Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 253 (2003) 121–132
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Circulation and biodistribution profiles of long-circulating PEG-liposomes of various sizes in rabbits

V.D. Awasthi*, D. Garcia, B.A. Goins, W.T. Phillips

Department of Radiology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA

Received 23 September 2002; received in revised form 9 December 2002; accepted 9 December 2002

Abstract

To determine the largest size of liposomes that can retain stealth behavior conferred by poly(ethylene glycol)-DSPE, neutral liposomes were studied in rabbits for their circulation and distribution. Five sizes (136.2, 165.5, 209.2, 275 and 318 nm) of liposomes (DSPC, Cholesterol, PEG-DSPE and α-tocopherol, 90:80:4.5:3.9 molar ratio) were made by extrusion technique and radiolabeled with technetium-99m (Tc-99m) to follow their distribution through 24 h. Although all liposomes showed prolonged circulation in blood, the amount still in circulation at 24 h was dependent on their size. Radioactivity accumulation in spleen progressively increased with increase in size of the liposomes. In the size range of ∼160–220 nm, liver uptake was minimum, spleen uptake was moderate while the amount of circulating liposomes was maximum. Gamma camera scintigraphy corroborated the distribution pattern of liposomes on necropsy. Images within 1 h showed high blood pool activities for liposomes of all sizes. However, at 24 h, the blood pool activity was diminished for 275 nm and negligible for 308 nm liposomes; the smaller sized liposomes (136.2–209.2 nm) continued to show high blood pool activity. The amounts of radioactivity still circulating at 24 h were 46.4, 50.4, 46.8, 36.2 and 14.5% for 136.2, 165.5, 209.2, 275 and 318 nm liposomes, respectively. Corresponding circulation $T_{1/2}$s were 21.7, 26.5, 24.9, 18.7 and 8.9 h, respectively. Thus, the optimum size of PEG-liposomes for prolonged circulation in rabbits is 160–220 nm. Beyond this range, the stealth property of PEG-liposomes is significantly compromised and the distribution is characterized by high RES accumulation.

Keywords: Liposomes; Technetium-99m; Liposome-encapsulated hemoglobin

1. Introduction

Liposomes have found useful applications in therapeutic (Fujii, 1996; Lasic, 1996) and diagnostic medicine (Goins et al., 1994; Phillips and Goins, 1995; Oyen et al., 1996; Torchilin, 1996; Phillips, 1999). Another nascent, but emerging application of liposomes is in emergency medicine where hemoglobin encapsulated inside the liposomes is being investigated for its oxygen carrying property (Rudolph et al., 1991; Goins et al., 1995; Tsuchida, 1995; Phillips et al., 1999; Szebeni et al., 1999). While the conventional goal in the first two applications is to cause maximum deposition of liposomes in the target tissue in order to maximize bioavailability at the target or contrast between normal and diseased tissue, as an oxygen carrier, it is desirable that liposome-encapsulated hemoglobin (LEH) has a very long circulation in blood without significant accumulation in any organ or tissue. With regard to drug delivery or diagnostic

* Corresponding author. Tel.: +1-210-567-1875; fax: +1-210-567-5549.
E-mail address: awasthi@uthscsa.edu (V.D. Awasthi).

0378-5173/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0378-5173(02)00703-2

imaging, several groups have investigated the optimal size of liposomes (Abra and Hunt, 1982; Allen et al., 1989; Mayer et al., 1989; Klibanov et al., 1991; Liu et al., 1992; Zou et al., 1995; Boerman et al., 1997; Oussoren et al., 1997; Nagayasu et al., 1999). However, the published literature is complicated by variations in component lipids, charge, animal species, production and analytical methods and the intended purpose, such that a direct extrapolation may not be possible to effectively address questions pertaining to the optimal size range for LEH. Small changes in size and encapsulation efficiency become more critical in the case of long-circulating liposomes meant for hemostatic intervention, such as LEH. Large amounts of LEH would be usually infused in situations demanding immediate oxygen carrying fluid replenishment during surgery, trauma or accidental blood loss. Thus, in order to maximize oxygen delivery and minimize additional lipid burden on already malfunctioning organs, an ideal LEH formulation should have a long in vivo circulation and maximum encapsulated hemoglobin per unit of lipid.

Large liposomes have short circulation half-life as they are eliminated very rapidly from circulation by a complement-mediated phenomenon (Bradley et al., 1998; Szebeni, 1998; Szebeni and Alving, 1999; Szebeni et al., 1999; Szebeni et al., 2000) and end up in the organs of reticuloendothelial system (RES); other factors such as composition of liposomes and administration method (Szebeni et al., 2000), have also been reported to influence complement activation that manifests itself as a pseudoallergic reaction. On the other hand, small liposomes (<200 nm) circumvent RES uptake due to their reduced recognition by circulating opsonins. Incorporation of polyethylene glycol (PEG)-lipids in the liposomes also inhibits liposome-induced complement activation (Ahl et al., 1997; Bradley et al., 1998). Liposomes of same size, but containing PEG-lipid, show significantly longer circulation in blood than liposomes without PEG-lipid. Bradley et al. have recently shown that PEG can reduce the complement depletion by liposomes of up to size 240 nm (Bradley et al., 1998). Incorporation of PEG-lipids, thus, tends to reduce the requirement of small size for prolonged circulation of liposomes. This ability of PEG-lipid has been linked to its ability to act as a steric hydrophilic barrier to opsonizing plasma proteins (Senior et al., 1991; Torchilin and Papisov, 1994).

It has been observed that large liposomes have better encapsulation efficiency because as the size of the liposomes increases, the entrapped volume increases for constant lamellarity (Perkins et al., 1993). For LEH, a large liposome in combination with long circulation half-life ($T_{1/2}$) is ideal. The goal of the present work was to determine the largest size of PEG-liposomes that would circulate for a long period in blood. Liposomes of different sizes consisting of distearoylphosphatidylcholine (DSPC), cholesterol and PEG-distearoylphosphatidylethanolamine (PEG-DSPE) were prepared. The circulation and tissue distribution of liposomes was followed in rabbits by labeling liposomes with gamma ray emitting radionuclide, technetium-99m (Tc-99m). Tc-99m labeling of liposomes was enabled by encapsulation of glutathione inside the liposomes (Phillips et al., 1992).

2. Materials and methods

2.1. Materials

The phospholipids, distearoylphosphatidylcholine (DSPC) and poly(ethylene glycol)$_{5000}$-distearoylphosphatidylethanolamine (PEG$_{5000}$-DSPE) were obtained from Avanti Polar Lipids (Pelham, AL). Cholesterol (C) was purchased from Calbiochem (La Jolla, CA) and α-tocopherol was purchased from Aldrich (Waukegan, IL). Glutathione was from Sigma (St. Louis, MO). The radiopharmaceutical, Tc-99m-sodium pertechnetate, was obtained commercially (Amersham Health Nuclear Pharmacy, San Antonio, TX). For animal experiments, anesthetics xylazine and ketamine were from Phoenix Scientific, Inc. (St. Joseph, MO) and Fort Dodge Animal Health (Fort Dodge, IO).

