# Synthesis of Diethylenetriaminepentaacetic Acid Conjugated Inulin and Utility for Cellular Uptake of Liposomes

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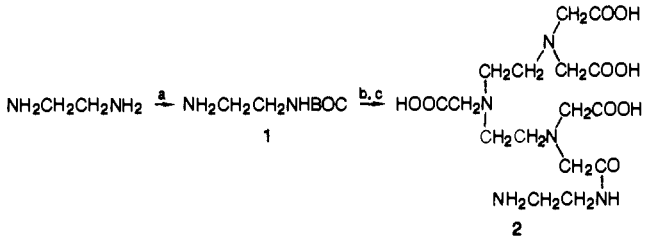
The synthesis, binding of radioactive cations, liposomal encapsulation, and biodistribution of the oxidized-inulin reaction product with ethylenediamine and diethylenetriaminepentaacetic acid (4) are described. The four-step synthesis of the inulin derivative proceeded in a good overall yield of 72%. The complex of the inulin derivative with either $^{67}Ga^{3+}$ or $^{111}In^{3+}$ was stable in vivo and did not readily distribute into tissues, being excreted primarily in urine after intravenous administration to mice. The liposome-entrapped inulin derivative can be loaded with radioactive heavy metal cations by mobile ionophores in high radiochemical yields of 80–91%. Following the intravenous administration of the liposomal encapsulation of the indium-111-labeled inulin derivative, the entrapped compound had a biodistribution characteristic of liposomes and allowed an estimation of the extent of the intracellular uptake of liposomes. The ability of the inulin derivative to chelate many different types of metals will allow the use of this probe for studying subtle differences in tissue distribution resulting from different drug targeting or delivery protocols in the same animal by multiple labeling techniques. Moreover, the chelate-conjugated inulin permits studies of the applications of drug delivery systems in primates or human subjects by noninvasive techniques such as γ-scintigraphic or nuclear magnetic resonance imaging methods.

The applications of inulin, a polysaccharide (5000 daltons), as a cytologic, macromolecular marker are well documented. Because of the macromolecular nature and the lack of specificity in binding with most cells, inulin can serve as an inert extracellular marker for studying the processes of receptor-mediated, endocytotic uptake or diffusion-controlled uptake by cells. Once inulin becomes internalized, inulin is a useful intracellular marker for quantitating cellular uptake, because it can neither diffuse easily out of a cell nor be degraded readily by intracellular enzymes. Consequently, inulin has been used as a reliable marker for studying the intracellular uptake of drug delivery systems, such as liposomes, in vitro and in vivo.[1,2] In addition, inulin is a nondiffusable marker for studying the processes of transport of drugs or drug carriers through diffusional barriers of layers of cells, such as the epithelial barrier in the skin[3] and stratum corneal,[4] and the endothelial layers of the blood–brain barrier.[5]

Invariably, in almost all the cytologic or biomedical studies, inulin labeled with either $^{3}H$ or $^{14}C$ has been employed. Because both $^{3}H$ and $^{14}C$ emit low-energy β-radiation, the detection of inulin requires elaborate and cumbersome procedures to solubilize the isolated specimen of tissues or cells for subsequent counting. It was not until recently that the synthesis of a γ-emitting $^{125}I$-labeled inulin has been developed.[6]

However, in many studies dealing with the transport, delivery, or targeting of drug delivery systems or drugs, experiments comparing the subtle differences between two or more delivery systems or drug formulations are often complicated by the variations among testing animals or human subjects. Ideally, an explicit demonstration of a subtle difference between two or more delivery systems may best be carried out in the same animal or human subject, with dual or multiple labeling techniques. The present study describes the synthesis and the potential applications of a versatile diethylenetriaminepentaacetic acid (DTPA) derivative of inulin, with liposomes as a model of a drug-delivery system.

