Neutral and Anionic Liposome-Encapsulated Hemoglobin: Effect of Postinserted Poly(ethylene glycol)-distearoylphosphatidylethanolamine on Distribution and Circulation Kinetics


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ABSTRACT

To prepare long-circulating liposomes, poly(ethylene glycol) (PEG)-lipid is usually mixed with other lipid components before vesicle formation. PEG-lipids can also be postinserted in the outer layer of liposomes after the preparation. In this study, PEG-distearoylphosphatidylethanolamine was incorporated by postinsertion technique into liposome-encapsulated hemoglobin (LEH) carrying neutral or negative charge. Postinsertion technique improved the encapsulation efficiency of hemoglobin from about 0.0017 to 0.017 (hemoglobin/phospholipid, molar ratio) for a similar lipid composition. Thus, neutral, anionic, PEG-neutral, and PEG-anionic LEHs were made and labeled with technetium-99m to follow their biodistribution. A small dose of LEH (~15 mg of phospholipid) was injected intravenously in rabbits, and its distribution was monitored by blood sampling, gamma camera imaging, and tissue radioactivity counting on necropsy. The 24-h blood levels of neutral, PEG-neutral, anionic, and PEG-anionic LEHs were 14, 40.3, 13.1, and 35.7% of injected dose, respectively; calculated \( T_{1/2} \) values of circulation were 8.9, 19.3, 9.6, and 16.5 h, respectively. PEGylation also influenced accumulation of LEH in the reticuloendothelial system. Liver uptake of neutral LEH dropped from 52.1 to 19.1%, whereas that of anionic LEH came down from 35.3 to 11.5% on PEGylation. In contrast, PEGylation increased the spleen uptake by 8.5- and 2.5-fold for neutral and anionic LEH, respectively. The results demonstrate that PEGylation by postinsertion not only improves the circulation \( T_{1/2} \) of LEH but also enhances hemoglobin content inside the vesicles for better oxygen-carrying capacity.

Hemoglobin vesicles or liposome-encapsulated hemoglobin (LEH) is being examined as an oxygen carrier that mimics membrane enclosed cellular structure of red blood cells (Rudolph, 1995; Sakai et al., 1996; Phillips et al., 1999). Compared with encapsulated hemoglobin, free unmodified hemoglobin has low oxygen carrying capacity and is rapidly eliminated from the body. Polymerized, cross-linked, or polymer-conjugated hemoglobins have improved biological behavior, but the problems of cytotoxicity and vasoconstrictive activity are still not fully resolved (Reiss, 2001; Squires, 2002). Second generation hemoglobin-based oxygen carriers have, however, limited toxicity as exemplified by PEG-hemoglobin (Vandegriff et al., 2003). On the other hand, LEH is characterized by spatial isolation of hemoglobin by an oxygen-permeable lipid layer that eliminates the toxicity associated with free modified or unmodified hemoglobin. In addition, coencapsulation of reductants, antioxidative enzymes, and oxygen-affinity modifiers inside liposomes is readily possible to enhance resuscitative capacity of LEH. Despite these desirable properties, a major impediment in the development of LEH has been the low encapsulation efficiency of hemoglobin inside the vesicles. To increase the encapsulation of proteins inside liposomes, anionic lipids, such as dimyristoyl phosphatidylglycerol (DMPG) and dipalmitoylphosphatidylglycerol (DPPG) are usually incorporated in the lipid composition (Drummond et al., 1999; Walde and Ichikawa, 2001). However, anionic liposomes rapidly interact with the biological system subsequent to their opsonization with complement and other circulating proteins (Harashima et al., 1998; Miller et al., 1998; Semple et al., 1998; Szebeni, 1998). Such an interaction has at least two acute consequences: a rapid uptake by the reticuloendothelial system (RES), and toxic effects, such as...
pseudoallergy that is manifested as vasoconstriction, pulmonary hypertension, dyspnea, and drop in circulating platelets and leukocytes. Because these reactions are mostly dependent on lipid dose, the problem becomes more challenging when huge quantities of liposomes need to be administered (as in the case of resuscitative LEH). Thus, a conflict occurs between the necessity to encapsulate maximum amounts of hemoglobin in the least amount of lipid using anionic lipids and to keep the charge-associated undesirable effects in check.