2.2. Preparation of liposomes

Liposomes of different sizes, containing 5% PEG-DSPE of total phospholipid concentration (DSPC: Chol:PEG$_{5000}$-DSPE:α-tocopherol, 90:80:4.5:3.9 molar ratio), were prepared by the method reported earlier (Awasthi et al., 1998). Briefly, the solution of lipids in chloroform was evaporated to a dry film

in a rotary film evaporator (Brinkmann Instruments, NY). After further exposing the lipid film to vacuum for 4–6 h, the dried lipid film was hydrated with a solution of sucrose (300 mM) in sterile water for injection. The suspension was lyophilized overnight and the dried mixture was again hydrated with 100 mM solution of glutathione in Dulbecco's phosphate buffered saline (PBS, pH 6.3). The lipid suspension was sequentially extruded through polycarbonate membranes of well-defined pore sizes in an extruder (Lipex Biomembranes Inc., Canada). Liposomes were sized after every critical extrusion to check the resulting particle size and the extrusion was stopped when the liposomes reached a desired particle size. The resultant liposomes were centrifuged in a Beckman LE-80L ultracentrifuge at 184,000 × *g* for 45 min to obtain a pellet. Supernatant liquid, containing extravesicular glutathione, was discarded. The liposome pellet was washed two times with PBS (pH 6.3). Finally, the liposomes were resuspended in 300 mM sucrose in PBS (pH 6.3). The liposomes were always stored at 4 °C. The phospholipid concentration of the liposomes was determined by the method of Stewart (1980). The amount of glutathione encapsulated inside the liposomes was estimated by a commercial assay kit (Bioxytech, France). The sizes of the five liposome preparations are given in Table 1.

2.3. Liposome size

The size of the liposomes was determined at 25 °C 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 diluted in PBS, pH 7.4 and sized for 2 min with detector at 90° angle and sample housed in a 25 °C bath. On each occasion the instrument performance was ascertained by running a standard polystyrene beads of known diameter. The data was analyzed by non-negatively constrained least squares (CONTIN) using dynamic light scattering software—9KDLSW, beta version 1.24 supplied with the instrument. The mean diameter and quadratic polydispersity were noted. Essentially, the liposomes were sized at least two times per sample after preparative stage. Since the size of the liposome preparations form an essential part of this study, every injected preparation was analyzed for the size and size distribution of the liposomes. The reported sizes for all the samples of each liposome preparation were averaged and expressed as mean ± S.E.M. There was no change in the size and pattern of particle distribution while the liposomes were kept at 4 °C during the study. For convenience, the liposomes were also classified as small, intermediate, large and largest (Table 1).

2.4. Radiolabeling of liposomes

Liposomes were labeled essentially by the method developed by Phillips et al. (1992). Liposomes (1 ml) were mixed with 1 ml of Tc-99m-hexamethyl propylene amine oxime (HMPAO) that was prepared by reconstituting the HMPAO kit (Ceretec, Amersham, IL) with 15 mCi of sodium pertechnetate in 5 ml of normal saline. After 30 min of incubation at room temperature the liposomes were passed through a PD-10 column (Pharmacia Biotech, Sweden) to separate any radioactivity that was not associated with the liposomes. Labeling efficiency was determined by counting liposomes before and after passing them through the column. There was negligible loss of labeling efficiency of liposome preparations during the study.

Table 1
Properties of liposomes injected in rabbits

Liposomes	Size (mean ± S.E.M.)	(Lipid) mg/ml	Lipid injected per animal (mg)	Tc-99m labeling efficiency (%)	Radioactivity injected per animal (mCi)
Small	136.2 ± 5.9	28.67	16.7 ± 0.2	87.5 ± 3.6	2.04 ± 0.25
Intermediate	165.0 ± 4.3	26.18	13.6 ± 1.7	79.8 ± 6.5	2.26 ± 0.44
Intermediate	209.2 ± 9.5	19.32	15.3 ± 2.0	84.4 ± 5.4	2.32 ± 0.64
Large	275.0 ± 23.6	27.21	11.2 ± 1.8	80.4 ± 6.6	1.71 ± 0.08
Largest	318.0 ± 6.0	26.92	14.8 ± 0.5	80.4 ± 2.3	2.15 ± 0.2

2.5. Animal biodistribution and imaging studies

The animal experiments were performed according to the NIH Animal Use and Care 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 ($n = 4$ per liposome preparation), weighing 2.5–3.0 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 Tc-99m-liposomes were administered in 2 ml volume; lipid dose and radioactivity injected are given in Table 1. After intravenous administration of Tc-99m-liposome, anterior whole body scintigrams (64 × 64 matrix) of the rabbits were acquired using a Picker Model Dyna 4 Gamma Camera (Cleveland, OH) interfaced to a Pinnacle computer (Medasys, Miami, FL). A low energy high-resolution collimator was used and the camera was peaked at 140 keV with ±20% window. Arterial blood samples (100 μl) were obtained at various times after liposome injection. After imaging at 24 h, the rabbits were euthanized by an overdose of euthanasia solution (Buthenesia, Veterinary Labs, Inc., Lenexa, KS). Various organs were excised, washed with saline, weighed and appropriate samples were counted in a Wallac Wizard 3″ 1480 automatic gamma counter (Perkin–Elmer, Gaithersburg, MD). Femur with bone marrow was taken as representative of bone. A diluted sample of injected liposomes served as a standard for comparison. Total blood volume, bone and muscle mass were estimated as 5.4, 10 and 40% of body weight, respectively (Frank, 1976; Petty, 1982).

2.6. Data analysis

All average values are given ± S.E.M. The data was statistically analyzed by univariate analysis of variance using SPSS software for Windows (Upper Saddle River, NJ). The acceptable probability for significance was $P < 0.05$. All calculations concerning radioactivity were performed with activity decay in consideration. To determine the $T_{1/2}$ of circulation, a linear fit of log-linear plot was performed for all liposomes, except 318 nm liposomes that were analyzed by biexponential model using method of residuals.

