Effect of Bilayer Composition on the Phase Behavior of Liposomal Suspensions Containing Poly(ethylene glycol)–Lipids

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ABSTRACT: Liposomes containing phospholipids with covalently attached poly(ethylene glycol) (PEG–lipids) are being developed for use as carriers in *in vivo* drug delivery. A critical design parameter for these liposomes is the maximum amount of PEG–lipids that can be incorporated into the phospholipid bilayer before it is converted into a micelle. In this paper, X-ray diffraction is used to determine this saturation limit of PEG–lipids for a variety of phospholipid bilayers with different tensile strengths and polymorphic properties. It is found that 15–20 mol % PEG–lipid can be incorporated into gel phase bilayers, liquid-crystalline bilayers, and bilayers containing equimolar cholesterol. However, the saturation limit of PEG–lipid in the bilayer is decreased to about 8 mol % when lysolipids are added to liquid-crystalline phase bilayers or when the gel phase bilayers are made with shorter hydrocarbon chains. These data indicate that the phase transition from lamellar to micellar phase for lipid suspensions containing PEG–lipids does not depend strongly on the tensile strength of the bilayer, but rather is determined primarily by the polymorphic properties of the lipid molecules. This study also measures the range and magnitude of the steric barrier provided by the incorporation of PEG–lipid into bilayers of different compositions. The steric barrier depends on the concentration of PEG–lipid in the bilayer, with the incorporation of 10 mol % PEG-2000 into gel, liquid-crystalline, and cholesterol-containing bilayers providing a barrier that extends about 65 Å from each bilayer surface.

1. Introduction

Liposomes containing lipids with covalently attached poly(ethylene glycol) (PEG–lipids) have proven useful in *in vivo* drug/agent delivery.[1–7] The presence of grafted PEG creates a steric barrier around these liposomes and allows them to effectively evade the host defense system.[4,8] This results in a prolonged blood circulation time of the PEG–liposomes, as compared to conventional liposomes. Because of their long blood circulation time and the leakiness of the microvasculature in solid tumors, PEG–liposomes can be used to deliver drugs to solid tumors.

It has been shown[9] that the blood circulation time of the PEG–liposomes reaches a maximum at a PEG–lipid concentration in the bilayer of about 10 mol %, an effect which is relatively insensitive to the phospholipid composition and cholesterol content of the liposome. Further increases in PEG–lipid concentration decrease the performance of PEG–liposomes as drug carriers. For one particular phospholipid (distearoylphosphatidylcholine), Kenworthy *et al.*[10] used X-ray diffraction, NMR, and calorimetry to show that, for PEG molecular weights higher than 750, increasing the PEG–lipid concentration above about 15 mol % leads to a solubilization of the lamellar phase and a phase transition to a nonbilayer (micelle) phase. This solubilization of the bilayer is most probably the explanation of the decreased blood circulation times measured at high PEG–lipid concentrations.[10]

Recent work has addressed how bilayer composition may influence the physical parameters that ultimately determine the phase transition from a lamellar to a micellar phase in the PEG–liposome system. Hristova and Needham[11] proposed a theoretical model for the phase behavior of mixtures of PEG–lipids and lipids in aqueous medium as a function of the molar percent of PEG–lipids. They predicted that the PEG–lipid/lipid/water system in excess water should exhibit a complex phase diagram, determined by the self-assembling properties of the lipids. Hristova and Needham[11] postulated that two factors could influence the nature of the phase behavior: (1) the material properties of the bilayer, such as bilayer tensile strength, and (2) lipid polymorphism, the ability of lipids to form different types of aggregates (such as bilayers or micelles) under different conditions.

In this paper we use X-ray diffraction analysis of bilayers of different compositions to test this theoretical treatment[11] and determine which of these two factors determines the maximum amount of PEG–lipid that can be incorporated into the bilayer. Kenworthy *et al.* have obtained phase diagrams[10] and pressure–distance relations[12] for PEG–lipids with PEGs ranging in mass from 350 to 5000 Da with the saturated phospholipid distearoylphosphatidylcholine (DSPC). Here we use one size of grafted PEG (PEG-2000) and obtain pressure–distance relations for a variety of phospholipids with different mechanical properties and different tendencies to form micellar phases. Using these pressure–distance relations, we determine the maximum concentration of PEG–lipid that can be incorporated into a bilayer phase, and thus infer the phase behavior of each system. These data also provide information on the range and magnitude of the steric barrier as a function of bilayer composition.

2. Phase Behavior of the PEG–Lipid/Lipid Mixtures in Aqueous Medium and Maximum Concentration of PEG–Lipids in the Bilayer

In this section we briefly review the theory developed in ref 11 and suggest a way to verify it.

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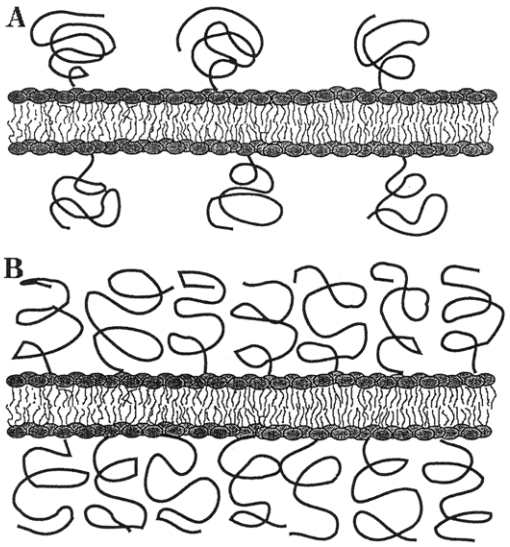

Figure 1. Schematic diagrams showing PEG–lipids in phospholipid bilayers. The solid circles represent the phospholipid head groups, the narrow lines represent the lipid hydrocarbon chains, and the wider curved lines represent the PEG that is covalently attached to the head groups of the PEG–lipids in the bilayer. Panel A depicts the mushroom regime at low PEG–lipid concentrations in the bilayer and (B) depicts the brush regime at high PEG–lipid concentrations.

