# Photoactivated Enhancement of Liposome Fusion†

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ABSTRACT: The photopolymerization of two-component large unilamellar liposomes (LUV) composed of 3:1 dioleoylphosphatidylethanolamine (DOPE) and either 1,2-bis[10-(2′-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphatidylcholine (bis-SorbPC) or 1-palmitoyl-2-[10-(2′-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphatidylcholine (mono-SorbPC) facilitated liposome fusion. Fusion was characterized by fluorescent assays for lipid mixing, aqueous contents mixing, and aqueous contents leakage. The rate and extent of the liposome fusion was dependent on the extent of photopolymerization, temperature, and the fusion initiation conditions, including the pH and the presence of $Mg^{2+}$ ions. Examination of the temperature dependence of fusion for unpolymerized and polymerized liposomes showed that an enhancement of the rate of fusion occurred in the temperature range $\Delta T_I$, which previous NMR studies have identified as the initial appearance of precursors to the formation of the inverted cubic phase [Barry, J. A., et al. (1992) *Biochemistry 31*, 10114]. The phase behavior and fusion characteristics of the DOPE/bis-SorbPC (3:1) membranes provide unequivocal evidence that liposome fusion is mediated via intermediates associated with the lamellar to $Q_{II}$ phase transition rather than the $H_{II}$ phase. Photopolymerization of SorbPC-containing liposomes forms poly-SorbPC, which enhances the lateral separation of the liposome components. The formation of enriched domains of polymorphic lipids, e.g., DOPE, causes isothermal induction of fusion by lowering the critical fusion temperature of the membranes.

*In vivo* delivery and buffering of drugs via liposomes as the carriers of either hydrophilic or lipophilic agents presently stimulate active research across many disciplines. The use of liposomes for drug delivery, gene transfer, and immunodiagnostic applications has been reviewed recently (Litzinger & Huang, 1992). Insight into the fusion of biological membranes has resulted in part from the study of a variety of methods devised to induce the fusion of liposomes: anionic liposomes in the presence of multivalent ions (Bentz & Nir, 1981; Düzgünes et al., 1981; Ohki et al., 1982; Bentz & Duzgunes, 1985; Carmona-Ribeiro et al., 1985; Bentz et al., 1988); liposomes containing polymorphic lipids, such as phosphatidylethanolamine (PE),[1] at temperatures near $T_H$ (Ellens et al., 1985, 1986a,b, 1989; Siegel et al., 1989a); neutral liposomes in the presence of dehydrating agents such as PEG (Parente & Lentz, 1986; Lentz et al., 1992; Massenburg & Lentz, 1993); so-called pH-sensitive liposomes at low pH or in the presence of multivalent cations (Connor et al., 1984; Ellens et al., 1984; Düzgünes et al., 1985; Nayar & Schroit, 1985; Straubinger et al., 1985; Leventis et al., 1987; Collins et al., 1989); formation of diacylglycerol (DAG) by the reaction of phospholipase C with PC-containing liposomes (Nieva et al., 1989; Luk et al., 1993); and cationic liposomes in the presence of anionic liposomes (Felgner et al., 1987; Stamatatos et al., 1988; Leventis & Silvius, 1990).

The delivery of therapeutic agents by liposomes to the cytoplasm of target cells would appear to require liposome−cellular membrane fusion. Fusion could occur either directly between the liposomal membrane and the plasma membrane or between the liposomal membrane and the endosomal membrane following liposome endocytosis. Fusion of cationic liposomes with the plasma membrane has been used for the transformation of cells by the delivery of liposome-encapsulated DNA to the cytoplasm (Felgner et al., 1987; Leventis & Silvius, 1990; Legendre & Szoka, 1992). Fusion of liposomes with both the plasma and endosomal membranes has been induced using the fusion proteins from both the influenza and Semliki Forest enveloped animal cell viruses (Gitman & Loyter, 1984; Stegmann et al., 1987). In these studies, virosomes were prepared by reconstituting the functional viral fusion proteins into liposomes. Fusion at the endosomal membrane has also been observed with pH-sensitive immunoliposomes (Wang & Huang, 1987, 1989). Both *in vitro* and *in vivo* transformation of target cells has been accomplished with pH-sensitive immunoliposomes by the delivery of DNA to the cytoplasm of target cells by fusion of the liposomal and endosomal membranes following uptake of the liposomes by endocytosis. Bentz et al. (1992) pointed out that the requirements for the delivery of the contents of pH-sensitive immunoliposomes across the endosomal membrane are different from those for mixing of the contents

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[1] Abbreviations: ANTS, 1-amino-3,6,8-naphthalenetrisulfonic acid disodium salt; DAG, diacylglycerol; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPE-Me, *N*-monomethyl-DOPE; 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, pH 9.5; $H_{II}$, inverted hexagonal phase; LUV, large unilamellar vesicles; MLV, large multilamellar vesicles; OD, optical density; PC, *sn*-glycero-3-phosphatidylcholine; PE, *sn*-glycero-3-phosphoethanolamine; $Q_{II}$, inverted cubic phase; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; bis-SorbPC, 1,2-bis[10-(2′-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphatidylcholine; mono-SorbPC, 1-palmitoyl-2-[10-(2′-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphatidylcholine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; $T_H$, lamellar liquid−crystalline/hexagonal phase transition temperature; $T_I$, lamellar liquid−crystalline ($L_\alpha$) phase to isotropic transition temperature; TLC, thin-layer chromatography.

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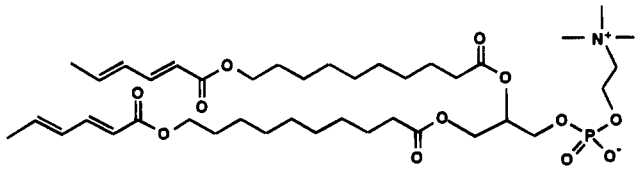
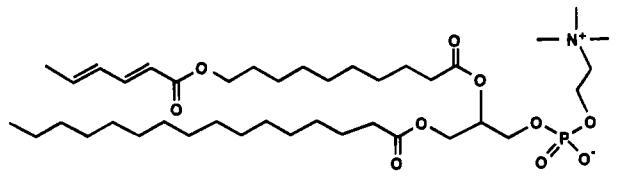
between two pH-sensitive liposomes. In the former case, successful delivery can occur even if fusion is accompanied by extensive leakage, while in the latter case, leakage is competitive with fusion.

The promotion of liposome fusion by added chemical agents is not necessarily appropriate for *in vivo* delivery. The use of light to enhance liposome fusion avoids the need for added chemical agents and provides both temporal and spatial control of the phenomena. It is the control of these variables that has proven so useful in photodynamic therapy. The successful use of light to treat disease could be greatly enhanced if light were used to release therapeutic agents from liposomes to other bilayer-bounded structures.

Photosensitive liposomes have been described in recent years [reviewed by O'Brien and Tirrell (1993)]. A variety of strategies has been employed, including the photochemical isomerization of chromophores in acyl chains (Kano et al., 1981; Pidgeon & Hunt, 1983, 1987), photocleavage of lipid chains (Kusumi et al., 1989; Anderson & Thompson, 1992), photoinduced change in the association of polyelectrolytes with liposomes (You & Tirrell, 1991), and the photopolymerization of lipids (Frankel et al., 1989; Lamparski et al., 1992). These methods generally were designed for liposome lysis. Only in the case of photopolymerization of liposomes has evidence for liposome fusion been reported (Bennett & O'Brien, 1993, 1994). The photopolymerization-induced destabilization of multilamellar liposomes (MLV) was proposed to occur through intraliposomal membrane contact following photoinduced domain formation (Lamparski et al., 1992). The photopolymerization of lipids has been demonstrated successfully with acryloyl, diacetylene, stryryl, dienoyl, sorbyl, and lipoyloxy groups and has recently been reviewed (O'Brien & Ramaswami, 1989; Singh & Schnur, 1993; O'Brien, 1994). Polymerization of bi- or multicomponent bilayers was shown to induce a lateral separation of the reactive and nonreactive bilayer components (Gaub et al., 1985; Tyminski et al., 1985, 1988; Armitage et al., 1993). Here we describe the effective use of the photopolymerization of sorbyl lipids to enhance fusion between LUV.



