Effects of Polyethyleneglycol Chain Length and Phospholipid Acyl Chain Composition on the Interaction of Polyethyleneglycol-phospholipid Conjugates with Phospholipid: Implications in Liposomal Drug Delivery

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Purpose. The purpose of this study was to investigate polyethyleneglycol(PEG)-phosphatidylethanolamine(PE) conjugate interaction with phospholipid bilayers, in an attempt to explain the dependence of liposome circulation time on formulation.

Methods. Differential scanning calorimetry, electron microscopy, dynamic light scattering and NMR were the major methods used in the study.

Results. Mixtures of PEG-phospholipid conjugates and phosphatidylcholine existed in three different physical states: a lamellar phase with components exhibiting some miscibility, a lamellar phase with components phase separated, and mixed micelles. Beyond 7 mol% of PEG(1,000–3,000)-dipalmitoyl phosphatidylethanolamine (DPPE), and 11 mol% PEG(5,000)-DPPE in dipalmitoyl phosphatidylcholine (DPPC), a strong tendency towards mixed micelle formation was observed. All concentrations of PEG(12,000)-DPPE and PEG(5,000)-DPPE beyond 8 mol% formed phase separated lamellae with phosphatidylcholine. Decreasing the acyl chain length from $C_{16:0}$ to $C_{14:0}$ caused a decrease in tendency towards micelle formation and phase separation. These tendencies increased upon increasing acyl chain length to $C_{18:0}$. Phase separation was at least partly due to PEG chain-chain interaction. This was supported by an increased fraction of PEG chains exhibiting a fast NMR transverse relaxation in DPPC/PEG(5,000)-DPPE mixtures as compared to that in distearoyl phosphatidylcholine (DSPC)/PEG(5,000)-dioleoyl-PE (DOPE).

Conclusions. These phenomena are discussed in relation to both bilayer and steric stabilization of liposomes, and the lack of prolonged circulation with certain formulations is discussed.

KEY WORDS: differential scanning calorimetry; drug delivery; long circulating liposomes; mixed micelles; NMR transverse relaxation; phase separation.

INTRODUCTION

The inclusion of PEG-PE conjugates in liposomes results in drastically prolonged circulation times (1,2). A number of mechanisms have been proposed to explain the evasion of the MPS by these novel liposomes, and the resulting prolonged circulation. Steric stabilization of colloids, which has been described for inorganic particles (3), is probably the most plausible hypothesis to date (2). Steric repulsion resulting from polymer coating leads to reduced particle-particle interaction, therefore causing an inhibition of the adsorption of various opsonins such as complement components (4) onto the liposome surface resulting in an increased biological stability. Both the flexibility of short PEG chains forming a dense "polymeric cloud" (5), and the surface charge and hydrophilicity of PEG may play an important role in imparting this long circulating effect (6). The existing theory of steric stabilization, however, does not explain the reduced protection of liposomes with PEG > 5,000 MW, and also the effect of phospholipid composition on the circulation times of theses liposomes. Most research aimed at understanding the mechanisms of long circulation have focused on studying the interactions of PEG on the liposome surface (5–8). To fully understand the mechanism of prolonged circulation, and also to enable the preparation of optimal pharmaceutical formulations of these liposomes, an understanding of the interactions of PEG-PE within the phospholipid membrane is equally important. This therefore is the focus of the current study.

MATERIALS AND METHODS

Materials

The phosphatidylcholines and phosphatidylethanolamines were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The monomethoxy polyethyleneglycol-succinimidyl succinates (PEG-OSu) were a generous gift from NOF corporation (Tokyo, Japan). HPLC grade chloroform was obtained from Sigma, and PEG-PE was prepared as described previously (1).

Preparation of Hydrated Lipid Dispersions

PEG-PE/PC liposomes were prepared according to the thin film hydration method of Bangham et al. (9). In every case, PC and PE of identical acyl chain length were used to ensure good mixing. Lipid concentrations were 1–2 mM. Neither sonication nor extrusion were used in the preparation.

Calorimetry

DSC scans were performed on a MC-2 Microcal high sensitivity calorimeter (Microcal Inc., Northampton, MA), at a heating rate of 0.5°C/min between the temperatures of 5–85°C. Each scan was run at least five times to ensure the attainment of equilibrium.

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Abbreviations: DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; DSPC, distearoyl phosphatidylcholine; DMPE, dimyristoyl phosphatidylethanolamine; DOPE, dioleoyl phosphatidylethanolamine; DPPE, dipalmitoyl phosphatidylethanolamine; DSPE, distearoyl phosphatidylethanolamine; DSC, differential scanning calorimetry; PC, phosphatidylcholine; PEG, polyethyleneglycol; PEG-PE, dioleoyl-N-(monomethoxy polyethyleneglycol succinyl) phosphatidylethanolamine.

