Labeling of Preformed Liposomes with Ga-67 and Tc-99m by Chelation

D. J. Hnatowich, B. Friedman, B. Clancy, and M. Novak

University of Massachusetts Medical Center and Clark University, Worcester, Massachusetts

We have synthesized a long-chain hydrocarbon covalently coupled to diethylenetriaminepenta-acetic acid (stearylamine-DTPA) and have incorporated this compound in liposomes during their preparation. The lipophilic hydrocarbon chain anchors the molecule in the lipid bilayer, exposing the DTPA groups on the surface for chelation. Ethanolic solutions of the lipids are evaporated to dryness under nitrogen in multidose vials; the lipids are suspended in the vial by adding a small volume of distilled water followed by sonication. The liposomes are then labeled by transcomplexation in the case of Ga-67 and by conventional stannous reduction in the case of Tc-99m, by adding the activities directly to the vial. These liposomes bind 95 ± 5% of Ga-67 and Tc-99m activity, as determined by paper chromatograph assay, eliminating the need for a purification step. The labeled liposomes release about 5% of their Ga-67 activity, and about 30% of their Tc-99m activity after 2 hr of incubation in 50% human plasma at 37°C. Activity released from liposomes labeled with Ga-67 or Tc-99m oxine is much greater under the same conditions. In normal mice the labeled liposomes show biodistributions that are comparable with that obtained with liposomes labeled by conventional techniques.

J Nucl Med 22: 810–814, 1981

One limitation to the study of liposomes labeled in the lipid phase with radionuclides, such as Ga-67 and Tc-99m, is that standard procedures for their preparation incorporate the oxine chelates into the lipid bilayer of the liposome during their preparation (*1*). The drawbacks to this approach are: (a) the oxine chelates of these metals tend to diffuse from the liposome, creating radiochemical impurities; and (b) the liposomes must be labeled during their preparation, thus eliminating any possibility of a "kit" preparation whereby the lipids may be stored for constitution and labeling as needed.

Using a stability assay by paper chromatography, we have established that immediately following purification by gel filtration of the oxine-labeled liposomes, an appreciable fraction of unbound activity is still present, suggesting that the oxine chelates continually migrate from the liposomes. We have also determined that in 50% plasma at 37°C, approximately 25% of the activity of Ga-67-oxine-labeled liposomes is released after 2 hr in this medium, while for Tc-99m-oxine-labeled liposomes the release is greater (*2*).

For the preparation of liposomes labeled with metallic radionuclides we have developed methods that do not suffer from these drawbacks. We have synthesized for this application a long-chain hydrocarbon linked by an amide bond to diethylenediaminepentaacetic acid (DTPA). This compound, octadecylamine-DTPA, has been added to lecithin and cholesterol during the preparation of liposomes. The hydrocarbon chain anchors the molecule into the liposome bilayer while the polar DTPA groups, being lipophobic, are present on the liposome surface. Liposomes prepared in this way may be labeled with either Ga-67 or Tc-99m at virtually 100% efficiency by standard methods, and the labeled product shows a stability considerably superior to that of the oxine-labeled liposomes.

Received Nov. 25, 1980; revision accepted April 24, 1981.

For reprints contact: D. J. Hnatowich, Dept. of Nuclear Medicine, Univ. of Massachusetts Med. Ctr., 55 Lake Ave. North, Worcester, MA 01605.

METHODS AND RESULTS

Preparation of octadecylamine-DTPA. The synthesis involves the preparation of the anhydride of DTPA and

its reaction in chloroform with octadecylamine (stearylamine).