*bis*-SorbPC$_{17,17}$



*mono*-SorbPC$_{16,17}$

## MATERIALS AND METHODS

*Materials.* Dioleoylphosphatidylethanolamine (DOPE), phosphatidylcholine (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 used without purification (one spot on TLC, 65:25:4, $CHCl_3$/MeOH/$H_2O$). The synthetic 1,2-bis[10-(2′-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphatidylcholine (bis-SorbPC) and 1-palmitoyl-2-[10-(2′-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphatidylcholine (mono-SorbPC) were prepared as described previously (Lamparski et al., 1992). 1-Aminonaphthalene-3,6,8-trisulfonic acid (disodium salt) (ANTS) and *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 vesicles (LUV) were prepared by freeze−thaw extrusion techniques (Hope et al., 1985; Mayer et al., 1986). Lipid stock solutions were mixed in benzene, and then lipid films (7−21 μmol) were prepared in 10 mL flasks by evaporating the solvent under a stream of nitrogen gas and drying under vacuum for several hours. The resulting lipid film was hydrated by the addition of the appropriate buffer followed by vortex mixing and brief irradiation in a bath-type sonicator. The lipid suspensions were freeze−thawed five times, employing alternating cycles between dry ice/isopropyl alcohol and warm water (30 °C) baths. The freeze−thawed lipid suspensions were stored overnight at −40 °C or were immediately extruded 10 times through two stacked 0.1 μm pore size Nucleopore polycarbonate filters at room temperature with nitrogen pressures of 300−500 psi using a stainless steel extrusion device (Lipex Biomembranes, Vancouver, BC, Canada). The sizes of the resulting populations of LUV were determined by quasi-elastic light scattering to be monodisperse with an average diameter of 120 ± 10 nm.

Fluorescence-labeled liposomes for lipid mixing assays were prepared containing either 1.0 or 0.1 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 (115 mM NaCl and 10 mM glycine, pH 9.5) to 13 mM before the formation of LUV by extrusion.

LUV for the ANTS/DPX fusion assays were prepared according to methods described previously (Ellens et al., 1985, 1989) with some modifications. LUV contained either (i) 25 mM ANTS and 60 mM NaCl or (ii) 90 mM DPX. Both solutions were buffered with 10 mM glycine at pH 9.5. The ANTS/DPX leakage assays were also performed according to established methods (Ellens et al., 1985). LUV contained either (i) 12.5 mM ANTS, 45 mM DPX, and 30 mM NaCl buffered with 10 mM glycine at pH 9.5 or (ii) glycine buffer. Encapsulated material was separated from unencapsulated material on Sephacryl S-300 (Pharmacia) gel filtration columns (1.6 × 20 cm) using glycine buffer as the eluent. In all cases, the encapsulated solutions were isoosmotic to the buffers used for gel filtration chromatography and for the subsequent fusion and leakage assays. In these experiments, the osmolarities of all buffers were determined to be 220 mosm/kg (Osmette S osmometer, Precision Instruments). The vesicle concentration was determined spectroscopically from the bis-SorbPC chromophore ($\epsilon$ = 47 100, $\lambda_{max}$ = 258 nm, methanol) or from the mono-SorbPC ($\epsilon$ = 23 500, $\lambda_{max}$ = 258 nm, methanol) chromophore by diluting 25 μL aliquots of the chromatographed LUV suspensions into 1 mL volumes of methanol.

*Liposome Photolysis.* LUV samples (3.0 mL) were placed 1 cm from a low-pressure mercury vapor pen lamp (254 nm) 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 attenuate the intensity of the 254 nm light incident on the sample. Photopolymerization was carried out continuously for each new sample, and photolysis times ranged from 0 to 3 min. The loss of monomeric mono-SorbPC or bis-SorbPC was monitored by the decrease in the UV absorption of the aqueous LUV suspensions at 258 nm. The extent of conversion of monomer (% polymerization) was determined using the following equation: % polymerization = $[(A_0 - A_t)/(A_0 - A_\infty)]100$, where $A_0$ is the initial absorbance, $A_t$ is the absorbance after $t$ min of irradiation, and $A_\infty$ is the absorbance after the complete conversion of monomer.

*Fluorescence Measurements.* Fluorescence was measured with a Spex Fluorolog 2 fluorimeter (Spex Industries, Inc., Edison, NJ). The cuvette holder was thermostated at various temperatures by a water circulator, and samples were thermally equilibrated for at least 5 min before the assays were initiated. For lipid mixing studies, excitation was 450 nm and emission was 530 nm; band slits were 2 mm for excitation and 4 mm for emission. For ANTS/DPX fusion and leakage studies, the excitation was 360 nm and emission was 520 nm; 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 Düzgünes et al. (1987). The residual fluorescence at 530 nm of the labeled LUV containing 1.0 mol % each of NBD-PE and Rh-PE was taken as 0% of maximum fluorescence. Lipid mixing results in an increase in the fluorescence of NBD-PE due to decreased energy transfer efficiency to its quencher, Rh-PE, as the fluorescent probes are diluted from labeled LUV into unlabeled LUV. In all cases, labeled LUV were mixed in a 1:9 molar ratio with unlabeled LUV. The total lipid concentrations was usually 300 $\mu$M, although a limited number of experiments were performed at 50 $\mu$M. Multiple rounds of random fusion between the LUV populations would result in membranes containing 0.1 mol % of each of the fluorescent phospholipids. The value for the theoretical maximum fluorescence was set by subjecting a mixture of the labeled and unlabeled LUV populations to five cycles of freeze−thawing (dry ice/isopropyl alcohol bath, 30 °C water bath) (MacDonald & MacDonald, 1983). Randomization of the lipids by this procedure was verified by preparing a separate LUV population containing 0.1 mol % of each probe, which would be the result of multiple rounds of fusion between labeled and unlabeled LUV.

The unlabeled LUV stock solution (0.81 $\mu$mol) was diluted to ca. 2.9 mL and then photolyzed for the requisite time. The unirradiated stock solution of labeled LUV (0.09 $\mu$mol) was then added to produce a 1:9 molar ratio of labeled and unlabeled liposomes of ca. 300 $\mu$M. Lipid mixing was initiated in the fluorimeter by injection of 75 $\mu$L of various stock solutions into the magnetically stirred liposome suspension, resulting in a final volume of 3.0 mL. Dark assays were performed in the same manner in the absence of photolysis of the unlabeled LUV. A 75 $\mu$L aliquot of 2.0 M acetic acid/sodium acetate (pH 4.5) buffer was added to achieve pH 4.5. A 75 $\mu$L aliquot of 0.5 M TES (pH 7.0) buffer produced a final pH of 7.5. The addition of a 75 $\mu$L aliquot of 0.5 M TES and 0.2 M $MgCl_2$ (pH 7.0) gave a pH of 7.5 and 5 mM $Mg^{2+}$. A 75 $\mu$L aliquot of 0.8 M $MgCl_2$ (pH 6.8) was added to achieve pH 9.5 and 20 mM $Mg^{2+}$.

*Fusion and Leakage Studies.* The ANTS/DPX fusion assay reports mixing of aqueous contents between liposome populations containing either ANTS or DPX by decreased ANTS fluorescence due to DPX quenching (Ellens et al., 1985). The ANTS- and DPX-containing liposomes were combined 1:9 at a lipid concentration of 300 $\mu$M. In order to avoid photobleaching of ANTS, only the DPX liposomes were exposed to 254 nm light. Following the exposure, the ANTS liposomes were added, and the mixture was thermally equilibrated at the temperature of interest for at least 5 min in the fluorimeter. The fluorescence scale was calibrated using the emission intensity of a 1:9 (mol/mol) mixture of ANTS and DPX liposomes in glycine buffer as 100% fluorescence (0% fusion) and the intensity of liposomes containing the coencapsulated ANTS/DPX as 0% fluorescence (100% fusion). Since ANTS is severely quenched by DPX, the latter value was close to zero.

The ANTS/DPX leakage assay employed LUV with coencapsulated ANTS and DPX. Leakage of the aqueous contents from 30 $\mu$M liposomes in the presence of 270 $\mu$M empty liposomes results in ca. $1 \times 10^4$-fold dilution of the probes into the extraliposomal medium, and yields increased fluorescence from ANTS due to the relief of its quenching by DPX (Ellens et al., 1984; Bentz et al., 1987). 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 lysis of the LUV by the addition of Triton X-100 to a final concentration of 0.5% (v/v) as 100% fluorescence (100% leakage). The 100% fluorescence value for each sample was determined since 254 nm exposure of the samples resulted in the bleaching of ANTS to a limited, but significant extent. The fusion and leakage assays were initiated in the same manner as the lipid mixing assays by injection of 75 $\mu$L aliquots of various stock solutions into the stirred LUV suspensions. The initial rates of fusion and leakage were calculated from the slopes of the fluorescence curves during the period immediately following the neutralization of liposome surface charge.