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Negative Stain Electron Microscopy

Platinum stabilized, formvar coated 300-mesh copper grids were placed on a drop of the test sample and left for 4 min at room temperature. The grid was then carefully dried and then placed on a drop of the stain 1% (w/v) potassium phosphotungstate pH 7.5 for 1 min and again dried. The stained grids were then examined in a JEOL JEM-100CX II electron microscope operating at 80kV. Electron micrographs were taken at 10–100,000X magnification. For size determination, standard latex beads were used as reference at similar magnification.

Nuclear Magnetic Resonance

All ^{1}H-NMR experiments were conducted using an Otsuka-Chemagnetics (Fort Collins, CO) CMXW-400 spectrometer operating at 401.102 MHz. The sample temperature was controlled at 25°C. The transverse relaxation time (T_2) of polyoxyethylene protons ($-(CH_2\text{-}CH_2\text{-}O)_n-$) was measured using a spin-echo pulse sequence (90_x-TE/2-180_y-TE/2-ACQ). The nominal pulse widths for the 90° and 180° pulses were 40 μs and 80 μs respectively. After Fourier transformation, the echo intensities of the polyoxyethylene proton resonance were integrated and T_2 calculated using nonlinear regression with a biexponential decay function.

Dynamic Light Scattering

Particle size distribution was analyzed by photon correlation spectroscopy using a Coulter Model N4SD sub micron particle analyzer (Hialeah, FL).

RESULTS

Short Chain PEG(1,000 and 3,000)-DPPE/DPPC

Results obtained for PEG(1,000 and 3,000)-DPPE were identical. Visual observation of turbidity of the dispersions showed gradual loss of turbidity between 5–10 mol% PEG(1,000 & 3,000)-PE, and drastic loss of turbidity beyond 10 mol%. Formulations containing at least 17 mol% PEG-PE appeared completely transparent.

DSC thermograms of DPPC/PEG(3,000)-PE mixtures are shown in Fig. 1. Phase transition cooperativity decreased with increasing concentration of PEG-PE up to 5 mol% (Fig. 1b). The presence of a single transition peak indicates uniform mixing of the PEG-PE within the phospholipid bilayer. By further increasing PEG-PE concentration to 10 mol%, a new peak appeared as a shoulder on the high temperature side of the main phase transition, indicating gradual transition from the original lamellar state to another state. Conversion from the lamellar state was complete by 17 mol% of PEG-PE (thermogram not