3. Results

The focus of the studies described here was to determine the size window of PEG-liposomes where PEG-DSPE maintains the long-circulating property of liposomes. Thus, liposomes of five different sizes and containing PEG-DSPE were prepared by extrusion through polycarbonate filters of well-defined pore sizes. The lipid composition of the liposomes was identical and all contained glutathione (10–12 mM) inside the bilayer. These liposomes were labeled with Tc-99m and injected in rabbits to investigate their circulation in blood and distribution in various organs. The properties of these five batches of liposomes and the injected labeled preparations are shown in Table 1. The labeling of liposomes with Tc-99m was reproducible and the efficiency of labeling was always over 75%. The liposome preparations were kept at 4 °C in between the studies and there was no loss of ability of liposomes to label with Tc-99m. The phospholipid concentrations of the preparations were comparable. The amount of radioactivity and the lipid injected in animals were in the range of 1.7–2.3 mCi and 11–17 mg per injection, respectively. The amount of phospholipid injected in every animal was determined by assaying Tc-99m-labeled liposomes aliquoted prior to injection in rabbits.

The accumulation of Tc-99m-radioactivity in various organs of rabbits is shown in Table 2. The major organs of accumulation of radioactivity were blood, spleen and liver; other organs accumulated negligible amount of activity (<0.1% on per gram basis). All liposome batches showed prolonged circulation in blood. Even the largest liposomes (d, ∼318.0±6.0 nm) were 13.7% ID in blood after 24 h of injection. However, as expected, the magnitude of blood borne radioactivity was dependent on the size of the liposomes. Small liposomes of size 136.2 ± 5.9 nm circulated to a lesser extent (37%) than the two intermediate sized liposomes (165.5 ± 4.3 and 209.2 ± 9.5 nm, 47.3 and 45.8% at 24 h, respectively). Radioactivity accumulation in spleen, however, progressively increased with increase in size of the liposomes (Table 2). Thus, it appears that spleen uptake is solely dependent on the size of the liposomes and shows a direct correlation (Fig. 1, $R^2 = 0.98$). In the size range of ∼160–220 nm, liver uptake is minimum, spleen uptake is moderate while the amount of circulating liposomes is highest. Close

Table 2
Distribution of Tc-99m-liposomes in rabbits at 24 h (percent of injected dose per organ and gram)

Organs	Liposome size (mean ± S.E.M.)				
	136.2 ± 5.9	165.5 ± 4.3	209.2 ± 9.5	275.0 ± 23.6	318.0 ± 6.0
Tc-99m radioactivity accumulation (% injected dose per organ ± S.E.M.)					
Blood	36.99 ± 2.76[a]	47.33 ± 1.52[a,b]	45.82 ± 5.01[a]	32.44 ± 2.77[a]	13.73 ± 1.38
Spleen	2.52 ± 0.32[a,b]	3.36 ± 0.35[a,b]	5.82 ± 1.47[a,b]	8.53 ± 0.73	9.06 ± 0.97
Liver	13.27 ± 0.34	10.96 ± 3.34[a]	11.14 ± 0.60[a]	12.08 ± 0.39	18.83 ± 0.68
Kidney	1.51 ± 0.09	1.87 ± 0.35	1.67 ± 0.06	1.47 ± 0.04	1.77 ± 0.08
Lung	0.38 ± 0.03	0.77 ± 0.12	0.56 ± 0.04	0.41 ± 0.06	0.53 ± 0.02
Heart	0.10 ± 0.01	0.27 ± 0.12	0.12 ± 0.01	0.08 ± 0.00	0.05 ± 0.00
Muscle	2.61 ± 0.94	5.70 ± 2.17	5.04 ± 0.56	3.06 ± 1.01	1.03 ± 0.23
Bone	0.42 ± 0.04	4.91 ± 0.57	1.58 ± 1.19	0.32 ± 0.05	3.20 ± 0.23
Skin	3.82 ± 1.21	2.23 ± 0.25	1.67 ± 0.19	2.45 ± 0.66	1.28 ± 0.17
Testis	0.25 ± 0.03	0.28 ± 0.02	0.33 ± 0.06	0.09 ± 0.01	0.07 ± 0.01
Brain	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
Recovery	61.89 ± 2.92	77.71 ± 5.01	73.79 ± 2.50	60.95 ± 3.22	49.56 ± 1.04
Tc-99m radioactivity accumulation (% injected dose per gram ± S.E.M.)					
Blood	0.24 ± 0.02[a]	0.30 ± 0.02[a,b]	0.25 ± 0.01[a]	0.21 ± 0.02[a]	0.09 ± 0.01
Spleen	2.72 ± 0.30[a,b]	2.49 ± 0.41[a,b]	4.06 ± 0.80[a,b]	9.23 ± 0.75	9.19 ± 0.82
Liver	0.13 ± 0.01	0.12 ± 0.04	0.12 ± 0.01	0.12 ± 0.01	0.18 ± 0.01
Kidney	0.09 ± 0.01	0.11 ± 0.02	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.00
Lung	0.04 ± 0.00	0.08 ± 0.01	0.06 ± 0.00	0.04 ± 0.01	0.05 ± 0.00
Heart	0.02 ± 0.00	0.04 ± 0.02	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
Muscle	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Bone	0.00 ± 0.00	0.04 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.02 ± 0.00
Skin	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
Testis	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.00	0.01 ± 0.00
Brain	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

$P < 0.05$.
[a] Vs. 318 nm.
[b] Vs. 275 nm.

scrutiny of the data in Table 2 also demonstrates that the muscle activity follows the pattern as that followed by blood, signifying high vascularization of this tissue. Kidney activity is due to the ongoing elimination of postmetabolic radioactivity that finally appears in urinary bladder visualized in scintigraphic images (Fig. 2).

Scintigraphic imaging of Tc-99m using gamma camera corroborated the distribution pattern of liposomes on necropsy. In general, the early scintigraphy at 1 h showed a typical distribution of liposomes with PEG-DSPE, i.e. high blood pool activity evidenced by radioactivity accumulated in heart (Fig. 2). Even large 318 nm liposomes also showed considerable blood pool activity at 1 h. Twenty-four hour images, however, demonstrated differences in radioactivity accumulation depending on the size of the liposomes. There was very slight difference in heart activity between first three formulations (136.2–209.2 nm), but the heart activity was diminished in 275 nm liposomes and was negligible in 308 nm liposomes (Fig. 2). Simultaneously, there was an increase in radioactivity accumulation in the organs of RES—liver and spleen. While interpreting images, a relative accumulation of radioactivity in various organs is important because the acquired image depends on the amount of radioactivity injected and the adjustment of contrast. Though the imaging during intervening times was feasible, it was not done in all cases and therefore has not been shown here. The bladder activity is due to the Tc-99m-HMPAO escaping from liposomes after their metabolism or degradation.

