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Factors influencing the retention and chemical stability of poly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles

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Abstract

Poly(ethylene glycol)(PEG)-lipid anchor conjugates can prolong the circulation lifetimes of liposomes following intravenous injection. In this work we investigate the influence of the lipid anchor and the nature of the chemical link between the PEG and lipid moieties on circulation lifetime. It is shown that incorporation of *N*-(monomethoxypoly(ethylene glycol)$_{2000}$-succinyl)-1-palmitoyl-2-oleoylphosphatidylethanolamide (MePEG$_{2000}$-S-POPE) into large unilamellar vesicles (LUVs) composed of distearoylphosphatidylcholine (DSPC) and cholesterol (DSPC/cholesterol/MePEG$_{2000}$-S-POPE, 50:45:5, mol/mol) results in only small increases in the circulation lifetimes as observed in mice. This is shown to be due to rapid removal of the hydrophilic coating in vivo, which likely arises from exchange of the entire PEG-lipid conjugate from the liposomal membrane, although chemical breakdown of the PEG-lipid conjugate is also possible. The chemical stability of four different linkages was tested, including succinate, carbamate and amide linkages between MePEG derivatives and the amino head group of PE, as well as a direct link to the phosphate head group of phosphatidic acid (PA). The succinate linkage was found to be the most labile. The anchoring capability of DSPE as compared to POPE in PEG-PE conjugates was also examined. It is shown that incorporation of MePEG$_{2000}$-S-DSPE conjugates into DSPC/cholesterol LUVs results in little loss of the PEG coating in vivo, long circulation lifetimes and reduced chemical breakdown of the PEG-lipid conjugate. This work establishes that DSPE is a considerably more effective anchor for PEG$_{2000}$ than POPE and that the chemical stability of PEG-PE conjugates is sensitive to the nature of the linkage and exchangeability of the PEG-PE complex. We suggest that retention of the PEG coating is of paramount importance for prolonged circulation lifetimes.

Keywords: Liposome; Poly(ethylene glycol); Drug delivery system; Stability; Exchange; Biodistribution

1. Introduction

The use of liposomes as drug delivery vehicles in vivo requires long circulation lifetimes. For example, increased circulation times result in enhanced tumor uptake and enhanced efficacy when the liposomes contain anticancer drugs [1–6]. It has been shown that the incorporation of ganglioside G_{M1} into the liposomes can improve circulation longevity [7]. Alternatively, incorporation of monomethoxypoly(ethylene glycol)-phosphatidylethanolamine (MePEG-PE) conjugates into liposomal systems can also significantly extend the circulation lifetimes of intravenously injected liposomes [8–12], often to an extent greater than that provided by G_{M1}. It has been proposed that MePEG-PE provides a 'steric stabilization' of the surface by virtue of the hydrophilic brush coat provided by the MePEG polymer [13–15]. This coat may inhibit serum protein binding to the liposomal surface [16,17] which would otherwise promote uptake by the reticuloendothelial system (RES), the circulating phagocytic cell populations or fixed populations such as those found in the liver and spleen.

The extent to which the PEG coating remains associated with the liposome after injection into the circulation has not yet been adequately addressed. The lipid moiety of the molecule must obviously be sufficiently lipophilic to firmly anchor the hydrophilic coat to the surface. In this regard, liposomally incorporated PEG-cholesterol or PEG-monostearate are relatively inef-

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fective at improving the circulation lifetimes of intravenously injected liposomes [11]. Presumably, the hydrophobic moiety in these compounds is an ineffective anchor and thus the hydrophilic coat is rapidly lost from injected LUVs. Reports on the anchoring properties of diacylphosphatidylethanolamine anchors have been conflicting. It has been suggested that the lipid moiety has little effect on 'stealth' characteristics of LUVs [18], while others indicate that the lipid anchor is a important factor [11]. The chemical stability of MePEG-PEs in vivo has not received detailed attention.

In this work we examine the influence of the lipid anchor and linkage chemistry on the ability of MePEG-PE to improve circulation lifetimes of LUV systems. We show that the anchoring capacity of PE anchors is extremely sensitive to the acyl chain composition, where distearoyl PE species are considerably more effective anchors than palmitoyloleoyl species. Second, depending on the type of linkage between the PEG and the PE, breakdown can occur either on the LUV surface or after release of PEG-PE from the LUV. These factors should be considered when discussing the usefulness or mechanisms of PEG-PEs incorporated into liposomes.

2. Materials and methods

2.1. Monomethoxypoly(ethylene glycol)-lipid (MePEG-lipid) synthesis

The overall chemical structures of the of the various MePEG-lipids synthesized are shown in Fig. 1, which include MePEG linked to phosphatidylethanolamine via succinate (MePEG-S-PE), carbamate (MePEG-C-PE) and amide (MePEG-A-PE) linkages, and directly to phosphatidic acid (MePEG-PA). All PEG-lipids were isolated as a single component on TLC, with similar R_f values, and showed ^1H NMR resonances characteristic of the MePEG and lipid groups.

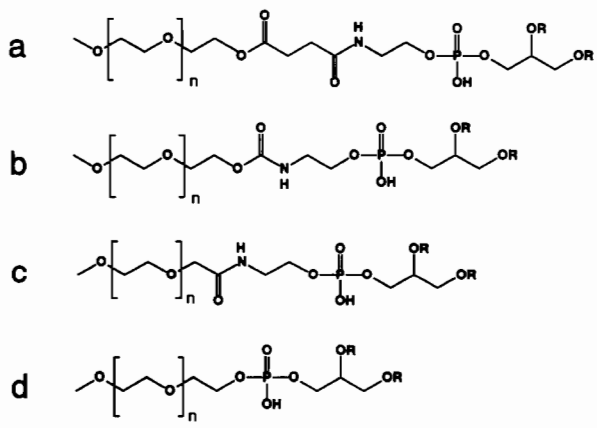

Fig. 1. Summary of PEG-lipid conjugate chemical structures. (a), succinate linkage: MePEG-S-PE; (b), carbamate linkage: MePEG-C-PE; (c), amide linkage: MePEG-A-PE; (d), direct linkage: MePEG-PA. (See Materials and methods for detailed descriptions of the compounds synthesized.)