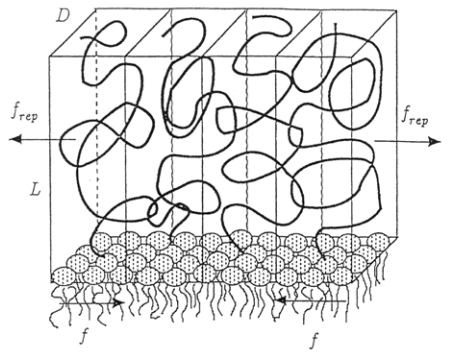

Figure 2. Schematic diagram showing that in bilayers the brush lateral force (denoted f_{rep}) is balanced by bilayer cohesive forces (denoted f).

2.1. Phase Transition Determined by the Material Properties of the Bilayer. The two regimes of grafted polymer behavior, as defined by de Gennes,[13] are presented in Figure 1, A and B. At low grafting concentrations each polymer chain is in its random coil conformation; this is the "mushroom" regime. At high grafting concentrations the chains interact laterally, so a uniform extended "brush" is formed. Since by definition there are no lateral interactions between polymers in the mushroom regime, if the phase transition to micelles were determined by polymer lateral interactions it should not occur until PEG–lipid concentrations are within the brush regime. In the brush regime there is energy stored in the brush which increases with the polymer molecular weight and with PEG–lipid concentration in the bilayer. This stored free energy is expressed as a lateral tension between the brushes. If the bilayer structure were stable, the lateral pressure in the two polymer layers (see Figure 2) would be felt as an isotropic tension in the bilayer. Just as in micropipet experiments[14,15] where applied tension leads to area change, the polymer would promote area expansion of the bilayer, which would be opposed by the cohesive forces in the bilayer (Figure 2). This tension would increase with PEG–lipid concentration and eventually reach the critical tension that the bilayer could support as a material. The maximum PEG–lipid concentration would therefore be the one at which the critical material parameters are attained; higher PEG–lipid concentrations could not be achieved. So the material properties define a saturation limit n_{sat}, such that increasing the PEG–lipid concentration above n_{sat} leads to a transition from bilayer to micellar phase.

To calculate the maximal concentration of PEG–lipid in the bilayer predicted by this formalism, Hristova and Needham[16] developed a simple lateral force model where at the maximum concentration of PEG–lipid in the bilayer the lateral steric repulsion between the polymer chains is balanced by the cohesive forces in the bilayer. Hristova and Needham[16] found that n_{sat} (that is, the maximum lateral tension the bilayer can withstand before rupture) is proportional to the area per lipid molecule A times the bilayer tensile strength τ_s to the $^3/_5$ power:

$$n_{sat} = \frac{A}{(12/15(\pi^2/12)^{1/3} kT\, Na^{4/3}/\tau_s)^{3/5}} \qquad (1)$$

If this prediction were true, one could control and manipulate this maximum PEG–lipid concentration in the bilayer by changing the lipid composition (e.g. incorporating cholesterol that is known to increase the tensile strength τ_s of the bilayer or using gel phase bilayers which have larger tensile strengths than liquid-crystalline bilayers[15]). To illustrate this, we calculate the ratio of n_{sat} for equimolar DSPC:cholesterol bilayers to n_{sat} for SOPC bilayers (lipid abbreviations are given in the Materials and Methods) using the Hristova and Needham formalism and values for tensile strength from Needham and Nunn[15] and Needham:[14]

$$\frac{n_{sat}^{DSPC:Ch}}{n_{sat}^{SOPC}} = \frac{A^{DSPC:Ch}}{A^{SOPC}} \left(\frac{\tau_s^{SOPC}}{\tau_s^{DSPC:Ch}} \right)^{3/5} \qquad (2)$$

where $A^{SOPC} = 65$ Å^2,[17] $A^{DSPC:Ch} = 39$ Å^2, $\tau_s^{SOPC} = 5.7$ dyn/cm,[15] and $\tau_s^{DSPC:Ch} = 41$ dyn/cm.[14] The calculated ratio is 2.0, indicating that this scheme predicts that twice as much PEG–lipid can be incorporated into DSPC:Ch bilayers as can be incorporated into SOPC or EPC bilayers (SOPC is a synthetic analog of EPC). Thus this theory implies that increasing the bilayer cohesion is a means of achieving a high density of grafting in these self-assembling lipid systems.

2.2. Phase Transition Determined by the Thermodynamics of a Self-Assembling PEG–Lipid/Lipid System (the Minimum Energy Requirement). If lipid molecules formed only bilayers, then the only scheme for the saturation limit would be the one determined by the material properties of the bilayer. In fact, lipid molecules are polymorphic, in that they can form different types of aggregates under different conditions. As pointed out by Israelachvili,[18] fully hydrated diacyl phospholipid molecules, such as diacylphosphatidylcholines, which have approximately a cylindrical shape (the excluded area in the head group approximately equals the excluded area in the hydrocarbon region), usually form bilayers. But changes in temperature, pH, or as discussed here, a high concentration of incorporated PEG–lipid can lead to enhanced lateral repulsion in the head group region and can induce a phase transition to a micellar phase. In the case of suspensions containing PEG–lipids, curving the

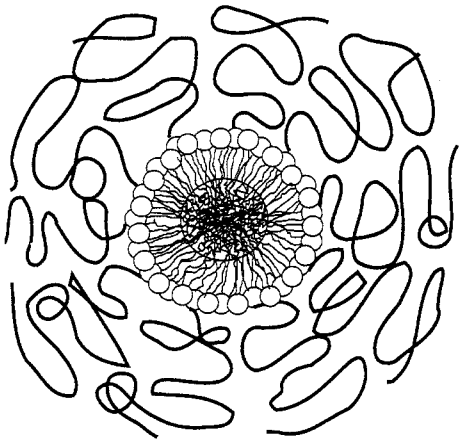

Figure 3. Schematic diagram of a micelle containing phospholipids and PEG–lipids.