The DTPA anhydride was prepared according to the method of Eckelman et al. (*3*). About 40 g of the acid were reacted with 38 ml of acetic anhydride in 50 ml of pyridine for 24 hr at 65°C. The resulting DTPA anhydride was purified by filtration, repeatedly washed with acetic anhydride and anhydrous ether, and dried in an oven at 50–60°C. The infrared spectra of the anhydride show the presence of the characteristic anhydride stretching vibrations at 1820 and 1770 cm^{-1} and the absence of the carbonyl stretching vibrations present in the spectra of the acid. A strong absorption at 1635 cm^{-1} indicates that the central carboxyl group is ionized, so that the DTPA anhydride is probably a zwitter ion. The melting point is 175–185°C, in good agreement with Eckelman et al. (*3*).

Octadecylamine was obtained commercially at 98% purity and used without further purification. About 1–2 g of the amine was dissolved in ~200 ml of anhydrous chloroform in a round-bottom flask. A 1.1 molar excess of the anhydride was added and the mixture was refluxed with stirring under a dry atmosphere for 1–2 days. The reaction product was insoluble in chloroform and was collected by filtration. The infrared spectra of the evaporated crude product show the presence of an anhydride linkage as well as an amide linkage. It was treated with boiling water to hydrolyze the remaining anhydride linkage and purified by recrystallization from hot ethanol. The infrared spectra of this product is characterized by a strong C·H stretching band at 2900 cm^{-1} due to the saturated hydrocarbon chain, by strong carbonyl stretching bands at 1690–1720 cm^{-1} due to the carboxylic acid groups, and by a band at 1640–1670 cm^{-1} due to the amide linkage. As expected, the characteristic anhydride stretching vibrations at 1770 and 1820 cm^{-1} are absent. On heating, the compound decomposes such that no sharp melting point was obtained. Elemental analysis was obtained commercially*; the calculated percentages are for the salt (i.e., octadecylpentetamic acid monooctadecylaminium salt): expected %C 65.67, %H 10.95, %N, 7.66; found %C 65.72, %H 10.84, %N 7.67.

Preparation of liposomes. Typically, 13 mg of phosphatidylcholine dipalmitoyl (lecithin), 1 mg of cholesterol, and 1 mg of octadecylamine-DTPA (molar ratio 23:3:2) were added in ethanol to a 5-ml multidose vial. The lipids were evaporated to dryness in a 50–60°C water bath under a constant stream of oxygen-free nitrogen. The vial was then sealed and stored in a freezer.

In preparation for labeling, 1 ml of nitrogen-purged distilled water was added to the vial and the lipids dislodged from the glass by vortexing. The inclusion of a small glass bead facilitates this process. The vial was then sonicated at 0°C in a bath sonicator,† no more than 5 min of sonication being necessary or desirable. The preparations were examined by light microscopy to confirm vesicle formation.

Labeling of liposomes with Ga-67 and Tc-99m. The contents of entire vials were used in these labeling studies unless otherwise indicated, thus the labeling was performed at a lipid concentration of ~15 mg/ml.

Initially liposomes were labeled with Ga-67 by adding the trichloride‡ at acid pH to the suspension, followed by neutralization. Subsequently labeling was achieved by transcomplexation by adding the desired activity in 0.1 *M* acetate buffer. Labeling with Tc-99m requires the reduction of pertechnetate, and this was accomplished by the stannous method. About 50 μg of $SnCl_2 \cdot 2H_2O$ was added from a 0.5 mg/ml solution in 0.1 *M* HCl. After about 5 min, the suspension was neutralized by adding 250 μl of 0.2 *M* phosphate buffer followed by a small volume of 1.0 *M* NaOH. The resulting pH was 6.6. After an additional 10 min, between 1–2 mCi of pertechnetate solution was added and the vial allowed to stand for at least 10 min. Labeling efficiency was determined by mixing the suspensions with an equal volume of human plasma and incubating for 10 min at 37°C in a shaking water bath. The mixture was then spotted on Whatman No. 1 paper for development in a 0.1 *M* acetate buffer eluant. Activity bound to liposomes remains at the origin, whereas all soluble forms of the activity migrate. Blank studies—in which preparations of $Ga(OH)_3$ and "reduced, hydrated, unbound" Tc-99m were analyzed in the same system—showed about 1–8% remaining at the origin (*2*). The percentage of activity in the symmetric origin peak with respect to that in the entire chromatogram was taken as labeling efficiency. Stability of the preparation was determined by measuring the activity still bound to the liposomes after prolonged incubations in 50% plasma at 37°C.

