Transbilayer Lipid Redistribution Accompanies Poly(ethylene glycol) Treatment of Model Membranes but Is Not Induced by Fusion†

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ABSTRACT: Small, unilamellar vesicles (SUV) or large, unilamellar vesicles (LUV) containing a small amount of *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) or the corresponding phosphatidylserine (NBD-PS) were made asymmetric in labeled lipid by reduction of outer leaflet probe with externally added sodium dithionite. Following removal of dithionite, transbilayer lipid redistribution (presumably due to lipid flip-flop) was indicated by a loss of fluorescence intensity upon readdition of dithionite. Vesicle rupture and fusion in the presence of PEG were measured by changes in the fluorescence of trapped Tb^{3+} complexed with dipicolinic acid (DPA) or by the increase of fluorescence from 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) coencapsulated with a quenching agent. NBD-PE redistributed slowly (∼2%/h) in all symmetrically labeled vesicles examined, while NBD-PS did not. NBD-PE redistribution was not accelerated by treatment of vesicles with PEG below concentrations that induced vesicle rupture or fusion, but was enhanced at or above these PEG concentrations. SUV prepared from hen egg yolk phosphatidylcholine (egg PC) or from dioleoylphosphatidylcholine (DOPC)/dilinolenoylphosphatidylcholine ($diC_{18:3}PC$) (85/15) mixtures were shown to fuse without rupturing in the presence of appropriate concentrations of PEG. Matching the osmolalities inside and outside the vesicle mitigated against rupture but did not prevent fusion. Under these conditions, NBD-PE flip-flop was proportional to the amount of fusion, but with different proportionality constants for the two lipid systems, while NBD-PS flip-flop did not occur. Redistribution of total mass from the outer to the inner leaflet during the fusion process could be detected in terms of a change in the ratio of dithionite-reducible probe to total probe. Both probes detected inwardly directed redistribution of lipid mass under conditions that induced fusion of SUV. We conclude that inward mass redistribution must accompany PEG-mediated SUV fusion, but that lipid flip-flop is not mechanistically related to the fusion process.

Poly(ethylene glycol) (PEG)[1] is used widely to mediate cell−cell fusion in the production of somatic cell hybrids (Davidson & Gerald, 1977) and in the fusion injection of macromolecules into cultured cells from erythrocytes (Davidson & Gerald, 1977) or liposomes (Szoka *et al.*, 1981). PEG is a hydrophilic polymer that causes dehydration of membrane surfaces through its interaction with water and its exclusion from a volume near the surface, rather than through its interaction with membranes (Arnold *et al.*, 1983, 1990). Dehydration brings about close contact between membranes, thus overcoming the hydration repulsion between membrane bilayers, which is considered to be the initial step in the fusion process (Rand & Parsegian, 1988). PEG-induced fusion of phospholipid vesicles is used as a model for understanding the molecular mechanisms of membrane fusion in biological systems (Lentz, 1994). PEG indeed forces close apposition between large, unilamellar vesicles (LUV);[1] the probability of fusion between these vesicles appears to be related to the closeness of approach (Burgess *et al.*, 1992). Nonetheless, PEG-induced close apposition of bilayers is not sufficient to cause fusion (Burgess *et al.*, 1991). For PEG-induced aggregation to result in fusion, membrane bilayers must be perturbed by the presence of a small amount of certain amphipaths (Lentz *et al.*, 1992), by high bilayer curvature (Lentz *et al.*, 1992; Talbot *et al.*, 1997), or by other manipulations that alter the packing and rates of molecular motions within contacting outer membrane leaflets (Wu *et al.*, 1996; Lee & Lentz, 1997a). The probability of fusion, then, appears to be related to the probability of molecular contact between apposing bilayers that are perturbed in such a way that formation of a fusion intermediate is favored (Massenburg & Lentz, 1993; Lentz, 1994).