*Light Scattering.* Liposome size distributions were measured using dynamic laser light scattering (Brookhaven BI-8000AT correlator with a 5 mW He−Ne laser light source, Brookhaven Instruments Corp.). LUV were examined at a total lipid concentration of 100 $\mu$M at an angle of 90°. 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

*Photopolymerization of SorbPC Membranes.* Liposomes of DOPE/bis-SorbPC and DOPE/mono-SorbPC in glycine buffer at 37 °C exhibit absorbance maxima at 258 nm due to the sorbyl chromophore. Photolysis of the liposomes with UV light (254 nm) diminishes this absorbance band. Both empty and DPX-containing LUV composed of either DOPE/bis-SorbPC (3:1) or DOPE/mono-SorbPC (3:1) at ca. 300 $\mu$M in 3 mL stirred suspensions were completely polymerized by 3.0 min exposures from the filtered emission of a

low-pressure Hg pen lamp (predominantly 254 nm light) placed 1 cm from the front face of a quartz cuvette. Polymerization of 50% of the bis-SorbPC or mono-SorbPC component was accomplished after 0.5 or 0.66 min of exposure, respectively, under the same conditions. The reaction rate is attenuated at a high conversion of monomer due to the decreased absorbance due to monomer absorbance (DeSchryver et al., 1975).

The photolysis of LUV in optically transparent medium, i.e., water, reveals that the absorbance decrease at 258 nm is accompanied by an increase in the absorbance at 195 nm, with a well-defined isosbestic region at 222 nm. The existence of the isosbestic region suggests that the photolysis yields principally one product (Lamparski et al., 1992). $^1$H NMR studies of the polymer products of photolysis of bis-SorbPC and mono-SorbPC LUV identified the principal photoproduct as the 1,4-polymer (Lamparski & O'Brien, 1995). The insensitivity of the photoreaction to the presence of oxygen and the direct proportionality of the rate of the reaction to light intensity indicate that the reaction proceeds by photoactivated addition (Lamparski & O'Brien, 1995). Prolonged photolysis of bis-SorbPC LUV results in the eventual disappearance of the band at 195 nm. This was inferred to be due to reaction of the residual double bond in the 1,4-polymer. Because of the possibility that this second photoreaction could lead to uncontrolled cross-linking of the sorbyl lipids, precautions were taken against this occurrence by both the use of short photolysis times ($\leq$3.0 min) and the attenuation of short wavelength light incident on the samples with a 230 nm high-pass filter. Recently, the photoactivated polymerization of LUV composed of mono-SorbPC was shown to produce oligomers and no high molecular weight polymers (Lamparski & O'Brien, 1995). The size of the bis-SorbPC polymers is not known, but their greater insolubility in organic solvents than the polymers from mono-SorbPC implies that considerable cross-linking occurs with the bis-SorbPC at short photolysis times.

Prior to photolysis, LUV samples (270 $\mu$M in ca. 3 mL) composed of DOPE and either bis-SorbPC or mono-SorbPC exhibited optical densities of ca. 2.7 or 1.7, respectively. Therefore, at the beginning of the photolysis, $\geq$98% of the 254 nm light was absorbed in both cases. The incident flux at the front face of the cuvette was determined to be $\sim 8 \times 10^{15}$ photons s$^{-1}$ by potassium ferrioxalate solution actinometry (Hatchard & Parker, 1956). Under these conditions, 50% conversion of the bis-SorbPC to poly(bis-SorbPC) required 30 s, and 50% conversion of mono-SorbPC required 40 s. Hence, the quantum yields for the reactions were 0.25 and 0.20, respectively.

*Phase Behavior of Mixed Lipid Systems.* Bilayers of PE are generally unstable at physiological pH unless they contain charged amphiphiles, such as a fatty acids (Litzinger & Huang, 1992), or $L_\alpha$-competent colipids, e.g., PA or PC (Stollery & Vail, 1977). Colloidally stable 0.1 $\mu$m LUV suspensions of 2:1, 3:1, and 4:1 mixtures of either DOPE/bis-SorbPC or DOPE/mono-SorbPC LUV can be prepared by extrusion (0.1 $\mu$m pore size Nucleopore membranes) at physiological pH in aqueous buffers (100 mM NaCl and 20 mM Tris, pH 7.4) at room temperature. However, under these same conditions, hydration and extrusion of 0.1 $\mu$m LUV of 2:1, 3:1, and 4:1 mixtures of DOPE and DOPC result in lipid suspensions that rapidly aggregate and precipitate. The presence of the sorbyl group in the middle of the mixed lipid bilayers apparently has a profound effect on the equilibrium phase behavior of LUV composed principally of DOPE. Table 1 gives the lamellar to nonlamellar phase transition temperatures for DOPE and its mixtures in various molar ratios with either DOPC or bis-SorbPC. Note that although DOPE/DOPC (3:1) systems readily adopt the $H_{II}$ phase, neither DOPE/bis-SorbPC (3:1) nor DOPE/mono-SorbPC (3:1) systems have been observed to adopt the $H_{II}$ phase at experimentally accessible temperatures.

Table 1: Lamellar to Nonlamellar Phase Transition Temperatures

| lipid system | conditions | $T_I$ (onset) (°C) | $T_H$ (peak) (°C) |
|---|---|---|---|
| DOPE/DOPC (4:1) | pH 4.5 | | 47 ± 2[a] |
| | pH 7.0 | <35[c] | |
| DOPE/DOPC (3:1) | pH 4.5 | | 60 ± 4[a] |
| | 5 mM $Mg^{2+}$/pH 7.4 | 45−50[a] | |
| DOPE/DOPC (2:1) | pH 4.5 | | ≥80[a] |
| | pH 7.4 | 65−70[b] | |
| DOPE/bis-SorbPC (3:1) | pH 7.4 | 49 ± 0.5[d] | >85[d] |
| DOPE/mono-SorbPC (3:1) | pH 7.4 | na | na |

[a] Ellens et al., 1989. [b] van Duijn et al., 1986. [c] Tilcock et al., 1982. [d] Barry et al. (1992) did not observe an $H_{II}$ phase below 85 °C. Higher sample temperatures were not examined.

*Lipid Mixing Studies.* Lipid mixing between labeled and unlabeled LUV populations was carried out with two protocols. In the first, the LUV were prepared at pH 7.4 (100 mM NaCl and 20 mM Tris) and were combined in a stirred and thermostated cell with or without UV photolysis. Aliquots were removed at 5 min intervals and were diluted 20-fold into the medium. Lipid mixing after different dark or photolyzed incubation periods was quantified by measuring the fluorescence emission spectra of the diluted aliquots. The spectra were corrected for dilution errors by normalization at the isoemissive point (569 nm). Lipid mixing was studied with either DOPE/bis-SorbPC (3:1) LUV or DOPC/bis-SorbPC (3:1) LUV. In the photolyzed assays, both LUV populations were irradiated simultaneously. Lipid mixing was found to be dependent on the extent of photopolymerization, the temperature, and the inclusion of PE in the bilayers (Bennett & O'Brien, 1993; Bennett et al., 1994). At 37 °C and 300 $\mu$M, the photolyzed DOPE/bis-SorbPC LUV underwent significantly greater lipid mixing than the dark LUV. Lipid mixing of both dark and photolyzed LUV was increased significantly by the addition of 5 mM $Mg^{2+}$. Control liposomes composed of DOPC/bis-SorbPC exhibited no lipid mixing under any conditions. Although this protocol was suitable for the determination of photoinduced lipid mixing, which is evidenced by a persistent change in fluorescence, it could not be applied to the measurement of aqueous content mixing due to the transient nature of the fusion event and the attendant changes in fluorescence. A second protocol, which allowed the temporal separation of the polymerization and fusion events, was required for the determination of the effect of different extents of polymerization on fusion.

