Optically controlled ligand delivery: 3. Photocleavage of 2-nitrobenzyl bonds at solid–liquid interfaces

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Optically controlled ligand delivery systems would be useful for the design and development of biosensors. We synthesized a *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer containing sidechains terminated in model ligand (Boc–Gly), bound via photocleavable 2-nitrobenzyl bonds. To study photocleavage at solid–liquid interfaces, the HPMA copolymer was covalently attached to 3-aminopropyl triethoxy silane (APS)-coated porous silica beads, which were modified with α,ω-diaminopoly(ethylene oxide) of molecular weight 1000 or 5000. The influence of the poly(ethylene oxide) (PEO) spacer on the rate of photolysis and on the minimization of non-specific adsorption was determined. The results show that the photolytic rates are faster for copolymer-derivatized silicas with PEO spacers compared to that without spacer and that the PEO spacer reduces the non-specific adsorption of HPMA copolymer on the silica surface.

(Keywords: ligand delivery; optical control; 2-nitrobenzyl bonds)

INTRODUCTION

The combination of the specificity of antibody–antigen (Ab–Ag) interactions and sensitivity of competitive binding assays using labelled Ag or labelled Ab can be applied to the development of biosensors[1]. Such fibre-optic, or waveguide-based sensors could be used in a continuous or semicontinuous mode[1]. One type of sensor is based on remotely controlled ligand delivery and total internal reflection fluorescence (t.i.r.f.) sensing[2].

This series of papers focuses on one key part of this sensor research: optical ligand delivery[3,4]. In the first paper HPMA copolymers with ligands (Boc–Gly, fluorescein and tetramethylrhodamine) bound via 2-nitrobenzyl bonds were synthesized and their photocleavage in solution studied[3]. The second paper focused on the synthesis of HPMA copolymer-derivatized silica beads with ligand, Boc–Gly, bound via the α-methylphenacyl group and on the study of their photocleavage, both in solution and at the solid–liquid interface[4].

Silica was used as a solid support because of its optical properties; especially its high optical transmission, low fluorescence and relatively high refractive index. The last of these permits the use of total internal reflection optics as a means to excite fluorescence on the solution side of the solid–liquid interface without exciting bulk fluorescence in the solution phase[5,6].

In this paper we present a HPMA copolymer containing sidechains terminated in model ligand (Boc–Gly) bound via 2-nitrobenzyl groups. This copolymer was used to study the photocleavage reaction at the solid–liquid interface. Copolymers at the solid (silica beads)–liquid interface were exposed to light, resulting in release of the bound ligand. The dependence of the rate of cleavage on the presence of the PEO spacer was determined.

EXPERIMENTAL

Materials

Porous silica beads, 3-aminopropyl triethoxy silane (APS)-coated, 30–40 mesh, 37.5 nm pore size, were obtained from Fluka. Glutaraldehyde (GLU), 25% solution in water, E.M. Grade, was supplied by Polysciences. Diamines: NH_2–PEO_{1000}–NH_2 and NH_2–PEO_{5000}–NH_2 were a kind gift from Dr S. Nagaoka (Toray Industries, Inc., Kanagawa, Japan).

Synthesis

N-(2-Hydroxypropyl)methacrylamide (HPMA) was prepared as described previously, melting point 69–70°C (reference 7, melting point 69–70°C). 4-[N-(tert-Butoxycarbonyl)glycyloxymethyl]-3-nitrobenzoic acid was prepared by a series of reactions as described previously, melting point 163–164°C (reference 8, melting point 159–160°C).

Copolymer **1** (*Figure 1*) was prepared by radical copolymerization of HPMA and N-(3-aminopropyl)-methacrylamide hydrochloride (mole ratio 85:15) in methanol (12.5 wt% of monomer, 0.6 wt% of AIBN as initiator) as described previously[3,4,9]. The content of monomeric units of N-(3-aminopropyl)methacrylamide was found to be 8.7 mol% by using the ninhydrin method[10]. The weight- and number-average molecular weights of copolymer **8** ($M_w = 51\,000$, $M_w/M_n = 1.6$) were estimated from the g.p.c. analysis on Sepharose 4B/6B[11].

