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Modulation of Poly(ethylene glycol)-Induced Fusion by Membrane Hydration: Importance of Interbilayer Separation†

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ABSTRACT: Large unilamellar vesicles composed of lipids with different hydration properties were prepared by the extrusion technique. Vesicles were composed of dioleoylphosphatidylcholine in combination with either 0.5 mol % monooleoylphosphatidylcholine or different molar ratios of dilauroylphosphatidylethanolamine. Fusion was revealed via a fluorescence assay for contents mixing and leakage, a fluorescent lipid probe assay for membrane mixing, and quasi-elastic light scattering to detect vesicle size growth. As the percentage of poorly hydrating phosphatidylethanolamine increased, the concentration of poly(ethylene glycol) (PEG) required to induce fusion decreased. From differential scanning calorimetry studies of membrane-phase behavior and X-ray diffraction monitoring of phase structure in PEG, it was concluded that PEG did not induce a hexagonal-phase transition or lamellar-phase separation. Electron density profiles derived from X-ray diffraction studies of multi- and unilamellar vesicles indicated that the water layer between vesicles had a thickness of approximately 5 Å at PEG concentrations at which vesicles were first induced to fuse. At this distance of separation, the choline headgroups from apposing bilayers are in near-molecular contact. Since pure phosphatidylcholine vesicles did not fuse at this interbilayer spacing, a reduction in the interbilayer water layer to a critical width of ~2 water molecules may contribute to but is not sufficient to produce PEG-mediated fusion of phospholipid membranes. Comparison of these results with other results from this laboratory also indicates that, while close contact between bilayers promotes fusion, near-molecular contact is apparently not absolutely necessary to bring about fusion. A tentative model is presented to account for these results.

It is well recognized that membrane fusion is important to numerous biological processes. In order to understand the mechanism behind these cellular processes, researchers have used model membrane systems to probe the detailed mechanism of the fusion event. While model membranes may not perfectly mimic their cellular counterparts, they should still provide insight into basic structural events necessary for membrane fusion. Among the structural events thought to contribute to fusion, bilayer dehydration (Wilschut et al., 1985b), local alterations in bilayer curvature (Nir et al., 1982; Lentz et al., 1987), changes in membrane "fluidity" (Wilschut

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et al., 1985a; Düzgünes et al., 1987), bilayer-phase separation (Hoekstra, 1982), and locally induced nonbilayer phases (Ellens et al., 1989) are a few that have been proposed. These studies have yielded significant information and greatly extended our understanding of the fusion process.

The short-range interactions of contacting membranes are controlled by the hydration forces acting between the lipid hydrophilic surfaces (LeNeveu et al., 1976, 1977; Marra & Israelachvili, 1985). The role of membrane hydration in fusion has been stressed in earlier model membrane studies (Hoekstra, 1982; Rupert et al., 1986), and overcoming the hydration repulsion between membranes in contact has been identified as a possible initial step in the fusion process (Rand & Parsegian, 1988). However, a crucial factor defining the role of membrane hydration in fusion, namely, the dimensions and nature of the fluid space at the point of fusion, has yet to be determined. If we are to understand fully the mechanism of fusion on a molecular scale, we must consider all the components of the destabilized intermediate state of membrane fusion, including the water layer.

We report here an examination of the relationship between poly(ethylene glycol) (PEG)[1]-mediated model membrane fusion and the dehydrated complex induced by PEG, using X-ray diffraction to monitor the fluid space between contacting membranes (McIntosh & Simon, 1986a) and established methods for determining lipid mixing, contents mixing, and size growth to monitor fusion (Parente & Lentz, 1986; Burgess & Lentz, 1992; Ellens et al., 1984, 1985; Lentz et al., 1992). Our goals were (i) to determine whether fusion occurred at a critical bilayer separation or critical osmotic stress, (ii) to quantitate the amount of water associated with the membrane in the intimately contacting intermediate state of fusion, and (iii) to define the nature of the water in the fusion intermediate. To achieve these goals, we selected three lipid systems which would be expected to have different hydration properties. The concentrations of PEG required to induce fusion in the three systems were determined, as was the fluid spacing between membranes in the presence of PEG. Our results provide evidence that fusion of simple phospholipid membranes is promoted when they come close to a common bilayer separation in which most of the water is removed and phospholipid headgroups from adjacent bilayers come into molecular contact. However, attaining this interbilayer separation appears to be neither a necessary nor a sufficient condition to induce fusion.

Experimental Procedures

Materials

Chloroform stock solutions of 1,2-dilauroyl-3-*sn*-phosphatidylethanolamine (DLPE),[1] 1,2-dioleoyl-3-*sn*-phosphatidylcholine (DOPC)[1] and 1-palmitoyl-2-[[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]oxy]carbonyl]-3-*sn*-phosphatidylcholine (DPHpPC)[1] were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). 1-Oleoyl-*sn*-glycero-3-phosphocholine (lysoPC)[1] was obtained from Sigma. Lipids were verified to be greater than 98% pure by thin-layer chromatography on Analtech GHL plates. Plates were developed in a 65:25:4 (v/v/v) $CHCl_3/CH_3OH/H_2O$ mixture and were stained with iodine vapors. DPHpPC was also viewed under near-UV light. The fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS)[1] and its quencher *N*,*N*′-xylylenebis(pyridinium bromide) (DPX)[1] were purchased from Molecular Probes (Junction City, OR). Both ANTS and DPX solutions were stored at 4 °C. DPX solutions were filtered after preparation through 0.22-μm GS filters (Millipore Corp., Bedford, MA) to remove a small amount of insoluble material. Poly(ethylene glycol) (average molecular weight 8000) was obtained from Fisher Scientific (lot 874229). Dodecyl octaethylene glycol monoether ($C_{12}E_8$)[1] and *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES)[1] were purchased from Calbiochem. Buffer solutions were filtered prior to use through a Nalgene disposable filter to remove dust which might interfere with the fluorescence measurements. All other chemicals were of the highest quality available.