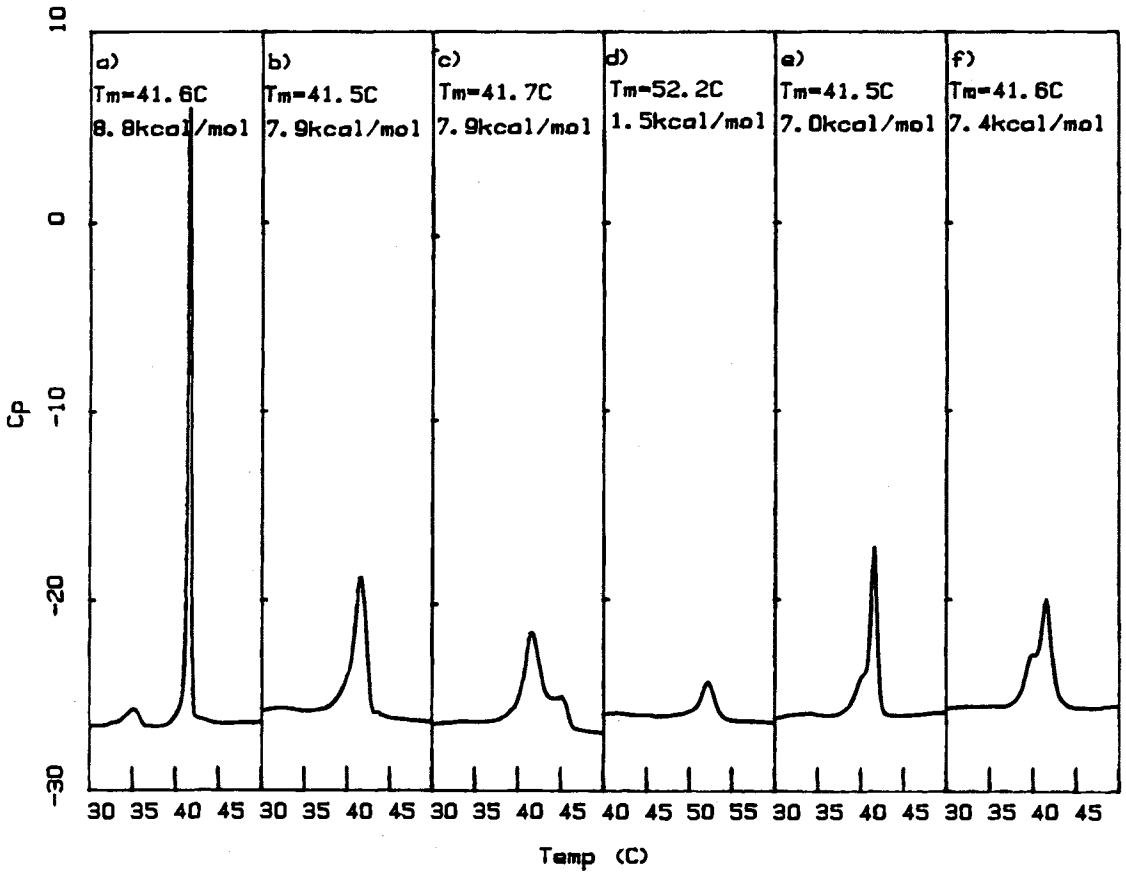

Fig. 1. DSC thermogram of DPPC bilayer containing 0 mol% of PEG-PE (a), 5 mol% of PEG(3,000)-DPPE (b), 10 mol% of PEG(3,000)-DPPE (c), thermogram of pure PEG(3,000)-DPPE (d), 5 mol% of PEG(12,000)-DPPE (e), and 17 mol% of PEG(12,000)-DPPE (f).

shown). Dynamic light scattering indicated marked particle size reduction by 17 mol% PEG(3,000)-PE with a significant fraction of particles < 40nm (Table I). This suggests that the shoulder observed with the DSC thermograms indicates bilayer solubilization to a mixed micellar state. Negative stain electron micrographs (Fig. 2) showed predominantly bilayer structures at low PEG-PE concentration, a mixture of rod shaped micelles and bilayer vesicles at 10 mol% (Fig. 2b), and a complete conversion to mixed micelles by 17 mol% (Fig. 2c).

Long Chain PEG(12,000)-DPPE/DPPC

Pure PEG(12,000)-DPPE formed transparent micellar solutions. Turbid suspensions existed even beyond 35 mol% of PEG(12,000)-DPPE.

DSC thermograms of DPPC/PEG(12,000)-PE mixtures are shown in Fig. 1 e and f. A shoulder, on the lower temperature side of the main phase transition was present at all concentrations of PEG(12,000)-DPPE. Pure PEG(12,000)-DPPE did not give any phase transition within the range of temperatures investigated, i.e., 10–90°C (thermogram not shown). The loss in phase transition cooperativity is reduced compared to short and intermediate chain PEG-PE, suggesting that PEG(12,000)-PE is not fully incorporated into the PC bilayer. We confirmed this by ultracentrifugation at 100,000 × g for 30 min. Analysis of the supernatant indicated that PEG-PE incorporation into the bilayer increased with PEG-PE concentration, and at 10 mol% PEG-PE, 50% was incorporated. Absence of DPPC in the supernatant confirmed negligible bilayer solubilization. Phase separation within the bilayer was still observed by DSC after separation of the free PEG(12,000)-PE. This confirms theoretical studies (10) suggesting that PEG-PE can be incorporated into the bilayer up to a certain concentration which is inversely proportional to the PEG MW. It was postulated (10), that at the maximum concentration, lateral steric repulsion between the polymer chains exceeds attractive forces between bilayer lipid molecules.

Dynamic light scattering showed a decrease in particle size with increase in concentration of PEG(12,000)-DPPE (Table I). Table I suggests that micelles of pure PEG(12,000)-PE coexisted with the lamellae since no bilayer solubilization was observed.

Electron microscopy (Figs. 2 e and f) shows a predominance of bilayer structures with concentrations as high as 35 mol% coexisting with micelles of pure PEG(12,000)-DPPE.