grafting surface would reduce the lateral tension in the polymer layer, with higher curvature producing more relaxation. The highest curvature would be obtained if the lipids packed into micelles (Figure 3). Micelle formation would cost additional energy due to "unfavorable packing" of the hydrocarbon chains. Although the chain-packing energy does not depend on the polymer molecular weight or grafting density, the energy stored in the polymer brush does depend crucially on them—the larger the PEG molecular weight or concentration of PEG–lipid, the larger the relaxation in the transition from a bilayer to a micelle. Thus, for large concentrations of high molecular weight PEG–lipids, the decrease in brush energy due to curvature of the surface would be greater than the hydrocarbon energy increase due to micelle packing and a phase transition from bilayers to micelles would occur. The concentration n_{tr} (the thermodynamic crossover) at which this transition would occur depends on the molecular weight and the grafting density of the polymer and on the structure of the hydrocarbon tails. For instance, the number of CH_2 groups and the number of double bonds in the hydrocarbon chains determine how "difficult" it is to pack the chains in a micelle.

To understand the mechanism of this phase transition, Hristova and Needham[11] analyzed the changes in the energy of the system which occur upon an increase in the concentration of the PEG–lipids in a suspension containing phospholipids that normally form bilayers. They found theoretically that upon an increase in the concentration of PEG–lipid in the sample the energy minimum gradually moves from a bilayer into a micellar phase. For concentrations lower than n_{tr} the dominant phase is the bilayer. Increasing the concentration above the thermodynamic crossover n_{tr} has the effect of decreasing the probability for formation of bilayers. Above n_{tr} a mixed phase exists where bilayers and micelles have the same energy. The PEG–lipid concentration in the bilayer is equal to the system concentration (i.e. concentration in the original anhydrous mixture) for concentrations less than n_{tr}. Above n_{tr} it is equal to n_{tr}, as dictated by the behavior of a mixed phase region.

Thus, in the first scheme the phase transition from bilayers to micelles is determined by attaining critical material parameters of the bilayer, whereas in the second scheme the minimum energy requirement is the driving force for the phase transition. Both schemes predict micelle formation and a mixed bilayer–micelle phase above a certain critical PEG–lipid concentration, as has been observed experimentally.[10] Also, in both schemes the PEG–lipid concentration in the bilayer increases up to this critical concentration and then levels off. Both schemes predict similar maximum concentrations of PEG–lipid in gel phase bilayers, consistent with the amount found experimentally.[10] In the first scheme this concentration is the saturation limit of the polymer–lipids in the bilayer n_{sat} and is determined by the tensile strength of the bilayer, whereas in the second scheme the critical concentration is the thermodynamic crossover n_{tr}.

Experimentally, we can identify which effect occurs by systematically varying the lipid composition. To test the first scheme, we compare the amount of PEG–lipids that can be incorporated into (1) gel and liquid-crystalline bilayers and (2) bilayers in the presence and absence of cholesterol. These experiments test the role of bilayer material properties, since gel and liquid-crystalline bilayers have different tensile strengths and cholesterol improves the cohesive properties of the lipid bilayer but does not induce micelle formation.[15] To test the second scheme, we compare the amount of PEG–lipids that can be incorporated into (1) bilayers with different hydrocarbon chain lengths and (2) bilayers containing lysophosphatidylcholine. Bilayers with longer hydrocarbon chains should be more stable and less likely to form micelles than those with shorter chains. Lysophosphatidylcholines have only one hydrocarbon tail, so are wedged shape and self-aggregate into micelles.[18] When incorporated into a lipid bilayer above a certain concentration, they induce micelle formation.[19] If the second scheme were correct, then the amount of PEG–lipid that could be incorporated into a bilayer phase should be a function of lipid hydrocarbon chain length and the amount of lysophosphatidylcholine in the suspension. Therefore, to distinguish the two possible models for bilayer to micelle transition for PEG–lipid suspensions, we use X-ray diffraction to determine the phase properties of the suspensions as a function of bilayer phase, cholesterol content, hydrocarbon chain length, and lysolipid content.

3. Materials and Methods

3.1. Materials and Sample Preparations. All phospholipids and cholesterol (Ch) were purchased from Avanti Polar Lipids (Alabaster, AL). The phospholipids were diacylphosphatidylcholines, including 1,2-distearoylphosphatidylcholine (DSPC), 1,2-dipalmitoylphosphatidylcholine (DPPC), egg phosphatidylcholine (EPC), PEG–lipids with PEG of molar mass 2000 Da covalently to either 1,2-distearoylphosphatidylethanolamine (DSPE-PEG), 1,2-dipalmitoylphosphatidylethanolamine (DPPE-PEG), or 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE-PEG), and a lysophosphatidylcholine, monooleoylphosphatidylcholine (MOPC). Host lipid bilayers in the gel phase at room temperature were composed of either DSPC or DPPC, which are saturated phospholipids containing 18 and 16 carbons per chain, respectively. Bilayers in the liquid-crystalline phase were composed of EPC, which typically contains one saturated chain and one unsaturated chain (usually an oleoyl chain). The PEG–lipids and lysophosphatidylcholine were chosen so that they had hydrocarbon chain compositions similar to that of the appropriate host bilayer.[10] Dextran with a mass-average molar mass of 503 000 Da was purchased from Sigma Chemical Co. (St. Louis, MO).

