*Biochimica et Biophysica Acta*, 1148 (1993) 77–84



BBAMEM 75966

# Polymer-derivatized technetium $^{99m}$Tc-labeled liposomal blood pool agents for nuclear medicine applications

Colin Tilcock [a], Quet Fah Ahkong [a] and Derek Fisher [b]

[a] *Department of Radiology, University of British Columbia, Vancouver, B.C. (Canada) and* [b] *Department of Biochemistry and Molecular Cell Pathology Laboratory, Royal Free Hospital School of Medicine, London (UK)*

(Received 8 July 1992)
(Revised manuscript received 29 January 1993)

Key words: Liposome; Poly(ethylene glycol); Circulation half-life; Technetium; Indium

By using the lipophilic chelator, dipalmitoylphosphatidylethanolamine-diethylenetriaminetetraacetic acid (DPPE-DTTA), lipid vesicles may be prepared labeled on their surface with technetium 99*m*. When technetium-labeled vesicles were injected intravenously into rabbits, the half-life for clearance of the label from the circulation was less than 30 min. By further incorporating a synthetic phosphatidylethanolamine-monomethoxypoly(ethylene glycol) 5000 conjugate (PE-MPEG) the circulation half-life of the radiolabel was increased, liver uptake decreased and exchange of technetium from the vesicle surface suppressed, depending upon both the DPPE-DTTA and PE-MPEG content. For vesicles containing 20 mol% DPPE-DTTA, incorporation of PE-MPEG had no effect upon the circulation half-life of the radiolabel, however, for vesicles containing 2 mol% DPPE-DTTA, incorporation of more than 4 mol% PE-MPEG increased the circulation half-life of the label to more than 12 h. Less than 2 mol% PE-MPEG or 8 mol% ganglioside GM1 were, however, ineffective at increasing the circulation half-life of surface-bound technetium. It was shown that unilamellar lipid vesicles with DPPE-DTTA can be lyophilized in the presence of external sucrose, subsequently rehydrated with no change in vesicle size and labeled with technetium. It is suggested that polymer-derivatized, technetium-labeled vesicles may prove a useful substitute for technetium-labeled red blood cells as a vascular marker in various nuclear medicine procedures and that lyophilization/rehydration provides a possible route to realization of such vesicles in a pharmaceutically useful form.

## Introduction

Technetium-99*m* labeled autologous red blood cells (RBCs) are routinely used in various nuclear medicine procedures such as the measurement of cardiac ejection fraction or detection of left-right shunts, determination of total RBC mass or in the detection of vascular malformations [1,2]. While in principle cardiac-gated, flow-sensitive or rapid-scan magnetic resonance techniques [3] could either complement or even supplant nuclear medicine for certain of these procedures, the continued use of radiolabeled RBCs would seem assured not least because RBCs possess one important pharmacokinetic property, namely, when undamaged, they have a half-life in the circulation of approx. 60 days [4]. Radiolabeled RBCs are therefore excellent markers of the vasculature, a property that is exploited when using technetium-labeled RBCs for the detection of, e.g., gastrointestinal bleed sites [5].

Correspondence to: C. Tilcock, Department of Radiology, University of British Columbia, 2211 Wesbrook Mall, Vancouver, B.C., Canada V6T 2B5.

When introduced into the circulation, lipid vesicles, by virtue of their size, can only be cleared from the circulation at sites of fenestrated or discontinuous epithelia, and so are taken up by the reticuloendothelial system (RES) and delivered principally to liver, spleen and, depending upon vesicle size, to bone marrow. The rate of clearance from the circulation is highly variable depending upon many factors including vesicle size, charge, lipid composition and total lipid dose [6–9], with the general trends being that increased vesicle size or the presence of charge favors rapid clearance and RES uptake. Increased lipid saturation increases the vesicle stability in vivo rendering them more resistant to lipid exchange with serum proteins and protecting against loss of internal contents whereas high lipid doses are considered to saturate the RES and hence lead to an extended half-life in the circulation.

Within this context, it is of interest that various studies have indicated that by either incorporating ganglioside GM1 within the vesicle [10,11] or by covalently coupling the neutral polymer polyethylene glycol to the vesicle surface [12–20], the lifetime of the vesicles in the circulation can be greatly extended and uptake by the RES suppressed. The specific goals of

this work were to examine whether ganglioside GM1 or covalently attached polymer could similarly extend the circulation half-life of radiolabeled lipid vesicles where the radionuclide is chelated at the membrane surface, to investigate the effect of both chelate and polymer concentration upon labeling and clearance from the circulation and to examine lyophilization/rehydration as a potential method of long-term storage of lipid vesicles.

## Materials and Methods

Dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylcholine (DPPC) and sphingomyelin (Sph) were obtained from Avanti Polar Lipids (Birmingham, AL). Carrier-free $^{99m}$Tc was obtained from a commercial $^{99}$Mo/$^{99m}$Tc generator (Nordion, Chalk River, ON). Cholesterol (Chol), stannous chloride, ascorbic acid and miscellaneous reagents were obtained from Sigma (St. Louis, MO). Solvents were obtained from BDH (Vancouver, BC).