Copolymer **2** (*Figure 2*) was prepared by polymer-analogous reaction of copolymer **1** with 4-[N-(tert-butoxycarbonyl)glycyloxymethyl]-3-nitrobenzoic acid by

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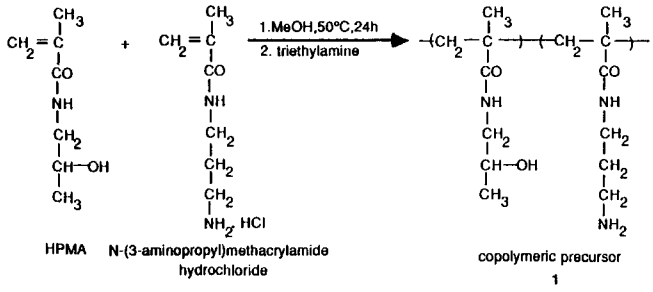

Figure 1 The preparation of copolymer 1

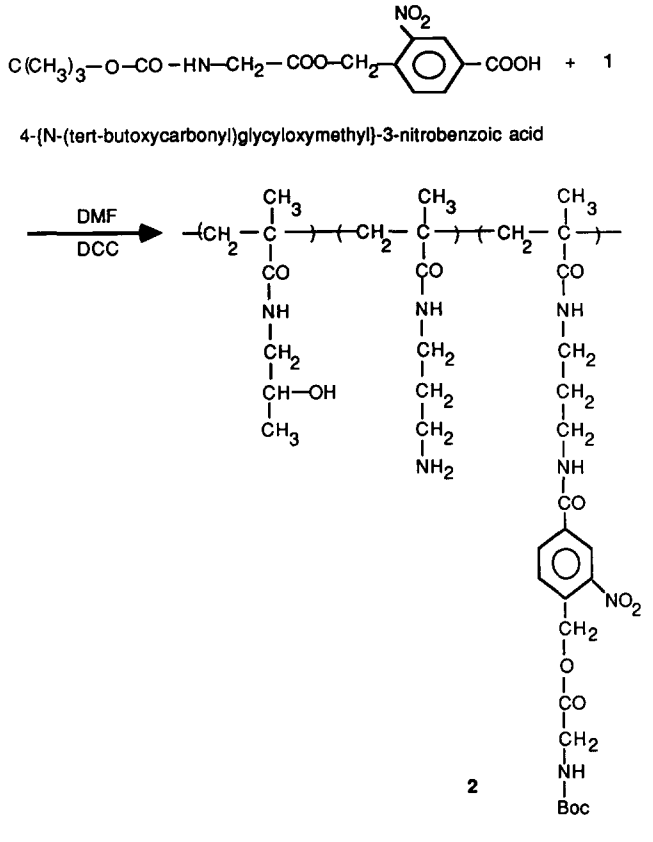

Figure 2 The preparation of copolymer 2

using the dicyclohexylcarbodiimide (DCC) method[3]. The content of remaining sidechains containing free amino groups in copolymer **2** was calculated to be 5.0 mol% by using the ninhydrin method[10]. Consequently, 3.7 mol% of sidechains in copolymer **2** were terminated with Boc–Gly bound via 2-nitrobenzyl groups.

Modification of silica beads

The silica beads were modified according to procedures previously described[4,12] (*Figure 3*). The concentration of amino groups on the modified beads (**3–8**) was determined by using N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP)[13].

Preparation of copolymer-derivatized silica beads:)–APS–GLU(glutaraldehyde)–CH_2–NH–copolymer **2** (**9**),)–APS–GLU–PEO_{1000}–GLU–CH_2–NH–copolymer **2** (**10**) and)–APS–GLU–PEO_{5000}–GLU–CH_2–NH–copolymer **2** (**11**).

