Interaction of Polyethyleneglycol-Phospholipid Conjugates with Cholesterol-Phosphatidylcholine Mixtures: Sterically Stabilized Liposome Formulations

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

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

Results. Studies performed in the absence of cholesterol indicated the formation of three distinct physical states depending on the chain length of PEG in PEG-PE. Mixed micelle formation was observed at concentrations of PEG(1,000)-DPPE above 7 mol% of lipid. Phase separated lamellae were observed at all concentrations of PEG(12,000)-DPPE (Bedu-Addo et al. Pharm. Res. **13**:710–717 (1996)). Upon incorporation of high concentrations of cholesterol >30 mol% into the lipid bilayer, the formation of phase separated lamellae was completely inhibited and the formation of mixed micelles significantly reduced. At high concentrations of PEG(1,000)-PE, solubilization of the bilayer occurred with preferential solubilization of cholesterol over phosphatidylcholine. Maximum steric stabilization (surface protection) was observed with low concentrations of short chain PEG-PE and high concentrations of cholesterol.

Conclusions. The study provides a physical mechanism for the following observations: the blood circulation time is significantly increased or decreased with liposomes highly enriched with cholesterol or PEG-PE respectively.

KEY WORDS: cholesterol; differential scanning calorimetry; drug delivery; electron microscopy; fluorescence quenching; long circulating liposomes; NMR.

INTRODUCTION

Large quantities of cholesterol (~2:1 phosphatidylcholine/cholesterol mole ratio) are commonly used in the pharmaceutical formulation of long circulating (sterically stabilized) liposomes containing polyethyleneglycol-phosphatidylethanolamine (PEG-PE) conjugates. The presence of cholesterol prevents the leakage of encapsulated drugs (1) and also further prolongs the blood circulation time of long circulating liposomes (2). Studies utilizing a number of physical methods including differential scanning calorimetry (DSC) (3), have indicated that cholesterol modulates the physical properties of phospholipid bilayers.

We have found that in the absence of cholesterol, head group differences between PEG-PE and PC play a significant role in the intermolecular interaction. Short chain PEG-PE (MW 1,000–3,000) beyond 7 mol% causes solubilization of the bilayer, resulting in mixed micelle formation. Inclusion of long chain PEG-PE (MW 12,000) within the DPPC bilayer induces the formation of phase separated lamellae at all concentrations due to PEG chain-chain interaction (4).

The purpose of this work is to study the role of cholesterol in stabilization of PC bilayers containing PEG-PE. The results have shed light on the optimal liposome formulations for prolonged circulation.

MATERIALS AND METHODS

Materials

The phosphatidylcholine and phosphatidylethanolamine as well as the N-(7-nitro-2-1, 3-benzoxadiazol-4-yl) diacyl phosphatidylethanolamine (NBD-PE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol of 99.9+% purity was obtained from Sigma (St. Louis, MO). Ovalbumin, rhodamine B conjugate was obtained from Molecular Probes, Inc. (Eugene, OR). PEG-PE of different PEG chain length were prepared as described (4).

Preparation of Hydrated Lipid Dispersions

Dispersions for DSC and NMR experiments were prepared according to (4). For fluorescence experiments, one mol% of the fluorescent label NBD-PE was mixed together with appropriate amounts of PEG-PE, PC and cholesterol. The hydrated lipid dispersion was sonicated in a bath sonicator. Liposome size distribution was about 120±20nm for all preparations. 1–2mM of lipid was used in each case.

Calorimetry

The procedure for DSC has been described (4). The effect of NBD-PE as a membrane probe, was investigated to ensure that the bilayer was not significantly perturbed, and that mixing and formation of different physical states (4) was not affected by the addition of the probe.

Negative Stain Electron Microscopy

The procedure has been described previously (4).

Nuclear Magnetic Resonance

The conditions for the ^{1}H-NMR experiments have been described (4). ^{1}H-NMR spectra were recorded for each of the

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

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following samples comprising either one, two, or three components of PC/cholesterol/PEG-PE. Spectra were obtained separately for PC, cholesterol, PEG-PE, PC/ cholesterol, PEG-PE/ cholesterol, PC/PEG-PE and for PC/cholesterol/PEG-PE. Lipid concentration was controlled at 5–8 mM in D_2O.

