression were estimated by Western blot analysis with PAE/KDR cells (2.5×10^5 VEGFR-2/cell) used as a standard. Two clones of VEGFR-2 transfected PAE cells, PAE/0.25-KDR and PAE/0.10-KDR, expressed, respectively, four and ten times less VEGFR-2/cell than PAE/KDR (Figure 4). We found that sensitivity of VEGFR-2 transfected PAE cells to SLT-VEGF/L declined as the VEGFR-2 density decreased (IC_{50} on Figure 4). PAE/0.10-KDR cells expressing ~ 25000 VEGFR-2/cell were approximately as sensitive as PAE/V cells, indicating that only a high level of VEGFR-2 expression conferred sensitivity of growing endothelial cells to SLT-VEGF/L.

Requirement for Catalytic Activity of SLT Moiety in SLT-VEGF Fusion Proteins. To establish the role of N-glycosidase activity of SLT moiety in the observed effects, we tested SLT-VEGF/Lci containing a catalytically inactive A-subunit. As was described before, SLT-VEGF/Lci did not inhibit protein synthesis but retained the ability to bind and activate VEGFR-2 (Figure 2). We found that 2.5 nM SLT-VEGF/Lci did not affect growth of PAE/KDR or PAE/V cells (Figure 5A), establishing that the ability to damage ribosomes was the basis of the SLT-

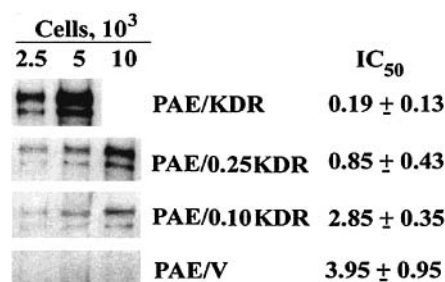


Figure 4. High level of VEGFR-2 is necessary to confer sensitivity of cells to SLT-VEGF/L. Three clones of VEGFR-2 transfected and control PAE/V cells were grown in six-well plates. Subconfluent cells were washed with PBS, detached with PBS containing 1 mM EDTA, counted in a Coulter Counter, and lysed in SDS-PAGE loading buffer. Cell lysates containing equal numbers of cells were analyzed by SDS-PAGE on 7.5% gels followed by Western blotting. IC₅₀ for each clone was determined as described in the legend for Figure 3B. Each experiment was done in triplicate.

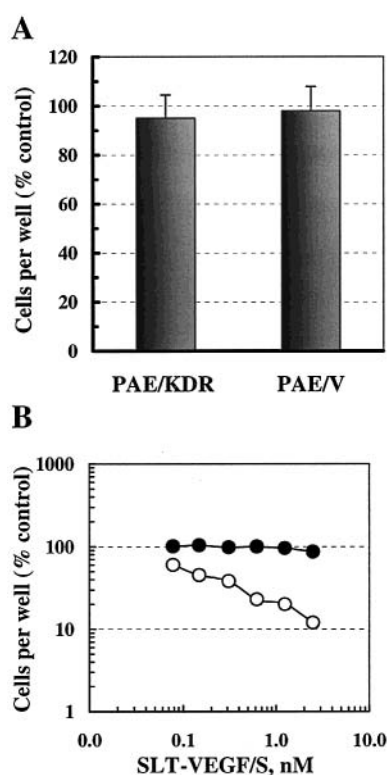


Figure 5. Cytotoxicity of SLT-VEGF proteins requires catalytic activity of SLT moieties. PAE/KDR and PAE/V cells were plated onto 24-well plates at densities of $(6-8) \times 10^3$ cells/well. Twenty hours later cells were exposed to (A) 2.5 nM SLT-VEGF/Lci containing a catalytically inactive SLT A-subunit, or (B) varying concentrations of SLT-VEGF/S containing a truncated SLT A-subunit. (○), PAE/KDR; (●), PAE/V. After a 72-h exposure to the fusion proteins, cells were counted in a Coulter Counter.

VEGF/L cytotoxicity. The role of N-glycosidase activity of SLT moiety of SLT-VEGF proteins was further explored using SLT-VEGF/S that, in comparison with SLT-VEGF/L, was less active in inhibition of cell-free translation, but more active in induction of VEGFR-2 tyrosine phosphorylation (see, Figure 2A and 2B). SLT-VEGF/S inhibited growth of PAE/KDR cell, while PAE/V cells were marginally affected (Figure 5B), indicating the VEGFR-2-mediated mechanism of action. Surprisingly, despite ~50-fold lower activity in a cell-free translation assay (Figure 2A), SLT-VEGF/S inhibited PAE/KDR growth in the same concentration range as SLT-VEGF/L (compare Figure 3A and 5B). Perhaps, lower catalytic

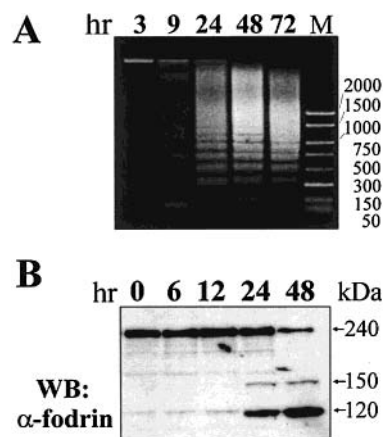


Figure 6. SLT-VEGF/L induces apoptosis in PAE/KDR cells. A, Apoptotic DNA fragmentation. Cells were plated onto six-well plates at a density of 2×10^5 cells/well and exposed to 2.5 nM SLT-VEGF 24 h later. After indicated periods of time DNA was isolated from cell lysates and fractionated on 1.5% agarose gel. B, Western blot analysis of α-fodrin. Cells were plated onto 24-well plates at a density of 4×10^4 cells/well and exposed to 2.5 nM SLT-VEGF 20 h later for indicated periods of time.

activity of SLT-VEGF/S was compensated by its ability to induce higher levels of VEGFR-2 tyrosine phosphorylation (Figure 2B) that might lead to a better uptake and/or processing of SLT-VEGF/S(VEGFR-2)₂ complexes. Unfortunately, a very low yield of SLT-VEGF/S prevented further experiments.

SLT-VEGF/L Induces Apoptosis in PAE/KDR Cells.

We found that SLT-VEGF induces rapid apoptotic changes in PAE/KDR cells. After 5–10 h of exposure to 2.5 nM SLT-VEGF, PAE/KDR cells progressively underwent morphological alterations resembling apoptosis (data not shown). An apoptotic DNA laddering was observed after a 9-h exposure, and little or no high molecular weight DNA was detectable after 24 or 48-h exposures (Figure 6A). Another hallmark of apoptosis, cleavage of 240 kDa α-fodrin into 150 kDa and 120 kDa fragments (35) was readily detectable after a 24-h exposure (Figure 6B).

Effects of SLT-VEGF/L on Protein Synthesis.

Native SLT-1, and presumably SLT-VEGF/L, inhibits protein synthesis by damaging ribosomes via depurination of A₄₃₂₄ in 28S rRNA. The resulting apoptosis may be initiated by collapse of protein synthesis. Alternatively, apoptosis may be initiated by ribosome damage through the recently discovered ribotoxic response mechanism that is mediated by activation of stress-response kinases and does not require significant inhibition of protein synthesis (36). To choose between these alternatives, we tested the effects of SLT-VEGF/L on protein synthesis in PAE/KDR cells. We found that ³⁵S-methionine incorporation per well with SLT-VEGF/L treated cells was progressively lower than that for untreated cells, with approximately 50% inhibition observed after a 20-h treatment (Figure 7A). Similar results have been reported recently for protein synthesis inhibition in SLT treated Caco-2 cells (37). However, this effect reflected mostly the difference in the cell numbers, since ³⁵S-methionine incorporation per cell varied only by 30–50% for both treated and control cells (Figure 7B). In contrast, cycloheximide treatment (0.1 mg/mL) rapidly inhibited protein synthesis on a per cell basis (Figure 7B). It was possible that even small alterations in protein synthesis might inhibit cell growth and be cytotoxic to PAE/KDR cells. However, this explanation appears unlikely since even an 80% inhibition of protein synthesis by cyclohex-

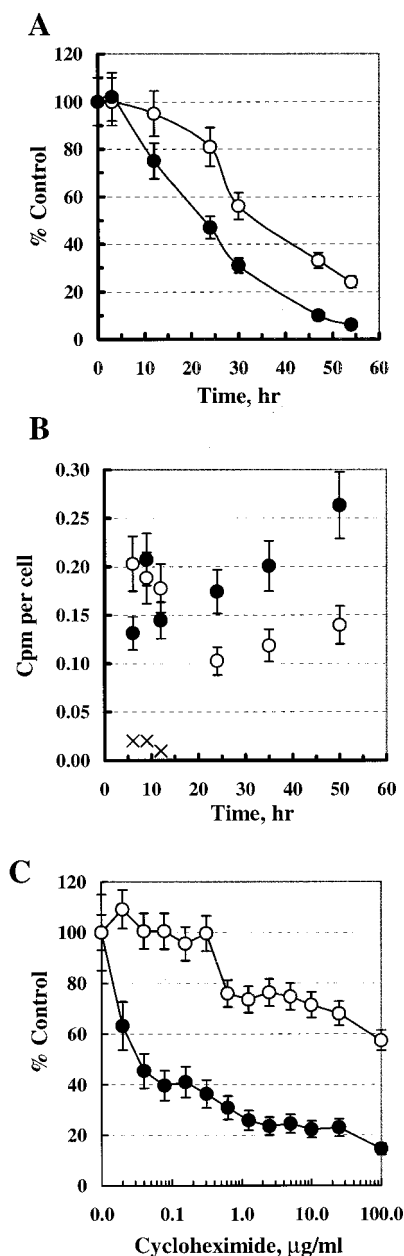


Figure 7. Protein synthesis inhibition in PAE/KDR. PAE/KDR cells were plated onto 24-well plates at densities of $6\text{--}10 \times 10^3$ cells/well and 20 h later exposed either to 2.5 nM SLT-VEGF/L or to varying amounts of cycloheximide. A, effects of SLT-VEGF/L on PAE/KDR growth (○) and ^{35}S -incorporation (●), B, effects of SLT-VEGF/L on ^{35}S -incorporation per cell for SLT-VEGF/L treated (○) and untreated cells (●). Effect of 0.1 mg/mL cycloheximide (X) is shown for comparison. C, Effects of cycloheximide on PAE/KDR growth (○) and ^{35}S -incorporation (●).

himide caused only a 40% inhibition of PAE/KDR cell growth (Figure 7C).

DISCUSSION

Growing endothelial cells at the sites of angiogenesis express high numbers of VEGF receptors (11–15) and therefore may be more sensitive to toxin-VEGF fusion proteins than quiescent endothelial cells. To test this hypothesis, we have constructed a protein containing the 293 amino acid catalytic A-subunit of Shiga-like toxin I fused to VEGF₁₂₁ (SLT-VEGF/L). Judging by its ability to inhibit protein synthesis in a cell-free system and to induce VEGFR-2 tyrosine autophosphorylation (Figure 2),

both SLT and VEGF moieties in the fusion proteins are folded into functionally active conformations. It is somewhat surprising that VEGF isoform containing 121 amino acid residues can accommodate a 293 amino acid N-terminal extension and retain 3-D structure suitable for interactions with VEGFR-2 (38).