In the second protocol, labeled and unlabeled liposomes were prepared at pH 9.5 (glycine buffer) and combined (1:9). Lipid mixing was initiated by using $H^+$, $Mg^{2+}$, or both to neutralize the initially negative bilayer surfaces of the LUV. With both the DOPE/bis-SorbPC (3:1) and DOPE/mono-SorbPC (3:1) systems, the rates and extents of lipid mixing increased in the following order: pH 7.5 < pH 7.5/5 mM Mg < pH 4.5 < pH 9.5/20 mM Mg. Large differences

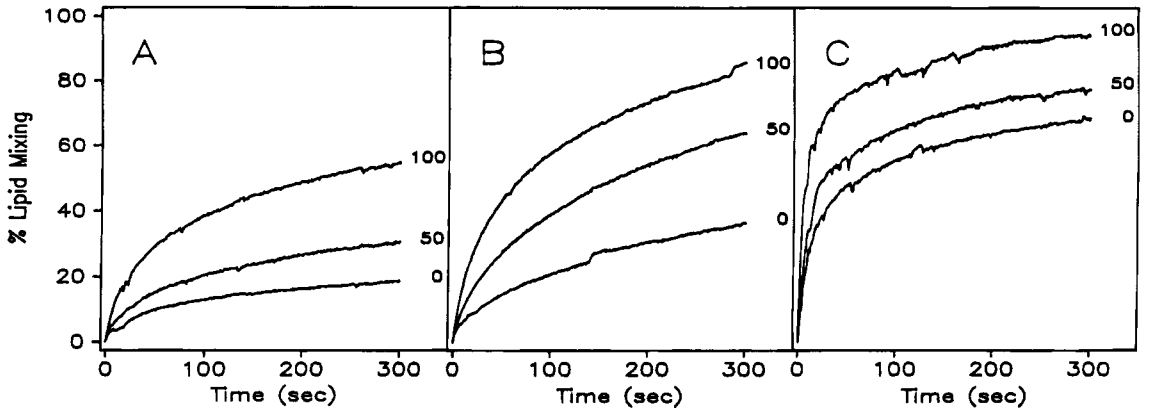



FIGURE 1: Effect of polymerization on lipid mixing between 120 nm LUV composed of DOPE/bis-SorbPC (3:1) at 37 °C and different initiation conditions: (A) 5 mM $Mg^{2+}$ at pH 7.5; (B) pH 4.5; (C) 20 mM $Mg^{2+}$ at pH 9.5. ANTS- and DPX-containing LUV were combined in a 1:9 molar ratio at 300 $\mu$M. Unpolymerized ANTS LUV were combined with DPX LUV whose bis-SorbPC component was either unpolymerized, 50% polymerized, or 100% polymerized, as indicated in the figure.

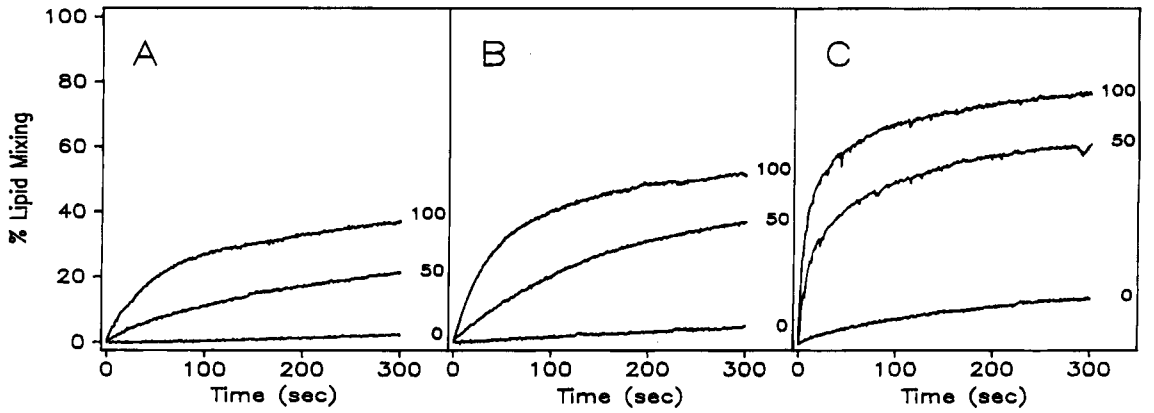



FIGURE 2: Effect of polymerization on lipid mixing between 120 nm LUV composed of DOPE/mono-SorbPC (3:1) at 50 °C and different initiation conditions: (A) 5 mM $Mg^{2+}$ at pH 7.5; (B) pH 4.5; (C) 20 mM $Mg^{2+}$ at pH 9.5. Conditions otherwise are identical to those given in Figure 1.

in lipid mixing were observed at pH 4.5 and 7.5 (data not shown). This was probably due to the presence of residual negative surface charge on the LUV at the higher pH due to incomplete protonation of the PE amino group. Allen et al. (1990) reported the inhibition of aggregation of egg PE liposomes in the region pH 7.4−8.0 and attributed the observation to a small residual surface charge. Figure 1 shows the effect of polymerization of DOPE/bis-SorbPC LUV on the time course of lipid mixing at 37 °C after initiation in the presence of 5 mM $Mg^{2+}$ at pH 7.5 (panel A), pH 4.5 (panel B), or 20 mM $Mg^{2+}$ at pH 9.5 (panel C). Time scans are shown for 0%, 50%, and 100% photopolymerization, as indicated in the figure. In all cases, lipid mixing increased in both rate and extent with increasing conversion of bis-SorbPC to poly(bis-SorbPC). Lipid mixing was also examined in this system at 20 °C, where the process was observed to be significantly retarded under all initiation conditions. A decrease in the lipid concentration to 50 $\mu$M did not alter either the rates or extents of lipid mixing, as determined at pH 4.5.

In Figure 2, the time course of lipid mixing is shown for DOPE/mono-SorbPC (3:1) under the same conditions as for Figure 1, except that the studies were performed at 50 °C. Again, the lipid mixing increased with increasing photopolymerization. Determination of the lipid mixing for DOPE/mono-SorbPC at lower temperatures (20 and 37 °C) showed only small amounts of mixing. Comparison of Figures 1 and 2 reveals that the effect of photopolymerization on the mono-SorbPC system was markedly smaller than the effect of photopolymerization of equivalent numbers of lipids on the bis-SorbPC system. The $T_m$ values for bis-SorbPC and mono-SorbPC are 28.8 and 36.1 °C, respectively (Lamparski et al., 1993). The ca. 7 °C difference in $T_m$ values for the two polymerizable lipids contributes to a ca. 15 °C shift to higher temperature in the fusion threshold of the DOPE/mono-SorbPC LUV. Substitution of the sn-1 fatty acyl tail of bis-SorbPC system to the saturated 16-carbon tail of mono-SorbPC remarkably attenuates the lipid mixing of the LUV at equivalent temperatures (e.g., 37 °C) where both systems are well above their $T_m$ values (data not shown).

*Fusion and Leakage Studies.* The experimental design, in which only one population of LUV was photolyzed, was motivated by a reluctance to photobleach the ANTS fluorophore during the photopolymerization of bis-SorbPC or mono-SorbPC. An alternative fluorescence assay for aqueous content mixing that employs the $Tb^{3+}$−dipicolinic acid complex ($Tb(DPA)_3^{3-}$) (Wilschut et al., 1980) is inconvenient since the excitation band of encapsulated DPA ($Ex_{max}$ = 278 nm) is screened by the intense absorption band of the sorbyl chromophore ($\lambda_{max}$ = 258 nm). Using the ANTS/DPX assay, the fusion behavior of ANTS- and DPX-containing LUV (combined 1:9) was compared under the same initiation conditions employed in the lipid mixing studies. Leakage was studied with ANTS/DPX-containing LUV in the presence of empty LUV (containing the same glycine buffer as composed the extraliposomal medium). In the fusion assay,