Beads **4**, **7** and **8** (250 mg) were treated with excess of copolymer **2** (125 mg) in DMSO. The azomethine bond (–CH=N–) was further reduced by reaction with 50 mg $NaCNBH_3$ in pH 4.5 citrate/phosphate buffer. At the end of reduction, the beads were further soaked in pH 4.5 buffer under shaking. After two days of soaking, the beads were dried and the binding efficiency was quantified by the use of amino-acid analysis.

Non-specific adsorption of copolymer **2** *to silica beads*

To estimate the extent of the non-specific adsorption of copolymer **2**, beads **3**, **5** and **6** were incubated with copolymer **2**, as described above. Beads with physically adsorbed copolymer **2** were numbered **12**, **13** and **14**, respectively.

Quantification of the surface-bound copolymer on silica surface

The amount of surface-bound (by covalent or physical bonds) copolymer **2** on silica beads was quantified by amino-acid analysis. During the hydrolysis procedure, the Boc group of the Boc–Gly bound to the copolymer **2** was removed by acidolysis and the glycine obtained could be quantified by high-pressure liquid chromatography (h.p.l.c.). Hydrolysis was performed according to Scotchler *et al.*[14]. All analyses were performed on a 1084 A Liquid Chromatograph (Hewlett–Packard) by using a Beckman Column No. 235330 (4.6 mm × 15 cm), Model Ultrasphere (5 μm diameter beads).

Photocleavage

Thirty milligrams of copolymer-**2**-derivatized silica beads (**9**, **10** and **11**) and 0.5 ml pH 7.2 PBS buffer or 0.5 ml pH 4.5 citrate/phosphate buffer were poured into

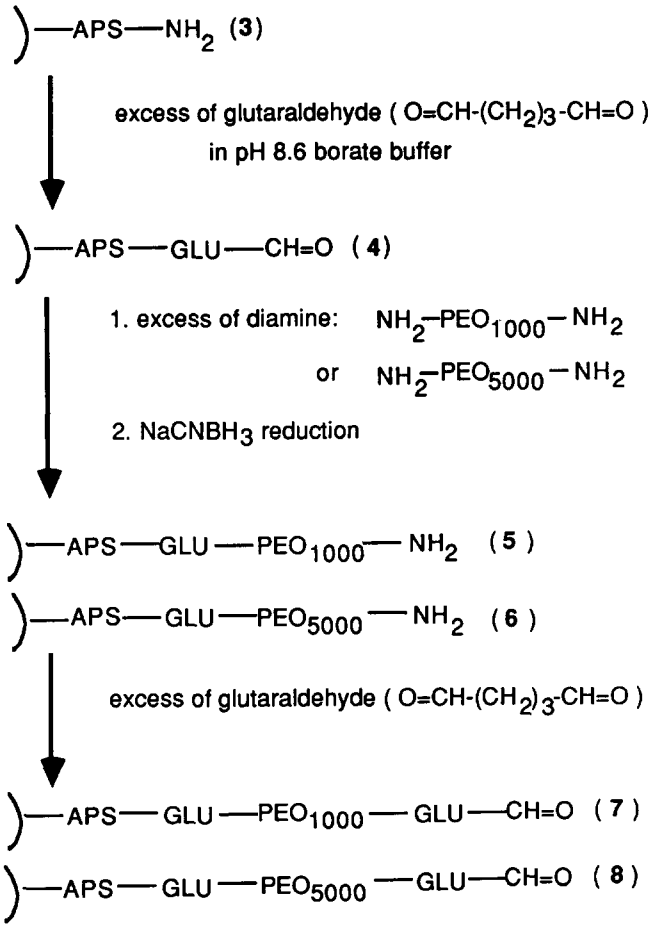

Figure 3 Modification of porous silica beads **3–8**

Table 1 The properties of the modified silica beads **3–8**

Silica beads[a] No.	Schematic structures	Content of amino groups (NH_2) (nmol mg^{-1})	Specific surface area ($m^2\ g^{-1}$)
3	)–APS–NH_2	14	33.4
4	)–APS–GLU–CH=O	0	–
5	)–APS–GLU–PEO_{1000}–NH_2	9	29.4
6	)–APS–GLU–PEO_{5000}–NH_2	10	31.0
7	)–APS–GLU–PEO_{1000}–GLU–CH=O	1	–
8	)–APS–GLU–PEO_{5000}–GLU–CH=O	2	30.6