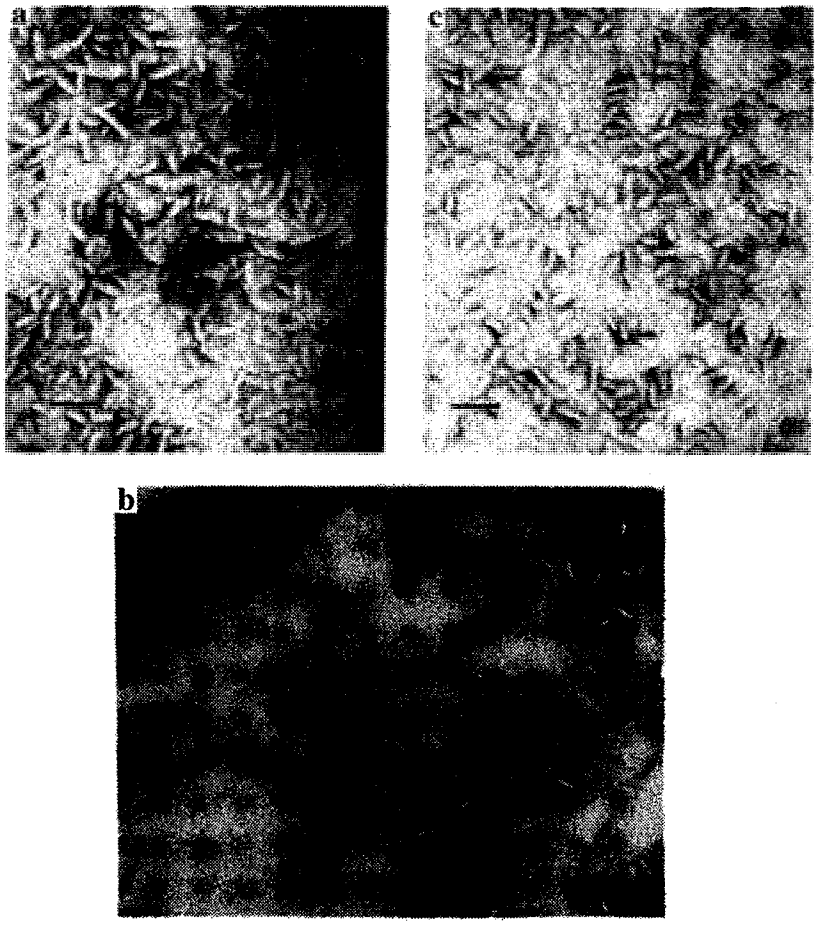

Fig. 2. Negative stain electron micrographs of PEG(1,000)-DPPE (a), 10 mol% PEG(1,000)-DPPE in DPPC (b), 17 mol% PEG(1,000)-DPPE in DPPC (c), PEG(12,000)-DPPE (d), 17 mol% PEG(12,000)-DPPE in DPPC (e), and 35 mol% PEG(12,000)-DPPE in DPPC (f). Scale bar is 100 nm for (a), (c), (d), (e) and (f). For (b) scale bar is 200 nm.

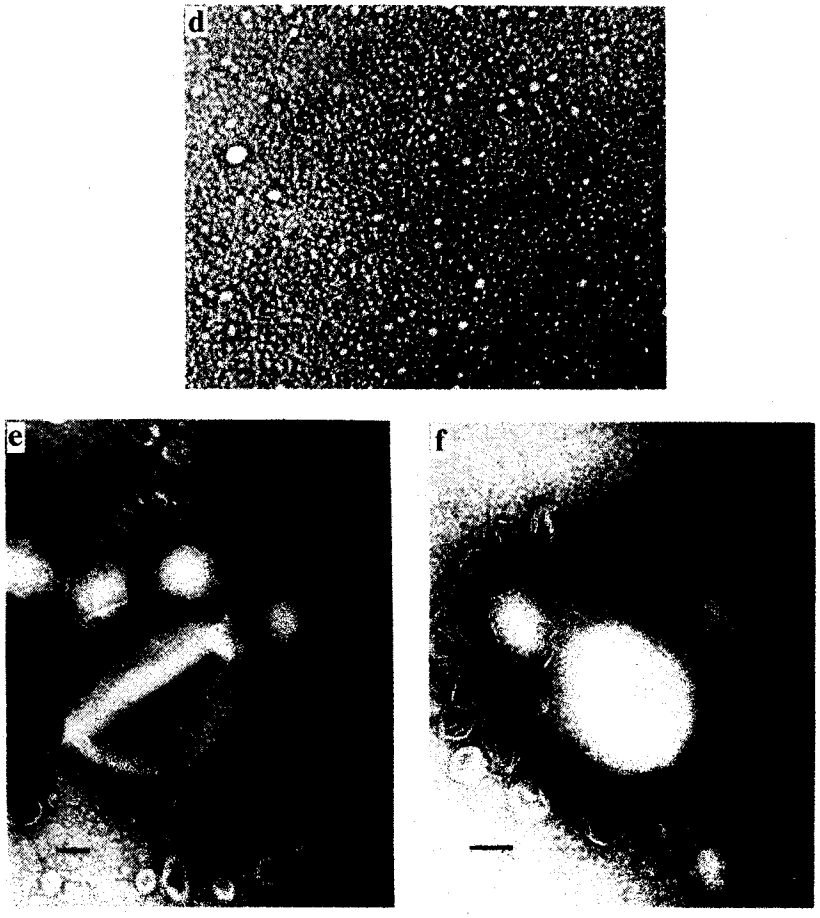

Fig. 2. Continued.

