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STABILIZATION AND REGULATED FUSION OF LIPOSOMES CONTAINING A CATIONIC LIPID USING AMPHIPATHIC POLYETHYLENEGLYCOL DERIVATIVES

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ABSTRACT

Current fusogenic liposomal delivery systems have limited applicability *in vivo* due to poor stability in the blood and rapid clearance from the circulation. This is particularly true for liposomes composed of dioleoylphosphatidylethanolamine (DOPE) and cationic lipids, currently being developed for systemic delivery of gene-based drugs. This paper describes a potential strategy to overcome these problems, involving the incorporation of exchangeable amphipathic polyethyleneglycol (PEG) derivatives to transiently stabilize fusogenic liposomes while in the circulation, but where the PEG coating dissipates to reveal fusogenic character at later times-after arrival at target sites. It is shown here that large unilamellar vesicles(LUVs) containing DOPE and the cationic lipid, *N,N*-dioleoyl-*N,N*-dimethylammonium chloride (DODAC) can be stabilized against serum-induced aggregation and fusion by inclusion of at least 2 mol% of PEG coupled to phosphatidylethanolamine (PEG-PE) or ceramide (PEG-Cer). However, low in vitro recovery of fusogenic activity was obtained for the PEG-PE-containing system, presumably due to electrostatic interactions between the negatively charged PEG-PE and the cationic lipid which prevent PEG-PE dissociation from the LUV. Improved recovery

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of fusogenic activity was achieved for LUVs stabilized by the neutral PEG-Cer derivatives, with shorter chain ceramides exhibiting more rapid recovery rates. Biddistribution studies showed that DOPE/DODAC (85: 15, mol/mol) LUVs were rapidly cleared from the circulation, whereas inclusion of 10 mol% PEG-Cer(C_{20}) resulted in significantly prolonged circulation time. Inclusion of shorter ceramide chain lengths resulted in decreased circulation times, consistent with increased exchangeability. These findings demonstrate the feasibility of developing a cationic liposome that is stable in the circulation, but retains its ability to fuse with membranes. This work represents the first step toward the rationale design of fusogenic cationic liposomes for the systemic delivery of gene-based drugs to target tissues, such as tumors.

INTRODUCTION

A major goal in the field of drug delivery is to develop a system capable of efficient localization at a target cell and subsequently of releasing encapsulated drugs either into the cell or in the vicinity of the cell in a controlled fashion. A variety of liposomal systems with different controlled release mechanisms have been proposed [11. Examples are pH-sensitive [2-6], temperature-sensitive **[7-9],** target-sensitive [l0], and fusogenic [1 l] liposomes. These liposomes are expected to increase extracellular and/or intracellular concentration of therapeutic agents at target sites. Although rationales for each of these liposomal systems have been demonstrated in well-defined ***in vitro*** systems, their ***in vivo*** applicability is severely limited by their low stability ***in vivo*** and rapid clearance from the blood [12].

Fusogenic liposomes are particularly attractive cytoplasmic drug delivery systems [13]. These systems are designed to fuse with a target cell membrane and thus facilitate intracellular delivery of encapsulated materials. Examples include the inclusion of a fusion protein or peptide in the liposome membrane [14-16] or the inclusion of a non-bilayer forming lipid as a component of the liposome membrane [17- 18]. The use of a non-bilayer forming lipid to promote the fusogenic activity of liposomes is based on studies indicating the involvement of intermediate non-bilayer structures, such as inverted micelles, in the membrane fusion process [19-22]. Dioleoylphosphatidylethanolamine (DOPE) is a non-bilayer, hexagonal H,t phase forming lipid, which has been widely used in fusogenic liposomal formulations (reviewed in 13). A stable conformation in membranes containing DOPE requires a second lipid component as a bilayer stabilizer.

Recently, DOPE-based liposomes containing cationic lipids have gained increased attention as delivery systems for plasmid DNA [24-27], oligonucleotides [28,29] and proteins [30]. In these systems, the cationic lipid acts as a stabilizer to maintain DOPE in a bilayer conformation. Previous studies have shown that large unilamellar vesicles (LUVs) containing DOPE and cationic lipids fuse with negatively-charged liposomes and with negatively-charged cell membranes [31]. The fusogenic activity of DOPE-based liposomes containing a cationic lipid is potentially advantageous for efficient intracellular delivery of encapsulated mate-

rials *in vivo.* However, this lipid composition is not 'compatible with long circulation times in the blood [32]. Several studies have shown that cationic liposomes are unstable in the presence of serum components, as indicated by rapid aggregation and fusion, in the presence of serum and rapid clearance from the blood, resultingin limited utility for systemic administration [32]. In order for these fusogenic liposomes to be used as a drug delivery system in vivo, they must be designed in such a way that they are stable in the blood, for an extended period of time, allowing the delivery system to reach a target cell, prior to-becoming fusogenic. This requires developing a mechanism to temporarily mask the fusogemc activity of cationic liposomes for defined periods of time following introduction into the circulation.