[a]For experimental details see reference 4. Reaction with glutaraldehyde was performed at pH 8.6

a u.v. quartz cylindrical cell with Teflon stopper. The cell was attached to a rotator (Clas-Col Co.) and a speed of 60 rev min^{-1} was used, so that during irradiation a centrifugal force gently agitated the beads to ensure even light access. The cell was irradiated with an LH 150 mercury lamp (200 W, Schoeffel Instr. Co). The light was first focused by a fused silica lens, then reflected by a dichroic-coating mirror (Oriel Corp.), which reflected only the wavelengths between 260 nm and 340 nm (the major intense emission peaks are 296 nm and 312 nm). At chosen time intervals, the supernatant was withdrawn and the beads were washed with water, ethanol, ether and dried. Then the beads were analysed by using amino-acid analysis. The decrease of the amount of surface-bound glycine after irradiation reflected the amount of photo-released ligand. Controls were kept in the dark.

RESULTS

Synthesis

To prepare the copolymer suitable for ligand delivery system, a HPMA and *N*-(3-aminopropyl)methacrylamide hydrochloride copolymer precursor **1** (*Figure 1*) was synthesized. Copolymer **2** (*Figure 2*), containing side-chains with 2-nitrobenzyl bonds, was prepared by reacting copolymer **1** with 4-[N-(tert-butoxycarbonyl)-glycyloxymethyl]-3-nitrobenzoic acid using the DCC method. The remaining free amino groups of copolymer **2** could be further reacted with the functionalized silica surface, i.e. silica beads with terminal aldehyde groups.

Modification of silica

The modification of porous silica beads is shown schematically in *Figure 3*, and their properties are given in *Table 1*[4,12]. The APS-coated beads **3** contained 14 nmol mg^{-1} of amino groups. After reaction with glutaraldehyde, practically all amino groups were converted to aldehyde groups (beads **4**). The latter were modified with PEO diamines. The resulting beads **5** and **6** contained 9 and 10 nmol mg^{-1} of terminal amino groups, respectively. Owing to the incomplete reaction of beads **5** and **6** with glutaraldehyde, beads **7** and **8** still contained less than 2 nmol mg^{-1} of unreacted amino groups on surfaces (*Table 1*). The surface areas of the beads did not show any dramatic change during these polymer-analogous reactions (*Table 1*).

Copolymer-derivatized silica beads

Silica beads **9**, **10** and **11** were synthesized by reacting copolymer **2** with silica beads **4**, **7** and **8**, respectively. The amounts of copolymer **2** on silica beads **9**, **10** and **11** were quantified by hydrolysing the beads with propionic/hydrochloric acid (50:50, v/v) for 1 h, followed by amino-acid analysis. The amounts of Boc–Gly bound on silica **9**, **10** and **11** are shown in *Table 2*. To estimate the non-specific adsorption of copolymer on the silica surface, the amounts of physically adsorbed copolymer **2** on silica beads **3**, **5** and **6** were included for comparison. The amounts of non-specific adsorption of HPMA copolymer were very limited. The results suggest that the amounts of covalently bound and of adsorbed HPMA copolymer decreased as the length (molecular weight) of the PEO spacer increased.

