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Preparation of liposomes entrapping a high specific activity of $^{111}In^{3+}$-bound inulin

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Targeting liposomes to specific tissues or cells require the unequivocal determination of the uptake of liposomes at the cellular level. The present report describes the preparation of liposomes entrapping a high specific activity of $^{111}In^{3+}$-bound inulin, and the potential applications of a multiple labeling technique for characterizing the extent of uptake of liposomes by tissues or different cells in a given tissue in vivo. The labeling method involves the application of the technique of acetylacetone-mediated, ionophoric loading of $^{111}In^{3+}$ into liposomes entrapping an inulin derivative to which a strong chelating agent, diethylenetriaminepentaacetic acid (DTPA), is bound. Subsequent ionophoric removal of the weakly bound $^{111}In^{3+}$ by incubating the previously $^{111}In^{3+}$-loaded liposomes with 10 mM nitrilotriacetic acid and 100 μM tropolone at room temperature for 20 min results in the preparation of liposomes entrapping $^{111}In^{3+}$-DTPA-inulin. Our method of preparation yields net efficiencies of converting 63–78% of the externally added $^{111}In^{3+}$ to liposome-entrapped $^{111}In^{3+}$-DTPA-inulin.

Introduction

Continued interest in the use of liposomes as drug carriers to enhance the therapeutic index of drug molecules has created a need for methods to determine the fate of liposomes at the cellular level in vivo. One of such protocols involves the use of entrapped labelled compounds or probes to estimate the extent of cellular uptake of liposomes. In order for these probes to reflect truly the degree of uptake of liposomal contents by different cells in a given tissue, there are several criteria or characteristics that these probes must possess. These are that the probe itself has a fast clearance rate from circulation, that it is not metabolized or readily distributed into tissues and that once it is internalized into intracellular compartments it remains associated with tissues for a long time without redistribution. Water-soluble macromolecular markers, such as ^{125}I-labelled polyvinylpyrrolidone and [^{3}H]-, [^{14}C]- or [^{125}I]inulin appear to fulfill the mandate [1–3].

However, the low efficiency of encapsulating these water-soluble markers by small unilamellar vesicles (SUV), which have been shown to be more effective than large unilamellar or multilamellar liposomes in targeting drugs to non-phagocytic cells in vitro [4] and in vivo [5], is one of the major drawbacks. Furthermore, recent studies on the fate of SUV in vivo indicate that the extent of uptake of SUV by different cells in the liver could

Abbreviations: DTPA, diethylenetriaminepentaacetic acid; SUV, small unilamellar vesicles; MLV, multilamellar vesicles; DPPC, dipalmitoylphosphatidylcholine.

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be markedly affected by the liposomal lipid dose [6–9]. An evaluation of how the efficacy of various targeting protocols may be influenced by the administered dose of liposomal lipid, particularly in the low liposomal lipid range, requires the application of liposomes with a high specific radioactivity (nCi per nmol liposomal lipid).

Moreover, an unequivocal demonstration of the subtle differences in the tissue or cellular uptake resulting from a certain targeting protocol and the control is best revealed in the same set of experimental animals. This may sometimes be achieved by multiple labeling techniques, using the same batch of liposomal preparation for both the targeting and control protocols in the same experimental animals simultaneously. The present report describes the use of an inulin derivative to which a strong chelating agent, diethylenetriaminepentaacetic acid, is bound for preparing liposomes entrapping a high specific activity of radiolabeled inulin, and the potential applications of a multiple labeling technique for characterizing the extent of uptake of liposomes by tissues or different cells in a given tissue in vivo.