During the 24 h period, blood samples were withdrawn at various times after Tc-99m-liposome

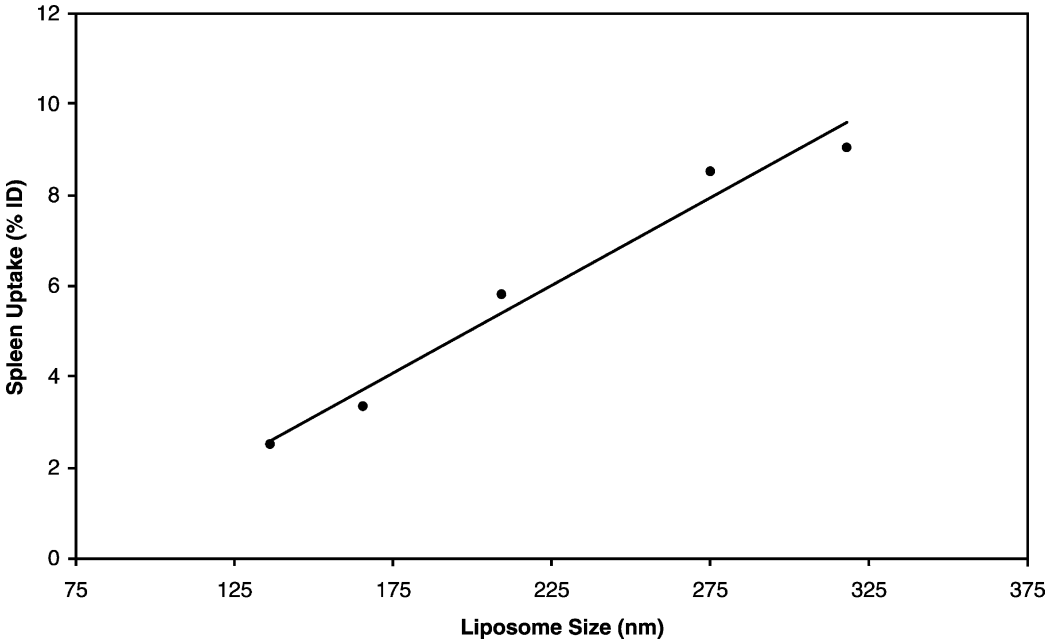

Fig. 1. Dependence of Tc-99m-liposome uptake by rabbit spleen on size of the liposomes.

injection. These samples were counted for circulating radioactivity. Fig. 3 shows the circulation profiles of the liposome preparations in blood. There was negligible difference in circulating activity for small, intermediate and large liposomes, however, the largest liposomes started deviating as soon as 1 h after injection. The amounts of radioactivity still circulating at 24 h were 46.4, 50.4, 46.8, 36.2 and 14.5% of baseline for 136.2, 165.5, 209.2, 275 and 318 nm liposomes, respectively. The 24 h arterial data corroborated very closely with blood borne activity from tissue distribution data described above ($R^2 = 0.95$). The difference in 24 h circulating radioactivity between 318 nm liposomes and other size liposomes was statistically significant ($P < 0.05$). Table 3 gives an estimated $T_{1/2}$ of these liposomes in conjunction with calculated captured volume. The assumptions made while calculating the captured volume were: (a) bilayer thickness is 5 nm; (b) PEG brush is 5 nm; and (c) liposomes are unilamellar.

From the results described here, we conclude that for neutral liposomes that have been modified by PEG-DSPE to show enhanced circulation, the optimum size ranges from 160 to 220 nm. The maximum size of PEG-liposomes that still demonstrated considerably long-circulation in rabbits was around 275 nm.

Table 3
Theoretical captured volume and estimated $T_{1/2}$ of liposomes

Liposome size (nm)	Theoretical entrapped volume (μl^3)	$T_{1/2}$ (h)
136.2	8.2×10^{-4}	21.7
165.5	16.1×10^{-4}	26.5
209.2	35.5×10^{-4}	24.9
275.0	86.9×10^{-4}	18.7
318.0	138.6×10^{-4}	8.9

4. Discussion

The two primary requirements for a successful LEH are a large load of oxygen carrying hemoglobin and a long in vivo circulation. Therefore, long-lived larger vesicles that can accommodate realistic quantities of hemoglobin (without increasing lipid dose) are important. In this report, role of particle size in determining the biodistribution of liposomes was studied