Table 2 The amounts of Boc–Gly bound on modified silica beads **9–14**

Beads No.	Amount of bound Boc–Gly: nmol per 50 mg of beads	Amount of bound Boc–Gly: nmol per m^2[a]
9[b]	267	178
10[b]	164	109
11[b]	128	85
12[c]	16	11
13[c]	10	7
14[c]	6	4

[a]The specific surface area of 30 $m^2\ g^{-1}$ of beads was used as a basis for calculation
[b]Covalent attachment to beads **4**, **7** and **8**, respectively
[c]Non-specific adsorption on beads **3**, **5** and **6**, respectively

Photocleavage

The rates of photocleavage of copolymer-derivatized silica beads **9**, **10** and **11** in pH 4.5 citrate/phosphate buffer are shown in *Figure 4*. In all three silica beads the patterns of photocleavage were similar. The half-lives of photolysis of **9**, **10** and **11** were approximately 2, 1 and 1 h, respectively. The rates of photolysis are similar for both beads with PEO_{1000} and PEO_{5000} spacers and faster than those of beads without PEO spacer. Photolysis for all three bead systems proceeded rapidly in the first hour, with more than 35% of ligand released.

The photolytic yield after 5 h irradiation for silica beads **9**, **10** and **11** in pH 7.2 PBS buffer were 78%, 87% and 85%, respectively (results not shown). These results were almost the same as the data obtained in pH 4.5 citrate/phosphate buffer (77%, 85% and 86% for silica **9**, **10** and **11**, respectively).

DISCUSSION

Immunoassays for clinical medicine often use the competitive binding principle[15], which requires the availability

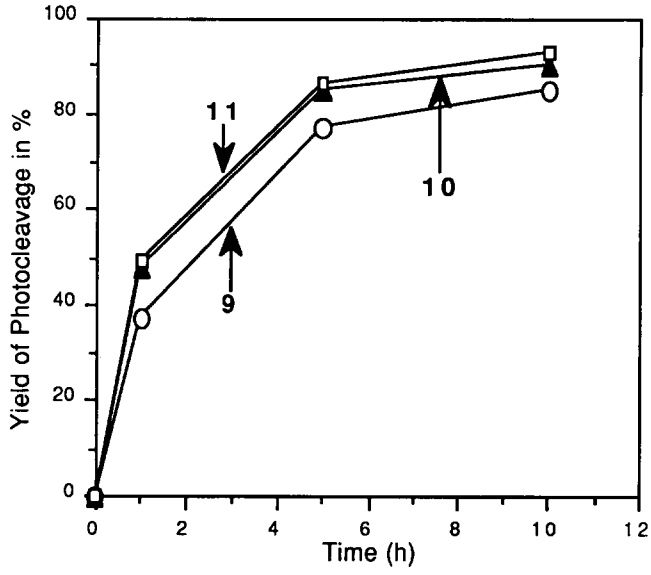

Figure 4 Photocleavage of copolymer-2-derivatized silica beads **9**, **10** and **11** in pH 4.5 citrate/phosphate buffer

of a labelled competing reagent with identical binding properties to the analyte. As our approach to remote sensing is optical, we have chosen to develop an on-demand, optically based, reagent delivery technology. Basically the ligand, e.g. fluorescein-labelled or radioisotope-labelled antigen, is coupled via a photolabile bond to a polymer matrix. Exposure to light of the proper intensity and wavelength results in bond breakage, providing released ligand, which then competes with circulating ligand for the antibody binding sites on the sensor surface[2]. In this way, an immunosensor that is remote and continuous can be developed[16].

This study focuses on means of releasing a model ligand (Boc–Gly) at the solid–liquid interface under optical control. Boc–Gly was attached to a surface-bound polymeric carrier via 2-nitrobenzyl bonds.

Type of bond between the carrier and ligand

The 2-nitrobenzyl group[17] is widely used in polymer-based peptide synthesis, both in solid[18] and in liquid phases[19–21], and in light-flash physiology[22–24] (the application of the light-flash technique to photosensitive molecules to study how intracellular messengers act). During irradiation of aromatic nitro compounds, which have a C–H bond in the *ortho*-position, the nitro group is reduced to a nitroso group[17] and an oxygen is inserted into the C–H bond at the *ortho*-position, followed by rearrangement to a more stable structure.