**Scheme I**[a]

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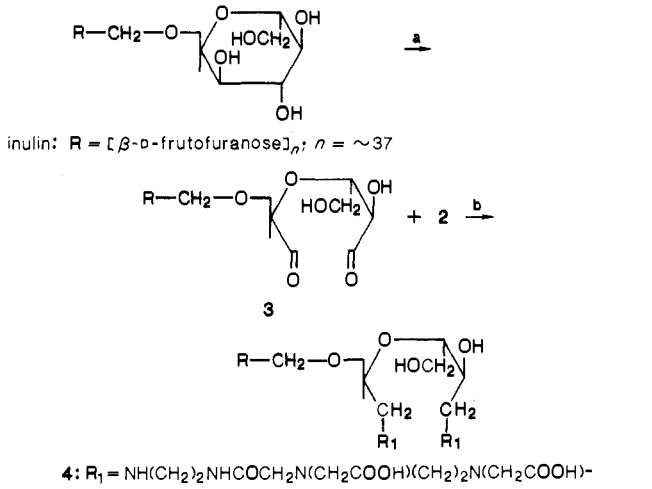
[a] Reagents and conditions: (a) BOCON, $ET_3N$, $CHCl_3$; (b) DTPA anhydride, $CHCl_3$, reflux; (c) $CF_3COOH$, 25 °C.

**Scheme II**[a]




[a] Reagent and conditions: (a) $NaIO_4$, $H_2O$, 4 °C; (b) $NaBH_3CN$, $H_2O$, 25 °C.

## Results

**Chemistry.** The oxidized-inulin reaction product with 1,2-ethylenediamine and diethylenetriaminepentaacetic acid (4) utilized in this study was prepared by the general methods outlined in Schemes I and II. All solvents used in the sequence of reactions were of reagent grade, and buffers also were made from deionized water to maintain

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**Table I.** Biodistribution of $^{111}$In-Labeled 4 and $^{67}$Ga-Labeled 4 in Mice at Various Times Postinjection[a]

| tissue | $^{111}$In-labeled 4 ($n$ = 4) | | | $^{67}$Ga-labeled 4 ($n$ = 4) | | |
|---|---|---|---|---|---|---|
| | 0.17 h | 1 h | 21 h | 0.17 h | 1 h | 21 h |
| blood | 21.1 ± 0.1 | 1.1 ± 0.4 | 1.0 ± 0.3 | 20.9 ± 3.8 | 4.5 ± 1.0 | 0.7 ± 0.3 |
| liver | 0.6 ± 0.0 | 0.3 ± 0.1 | 0.4 ± 0.0 | 1.3 ± 0.1 | 1.4 ± 0.0 | 2.1 ± 0.5 |
| kidneys | 24.4 ± 2.7 | 1.9 ± 0.3 | 0.8 ± 0.1 | 4.4 ± 1.6 | 1.7 ± 0.1 | 0.9 ± 0.0 |
| spleen | 0.2 ± 0.1 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.1 | 0.0 ± 0.0 | 0.1 ± 0.0 |
| intestine | 2.2 ± 0.1 | 0.8 ± 0.4 | 2.2 ± 1.8 | 2.4 ± 0.0 | 1.4 ± 0.4 | 5.8 ± 1.4 |
| skin | 15.6 ± 2.2 | 5.8 ± 0.8 | 21.0 ± 0.2 | 15.1 ± 2.4 | 7.7 ± 0.7 | 1.4 ± 0.3 |
| tail | 3.8 ± 0.5 | 2.0 ± 1.0 | 2.1 ± 0.6 | 3.7 ± 0.4 | 2.0 ± 0.9 | 2.3 ± 0.6 |
| leg muscle | 3.2 ± 0.5 | 0.8 ± 0.2 | 0.2 ± 0.0 | 3.1 ± 0.4 | 1.3 ± 0.4 | 0.3 ± 0.2 |
| carcass | 14.2 ± 0.2 | 4.1 ± 0.5 | 0.8 ± 0.1 | 19.3 ± 0.8 | 9.1 ± 0.4 | 3.0 ± 0.2 |
| excretion | 3.6 ± 2.5 | 50.7 ± 4.1 | 77.1 ± 2.7 | 3.2 ± 2.9 | 50.0 ± 5.2 | 69.5 ± 5.3 |

[a] Values are percentages of injected dose ± standard deviation.