$MePEG_{2000}$-S-POPE and $MePEG_{2000}$-S-DSPE were synthesized as follows. Monomethoxypoly(ethylene glycol) ($MePEG_{2000}$-OH) was treated in pyridine with ten equivalents of succinic anhydride at room temperature for two days. The solution was diluted with water, acidified, extracted with methylene chloride, and the organic extracts were dried over magnesium sulfate, filtered, and the solvent removed. The resulting residue was subjected to silica gel column chromatography in methylene chloride/methanol (96:4, v/v) and $MePEG_{2000}$-succinate isolated. The dry $MePEG_{2000}$-S, DCC, and NHS were dissolved in chloroform, stirred for 1 h and filtered. Dry 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) or 1,2-distearoyl phosphatidylethanolamine (DSPE) was dissolved in warm chloroform and added to the filtrate, then triethylamine was added and the reaction mixture stirred for half an hour. Combined organic extracts were dried over magnesium sulphate, filtered and the solvent removed under reduced pressure. The residue was subjected to silica gel column chromatography. Fractions collected were analyzed by TLC using methanol/chloroform as the solvent (15:85, v/v) and visualized by exposure to iodine. Fractions containing pure $MePEG_{2000}$-S-POPE or $MePEG_{2000}$-S-DSPE were combined, taken up into distilled water, centrifuged at $1500 \times g$ for 30 min and the supernatants dialyzed against distilled water overnight. The resultant solutions were lyophilized to yield a white powder.

The synthesis of $MePEG_{2000}$-[^{14}C]S-POPE was carried out as described for $MePEG_{2000}$-S-POPE with the exception that [1,4-^{14}C]succinic anhydride was reacted first before addition of an excess of unlabelled succinic anhydride. The product was isolated by preparative TLC using two successive plates run in methanol/chloroform (15:85, v/v). The $MePEG_{2000}$-S-POPE component was extracted from the appropriate scraped bands with methanol and then methanol/water (1:1, v/v) to yield $MePEG_{2000}$-[^{14}C]S-POPE with specific activity 1.74 mCi/mmol. The extract was dispersed in water, centrifuged at $1500 \times g$ for 30 min, frozen and lyophilized.

The synthesis of $MePEG_{2000}$-S-[^{3}H]DSPE first required making $MePEG_{2000}$-S-DOPE similarly to the procedure described for $MePEG_{2000}$-S-POPE. The $MePEG_{2000}$-S-DOPE and Pd-C were then added with methanol to a vial which was sealed and flushed with nitrogen. Sodium borotritide (490 mCi/mmol) was injected and the mixture stirred for 1 h. Sodium borohydride was added and the solution stirred for 3 h. After carefully opening in a fume hood, the suspension was

acidified with a drop of hydrochloric acid (10%), more methanol added, and then centrifuged. The supernatant was filtered through celite, diluted with water and extracted with methylene chloride. The organic fractions were dried over magnesium sulfate, filtered, and the $MePEG_{2000}$-S-[^{3}H]DSPE was purified by preparative TLC as described above (99% of the remaining radioactivity was located in the $MePEG_{2000}$-S-DSPE component, specific activity 140 mCi/mmol).

To synthesize [^{3}H]$MePEG_{2000}$-S-DSPE, a sample of [^{3}H]$MePEG_{2000}$-OH (Amersham, custom synthesis) was used as the starting material with the remainder of the synthetic procedure followed that of $MePEG_{2000}$-S-DSPE. Purification was carried out using preparative TLC plates as described above to yield [^{3}H]Me-PEG_{2000}-S-DSPE (specific activity 44 mCi/mmol). [^{3}H]$MePEG_{5000}$-S-POPE (specific activity 156 mCi/mmol) was synthesized similarly to [^{3}H]$MePEG_{2000}$-S-DSPE.

$MePEG_{2000}$-S-(1-palmitoyl-2-(4-pyrenyl)-butyroyl)PE was made by first synthesizing $MePEG_{2000}$-S-(1-palmitoyl-2-hydroxy)-phosphatidylethanolamine as the starting species using the procedure described for Me-PEG_{2000}-S-POPE. A solution of pyrenebutyric acid and DCC in alcohol free chloroform was allowed to stir at room temperature for 1 h, filtered and then added to the reaction mixture. After work up, the product was isolated by column chromatography and preparative thin-layer chromatography as before to yield a pure fluorescent compound, $MePEG_{2000}$-S-(1-palmitoyl-2-(4-pyrenyl)-butyroyl)PE ($MePEG_{2000}$-S-PPBPE).

$MePEG_{2000}$-C-POPE was made according to the following procedure. Dry $MePEG_{2000}$-OH was dissolved in chloroform/toluene (50 : 2, v/v), reacted with triphosgene and the product precipitated by addition of ether. The precipitate, $MePEG_{2000}$-chloroformate ($MePEG_{2000}$-C-Cl), was filtered and dried under vacuum. The $MePEG_{2000}$-C-Cl and dry POPE were dissolved in chloroform and treated with triethylamine. The solvent was removed under vacuum and the residue dissolved in water. The aqueous solution was acidified and extracted with methylene chloride. The combined organic fractions were dried over magnesium sulphate, filtered, solvent removed and the residue subjected to column chromatography using silica gel and methanol/methylene chloride to yield pure $MePEG_{2000}$-carbamate-POPE ($MePEG_{2000}$-C-POPE). The product was dispersed in water, centrifuged at $1500 \times g$ for 30 min and the supernatant lyophilized to yield a white powder.

$MePEG_{2000}$-A-POPE and $MePEG_{2000}$-A-DSPE were synthesized as follows. $MePEG_{2000}$-acetic acid was synthesized by an adaption of the procedure in Sessler et al. [19]. Briefly, $MePEG_{2000}$-OH was added to a solution of sodium dichromate in dilute sulfuric acid (10%) and the solution stirred at room temperature overnight. The solution was extracted with methylene chloride and the combined organic extracts washed with sodium hydroxide solution (1 M). The organic fraction was dried over magnesium sulphate, filtered and the solvent removed under reduced pressure. The residue was dissolved in a minimum of chloroform and precipitated with ether. The precipitate was filtered and dried, yielding $MePEG_{2000}$-acetic acid as a pale blue powder (colour due to complexed chromium). $MePEG_{2000}$-A-POPE and $MePEG_{2000}$-A-DSPE were then prepared using the same procedure as the succinate analogs, substituting $MePEG_{2000}$-acetic acid for $MePEG_{2000}$-succinate.

To synthesize $MePEG_{2000}$-POPA, a mixture of 1-palmitoyl-2-oleoylphosphatidic acid (POPA), Me-PEG_{2000}-OH and 2,6,6-triisopropylbenzenesulphorylchloride (TIPBSC) was suspended in pyridine. The reaction mixture was protected from light and allowed to stir overnight. Water was added and the mixture allowed to stir for a further 3 h. The solution was diluted with water, acidified and extracted with methylene chloride. After removal of the solvent, the residue was dispersed in water, filtered, centrifuged and the supernatant lyophilized. The resultant powder was subjected to column chromatography using silica gel and methanol/chloroform. Pure fractions were combined, taken up in water, centrifuged and lyophilized to yield a white powder.

