Poly(ethylene glycol)−Lipid Conjugates Promote Bilayer Formation in Mixtures of Non-Bilayer-Forming Lipids†

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ABSTRACT: The influence of poly(ethylene glycol)−lipid conjugates on phospholipid polymorphism has been examined using ^{31}P-NMR and freeze−fracture electron microscopy. An equimolar mixture of dioleoylphosphatidylethanolamine (DOPE) and cholesterol adopts the hexagonal (H_{II}) phase when hydrated under physiological conditions but can be stabilized in a bilayer conformation when a variety of PEG−lipid conjugates are included in the lipid mixture. These PEG conjugates produced an increase in the bilayer to hexagonal (H_{II}) phase transition temperature and a broadening of the temperature range over which both phases coexisted. Further, the fraction of phospholipid adopting the bilayer phase increased with increasing mole fraction of PEG−lipid such that at 20 mole % DOPE−PEG_{2000} no H_{II} phase phospholipid was observed up to at least 60 °C. Increasing the size of the PEG moiety from 2000 to 5000 Da (while maintaining the PEG−lipid molar ratio constant) increased the proportion of lipid in the bilayer phase. In contrast, varying the acyl chains of the PE anchor had no effect on polymorphic behavior. PEG−lipid conjugates in which ceramide provides the hydrophobic anchor also promoted bilayer formation in DOPE:cholesterol mixtures but at somewhat higher molar ratios compared to the corresponding PEG−PE species. The slightly greater effectiveness of the PE conjugates may result from the fact that these derivatives also possess a net negative charge. Phosphorus NMR spectroscopy indicated that a proportion of the phospholipid in DOPE:cholesterol:PEG−PE mixtures experienced isotropic motional averaging with this proportion being sensitive to both temperature and PEG molecular weight. Surprisingly, little if any isotropic signal was observed when PEG−ceramide was used in place of PEG−PE. Consistent with the ^{31}P-NMR spectra, freeze−fracture electron microscopy showed the presence of small vesicles (diameter < 200 nm) and lipidic particles in DOPE:cholesterol mixtures containing PEG−PE. We conclude that the effects of PEG−lipid conjugates on DOPE:cholesterol mixtures are 2-fold. First, the complementary "inverted cone" shape of the conjugate helps to accommodate the "cone-shaped" lipids, DOPE and cholesterol, in the bilayer phase. Second, the steric hindrance caused by the PEG group inhibits close apposition of bilayers, which is a prerequisite for the bilayer to H_{II} phase transition.

INTRODUCTION

The polymorphic behavior of lipids in organized assemblies can be explained qualitatively in terms of the dynamic molecular shape concept [reviewed in Cullis et al. (1991)]. When the effective cross-sectional areas of the polar headgroup and the hydrophobic region buried within the membrane are similar, the lipids have a cylindrical shape and tend to adopt a bilayer conformation. Cone-shaped lipids which have polar headgroups that are small relative to the hydrophobic component, such as unsaturated phosphatidylethanolamines, prefer non-bilayer phases such as inverted micelles or the hexagonal (H_{II}) phase. Conversely, lipids with headgroups that are large relative to their hydrophobic domain, such as lysophospholipids, have an inverted cone shape and tend to form micelles in aqueous solution. Generally the phase preference of a mixed lipid system depends on contributions from all components to the net dynamic molecular shape. As a result a combination of cone-shaped and inverted cone-shaped lipids can adopt a bilayer conformation under conditions where neither lipid in isolation exists in this phase (Madden & Cullis, 1982).

A more formal model of polymorphic behavior is based on the intrinsic curvature hypothesis (Kirk et al., 1984; Gruner, 1985). This model explains phospholipid polymorphism in terms of two opposing forces. In considering the $L_\alpha-H_{II}$ phase transition, for example, the lipid monolayers essentially exist in a planar arrangement within the bilayer phase and as tightly rolled cylinders in the H_{II} phase. For monolayers that have an inherent tendency to curl, minimization of the elastic free energy is achieved when the radius of curvature equals the "equilibrium" or "intrinsic" curvature (R_o). Monolayer curling, however, results in an increase in hydrocarbon-packing free energy because the fatty acyl chains can no longer adopt the same mean relaxed length. In the H_{II} phase, for example, some of the hydrocarbon chains must extend to occupy voids between the lipid cylinders. Factors that decrease the intrinsic radius of curvature (R_o), such as increased volume occupied by the hydrocarbon chains upon introduction of double bonds, provide a larger contribution to the elastic free energy component and hence tend to promote H_{II} phase formation. In addition, as R_o decreases, the diameter of lipid cylinders formed in the H_{II}

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phase also decreases, reducing the constraints on hydrocarbon chain packing. Conversely, an increase in the size of the headgroup increases R_o, and promotes bilayer formation or stabilization. Further, introduction of apolar lipids that can fill the voids between inverted lipid cylinders also promotes H_{II} phase formation (Gruner, 1989; Sjoland et al., 1989).

These models of lipid polymorphism, in addition to providing insight into the structure and function of biological membranes, can be used to predict the structures adopted by complex lipid mixtures. Synthetic conjugates in which a hydrophilic polymer, such as poly(ethylene glycol) (PEG), is attached to the headgroup of a phospholipid, such as phosphatidylethanolamine (PE),[1] have previously been used to increase the blood circulation life times of liposomal drug delivery systems [reviewed in Woodle and Lasic (1992)]. This increased liposome circulation half-life has been correlated with an enhanced ability to accumulate at tumor sites *in vivo* (Papahadjopoulos et al., 1991; Allen et al., 1991). While the utility of such systems is obvious, the influence of PEG−lipid conjugates on membrane properties is still incompletely understood. In aqueous solution, PEG−lipid conjugates form micelles as a result of the packing constraints imposed by their large hydrophilic moiety (Woodle & Lasic, 1992). Consequently we predicted that such conjugates would promote bilayer formation in mixtures of non-bilayer-forming lipids, analogous to previous results showing bilayer formation by unsaturated PEs in the presence of detergents (Madden & Cullis, 1982). In this paper we have used ^{31}P-NMR and freeze−fracture electron microscopy to characterize the effects of PEG−lipid conjugates on the polymorphic behavior of DOPE:cholesterol mixtures. A companion paper (Holland et al., 1996) extends this research to examine the ability of PEG−lipid conjugates to inhibit fusion between vesicle systems. Further, this latter paper demonstrates that removal of the PEG−lipid, via spontaneous exchange to an acceptor liposome, allows recovery of vesicle fusogenic activity.