One way to circumvent the physiological response to liposome administration is to conceal the liposome surface with hydrophilic polymers by incorporating polyethylene glycol-linked phosphatidylethanolamines (PEG-PE) in the bilayer structure. Inclusion of PEG-PE improves storage stability, remarkably reduces RES uptake, and decreases dependence on small size to achieve prolonged circulation of liposomes. It is believed that a hydrophilic PEG coating on the liposome surface creates a steric barrier, enabling liposomes to circulate longer (Torchilin and Papisov, 1994). Secondary to the steric hindrance, inhibition of liposome-induced complement activation may also be partly responsible for the beneficial effects of PEG-PE (Ahl et al., 1997; Devine and Bradley, 1998).

Incorporation of PEG-PE in the liposome bilayer is most easily done when preparing lipid phase just before its hydration with an aqueous phase. However, this technique results in the PEG brush or mushroom occupying space inside the liposomes. Theoretically, in a liposome with size of 200 nm and a PEG brush of 5 nm, there is a net reduction of ~15% space available for the encapsulated drugs. The smaller the size or the greater the lamellarity of the liposomes, the greater is the impact of PEG on total usable space for encapsulated material. In addition, the same steric hindrance that makes PEG useful may inhibit the encapsulation of substances by exclusion phenomenon (Nicholas et al., 2000). Realization of these problems has led to the development of a technique where PEG-PE is inserted in the outer layer of liposomes after they have undergone final manufacturing stages (Uster et al., 1996; Sakai et al., 1997; Sakai et al., 2000). Such a technique, called postinsertion, is especially useful in the case of LEH. As a part of optimization of LEH formulation, in this article we present results of inserting PEG-DSPE in the outer lipid layer of LEH and the in vivo implications of PEGylation of neutral and anionic LEH in rabbits.

Materials and Methods

The phospholipids, distearoylphosphatidylcholine (DSPC), DMPG, and poly(ethylene glycol)5000-distearoylphosphatidylethanolamine (PEG5000-DSPE) were obtained from Avanti Polar Lipids (Pelham, AL). Cholesterol (Chol) was purchased from Calbiochem (La Jolla, CA), and α-tocopherol was purchased from Aldrich (Waukegan, IL). Glutathione, octyl-β-glucoside, and pyridoxal-5'-phosphate were from Sigma-Aldrich (St. Louis, MO). The radiopharmaceutical, 99mTc-sodium pertechnetate, was a kind gift from U.S. Army (Walter Reed Army Institute of Research, Biological Resources Division, Washington, DC). Because carboxyl-hemoglobin (CO-Hb) is more stable than the O₂-Hb and can tolerate processing at elevated temperature (~55°C) and shear, CO-Hb was used in LEH manufacturing. Immediately after thawing O₂-Hb was carbonylated with carbon monoxide (CO) under aseptic conditions (Sakai et al., 1997).