Amphipathic polyethyleneglycol (PEG) derivatives have been-used to 'steritally stabilize' liposomes and to reduce specific and nonspecific interactions with serum proteins (reviewed in 33-35). Such sterically stabilized liposomes show reduced interactions with cells in the reticuloendothelial system (located mainly in the liver and spleen) and thus prolonged circulation times in the blood [36-. 41]. Previous studies from this laboratory [42] have shown that a PEG coating can also inhibit fusion between LUVs. Furthermore, these studies have also shown that variations in the acyl chain length of the hydrophobic anchor can be used to modulate the affinity of the amphipathic PEG anchor molecule for the liposome membrane [43].

These observations lead to the possibility that amphipathic PEG derivatives can be designed, not only to stabilize cationic liposomes against fusion and rapid clearance ***in vivo,*** but also to dissociate from the carrier at some later time, allowing liposomes to fuse with target cells. The feasibility of this approach has been demonstrated elsewhere for ***in vitro*** applications [42]. Here, this approach has been extended to ***in vivo*** situations. A series of amphipathic PEG derivatives were incorporated into liposomes composed of DOPE and a cationic lipid, *N,N*-dioleoyl-*N,N*-dimethylammonium chloride (DODAC), to stabilize these cationic liposomes in the blood. These liposomes were examined with respect to their fusogenic activity and biodistribution.

MATERIALS AND METHODS

Dioleoylphosphatidylethanolamine (DOPE) and polyethyleneglycol (PEG) (Mr. 2000) conjugated to distearoylphosphatidylethanolamine (PEG-DSPE) and dimyristoylphosphatidylethanolamine (PEG-DMPE) were obtained from Northem Lipids Inc. (Vancouver, BC, Canada). *N,N*-dioleoyl-*N,N*-dimethylammonium chloride (DODAC) was kindly provided by Dr. Steven M. Ansell of Inex Pharmaceuticals Corp. (Vancouver, BC, Canada). [^{3}H]-cholesterylhexadecyl ether was obtained from New England Nuclear (Ontario, Canada). N-[7-nitrobenz-2-oxa- 1,3-diazol-4-yl]-dioleoylphosphatidylethanolamine (NBD-PE) and N-[lissamine-rhodamine (-sulfonyl]dioleoyl phosphatidylethanolamaine (Rh-PE) were obtained 'from Avanti Polar Lipids (Pelham, AL). All other chemicals were of reagent grade.

Synthesis of 1-O-(monomethoxypolyethyleneglycol succinoyl)-ceramide

A series of PEG (Mr. 2,000) derivatives conjugated to ceramide (PEG-Cer) was prepared by conjugating monomethoxypolyethyleneglycol (MePEG)-succinate with various ceramides having different acyl chain lengths (C = 8, 14, 16, 20 and 24).

MePEG-succinate was first synthesized as follows. MePEG (Mr. 2,000) (Sigma Chemicals) (1 mol part) dissolved in CH_2Cl_2 was incubated in the presence of succinic anhydride (3 mol parts), triethylamine (2 mol parts) and 4-dimethylaminopyridine (1 mol part) under nitrogen for 16 h. The mixture was diluted with water, acidified with 1 N HCl and the organic layer separated. The aqueous layer was further extracted twice with CH_2Cl_2. The combined organic extracts were dried ($MgSO_4$) and then evaporated to dryness. The crude product of MePEG-succinate was-purified by silica gel column chromatography to give a white solid after trituration with diethyl etber.

N-hydroxysuccinimide esters of octahoic acid (C_8), myristic acid (C_{14}), eicosanoic acid (C_{20}), and tetracosanoic acid (C_{24}) were synthesized using the procedure of Lapidot et al 144]. Each N-hydroxysuccinimide ester (1 mol part) was added to a solution of D-sphingosine (1 mol part) (Avanti Polar Lipids, Inc.), in anhydrous CH_2Cl_2 (68 ml) and triethylamine (1.4 mol part), and the mixture was incubated with stirring under nitrogen at 4O(C for 6 h until all the D-sphingosine had reacted as analyzed by thin layer chromatography. The reaction mixture was concentrated in vacuo and the residue triturated with methanol. The pure ceramide was obtained by recrystallization twice from acetone. For ceramide (C_{16}), egg ceramide (Avanti Polar Lipids, Inc.) was used for the conjugation reaction with MePEG-succinate.

The conjugation reaction for MePEG-succinate with the above series of ceramide was performed as follows. Ceramide (1 mol part), dicyclohexylcarbodiimide (1.2 mol part) and 4-dimethylaminopyridine (1.2 mol part) were dissolved'in warm anhydrous CH_2Cl_2. MePEG-succinate (1 mol part) dissolved in CH_2Cl_2 was then added dropwise to the above reaction mixture, and the resulting mixture was incubated for 8 h at 40°C under nitrogen with stirring. The reaction mixture was cooled to room temperature, precipitated dicyclohexyurea was filtered off, and the filtrate concentrated in vacuo. Trituration of the residue with diethylether removed most of the dicyclohexylcarbodiimide, 4-dimethylaminopyridine, and unreacted ceramide. The obtained crude product was chromatographed on a silica gel column. The resulting solid was dissolved in distilled water and dialyzed at 4°C against distilled water overnight. The purified PEG-Cer was subsequently obtained by lyophilization as a white powder.