All lipids utilized in the above synthetic procedures were from Avanti Polar Lipids. Unless indicated otherwise, all other materials were from Sigma. Column chromatography was carried out using silica gel 60 (70–230 Mesh ASTM) (Merck). Analytical TLC employed aluminum backed silica gel 60-F_{254}, 0.2 mm thick (Merck) and preparative TLC employed glass backed silica gel 60, 0.5 mm thick (Merck).

2.2. Preparation of large unilamellar vesicles (LUVs)

The production of LUVs was carried out as previously described [20]. Briefly, lipid mixtures composed of distearoylphosphatidylcholine (DSPC) and cholesterol (55 : 45, mol/mol), DSPC, cholesterol and MePEG-PE (50 : 45 : 5, mol/mol), or DSPC, cholesterol and G_{M1} (45 : 45 : 10, mol/mol), each with trace amounts of [^{14}C]- or [^{3}H]cholesteryl hexadecyl ether (CHE) as a non-metabolizable and non-exchangeable liposome marker [21] were freeze-dried from benzene/methanol solution and hydrated in physiological sterile Hepes-buffered saline (HBS) (20 mM Hepes, 150 mM NaCl, pH 7.4). The sample was freeze-thawed five times and then extruded at 65°C ten times through two stacked 100 nm pore size polycarbonate filters (Costar/Nuclepore, Canada) employing an Extruder (Lipex Biomembranes, Canada). Liposome size ranged from 95 to 115 nm as determined by

quasi-elastic light scattering on a NICOMP Model 270 submicron particle sizer. The resultant LUVs were loaded onto a conventional Bio-Gel A-15m (200–400 mesh) (Bio-Rad, Canada) 10×1 cm column equilibrated with HBS to remove unincorporated MePEG-PE, and the pooled liposome peak diluted with HBS to an appropriate concentration. All initial liposome preparations were checked for concentration by determination of phosphorous [22] using a Shimadzu UV-visible recording spectrophotometer at 815 nm, and thereafter by scintillation counting using a Beckman LS3801 with Pico-Fluor 40 scintillation fluid (Packard). [^{14}C]- and [^{3}H]CHE was from NEN/DuPont, DSPC from Avanti Polar Lipids, and cholesterol and other chemicals were from Sigma.

2.3. Exchange studies

The MePEG-PE to liposome radiolabel ratios for 5 mM (total lipid) preparations incorporating either $MePEG_{2000}$-[^{14}C]S-POPE, [^{3}H]$MePEG_{2000}$-S-DSPE, or $MePEG_{2000}$-S-[^{3}H]DSPE were taken to determine the starting ratio for the exchange studies. Then 500 μl of the liposome preparation was diluted in 500 μl of either HBS or normal mouse serum (Cedar Lane, Canada). For $MePEG_{2000}$-S-POPE, an additional incubation involving 500 μl of liposomes with 200 μl of mouse serum and 300 μl of HBS was carried out. These mixtures were incubated at 37°C and at various times, two 50 μl aliquots were removed and passed down 1 ml Bio-Gel A-15m spin columns to separate liposomes in the void volume from serum and unincorporated MePEG-PE radiolabel. (The use of these spin columns to separate liposomes from serum components has been previously described [23]). The peak two liposome fractions from both columns were counted and the MePEG-PE to liposome radiolabel ratios were determined by a corrected DPM dual label determination.

2.4. Chemical stability studies

5 mg of the MePEG-lipid indicated was dissolved first in 200 μl of water, and then incubated in 1000 μl normal mouse serum at 37°C. Additional incubations involving 500 μl of liposomes (20 mM total lipid) composed of DSPC/cholesterol/MePEG-lipid in 500 μl serum at 37°C were also carried out. At various times, aliquots from the micellar or liposomal incubations were removed and lipid components extracted by the following procedure. 80 μl of sample was added to 920 μl of water. To this was added 2.1 ml of methanol and 1.0 ml of chloroform. After mixing, 1.0 ml of water was added, vortexed, and then an additional 2.0 ml of chloroform was added. After thorough vortexing, the sample was allowed to sit for 10 min before centrifuging at $1500 \times g$ for 1 h. The organic layer was isolated, concentrated, and then spotted on 0.25 mm thick, silica gel 60, 5×10 cm TLC glass plates (Merck). The solvent system used to develop the plates was chloroform/methanol (85:15, v/v), and were visualized with iodine vapor. Where [^{3}H]$MePEG_{5000}$-S-POPE was used, 0.5 cm sections of each running lane were scraped and extracted with 3×1.0 ml chloroform/methanol/water (50:40:10, v/v). The extract was placed in scintillation vials, solvent removed, 5.0 ml scintillation fluid added, and left overnight before counting. Where $MePEG_{2000}$-S-PPBPE was used, fluorescence associated with scraped sections was extracted as above, solvent removed, and resuspended in 4.0 ml of 0.5% (w/v) sodium cholate detergent. Fluorescence was read on a Perkin Elmer LS50 luminescence spectrometer operating at 600 V using an excitation wavelength of 339 nm (2.5 mm slit width), emission wavelength of 377 nm (2.5 mm slit width), and filter set at 350 nm. TLC standards used to aid identification included the appropriate free MePEG-OHs, extracted serum, free 4-(1-pyrenyl)butyric acid, and the appropriate MePEG-lipids.

2.5. Biodistribution and circulation longevity studies

The LUV preparations employing trace [^{3}H]- or [^{14}C]CHE as liposome markers were injected via lateral tail vein in a volume of 200 μl (1 μmol total lipid) into 25 g CD-1 mice (Charles River, Canada). At various times, the mice were sacrificed and blood withdrawn by cardiac puncture and collected in microtainer tubes with EDTA (Becton-Dickinson, Canada). After centrifuging at $1500 \times g$ for 10 min, the plasma was isolated and showed no hemolysis. Two 100 μl samples from each mouse were counted directly in 5.0 ml scintillation fluid. The percentage recovery of liposomes remaining in circulation was based on a plasma volume of 4.55% of individual mouse body weight. Liver and spleen tissue were homogenized by Polytron to 20% and 10% in saline, respectively. 200 μl of tissue homogenate was solubilized with 500 μl Solvable (NEN/DuPont) for 2 h at 60°C, after which the samples were cooled and treated overnight with 200 μl hydrogen peroxide. 5 ml scintillation fluid was then added before counting. Liver and spleen associated liposomes are expressed as percent injected dose per tissue (total organ weight). Where in vivo exchange studies were carried out, two 50 μl aliquots of plasma were passed down spin columns as described above and the MePEG-lipid to liposome ratio determined.