EXPERIMENTAL PROCEDURES

Materials. All phospholipids including PEG−PE conjugates were purchased from Avanti Polar Lipids, Birmingham, AL, except for 1-*O*-methyl(poly(ethoxy)-*O*-succinyl-*O*-(egg)-ceramide which was obtained from Northern Lipids Inc., Vancouver, BC, Canada. Di[1-^{14}C]palmitoylphosphatidylcholine was purchased from Du Pont, Mississuaga, Ontario, Canada. [^{3}H]DSPE−PEG$_{2000}$ was synthesized as described previously (Parr et al., 1994). Other reagents were purchased from Sigma, St. Louis, MO.

Preparation of Multilamellar Vesicles and Large Unilamellar Vesicles. Lipid components were mixed in 1−2 mL of benzene:methanol (95:5, v/v) and then lyophilized for a minimum of 5 h at a pressure of <60 mTorr using a Virtis lyophilizer equipped with a liquid N_2 trap. Multilamellar vesicles (MLVs) were prepared by hydrating the dry lipid mixtures in 150 mM NaCl, buffered with 10 mM Hepes-NaOH, pH 7.4 (Hepes-buffered saline, HBS). Mixtures were vortexed to assist hydration. To produce large unilamellar vesicles (LUVs), MLVs were first frozen in liquid nitrogen and then thawed at 30 °C with this cycle repeated five times. LUVs were then produced by extrusion of the frozen and thawed MLVs 10 times through two stacked polycarbonate filters of 100 nm pore size at 30 °C and pressures of 200−500 psi (Hope et al., 1985).

^{31}P-NMR Spectroscopy. ^{31}P-NMR spectra were obtained using a temperature-controlled Bruker MSL200 spectrometer operating at 81 MHz. Free induction decays were accumulated for 2000 transients using a 4 μs, 90° pulse, 1 s interpulse delay, a 20 kHz sweep width, and Waltz decoupling. A 50 Hz line broadening was applied to the data prior to Fourier transformation. Samples were equilibrated at the indicated temperature for 30 min prior to data accumulation. Lipid concentrations of 30−70 mM were used.

Freeze−Fracture Electron Microscopy. MLVs were prepared by hydrating a mixture of DOPE:cholesterol:DOPE−PEG$_{2000}$ (1:1:0.1 mole ratio) with HBS. A portion of the mixture was extruded as described above to produce LUVs. Glycerol was added to both MLVs and LUVs to a final concentration of 25% (v/v), and samples were rapidly frozen in liquid Freon. The samples were fractured at −110 °C and $<10^{-6}$ Torr in a Balzers BAF400 unit. Replicas were prepared by shadowing at 45° with a 2 nm layer of platinum and coating at 90° with a 20 nm layer of carbon. The replicas were cleaned by soaking in hypochlorite solution for up to 48 h and were visualized in a Jeol JEM-1200 EX electron microscope.

Size Exclusion Chromatography. LUVs composed of DOPE:cholesterol:DSPE−PEG$_{2000}$ (1:1:0.1 mole ratio) with trace amounts of [^{14}C]DPPC and [^{3}H]DSPE−PEG$_{2000}$ were chromatographed at a flow rate of approximately 0.5 mL/min on a column of Sepharose CL-4B (42 × 1.6 cm) equilibrated with HBS. The column was pretreated with 10 mg of egg PC, which had been suspended in HBS by bath sonication, to eliminate nonspecific adsorption of lipid to the column. Micelles were prepared by hydrating DSPE−PEG$_{2000}$ containing a trace amount of [^{3}H]DSPE−PEG$_{2000}$ with HBS and chromatographed as described for LUVs.

RESULTS

^{31}P-NMR Spectroscopy. ^{31}P-NMR spectroscopy has been widely used to examine lipid polymorphism and provides results that are in good agreement with X-ray crystallographic techniques (Tilcock et al., 1986a,b). Phospholipids give rise to characteristic ^{31}P-NMR spectral line shapes as a result of the different degrees of motional freedom that exist in different phases. This makes it possible to distinguish, for example, between phospholipids adopting a bilayer conformation and those in the hexagonal H_{II} phase. In this section we describe studies using ^{31}P-NMR to determine the effects of various PEG−lipid conjugates on the phase properties of non-bilayer-forming lipids. Our goals were first to determine if PEG−lipid conjugates could stabilize DOPE and cholesterol into a bilayer conformation and second to determine the effects of PEG−lipid molar ratio, PEG molecular weight, and the nature of the lipid anchor on bilayer stabilizing properties.

The effect of poly(ethylene glycol)$_{2000}$ conjugated to DOPE (DOPE−PEG$_{2000}$), on the phase preference of an equimolar mixture of DOPE and cholesterol is shown in Figure 1. In the absence of DOPE−PEG$_{2000}$ the mixture adopted the

[1] Abbreviations: PE, phosphatidylethanolamine; DMPE, dimyristoylphosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; DSPE, distearoylphosphatidylethanolamine; HBS, Hepes-buffered saline; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; PEG$_n$, 1-*O*-methylpoly(ethylene glycol) (n = average mol wt); RES, reticuloendothelial system.

