Poly(ethylene glycol)−Lipid Conjugates Regulate the Calcium-Induced Fusion of Liposomes Composed of Phosphatidylethanolamine and Phosphatidylserine[†]

John W. Holland,[‡,§] Cathy Hui,[§] Pieter R. Cullis,[||] and Thomas D. Madden*[,§]

Department of Pharmacology and Therapeutics and Department of Biochemistry, University of British Columbia, Vancouver, BC, V6T lZ3, Canada

Received August 22, 1995; Revised Manuscript Received December 18, 1995[⊗]

ABSTRACT: The effect of poly(ethylene glycol)−lipid (PEG−lipid) conjugates on liposomal fusion was investigated. Incorporation of PEG−lipids into large unilamellar vesicles (LUVs) composed of equimolar phosphatidylethanolamine (PE) and phosphatidylserine (PS) inhibited calcium-induced fusion. The degree of inhibition increased with increasing molar ratio of the PEG conjugate and with increasing size of the PEG moiety. Inhibition appeared to result from the steric barrier on the surface of the liposomes which opposed apposition of bilayers and interbilayer contact. In the presence of a large excess of neutral acceptor liposomes, however, fusogenic activity was restored. The rate of fusion under these conditions depended on the initial molar ratio of the PEG conjugate in the PE:PS vesicles and the length and degree of saturation of the acyl chains which composed the lipid anchor. These results are consistent with spontaneous transfer of the PEG−lipid from PE:PS LUVs to the neutral lipid sink reducing the steric barrier and allowing fusion of the PE:PS LUVs. The primary determinant of the rate of fusion was the rate of transfer of the PEG−lipid, indicating that liposomal fusion could be programmed by incorporation of appropriate PEG−lipid conjugates. Interestingly, increasing the size of the PEG group did not appear to affect the rate of fusion. The implications of these results with respect to the design of fusogenic liposomal drug delivery systems are discussed.

Theories on the mechanism(s) whereby biological membranes undergo fusion have developed largely from studies of the corresponding process in model systems such as phospholipid vesicles. Such studies have revealed a correlation between the ability of phospholipids to adopt the hexagonal H_{II} phase under defined conditions of temperature, pH, divalent cation concentration and ionic strength, and the fusogenicity of the same mixture [reviewed in Cullis et al. (1991)]. This correlation probably reflects the need to adopt, at least transiently, the highly curved non-bilayer structures proposed as intermediates in fusion (Cullis & Hope, 1978; Siegel, 1986a,b; Ellens et al., 1986a,b, 1989) rather than a direct involvement of H_{II} phase lipid *per se*.

In the preceding paper (Holland et al., 1996) it was shown that the polymorphic properties of DOPE:cholesterol (1:1) mixtures could be dramatically influenced by inclusion of poly(ethylene glycol)−lipid conjugates (PEG−lipids). The bilayer to hexagonal transition temperature (T_h) was increased and the transition occurred over a much broader temperature range than was observed in the absence of PEG−lipid. It was proposed that two factors contributed to this bilayer stabilizing effect. The first derives from the dynamic molecular shape hypothesis (Cullis et al., 1991) wherein the complementary "inverted cone" shape of the PEG conjugate would help to accommodate the "cone-shaped" DOPE and cholesterol within a bilayer structure. Second, close bilayer apposition, a prerequisite to the transition from bilayer to H_{II} phase, would be inhibited by the steric hindrance resulting from hydrophilic PEG chains on the liposome surface.

Since close apposition of bilayers is a prerequisite for both the bilayer to H_{II} phase transition and membrane fusion (Siegel, 1986a,b; Ellens et al., 1986a; Allen et al., 1990), it may be hypothesized that incorporation of PEG−lipids into large unilamellar vesicles (LUVs)[1] would inhibit their fusion. In this paper the effects of PEG−lipids on calcium-induced fusion of LUVs composed of phosphatidylethanolamine (PE) and phosphatidylserine (PS) are described. The results indicate not only that PEG−lipids inhibit fusion of PE:PS LUVs when incorporated into the bilayer but also that fusogenic activity is recovered under conditions that allow spontaneous transfer of the PEG−lipid out of the PE:PS vesicles. The rate at which fusogenic activity is recovered is shown to be controlled to a large extent by the same parameters that regulate spontaneous transfer of lipids between bilayers.

EXPERIMENTAL PROCEDURES

Materials. All phospholipids including fluorescent probes and PEG−PE conjugates were purchased from Avanti Polar Lipids, Birmingham, AL. 1-*O*-Methylpoly(ethoxy)-*O*-succinyl-*O*-(egg)ceramide was obtained from Northern Lipids,

[†] This work was supported by Inex Pharmaceuticals Corporation, Vancouver, BC, Canada.

[‡] Present address: Department of Pharmacy, University of Sydney, NSW 2006, Australia.

[§] Department of Pharmacology and Therapeutics.

[||] Department of Biochemistry.

[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1996.

[1] Abbreviations: PE, phosphatidylethanolamine; PS, phosphatidylserine; DOPE, dioleoylphosphatidylethanolamine; DMPE, dimyristoylphosphatidylethanolamine; DPPE, dipalmitoylphosphatidylethanolamine; DSPE, distearoylphosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; HBS, Hepes-buffered saline; LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; PEG_n, 1-*O*-methylpoly(ethylene glycol) (n = average mol wt).

