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

John W. Holland, Pieter R. Cullis, and Thomas D. Madden

Department of Pharmacology and Therapeutics and Department of Biochemistry, University of British Columbia, Vancouver, BC, V6T 1Z3, Canada

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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 phosphatidyl-ethanolamines, 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\(_{II}\)
Bilayer Stabilization by PEG–Lipid Conjugates

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[1-31P]palmitoylphosphatidylcholine was purchased from Du Pont, Mississauga, Ontario, Canada. [31P]dipalmitoylphosphatidylethanolamine was prepared by hydrating the dry lipid, via spontaneous exchange to an increase the blood circulation life times of liposomal drug delivery systems [reviewed in Woodle & 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 [31P]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.

RESULTS

31P-NMR Spectroscopy. 31P-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.

Size Exclusion Chromatography. LUVs composed of DOPE:cholesterol:DSPE–PEG2000 (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.

31P-NMR Spectroscopy. 31P-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 31P-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 H2 phase. In this section we describe studies using 31P-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–PEG2000), on the phase preference of an equimolar mixture of DOPE and cholesterol is shown in Figure 1. In the absence of DOPE–PEG2000 the mixture adopted the

1 Abbreviations: PE, phosphatidylethanolamine; DMPE, dimyristoylphosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; DSPE, distearoylphosphatidylethanolamine; HBS, Heps-buffered saline; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; PEGn, 1-O-methyl(poly(ethylene glycol)) (n = average mol wt); RES, reticuloendothelial system.
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)
showed limited ability to stabilize DOPE:cholesterol in a bilayer organization. A broad transition from the bilayer to HII 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, however, caused a marked increase in bilayer stability. The bilayer to HII transition temperature increased to approximately 30 °C, and the isotropic signal was barely discernible. The broadening of the bilayer to HII transition noted above is particularly evident here with HII 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 HII 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 31P-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 HII 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 HII 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 31P-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 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.](image1)

![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.](image2)
The results presented here provide a clearer understanding of the polymorphic behavior of lipid mixtures containing PEG–PE 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.

Diöleoylphosphatidylethanolamine (DOPE) undergoes a transition from the bilayer to hexagonal \( H_\text{II} \) phase at \( 8-10 \) °C and under physiological conditions, therefore, normally exists in the \( H_\text{II} \) phase (Cullis & de Kruijff, 1976; Tilcock et al., 1982; Epand, 1985). The addition of cholesterol to DOPE lowers this transition temperature such that only \( H_\text{II} \) phase lipid is observed above 0 °C. This ability of cholesterol to promote \( H_\text{II} \) 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 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 tubular characteristic of lipid adopting the \( H_\text{II} \) 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 \( H_\text{II} \) 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 \( ^{31}\text{P}-\text{NMR} \) 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 \( ^{31}\text{P}-\text{NMR} \) 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 \( ^{4\text{C}}\text{DPPC} \) and \( ^{4\text{H}}\text{DSPE–PEG}_{2000} \). 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.

**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.
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 & de Kruijff, 1979; Israelachvili et al., 1980; Cullis et al., 1985) where the cone-shaped lipids, DOPE and cholesterol, which normally adopt a HII 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 HII phase transition is shifted to higher temperatures.

In addition to increasing the bilayer to HII 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 HII 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 HII 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–PEG1900 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 6:** Freeze–fraction 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 HII 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.
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 31P-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 31P-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 31P-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 & Cecc, 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–PEG2000 was incorporated into the bilayer compared to similar vesicles containing POPE–PEG2000 (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-prefering 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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