Effect of Liposomal Composition on Photoactivated Liposome Fusion†

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ABSTRACT: Bennett and O'Brien [(1995) *Biochemistry 34,* 3102] showed that the ultraviolet light exposure of two-component large unilamellar liposomes (LUV) composed of a 3:1 molar mixture of dioleoylphosphatidylethanolamine (DOPE) and 1,2-bis[10-(2′-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphatidylcholine (bis-SorbPC) facilitated liposome fusion. The rate and extent of liposome fusion was dependent on the extent of photopolymerization, the temperature, and the pH. Examination of the temperature dependence of fusion of photolyzed and unphotolyzed liposomes demonstrated that an enhancement of the rate of fusion occurred in the temperature range associated with the initial appearance of precursors to the inverted cubic (Q_{II}) phase [Barry et al. (1992) *Biochemistry 31,* 10114]. Here, the effect of the molar lipid ratio of the DOPE/bis-SorbPC liposomes on the temperature for the onset of fusion, i.e. the critical fusion temperature, was characterized by changing the relative amounts of unreactive polymorphic lipid and reactive lamellar lipid. In each case, photopolymerization of bis-SorbPC lowered the critical fusion temperature by ca. 15−20 °C. The photoreaction of the bis-SorbPC-containing LUV yields cross-linked poly-SorbPC, enhancing the lateral separation of the DOPE and the polylipid and causing isothermal induction of liposome fusion by lowering the temperature for the onset of fusion. Evidence is presented to support the hypothesis that the critical temperature for fusion of two LUV populations depends on the molar ratio of the monomeric lipids in heterodimers of the two LUV. This analysis indicates that the photopolymerization of appropriately designed LUV can decrease the critical fusion temperature from above to below 37 °C.

The delivery and buffering of therapeutic agents with liposomes currently stimulates active research in many areas. The large aqueous interiors of liposomes provide the opportunity to deliver large local concentrations of therapeutics to target cells, as long as the liposomes are properly designed to avoid nonspecific uptake by systemic cells (Lasic & Martin, 1995). The delivery of therapeutic agents to the cytoplasm of target cells would appear to require liposome−cell fusion. Cytoplasmic delivery could occur via fusion between the liposomal membrane and the endosomal membrane following endocytosis, as has been observed with pH-sensitive immunoliposomes (Wang & Huang, 1987, 1989). Alternatively, the contents of the liposomes could be released in the vicinity of the target cell and then be transported across the cellular membrane as proposed in the case of drug delivery to murine lung tumor *in vivo* (Allen, 1994).

The study of liposome−liposome fusion and liposome−cell fusion has been facilitated by the development of several fusion assays (Bentz et al., 1983). Several methods to induce the fusion of liposomes are now known: addition of multivalent ions to anionic liposomes (Bentz & Nir, 1981; Düzgunes et al., 1981; Bentz et al., 1985), incubation of liposomes containing polymorphic lipids such as phosphatidylethanolamine (PE)[1] at temperatures near T_H (Ellens et al., 1985, 1986a,b, 1989; Siegel et al., 1989), exposure of pH-sensitive liposomes to the low pH of the endosome (Connor et al., 1984; Ellens et al., 1984; Düzgunes et al., 1985; Straubinger et al., 1985; Leventis et al., 1987; Collins et al., 1989), and addition of cationic liposomes to suspensions of anionic membranes (Felgner et al., 1987; Stamatatos et al., 1988; Leventis & Silvius, 1990). Bentz et al. (1992) pointed out that the delivery of the contents of pH-sensitive liposomes to the cytoplasm following endocytosis is different from the mixing of contents between two pH-sensitive liposomes, since cytoplasmic delivery can occur by either stable fusion of the liposome with the endosomal membrane or mere destabilization of the endosomal membrane and the subsequent extensive leakage of the liposomal contents into the cytoplasm; by contrast, leakage is completely antithetical to fusion between liposomes. Cytoplasmic delivery from liposomes to cells may be approximated by the measurement of liposome−liposome interactions, despite the fact that between two liposomes leakage and fusion compete. Thus, both fusion and leakage assays should be performed since either destabilization process could lead to cytoplasmic delivery of therapeutic agents. Fusion assays may be

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[1] Abbreviations: ANTS, 1-amino-3,6,8-naphthalenetrisulfonic acid disodium salt; DAG, diacylglycerol; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPX, *N,N′-p*-xylylenebis(pyridinium bromide); EDTA, ethylenediaminetetraacetic acid tetrasodium salt; glycine buffer, 115 mM NaCl, 10 mM glycine, and 0.1 mM EDTA at pH 9.5; H_{II}, inverted hexagonal phase; LUV, large unilamellar liposomes; MLV, multilamellar liposomes; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dioleoylphosphatidylethanolamine; OD, optical density; PC, *sn*-glycero-3-phosphatidylcholine; PE, *sn*-glycero-3-phosphatidylethanolamine; Q_{II}, inverted cubic phase; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; bis-SorbPC, 1,2-bis[10-(2′,4′-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphatidylcholine; T_f, critical fusion temperature; T_H, lamellar liquid crystalline/inverted hexagonal phase transition temperature; T_l, lamellar liquid crystalline (L_α) phase to isotropic transition temperature; TLC, thin-layer chromatography.

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initiated by subjecting liposomes to low-pH conditions to mimic delivery of therapeutic agents to a cell via fusion with the endosomal membrane following endocytosis. The addition of chemical agents to facilitate liposome fusion is not necessarily suitable for *in vivo* delivery. The use of radiant energy to enhance liposome fusion avoids the need for added chemical agents and offers the further advantages of temporal and spatial control of the fusion event. It is the control of these variables that has proven so useful in photodynamic therapy. The use of light to treat disease could be enhanced if the radiant energy were used to release therapeutic agents from liposomes.