Materials and Methods

Inulin was obtained from Sigma. Sephadex G-25 and G-50, Sepharose 4B(CL) and 6B were from Pharmacia, and AG1-X8 was from Bio-Rad. Indium-111 chloride was supplied by Medi + Physics, Richmond, CA. The purification of $^{111}In^{3+}$ and the preparation of AG1-X8 (phosphate form) were carried out as described previously [10,11]. The specific activity of $^{111}In^{3+}$ was 1 mCi per 11–55 pmol. Trifluoroacetic acid and *t*-butoxycarbonyloxyimino-2-phenylacetonitrile were from Pierce Chemical Co. All phospholipids were from Avanti Polar Lipids, Inc. Other chemicals were of analytical grade.

The detailed procedure of the synthesis of DTPA-inulin is described elsewhere [13]. Briefly, DTPA-inulin was prepared by four steps of synthesis, starting from the preparation of DTPA-conjugated ethylenediamine, and periodate-oxidized inulin. DTPA-conjugated ethylenediamine was prepared by reacting DTPA anhydride with a mono *t*-butoxycarbonyl derivative of ethylenediamine followed by deblocking the mono *t*-butoxycarbonyl group in trifluoroacetic acid and subsequent purification in a AG1-X8 (formate form) cation-exchange resin. DTPA-inulin was obtained by reacting the periodate-oxidized inulin with DTPA-conjugated ethylenediamine followed by purification in cellulose CM-52 cation-exchange chromatography. The tight binding of DTPA-inulin with heavy metal cations, such as $^{111}In^{3+}$ or $^{67}Ga^{3+}$, was ascertained by quantitating the complex of DTPA-inulin with $^{111}In^{3+}$ eluted in the void volume from a Sephadex G-25 column (0.8 × 110 cm) after incubating DTPA-inulin with $^{111}In^{3+}$-nitrilotriacetic acid at room temperature for 15 min prior to passing through the Sephadex G-25 column. The concentration of DTPA-inulin was estimated by the phenolsulfuric acid assay [14].

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 (pH 7.4) isotonic buffer containing 1 mM DTPA-inulin (or 1 mM nitrilotriacetic acid, or both compounds). Sonication was carried out in a model G112 SPIT bath sonicator (Laboratory Supplies Co.) at 60–80 W in a 50 °C water bath, using 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 as that described above. The MLV were annealed at 65 °C for 30 min prior to the separation from the unentrapped DTPA-inulin (or nitrilotriacetic acid, or both compounds) by passing through a Sepharose 4B column (0.8 × 70 cm) that was equilibrated and eluted with 0.154 M NaCl, 0.02% NaN_3, 5 mM sodium phosphate (pH 7.4). The MLV were collected in the void volume.

SUV were prepared by sonicating 20 mg of either the dry powder of L-α-dipalmitoylphosphatidylcholine (DPPC) or thin film bovine brain sphingomyelin/cholesterol (2:1; mol/mol) in 0.106 M sodium phosphate (pH 7.4) isotonic buffer containing 1 mM DTPA-inulin (or 1 mM nitrilotriacetic acid, or both compounds) in a Branson 350 sonicator for 15 min as described previously [15]. The SUV were then purified by passage through a Sepharose 6B column (0.8 × 70 cm),

equilibrated and eluted with 0.154 M NaCl, 0.02% NaN_3, 5 mM sodium phosphate (pH 7.4). SUV were recovered at the void volume. The concentration of phospholipid was determined by either the ferrothiocyanate assay [16] or the phosphate analysis after perchloric acid ashing [17].

Radiolabeling of liposome-entrapped DTPA-inulin with $^{111}In^{3+}$ was carried out essentially by the same method for externally loading $^{111}In^{3+}$ by acetylacetone to liposome-entrapped nitrilotriacetic acid as that described previously [10,12]. 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 0.106 M sodium phosphate (pH 7.4) isotonic buffer as reported previously [11,15]. Percent loading was estimated from the radioactivity associated with the purified liposomes and the total radioactivity applied to the column. In some cases, encapsulation of $^{111}In^{3+}$ was achieved by sonicating the lipid and the mixture of 1 mM DTPA-inulin, 1 mM nitrilotriacetic acid and 20–30 pCi $^{111}In^{3+}$ followed by the same procedure of purification by Sepharose 6B gel filtration chromatography as that described above.