0006-2960/96/0435-2618$12.00/0

Vancouver, BC, Canada. Other reagents were purchased from Sigma, St. Louis, MO.

Preparation of Multilamellar Vesicles and Large Unilamellar Vesicles. Lipid components were mixed in benzene:methanol (95:5, v/v) at a lipid concentration of approximately 50 mg/mL 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). To assist lipid hydration the suspension was vortexed and then frozen (liquid N_2) and thawed (30 °C) five times (Mayer et al., 1986). Large unilamellar vesicles (LUVs) were produced by extrusion of the frozen and thawed MLVs ten times through two stacked polycarbonate filters of 100 nm pore size at 30 °C and pressures of 200−500 psi (Hope et al., 1985) using an Extruder (Lipex Biomembranes, Vancouver, BC, Canada).

Vesicle Fusion Assay. Fusion between LUVs was followed by measuring lipid mixing using a modification of the fluorescence resonance energy transfer (FRET) assay of Struck et al. (1981). Large unilamellar vesicles (DOPE:POPS) were prepared containing the fluorescent lipids, *N*-(7-nitro-2-oxa-1,3-diazol-4-yl)dioleoylphosphatidylethanolamine (NBD−PE) and *N*-(lissamine rhodamine B sulfonyl)-dipalmitoylphosphatidylethanolamine (Rh−PE) at 0.5 mole % each. These fluorescent labeled LUVs (50−60 μM) and a 3-fold excess of unlabeled PE:PS LUVs were mixed in the fluorimeter at 37 °C or in sealed cuvettes in a dark water bath at 37 °C for the longer time courses. For measurements of fusion after PEG−lipid transfer, an excess of vesicles prepared from 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) was added as a sink for the PEG−lipid. Fluorescence emission intensity was measured at 517 nm (slit width 5 nm) with excitation at 465 nm (slit width 5 nm) both before and after the addition of Triton X-100 (final concentration of 0.5% or 1% when POPC vesicles were employed as a sink for PEG−lipid) using a Perkin-Elmer LS-50 spectrofluorimeter. Data are presented as either uncorrected fluorescence intensity for short-term assays (≤l h) or as percentage fusion. Light-scattering controls were performed by replacing LUVs labeled with 0.5 mole % probes with unlabeled vesicles. Maximum fusion was determined using mock fused vesicles containing 0.125 mole % of each fluorescent probe. The percentage fusion was calculated as

$$\% \text{ fusion} = \frac{\left(\frac{F_t - L_t}{F_T - L_T}\right) - \left(\frac{F_o - L_o}{F_T - L_T}\right)}{\left(\frac{M_t - L_t}{M_T - L_T}\right) - \left(\frac{F_o - L_o}{F_T - L_T}\right)} \times 100$$

where F_t = fluorescence intensity at time t; F_o = fluorescence intensity at zero time; F_T = fluorescence intensity in the presence of Triton X-100. M and L represent the same measurements for the mock fused control and the light-scattering control respectively. Changes in fluorescence of the mock fused control indicated that exchange of the fluorescent probes over 24 h accounted for 10% of the fluorescence change observed but was negligible over the first hour.

Other Procedures. Phospholipid concentrations were determined by assaying for phosphate using the method of Fiske and Subbarow (1925). Liposome size distributions were determined by quasielastic light scattering (QELS) using a Nicomp 370 particle sizer (Madden et al., 1988).

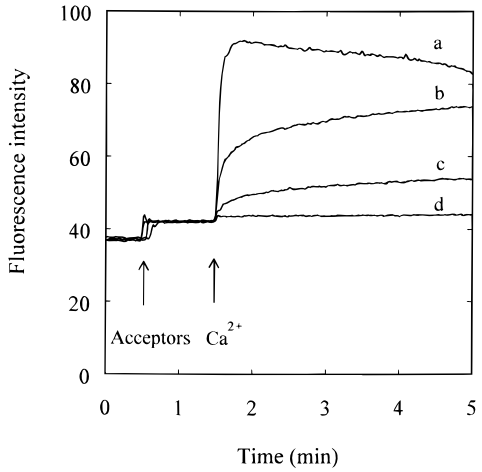

FIGURE 1: Inhibition of PE:PS vesicle fusion by DMPE−PEG_{2000}. Liposomes were prepared from equimolar mixtures of DOPE and POPS containing (a) 0, (b) 0.5, (c) 1, or (d) 2 mole % DMPE−PEG_{2000}. In addition to the above lipids, labeled liposomes also contained the fluorescent lipids NBD−PE and Rh−PE at 0.5 mole % each. Fluorescent labeled liposomes (final concentration 60 μM) were incubated at 37 °C for 30 s before the addition of a 3-fold excess of unlabeled liposomes followed l min later by $CaCl_2$ (final concentration 5 mM).