As a control, liposomes were prepared in the identical manner described above except that octadecylamine was used in place of octadecylamine-DTPA. Table 1 presents the results of dialysis experiments in which Ga-67-la-

TABLE 1. GALLIUM-67 ACTIVITY (μCi) PRESENT IN DIALYSIS BAG FOR PREPARATIONS OF OCTADECYLAMINE AND OCTADECYLAMINE-DTPA LIPOSOMES COMPARED WITH DIALYSIS TIME, CORRECTED FOR DECAY

Time	Octadecylamine liposomes	Octadecylamine-DTPA liposomes
0 min	207	191
10 min	200	191
30 min	163	191
6 hrs	57	203
16 hrs	44	227
24 hrs	42	226

TABLE 2. GALLIUM-67 ACTIVITY BOUND TO OCTADECYLAMINE AND OCTADECYLAMINE-DTPA LIPOSOMES IN SALINE SUSPENSIONS AND IN 50% HUMAN PLASMA AT 37°C AT TWO TIME POINTS*

Time	Octadecylamine liposomes		Octadecylamine-DTPA liposomes	
	Saline	50% plasma	Saline	50% plasma
5 min	75 (73–77)	13 (12–15)	94 (90–98)	90 (84–97)
18 hrs	70 (69–71)	12 (10–13)	91 (85–96)	75 (72–77)

* Average of two to three determinations, with range in parentheses.

beled liposome suspensions were dialyzed against phosphate-buffered saline. Periodically the bags were removed, rinsed, and counted in an ionization counter. The Ga-67 activity in the octadecylamine liposomes was present in some dialyzable chemical form, since activity decreased uniformly with time, whereas no decrease was observed in the case of octadecylamine-DTPA liposomes.

The results of a paper-chromatographic analysis of these two preparations are presented in Table 2. The 70–75% bound Ga-67 activity present on the octadecylamine liposomes in saline probably reflects nonspecific binding, which is released in 50% plasma. Activity bound to the octadecylamine-DTPA liposomes does not show this release. The stability over time of Ga-67-labeled octadecylamine-DTPA liposomes can be seen from Table 3, where the half-time of activity release may be estimated as roughly 30 hr.

Since labeling efficiency must be directly related to the concentration of DTPA groups in suspension, the fraction of bound Ga-67 activity will be related to the concentration of liposomes labeled. As shown in Table 4, when less than about 20% (~3 mg) of a typical liposome suspension was labeled in 1 ml, there was reduced binding. The lower limit of liposome concentration that still provided adequate labeling was found to vary about this average value and is probably related to the concentration of trace metals in the radioactivity sources.

TABLE 3. GALLIUM-67 ACTIVITY BOUND TO OCTADECYLAMINE-DTPA LIPOSOMES FOLLOWING INCUBATION IN 50% PLASMA AT 37°C*

Time	Liposome activity (%)
10 min	94 (92–96)
1 hr	93 (92–93)
3 hr	90 (85–95)
6 hr	71 (61–84)
50 hr	35 (29–40)

* Average of two to three determinations, with range in parentheses.

The effect of octadecylamine-DTPA concentration in liposomes on their surface charge was investigated. Liposomes prepared with varying weight percentages of octadecylamine-DTPA were labeled with Ga-67 with a labeling efficiency of greater than 90% and were applied to a 1 × 8.5-cm column of AG 1 × 4 anion-exchange resin, 100–200 mesh, and eluted with distilled water pH 5.5 at a flow rate of 6 ml/min. Figure 1 plots the percentage of labeled liposomes eluting in the void volume against the weight percent of octadecylamine-DTPA in lecithin and cholesterol liposomes. As observed earlier (*2*), the fraction of activity eluting from the column decreased with increasing negative charge.