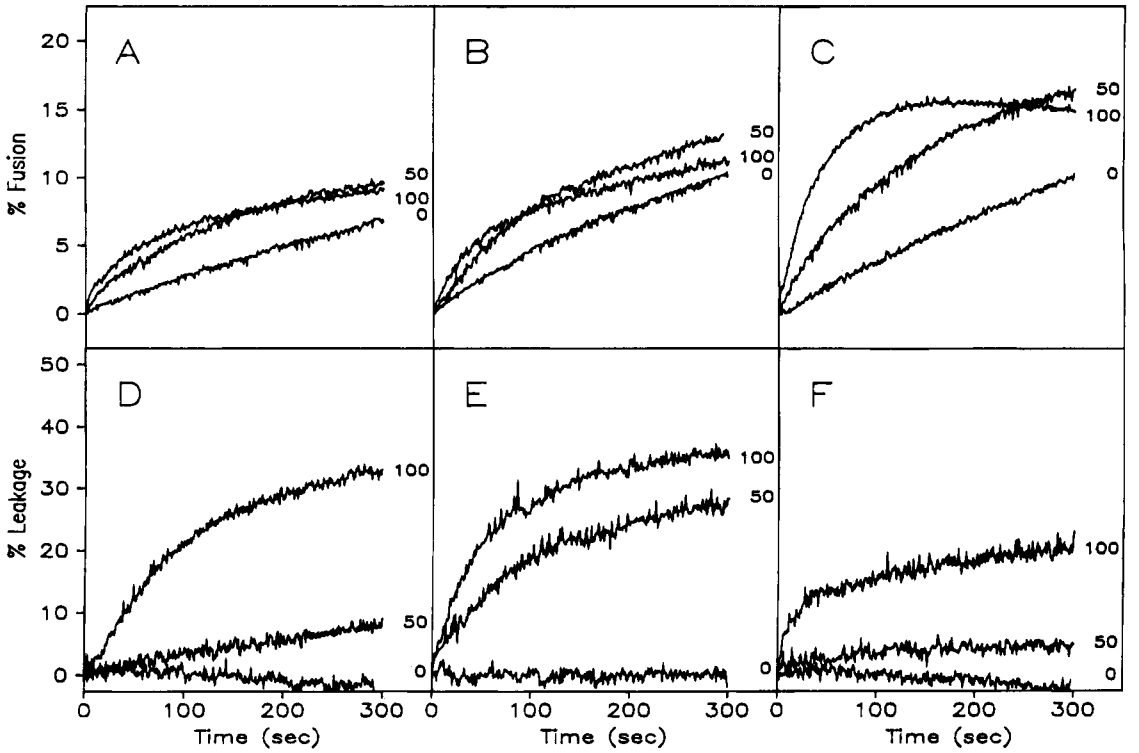



FIGURE 3: Effect of polymerization on fusion (aqueous content mixing) and leakage of 120 nm LUV composed of DOPE/bis-SorbPC (3:1) at 40 °C. Panels A and D show fusion and leakage, respectively, in the presence of 5 mM $Mg^{2+}$ at pH 7.5. Panels B and E show fusion and leakage, respectively, at pH 4.5. Panels C and F show fusion and leakage, respectively, in the presence of 20 mM $Mg^{2+}$ at pH 9.5. Unpolymerized ANTS LUV were combined in a 1:9 ratio at 300 $\mu$M with DPX LUV whose bis-SorbPC component was either unpolymerized, 50% polymerized, or 100% polymerized, as indicated in the figure.

only the 9-fold excess DPX-containing LUV were photolyzed; in the leakage assay, only the 9-fold excess empty LUV were photolyzed. Figure 3 shows fusion and leakage for DOPE/bis-SorbPC (3:1) LUV at 40 °C in the presence of 5 mM $Mg^{2+}$ at pH 7.5 (panels A and D, respectively), pH 4.5 (panels B and E, respectively), and 20 mM $Mg^{2+}$ at pH 9.5 (panels C and F, respectively). The shapes of the fusion curves are readily understandable in light of their respective leakage traces. In all cases, fusion is rapid initially, but with time decreases in rate due to simultaneous and competitive leakage. In some cases, the extent of fusion climbs to a maximum value and then decreases due to leakage. Under such conditions, the rate of leakage initially lags behind the rate of fusion and eventually overcomes the fusion rate.

The extents of fusion and leakage under the different conditions are inversely related: at pH 4.5, both fusion and leakage are intermediate compared to the other initiation conditions; at pH 9.5/20 mM Mg, fusion is greatest and leakage is lowest; and at pH 7.5/5 mM Mg, fusion is lowest and leakage is greatest (Figure 3). Although lipid mixing is greater for pH 9.5/20 mM Mg than for either of the other conditions, it is the rate of leakage, not the rate of aggregation (as evidenced by lipid mixing), that controls the resulting rate of productive fusion (as evidenced by aqueous contents mixing). The similarity in the behavior of the curves for 50% and 100% photopolymerization under the different initiation conditions is striking: under all conditions, the extent of fusion at 50% photopolymerization surpasses that at 100% photopolymerization after times of no more than 4 min, and the extent of leakage at 50% photopolymerization is lower than that for 100% photopolymerization. Fusion measured at pH 4.5 and 50 $\mu$M was similar to that observed at 300 $\mu$M. Due to the noise in the fluorescence time scans at the lower concentration, these studies focused on the higher concentration regime. The fusion curves measured from scan to scan and even in different preparations were reproducible.

The effect of photopolymerization on the time course of fusion of DOPE/mono-SorbPC (3:1) LUV at 50 °C and three different initiation conditions is shown in Figure 4. Fusion is shown under the same conditions as those employed for the lipid mixing assays presented in Figure 2. A strong correlation between the lipid mixing and fusion assays exists for the DOPE/mono-SorbPC (3:1) system. In both studies, LUV interaction increased in the same order: pH 7.5/5 mM Mg < pH 4.5 < pH 9.5/20 mM Mg. The differences in the rates and extents of fusion of photolyzed or dark-adapted DOPE/mono-SorbPC LUV are significantly smaller than those observed in the DOPE/bis-SorbPC (3:1) system.

Figure 5A–C shows the effect of temperature on the fusion of DOPE/bis-SorbPC (3:1) LUV at pH 4.5 and at different extents of photopolymerization: 0% (panel A), 50% (panel B), and 100% (panel C). Figure 5D–F shows the corresponding effect of temperature on the leakage of DOPE/bis-SorbPC (3:1) LUV under the same conditions. Evidently, fusion can proceed in the relative absence of leakage at low temperatures for both dark-adapted and photolyzed LUV. As the temperature is raised, leakage increases progressively. While the initial rates of fusion increase as the temperature is raised, the extents are diminished. In Figure 6, the initial rates of fusion and leakage versus temperature are shown for the same systems as in Figure 5. The rates used in Figure 6 were extracted from the initial slopes of the fusion and leakage curves. Both the fusion and leakage curves are shifted to lower temperature with increasing extents of photopolymerization. The temperature threshold for the

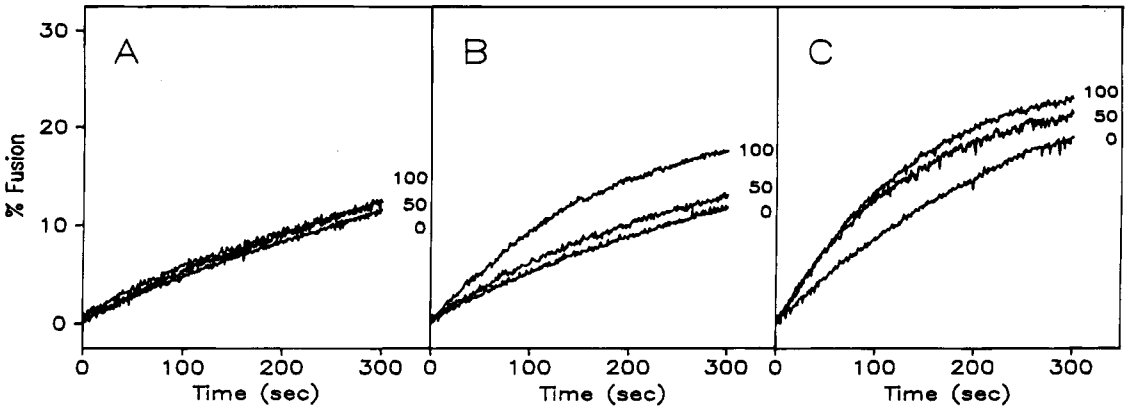



FIGURE 4: Effect of polymerization on fusion of 120 nm LUV composed of DOPE/mono-SorbPC (3:1) at 50 °C. Panel A shows fusion in the presence of 5 mM $Mg^{2+}$ at pH 7.5. Panel B shows fusion at pH 4.5. Panel C shows fusion in the presence of 20 mM $Mg^{2+}$ at pH 9.5. Conditions otherwise are identical to those given in Figure 3.

