Biochimica et Biophysica Acta, 1105 (1992) 193–200

BBAMEM 75579

Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes

M.C. Woodle [a], K.K. Matthay [b], M.S. Newman [a], J.E. Hidayat [b], L.R. Collins [a], C. Redemann [a], F.J. Martin [a] and D. Papahadjopoulos [c]

[a] *Liposome Technology, Inc., Menlo Park, CA (USA),* [b] *Department of Pediatrics, University of California, San Francisco, CA (USA) and* [c] *Cancer Research Institute, University of California, San Francisco, CA (USA)*

(Received 16 August 1991)

Key words: Liposome; Clearance; Poly(ethylene glycol); Desferoxamine; Gallium-67 desferoxamine; (Mouse); (Rat)

Efforts to overcome rapid uptake of liposomes by cells of the mononuclear phagocytic system (MPS) have identified that lipids derivatized with the hydrophilic polymer poly(ethylene glycol) (PEG) have many advantages. The structure-function relationship of PEG-derivatized phosphatidylethanolamine (PEG-PE) has been examined by studies of blood lifetime and tissue distribution in both mice and rats. Liposomes composed of phosphatidylcholine (PC), cholesterol, and 7.5 mol% of PEG-PE show prolonged circulation and reduced MPS uptake when the PEG has a molecular weight in the range of 1000 to 5000. Up to 35% of the injected dose remains in the blood and less than 10% is taken up by the MPS (liver plus spleen) after 24 h in the best cases as compared to 1% and 40%, respectively, for liposomes without PEG-PE. Prolonged circulation with PEG-PE is independent of cholesterol, degree of saturation in either the PC or the PE lipid anchor, lipid dose, or addition of other negatively charged lipids, phosphatidylglycerol or cholesterol sulfate. This versatility in lipid composition and dose without alteration of blood lifetime or tissue distribution is essential for controlling drug dosage and release properties in a liposome-based therapeutic agent.

Introduction

Liposomes have proven valuable both for model studies of structure-function relationships in biological membranes, as well as a carrier system for drug delivery. However, rapid uptake following intravenous administration by cells of the mononuclear phagocytic system (MPS), originally referred to as the reticuloendothelial system (RES), has greatly limited drug delivery efforts (for review articles see Refs. 1–3). Advances in prolonged circulation and reduced MPS uptake have been achieved only within the last few years through incorporation of a few specific natural glycolipids [4–6]. These compositions showed markedly improved characteristics compared with the best previous alternative, small neutral unilamellar vesicles (SUV) composed of distearoylphosphatidylcholine and cholesterol [2]. Such SUV have limitations for drug delivery in several respects: a low encapsulated aqueous volume [7], poor particle stability due to the lack of charge stabilization [8], and a dependence on saturated acyl chains of the phospholipid. These properties make liposome preparation difficult and control of drug release impossible. The new liposome formulations, referred to as Stealth® * liposomes [9], have been shown recently to provide substantial improvements in therapeutic applications [10,11]. However, these formulations also may be limited with respect to pharmaceutical development. This results from the fact that they employ natural glycolipids, such as the monosialoganglioside (G_{M1}) or hydrogenated plant phosphatidylinositol (HSPI), in combination with bulk lipids having a high temperature phase transition. A specific limitation of G_{M1} is that it only occurs in relatively small

Abbreviations: M-PEG, methoxypoly(ethylene glycol); PEG, poly(ethylene glycol); IV, iodine value; EPC, egg phosphatidylcholine; PHEPC, partially hydrogenated egg phosphatidylcholine; HSPC, hydrogenated soy phosphatidiylcholine; EPG, egg phosphatidylglycerol; EPE, egg phosphatidylethanolamine; POPE, palmitoyloleylphosphatidylethanolamine; DSPE, distearoylphosphatidylethanolamine; HSPE, hydrogenated soy phosphatidylethanolamine; C, cholesterol; CS, sodium cholesterol sulfate; ^{67}Ga-DF, ^{67}gallium desferoxamine.

Correspondence: M.C. Woodle, Liposome Technology, Inc. 1050 Hamilton Ct., Menlo Park, CA 94025, USA.

* Stealth® is a registered trademark of Liposome Technology, Inc.

quantities and is difficult to purify or synthesize. In the case of HSPI, it has a reduced solubility in many solvents complicating the formation of uniform liposomes and making their preparation difficult. In any event, the findings with these lipids led to a search for the underlying mechanism of action and thereby the development of better alternatives. The efforts have culminated in recent reports of synthetic lipids derivatized with the hydrophilic polymer poly(ethylene glycol) (PEG) showing that their incorporation in liposomes results in prolonged circulation and reduced MPS uptake [12–16]. The term 'sterically stabilized' has been proposed for a whole variety of liposomes which show prolonged blood circulation and dose independence in their pharmacokinetics mediated by lipids with specific hydrophilic groups [16–18].