3. Results

3.1. PEG_{2000}-S-POPE is lost from the liposome surface both in vivo and in vitro

The first series of experiments were designed to ascertain the influence of $MePEG_{2000}$-S-POPE on the circulation lifetimes of 100 nm diameter DSPC/cholesterol (55:45 mol/mol) LUVs in mice. As shown in Fig. 2, the incorporation of 5 mol% $MePEG_{2000}$-S-POPE results in a relatively modest increase in LUV circulation lifetimes. At 24 h, the LUV preparation incorporating PEG-POPE is almost completely cleared. This may be contrasted with previous reports [11] that incorporation of 5 mol% PEG-PE can result in up to 30% of DSPC/cholesterol LUVs remaining in the circulation at 24 h, and the third curve where liposomes incorporating $MePEG_{2000}$-S-DSPE have much greater circulation levels (over 20% at 24 h). In order to determine whether this could be due to the loss of the PEG coating arising from interactions with serum protein factors, the DSPC/cholesterol LUVs (labelled with [^{3}H]CHE as a non-exchangeable liposome marker) incorporating $MePEG_{2000}$-[^{14}C]S-POPE were incubated with normal mouse serum at 37°C and the retention of radiolabel monitored. As shown in Fig. 3A, this incubation results in rapid loss of the MePEG-PE radiolabel. While the MePEG-PE content of the LUVs is relatively unaffected when incubated in HBS alone, the MePEG-PE radiolabel rapidly drops when incubated in the presence of either 20% or 50% serum. After 24 h in 50% serum, the MePEG-PE to liposome marker ratio has dropped to nearly 50% of its initial value, suggesting that practically all of the MePEG-PE

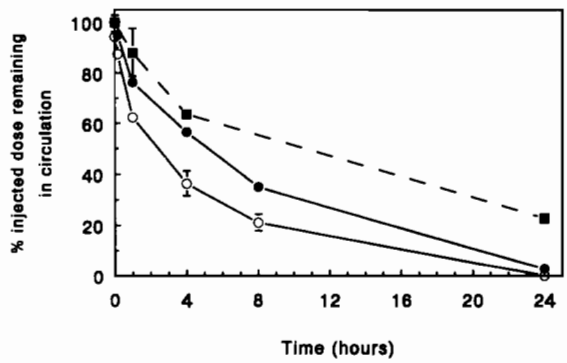

Fig. 2. Circulation lifetime of DSPC/cholesterol/$MePEG_{2000}$-S-POPE liposomes. Large unilamellar vesicles composed of DSPC/cholesterol (55:45) (○), DSPC/cholesterol/$MePEG_{2000}$-S-POPE (50:45:5) (●), (or DSPC/cholesterol/$MePEG_{2000}$-S-DSPE (50:45:5) (■), dashed line, taken from Fig. 6A) were injected via the lateral tail vein into 25 g CD-1 mice (5 mM total lipid in 200 μl, 1 μmol total lipid/mouse). At various times, the mice were sacrificed and plasma isolated. The marker [^{3}H]CHE was used to determine liposome recovery ([^{14}C]CHE for (■)). Results shown represent the mean ± S.E. of four animals.

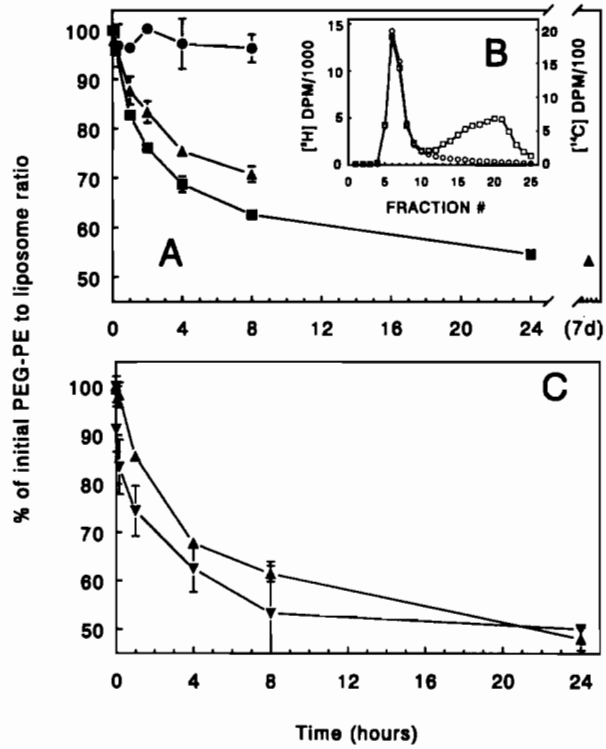

Fig. 3. Loss of PEG coating from the surface of the LUV. (A) In vitro incubation of DSPC/cholesterol/$MePEG_{2000}$-[^{14}C]S-POPE (50:45:5, mol/mol) large unilamellar vesicles in normal mouse serum at 37°C. 500 μl of the LUV preparation (5 mM total lipid) was incubated in the presence of 500 μl HBS (●), 200 μl serum plus 300 μl HBS (▲), or 500 μl serum (■), representing 0%, 20%, and 50% serum concentrations, respectively. At various times, two 50 μl aliquots of the incubation mixture were removed and liposomes separated from the free PEG-PE and serum components by loading onto a 1 ml Bio-Gel A-15m spin column. The ^{14}C (PEG-PE)/^{3}H (CHE, liposome marker) ratios were obtained from the peak two liposome fractions from both columns and expressed as a percentage (±S.D.) of the initial ratio before incubation. (B) Bio-Gel A-15m (200–400 mesh) spin column profile of DSPC/cholesterol/$MePEG_{2000}$-[^{14}C]S-POPE LUVs in 50% serum at 37°C for 24 h. (○), the ^{3}H DPM (liposome) label, and (□), the ^{14}C DPM (PEG-PE) label measured for 30 μl of each collected fraction. (C) The in vivo loss of the PEG-PE coating from injected liposomes. The ^{14}C (PEG-PE)/^{3}H (CHE) ratio was determined for liposomes recovered from mice injected with DSPC/chol/PEG-PE liposomes both before (▲) and after (▼) separation of liposomes from plasma components via spin column. Results represent the means of ^{14}C (PEG-PE)/^{3}H (CHE) ratios obtained from the four mice before, or after the peak two liposome fractions from two columns for each mouse plasma sample, and expressed as a percentage (±S.D.) of the initial ratio before injection.

radiolabel in the outer monolayer has been removed. This is supported by the results of Fig. 3B, which shows the spin column profile for the 24 h time point. There is an exact overlap of the MePEG-PE and liposomal peaks, and good separation from the MePEG-PE no longer associated with the liposomes. Integration of the two peaks yields a 52:48 ratio for liposome associated to free MePEG-PE. After an additional 6 days at room temperature in the 20% incubation, this ratio was also

nearly 50%. Given our data, it is not unreasonable to assume that little more than 50% of the label is available for exchange. In addition, studies with Me-PEG_{1900}-carbamate-DSPE at 4 mol% indicate that the PEG extends outward from the surface in a brush formation appoximately 5 nm [14]. Although direct evidence is lacking, it has been suggested that the distribution should be approximately equal between the two leaflets of the bilayer as long as the radius of the vesicle is large (greater than a factor of 10) relative to the length of the polymer [15] as is the case here.