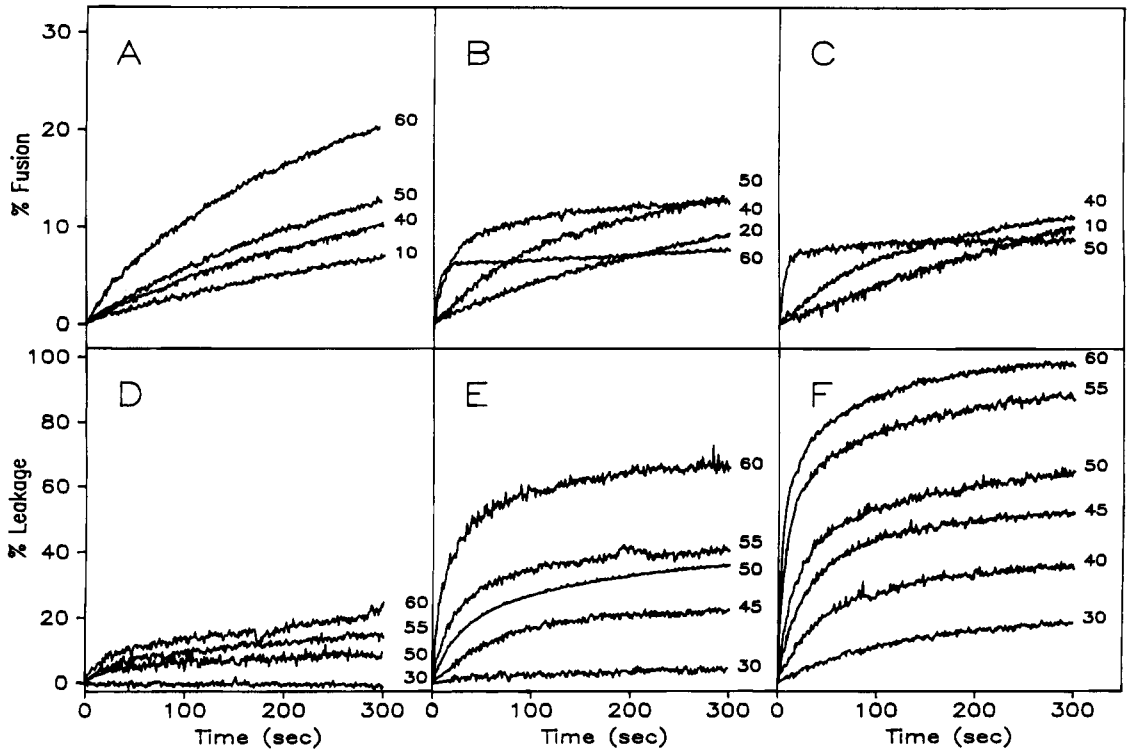



FIGURE 5: Temperature dependence of fusion and leakage of 120 nm LUV composed of DOPE/bis-SorbPC (3:1) at pH 4.5 and at different extents of polymerization. Panels A–C are for the fusion of unpolymerized ANTS-containing LUV combined 1:9 at 300 $\mu$M with DPX LUV whose bis-SorbPC component is either (A) unpolymerized, (B) 50% polymerized, or (C) 100% polymerized. Panels D–F are for the leakage of unpolymerized ANTS/DPX-containing LUV combined 1:9 at 300 $\mu$M with empty LUV whose bis-SorbPC component is either (D) unpolymerized, (E) 50% polymerized, or (F) 100% polymerized.

onset of rapid fusion, which is hereafter termed the critical fusion temperature, was nearly 65 °C for the dark-adapted LUV. But after 50% photopolymerization, the critical fusion temperature was reduced to 45 °C, and upon complete photopolymerization it was 40 °C. Therefore, at temperatures between 40 and 60 °C, photopolymerization of bis-SorbPC represents a mechanism to isothermally induce liposome fusion by decreasing the critical fusion temperature by 20 to 25 °C.

Figure 7A–C shows the effect of temperature on the fusion of DOPE/mono-SorbPC (3:1) LUV under the same conditions that were used in Figure 5A–C. The initial rates of fusion versus temperature are shown in Figure 8. All the curves for the mono-SorbPC systems are shifted to higher temperature than that observed for the bis-SorbPC system shown in Figure 6. Furthermore, the effect of photopolymerization on the curves is relatively small compared to those for the bis-SorbPC system.

## DISCUSSION

The fusion kinetics of certain liposome systems, composed of a subpopulation of those lipids capable of undergoing $L_\alpha$ to $H_{II}$ phase transition that can adopt an intermediate $Q_{II}$ phase on the first heating scan, are strongly enhanced in the temperature range near, but below, $T_H$ (Ellens et al., 1986b, 1989). Precursors to the $Q_{II}$ phase can be detected by $^{31}P$ NMR as narrow isotropic resonances (Gagné et al., 1985; Ellens et al., 1989; Barry et al., 1992) and are believed to correspond to intermediate structures between the lamellar and nonlamellar phases (Siegel et al., 1989c, 1994). Several intermembrane structures have been proposed that could account for the resonances, including inverted micellar

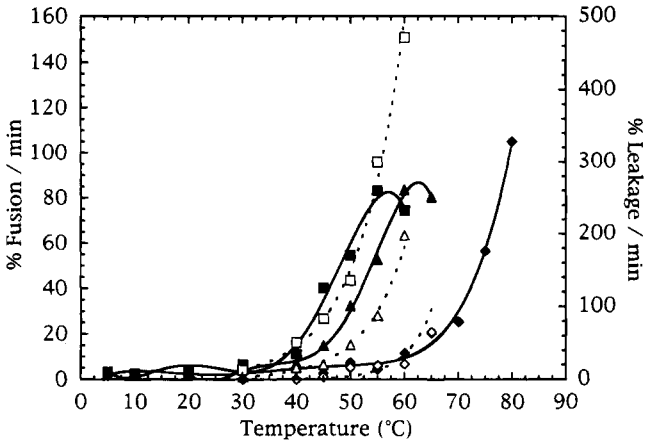



FIGURE 6: Initial rates of fusion and leakage of 120 nm LUV composed of DOPE/bis-SorbPC (3:1) at pH 4.5 and 300 $\mu$M as a function of temperature. The curves at the extreme right show the initial rate of fusion (◆) and leakage (◇) for unpolymerized liposomes. The middle curves show the initial rate of fusion (▲) and leakage (△) for unpolymerized ANTS-containing LUV (in the case of fusion) or ANTS/DPX-containing LUV (in the case of leakage) in the presence of a 9-fold excess of 50% polymerized DPX-containing LUV (fusion) or empty LUV (leakage). The extreme left curves show the initial rate of fusion (■) and leakage (□) for unpolymerized ANTS-containing LUV (fusion) or ANTS/DPX-containing LUV (leakage) in the presence of a 9-fold excess of 100% polymerized DPX-containing LUV (fusion) or empty LUV (leakage). The initial rate is the initial slope of the fusion or leakage curve, as 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.

intermediates (IMI) (Siegel, 1984, 1986a,b), stalks (Markin et al., 1984; Chernomordik et al., 1987; Siegel, 1993a), and fusion pores or interlamellar attachments (ILA) (Siegel, 1986c; Siegel et al., 1988). IMI and stalks have each been proposed as the first-formed fusion intermediates that evolve into ILA. Siegel has calculated that both IMI and stalks should have lifetimes too short to allow their observation even by fast-freezing electron microscopy techniques and that ILAs therefore are more likely to be responsible for isotropic $^{31}$P NMR signals.

Processes that trigger the phase separation of PEs from other lipids yield enriched domains of PE and modify its local phase behavior. Since PEs are significantly less hydrated than PC (Parsegian et al., 1979; Rand, 1981), the formation of domains of PE in bilayer membranes facilitates the close approach of these regions of the bilayer surfaces. Contact between bilayers is a prerequisite for bilayer destabilization and liposome fusion (Ellens et al., 1984).

The polymerization of bi- or multicomponent liposomes has been usefully employed to form domains of enriched lipids for the insertion of transmembrane proteins into partially polymerized liposomes (Tyminski et al., 1985, 1988), for the efficient photoinduced destabilization of oligolamellar liposomes (Frankel et al., 1989; Lamparski et al., 1992), and for the enhancement of energy transfer between membrane surface-bound dyes (Armitage et al., 1993). In each of these cases, the covalent linking of the polymerizable lipids separates the lipids into polymeric and monomeric domains.