*Preparation of dipalmitoylphosphatidylethanolamine-diethylenetriaminetetraacetic acid (DPPE-DTTA)*

Following the procedure of Grant et al. [21], dipalmitoylphosphatidylethanolamine (DPPE), 1 g, was dissolved with warming to clarity in 100 ml of anhydrous pyridine. 5 g of DTPA dianhydride was dissolved in 100 ml of anhydrous pyridine and added dropwise with vigorous stirring to the lipid solution. The reaction mixture was refluxed for 70 min, 50 ml of water added, then refluxed for a further 70 min. After cooling, the reaction mixture was rotary evaporated to a film and residual solvent removed under reduced pressure (< 0.01 mmHg) for 4 h. The reaction mixture was dissolved in chloroform/methanol/water/acetic acid (65:25:4:1, v/v) and purified by chromatography on silicic acid.

*Preparation of tresylmonomethoxypoly(ethylene glycol) 5000 (TMPEG)*

Tresylmonomethoxypoly(ethylene glycol) 5000 was prepared from poly(ethylene glycol) 5000 monomethoxyether and tresyl chloride as previously described [22] or by a modification of the procedure of Harris et al. [23] for the preparation of tosyl-PEG as follows. Poly(ethylene glycol) 5000 monomethyl ether (5 g, 1 mmol) was dissolved in dry dichloromethane (10 ml) and cooled to 0°C. Dry triethylamine (357 μl, 2.7 mmol) was added at 0°C followed by tresyl chloride (300 μl, 2.7 mmol) from a freshly opened ampoule. The reaction mixture was stirred for 2 h at 0°C and the product precipitated three times from dry diethyl ether (150 ml). Residual ether was removed by rotary evaporation followed by storage under reduced pressure (< 0.1 mmHg) for 18 h at room temperature.

*Preparation of phosphatidylethanolamine-monomethoxypoly(ethylene glycol) (PE-MPEG)*

Phosphatidylethanolamine-monomethoxypoly(ethylene glycol) (PE-MPEG) was synthesized by a modification of the method of Yoshida and Gote [24], replacing cyanuric acid-activated MPEG with tresyl MPEG as follows. DPPE (138 mg, 200 μmol) and TMPEG (1.65 g, 300 μmol) were dissolved in dry chloroform (20 ml) and refluxed in the presence of sodium carbonate (2 g) until the mixture gave a negative ninhydrin color reaction (15–21 h). The MPEG derivative was precipitated thee times from dry diethyl ether (150 ml) and dried under reduced pressure as above.

*Preparation of unilamellar lipid vesicles for technetium labeling*

Unilamellar lipid vesicles of 100 nm average diameter were prepared as follows. Lipids were combined from stock solutions in chloroform and solvent was removed initially by rotary evaporation then by storage under reduced pressure (< 0.01 mmHg) for at least 2 h. The combined lipids were dispersed at a concentration of either 20 μmol/ml (DPPC/DPPE-DTTA, 8:2 only) or 100 μmol/ml (all other samples), in degassed 0.9% saline by vortex mixing to form multilamellar vesicles (MLVs). These MLVs were freeze-thawed five times in liquid nitrogen then extruded at 60°C by ten passes through two stacked 0.1 μm polycarbonate filters under nitrogen pressure using a thermobarrel extruder device [25]. Vesicles were stored at 4°C until required. Vesicle sizes were determined by quasi-elastic light scattering using a Nicomp model 270 particle sizer (Goleta, CA).

*Technetium-99m labeling of lipid vesicles, separation on minicolumns and stability in plasma*

To 0.5 ml of lipid vesicles prepared above was added 100 MBq of pertechnetate in 0.9% saline derived from a commercial molybdenum generator. Stannous chloride (6.7 mg) was freshly dissolved in 2 ml of degassed 0.9% saline containing 10 mg/ml ascorbic acid and 5 μl of the solution quickly added to the lipid vesicles with pertechnetate. For samples composed of DPPC/DPPE-DTTA (8:2) the stannous chloride was dissolved in 1 ml of degassed saline with ascorbate and 10 μl added to the vesicles with pertechnetate. The mixture was allowed to stand for at least 10 min at room temperature prior to use.

*Ionophoretic loading of pre-formed vesicles with indium-111*

Unilamellar vesicles (100 nm diameter) composed of either Sph/Chol (64:32) or Sph/Chol/PE-MPEG (64:32:4) were prepared by freeze-thaw extrusion as detailed above at a lipid concentration of approx. 50 mM in a buffer containing 250 mM sucrose, 15 mM

DTPA and adjusted to pH 7.4 with arginine. The untrapped buffer was exchanged for external buffer (250 mM sucrose, 20 mM Hepes adjusted to pH 7.4 with arginine) by passing the lipid dispersion through a Sephadex G-50 column pre-equilibrated with the external buffer. The lipid vesicles eluted in the void volume and were diluted with external buffer to a final lipid concentration of approx. 5 mM before mixing with a dry film of the ionophore A23187 (25 mg). After 10 min incubation at 47°C, carrier free $^{111}$In (approx. 20 MBq) was next added to the vesicles and following a subsequent hour at 47°C, the entrapped $^{111}$In was separated from the free $^{111}$In using a 1 ml Bio-Gel A15 minicolumn with external buffer as eluant. The radiolabeled vesicles eluted in the void volume and were used for the imaging studies.