Preparation of LEH. LEHs (DSPC/Chol/α-tocopherol, 51:4:4.6:2.2 and DSPC/Chol/DMPG/α-tocopherol, 46:42:9:8:2.2) were prepared by microfluidization technique. Briefly, a solution of lipids in chloroform was evaporated to a dry film in a rotary film evaporator (Brinkmann Instruments, Westbury, NY). After further drying the lipid film for 4–6 h under vacuum, the dried lipid film was hydrated with sterile water for injection. The suspension was lyophilized overnight, and the dried mixture was again hydrated with 38% solution of CO-hemoglobin containing glutathione (100 mM) and pyridoxal-5’ phosphate (18 mM). The mixture was thoroughly mixed at room temperature to form a homogeneous suspension of large liposomes. The particle size of the liposomes was reduced in a Microfluidizer (M110-T; Microfluidics Corp., Newton, MA) by passing the suspension 10 times through a 200-μm interaction chamber. The bulk of the unencapsulated material was separated from LEH by tangential ultrafiltration through a 300-kDa cartridge (Millipore Corporation, Bedford, MA) using phosphate-buffered saline (PBS, pH 7.4) as a wash fluid. After filtration, the neutral and negative LEH preparations were each divided into two equal halves. One-half of each preparation was PEGylated by postinsertion, whereas the other half was further processed without PEGylation. For PEGylation, 1% (w/v) aqueous solution of PEG5000-DSPE (50 ml for each 100 ml of LEH batch) was added to a dilute suspension of LEH, such that the concentration of PEG5000–DSPE was below its critical micelle concentration (Sou et al., 2000). The mixture was stirred for 1 h at 55°C under CO atmosphere to enable insertion. The insertion of PEG5000–DSPE into the outer layer of LEH was monitored by the assay reported earlier (Shimada et al., 2000). Approximately 28% of the added PEG5000–DSPE was incorporated into the bilayer. To convert the encapsulated CO-Hb back to O₂-Hb, the PEGylated and non-PEGylated LEHs were exposed to bright visible light from a 500-W halogen lamp under saturating oxygen atmosphere at 4–8°C (Sakai et al., 1996). To concentrate, the preparations were centrifuged in an LE-80L ultracentrifuge (Beckman Coulter, Fullerton, CA) at 184,000g for 45 min to obtain LEH pellets. The pellets were washed twice with PBS (pH 7.4) and finally, resuspended in PBS (pH 7.4).

Characterization of LEHs (Table 1). The phospholipid concentration of the liposomes was determined by the method of Stewart (1980). The oxygen affinity (p50) of encapsulated hemoglobin was measured using a Hemoximeter (TCS Scientific Corp., Cortland, NY). Methemoglobin content was measured by a cyanomethemoglobin method (Matsuoka, 1997). The size of the liposomes was determined by photon correlation spectroscopy using a Brookhaven particle size analyzer equipped with argon laser, BI-9000AT digital correlator, and BI-200SM goniometer (Holtsville, NY). Each sample was sized for 2 min with detector at 90° angle and sample housed in a 25°C bath. The data were analyzed by non-negatively constrained least-squares (CONTIN) using dynamic light scattering software 9KDLSW, beta version 1.24, supplied with the instrument.

Radiolabeling of LEH. LEHs were labeled essentially by the method developed by Phillips et al. (1992). LEHs (1 ml) were mixed with 1 ml of 99mTc-hexamethyl propylene amine oxime (HMPAO) that was prepared by reconstituting the HMPAO kit (Ceretec; Nycomed-Amersham, Arlington Heights, IL) with 15 mCi of sodium 99mTc-percetenate in 5 ml of normal saline. After 30 min of incubation at room temperature, the LEHs were passed through PD-10 columns (Amersham Biosciences Inc., Piscataway, NJ) to separate any radioactivity that was not associated with the LEH. Labeling efficiency was determined by counting LEHs before and after passing them through the column. Both PEGylated and non-PEGylated LEHs labeled with similar efficiency (Table 1). Also, negligible loss of labeling efficiency was observed during
the study period of approximately 1 month when the LEH preparations were stored at 4–8°C.