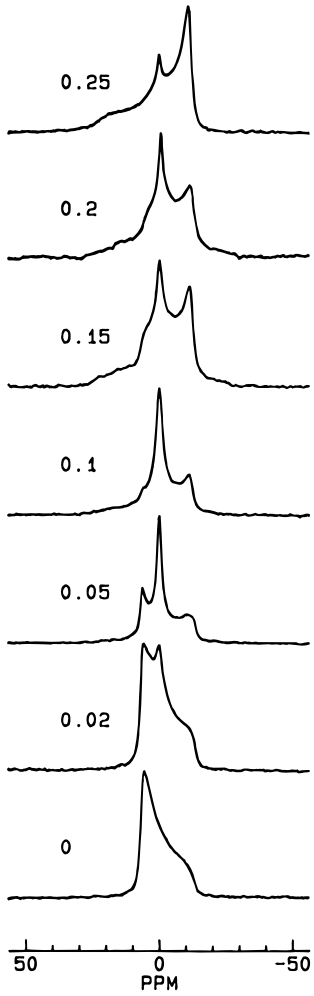

FIGURE 1: Bilayer stabilization by DOPE−PEG_{2000}: influence of molar ratio. Multilamellar vesicles were prepared as described under Experimental Procedures from mixtures of DOPE:cholesterol:DOPE−PEG_{2000} 1:1:*N*, where *N* is the proportion of DOPE−PEG_{2000} as indicated on the figure. ^{31}P-NMR spectra were obtained at 20 °C after the sample had equilibrated for 30 min.

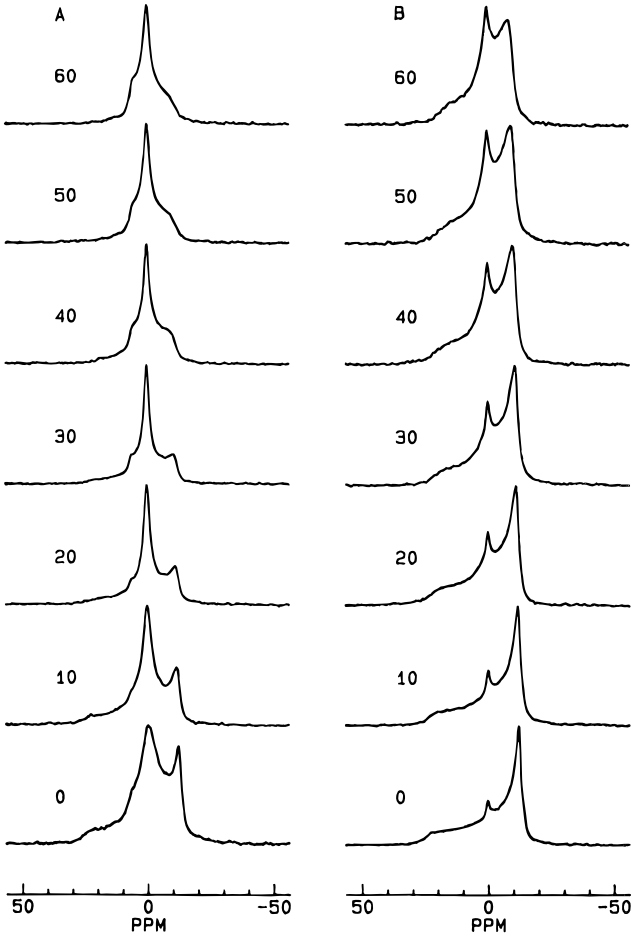

FIGURE 2: Temperature dependence of bilayer stabilization by DOPE−PEG_{2000}. Multilamellar vesicles were prepared as described under Experimental Procedures from mixtures of DOPE:cholesterol:DOPE−PEG_{2000} at a ratio of (A) 1:1:0.1 or (B) 1:1:0.25. The samples were cooled to 0 °C, and ^{31}P-NMR spectra were obtained from 0 to 60 °C at 10 °C intervals. The samples were equilibrated at each temperature for 30 min prior to data accumulation.

hexagonal (H_{II}) phase at 20 °C as determined from the characteristic ^{31}P-NMR line shape with a low-field peak and high-field shoulder (Cullis & deKruijff, 1979). As the molar ratio of DOPE−PEG_{2000} in the mixture was increased, the peak corresponding to H_{II} phase phospholipid decreased and a high-field peak with a low-field shoulder, characteristic of bilayer phase phospholipid (Cullis & deKruijff, 1979), appeared. When DOPE−PEG_{2000} was present at 20 mole % phospholipid, the mixture was almost exclusively bilayer with no evidence of H_{II} phase lipid.

In addition to the peaks corresponding to H_{II} phase and bilayer phase, a third peak indicative of isotropic motional averaging was observed in the presence of DOPE−PEG_{2000} (Figure 1). The size of the isotropic signal varied with the amount of DOPE−PEG_{2000} present and, as shown in subsequent figures, the nature of the PE−PEG species. The signal was largest at concentrations of DOPE−PEG_{2000} that allowed H_{II} and bilayer phases to coexist and diminished when either H_{II} or bilayer phase predominated. Such a signal may be produced by a number of macromolecular structures which allow isotropic motional averaging on the NMR time scale: these include the micellar, small vesicular, cubic, and rhombic phases.

The effect of temperature on the phase properties of DOPE:cholesterol:DOPE−PEG_{2000} mixtures is illustrated in Figure 2. When DOPE−PEG_{2000} was present at 9 mole % phospholipid, a large isotropic signal dominated the spectrum at all temperatures. The predominant, nonisotropic phase at 0 °C was bilayer. As the temperature was increased, however, the high-field peak diminished and a shoulder corresponding to the low-field peak of the H_{II} phase could be discerned superimposed on the isotropic component. The apparent bilayer to hexagonal phase transition occurred at 40−50 °C but was almost obscured by the large isotropic signal. DOPE alone exhibits a sharp bilayer to H_{II} transition over an interval of approximately 5 °C [see Figure 1 in Tilcock et al. (1982)]. In comparison the transition for mixtures of DOPE:cholesterol:PE−PEG_{2000} was broad with both phases present over a temperature range of almost 40 °C (see also Figure 3).

For DOPE:cholesterol:DOPE−PEG_{2000} mixtures containing 20 mole % (phospholipid) of the PEG conjugate, there was no evidence of H_{II} phase phospholipid at temperatures up to 60 °C (Figure 2). Further, at all temperatures the isotropic signal was markedly reduced compared to the same mixture containing 9 mole % (phospholipid) DOPE−PEG_{2000}.