The nature of the fusion intermediate and the details of the molecular rearrangements that must accompany fusion have been the subject of much debate and investigation (Lentz, 1994). The most likely model at this time is that fusion proceeds *via* a "stalk" or "single bilayer septum" (or both) that forms at the point of contact between two closely apposed bilayers (Kozlov *et al.*, 1989; Chernomordik *et al.*, 1995). The stalk model predicts that contacting outer leaflets of fusing membranes will "fuse" or exchange lipids as a first step in the fusion process. A "hemi-fused" intermediate with exchanged lipids has been demonstrated between red blood cells and fibroblasts expressing an engineered influenza virus hemagglutinin anchored to the membrane by a lipid linker

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[1] Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; $diC_{18:3}PC$, dilinolenoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPA, dipicolinic acid; PEG, poly(ethylene glycol); LUV, large, unilamellar vesicles; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; NBD-PS, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylserine; SUV, small, unilamellar vesicles; TES, N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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(Kemble *et al.*, 1994). Transfer of a detergent-like dye molecule, octadecylrhodamine, between the outer leaflets of red blood cells and hemagglutinin-expressing fibroblasts has been observed to precede by tens or hundreds of seconds the connection of cell compartments as measured by movement of a water-soluble dye (Zimmerberg *et al.*, 1994). We have recently demonstrated that transfer of fluorescently labeled lipids between the outer leaflets of PEG-aggregated synthetic lipid vesicles occurs on a time scale of tens of seconds at 23 °C, and also precedes by hundreds of seconds the slower transfer of lipids between membrane inner leaflets and the simultaneous merging of trapped aqueous compartments (Lee & Lentz, 1997b). Apparently, the initial outer leaflet merged complex must reorganize or mature in some way to allow the formation and/or spreading of a fusion pore. Little is known about this maturation process. A process that may well occur during this maturation is redistribution of lipids between the inner and outer leaflets of fusing membranes. This could be especially important in relieving the asymmetric packing strain that we have found necessary to facilitate the PEG-mediated fusion process (Wu *et al.*, 1996; Lee & Lentz, 1997a) or the asymmetric mass distribution inherent in fusogenic SUV membranes (Lentz *et al.*, 1987; Talbot *et al.*, 1997). We report here that PEG treatment of two phosphatidylcholine SUV preparations facilitates inner to outer leaflet movement of a fluorescent phosphatidylethanolamine but that this redistribution is not inherent to the fusion process in the absence of membrane rupture. In addition, we show that outer leaflet to inner leaflet redistribution of total lipid mass does accompany PEG-mediated fusion of SUV membranes.

MATERIALS AND METHODS

Materials

Chloroform stock solutions of egg yolk phosphatidylcholine (egg PC)[1], dioleoylphosphatidylcholine (DOPC),[1] dilinolenoylphosphatidylcholine (di$C_{18:3}$PC)[1], *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE)[1], and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylserine (NBD-PS)[1] were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL), and used without further purification. The disodium salt of 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS)[1] and *N*,*N*′-*p*-xylylenebis(pyridinium bromide) (DPX)[1] were purchased from Molecular Probes (Engene, OR). Carbowax PEG 8000 (molecular weight 7000−9000) was from Fisher Scientific (Fairlane, NJ), and was purified as described previously (Lentz *et al.*, 1992). *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES)[1] was from Research Organics, Inc. (Cleveland, OH). Dodecyl octaethylene glycol monoether ($C_{12}E_8$)[1] was from Calbiotech (LaJolla, CA). All other reagents were of the highest purity commercially available.