Methods

Vesicle Preparation. Large unilamellar extrusion vesicles (LUVET)[1] were prepared by the method of Hope et al. (1985). Lipid was dissolved in cyclohexane and the solvent removed under vacuum. Dried lipid samples were suspended in a buffer at a temperature above the gel- to liquid-crystalline-phase transition. The large multilamellar vesicles which formed were allowed to equilibrate and fully hydrate above their phase transition for approximately 30 min. Vesicles containing DLPE were incubated at pH 8.5 to ensure proper hydration. The vesicles were then forced 7 times through a 0.1-μm polycarbonate filter (Nucleopore) above their phase transition under a pressure of approximately 200 psi of argon. For DOPC/lysoPC vesicles, this procedure yielded a fairly homogeneous population of unilamellar vesicles with a mean diameter of ~1200 Å (mean diameter determined by quasi-elastic light scattering, QELS).[1] DLPE/DOPC vesicles were found to have a mean diameter of ~1300 Å by QELS and to be bi- or trilamellar (trapped volume, 1.05 μL/μmol of lipid).

The concentrations of all vesicle samples were determined by phosphate analysis using a modification of the procedure of Chen et al. (1956). For lipid mixing experiments, vesicles were prepared in 2 mM TES buffer containing 100 mM NaCl and 1 mM EDTA,[1] pH 7.4 (pH 8.5 for DLPE/DOPC vesicles). DLPE/DOPC vesicles were adjusted to pH 7.4 following extrusion. For the contents mixing experiments, vesicles were prepared in buffers containing 25 mM ANTS (or 90 mM DPX), 40 mM NaCl, and 10 mM TES, pH 7.4 (pH 8.5 for DLPE/DOPC vesicles). For contents leakage experiments, the buffer contained 12.5 mM ANTS, 45 mM DPX, 40 mM NaCl, and 10 mM TES, pH 7.4 (pH 8.5 for DLPE/DOPC vesicles). Vesicles were eluted from a Sephadex G-75 column (0.8 × 10 cm) with 2 mM TES buffer containing 100 mM NaCl and 1 mM EDTA, pH 7.4 (pH 8.5 for DLPE/DOPC vesicles), to remove untrapped ANTS or DPX immediately before use. DLPE/DOPC vesicles were adjusted to pH 7.4 following elution from the column. Osmolarities of all buffers were monitored using a μOsmette freezing point depression microosmometer (Precision Systems, Sudbury, MA). In this way, the osmotic strengths of the trapped and extravesicular buffers were assured to be equal.

Fluorescence. All fluorescence measurements were made on an SLM 4800 spectrofluorometer (Urbana, IL) equipped

[1] Abbreviations: PEG, poly(ethylene glycol); ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt; DPX, *N*,*N*′-xylylenebis(pyridinium bromide); TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid tetrasodium salt; $C_{12}E_8$, dodecyl octaethylene glycol monoether; DLPE, 1,2-dilauroyl-3-*sn*-phosphatidylethanolamine; DOPC, 1,2-dioleoyl-3-*sn*-phosphatidylcholine; lysoPC, 1-oleoyl-*sn*-glycero-3-phosphocholine; DPHpPC, 1-palmitoyl-2-[[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)-phenyl]ethyl]oxy]carbonyl]-3-*sn*-phosphatidylcholine; LMV, large multilamellar vesicle(s); LUV, large unilamellar vesicle(s); LUVET, large unilamellar vesicle(s) made by the rapid extrusion technique; DSC, differential scanning calorimetry; DOG, dioleoylglycerol.

with a modified, three-position, multitemperature cuvette holder (Barrow & Lentz, 1985) and 200-W Hg–Xe or 150-W Xe arc lamps mounted horizontally in a Photon Technology International (Princeton, NJ) lamp housing. The 366-nm mercury line was used to excite DPHpPC for lifetime measurements, while emission was monitored through a 3-mm high-pass KV-450 filter (50% transmittance at 450 nm; Schott Optical Glass, Duryea, PA). Vertically polarized and modulated light from the Pockel cell was rotated to 35° from vertical by placing a Soliel-Babinet compensator (Karl Lambrecht, Chicago, IL) rotated 17.5° from vertical and set for half-wave at 366 nm in the excitation path. This allowed us to excite with linearly polarized light at the magic angle so as to avoid errors in lifetime calculations without the loss of intensity inherent in the use of polarizers.

Fluorescence intensity measurements of ANTS were made at an excitation wavelength of 384 nm using the 150-W Xe arc lamp. Emission was observed through a 2-mm OG-515 filter (50% transmittance at 515 nm; Schott Optical Glass).

Lipid Mixing Assay. Mixing of membrane components induced by PEG was demonstrated by using the DPHpPC fluorescent lifetime lipid mixing assay (Parente & Lentz, 1986). A detailed description of the assay is found in Burgess and Lentz (1992). Briefly, vesicles containing a high surface concentration of DPHpPC were mixed with vesicles free of probe, and the transfer of probe between vesicle populations was monitored through the resulting increase in probe fluorescence lifetime. Once the calibration curves were established for a particular lipid system (Burgess & Lentz, 1992), probe vesicles (containing 10 mol % DPHpPC) and blank vesicles (in the ratio of 1:10 probe to blank) were injected into a cuvette containing a solution of PEG. The phase angle of the sample was continuously monitored for 30 min or until change had ceased. The phase angle of an isochronal reference fluorophore at 23 °C (DPH in heptane; $\tau = 6.75$ ns, 2×10^{-7} M; Barrow & Lentz, 1983) was measured before and after the time-course to determine the average phase angle of the reference (there was no change over the time period of the experiment), and the sample phase angles were converted to lifetimes using the method of Spencer and Weber (1969). The average asymptotic lifetime was compared to the calibration curve to obtain an average lipid:probe (L:P) ratio for the sample. The data are presented as the percent of change in lipid:probe ratio expected for complete lipid mixing, 11% being the value expected for one "ideal" (i.e., uniform) round of fusion (each probe vesicle fusing with a probe free vesicle). The standard error (0.05 ns) associated with the measured lifetime of a given lipid mixing experiment was used to determine the error in the extent of lipid mixing for each PEG concentration.