In this report we are presenting data on the compositional requirements of PEG lipid derivatives in reducing MPS uptake and prolonging the blood circulation of liposomes in mice, as well as in rats. An efficient method for blood lifetime measurements in rats was developed [19] giving results comparable to those obtained in mice, indicating that the pharmacokinetic properties of these novel lipids are not species-dependent. The results show that synthetic lipids with polymeric headgroups, amenable to large scale pharmaceutical processing, can be optimized to improve the properties of long circulating liposomes. These new properties include compositional flexibility and dose-independent kinetics that will be valuable assets in the development of liposomes carrying therapeutic agents.

Materials and Methods

All reagents purchased were of analytical grade purity. Several methoxypoly(ethylene glycol) (M-PEG) derivatives of phosphatidylethanolamine (PE) were prepared as follows. The derivatives were prepared by first drying 10 mmoles of M-PEG (Aldrich Chemical, Milwaukee, WI) in 75 ml of benzene by reflux with a Dean-Stark moisture trap. Then 11 mmoles of carbonyldiimadazole (Aldrich) was added and refluxed for 2 h. Finally, 9.36 mmoles of 99% pure distearoylphosphatidylethanolamine (Calbiochem, San Diego, CA) and 20 mmoles of triethylamine were added and refluxed for 20 h. The impure product was obtained as a syrup after evaporation of the solvent. The product was purified by chromatography on C-18 reversed phase silica gel (J.T Baker, Phillipsburg, NJ). The reaction mixture was applied to a column as a solution in 4:1 ethanol/water and the product eluted with the same solvent mixture. Reuse of the column was possible after washing with t-butanol. Structure and purity of the product was determined by NMR and TLC. Samples showing a single spot on TLC were used. Derivatives of distearoylphosphatidylethanolamine (PEG-DSPE) were prepared with M-PEG moieties of several different molecular masses: 120, 750, 1900, and 5000 daltons. In addition, derivatives with ^{1900}M-PEG were prepared with egg PE (Avanti Biochemicals, Birmingham, AL) (PEG-EPE), 1-palmityl-2-oleyl PE (Avanti Biochemicals) (PEG-POPE), and hydrogenated soy PE (Natterman, Koln, Germany) (PEG-HSPE). All these derivatives, M-PEG carbamyl PE (PEG-PE), are negatively charged molecules in aqueous solution at neutral pH due to ionization of the phosphate and have been purified as either the imidazole or sodium salts.

Extruded multilamellar liposomes were prepared according to procedures already described [20,21]. Briefly, thin films of lipids were prepared by roto-evaporation of lipid mixtures in chloroform using one or more of the following lipids: 99% egg phosphatidylcholine (EPC) (Avanti Biochemicals), 99% partially hydrogenated egg phosphatidylcholine (PHEPC) with iodine values from 1 to 40 (Asahi Chemicals, Japan) [22]; hydrogenated soy phosphatidylcholine (HSPC) (Natterman, Koln, Germany); 95% egg phosphatidylglycerol (EPG) (Avanti Biochemicals); USP cholesterol (C) (Croda, Fullerton, CA); cholesterol sulfate (CS) (Sigma Chemical, St. Louis MO) and PEG-PE prepared as described above. In some cases the liposomes were labeled by incorporation of small traces of ^{14}C-labeled cholesterol oleate (New England Nuclear, Boston, MA) into the lipid film. The lipid films were hydrated with 0.9% saline (injection grade) containing 25 or 5 mM desferoxamine mesylate (DF) (Sigma) by shaking above the phase transition temperature (22°C for EPC or PHEPC IV 20–40 and 60°C for HSPC or PHEPC IV 1–10) followed by three cycles of freezing in a solid CO_2-acetone bath and thawing in warm water before extrusion through Nucleopore defined pore filters [20] with 0.4, 0.2 and 0.1 μm pores. Typically, the extrusion was repeated until the mean diameter was below 100 nm and the standard deviation of the distribution $\leq$ 30 nm as determined by dynamic light scattering (Gaussian distribution analysis with NICOMP model 200 using 'vesicle' mode). The samples gave good fits with a unimodal population. The liposomes were labeled with ^{67}Ga (New England Nuclear) as in previous methods [23,24] with modifications and determination of free label by gel permeation chromatography described elsewhere (Woodle, M.C., J. Nuclear Med. Biol., submitted). Samples with 90% or greater entrapped label were used.

Studies in mice were performed with normal female Swiss-Webster mice 6 to 8 weeks old as before [6]. Briefly, groups of three mice were injected in the tail vein with 0.2 to 0.3 ml samples containing approx. 10^5 to 10^6 cpm of ^{67}Ga-DF encapsulated in 0.1 to 2 μmole phospholipid, typically 1 μmole. At designated times the tissue distribution of label was determined by counting entire organs and the remainder of the ani-

mal. The tail was counted separately and blood content correction factors previously determined were used [6]. Total label remaining in vivo (total recovery) excluded excreted label and gives an indication of label leakage during circulation since free ^{67}Ga-DF is removed rapidly from blood by the kidneys.