SLT-VEGF/L was tested on growing and quiescent endothelial cells and on endothelial cells expressing different levels of VEGFR-2. We report here that SLT-VEGF/L inhibits growth of PAE/KDR cells expressing 2.5×10^5 VEGFR-2 receptors/cell with an IC_{50} of ~ 0.2 nM and induces massive apoptosis at concentrations ≥ 1 nM (Figure 3A). These effects are mediated by VEGFR-2, since parental cells lacking VEGFR-2 are only marginally affected by SLT-VEGF/L (Figure 3A).

However, the presence of VEGFR-2 appears to be necessary but not sufficient for the sensitivity of endothelial cells to SLT-VEGF/L. We found that quiescent PAE/KDR cells are not affected by a 72-h exposure to 20 nM SLT-VEGF/L, while growing PAE/KDR cells are killed by a 5-min exposure (Figure 3A and Figure 3C). Resistance of quiescent PAE/KDR to SLT-VEGF/L may be due to the lower metabolic rates in quiescent cells. Indeed, a lower sensitivity of quiescent endothelial cells to DT-VEGF conjugate was reported previously (17). Sensitivity to SLT-VEGF/L for growing endothelial cells expressing lower numbers of VEGFR-2 ($\sim 6 \times 10^4$ and 2.5×10^4 receptors per cell) progressively declines to the level of PAE/V cells lacking VEGFR-2 (Figure 4). These results are in agreement with our previous finding that human umbilical vein endothelial cells (HUVEC) and mouse MS1 endothelial cells expressing $(3\text{--}5) \times 10^4$ VEGFR-2/cell are not sensitive to SLT-VEGF/L at concentrations as high as 20 nM (28). Taken together the requirements for growth and for high number of VEGFR-2 suggest that growing endothelial cells at the sites of angiogenesis cells would be more sensitive to SLT-VEGF/L than quiescent endothelium.

It should be noted that the lack of sensitivity of HUVEC to SLT-VEGF/L is in contrast to the reported high sensitivity of these cells to DT-VEGF proteins (IC_{50} of 36 pM) (18). The differential sensitivity of HUVEC may reflect differences in internalization, and/or intracellular processing of DT-VEGF and SLT-VEGF/L proteins (39–41). Furthermore, DT and SLT target different steps in protein biosynthesis, whereby DT catalyzes ADP-ribosylation of EF-2, while SLT damages ribosomes. However, the high activity of DT-VEGF against HUVEC raises a question whether this protein would discriminate between endothelial cells with low and high numbers of VEGFR-2 in a manner similar to SLT-VEGF/L.

Our finding that catalytically inactive SLT-VEGF/Lci does not affect PAE/KDR cells indicates that enzymatic inactivation of ribosomes is crucial for the cytotoxic activity of SLT-VEGF/L. However, we found that a collapse of protein synthesis is not required for SLT-VEGF/L induced onset of growth inhibition and apoptosis. Indeed, in the presence of 2.5 nM SLT-VEGF/L protein synthesis per PAE/KDR cell is inhibited marginally after 12 h, and only by 30% after 50 h (Figure 7B). Nevertheless, under the same conditions, apoptotic DNA laddering is readily detectable in treated cells after 9 h, and a 50-h treatment kills $\sim 30\%$ of plated cells (Figure 3A).

There is evidence that bacterial toxins and immunotoxins may induce apoptosis not only through shortage of de novo synthesized proteins, but also through other mechanism(s) (37, 42–44). Moreover, the inhibitory effect of toxins on protein synthesis may be overestimated

because many studies rely on comparison of ^{35}S -incorporation per well of treated vs control cells (see, for example 18, 43). In such comparison a decrease in ^{35}S -incorporation per well reflect both a decrease in a number of treated cell and a possible decrease in protein synthesis in each cells. Recently, Iordanov et al., discovered a ribotoxic stress response that can be initiated by site-specific damage of 28S rRNA by ricin and α -sarcin and by several other agents (36, 45, 46). This response includes activation of stress-activated protein kinases that initiate signal transduction cascades leading to apoptotic death (reviewed in 47). Indeed, it was shown recently, that treatment of Caco-2 cells with SLT holotoxin induces expression of MAPK phosphatase (MKP-1) and activation of JNK (37). Alternatively, a surveillance mechanism may connect damage of ribosomes to transcription machinery that activate expression of proapoptotic cytokines. Indeed, it has been shown recently that treatment of monocytes, macrophages, and tumor cells with native SLT-1 induces cytokine production (48). Finally, existence of fine-tuned mechanisms for monitoring the integrity of ribosomes is suggested by the recent finding that ribosomal S6 protein is dispensable for growth but not proliferation (49). Regardless of molecular mechanism(s) of action, SLT-VEGF/L displays remarkably selective cytotoxicity against growing endothelial cells overexpressing VEGFR-2. Experiments are now in progress to determine efficacy of SLT-VEGF/L in animal angiogenesis models.

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Identification and Activities of Human Carboxylesterases for the Activation of CPT-11, a Clinically Approved Anticancer Drug

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CPT-11 is a clinically approved anticancer drug used for the treatment of advanced colorectal cancer. Upon administration, the carbamate side chain of the drug is hydrolyzed, resulting in the release of SN-38, an agent that has approximately 1000-fold increased cytotoxic activity. Since only a very small percentage of the injected dose of CPT-11 is converted to SN-38, there is a significant opportunity to improve its therapeutic efficacy and to diminish its systemic toxicity by selectively activating the drug within tumor sites. We envisioned that a mAb-human enzyme conjugate for CPT-11 activation would be of interest, particularly since the conjugate would likely be minimally immunogenic, and the prodrug is clinically approved. Toward this end, it was necessary to identify the most active human enzyme that could convert CPT-11 to SN-38. We isolated enzymes from human liver microsomes based on their abilities to effect the conversion and identified human carboxylesterase 2 (hCE-2) as having the greatest specific activity. hCE-2 was 26-fold more active than human carboxylesterase 1 and was 65% as active as rabbit liver carboxylesterase, the most active CPT-11 hydrolyzing enzyme known. The anti-p97 mAb 96.5 was linked to hCE-2, forming a conjugate that could bind to antigen-positive cancer cells and convert CPT-11 to SN-38. Cytotoxicity assays established that the conjugate led to the generation of active drug, but the kinetics of prodrug activation ($48 \text{ pmol min}^{-1} \text{ mg}^{-1}$) was insufficient for immunologically specific prodrug activation. These results confirm the importance of hCE-2 for CPT-11 activation and underscore the importance of enzyme kinetics for selective prodrug activation.

INTRODUCTION

The anticancer drug CPT-11 (irinotecan) is a water-soluble derivative of camptothecin and is clinically approved for the treatment of colorectal carcinoma (reviewed in refs 1–5). As with other camptothecin family members, CPT-11 inhibits DNA topoisomerase 1, an enzyme involved in relaxation of supercoiled DNA. This leads to the generation of DNA single strand breaks and eventual cytotoxic activity. Although CPT-11 has demonstrated significant antitumor activities in the clinic, the drug has several serious side effects including gastrointestinal toxicity and neutropenia (1–5). There is a great deal of interest in understanding the biochemical basis for the dose-limiting toxicities of CPT-11 and to find improved forms and delivery mechanisms for the drug.

One of the distinguishing features of CPT-11 is that it is metabolized to SN-38 (Figure 1), an agent that is more potent in assays for topoisomerase 1 inhibition and in cytotoxicity activity on transformed cell lines (6, 7). Early on, it was found that carboxylesterases effected the conversion of CPT-11 to SN-38 and that enzymes from several species carried out the reaction (8, 9). In these studies, the most active enzyme was from guinea pig livers and had very low specific activity ($4.5 \text{ nmol min}^{-1} \text{ mg}^{-1}$). The human enzyme used for comparison had only 3% of this activity. It is noteworthy that carbamates in general and CPT-11 in particular can act as serine carboxylesterase inhibitors, due to the stability of the active site serine-carbamate intermediates formed (10).

Consequently, even though carboxylesterases are widespread throughout the body (11–14), only 2–5% of the injected dose of CPT-11 in people is converted to SN-38. Most of the drug is excreted through the liver and kidneys without modification of the carbamate bond (1–5). If SN-38 could be intratumorally generated from CPT-11 in an efficient manner, it might be possible to improve CPT-11 therapeutic efficacy and at the same time minimize the severe toxicities associated with this drug.

Several methods can be envisioned to enhance intratumoral CPT-11 activation. One approach that has recently been explored involves gene therapy (15) in which the gene encoding an enzyme that can activate CPT-11 is transfected into the target cancer cell population. Candidate genes include those corresponding to rabbit liver carboxylesterase (16, 17), human and horse butyrylcarboxylesterase (18), and two forms of human carboxylesterase known as hCE-1 (16, 19, 20) and hCE-2 (21), all of which convert CPT-11 to SN-38. Of these enzymes, rabbit liver carboxylesterase (rCE) was regarded as being the most effective, since it catalyzed CPT-11 carbamate hydrolysis 100–1000-fold more efficiently than hCE-1 (16, 17). Human rhabdomyosarcoma cells transfected with the gene for rCE were sensitized to CPT-11 to a greater extent than were nontransfected cells or cells transfected with the gene encoding hCE-1. These results were confirmed in vivo in mice with subcutaneous tumor implants (16). Other studies have shown that some cell lines transfected with hCE-1 were modestly more sensitive to CPT-11 than control cells (19, 20).

Another method to enhance the activation of CPT-11 within tumors would be to utilize a prodrug activation strategy known commonly as ADEPT, antibody directed

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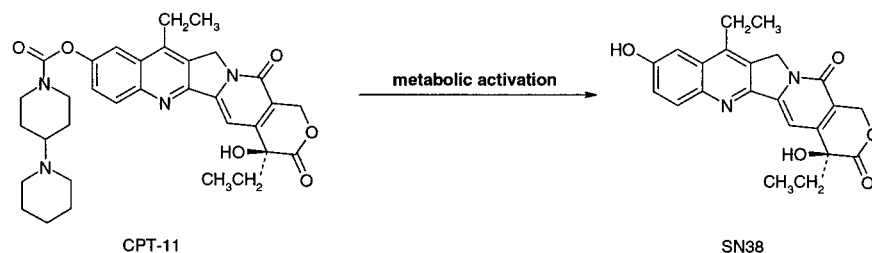


Figure 1. Structure of the prodrug CPT-11 and its conversion to the active metabolite SN-38.

enzyme prodrug therapy (reviewed in refs 22–23). This is a two-step targeting strategy, involving the administration of a monoclonal antibody (mAb)–enzyme conjugate that binds to tumor associated antigens, followed at an appropriate time by a prodrug that is activated by the targeted enzyme. For the purposes of CPT-11, the enzyme would be selected for its ability to efficiently hydrolyze the carbamate bond present within the drug and to generate cytotoxic levels of SN-38 intratumorally. Such an enzyme/prodrug combination would be distinguished from others that have been reported, since the human enzyme would likely be minimally immunogenic, and the prodrug for activation is already clinically approved. Toward developing such a system, we noted that a detailed search for suitable human enzymes has not been published. Here, we report the purification and characterization of CPT-11 activating enzymes from human liver along with their applications for specific prodrug activation.