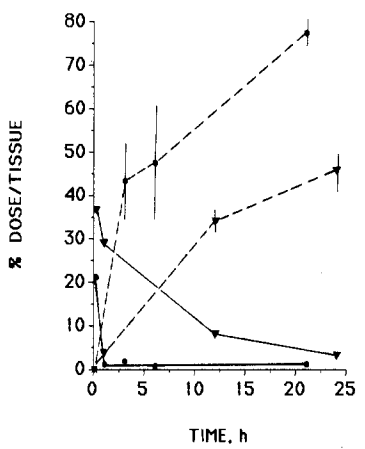


**Figure 1.** Blood clearance (—) and urine excretion (- -) of $^{111}$In-labeled 4 (●) and $^{111}In^{3+}$-NTA (▼) from BALB/c mice. A volume of 50–60 μL of phosphate saline buffer containing either of the indium-111 samples was injected into the mouse via the tail vein. Results are expressed as the percentage of the total injected dose ± standard deviation ($n$ = 3–4).

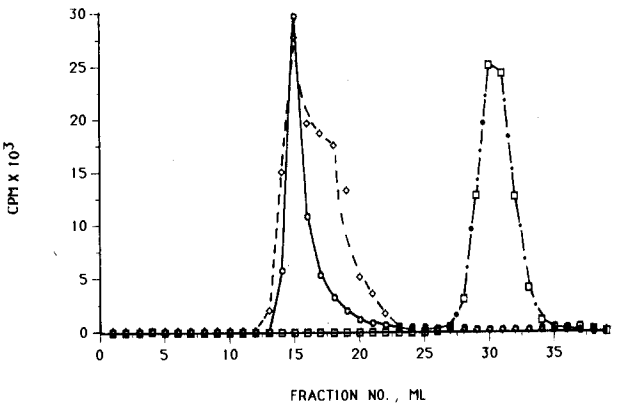


**Figure 2.** Profiles of $^{111}In^{3+}$-labeled 4 (□) and SM/CH (2:1, mol/mol) MLV-entrapped, $^{111}In^{3+}$-labeled 4 (○) in a Sepharose 6B column. For the purpose of comparison, the concentration of liposome (◇) as measured by the fluorescence intensity of diphenylhexatriene has been scaled up 500 times.

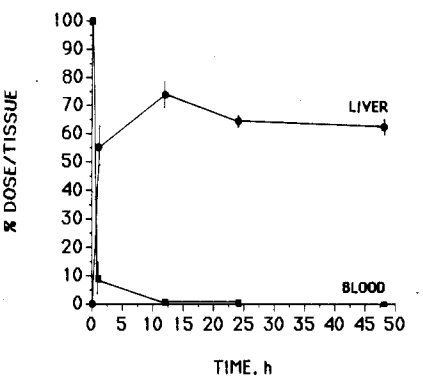


**Figure 3.** Blood clearance (■) and liver uptake (●) of SM/CH (2:1, mol/mol) MLV-entrapped, $^{111}$In-labeled 4 in mice after intravenous injection. The injected dose was 1–2 μg of phospholipid/g body weight. Results are expressed as the percentage of total injected dose ± standard deviation ($n$ = 4–5).