Studies in rats were performed with male and female adult Sprague-Dawley rats as described [12,19]. Animals of 250–400 g were maintained under standard conditions with food and water ad libitum. The animals were surgically prepared for intravenous administration and arterial blood sampling using a mixture of nitrous oxide 2100 cm^3/min, oxygen 400 cm^3/min, and 2–5% Isoflurane (Aerrane) for anesthesia. A 300–400 μl sample was administered via a femoral venous cannula, which was removed and the vein tied off. Whole blood samples of 400 μl were obtained at various times which has little effect on blood properties [25] via a chronically implanted femoral arterial cannula externalized at the back of the neck. The animal was awake and unrestrained during the blood drawing procedures except for the initial two time points, 1 and 15 min. The blood samples were distributed into 100-μl aliquots for measurements of ^{67}Ga by gamma counter (Beckman model 5000). When ^{14}C containing cholesterol oleate was included the blood levels of label remaining were determined by scintillation counting of 100-μl aliquots of the blood samples bleached by addition of 0.1 ml 30% hydrogen peroxide 1 h before addition of scintillation cocktail (Beckman ReadyGel). In some studies, a chronic jugular venous cannula was implanted surgically (the day before) for blood sampling, and the dose administered by tail vein injection; no differences were observed in the results. Tissues were removed surgically after the final blood sample was obtained and the levels of ^{67}Ga radioactivity determined by gamma counter.

Results

Tissue distribution measurements in mice after intravenous administration of liposomes were performed using samples labeled by ^{67}Ga-DF in the aqueous compartment as described previously [6]. MPS uptake is approximated by the sum of label in liver and spleen, neglecting the bone marrow which represents very little uptake when expressed as percent of injected dose (Woodle, M.C. and Newman, M.S., unpublished observations). The results, given in Table I, show that incorporation of ^{1900}PEG-DSPE into liposomes results in prolonged circulation and reduced MPS uptake comparable or exceeding that found with formulations containing G_{M1} [5,6]. Since the earlier studies revealed a striking dependence of prolonged circulation on 'rigid' bilayers composed of high temperature phase transition lipids [6], tests for a similar dependence with ^{1900}PEG-DSPE were performed by incorporation into liposomes prepared from a series of partially hydrogenated egg PC. Increasing the extent of hydrogenation, characterized by a decreasing iodine value, results in an increase in the phase transition temperature from below room temperature at an iodine value of 40 to roughly 50°C at an iodine value of 1 (fully hydrogenated) [22]. The results show that the prolonged

TABLE I

Tissue distribution 24 h after intravenous injection in Swiss-Webster mice

Lipid composition [a]	% Injected dose per tissue [b]			L+S [c]/blood
	blood	liver + spleen	total [d]	
EPG:PC:C [e]	0.7 ± 0.3	38.9 ± 3.8	59.3 ± 5.9	55.6
DSPC:C (10:5)	11.0 ± 2.6	25.7 ± 3.4	60.0 ± 3.4	2.3
G_{M1}:EPC:C	13 ± 1.6	15.4 ± 2.3	53.5 ± 6.6	1.2
G_{M1}:DSPC:C	17.5 ± 1.5	10.5 ± 1.2	61.6 ± 6.6	0.6
G_{M1}:PG:PC:C (1:1:10:5)	8.8 ± 2.7	19.8 ± 6.7	59.6 ± 2.0	2.3
PEG-DSPE:DSPC:C [f]	21.5 ± 5.9	18.0 ± 3.8	70.3 ± 2.0	1.2
PEG-DSPE:HSPC:C	20.5 ± 6.1	7.2 ± 0.6	55.1 ± 4.9	0.4
PEG-DSPE:HEPC (IV 1):C	19.1 ± 1.9	7.2 ± 0.5	61.1 ± 0.2	0.4
PEG-DSPE:PHEPC (IV 20):C	24.6 ± 3.5	7.5 ± 2.3	61.9 ± 4.8	0.3
PEG-DSPE:PHEPC (IV 40):C	17.2 ± 0.4	9.4 ± 0.7	57.6 ± 5.2	0.6
PEG-DSPE:EPC:C	18.6 ± 1.8	13.0 ± 1.0	63.4 ± 3.3	0.7
PEG-DSPE:EPG:EPC:C (1:1:10:5)	12.5 ± 1.3	10.2 ± 0.6	56.5 ± 3.3	0.8

[a] Molar ratio is 1:10:5 except where indicated; 80 nm mean diameter particle size distribution; IV value in parenthesis describes degree of acyl chain unsaturation determined from iodine uptake value [19].

[b] Results are based on Ga-DF aqueous entrapped label and expressed as the mean ± S.D. from at least three animals.

[c] L+S refers to the sum of label in liver and spleen.

[d] Total is the sum of label in all organs and the remaining carcass.

[e] Data from earlier work (Gabizon and Papahadjopoulos (1988) [6]).

[f] Results obtained with nine animals.