Dynamic Light Scattering

The method of particle size distribution analysis has been described (4).

Analysis of Solubilization

Mixed micelles were separated from the lipid bilayers similar to the method described by Urbaneja et al. (5). The filtrate was collected and analyzed for cholesterol using the Libermann-Burchard test (6). PEG-PE and PC in the filtrate were separated using preparative TLC and quantified by phosphate assay (7).

Fluorescence Measurements

Fluorescence measurements were performed similar to Torchilin et al (8). The conjugate for liposomal NBD-PE quenching was Lissamine™ rhodamine B conjugated to ovalbumin. The Rh-Ova conjugate contained 5 rhodamine residues per conjugate. One ml of NBD-containing liposomes of 0.8 μM concentration in PBS, pH 7.5 was added to a 2 ml cuvette, continuously stirred and initial fluorescence recorded at room temperature. Increasing quantities of 0.2 mM Rh-OVA solution in PBS were added to the cuvette with continuous stirring and the residual fluorescence recorded after 10 min of incubation. NBD was excited at 460 nm with emission at 534 nm. Fluorescence of Rh-OVA was measured at 555 nm excitation and 579 nm emission. Fluorescence intensity measurements were made using a LS-5B Perkin Elmer fluorescence spectrometer (Norwalk, CT).

RESULTS

Characterization of PEG-PE Interaction with the Phospholipid Membrane by DSC

Unless specifically stated, the results are presented according to PC:cholesterol or PC:cholesterol:PEG-PE molar ratios. With PEG(1,000−3,000)-PE, formation of a new peak occured by 10:0.7 PC/PEG-PE signifying the beginning of conversion of the bilayer phase to the mixed micellar phase (4). Addition of cholesterol to PC:PEG(1,000)-PE mixtures modifies the DSC thermograms, and a high temperature shoulder (new peak) signifying solubilization of the bilayer to mixed micelles was not observed at PEG-PE concentrations ≤10:0.5:1 molar ratios (Fig. 1b). Formation of the shoulder was observed at higher PEG-PE concentrations, however, indicating that above a 10:0.5:1 molar ratio of PEG-PE, solubilization of the bilayer occurred leading to mixed micelle formation. 10:1 PC/