EXPERIMENTAL METHODS

Materials and General Methods. Pharmaceutical grade CPT-11 was obtained as a pure preparation (by HPLC as indicated below) at 20 mg/mL. SN-38 was a gift from Dr. Ruiwen Zhang at the University of Alabama in Birmingham. The mAb 96.5 is of the IgG2a isotype and binds to the p97 antigen, which is expressed on most melanoma cells (24). The 3677 human melanoma (24) and L2987 human lung adenocarcinoma (25) cell lines were established from surgically resected tumors. A pathogen-free frozen human liver was obtained from the Northwest Tissue Center, Seattle, WA, and was stored at -80°C . Protein assays were conducted using the Pierce Micro BCA assay. The purification of guinea pig liver microsomal esterase has been previously described (26). Rabbit liver carboxylesterase was purchased from Sigma and purified by size exclusion chromatography to a high degree of homogeneity as evidenced by both SDS–PAGE and isoelectric focusing.

Enzyme Assays. With *p*-Nitrophenylacetate as Substrate. Protein samples were added to 1 mL of a 0.1 mM solution of *p*-nitrophenylacetate in phosphate buffered saline pH 7.2 (PBS), and the change in absorbance at 405 nm was recorded for 1 min. The protein samples were diluted to ensure that the measurements were linear during this time period. A change in absorbance of 1 OD represents the generation of 90 nmol of *p*-nitrophenol.

With CPT-11 as Substrate. Protein samples were added to a solution of CPT-11 in water so that the final CPT-11 concentration was 8 μM . Incubation was carried out at 37°C for 15–48 h, at which point HCl (final concentration 60 mM) was added to reform the lactone ring. Analysis by HPLC was performed as follows: injected 0.1 mL onto a C-18 column (4.6×75 mm) using a mobile phase of 60 mM ammonium carbonate, 4 mM tetrabutylammonium phosphate, 24% acetonitrile (pH 5.0) at 1 mL/min, and a detector set at 365 nm. The amount of

CPT-11 consumed and SN-38 generated was quantified using standard solutions of these agents that were treated identically to the test samples. The conversion of CPT-11 to SN-38 was linear over this time period.

Enzyme Purification. *Step 1. Microsome Purification.* A 300 g portion of a human liver was thawed and blended in a homogenizer with 500 mL of 1.5% KCl. The mixture was centrifuged at 8700g for 4 h at 4°C , and the clear supernatant was further centrifuged at 105000g for 1 h at 4°C .

Step 2. Ammonium Sulfate Precipitation. The microsome pellet was suspended in 100 mL of tris[hydroxymethyl]aminomethane (Tris) at pH 8.0, 1.5 g of saponin was added, and the mixture was gently rotated for 1 h at 4°C . Saturated aqueous ammonium sulfate was slowly added to a final concentration of 30%, and the precipitated material was removed by centrifugation at 11300g for 30 min at 4°C . To the supernatant was slowly added solid ammonium sulfate to a final concentration of 70%, and the precipitated material was removed by centrifugation at 11300g for 30 min at 4°C . The pellet was resuspended in and dialyzed against 10 mM Tris pH 8.0 overnight.

Step 3. Anion Exchange Chromatography. The dialysate from step 2 was applied to a 46 mL Q Sepharose Fast Flow column (Amersham Pharmacia), and the column was washed with 10 mM Tris pH 8.0 buffer until there was no absorbance at 280 nm. Bound proteins were eluted with a gradient of 10 mM Tris pH 8.0 to 10 mM Tris, 500 mM NaCl, pH 8.0, over 3 h at a flow rate of 5 mL/min. The fractions (10 mL each) were monitored for both *p*-nitrophenylacetate and CPT-11 hydrolysis activities. Significant activity was obtained in the unbound material, and in fractions 52–62.

Step 4. Hydrophobic Interaction Chromatography. The unbound material and fractions 52–62 from step 3 were separately diluted with 1.5 vol of 2 M ammonium sulfate in PBS, applied to a 1.5×12 cm phenylsepharose column (Amersham Pharmacia), and the column was washed until there was no absorbance at 280 nm in the eluant. The bound material was eluted with a 400 mL linear gradient from 0.8–0 M ammonium sulfate in PBS. Fractions that contained enzymatic activity were pooled and concentrated to 2 mL by ultrafiltration.

Step 5. Size Exclusion Chromatography. The material from step 4 was applied to a 2×65 cm Superdex 200 PS column (Amersham Pharmacia) and eluted with PBS. Fractions (3 mL each) were monitored for enzyme activity and purity by SDS–PAGE.

Sequence Analysis. The purified proteins (0.15–0.3 mg) were dialyzed against 10 mM Tris pH 8.0, concentrated to dryness on a vacuum centrifuge, and were dissolved in 0.03–0.1 mL of cyanogen bromide (Aldrich) in 70% aqueous formic acid (Fisher, sequencing grade). After 24 h at room temperature, 1.5 mL of water was added, and volatile material was evaporated in a vacuum centrifuge. This was repeated twice with 0.3 mL of water

Table 1. Purification Scheme for HCE-2 from Human Liver Microsomes Specific Activity

step	total protein (mg)	specific activity		total activity ^a (pmol/min)	fold purification
		<i>p</i> -nitrophenylacetate ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	CPT-11 ($\text{pmol min}^{-1} \text{mg}^{-1}$)		
ammonium sulfate	786	1.6	0.9	707	
anion exchange	94	3.2	4.3	404	4.8
hydrophobic interaction	4	53	58	232	64
size exclusion	1.4	60	92	129	102

^a Based on CPT-11 as the substrate.

to remove traces of acid. The residual material was dissolved in PBS (0.1 mL), applied to a 10% NuPage gel (Novex), and the gel was run using Novex MES running buffer. The gels were blotted onto PVDF filters, which were then stained with Coomassie Blue. The bands were cut out and sequenced by Sandy Kielland at the Protein Microsequencing Laboratory, University of Victoria, Victoria, BC, Canada.

Preparation of 96.5-hCE-2. A solution of the 96.5 mAb (0.29 mL at 7.0 mg/mL in PBS) was diluted with 0.05 mL of 0.5 M borate containing 0.5 M NaCl at pH 8.0, and 9 μL of *N*-(γ -maleimidobutyryloxy)succinimide ester (GMBS, Pierce) at 10 mM in DMSO was added. After 30 min at 37 °C, the solution was applied to a Sephadex G-25 PD 10 column (Amersham Pharmacia), and the modified protein was eluted in a 1.8 mL volume with 100 mM sodium phosphate containing 0.5 M NaCl at pH 7.2. A total of 1.77 mg of protein was obtained, based on the absorbance at 280 nm ($E_{0.1\%} = 1.4$). The maleimide content was determined by diluting 0.3 mL of the protein with 0.6 mL of PBS. To this was added 10 μL of a 1 mM aqueous solution of 2-mercaptoethanol, followed 1 min later by 25 μL of 50 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB). The difference in absorbance at 412 nm ($\epsilon = 14\,150$) as compared to a control containing no protein indicated that there were a total of 0.7 maleimides/mAb. The modified mAb was kept at 4 °C prior to being added to the enzyme.

hCE-2 (0.8 mL at 0.83 mg/mL in PBS; 33 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ specific activity using *p*-nitrophenylacetate as substrate) was diluted with 0.2 mL of 0.5 M sodium borate containing 0.5 M NaCl at pH 8.0. The protein was cooled on ice and treated with 53 μL of 20 mM 2-iminothiolane in water. After 180 min at 4 °C, low molecular weight agents were separated on a Sephadex G-25 PD 10 column, and the protein was collected in 2 mL of 100 mM sodium phosphate containing 0.5 M NaCl at pH 7.2. The protein concentration was 0.49 mg/mL, based on absorbance at 280 nm ($E_{0.1\%} = 1.01$). Sulfhydryl group analysis, determined by the addition of 25 μL of DTNB using the difference in absorbance at 412 nm ($\epsilon = 14\,150$), indicated that there were 2.5 SH groups/protein molecule.

The proteins were combined and concentrated to 1 mL by ultrafiltration. After 15 min at room temperature, 10 μL of 10 mM *N*-ethylmaleimide in DMSO was added, followed 5 min later by 20 μL of 10 mM 2-mercaptoethanol in water. The solution was further concentrated to 0.2 mL and then applied to a 7.8 $\times$ 300 mm Bio-Sil size-exclusion chromatography column (Biorad), which was eluted with PBS. Fractions that contained mainly monomeric conjugate mixed with unconjugated 96.5 mAb were gel filtered into 25 mM Tris buffer containing 125 mM NaCl at pH 7, and then applied to a 1 mL Q Sepharose column equilibrated with the same Tris buffer. Under these conditions, unconjugated mAb bound very poorly to the resin. The column was washed until there was no absorbance at 280 nm, and bound material was

eluted with 25 mM Tris buffer containing 1 M NaCl at pH 7. The resulting conjugate was almost entirely monomeric, with very small amounts of unconjugated 96.5 and aggregated protein. The specific activity with *p*-nitrophenylacetate was 5.2 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ of total protein, or 17.1 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ hCE-2 component.

Binding Studies. 3677 cells were suspended in polypropylene tubes (10^5 cells/tube in 0.6 mL of RPMI culture medium containing 10% fetal bovine serum), and 96.5-FITC (1 $\mu\text{g/mL}$ final concentration) was added with or without 96.5-hCE-2 at 0, 10, 25, and 50 $\mu\text{g/mL}$ final 96.5 concentration. After 1 h at 4 °C, the cells were washed, and fluorescence was determined on a fluorescence activated cell sorter. The competition of binding was compared to cells that were treated with 96.5-FITC (1 $\mu\text{g/mL}$ final concentration) together with unconjugated 96.5 at 0, 10, 25, and 50 $\mu\text{g/mL}$. 96.5-hCE-2 and unconjugated 96.5 were indistinguishable in this competition binding assay.

Cytotoxicity Assays. CPT-11 and SN-38 were made as 10 mM stocks in DMSO and diluted in media just prior to use. Cells (5×10^3 cells/well) were plated into 96-well tissue culture plates in 0.1 mL of RPMI medium containing 10% fetal bovine serum. To the wells were added 0.05 mL of the drugs at various concentrations, followed by 0.05 mL of the enzyme, 96.5-hCE-2, or PBS in media for the times indicated in the figure legends. The plates were centrifuged at 200g for 5 min, media was aspirated off, and the cells were washed three additional times by centrifugation and aspiration. Additional media (0.15 mL) was added, and incubation was continued for a total of 72 h, at which time the cells were treated with XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide, Sigma) and evaluated for toxicity according to previously described methods (27, 28).