*Imaging*

New Zealand white rabbits (1.5–2 kg) were anesthetized with i.m. ketamine/xylazine (80:10 mg/kg) and injected via the ear vein with radiolabeled vesicles diluted with 0.9% NaCl such that the total injected dose was approx. 30 MBq (corresponding to approx. 20 $\mu$mol of lipid) in an injection volume of 0.2 ml. Planar radionuclide scans were obtained using a Siemens low-energy mobile digital camera equipped with an appropriate collimator. Images were acquired using a 128 by 128 matrix for consecutive one or two minute periods for up to 1 h post-administration. Regions of interest centered upon heart, liver, kidney and bladder were identified and counts from those regions determined with commercial software. Data was normalized by assuming that the counts per pixel in a given region of interest, over the period 1–2 min post-injection, represent 100%.

*Lyophilization*

Lipid vesicles (final concentration 10 mM) were frozen in liquid nitrogen with or without sucrose (final concentration 150 mM) and lyophilized for 16 h at room temperature using a Labconco lyph-lock 4.5 liter bench top freeze dry system. The dry amorphous powder was rehydrated with shaking in distilled water at 37°C before the reconstituted vesicles were sized or labeled with technetium.

## Results

*Vesicles with entrapped indium-111*

Initial experiments were undertaken to demonstrate that the PE-MPEG derivatize synthesized here via a novel tresylation route was effective at increasing the circulation half-life of lipid vesicles. The system chosen was unilamellar lipid vesicles composed of sphingomyelin/cholesterol containing $^{111}$In as gamma emitter. This particular lipid composition was chosen because of studies reporting that vesicles of this composition were stable in the circulation [26]. It was observed that following i.v. administration, neutral Sph/Chol vesicles containing $^{111}$In-DTPA were rapidly cleared from the circulation with a half-life of approx. 15–20 min and delivered to the liver. When the vesicle composition was modified by inclusion of 4 mol% PE-MPEG 5000, the rate of clearance from the circulation was greatly decreased with > 85% of the injected dose still present in the circulation at 30 min post-administration. These results, increased circulation half-life and decreased RES uptake, are entirely consistent with the literature [12–20] and demonstrate that the PE-MPEG conjugates synthesized here were effective at modifying the biodistribution behavior of the vesicles.

*Vesicles with surface-attached technetium-99m. Choice of chelate concentration*

For a variety of reasons including decreased radiation dose to subject, as well as the more pragmatic considerations of reduced cost and ready availability from commercial generators, the use of $^{99m}$Tc would be favored over $^{111}$In for use in a liposomal blood pool agent. Having first demonstrated that our chelate was effective at increasing the circulation half-life, these studies were extended to lipid vesicles labeled with $^{99m}$Tc using a lipophilic chelator (PE-DTTA) incorporated within the lipid matrix of the vesicle.

Initial studies of the effect of covalently-attached polymer upon vesicle labeling and biodistribution were first undertaken using vesicle systems containing 20 mol% of the lipophilic chelator PE-DTTA, in the absence and presence of 4 mol% PE-MPEG 5000. For vesicles in the absence of polymer it was observed that there was a progressive decrease in the signal from the region bounded by the heart and an initial rapid increase in signal from the liver that slowly increased still further over the time course of the measurement. Signal associated with the region bounded by the kidney remained constant over the observation period whereas signal in the bladder increased more than 2-fold over 30 min. Similar distribution behavior was observed when using vesicles containing 20 mol% PE-DTTA and 4 mol% PE-MPEG 5000 and in fact when clearance rates from the heart are compared (Fig. 1), it is evident that within experimental error, the presence of polymer on the membrane surface made no measurable difference to the initial clearance rates of the vesicles from the circulation, a result apparently in disagreement with the results already discussed for vesicles with entrapped $^{111}$In.