Samples were prepared by codissolving the appropriate lipid mixtures in chloroform and removing the chloroform with a rotary evaporator. The dry lipid mixtures were incubated above the lipids' melting transition with the appropriate dextran–buffer (0.1 M NaCl, 20 mM HEPES, pH 7) solution. To ensure complete equilibration, the lipid suspensions were periodically vortexed and cycled through the bilayer's main phase transition at least three times.[12]

3.2. X-ray Diffraction/Osmotic Stress Method. X-ray diffraction can be used to distinguish bilayers from micellar phases, since multilamellar phosphatidylcholine bilayers (liposomes) routinely give a series of low-angle reflections that index as orders of a lamellar repeat period, whereas micelles give no sharp low-angle reflections. In the case of lipid suspensions containing relatively large concentrations of PEG–lipids, the disorder in the sample makes it difficult to record the discrete lamellar diffraction from liposomes in excess buffer.[10,12] Therefore, to improve the orderliness of the specimens, osmotic stresses were applied by incubating the suspensions in dextran solutions of known osmotic pressures.[20,21] Moreover, by determining by X-ray diffraction the distance between bilayers for each osmotic pressure, pressure–distance relations are obtained.[4,8,12]

The lipid/dextran suspensions were sealed in Quartz X-ray capillary tubes and mounted in either a point collimation or mirror–mirror X-ray camera. Exposure times were between 5 and 24 h. The scattered radiation was recorded at room temperature on a stack of Kodak DEF-5 X-ray films which were densitometered with a Joyce-Loebl microdensitometer.[22–25] For lamellar diffraction patterns, the integrated intensities ($I(h)$ for order h) were obtained by measuring the area under each diffraction peak. The structure amplitudes $F_u(h)$ were set equal to $(h^2I(h))^{1/2}$.[26]

The X-ray experiments provide the repeat period d of the lamellar phase. To compare directly the results for different bilayer compositions, it was necessary to take into account that the thickness of the bilayer is different for each bilayer system. Information about the bilayer thickness and the separation between bilayers can be obtained from electron density profiles, which were calculated from the recorded diffraction patterns by[27]

$$\rho(x) = \frac{2}{d}\sum_{1}^{h_{max}} (\pm)|F_u(h)| \cos(2\pi hx/d) \qquad (3)$$

where x is the distance from the bilayer center. The phases (+ or − for each diffraction order), have been obtained previously for these lipid systems.[10] The thickness of the bilayer is defined as the total thickness of the phosphatidylcholine bilayer,[24,25,28,29] assuming that the head group conformation is the same as in single crystals of DMPC[30] and the high-density head group peak in the electron density profile is located between the phosphate moiety and the glycerol backbone, 5 Å from the edge of the bilayer.[31,32] Thus we estimate the total bilayer thickness to be the distance between the high electron density peaks plus 10 Å. The interbilayer separation is defined as the difference between the lamellar repeat period and the bilayer thickness.

4. Results

Gel and Liquid-Crystalline Bilayers. Parts A and B of Figure 4 show plots of the logarithm of applied pressure (log P, where P is in dyn/cm^2) versus repeat period and fluid spacing, respectively, for DSPE-PEG/DSPC taken from Kenworthy *et al.*[12] These bilayers were in the gel phase at room temperature (see ref 10). For each concentration of PEG–lipid, the distance between bilayers decreased with increasing applied pressure (Figure 4B). Each pressure–distance curve provides the range and magnitude of the steric repulsion caused by the covalently attached PEG-2000.[12] For example, at a concentration of 10 mol %, the pressure–distance curve reached a maximum interbilayer distance of about 130 Å at the lowest applied pressures, indicating that the PEG extended about 65 Å from each bilayer surface. At a constant applied osmotic pressure, the distance between bilayer increased with increasing molar percent PEG–lipid up to 20–30 mol %. Upon a further increase in the PEG–lipid concentration the separation between the bilayers did not change, indicating that the maximum concentration of PEG–lipid in the bilayer had been obtained. Calculations show that this limit of the PEG–lipid concentration in the bilayer was reached in the brush regime.[12] Kenworthy *et al.*[10] have used a variety of other techniques including absorbance measurements, NMR, and differential scanning calorimetry to show that at 20–30 mol % PEG–lipid, concentrations at which saturation occurred, micelles formed. They showed that over a broad range of PEG–lipid concentrations (about 20–50 mol %) bilayers and micelles coexisted. This is in agreement with our predictions presented in section 2. A pure micellar phase was formed for PEG–lipid concentrations greater than about 50 mol %.[10]

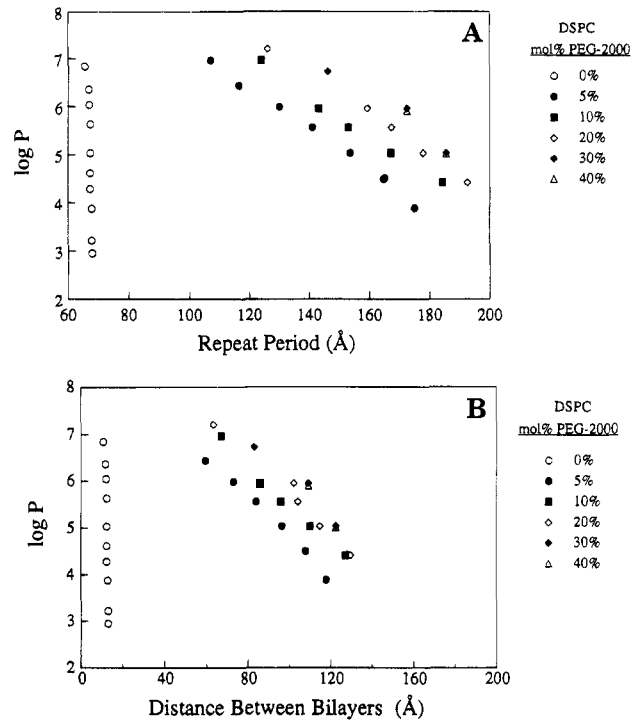

Figure 4. Plots of the logarithm of applied pressure (log P) (applied pressure is in dyn/cm^2 in all the plots) versus (A) the lamellar repeat period and (B) the distance between bilayer surfaces for gel phase DSPC bilayers containing various concentrations of PEG–lipid. Data taken from Kenworthy *et al.*[12]