TABLE II

Effect of lipid dose on tissue distribution of liposome label 24 h after intravenous injection in Swiss-Webster mice

Lipid compositions [a]	% Injected dose per tissue [b]			L + S [c] / blood
	blood	liver + spleen	total [d]	
DSPC:C				
4 μmol/kg	3.1 ± 0.7	42.0 ± 4.4	72.4 ± 2.1	13.5
40 μmol/kg	7.6 ± 6.1	23.7 ± 4.3	56.4 ± 3.4	3.1
PEG-DSPE:DSPC:C				
4 μmol/kg	19.4 ± 1.7	19.7 ± 0.9	91.7 ± 4.8	1.0
40 μmol/kg	18.1 ± 1.5	17.6 ± 0.8	76.4 ± 1.2	1.0

[a] Molar ratio is 10:5 (DSPC:C) and 1:10:5 (PEG-DSPE:DSPC:C); 80 nm mean diameter particle size distribution.
[b] Results are based on Ga-DF aqueous entrapped label and expressed as the mean ± S.D. from at least three animals.
[c] L + S refers to the sum of label in liver and spleen.
[d] Total is the sum of label in all organs and the remaining carcass.

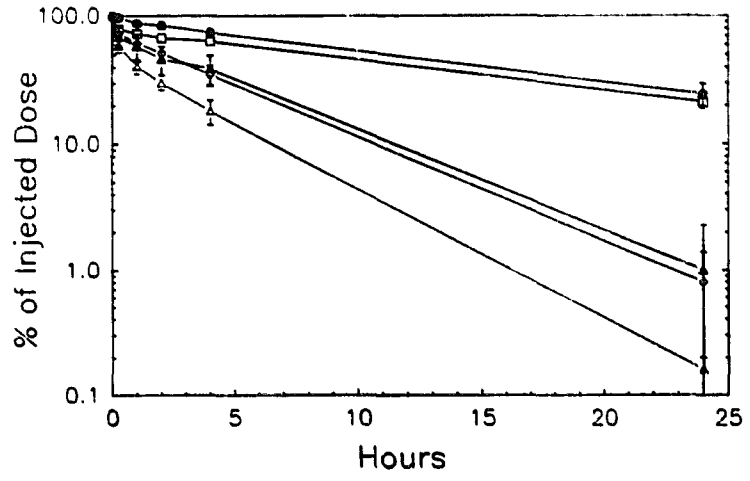

Fig. 1. Blood circulation in rats of ^{67}Ga-desferal labeled liposomes after intravenous administration. Solid circles: ^{1900}PEG-DSPE:PHEPC (IV 40):C; open squares: ^{5000}PEG-DSPE:PHEPC (IV 40):C; solid triangles: ^{120}PEG-DSPE:PHEPC (IV 40):C; and open triangles: EPG:PHEPC (IV 40):C all at 0.15:1.85:1 mole ratio. Open diamonds: PHEPC (IV 40):C at 2:1 mole ratio.

blood circulation and reduced MPS uptake is completely independent of the bulk lipid phase transition temperature.

Additionally, investigation of the lipid dose over the range from 4 to 40 μmol lipid per kg was performed comparing DSPC:C with and without PEG-DSPE. The results, shown in Table II, demonstrate a striking dependence of MPS uptake and Blood circulation of DSPC:C on lipid dose. In contrast, addition of PEG-DSPE eliminates this lipid dose dependence.

Similar measurements, but also including blood circulation kinetics over the first 24 h, were performed with rats. The blood circulation kinetics, using the Ga-DF labeling method, obtained with PEG-DSPE containing liposome formulations are shown in Fig. 1 along with those from two control formulations. The results show that clearance of PEG-DSPE containing formulations from the blood occurs as a single-exponential process. Prolonged circulation with a single clearance rate and up to 36% of the injected dose remaining in the blood after 24 h has been observed consistently in many studies using several preparations of ^{1900}PEG-DSPE in the same liposome composition. In some cases, a biexponential fit was obtained even with formulations containing a PEG derivative, Table III. In these cases, the fit gave a small component with a much faster rate. This could result from a small population with a faster clearance or, more likely, from small amounts of free label either present or released immediately upon dilution into the blood. In preliminary studies, prior to completion of the method used for removal of unentrapped label, the presence of unentrapped label resulted in a significantly greater component with a fast clearance which was not observed in samples with 10% or less unentrapped label. In addition, in vitro measures of plasma induced release have shown that these novel liposomes are resistant to plasma, but initial release occurs at a low level (Lasic, D.D., unpublished results). The combination of

TABLE III

In vivo tissue distribution and blood lifetime of liposome label 24 h after intravenous injection in Sprague-Dawley rats

Lipid composition [a]	% Injected dose per tissue [b]			Lifetime (h) [d] ($t_{1/2}$)
	blood	liver + spleen	L + S [c] / blood	
PG:PC:C	0.2 ± 0.02	14.7 ± 1.1	73.5	0.17, 2.9
PC:C (2:1)	0.8 ± 0.6	30.7 ± 1.3	38.4	3.4
HSPC:C (2:1)	26.8 ± 2.9	24.6 ± 6.2	0.92	14.3
PEG-DSPE:HSPC:C	21.1 ± 1.3	24.9 ± 3.0	1.2	0.17, 14.2
PEG-DSPE:PC:C				
70 μmol/kg	21.2 ± 6.8	7.3 ± 0.9	0.38	15.8
3 μmol/kg	23.4 ± 2.0	8.4 ± 1.5	0.36	12.4