Because the chelator PE-DTTA is negatively charged under the experimental conditions and, given previous observations that the presence of charge on a vesicle surface facilitates RES uptake [6,9], it was considered that the presence of the relatively large amount

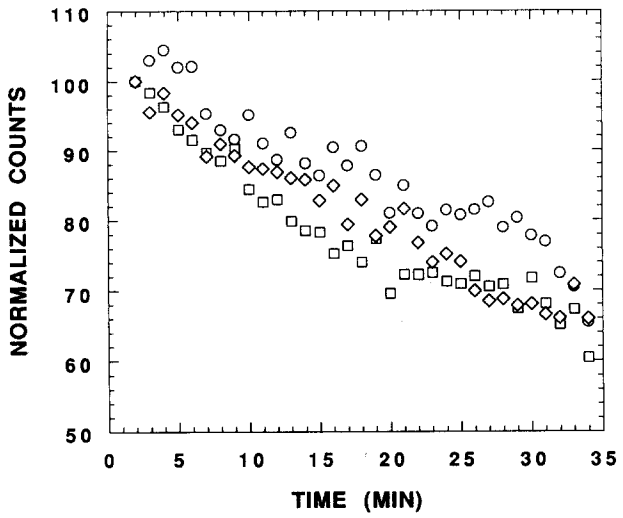


Fig. 1. Clearance of $^{99m}$Tc-labeled 100-nm diameter vesicles from the circulation. Results are normalized by assuming counts in the region bounded by the heart in the period 1–2 min after i.v. administration to represent a value of 100%. Results are shown for (○) DPPC/PE-DTTA (80:20) without surface polymer and (□,◇) two separate DPPC/PE-DTTA/PE-MPEG (76:20:4) vesicle preparations in different animals. Data are corrected for the decay of the isotope over the time-course of the measurement.

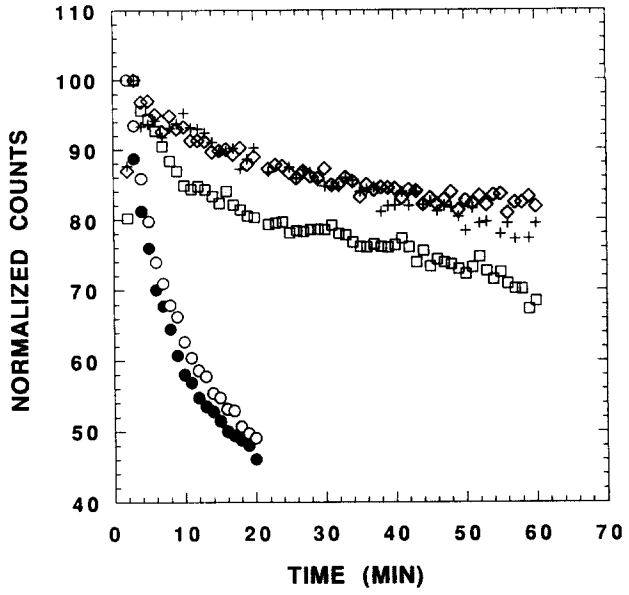


Fig. 2. Distribution of $^{99m}$Tc-labeled 100-nm diameter vesicles in the circulation following i.v. administration. Results are normalized by assuming counts in the region bounded by the heart in the period 1–2 min after i.v. administration to represent a value of 100%. Results are shown for 100-nm diameter vesicles composed of (●) DPPC/DPPE-DTTA (98:2) without surface polymer, (○) DPPC/DPPE-DTTA/GM1 (90:2:8), (□) DPPC/DPPE-DTTA/PE-MPEG (96:2:2), (◇) DPPC/DPPE-DTTA/PE-MPEG (94:2:4) and (+) DPPC/DPPE-DTTA/PE-MPEG (92:2:6). Data are corrected for the decay of the isotope over the period of the measurement.

of charge conferred by 20 mol% DPPE-DTTA was perhaps dominating the biodistribution behavior, despite the presence of polymer on the vesicle surface. Because steric packing constraints limit the amount of polymer that can be incorporated within the membrane [14,27], rather than increase the polymer content of the vesicles it was decided to decrease the surface charge by reducing the content of chelator within the membranes arbitrarily from 20 to 2 mol% of the lipid composition. In light of previous studies indicating that ganglioside GM1 can markedly increase the circulation half-life of lipid vesicles [10,11], we compared the efficacy of GM1 and PEG in this application.

*Effect of ganglioside GM1 and surface-bound polymer on the biodistribution of $^{99m}$Tc-labeled vesicles*

The effect of incorporation of either ganglioside GM1 or PE-PEG 5000 within the vesicles upon the clearance from the circulation is illustrated in Fig. 2. It was observed that for vesicles containing 2 mol% DTTA but no polymer, the vesicles cleared from the circulation with a half-life of approx. 20 min. Very similar clearance kinetics were observed for vesicles containing 8 mol% ganglioside GM1. However, for vesicles containing 2 or more mol% PE-PEG 5000 the rate of clearance of the vesicles from the circulation was greatly decreased. Assuming a monoexponential decrease with time the calculated half-life for clearance for the system containing 2 mol% PE-PEG was approx. 3 h, and for vesicles containing 4 or 6 mol% PE-PEG 6000, the calculated half-lives were greater than 12 h (see also Table I). Calculated half-lives for vesicles of various composition at Sn/DTTA ratios of 0.36 and 0.72 are presented in Table I. The efficiency of labeling is critically dependent upon the Sn/DTTA ratio. Too high a Sn/DTTA ratio results in excessive formation of stannous and technetium hydroxides, whereas too low a Sn/DTTA ratio causes incomplete reduction of added pertechnetate (results not shown). In both cases