Methods

Vesicle Preparation. Appropriate quantities of chloroform stocks of phospholipid were dried under a stream of nitrogen. The dried lipid was dissolved in cyclohexane and dried under vacuum overnight to produce a white powder, and this was suspended in the appropriate assay buffer at room temperature (20−22 m °C) at a concentration of 15 mM. For the ANTS/DPX contents mixing assay, lipids were suspended in buffer containing 25 mM ANTS (or 90 mM DPX), 40 mM NaCl, and 10 mM TES, pH 7.4. Vesicles for ANTS/DPX leakage experiments were prepared in a buffer containing 12.5 mM ANTS, 45 mM DPX, 40 mM NaCl, and 10 mM TES, pH 7.4. For the Tb/DPA contents mixing measurements, lipid was suspended in 150 mM DPA (or 15 mM $TbCl_3$, 150 mM sodium citrate), 2 mM TES, and 2 mM L-histidine, pH 7.4. Tb/DPA leakage experiments employed vesicles prepared in buffer containing 7.5 mM $TbCl_3$, 75 mM sodium citrate, 75 mM DPA, 2 mM TES, and 2 mM L-His. Multilamellar vesicles resulting from suspending lipids in these different buffers were subjected to sonic disruption in a Heat Systems Model 350 Sonicator (Plainview, NY) to prepare small unilamellar vesicles (SUV)[1]. SUV preparations were fractionated by centrifugation at 70 000 rpm for 25 min at 4 °C in a Beckman TL-100 ultracentrifuge (Palo Alto, CA). The hydrodynamic diameters of vesicles prepared in this way were 38.4 nm for egg PC and 44.4 nm for 85/15 DOPC/di$C_{18:3}$PC SUV, as determined by dynamic light scattering (Lentz *et al.*, 1992). Large unilamellar vesicles (LUV)[1] were prepared by extruding multilamellar vesicles 7 times through a 0.1 μm polycarbonate filter under a pressure of about 75 psi of argon to generate LUV (Mayer *et al.*, 1986). Egg PC LUV had an average hydrodynamic diameter of 153 nm. LUV or fractionated SUV were passed down a Sephadex G-75 column equilibrated in 100 mM NaCl, 2 mM Tes, and 1 mM EDTA, pH 7.4, for the ANTS/DPX assay. For the Tb/DPA assay, the external buffer of LUV was replaced with an osmotically balanced buffer (200 mM NaCl, 4 mM L-histidine, 4 mM TES, 0.2 mM EDTA, and 0.4 mM $CaCl_2$), and that of SUV was replaced with either an osmotically balanced buffer or a hypoosmotic buffer (100 mM NaCl, 2 mM L-histidine, 2 mM TES, and 1 mM EDTA, pH 7.4) (Wilschut et al. 1980) with no observable differences in results.

Leakage and Contents Mixing Assays. The ANTS/DPX contents mixing and leakage assays were based on those originally proposed by Ellens *et al.* (1984) and were carried out in a buffer containing 100 mM NaCl and 10 mM TES, pH 7.4, as previously described in detail (Lentz *et al.*, 1992). The percent contents mixing was calculated as the percent of the signal expected from one ideal round of fusion between pairs of vesicles after accounting for leakage of contents and the probability that two fusing vesicles contained ANTS and DPX (Lentz *et al.*, 1992).

The Tb/DPA contents mixing and leakage assays were carried out in an osmotically balanced buffer (185 mM NaCl, 4 mM L-histidine, 4 mM TES, 2 mM EDTA, and 20 mM $CaCl_2$). These assays were based on assays originally proposed by Wilshut *et al.* (1980) but were adapted to monitor PEG-mediated fusion as described in detail elsewhere (Talbot *et al.*, 1997). The concentration of lipid used was 0.7 mM. When *leakage of contents* occurred, there was a drop in fluorescence due to the quenching of the Tb/DPA complex by $CaCl_2$ and EDTA in the external aqueous environment. The loss of remaining contents could then be occasioned by addition of sodium cholate to a final concentration of 11.6 mM. Zero percent leakage was characterized by the fluorescence intensity of vesicles containing co-encapsulated Tb/DPA in buffer without PEG; 100% leakage was characterized by the fluorescence intensity of a co-encapsulated Tb/DPA vesicle sample treated with PEG and sodium cholate. Both measurements were made relative to