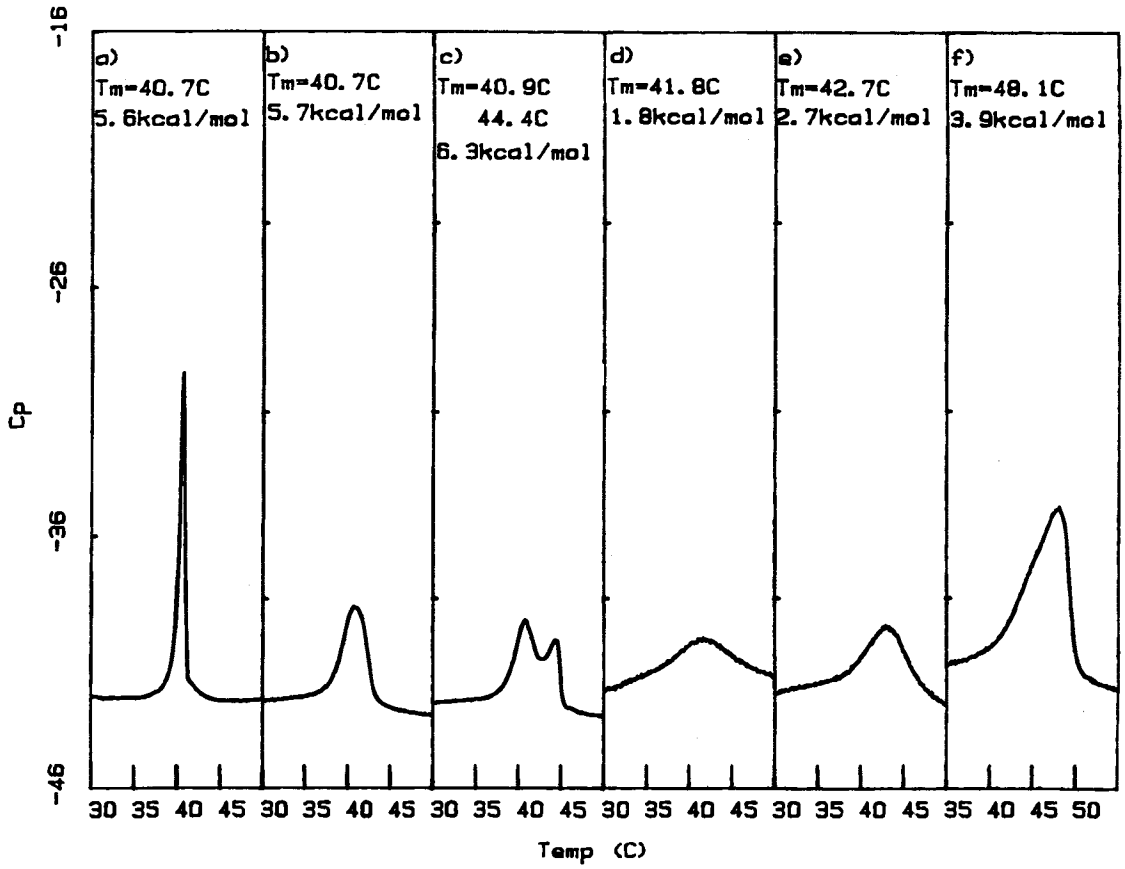

Fig. 1. DSC thermogram of DPPC/cholesterol/PEG(1,000)-DPPE mixtures at molar ratios of 10:0.5:0 (a), 10:0.5:1 (b), 10:0.5:2 (c), 10:3:0 (d), 10:3:2 (e), and 10:3:9 (f).