To ensure that the $^{111}In^{3+}$ was indeed chelated by DTPA-inulin, an unloading procedure of inducing the release of the weakly chelated $^{111}In^{3+}$ from liposomes was adopted. Liposomes entrapping $^{111}In^{3+}$-DTPA-inulin and/or $^{111}In^{3+}$-nitrilotriacetic acid were incubated with various concentration of tropolone in 0.106 M sodium phosphate (pH 7.4) in the presence of 1–10 mM 'external' nitrilotriacetic acid. Aliquots in the microliter range were taken out and applied to an AG1-X8 (phosphate form) (0.8 × 14 cm) column to assess time dependence of the release of the entrapped $^{111}In^{3+}$. The extent of release was estimated from the radioactivity associated with the eluted liposomes and the total radioactivity applied to the column [11,15]. The extent of the leakage of $^{111}In^{3+}$ from liposomes was also estimated and confirmed by Sephadex G-50 column chromatography. The columns in both methods were equilibrated and eluted with 0.106 M sodium phosphate (pH 7.4) isotonic buffer. Percent unloading of $^{111}In^{3+}$ from liposomes was estimated from the radioactivity dissociated from the liposomes and the total radioactivity applied to the column.

Result

Fig. 1 depicts the elution profiles of $^{111}In^{3+}$-nitrilotriacetic acid alone, $^{111}In^{3+}$-DTPA-inulin alone, and the 15-min incubation mixture of $^{111}In^{3+}$-nitrilotriacetic acid with DTPA-inulin in a Sephadex G-25 column. The transfer of $^{111}In^{3+}$ from $^{111}In^{3+}$-nitrilotriacetic acid to DTPA-inulin (Fig. 1a) suggests that the high metal binding affinity of DTPA to many metal cations (K_a = 10^{29}, 10^{27} and 10^{25} for In^{3+}, Fe^{3+} and Ga^{3+}, respectively [18]) permits the inulin derivative to compete effectively with nitrilotriacetic acid for $^{111}In^{3+}$. However, at a very high concentration of nitrilotriacetic acid or a long period of incubation, a gradual translocation of $^{111}In^{3+}$ from DTPA-inulin to nitrilotriacetic acid can occur (Fig. 2).

Fig. 3 shows that the transport of externally added $^{111}In^{3+}$ to the interior of SUV, where DTPA-inulin was entrapped, by either acetylacetone or tropolone, took place rapidly. An efficiency of 60–75% of the externally added $^{111}In^{3+}$ was loaded into the liposomes at 15 min after the onset of the loading process. Both ionophores were able to load about 90% of the externally

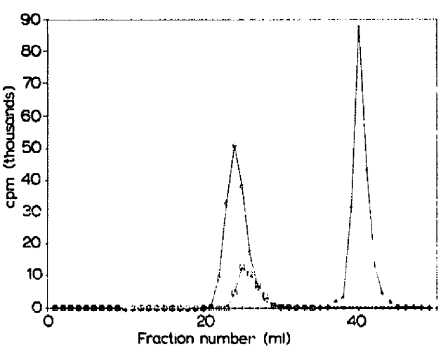

Fig. 1. Profiles of various $^{111}In^{3+}$ derivatives eluted from a Sephadex G-25 column. The Sephadex column (0.8 × 110 cm) was eluted by 0.154 M NaCl, 0.02% NaN_3 and 5 mM sodium phosphate (pH 7.4) with the void and bed volumes at 25 ml and 55 ml, respectively. The samples were: $^{111}In^{3+}$-DTPA-inulin (□); the mixture of 1 mM DTPA-inulin, 1 mM nitrilotriacetic acid and a trace amount of $^{111}In^{3+}$ (◇) in 0.154 M NaCl, 5 mM sodium citrate (pH 5.6); and 1 mM nitrilotriacetic acid and a trace amount of $^{111}In^{3+}$ in 0.9% NaCl, 5 mM sodium citrate (pH 5.6) (+).