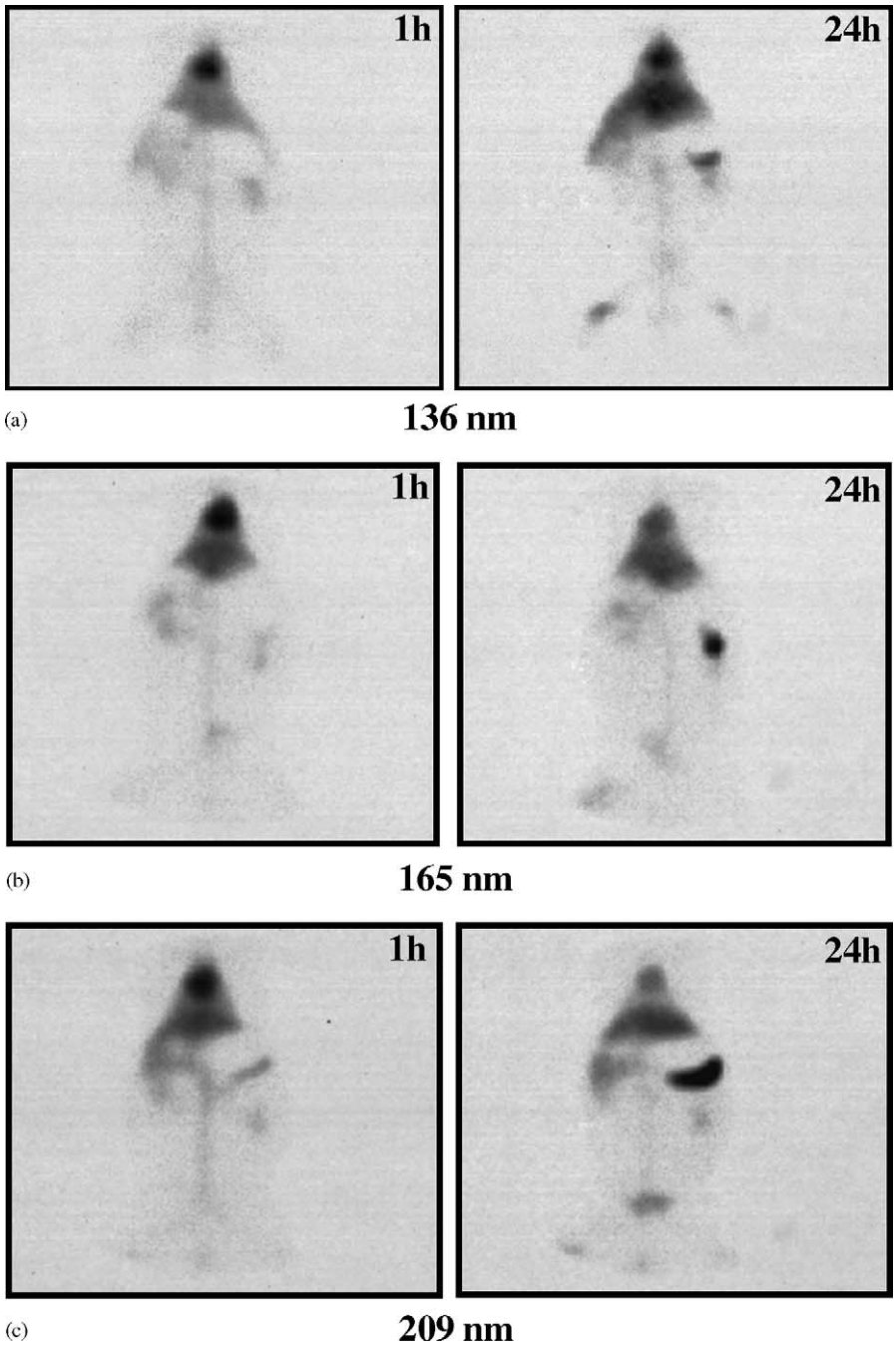

Fig. 2. Gamma camera acquired images of rabbits injected with Tc-99m-liposomes of various sizes—(a) 136 nm, (b) 165 nm, (c) 209 nm, (d) 275 nm and (e) 318 nm. Representative images at 1 and 24 h after liposome administration are shown.

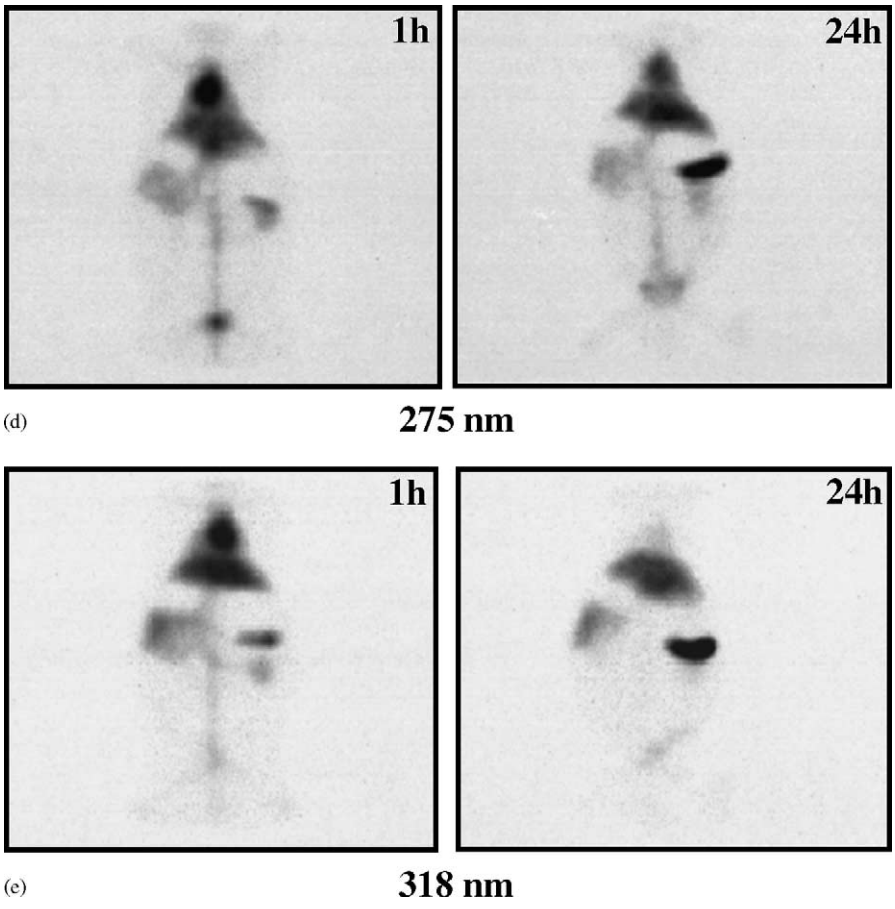

Fig. 2. (*Continued*).

to find the largest size of liposomes that would still hold the long circulating property conferred by the presence of PEG-DSPE. Since the published literature carries variations in lipid composition, methods to track the liposome disposition and the animal model, there was a need to establish optimum size window for LEH. This investigation utilizes a gamma ray emitting radionuclide (Tc-99m, $T_{1/2}$ 6 h) to follow the biodistribution of liposomes (Phillips et al., 1992). Presence of a gamma-emission of 140 keV helps in monitoring distribution through 24 h without a need to sacrifice animals at intermediate time points. The radiolabeling of liposomes is performed with a lipophilic chelate Tc-99m-HMPAO that upon crossing the bilayer is rendered hydrophilic by encapsulated glutathione (Phillips et al., 1992; Phillips and Goins, 1995; Phillips, 1999; Goins and Phillips, 2001). The hydrophilic Tc-99m-HMPAO is incapable of migrating back and remains trapped inside the liposomes. This is unlike labeling the exposed lipid bilayer using pentetate-stearyleamine (Litzinger et al., 1994) or hydrazinonicotinate-DSPE (Laverman et al., 1999) and encapsulating leakable radioactive compounds (Espinola et al., 1979; Morgan et al., 1985; Maruyama et al., 1992) that may add an avoidable artifact, influencing apparent versus real distribution of liposomes in vivo.

The amount of liposomes in circulation after intravenous administration is determined by various factors such as: (1) size; (2) surface charge; (3) composition; (4) presence of surface modifying lipids; and (5) the host pathophysiology. In general, $T_{1/2}$ of conventional