Previously, we demonstrated that 254 nm exposure of DOPE/bis-SorbPC (3:1) oligolamellar liposomes at pH 7.4 and 25 °C resulted in leakage of the self-quenched marker calcein (Lamparski et al., 1992). The leakage of calcein was attributed to photopolymerization of bis-SorbPC resulting, in enriched domains of DOPE that facilitated the formation of fusion pores between the bilayers comprising the oligolamellar liposomes. Photoinduced leakage could be readily observed when 30−50% of the bis-SorbPC was photopolymerized. A $^{31}$P NMR temperature study of the effect of photopolymerization on the phase behavior of DOPE/bis-SorbPC (3:1) membranes showed a progressive change from a lamellar signal to an isotropic signal upon single slow heating of the hydrated sample (Barry et al., 1992). A completely isotropic NMR signal was observed at 61.9 °C. This signal was ascribed to a precursor of an inverted cubic phase, which upon thermal cycling became a well-defined *Pn3m* cubic phase. The temperature, $\Delta T_I$, for the initial appearance of the isotropic signal decreased with increasing extents of photopolymerization of the bis-SorbPC. If the isotropic signal signifies the presence of ILAs or other precursors of the cubic phase, then the temperature for the first appearance of these intermediate lipid structures is decreased by the photolysis of the membranes. Since an ILA constitutes a permeability pore through two bilayers that were originally distinct, the temperatures for the onset of ILA formation and measurable liposome fusion or leakage should be similar. In fact, the data reported here (Figure 6) show good agreement between the critical fusion temperature and $\Delta T_I$ (Table 2).

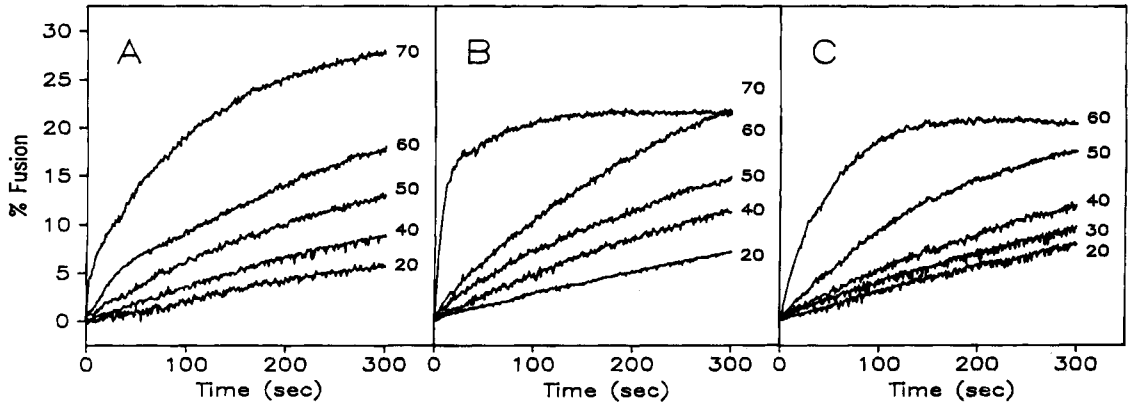



FIGURE 7: Temperature dependence of fusion of 120 nm LUV composed of DOPE/mono-SorbPC (3:1) at pH 4.5 and different extents of polymerization. Panels A−C are for the fusion of unpolymerized ANTS-containing LUV combined 1:9 at 300 $\mu$M with DPX-LUV whose bis-SorbPC component is either (A) unpolymerized, (B) 50% polymerized, or (C) 100% polymerized. No leakage data are shown on this plot.

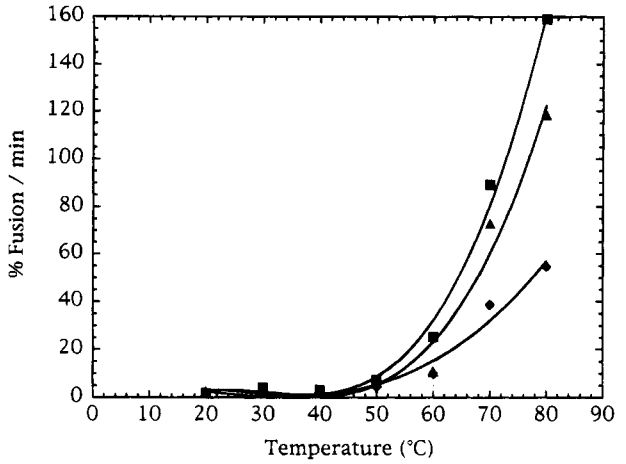



FIGURE 8: Initial rates of fusion of 120 nm LUV composed of DOPE/mono-SorbPC (3:1) at pH 4.5 and 300 $\mu$M as a function of temperature. The curve at the extreme right shows the initial rate of fusion (◆) for unpolymerized liposomes. The middle curve shows the initial rate of fusion (▲) for unpolymerized ANTS-containing LUV in the presence of a 9-fold excess of 50% polymerized DPX-containing LUV. The extreme left curve shows the initial rate of fusion (■) for unpolymerized ANTS-containing LUV in the presence of a 9-fold excess of 100% polymerized DPX-containing LUV. The initial rate is the initial slope of the fusion or leakage curve, as in Figure 7.

Table 2: Effect of bis-SorbPC Photopolymerization on the Temperature at Which Isotropic $^{31}$P NMR Resonances Are First Observed in DOPE/bis-SorbPC (3:1) Multilamellar Suspensions

| % loss of monomer | DOPE/monomeric PC[a] | $\Delta T_I$[b] (°C) |
|---|---|---|
| 0 | 3.0:1 | 48.6−61 |
| 33 | 4.3:1 | 44.3−57 |
| 51 | 6.1:1 | 42.2−55 |
| 90 | 31:1 | 37.5−38.7 |

[a] Values calculated for the total lipid present in the system. [b] Data are from Barry et al. (1992).

The evolving understanding of liposome fusion has focused attention on the formation of lipid structures intermediate between the lamellar and nonlamellar phases. Since many fusion-competent lipid systems exhibit both inverted cubic ($Q_{II}$) and inverted hexagonal ($H_{II}$) phases at appropriate concentrations and temperatures, it has been difficult to unambiguously ascribe fusion to the onset of either the $Q_{II}$ or $H_{II}$ phase. Since the DOPE/bis-SorbPC (3:1) lipid system has been observed to exhibit lamellar and inverted cubic phases but not an $H_{II}$ phase (Barry et al., 1992), the observed fusion described here cannot be due to the onset of the $H_{II}$ phase. The phase behavior and fusion characteristics of the DOPE/bis-SorbPC (3:1) membranes provide unequivocal evidence that liposome fusion is mediated via intermediates associated with the lamellar to $Q_{II}$ phase transition rather than to the $H_{II}$ phase.

In the present study, photopolymerization of LUV composed of either DOPE/bis-SorbPC (3:1) or DOPE/mono-SorbPC (3:1) was employed to induce the fusion of the bilayers of distinct LUV populations. LUV were prepared at pH 9.5, where the PE component is negatively charged due to deprotonation of the amino group of the ethanolamine head group ($pK = 9.5$) (Stollery & Vail, 1977). Electrostatic repulsion between LUV at pH 9.5 prevents their aggregation and allows the experimental separation of bilayer polymerization and LUV fusion. Aggregation and membrane contact between liposomes was initiated by neutralizing the LUV with either $H^+$ or $Mg^{2+}$. These experiments compared the extent of interaction between LUV populations, as evidenced by lipid mixing, aqueous content mixing, and aqueous content leakage, where neither population was photopolymerized or where just one population was photopolymerized to varying extents. The behavior of two-component LUV containing either bis-SorbPC or mono-SorbPC was studied to evaluate the importance of either a cross-linked or linear polymer matrix, respectively, to the extent of lipid phase separation and subsequent bilayer interaction.