Table I. Vesicle Size Distribution[a] of Various Formulations

Host Lipid	PEG-PE		Size distribution[b]
	PEG M.W.	mol%	
DPPC		0	3000 ± 310nm
	3000	5	2000 ± 350nm (60%), 580 ± 170nm (19%), 110 ± 30nm (8%), 30±5nm (13%)
	3000	17	40 ± 10nm (20%), 5 ± 0.5nm (80%)
	5000	5	1850 ± 460nm (70%), 370 ± 190nm (30%)
	5000	12	190 ± 150nm (broad)
	5000	17	80 ± 70nm (broad)
	12000	5	660 ± 150nm (75%), 140 ± 40nm (25%)
	12000	17	280 ± 260nm (broad)
DSPC		0	2600 ± 310nm
	5000	5	1380 ± 300nm (48%), 250 ± 90nm (6%), 45 ± 10nm (48%)
	5000	17	150 ± 140nm (broad)
DMPC		0	2890 ± 360nm
	1000	5	250 ± 140nm (99%), 15 ± 5nm (1%)
	1000	17	150 ± 40nm (97%), 3 ± 0.5nm (3%)
	12000	17	1500 ± 350nm (6%), 300 ± 100nm (93%), 20 ± 5nm (1%)

[a] Sizes obtained by dynamic light scattering.

[b] Mean ± s.d. In case of more than one population, number in parenthesis indicates percentage of total population falling within the indicated size range.

Intermediate Chain PEG(5,000)-DPPE

Turbidity observations indicated slight loss of turbidity by 12 mol% and a drastic loss of turbidity at higher concentrations.

Results for increasing PEG(5,000)-DPPE/DPPC mixtures (Fig. 3) are particularly interesting as each of the previously observed physical states exist. Below concentrations of 8 mol%, a single transition peak was seen. Beyond 8 mol% of PEG-PE, the formation of a shoulder on the low temperature side of the phase transition similar to PEG(12,000)-PE indicated phase separation. Above 11 mol%, a new shoulder on the high temperature side of the phase transition peak similar to PEG(1,000 & 3,000)-PE, indicated bilayer solubilization. No phase transition was observed for pure PEG(5,000)-DPPE.

Particle size analysis is shown in Table I and indicates reduced bilayer solubilization compared to PEG(3,000)-PE.

Intermediate Chain PEG(5,000)-DSPE in Bilayers Containing Long Acyl Chain DSPC

The effect of PC acyl chain length was investigated by incorporating PEG(5,000)-DSPE into DSPC bilayers. Fig. 4 shows that in contrast to PEG(5,000)-PE/DPPC, poor mixing of the liposome components occurred at all concentrations. Apparently, increase in PC acyl chain length and bilayer rigidity decreased miscibility of the PEG-PE leading to greater extent of phase separation.

A more rapid loss of turbidity was observed with the DSPC ($C_{18:0}$) system as compared to the DPPC ($C_{16:0}$), and dynamic light scattering (Table I) showed significant micelle formation at concentrations as low as 5 mol% PEG(5,000)-DSPE.

Short and Long Chain PEG-PE in Bilayers Containing Short Acyl Chain DMPC

Interaction of PEG-PE with PC was also studied with the $C_{14:0}$ acyl chain length system. The DSC thermograms of the $C_{14:0}$ system were similar to their $C_{16:0}$ counterparts (thermograms not shown). However, mixed micelle formation and phase separation with PEG(1,000)-DMPE/DPPC and PEG(12,000)-DMPE/DPPC, respectively, were delayed until concentrations above 10 mol% of PEG-PE. Interaction between shorter acyl chains improved the mixing of PEG-PE with PC.

Delayed solubilization was also shown by dynamic light scattering (Table I).

Effect of Unsaturation in PE Linker on PEG-PE Interaction with PC

The effect of PEG chain-chain interaction in causing phase separation was investigated by replacing DSPE with DOPE which contains fatty acid chain $C_{18:1}$ therefore introducing unsaturation into the linker grafting PEG(5,000) to lamellae composed of DSPC (Fig. 4 e, f). The formation of any shoulders