ANTS/DPX Assay for Contents Mixing and Leakage. We used the ANTS/DPX assay (Ellens et al., 1984, 1985) to determine the extent of internal aqueous contents leakage and mixing. A detailed description of the modified assay and of controls essential for monitoring PEG-induced fusion is given in Lentz et al. (1992). For the leakage assay, ANTS and DPX were coencapsulated in one vesicle population (0.5 mM final lipid concentration) such that DPX quenched over 85% of ANTS fluorescence. When leakage occurred, there was an increase in fluorescence due to dilution of ANTS and DPX in solution and loss of quenching efficiency. For the contents mixing assay, equal volumes of 0.25 mM ANTS-containing vesicles and 0.25 mM DPX-containing vesicles were used. Mixing of vesicle contents exposed ANTS to DPX at quenching concentrations and resulted in a loss of fluorescence. It is important to note that the concentrations of vesicles used were such that leakage of vesicle contents did not result in a loss of ANTS fluorescence, making this a rigorous assay for vesicle fusion. The observed fluorescence change associated with contents mixing was processed as described in detail in Lentz et al. (1992) to obtain the percentage of the fluorescence change expected for one "ideal" round of fusion (i.e., every ANTS vesicle fusing with a DPX vesicle). One ideal round of fusion results in the largest possible fluorescence change. Thus, it is not surprising that small values (12–25%) are routinely observed and that a value of 100% is never observed.

Calorimetry. Differential scanning calorimetry (DSC)[1] measurements of the phase behavior of extrusion vesicles were performed using an MC-2 high-sensitivity calorimeter (Microcal, Inc., Northampton, MA). Samples were degassed for at least 45 min prior to being loaded in the calorimeter. Scans performed in the presence of PEG required PEG at the same concentration to balance the heat capacity of the reference cell. The scan in the absence of PEG was done in the buffer described above for lipid exchange experiments. The scan rate for all experiments was 45 °C/h.

X-ray Diffraction. Multilamellar vesicles and LUVET were prepared as described above using 10–15 mg of lipid for each sample. Samples were placed in PEG solutions and allowed to equilibrate overnight. Vesicle/PEG suspensions were centrifuged in a microfuge for less than 2 min to concentrate the vesicles. In the presence of PEG, the vesicles floated to the top of the tube, while a loose pellet was formed after more extensive centrifugation in buffer in the absence of PEG. The concentrated vesicle suspensions were sealed in quartz glass capillary tubes, which were mounted in a point-collimation X-ray camera. Since PEG is excluded from the membrane surface (Arnold et al., 1990), it withdraws water from the membrane surface, thereby exerting an osmotic pressure that forces the lipid bilayers together (Evans & Needham, 1988). The applied osmotic stress for each PEG concentration was calculated from the expression given in Parsegian et al. (1986). It should be noted that the low centrifugation force used in sample handling would not contribute significantly to the osmotic stress on a vesicle. In confirmation of this, the X-ray scattering pattern and lamellar repeat period were not altered as a result of centrifugation.

For controlled vapor pressure experiments, a drop of vesicles in buffer was applied to a narrow strip of aluminum foil and allowed to dry. The aluminum foil substrate was given a convex curvature and placed in a chamber kept at a controlled relative humidity by equilibration with a saturated salt solution (McIntosh et al., 1987). Vapor pressures of 0.93 and 0.86 were used in our experiments. The applied osmotic pressure at these vapor pressures was calculated according to Parsegian et al. (1986). The specimen was mounted on a line-focused single-mirror X-ray camera so that the X-ray beam was oriented at a grazing angle relative to the oriented multilayers.

For all samples, X-ray diffraction patterns were recorded on Kodak DEF 5 X-ray film. DOPC/lysoPC vesicles were recorded at 23 °C, and DLPE/DOPC vesicles were recorded at 30 °C. Films were processed by standard techniques, and the extent of exposure of individual reflections was determined with a Joyce-Loebl Model MKIIIC microdensitometer. For the unoriented samples, the densitometer trace was taken in a radial direction from the center of the film, whereas for the oriented samples the trace was taken through the center of each reflection. After background subtraction, integrated intensities, $I(h)$, were obtained for each order h by measuring the area under each diffraction peak. For unoriented patterns,

the structure amplitude, $F(h)$, was set equal to $[h^2I(h)]^{1/2}$ (Blaurock & Worthington, 1966; Herbette et al., 1977). For the oriented, line-focused patterns, there was no appreciable arcing of the reflections, which were of uniform height. In this case, the intensities were corrected by a single factor of h due to cylindrical curvature of the multilayers (Herbette et al., 1977), so that $F(h) = [hI(h)]^{1/2}$. The validity of this correction factor for this specimen geometry has been demonstrated previously (McIntosh et al., 1987).

Electron density profiles on a relative electron density scale were calculated from

$$\rho(x) = (2/d)\sum_h \exp[\phi(h)]F(h) \cos(2\pi xh/d) \quad (1)$$

where d is the lamellar repeat period and $\phi(h)$ is the phase angle for each order h. Electron density profiles were calculated at a resolution of $d/2h_{max} \cong 7$ Å. Phase angles were determined from a sampling theorem analysis as described in detail previously (McIntosh & Holloway, 1987).