RESULTS

The purification protocol of human liver microsomal proteins that effected the conversion of CPT-11 to SN-38 (Figure 1) is shown in Table 1. Throughout protein purification, an HPLC-based assay was used to monitor this hydrolysis reaction, and the data were supplemented with total esterase activity using *p*-nitrophenylacetate as a substrate along with SDS-PAGE analysis. In establishing the HPLC assay, it was found as reported earlier (10), that the conversion of CPT-11 to SN-38 was more facile at CPT-11 concentrations that minimally inhibited the enzymatic hydrolysis of *p*-nitrophenylacetate. This is most likely due to the stability of the active site serine carbamate formed during the course of CPT-11 hydrolysis (10). Anion-exchange chromatography of the ammonium sulfate precipitated proteins led to two separate populations of protein that hydrolyzed CPT-11, one that bound to the resin and was eluted with a salt gradient, and one that did not bind to Q Sepharose. The subsequent purification steps, illustrated in Table 1 for the material that bound to the anion-exchange resin, were used for

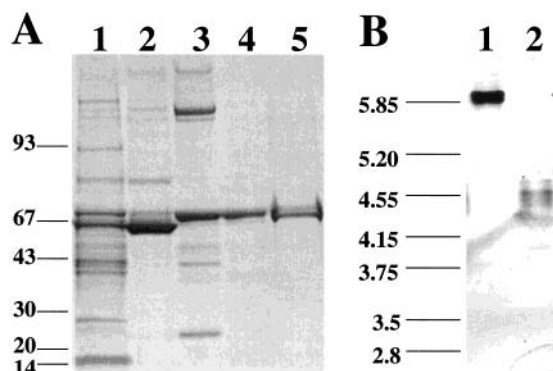


Figure 2. Analysis of purified proteins by gel electrophoresis. (A) The purification of carboxylesterases from human liver microsomes was monitored by SDS-PAGE (10% polyacrylamide). Lanes 1–4 illustrate the purification of hCE-2. Lane 1, ammonium sulfate precipitated protein; lane 2, after anion-exchange chromatography; lane 3, after hydrophobic interaction chromatography; lane 4, after size exclusion chromatography. Lane 5, hCE-1 using the same purification steps. Molecular weight standards gave bands as indicated. (B) Isoelectric focusing gel (pH 3–7) of purified proteins. Lane 1, hCE-1; lane 2, hCE-2. The IEF protein standards were obtained from Amersham Pharmacia. Both gels were stained with Coomassie Blue.

both populations. After hydrophobic interaction chromatography on phenyl Sepharose, followed by size exclusion chromatography, the resulting protein was homogeneous by SDS-PAGE (Figure 2A) and had a diffuse pI, most likely due to post-transcriptional protein modification, centered at pH 4.4 (Figure 2B). The active protein that did not bind to the Q Sepharose resin was similarly purified and had a pI value of approximately 6.0. The two purified proteins had apparent molecular masses of 65 kDa.

Attempts to identify the proteins by N-terminal sequencing failed since both proteins apparently had blocked amino termini. For that reason, the proteins were fragmented with cyanogen bromide, and some of the fragments that were separated by SDS-PAGE were subjected to N-terminal sequence analysis using Edman degradation chemistry. The results (Figure 3) indicate that the primary amino acid sequence of the lower activity enzyme corresponds to human carboxylesterase 1 (hCE-1, Figure 3A), and the higher activity protein corresponds to human carboxylesterase 2 (hCE-2, Figure 3B). In both cases, there was 100% homology between the sequenced peptides and the published sequences for hCE-1 and hCE-2 (16, 29).

The specific activities of the purified human carboxylesterases were compared to highly purified samples of rabbit liver carboxylesterase (rCE) and guinea pig liver carboxylesterase (gpCE). The latter two enzymes were chosen based on reports describing them as being proficient at catalyzing the hydrolysis of CPT-11 (9, 16). Unexpectedly, hCE-2 had 66% of the activity of rCE and gpCE on CPT-11. Consistent with previous reports, hCE-1 was significantly less active than hCE-2 and the other enzymes tested (9, 16, 17).

In vitro studies were carried out to compare the enzymes for their abilities to enhance the cytotoxicity of CPT-11. Tumor cell lines were exposed to varying concentrations of SN-38, CPT-11, or CPT-11 in the presence of purified carboxylesterases for a total of 24 h. Cell viability was measured 2 days later using XTT, a tetrazolium dye that measures mitochondrial membrane potential (27, 28). As expected (6, 30), SN-38 was approximately 1000-fold more potent than CPT-11 (Figure

A

MWLRATLTLASASAAWGHPSPPVVDTHVHGKVLGKFSLEGFAQPVAFILGIP
FAKPLGLPLRFTPPQAPWFSVKNATSYPPMCTQDPKAGQLLSELFNTNRKEN
IPLKLSDECLYLNITPADLTKNRLPVMVWVHGGGLMVGAASYDGLALAAHE
NVVVVTIQYRLGIWGGFFSTGDEHSRGNWGHLDQVAALRWVQDNIAFSFGNPG
SVTIFGESAGGESVSVLVLSPLAKNLFHRAISEGVALTSVLVKKGDVVKPLAEQI
AITAGCKTTTSAVVMVHCLRQKTEELLETTLKMKFLSLDLQGGDPRESQPLGLTV
IDGMLLLKTPEELQAERNFHTVPYVMGINKQEGFWLIPMLQMSYPLSEGQLDQ
KTAMSLWLKSYPLVCIKELIPEATEKYLGGTDDTVKKKDLFLDLIADVMFVGPS
VIVARNHRDAGAPTYMYEFQYRPSFSSDMKPKTVIGDHGDELFSVFGAPFLKE
GASEEILRLSKVMKFWANFARNGNPNGEGLPHWPEYNQKEGYLQIGANTQA
AQKLKDKVEAFWNTLFAKKAKEKPPQTEHIEL

B

QDSASPIRTHHTGQVLGSLVHVKGANAGVQTLGIPFAKPLGLPLRFAPPEPPE
SWSGVRDGTTHPAMCLQDLTAVESEFLSQFNMTFSPDSMSSEDECLYSITPA
HSHEGSNLPVVMVWVHGGALVFGMASLYDGSMLAALENVVVVIIQYRLGVLGFF
STGDKHATGNWGYLDQVAALRWVQDNIAHFGGNPDRTVIFGESAGGTSVSSL
VVSPISQGLFHGAIMEESGVALLPGLIASADVISTVANLSACDQVDSEALVGCLR
GKSKEELAINKPFKMPGVVDGVFLPRHPQELLASADFQVPVSIQVGNNEFG
WLIPKVMRIYDTQKEMDREASQAALQKMLTLLMLPPTFGDLLREEYIGDNGDPQ
TLQAQFOEMMADSMFVIPALQVAHFQCSRAPVYFYEFGHQPWSLKNIRPPHM
KADHGDELFPVFRSFFGGNYIKFTEEEELSRKMMKYWANFARNGNPNGEGL
PHWPLFDQEEQYLQNLQPAVGRALKAHRLQFWKKALPKIQIELEEFERHTE
L

Figure 3. Sequence data from purified proteins. The peptide sequences determined from cyanogen bromide fragments of the purified proteins are underlined and bold. The sequences were 100% homologous to the sequences within (A) hCE-1 (16) and (B) hCE-2 (29).

4). The degree to which CPT-11 was activated on both L2987 human lung adenocarcinoma (Figure 4A,B) and on 3677 human melanoma (Figure 4C) cells was greater for hCE-2, rCE, and gpCE than for hCE-1. In addition, it was found that the extent of prodrug activation for hCE-2, rCE, and gpCE was equivalent and dose-dependent on the L2987 human lung adenocarcinoma cell line. The cytotoxic activity was greater at 5 μ g/mL carboxylesterase (Figure 4B) than at 1 μ g/mL (Figure 4A). On 3677 cells, hCE-2 was slightly less effective than rCE and gpCE (Figure 4C). These data show that hCE-2 activates CPT-11 under the conditions of the cytotoxicity assays.

Conjugates of hCE-2 were prepared by linking the enzyme to the 96.5 mAb, a murine IgG2a that binds to the p97 antigen on most melanomas and on several carcinomas (24). The initial approach used was to react hCE-2 with *N*-(γ -maleimidobutyryloxy)succinimide ester (GMBS), which reacts with reactive amines such as those present on lysine side chains. However, the conjugates formed by combining hCE-2–GMBS adducts with antibody sulphydryl groups suffered from approximately 90% reductions in enzyme activity. An alternative approach was more successful, in which sulphydryl groups were appended to hCE-2 using 2-iminothiolane, and then the modified protein was reacted with maleimide-substituted 96.5. The 96.5-hCE-2 conjugate was then subjected to a two-step purification procedure that involved gel filtration to remove aggregates and free enzyme, followed by anion-exchange chromatography to separate most of the unconjugated mAb. Analysis by SDS-PAGE indicated that the conjugate was >75% monomeric. As has been previously shown with 96.5 enzyme conjugates (24, 25), binding studies using flow cytometric analysis indicated that 96.5-hCE-2 bound selectively to antigen positive 3677 melanoma cells and had unimpaired binding activity as compared to unmodified 96.5 (see Experimental Methods). 96.5-hCE-2 retained 52 and 69% of the specific activity with *p*-nitrophenylacetate and CPT-11 as substrates, respectively, as compared to the hCE-2 that was used for the conjugation (Table 2).

In vitro cytotoxicity experiments were conducted on 3677 melanoma cells (p97 antigen positive) that were

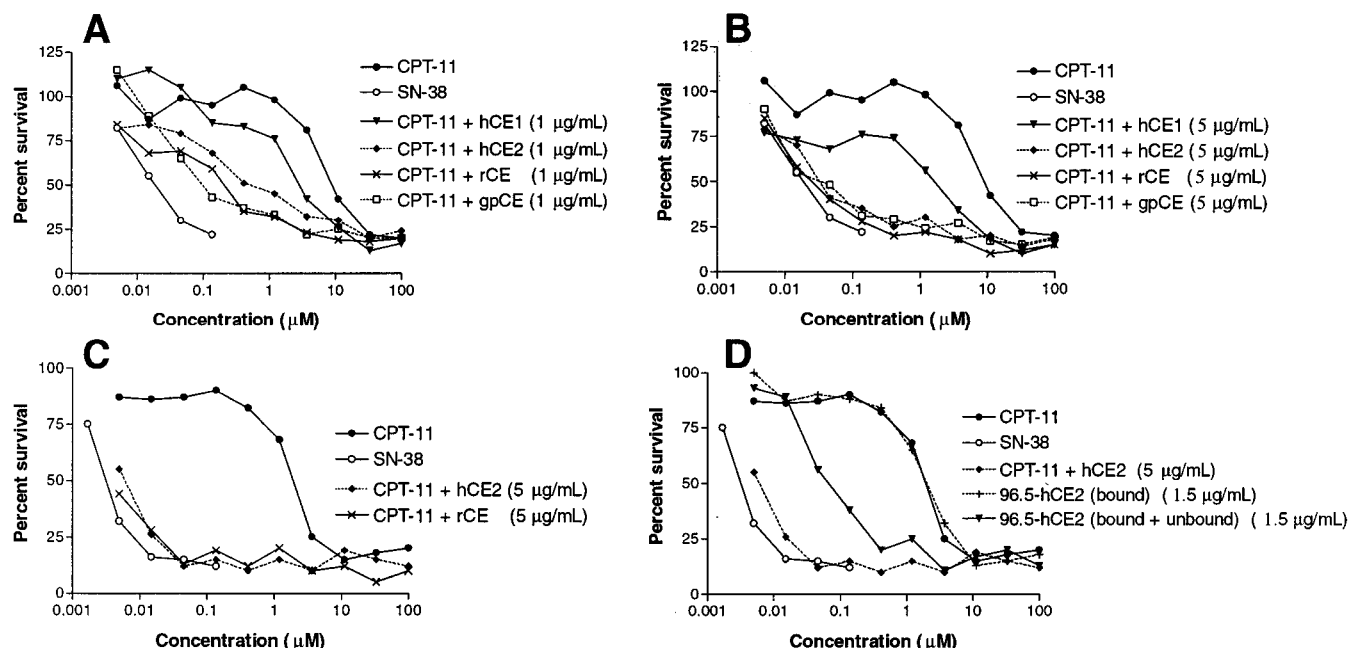


Figure 4. In vitro cytotoxicity assays. (A) and (B) L2987 human lung adenocarcinoma and (C) and (D) 3677 human melanoma cells were exposed to drugs with or without purified carboxylesterases for 24 h, washed, and the cytotoxic effects were determined 48 h later using XTT as an indicator of cell viability. For (D), 96.5-hCE-2 was used at 5 $\mu\text{g/mL}$ total protein, representing 1.5 $\mu\text{g/mL}$ hCE-2. Conjugate treated cells were either exposed to conjugate throughout the period of CPT-11 treatment (bound + unbound), or were washed to removed unbound conjugate prior to CPT-11 treatment (bound).