Parts A and B of Figure 5 show plots of log P versus repeat period and fluid spacing, respectively, for POPE-PEG/EPC bilayers in the liquid-crystalline phase. For this system we were unable to obtain diffraction patterns with high enough resolution to construct the electron density profile. On the basis of experience with other bilayer systems,[8,10] we assumed that the width of the EPC bilayer was not appreciably changed by the incorporation of DOPE-PEG. Thus we assumed a constant bilayer thickness, the same as that of the pure lipid bilayer. Therefore, to obtain the bilayer separation from the repeat period we subtracted the EPC bilayer thickness of 48 Å, as previously measured.[23] The data for the POPE-PEG/EPC system (Figure 5B) show the same characteristic features seen with the gel phase DSPE-PEG/DSPC system (Figure 4B). Specifically, at the lowest applied pressure the incorporation of 10 mol % PEG-2000 increased the interbilayer distance to about 130 Å, similar to that found for DSPC (Figure 4B). Again, this indicates that the PEG extended about 65 Å from each bilayer surface. For a constant applied pressure the distance between bilayers increased up to about 15 mol % polymer–lipid and then further in-

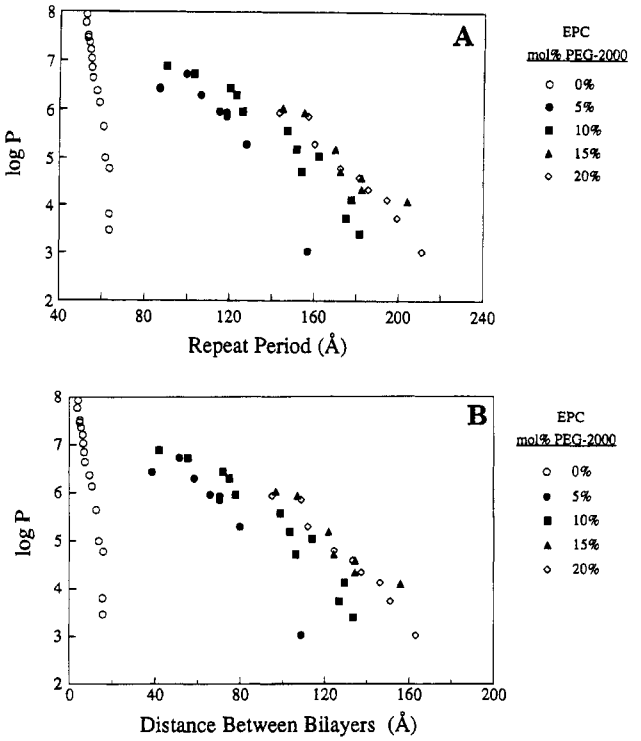

Figure 5. Plots of the logarithm of applied pressure (log P) versus (A) the lamellar repeat period and (B) the distance between bilayer surfaces for liquid-crystalline phase EPC bilayers containing various concentrations of PEG–lipid. Data for EPC bilayers with 0 mol % PEG–lipid were taken from ref 22.

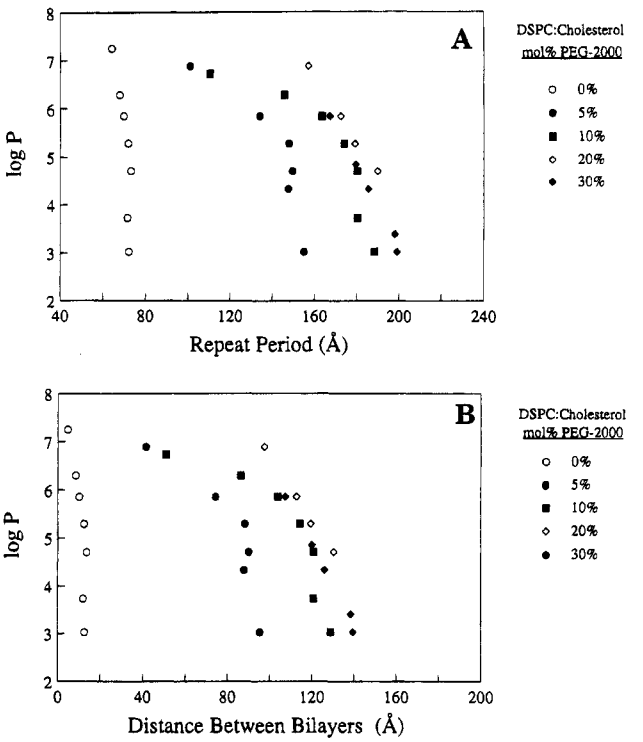

Figure 6. Plots of the logarithm of applied pressure (log P) versus (A) the lamellar repeat period and (B) the distance between bilayer surfaces for DSPC:cholesterol bilayers containing various concentrations of PEG–lipid.

creases in POPE-PEG concentration did not change the spacing (Figure 5B). Again, this was an indication of saturation of PEG–lipid in the bilayer. Moreover, in order to obtain measurable patterns for 20 mol % polymer–lipid we had to increase the exposure time about 2 times as compared to 10 mol % polymer–lipid, and at log P = 4, lamellar diffraction could not be recorded for PEG–lipid concentrations greater than about 30 mol %. This indicates that for PEG–lipid concentrations above about 15 mol % (bilayer saturation) a mixed phase region of bilayers and micelles was formed. The saturation and the gradual disappearance of the bilayer phase indicate that concentrations in the range 15–30 mol % were within the region of bilayer and micelle coexistence, as described in section 1.