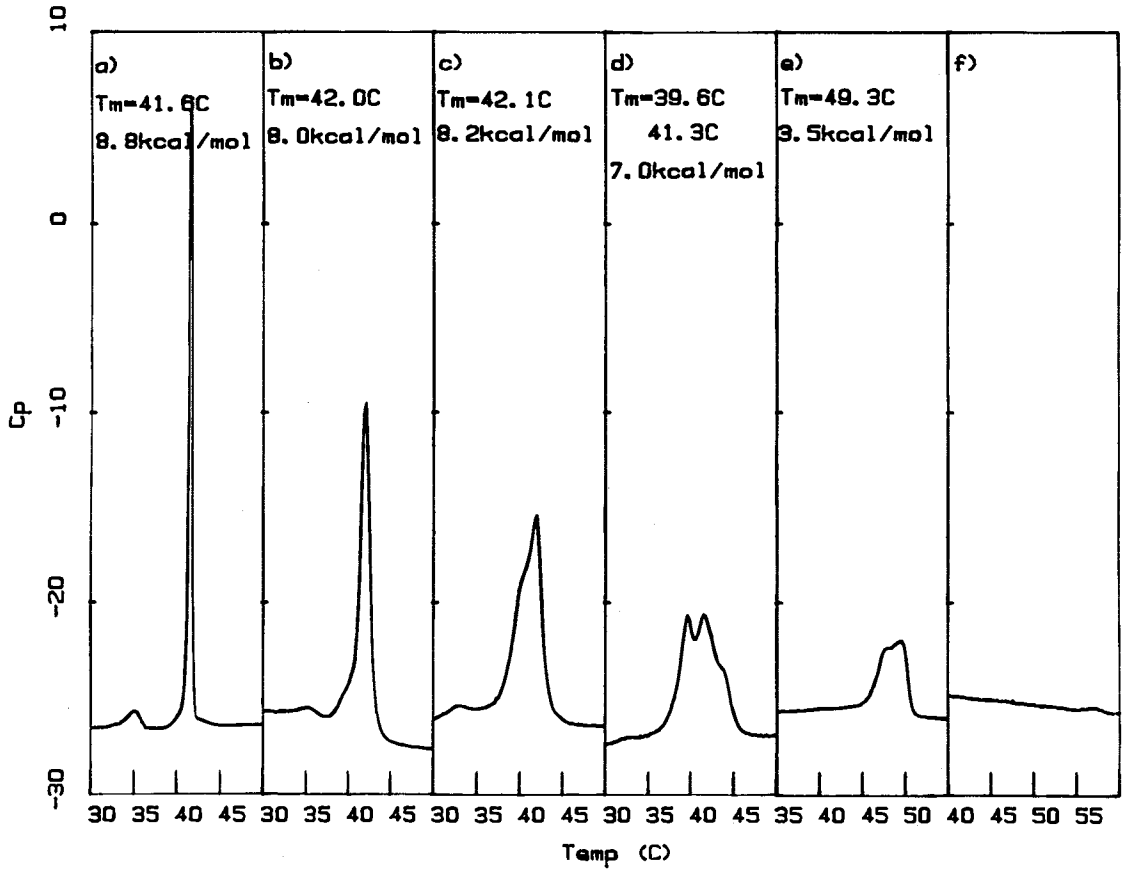

Fig. 3. DSC thermogram of DPPC bilayer containing 0 mol% of PEG-PE (a), 5 mol% of PEG(5,000)-DPPE (b), 9 mol% of PEG(5,000)-DPPE (c), 14 mol% of PEG(5,000)-DPPE (d), 44 mol% of PEG(5,000)-DPPE (e), and thermogram of pure PEG(5,000)-DPPE (f).