RESULTS

Effect of PE−PEG_{2000} on Fusion of PE:PS LUVs. The fusion assay developed by Struck et al. (1981) relies on resonance energy transfer between the fluorescent lipids, NBD−PE and Rh−PE. The efficiency of this energy transfer is very sensitive to the relative membrane concentrations of the two probes, and, in the case where both are present at 0.5 mole %, results in marked quenching of the emission signal from NBD−PE. If vesicles containing these molar ratios of fluorescent lipids fuse with unlabeled vesicles, however, lipid mixing will occur resulting in dilution of the fluorescent probes and recovery of the emission signal from NBD−PE. This behavior is illustrated in Figure 1. When unlabeled LUVs composed of DOPE:POPS (1:1) were added to fluorescent labeled LUVs of similar composition, there was a small jump in apparent fluorescence intensity due to increased light scattering but no fusion (Figure 1, trace a). Upon addition of 5 mM Ca^{2+}, however, a rapid increase in fluorescence intensity was observed, consistent with lipid mixing as a result of membrane fusion. Fusion was complete within a few seconds, and there followed a slow decrease in measured fluorescence. Inspection of the cuvette revealed the presence of visible aggregates that settled despite stirring, resulting in the observed decrease in fluorescence intensity. When PEG_{2000} conjugated to dimyristoylphosphatidylethanolamine (DMPE−PEG_{2000}) was included in both the labeled and unlabeled PE:PS LUVs, however, inhibition of fusion was observed. As shown in Figure 1 (traces b−d) inhibition was dependent on the molar ratio of DMPE−PEG_{2000} in the vesicles with as little as 2 mole % being sufficient to prevent Ca^{2+}-induced fusion.

Effect of PE−PEG Loss on Fusion. Phospholipids conjugated to PEGs of molecular masses 750−5000 Da can undergo spontaneous transfer between liposomes. The half-

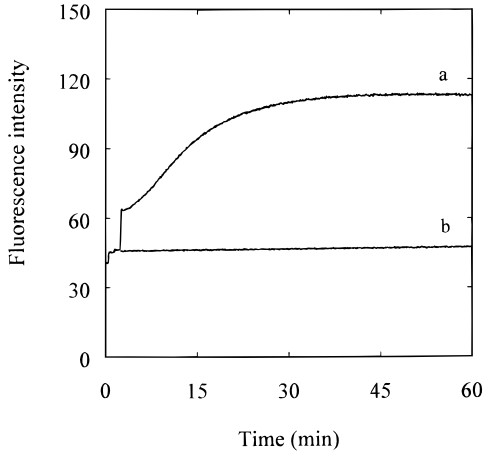

FIGURE 2: Recovery of fusogenic activity after PEG−PE removal. Fusion between fluorescent labeled and unlabeled PE:PS LUVs containing 2 mole % DMPE−PEG_{2000} was assayed as described under Figure 1 except that 1 min after addition of $CaCl_2$ a 12-fold excess (over labeled vesicles) of POPC liposomes (curve a) or an equivalent volume of buffer (curve b) was added.

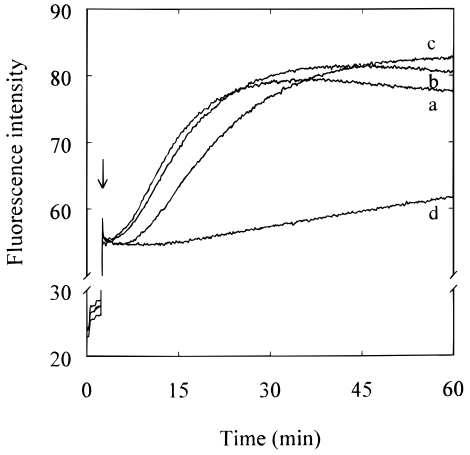

FIGURE 3: Recovery of fusogenic activity; dependence on DMPE−PEG_{2000} molar ratio. Fusion between fluorescent labeled and unlabeled PE:PS LUVs containing (a) 2, (b) 3, (c) 5, or (d) 10 mole % DMPE−PEG_{2000} was assayed as described under Figure 2 except that POPC liposomes were added as a 36-fold excess over labeled vesicles.

time for transfer of these conjugates can vary from minutes to hours and depends on both the size of the PEG group (Silvius & Zuckermann, 1993) and the nature of the acyl chains that anchor the conjugate in the bilayer (Silvius & Leventis, 1993). We therefore examined the behavior of PE:PS LUVs containing 2 mole % DMPE−PEG_{2000} under conditions where the PEG−lipid would be expected to transfer out of the liposomes. Following addition of calcium chloride (5 mM) to PE:PS liposomes containing 2 mole % DMPE−PEG_{2000}, a 12-fold excess (over fluorescent labeled vesicles) of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) liposomes were added as a sink for the PEG-PE. Full equilibration between all of the vesicles would be expected to reduce the molar ratio of DMPE−PEG_{2000} in the outer monolayer of the PE:PS vesicles to 0.5 mole %. As shown in Figure 2 (curve a), while fusion was initially blocked by the presence of DMPE−PEG_{2000}, the addition of POPC liposomes, to act as a sink, led to recovery of fusogenic activity following a short time lag. It should be noted that the apparent jump in fluorescence intensity that occurred when POPC liposomes were initially added to PE:PS vesicles (curve a) resulted from increased light scattering, not fusion. Control experiments demonstrated that no fusion occurred in the absence of POPC liposomes (Figure 2, curve b). Experiments were also undertaken to determine if fusion occurred between PE:PS vesicles and POPC liposomes. In this case, the incubation mixture contained only fluorescently labeled PE:PS vesicles and neutral acceptor liposomes; addition of calcium, therefore, would lead to recovery of fluorescence signal only as the result of fusion between PE:PS vesicles and POPC liposomes. No evidence of fusion between anionic and neutral vesicles was obtained.