Several strategies for the design of photosensitive liposomes have been described in recent years [reviewed by O'Brien and Tirrell (1993)]: (1) photochemical isomerization of chromophores in the acyl chains (Kano et al., 1981; Pidgeon & Hunt, 1983, 1987; Song et al., 1995), (2) photocleavage of lipid chains (Kusumi et al., 1989; Anderson & Thompson, 1992), (3) photoinduced change in the association of polyelectrolytes with liposomes (You & Tirrell, 1991), and (4) photopolymerization of lipids (Frankel et al., 1989; Lamparski et al., 1992). These methods were generally designed to favor liposomal lysis. Only in the case of the photopolymerization of liposomes has evidence for liposome fusion been reported (Bennett & O'Brien, 1994, 1995). The photolysis of large unilamellar liposomes (LUV), consisting of a 3:1 molar ratio of DOPE and bis-SorbPC, induced lateral separation of reactive and nonreactive components and facilitated fusion of the LUV. The destabilization event was proposed to occur through interliposomal membrane contact following photoinduced domain formation. It was shown that the rate and extent of liposome fusion was dependent on the extent of photopolymerization, temperature, and pH. Here, we show that the composition of the liposomal bilayer, i.e. the ratio of polymerizable lipids to polymorphic lipids, has a significant effect on the threshold temperature for the fusion of the reactive liposomes.

MATERIALS AND METHODS

Materials. Dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylcholine (DOPC), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dioleoylphosphatidylethanolamine (NBD-PE), and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL) and were used without purification (one spot on TLC, 65:25:4 $CHCl_3$/MeOH/H_2O). The synthetic lipid 1,2-bis[10-(2′-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphatidylcholine (bis-SorbPC) was prepared as described previously (Lamparski et al., 1992). 1-Aminonaphthalene-3,6,8-trisulfonic acid (disodium salt) (ANTS) and *N*,*N*′-*p*-xylylenebis(pyridinium bromide) (DPX) were obtained from Molecular Probes, Inc. (Junction City, OR). Water was distilled and then purified by a MilliQ filtration system (Millipore Corp., Bedford, MA).

Liposome Preparation. Large unilamellar liposomes (LUV) were prepared by freeze/thaw and extrusion as described previously (Bennett & O'Brien, 1995). The sizes of the LUV populations were determined by quasi-elastic light scattering to be relatively monodisperse with an average diameter of 120 ± 10 nm.

Fluorescence-labeled liposomes for lipid mixing assays were prepared containing 1 mol % each of NBD-PE and Rh-PE. The fluorophores were added to weighed lipid films from an equimolar chloroform stock solution of NBD-PE and Rh-PE. The mixed lipid films were hydrated with glycine buffer (155 mM NaCl, 10 mM glycine, and 0.1 mM EDTA at pH 9.5) to a concentration of ca. 13 mM lipid before the formation of LUV by extrusion.

LUV for the ANTS/DPX fusion assays were prepared according to the method of Ellens et al. (1985, 1989) with modifications described by Bennett and O'Brien (1995). LUV contained either 25 mM ANTS or 90 mM DPX and were buffered by 10 mM glycine at pH 9.5. LUV for the ANTS/DPX leakage assays were also prepared according to established methods (Ellens et al., 1985; Bennett & O'Brien, 1995). LUV contained either 12.5 mM ANTS and 45 mM DPX, buffered with 10 mM glycine at pH 9.5, or glycine buffer at pH 9.5. Encapsulated material was separated from unencapsulated material on Sephacryl S-300 (Pharmacia) gel-filtration columns (1.6 × 20 cm) with glycine buffer as eluent. The buffers used were isoosmotic to the solutions of fluorophores, having osmolarities of 220 mosM/kg (Osmette S Osmometer, Precision Instruments).

Liposome Photolysis. LUV samples (3.0 mL) were placed 1 cm from a low-pressure mercury vapor pen lamp (predominantly 254 nm light) in a stirred 3.5 mL fluorescence quartz cuvette that was thermostated at 37 °C. A Corning CS-9-54 filter (>230 nm) was used to minimize the intensity of short-UV light incident on the sample. Photolysis times ranged from 0 to 2 min for the DOPE/bis-SorbPC sample (4:1), 0 to 3 min for the 3:1 sample, and 0 to 4 min for the 2:1 sample. The different photolysis times are a consequence of optical densities of samples of each lipid composition. The extent of polymerization was calculated as previously described by Bennett and O'Brien (1995).

Fluorescence Measurements. Fluorescence was measured with a Spex Fluorolog 2 fluorimeter (Spex Industries, Inc., Edison, NJ) that was equipped with a thermostated cuvette holder and a magnetic stirring assembly. Samples were thermally equilibrated for at least 5 min before assays were initiated. The lipid mixing studies utilized an excitation wavelength of 450 nm and an emission wavelength of 530 nm with band slits of 2 mm for excitation and 4 mm for emission. The ANTS/DPX fusion and leakage studies were performed with an excitation wavelength of 360 nm and an emission wavelength of 520 nm. The band slits were 8 mm for both excitation and emission.

Lipid Mixing Studies. Lipid mixing between different LUV populations was measured by the NBD-PE/Rh-PE assay (Struck et al., 1981) following the modifications of Düzgunes et al. (1987). The residual fluorescence at 530 nm of the labeled LUV containing 1 mol % each of NBD-PE and Rh-PE was taken as 0% fluorescence. Lipid mixing between labeled LUV and unlabeled LUV results in an increase in NBD-PE fluorescence since there is decreased energy transfer to Rh-PE as the probes are diluted from labeled LUV to unlabeled LUV upon vesicle fusion. Labeled LUV were mixed in a 1:9 molar ratio with unlabeled LUV. The value for the theoretical maximum fluorescence was established by measuring the emission from a mixture of labeled and unlabeled LUV in their respective experimental concentrations that had been subjected to five freeze/thaw cycles (dry ice/isopropyl alcohol bath, 35 °C water bath) in order to randomize the lipid in the two LUV populations (MacDonald & MacDonald, 1983). The unlabeled LUV

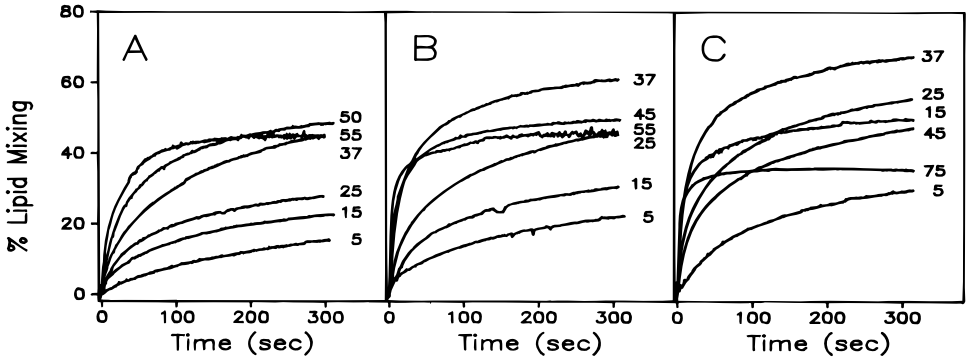

FIGURE 1: Temperature dependence of lipid mixing of 120 nm LUV composed of DOPE/bis-SorbPC (4:1) at pH 4.5 and different extents of photopolymerization. Panels A−C show the lipid mixing of unphotolyzed labeled LUV combined in a ratio of 1:9 with unlabeled LUV (total concentration of 300 μM) whose bis-SorbPC component is either (A) unphotopolymerized, (B) 50% photopolymerized, or (C) 100% photopolymerized.