Quasi-Elastic Light Scattering (QELS).[1] After treatment with PEG, vesicles for light-scattering measurements were separated from the polymer by elution from a Pharmacia Sepharose CL 4B (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) gel filtration column maintained at 48 °C in an incubator oven using a buffer consisting of 100 mM NaCl, 2 mM TES, and 1 mM EDTA, pH 7.4, according to the procedure described by Burgess et al. (1991a). Light-scattering measurements were made on a multiangle instrument constructed by us (Lentz et al., 1992). The heart of the instrument was a Model 170 computing autocorrelator (Particle Sizing Systems, Inc., Santa Barbara, CA). The data collection was controlled and displayed by software located in an external personal computer. The software supplied by Particle Sizing Systems permitted both a cummulants and a multi-Gaussian distributional analysis. All of the measurements performed on this instrument were obtained at an angle of 90°, and the cummulants analysis was used whenever appropriate. The results of the analyses reported here are all intensity-weighted diameters which are calculated from the Z-average diffusion coefficients. Polystyrene beads (lot 90817; Polyscience, Inc., Warrington, PA; nominal diameter 1200 Å from electron microscopy measurements) prepared in the presence of 0.06% Triton X-100 (lot 95c-0058; Sigma Chemical Co., St. Louis, MO) were measured to be 1229 ± 40 Å in diameter. The instrumental precision of measurements performed on a single sample of small unilamellar vesicles (whether this was kept in the sample cuvette or removed and replaced several times) was ± 14 Å. Measurements of diameters of different vesicle preparations were reproducible to within ±1–2% for samples that were never treated with PEG and to within ±3–4% for vesicles treated with ≤35 wt % PEG). Furthermore, the diameters we obtained for phosphatidylcholine LUVET were identical to those published in earlier reports (Mayer et al., 1986).

Results

Fusion Studies of PE Systems. Initially, we examined several lipid systems containing PE alone or mixed with other lipids. The systems included DLPE, DLPE/DOPC (95:5), and DLPE/lysoPC (99.5:0.5). In all cases, we were unable to detect mixing of internal contents of these vesicles even in the presence of 30 wt % PEG, at which point they had leaked 50–80% of their contents. The lack of fusion was confirmed for DLPE LUVET using QELS size growth measurements: these vesicles were ~1200 Å when first extruded at pH 8.5, ~1300 Å after being adjusted to pH 7.4, and ~1300 Å after being treated with 30 wt % PEG. Nonetheless, other PE-containing vesicles that we examined did fuse in the presence of PEG, namely, DLPE/DOPC (85:15), DLPE/DOPC (65:35), and DOPC/lysoPC (99.5:0.5). Detailed studies of these systems are described below.

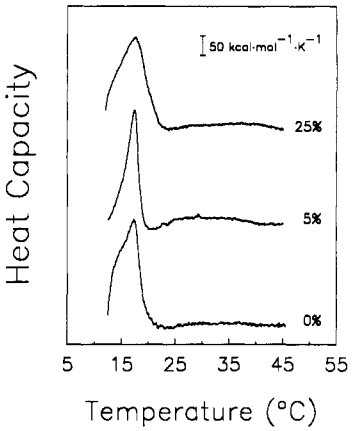

FIGURE 1: Heat capacity profile of DLPE/DOPC (65:35) LUVET in the presence of PEG. Heating scans in the presence of 0, 5, and 25 wt % PEG are shown. Lipid concentration for all scans was ~2 mM. Samples were degassed at least 45 min prior to loading. The scan rate for all experiments was 45 °C/h.

Differential Scanning Calorimetry of DLPE/DOPC LUVET. DLPE was chosen for this study because it forms a lamellar, and not a hexagonal, phase over a broad temperature range (McIntosh & Simon, 1986b). Nonetheless, our X-ray data indicated that some DLPE/DOPC samples, after long (more than 2 days) incubation below 30 °C in the presence of PEG, underwent a transition to a dehydrated crystalline phase, as previously reported (Seddon et al., 1983). The possibility of the coexistence of multiple phases had to be resolved in order to avoid ambiguities in interpretation of fusion and X-ray experiments. For this reason, DSC measurements on DLPE/DOPC LUVET were performed to confirm that DLPE/DOPC-phase separation and hexagonal-phase formation were not induced in the presence of PEG. The scans shown in Figure 1 are heating scans for DLPE/DOPC (65:35) LUVET in the presence of various concentrations of PEG. Scans were carried out from 4 to 65 °C; however, data are presented only for temperatures up to 45 °C, since all fusion and X-ray experiments were performed below 40 °C. In addition, the lowest temperature for which we can present data was 7 °C, since the instrument requires a finite time in order to reach steady state once a scan has been initiated. Despite the truncation of the low-temperature base line, these scans show no evidence of phase separation between 25 and 65 °C, effectively ruling out the possibility of hexagonal-phase formation, or dehydrated crystalline phase. If an enriched PE phase had formed under our conditions in the presence of PEG, the melting of this phase would have been detected. X-ray diffraction patterns also did not reveal any evidence of a hexagonal phase in DLPE/DOPC mixtures in the presence of PEG. Finally, Seddon et al. (1983) reported two dehydrated crystalline phases for DLPE with transitions of 36 and 43 °C. We could not detect these transitions for LUVET or large multilamellar vesicles (LMV)[1] in the presence or absence of PEG. These results show that any fusion event between DLPE/DOPC vesicles induced by PEG cannot proceed via a mechanism involving significant amounts of crystalline-, gel-, or hexagonal-phase formation. However, our results cannot rule out completely the possibility of local bilayer perturbations in the intimate membrane contact region which might support such structures.

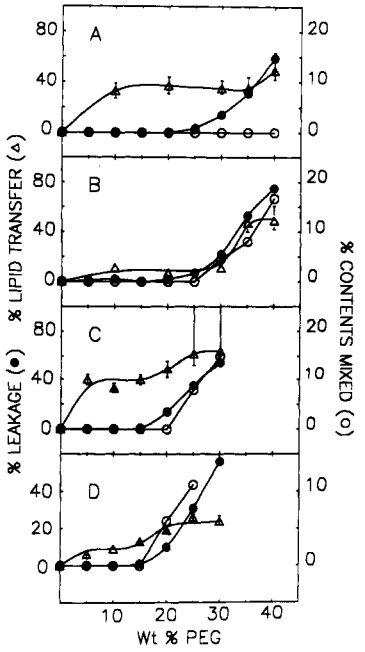

FIGURE 2: Summary of fusion assay results for extruded vesicles. Extruded vesicles composed of (A) DOPC, (B) DOPC/lysoPC (99.5:0.5), (C) DLPE/DOPC (65:35), or (D) DLPE/DOPC (85:15) were incubated in various concentrations of PEG at either 23 °C (A and B) or 40 °C (C and D), and their response was monitored using assays for contents mixing (open circles), contents leakage (closed circles), and lipid mixing (open triangles). The data in frame A are included for comparison and are adapted from Burgess et al. (1991a).