Liposome Preparation

LUVs composed of DOPE and DODAC with or without an amphipathic PEG derivative at specified molar ratios were prepared by the extrusion method

as described previously [45,46]. Briefly, the solvent-free lipid mixture containing [^{3}H]-cholesterylhexadecyl ether, as a nonexchangeable and nonmetabolizable lipid marker [47], (1 μCi/mg lipid) was hydrated with distilled water overnight. Normally, the liposome suspension (5 mg lipid per ml) was extruded, at room temperature, 10 times through stacked Nuclepore membranes (0.1 (m pore size)-(Nuclepore, Pleasanton, CA) using an extrusion device obtained from Lipex Biomembranes, Inc. (Vancouver, BC, Canada) to generate liposomes with homogeneous size distributions. Liposome size was determined by quasielastic light scattering (QELS) using a Nicomp model 370 particle sizer and expressed as average diameter with standard deviation.

Freeze-fracture Electron Microscopy of Serum-treated Liposomes

LUVs were prepared as described above and incubated in 50% (vol/vol) mouse serum at 37°C for 30 min. Untreated and serum-treated LUVs were mixed with glycerol (25%, vol/vol) and frozen from 20°C in a Freon slush suspended in liquid nitrogen. Samples were fractured and replicated using a Balzer's BAF 400D apparatus, and micrographs of replicas were obtained using a JEOL. Model JEM- 1200EX electron microscope.

Liposome Biodistribution

^{3}H-labeled LUVs with various lipid compositions were injected intravenously into female CD-l mice [8-10 weeks old, Charles River Laboratories, Canada) at a dose of 1 mg lipid per mouse in 0.2 ml of distilled water. At specified time intervals, mice were killed by overexposure to carbon dioxide, and blood was collected via cardiac puncture in EDTA-treated 1.5ml microcentrifuge tubes (Becton Dickinson, Canada) and centrifuged (12,000 rpm, 2 min, 4°C) to pellet plasma cells. Major organs, including the spleen, liver, lung, heart and kidney, were collected, weighed and homogenized in distilled water. Fractions of the plasma and tissue homogenates were transferred to glass scintillation vials, solubilized with Solvable (NEN) at 50°C according to the manufacturer's instructions, decolorized with hydrogen peroxide, and analyzed for ^{3}H radioactivity in scintillation fluid in a Beckman counter. Data were expressed as a percentage of the total injected dose of ^{3}H-labeled LUVs in the plasma and each organ. LUV levels in the plasma were determined by assuming that the plasma volume of a mouse is 5% of the total body weight. The amount of LUVs in each organ was corrected for plasma contamination as described elsewhere [48].

Lipid Mixing Fusion Assays

Liposome fusion, monitored as lipid mixing, was determined by a resonance energy transfer assay [49] using NBD-PE and Rh-PE as an energy donor and acceptor, respectively. Three sets of LUVs were prepared for the assay, including labeled test LUVs containing 0.5 mol% NBD-PE and 0.5 mol% Rh-PE, nega-

tively-charged target LUVs composed of DOPC and POPS (85 : 15, mol/mol), and POPC LUVs used as an acceptor for the amphipathic PEG derivatives. These LUVs were prepared by the extrusion method as described above and had average diametersof approximately 100 nm, The lipid concentration of each liposome preparation was determined by the phosphorus analysis of the phospholipid component [SO]. Liposome preparations were preincubated at 37°C before the assay. The assay was initiated by sequential addition of labeled LUVs, target LUVs, and acceptor LUVs in the fluorimeter cuvette at a ratio of 1: 3 : 10 (mol/mol) and at a final lipid concentration of 0.6 mM. The fluorescence intensity was recorded using a Perkin Elmer LS50 luminescence spectrophotometer. Fluorophore excitation was set at 465 nm, and fluorescence emission was monitored at 535 nm with an emission filter placed at 530 nm to reduce intensity due to light scattering. The 'incubation mixture was continuously stirred and thermostated to 37°C during the measurement. The fluorescence intensity was corrected for the background intensity obtained using unlabeled test LUVs in a separate experiment. The maximal fluorescence was obtained by the addition of Triton X-100 at a final concentration of 0.5%' and was considered as the maximal lipid mixing. Data are expressed as a percentage of the maximal lipid mixing of labeled test LUVs.

RESULTS

Stabilization of DOPE/DODAC LUVs by Amphipathic PEG Derivatives

This study was initiated to examine if inclusion of an amphipathic PEG derivative in the lipid composition is able to prevent serum-induced aggregation and fusion of DOPE-based cationic liposomes. This was examined by QELS and freeze-fracture electron microscopic analyses of mouse serum-treated LUVs. In this study, LUVs composed of DOPE/DODAC (85 : 15, mol/mol), DOPE/DODAC/PEG-DSPE (83 : 15 : 2, mol/mol), and DOPE/DODAC/PEG-Cer(C_{20}) (83 : 15 : 2, mol/mol) were prepared by the extrusion method and had similar average diameters (100 nm) as determined by QELS. Figures la-c are freeze-fracture electron micrographs of untreated LUVs, showing unilamellar liposomes with relatively narrow size ranges. However, pretreatment of DOPE/DODAC LUVs with mouse serum resulted in the formation of massive aggregates (Fig. 1d). On the other hand, both DOPE/DODAC/PEG-Cer(C_{20}) and DOPE/DODAC/PEG-DSPE LUVs did not show any aggregation when these LUVs were pretreated with mouse serum (Figs. le, 1f). QELS analyses also rendered no size increase on exposure to serum (data not shown). These results clearly show the effectiveness of amphipathic PEG derivatives for stabilizing DOPE-based cationic liposomes in the presence of serum components.