To confirm that recovery of fusogenic activity was dependent on removal of PEG−PE, we examined the influence of initial PEG−lipid molar ratio on the duration of the lag phase prior to fusion (Figure 3). Liposomes containing equimolar PE and PS were prepared with 2, 3, 5, or 10 mole % DMPE−PEG_{2000}. Fluorescent labeled and unlabeled vesicles were mixed at a ratio of 1:3, and after the addition of 5 mM $CaCl_2$, a 36-fold excess (over labeled vesicles) of POPC liposomes were added. As shown in Figure 3, there is a progressive increase in the lag period prior to fusion as the molar ratio of DMPE−PEG_{2000} initially present in the PE:PS LUVs is increased from 2% to 5%. Again it should be noted that the apparent jump in fluorescence intensity seen at time zero (arrow) for all samples results from light scattering by the POPC vesicles and not fusion. In the case of vesicles prepared with 10 mole % PEG−lipid, we would anticipate that full equilibration with the POPC "acceptor" vesicles would reduce the molar ratio in the outer leaflet of the PE:PS LUVs to about l mole %. As indicated in Figure l, this should be sufficient largely to prevent fusion, and in agreement we see little increase in fluorescence intensity for this sample (Figure 3, trace d).

Effect of PEG−PE Acyl Chain Composition on Fusogenic Activity. Since fusion is dependent on migration of the PEG−PE out of the PE:PS LUVs, it may be expected that the rate at which fusogenic activity is recovered would depend on the rate at which this loss occurs. One factor that affects the rate of spontaneous lipid transfer from one membrane to another is the acyl chain length of the migrating lipid species. We therefore compared the recovery of fusogenic activity of PE:PS LUVs containing 2 mole % DMPE−PEG_{2000} with those containing 2 mole % DPPE−PEG_{2000} or 2 mole % DSPE−PEG_{2000} following addition of an acceptor vesicle sink. As can be seen from Figure 4A, increasing acyl chain length from 14 to 16 carbons caused a dramatic increase in the lag period before fusion was initiated. Although similar levels of fusion occurred using either DMPE−PEG_{2000} or DPPE−PEG_{2000}, this process was essentially complete in 40 min when DMPE−PEG_{2000} was the stabilizer but took 24 h when DPPE−PEG_{2000} was used. The implied decrease in rate of transfer (30−40-fold) is consistent with previous measurements of the effect of acyl chain length on rates of spontaneous phospholipid migration (Silvius & Leventis, 1993). Increasing the acyl chain length to 18 carbons (DSPE−PEG_{2000}, Figure 4A) extended the lag even further, and after 24 h the level of fusion was only 20% of maximum. Extended observations beyond 24 h were not performed because of potential complications arising from exchange of the fluorescent probes.

A second factor that affects the rate of spontaneous transfer of phospholipids between bilayers is the degree of unsaturation of the acyl chains. The rate of fusion of LUVs

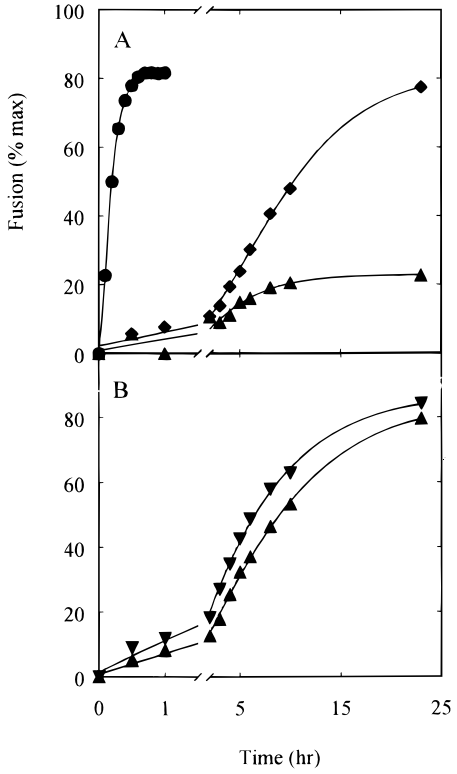

FIGURE 4: Programmable fusion. Fusion between fluorescent labeled and unlabeled PE:PS LUVs containing 2 mole % of the indicated PE−PEG_{2000} was assayed as described under Figure 2. The percentage of fusion was calculated as described under Experimental Procedures. (A) DMPE−PEG_{2000} (●); DPPE−PEG_{2000} (◆); DSPE−PEG_{2000} (▲). (B) DOPE−PEG_{2000} (▲); egg ceramide−PEG_{2000} (▼).