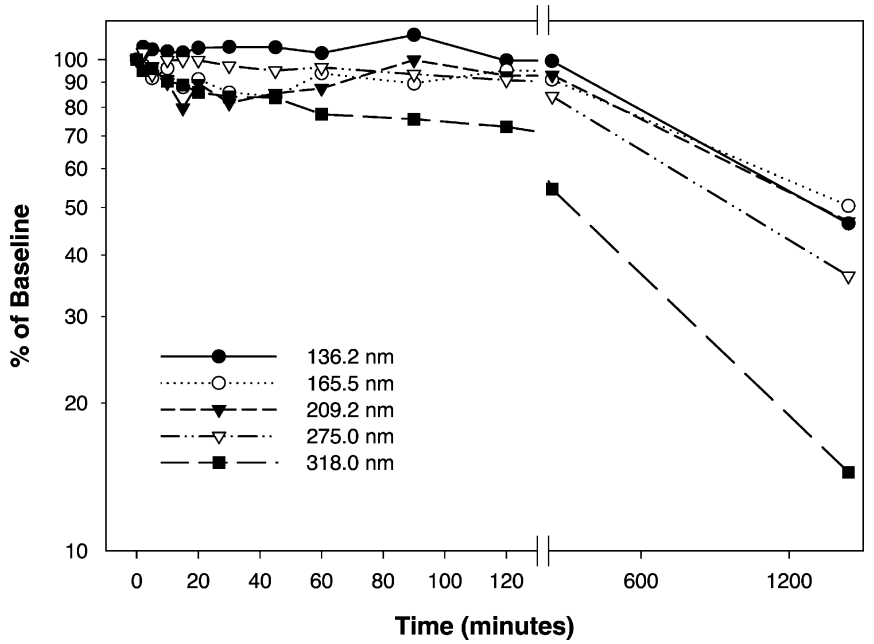

Fig. 3. Circulation kinetics of Tc-99m-liposomes of various sizes in rabbits. Inset shows blood activity at 4 and 24 h after injection.

liposomes decreases with increasing size, negative charge density and fluidity in the bilayer. However, liposome size and surface charge both override the state of bilayer stability in determining liposome clearance (Gregoriadis, 1995). The exact mechanism for the effect of each of these factors is not well understood, however, adsorption of plasma proteins on the liposome surface has been suggested as a critical step (Lasic and Martin, 1995). A liposome of large size may facilitate multiligand interactions or may be opsonized by a protein specific for large liposomes (Harashima et al., 1994). Nevertheless, very small liposomes ($d \sim 0.06$ um) have been shown to accumulate in liver more than the intermediate size liposomes (Abra and Hunt, 1982). We also found that liposomes of small size (136 nm) deposited in the liver slightly more (about 20% more) than the liposomes of intermediate size (165–209 nm). It has been proposed that elevated liver accumulation of very small liposomes is due to their access through the fenestrated hepatic endothelium to the hepatocytes (Liu et al., 1992). However, using fluorescently labeled PEG-liposomes, Litzinger et al. could not detect small liposomes (70 nm) associated with hepatocytes in mice (Litzinger et al., 1994).

It may be safely assumed that for all other factors remaining the same, the larger the liposome size, the greater is the captured volume, defined as milliliter/millimole of lipid (Perkins et al., 1993). There exists a direct physical proportionality between the capture volume and the quantity of substance encapsulated. Although, polyethylene glycol-phosphatidylethanolamine (PEG-PE) reduces the requirement of small size for long circulation, its influence is restricted within a size range and other parameters. From the circulation of various sized liposomes, it was clear that a size range of 210–275 nm is the optimum size where PEG-liposomes still circulate for a prolonged time. Above this range the circulation half-life is limited while below this range the captured volume is considerably reduced (Table 3). Earlier, Maruyama et al. have also shown that circulation of DSPC vesicles drops off rapidly over 300 nm, however, the circulation was studied in mice only up to 3 h after injection of PEG-DSPE liposomes encapsulating ^{125}I-tyraminylinulin (Maruyama et al., 1992).

Liposomes consisting of cholesterol and PC are quickly eliminated from circulation by the phagocytic cells of RES (Allen and Hansen, 1991). In order to prevent this rapid accumulation by mononuclear

phagocytic system (MPS), liposomes have been modified with certain lipids, such as monosialoganglioside (GM1), palmityl-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 Papahadjoulos, 1992; Litzinger and Huang, 1992; Liu et al., 1992; Litzinger et al., 1994). Of these, PEG-PE has commanded special attention and is the most widely used modifier for prolonging circulation of liposomes in vivo. Incorporation of PEG-PE up to 10% molar concentration remarkably prolongs circulation of liposomes (Allen et al., 1991; Klibanov et al., 1991; Maruyama et al., 1992; Torchilin and Papisov, 1994; Phillips et al., 1999). In contrast to the classical liposomes, these PEG-modified liposomes have nonsaturable, log-linear kinetics (Drummond et al., 1999). 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; Allen et al., 1991; Torchilin and Papisov, 1994). In general, creation of a surface coating that forms a hydrophilic or steric barrier similar to the glycocalyx of red blood cells has been suggested to alter the interaction of PEGylated liposomes with plasma components such as complement, immunoglobulin and other opsonins (Allen and Hansen, 1991; Allen et al., 1991; Torchilin and Papisov, 1994). The subdued opsonization in turn results in reduced RES uptake and prolonged blood sojourn. A recent in vitro report, however shows that the serum protein associated with PEG-liposomes is more than the liposomes without PEG and that the surface-associated serum proteins inhibit uptake of PEG-liposomes by mouse bone marrow macrophages (Johnstone et al., 2001). It is interesting to note that PEG-PE, like PG, imparts negative surface potential to the liposomes, but reduces electrophoretic mobility of the liposomes because of the hydrodynamic drag contributed by the hydrophilic head group (Woodle et al., 1992).

Of the major organs of liposome accumulation, accumulation in spleen was in direct correlation with the size of the liposomes (Fig. 1). Litzinger and Huang (Litzinger and Huang, 1992) reported that DOPC liposomes stabilized with PEG_{5000}-DOPE accumulated in spleen of mice to a lesser extent than the DOPC liposomes without PEG_{5000}. Contrary to this, we have found that the uptake of PEG-liposomes (DSPC/Chol/PEG-DSPE) by rabbit spleen is higher as compared to that of neutral liposomes (DSPC/Chol) without PEG (unpublished data). This apparent discrepancy may be due to the difference in the way various animal species handle PEG-liposomes or the difference in liposome composition. The use of the rabbits for studying PEG-liposome size vis-à-vis in vivo performance is unique because most related studies have been performed in either mice or rats. Based on comparison of a PEG-liposome formulation developed for diagnostic imaging purposes (Laverman et al., 2000), it appears that rats show unusually high uptake in RES as compared to rabbits and humans and rabbit seemed to be a better model for studying biodistribution of liposomes in humans. From our own experience with PEG-liposomes, rats tend to have much greater spleen uptake of all liposome formulations >100 nm in size, while rabbits have more formulation and size-dependent spleen uptake of liposomes. Based on a comparison of liposome-encapsulated doxorubicin in small animals and humans, it has been roughly estimated that a circulation $T_{1/2}$ of 12–20 h in rats or mice translates into 40–60 h in humans (Woodle et al., 1995). Another factor that may be responsible for the difference in observation is the use of ^{111}I-DTPA-SA to track the liposomes by Litzinger and Huang. We have previously shown that the same liposomes labeled with Tc-99m and ^{111}I simultaneously, show difference in radioisotopic distribution in spleen, with ^{111}I activity accumulating more than Tc-99m activity (Awasthi et al., 1997).