Modulation of Fusogenic Activity of DOPE/DODAC LUVs by Amphipathic PEG Derivatives

A previous study from this laboratory [43] has shown that the Ca^{2+}-induced fusion of LUVs composed of phosphatidylethanolamine (PE) and phosphatidyl-

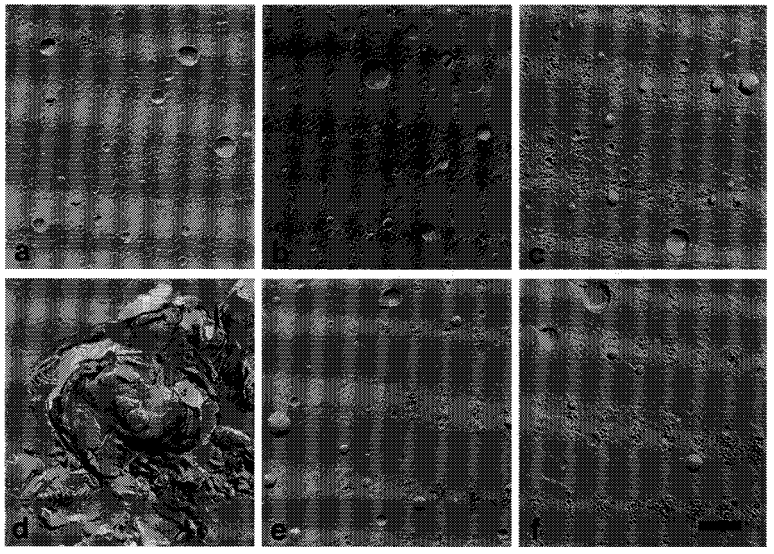

Figure 1. Freeze-fracture electron micrographs of serum-treated DOPE/DODAC LUVs containing an amphipathic PEG derivative. Freeze-fracture electron micrographs were obtained for untreated (a-c) and serum-treated (d-f) LWs. Lipid compositions of LUVs are: (a and d) DOPE/DODAC (85 : 15 **mol :** mol), (b and e) DOPE/DODAC/PEG-DSPE (83 : 15 : 2 mol: **mol :** mol), and (c and f) DOPE/DODAC/PEG-Cer(C_{20}) (83 : 15 : 2 **mol :** mol : mol). Bar represents a length of 200 nm.

serine (PS) can be inhibited by the presence of 2 mol% PEG-PE. Furthermore, for short chain PEG-PE derivatives which can readily exchange between the outer monolayers of liposomes, the addition of 'acceptor' PC liposomes results in renewed Ca^{2+}-induced fusion as the PEG-PE fusion inhibitor exchanges into the acceptor liposomes. The aim of the studies of this section was to examine whether fusion of cationic liposomes could be regulated by amphipathic PEG derivatives in a similar manner.

LUV fusion was examined in the presence of acceptor liposomes using the lipid mixing assay. As shown in Fig. 2a, DOPE/DODAC LUVs fuse avidly with negatively charged PS-containing LUVs. The lipid-mixing fluorescence maximum observed was only -45% of the absolute maximum, which may arise due to attenuation of NBD-PE fluorescence by high concentrations of the negatively-charged PS. This fusion resulted in the formation of large aggregates which precipitated out of solution over time. As shown elsewhere [54], freeze-fracture studies on the aggregates formed by fusion between DOPE-DODAC and PS-containing LUVs reveal the presence of large structures consistent with complete fusion between vesicles, rather than hemifusion. The presence of 5 mol% PEG-DMPE or PEG-DSPE substantially inhibits lipid mixing of DOPE/DODAC LUVs with PS-containing LUVs (Fig. 2b). This is similar to the ability of PEG-DMPE to inhibit Ca^{2+}-induced fusion of PE/PS liposomes [42]. However, in contrast tothebehavior of the PE/PS system, the presence of POPC acceptor LUVs, which act as a 'sink' for the PEG-DMPE, does not result in fusion.