containing 2 mole % DOPE−PEG_{2000} is shown in Figure 4B. The presence of a double bond increased the rate of recovery of fusogenic activity in the presence of a sink over that exhibited by vesicles containing the corresponding saturated species (DSPE−PEG_{2000}, Figure 4A). The rate of fusion of PE:PS LUVs containing DOPE−PEG_{2000} was, in fact, similar to that seen for systems containing DPPE−PEG_{2000}. Figure 4B also shows the rate of fusion obtained when the neutral PEG−lipid species, egg ceramide−PEG_{2000}, was employed as the protective component. The fusion rate was somewhat faster than observed with either DPPE−PEG_{2000}- or DOPE−PEG_{2000}-containing systems. Although differences in the interaction of the two lipid anchors with neighboring phospholipids make direct comparison of interbilayer transfer rates and hence fusion difficult, the results indicate that the presence of a negative charge (as is present on the PE−PEG lipids) is not required for desorption of the conjugate. Further the protective ability of egg ceramide conjugates suggests that the hydrophilic PEG moiety is largely responsible for inhibition of Ca^{2+}-induced fusion rather than the nature of the lipid anchor.

Effect of PEG Molecular Weight on Fusogenic Activity. Given that the presence of PEG conjugated to the liposome surface results in a steric barrier that inhibits close bilayer apposition and subsequent fusion, the magnitude of this barrier should increase with increasing PEG molecular weight. We therefore prepared PE:PS (1:1) LUVs containing a longer chain PEG conjugate (DMPE−PEG_{5000}) and examined the concentration dependence of fusion inhibition (Figure 5A). The results are similar to those obtained with DMPE−PEG_{2000} except that the longer chain PEG conjugate

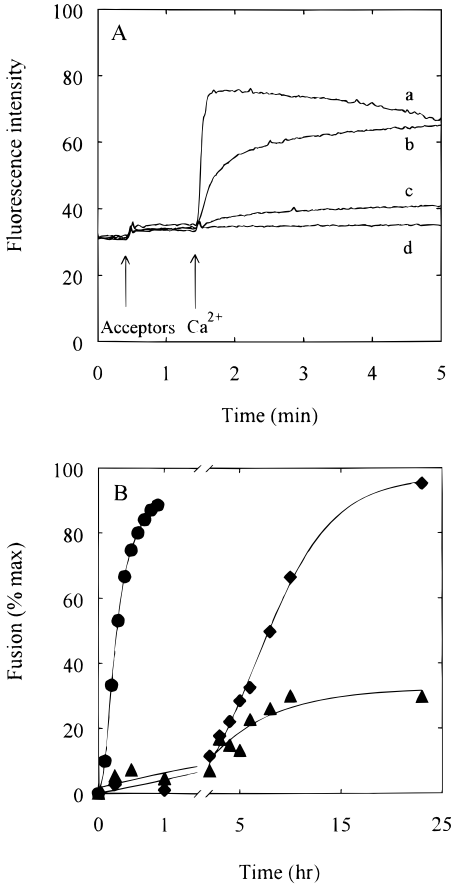

FIGURE 5: Influence of PEG molecular weight on fusion. (A) Assays were carried out as described in Figure 1. PE:PS LUVs contained (a) 0, (b) 0.25, (c) 0.5, or (d) 1 mole % DMPE−PEG_{5000}. (B) Assays were performed as described under Figure 4. PE:PS LUVs contained 1 mole % of DMPE−PEG_{5000} (●), DPPE−PEG_{5000} (◆), or DSPE−PEG_{5000} (▲).

confers protection against fusion at much lower molar ratios. For example, whereas 2 mole % DMPE−PEG_{2000} is required to completely inhibit fusion, only 1 mole % DMPE−PEG_{5000} achieves the same effect.

The influence of acyl chain composition on the rate of fusion of PE:PS LUVs in the presence of excess "acceptor" liposomes was also examined for the longer chain PEG conjugate. As observed for the PE−PEG_{2000} series, there was a marked dependence of acyl chain length on vesicle fusion rate (Figure 5B). Essentially complete fusion was observed in less than 60 min for PE:PS LUVs containing DMPE−PEG_{5000}, while vesicles containing DPPE−PEG_{5000} exhibited a similar extent of fusion only after about 24 h. In the case of PE:PS vesicles containing DSPE−PEG_{5000}, somewhat higher levels of fusion were observed at 24 h compared to systems containing DSPE−PEG_{2000} (cf. Figure 4), but even so this represented less than 30% of maximal fusion. Interestingly, the rates of fusion observed for PE:PS LUVs containing 1 mole % of the PE−PEG_{5000} series were similar to those seen for vesicles containing 2 mole % of the PE−PEG_{2000} series. The initial molar ratios of these two PE−PEG series were selected on the basis of their relative abilities to inhibit fusion (cf. Figures 1 and 5A), and it was our expectation that faster rates of fusion would be seen in systems containing the PEG_{5000} moiety as a result of faster inter-bilayer transfer of these longer chain conjugates (Silvius & Zuckermann, 1993). A comparison of Figures 4 and 5B, however, indicates that this is not the case. To