metal-free conditions. The synthesis of **2** as outlined in scheme I was achieved in a 72% yield by a coupling reaction between the N-protected ethylenediamine and DTPA anhydride.[7] The N-protected ethylenediamine was previously prepared in a 90% yield using a *tert*-butoxycarbonyl (Boc) protecting group and ethylenediamine according to the method of Itoh et al.[8] Scheme II delineates the preparation of the compound **4** in a 72% yield from the condensation of excess molar ratio of **2** with inulin dialdehyde **3** followed by $NaBH_3CN$ reduction of the imine bond. Compound **3** was previously obtained by oxidation of inulin at 4 °C by use of sodium meta periodate. Periodate consumption was assayed by the arsenite method, indicating approximately two oxidations per inulin molecule.[9]

**Biological Studies.** As presented in Figure 1, the clearance of $^{111}$In-labeled 4 from blood is rapid, as only 1% remains after 1 h with a liver to blood ratio of 0.27 and remains at approximately this level up to 21 h. The injected $^{111}$In-labeled 4 is subsequently recovered in the urine, where 70% or more of the injected dose appears within 21 h. This indicates that the free $^{111}$In-labeled 4 is rapidly cleared from blood circulation and excreted. In contrast, the effect of protein binding of $^{111}In^{3+}$ not only prolonged the resident time of indium-111 in the circulation but also slowed down the rate of excretion of indium-111, when the indium-111 as $^{111}In^{3+}$–NTA was injected intravenously to mice. The lack of uptake of the indium-111- or gallium-67-labeled 4 by tissues is evident in Table I, indicating that a very insignificant amount of metal cations that have been bound with 4 are dissociated in vivo.

Figure 2 shows that the radioactivity of $^{111}In^{3+}$-labeled 4 alone moves as a single peak (from fractions 27–35) in Sepharose 6B gel filtration chromatography. On the other hand, the liposome-encapsulated 4 moves along with the multilamellar liposomes, having a peak in the void volume (fractions 13–15) of the column. Following the administration of the indium-111-labeled 4 entrapped in bovine brain sphingomyelin (SM)/cholesterol (CH) (2:1, mol/mol) multilamellar vesicles (MLV), a very rapid blood clearance and the concomitant liver uptake of the MLV-entrapped 4 are evident (Figure 3). There is a decline in liver levels

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Table II. Time Course of the Biodistribution of SM/CH (2:1, mol/mol) MLV-Entrapping $^{111}$In-Labeled 4 in BALB/c Mice[a]

| tissue | 1 h ($n$ = 3) | 12 h ($n$ = 4) | 24 h ($n$ = 2) | 48 h ($n$ = 5) |
|---|---|---|---|---|
| blood | 9.1 ± 6.3 | 0.5 ± 0.0 | 0.4 ± 0.7 | 0.4 ± 0.0 |
| liver | 55.1 ± 6.6 | 73.6 ± 4.8 | 64.2 ± 1.9 | 62.4 ± 2.3 |
| kidneys | 0.5 ± 0.3 | 0.4 ± 0.0 | 0.5 ± 0.2 | 0.4 ± 0.0 |
| spleen | 3.9 ± 0.4 | 4.0 ± 0.6 | 1.9 ± 1.9 | 2.9 ± 0.8 |
| heart | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| lung | 0.4 ± 0.1 | 0.1 ± 0.0 | 0.4 ± 0.3 | 0.2 ± 0.2 |
| intestine | 0.7 ± 0.2 | 1.0 ± 0.3 | 0.8 ± 0.3 | 0.6 ± 0.2 |
| fat | 0.3 ± 0.2 | 0.1 ± 0.0 | 0.4 ± 0.2 | 0.1 ± 0.1 |
| skin | 1.6 ± 0.6 | 1.1 ± 0.2 | 2.0 ± 1.0 | 2.8 ± 1.8 |
| tail | 3.7 ± 0.3 | 2.7 ± 0.7 | 0.9 ± 0.5 | 2.8 ± 1.7 |
| leg muscle | 0.3 ± 0.0 | 0.4 ± 0.2 | 0.2 ± 0.1 | 0.3 ± 0.1 |
| carcass | 4.0 ± 1.0 | 2.0 ± 0.3 | 2.1 ± 0.3 | 1.3 ± 0.1 |
| brain | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.2 ± 0.2 | 0.2 ± 0.0 |
| stomach | 0.8 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.0 ± 0.0 |
| excretion | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |

[a] Values are percentages of injected dose ± standard deviation. The injected dose was 1–2 μg of phospholipid/g body weight.

during the initial time period. This phenomenon was also observed when MLV-entrapped $^3$H- or $^{14}$C-labeled inulin was administrated.[10] It appears that after the initial rapid uptake by the liver, the amount of MLV-entrapped 4 in the liver remained relatively constant over a period of 48 h. In addition, some of the injected radioactivity of the MLV-entrapped indium-111-labeled 4 was found in the spleen and other tissues (Table II).

## Discussion

The four-step synthesis of the water-soluble DTPA-conjugated inulin proceeded in a good overall yield of 72%. The radiolabeling of liposome-entrapped 4 also proceeded in high radiochemical yields of greater than 85%. The liposome-entrapped 4 can be loaded with not only $^{111}In^{3+}$ but also $^{67}Ga^{3+}$. It is significant that not only do the radioactive cations remain tightly bound with 4 after intravenous injection, but also the $^{111}In^{3+}$ bound with MLV-entrapped 4 does not redistribute to other tissues after being taken up by the liver for at least 48 h. The high binding constant of DTPA with many heavy metal cations[11] and the stability of the complex of 4 with $^{111}In^{3+}$ and $^{67}Ga^{3+}$ in vivo make 4 a versatile marker for many applications in studying transport and delivery of drugs or drug delivery systems. It is equally significant to note the versatility of 4 in that it may be labeled by other metals for imaging by techniques of the nuclear magnetic resonance (NMR) imaging.

Two different patterns of organ distribution and of tissue residence time for radiolabeled free and MLV-entrapped 4 are revealed. This demonstrates the alteration of the tissue uptake of inulin as a result of the entrapment with the liposomal drug delivery system. Because of the macromolecular size and the hydrophilic nature of inulin and the fact that inulin is not metabolized to any significant extent, the tissue distribution allows for the estimation of the intracellular delivery of drugs by the liposomal drug delivery system. The rapid clearance from the blood, the lack of uptake by tissues of the free radiolabeled 4, and the ability to chelate many different types of metals allows, the use of this probe in studying subtle differences in tissue distribution resulting from different drug targeting or delivery protocols in the same animal by the multiple-labeling technique. Moreover, the chelate-conjugated inulin permits studies of the applications of drug delivery systems in primates or human subjects by noninvasive techniques such as γ-scintigraphic or nuclear magnetic resonance imaging methods.

## Experimental Section

**Chemicals.** Inulin and $NaBH_3CN$ were a obtained from Sigma. Ethylenediamine was from Aldrich. Periodic acid was purchased from MCB. Sephadex G 25 and G 50 and Sepharose 4B(CL) and 6B were from Pharmacia, and AGI-X8 was from Bio-Rad. Indium-111 chloride and Galium-67 citrate were supplied by Medi+Physics, Richmond, CA. The purification of $^{111}In^{3+}$ and $^{67}Ga^{3+}$ and the preparation of AGI-X8 (phosphate form) were carried out as described previously.[18] Trifluoroacetic acid and 2-[[(*tert*-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile (BOC-ON) were from Pierce Chemical Co. All phospholipids were from Avanti Polar Lipids, Inc. Other chemicals were of analytical grade.