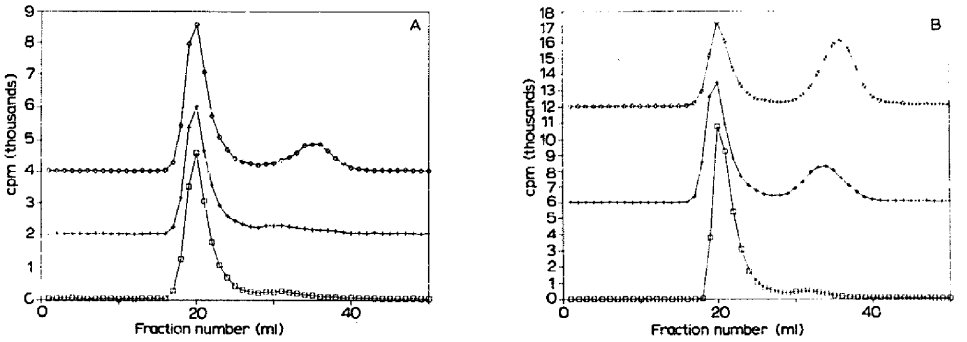

Fig. 2. The concentration and time dependence of the translocation of $^{111}In^{3+}$ from DTPA-inulin to nitrilotriacetic acid. A trace amount of $^{111}In^{3+}$ was incubated with 0.154 M NaCl, 2.5 mM DTPA-inulin and 5 mM sodium citrate (pH 5.6) in the absence (□) or presence of 1 mM (+) or 100 mM (◇) nitrilotriacetic acid for (A) 1 h and (B) 18 h. The separation of $^{111}In^{3+}$-DTPA-inulin (fraction No. 20) from $^{111}In^{3+}$-nitrilotriacetic acid (fraction No. 35) was achieved in a Sephadex G-25 column (0.8×70 cm) equilibrated and eluted by 1 M acetic acid. The base lines of the curves from the samples containing nitrilotriacetic acid are elevated.

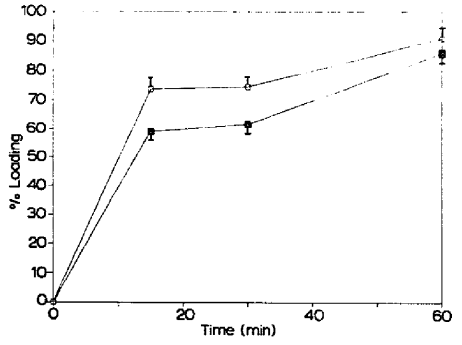

Fig. 3. Time dependence of the efficiency of loading $^{111}In^{3+}$ to DPPC or bovine brain sphingomyelin/cholesterol (2:1; mol/mol) SUV entrapping DTPA-inulin. The loading solution was prepared by mixing 10–20 μl $^{111}In^{3+}$ (1–5 pCi) in 3 mM HCl with 100–200 μl of either 10 mM Tris buffered (pH 7.6) 0.154 M NaCl, containing an appropriate amount of acetylacetone or 10 mM Hepes-buffered (pH 7.4) 0.154 M NaCl, containing an appropriate amount of tropolone. Liposomes were loaded with $^{111}In^{3+}$ by adding 140–200 μl loading solution of $^{111}In^{3+}$/acetylacetone (□) or $^{111}In^{3+}$/tropolone (■) to 1 ml SUV (1–5 mg/ml) and incubating at room temperature for various periods prior to removing the unloaded $^{111}In^{3+}$ ionophore by passage through an AG1-X8 column (0.8×14 cm) equilibrated and eluted by 0.106 M sodium phosphate buffer (pH 7.4). The final concentrations of acetylacetone and tropolone in the incubation mixture of liposomes and ionophore were 30 mM and 33 μM, respectively. Each point is an average of at least three measurements.