Table 5 lists the results obtained on labeling both liposome types with Tc-99m. As in the Ga-67 case, activity bound to the octadecylamine liposomes is quickly released in 50% plasma, in contrast to the octadecylamine-DTPA liposomes. The half-time for release of activity from the octadecylamine-DTPA liposomes labeled with Tc-99m may be estimated from the table to be ~4 hr. As was the case with the Ga-67-labeled liposomes, labeling efficiency with Tc-99m also depends on the fraction of the liposome suspension removed for labeling. Attempts to label less than half the suspension resulted in decreased labeling efficiency.

Biodistribution studies in mice. Biodistributions in normal CD-1 male Fisher mice have been obtained with both the Ga-67- and Tc-99m-labeled octadecylamine-DTPA liposomes, and with the labeled octadecylamine-DTPA compound itself. In the case of the liposomes, each mouse received about 0.6 mg of lipids in about 0.2 ml, whereas for the labeled octadecylamine-DTPA study each received 0.2 ml of a 5% solution of human serum albumin containing about 100 μg of the amidic acid. Following etherization of each animal at 30 min, a blood sample was obtained by cardiac puncture and tissue samples were removed for weighing and subsequent counting in a NaI(Tl) well counter. Samples of bone were obtained from the intact femur. Standards of the injected solution were counted along with the tissue samples so that results could be expressed as percentage injected dose per gram wet weight, normalized to a 25-g mouse.

Results of the animal biodistribution studies are

TABLE 4. GALLIUM-67 ACTIVITY BOUND TO OCTADECYLAMINE-DTPA LIPOSOMES AFTER 10 MIN IN 50% PLASMA COMPARED WITH THE FRACTION OF THE 1.0 ml LIPOSOME SUSPENSION REMOVED FOR LABELING*

Fraction:	0.0	0.05	0.10	0.20	0.40
Activity (%):	10 (8–12)	8 (7–8)	22 (19–24)	97 (94–100)	96 (95–96)

* Average of two determinations, with range in parentheses.

presented in Table 6. Both the Ga-67 and Tc-99m-labeled liposomes show the accumulation of activity in liver and spleen expected for these liposomes, in contrast to that observed for the labeled compounds themselves.

DISCUSSION

These findings show clearly that the use of small amounts of octadecylamine-DTPA in lecithin liposomes provides a preparation that may be effectively and rapidly labeled with Ga-67 or Tc-99m by chelation. In the case of both nuclides, the stability of the preparation is substantially improved over oxine methods of labeling. For example, after 2 hr in 50% plasma at 37°C, liposomes labeled by conventional methods show about 25% dissociation in the case of Ga-67 and about 80% in the case of Tc-99m (*2*). This is in addition to any free activity that is present immediately following gel chromatography or some similar purification step. By contrast, with the DTPA-containing liposomes these values are about 5 and 30%, respectively.

The dissociation we have observed is not due to instability of the amide bond. Radiolabeled DTPA, the product of amide-bond instability, has an R_f of 0.9 in our chromatographic assay system, and such a product was not observed in this study. Nor is the dissociation due to release of the label from the chelate. In a recent study with EDTA- and DTPA-coupled albumin microspheres, prepared in the same manner as octadecylamine-DTPA, we observed no dissociation of Ga-67-labeled particles over 24 hr in 50% plasma at 37°C (*4*). The most plausible explanation is that the dissociation results from the adverse effects of plasma on liposomes. Lecithin liposomes have recently been shown to lose about 50% of their activity in the aqueous phase after 1 hr in 50% serum and plasma (*5*). Consequently, the dissociation observed in this work may represent a limit on stability that is achievable with liposomes.