**Animal Biodistribution and Imaging Studies.** The animal experiments were performed according to the National Institutes of Health Animal Care and Use Guidelines and were approved by the Institutional Animal Care Committee of the University of Texas Health Science Center at San Antonio. Male New Zealand White rabbits (n = 4/LEH preparation), weighing 2.5 to 3.0 kg, were anesthetized by intramuscular injection of ketamine/xylazine mixture (50 and 10 mg/kg body weight, respectively). Patency of arterial and venous lines was established by an angiocath and a butterfly, respectively. The 99mTc-LEHs were administered in 2-ml volume; lipid dose, and radioactivity injection are given in Table 1. After intravenous administration of 99mTc-LEH, anterior whole body scintigrams were acquired by a gamma camera (Picker, Cleveland, OH) interfaced to a Pinnacle Liminator camera (Picker, Cleveland, OH) interfaced to a Pinnacle Limator was used and the camera was peaked at 140 KeV with ±20% window. Arterial blood samples (100 µl) were obtained at various times after LEH injection. After imaging at 24 h, the rabbits were euthanized by an overdose of an euthanasia solution (Buthenesia; Veterinary Labs, Inc., Lenexa, KS). Various organs were excised, washed with saline, weighed, and appropriate tissue samples were counted in a gamma counter (PerkinElmer Life and Analytical Sciences, Boston, MA). Femur with bone marrow was taken as representative of bone. Total blood volume and bone and muscle mass were estimated as 5.4, 10, and 40% of body weight, respectively (Awasthi et al., 2003). A diluted sample of injected LEHs served as a standard for comparison.

**Data Analysis.** All average values are given ± standard error of mean. The data were statistically analyzed by the univariate analysis of variance using SPSS software for Windows (SPSS Science, Upper Saddle River, NJ). For multiple comparisons, Bonferroni’s post hoc test was applied. The acceptable probability for significance was p < 0.05. To determine the t1/2 of circulation, a linear fit of log-linear plot was performed for all LEH preparations, and the circulation data were analyzed by the method of residuals (Awasthi et al., 2003). For quantitative analysis of scintimages, regions of interest were drawn around the organs, and the total computed counts in an organ was normalized with the total number of counts registered in the whole image. The results are expressed as percentage of whole body.

**Results**

The purpose of this study was to ascertain how the postsedimentation of PEG–DSPE influences the distribution and circulation of LEH with different surface charges. Two types of LEHs were prepared: one with negative charge (anionic) and the other with no charge (neutral). The physical characteristics of the preparations are shown in Table 1. All the preparations were comparable in their size distribution, p50, lipid content, and other properties. The methemoglobin content of the LEHs was <5%. The size of neutral LEH seemed to increase with time; therefore, its average size is significantly more than the other three preparations. The LEHs were labeled with 99mTc to monitor their distribution by gamma camera imaging and counting tissue-associated radioactivity on necropsy.

The accumulation of 99mTc-LEHs in various organs of rabbits is shown in Table 2. The major organs of accumulation of radioactivity were blood, spleen, and liver (Fig. 1); other organs accumulated negligible amount of activity. The 24-h blood activity showed that PEGylated LEHs, both neutral and anionic, had a prolonged circulation in blood PEG-neutral LEH reporting slightly better than the PEG-anionic LEH. On the other hand, the non-PEGylated neutral and anionic LEHs were cleared rapidly from the circulation (13–14% in blood at 24 h). Spleen activity was significantly lower in rabbits injected with non-PEGylated LEHs compared with PEGylated LEHs. It seems that PEGylation abolishes the effect of charge on spleen uptake that was found significantly different between the non-PEGylated neutral and anionic LEHs (neutral < anionic; p < 0.05). Liver accumulated the majority of activity in neutral (52.13%) and anionic (35.3%) LEHs. In contrast, PEGylated LEHs accumulated to the extent of 19% (PEG-neutral) and 12% (PEG-anionic) in liver. A similar pattern was observed in kidneys. Two other organs of significant accumulation were muscle and skin and both seem to follow the pattern shown by bloodborne activity. In general, regardless of PEGylation, LEHs without charge stayed in the system more than the negatively charged LEH. This was clear by the total recovered activity (>70% for neutral LEHs compared with about 60% for anionic LEHs). Although there was a difference in spleen and liver uptake of neutral and anionic LEHs, PEGylation nullified the influence of charge on accumulation in blood, spleen, and liver (Fig. 1).