The in vivo exchange results presented in Fig. 3C confirm that the MePEG-PE is lost from the surface of the injected LUVs. Here, plasma samples were taken at various times from mice which had received [^{3}H]CHE labelled liposomes incorporating Me-PEG_{2000}-[^{14}C]S-POPE and were counted to compare the MePEG-PE to liposome ratios both before and after separation of the liposomes from the plasma. In agreement with the in vitro data, this ratio drops significantly with time indicating rapid loss of the Me-PEG-PE. The differences in the ratios for unseparated and separated liposomes can be attributed to Me-PEG-PE which is no longer associated with the liposomes but which continues to circulate for a short period of time and to the short time required to isolate plasma and elute the spin columns. The level of Me-PEG-PE still associated with the liposome approaches 50% by 8 h, and by 24 h has completely leveled off. The faster rate of loss of this component from the outer monolayer in vivo versus in vitro is expected given the greater plasma/serum to liposome ratio in vivo. It would also appear that the rate of loss of MePEG-PE is faster than the clearance rate of the LUVs initially containing this component. If only approximately 50% of the label is available for exchange, label loss leads clearance by a significant amount. For example, at 1 h post injection 76% of the vesicles remain in the blood whereas, after serum removal, 75% of the label remains. This indicates that 50% of the available exterior label, then 50% of the outer label has been lost and 24% of the vesicles have been cleared. At 4 h, by the same arguments, 75% of the outer label has been removed while 45% of liposomes have been cleared. Thus, at these early time points outer label removal leads clearance by 25–30%. It is also of interest to compare the clearance rate to the control. One can see that when at least some proportion of the PEG coating remains, these liposomes have a slower clearance rate than the control. However, beyond some critical value of PEG loss between 4 and 8 h after which there is very little PEG remaining on the surface, the clearance of these liposomes quickly approaches that of the control.

3.2. Chemical stability of various linker groups in the MePEG-PE conjugate

There are two possible mechanisms for the loss of the MePEG-PE radiolabel from the LUVs in vitro and in vivo. These are cleavage of the MePEG moiety from the lipid anchor or exchange of the entire MePEG-PE out of the LUV. Chemical breakdown was monitored by thin-layer chromatography (TLC) after incubation of MePEG-PE micelles in normal mouse serum. The data of Fig. 4 shows the effect of different chemical linkages between MePEG and the lipid anchor on the chemical stability of MePEG-PE. All four versions

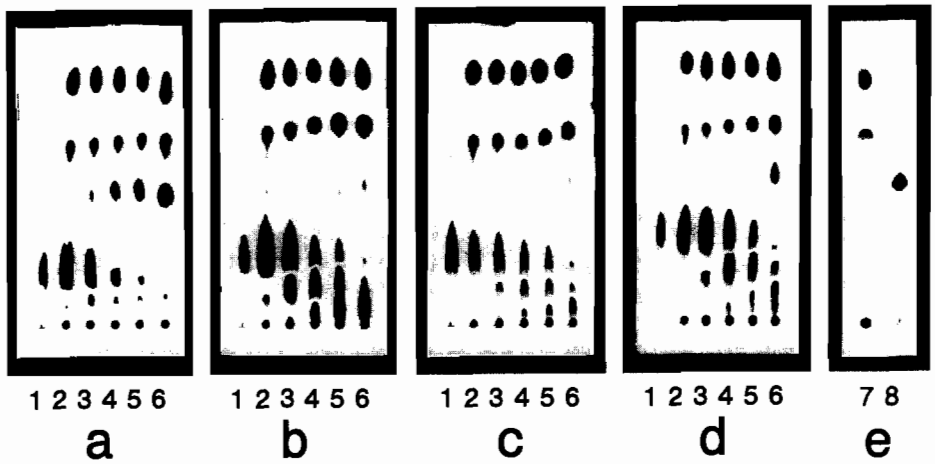

Fig. 4. Thin-layer chromatography of the results of incubation of micellar PEG-PE in serum at 37°C. (a), $MePEG_{2000}$-S-POPE; (b), $MePEG_{2000}$-C-POPE; (c), $MePEG_{2000}$-A-POPE; (d), $MePEG_{2000}$-POPA. 5.0 mg of the PEG-PE indicated was first dissolved in 200 μl of water, then incubated with an additional 1000 μl of normal mouse serum at 37°C. At various times, 80 μl aliquots were withdrawn, extracted, and concentrated. The samples were run on 0.25 mm silica plates developed with chloroform/methanol (85 : 15, v/v) and spots visualized with iodine vapor. Lanes were: (1), before incubation; (2), 5 min incubation; (3), 1 h; (4), 4 h; (5), 8 h; (6), 24 h. (e) shows TLC standards normal mouse serum extract (7) and free $MePEG_{2000}$-OH (8).

degrade significantly after exposure to serum at 37°C. The succinate linked version rapidly generates a compound which corresponds to free MePEG, while the other three (carbamate, amide and direct linked) versions show very little of this product. The breakdown of the succinate ester linkage is perhaps not unexpected given a similar phenomenon observed in the corresponding MePEG-protein conjugates [24], however, this has not been previously reported for PEG-lipid conjugates. In addition, all four versions show the appearance of a product which is slightly more polar than the starting MePEG-lipid, which in turn is broken down to yield a product with further increased polarity by 24 h. When these four compounds were incorporated into liposomes, incubation in serum yielded the same patterns of breakdown products, but the rates at which these products are formed was significantly slower (results not shown).