Table 2. Activities of Purified Carboxylesterases

protein ^a	specific activity	
	<i>p</i> -nitrophenylacetate ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	CPT-11 ($\text{pmol min}^{-1} \text{mg}^{-1}$)
hCE-1	10	3.5
hCE-2	60	92
rCE	28	142
gpCE	46	140
96.5-hCE-2 ^b	17	48

^a The abbreviations used for the proteins are hCE-1, human carboxylesterase 1; hCE-2, human carboxylesterase 2; rCE, rabbit liver carboxylesterase; gpCE, guinea pig liver carboxylesterase; 96.5-hCE-2, the conjugate formed between the 96.5 IgG and hCE-2. ^b The specific activity is based on the hCE-2 component of the conjugate, which had 33 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ and 70 $\text{pmol min}^{-1} \text{mg}^{-1}$ activity on *p*-nitrophenylacetate and CPT-11, respectively.

exposed to 96.5-hCE-2 prior to treatment with CPT-11. If the cells were extensively washed prior to prodrug treatment, there was no enhancement in the cytotoxic effects of CPT-11 (Figure 4D). In contrast, cells that were treated with the combination of 5 μg of 96.5-hCE-2/mL (contains 1.5 $\mu\text{g/mL}$ hCE component) and CPT-11 for 24 h were sensitized to CPT-11, albeit to a slightly lesser extent than corresponding cells treated with free hCE at 5 $\mu\text{g/mL}$. These results indicate that the enzyme within the conjugate is active and capable of activating CPT-11 under the conditions used in the cytotoxicity experiments, but that the amount of antigen-bound activity is too low to achieve detectable levels of prodrug activation. Several experiments were conducted using increased conjugate concentrations, exposing cells at high density with saturating concentrations of conjugate (31), using other mAb targeting vehicles for hCE-2, and using assays involving the binding of biotinylated hCE-2 to mAb-streptavidin conjugates. The results were invariable in that bound enzyme conjugates did not release cytotoxic concentrations of active drug. We conclude that the activity of hCE-2 is insufficient for targeting strategies utilizing

mAb-hCE-2 conjugates for antigen-specific CPT-11 activation.

DISCUSSION

The use of mAb-enzyme conjugates for prodrug activation at tumor sites has received considerable attention, and several targeted enzyme/prodrug combinations have demonstrated significant levels of antitumor activity in preclinical cancer models (reviewed in refs 22–23). The most advanced systems include bacterial enzymes, such as carboxypeptidase G2 and β -lactamase for the activation of nonclinically approved anticancer prodrugs (reviewed in refs 22, 23). One of the shortcomings of using such proteins is their expected immunogenicity in people, as evidenced by the anti-CEA mAb conjugate A5B7-mAb-carboxypeptidase G2 where all patients treated mounted significant immune reactions against the foreign protein (32). An additional complication in such systems is that the prodrugs are not clinically approved, and have unknown toxicities, pharmacokinetics, and biological effects. A preferable combination would be to utilize mAb-human enzyme conjugates for the activation of clinically approved anticancer prodrugs. No such examples exist, although there have been reports of activating clinically approved anticancer prodrugs with foreign proteins (33) and activating nonapproved anticancer prodrugs with human proteins (34, 35, reviewed in ref 26).

In using a human enzyme for the activation of the clinically approved anticancer drug CPT-11, we were aware that the known enzymes that converted CPT-11 to SN-38 had very poor activities. With the intent of exploring which enzyme might be the most efficient, we began by purifying enzymes from human liver microsomes on the basis of their abilities to effect the hydrolysis reaction. This search led us to hCE-1, one of the most abundant of the microsomal carboxylesterases (16, 17), and to hCE-2, which was reported during the course of our work as being more active than hCE-1 with CPT-11 as a substrate (21). In our hands,

hCE-2 was 26-fold more active than hCE-1 with CPT-11 and was almost as active as rCE, the highest activity enzyme known for the hydrolysis of CPT-11 (16, 19). Previous studies have shown that cancer cell lines transfected with the genes for either rCE (16, 17) or with hCE-1 (19, 20) were rendered significantly more sensitive to CPT-11 treatment as compared to the parental cells. Our results would suggest that the gene for hCE-2 might have considerably greater potential for gene therapy for CPT-11 activation, since it is more active than hCE-1 and would not be expected to induce an immune reaction like rCE.

It is significant that in vitro potentiation of CPT-11 activity could be obtained by gene therapy with hCE-1 and with rCE2 (16, 17, 19, 20) but that we were unable to demonstrate any such activity on cells that were exposed to 96.5-hCE-2. Another conjugate, 96.5- β -lactamase, has been used successfully for specific activation of many anticancer prodrugs under conditions involving subsaturating concentrations and prodrug exposure for very short periods of time (24, 25). Here, we were unable to effect prodrug activation even at saturating 96.5-hCE-2 concentrations and with prodrug exposures for as long as 24 h. We believe the reason for the discrepancy between these two systems lies in the kinetics of the enzymes used. While β -lactamase has specific activities in the range of $315 \mu\text{mol min}^{-1} \text{mg}^{-1}$ with a doxorubicin prodrug (25) to $1470 \mu\text{mol min}^{-1} \text{mg}^{-1}$ for a melphalan prodrug (36), hCE-2 has activity 10^6 – 10^7 -fold lower than that. The fact that hCE-1 and rCE have proven useful in gene therapy studies might be attributed to very high level expression of the protein inside the target cells as compared to the amount that can be delivered through a mAb-enzyme conjugate, and to possible differences between intracellular and extracellular generation of SN-38.

We conclude that the activity of hCE-2 must be significantly increased to be effective for ADEPT. One of the lowest activity enzymes successfully used to activate an anticancer prodrug in an immunologically specific manner was penicillin G-amidase for the activation of a prodrug of doxorubicin (31). The specific activity of the enzyme was $12.7 \text{ nmol min}^{-1} \text{mg}^{-1}$, which is only 140-fold greater than hCE-2. It is reasonable to expect that directed evolution of hCE-2 toward CPT-11 hydrolysis should achieve catalytic efficiencies in this range or higher. Such studies are warranted, given the clinical need to improve the therapeutic window for CPT-11 and the advantages in using a potentially nonimmunogenic conjugate for doing so.

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TECHNICAL NOTES

DOTA Tris(phenylmethyl) Ester: A New Useful Synthon for the Synthesis of DOTA Monoamides Containing Acid-Labile Bonds

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The synthesis of DOTA tris(phenylmethyl) ester **2**, a new monoreactive derivative of DOTA, is described. This versatile synthon can be easily coupled to compounds bearing an amino group and then deprotected to DOTA monoamide under mild and neutral conditions by catalytic hydrogenolysis. Accordingly, compound **2** has been used in the synthesis of a DOTA monoamide gadolinium complex containing two palmitic esters, which is a component of mixed micelles as MRI contrast agents.

INTRODUCTION

The 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) is a well-studied ligand which forms extremely stable complexes with a variety of metals (1–5). Such metal complexes have found a widespread use in therapy and diagnostic imaging (6–9). For example, the gadolinium complex of DOTA is currently used as extravascular contrast agent for magnetic resonance imaging (MRI) (10, 11).

The search for more and more specific therapeutic and diagnostic entities incorporating a metal complex has led to the conjugation of the DOTA moiety to a variety of biomolecules [e.g., peptides (12, 13), bile acids (14, 15), proteins (16)] which should act as carriers, delivering the product to a selected district of the body.

Since the conversion of a carboxyl moiety of DOTA into a carboxamide does not dramatically affect the stability of the final metal complex (13, 17), the easiest way to conjugate DOTA to a carrier is by formation of an amide bond. In this respect the activation with isobutyl chloroformate (13, 18) or by formation of a sulfo-NHS active ester (16) have been reported. The statistical polyactivation of the four carboxylic groups of DOTA is the main drawback of this strategy, and to overcome this problem a triprotected derivative should be employed. Indeed, the synthesis of DOTA tris(*tert*-butyl) ester **1** has been recently reported (12, 19), and conjugation of **1** to a somatostatin derivative gives rise, after acid deprotection of the *tert*-butyl esters, to a DOTA monoamide analogue of OctreoScan (12).

In the past we took advantage of **1** for the coupling of DOTA to bile acid derivatives (14, 15) in order to obtain gadolinium complexes for the magnetic resonance imaging (MRI) of the liver (Figure 1).

Recently, we focused our attention on the synthesis of lipophilic gadolinium complexes which were found to be very useful agents for magnetic resonance angiography (MRA) when formulated as mixed micelles (20, 21). Mixed micelles are multicomponent micelles containing a phos-

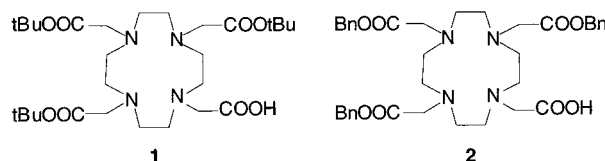


Figure 1. Structures of DOTA tris(*tert*-butyl) ester **1** and DOTA tris(phenylmethyl) ester **2**.

pholipid, a biocompatible nonionic surfactant (e.g., Synperonic F-108), and a lipophilic gadolinium complex, such as **8** or **9** (Figure 2). In particular, compound **8** was found very interesting. The mixed micelles containing **8** show high relaxivities and long blood permanence in rats, two essential features for a MRA agent. The only drawback of **8** is its elimination. In fact, 7 days after injection in rats of the formulation only 50% of injected dose is eliminated by the liver while the remaining is still present into the animal body. To increase the elimination, the synthesis of **9** was taken into account. Compound **9** contains two palmitic esters which should be hydrolyzed in vivo, giving rise to a small, hydrophilic gadolinium complex more easily eliminable.

We now report here the synthesis of DOTA tris(phenylmethyl) ester **2**, which is a valid alternative to compound **1** especially in all those cases in which the conjugated counterpart cannot survive acid deprotection conditions, as assessed for example in the synthesis of **9**.