stock solution was diluted to 270 μM in ca. 2.9 mL and then photolyzed at 254 nm for the requisite time. The unirradiated stock of labeled LUV was then added to produce a 1:9 molar ratio of labeled and unlabeled liposomes of 300 μM. Solutions were thermostated for 5 min before the start of each assay. Lipid mixing was initiated by addition of 75 μL of 2.0 M acetic acid/sodium acetate (pH 4.5) buffer under conditions of stirring to yield a pH of 4.5. In a similar manner, a 75 μL aliquot of 0.5 M TES and 0.2 M $MgCl_2$ at pH 7.0 was used to achieve a pH of 7.5 and a Mg^{2+} concentration of 5 mM.

Fusion and Leakage Studies. The ANTS/DPX fusion assay reports mixing of aqueous contents between liposome populations containing either ANTS or DPX by DPX quenching of ANTS fluorescence (Ellens et al., 1985, 1989). The DPX LUV stock solution was diluted to 270 μM in ca. 2.9 mL of glycine buffer and was irradiated by 254 nm light for the requisite time. Following exposure, the ANTS-containing LUV were added in a 1:9 molar ratio to make 300 μM total lipid and the mixture was thermally equilibrated for 5 min in the fluorimeter before initiation of the fusion event. The ANTS-containing LUV were not irradiated in order to prevent bleaching of the ANTS fluorophore. The fluorescence scale was calibrated using the emission intensity of a 1:9 molar mixture of ANTS and DPX-containing liposomes as 100% fluorescence (0% fusion) and the intensity of a coencapsulated population of ANTS/DPX-containing LUV as 0% fluorescence (100% fusion). The ANTS/DPX leakage assay reports relief quenching of co-encapsulated ANTS and DPX upon release from the LUV. Leakage of the aqueous contents from liposomes results in ca. 10^4-fold dilution of the probes into the surrounding medium and increased ANTS fluorescence (Ellens et al., 1985). The assay was calibrated using the initial intensity of the ANTS/DPX liposomes in the pH 9.5 glycine buffer as 0% fluorescence (0% leakage) and the intensity of the same solution following addition of Triton X-100 to a final concentration of 0.5% (w/v) as 100% fluorescence (100% leakage). The fusion and leakage assays were initiated by addition of 75 μL of either a 2.0 M acetic acid/sodium acetate (pH 4.5) buffer solution, a 0.5 M TES and 0.2 M $MgCl_2$ buffer solution (pH 7.5), or a 0.8 M $MgCl_2$ (pH 6.8) buffer solution to stirred suspensions of the liposomes in the fluorimeter.

Light Scattering. Liposome size distributions were measured using quasi-elastic light scattering (Brookhaven BI-8000AT correlator with a 5 mW He−Ne polarized laser source, Brookhaven Instruments Corp.). LUV were examined at a total lipid concentration of 100 μM at angles of 60, 90, and 120°. Two fitting methods, nonnegative least squares and CONTIN, were used to extract the set of exponential functions that made up the autocorrelation functions (Kölchens et al., 1993).

RESULTS

In earlier work, the temperature dependence of lipid mixing, liposome fusion, and aqueous contents leakage of photolyzed DOPE/bis-SorbPC (3:1) LUV was investigated (Bennett & O'Brien, 1995). Here, we report the effect of the lipid composition on the lipid mixing, liposome fusion, and aqueous contents leakage of the photosensitive LUV composed of DOPE/bis-SorbPC. The composition was varied by changing the relative amounts of the unreactive polymorphic DOPE and the photoreactive bis-SorbPC.

Lipid Mixing Studies. These studies measure lipid mixing between photolyzed and dark LUV. Only the unlabeled liposomes were irradiated to avoid photobleaching of the fluorescent probes. Labeled and unlabeled liposomes were prepared at pH 9.5 in glycine buffer where the PE component of the LUV is partially deprotonated so that the bilayers are negatively charged. Lipid mixing was initiated by combining labeled and unlabeleled LUV in a 1:9 ratio and then adding either H^+ ions, Mg^{2+} ions, or both to neutralize the initially negatively charged bilayer surfaces. Figure 1 shows the effect of temperature on the lipid mixing of DOPE/bis-SorbPC (4:1) LUV at pH 4.5 with samples that have been photopolymerized to different extents: 0% (panel A), 50% (panel B), and 100% (panel C). The mixing of lipids between LUV populations increased in both rate and extent with increasing photoconversion of bis-SorbPC to poly(bis-SorbPC) as well as with increasing temperature, except at high temperatures (ca 55 °C) where the overall extent was diminished, even though the initial rate continued to increase. Similar results were found when LUV composed of either 3:1 or 2:1 DOPE/bis-SorbPC were used (data not shown). Diminished extents of lipid mixing at high degrees of photopolymerization may be due to a decrease in the formation of precursors to the cubic phase at high extents of conversion, which could lead to overall less effective interactions between liposomes, although the initial interactions are kinetically much faster (Barry et al., 1992). The initial rates of lipid mixing derived from the data in Figure

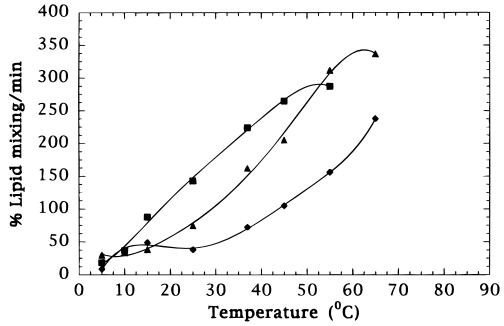

FIGURE 2: Effect of photopolymerization on the initial rates of lipid mixing of 120 nm LUV composed of DOPE/bis-SorbPC (4:1) at pH 4.5 and 300 μM as a function of temperature. The curves display the initial rates of lipid mixing extracted from the data in Figure 1. The curve at the extreme right is for (◆) unphotopolymerized NBD-PE and Rh-PE containing LUV in the presence of a 9-fold excess of unphotopolymerized LUV; the middle curve is for (▲) unphotopolymerized NBD-PE and Rh-PE containing LUV in the presence of a 9-fold excess of unlabeled LUV whose bis-SorbPC component is 50% photopolymerized, and the extreme left curve (■) is for unphotopolymerized NBD-PE/Rh-PE LUV in the presence of a 9-fold excess of unlabeled LUV whose bis-SorbPC component is 100% photopolymerized. All the initial rates are expressed in percent lipid mixing per minute.