We next examined the influence of headgroup size on the bilayer-stabilizing properties of DOPE−PEG. As illustrated in Figure 3, DOPE−PEG_{2000} at 5 mole % (phospholipid)

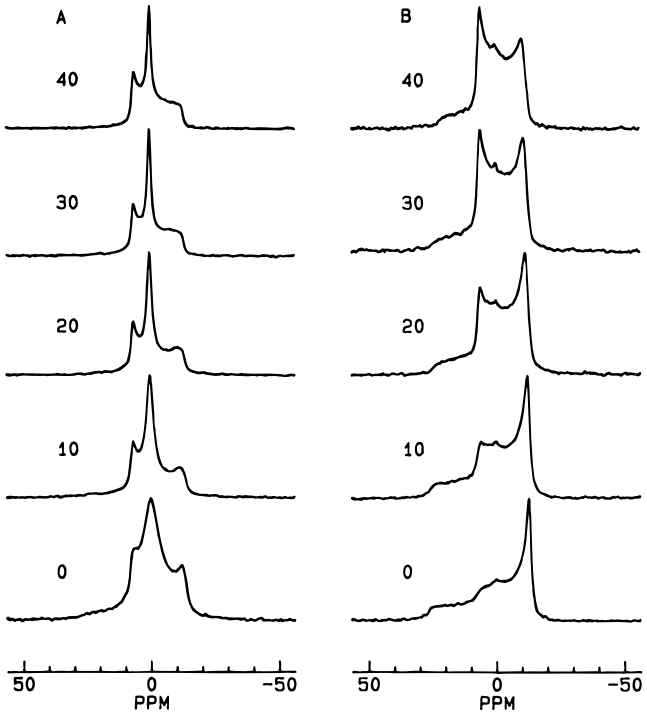

FIGURE 3: Effect of headgroup size on the bilayer stabilizing ability of DOPE−PEG. Multilamellar vesicles were prepared from either (A) DOPE:cholesterol:DOPE−PEG_{2000}, 1:1:0.05, or (B) DOPE:cholesterol:DOPE−PEG_{5000}, 1:1:0.05. Other conditions were the same as for Figure 2.

showed limited ability to stabilize DOPE:cholesterol in a bilayer organization. A broad transition from the bilayer to H_{II} phase was centered at approximately 10 °C, but a large proportion of the lipid adopted non-bilayer phases at all temperatures examined. Increasing the size of the headgroup by using poly(ethylene glycol)$_{5000}$ conjugated to DOPE (DOPE−PEG_{5000}) in place of DOPE−PEG_{2000}, at the same molar fraction, however, caused a marked increase in bilayer stability. The bilayer to H_{II} transition temperature increased to approximately 30 °C, and the isotropic signal was barely discernible. The broadening of the bilayer to H_{II} transition noted above is particularly evident here with H_{II} phase lipid present at 0 °C and bilayer phase lipid present at 40 °C.

The bilayer-stabilizing abilities of PE−PEG_{2000} conjugates differing only in acyl chain composition are shown in Figure 4. PEG_{2000} conjugated to dimyristoylphosphatidylethanolamine (DMPE−PEG_{2000}), dipalmitoylphosphatidylethanolamine (DPPE−PEG_{2000}), or distearoylphosphatidylethanolamine (DSPE−PEG_{2000}) showed a similar ability to stabilize an equimolar mixture of DOPE and cholesterol. The bilayer to H_{II} phase transition was raised to approximately 40−50 °C by the incorporation of 9 mole % PE−PEG. These results are similar to those presented in Figure 2, which were obtained using a PE−PEG_{2000} with the same headgroup but unsaturated acyl chains (DOPE−PEG_{2000}) at the same molar ratio. The size of the isotropic signal varied somewhat for the different PE−PEG_{2000} species, being smallest with DSPE−PEG_{2000} and largest with DOPE−PEG_{2000} (cf. Figures 2 and 4).

The spectra reported above were all obtained using PEG conjugated to phosphatidylethanolamine through a carbamate linkage. We also examined the use of ceramide as an alternative anchor for the hydrophilic polymer. Shown in Figure 5 are the ^{31}P-NMR spectra obtained using mixtures of DOPE:cholesterol:egg ceramide−PEG_{2000} (1:1:0.1 and 1:1:0.25 mole ratios) over the temperature range 0−60 °C. At the lower molar ratio of PEG−ceramide both bilayer and H_{II} phase lipid are evident at most temperatures. However, at the higher PEG−ceramide molar ratio the spectra are exclusively bilayer up to 60 °C, at which point a low-field shoulder corresponding to H_{II} phase lipid is visible. Unlike the spectra obtained using PE−PEGs, there was almost no isotropic signal when PEG−ceramide was used.

Freeze−Fracture Electron Microscopy. One of the interesting features in several of the ^{31}P-NMR spectra was the narrow signal at 0 ppm, indicative of isotropic motional averaging. As noted previously, this signal can arise from a number of phospholipid phases such as micellar, small vesicular, cubic, and rhombic phase structures. We therefore used freeze−fracture electron microscopy to investigate this aspect further. An electron micrograph of MLVs prepared by hydrating a mixture of DOPE:cholesterol:DOPE−PEG_{2000} (1:1:0.1) with HBS at room temperature is shown in Figure 6A. This lipid mixture corresponds to the NMR spectra