TABLE I

*Effect of PE-MPEG content and Sn/DTTA ratio on the circulation half-life in rabbits of $^{99m}$Tc-labeled 100-nm vesicles containing 2 mol% PE-DTTA*

| % PE-PEG | Sn/DTTA ratio | Half-life (h) [a] |
|---|---|---|
| 0 | 0.36 | $0.5 \pm 0.2$ ($n = 5$) |
| 2 | 0.36 | 3.2, 2.6 |
| 2 | 0.72 | $0.9 \pm 0.3$ ($n = 3$) |
| 4 | 0.36 | $> 12$ ($n = 3$) |
| 4 | 0.72 | 2.6, 2.2 |
| 5 | 0.36 | $> 12$ ($n = 1$) |
| 6 | 0.36 | $> 12$ ($n = 3$) |
| 6 | 0.72 | $1.7 \pm 0.4$ ($n = 3$) |

[a] Half-lives were calculated assuming a monoexponential clearance from the circulation and are corrected for the decay of the isotope. Results are presented as mean ± S.D. ($n$ = number of samples) where appropriate.

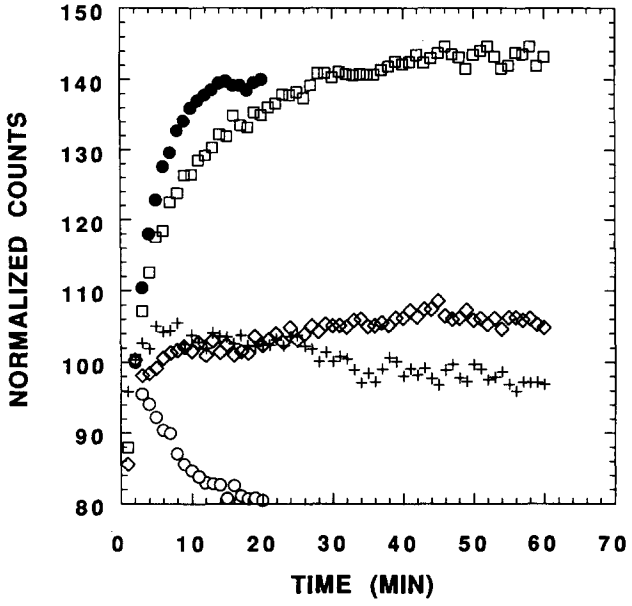


Fig. 3. Distribution of $^{99m}$Tc-labeled 100-nm diameter vesicles in the liver following i.v. administration. Results are normalized by assuming counts in the region bounded by the liver in the period 1–2 min after i.v. administration to represent a value of 100%. Results are shown for 100-nm diameter vesicles composed of (●) DPPC/DPPE-DTTA (98:2) without surface polymer, (○) DPPC/DPPE-DTTA/GM1 (90:2:8), (□) DPPC/DPPE-DTTA/PE-MPEG (96:2:2), (◇) DPPC/DPPE-DTTA/PE-MPEG (94:2:4) and (+) DPPC/DPPE-DTTA/PE-MPEG (92:2:6). Data are corrected for the decay of the isotope over the time-course of the measurement.

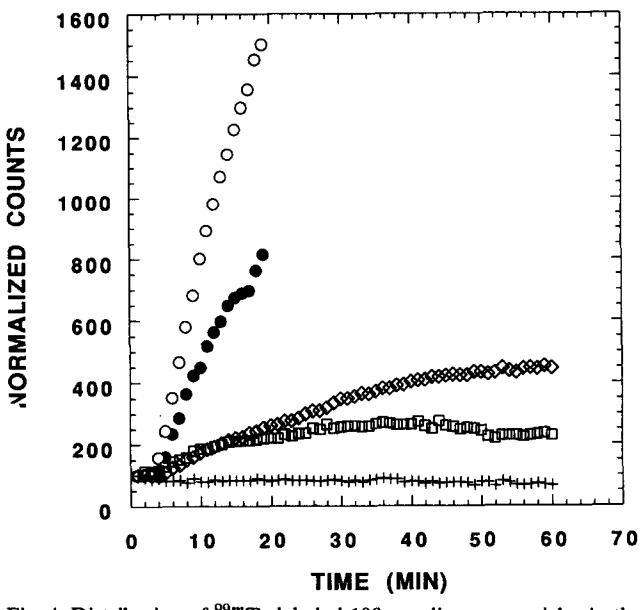


Fig. 4. Distribution of $^{99m}$Tc-labeled 100-nm diameter vesicles in the bladder following i.v. administration. Results are normalized by assuming counts in the region bounded by the bladder in the period 1–2 min after i.v. administration to represent a value of 100%. Results are shown for 100-nm diameter vesicles composed of (●) DPPC/DPPE-DTTA (98:2) without surface polymer, (○) DPPC/DPPE-DTTA/GM1 (90:2:8), (□) DPPC/DPPE-DTTA/PE-MPEG (96:2:2), (◇) DPPC/DPPE-DTTA/PE-MPEG (94:2:4) and (+) DPPC/DPPE-DTTA/PE-MPEG (92:2:6). Data are corrected for the decay of the isotope over the period of the measurement.

inefficient labeling and shortened circulation half-lives result.