EXPERIMENTAL PROCEDURES

General. Organic and inorganic reagents were purchased from Merck KGaA (Darmstadt, Germany) and Fluka A.G. (Buchs, Switzerland) except for 1-propane-phosphonic acid cyclic anhydride **12** (Lancaster, Mülheim am Main, Germany). 1,4,7,10-Tetraazacyclododecane **3** was synthesized following our patented procedure (22). Resin Amberlite XAD 16.00 was purchased from Rohm and Haas Italia Srl (Gessate, Italy). Thin-layer chromatography (TLC) was carried out on silica gel plates (Merck KGaA, Silica 60 F254, 0.2 mm), and spots were visualized with 1% KMnO₄ in 1 N NaOH. Flash chromatography was performed on Merck KGaA silica

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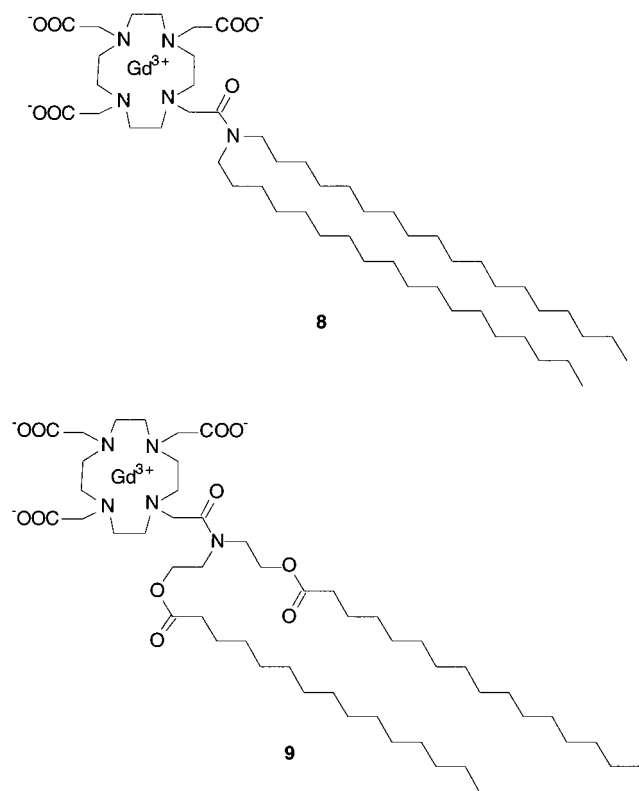


Figure 2. Structures of the lipophilic gadolinium complexes **8** and **9**.

gel 60 (230–400 mesh). IR spectra were recorded on KBr cells on a Perkin-Elmer Infrared 882 spectrometer. ^1H and ^{13}C NMR spectra (in CDCl_3 , if not differently specified) were recorded on a Bruker AC 200 spectrometer. Electrospray ionization was performed on a Finnigan TSQ 700 triple quadrupole mass spectrometer fitted with a Finnigan ESI interface. Melting points were determined in open capillaries with a Büchi B-540 apparatus and are uncorrected. Elemental analysis were performed by Redox Laboratories (Monza, Italy).

1,4,7,10-Tetraazacyclododecane-1-acetic acid 1,1-Dimethylethyl Ester (4). A solution of *tert*-butyl bromoacetate (25.3 g, 130 mmol) in CHCl_3 (500 mL) was added dropwise in 7 h to a solution of 1,4,7,10-tetraazacyclododecane **3** (112.3 g, 650 mmol) in CHCl_3 (2 L) maintained under nitrogen at room temperature. After 14 h, the solution was concentrated to 800 mL, washed with H_2O (9×200 mL) to eliminate the excess of **3**, dried over Na_2SO_4 , and evaporated to give **4** (39 g, 99%) as a pale yellow oil: IR 1728 cm^{-1} ; ^1H NMR δ 1.12 (s, 9H, *t*Bu), 2.26 (m, 8H, CH_2N), 2.43 (m, 8H, CH_2N), 2.93 (s, 2H, CH_2COO); ^{13}C NMR δ 27.77 (CH_3), 44.9, 45.7, 46.6, 51.4 (CH_2N), 56.7 (CH_2COO), 80.4 (C), 170.4 (CO); MS m/z 287 [$\text{M} + \text{H}$] $^+$. Anal. Calcd for $\text{C}_{14}\text{H}_{30}\text{N}_4\text{O}_2$: C, 56.20; H, 10.06; N, 18.56. Found: C, 56.36; H, 10.34; N, 17.84.

1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid 1,1-dimethylethyl tris(phenylmethyl) ester adduct with NaCl (5). A solution of **4** (36 g, 126 mmol) in DMF (200 mL) was added dropwise in 7 h to a stirred suspension of benzyl bromoacetate (94.96 g, 414 mmol) and K_2CO_3 (86.8 g, 628 mmol) in DMF (250 mL) maintained under nitrogen at room temperature. After 14 h the suspension was filtered and the orange solution evaporated to dryness. The residue was dissolved in EtOAc (500 mL), washed with H_2O (3×400 mL) then with brine (2×300 mL). The organic phase was separated, dried over Na_2SO_4 and evaporated. The resi-

due was purified by flash chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 15:1) to give **5** (51 g, 51%) as a sticky pale orange solid: IR 1730 cm^{-1} ; ^1H NMR δ 1.29 (s, 9H, *t*Bu), 2.19–3.26 (m, 24H, CH_2N), 4.94–4.98 (m, 6H, CH_2Ph), 7.13–7.16 (m, 15H, Ph); ^{13}C NMR δ 27.8 (CH_3), 48–53 (broad signal, CH_2N), 55.0, 55.6 (CH_2COO), 66.8 (CH_2Ph), 82.1 (C), 128.2, 128.4, 128.4, 134.9, 135.0 (Ph), 172.9, 173.4 (CO); MS m/z 754 [$\text{M} + \text{Na}$] $^+$. Anal. Calcd for $\text{C}_{41}\text{H}_{54}\text{ClN}_4\text{NaO}_8$: C, 62.38; H, 6.91; N, 7.10; Na, 2.91; Cl, 4.49. Found: C, 61.77; H, 6.74; N, 6.90; Na, 2.90; Cl, 4.95.

1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic Acid Tris(phenylmethyl) Ester (2). Compound **5** (47.11 g, 60 mmol) was dissolved in dioxane (500 mL), and then 12 N HCl (500 mL) was added under nitrogen at room temperature, obtaining a white precipitate. After stirring for 16 h, the pale yellow suspension was evaporated and the residue dissolved in H_2O (700 mL) by ultrasound sonication. The solution (pH 2) was loaded onto an Amberlite XAD-16.00 resin column (900 mL) and eluted with a $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ gradient. The product elutes with 40% $\text{CH}_3\text{CN}/\text{H}_2\text{O}$. The fractions containing the product were concentrated to remove CH_3CN and then extracted with EtOAc. The organic phase was dried over Na_2SO_4 and evaporated. The pale yellow residue was triturated with EtOAc (150 mL) to give **2** (21 g, 52%) as a white solid: mp $108\text{--}110\text{ }^\circ\text{C}$; IR 1735 cm^{-1} ; ^1H NMR δ 2.64 (m, 8H, CH_2N), 2.90 (m, 4H, CH_2N), 3.34–3.58 (m, 12H, CH_2N), 5.01 (m, 6H, CH_2Ph), 7.24 (m, 15H, Ph); ^{13}C NMR δ 48.1, 50.2, 53.1, 53.2, 55.4, 55.6, 56.1 (CH_2N), 66.1, 66.3 (CH_2Ph), 128.1, 128.2, 128.3, 128.4, 128.5, 135.2 (Ph), 166.5, 170.4, 171.1 (CO); MS m/z 697 [$\text{M} + \text{Na}$] $^+$. Anal. Calcd for $\text{C}_{37}\text{H}_{46}\text{N}_4\text{O}_8$: C, 65.86; H, 6.87; N, 8.30. Found: C, 66.00; H, 7.03; N, 8.33.

1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic Acid Phenylmethyl Ester (6). This product elutes with 10% $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ from the column used for purification of **2**. Evaporation of the eluate gave **6** (3 g, 10%) as a pale yellow solid: mp $87\text{--}92\text{ }^\circ\text{C}$ (dec); IR 1737 cm^{-1} ; ^1H NMR δ (CD_3OD) 3.14–3.85 (m, 24 H, CH_2N), 5.13 (s, 2H, CH_2Ph), 7.36 (m, 5H, Ph); ^{13}C NMR δ (CD_3OD) 49.6, 50.1, 52.6, 53.0, 54.3, 54.9, 58.2 (CH_2N), 67.8 (CH_2Ph), 129.7, 129.9, 137.4 (Ph), 170.4, 172.4, 175.0 (CO); MS m/z 517 [$\text{M} + \text{Na}$] $^+$. Anal. Calcd for $\text{C}_{23}\text{H}_{34}\text{N}_4\text{O}_8$: C, 55.85; H, 6.93; N, 11.33. Found: C, 55.84; H, 7.09; N, 11.08.

1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic Acid 1,4-bis(Phenylmethyl) Ester (7). This product elutes with 20% $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ from the column used for purification of **2**. Evaporation of the eluate gave **7** (3 g, 8.5%) as a pale yellow solid: mp $78\text{--}82\text{ }^\circ\text{C}$ (dec); IR 1736 cm^{-1} ; ^1H NMR δ ($\text{DMSO}-d_6$) 2.71–3.63 (m, 24H, CH_2N), 5.10 (s, 4H, CH_2Ph), 7.35 (m, 10H, Ph); ^{13}C NMR δ ($\text{DMSO}-d_6$) 49.3, 49.9, 50.8, 51.1, 54.2, 55.6 (CH_2N), 65.3 (CH_2Ph), 127.9, 128.4, 136.0 (Ph), 169.2, 170.9 (CO); MS m/z 607 [$\text{M} + \text{Na}$] $^+$. Anal. Calcd for $\text{C}_{30}\text{H}_{44}\text{N}_4\text{O}_8$: C, 61.20; H, 7.54; N, 9.52. Found: C, 61.52; H, 6.97; N, 9.43.

10-[2-[Bis(2-[(1-oxohexadecyl)oxy]ethyl)amino]-2-oxoethyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic Acid Tris(Phenylmethyl) Ester Adduct with NaCl (13). A 50% EtOAc solution of 1-propanephosphonic acid cyclic anhydride **12** (23) (16.9 g, 26.6 mmol) was added to a solution of **2** (13 g, 19.3 mmol), hexadecanoic acid iminodi-2,1-ethanediyl ester hydrochloride **11** (24) (12 g, 19.4 mmol) and Et_3N (12.6 mL, 90.5 mmol) in CH_2Cl_2 (600 mL). The reaction mixture was stirred under nitrogen at room temperature for 24 h, and then more 50% EtOAc solution of **12** (6.1 g, 9.6 mmol) was added. After another 24 h, the mixture was washed with brine (2×300 mL), dried over Na_2SO_4 , and evaporated.

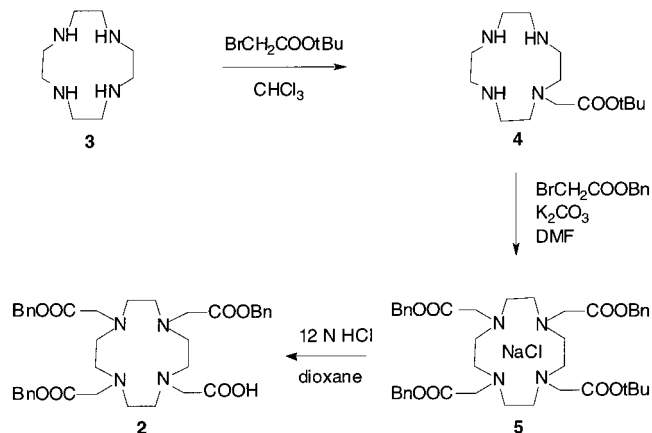
The residue was purified by flash chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1) to give **13** (12 g, 48%) as a yellow oil: IR 1735 cm^{-1} ; ^1H NMR δ 0.81 (t, 6H, $J = 6.7\text{ Hz}$, CH_3), 1.19 (m, 48H, aliphatic chain), 1.49 (m, 4H, $\text{CH}_2\text{-CH}_2\text{COO}$), 2.20 (m, 4H, CH_2COO), 2.26–3.56 (m, 28H, CH_2N), 4.14 (m, 4H, CH_2OOC), 5.07 (m, 6H, CH_2Ph), 7.27 (m, 15H, Ph); ^{13}C NMR δ 13.9 (CH_3), 22.5, 24.3, 24.6, 29.0, 29.1, 29.2, 29.3, 29.5, 31.7, 33.9 (aliphatic chain CH_2), 46.4, 46.8 (CONCH_2), 48–53 (broad signals, tetraazacyclo CH_2), 55.0 (NCH_2CO), 61.8 (CH_2OOC), 66.7, 66.8 (CH_2Ph), 126.9, 128.0, 128.3, 128.4, 135.1, 135.2 (Ph), 172.0, 173.2, 173.4 (CO); MS m/z 1261 [$\text{M} + \text{Na}$] $^+$. Anal. Calcd for $\text{C}_{73}\text{H}_{115}\text{ClN}_5\text{NaO}_{11}$: C, 67.59; H, 8.94; N, 5.40; Cl, 2.73; Na, 1.77. Found: C, 66.91; H, 9.09; N, 5.34; Cl, 3.10; Na, 1.92.