[a] Molar ratio is 1:10:5 except where indicated; particle size distribution ≤ 100 nm mean diameter; PC refers to PHEPC (IV 40).
[b] Results are based on Ga-DF aqueous entrapped label and expressed as the mean ± S.D. from at least three animals.
[c] L + S refers to the sum of label in liver and spleen.
[d] Halflife of blood levels from fit to a single exponential or in some cases to a biexponential in which two halflives are reported (RSTRIP, MicroMath 1987, SLC, UT) least squares program polyexponential curve stripping.

these two sources of unentrapped label may have contributed to observations of biphasic kinetics in those samples. In any case, the results obtained do not permit a detailed pharmacokinetic analysis due to the limited number of time points but rather a comparison of the blood clearance kinetics obtained with the various formulations. The data indicate that the lipid compositions can be divided up into two general categories: either with or without prolonged circulation. Only small differences in clearance within these categories are observed.

With these same preparations, usually less than 10% was taken by the major organs of the MPS after 24 h, as shown in Table III. This level of uptake by the MPS after 24 h is relatively low compared with that obtained with G_{MI}, Table I, typically not lower than 13%. Table III shows a number of studies performed to relate the results obtained in rats with those obtained in mice. The formulations studied show results generally comparable to those obtained in mice (Tables I and II versus III). An examination of the lipid dose dependence of ^{1900}PEG-DSPE : PHEPC (IV40) : C showed no effect on the blood clearance or MPS uptake over the range from 3 to 70 μmol lipid/kg. Also reported in Table III is the blood circulation halflife (calculated from data as shown in Fig. 1) and a ratio of the label recovered in the MPS to that remaining in the blood which decreases with prolonged circulation and/or reduced MPS uptake [5,6].

Free ^{67}Ga-DF label is rapidly excreted into the urine ($t_{1/2}$ for aqueous ^{67}Ga-DF = 3 min) and therefore, any free Ga-DF originally present in the formulation or that leaked during circulation can be determined. The studies in mice rely on an indirect measure of the label excreted in the urine by accessing total label remaining in vivo, typically between 55 and 75% of the injected label after 24 h, which is determined by adding the label found in each tissue measured with that remaining in the carcass [5,6]. When this recovery level dropped below about 40% the results were considered inaccurate. In one case, a higher total recovery was observed, the low lipid dose with PEG-DSPE shown in Table II. The explanation for this difference is not clear especially since leakage might be expected to increase with a greater dilution of the liposomes in the blood. Note, though, that both the DSPC : C and the liposomes containing PEG-DSPE show increased recovery at the lower dose, Table II. Nevertheless, the potential for misinterpretation of the results occurs only when the total recovery is low. Consequently, no consistent correlation of the total label recovered, and thus leakage rate, is found with liposome composition; the leakage remains within a fairly narrow range. In the studies with rats, measures of leakage were performed by determination of the label excreted in the urine over 24 h, using either metabolic cages or cages with metal walks rather than bedding. Excretion of typically 5 to 10%, with a high of 20%, of the injected label in the urine and up to 3% in the feces was observed, in agreement with the consistent total recovery regardless of lipid composition in mice, Table I, and previous results [23].

Further investigation of several aspects of liposome composition and structure of the PEG lipid derivative

TABLE IV

Effect of PEG molecular height on in vivo tissue distribution 24 h after intravenous injection and blood lifetime in rats

Lipid composition PEG-PE : PC : C [a]	Size [b] (nm)	% Injected dose per tissue [c]			Lifetime (h) [e] ($t_{1/2}$)
		blood	liver + spleen	L + S [d]/blood	
^{120}PEG-DSPE	100	1.0 ± 1.3	12.9 ± 1.4	12.9	5.6
^{120}PEG-DSPE (0.3 : 1.7 : 1)	100	4.7 ± 0.3	14.2 ± 0.2	3.02	0.7, 6.0
^{750}PEG-DSPE [f]	100	3.2 ± 1.3	9.7 ± 9.1	3.03	0.09, 6.4
^{1900}PEG-DSPE	100	21.2 ± 6.8	7.3 ± 0.9	0.38	15.8
	150 [f]	14.4 ± 4.2	25.0 ± 3.9	1.74	10.0
	250	8.7 ± 2.1	18.4 ± 2.5	2.11	7.3
^{1900}PEG-HSPE	100	15.5 ± 2.6	17.0 ± 3.6	1.10	9.2
^{5000}PEG-DSPE	100	21.0 ± 1.5	9.6 ± 2.0	0.46	12.7

[a] Lipid composition and molar ratio (0.15 : 1.85 : 1) was held constant except where indicated; PC represents PHEPC (IV 40).
[b] Mean diameter measurement of particle size distribution.
[c] Results are based on Ga-DF aqueous entrapped label and expressed as the mean ± S.D. from three animals except as noted.
[d] L + S refers to the sum of label in liver and spleen.
[e] Halflife of blood levels from fit to a single exponential or in some cases to a biexponential in which two halflives are reported (RSTRIP, MicroMath 1987, SLC, Utah), least squares program polyexponential curve stripping.
[f] Average and range results from two animals.