Phospholipid:Cholesterol Bilayers. X-ray diffraction experiments were performed on multilamellar DSPE-PEG:DSPC:cholesterol liposomes with the following mole ratios: 0:50:50, 5:45:50, 10:40:50, 20:30:50, and 30:20:50. Thus, the ratio between the phospholipid (PEG–lipid or DSPC) and cholesterol was always 1:1. A plot of log P versus repeat period for these systems is shown in Figure 6A. No lamellar diffraction could be recorded from specimens containing higher concentrations of PEG–lipid. To determine the bilayer width for DSPC:cholesterol bilayers, we calculated electron density profiles, as shown in Figure 7. These profiles had a head group peak separation across the bilayer of 49 Å, giving 59 Å for the bilayer thickness. After subtracting this bilayer thickness, we obtained the relation log P versus fluid separation shown in Figure 6B. Again, at a constant applied pressure the distance between bilayers increased with a PEG–lipid concentration up to about 20 mol %. Note that the pressure–distance relations for PEG–lipid with DSPC:cholesterol bilayers (Figure 6B) were similar to those for PEG–

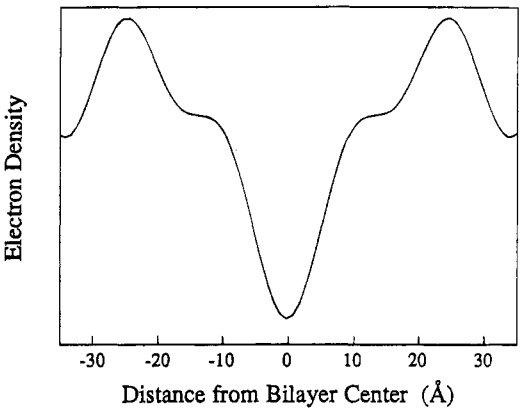

Figure 7. Electron density profile of an equimolar DSPC:cholesterol bilayer. The bilayer center is at the middle of the profile. The high-density peaks at approximately ±25 Å correspond to the phospholipid head groups, the low-density region in the center of the profile corresponds to the hydrocarbon interior of the bilayer, and the medium-density regions at the outer edges of the profile correspond to the fluid spaces between adjacent bilayers.

lipid with liquid-crystalline phase EPC (Figure 5B). Specifically, the incorporation of 10 mol % PEG-2000 increased the interbilayer distance to about 130 Å, similar to that found for DSPC (Figure 4B) and EPC bilayers (Figure 5B).

Phospholipid:Lysopholipid Bilayers. To test whether lipid polymorphism was a determining factor in the saturation of the bilayer with PEG–lipid, we perturbed the lipid matrix by incorporating lysophosphatidylcholine. Mixtures of POPE-PEG:EPC:MOPC in the following mole ratios were used: 0:50:50, 5:45:50, 8:42:50, 10:40:50, and 20:30:50. For all these samples the ratio between the number of phospholipids (POPE-PEG or EPC) and lysolipids (MOPC) was 1:1. Parts A and B of Figure 8 show plots of log P versus repeat

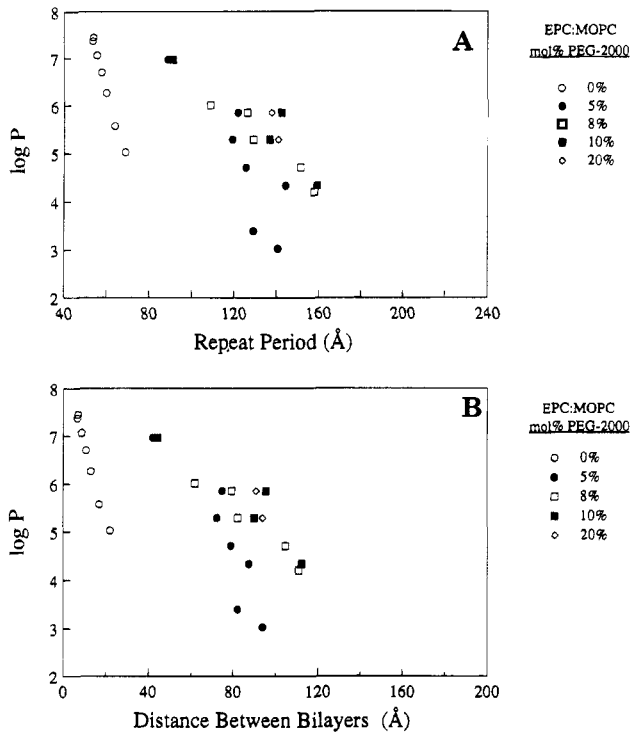

Figure 8. Plots of the logarithm of applied pressure (log P) versus (A) the lamellar repeat period and (B) the distance between bilayer surfaces for EPC:MOPC bilayers containing various concentrations of PEG–lipid. Data for EPC:MOPC bilayers containing 0 mol % PEG–lipid were taken from ref 33.

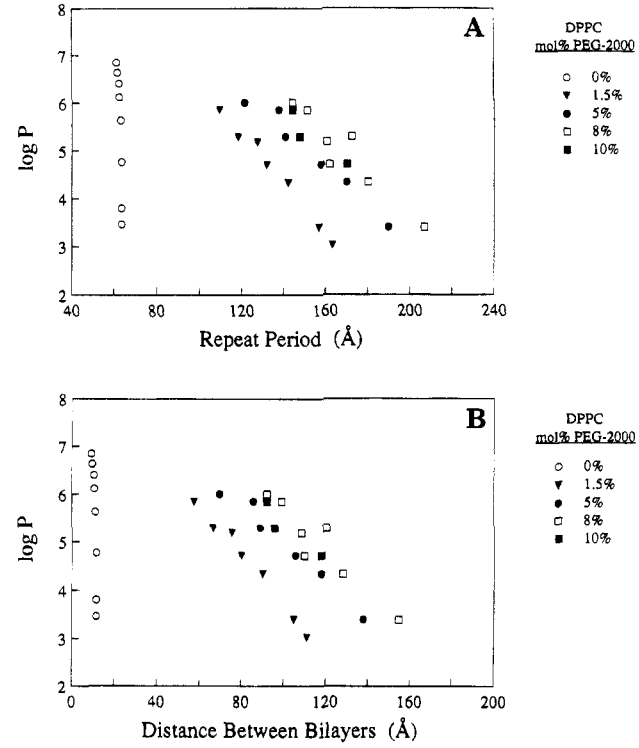

Figure 9. Plots of the logarithm of applied pressure (log P) versus (A) the lamellar repeat period and (B) the distance between bilayer surfaces for gel phase DPPC bilayers containing various concentrations of PEG–lipid. Some of the data for DPPC bilayers containing 0 mol % PEG–lipid were taken from ref 23.