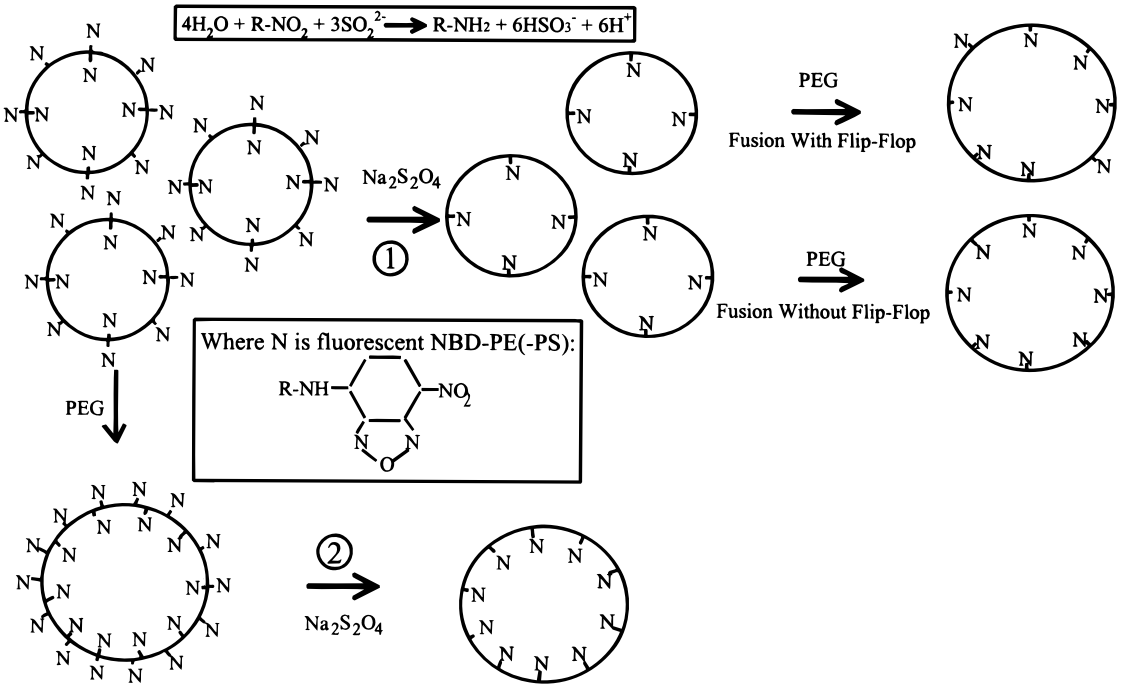

FIGURE 1: Schematic of NBD-PE(-PS) redistribution assays. Sodium dithionite was used to reduce exposed NBD-PE (NBD-PS) to a nonfluorescent form. This produced, after removal of external dithionite, asymmetrically labeled vesicles. In the inner to outer leaflet probe redistribution assay, these asymmetric vesicles were treated with PEG, and exposed probe was detected by a further reduction in fluorescence in the presence of external dithionite. Figure 2 illustrates this experiment. In the outer to inner leaflet lipid redistribution assay, the ratio of inner leaflet to total probe after fusion (reduction 2) was compared to this ratio before fusion (reduction 1).

the fluorescence of detergent-released vesicles. Thus, percent leakage was calculated as:

$$\%\ \text{leakage} = [(\text{loss of fluorescence at 0\% PEG due to detergent}) - (\text{loss of fluorescence at } X\% \text{ PEG due to detergent})]/(\text{loss of fluorescence at 0\% PEG due to detergent})$$

The *contents mixing* assay was carried out by mixing equal volumes of vesicles (0.35 mM lipid) containing either $TbCl_3$ or DPA, then incubating these vesicles for 5 min with buffer containing PEG (a time sufficient to complete fusion; Lentz *et al.*, 1992), and finally reading the fluorescence intensity of this mixture without dilution (Talbot *et al.*, 1997). The final lipid concentration was 0.35 mM for each vesicle species. Next, detergent was added to release the contents of the vesicles to the external aqueous environment, and the resulting loss of fluorescence intensity was compared to the intensity loss observed in comparably treated vesicles containing coencapsulated Tb/DPA. The percent contents mixing was calculated as the percent of the signal expected for one ideal round of fusion between pairs of vesicles (Talbot *et al.*, 1997):

$$\%\ \text{contents mixing} = [2 \times (\text{loss of fluorescence of Tb SUV and DPA SUV at } X\% \text{ PEG due to detergent})]/(\text{loss of fluorescence of Tb/DPA SUV at } X\% \text{ PEG due to detergent})$$

In this expression, the factor of 2 accounts for the probability that two fusing vesicles contained Tb^{3+} and DPA. Leakage of contents is taken into account by always comparing the signal from separately encapsulated Tb^{3+} and DPA vesicles with that from coencapsulated Tb^{3+}/DPA vesicles; leakage reduces the signal from the latter vesicles just as it reduces the signal from fused vesicles.