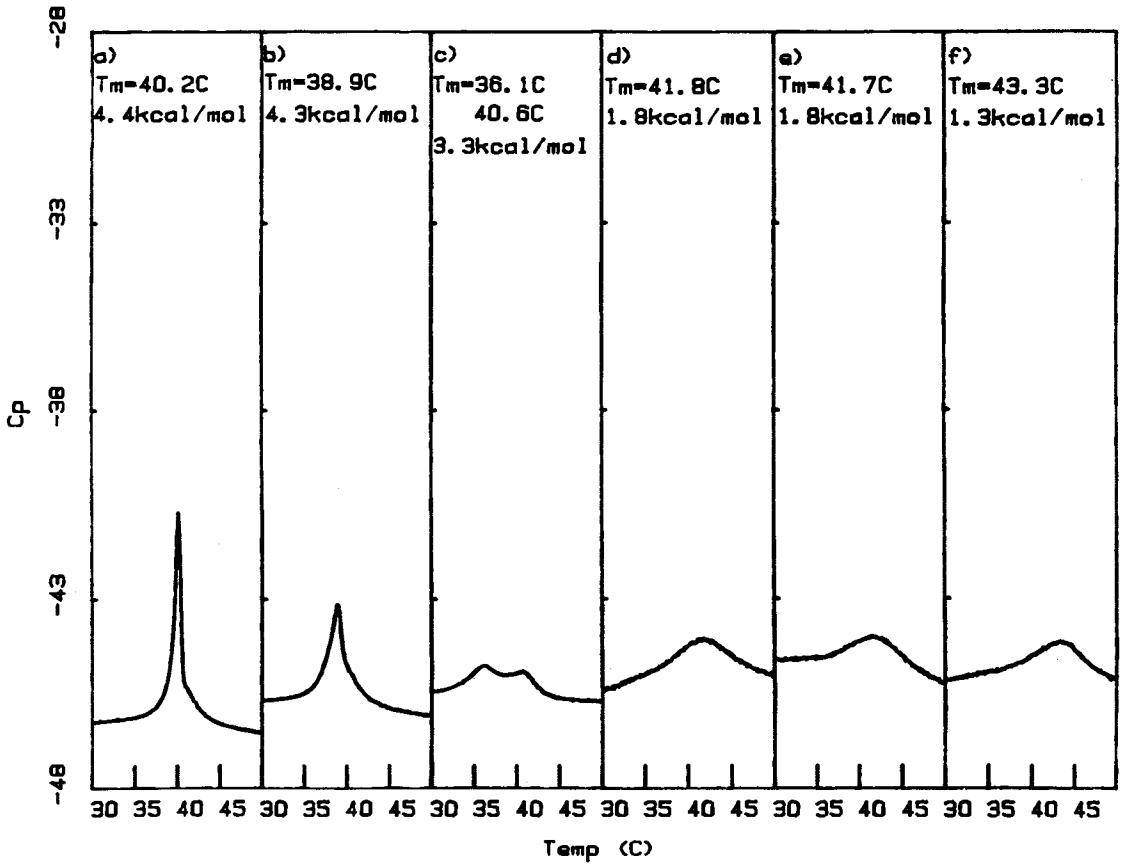

Fig. 2. DSC thermogram of DPPC/cholesterol/PEG(12,000)-DPPE mixtures at molar ratios of 10:1:0 (a), 10:1:1 (b), 10:1:4 (c), 10:3:0 (d), 10:3:3 (e), and 10:3:9 (f).

cholesterol molar ratios provided identical results to those obtained with a 10:0.5 molar ratio. By the ratio of 10:3 PC/cholesterol, formation of the shoulder was completely inhibited. However, contrary to the known effect of the addition of PEG-PE and cholesterol to PC bilayers, both of which lead to a loss in phase transition enthalpies and cooperativity, the enthalpy of the main phase transition increased drastically beyond the 10:3:9 molar ratio (Fig. 1f).

For long chain PEG(12,000)-DPPE, DSC thermograms are shown in Fig. 2. At 10:1 PC/cholesterol molar ratio, phase separation did not occur until the PEG-PE concentration reached 10:1:1 molar ratio were exceeded (Fig 2c). At 10:3 PC/cholesterol molar ratio, phase separation was completely inhibited up to PEG-PE concentration of 10:3:9 (Fig 2f). Contrary to short chain PEG, no increase in enthalpy of the main phase transition was obtained with increase in PEG-PE concentration.

Study of Particle Size Distribution by Light Scattering

Decrease in vesicle size of the suspensions was observed both with an increase in concentration of PEG-PE (all chain lengths), and decrease in PEG chain length as shown in Table I. Distinct loss of turbidity occured beyond 10:0.5:1 and 10:1:1 concentrations of PEG(1,000)-PE. Loss of turbidity coincided with the formation of shoulders observed by the high sensitivity DSC, and also the formation of large populations of tiny vesicles (micelles) observed by dynamic light scattering.

Table I. Vesicle Size Distribution of Lipid Dispersions Containing DPPC/cholesterol/PEG-PE

PEG MW	Molar Ratio[a]	Size Distribution[b]
1,000	10:0.5:0.5	730 ± 160 nm (78%), 130 ± 30nm (20%), 40 ± 10nm (2%)
	10:0.5:2	480 ± 100nm (10%), 65 ± 10nm (90%)
	10:1:2	280 ± 100nm (20%), 80 ± 20nm (80%)
	10:3:2	1970 ± 220nm (80%), 260 ± 60nm (20%)
	10:3:9	265 ± 100nm (12%), 20 ± 5nm (88%)
12,000	10:1:0.5	1580 ± 400nm (84%), 220 ± 35nm (16%)
	10:1:2	1020 ± 250nm (72%), 170 ± 90nm (28%)
	10:3:9	370 ± 60nm (37%), 105 ± 30nm (63%)

[a] Molar ratio refers to DPPC/cholesterol/PEG-PE.

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

With PEG(12,000)-DPPE, no loss of turbidity was observed.

Interaction of Short and Long Chain PEG-PE Studied by Using ^{1}H-NMR

Fig. 3 compares ^{1}H-NMR spectra of short chain PEG(1,000)-PE (Figs. 3a, c, and e) and long chain PEG(12,000)-PE (Figs. 3b, d, and f) in mixtures of PC/PEG-PE (Figs. 3a and b), PEG-PE/cholesterol (Figs. 3c and d), and PC/cholesterol/PEG-PE (Figs. 3e and f). The presence of high and low field choline peaks (Fig. 3a) suggests heterogeneity of the choline head group population within the mixture. The narrower high field peak was due to the choline present within the mixed micelles, and the broader low field peak was from choline present within the bilayer. Fig. 3b shows the existence of only the broader choline peak indicating that with PEG(12,000)-PE there was no choline existing in a micellar phase. Fig. 3a shows high resolution peaks within the $(CH_2)_n$ peak region again indicating the presence of CH_2 in both mixed micelles and bilayers. A high resolution spectrum in Fig. 3c indicates a large micellar population of cholesterol which was not present with long chain PEG(12,000)-PE (Fig. 3d). Comparison of Fig. 3e with Figs. 3a and c shows that high cholesterol solubilization occurred in the three component mixture also. Due to the distribution of line widths and shapes and varying concentrations of PEG-PE and cholesterol used, no quantitation was attempted from the NMR spectra.