In summary, we found that PEG-liposomes in the size range of 160–210 nm have prolonged circulation in blood. A similar conclusion was drawn by Drummond et al. based on previously published reports (Drummond et al., 1999). Although, 160–210 nm range seems ideal for LEH, we are currently focusing on encapsulating hemoglobin in the liposome size of approximately 240 nm. Keeping in mind that this investigation was done with lipid dose of about 5.2 mg/kg body weight, it is fair to assume that larger lipid doses that are more relevant in case of LEH administrations would nullify the effect of 30 nm larger size. It has been shown that lipid dose determines the circulation of liposomes (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). Such would be the case of liposomes encapsulating hemoglobin for carrying oxygen where several grams of lipid would be infused.

Acknowledgements

This work was supported by a grant from the Office of Naval Research, Washington, DC, USA.

References

Abra, R.M., Hunt, C.A., 1982. Liposome disposition in vivo. IV. The interaction of sequential doses of liposomes having different diameters. Res. Commun. Chem. Pathol. Pharmacol. 36, 17–31.

Ahl, P.L., Bhatia, S.K., Meers, P., Roberts, P., Stevens, R., Dause, R., Perkins, W.R., Janoff, A.S., 1997. Enhancement of the in vivo circulation lifetime of L-a-distearoylphosphatidylcholine liposomes: importance of liposomal aggregation versus complement opsonization. Biochim. Biophys. Acta 1329, 370–382.

Allen, T., Hansen, C., 1991. Pharmacokinetics of stealth versus conventional liposomes: effect of dose. Biochim. Biophys. Acta 1068, 122–141.

Allen, T.M., Hansen, C., Rutledge, J., 1989. Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. Biochim. Biophys. Acta 981, 27–35.

Allen, T.M., Hansen, C., Martin, F., Redemann, C., Yau-Young, A., 1991. Liposomes containing synthetic lipid derivative of poly(ethylene) show prolonged circulation half-lives in vivo. Biochim. Biophys. Acta 1066, 29–36.

Awasthi, V.D., Goins, B., Klipper, R., Loredo, R., Korvick, D., Phillips, W.T., 1997. Comparison of dual radiolabeled liposomes (DRL), Tc-99m-MDP and Ga-67 citrate for imaging osteomyelitis in rabbit model. J. Nucl. Med. 38, 182P.

Awasthi, V., Goins, B., Klipper, R., Loredo, R., Korvick, D., Phillips, W.T., 1998. Imaging experimental osteomyelitis using radiolabeled liposomes. J. Nucl. Med. 39, 1089–1094.

Boerman, O.C., Oyen, W.J.G., van Bloois, L., Koenders, E.B., van der Meer, J.W.M., Corstens, F.H.M., Storm, G., 1997. Optimization of technetium-99m-labeled PEG liposomes to image focal infection: effects of particle size and circulation time. J. Nucl. Med. 38, 489–493.

Bradley, A.J., Devine, D.V., Ansell, S.M., Janzen, J., Brooks, D.E., 1998. Inhibition of liposome-induced complement activation by incorporated poly(ethylene glycol)-lipids. Arch. Biochem. Biophys. 357, 185–194.

Drummond, D.C., Meyer, O., Hong, K., Kirpotin, D.B., Papahadjoulos, D., 1999. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. Pharmacol. Rev. 51, 691–743.

Espinola, L.G., Beaucaire, J., Gottschalk, A., Caride, V.J., 1979. Radiolabeled liposomes as metabolic and scanning tracers in mice. II. In-111 oxine compared with Tc-99m DTPA, entrapped in multilamellar lipid vesicles. J. Nucl. Med. 20, 434–440.

Frank, D.W., 1976. Physiological data of laboratory animals. In: Melby Jr., E.C. (Ed.), Handbook of Laboratory Animal Science, vol. 3. CRC Press, Boca Raton, FL, pp. 23–64.

Fujii, G., 1996. Liposomal amphotericin B (AmBisome): realization of the drug delivery concept. In: Rosoff, M. (Ed.), Vesicles. Marcel Dekker, New York, pp. 491–520.

Gabizon, A., Papahadjoulos, D., 1992. The role of surface charge and hydrophilic groups on liposome clearance in vivo. Biochim. Biophys. Acta 1103, 94–100.

Goins, B.A., Phillips, W.T., 2001. The use of scintigraphic imaging as a tool in the development of liposome formulations. Prog. Lipid Res. 40, 95–123.

Goins, B.A., Klipper, R., Rudolph, A.S., Phillips, W.T., 1994. Use of technetium-99m-liposomes in tumor imaging. J. Nucl. Med. 35, 1491–1498.

Goins, B.A., Klipper, R., Sanders, J., Cliff, R.O., Rudolph, A.S., Phillips, W.T., 1995. Physiological responses, organ distribution, and circulation kinetics in anesthetized rats after hypovolemic exchange transfusion with technetium-99m-labeled liposome-encapsulated hemoglobin. Shock 4, 121–130.

Gregoriadis, G., 1995. Fate of liposomes in vivo and its control: a historical perspective. In: Lasic, D.D., Martin, F. (Eds.), Stealth Liposomes. CRC Press, Boca Raton, FL, pp. 7–12.

Harashima, H., Sakata, K., Funato, K., Kiwada, H., 1994. Enhanced hepatic uptake of liposomes through complement activation depending on the size of liposomes. Pharm. Res. 11, 402–406.

Johnstone, S.A., Masin, D., Mayer, L., Bally, M.B., 2001. Surface-associated serum proteins inhibit the uptake of phosphatidylserine and poly(ethylene glycol) liposomes by mouse macrophages. Biochim. Biophys. Acta 1513, 25–37.

Klibanov, A.L., Maruyama, K., Bekerleg, A.M., Torchilin, V.P., Huang, L., 1991. Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target. Biochim. Biophys. Acta 1062, 142–148.

Lasic, D.D., 1996. Liposomes in drug delivery. In: Rosoff, M. (Ed.), Vesicles. Marcel Dekker, New York, pp. 447–476.

Lasic, D.D., Martin, F. (Eds.), 1995. Stealth Liposomes. CRC Press, Boca Raton, FL.

Laverman, P., Dams, E., Oyen, W., Storm, G., Koenders, E., Prevost, R., van der Meer, J., Corstens, F., Boerman, O., 1999. A novel method to label liposomes with ^{99m}Tc by the hydrazino nicotinyl derivative. J. Nucl. Med. 40, 192–197.

Laverman, P., Brouwers, A.H., Dams, E.T.M., Oyen, W.J.G., Storm, G., Rooijen, N.V., Corstens, F.H.M., Boerman, O.C., 2000. Preclinical and clinical evidence for disappearance of long-circulating characteristics of polyethylene glycol liposomes at low lipid dose. J. Pharmacol. Exp. Ther. 293, 996–1001.