10-[2-Bis[2-[(1-oxohexadecyl)oxy]ethyl]amino]-2-oxoethyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic Acid (14**)**. Pd (10% on carbon (1.43 g) was added to a solution of **13** (10 g, 7.7 mmol) in EtOH (150 mL), and the suspension was stirred over 12 h under a hydrogen atmosphere at room temperature. The mixture was filtered over Millipore FT 0.45 μm and the solution evaporated under reduced pressure. The residue was suspended in H_2O (200 mL), stirred for 1 h, and then centrifuged (10 $^\circ\text{C}$, 8000 rpm, 30 min). The solution was decanted away to eliminate the dissolved NaCl, and the remaining solid was dried (1.3 kPa, P_2O_5) to give **14** (7.4 g, 98%) as a white solid: mp $82\text{ }^\circ\text{C}$ (dec); IR 1736 cm^{-1} ; ^1H NMR δ 0.84 (t, 6H, $J = 7\text{ Hz}$, CH_3), 1.22 (m, 48H, aliphatic chain), 1.55 (m, 4H, $\text{CH}_2\text{CH}_2\text{COO}$), 2.25 (m, 4H, CH_2COO), 2.5–3.9 (broad m, 28H, CH_2N), 4.15 (m, 4H, CH_2OOC); ^{13}C NMR δ 13.9 (CH_3), 22.5, 24.4, 24.7, 29.1, 29.2, 29.6, 31.8, 34.0, 34.8 (aliphatic chain CH_2), 46.9–58.0 (broad signals, CH_2N), 61.7 (CH_2OOC), 173.3, 173.4 (CO); MS m/z 991 [$\text{M} + \text{Na}$] $^+$. Anal. Calcd for $\text{C}_{52}\text{H}_{97}\text{N}_5\text{O}_{11}$: C, 64.50; H, 10.10; N, 7.23. Found: C, 64.07; H, 9.78; N, 7.22.

RESULTS AND DISCUSSION

The use of compound **1** for the synthesis of **9** is hampered by the fact that the acid deprotection of *tert*-butyl esters leads to a significant hydrolysis of the palmitic esters too. For this reason we planned the synthesis of synthon **2** (Scheme 1), since the benzyl esters

Scheme 1. Synthesis of DOTA Tris(phenylmethyl) Ester **2**



can be deprotected by catalytic hydrogenolysis without affecting the esters with the palmitic residues.

Reaction of an excess of 1,4,7,10-tetraazacyclododecane **3** (5 mol equiv) with *tert*-butyl bromoacetate gave the monoalkylated derivative **4** in nearly quantitative yield.

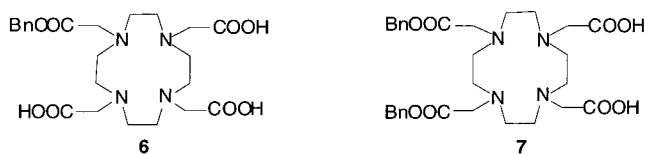


Figure 3. Structures of the byproducts recovered during the purification of **2**.

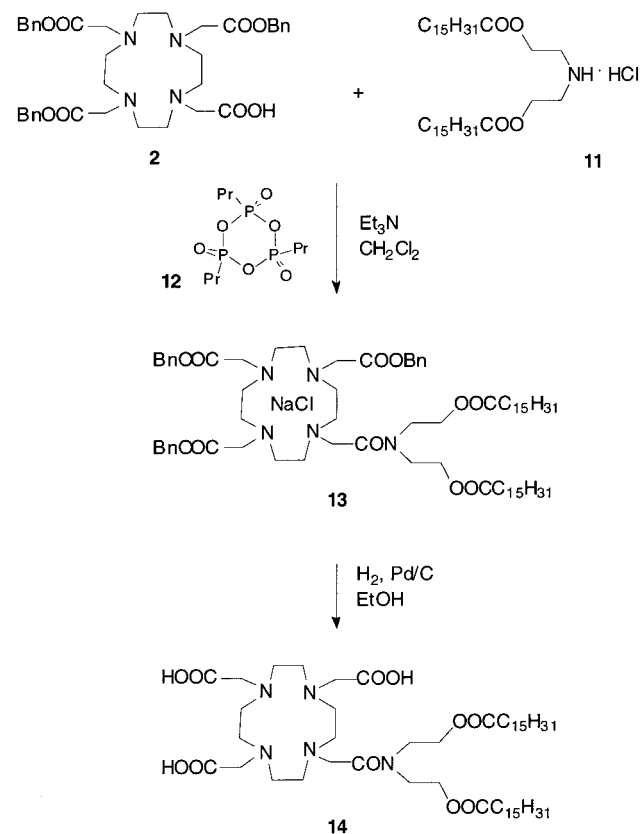
Peralkylation of **4** with benzyl bromoacetate in DMF and K_2CO_3 gave tetraester **5** as NaCl adduct, after workup with brine. The ability of DOTA tetraesters to coordinate sodium ions has been already assessed (25, 26). The presence of NaCl made the purification of **5** easier by flash chromatography on silica gel, avoiding the use of large amounts of ammonia in the eluent.

Deprotection of the *tert*-butyl ester of **5** with 12 N HCl in dioxane gave compound **2**, which was purified and desalted by elution through an Amberlite XAD 16.00 resin column with $\text{CH}_3\text{CN}/\text{H}_2\text{O}$.

Minor amounts of DOTA benzyl ester **6** and 1,4-dibenzyl ester **7** were also recovered during the purification of **2**. The structure of **7** was assigned on the basis of symmetry and the ^{13}C NMR spectrum (Figure 3). In fact, the ^{13}C NMR spectrum of **7** shows six different methylene groups on nitrogen, while the corresponding 1,7-dibenzyl ester should have only four.

The usefulness of intermediate **2** was proved in the synthesis of ligand **14** (Scheme 2). DOTA tribenzyl ester

Scheme 2. Synthesis of Ligand **14**



2 was coupled to amine hydrochloride **11** (24) using 1-propanephosphonic acid cyclic anhydride **12** as condensing agent (23) to obtain **13** as NaCl adduct. Cleavage of the benzyl ester protections of **13** by hydrogenolysis yielded the triacid **14**. Complexation of **14** with $(\text{CH}_3\text{-COO})_3\text{Gd}$ in $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ at $50\text{ }^\circ\text{C}$ gave the complex **9** in quantitative yield.

Mixed micelles containing complex **9** show a better elimination profile than those containing complex **8**. In fact, 7 days after injection in rats, 67% of the injected dose is eliminated (for complex **8** was only 50%). Moreover, 27% of injected dose is found in urine, and this is an indirect evidence of a partial hydrolysis in vivo of complex **9** (21).

In conclusion, compound **2** can be easily coupled to a variety of carriers bearing an amino group and then deprotected to DOTA monoamide under mild and neutral conditions by catalytic hydrogenolysis. This is a considerable advantage over compound **1** which requires acidic conditions for the deprotection. Indeed, this was proved with the synthesis of gadolinium complex **9**, which is a promising component of mixed micelles for MRI coronary angiography.

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Hyaluronic Acid-*N*-hydroxysuccinimide: A Useful Intermediate for Bioconjugation

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Hyaluronic acid (HA) is an abundant nonsulfated glycosaminoglycan component of synovial fluid and the extracellular matrix. HA is an important building block for biocompatible and biointeractive materials with applications in drug delivery, tissue engineering, wound repair, and viscosupplementation. Herein we describe the synthesis and characterization of HA-*N*-succinimide, an activated ester of the glucuronic acid moiety. This HA-active ester intermediate is a precursor for fluorescent probes, drug-polymer conjugates, and cross-linked hydrogels. As a demonstration, we used HA-NHS to prepare HA-BODIPY by coupling with the hydrazide derivative of the fluor. Intracellular uptake of HA-BODIPY into human ovarian cancer cells, which overexpress cell-surface HA receptors, was visualized using confocal microscopy.

Hyaluronic acid (hyaluronan, HA), a (β -1,4)-linked D-glucuronic acid and (β -1,3) *N*-acetyl-D-glucosamine polysaccharide (Figure 1), is a nonsulfated glycosaminoglycan (GAG) found in the extracellular matrix (ECM). HA plays an important role in the structure and organization of ECM, including the maintenance of extracellular space, the transport of ion solutes and nutrients, and the preservation of tissue hydration. HA concentrations increase whenever rapid tissue proliferation, regeneration, and repair occur. HA also binds specifically to proteins in ECM, on cell-surface receptors, and within the cell cytosol. These protein-ligand interactions are important in stabilizing the cartilage ECM (1, 2), regulating cell adhesion and motility (3, 4), mediating proliferation and differentiation (5), and the action of growth factors (6). HA has also been implicated in morphogenesis and embryonic development (7), in cancer (8–14), in modulating inflammation (12), in stimulating angiogenesis and healing (15), and as a protective coating (16). HA and its chemically modified derivatives (17) have emerged as versatile building blocks for novel biomaterials (18).

Chemical modification of HA has employed the carboxyl group, the hydroxyl groups, the reducing end, and in some cases partially *N*-deacetylated materials. The attempted carbodiimide-mediated coupling of primary amines to HA resulted in negligible coupling due to the protonation of the required nucleophilic nitrogen at the reaction pH. The major product was the *N*-acylurea adduct. Recently, a modified method for carbodiimide/active ester-mediated coupling of polyfunctional amines to HA was developed in which HA was reacted with 30-fold excess of the amine component at pH 6.8 in the presence of EDCI and 1-hydroxybenzotriazole (HOBt) with DMSO/H₂O (1:1) as solvent (19). Antitumor agents have been coupled to HA to provide polymeric conjugates that deliver chemotherapeutics to target tissues. For

example, adriamycin (20), mitomycin C, and epirubicin (21) were coupled to HA using carbodiimide chemistry, and butyric acid-HA was synthesized by reaction of butyric anhydride with HA (22).

In this technical note, we describe a convenient route to the HA-*N*-succinimide active ester (Figure 1). Ion-exchange (23) and lyophilization afforded the HA tetrabutylammonium salt (HA-TBA). Next, 2 mol equiv (based on the calculated number of glucuronic acid residues present) of purified *N*-hydroxysuccinimido diphenyl phosphate (SDPP), mp 89–90 °C (24), were added to a solution of 2 wt % HA-TBA in DMSO. The reaction was stirred for either 2 h or overnight at room temperature, and the product was precipitated by addition of 3 volumes of hexane followed by excess acetone. The residue was dissolved in 2 mL of H₂O and purified on a Sephadex G-25 (20–80 μ m) (Sigma, St. Louis, MO) gel filtration column. In our experience, SDPP activation gave superior yields of HA-*N*-succinimide active ester (HA-NHS) in less time and under milder conditions than those reported previously (19, 23, 25–28).