Monitoring Fusion of LUVET. Vesicles composed of DOPC, DOPC/lysoPC (99.5:0.5), DLPE/DOPC (65:35), and DLPE/DOPC (85:15) were incubated with various concentrations of PEG, and their response was monitored using established methods for lipid exchange and contents mixing/leakage. Figure 2 shows the results of these experiments. As we have previously reported (Burgess et al., 1991a), the control DOPC vesicles did not experience mixing of contents at any PEG concentration, even though substantial lipid exchange occurred and a good fraction of their contents was lost (Figure 2A). Treatment of these vesicles with PEG also failed to elicit any change in vesicle diameter (Burgess et al., 1991a). For DOPC/lysoPC (99.5:0.5) LUVET, however, contents mixing was observed at ≥35 wt % PEG (panel B). As noted under Methods, our contents mixing assay is designed to be insensitive to leakage, so that the observation of quenching of ANTS by DPX must reflect their coencapsulation in a single vesicle compartment, offering clear and irrefutable evidence for fusion of two compartments. Observation of contents mixing was accompanied by significant increases in lipid mixing and contents leakage and by a measurable increase in vesicle size (~1400–1800 Å; Burgess et al., 1991a). The correlation of observable contents mixing with a small but detectable increase in lipid transfer was evident in all three lipid systems for which fusion occurred (see panels B–D). The DLPE/DOPC (85:15) vesicles also increased in diameter from 1360 to 1830 Å after treatment with 20 wt % PEG. Contents mixing and increases in lipid mixing and contents leakage occurred for DLPE/DOPC (65:35) LUVET at PEG concentrations ≥25 wt %, and for DLPE/DOPC (85:15) LUVET at ≥20 wt % PEG. The observation of 10–20% contents mixing concomitant with an increase in lipid mixing and a small but significant increase in vesicle diameter was taken as very strong evidence for vesicle fusion. The fact that these effects were specific to certain lipid systems and did not occur for the DOPC control indicates that the fusion event was not the result of the nonspecific dehydrating influence of PEG. Finally, the PEG concentrations at which fusion occurred were different for each system (Table II).

Table I: X-ray Repeat Periods and Fluid Spaces for Lipid/PEG Suspensions

lipid	[PEG] [% (w/w)]	repeat period (Å)	fluid space (Å)
DOPC	10	56.7	9.6
	20	54.2	7.1
	30	52.4	5.3
	35	52.1	5.0
	40	51.2	4.1
DOPC/lysoPC	35	52.4	5.1
DLPE/DOPC (65:35)	0	57.1	10.8
	10	55.0	8.7
	20	51.8	5.5
	25	51.4	5.1
	30	49.7	3.4
DLPE/DOPC (85:15)	5	53.6	8.0
	15	52.3	6.7
	20	51.0	5.4

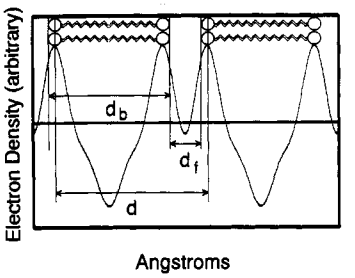

FIGURE 3: Electron density profile of DOPC/lysoPC LUVET in 35% (w/w) PEG solution, calculated as described in the text. The repeat period, d, is defined as the distance between high-density peaks corresponding to lipid headgroups in the same orientation; the bilayer thickness, d_b, is the distance between high-density peaks for the same bilayer plus 10 Å (see text), and the fluid space, d_f, is $d - d_b$.

X-ray Diffraction Studies of LUVET. LMV and LUVET of the lipid systems described above were incubated in various concentrations of PEG and their X-ray diffraction patterns recorded. For LUVET in water or low PEG concentrations (<10%), the diffraction patterns consisted of several broad bands, whereas for LMV under these conditions the diffraction patterns contained several sharp reflections which indexed as orders of a lamellar repeat period. For PEG concentrations ≥10%, the diffraction patterns for LMV and LUVET were similar, as both contained sharp lamellar reflections with the same repeat periods (Table I), although more orders of diffraction were often detectable with LMV. Electron density profiles were calculated for specimens which contained 4 or more lamellar orders of diffraction. The electron density profile for DOPC/lysoPC LUVET in 35% (w/w) PEG is shown in Figure 3. In this profile, the high-density peaks correspond to the lipid headgroups, the low-density region between these peaks corresponds to the lipid hydrocarbon chains, and the medium-density regions at the outer edges of the profile correspond to the fluid spaces between adjacent bilayers. The distance between headgroup peaks for a single bilayer was found to be 37.1 Å for DOPC, 37.3 Å for DOPC/lysoPC, 36.3 Å for 65:35 DLPE/DOPC (65:35), and 35.6 Å for DLPE/DOPC (85:15). Note that the headgroup separations for the DLPE/DOPC bilayers are between the value obtained for DOPC and the value of 33.0 Å previously obtained for liquid-crystalline DLPE (McIntosh & Simon, 1986b). We note also that sharp, LMV-like diffraction patterns were obtained from membranes that fused and from membranes that did not fuse (i.e., DOPC or the other systems below the fusing concentration of PEG).

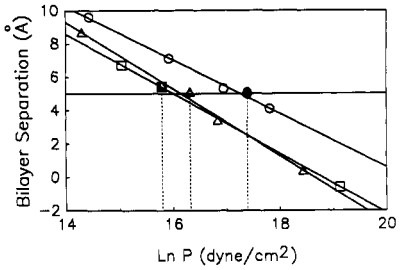

FIGURE 4: Summary of X-ray diffraction results for model membranes. DOPC (open circles), DOPC/lysoPC (99.5:0.5) (closed circle), DLPE/DOPC (65:35) (open triangles), or DLPE/DOPC (85:15) (open squares) vesicles were incubated in various concentrations of PEG and their X-ray diffraction patterns obtained. The bilayer separations were calculated from repeat periods (obtained from diffraction patterns) and bilayers thicknesses (determined from electron density profiles; see Methods and Figure 3). Dotted lines represent the natural logarithm of the osmotic pressures at which fusion was first observed for (from left to right) DLPE/DOPC (85:15), DLPE/DOPC (65:35), and DOPC/lysoPC (99.5:0.5). The horizontal solid line is for reference only, indicating a bilayer separation of 5 Å.