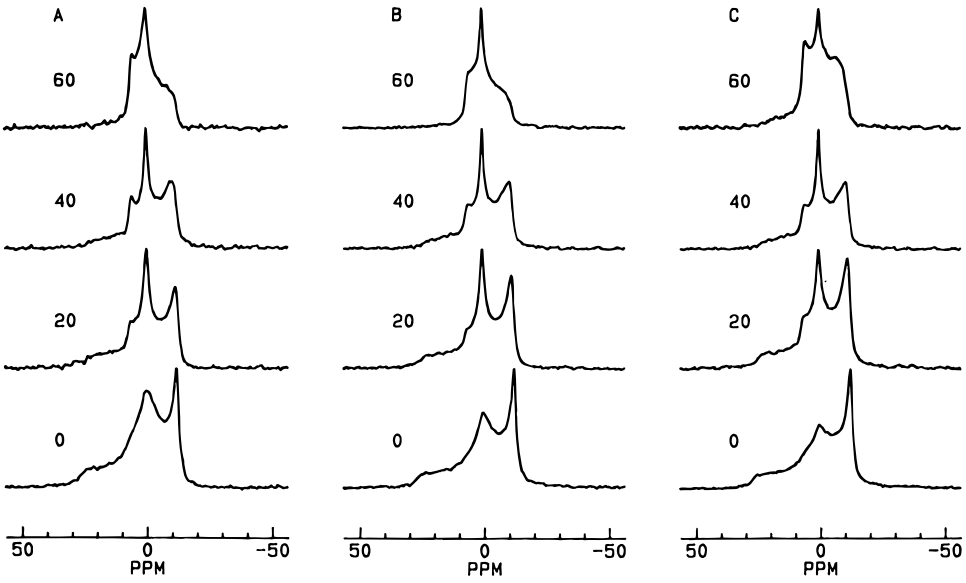

FIGURE 4: Effect of acyl chain composition on the bilayer stabilizing ability of PE−PEG_{2000}. Multilamellar vesicles were prepared as described under Experimental Procedures from (A) DOPE:cholesterol:DMPE−PEG_{2000}, 1:1:0.1; (B) DOPE:cholesterol:DPPE−PEG_{2000}, 1:1:0.1, or (C) DOPE:cholesterol:DSPE−PEG_{2000}. Other conditions were the same as for Figure 2.

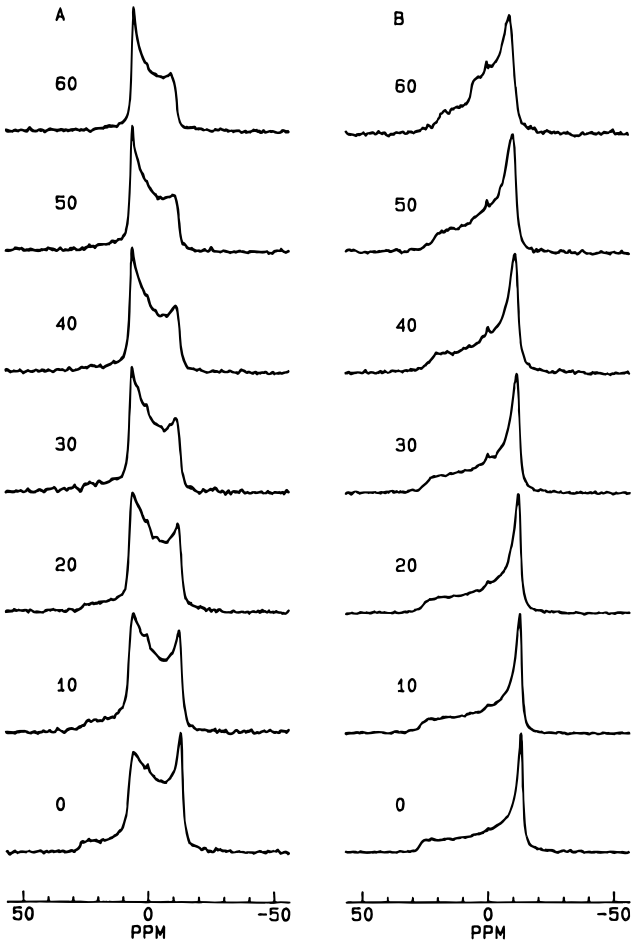

FIGURE 5: Bilayer stabilization by PEG−ceramide. Multilamellar vesicles were prepared as described under Experimental Procedures from DOPE:cholesterol:egg ceramide−PEG_{2000} at a ratio of (A) 1:1:0.1, or (B) 1:1:0.25. Other conditions were the same as for Figure 2.

shown in Figure 2A, which show evidence of bilayer, H_{II}, and isotropic phases. A number of different structures are visible in the micrograph. While much of the lipid is present as large spherical vesicles 400−600 nm in diameter, many of the vesicles exhibit indentations. In some cases these indentations appear to be randomly distributed while in other vesicles they are organized in straight or curved lines. Cusp-like protrusions are also visible on the concave surfaces of some vesicles. These features are commonly referred to as lipidic particles (Verkleij, 1984) and may represent an intermediate structure formed during fusion of bilayers. The large vesicles present within the sample would be expected to give rise to a bilayer ^{31}P-NMR spectrum with a small isotropic component arising from phospholipids within the lipidic particles. This would be consistent with earlier studies that examined the phase behavior of N-methylated phosphatidylethanolamines (Gagne et al., 1985). A number of smaller vesicles around 100−200 nm diameter can also be seen in Figure 6A. These may have formed spontaneously on hydration of the lipid mixture, or they may have been produced by vesiculization of larger structures. These vesicles are sufficiently small for lipid lateral diffusion, or tumbling of the vesicles in suspension, to produce motional averaging on the NMR time scale (Burnell et al., 1980) giving rise to an isotropic signal (see Figure 2A). To the left of center in Figure 6A is a large aggregate showing evidence of several different structures. The right side of the aggregate is characterized by what appear to be closely packed lipidic particles. The upper left hand side shows a distinct organization into three-dimensional cubic arrays, and the lower left hand region (arrowed) has the appearance of stacked tubes characteristic of lipid adopting the H_{II} phase (Hope et al., 1989). Freeze−fracture electron microscopy reveals similar structures in mixtures of DOPE:cholesterol:PEG−ceramide (1:1:0.1). In the upper section of the large aggregate shown in Figure 6B, for example, closely stacked tubes characteristic of the hexagonal H_{II} phase are readily apparent. Again within this lipid mixture lamellar structures are also present (Figure 6C). Qualitatively, therefore, the structures seen by freeze−fracture electron microscopy are consistent with the corresponding ^{31}P-NMR results.