period and fluid spacing, respectively, for these mixtures. The bilayer thickness of the 1:1 EPC:MOPC bilayer was shown to be 47 Å.[33] Note that the pressure–distance data were nearly the same for bilayers containing 8, 10, and 20 mol % PEG–lipid, implying that saturation of the bilayer occurred at about 8 mol % PEG–lipid. Moreover, the lamellar intensities were extremely weak for 20 mol % PEG–lipid, indicating that at this PEG–lipid concentration the sample consisted primarily of micelles. No lamellar diffraction data were recorded for concentrations greater than 20 mol % PEG–lipid. In addition, at a given pressure (for instance log P = 4), the maximum distance between bilayers was smaller for the EPC:MOPC system than for DSPC (Figure 4B), EPC (Figure 5B), or DSPC:cholesterol (Figure 6B) bilayers. Thus, taken together, these data indicate that significantly less PEG–lipid was incorporated into EPC:MOPC bilayers than was incorporated into DSPC, EPC, or DSPC:cholesterol bilayers.

Bilayers with Different Hydrocarbon Chain Lengths. If the lamellar to micellar phase transition were determined by lipid polymorphism, then one would expect that the hydrocarbon chain length would have an effect on n_{tr}. That is, a decrease in chain length should decrease n_{tr}, since the lipid molecules should pack more easily into micelles the shorter the lipid hydrocarbon chain length. To test this idea, we performed X-ray diffraction on DPPE-PEG/DPPC multilamellar suspensions. Parts A and B of Figure 9 show plots of log P versus the lamellar repeat period and the distance between bilayers for DPPE-PEG/DPPC bilayers. A bilayer thickness of 52 Å was used.[23] The pressure–distance data were similar for bilayers containing 8 and 10 mol % PEG–lipid, implying that saturation of the bilayer occurred at about 8 mol % PEG–lipid. Moreover, the lamellar intensities were extremely weak for 10 mol % PEG–lipid, indicating that at this PEG–lipid concentration the sample consisted primarily of micelles. These data indicate that smaller amounts of PEG–lipid were incorporated into DPPC bilayers (Figure 9B) than into DSPC bilayers (Figure 4B), indicating that decreasing the hydrocarbon chain length decreased n_{tr}.

5. Discussion

As noted above, the theoretical treatment of Hristova and Needham[11] has two schemes for the saturation limit of PEG–lipids in bilayers, the first depending on the tensile strength of the bilayer and the second depending on the polymorphic properties of the lipid molecules.

With regard to the first scheme, the X-ray diffraction data presented in this paper indicate that approximately the same amount of PEG–lipid can be incorporated into gel (Figure 4B), liquid-crystalline (Figure 5B), and bilayers containing equimolar cholesterol (Figure 6B), even though the tensile strengths of gel bilayers and cholesterol-containing bilayers are significantly higher than the tensile strength of liquid-crystalline phase bilayers.[34] Specifically, as detailed above, the first scheme predicts that twice as much PEG–lipid can be incorporated into DSPC:cholesterol bilayers as into EPC bilayers. Thus, the experimental evidence does not favor this first scheme. Yet the theoretical treatment of the thermodynamics of the system shows[11] that if the transition were determined by lipid polymorphism, then the saturation limit should be the same.

In contrast, the amount of PEG–lipid in the bilayer is measurably reduced for bilayers containing lysophosphatidylcholine (Figure 8B) or for gel bilayers composed of shorter chained lipids (Figure 9B). These data are

consistent with the second scheme, since both the incorporation of lysolipids and the reduction of the hydrocarbon chain length should increase the tendency to form micelles. Moreover, the smaller amount of PEG–lipid in DPPC as compared to DSPC bilayers is not predicted by the first scheme, since the tensile strengths would be expected to be similar for these gel phase bilayers.

Taken together, the data presented in this paper indicate that the polymorphic behavior of the PEG–lipid and phospholipid molecules is the primary factor that determines the maximum amount of PEG–lipid that can be incorporated into lipid bilayers. We argue that the shape of the PEG–lipid molecule is the major reason that these molecules can convert phospholipid bilayers to micelles. PEG–lipids, with attached PEGs greater than 750 Da, are cone-shaped and form micelles in excess water.[10] In these regards these PEG–lipids are similar to detergents and lysolipids, molecules that also tend to solubilize bilayers.[35] For instance, it has been shown that the incorporation of greater than equimolar lysolecithin into lecithin bilayers destabilizes the bilayer and leads to micelle formation.[36]

From the pressure–distance data (Figures 4B, 5B, 6B, 8B, and 9B) one can evaluate the critical concentration of the phase transition, i.e. the thermodynamic crossover n_{tr}. For each set of pressure–distance curves obtained as a function of PEG–lipid concentration, n_{tr} is the lowest concentration for the which pressure–distance curves overlap. For DSPE-PEG/DSPC, POPE-PEG/EPC, and DSPE-PEG/DSPC/Ch n_{tr} is about 15–20 mol %, whereas for POPE-PEG/EPC/MOPC and DPPE-PEG/DPPC n_{tr} is about 8 mol %.

Thus the experimental data support the scenario and the basic features of the bilayer to micelle transition as predicted in ref 11. The discrepancy between the n_{tr} values of 7 mol % predicted theoretically[11] and the value of 15–20 mol % determined experimentally is probably due to the fact that some numerical parameters used in ref 11 are crudely evaluated or based on mean-field theory calculations of chain conformation in bilayers and micelles.[37,38]

The data in Figures 4B, 5B, and 6B show that, for a given concentration of PEG–lipid, the pressure–distance relations, and thus the magnitudes and ranges of the repulsive pressures, are similar for gel and liquid-crystalline bilayers and bilayers containing cholesterol. This means that the steric barrier around the liposome is similar for these different systems. These data are consistent with the model that the steric barrier around the liposome is the critical factor for the increased blood circulation time of PEG–liposomes,[4,8,9,39] since the blood circulation time is similar for gel, liquid-crystalline, and cholesterol-containing bilayers.[9] The solubilization of all of these bilayer systems by 15–20 mol % PEG–lipids explains why these high concentrations of PEG–lipids deteriorate the *in vivo* performance of the drug-carrying liposomes.[9] Finally, the similarities of the pressure–distance data from gel, liquid-crystalline, and cholesterol-containing bilayers (Figures 4B, 5B, and 6B) are also consistent with the model developed earlier[16] that shows that the repulsive steric pressure[12] should be nearly independent of the area per lipid molecule.