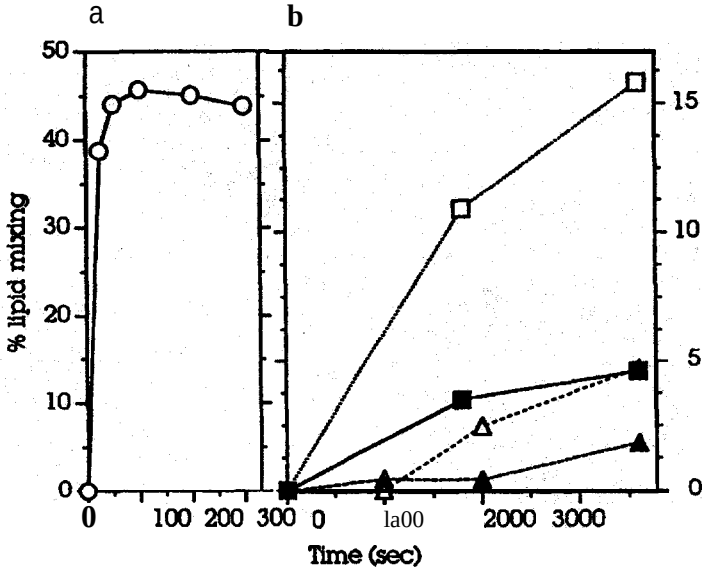

Figure 2. Lipid mixing of DOPE/DODAC LUVs containing an amphipathic PEG derivative. Lipid compositions of LUVs are: (a) (0) DOPE/DODAC (85: 15 mol:mol), (b) (A) DOPE/DODAC/PEG-DSPE (80 : 15 : 5 **mol :** mol : mol), (■) DOPE/DODAC/PEG-DMPE (80 : 15 : 5 **mol : mol :** mol); (□) **DOPE/DODAC/PEG-Cer(C_{14})** (80 : 15 : 5 **mol :** mol : mol), and (A) DOPE/DODAC/**PEG-Cer(C_{20})** (80: 15 : 5 mol: mol : mol). Data are expressed as a percentage of maximal lipid mixing obtained in the presence of 0.1 % Triton X-100. These lipid-mixing assays were conducted for DOPE-DODAC (PEG-containing) LUVs in the presence of negatively-charged DOPC/POPS (85/15; mol/mol) target LUVs and acceptor POPC LUVs. The ratio of the DODAC containing LUVs to target and acceptor LUVs was 1:3: 10 (mol:mol:mol).

A possible explanation is that the PEG-PE molecules, which carry a negative charge, exhibit reduced exchangeability when incorporated into liposomes containing a positively charged lipid. In order to examine this possibility, a neutral PEG-containing lipid, **PEG-Cer(C_{14})** was synthesized and incorporated into DOPE/DODAC LUVs. As shown in Fig. 2b, inclusion of **PEG-Cer(C_{14})** at 5 mol% of the lipid mixture inhibited fusion as assayed by lipid mixing (Fig. 2b). It is also noted that, in contrast to PEG-DMPE, the presence of acceptor POPC LUVs results in partial recovery of fusion in the **PEG-Cer(C_{14})**-containing system, presumably due to an enhanced ability of the neutral **PEG-Cer(C_{14})** molecules to leave DODAC-containing liposomes. Also shown in Fig. 2b is that **PEG-Cer(C_{20})** suppressed lipid mixing of DOPE/DODAC LUVs to a greater extent than **PEG-Cer(C_{14})**. These results indicate that the fusogenic activity of DOPE/DODAC LUVs can be modulated by inclusion of amphipathic PEG derivatives with different charge properties and acyl chain length of the hydrophobic anchor.

Biodistribution of DOPE/DODAC LUVs Containing Amphipathic PEG Derivatives

The effect of amphipathic PEG derivatives on the biodistribution of DOPE/DODAC LUVs was examined. In this study, DOPE/DODAC LUVs with or with-

out the amphipathic PEG derivative were prepared to include [^{3}H]cholesterylhexadecyl ether as a lipid marker, and the biodistribution was examined in mice at 1 h after injection. This short time interval was chosen because of the relatively rapid clearance of LUVs containing cationic lipids as compared to LUVs composed of neutral lipids. LUVs tested in this study included DOPE/DODAC (85 : 15, mol/mol), DOPE/DODAC/PEG-DSPE (80: 15 : 5, mol/mol), and DOPE/DODAC/PEG-Cer (80: 15 : 5, mol/mol). PEG-Cer molecules with different acyl chain lengths were also used to examine the effect of the hydrophobic anchor on liposome biodistribution. These LUV formulations had similar average diameters as determined by QELS, ranging from 89 to 103 nm.

Table 1 shows lipid levels in the plasma, spleen, liver, lung, heart and kidney. At 1 h after injection, DOPE/DODAC LUVs were almost completely cleared from the blood and accumulated primarily in the liver and spleen. Only a small fraction of LUVs was found in the lung, heart and kidney. Inclusion of amphipathic PEG derivatives at 5 mol% of the lipid mixture resulted in increased LUV plasma levels and concomitantly decreased liver accumulation in a manner which depended on the acyl chain composition. DOPE/DODAC/PEG-DSPE LUVs showed the highest plasma level (50%) and the lowest liver accumulation (35%) at 1 h after injection. Among the various PEG-Cer derivatives with different acyl chain lengths, inclusion of PEG-Cer(C_{20}) resulted in the highest LUV level (30%) in the plasma, while only a marginal increase in the circulation levels was obtained for PEG-Cer(C_8). The ability of PEG-Cer to increase the plasma level of DOPE/DODAC LUVs therefore directly correlates with the acyl chain length of the ceramide anchor with the higher levels obtained with the longer acyl chain lengths.