1 are plotted as a function of temperature in Figure 2. Significant increases in the rates of lipid mixing were found in each case as the temperature was increased. Increased photoexposure of the LUV shifted the lipid mixing curves to lower temperatures. Thus, a rate of lipid mixing of 100%/min was achieved at ca. 43 °C prior to photopolymerization, at ca. 29 °C at 50% photopolymerization, and ca. 18 °C at 100% photopolymerization. The readiness of dark LUV to interact with photolyzed LUV was substantially enhanced by increased photopolymerization of their bis-SorbPC component. A difference of nearly 25 °C was observed for the dependence of temperature of the lipid mixing curves between the unexposed and the completely photopolymerized LUV. Thus, photopolymerization of bis-SorbPC provides a means to isothermally induce liposome−liposome interaction. Figure 3 shows the initial rates of lipid mixing as a function of temperature at pH 4.5 for 3:1 DOPE/bis-SorbPC (panel A) and 2:1 DOPE/bis-SorbPC (panel B). Altering the DOPE/bis-SorbPC LUV composition by including more bis-SorbPC increases the temperature at which lipid mixing occurs as expected for the more stable PC-rich LUV.

Fusion and Leakage Studies. In these experiments, only one population of liposomes was photopolymerized; therefore, the observed effects are always due to interactions between light and dark LUV. The fusion of ANTS- and DPX-containing LUV (combined 1:9) was monitored using the ANTS/DPX assay as a function of time. The LUV were prepared at pH 9.5 in glycine buffer, and LUV interaction was initiated by addition of either H^+ ions, Mg^{2+} ions, or both to neutralize the initially negatively charged bilayer surfaces of the LUV. Figure 4 shows the effect of photopolymerization on fusion and leakage of LUV composed of DOPE/bis-SorbPC (4:1) at 37 °C and two different pH conditions. Panels A and C depict fusion and leakage, respectively, of LUV due to addition of 5 mM Mg^{2+} and H^+ to pH 7.5. Panels B and D are for fusion and leakage, respectively, due to addition of H^+ to pH 4.5. Liposomes were photopolymerized to extents of 100, 50, and 0% as indicated in the figure. Note that for both initiation conditions the initial rates of both fusion and leakage increase

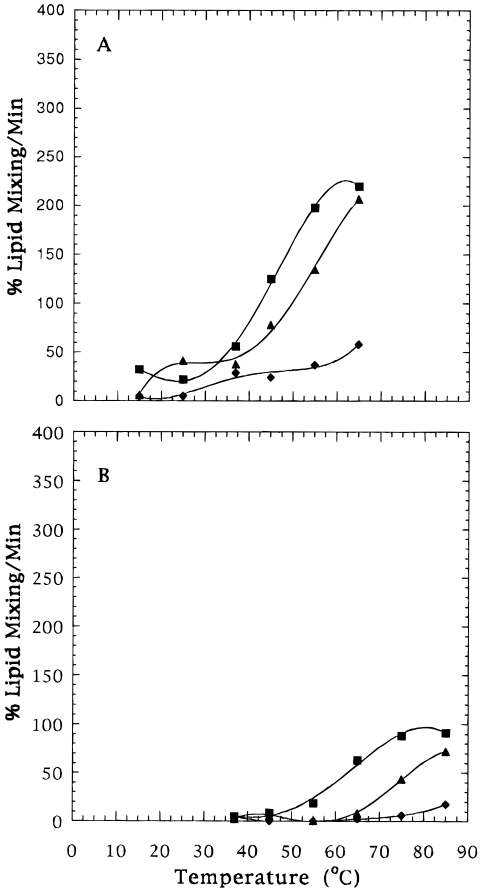

FIGURE 3: Effect of photopolymerization on the initial rates of lipid mixing of 120 nm LUV. Panel A depicts lipid mixing for LUV composed of DOPE/bis-SorbPC in a 3:1 molar ratio; panel B is for a 2:1 molar ratio at pH 4.5 and 300 μM total lipid as a function of temperature. The initial rates were determined as in Figure 2 (data not shown). The symbols are as in Figure 2.

with increasing photopolymerization. Figure 5 (panels A−C) shows the effect of temperature on the fusion of DOPE/bis-SorbPC (4:1) LUV at pH 4.5 and at different extents of polymerization: 0% (panel A), 50% (panel B), and 100% (panel C). Figure 5 (panels D−F) shows the corresponding effect of temperature on leakage of DOPE/bis-SorbPC (4:1) LUV. At temperatures below 25 °C, both the initial rates and overall extents of leakage and fusion are low. As the sample temperature was increased, the *initial rates* of both fusion and leakage increased, and the *extent* of leakage increased. Hence, the overall extent of fusion also decreased. Since leakage can occur concomitantly with fusion, increased extents of leakage can diminish the extent of fusion at higher temperatures. Photopolymerization of one population of LUV increased both the initial rates of fusion and leakage and the extent of leakage at higher temperatures. Similar trends were seen for LUV composed of either a 3:1 or 2:1 ratio of DOPE/bis-SorbPC (data not shown). In Figure 6, the initial rates of DOPE/bis-SorbPC (4:1) LUV fusion and leakage are plotted as a function of temperature. The data presented in Figure 6 were extracted from the initial slopes of the fusion and leakage curves shown in Figure 5. Bennett and O'Brien (1995) previously observed a temperature threshold for the onset of rapid LUV fusion. For convenience of comparison and discussion, the temperature threshold for rapid fusion was defined as a critical fusion temperature, T_f. The data in Figure 6 indicate the T_f is shifted to lower temperatures with increasing extents of photo-