An important part of using gamma ray emitting radionuclide (99mTc) is the capability of imaging the distribution of LEH in vivo without sacrificing the animal at intermediate time points. Figure 2 shows the early (1 h) and late (24 h) images of rabbits after injection of 99mTc-LEH. Essentially, the scintigraphic images provided us with the same information that was obtained by sacrificing the animal and counting various organs for radioactivity. Circulating activity in the blood is estimated by evaluating the activity seen in the heart in the images (Fig. 2). Early images demonstrate rapidly diminishing heart activity when non-PEGylated LEHs were injected (Fig. 2, A and B, left). On region of interest analysis it was found that at 1 h, neutral LEH was approximately 7% of whole body activity, whereas anionic LEH was about 8.1% (Fig. 3). The corresponding values for PEGylated LEHs were 12.2 and 11.7%, respectively. The liver uptake of non-PEGylated LEHs was already exceeding that of PEGylated LEHs at 1 h, although blood pool activity partially contributes to the apparent liver uptake in images (Fig. 3).

![Table 1](image)

<table>
<thead>
<tr>
<th>LEH</th>
<th>Size, Diameter [Lipid]</th>
<th>Lipid Injected per Animal</th>
<th>99mTc-Labeling Efficiency %</th>
<th>p50 Met-Hb %</th>
<th>Endotoxin [%]</th>
<th>[Hb] g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral 266.6 ± 35.5</td>
<td>27.44</td>
<td>17.95 ± 1.4</td>
<td>85.63 ± 1.22</td>
<td>25.45</td>
<td>ND</td>
<td>&gt;2.5 &lt;12.5</td>
</tr>
<tr>
<td>PEG-neutral 189.8 ± 20.3</td>
<td>25.72</td>
<td>18.57 ± 2.3</td>
<td>86.35 ± 1.85</td>
<td>25.94</td>
<td>3.8</td>
<td>&gt;2.5 &lt;12.5</td>
</tr>
<tr>
<td>Anionic 151.2 ± 17.7</td>
<td>29.44</td>
<td>14.58 ± 1.6</td>
<td>85.20 ± 4.68</td>
<td>21.91</td>
<td>ND</td>
<td>&gt;2.5 &lt;12.5</td>
</tr>
<tr>
<td>PEG-anionic 135.7 ± 5.4</td>
<td>29.63</td>
<td>17.72 ± 0.7</td>
<td>76.00 ± 2.94</td>
<td>21.42</td>
<td>4.9</td>
<td>&gt;0.5 &lt;2.5</td>
</tr>
</tbody>
</table>

ND, not determined.
images of animals injected with non-PEGylated LEHs were characterized by high liver uptake and negligible heart activity. PEGylation, on the other hand, substantially enhanced the blood borne activity. Again, as was observed in tissue distribution studies, PEGylation increased LEH accumulation in spleen but reduced that in liver. Little bladder activity that showed up in the 24-h images seems to be due to the excretion of hydrophilic 99mTc-chelates after metabolic degradation of liposome structure and 99mTc-HMPAO.

Simultaneous to the dynamic and static image acquisition of animals, blood samples were withdrawn at intermittent times during the 24-h period of study. These samples were counted for circulating radioactivity. Figure 4 shows the circulation profiles of the LEH preparations in blood. The amounts of radioactivity still circulating at 24 h were 14.4, 20.1, 44.8, and 39.5% for neutral, anionic, PEG-neutral, and PEG-anionic LEH, respectively. The 24-h arterial data correlated very closely with bloodborne activity from tissue distribution data described above. All the preparations seemed to drop from circulation in a biphasic pattern. Compared with