Optimization of DOPE/DODAC LUVs for Prolonged Circulation Times

The effect of increasing concentrations of the amphipathic PEG derivative in the lipid mixture on the biodistribution of DOPE/DODAC LUVs was examined using PEG-Cer(C_{20}). In this study, PEG-Cer(C_{20}) was included in DOPE/DODAC LUVs at increasing concentrations (O-10 mol%), while the DODAC concentration remained at 15 mol% of the lipid mixture. LUVs were prepared by the extrusion method and hadsimilar average diameters ranging from 102 to 110 nm. ^{3}H-labeled LUVs were injected i.v. into mice, and biodistribution was examined at 1 h after injection. Fig. 3 shows LUV levels in the plasma and liver at 1 h after injections, plotted as a function of the PEG-Cer(C_{20}) concentration. It is clear that increasing the concentration of PEG-Cer in the lipid mixture resulted in a progressive increase in the LUV level in the plasma, accompanied by decreased accumulation in the liver. The time dependence of plasma and liver levels of DOPE/DODAC LUVs with or without 10 mol% PEG-Cer(C_{20}) are shown in Fig. 4. Inclusion of PEG-Cer(C_{20}) in the lipid composition resulted in dramatically prolonged circulation times.

The effect of increasing concentrations of DODAC on the biodistribution of DOPE/DODAC LUVs was then examined. In this study, liposomes containing

Table I. Effect of amphipathic PEG derivatives on biodistribution of DOPE/DODAC liposomes[a]

PEG-DERIVATIVE[b]	MEAN DIAMETER	% INJECTED DOSE[c]					
		PLASMA	LIVER	SPLEEN	LUNG	HEART	KIDNEY
None	103 (29)	0.8 (0.4)	64.4 (2.0)	3.1 (1.8)	1.2 (0.2)	0.2(0.0)	0.3 (0.0)
PEG-DSPE	95 (26)	59.1 (8.2)	34.7 (2.1)	2.9 (0.1)	1.9 (0.8)	1.7 (0.4)	1.2 (0.5)
PEG-CER(C8)	89 (24)	6.5 (1.9)	62.8 (3.4)	4.2 (1.0)	0.5 (0.3)	0.3 (0.1)'	0.3 (0.1)
PEG-CER(C14)	93 (25)	5.9 (0.5)	55.9 (1.0)	3.3 (0.2)	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)
PEG-CER(C16)	93 (24)	13.9 (2.1')	57.5 (2.0)	2.6 (0.1)	0.0 (0.0)	0.2 (0.1)	0.0 (0.0)
PEG-CER(C20)	101 (24)	29.8 (4.8)	51.0 (2.2)	1.9 (0.2)	0.0 (0.0)	0.3 (0.1)	0.0 (0.0)
PEG-CER(C24)	92 (28)	26.7 (0.8)	46.7 (7.6)	5.7 (1.2)	1.0 (0.2)	6.9 (0.2).	0.4 (0.1)

[a] ^{3}H-labeled liposomes composed of DGPE/DODAC (85: 15, mol/mol) and DOPE/DODAC (80:15, mol/mol) additionally containing the indicated amphipathic PEG derivative at ,5 mol% of the lipid mixture were injected iv. into mice.Biodistribtition was examined at 1 hr after injection.
[b] the 'indicated amphipathic FEG derivative was included at 5 mol% of the lipid mixture.
'data are expressed as percentages of injected dose of liposomes with SD ($n = 3$);