Vesicle Characterization by Electron Microscopy

Electron micrographs of 10:3:1 and 10:3:9 PC/chol/PEG-PE mixtures are shown in Fig. 4. At low concentrations of PEG(1,000)-PE (10:3:1), the mixture existed as a multilamellar suspension predominantly, and at the higher PEG-PE concentration, a large micellar population existed. With PEG(12,000)-PE, a predominance of lamellar structures existed in both cases.

Analysis of Solubilization

An analysis of the filtrate of 10:3:9 PC/cholesterol/PEG(1,000)-PE yielded the following percentages of the original individual components used in the preparation: approximately 80% cholesterol, 55% PC and 80% PEG-PE.

Fluorescence Experiments

Experiments performed by Torchilin et al. (8) show that liposomal NBD interacts with Rh-OVA. Excitation energy is transferred from NBD to the OVA-conjugated Rh, resulting in the quenching of NBD fluorescence. Sonicated DPPC liposomes containing increasing concentrations of PEG(1,000 and 12,000)-DPPE, and also liposomes containing cholesterol at 10:3 PC: cholesterol molar ratio were treated with increasing concentrations of Rh-OVA. The results are shown in Fig. 5. Fluorescence quenching was reduced in the liposomes containing PC: PEG(1,000)-PE at a 10:0.2 molar ratio. This protection against NBD fluorescence quenching was decreased at a