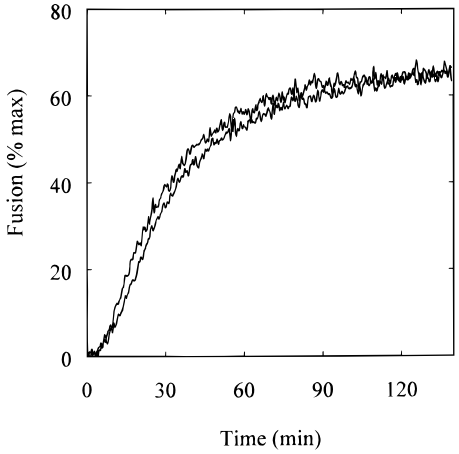

FIGURE 6: Recovery of PE:PS vesicle fusogenic activity; comparison of DMPE−PEG_{2000} and DMPE−PEG_{5000} at equal oxyethylene molar ratio. PE:PS LUVs contained either 2 mole % PEG_{5000} (upper curve) or 5 mole % PEG_{2000} (lower curve). Other conditions were as described under Figure 3.

examine this aspect further we compared fusion rates under conditions where the initial surface density of ethylene glycol groups was similar. Figure 6 shows the fusion of PE:PS (1:1) LUVs containing 5 mole % DMPE−PEG_{2000} or 2 mole % DMPE−PEG_{5000} after addition of an excess of acceptor vesicles to act as a sink for the PEG−lipid. The rates observed are very similar suggesting that DMPE−PEG_{5000} did not transfer appreciably faster than DMPE−PEG_{2000}. This observation is considered further in the Discussion.

DISCUSSION

Calcium-induced fusion of liposomes containing phosphatidylserine has been studied extensively as a model of membrane fusion [reviewed in Wilschut 1991)]. It has been proposed that Ca^{2+} acts either by neutralizing the charge repulsion between negatively charged liposomes, thereby promoting dehydration and bilayer contact through the formation of interbilayer Ca^{2+}−PS complexes (Portis et al., 1979), or by inducing formation of non-bilayer fusion intermediates (Tilcock et al., 1984). It is of interest that in mixed lipid systems containing PS, fusion can occur without lateral phase separation (Bally et al., 1983; Silvius & Gagne, 1984; Duzgunes et al., 1984; Tilcock et al., 1984).

Regardless of the structures formed during or after fusion, the initial step must involve close apposition of bilayers so that membrane contact can occur. The presence of bulky hydrophilic groups on the membrane surface, however, may be expected to interfere with this close approach. Measurements of bilayer repeat spacings in multilamellar vesicles containing DSPE−PEG_{1900}, for example, indicate that the hydrophilic polymer extends approximately 5 nm beyond the bilayer surface (Needham et al., 1992) whereas for fusion to occur the bilayers must approach to within 1−2 nm before contact can be established. Consistent with this earlier work we show that incorporation of PEG−lipid conjugates into PE:PS LUVs blocks Ca^{2+}-induced fusion, with as little as 1 mole % PE−PEG_{5000} or 2 mole % PE−PEG_{2000} being sufficient to achieve this effect. These molar ratios, which represent approximately 425 and 850 PEG molecules, respectively, on the outer surface of 100 nm vesicles, are in good agreement with recent theoretical estimates of the polymer grafting density required for steric protection of liposomes (Torchilin et al., 1994).

The use of hydrophilic polymers to provide a steric barrier has previously been applied to liposomal drug delivery systems (Papahadjopoulos et al., 1991). Following intravenous administration, the major route of liposome clearance from the blood involves uptake by free and fixed macrophages of the reticuloendothelium system (RES). It appears that recognition and accumulation of circulating liposomes by the RES are triggered by binding of plasma proteins to the vesicle surface. Several studies have shown that incorporation of PEG−lipid conjugates or monosialoganglioside G_{M1} into the liposomal membrane reduces the level of protein binding resulting in much longer blood circulation lifetimes (Senior et al., 1991; Chonn et al., 1992; Litzinger et al., 1994; Torchilin et al., 1994). Similarly, incorporation of PEG−lipid conjugates inhibits streptavidin-induced aggregation of liposomes containing biotinylated lipids (Klibanov et al., 1991; Mori et al., 1991; Litzinger et al., 1994), and liposome cross-linking during protein coupling (Harasym et al., 1995).

While polymer−lipid conjugates can enhance the blood circulation times of liposomal drug delivery systems, to derive maximum advantage from the steric barrier they provide it is clearly necessary that the conjugate remain associated with the liposome. Measurements of spontaneous transfer rates for various hydrophilic polymers conjugated to phosphatidylethanolamine indicate, however, that these rates are dramatically increased over the unconjugated phospholipid (Silvius & Zuckermann, 1993). This is consistent with results presented here, where it is shown that while PEG−lipid conjugates can prevent Ca^{2+}-induced fusion of PE:PS LUVs, fusogenic activity is restored upon addition of acceptor liposomes to which the PEG−lipid can exchange. That the recovery of fusogenic activity represents a decrease in the surface density of the polymer is supported by the observation that there is a lag period between the addition of acceptor liposomes and the onset of fusion and that the length of this delay and the subsequent rate of fusion are dependent on the initial concentration of the conjugate and the nature of the lipid anchor. Increasing the hydrophobicity of the lipid anchor by increasing acyl chain length decreases the rate at which fusion occurs and increases the lag period prior to the initiation of fusion. Conversely, introduction of a double bond has the opposite effect. The magnitude of the changes in fusion rate are comparable to reported changes in rates of spontaneous phospholipid transfer due to similar changes in acyl chain composition (Nichols, 1985; Ferrell et al., 1985; Homan & Pownall, 1988; Silvius & Leventis, 1993) and suggest that the rate of transfer of the conjugate is the primary determinant of the rate at which fusogenic activity is restored.