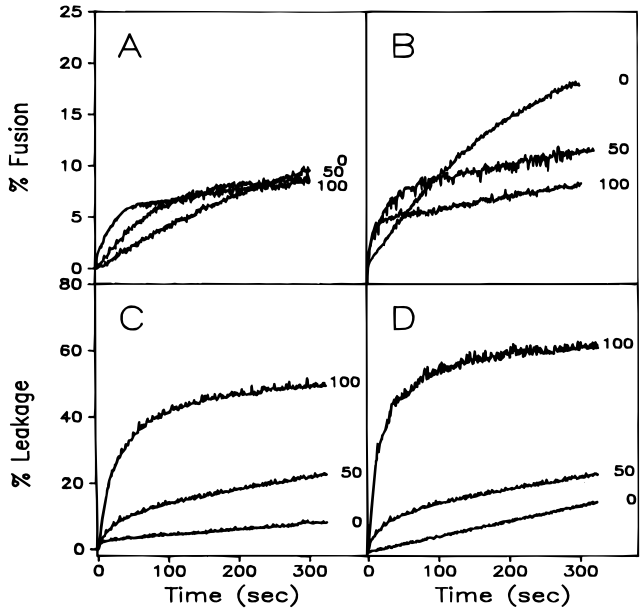

FIGURE 4: Effect of photopolymerization on fusion and leakage between 120 nm LUV composed of DOPE/bis-SorbPC (4:1) at 37 °C and different initiation conditions. Panels A and B are for fusion under the initiation conditions of (A) 5 mM Mg^{2+} and pH 7.5 and (B) pH 4.5. Panels C and D are for leakage under the conditions of (C) 5 mM Mg^{2+} and pH 7.5 and (D) pH 4.5. The LUV were polymerized to different extents, 0, 50, or 100%, as indicated in the figure.

polymerization. This is more clearly seen in the Arrhenius plot of the fusion data (Figure 7A), which shows a relatively low fusion rate at lower temperatures, in contrast to the increasing fusion rates at higher temperatures. The point of the initial increase in the fusion rate with increasing temperatures is taken to be T_f. This value for dark DOPE/bis-SorbPC (4:1) LUV was ca. 44 °C, whereas it was 25 °C following complete photopolymerization of the LUV. Similar changes in the temperature dependence of leakage behavior of the LUV were observed. Thus, photopolymerization of LUV composed of DOPE/bis-SorbPC can induce liposome fusion and release of internal contents by decreasing the critical fusion temperature by ca. 15−20 °C.

The effect of variations in the DOPE:bis-SorbPC ratio on the observed initial rate of fusion is shown in the Arrhenius plots in Figure 7. The data from dark and photolyzed LUV composed of DOPE/bis-SorbPC in ratios of 4:1 (panel A), 3:1 (panel B), and 2:1 (panel C) each exhibit a temperature region that marks the onset of increasing rates of fusion. Table 1 summarizes the measured T_f values for each of the liposome compositions studied. The value of T_f decreased as the content of the polymorphic DOPE increased relative to the monomeric bis-SorbPC by either changing the starting composition of the LUV and/or photochemically cross-linking the bis-SorbPC. Figure 7 also shows the temperature range for the maximum difference in the fusion rate between dark and photolyzed LUV. This relatively narrow temperature range occurs when the photolyzed LUV exhibit their maximum fusion rate and the dark LUV are still at a temperature below T_f. The maximum increase in the fusion rate upon photolysis is 7-fold for the 4:1 DOPE/bis-SorbPC liposomes, 20-fold for the 3:1 DOPE/bis-SorbPC liposomes, and 70-fold for the 2:1 DOPE/bis-SorbPC liposomes. Since each liposome composition attains a similar maximum initial rate of fusion, i.e. ca. 10^2 % fusion/min, the differences in the magnitude of the photoeffect on fusion are a consequence of the faster rate of dark fusion of LUV with a higher DOPE content, in particular the 4:1 DOPE/bis-SorbPC liposomes. Thus, the LUV that fuse at the lowest temperatures show the smallest photoactivated effect, whereas the more thermally stable 2:1 DOPE/bis-SorbPC liposomes display the greatest photoenhancement of liposome fusion.

The initial step in fusion of two liposomes is the formation of an aggregated dimer of liposomes. Bennett and O'Brien (1995) concluded that aggregation of 3:1 DOPE/bis-SorbPC liposomes was not rate-limiting by comparing the fusion at two different lipid concentrations, i.e. 50 and 300 μM. The interaction between a dark ANTS-containing LUV with a photolyzed DPX-containing LUV forms a heterodimer. The estimated critical fusion temperatures are obtained from the initial rates of fusion (up to 6 s) and therefore depend primarily on fusion events occurring between aggregated heterodimers. The putative initially formed fusion intermediates (stalks) are necessarily composed of a mixture of the lipids contributed by the cis monolayers of both liposomes of an aggregated dimer (Siegel, 1993). The calculated ratios of DOPE to total monomeric PC composing the stalks of fusing heterodimers for 4:1, 3:1, and 2:1 DOPE/bis-SorbPC LUV are shown in Table 1. The data indicate that the T_f for two LUV populations varies with the molar ratio of DOPE and monomeric bis-SorbPC in the aggregated dimer. In the 4:1, 3:1, and 2:1 DOPE/bis-SorbPC systems, when the DOPE to monomeric bis-SorbPC ratio is about 4:1 (0% polymerization of the 4:1 system, 50% polymerization of the 3:1 system, and 100% polymerization of the 2:1 system, respectively), the T_f value is 40−45 °C. In the 3:1 and 2:1 systems, when the DOPE to monomeric PC ratio is about 3:1 (0% polymerization of the 3:1 system or 50% polymerization of the 2:1 system, PE:PC = 2.6, respectively), the T_f value is 55−60 °C. In the 4:1 system when the PE:PC ratio is 5.3:1 (50% polymerization), the T_f value is 30 °C, whereas in the 3:1 system, when the PE:PC ratio is 6:1 (100% polymerization), the T_f value is 35 °C. Finally, in the 100% photopolymerized 4:1 system, when the PE:PC ratio is 8:1, the T_f value is 25 °C. These data are plotted in Figure 8 as the measured T_f vs the calculated molar ratio of DOPE to total monomeric lipid. This analysis assumes that monomeric lipid, rather than polylipid, is more likely to participate in the nonlamellar phase intermediates necessary to allow for fusion between two liposomes, since the lipids involved in such structures must assume high curvatures. Interestingly, when the best fit linear regression to the fusion data in Figure 8 is extrapolated to a DOPE:monomeric lipid mole ratio of 1, which corresponds to pure DOPE in the area of contact, the predicted temperature of 7 °C closely corresponds to the accepted T_H value for pure DOPE systems (8−10 °C) (Lewis et al., 1989). This temperature also marks the point of appearance of nonlamellar precusors to the inverted cubic phase, because the repetitively thermal cycling of a sample of DOPE through the transition temperature converts DOPE into an inverted cubic phase (Shyamsunder et al., 1988).