Copolymer-derivatized silica beads

PEO spacers were chosen for this study because PEO has a unique protein-resistant property at solid–liquid interfaces, probably due to its low interfacial free energy with water, unique solution properties and molecular conformation in aqueous solution, hydrophilicity, high surface mobility and steric stabilization effects[12,25–27]. The results (*Table 2*) demonstrate that the PEO spacer reduces the non-specific adsorption of HPMA copolymer **2** on modified silica surfaces. The degree of non-specific adsorption of HPMA copolymer on silica surfaces may be dependent on several factors, e.g. on the density of PEO chains on the surface and on their molecular weight. Because of the similar specific surface areas of beads studied (*Table 1*), similar content of amino groups at the surface of silica beads **5** and **6** (*Table 1*), and the low possibility for loop formation during PEO attachment (under experimental conditions used)[4,12], the packing density of PEO chains on surfaces of beads **5** and **6** should be similar. Consequently, the decrease of non-specific adsorption of copolymer **2** with increasing length of PEO spacers, reflects the molecular weight influence. The amount of covalently bound copolymer **2** on the silica beads also decreased as the length of PEO spacer increased. This is probably due to the lower concentration of free aldehyde groups on silica beads **7** and **8** than on silica beads **4**.

Photocleavage at the solid–liquid interface

At the solid–liquid interface the photorelease of Boc–Gly from beads **9**, **10** and **11** was evaluated in pH 7.2 PBS buffer and pH 4.5 citrate/phosphate buffer (*Figure 4*). The photolytic rates in pH 4.5 and pH 7.2 buffers were almost the same (5 h irradiation). The rates of photolysis for **10** and **11** (with PEO spacer) were similar, but were distinctly higher compared with **9** (without PEO spacer). These results are consistent with our previous data using photocleavable α-methylphenacyl bonds[4]. The differences in photolytic rates could be due to the change of microenvironment at the solid–liquid interface, resulting in a change in quantum yield of this photolytic reaction[4]. One possible explanation is related to the flexibility of the PEO spacer[28,29]. The higher mobility of the PEO spacer might make the surface-bound polymer more flexible. Under the photolytic condition, i.e. a non-polarized light source, the more mobile polymer would have a higher efficiency of light absorption and result in a higher photolytic rate. The short interval of fast and nearly linear ligand release (the first hour of irradiation) would be most suitable for ligand delivery applications.

Several factors should be optimized for the development of an optically controlled ligand delivery system: the amount of photocleavable ligand chemically bound on the surface; minimization of non-specific adsorption; and the rate of photolysis[4]. For silica beads **9**, the amount of ligand loaded on the silica surface was the largest, yet the rate of photolysis was lowest and the ability to prevent non-specific adsorption was poorest. On the other hand, although silica **11** showed the highest rate of photolysis and good capacity for preventing non-specific adsorption, the amount of ligand chemically bound on the surface was the lowest.

CONCLUSIONS

The amino-acid analysis method was successfully applied to quantify surface-bound amino-acid ligand and the rate of photolysis at the solid–liquid interface. This method can be potentially extended as a general analytical tool to monitor the photocleavage patterns for peptide ligands.

The amount of covalently bound HPMA copolymer or ligand, i.e. Boc–Gly, on the silica surface decreases as the length of PEO spacer increases.

The PEO spacer reduces the non-specific adsorption of HPMA copolymer on the modified silica surface.

The photolytic rates of 2-nitrobenzyl bonds are faster

for copolymer-derivatized silicas with PEO spacers than for those without spacers.

It appears that the amount of photocleavable ligand chemically bound on the surface, minimization of non-specific adsorption and the photolytic rate are three major factors to be optimized before developing a ligand-delivery-system-based biosensor.

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