Increasing the size of the PEG moiety increases the effectiveness of the steric barrier and reduces the molar ratio required to completely inhibit fusion. However, for PE:PS LUVs containing either DMPE−PEG_{2000} or DMPE−PEG_{5000} there was little difference in the rates at which fusogenic activity was recovered upon addition of a sink. This is surprising given that the conjugate with the larger PEG moiety would be expected to transfer out of the vesicles more rapidly than the one with the smaller PEG group. Silvius and Zuckermann (1993), for example, reported a 1.7-fold increase in transfer rate going from DMPE−PEG_{2000} to DMPE−PEG_{5000}. It may be that the higher molar ratios of PEG−lipid used in our experiments lead to interactions between the hydrophilic chains at the vesicle surface. The

rate-limiting step in lipid exchange is believed to be desorption from the bilayer. If following this step, however, diffusion of the PEG−lipid away from the liposome surface were slowed by "tangling" or steric hindrance due to adjacent PEG conjugates, then this would be expected to increase the likelihood of bilayer reinsertion of the lipid anchor and hence reduce the net rate of PEG−lipid loss from the vesicle. Such a steric barrier to diffusion away from the liposome surface might be more pronounced in the case of the longer chain PEG moiety resulting in similar net transfer rates for DMPE−PEG_{2000} and DMPE−PEG_{5000}.

The LUVs used in these experiments were produced by incorporating the PEG−lipid conjugate into the dried lipid film prior to hydration. Consequently the conjugate would be distributed in both the inner and outer monolayers. These two pools of PEG−lipid probably remain distinct as trans-bilayer movement of the conjugate is highly unlikely. Only the pool in the outer monolayer contributes to the steric barrier that inhibits bilayer apposition and only this pool is free to transfer between bilayers. It is apparent from the results that the presence of PEG−lipid in the inner monolayer does not prevent fusion. This is not surprising in view of current models of membrane fusion [e.g. Siegel (1993)] which, in the initial stages at least, involve interaction between lipids in the cis-monolayers (i.e., the apposed outer monolayers of vesicles). It has been suggested previously, however, that modulating trans-monolayer properties may alter the rate of lipid equilibration between these two leaflets (Walter & Siegel, 1993). The extent of lipid mixing observed in the present study, however, indicates that inner monolayer lipids are also involved.

If we extrapolate the present results to the situation where PEG−lipids are used to suppress liposome uptake by the RES, then it would be predicted that the rate of loss of PEG conjugate, and hence the circulation lifetime of the liposome, would be strongly influenced by the acyl composition of the lipid anchor. In support of this contention it has been shown that liposomes containing DSPE−PEG_{2000} have longer circulation lifetimes than those with an equivalent concentration of POPE−PEG_{2000} (Parr et al., 1994).

In the preceding paper we show that PEG−lipids can stabilize non-bilayer-forming lipid mixtures within a bilayer organization (Holland et al., 1996). Taken together with the present work demonstrating that PEG conjugates can reversibly inhibit vesicle fusion, these results have important implications with respect to the development of fusogenic drug delivery vehicles. As discussed above, the incorporation of PEG conjugates can result in greatly increased liposome blood circulation times and enhanced accumulation at disease sites. Conventional liposomes, however, are designed to be mechanically resilient and resistant to leakage or fusion. As a result encapsulated drugs may not be released from the carrier even after it has been deposited at the disease site and hence would not be available to the target cells or organisms (Mayer et al., 1995). We have shown that stable vesicles can be generated from lipid mixtures that do not normally adopt a bilayer organization (DOPE:Chol or PE:PS in the presence of Ca^{2+}) by the inclusion of PEG−lipid conjugates. These systems would be suitable for drug delivery applications and would initially be stable in the circulation following intravenous administration. As with conventional liposomes, clearance by the RES would be reduced as a result of the hydrophilic polymer coating, allowing enhanced accumulation at sites of disease. Unlike conventional delivery systems, however, loss of the PEG−lipid would cause the vesicles to become unstable and would promote fusion with adjacent membranes. This would ensure that the encapsulated drug was released at the disease site or, preferably, introduced directly to the target cell or organism. Further, given that the rate of exchange of the PEG conjugate is determined by the nature of the hydrophobic anchor, it would be possible to program the rate of destabilization to ensure that the inherent fusogenic character of the vesicles was expressed only after they had exited the circulation and had achieved maximal levels at the target site(s).

In conclusion, while a number of studies have demonstrated that incorporation of hydrophilic polymers into liposomes provides a steric barrier that can minimize interaction with blood components *in vivo* and thereby extend circulation lifetimes, the results herein show that PEG−lipid conjugates can also be used to regulate membrane fusion and may be of utility in the development of programmable fusogenic liposomes for intracellular drug delivery.