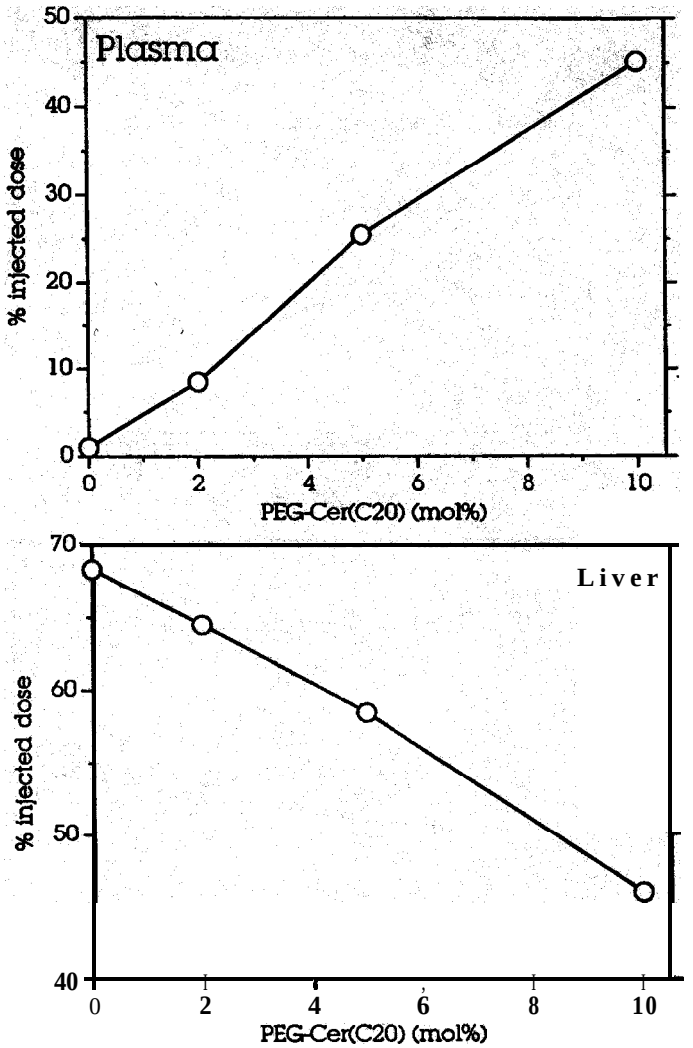

Figure 3. Effect of increasing concentrations of PEG-Cer(C_{20}) on LUV levels in the plasma and liver. ^{3}H-labeled LUVs composed of DOPE, DODAC, and PEG-Cer(C_{20}) with various molar ratios were injected i.v. into mice. LUV levels in the plasma and liver were examined at 1 hr after injectkn. Molar ratios and average diameters of DOPE/DODAC/PEG-Cer(C_{20}) LUVs are: 85 : 15:0, 103 (29) nm: **83:15:2,** 110 **(42)** nm: 80: 15:5, 109 (38) nm: 75: 15: 10, 102 (38) nm. Data were expressed as percentages of injected dose in the plasma and liver with SD (n = 3).

10 mol% PEG-Cer(C_{20}) and DODAC at various concentrations (15,30,50 mol%) of the lipid mixture were prepared by the extrusion method and had similar average diameters ranging from 103 to 114 nm. It is clear from Fig. 5 that increasing the DODAC concentration in the lipid mixture resulted in markedly decreased levels in the plasma and concomitantly increased liver accumulation of both LUV formulations.

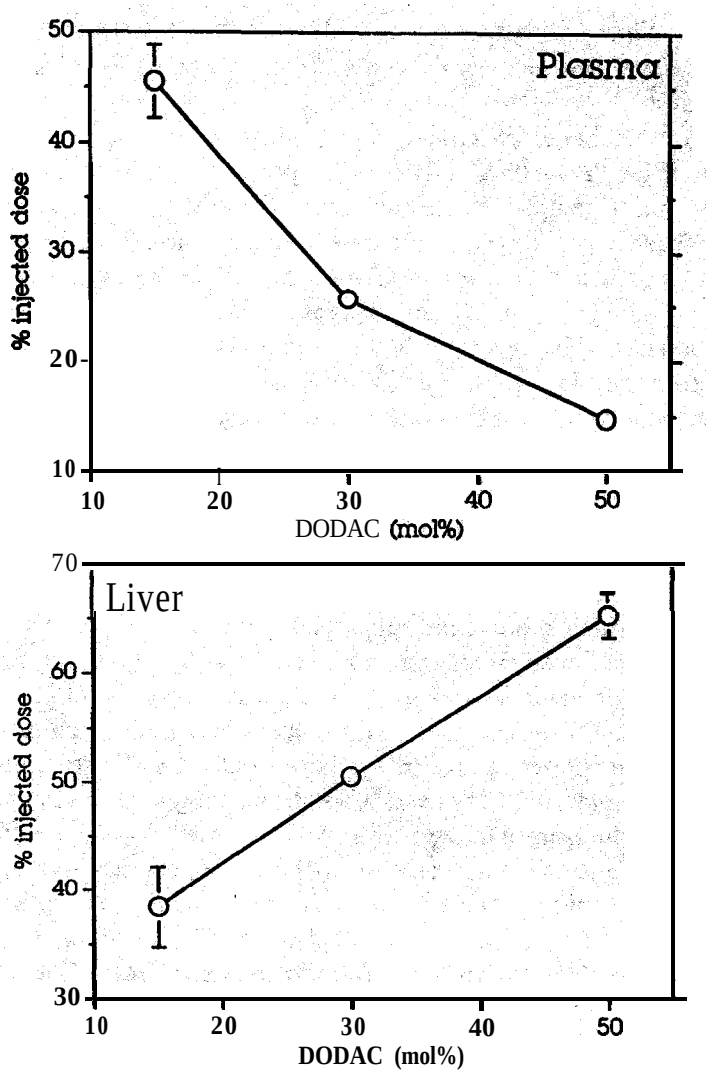

Figure 5. Effect of increasing concentrations of DODAC on LUV levelsinthe plasma and liver. 'H-labeled LUVs composed of DOPE, DODAC with 10 mol% PEG-Cer(C_{20}) with various molar ratios were injected i.v. into mice. LUV levels in theplasma,and~$ver were examined at 1 hr after injection. Molar ratios and average diameters of DOPE/DODAC/PEG-Cer(C_{20}) ***LUVs*** used were: 75: 15: 10, 102 (38) nm; 60:30: 10, 111 (38) nm; 40:50: 10, 114 (33) nm. Data were expressed as percentages of injected dose in the plasma and liver with SD (n = 3).

component leads to short circulation times and thus limits their utility as a drug delivery system [32]. In vivo stabilization of cationic liposomes represents the first step toward the -development of more controllable fusogenic systems. Our approach is based on the development of PEG-stabilized LUVs in which the fusogenic properties are transiently. masked by the presence of an-exchangeable polymer coating_ In- the present study, a series of amphipathic PEG derivatives was

used to 1) stabilize DOPE-based cationic liposomes in the presence of serum proteins, 2) regulate their fusion characteristics and 3) extend their blood circulation time after systemic administrations.