The electron density profiles can be used to estimate the fluid separation between adjacent bilayers. As discussed previously (McIntosh et al., 1987), the definition of fluid layer thickness is somewhat arbitrary for several reasons. First, the bilayer surface is not smooth at the level of molecular resolution (this is particularly true for bilayers containing two different types of lipid headgroups); second, the lipid headgroups are mobile; and, third, water penetrates into the headgroup region. As done in the past (McIntosh & Simon, 1986a; McIntosh et al., 1987, 1989), we define the bilayer thickness as the total thickness of the bilayer assuming the conformation of the PC headgroup is the same as it is in single crystals (Pearson & Pascher, 1979). For electron density profiles at this resolution, the edge of the bilayer is about 5 Å from the center of the headgroup peaks in the electron density profiles (McIntosh & Simon, 1986a; McIntosh et al., 1987, 1989), so that we define the bilayer thickness to be the distance between headgroup peaks in the electron density profiles plus 10 Å. We calculate the fluid spacing between bilayers to be the lamellar repeat period minus this bilayer thickness (Figure 3). The fluid spaces between bilayers for the three fusing model membrane systems examined in this study are summarized in Table I as a function of PEG concentration. DOPC vesicles, which do not fuse in the presence of PEG, were found to come to a similar bilayer separation at 35 wt % PEG (Table I) as did DOPC/lysoPC vesicles, which did fuse in the presence of 35 wt % PEG.

Figure 4 shows the fluid spacing between multilayers or aggregated vesicles as a function of the natural logarithm of the applied osmotic pressure. Osmotic pressures for which ln $P < 18$ correspond to experiments with liposomes in PEG solutions, whereas pressures for which ln $P > 18$ correspond to data obtained at defined, reduced vapor pressure. The dotted lines indicate the osmotic pressures corresponding to the PEG concentrations at which fusion was initially observed. This represents the minimum concentration of PEG needed to induce fusion in the respective systems. A summary of these data is given in Table II. As is evident, there was not a single "critical" osmotic pressure necessary to induce fusion. The osmotic pressure required to induce vesicle fusion decreased with increasing PE content (or decreasing hydration repulsion; see Table II). For each system, the fluid spacing between adjacent membranes at the PEG concentration where fusion was first observed was ~5 Å (see Table II). This suggests that, for these three systems, there is a *critical bilayer separation* that must be approached for fusion to occur. On the other hand, the data in Figure 2A demonstrate that DOPC LUVET do not fuse at PEG concentrations up to 40 wt %. This demonstrates that bringing membranes into close contact alone is not sufficient for the fusion process to occur.

Table II: Summary of Fusion and X-ray Data for Systems Studied

lipid	[PEG] for fusion (wt %)	ln P (dyn/cm^2)	fluid space (Å)
DOPC/lysoPC (99.5:0.5)	35	17.39	4.9
DLPE/DOPC (65:35)	25	16.32	5.1
DLPE/DOPC (85:15)	20	15.80	5.4

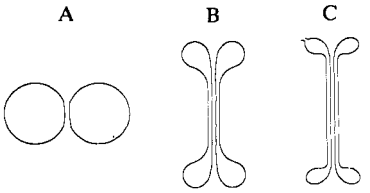

FIGURE 5: Hypothetical structure of vesicles aggregated in PEG. Vesicles suspended in low concentrations of PEG aggregate but do not deform (A). As the concentration of PEG increases, the interior of the vesicles becomes dehydrated, and membranes come into intimate contact, at which point fusion may occur (B). Leakage could occur via the distorted bilayers associated with the highly curved edges of these stacked vesicles. Additional osmotic pressure may induce increased membrane curvature, possibly resulting in vesicle rupture (C).

Discussion

PEG-Induced Bilayer Distortion, Rupture, and Relation to Fusion. X-ray diffraction patterns from LUVET in high concentrations of PEG (≥10 wt %) contain sharp lamellar reflections, indistinguishable from the patterns obtained from LMV under the same conditions. Two observations, however, suggest that PEG-induced LMV formation cannot account for the sharp patterns observed. First, our QELS data indicate that nonfusing vesicles (DOPC and DOPC/lysoPC below 35 wt % PEG) did not increase in size at concentrations of PEG for which sharp diffraction patterns were observed. Even vesicles that fused did so with a small increase in diameter (~40–60%) characteristic of one or two rounds of fusion rather than with the dramatic change in size that would be expected for formation of LMV. Second, vesicles treated with "sub-fusing" concentrations of PEG maintain their trapped volumes and their outer leaflet:inner leaflet mass ratios as detected by NMR; even fused vesicles remain substantially unilamellar (Massenburg and Lentz, unpublished results). In order to reconcile these observations with the observation of sharp diffraction patterns, we suggest that PEG dehydrates both the inside and the outside of the vesicle so that, in the presence of PEG, the fluid spaces between adjacent vesicles are the same as fluid spaces inside vesicles. If the fluid space inside the vesicles were different from that between adjacent vesicles, we would observe either much larger repeat periods (since the unit cell would contain two bilayers) or broad, undefined reflections like those obtained with LUVET at low PEG concentrations. Thus, we picture LUVET preparations in the presence of ≥10 wt % PEG to be stacks of flattened vesicles, as illustrated in Figure 5. The high radius of curvature at the ends of such flattened vesicles could cause disruption of lipid packing, leading to leakage of internal contents (Figure 5). At very high PEG concentrations, the membrane tension in these curved regions would be expected to be very great (Needham & Nunn, 1990), and could exceed the bilayer tensile strength, causing vesicle rupture (see Figure 5). The precise relationship between contents leakage, bilayer rupture,

and the fusion event remains to be established. While contents leak extensively at the PEG concentrations which induce fusion in the vesicles under study here, this is not the case in all the vesicle systems we have examined (Massenburg and Lentz, unpublished observations). Even for the systems examined here, however, leakage of contents is not complete, and vesicle contents do mix, suggesting that at least a portion of the vesicle interior is not completely dehydrated and at least some vesicles retain some contents which can mix upon fusion with other vesicles.