**Chemical Procedures.** Melting points were determined in open capillary tubes with a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analysis were performed by MicAnal Organic Microanalysis Laboratory, Tuscon, AZ, and were within ±0.4% of the theoretical values. $^1$H NMR spectra were recorded at ambient temperature on a JEOL instrument with $Me_4Si$ as internal standard in chloroform or with NaOD in $D_2O$. Mass spectra were obtained on Hewlett-Packard 5985 A quadrupole mass spectrometer (Hewlett-Packard, Palo Alto, CA), coupled to gas chromatograph via an all-gas jet separator. All TLC data were obtained on Analtech silica gel chromatography plates, and the spots were routinely detected by ninhydrin spray. The eluents used were solvent A, $CH_2Cl_2$, and solvent B, $CH_2Cl_2$–$CH_3OH$–$NH_4OH$, 17:4:2 mL. Column chromatography was carried out with silica gel (60–200 mesh) from J. T. Baker. Spectrapor tubing (*mw* cutoff 3500) was used for all dialysis procedures.

**[*N*-(*tert*-Butoxycarbonyl)amino]ethyleneamine (1).** A solution of BOCON (4.9 g, 0.02 mol) in 10 mL of $CHCl_3$ was added by drops from a dropping funnel to a stirred solution of ethylenediamine (7.2 g, 0.12 mol) and triethylamine (4.18 mL, 0.03 mol) in 20 mL of $CHCl_3$ at room temperature. The mixture was allowed to stir for 4 h at room temperature. TLC analysis (solvent A) revealed two spots that were ninhydrin positive. The solution was filtered to remove the precipitate formed. The solvent was evaporated on the rotovap (25 mmHg, 50–60 °C), and the reaction mixture was further dried in vacuo (1 mmHg, 12 h). The mixture was dissolved in 1.5 mL of $CH_3OH$ and applied to a short silica gel column (12 × 7 cm) that had been equilibrated with $CH_2Cl_2$. The column was eluted with solvent A (600 mL) to remove any bis(*tert*-butoxycarbonyl)ethylenediamine by product. The

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(*tert*-butoxycarbonyl)ethylenediamine product was eluted by using solvent B and collected in 8.5-mL fractions. The desired fractions were traced by ninhydrin-positive spray reactions. The fractions containing the product were combined and evaporated to dryness. It was then dissolved in a minimum amount of hot 2-propanol, cooled to room temperature, and then left to crystallize overnight to yield the desired product **1** (2.87 g, 90%): mp 129–130 °C; $R_f$ 0.59 (solvent A); NMR ($CDCl_3$) $\delta$ 5.75 (2 H, s, $NH_2$), 3.35 (4 H, m, $CH_2CH_2$), 1.59 (9 H, s, $(CH_3)_3CO$); CIMS, *m/e* 160 [$M^+$].

**[(Carboxymethyl)iminobis(ethylenenitrilo)]tetraacetic Acid Mono(2-aminoethylamide) (2).** A solution of compound **1** (20 mL, 0.009 mol) was added by drops to diethylenetriaminepentaacetic acid anhydride (DTPA anhydride)[7] (9.6 g, 0.0027 mol) in 80 mL of anhydrous $CHCl_3$, and the mixture was refluxed for 24 h to an almost clear solution. It was then filtered by use of Whatman filter paper no. 1, and the filtrate was concentrated in vacuo. The reaction mixture was then taken up in 50 mL of deionized water, refluxed for 2 h to hydrolyze the anhydride, and then freeze-dried. The dried mixture was treated without further purification with $CF_3COOH$ (5.0 mL, 0.065 mol) for a reaction time of 3 h. The reaction mixture was quenched with 10 mL of deionized water and neutralized to pH 7.0 with 1 N NaOH. The freeze-dried mixture (2.8 g/run) was dissolved in 0.3 N NaOH and applied to an AG1-X8 (formate form) anion-exchange column (2.5 × 45 cm), which had been equilibrated with and washed by deionized water. The column was eluted initially by 260 mL of $H_2O$ and then by a linear gradient of 500 mL of $H_2O$ and 500 mL of 5 M formic acid at a flow rate of 3.0 mL/min, collecting 8.5 mL/fraction. Two ninhydrin-positive peaks were identified. The product recovered from the second ninhydrin-positive peak (fractions 121–150) was pooled and freeze-dried. It was then further purified by crystallization from 10 mL of $H_2O$ at pH 3.0 (adjusted with concentrated formic acid) in a yield of 72% of **2** formate: mp 230 °C dec; $^1H$ NMR ($NaOD/D_2O$) 4.21 (8 H, s, $NCH_2COOH$), 3.75 (2 H, s, $CH_2C{=}ON$), 3.65 (8 H, t, $(NCH_2CH_2N)_2$), 3.40 (4 H, t, $CH_2CH_2NH^+$); CIMS, *m/e* M + $NH_3$ [447]. Anal. ($C_{19}H_{35}N_5O_{15}$) C, H.