added $^{111}In^{3+}$ into liposomes at 1 h after the onset of the loading process (Fig. 3). Fig. 4 shows that after purification by the AG1-X8 cation-exchange chromatography, $^{111}In^{3+}$-loaded SUV moved as a single peak at the void volumes of a Sepharose 6B

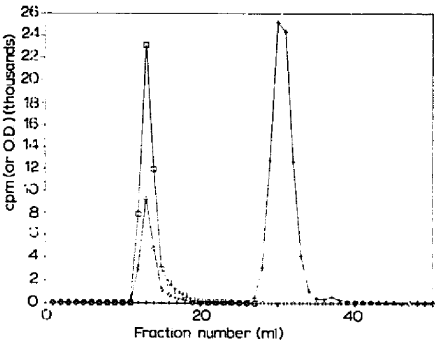

Fig. 4. Profiles of intact SUV (□) and $^{111}In^{3+}$-DTPA-inulin (+) eluted from a Sepharose 6B column. The bovine brain sphingomyelin/cholesterol (2:1; mol/mol) SUV-entrapped $^{111}In^{3+}$, 1 mM DTPA-inulin and 1 mM nitrilotriacetic acid. The column (0.8×70 cm) was equilibrated and eluted with 0.154 M NaCl, 0.02% NaN_3, 5 mM sodium phosphate (pH 7.4). The void and bed volumes of the column are 15 ml and 35 ml, respectively. An arbitrary scale of the concentration of DTPA-inulin (◇) monitored by absorbance at 239 nm is used in the plot.

TABLE I

EFFECT OF THE TROPOLONE CONCENTRATION ON THE TROPOLONE-MEDIATED RELEASE OF $^{111}In^{3+}$ FROM BOVINE BRAIN SPHINGOMYELIN/CHOLESTEROL (2:1; mol/mol) SUV ENTRAPPING $^{111}In^{3+}$-DTPA-INULIN OR $^{111}In^{3+}$-NITRILOTRIACETIC ACID

Liposomes were loaded with $^{111}In^{3+}$ by acetylacetone according to the procedure described in the legend of Fig. 3. The loading time was 1 h. The tropolone-mediated release of $^{111}In^{3+}$ was induced by incubating previously $^{111}In^{3+}$-loaded SUV with 10 mM nitrilotriacetic acid, 0.106 M sodium phosphate buffer (pH 7.4), containing various concentrations of tropolone, at room temperature for 20 min. The percent of release was determined by AG1-X8 chromatography, as described in Materials and Methods.

Tropolone (μM)	Entrapped marker	
	$^{111}In^{3+}$-DTPA inulin ($n = 3$)	$^{111}In^{3+}$-nitrilotriacetic acid ($n = 3$)
50	12.6 ± 0.4	95.3 ± 1.9
100	13.4 ± 0.7	97.2 ± 0.1
500	37.9 ± 7.8 [a]	97.8 ± 1.8

[a] $n = 6$.

column. A similar extent of purification of $^{111}In^{3+}$-loaded MLV by AG1-X8 chromatography was also observed (data not shown). In addition,