To determine whether DOPE:cholesterol:DOPE−PEG_{2000} (1:1:0.1) could form stable large unilamellar vesicles (LUVs), an aliquot of the same sample shown in Figure 6A was extruded through 100 nm pore size polycarbonate filters as described under Experimental Procedures. Figure 6D shows a freeze−fracture electron micrograph of this extruded sample in which it can be seen that the lipid is predominantly organized into vesicles of approximately 100 nm diameter. Closer inspection reveals the presence of occasional larger vesicles and some of tubular shape. Overall the fairly uniform size distribution is typical of that obtained when LUVs are produced by extrusion. As indicated above, the isotropic signal seen in ^{31}P-NMR spectra of DOPE:cholesterol:PEG−PE mixtures could arise from phospholipids in several macromolecular structures, including micelles. Lipid micelles would not be readily identified by freeze−fracture electron microscopy and it has previously been shown that PEG−PE conjugates in isolation adopt such a micellar organization on hydration (Woodle & Lasic, 1992). We tested for the presence of micelles, therefore, by subjecting a suspension of LUVs to size exclusion chromatography on Sepharose CL-4B. The LUVs were of the same composition used for the freeze−fracture studies (Figure 6D) except that DSPE−PEG_{2000} was used in place of DOPE−PEG_{2000} and they contained trace amounts of [^{14}C]DPPC and [^{3}H]DSPE−PEG_{2000}. As shown in Figure 7, both the phospholipid and PEG−PE markers eluted in a single peak corresponding to the column void volume. In a separate control experiment, also shown in Figure 7, we demonstrated that PEG−PE micelles eluted within the included volume of the column and would have been clearly resolved if present in the liposomal preparation.

DISCUSSION

The results presented here provide a clearer understanding of the polymorphic behavior of lipid mixtures containing PEG−lipid conjugates and have important implications with respect to the development of vesicle systems that exhibit controlled fusion. These two areas will be discussed in turn.

Dioleoylphosphatidylethanolamine (DOPE) undergoes a transition from the bilayer to hexagonal (H_{II}) phase at 8−10 °C and under physiological conditions, therefore, normally exists in the H_{II} phase (Cullis & deKruijff, 1976; Tilcock et al., 1982; Epand, 1985). The addition of cholesterol to DOPE lowers this transition temperature such that only H_{II} phase lipid is observed above 0 °C. This ability of cholesterol to promote H_{II} phase formation has been demonstrated previously using mixtures of unsaturated PE with phosphatidylcholine (Tilcock et al., 1982) or phosphatidylserine (Bally et al., 1983). We show here, however, that