In addition to fusion between bilayers from adjacent vesicles, the dehydration of the inside of the vesicle by PEG could also promote fusion of bilayers from apposing walls of the same vesicle. Such intravesicle fusion events would tend to *decrease* vesicle size and increase contents leakage, but would not affect lipid mixing or contents mixing. Thus, there may be *more* membrane fusion events taking place (intravesicle as well as intervesicle fusion events) than might be predicted on the basis of lipid mixing and contents mixing assays. Supporting evidence for such an event was obtained when treating DLPE/DOPC/dioleoylphosphatidylglycerol (85:10:5) vesicles with PEG. Before exposure to PEG, these vesicles had a diameter of 1200 Å. At a PEG concentration similar to that necessary for fusion of DLPE/DOPC (85:15) vesicles, QELS repeatedly revealed a significant population (40% by intensity weighting) of smaller vesicles (950-Å diameter) whose size was consistent with intravesicle fusion of untreated vesicles. The mean vesicle diameter of the remaining 60% of the vesicles was over 3000 Å, suggesting that intervesicle fusion had also occurred. Similarly, a minor contamination of smaller vesicles (~10% intensity) was once observed in a population of DLPE/DOPC (85:15) vesicles treated with 20 wt % PEG.

Role of Membrane Hydration in Fusion. The association of water with lipid bilayers creates a large repulsive force which opposes the close approach of adjacent membranes (LeNeveu et al., 1977). The amount of work required to remove these water molecules is significant (LeNeveu et al., 1977; McIntosh et al., 1987). In fact, hydration repulsion has been suggested as a major barrier to membrane fusion (Rand & Parsegian, 1988), such that overcoming this repulsion is viewed by some to be the rate-determining step in the fusion process (Wilschut et al., 1985b). Rand et al. (1988) have reported that increasing phosphatidylethanolamine content in phosphatidylcholine membranes decreased the hydration repulsion between bilayers. In the study reported here, we have followed up on the observation of Rand et al. and have shown that increasing surface concentrations of DLPE in DOPC membranes decreased the concentration of PEG needed to induce fusion. Rather than there being a critical PEG concentration needed to induce fusion, our results have shown that vesicles containing mixtures of DLPE and DOPC can be induced to fuse when they are forced by the presence of PEG to approach each other to within a critical mean interbilayer separation of ~5 Å. McIntosh et al. (1987) have noted for egg phosphatidylcholine bilayers a distinct upward break in the pressure–distance relation at an interbilayer spacing of ~5 Å. They attributed this break to the onset of steric repulsion between the mobile lipid headgroups, which can extend 2–3 Å into the fluid space between bilayers. In short, bilayers at this 5-Å separation are essentially in molecular contact and, as such, are expected to have properties different from those of bilayers separated by a substantial water layer.

At the osmotic pressure where fusion occurred for DOPC/lysoPC (99.5:0.5) vesicles, the amount of water associated with hen egg phosphatidylcholine bilayers is reported to be ~13 water molecules/lipid (McIntosh et al., 1987). This report is based on the controlled hydration studies of Jendrasiak and Hasty (1974). We were unable to quantitate the number of water molecules per lipid for other systems because controlled hydration studies for phosphatidylethanolamine/phosphatidylcholine mixtures have not been carried out. Jendrasiak and Hasty (1974) showed that for pure phosphatidylcholine membranes, each lipid molecule has associated with it two water molecules which are tightly bound and an additional six to seven water molecules which are less tightly bound. Presumably, these molecules of water are important to membrane structural integrity, and their perturbation is expected to have consequences in terms of the structure and properties of the lipid bilayer. In this regard, the reduction of the number of water molecules between bilayers dehydrated and brought into contact by PEG apparently promotes rapid transfer of lipids between bilayers (Burgess et al., 1991b). It appears also from the altered activation thermodynamics of lipid transfer that excursions of lipid molecules into the interbilayer space become more probable for bilayers dehydrated by even low concentrations (10 wt %) of PEG (Wu & Lentz, 1991). Water molecules beyond the bound waters are presumed to have less direct effect on bilayer properties aside from the obvious effect of being necessary for the hydrophobic association of the lipid acyl chains.

A Hypothesis for the Structural Requirements of Fusion. While it appears to be necessary for membranes to come into near-molecular contact in order to fuse (see Figure 4), it should be pointed out that the membranes examined here constitute a rather special and limited set of membranes chosen specifically to display a range of hydration properties. Not all the model membranes we have studied need be brought to molecular contact in order for fusion to be observed. For instance, Lentz et al. (1992) have shown that incorporation of 0.5 mol % palmitic acid allows fusion of phosphatidylcholine vesicles at much lower PEG concentrations (20 wt %) than we have observed are necessary to fuse the DOPC/lysoPC vesicles studied here. It is highly unlikely that such small surface concentrations of palmitic acid could alter the interbilayer hydration potential, since Katsaras and Stinson (1990) have shown that up to 40 mol % palmitic acid did not appreciably change the fluid space between dipalmitoylphosphadidylcholine bilayers. Thus, the phosphatidylcholine vesicles containing 0.5 mol % palmitic acid likely fused at somewhat larger interbilayer separations (~7 Å) than the 5 Å reported here as a necessary condition for fusion of DOPC/lysoPC or DOPC/DLPE vesicles. Thus, we must conclude that, while approach to molecular contact appears to favor fusion, molecular contact seems not to be an absolute requirement for fusion.