DISCUSSION

Processes that cause the phase separation of lipids, such as DOPE, from other lipids can modify the local phase behavior of the enriched domains of PE. The extent of hydration of PE is considerably less than that of PC

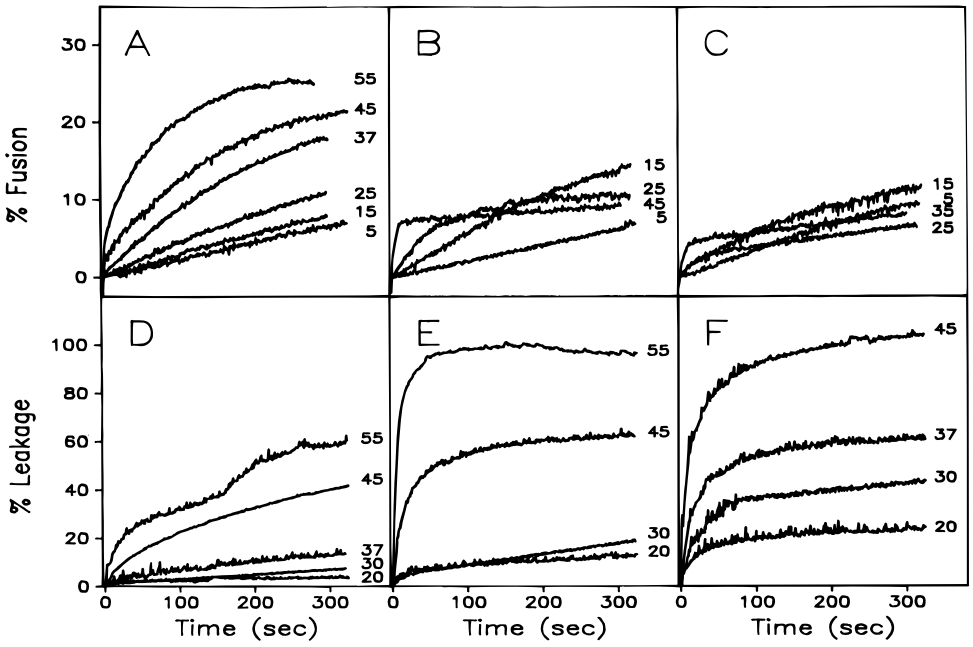

FIGURE 5: Temperature dependence of fusion and leakage of 120 nm LUV composed of DOPE/bis-SorbPC (4:1) at pH 4.5 at different extents of polymerization. Panels A−C are for the fusion of unphotopolymerized, ANTS-containing LUV combined 1:9 (total concentration of 300 μM) with DPX-containing LUV whose bis-SorbPC component is either (A) unphotopolymerized, (B) 50% polymerized, or (C) 100% polymerized. Panels D−F are for the leakage of unphotopolymerized ANTS/DPX-containing LUV combined 1:9 (total concentration of 300 μM) with empty LUV whose bis-SorbPC component is either (D) unphotopolymerized, (E) 50% polymerized, or (F) 100% polymerized.

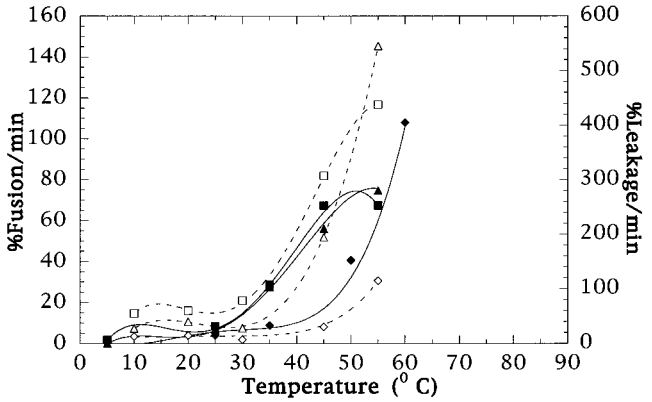

FIGURE 6: Initial rates of fusion and leakage of 120 nm LUV composed of DOPE/bis-SorbPC (4:1) at pH 4.5 and 300 μM as a function of temperature. The curves at the extreme right show the initial rate of fusion (◆) and leakage (◇) for unphotopolymerized liposomes. The middle curves show the initial rate of fusion (▲) and leakage (△) for unphotopolymerized ANTS-containing (for fusion) or ANTS/DPX-containing LUV (for leakage) in the presence of a 9-fold excess of 50% photopolymerized DPX-containing (for fusion) or empty LUV (for leakage). The extreme left curves show the initial rate of fusion (■) and leakage (□) for unphotopolymerized ANTS-containing (fusion) or ANTS/DPX-containing LUV (leakage) in the presence of a 9-fold excess of 100% photopolymerized DPX-containing LUV (fusion) or empty LUV (leakage). The rates were obtained from the initial slope of the fusion or leakage curves in Figure 5. All the initial rates are expressed in percent per minute, but for ease of comparison, fusion and leakage are presented on different scales.

(Parsegian et al., 1979). Therefore, the formation of domains of DOPE facilitates the close approach of these regions of the lipid membrane surface. Since bilayer contact is required for bilayer destabilization and liposome fusion (Ellens et al., 1984), the phase separation of DOPE enhances the probability of liposome fusion. We have previously shown that the cross-linking polymerization of bi- or multicomponent lipid membranes can effect phase separation of the unreactive lipid(s) from the growing polymeric domains [reviewed by Armitage et al. (1996)]. Thus, polymerization-induced phase separation of lipids was employed for the insertion of transmembrane proteins into partially polymerized liposomes (Tyminski et al., 1988), for the efficient photodestabilization of oligolamellar liposomes (Frankel et al., 1989; Lamparski et al., 1992), and for the enhancement of energy transfer between donor and acceptor dyes associated with the membrane surface (Armitage et al., 1993). Other laboratories have utilized lipid polymerization to create enriched lipid domains in bilayer membranes (Ringsdorf et al., 1988).