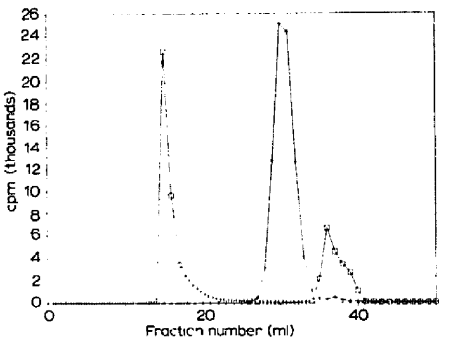

Fig. 5. Profile of tropolone-mediated released radioactivity eluted from a Sepharose 6B column. Bovine brain sphingomyelin/cholesterol (2:1; mol/mol) SUV entrapping $^{111}In^{3+}$ and 1 mM DTPA-inulin was incubated with 10 mM nitrilotriacetic acid containing 500 μM tropolone at room temperature for 20 min prior to analysis by Sepharose 6B gel filtration chromatography (□). The column chromatography procedure was the same as that described in Fig. 4. For comparison, the profile of $^{111}In^{3+}$-DTPA-inulin (+) is also plotted.

Fig. 4 illustrates the need of a relatively long Sepharose 6B column to ensure complete separation of unentrapped DTPA-inulin from SUV.

Table I demonstrates that an almost complete ionophore-mediated release of the entrapped $^{111}In^{3+}$ from the SUV-entrapping $^{111}In^{3+}$-nitrilotriacetic acid can be induced by incubating the SUV with 10 mM nitrilotriacetic acid, 0.106 M sodium phosphate (pH 7.4) isotonic buffer in the presence of 50 μM tropolone. In contrast, the release of $^{111}In^{3+}$ from the SUV-entrapped $^{111}In^{3+}$-DTPA-inulin remained relatively constant at a level of 13% of the entrapped $^{111}In^{3+}$, when the ionophore concentration was below 100 μM tropolone. At, or above, a tropolone concentration of 500 μM, the release of $^{111}In^{3+}$ from the SUV-entrapping $^{111}In^{3+}$-DTPA-inulin became signifi-

TABLE II

PERCENT LOADING AND UNLOADING $^{111}In^{3+}$ TO AND FROM DPPC SUV AND FROM BOVINE BRAIN SPHINGOMYELIN/CHOLESTEROL (2:1, mol/mol) MLV ENTRAPPING DIFFERENT CHELATING MOLECULES

Liposomes were loaded with $^{111}In^{3+}$ by acetylacetone according to the procedure described in the legend of Fig. 3. The loading time was 1 h. Ionophore-mediated release of $^{111}In^{3+}$ was induced by incubating the previously $^{111}In^{3+}$-loaded liposomes with 100 μM tropolone, 10 mM nitrilotriacetic acid, 0.106 M sodium phosphate buffer (pH 7.4) at room temperature for 20 min. The unloading efficiency was determined by AG1-X8 chromatography, as described in Materials and Methods. $^{111}In^{3+}$ was encapsulated in DPPC SUV by sonicating the lipid in solution containing the entrapped marker followed by Sepharose 6B purification, as described in Materials and Methods.

Entrapped markers ($n = 3$)	Loading for MLV ($n = 4$) (%)	Unloading (%)	Net loading efficiency (%)
DTPA-conjugated inulin (1 mM)			
SUV	91.6 ± 2.7	13.3 ± 0.21	78.02
MLV	89.5 ± 3.7	26.5 ± 1.5	63
Nitrilotriacetic acid (1 mM)			
SUV	89.3 ± 0.7	98.3 ± 0.8	0
MLV	90.4 ± 0.3	99.1 ± 0.1	0
Nitrilotriacetic acid (1 mM) + DTPA-conjugated inulin (1 mM)			
SUV	82.5 ± 1.5	72.8 ± 2.7	9.7
MLV	83.8 ± 0.5	66.1 ± 1.5	17.7
Nitrilotriacetic acid (1 mM) + DTPA-conjugated inulin (1 mM) + $^{111}In^{3+}$			
SUV	–	4.9 ± 0.5	–

cant. Furthermore, Fig. 5 shows that the released radioactivity was in the form of $^{111}In^{3+}$ and not in the form of $^{111}In^{3+}$-DTPA-inulin, suggesting that the radioactivity was not released from the degraded liposomes.