The incorporation of small amounts of octadecylamine-DTPA in liposomes results in only small numbers of chelating groups available for chelation. At the lipid concentration used in this work, no more than about 250 μg of DTPA groups are present on the outside surfaces of unilaminar liposomes, and much less in the case of multilaminar liposomes. The small number of chelating groups most likely explains the decreasing labeling efficiency observed for both Ga-67 and Tc-99m with decreasing fractions of lipids removed for labeling. Since both radionuclides used in this study are free of added carrier, only small numbers of chelating groups are necessary to achieve 100% labeling efficiency in the absence of competition from other species. Most likely the lower limit on the concentration of chelating groups

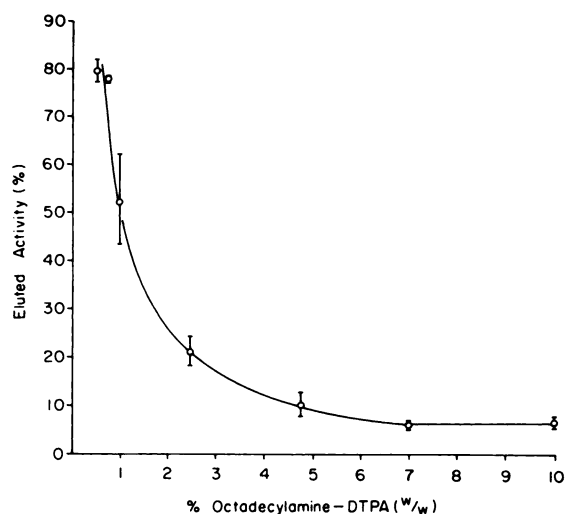

FIG. 1. Effect of weight percent of octadecylamine-DTPA in lecithin liposomes on their negative surface charge as determined by anion-exchange chromatography. Each datum is average of two to four determinations on one or two preparations.

TABLE 5. TECHNETIUM-99m ACTIVITY (%) BOUND TO OCTADECYLAMINE AND OCTADECYLAMINE-DTPA LIPOSOMES COMPARED WITH INCUBATION TIME IN 50% PLASMA AT 37°C

Time	Octadecylamine liposomes*	Octadecylamine-DTPA liposomes†
0 min	54 (48–60)	91 (84–97)
10 min	19 (19–19)	85 (74–95)
30 min	21 (19–22)	80 (71–94)
60 min	18 (17–19)	78 (68–92)
2 hr	19 (15–23)	67 (55–78)

* Average of two to three determinations.
† Average of 10–11 preparations, with range in parentheses.

TABLE 6. BIODISTRIBUTIONS IN NORMAL MICE AT 30 MIN FOR Ga-67- AND Tc-99m-LABELED OCTADECYLAMINE-DTPA LIPOSOMES AND FOR LABELED OCTADECYLAMINE-DTPA (N = 5–6)*

Organ	Ga-67 liposomes	Ga-67 octadecylamine-DTPA	Tc-99m liposomes	Tc-99m octadecylamine-DTPA
Liver	34.7 (3.9)	2.3 (1.0)	23.9 (2.2)	18.4 (1.4)
Heart	0.95 (0.17)	2.6 (1.4)	1.3 (0.2)	1.3 (0.2)
Lung	8.9 (1.8)	3.5 (0.7)	4.8 (0.5)	2.7 (0.5)
Kidney	0.85 (0.16)	3.4 (0.9)	3.3 (0.6)	11.7 (4.6)
Spleen	72.8 (8.8)	3.4 (1.1)	80.9 (10.5)	4.5 (0.9)
Brain	0.04 (0.12)	0.46 (0.18)	0.22 (0.04)	0.16 (0.09)
Muscle	0.26 (0.06)	2.7 (1.2)	0.22 (0.04)	0.69 (0.12)
Bone	0.80 (0.17)	9.8 (3.2)	2.4 (1.8)	2.9 (0.4)
Blood	5.5 (0.36)	7.8 (1.5)	13.3 (2.2)	5.1 (0.5)

* % dose/g normalized to 25-g mouse, one s.d. of the mean in parentheses.

that provides effective chelation is related to the level of trace metals in each preparation. We have observed reduced labeling efficiency with certain batches of Ga-67 acetate, and this we attribute to the presence of increased trace metals in the corresponding Ga-67 trichloride samples. The reduced labeling efficiency of the Tc-99m-labeled octadecylamine-DTPA liposomes, as compared with the Ga-67 product, may also be related to this phenomenon, particularly since stannous ions are purposely introduced at levels that could compete with the reduced Tc-99m for the binding sites.