Additional experiments were performed to characterize the breakdown products. The [^{3}H]MePEG$_{5000}$-S-POPE was incubated in micellar form in serum as described above, and the TLC lanes were counted for radioactivity. The results (not shown) confirmed that the apparent free MePEG spot contained [^{3}H]MePEG$_{5000}$-OH. When MePEG$_{2000}$-S-(1-palmitoyl-2-(4-pyrenyl)-butyroyl)PE (MePEG$_{2000}$-S-PPBPE) was incubated in serum, fluorescence (due to the pyrene moiety) was observed in the more polar spots as well as the spot corresponding to free pyrene butyric acid (data not shown). This suggests that the more polar spots are lyso-MePEG-lipid compounds which have had one or both of the acyl chains cleaved from the conjugate. In addition, heat inactivation of serum or addition of EGTA was found to significantly reduce the rate of breakdown. As controls, extraction efficiencies from serum or buffer over all time points and conditions were checked and found to be consistently above 90%. All versions of the MePEG-lipid conjugates remain relatively stable in HBS (pH 7.4) over 24 h, although the succinate version did show some slow breakdown. Lower pH values (pH 2) resulted in increased appearance of lyso compounds, probably due to acid catalyzed hydrolysis at the *sn*-1 and *sn*-2 positions [21].

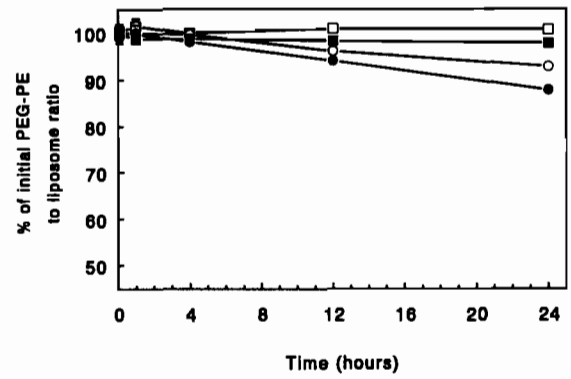

Fig. 5. In vitro incubation in normal mouse serum at 37°C of DSPC/cholesterol large unilamellar vesicles incorporating 5 mol% MePEG$_{2000}$-S-DSPE. 500 μl of the LUV preparations (5 mM total lipid) incorporating [^{3}H]MePEG$_{2000}$-S-DSPE (circles) or MePEG$_{2000}$-S-[^{3}H]DSPE (squares) was incubated in the presence of 500 μl HBS (open symbols), or 500 μl serum (closed symbols). At various times, two 50-μl aliquots of the incubation mixture were removed and liposomes separated from free components by loading onto a 1 ml Bio-Gel A-15m spin column. The ^{3}H (PEG-PE)/^{14}C (CHE) ratios were obtained from the peak two liposome fractions from both columns and expressed as a percentage (±S.D.) of the initial ratio before incubation.

3.3. PEG$_{2000}$-DSPE is retained in DSPC / cholesterol LUVs and exhibits enhanced chemical stability

The chemical breakdown of MePEG-lipids can occur either on the surface of the liposome or after exchange of the whole molecule out of the LUV. In order to reduce the possibility of exchange, MePEG$_{2000}$-PEs were synthesized with a DSPE anchor which may be expected to result in improved retention. The exchange of either [^{3}H]MePEG$_{2000}$-S-DSPE or MePEG$_{2000}$-S-[^{3}H]DSPE from [^{14}C]CHE labelled LUVs in serum or HBS at 37°C is illustrated in Fig. 5. The acyl chain label remains associated with the liposome in both HBS and in serum. However, the MePEG

Table 1
Biodistribution of DSPC/cholesterol large unilamellar vesicles incorporating G_{M1} or PEG-PE 24 h after injection

Liposome composition (molar ratio)		% of injected dose recovered per total tissue		
		blood	liver	spleen
DSPC/chol	(55:45)	0.20 ± 0.10	56.4 ± 3.3	7.76 ± 1.50
DSPC/chol/G_{M1}	(45:45:10)	11.3 ± 0.5	25.4 ± 0.4	1.90 ± 0.09
DSPC/chol/MePEG$_{2000}$-S-POPE	(50:45:5)	3.31 ± 1.15	42.2 ± 2.8	3.85 ± 0.45
DSPC/chol/MePEG$_{2000}$-A-POPE	(50:45:5)	2.57 ± 0.92	42.6 ± 2.8	3.49 ± 0.49
DSPC/chol/MePEG$_{2000}$-S-DSPE	(50:45:5)	16.3 ± 1.1	24.9 ± 2.6	1.60 ± 0.10
DSPC/chol/MePEG$_{2000}$-A-DSPE	(50:45:5)	18.0 ± 0.9	18.8 ± 1.4	1.41 ± 0.07

The 5 mM 100 nm LUV preparations were injected via lateral tail vein in a volume of 200 μl (1 μmol total lipid) into 25 g CD-1 mice. At 24 h, the mice were killed and plasma, liver, and spleen isolated. The percentage recovery of liposomes remaining in circulation was based on a plasma volume of 4.55% of individual mouse body weight. Liposomes associated with liver and spleen tissues were determined based on total organ weight. Each preparation employed trace [^{3}H]CHE as a liposome marker, and the results represent the mean of four animals ± S.E.

leaves the liposome to a measurable extent, resulting in approximately 7% loss in HBS over 24 h. In serum, the loss of this label is somewhat greater, up to approximately 12% loss over 24 h, although this rate is much reduced compared to $MePEG_{2000}$-S-POPE. Thus, the results of Fig. 5 demonstrate that a primary factor for retention of the MePEG coating is the lipid anchor and that these compounds are relatively chemically stable if they remain associated with the liposome. However, it also appears that slow hydrolysis of the succinate bond can occur on the liposome surface leaving the lipid anchor behind.

The ability of $MePEG_{2000}$-S-DSPE, when incorporated into DSPC/cholesterol LUVs, to prolong the circulation lifetime is significantly improved over that observed for $MePEG_{2000}$-S-POPE. Using [^{14}C]CHE labeled liposomes incorporating [^{3}H]$MePEG_{2000}$-S-DSPE or $MePEG_{2000}$-S-[^{3}H]DSPE, approximately 20% of the injected dose remains in the circulation at 24 h as shown in Fig. 6, with the two preparations exhibiting very similar clearance behavior. When the $^{3}H/^{14}C$ ratio was checked at various times both before and after separation of liposomes from plasma components (Fig. 6B and 6C), both the MePEG and acyl moiety labels of the $MePEG_{2000}$-S-DSPE are shown to remain associated with the liposome. It may, however, be more accurate to say that the liposomes recovered, which are representative of those still in circulation, have retained most of their PEG coating. It is probable that liposomes which have lost their protective coating would have been rapidly cleared.