The presence of the polymer on the membrane surface not only extends the half-life of the vesicles in the circulation but also modifies the biodistribution behavior of the vesicles as illustrated in Fig. 3. For both vesicles without polymer and those containing 2% PE-PEG 5000 it was observed that the counts associated with the liver increased significantly over the time course of the measurement presumably reflecting RES uptake whereas for vesicles prepared with greater amounts of PEG (4 or 6%) the increase in counts associated with the liver was much less. For vesicles containing ganglioside GM1 there was actually a decrease in counts associated with the liver over 20 min. It is likely that GM1 counts seen in the liver at early time points are actually due to liposomes in the blood content of the liver and not due to liposomes taken up by the liver. The reason for the decrease in GM1 counts with time most probably reflects the rapid renal clearance of technetium as illustrated in Fig. 4.

The clearance of counts to the bladder of the animals is presented in Fig. 4 which shows that the loss of technetium and subsequent renal clearance was greatest for vesicles containing ganglioside GM1 and that with increasing polymer content, renal clearance was progressively suppressed. For vesicles containing 6 mol% PEG there was no discernible renal clearance over 1 h post administration.

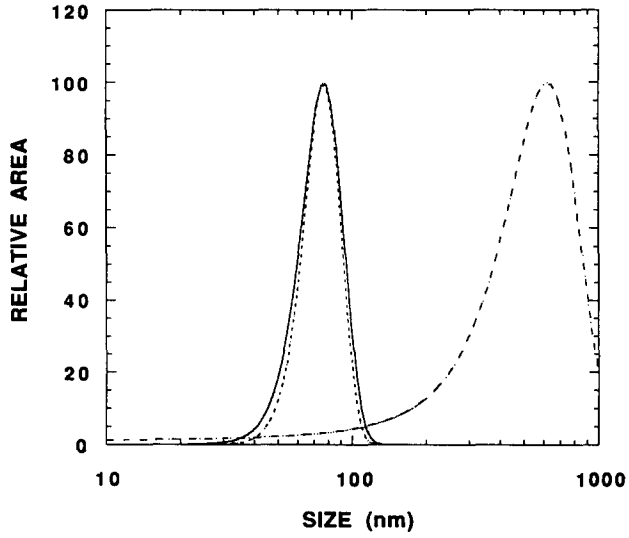


Fig. 5. The effect of lyophilization and rehydration upon the size of unilamellar lipid vesicles. Shown are quasi-elastic light scattering analyses of (———) DPPC/DPPE-DTTA (80:20) vesicles before lyophilization, and after lyophilization and subsequent rehydration in (— — —) the absence of sucrose and (- - - - -) the presence of 150 mM external sucrose.

*Lyophilization and rehydration of lipid vesicles*

Fig. 5 shows the effect of lyophilization and rehydration upon the average diameter and size distribution of lipid vesicles containing 2 mol% PE-DTTA both in the absence and presence of 150 mM external sucrose. In the absence of sucrose and upon subsequent rehydration, it was observed that the size of the vesicles had increased markedly, presumably as a result of vesicle fusion [28]. However, when lyophilized in the presence of external sucrose and subsequently rehydrated, it was observed that there was essentially no change in either the average size or size distribution of the vesicles. Significantly, the presence of sucrose on the vesicle exterior had no effect on the subsequent binding of technetium (results not shown).

## Discussion

The results presented here clearly demonstrate the principle that polymer-derivatization of the membrane surface can extend the circulation half-life of technetium-labeled vesicles, without recourse to RES blockade, when using a lipophilic chelator to anchor the radionuclide at the membrane surface. The presence of the polymer extends the circulation half-life, modifies the biodistribution pattern suppressing liver uptake and apparently protects against loss of technetium from the membrane surface when injected intravenously. Interestingly, ganglioside GM1, which has been previously shown to increase the circulation half-life of lipid vesicles [10,11] was not effective in this application. In part this may be due to the fact that GM1 presents less of a steric barrier than PEG to the binding of plasma proteins [14,18,27] against which $^{99m}$Tc at the vesicle surface may exchange. Additionally it may be considered that the reduced pertechnetate binds to the *N*-acetylneuraminic acid residue of the GM1 headgroup, but that the association is weak resulting in rapid clearance of $^{99m}$Tc from the vesicle surface in the circulation.