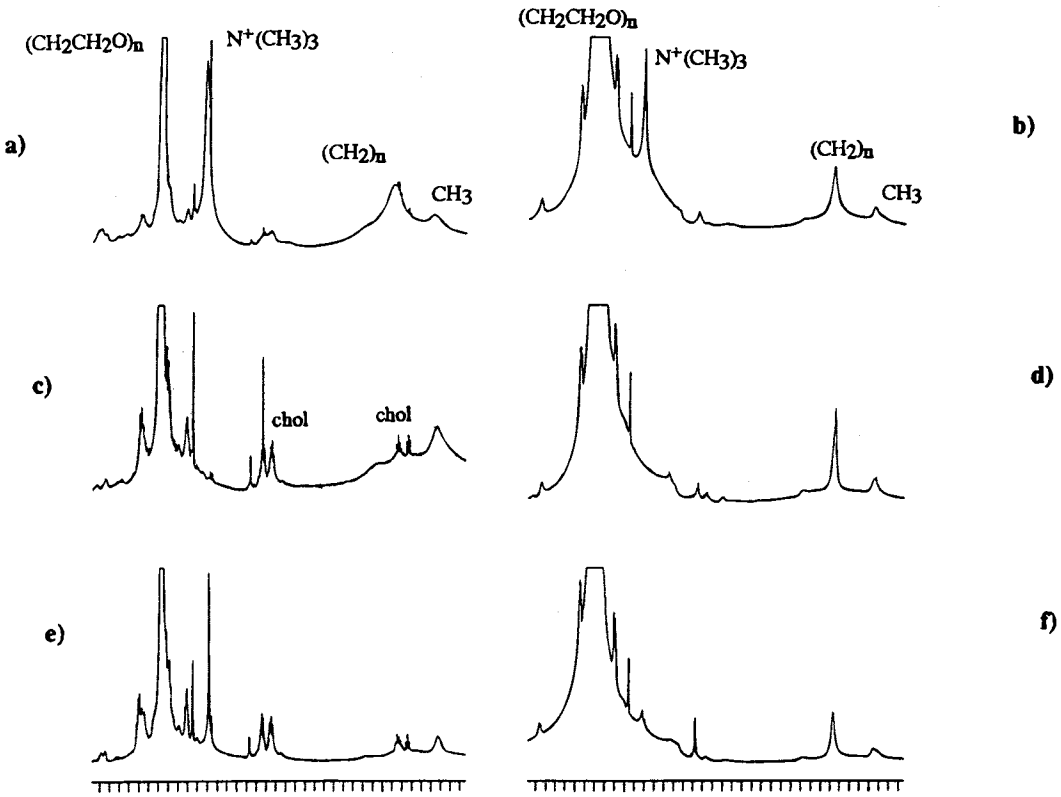

Fig. 3. ^{1}H-NMR spectra of mixtures of PC/PEG(1,000)-DPPE (a), PC/PEG(12,000)-DPPE (b), cholesterol/PEG(1,000)-DPPE (c), cholesterol/PEG(12,000)-DPPE (d), DPPC/cholesterol/PEG(1,000)-DPPE (e), and DPPC/cholesterol/PEG(12,000)-DPPE (f).

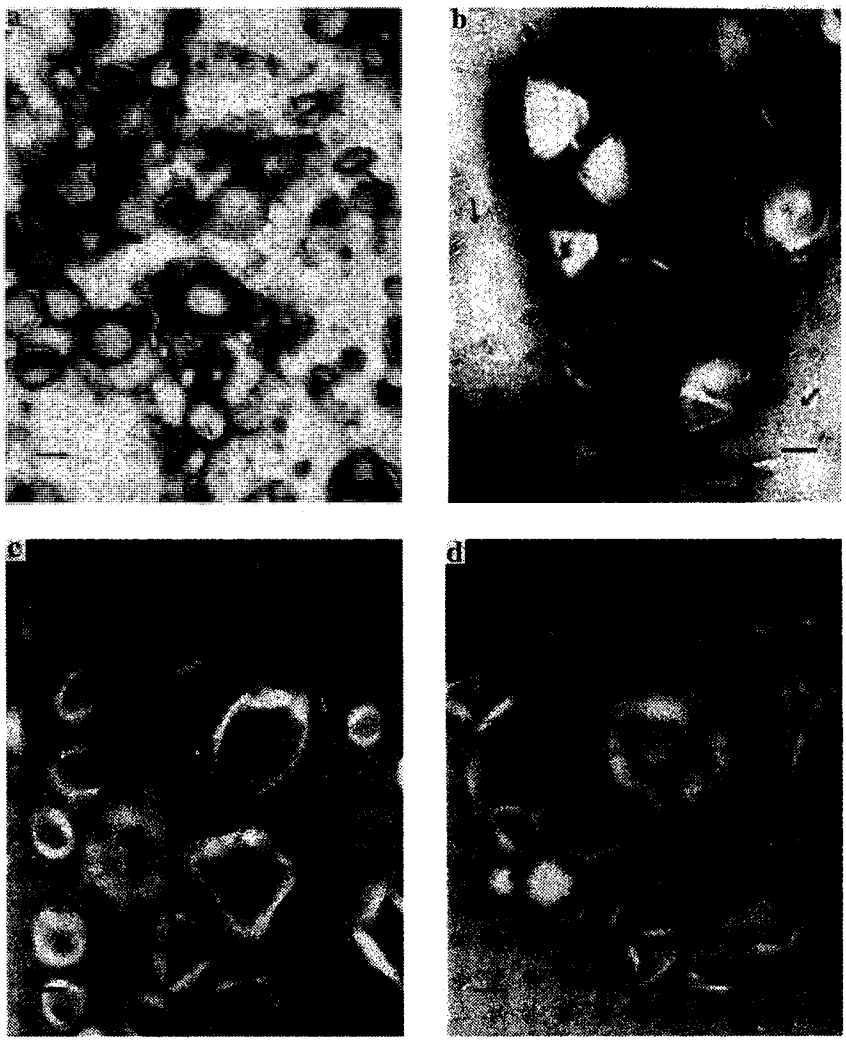

Fig. 4. Negative stain electron micrographs of DPPC/cholesterol/PEG(1,000)-DPPE at molar ratios of 10:3:1 (a), 10:3:9 (b). Negative stain electron micrographs of DPPC/cholesterol/PEG(12,000)-DPPE at molar ratios of 10:3:1 (c), and 10:3:9 (d). The mixed micelles are indicated by arrows. Scale bar is 400nm for (a), 200nm for (b), and 100nm for (c) and (d).