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References and Notes

(1) Blume, G.; Cevc, G. *Biochim. Biophys. Acta* **1990**, *1029*, 91.
(2) Klibanov, A. L.; Maruyama, K.; Torchilin, V. P.; Huang, L. *FEBS Lett.* **1990**, *268*, 235.
(3) Mori, A.; Klibanov, A. L.; Torchilin, V. P.; Huang, L. *FEBS Lett.* **1991**, *284*, 263.
(4) Needham, D.; Hristova, K.; McIntosh, T. J.; Dewhirst, M.; Wu, N.; Lasic, D. *J. Liposome Res.* **1992**, *2*, 411.
(5) Papahadjopoulos, D.; Allen, T.; Gabizon, A.; Mayhew, E.; Matthay, K.; Huang, S. K.; Lee, K.; Woodle, M. C.; Lasic, D. D.; Redemann, C.; Martin, F. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11460.
(6) Senior, J.; Delgado, C.; Fisher, D.; Tilcock, C.; Gregoriadis, G. *Biochim. Biophys. Acta* **1991**, *1062*, 77.
(7) Chonn, A.; Semple, S. C.; Cullis, P. R. *J. Biol. Chem.* **1992**, *267*, 18759.
(8) Needham, D.; McIntosh, T.; Lasic, D. *Biochim. Biophys. Acta* **1992**, *1108*, 40.
(9) Woodle, M.; Lasic, D. *Biochim. Biophys. Acta* **1992**, *1113*, 171.
(10) Kenworthy, A.; Simon, S. A.; McIntosh, T. J. *Biophys. J.* **1995**, *68*, 1903.
(11) Hristova, K.; Needham, D. *Macromolecules* **1995**, *28*, 991.
(12) Kenworthy, A.; Hristova, K.; Needham, D.; McIntosh, T. J. *Biophys. J.* **1995**, *68*, 1921.
(13) de Gennes, P. G. *Macromolecules* **1980**, *13*, 1069.
(14) Needham, D. In *Permeability and Stability of Lipid Bilayers*; Disalvo, E. A., Simon, S. A., Eds.; CRC Press, Inc.: Boca Raton, FL, 1995.
(15) Needham, D.; Nunn, R. *Biophys. J.* **1990**, *58*, 997.
(16) Hristova, K.; Needham, D. *J. Colloid Interface Sci.* **1994**, *168*, 302.
(17) Marsh, D. *Handbook of Lipid Bilayers*; CRC Press, Inc.: Boca Raton, FL, 1990.
(18) Israelachvili, J. *Intermolecular and Surface Forces*; Academic Press: San Diego, CA, 1985.
(19) Mitov, M. IV Liquid Crystal Conference Soc. Countries, Tbilisi, 1981.
(20) Parsegian, V. A.; Rand, R. P.; Fuller, N. L.; Rau, D. C. *Methods Enzymol.* **1986**, *127*, 400.
(21) Parsegian, V. A.; Rand, P. R. *Biochim. Biophys. Acta* **1989**, *988*, 351.
(22) McIntosh, T. J.; Magid, A. D.; Simon, S. A. *Biochemistry* **1987**, *26*, 7325.
(23) McIntosh, T. J.; Simon, S. A. *Biochemistry* **1986**, *25*, 4058.
(24) McIntosh, T. J.; Magid, A. D.; Simon, S. A. *Biochemistry* **1989**, *28*, 7904.
(25) Simon, S. A.; McIntosh, T. J. *Proc. Nat. Acad. Sci. U.S.A.* **1989**, *86*, 9263.
(26) Herbette, L.; Marquardt, J.; Scarpa, A.; Blasie, J. K. *Biophys. J.* **1977**, *20*, 245.
(27) Blaurock, A. E. *J. Mol. Biol.* **1971**, *56*, 35.
(28) McIntosh, T. J.; Magid, A. D.; Simon, S. A. *Biophys. J.* **1990**, *57*, 1187.
(29) McIntosh, T. J.; Magid, A. D.; Simon, S. A. *Biophys. J.* **1989**, *55*, 897.
(30) Pearson, R. H.; Pascher, I. *Nature* **1979**, *281*, 499.
(31) Hitchcock, P. B.; Mason, R.; Thomas, K. M.; Shipley, G. G. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *71*, 3036.
(32) Lesslauer, W.; Cain, J. E.; Blasie, J. K. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 1499.
(33) McIntosh, T. J.; Advani, S.; Burton, R. E.; Simon, S. A.; Zhelev, D. V.; Needham, D. *Biochemistry* **1995**, *34*, 8520.
(34) Evans, E.; Needham, D. *Faraday Discuss. Chem. Soc.* **1986**, *81*, 267.
(35) Safran, S. A.; Pincus, S. P.; Andelman, D. *Science* **1990**, *248*, 354.
(36) van Echteld, C. J. A.; de Kruijff, B.; Mandersloot, J. G.; de Gier, J. *Biochim. Biophys. Acta* **1981**, *649*, 211.
(37) Ben-Shaul, A.; Szleifer, I.; Gelbart, W. *J. Chem. Phys.* **1985**, *83*, 3612.
(38) Szleifer, I.; Ben-Shaul, A.; Gelbart, W. *J. Chem. Phys.* **1986**, *85*, 5345.
(39) Lasic, D. D.; Martin, F. J.; Gabizon, A.; Huang, S. K.; Papahadjopoulos, D. *Biochim. Biophys. Acta* **1986**, *1071*, 187.

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