For the purposes of a blood pool agent, it is not necessary that the biodistribution of the lipid vesicle and label be the same, it is only important that the label remain in the circulation for a sufficiently long period. To be effective as a vascular marker it would only be necessary that the label exhibit a half-life in the circulation of approx. 12 h. This would be quite adequate for a blood pool imaging agent because the limitation is not the half-life of the label in the circulation, but rather the decay of the technetium. By six half-lives (36 h for technetium) counts will have decayed to approx. 1% of the original amount, thus even for technetium-labeled RBCs which have a 60-day half-life, there is little point in imaging the patient later than a day post-administration. Results so far would indicate that for $^{99m}$Tc-labeled polymer-derivatized vesicles, a 12 h half-life is readily attainable (Table I), consistent with literature values for the circulation half-life of other polymer derivatized vesicle systems [12–20].

For the particular lipophilic negatively-charged chelator PE-DTTA, whether or not surface-bound polymer is effective at extending the circulation half-life depends upon the composition of the membrane. When the PE-DTTA content is 20 mol%, covalently attached MPEG does not extend the circulation half-life. It is conjectured that at this PE-DTTA content, the presence of the polymer on the vesicle cannot mask the negative charge of the PE-DTTA at the membrane surface, which dominates the biodistribution characteristics of the vesicles in a manner analogous to previous studies of PEG on negatively charged phosphatidylserine vesicles [16]. However, when the PE-DTTA content is 2 mol%, surface MPEG extends the circulation half-life from $< 0.5$ h to $> 12$ h depending upon the polymer content.

Both in vitro and in vivo studies indicate that selection of the appropriate reducing conditions is critical to efficient and stable labeling and that for these systems, a Sn/DTTA ratio of 0.36 : 1 worked best. These results also suggest that between 4 and 6 mol% of the MPEG-5000 should be included within the vesicle composition to both maximize circulation half-life, minimize liver uptake and minimize technetium excretion. The choice of polymer molecular weight chosen for this study was made upon the basis of two criteria, ready availability of the starting monomethoxy derivative and ease of purification of the tresyl derivative by precipitation. However, there is no reason in principle why other molecular weights of polymer should not be used although it is reasonable to suggest on the basis of previous studies [17,27] that loss of $^{99m}$Tc from the vesicle surface would be best prevented by using a polymer which provides the most effective steric barrier to close approach of plasma components. Thus it is probable that for poly(ethylene glycol), molecular weights greater than approx. 2000–5000 would be favored in this application.

Preliminary studies suggest that lyophilization of the vesicles with surface chelator may be a valid approach to long term storage. By addition of sucrose to the vesicle exterior, upon subsequent rehydration no significant change in vesicle dimension was observed. Sucrose would be innocuous within the clinical context and importantly, does not appear, at least in preliminary studies, to compete with surface chelator for technetium. This raises the distinct possibility of preparing lyophilized vesicles with external sucrose, reducing agent and perhaps antioxidant, ready for addition of pertechnetate in saline from a generator. Rehydration with swelling of the lipid vesicles and labeling of the vesicle surface occur simultaneously

within 5–10 min after which the agent would be ready for injection. In previous studies of rehydration of vesicles with sugars it has been noted that to protect against loss of internal contents it is necessary to prepare vesicles in the presence of the sugars so that the sugar is present on both the interior and exterior of the vesicle [28]. For vesicles with lipophilic chelator, the radiolabel is on the membrane surface and not trapped within the vesicle, loss of internal contents is therefore not a consideration.

In general terms, in designing radiolabeled vesicles as a marker of blood pool there are two approaches. Either the radiolabel may be entrapped within the vesicle interior or else attached to the vesicle surface; each approach has unique merits. By attaching the radionuclide to the membrane surface, preparation of the vesicle is simplified in that binding to the membrane surface is quantitative and rapid, there is no non-entrapped chelating agent to be removed prior to radiolabeling, there is no material to be lost if the vesicles are ruptured in the circulation and additionally, lyophilization/rehydration can be readily achieved by addition of sucrose to the exterior of pre-formed vesicles, i.e., there are no viscosity problems associated with vesicle preparation because the sugar is added afterwards. However, the disadvantage of this surface-chelation approach is that the radionuclide is completely exposed to interaction with plasma components and can be exchanged away from the membrane surface.

By entrapping the radionuclide inside the vesicle, interaction with circulating proteins or lipoproteins is essentially eliminated so long as the vesicle remains intact. Complete retention of internal contents for extended times in the circulation is, however, not a trivial goal [9]. Preparation of the vesicles with entrapped label is also technically more complex than surface-chelation. Ionophoretic loading techniques that have been developed for the transport of various radioactive cations such as $^{111}In$ or $^{67}Ga$ across a lipid bilayer [29,30] are not appropriate to technetium because of non-specific binding of the reduced pertechnetate to the membrane surface and subsequent rapid removal in plasma (unpublished results). However, it is quite feasible to transport pertechnetate across the lipid bilayer using hexamethylpropyleneamineoxime (HMPAO). This approach has been used to radiolabel lipid vesicles with entrapped hemoglobin as a deposition marker of this blood substitute [31] but the present high cost of HMPAO would make this method commercially unattractive as a means of preparing a blood pool agent.