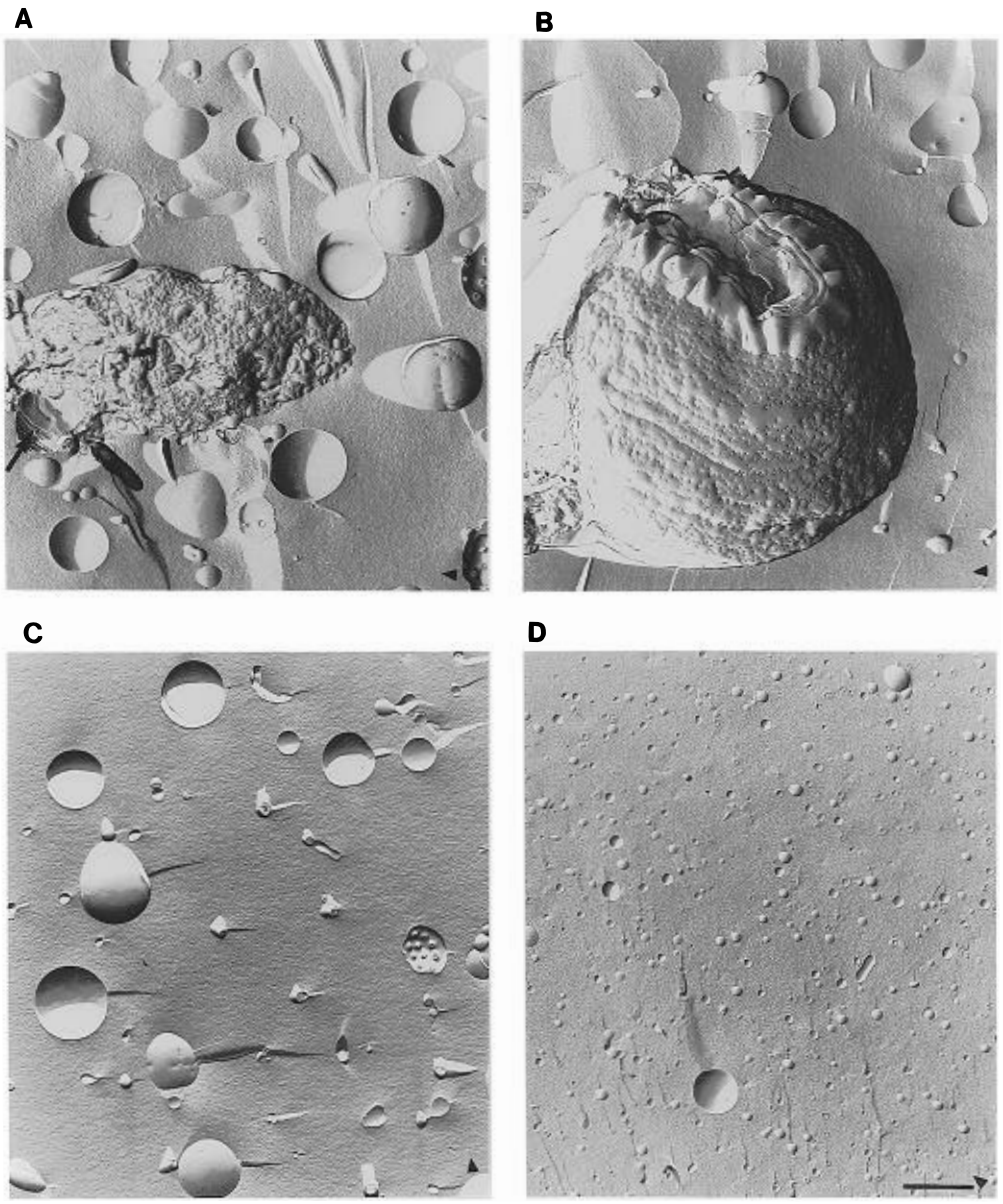

FIGURE 6: Freeze−fracture electron microscopy. (A) DOPE:cholesterol:DOPE−PEG_{2000} (1:1:0.1) was hydrated in HBS as described under Experimental Procedures. An area characteristic of stacked H_{II} phase tubes is marked by the arrow. (B, C) These two electron micrographs show representative structures in a mixture of DOPE:cholesterol:PEG−ceramide (1:1:0.1). (D) DOPE:cholesterol:DOPE−PEG_{2000} (1:1:0.1) was hydrated in HBS as for A and then extruded through 100 nm pore size polycarbonate filters as described under Experimental Procedures. The bar shown in D represents 500 nm for all micrographs, and the arrowheads indicate the direction of shadowing.

the addition of various PE−PEG species to DOPE:cholesterol results in a concentration and temperature dependent stabilization of the mixture into a bilayer conformation. This polymorphic behavior can be explained in terms of the dynamic molecular shape concept (Cullis & deKruijff, 1979; Israelachvili et al., 1980; Cullis et al., 1985) where the cone-shaped lipids, DOPE and cholesterol, which normally adopt a H_{II} structure, are stabilized in a bilayer organization by the complementary inverted cone shape of the PEG−PE. Alternatively, using the more formal concept of equilibrium radius of curvature, R_o (Gruner, 1985), the increase in headgroup area of a small fraction of the lipid increases R_o for the lipid mixture. The corresponding increase in hydrocarbon packing energy as the lipid monolayers attempt to curl to express their intrinsic curvature counteracts the decrease in elastic free energy that would result. Consequently the bilayer to H_{II} phase transition is shifted to higher temperatures.

In addition to increasing the bilayer to H_{II} transition temperature, the presence of PEG−lipid conjugates considerably broadens the transition compared to that seen for DOPE alone. The mechanism by which lipids reorganize from bilayer to H_{II} phase is uncertain, but theoretical and experimental evidence suggests that close apposition of the bilayers followed by membrane contact is necessary (Ellens et al., 1986a,b; Siegel, 1986; Allen et al., 1990). The inhibition and/or broadening of the bilayer to H_{II} transition produced by PEG−lipid conjugates probably results from steric hindrance to bilayer apposition. Measurements of interbilayer repulsion for multilamellar vesicles composed of SOPC:cholesterol (2:1) with 4 mole % DSPE−PEG_{1900} indicate that the PEG moiety extends approximately 5 nm from the bilayer surface and causes a repulsive pressure opposing the close approach of two membranes (Needham et al., 1992). Thus the stabilizing effect of the PEG−lipid can be seen to be 2-fold; not only does its shape help to

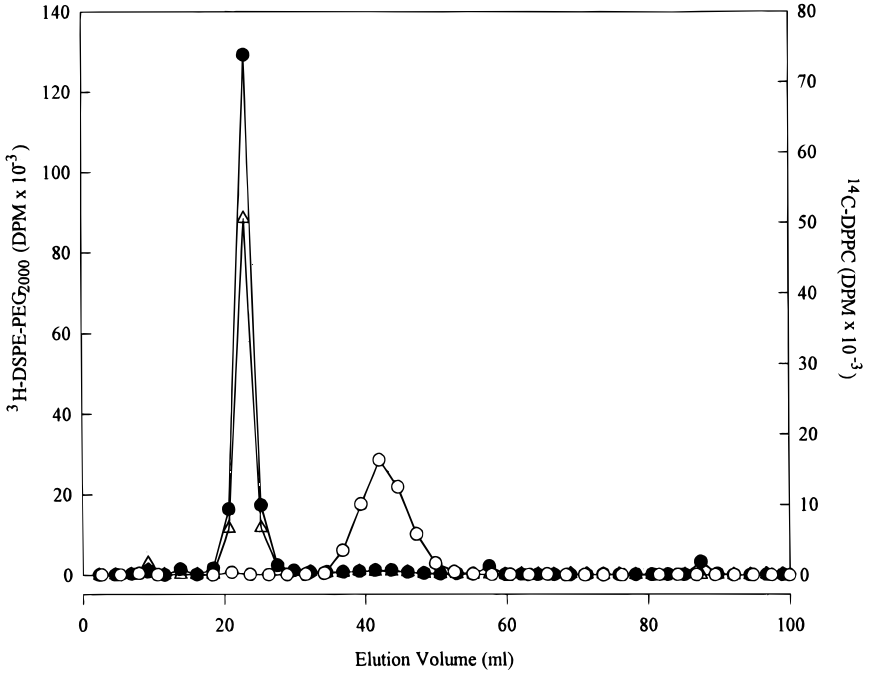

FIGURE 7: Gel exclusion chromatography of LUVs prepared from DOPE:cholesterol:DSPE−PEG$_{2000}$ and micelles composed of DSPE−PEG$_{2000}$. LUVs were prepared as described under Experimental Procedures from DOPE:cholesterol:DSPE−PEG$_{2000}$ (1:1:0.1) with trace amounts of [^{14}C]DPPC (△) and [^{3}H]DSPE−PEG$_{2000}$ (●). They were chromatographed as described under Experimental Procedures. In a separate experiment, micelles were prepared from DSPE−PEG$_{2000}$ labeled with [^{3}H]DSPE−PEG$_{2000}$ (○) and chromatographed on the same Sepharose CL-4B column.

accommodate non-bilayer-forming lipids in bilayer structures but the large hydrophilic domain acts to protect those bilayer structures from destabilization by inhibiting the approach of other lipid surfaces.