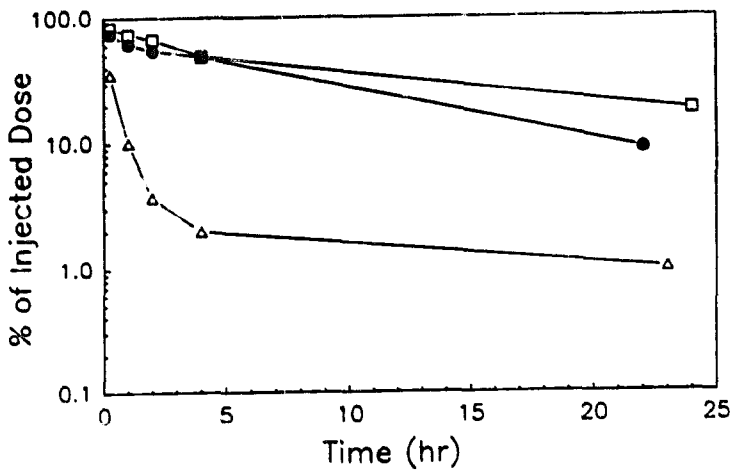

Fig. 2. Blood circulation in rats of cholesterol [^{14}C]oleate labeled liposomes after intravenous administration. All samples contained the PEG derivative or a negatively charged lipid with phosphatidylcholine and cholesterol in a lipid mole ratio of 0.15:1.85:1, respectively. Circles: ^{1900}PEG-DSPE:HSPC:C with a mean diameter of 228 nm; squares: ^{1900}PEG-POPE:PHEPC (IV 40):C with a mean diameter of 110 nm; and triangles: HEPG:HSPC:C with a mean diameter of 230 nm.

were undertaken through studies of blood lifetime and tissue distribution in rats, shown in Fig. 2 and Table IV. Fig. 2 shows blood circulation kinetics of formulations labeled with a lipid marker, cholesterol [^{14}C]-oleate, also demonstrating independence of the results from method of liposome labeling. One important issue is the role of the acyl chains of the PEG derivatized lipid. A PEG derivative of POPE (PEG-POPE), a lipid with a low temperature phase transition [26], was incorporated in a 'fluid' liposome composed of PHEPC (IV 40) and cholesterol. The results with this formulation, shown in Fig. 2 using the lipid label cholesterol oleate, show no significant difference with those described earlier (Fig. 1) utilizing a PEG derivative of the 'solid' lipid (DSPE) and labeled with the aqueous Ga-DF marker.

Studies on the effect of varying the length of the PEG moiety are described in Table IV. An increase in blood clearance was found when the PEG molecular mass was decreased to 750 daltons or less. Increasing the PEG molecular mass from 1900 to 5000 daltons had no effect. While a PEG molecular mass greater than 750 daltons appears essential for prolonged circulation, increasing the mole content of lower molecular mass derivatives may also increase blood circulation as shown by the results obtained with the 120 dalton derivative in Table IV. Based on these results, the ^{1900}PEG-DSPE has been used for all further studies and the molecular mass superscript for that derivative will be dropped.

Several other parameters of the liposome composition can also be varied when the PEG-derivative is incorporated without adversely reducing their blood circulation (Table V). Two important aspects of liposome composition besides phospholipid phase transition temperature are cholesterol content and the presence of other charged lipids. Results obtained with a PEG-DSPE containing formulation lacking cholesterol shows no dependence of prolonged circulation on cholesterol presence. Addition of other negatively charged lipids, either EPG or cholesterol sulfate, to liposomes containing PEG-DSPE also showed no adverse effect on blood circulation or MPS uptake. This was true even when EPG was added at 15 mol%, three times more than that of the PEG-DSPE.

Another issue is the importance of particle size. The effect of increasing particle size was examined using the Ga-DF aqueous label and the results are shown in Table IV. The blood levels after 24 h are 14.4 and 9% of the injected dose for samples with mean diameters of 150 and 250 nm, respectively (Table IV). The results also show an increase in the MPS uptake with increas-

TABLE V

Effect of additional negative lipids with PEG-DSPE on in vivo tissue distribution after 24 h and blood lifetime in rats

Lipid composition [a]	Size [b] (nm)	% injected dose per tissue [c]			Lifetime (h) [e] ($t_{1/2}$)
		blood	liver + spleen	L + S [d]/blood	
PEG-PE:PC:C	100	21.2 ± 6.8	7.3 ± 0.9	0.38	15.8
PEG-PE:PC (0.15:1.85)	80	22.3 ± 2.9	14.1 ± 1.7	0.63	14.7
PEG-PE:PG:PC:C (0.15:0.45:1.4:1)	58	39.7 ± 11.2	9.8 ± 0.9	0.25	14.9
PEG-PE:PC:CS:C (0.15:1.85:0.15:0.85)	62	24.9 ± 2.9	9.9 ± 0.65	0.40	1.5, 18.2

[a] Molar ratio is 1:10:5 except where indicated. PC refers to PHEPC IV 40 and PEG-PE to ^{1900}PEG-DSPE.
[b] Mean diameter of particle size distribution.
[c] Results are the mean ± S.D. from at least three animals.
[d] L + S refers to the sum of label in liver and spleen.
[e] Halflife of blood levels from fit to a single exponential or in some cases to a biexponential in which two halflives are reported (RSTRIP, MicroMath 1987, SLC, Utah), least squares program polyexponential curve stripping.

ing particle size above 150 nm. Also shown in Fig. 2 using the lipid label are the results of PEG-DSPE incorporated in a 'rigid' liposome composed of HSPC and cholesterol with a larger mean particle size, 228 nm. These results are consistent with slight increases in blood clearance when the mean particle diameter is greater than 150 nm. In addition, they indicate that the results are independent of the liposome label method.