Lastly, although the emphasis of this work has been the design of radiolabeled nuclear medicine blood pool agents, exactly the same methodologies and procedures would be applicable to the design of long-lived vesicle-based perfusion agents for magnetic resonance imaging applications, where instead of using a radioactive cation, a paramagnetic metal such as Mn or Gd could be chelated to the vesicle surface.

## Acknowledgments

This work was supported in part by the Medical Research Council of Canada and by travel grants from the British Council and NATO. We thank Terri Rihela for technical assistance and Dr. Don Lyster for valuable criticism. C.T. is an MRC Scholar.

## References

1 Strauss, H.W. and Pitt, B. (1978) Circulation 59, 645–664.
2 Swanson, D.P. (1990) in Pharmaceuticals in Medical Imaging (Swanson, D.P., Chilton, H.M. and Thrall, J.H., eds.), pp. 616–643, Macmillan, New York.
3 Wehrli, F.W. (1991) Fast-scan magnetic resonance. Principles and Applications, Raven Press, New York.
4 Ness, P.M. and Stengle, J.M. (1974) in The Red Blood Cell (Surgenor, D.M., ed.), pp. 22–24, Academic Press, New York.
5 Winzelberg, G.G., McKusick, K.A., Froelich, G., Callahan, R.J. and Strauss, H.W. (1982) Semin. Nucl. Med. 12, 129–146.
6 Juliano, R.L. and Stamp, D. (1975) Biochem. Biophys. Res. Commun. 63, 651–658.
7 Abra, R.M. and Hunt, C.A. (1981) Biochim. Biophys. Acta 666, 493–503.
8 Hwang, K.J. (1987) in Liposomes: From Biophysics to Therapeutics (Ostro, M., ed.), pp. 109–156, Marcel Dekker, New York.
9 Senior, J. (1987) Crit. Rev. Ther. Drug. Carrier Syst. 3, 123–193.
10 Allen, T.M. and Chonn, A. (1987) FEBS Lett. 223, 42–46.
11 Gabizon, A. and Papahadjopoulos, D. (1988) Proc. Natl. Acad. Sci. USA 85, 6949–6953.
12 Klibanov, A.L., Muruyama, K., Torchilin, V.P. and Huang, L. (1990) FEBS Lett. 268, 235–238.
13 Blume, G. and Cevc, G. (1990) Biochim. Biophys. Acta 1029, 91–97.
14 Allen, T.M., Austin, G.A., Chonn, A. Lin, L. and Lee, K.C. (1991) Biochim. Biophys. Acta 1061, 56–64.
15 Senior, J., Delgado, C., Fisher, D., Tilcock, C. and Gregoriadis, G. (1991) Biochim. Biophys. Acta 1062, 77–82.
16 Klibanov, A.L., Maruyama, K., Beckerleg, A.M., Torchilin, V.P. and Huang, L. (1991) Biochim. Biophys. Acta 1062, 142–148.
17 Allen, T.M., Hansen, C., Martin, F., Redemann, C. and Yau-Young, A. (1991) Biochim. Biophys. Acta 1066, 29–36.
18 Papahadjopoulos, D., Allen, T.M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S.K. Lee, K.-D., Woodle, M.C., Lasic, D.D., Redemann, C. and Martin, F.J. (1991) Proc. Natl. Acad. Sci. USA 88, 11460–11464.
19 Mori, A., Klibanov, A.L., Torchilin, V.P. and Huang, L. (1991) FEBS Lett. 284, 263–266.
20 Woodle, M.C. and Lasic, D.D. (1992) Biochim. Biophys. Acta 1113, 171–199.
21 Grant, C.W., Karlik, S. and Florio, E. (1989) Magn. Reson. Med. 11, 236–243.
22 Delgado, C., Patel, J.N., Francis, G.E. and Fisher, D. (1990) Biotechnol. Appl. Biochem. 12, 199–128.
23 Harris, J.M., Struck, E.C., Case, M.G., Paley, M.S., Yalpani, M., Van Alstine, J.M. and Brooks, D.E. (1984) J. Polym. Sci. Polymer Chemistry Edn. 22, 341–352.
24 Yoshida, H. and Gote, H. (1990) EPA 0 354 855 A2.

25 Mayer, L.D., Hope, M.J. and Cullis, P.R. (1986) Biochim. Biophys. Acta 858, 161–168.
26 Hwang, K.J., Luk, K.S. and Baumier, P. (1980) Proc. Natl. Acad. Sci. USA 77, 4030–4034.
27 Yoshioka, H. (1991) Biomaterials 12, 861–864.
28 Madden, T.C., Bally, M.B., Hope, M.J., Cullis, P.R., Schieren, H.P. and Janoff, A.S. (1985) Biochim. Biophys. Acta 817, 67–74.
29 Mauk, M. and Gamble, R.C. (1979) Anal. Biochem. 94, 302–307.
30 Baumier, P.L. and Hwang, K.J. (1982) J. Nucl. Med. 23, 810–815.
31 Rudolph, A.S., Klipper, R.W., Goins, B. and Phillips, W.T. (1991) Proc. Natl. Acad. Sci. USA 88, 10976–10980.