Measurements of *lipid redistribution across the bilayer* were carried out using a method for producing and detecting vesicles asymmetrically labeled with NBD-PE, as described by McIntyre & Sleight (1991). A modification of this method utilizes NBD-PS, which is purported to translocate less rapidly than NBD-PE (Meers *et al.*, 1996). NBD-PE has been used previously to monitor outward and inward lipid translocation in phosphatidylcholine vesicles (Moss & Bhattacharya, 1995), although not by exactly the same procedures used here. For *measurement of inner to outer leaflet movement* ["flip", as defined by Moss & Bhattacharya (1995)], vesicles were prepared from a lipid mixture containing 0.75 mol % NBD-PE (or NBD-PS) in buffer containing 185 mM NaCl, 3.7 mM L-His, 3.7 mM TES, 2.0 mM EDTA, and 20 mM $CaCl_2$, pH 7.4, at a final lipid concentration of 15 mM. NBD-PE(-PS) in the outer leaflet of these vesicles was reduced by the addition of 60 μL of a freshly prepared 1 M $Na_2S_2O_4$ (sodium dithionite) stock solution in 1 M Tris buffer, pH 10.0, to 2.7 mL of a 10 mM vesicle sample. Incubation for 15 min at 23 °C reduced the outer leaflet NBD probe and created asymmetrically labeled vesicles (see Figure 1). The vesicles were passed over a Sephadex G-75 column (1 × 14 cm) equilibrated with 200 mM NaCl, 4 mM TES, 4 mM L-histidine, 0.2 mM EDTA, and 0.4 mM $CaCl_2$, pH 7.4, to remove any $Na_2S_2O_4$ in solution. After incubation of asymmetrically labeled vesicles with PEG for 5 min, the vesicles were diluted 10-fold with buffer to minimize the influence of PEG on the measurement of NBD fluorescence. NBD fluorescence intensity was measured using an SLM 48000 spectrofluorometer (SLM-Aminco, Rochester, NY) equipped with a focused 450-W xenon arc lamp. NBD was excited at 470 nm (slits = 4) and emission was detected after

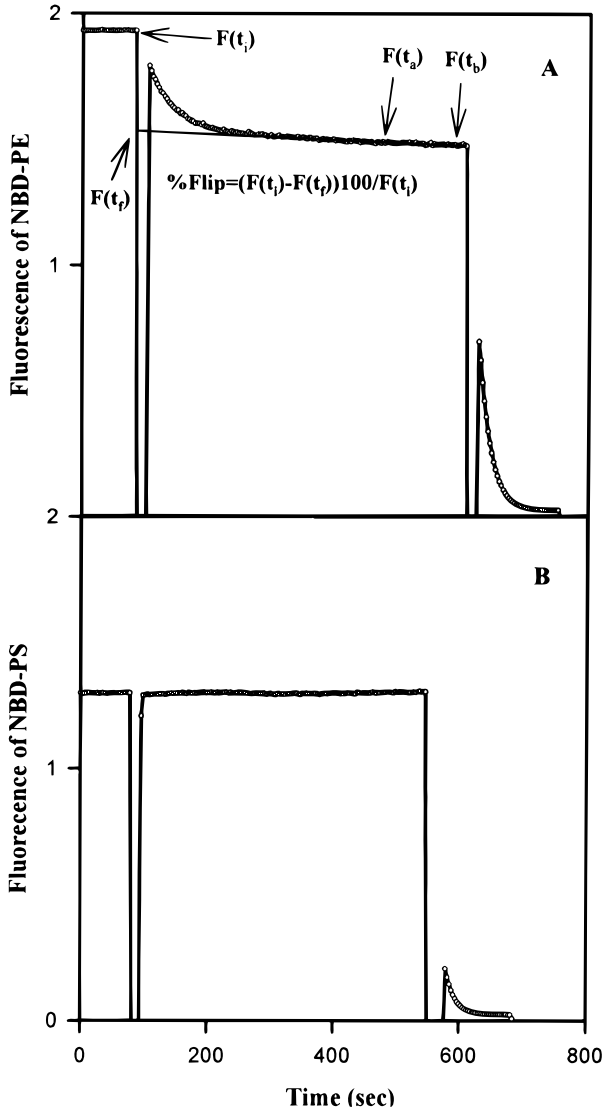

FIGURE 2: Measurement of PEG-induced outward movement of NBD-PE (frame A) or NBD-PS (frame B). (A) Following incubation with 22.5 wt % PEG, the NBD fluorescence from asymmetrically labeled, osmotically balanced egg PC SUV (0.07 mM) was recorded. At time t_i, dithionite (25 μL from a 1 M stock) was added to 3 mL of vesicles, and the time course of reduction of exposed NBD was monitored in terms of a loss in fluorescence intensity. After a steady-state rate of decrease was reached, the fluorescence intensity was extrapolated back to t_i to obtain $F(t_i)$, the final fluorescence of NBD following reduction of exposed NBD probe. The percent inner to outer leaflet redistribution (termed "flip") was then calculated as shown in the figure. Sodium cholate was added at roughly 600 s to confirm that complete reduction of probe would eliminate all fluorescence. (B) NBD-PS redistribution in asymmetrically labeled 85/15 DOPC/di$C_{18:3}$PC SUV treated with 17.5 wt % PEG.