Table 2

<table>
<thead>
<tr>
<th>Organ</th>
<th>Neutral</th>
<th>PEG-Neutral</th>
<th>Anionic</th>
<th>PEG-Anionic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>13.99 ± 3.59*</td>
<td>40.27 ± 2.14</td>
<td>13.12 ± 4.73*</td>
<td>35.70 ± 1.63</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.61 ± 0.10*</td>
<td>0.52 ± 0.07</td>
<td>0.56 ± 0.06*</td>
<td>0.61 ± 1.21</td>
</tr>
<tr>
<td>Liver</td>
<td>52.13 ± 8.93*</td>
<td>19.12 ± 1.47</td>
<td>35.26 ± 7.44*</td>
<td>11.51 ± 1.58</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.40 ± 0.42</td>
<td>0.85 ± 0.24</td>
<td>0.38 ± 0.37</td>
<td>0.61 ± 0.05</td>
</tr>
<tr>
<td>Lung</td>
<td>0.33 ± 0.03</td>
<td>0.88 ± 0.06</td>
<td>0.49 ± 0.07</td>
<td>0.62 ± 0.03</td>
</tr>
<tr>
<td>Heart</td>
<td>0.06 ± 0.01</td>
<td>0.14 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td>0.11 ± 0.00</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.74 ± 0.33</td>
<td>0.87 ± 0.54</td>
<td>0.28 ± 0.25</td>
<td>0.89 ± 0.03</td>
</tr>
<tr>
<td>Femur</td>
<td>0.35 ± 0.02</td>
<td>0.38 ± 0.04</td>
<td>0.46 ± 0.07</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>Skin</td>
<td>0.22 ± 0.51</td>
<td>0.27 ± 1.03</td>
<td>0.15 ± 0.11</td>
<td>0.29 ± 0.24</td>
</tr>
<tr>
<td>Testis</td>
<td>0.14 ± 0.03</td>
<td>0.31 ± 0.06</td>
<td>0.14 ± 0.05</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>Brain</td>
<td>0.02 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.02 ± 0.00</td>
<td>0.04 ± 0.00</td>
</tr>
<tr>
<td>Recovery</td>
<td>73.99 ± 6.13</td>
<td>76.35 ± 5.23</td>
<td>56.91 ± 3.15</td>
<td>60.48 ± 2.16</td>
</tr>
</tbody>
</table>

Percentage of injected dose per gram

<table>
<thead>
<tr>
<th>Organ</th>
<th>Neutral</th>
<th>PEG-Neutral</th>
<th>Anionic</th>
<th>PEG-Anionic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.08 ± 0.02*</td>
<td>0.24 ± 0.02</td>
<td>0.09 ± 0.03*</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.64 ± 0.06*</td>
<td>4.99 ± 0.22</td>
<td>1.77 ± 1.14*</td>
<td>5.79 ± 0.76</td>
</tr>
<tr>
<td>Liver</td>
<td>0.54 ± 0.10*</td>
<td>0.19 ± 0.03</td>
<td>0.46 ± 0.10</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>Kidney</td>
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<td>0.10 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Lung</td>
<td>0.04 ± 0.00</td>
<td>0.09 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.07 ± 0.00</td>
</tr>
<tr>
<td>Heart</td>
<td>0.01 ± 0.00</td>
<td>0.03 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Femur</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Skin</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>Testis</td>
<td>0.04 ± 0.01</td>
<td>0.07 ± 0.02</td>
<td>0.02 ± 0.00</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Brain</td>
<td>0.00 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.01 ± 0.00</td>
</tr>
</tbody>
</table>

*p < 0.05 versus PEG-neutral.
**p < 0.05 versus PEG-anionic.

Fig. 1. Distribution of 99mTc-LEH in various organs of rabbits (data from Table 2). *, p < 0.05. The data were analyzed by univariate analysis of variance followed by Bonferroni’s post hoc test for multiple comparisons.
PEGylated LEHs, the first phase in case of non-PEGylated LEHs was steeper. There was minimal difference among PEG-neutral, PEG-anionic, and anionic LEHS during the 1st h of injection, but neutral LEH showed significantly less circulating activity as early as 5 min (Fig. 4, inset). More than 50% of neutral or anionic LEH disappeared from circulation within 6 h of injection, whereas the PEGylated LEHs achieved the same level only after 15 to 20 h. The estimated $t_{1/2}$ of neutral, anionic, PEG-neutral, and PEG-anionic LEHs were 8.9, 9.6, 19.3, and 16.5 h, respectively.
Discussion