3.4. *Biodistributions of DSPC / cholesterol LUVs containing different species of $MePEG_{2000}$-PE*

The final series of experiments were performed to characterize the biodistribution at 24 h for DSPC/cholesterol LUVs incorporating various species of $MePEG_{2000}$-PE varying in acyl chain composition or PEG-PE chemistry at a liposome dose level of 1 μmol total lipid per mouse. The results are shown in Table 1. In the absence of a PEG-PE coating, DSPC/cholesterol LUVs are almost completely removed from the circulation at 24 h (less than 1% of the injected dose remains), with high levels accumulated in the liver and spleen. The incorporation of 10 mol% G_{M1} significantly increases the circulation levels to approximately 11% of the injected dose remaining while decreasing the amount found in the liver and spleen at 24 h by factors of 2 and 4, respectively. Incorporation of 5 mol% $MePEG_{2000}$-S-POPE and $MePEG_{2000}$-A-POPE have smaller effects than G_{M1} in altering the LUV biodistribution. However, the presence of either $MePEG_{2000}$-S-DSPE or $MePEG_{2000}$-A-DSPE greatly increased the circulation levels present at 24 h to almost 20%, higher than achieved with G_{M1}, while the accumulation by the liver and spleen is reduced to an equal or better extent as G_{M1}. While there is little difference in biodistribution behavior between the succinate and amide versions for MePEG-POPE, the data for DSPE anchored species suggest that the amide linkage may be slightly superior in both improved circulation lifetimes and reduced liver and spleen uptake.

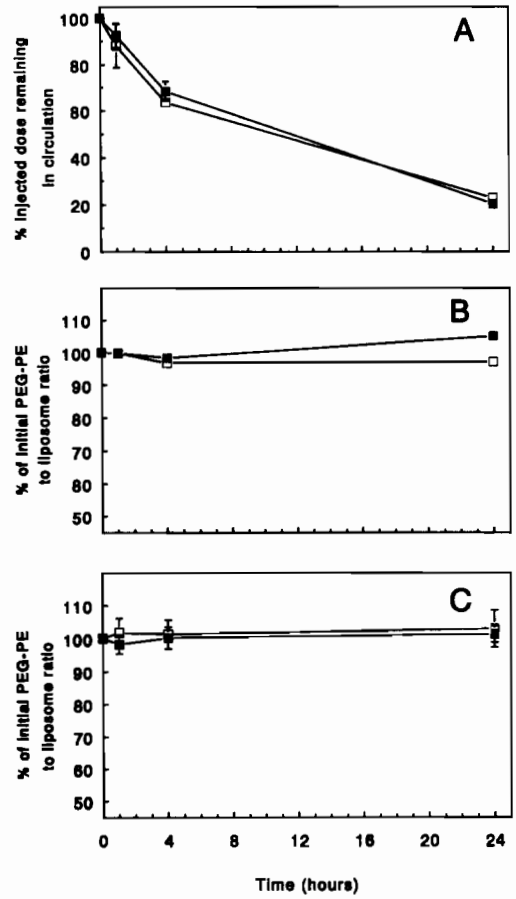

Fig. 6. Circulation lifetime of DSPC/cholesterol/$MePEG_{2000}$-S-DSPE liposomes and in vivo exchange of $MePEG_{2000}$-S-DSPE from injected liposomes. (A) Circulation lifetime. Large unilamellar vesicles composed of DSPC/cholesterol/[^{3}H]$MePEG_{2000}$-S-DSPE (50:45:5) (□) or DSPC/cholesterol/$MePEG_{2000}$-S-[^{3}H]DSPE (50:45:5) (■) were injected via lateral tail vein into 25 g CD-1 mice (5 mM total lipid in 200 μl, 1 μmol total lipid/mouse). At various times, the mice were sacrificed and plasma isolated. The marker [^{14}H]CHE was used to determine liposome recovery. Results shown represent the mean ± S.E. of four animals. (B) The $^{3}H/^{14}C$ ratio for plasma isolated from A before separation of liposomes from plasma components via spin column. (□), [^{3}H]$MePEG_{2000}$-S-DSPE, and (■), $MePEG_{2000}$-S-[^{3}H]DSPE preparations. Results represent the means of ratios obtained from the four mice and expressed as a percentage (± S.D.) of the initial ratio before injection. (C) The $^{3}H/^{14}C$ ratio for plasma isolated from (A) after separation of liposomes from plasma components via spin column. (□), [^{3}H]$MePEG_{2000}$-S-DSPE, and (■), $MePEG_{2000}$-S-[^{3}H]DSPE preparations. Results represent the means of ratios of the peak two liposome fractions from two spin columns from four mice, and expressed as a percentage (± S.D.) of the initial ratio before injection.

4. Discussion

The use of liposomes as drug delivery vehicles depends upon their ability to remain in circulation for extended periods of time. The incorporation of PEG-lipids clearly allows extended circulation lifetimes to be achieved. However, the results presented here emphasize two major points. First, relatively subtle changes in the acyl chain composition of the PE anchor can significantly influence retention of the PEG-PE in the outer monolayer of the liposome. Second, significant chemical breakdown of PEG-PE conjugates may occur, particularly after the PEG-PE is lost from the LUV surface.

The influence of acyl chain composition on PEG-PE retention and related clearance behavior is particularly profound. As shown here, when $MePEG_{2000}$-S-POPE is incorporated into DSPC/cholesterol LUVs, the circulation lifetime is only modestly increased. This increase is less than that reported for $MePEG_{2000}$-DSPE [11,18], but comparable to other studies using $MePEG_{2000}$-DOPE [25]. The poor performance of PEG-POPE is due to rapid removal of the exterior PEG coating, with a half-time of approximately 2 h in vitro (50% mouse serum at 37°C) and approximately 1 h in vivo. This may be compared with the rate of clearance of the injected LUVs, which exhibit a half-life in the circulation of approximately 5 h. The fact that loss of the hydrophilic coating precedes liposome clearance suggests that loss of the PEG coating hastens clearance. As shown here, the loss of the PEG-POPE coating is primarily due to exchange of the entire PEG-POPE molecule out of the external monolayer.

The use of DSPE as the lipid anchor in place of POPE results in a dramatic improvement on the retention of the PEG coating. When $MePEG_{2000}$-S-DSPE is incorporated into LUVs and incubated in 50% mouse serum, approximately 90% of the PEG-PE remains associated with the LUVs after 24 h. The DSPE anchor also exhibits markedly superior properties in vivo. The circulation half-life of LUVs incorporating $MePEG_{2000}$-S-DSPE is approximately 10 h, with over 20% of the injected dose remaining in circulation at 24 h. In addition, the LUVs recovered from the circulation even up to 24 h show no exchange or breakdown of the $MePEG_{2000}$-S-DSPE, although any liposomes which have lost their PEG coating would likely have been cleared from the circulation.