**Synthesis of Oxidized-Inulin Reaction Product with Ethylenediamine and Diethylenetriaminepentaacetic Acid (4).** Inulin (1.0 g) was dissolved in 90 mL of deionized $H_2O$ and cooled to 4 °C, 10 mL of (fresh) 0.1 M sodium metaperiodate was added, and the solution was incubated at 4 °C for 20 min in the dark. Periodate consumption was assayed by the arsenite method, indicating approximately two oxidations per inulin molecule.[9] The oxidation was terminated by the addition of an excess of ethylene glycol, and the solution was dialyzed at 4 °C against 5 mM citrate buffer, pH 5.0, and then deionized $H_2O$ repeated five times. To this dialyzed solution was added compound **2** (1.0 g, 0.002 mol) dissolved in 0.2 N NaOH. The solution was stirred at pH 8.1 for 1 h. To this solution was added $NaBH_3CN$ (0.11 g, 0.002 mol), and the pH was adjusted to 5.4. The solution was stirred for 16 h at room temperature. It was degassed under reduced pressure and then dialyzed twice against 20 mL of 5 mM citrate buffer, pH 5.0, and seven times against 20 mL of deionized water. The product was recovered by lyophilization. It was then further purified by gel filtration on a Sephadex G-25 column (2.0 × 80 cm) at a flow rate of 0.2 mL/min, collecting 3 mL/fraction. The eluent was monitored by UV with the wavelength setting at 220 nm. The chelate-coupled sugar fractions were recovered at fractions 11–33, while the unreacted **2** appeared at fractions 65–78. The combined sugar fractions were lyophilized, giving an 84% (1.19 g) yield. The product was further purified by a cellulose CM-52 cation-exchange column (0.9 × 67 cm) at a flow rate of 0.2 mL/min (1 mL/fraction), eluting by a linear salt gradient of 400 mL of 0.01 M NaCl, 0.04 M NaAc, pH 4.0, and 400 mL of 0.1 M NaCl, 0.04 M NaAc, pH 4.0. The linear gradient was established by a Harvard peristaltic pump and gradient mixer (Bethesda Research Laboratories). Aliquots of the chelate-coupled sugar fractions were identified by the anthrone reagent assay.[12] The product eluted at fraction 61 and was further confirmed by chelating $^{111}In^{3+}$ and eluting the mixture through a Sephadex G-25 column. Calibration of the CM-52 column with oxidized inulin showed it to peak at fraction 25. The DTPA-conjugated inulin was thus separated from the unreacted inulin. The fractions of DTPA-conjugated inulin were pooled, lyophilized, and stored at −20 °C.

**Metal Binding.** The tight binding of **4** with $^{111}In^{3+}$ or $^{67}Ga^{3+}$ was ascertained by quantitating the complex of **4** with $^{111}In^{3+}$ (or $^{67}Ga^{3+}$) eluted in the void volume from a Sephadex G-25 column (0.8 × 70 cm) after incubating **4** with $^{111}In^{3+}$–nitrilotriacetic acid (NTA) at room temperature for 15 min prior to passing through the Sephadex G-25 column. The radioactivity of $^{111}In^{3+}$ or $^{67}Ga^{3+}$ in each fraction was monitored by $\gamma$-counting, and the concentration of **4** was estimated by phenol–sulfuric acid assay.[12]