The two primary requirements for a successful LEH product are a large load of oxygen carrying hemoglobin and a long in vivo circulation. Therefore, long-lived vesicles that can accommodate realistic quantities of hemoglobin are important. We found that a size range of 210 to 240 nm is optimum for prolonged circulation conferred by PEG to the liposomes (Awasthi et al., 2003). Here, we investigated the effect of postinserted PEG-DSPE on biodistribution of small doses of LEHs with different surface charges. A gamma ray emitting radionuclide (99mTc, t\textsubscript{1/2} of 6 h) was used to follow the distribution of LEHs. Presence of a gamma-emission of 140 KeV helps in monitoring distribution through at least 24 h without a need to sacrifice animals at intermediate time points. The radiolabeling of liposomes is performed with a lipophilic chelate 99mTc-HMPAO that upon crossing the bilayer is rendered hydrophilic by encapsulated glutathione (Phillips et al., 1992; Phillips, 1999). The hydrophilic 99mTc-HMPAO is incapable of migrating back and remains trapped inside the liposomes. Use of a suitable radionuclide for such studies is critical to reflect actual distribution of liposomes.

Among several factors governing pharmacokinetics of liposomes, surface charge has a major influence on circulation and toxicity of liposomes administered intravenously. In general, circulation t\textsubscript{1/2} of conventional liposomes decreases with increasing size, negative charge density, and fluidity in the bilayer. However, both liposome size and surface charge are more important factors than the state of bilayer stability in determining the liposome clearance (Gregoriadis, 1995). Opsonization of liposomes by plasma proteins has been suggested as a critical step in the clearance of liposomes from circulation (Lasic and Martin, 1995). Large liposomes consisting of cholesterol and phosphatidylcholine are rapidly eliminated from circulation by the phagocytic cells of RES (Allen and Hansen, 1991). To prevent this rapid accumulation by mononuclear phagocytic system, liposomes have been modified with certain lipids, such as monosialoganglioside, palmitoyl-d-glucoronic acid, and PEG-PE. Liposomes of the same size but containing one of these lipids show significantly longer circulation in blood than the liposomes without these lipids (Allen et al., 1989; Klibanov et al., 1991; Gabizon and Papahadjoulas, 1992; Litzinger and Huang, 1992; Liu et al., 1992). Of these, PEG-PE is the most widely used lipid modifier for prolonging circulation of liposomes in vivo. Incorporation of PEG-PE up to 10% molar concentration remarkably prolongs circulation of liposomes (Allen and Hansen, 1991; Klibanov et al., 1991; Maruyama et al., 1992; Torchilin and Papiosov, 1994; Phillips et al., 1999). In contrast to the classical liposomes, these PEG-modified liposomes have nonsaturable, log-linear kinetics (Dummond et al., 1999).

Despite the attractive properties rendered by the PEG-lipids, their incorporation in liposomes has been found to diminish encapsulation efficiency (Nicholas et al., 2000). Conventionally, PEG-lipid is added to the lipid mixture during the initial stages of the liposome-manufacturing process, and it is incorporated into both layers of a unilamellar liposome. As illustrated in Fig. 5, the conventional PEGylation requires more PEG-lipid than is needed for useful stealthing of a liposome; thus, under-using an expensive lipid. In multilamellar liposomes, the magnitude of wastage is more because inner bilayers do not materially contribute to the in vivo behavior of the liposomes. Also, PEGylation of the inner layer tends to exclude the encapsulated molecules by steric hindrance, the same mechanism that helps enhance circulation of PEG-liposomes in vivo. This untoward exclusion reduces the encapsulation efficiency, especially of macromolecules, such as hemoglobin (Fig. 5). The inhibitive influence of PEG-lipid is a function of length of PEG chain and the amount of PEG-lipid (Nicholas et al., 2000). These undesirable effects may be eliminated if PEG-lipids can be stably inserted into only the outer lipid layer of the liposomes. Using amphiphilic nature of PEG-lipids, several investigators have inserted PEG-lipids successfully into the outer lipid layer to prepare stealth liposomes (Uster et al., 1996; Sakai et al., 1997; Sakai et al., 2000). The mechanistic basis of PEG-lipid insertion into liposome bilayer has been discussed elsewhere (Sou et al., 2000). Basically, amphiphilic PEG-DSPE exists as a monomer below its critical micelle concentration and intercalates into the outer lipid layer of liposomes. The degree of incorporation is a function of PEG-chain