It is conceivable that if the levels of trace metals in these preparations were substantially reduced, the weight of octadecylamine-DTPA incorporated into the liposomes could also be reduced without affecting labeling efficiency. Since the vast majority of DTPA groups are uncomplexed and negatively charged at neutral pH, their presence will affect liposome charge; so reducing their concentration is advantageous. However, at the level used in this study, the charge introduced by the presence of octadecylamine-DTPA is small. This is apparent from Fig. 1, where the negative charge on the liposome surface was found to increase, as expected, with increasing weight of octadecylamine-DTPA in lecithin liposomes. Even assuming that 100% of liposomes free of octadecylamine-DTPA would elute under identical chromatographic conditions, the incorporation of 1% (w/w) octadecylamine-DTPA (molar ratio 16:3:0.2) has resulted in an increased negative charge that is a small fraction of that found with cardiolipin liposomes; these, when containing 1% octadecylamine-DTPA, show only 4% ± 1 elution.

In summary, the use of octadecylamine-DTPA in lecithin liposomes has been shown to provide a product that is efficiently labeled and, once labeled, shows high stability in 50% plasma at 37°C for prolonged periods, relative to conventionally labeled liposomes. Because of the high labeling efficiency, a purification step is not required, and the preparation is therefore by "kit" methods performed in a single vial. In the case of Tc-99m-labeled liposomes, an alternative "kit" preparation has been reported using a labeling procedure similar to that used in this work for the octadecylamine liposome blank studies (*6*). Using this method, we have been unable to achieve a label that is stable by the above criteria.

FOOTNOTES

* Galbraith Laboratories, Inc., Knoxville, TN.

† Bronsonic Model 10-002, 25 watts.

‡ New England Nuclear Corp., North Billerica, MA.

ACKNOWLEDGMENTS

The authors express their appreciation to Linda Carreaux for her assistance in the completion of this manuscript. This work was supported by the National Cancer Institute, Grant No. 1 RO1 CA26968.

REFERENCES

1. ESPINOLA LG, BEAUCAIRE J, GOTTSCHALK A, et al: Radiolabeled liposomes as metabolic and scanning tracers in mice. II. In-111 oxine compared with Tc-99m DTPA, entrapped in multilamellar lipid vesicle. *J Nucl Med* 20:434–440, 1979

2. HNATOWICH DJ, CLANCY B: Investigations of a new, highly negative liposome with improved biodistribution for imaging. *J Nucl Med* 21:662–669, 1980

3. ECKELMAN WC, KARESH SM, REBA RC: New compounds: Fatty acid and long chain hydrocarbon derivatives containing a strong chelating agent. *J Pharmacol Sci* 64:704–706, 1975

4. HNATOWICH DJ, SCHLEGEL P: Albumin microspheres labeled with Ga-67 by chelation. Concise communication. *J Nucl Med* 22:623–626, 1981

5. FINKELSTEIN MC, WEISSMAN G: Enzyme replacement via liposomes. Variations in lipid composition determine liposomal integrity in biological fluids. *Biochem Biophys Acta* 587:202–216, 1979

6. RICHARDSON VJ, JEYASINGH K, JEWKES RF, et al: Possible tumor localization of Tc-99m-labeled liposomes: Effects of lipid composition, charge, and liposome size. *J Nucl Med* 19:1049–1054, 1978