Table II summarizes the efficiencies of loading and unloading $^{111}In^{3+}$ to and from SUV and MLV, when 0.106 M sodium phosphate (pH 7.4) isotonic buffer containing 1 mM DTPA-inulin and/or nitrilotriacetic acid were entrapped in the aqueous compartment of liposomes. The finding of a high level of ionophore-mediated release of $^{111}In^{3+}$ from the $^{111}In^{3+}$-loaded liposomes within which both nitrilotriacetic acid and DTPA-inulin were co-encapsulated was unexpected. In contrast, only 4.9% of the total SUV-entrapped $^{111}In^{3+}$ can be released by incubating 100 μM tropolone/10 mM nitrilotriacetic acid mixture with a different preparation of SUV in which $^{111}In^{3+}$-DTPA-inulin (1 mM DTPA-inulin plus 20–30 μCi $^{111}In^{3+}$) and 1 mM nitrilotriacetic acid were encapsulated by the conventional procedure of probe sonication/gel filtration chromatography.

Discussion

Inulin is a useful marker for determining the intracellular uptake of liposomes by tissues or by different cell types in a given tissue. In addition to having all the characteristics of inulin, DTPA-inulin possesses the appealing feature of being able to become a radioactive derivative of inulin, after it is encapsulated by liposomes. In contrast to the methods of encapsulating a high level of pre-labeled radioactive inulin, the method described here allows efficient encapsulation of a high level of radioactive cations into liposomes without the need of either using a large quantity of liposomal lipid or using a very high level of pre-labeled radioactive inulin to begin with. The loss of radioactive inulin and the exposure of workers to the radioactivity during the process of encapsulation are minimized.

In addition, the present method is particularly useful for studying the roles of tissues (or cells in a given tissue, such as the liver) in the uptake of SUV under conditions whereby the experimental animal is not saturated with the administered dose of liposomal lipid. Furthermore, the capability of chelating a variety of metal cations ($^{40}Ca^{2+}$, $^{59}Fe^{3+}$, $^{67}Ga^{3+}$, $^{111}In^{3+}$ for example) by DTPA permits experimental protocols that may be utilized in studying and comparing the effects of certain liposome-bound targeting molecules (glycolipids, glycoproteins, hormones and monoclonal antibodies, for example) on the tissue or cellular distribution of liposomes in the same experimental subjects simultaneously. Moreover, the principle of the present method allows the possibility of loading metal cations with useful spectroscopic properties into the liposome-entrapped DTPA-inulin for studying the fate of liposomes in vivo by other methods, such as nuclear magnetic resonance imaging.

The main thrust of the present method relies on the ability of an ionophore to transport metallic cations in and out of the liposomes, and on the metal-binding affinity of the chelating molecules situated in the inner and the outer aqueous compartments of liposomes. Obviously, not only do the stability constants of the metal complexes but also the on-off rates of the chelating molecules to release the metallic cations play an important role in regulating the direction of the net transport of the metallic cations across liposomal bilayers. During the ionophoric loading process, the purpose of the presence of 1 mM nitrilotriacetic acid or DTPA-inulin in the inner aqueous compartment of liposomes is to act as an infinite sink to trap the incoming $^{111}In^{3+}$ (11 pmol per mCi).

It has been show that not all the loaded $^{111}In^{3+}$ ends up bound to the entrapped chelating molecule [12,19]. A small portion of $^{111}In^{3+}$ could be imbedded in the liposomal lipid bilayer as a complex of $^{111}In^{3+}$-ionophore-lipid and/or $^{111}In^{3+}$-ionophore. The objective of implementing an ionophore-mediated release after loading $^{111}In^{3+}$ to the liposomes is to ensure that all the remaining $^{111}In^{3+}$ cations that do not end up binding to DTPA-inulin are removed. The probability of the nonspecific, extracellular binding of the entrapped $^{111}In^{3+}$ not being chelated by DTPA-inulin [2,20] is, thus, minimized by the ionophore-mediated unloading process.