![Fig. 4. Circulation kinetics of 99mTc-LEH in rabbits. An aliquot of arterial blood was sampled at various times for radioactivity counting after injecting radiolabeled preparations. The amount of radioactivity at any particular time is given in terms of percentage radioactivity present in a sample withdrawn immediately after LEH injection.](image-url)
length, fatty acid, temperature and concentration of lipids (Sou et al., 2000). Dr. Tsuchida and group have used PEG-linked lipids to show the utility of postinsertion technique in formulation of anionic-LEH, whereas in this work we used PEG5000-DSPE in a different formulation of LEH. Apparently, a larger head group reduces the net insertion of PEG-lipid but confers better stealth property to the liposomes.

Of the major organs of liposome accumulation, accumulation in spleen is dependent largely on the PEGylation state of LEH (Fig. 2, A and B) and partly on the charge of the liposomes. On the other hand, liver uptake and circulating activity seem to be inversely related (Table 2). In a separate study, we found that uptake of PEG-liposomes by rabbit spleen is directly related to the size of the liposomes and that the liver uptake of PEG-liposomes is dependent solely on liposome size (Awasthi et al., 2003). Because liposomes without charge have a tendency to coalesce and increase in size on storage (Table 1), neutral liposomes without PEG-DSPE seem to accumulate in liver more than the anionic LEH without PEG-DSPE. Rapid metabolic turnover of anionic lipid in the RES might also be the reason of 99mTc-neutral liposomes being recovered more than the 99mTc-anionic liposomes (Table 2).

Inclusion of anionic lipid in liposome composition is practiced for two main reasons. First, it improves the shelf stability of the preparation by providing a charge-based repulsion of liposomes in suspension. Second, anionic lipids interact with cationic domains of protein or drugs and thus increase their encapsulation. In LEH, it is desirable to encapsulate large amounts of hemoglobin within a minimum amount of lipid. This factor is quantified on a weight basis as hemoglobin-to-lipid ratio (Hb/lipid). In a recent work, Tsuchida and coworkers achieved a Hb/lipid of 1.61 using dipalmitoyl phosphatidylglycerol/DPPC/cholesterol (1:5:5, molar ratio) (Phillips et al., 1996; Laverman et al., 2000). A large dose of liposomes saturated the endocytotic elimination pathways or the plasma opsonizing factors and tends to increase the circulating liposomes in blood (Drummond et al., 1999).

From the circulation data (Fig. 3), it is clear that postinserted PEG-neutral LEH has significantly longer circulation $t_{1/2}$ than the other non-PEGylated preparations. Using 99mTc-labeled LEH with postinserted PEG-DSPE, we show scintigraphic images (Fig. 2) that are comparable with the images previously published using similar PEG-liposomes made by conventional technique (Goins et al., 1996). The mechanism behind prolonged blood circulation and reduced RES uptake of PEG-liposomes is not clear, although several related hypotheses have been proposed to explain the phenomenon (Allen and Hansen, 1991; Torchilin and Papisov, 1994). In general, creation of a surface coating that forms a hydrophilic or steric barrier has been suggested to alter the interaction of PEGylated liposomes with plasma components such as complement, immunoglobulin and other opsonins (Allen and Hansen, 1991; Torchilin and Papisov, 1994). The subdued opsonization, in turn, results in reduced RES uptake and prolonged circulation. It has been shown that lipid dose determines the circulation $t_{1/2}$ of liposomes (Oja et al., 1996; Laverman et al., 2000). A large dose of liposomes saturates the endocytotic elimination pathways or the plasma opsonizing factors and tends to increase the circulating liposomes in blood (Drummond et al., 1999).

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