Discussion

Results obtained with two animal species and a wide variety of lipid compositions show that liposomes containing PEG-derivatized lipids invariably have sharply reduced MPS uptake and prolonged blood circulation. A single rate for removal from the blood has been observed indicating that a single process may be responsible for their clearance from the plasma. The process (or processes if in fact there are more than one) is apparently dependent only on the PEG coating rather than the liposome bilayer composition as shown by a consistent finding of about 15 h halflife for the clearance regardless of phospholipid phase transition or cholesterol content (with the same particle size and mole content of ^{1900}PEG-DSPE, Tables I and III). Further evidence is provided by the virtually identical results obtained when POPE was used as the lipid anchor for the PEG (Fig. 2). A similar effect appears to apply even when additional negative surface charge is present by incorporation of either EPG or cholesterol sulfate (Table V).

Studies with different PEG derivatives showed that a molecular mass greater than 750 daltons is required when incorporated in the liposomes at 5 mol%. At the relatively low levels used, increasing the PEG molecular mass from 2000 to 5000 gives no significant increase in circulation or decreased uptake. Increasing the mol% of the higher molecular mass PEG derivatives is not expected to have a dramatic effect, or else increasing the molecular mass would have further increased circulation. Perhaps more importantly, a mol% of ^{1900}PEG-DSPE higher than 20% leads to bilayer destabilization and at very high contents mixed micelles [27]. With the low molecular mass PEG derivative, though, the same low levels as used with higher molecular masses only marginally increase circulation compared with PC:C. Increasing the mol% to 10% with the smallest derivative, ^{120}PEG-DSPE, increased the circulation slightly, from 1.6 to 4.7% of the injected dose remaining in the blood after 24 h. In this case, a limited circulation was obtained which could also be useful for some therapeutic agents. For example, in some diagnostic regimens a limited duration in the blood followed by clearance may be preferred.

Liposome particle size is known to have a substantial role in determining blood clearance, especially with the neutral and 'rigid' DSPC:C formulation [28]. Such striking dependence on particle size was not observed when the ^{1900}PEG lipid derivative was incorporated at 5 mol%. Nevertheless, the blood levels after 24 h show a reduction from more than 20% to about 10% of the injected dose when the mean particle diameter was increased from 100 to 250 nm.

The extent of prolonged circulation achieved in these studies by incorporation of ^{1900}PEG-DSPE is substantially greater than some reports using other PEG lipid derivatives [13,15]. A possible explanation for a relatively shorter circulation time in the case of PEG coupling to the surface of existing liposomes [15], is that complete surface coating may not have been achieved by that method. In the other case [13], it is more likely that a larger particle size contributed to a shorter circulation time. Although particle size was not reported [13], the extrusions were performed with 0.2 μm pore filters which should result in an approx. 200 nm mean diameter liposomes. An alternative may be differences due to the coupling chemistry utilized and their stability in vivo. This latter explanation appears unlikely, though, since results obtained by Blum and Ceve [14] using a different coupling chemistry are in good agreement with our results.

The wide range of versatility in liposome composition without affecting the extent of MPS uptake in vivo may be explained, at least partially, as the result of the unique properties of the PEG derivatives [27]. The potential for colloid steric stabilization by the PEG coating achieved has been proposed recently by Lasic, D.D. and co-workers [17,29,30]. Steric stabilization could be expected to reduce MPS uptake by reducing interactions with cells as well as with biological molecules and plasma components [17,18,31]. Evidence of the latter can be found in the results reported here, with the PEG-DSPE containing liposomes lacking cholesterol, in agreement with the previous investigations of PEG coating on in vitro stability [14,15]. The formulation lacking cholesterol showed no reduction in circulation, (Table V) or increased loss of liposome label contrary to earlier findings with G_{M1}-DSPC mixtures [4,5]. Nevertheless, steric stabilization may not explain fully the dependance on particle size and it is possible that other processes may be operating with particles greater than 200 nm. Many aspects of the steric stabilization model remain to be explored such are role of PEG as compared with other hydrophilic polymers. These are currently being investigated.