10:1 molar ratio. Interestingly, with PEG(12,000)-DPPE, 10:0.2 molar ratios provided some protection, however, beyond 10:1 molar ratio no protection was observed. Addition of cholesterol provided increased protection in each case. For PEG(12,000)-PE, however, this protection remained considerably lower than that provided by PEG(1,000)-PE despite minimal phase separation and micelle formation as detected by DSC.

DISCUSSION

Cholesterol is known to have remarkable effects on the physical properties of phospholipid membranes. It reduces surface compressibility by a factor of 3 to 4, and also increases bilayer cohesive strength (9).

Fig. 1 indicates that even at low cholesterol concentrations of 10:0.5 molar ratio bilayer cohesive strength is increased, resulting in decreased bilayer solubilization by PEG(1,000)-PE. Dynamic light scattering however indicates that at low PEG(1,000)-PE concentrations a small degree of solubilization of the bilayer still occurs. Addition of cholesterol was also observed to inhibit the formation of phase separated lamellae with PEG(12,000)-PE (Fig. 2). The planar hydrophobic steroid ring of cholesterol is relatively inflexible and restricts flexibility of the hydrocarbon chain (10). Also, the most significant cholesterol interaction occurs within the bilayer hydrocarbon chains. Cholesterol therefore acts as a spacer pushing apart lipid chains and reducing PEG chain-chain interaction. This leads to the observed reduced and broadened main phase transition, and also the inhibition of phase separation. As could be expected, transition enthalpies decrease with increasing concentrations of cholesterol (Fig. 2).

The results of NMR studies have shed some light into the mechanism of increase in transition enthalpy with PEG(1,000)-PE in PC/cholesterol mixtures (Fig. 1). A 10:1 PC/PEG(1,000)-PE molar ratio, results in the superposition of narrow peaks typical of mixed micellar PC (11), and broader resonance typical of lamellar PC (Fig. 3a). This is in perfect agreement with DSC data indicating partial solubilization at the above concentration of PEG(1,000)-PE. Similar observations of high resolution Lorentzian line formation with micelles have been reported

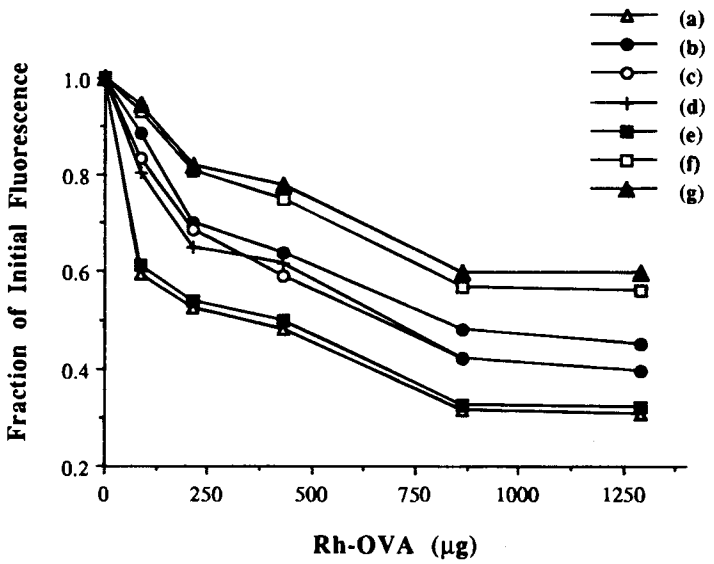

Fig. 5. Quenching of liposomal NBD fluorescence with Rh-OVA at increasing Rh-OVA concentrations in liposomes containing PC/cholesterol/PEG(1,000)-PE at indicated molar ratio. (a), 10:3:0; (b), 10:0:0.2; (c), 10:0:1; PC/cholesterol/PEG(12,000)-PE at (d), 10:0:0.2; (e), 10:0:0.1; and PC/cholesterol/PEG(1,000)-PE at (f), 10:3:0.2 and (g) 10:3:1. Negligible standard deviation was obtained in each case.