In a previous study, Bennett and O'Brien (1995) showed that the photoexposure of two-component LUV composed of a 3:1 molar mixture of DOPE and bis-SorbPC facilitated liposome fusion between the photolyzed and dark liposomes. The fusion of these LUV was demonstrated by examination of the effect of photoexposure on three processes: lipid mixing, aqueous contents mixing, and aqueous contents leakage. The rates and extents of liposome fusion were dependent on the degree of bis-SorbPC polymerization, the sample temperature, the presence of Mg^{2+}, and the pH. The photopolymerization of 3:1 DOPE/bis-SorbPC membranes reduced the critical fusion temperature by ca. 20 °C. In contrast, the effect of photopolymerization of a 3:1 DOPE/mono-SorbPC membrane was much more modest. These contrasting effects of bilayer polymerization of membranes composed in part of bis-SorbPC or mono-SorbPC were ascribed to the different effects of cross-linking and linear polymerizations, respectively. Cross-linking polymerizations of bilayers more effectively induce domain formation in the membrane (Armitage et al., 1996). Bennett and O'Brien proposed that photoreaction of the bis-SorbPC-containing liposomes resulted in the formation of cross-linked poly-SorbPC and thereby enhanced the lateral separation of the DOPE from the polylipid and lowered the temperature for the onset of liposome fusion. The polymerization could

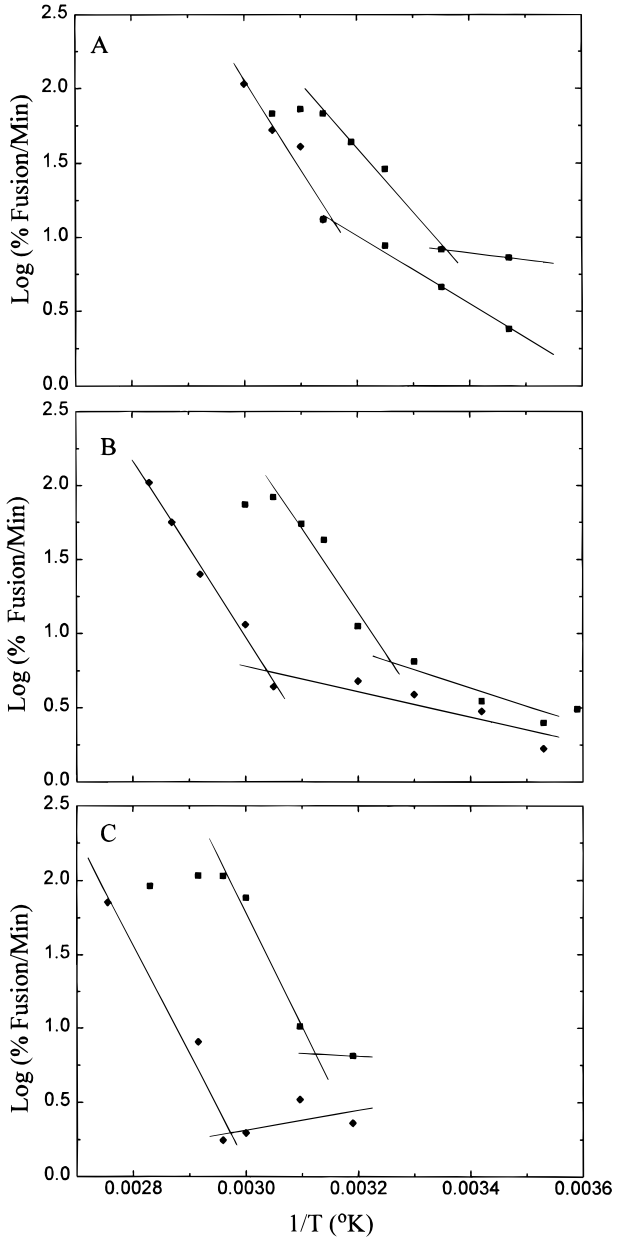

FIGURE 7: Arrhenius plots of the log of the initial rate of fusion vs temperature^{-1} for 120 nm LUV composed of DOPE/bis-SorbPC in the following initial molar ratios, 4:1 (A), 3:1 (B), and 2:1 (C), or at pH 4.5 and 300 μM lipid. The rates were obtained from the initial slope of the fusion curves for each of these LUV compositions. Data for unexposed (◆) and 100% photopolymerized (■) LUV are shown.

facilitate both liposome aggregation and the subsequent fusion steps. Greater liposome interaction and adhesion is a consequence of decreased hydration of the enriched PE domains discussed earlier. Thus, photopolymerization leads to a decreased intrinsic radius of curvature, R_o, of the monolayer composed of the PE-rich domains (Gruner, 1985, 1989). The decreased R_o is due to both the ratio of DOPE to monomeric PC and the hydration.

Partial characterization of the phase behavior of 3:1 DOPE/bis-SorbPC membranes by ^{31}P NMR and X-ray diffraction by Barry et al. (1992) showed that this lipid system exhibits only lamellar and inverted cubic (Q_{II}) phases in the relevant temperature range for the onset of liposome fusion. The inverted hexagonal (H_{II}) phase was not observed below 80 °C. In fact, the temperature for the initial appearance of an isotropic signal in the ^{31}P NMR spectra of 3:1 DOPE/bis-SorbPC membranes agrees well with the critical fusion temperature for these membranes (Bennett & O'Brien, 1995). These data provide unequivocal evidence that liposome fusion in DOPE/bis-SorbPC membranes is mediated by intermediates associated with the L_α to Q_{II} phase transition. Ellens et al. (1989) reported that liposomes composed of N-methylated DOPE or DOPE and DOPC exhibit rapid fusion and leakage in a narrow temperature range associated with the appearance of precursors to the inverted cubic phase. They proposed that both membrane fusion and the L_α to inverted cubic phase transition proceed by a common set of intermembrane intermediates. Both the observations of Bennett and O'Brien (1995) and the data described in this report are consistent with the hypothesis advanced by Ellens et al. (1989).