Fusion of liposomes has been described by a mass action kinetic model in which the first step is the aggregation of two stable vesicles to form a dimer, and the second step is the actual fusion process that produces the fusion product (Bentz et al., 1983). The latter step includes destabilization of the two vesicles and their communion via the fusion process. The kinetics of the overall fusion process depends on the rates of both the aggregation and the fusion event. In applying this model to a given system, the initial rates of fusion are assumed to reflect only the initial formation of fused dimers. The temperature dependence of the kinetics of destabilization of membranes following aggregation has been correlated with the nonlamellar phase transition temperature of the lipid mixture composing the area of contact. Bentz et al. (1987) demonstrated that the $L_\alpha/H_{II}$ phase transition was relevant to the destabilization of asymmetric membranes, even if only one of the membranes was composed of $H_{II}$-competent lipids. The authors compared the abilities of different PE-containing liposomes to destabilize one another when the temperature was above the $T_H$ of the lipids composing one liposome population and below the $T_H$ of the other population. The kinetics of destabilization of $L_\alpha$-competent membranes by $H_{II}$-competent membranes increased as the $T_H$ of the $H_{II}$-competent membranes decreased. The rates of leakage from $L_\alpha$-competent membranes were particularly enhanced at temperatures near the $T_H$ value associated with the lipid mixture comprising the area of contact between the two membranes.

Our results support the hypothesis that the relevant parameter controlling the rate of fusion of apposed membranes is the nonlamellar phase transition temperature of the lipids in the area of contact. In the photolyzed fusion assays, aqueous content mixing can be observed only upon contact of a dark-adapted ANTS LUV with a photopolymerized DPX LUV. Within the area of contact between these asymmetric membranes, we believe that the polymerized PC would be largely excluded. Hence, the lipid mixture in the contact region is likely to be composed of the average ratio of DOPE to monomeric PC in the aggregated heterodimer. Where the DPX LUV are 50% photopolymerized, this ratio is ca. 4:1; where the DPX LUV are completely photopolymerized, it is ca. 6:1. The corresponding onset $\Delta T_I$ values are 44.3 and 42.2 °C, respectively (Table 2). A subtle difference exists between the fusion and leakage assays. In the leakage assays, contact between the dark-adapted ANTS/DPX LUV and any other LUV can result in leakage. The temperature dependence for any leakage resulting from contact between two ANTS/DPX LUV must follow the curve at the extreme right of Figure 6. Since the ANTS/DPX LUV represent only 10% of the LUV, this type of leakage should make only a small contribution to the overall temperature dependence in the photolyzed assays. The occurrence of leakage resulting from the formation of homodimers of ANTS/DPX LUV might

explain why the photolyzed leakage curves are shifted to higher temperatures than the fusion curves.

Photopolymerization of DOPE/bis-SorbPC membranes is proposed to induce fusion by facilitating both aggregation and subsequent fusion. Polymerization induces lipid phase separation with consequent decreased hydration of PE-rich domains and decreased $R_0$ (see the following) of the lipids composing the PE-rich domains. Since the monomeric domains contain only small fractions of PC compared to the polymeric PC domains, the surface hydration of these regions is diminished. The decreased intrinsic radius of curvature, $R_0$, of the lipids composing the PE-rich domains (Gruner, 1985, 1989) is a consequence of both the increased ratio of DOPE to monomeric PC (see Table 2) and the decreased membrane hydration. Membrane adhesion of these membranes is promoted since repulsive hydration forces are weaker and attractive van der Waals forces are stronger in the PE interfaces than in the PC interfaces. After adhesion, the formation of fusion intermediates is enhanced by the decreased $R_0$ of the lipids composing the apposed leaflets of the membranes.

The crucial question in this work was how the different extents of photopolymerization of one liposome population, at a given temperature, affect the stability of an aggregated heterodimer of LUV at the site of bilayer contact and whether the extent of destabilization could be used to control whether fusion or leakage ensues. The critical temperature for fusion of the DOPE/bis-SorbPC (3:1) LUV was shifted from ca. 65 °C for dark-adapted LUV to ca. 45 or 40 °C for 50% or 100% photopolymerized LUV, respectively. The change in the temperature threshold for leakage was similar. The effectiveness of photopolymerization in decreasing the fusion threshold of these LUV by 20 °C to 25 °C provides a potentially useful strategy for triggering the fusion of liposomes with cellular membranes at physiological pH. Consider, for example, the fusion of liposomes with endosomal membranes that have a relatively high content of PC. These circumstances would probably shift the fusion threshold to higher temperatures. The composition of the photosensitive liposomes could be changed in a variety of ways to lower the critical fusion temperature with PC-rich membranes to 37 °C. These include a reduction in the proportion of the polymerizable PC, bis-SorbPC, or a change in the chain length of the SorbPC, which is known to alter its main phase transition temperature, $T_m$ (Lamparski et al., 1993). The polymorphic component (e.g., DOPE) could be replaced by a lipid exhibiting a lower $T_H$ (e.g., plasmenylethanolamine). Recently, Glaser and Gross (1994) reported that the substitution of plasmenylethanolamine for PE promoted calcium-induced fusion between liposomes also containing PC and PS. Finally, the critical temperature for the onset of fusion could be modified by the addition of other lipids, e.g., diacylglycerol (DAG), to the photosensitive LUV.

The photoactivated polymerization of mono-SorbPC produces only short linear oligomers of 3−10 repeat units, and in this case the photoreaction does not necessarily produce a dramatic phase separation of the membrane components. Although the polymer size produced by photopolymerization of bis-SorbPC is unknown, it formed cross-linked polymers. The size of the polymer domains may be as large as the square of the linear oligomers formed from mono-SorbPC. Regardless of the polymer domain size, subtle changes in the composition of membranes have previously been shown to exert dramatic influences—both inductive and inhibitive—on membrane fusion. Siegel and co-workers (Siegel et al., 1989a,b) showed that just 2 mol % of DAG was sufficient to decrease the $T_H$ value of *N*-monomethyl-DOPE (DOPE-Me) membranes by ca. 15 °C. The temperature of the onset of the appearance of isotropic resonances was lowered by a similar extent, and a sharp increase in the fusion rate accompanied the addition of DAG. Addition of either DAG or hexadecane to phosphatidylserine vesicles was also shown to increase the rates of divalent cation-induced vesicle fusion (Walter et al., 1994). Chernomordik et al. (1993) reported that lysoPC at subsolubilizing concentrations inhibited $Ca^{2+}$-, GTP-, and pH-dependent biological fusion processes, and Yeagle et al. (1994) demonstrated that very low concentrations of lysoPC (1−9 mol %) inhibited the fusion and leakage of liposomes composed of DOPE-Me. In these studies, the authors attribute the effect of the low-level perturbants on the rates of fusion to their abilities to either stabilize or destabilize the formation of stalk structures (Siegel, 1993b; Siegel et al., 1994).

The potential utility of photosensitive liposomes depends on the number of photons per liposome required to modify the bilayer properties. This value is the quotient of the number of lipids per liposome that must react to produce the required change in the liposome properties and the quantum yield of the photoreaction. The photoactivated polymerization of bis-SorbPC has a quantum yield of 0.25. Hence, reaction of 50% of the polymerizable lipids in 120 nm diameter DOPE/bis-SorbPC (3:1) LUV (composed of ca. $1.3 \times 10^5$ lipids) requires ca. $8 \times 10^4$ photons per liposome. Although this appears to be a relatively large number, it is readily achieved in 1 min or less under the experimental exposure conditions used here, which are well within the range used in photodynamic therapy.

Much higher efficiencies for the photopolymerization of liposomes may be possible by the use of chain polymerization rather than the stepwise UV-photoactivated polymerization used in these studies. Radical-initiated polymerization of mono-SorbPC or bis-SorbPC liposomes proceeds via a chain reaction and produces high molecular weight polymers having repeat units numbering from 50 to 600 (Lamparski & O'Brien, 1995). Hence, radical chain polymerization of either DOPE/bis-SorbPC or DOPE/mono-SorbPC LUV should effect more dramatic changes in the membrane properties than photoactivated polymerization. Consequently, large shifts in the initial rates of fusion versus temperature would be expected at low conversion of bis-SorbPC to polymer. This is particularly important because the photosensitized radical polymerization of bis-SorbPC has been achieved using membrane-bound cyanine dyes and visible light (Armitage et al., 1994). This polymerization method and its effect on the stability of liposomes composed in part of SorbPC lipids will be reported in due course. Thus, visible light-induced fusion of DOPE/bis-SorbPC liposomes under physiologically relevant conditions is possible using photosensitized polymerization.

These studies of the photoactivated fusion of liposomes demonstrate a significant enhancement in the fusion of LUV. The results complement the previous demonstration of photoinduced destabilization of oligolamellar liposomes. Both methods rely on the temporal and spatial characteristics of light to deliver reagents from liposomes to other bilayer-

bound structures in the case of LUV or to release reagents to the aqueous medium surrounding MLV.

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