The degree of substitution was calculated by integration of the succinimide ester methylenes (¹H NMR, broad singlet at δ = 2.62 ppm, 4H) relative to the methyl of the acetamido moiety of the GlcNAc residues resonance (δ = 1.95 ppm, 3H) (25). The degrees of substitution were 50 mol % and 80 mol % with the reaction time of 2 and 12 h, respectively. In some cases, triethylamine facilitated the carboxyl group activation reaction by SDPP (24). In the present example, however, use of triethylamine as a cosolvent led to ion exchange and isolation of the HA-triethylamine salt instead of HA-NHS.

The HA-NHS active ester is a versatile precursor for bioconjugation with reporter and effector molecules. BODIPY-FL hydrazide (Molecular Probes, Inc., Portland, OR) was used to demonstrate this versatility. Thus, 20 mg of HA-NHS (50 mol %) was dissolved in 2 mL of H₂O while stirring at room temperature; 1 mg of BODIPY-FL hydrazide in 0.5 mL of DMSO was then added with 3 drops of 0.1 N Na₂CO₃ buffer (pH 8.0). The reaction was stirred at room temperature for 3 days in the dark. Next, 2-aminoethanol (100 μ L) was added to

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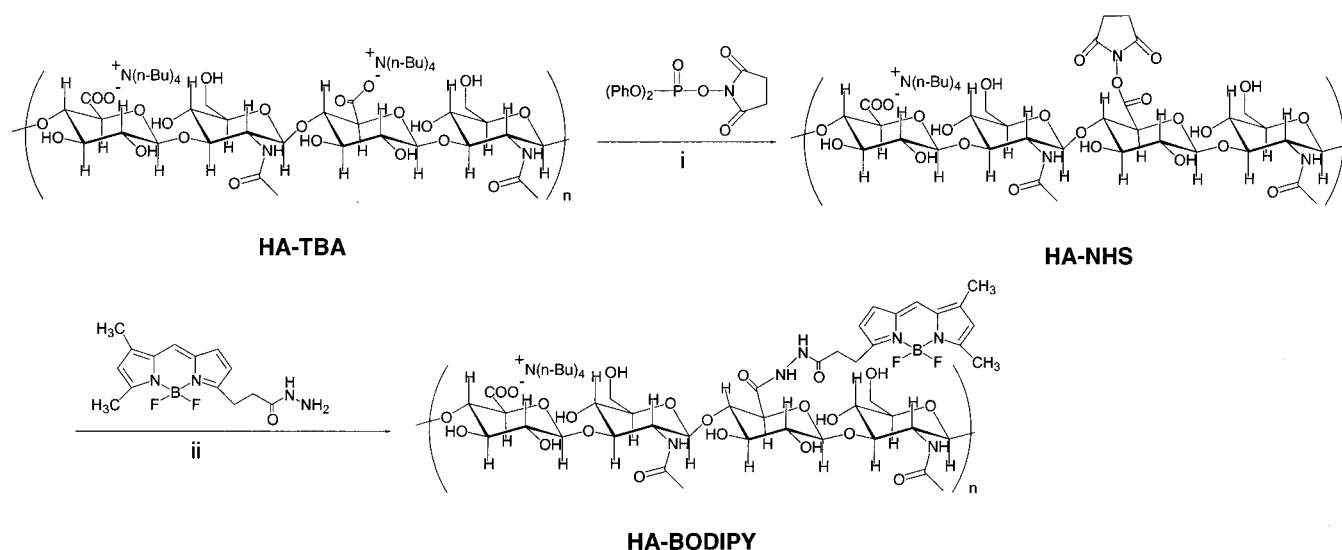


Figure 1. Synthesis of HA-NHS active ester and HA-BODIPY probe: (i) SDPP, DMSO, rt; (ii) DMSO/H₂O, rt.

consume any unreacted active ester. Solvents were removed by lyophilization, and the residue was dissolved in 1 mL of H₂O and purified by gel filtration. The purity of HA-BODIPY was measured at 210 nm (HA absorption) and 502 nm (BODIPY absorption) using analytical gel permeation chromatography (GPC) (Figure 2a). The low molecular weight HA-NHS has high efficiency of BODIPY coupling than the high molecular weight chains. The degree of substitution of BODIPY determined spectrophotometrically was 4.5 wt %. Interestingly, the HA-BODIPY conjugate had a 5-nm blue shifted absorbance with excitation at 488 nm as compared to the BODIPY hydrazide reagent (Figure 2b). In addition, a broadening of the fluorescence emission was observed (Figure 2c). It is possible that π -stacked or hydrophobically interacting aggregates could be formed in aqueous solution when a hydrophobic fluorophore is conjugated to a water-soluble polymer, thus altering the absorbance and emission of the fluorophore. (29). At the low modifications employed, this does not appear to be a problem, as a soluble, homogeneous derivative was obtained. This is consistent with previous work with HA-taxol (30) and HA-FITC-taxol derivatives (31).

HA-BODIPY was readily internalized by human ovarian cancer cells (SK OV-3), which overexpresses the HA receptor CD44 on the cell surface. Thus, SK OV-3 cells were incubated in a cell culture flask, harvested by trypsinization, and transferred into an 8-well cell culture slide. Cells (20,000) were seeded in each well of the slide and cultured for 48 h. The culture medium was replaced with medium containing HA-BODIPY probe (100 μ g/mL HA). Cells were cultured with the conjugates for various lengths of time. Unbound probe was removed by washing the cell layer three times with Dulbecco's phosphate-buffered saline (DPBS) (Sigma, St. Louis, MO). Cells were fixed with 3% paraformaldehyde for 10 min at room temperature and washed again with DPBS. Internalization of BODIPY-HA was visualized by fluorescence confocal microscopy (Figure 3). Initially, BODIPY-HA can be seen on the cell membranes, and with time it is gradually endocytosed and accumulates in the cytoplasm. Thus, HA and probe-modified HA bind to the cell surface via interactions with CD44 or RHAMM, and a portion subsequently undergoes endocytosis (4, 30, 31). Internalization of [³H]-labeled HA previously revealed that intracellular degradation of HA occurs within a low pH environment (32), such as that of the lysosome. In the

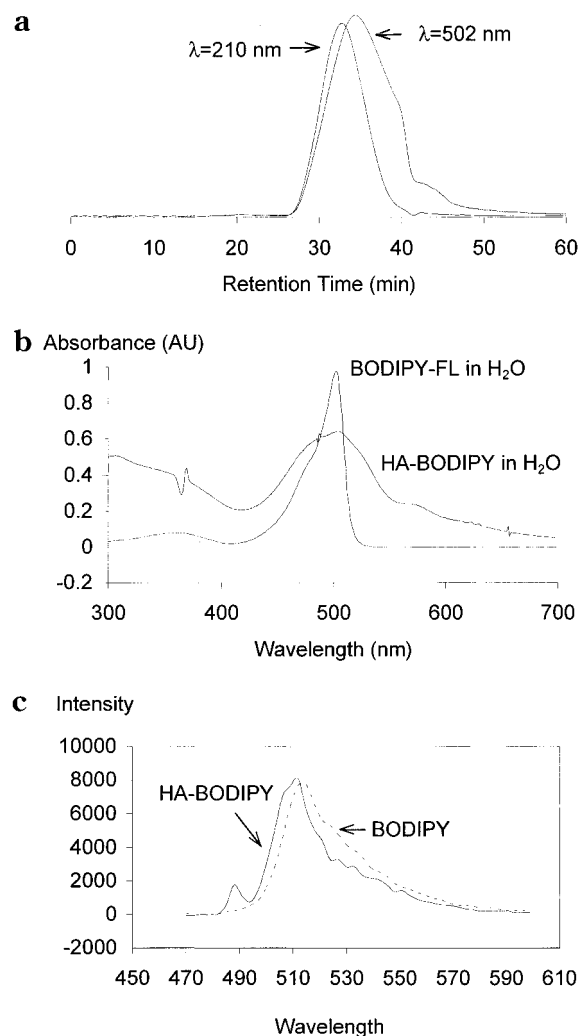


Figure 2. (a) GPC profile of HA-BODIPY detected at 210 and 502 nm. GPC was carried out on the following system: Waters 515 HPLC pump, Waters 410 differential refractometer, Waters 486 tunable absorbance detector, Waters Ultrahydrogel 250 and 2000 columns (7.8 mm ID $\times$ 30 cm) (Milford, MA). The GPC eluent was 150 mM phosphate buffer/MeOH = 80:20 (v/v), pH 6.5, and the flow rate was 0.5 mL/min. (b) UV/vis spectra of BODIPY and HA-BODIPY. (c) Fluorescence spectra of BODIPY and HA-BODIPY.

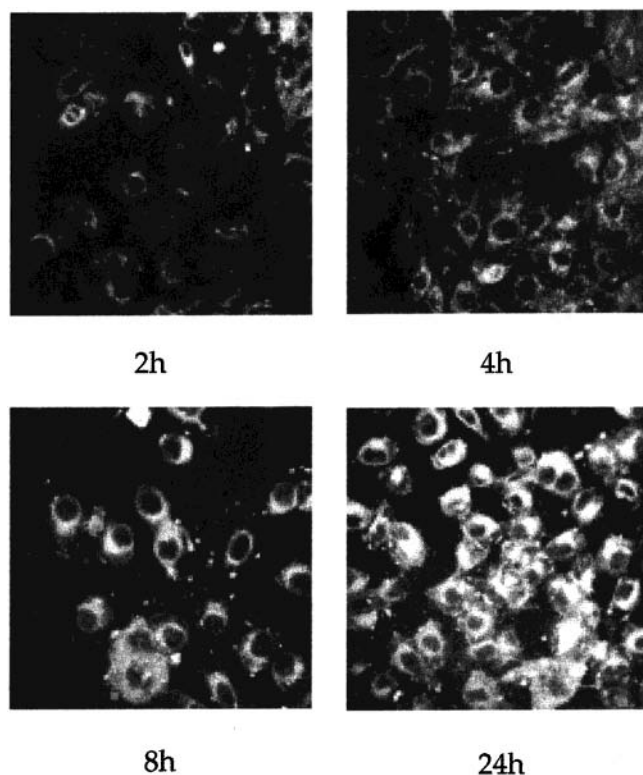


Figure 3. Time course internalization of HA-BODIPY by SK OV-3 cells. Cells were examined using an inverted microscope (Nikon) and a Bio-Rad (Hercules, CA) MRC 1024 laser scanning confocal microscope. A coverslip was mounted on a microscope slide containing fixed cells with ProLong Antifade Kit (Molecular Probes, Eugene, OR) as the mounting medium. Cell images were collected by using a 60 × oil immersion objective, and no postacquisition enhancement of images were performed. Image acquisition used the BHS block of filters (excitation 488 nm and emission through a 522 nm 32 band-pass filter). Fluorescence images were scaled to 256 Gy levels.

present study, the uptake and degradation of HA in cellular compartments resulted in the release of fluorescent molecules into the cytoplasm.

In conclusion, a new method was developed to prepare an HA-active ester derivative. This HA-active ester intermediate will find numerous applications in biomedical research, including the preparation of fluorescent probes, drug-polymer conjugates, and cross-linked hydrogels.

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