It is probable that the isotropic NMR signal observed in the 3:1 DOPE/bis-SorbPC membranes indicates the presence of so-called interlamellar attachments (ILA) or other precursors of the cubic phase. These precursors have been discussed by others in the context of inverted micellar intermediates (IMI) (Siegel, 1986a,b), stalks (Markin et al., 1984; Chernomordik et al., 1987; Siegel, 1993), and fusion pores or ILA (Siegel, 1986c; Siegel et al., 1988). The maximum rate of fusion observed in the photolyzed liposomes (Figure 7) was 1.5 ± 0.2% fusion/s. The calculated number of ANTS probe molecules per LUV is 10^4, based on the average size of the liposomes and the initial concentration of ANTS in the liposomes. The maximum initial fusion rate indicates that ca. 150 ANTS molecules are quenched per second. Quenching is a consequence of aqueous contents mixing between ANTS LUV and DPX LUV. Although the nature of the comixing that is necessary to achieve quenching is uncertain, it does appear that upward of 10^2 molecules/s can pass through the fusion pore connecting the LUV. The reasonableness of this value obviously depends on the dimensions of the fusion pore. Unfortunately, the pore size for DOPE/bis-SorbPC membranes is unknown at this time.

The crucial question addressed in this work is the effect of the initial lipid composition on the measured critical fusion temperatures following different extents of polymerization of one of the LUV populations. An increase in the DOPE to monomeric PC ratio will decrease the R_o and should lower the fusion temperature. Conversely, a decrease in this ratio should have the opposite effect, which is in fact what was observed in the experiments summarized in Figures 6 and 7. The polymerization of LUV composed of 4:1 or 3:1 DOPE/bis-SorbPC decreased the T_f by ca. 15−20 °C. Particularly noteworthy is the lower-temperature range for fusion found for the 4:1 and the 3:1 DOPE/bis-SorbPC membranes. This range spans the physiological temperature such that rapid fusion is not observed at 37 °C until the LUV are photopolymerized. Thus, photopolymerization of properly constituted LUV provides a potentially useful strategy for initiating the fusion of liposomes at physiological temperatures.

The fusion assays report the heterofusion between a photolyzed and dark LUV. The fusion event occurs following formation of a heterodimer of LUV, where the initial fusion intermediates are necessarily composed of a mixture of lipids from the cis monlayers of each liposome in the heterodimer. The critical fusion temperature should therefore

Table 1: Effect of Different Extents of Photopolymerization of bis-SorbPC on the Calculated Molar Ratio of DOPE to the Remaining Monomeric PC (PE/PC) in a Heterodimer of Two LUV Undergoing Productive Fusion and the Estimated Critical Fusion Temperatures (T_f)[a]

	original DOPE:bis-SorbPC ratio					
	4:1		3:1		2:1	
extent of polymerization (%)	PE/PC	T_f (°C)	PE/PC	T_f (°C)	PE/PC	T_f (°C)
0	4:1	45	3:1	55	2:1	65
50	5.3:1	30	4:1	40	2.6:1	55
100	8:1	25	6:1	35	4:1	45

[a] In all cases, only the DPX LUV were photopolymerized.

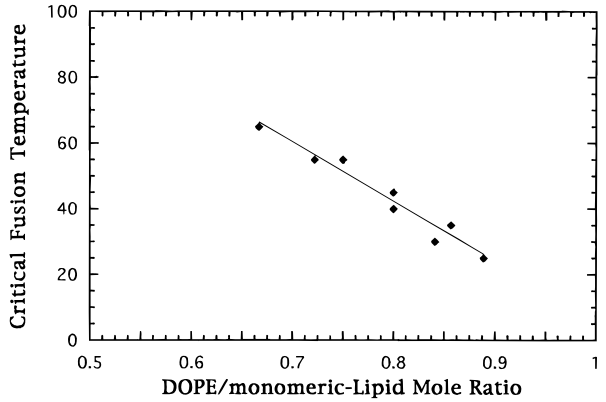

FIGURE 8: Effect of photopolymerization on the calculated molar ratios of DOPE to the total monomeric lipid in a heterodimer of two LUV vs measured critical fusion temperatures (T_f) for these LUV compositions. The data are taken from Table 1.

depend on the average R_o value of the participating lipids. The data in Table 1 and Figure 8 provide clear evidence supporting this hypothesis. Thus, by proper choice of the reactive liposome starting composition, the nonreactive liposome composition, and the photopolymerization conditions, a particular fusion temperature can be achieved. Indeed, one would anticipate that a similar plot of critical fusion temperature vs lipid composition could be constructed for any liposome composition.

Consider the problem of endosomal delivery where the LUV must interact with a membrane that is relatively rich in PC. These circumstances will probably shift the fusion threshold to higher temperatures. Among the photosensitive LUV studied to date, the 4:1 DOPE/bis-SorbPC membranes have the lowest T_f values. However, even the 4:1 membranes will probably not be capable of destabilizing a PC-rich membrane at physiological temperatures; therefore, other modifications will be required to decrease the average R_o value in the monomeric lipid domains comprising the fusion precursors (i.e. stalks). One possibility is to further increase the DOPE to polymerizable lipid ratio. We have shown here that decreasing the relative concentration of bis-SorbPC has the effect of lowering the temperature at which fusion occurs. Other possibilities include changing the chemical composition of the photosensitive LUV by either substitution for the DOPE, or the bis-SorbPC, or both or the addition of another polymorphic lipid to the LUV. The DOPE could be replaced all or in part by a polymorphic lipid that exhibits a lower T_H, such as plasmenylethanolamine (Glaser & Gross, 1994). The addition of a new polymorphic component, such as diacylglycerol (DAG), to the photosensitive LUV could also be used to change the critical fusion temperature. Previously, it was shown that the addition of 2 mol % of DAG was sufficient to decrease the T_H value of DOPE-Me membranes by ca. 15 °C, thereby increasing the fusion rate at a fixed temperature (Siegel et al., 1989). Photopolymerizable bis-SorbPCs with a different chain length and T_m are known and could also prove useful for reducing the average R_o value (Lamparski et al., 1993).

Photosensitive liposomes can be designed for efficient destabilization under a variety of experimental conditions. In effect, they are analogous to pH-sensitive liposomes and possess the added benefit of spatial and temporal control over the delivery mechanism. This potential advantage comes at the expense of requiring the delivery of light to the therapeutic site. Fortunately, the therapeutic use of light via endoscopy is well-advanced thanks to the active development of photodynamic therapy. Future experiments planned in this laboratory will seek to establish the conditions that favor destabilizing interactions of photosensitive liposomes with PC-rich membranes, including endosomal membranes.

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