Not only is approach to molecular contact not absolutely necessary for fusion, it seems that the close approach of apposing bilayers is not sufficient by itself to cause fusion of simple model membranes, since pure DPPC (Lentz et al., 1991), DOPC (Burgess et al., 1991a; see also Figure 2), and DLPE vesicles (see first section under Results) did not fuse at high PEG concentrations. We can conclude that membranes that do not fuse when brought into near-molecular contact lack an essential structural element that is present in membranes that do fuse. It is this structural requirement or several cooperative requirements which have been so elusive in the study of membrane fusion. In trying to define the structural requirements for fusion, it is important to emphasize that the studies we report here and elsewhere have employed model membranes composed of pure phospholipids or mixtures

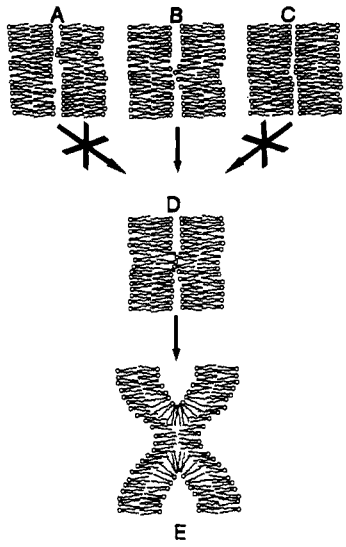

FIGURE 6: Hypothesis for the structural requirements of fusion. Membranes approaching molecular contact but without packing defects in their bilayers will not fuse. This is illustrated by structure C, which is meant to represent contacting phosphatidylcholine bilayers. Mixed-component bilayers can contain packing defects, as illustrated here in terms of mixed membranes composed of DLPE (small circles for headgroups) and DOPC (large circles for headgroups). Thermal motions of approaching bilayers can bring discrete regions into molecular contact (A), but these bilayers will not fuse unless packing defects occur at the point of contact (B). Within the context of this hypothesis, we speculate that, at the point of molecular contact, lipids from one bilayer may occupy the hydrophobic gap in the adjacent bilayer (D). This lipid penetration of adjacent bilayers could lead naturally to formation of a "single bilayer septum" (E), a structure that has been suggested previously as a likely intermediate in the fusion process (Chernomordik et al., 1987; Helm et al., 1989). The single-bilayer septum is not an essential feature of the current proposal but is shown only to illustrate the topological feasibility of the "fluctuating" contact between disrupted bilayers" proposal.

thereof. The structural requirements for fusion of these membranes may be different from those in biological membranes. The presence of glycolipids, cholesterol, and other amphipathic compounds as well as membrane proteins would be expected to affect the close approach of membranes as well as the structure of the interbilayer region at the point of close approach and fusion. Nonetheless, studies of simple membrane systems should provide insights as well as a basis for future studies of more complex biological membrane systems.

As discussed above, we have ruled out dehydration alone as being sufficient to induce membrane fusion. We have shown that large vesicles composed of pure phosphatidylcholine require small amounts of an additional amphipathic component to support fusion even in the presence of high concentrations of PEG (Lentz et al., 1992). Moderately curved, pure phosphatidylcholine vesicles, however, can be induced to fuse by treatment with somewhat lower concentrations of PEG while more highly curved vesicles probably rupture at even lower PEG concentration before they fuse (Lentz et al., 1992). We show here that binary lipid systems composed of certain mixtures of phosphatidylethanolamine and phosphatidylcholine are capable of fusing when brought into intimate contact in the presence of PEG. It is interesting that pure DLPE or DLPE/DOPC (95:5) vesicles did not fuse (see first section under Results), suggesting that bilayers rich in phosphatidylethanolamine, like pure phosphatidylcholine bilayers, do not possess the structural alterations necessary for fusion. Thus, the bilayer structural alterations necessary for fusion seem to occur in appropriate mixtures of two dissimilar phosphatides as well as in simple phosphatidylcholine bilayers either containing small quantities of appropriate amphipathic compounds or else forced into highly curved bilayers.

The nature of the structural alterations common to these various fusing systems is uncertain, but our results place some limits on the possibilities. There is no evidence for lamellar-phase separation or hexagonal-phase formation in the lipid mixtures studied here (see Figure 1). Similarly, Lentz et al. (1992) reported that amphipathic compounds that promoted fusion of DPPC vesicles in the presence of PEG did not stabilize hexagonal phases. There is nothing in our results, then, to suggest that formation of local structures related to hexagonal or other nonbilayer phases is a critical step in PEG-induced fusion of LUVET. However, our results do support the possibility that incompatible packing of two lipid species is important in PEG-mediated fusion. Thus, DOPC has an extensively hydrated headgroup of large cross-sectional area, while DLPE's headgroup is very weakly hydrated and occupies less area in the plane of the bilayer. In this regard, we found that the addition of lysoPC to DLPE membranes did not result in fusion, although the presence of lysoPC promoted fusion of DPPC and DOPC membranes (Lentz et al., 1992; Burgess et al., 1991a). This might be explained by phosphatidylethanolamine and lysoPC having compatible structural packing geometries. Phosphatidylethanolamine can be described as having a cone shape while lysoPC can be classified as having an inverted cone average structure (Israelachvili et al., 1980). These molecules would be expected to pack in a complementary fashion in the same membrane without introducing structural defects. Thus far, the only "structural defects" that seem common to the several membrane systems that we have observed to fuse in the presence of PEG are simple molecular packing mismatches. While this is a reasonable proposal on the basis of the results we have so far, more effort will be necessary to test whether this concept can be generalized and then to define more precisely the nature of the packing defects that lead to fusion.

Finally, we summarize our observations with a simple hypothesis for the molecular events leading to fusion. It may be that bilayers need only *approach* molecular contact and that the remaining gap can be bridged by thermal motions of the bilayer or of groups of lipids, resulting in transitory molecular contact between discrete regions of apposed bilayers. *The probability of fusion occurring between bilayers would, then, be proportional to both the closeness in interbilayer approach and the degree of bilayer packing disruption* induced by an appropriate perturbing influence. This hypothesis (illustrated in Figure 6) provides a possible, but certainly not an exclusive, explanation for why certain membranes can be induced to fuse by concentrations of PEG insufficient to bring about molecular contact between bilayers. This hypothesis, which is consistent with our results, is hoped to serve as a stimulus for further experimentation.

Registry No. PEG, 25322-68-3; DLPE, 59752-57-7; DOPC, 4235-95-4; lyso-PC, 19420-56-5.

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