REFERENCES

Allen, T. M., Hong, K., & Papahadjopoulos, D. (1990) *Biochemistry 29*, 2976−2985.

Bally, M. B., Tilcock, C. P. S., Hope, M. J., & Cullis, P. R. (1983) *Can. J. Biochem. Cell Biol. 61*, 346−352.

Chonn, A., Semple, S. C., & Cullis, P. R. (1992) *J. Biol. Chem. 267*, 18759−18765.

Cullis, P. R., & Hope, M. J. (1978) *Nature 271*, 672−674.

Cullis, P. R., Tilcock, C. P., & Hope, M. J. (1991) In *Membrane Fusion* (Wilschut, J., & Hoekstra, D., Eds) pp 35−64, Marcel Dekker, Inc., New York.

Duzgunes, N., Paiement, J., Freeman, K. B., Lopez, N. G., Wilschut, J., & Papahadjopoulos, D. (1984) *Biochemistry 23*, 3486−3494.

Ellens, H., Bentz, J., & Szoka, F. C. (1986a) *Biochemistry 25*, 285−294.

Ellens, H., Bentz, J., & Szoka, F. C. (1986b) *Biochemistry 25*, 4141−4147.

Ellens, H., Siegel, D. P., Alford, D., Yeagle, P. L., Boni, L., Lis, L. J., Quinn, P. J., & Bentz, J. (1989) *Biochemistry 28*, 3692−3703.

Ferrell, J. E., Jr., Lee, K.-J., & Huestis, W. (1985) *Biochemistry 24*, 2857−2864.

Fiske, C. M., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375−400.

Gabizon, A. (1992) *Cancer Res. 52*, 891−896.

Harasym, T. O., Tardi, P., Longman, S. A., Ansell, S. M., Bally, M. B., Cullis, P. R., & Choi, L. S. (1995) *Bioconjugate Chem. 6*, 187−194.

Holland, J. W., Cullis, P. R., & Madden, T. D. (1996) *Biochemistry 35*, 2610−2617.

Homan, R., & Pownall, H. J. (1988) *Biochim. Biophys. Acta 938*, 155−166.

Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55−65.

Klibanov, A. L., Maruyama, K., Beckerleg, A. M., Torchilin, V. P., & Huang, L. (1991) *Biochim. Biophys. Acta 1062*, 142−148.

Litzinger, D. C., Buiting, A. M. J., van Rooijen, N., & Huang, L. (1994) *Biochim. Biophys. Acta 1190*, 99−107.

Madden, T. D., Tilcock, C. P. S., Wong, K., & Cullis, P. R. (1988) *Biochemistry 27*, 8724−8730.

Mayer, L. D., Hope, M. J., Cullis, P. R., & Janoff, A. S. (1986) *Biochim. Biophys. Acta 817*, 193−196.

Mayer, L. D., Masin, D., Nayar, R., Boman, N. L., & Bally, M. B. (1995) *Br. J. Cancer 71*, 482−488.

Mori, A., Klibanov, A. L., Torchilin, V. P., & Huang, L. (1991) *FEBS Lett. 284*, 263−266.

Needham, D., McIntosh T. J., & Lasic, D. D. (1992) *Biochim. Biophys. Acta 1108*, 40−48.

Nichols, J. W. (1985) *Biochemistry 24*, 6390−6398.

Papahadjopoulos, D., Allen, T. M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S. K., Lee, K. D., Woodle, M. C., Lasic, D. D., Redemann, C., & Martin, F. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 11460−11464.
Parr, M. J., Ansell, S. M., Choi, L. S., & Cullis, P. R. (1994) *Biochim. Biophys. Acta 1195*, 21−30.
Portis, A., Newton, C., Pangborn, W., & Papahadjopoulos, D. (1979) *Biochemistry 18*, 780790.
Senior, J., Delgado, C., Fisher, D., Tilcock, C., & Gregoriadis, G. (1991) *Biochim. Biophys. Acta 1062*, 77−82.
Siegel, D. P. (1986a) *Biophys. J. 49*, 1155−1170.
Siegel, D. P. (I986b) *Biophys. J. 49*, 1171−1183.
Siegel, D. P. (1993) *Biophys. J. 65*, 2124−2140.
Silvius, J. S., & Gagne, J. (1984) *Biochemistry 23*, 3232−3240.
Silvius, J. S., & Leventis, R. (1993) *Biochemistry 32*, 13318−13326.
Silvius, J. S., & Zuckermann, M. J. (1993) *Biochemistry 32*, 3153−3161.
Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093−4099.
Tilcock, C. P. S., Bally, M. B., Farren, S. B., Cullis, P. R., & Gruner, S. M. (1984) *Biochemistry 23*, 2696−2703.
Torchilin, V. P., Omelyanenko, V. G., Papisov, M. I., Bogdanov, A. A., Jr., Trubetskoy, V. S., Herron, J. N., & Gentry, C. A. (1994) *Biochim. Biophys. Acta 1195*, 11−20.
Walter, A., & Siegel, D. P. (1993) *Biochemistry 32*, 3271−3281.
Wilschut, J. (1991) In *Membrane Fusion* (Wilschut, J., & Hoekstra, D., Eds) pp 89−126, Marcel Dekker, Inc., New York.

BI952000V