The 13–27% of the loaded radioactivity being removed by the subsequent unloading process (Table II) perhaps represents the portion of the bilayer-bound entrapped $^{111}In^{3+}$. The application

of the protocol of incubating liposomes with 10 mM nitrilotriacetic acid, 0.106 M sodium phosphate (pH 7.4) isotonic buffer in the presence of 100 μM tropolone to unload the non-DTPA-bound $^{111}In^{3+}$ from liposomes was strictly a kinetic consideration of the time factor required. Other unloading conditions using lower concentrations of the ionophore and nitrilotriacetic acid appeared to be equally efficient, except that a longer period than 20 min was required to complete the unloading process. Furthermore, high concentrations of tropolone may mediate the release of $^{111}In^{3+}$ from liposome-entrapped $^{111}In^{3+}$-DTPA-inulin, if a high concentration of a strong chelating molecule exists on the outside of liposomes (Table I). Caution should be taken to ensure the removal of excess tropolone using the AG1-X8 chromatography [11].

It is likely that at concentrations of 500 μM tropolone or more, there are enough tropolone molecules to compete with DTPA-inulin for $^{111}In^{3+}$. Once bound with $^{111}In^{3+}$, the tropolone-$^{111}In^{3+}$ complex (partition coefficient = 11.7) becomes 5-times more soluble in the lipid bilayer than tropolone itself (partition coefficient = 2.2) [12]. Subsequent ionophoric transport of $^{111}In^{3+}$ by tropolone to nitrilotriacetic acid (20 mM) on the other side of the lipid bilayer can result in the release of $^{111}In^{3+}$ from entrapped $^{111}In^{3+}$-DTPA-inulin at high concentrations of tropolone. Presumably, at 100 μM tropolone or less, the concentration of tropolone is below the critical concentration needed for competing with DTPA-inulin for $^{111}In^{3+}$, thereby lacking the release of $^{111}In^{3+}$ from entrapped $^{111}In^{3+}$-DTPA-inulin. Thus, the reason that the release of $^{111}In^{3+}$ from liposome-entrapped $^{111}In^{3+}$-DTPA-inulin occurred at a concentration of 500 μM tropolone and not at or below 100 μM was perhaps a net effect of the competitive binding of $^{111}In^{3+}$ among various chelating molecules, the change of the partition coefficient of tropolone and the ionophoric properties of tropolone.

It is interesting to point out that as much as 66–73% of the loaded $^{111}In^{3+}$ was ionophorically unloaded from the liposomes in which both 1 mM nitrilotriacetic acid and 1 mM DTPA-inulin were entrapped prior to the loading of $^{111}In^{3+}$ (Table II). This is quite a contrast to the low percentage of tropolone-mediated release of the loaded $^{111}In^{3+}$ from the liposomes entrapping 1 mM DTPA-inulin alone. Similarly, when $^{111}In^{3+}$-DTPA-inulin and 1 mM nitrilotriacetic acid were entrapped by the conventional method of probe sonication, less than 5% of the entrapped radioactivity could be released by tropolone. These observations are consistent with the notion that at concentrations of 1 mM nitrilotriacetic acid, 1 mM DTPA-inulin and 20–25 mg lipid per ml, these chelating molecules were hardly co-encapsulated in the same inner aqueous compartment of a single small unilamellar vesicle or multilamellar vesicle. The high percentage of the ionophore-mediated release of $^{111}In^{3+}$ from the $^{111}In^{3+}$-loaded liposomes in which both 1 mM nitrilotriacetic acid and DTPA-inulin were encapsulated may suggest that $^{111}In^{3+}$ is loaded more favorably into the aqueous compartment entrapping nitrilotriacetic acid than that entrapping DTPA-inulin. Alternatively, more nitrilotriacetic acid molecules than DTPA-inulin molecules were entrapped in the liposomes.

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