All of the PEG−lipid conjugates examined in this study showed bilayer stabilizing abilities. While the extent of this ability varied with headgroup size, the composition of the lipid anchor appeared to be of much lesser importance. Increasing the size of the PEG moiety reduced the concentration of PE−PEG required to achieve an equivalent degree of stabilization. This is expected given that the larger polymer would offer a greater steric barrier. Measurements of agglutination of liposomes containing PE−PEGs of differing polymer chain length demonstrated that inhibition of agglutination was proportional to PEG molecular weight (Mori et al., 1991). In contrast, changes in length or degree of unsaturation of the acyl chains of the PE anchor had little effect on bilayer stability, and even when the anchor was changed to a ceramide, instead of a PE, only a modest decrease in bilayer-stabilizing ability was noted (cf. Figures 2 and 5).

Interestingly the ^{31}P-NMR spectra obtained from mixtures of DOPE and cholesterol with PEG−ceramide showed little evidence of phospholipid experiencing isotropic motional averaging. In contrast, when PEG−PEs were used at the same molar ratio, a considerable portion of the phospholipid adopted structures that allowed such isotropic motion on the NMR time scale. Covalent attachment of tresyl-PEG to preformed DOPE:DOPC (3:1) MLVs has also been reported to induce a large isotropic signal in the ^{31}P-NMR spectrum (Tilcock et al., 1992). The differences in NMR spectral profile for the two PEG−lipid species could be explained if the structures giving rise to the isotropic signal contained only the PEG−lipid conjugate (or conjugate plus cholesterol). In this case no contribution to the ^{31}P-NMR spectra would arise when PEG−ceramide was used because the conjugate lacks a phosphate group. In many cases, however, the isotropic signal observed in mixtures of DOPE:cholesterol and PEG−PE is too large to be accounted for by the PEG−PE alone. In addition, evidence suggests that in mixed lipid systems where different polymorphic phases coexist near-ideal lipid mixing is maintained (Tilcock et al., 1982), especially when cholesterol is present (Tilcock et al., 1984). Freeze−fracture electron micrographs of MLVs composed of DOPE:cholesterol:PE−PEG indicate that the isotropic signal probably arises from a combination of small vesicles (diameter < 200 nm) and lipidic particles. There was no evidence for PE−PEG micelles which might also have contributed to the isotropic signal. This is important because a previous report had indicated that PEG−DSPE micelles are formed when PEG−DSPE is present above 5−7 mol % in sphingomyelin:egg PC:cholesterol liposomes (Allen et al., 1991). Lipidic particles appear to represent interlamellar attachment sites (ILAs) which are intermediates in lamellar to inverse hexagonal phase transitions and membrane fusion (Siegel et al., 1989). Why ILAs and/or small vesicles do not appear to form readily in MLVs containing PEG−ceramide, as compared to PE−PEG, is not clear. The presence of a negative charge on the PEG−PE may be important, however PE−PEGs with a larger PEG group showed reduced isotropic signals (Figure 2B), so charge alone cannot be responsible. This phenomenon clearly warrants further investigation.

Interest in the influence of PEG−lipids on membrane properties has arisen largely as a result of recent developments in liposomal drug therapy. For many years the full therapeutic potential of liposomal delivery systems remained unrealized due to the relatively rapid removal of such carriers from the circulation by cells of the reticuloendothelial system (RES) (Gregoriadis, 1976). Uptake by the RES can be inhibited, however, by incorporation of PEG−lipid conjugates within the liposomal membrane. Such surface-coated

or "sterically stabilized" liposomes display extended lifetimes in the blood and greater accumulation at tumor sites (Klibanov et al., 1990; Blume & Cevc, 1990; Allen et al., 1991; Papahadjopoulos et al., 1991). This has been shown to enhance the effectiveness of entrapped anticancer drugs (Huang et al., 1992; Mayhew et al.,1992; Williams et al., 1993). It is of interest to note, however, that PEG−lipid conjugates display greatly enhanced rates of spontaneous exchange between liposomes compared to the nonderivatized lipid. The half-time for transfer is dependent on the size of the PEG moiety and the length and degree of saturation of the lipid fatty acyl chains and can vary from minutes to hours (Silvius & Leventis, 1993; Silvius & Zuckermann, 1993). Consequently, PEG−lipid conjugates with long, saturated acyl chains would be expected to remain liposome-associated longer *in vivo*, compared to conjugates with short or unsaturated acyl chains, and thereby provide greater enhancement of vesicle circulation half-life. This prediction was recently confirmed for liposomes composed of DSPC: cholesterol which showed a longer blood circulation time when DSPE−PEG_{2000} was incorporated into the bilayer compared to similar vesicles containing POPE−PEG_{2000} (Parr et al., 1994).

While liposomes, and particularly sterically stabilized systems, can achieve enhanced drug delivery to tumor sites, this is of little benefit if the drug remains encapsulated within the carrier and is not available to the tumor cells. In theory this latter requirement could best be achieved using vesicles that fuse with the cancer cell plasma membrane, thereby introducing the entrapped drug directly into the cell cytoplasm. The ability of PEG−lipid conjugates to both stabilize non-bilayer lipid mixtures in a bilayer organization and to undergo spontaneous transfer between membranes has exciting implications with respect to the development of such fusogenic drug delivery vehicles. As we have shown here, PEG−lipid conjugates can stabilize non-bilayer-preferring lipid mixtures in a bilayer organization and these mixtures can be extruded to form large unilamellar vesicles suitable for drug delivery applications. Following intravenous administration, loss of the PEG−lipid would occur via spontaneous exchange, causing destabilization of the vesicles and rendering them fusogenic. The rate of PEG−lipid loss would influence both the circulation half-life and the rate of onset of fusogenic activity. Further, this rate of PEG−lipid loss could be modulated by altering the length and degree of saturation of the acyl chains. In a companion paper we examine the ability of PEG−lipid conjugates to act as exchangeable regulators of liposome fusion (Holland et al., 1996).

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