**Liposome Preparation.** Multilamellar vesicles (MLV) were prepared by bath sonication of the dried thin film of lipid mixture or the dried powder of a pure phospholipid in the presence of 0.106 M sodium phosphate buffered isotonic saline, pH 7.4, containing 1 mM of **4**. Sonication was carried out in a Model G112 SPIT bath sonicator (Laboratory Supplies Co.) at 60–80 W in a 50 °C water bath, with use of a glass tube (13 × 100 mm), for 5 min. The suspension was freeze-dried over a 2-h period, reconstituted with 1 mL of deionized water, and resonicated for 5 min by the same procedure mentioned above. The MLV were annealed at 65 °C for 30 min prior to the separation from the unentrapped DTPA-conjugated inulin (or NTA, or both compounds) by passing through a Sepharose 6B column (0.8 × 70 cm) that was equilibrated and eluted with 5 mM sodium phosphate isotonic saline buffer, pH 7.4. The MLV were collected in the void volume. The concentration of liposomes in each fraction was esimated by fluorescence assay with diphenylhexatriene.[13]

Radiolabeling of liposome-entrapped DTPA-conjugated inulin with $^{111}In^{3+}$ was carried out essentially by the same method for externally loading $^{111}In^{3+}$ by acetylacetone to liposome-entrapped chelating molecules described previously.[14,15] The $^{111}In^{3+}$-loaded liposomes were subsequently purified by passage over a column of AG1-X8 (0.8 × 14 cm) that was equilibrated and eluted with isotonic phosphate buffer as reported previously.[14,15] To ensure that the $^{111}In^{3+}$ was indeed chelated by DTPA-conjugated inulin, an unloading procedure of inducing the release of the nonspecifically bound $^{111}In^{3+}$ from liposomes by tropolone, an ionophore, was adopted. Liposomes entrapping $^{111}In^{3+}$-DTPA-conjugated inulin were incubated with 0.106 M sodium phosphate, pH 7.4, in the presence of 100 $\mu$M tropolone and 10 mM NTA at room temperature for 20 min to induce the release of $^{111}In^{3+}$ ions, which are not chelated by DTPA-conjugated inulin. Liposomes were further purified by AG1-X8 ion-exchange chromatography described above.

**Biological Studies.** The biodistribution studies were performed in groups of three or four BALB/c mice (mean body weight about 25 g). Each mouse was injected intravenously through a tail vein with 50–100 $\mu$L of sample solution containing approximately 100 000 cpm radioactivity and housed in separated cages. At time points ranging from 10 min to 48 h as shown in the Tables I and II, the mice were sacrificed by cervical dislocation, and the blood and the cumulated excretion were collected. The various organs and tissues of each mouse were isolated, rinsed with saline, blotted, and weighed. Radioactivity was counted in a Packard dual channel $\gamma$ well counter with appropriate energy settings for $^{111}In$ and $^{67}Ga$. The blood volume, as determined by indium-111-labeled erythrocytes, was 8.0 ± 0.1%. In all tissue distribution studies, the radioactivity due to the blood background in each sample was subtracted according to the method described previously.[16] The sample solution injected into mice included the free and liposome-entrapped $^{67}Ga^{3+}$- or $^{111}In^{3+}$-labeled **4** and $^{67}Ga^{3+}$- or $^{111}In^{3+}$-NTA. The injected dose of liposomal lipid was determined by measuring the lipid concentration with the ferrothiocyanate assay[17] or the phosphate analysis after perchloric acid ashing.[18]

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**Registry No.** **1**, 57260-73-8; **2**, 112987-96-9; BOCON, 58632-95-4; DTPA anhydride, 23911-26-4; $H_2N(CH_2)_2NH_2$, 107-15-3; $BOCNH(CH_2)_2NHBOC$, 33105-93-0; inulin, 9005-80-5.