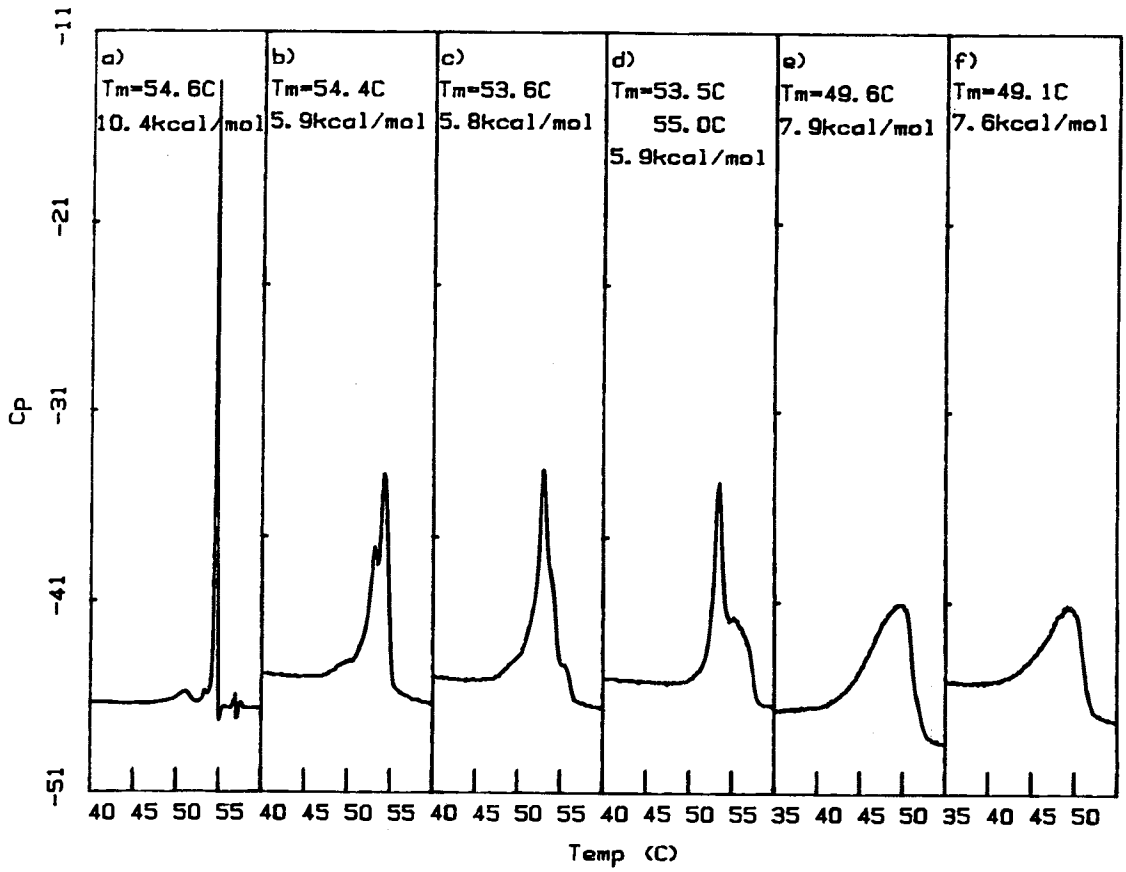

Fig. 4. DSC thermogram of DSPC bilayer containing 0 mol% of PEG-PE (a), 5 mol% of PEG(5,000)-DSPE (b), 10 mol% PEG(5,000)-DSPE, (c), 17 mol% PEG(5,000)-DSPE (d), 10 mol% PEG(5,000)-DOPE (e), and 17 mol% PEG(5,000)-DOPE (f).

on the phase transition peak signifying micelle formation or phase separation was not observed up to concentrations of 35 mol%. With PEG(5,000)-DSPE/DSPC (Fig. 4 b, c) phase separation and micelle formation were prominent.

NMR Transverse Relaxation Time Measurements

The NMR signal of the polyoxyethylene protons exhibited two T_2 components in all cases. The spin-echo intensities were best fit utilizing nonlinear regression of the equation

$$I(t) = I_f e^{-t/T2f} + I_S e^{-t/T2s}$$

where I(t) is the total intensity as a function of spin echo time, and I_f and I_s are the fractions of intensity contributed by the fast and slow T_2 components, respectively. T_{2f} and T_{2s} are the values of fast and slow transverse relaxation times. In the PEG(5,000)-DPPE/DPPC formulation, there was a higher fraction of PEG chains showing fast T_2 relaxation time, i.e. more restricted chain motion, than in the PEG(5,000)-DOPE formulation (Table II). Restriction in chain motion was not the result of inter-liposome interaction since both concentrated and dilute dispersions gave similar results.

DISCUSSION

This study classifies the PEG-PE into three groups: short chain PEG-PE with PEG MW 1,000–3,000, intermediate chain length PEG of MW 5,000, and long chain PEG of MW 12,000. An absence of phase transitions was observed for pure PEG(5,000)-PE and PEG(12,000)-PE. This probably is due to the formation of micelles with low aggregation number due to the bulky PEG head group, resulting in small circular micelles of high curvature (as seen in electron micrographs) which do not posses the cooperativity required to show a phase transition in DSC thermograms.

The effect of PEG MW on PEG-PE interaction with phospholipid was studied using PEG(1,000, 3,000, 5,000, and 12,000)-PE with DPPC. Short chain PEG-PE > 7 mol% led to bilayer solubilization. With PEG(12,000)-PE, the results do not suggest any mixed micelle formation. The low temperature shoulder on the main phase transition peak at all concentrations indicates a demixing of PEG-PE and PC within the gel phase leading to the formation of phase separated lamellae. Intermediate chain length PEG(5,000)-DPPE/DPPC mixtures exist in each of the three physical states already mentioned. Most suitable for drug delivery purposes is the bilayer state with liposome components exhibiting some miscibility, at low concentrations of short and intermediate chain PEG-PE.

The occurrence of phase separated lamellae with PEG ≥ 5,000 is a result of the solution properties of PEG. PEG becomes more hydrophobic with increase in MW. The unperturbed radius of gyration of the polymer is proportional to the ideality of the solvent for the polymer (11). As PEG MW increases, water

Table II. Percentages of Fast and Slow Transverse Relaxation Components and Values of T_2 in Concentrated and Dilute Dispersions of 10 mol% PEG(5,000)-DPPE/DPPC and 10 mol% PEG(5,000)-DOPE/DSPC[a]

	PEG5,000-DPPE/DPPC		PEG5,000-DOPE/DSPC	
	Concentrated	Dilute	Concentrated	Dilute
Percentage of fast component I_f	52.5 ± 0.3	50.7 ± 1.1	36.1 ± 0.6	25.6 ± 3.1
Percentage of slow component I_s	47.5 ± 0.3	49.3 ± 1.1	63.9 ± 0.6	74.4 ± 3.1
Fast relaxation time T_{2f}(msec)	9.8 ± 0.3	15.2 ± 0.9	14.5 ± 0.1	22.4 ± 2.0
Slow relaxation time T_{2s} (msec)	396.4 ± 4.9	437.0 ± 5.8	362.2 ± 0.9	361.9 ± 9.5

[a] Concentrated and dilute dispersions refer to 6 and 1.1 mM total lipid.

becomes a poor solvent, the segments attract each other, the intramolecular expansion factor $\alpha < 1$ leading to coil shrinkage ($\alpha = 1$ for an ideal solvent) (11). Increased PEG chain-chain interaction, even at low PEG(12,000)-PE concentrations due to the increased van der Waals forces for long PEG chains and also inter and intra-chain hydrogen bonding is proposed to lead to PEG chain entanglement causing formation of the observed PEG-PE enriched and PEG-PE poor domains. Dehydration due to the PEG may augment this phase separation effect as observed by Lehtonen and Kinnunen using free PEG(6,000) (12).