A variety of long circulating liposome formulations have been identified with substantial advantages for therapeutic applications. They are based on a synthetic lipid which is not only easier to prepare and therefore pharmaceutically realistic, but also provides substantial improvements compared with the best previous formulations containing G_{M1}. These include a reduced MPS

uptake and essentially complete flexibility regarding the composition of the other lipid components. In fact, recent results with these new liposome formulations have shown substantial therapeutic benefits [16]. By eliminating the requirement for lipids with a high temperature phase transition, these formulations allow greater control of leakage rates and other important bilayer properties. The ability to use fluid bilayers also has many advantages in terms of preparation, drug compatibility, and stability. Addition of other negatively charged lipids, such as EPG or cholesterol sulfate, provides further advantages. For example, ionic interactions are commonly used for increasing drug loading and particle stability. Use of different PEG molecular mass derivatives and lipid formulations can provide control in blood circulation, allowing adjustment from short to long circulation as desired, and in release rate of therapeutic agents, both of which may prove essential for therapeutic applications.

Acknowledgements

This work was supported in part by grants from the United States National Institute of Health and National Cancer Institute No. RO1-39448 (K.K.M.) and by an extramural research contract from Liposome Technology Inc. (K.K.M.).

References

1 Gregoriadis, G. (1988) Liposomes as Drug Carriers Recent Trends and Progress, J. Wiley and Sons, New York.
2 Senior, J.H. (1987) CRC Critical Reviews in Therapeutic Drug Carrier Systems 3, 123–193.
3 Papahadjopoulos, D., Lopez, N. and Gabizon, A. (1989) In Liposomes in the Therapy of infectious diseases and cancer (Lopez-Berestein, G. and Fidler, I.J., eds.), pp. 135–154, Alan R. Liss, New York.
4 Allen, TM. and Chonn, A. (1987) FEBS Lett 223, 42–46.
5 Allen, TM., Hansen, C. and Rutledge, J. (1989) Biochim. Biophys. Acta 981, 27–35.
6 Gabizon, A. and Papahadjopoulos, D. (1988) Proc. Natl. Acad. Sci. USA 85, 6949–6953.
7 Lichtenberg, D., Freire, E., Schmidt, C.F., Barenholz, Y., Felgner, P.L. and Thompson, T.E. (1981) Biochemistry 20, 3462.
8 Crommelin, D.J.A. (1984) J. Pharm. Sci. 73, 1559–1563.
9 Allen, T.M. (1989) in Liposomes in the Therapy of Infectious Diseases and Cancer (Lopez-Berestein, G. and I.J. Fidler, eds.), pp. 405–415, Alan R. Liss, New York.
10 Gabizon, A., Shiota, R. and Papahadjopoulos, D. (1989) J. Natl. Cancer Inst. 81, 1484–1488.
11 Gabizon, A., Price, D.C., Huberty, J., Bresalier, R.S. and Papahadjopoulos, D. (1990) Cancer Res. 50, 6371–6378.
12 Woodle, M.C., Newman, M., Collins, L., Redemann, C. and Martin, F. (1990) Proc. Int. Symp. Control. Rel. Bioact. Mater. 17, 77–78.
13 Klibanov, A.L., Maruyama, K. Torchillin, V.P. and Huang, L. (1990) FEBS Lett. 268, 235–237.
14 Blume, G. and Cevc, G. (1990) Biochim. Biophys. Acta 1029, 91–97.
15 Senior, J., Delgado, C., Fisher, O., Tilcock, C. and Gregoriadis, G. (1991) Biochim. Biophys. Acta 1062, 77–82.
16 Papahadjopoulos, D., Allen, T., 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.
17 Lasic, D.D., Martin, F.J., Gabizon, A., Huang, S.K. and Papahadjopoulos, D. (1991) Biochim. Biophys. Acta 1070, 187–192.
18 Kronberg, B., Dahlman, A., Carlfors, J. and Karsson, J. (1990) J. Pharm. Sci. 79, 667–671.
19 Woodle, M.C., Newman, M.S., Collins, L.R. and Martin, F.J. (1990) Biophys. J. 57, 261a.
20 Olsen, F., Huat, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 557, 9–23.
21 Woodle, M.C. and Papahadjopoulos, D. (1988) Methods Enzymol. 171, 193–217.
22 Lang, J., Vigo-Pelfrey, C. and Martin F.J. (1990) Chem. Phys. Lipids, 53, 91–101.
23 Gabizon, A., Huberty, J., Straubinger, R.M., Price, D.C. and Papahadjopoulos, D. (1988–89) J. Liposome Res 1, 123–135.
24 Ogihara-Umeda, I. and Kojima, S. (1988) J. Nucl. Med. 29, 516–523.
25 Hulse, M., Feldman, S. and Bruckner, J.V. (1981) J. Pharmacol. Ther. 218, 416–420..
26 Marsh, D. (1990) Handbook of Lipid Bilayers, CRC Press, Boca Raton.
27 Woodle, M.C., Lasic, D.D., Collins, L.R., Allen, T.M. and Martin, F.J. (1991) Biophys J. 59, 497a.
28 Senior, J., Crawley, J.C.W. and Gregoriadis, G. (1985) Biochim. Biophys. Acta 839, 1–8.
29 Martin, F.J. and Lasic, D.D. (1991) Biophys. J. 59, 497a.
30 Needham, D. and McIntosh, T.J. (1991) Biophys J. 59, 500a.
31 Gabizon, A. and Papahadjopoulos, D. (1992) Biochim. Biophys. Acta 1103, 94–100.