(11). Cholesterol does not resonate in PC-cholesterol mixtures due to the restricted motion of the sterol ring in the bilayer (12). However, with the addition of short chain PEG-PE high cholesterol resonance was observed, indicating solubilization of cholesterol and its extraction from the bilayer (Figs. 3c and e). This is not observed with long chain PEG-PE (Figs. 3d and f). Reduction in the high resolution (high field) choline peak (Fig. 3e) in the presence of cholesterol compared with that of Fig. 3a in the absence of cholesterol, is explained by the high concentration of cholesterol existing in the mixed micelles formed from bilayer solubilization by PEG(1,000)-PE. The narrowing of the low field choline peak corresponding to the vesicular choline in Fig. 3e, is however due to the fact that all spectra for samples incorporating cholesterol were recorded at 43°C due to high sample viscosity and lack of significant resolution at room temperature.

Preferential solubilization of cholesterol over PC (80 vs. 55%) at high PEG(1,000)-PE concentrations was confirmed by analysis of the micellar population. Cholesterol is therefore extracted from the bilayer into mixed micelles (Fig. 4) resulting in an increase in enthalpy and cooperativity of the PC transition. Absence of mixed micellar phase transition at high cholesterol and PEG(1,000)-PE is most probably due to the high cholesterol concentration within these micelles abolishing the transition endotherm of low enthalpy. Absence of distinct loss of turbidity is due to the incomplete solubilization, and also as suggested (5), probably micelles containing high cholesterol concentrations display scattering properties which differ from those exhibited by PC and PEG-PE mixed micelles.

These results suggest an increased steric stabilization at low PEG-PE and high cholesterol concentrations. To confirm this, an investigation of the efficiency of surface protection was performed utilizing fluorescence energy transfer. Steric stabilization was evaluated by the extent of excitation energy transfer from liposomal NBD to the OVA-bound Rh upon collision with the liposome surface resulting in NBD quenching. As expected, the highest degree of quenching occured with liposomes containing no PEG-PE and therefore no surface protection. Fluorescence quenching decreased with 10:0.2 molar ratio of PC/PEG(1,000)-PE. At 10:1 molar ratio micelle formation occured, and surface protection was decreased causing an increase in NBD fluorescence quenching. Fig. 5 shows that at 10:1 PC/PEG(12,000)-PE molar ratio, NBD fluorescence diminished identically to that of the plain, non PEG coated liposomes. Phase separation is distinct in this case (4). However, with lower concentrations of PEG(12,000)-PE of 10:0.2 molar ratios phase separation is minimal, and moderate protection was observed.

Addition of cholesterol improved surface protection. However, even in the presence of cholesterol PEG(12,000)-PE did not offer optimal surface protection (fluorescence pattern identical to that of 10:1 PC/PEG(1,000-PE) in both cases). This is probably due to the fact that reduction in the intramolecular expansion factor with increase in molecular weight could lead to coil shrinkage (4, 13) and reduced PEG chain flexibility. Minimal phase separation is also possible, which might not have been detected by the high sensitivity DSC due to broad peak width.

These results have direct implications on the use of liposomes for drug delivery. We suggested (4) that phase separation creates domains less enriched with PEG-PE or "bald spots" leading to the rapid clearance of liposomes by opsonin adsorption and lipid exchange with blood lipoproteins. The addition of cholesterol significantly reduces the tendency towards the formation of phase separated lamellae as shown by DSC. Short chain PEG is more hydrophilic and flexible, and is seen to provide better steric stabilization of liposomes. In the presence of cholesterol, bilayer solubilization by PEG(1,000)-PE is much reduced. However due to the preferential solubilization of cholesterol over PC, at high PEG(1,000)-PE concentrations, choles-

terol is extracted from the bilayer and solubilized into mixed micelles. High drug leakage can be expected in this case.

These results explain *in vivo* results obtained by Maruyama et al (14). Circulation times of liposomes containing DSPC: cholesterol at 1:1 molar ratio and containing 0 mol% and 6 mol% of PEG-DSPE of varying chain lengths were examined at 3 h after i.v.. injection. For liposomes containing no PEG-PE, PEG(1,000)-PE and PEG(12,000)-PE, the blood/mononuclear phagocyte system (MPS) ratios were 0.4, 4.4, and 1.8 respectively, indicating that short chain PEG-PE provides better surface protection than the long chain PEG-PE.

This study sheds light on the role of cholesterol in stabilizing liposomal formulations. The most suitable formulations for prolonged circulation should contain cholesterol concentrations >30 mol% and low concentrations ≤ 10 mol% of short chain PEG-PE.

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