Freeze-fracture electron micrographic analyses reveal extensive aggregation and fusion of DOPE/DODAC LUVs in the presence of serum. In addition, these LUVs are almost completely cleared from the blood within the first hour after injection. These observations illustrate the problems inherent in DOPE-based cationic liposomes when used for *in vivo* applications. We have recently demonstrated that cationic liposomes have increased affinity for negatively charged serum components, thus resulting in their accelerated destabilization and rapid clearance from the blood [52]. Alternatively, the generation of large heterogeneous fused aggregates of cationic liposomes in the presence of serum proteins (as shown here) or plasmid DNAs [53] enhances the rapid elimination of these particles from the circulation and inhibits their ability to extravasate into interstitial tissue.

The effectiveness of amphipathic PEG derivatives in stabilizing DOPE/DODAC LUVs. is demonstrated by the fact that incorporation of PEG-DSPE or PEG-Cer in the cationic LUVs prevents serum-induced aggregation. In addition, biodistribution studies on these amphipathic PEG-stabilized DOPE/DODAC LUVs showed prolonged circulation times in the blood and concomitantly reduced accumulation in the liver. Two possible mechanisms account for prolonged circulation times of DOPE-based cationic liposomes containing amphipathic PEG derivatives. First, the presence of a PEG polymer on the LUV surface prevents close apposition of neighboring LUVs and subsequent aggregation or fusion, thus stabilizing fusogenic liposomes in the bilayer configuration. Second, the PEG polymer may prevent specific and nonspecific interactions of liposomes with serum components, inhibiting rapid opsonization and reticulendothelial cell uptake [52].

The charge and acyl chain length of the hydrophobic anchor of the PEG derivative play important roles in determining the fusogenic activity and biodistribution of DOPE/DODAC LUVs. The results presented here show that LUVs with suppressed fusogenic activity exhibit longer circulation times in the blood. However, in order for the LUVs to undergo fusion with a target membrane, the PEG derivative must dissociate from the LUV surface. This study shows that the rate of dissociation of the amphipathic PEG-anchor molecules from the cationic LUV membrane is sensitive to both the charge and the chain length of the lipid anchor. With regard to the effect of charge, the leaving rates of the negatively charged PEG-PE molecules are slower when the liposomes contain a cationic lipid such as DODAC. This can obviously be rationalized on the basis of attractive electrostatic interactions between PEG-PE molecules and DODAC in the LUV membrane, reducing the proportion in the free, monomer form.

As a result of the influence of charge on the PEG derivative exchangeability, it is experimentally more convenient to employ neutral amphipathic PEG derivatives, such as PEG-Cer, where the activity of PEG-Cer in suppressing the fusogenic activity of DOPE/DODAC LUVs is a function only of the ceramide acyl chain length, The activity of PEG-Cer in prolonging the circulation time of DOPE/

DODAC LUVs in the blood is consistent with regulation of the rate at which the amphipathic PEG derivative dissociates from the liposome membrane by the acyl chain length of the hydrophobic anchor. The amphipathic PEG derivative with a longer acyl chain will dissociate more slowly from the liposome membrane, resulting in observed prolonged stabilization in vitro (Fig. 2) and prolonged circulation times in the blood (Fig. 4) of DOPE/DODAC LUVs. Thus, the stability and circulation time in the blood of these LUVs can be designed for particular applications by altering the hydrophobic anchor of the amphipathic PEG derivative and its concentration in the lipid- mixture. This leads to the concept of novel cationic liposomal systems with regulated stability properties, leading in turn to regulated fusion and drug release characteristics. In this system, cationic liposomes are stabilized for a predetermined period of time and subsequently activated for fusion and drug release through the dissociation of the amphipathic PEG derivative from the liposome membrane. The major advantage of such systems is that the long circulation times allow preferential "passive" targeting of LUVs to disease sites such as tumors, sites of infection and sites of inflammation [55], whereas the latent fusogenic character should facilitate intracellular delivery into target cells. The transiently stabilized fusogenic LUV systems described here should provide a system applicable in the delivery of a wide variety of therapeutic agents.

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ABBREVIATIONS

DODAC, N,N-dioleoyl-N,N-dimethylammonium chloride; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPS, dioleoylphosphatidylserine; DMPE, dimyristoylphosphatidylethanolamine; DSPE, distearoylphosphatidylethanolamine; LUV, large unilamellar vesicle' NBD-PE, N-[7-nitrobenz-2-oxa-l,3-diazol-4-y1]-dioleoyl-phosphatidylethanolamine; PC, phosphatidylcholine; PEG, polyethyleneglycol; PEG-Cer, 1-0-(monomethoxypolyethyleneglycol succinoyl)-ceramide; POPC, palmitoyloleoylphosphatidylcholine; POPS, palmitoyloleoylphosphatidylserine; PS, phosphatidylserine; QELS, quasielastic light scattering; Rh-PE, N-[lissamine-rhodamine P-sulfonylldioleoylphosphatidylethanolamine.

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