PEG chain-chain interaction was investigated by introducing cis double bonds into the PE linker in an attempt to space out the PEG chains. Fig. 4 compares thermograms of PEG(5,000)-PE/DSPC with a saturated and unsaturated linker. DOPE, as a result of the cis double bond at C_9 which imparts a kink of 30° into the acyl chains, alters lipid packing by spacing out the PC acyl chains. Free volume and rotational degree of freedom are increased, resulting in increased bilayer fluidity as seen from the reduced T_m. T_m of the PEG-DOPE/DSPC mixture still remains > 10°C above that of DPPC indicating that the bilayer still has greater rigidity. Increased spacing between the PEG-DOPE, prevents PEG chain entanglement and is therefore responsible for abolishing the phase separation.

To further prove our hypothesis that the phase separation is caused by increased PEG chain-chain interactions, we proceeded to perform NMR spin-spin relaxation experiments to determine the motional restriction of the PEG chains in PEG(5,000)-DOPE/DSPC and PEG(5,000)-DPPE/DPPC mixtures. The spin-spin or transverse relaxation involves the transfer of excitation energy among nuclear spins, and is characterized by the time constant T_2. PEG chains in close contact with each other would exhibit an efficient transverse relaxation or short T_2. Strong PEG chain-chain interactions with PEG-DPPE leads to the formation of PEG-rich and PEG-poor domains within the bilayer, causing the NMR signal to exhibit two T_2 components, T_{2fast} and T_{2slow}, respectively. With PEG-DOPE, the kink in the acyl chain prevents efficient PEG chain-chain interaction and clustering leading to reduced fraction with T_{2fast} component.

Changes in phospholipid composition alter the miscibility of PEG-PE with the host PC as seen in Fig. 4. The tendency towards both phase separation and micelle formation are clearly regulated by the lateral packing of the PC acyl chains. With increase in PC acyl chain length, fatty acid chain-chain interaction is increased, and so is the van der Waals cohesive force. This should result in more tightly packed PC and PEG-PE molecules and a greater tendency towards PEG chain-chain entanglement and micelle formation and vice versa. The extent of demixing of PEG-PE in bilayers, therefore, decreases in the order $C_{18:0} > C_{16:0} > C_{14:0}$.

The above observations have important implications on the use of PEG-PE in the pharmaceutical formulation of long circulating liposomes for drug delivery. The steric barrier created by PEG-PE greatly prolongs the liposome blood circulation time by reducing opsonin adsorption. Depth of the steric barrier is directly proportional to PEG chain length (13), and by this mechanism and also by the de Gennes model (7), high molecular weight PEG would be expected to produce longer circulating liposomes. PEG >5,000 MW in certain formulations is however, unsuitable for preparing long circulating liposomes (14). Formation of phase separated lamellae could be the reason for this. Lipid phase separation generates domains less enriched with PEG-PE and could lead to a less sterically stabilized liposome which would be rapidly cleared due to opsonin adsorption to these "bald" spots. Phase separation could also lead to destabilization by lipoproteins, and leakage of the encapsulated drug. This study also explains the *in vivo* observations in which with fluid liposomes composed of egg PC, circulation times increased with an increase of the steric barrier of the PEG chain which is directly proportional to chain length. PEG(5,000) > 2,000 > 1,000 (14), and PEG(5,000) > 2,000 > 750 (13). With rigid liposomes composed of DSPC (14,15), circulation times decreased in the order PEG(1,000) ~ 2,000 > 5,000 ~ 12,000. Close molecular packing allows PEG chain entanglement with the longer PEG chains leading to phase separated bilayer which is not condusive for long circulation. On the other hand, weak acyl chain interactions in the fluid bilayer would allow low level of PEG chain entanglement even with longer PEG chains. In the latter case, liposomal circulation time would be proportional to the PEG chain length.

Mixed micelles existing with short chain PEG-PE beyond 7 mol% would be unsuitable for use as a long circulating drug delivery system due to the rapid leakage of encapsulated drug.

This study explains why as observed by us and other investigators, the most stable and longest circulating liposomes formulations consist of low concentrations, ≤ 6 mol%, of short chain PEG-PE. With matrix PC of $C_{16:0}$ or less, intermediate chain length PEG-PE (≤ MW 5,000) would also be suitable at concentrations not exceeding 7 mol%. The study also demonstrates the predictive value of physical methods in determining the optimal pharmaceutical formulations of these liposomes which is presently done by a trial-and-error fashion.

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