Litzinger, D.C., Huang, L., 1992. Amphipathic poly(ethylene glycol) 5000-stabilized dioleoylphosphatidylethanolamine liposomes accumulate in spleen. Biochim. Biophys. Acta 1127, 249–254.

Litzinger, D.C., Buiting, A.M.J., van Rooijen, N., Huang, L., 1994. Effect of liposome size on the circulation time and intraorgan distribution of amphipathic poly(ethylene glycol)-containing liposomes. Biochim. Biophys. Acta 1190, 99–107.

Liu, D., Mori, A., Huang, L., 1992. Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of GM1-containing liposomes. Biochim. Biophys. Acta 1104, 95–101.

Maruyama, K., Yuda, T., Okamoto, A., Kojima, S., Suginaka, A., Iwatsuru, M., 1992. Prolonged circulation time in vivo of large unilamellar liposomes composed of distearoyl phosphatidylcholine and cholesterol containing amphipathic poly(ethylene glycol). Biochim. Biophys. Acta 1128, 44–49.

Mayer, L.D., Tai, L.C.L., Ko, D.S.C., Masin, D., Ginsberg, R.S., Cullis, P.R., Bally, M.B., 1989. Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice. Cancer Res. 49, 5922–5930.

Morgan, J.R., Williams, L.A., Howard, C.B., 1985. Technetium-labelled liposome imaging for deep-seated infection. Br. J. Radiol. 58, 35–39.

Nagayasu, A., Uchiyama, K., Kiwada, H., 1999. The size of liposomes: a factor which affects their targeting efficiency to tumors and therapeutic activity of liposomal antitumor drugs. Adv. Drug Deliv. Rev. 40, 75–87.

Oussoren, C., Zuidema, J., Crommelin, D.J.A., Storm, G., 1997. Lymphatic uptake and biodistribution of liposomes after subcutaneous injection. II. Influence of liposomal size, lipid composition and lipid dose. Biochim. Biophys. Acta 1328, 261–272.

Oyen, W.J.G., Boerman, O.C., Storm, G., Bloois, L.V., Koenders, E.B., Crommelin, D.J.A., Meer, J.W.M.V.D., Corstens, F.H.M., 1996. Labelled stealth liposomes in experimental infection: an alternative to leukocyte scintigraphy? Nucl. Med. Comm. 17, 742–748.

Perkins, W.R., Minchey, S.R., Ahl, P.L., Janoff, A.S., 1993. The determination of liposome captured volume. Chem. Phys. Lipids 64, 197–217.

Petty, C., 1982. Research Techniques in the Rats. Charles C. Thomas, Springfield, IL.

Phillips, W.T., 1999. Delivery of gamma-imaging agents by liposomes. Adv. Drug Deliv. Rev. 37, 13–32.

Phillips, W., Goins, B., 1995. Targeted delivery of imaging agents by liposomes. In: Torchilin, V.P. (Ed.), Handbook of Targeted Delivery of Imaging Agents. CRC Press, Boca Raton, FL, pp. 149–173.

Phillips, W.T., Rudolph, A.S., Goins, B., Timmons, J.H., Klipper, R., Blumhardt, R., 1992. A simple method for producing a technetium-99m-labeled liposome which is stable in vivo. Nucl. Med. Biol. 19, 539–547.

Phillips, W., Klipper, R., Awasthi, V., Rudolph, A., Cliff, R., Kwasiborski, V., Goins, B., 1999. Polyethylene glycol-modified liposome-encapsulated hemoglobin: a long circulating red cell substitute. J. Pharmacol. Exp. Ther. 288, 665–670.

Rudolph, A.S., Klipper, R.W., Goins, B., Phillips, W.T., 1991. In vivo biodistribution of a radiolabeled blood substitute: 99mTc-labeled liposome-encapsulated hemoglobin in an anesthetized rabbit. Proc. Natl. Acad. Sci. U.S.A. 88, 10976–10980.

Senior, J., Delgado, C., Tilcock, C., Gregoriadis, G., 1991. Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles. Biochim. Biophys. Acta 1062, 77–82.

Stewart, J.C.M., 1980. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal. Biochem. 104, 10–14.

Szebeni, J., 1998. The interaction of liposomes with the complement system. Crit. Rev. Ther. Drug Carrier Syst. 15, 57–88.

Szebeni, J., Alving, C.R., 1999. Complement-mediated acute effects of liposome-encapsulated hemoglobin. Artif. Cells Blood Substit. Immobil. Biotechnol. 27, 23–41.

Szebeni, J., Fontana, J.L., Wassef, N.M., Mongan, P.D., Morse, D.S., Dobbins, D.E., Stahl, G.L., Bunger, R., Alving, C.R., 1999. Hemodynamic changes induced by liposomes and liposome-encapsulated hemoglobin in pigs: a model for pseudoallergic cardiopulmonary reactions to liposomes. Role of complement and inhibition by soluble CR1 and anti-C5a antibody. Circulation 99, 2302–2309.

Szebeni, J., Baranyi, L., Savay, S., Bodo, M., Morse, D.S., Basta, M., Stahl, G.L., Bunger, R., Alving, C.R., 2000. Liposome-induced pulmonary hypertension: properties and mechanism of a complement-mediated pseudoallergic reaction. Am. J. Physiol. 279, H1319–H1328.

Torchilin, V.P., 1996. Liposomes as delivery agents for medical imaging. Mol. Med. Today 2, 242–249.

Torchilin, V.P., Papisov, M.I., 1994. Why do polyethylene glycol-coated liposomes circulate so long? J. Liposome Res. 4, 725–739.

Tsuchida, E., 1995. Artificial Red Cells: Materials, Performances and Clinical Study as Blood Substitutes. Wiley, Chichester, UK.

Woodle, M.C., Collins, L.R., Sponsler, E., Kossovsky, N., Papahadjoulos, D., Martin, F.J., 1992. Sterically stabilized liposomes: reduction in electrophoretic mobility but not electrostatic surface potential. Biophys. J. 61, 902–910.

Woodle, M.C., S.N.M., Working, P.K., 1995. Biological properties of sterically stabilized liposomes. In: Lasic, D.D., Martin, F. (Eds.), Stealth Liposomes. CRC Press, Boca Raton, FL, pp. 103–117.

Zou, Y., Ling, Y.-H., Reddy, S., Priebe, W., Perez-Solerr, R., 1995. Effect of vesicle size and lipid composition on the in vivo tumor selectivity and toxicity of the non-cross-resistant anthracycline annamycin incorporated in liposomes. Int. J. Cancer 61, 666–671.