Previous work [26] examining the intervesicular exchangeability of several PEG-lipids in vitro showed that transfer of saturated diacyl conjugates of $MePEG_{2000}$ decreased exponentially with increasing chain length. In addition, transfer of POPE derivatives of $MePEG_{2000}$ and $MePEG_{5000}$ was found to be 30–40-fold slower than the corresponding DMPE derivatives. Thus, the increase in LUV retention in vitro and in vivo between POPE and DSPE anchors observed here is not unreasonable.

The chemical stability studies on pure (micellar) PEG-lipids indicate that the succinate linkage is labile in mouse serum, generating free MePEG-OH by 1 h with complete hydrolysis of this linkage within 24 h. A variety of other linkages proved to be more stable in this respect, including carbamate, amide, and direct linkages. The results presented here also indicate that the succinate bond is protected by retention of the PEG-lipid in the LUV. For $MePEG_{2000}$-S-DSPE, a slow loss of the PEG headgroup as compared to the lipid anchor (which is completely retained) indicates that it is possible to remove the PEG from the LUV surface and leave the lipid anchor behind; however, this effect is small compared to loss of the POPE anchored version which is almost completely removed from the outer monolayer within several hours.

The rationale for the use of PEG-lipids is their ability to significantly reduce the rate of clearance of liposomes from the circulation. At a practical level, the results presented here demonstrate that chemical stability and lipid anchoring ability are determining factors for the ability of PEG-lipids to provide improved circulation lifetimes for LUVs in vivo. While it has been reported that the nature of the anchor of PEG-lipids influences the circulation lifetimes of injected liposomes [11] and that the LUV lipid composition can also affect the circulation lifetimes of liposomes incorporating PEG-PEs [27,28], others suggest that different PEG anchors are equivalent and that the lipid composition may be varied with little effect if PEG-PEs are incorporated [18]. Our results conclusively demonstrate the importance of the PEG-lipid anchor.

In summary, the lipid anchor is a primary factor in the retention of a PEG polymeric coating for LUVs. Chemical breakdown of the conjugate largely occurs after exchange out of the LUV but can occur on the LUV surface depending upon the PEG-lipid linkage. The use of a strong membrane anchor (DSPE) and chemically stable conjugate bond (amide) results in retention of the polymeric coating and greatly enhanced circulation lifetimes. We conclude that since the major rationalization for the use of PEG-lipids in liposomes is the prolonged circulation lifetimes and hence the greater chance of accumulation in targets other than the RES, by whatever detailed mechanisms PEG-PE is proposed to work, this requires that the PEG coating is retained and should be a consideration in any practical discussion of the use of PEG-lipids.

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References

[1] Gabizon, A. and Papahadjopoulos, D. (1988) Proc. Natl. Acad. Sci. USA 85, 6949–6953.

[2] Oku, N., Namba, Y. and Okada, S. (1992) Biochim. Biophys. Acta 1126, 255–260.

[3] Huang, S.K., Lee, K-D., Hong, K., Friend, D.S. and Papahadjopoulos, D. (1992) Cancer Res. 52, 5135–5143.

[4] Huang, S.K., Mayhew, E., Gilani, S., Lasic, D.D., Martin, F.J. and Papahadjopoulos, D. (1992) Cancer Res. 52, 6774–6781.

[5] Mayhew, E.G., Lasic, D., Babbar, S. and Martin, F.J. (1992) Int. J. Cancer 51, 302–309.

[6] Vaage, J., Mayhew, E., Lasic, D. and Martin, F. (1992) Int. J. Cancer 51, 942–948.

[7] Allen, T.M. and Chonn, A. (1987) FEBS Lett. 223, 42–46.

[8] Klibanov, A.L., Maruyama, K., Torchilin, V.P. and Huang, L. (1990) FEBS Lett. 268, 235–237.

[9] Blume, G. and Cevc, G. (1990) Biochim. Biophys. Acta 1029, 91–97.

[10] Senior, J., Delgado, C., Fisher, D., Tilcock, C. and Gregoriadis, G. (1991) Biochim. Biophys. Acta 1062, 77–82.

[11] Allen, T.M., Hansen, C., Martin, F., Redemann, C. and Yau-Young, A. (1991) Biochim. Biophys. Acta 1066, 29–36.

[12] Allen, T.M. and Hansen, C. (1991) Biochim. Biophys. Acta 1068, 133–141.

[13] Lasic, D.D., Martin, F.J., Gabizon, A., Huang, S.K. and Papahadjopoulos, D. (1991) Biochim. Biophys. Acta 1070, 187–192.

[14] Needham, D., McIntosh, T.J. and Lasic, D.D. (1992) Biochim. Biophys. Acta 1108, 40–48.

[15] Woodle, M.C. and Lasic, D.D. (1992) Biochim. Biophys. Acta 1113, 171–199.

[16] Chonn, A., Semple, S.C. and Cullis, P.R. (1992) J. Biol. Chem. 267, 18759–18765.

[17] Funato, K., Yoda, R. and Kiwada, H. (1992) Biochim. Biophys. Acta 1103, 198–204.

[18] Woodle, M.C., Matthay, K.K., Newman, M.S., Hidayat, J.E., Collins, L.R., Redemann, C., Martin, F.J. and Papahadjopoulos, D. (1992) Biochim. Biophys. Acta 1105, 193–200.

[19] Sessler, J.L., Magda, D. and Furuta, H. (1992) J. Org. Chem. 57, 818–826.

[20] Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1985) Biochim. Biophys. Acta 812, 55–65.

[21] Derksen, J.T.P., Morselt, H.W.M. and Scherphof, G.L. (1987) Biochim. Biophys. Acta 931, 33–40.

[22] Bartlett, G. (1959) J. Biol. Chem. 234, 466–468.

[23] Chonn, A, Semple, S.C. and Cullis, P.R. (1991) Biochim. Biophys. Acta 1070, 215–222.

[24] Zalipsky, S., Seltzer, R. and Menon-Rudolph, S. (1992) Biotechnol. Appl. Biochem. 15, 100–114.

[25] Mori, A., Klibanov, A.L., Torchilin, V.P. and Huang, L. (1991) FEBS Lett. 284, 263–266.

[26] Silvius, J.R and Zuckermann, M.J. (1993) Biochemistry 32, 3153–3161.

[27] Maruyama, K., Yoda, T., Okamoto, A., Kojima, S., Suginaka, A. and Iwatsuru, M. (1992) Biochim. Biophys. Acta 1128, 44–49.

[28] Litzinger, D.C. and Huang, L. (1992) Biochim. Biophys. Acta 1127, 249–254.