passing through both a monochromator set at 540 nm (slits = 4) and an OG515 filter (Schott Optical Glass, Duryea, PA). Addition of 25 μL of 1 M Na_2SO_4 stock reduced any NBD probe that translocated across the bilayer and led to a loss of fluorescence. Figure 2A illustrates this. Percent "flip" was calculated by dividing the loss of fluorescence intensity due to reduction of redistributed lipid by the initial fluorescence intensity as illustrated in Figure 2A. In every case, a control sample was handled identically except that it was never treated with PEG. The spontaneous redistribution in this sample was subtracted from that observed in the PEG-treated sample to obtain the PEG-induced flip.

Measurement of *lipid mass redistribution* was accomplished by monitoring the ratio of unexposed to total NBD-PE (NBD-PS) before and after treatment with PEG. As in measurements of inner to outer leaflet lipid movment, fluorescence measurements were made on 10-fold diluted samples to avoid interference from fluorescent impurities in PEG. Exposed NBD-PE was proportional to the reduction in fluorescence intensity on treatment of intact NBD-PE-containing vesicles with dithionite. Total NBD-PE(-PS) in the same vesicle preparation was obtained as proportional to the total fluorescence intensity loss upon reduction of NBD-PE(-PS) in the presence of 11.6 mM sodium cholate to disrupt the vesicle bilayer. Assuming that the transbilayer distribution of NBD-PE(-PS) was proportional to the transbilayer distribution of total lipid mass, the percent mass movement to the inner leaflet associated with PEG-mediated fusion is given by

$$\% \text{ mass movement} = \{(\text{unexposed/total NBD})_{\text{PEG}} - (\text{unexposed/total NBD})_{\text{before PEG}}\} \times 100$$

RESULTS

Figure 2A illustrates the data obtained when monitoring NBD fluorescence to detect the inner to outer leaflet redistribution of NBD-PE in osmotically balanced (see Figure 3B) egg PC SUV treated with 22.5 wt % PEG. The exponential loss of fluorescence following dithionite addition is due to NBD reduction. Note that this curve approaches an asymptote of non-zero slope. This could reflect slow leakage of either dithionite [as suggested by Langner & Hui (1993)] or NBD-PE across the bilayer. According to Meers *et al.* (1996), NBD-PS is expected to undergo transbilayer redistribution much more slowly than NBD-PE. This was confirmed by the experiment with NBD-PS-containing vesicles shown in Figure 2B, in which the slope of the long-time asymptote is zero. Thus, NBD-PE appears to redistribute slowly across an asymmetrically labeled bilayer, while NBD-labeled PS does not. It was necessary to correct for this slow redistribution of NBD-PE as shown in Figure 2A when calculating "% flip", as defined under Methods.

Figure 2 also demonstrates another key result. While treatment of egg PC SUV with 22.5 wt % PEG promoted NBD-PE flip (frame A), treatment of 85/15 DOPC/di$C_{18:3}$PC SUV with 17.5 wt % PEG did not induce NBS-PS transbilayer movement (frame B). Similarly, no NBD-PS transbilayer movement was observed when osmotically treated egg PC SUV were treated with 22.5 wt % PEG (see diamond in Figure 3B). The different responses of NBD-PE and NBD-PS to PEG treatment under nonrupturing conditions seem to be a general result and will prove essential to understanding the relationship of fusion to lipid transbilayer redistribution.

Because loss of bilayer integrity might be expected to encourage transbilayer lipid redistribution, it was important to locate systems for which substantial fusion can be observed reproducibly in the absence of leakage of contents. Although we have reported on two systems that meet this requirement (Massenburg & Lentz, 1993; Wu *et al.*, 1996), both involved dipalmitoylphosphatidylcholine membranes and required that experiments be performed at or above 45 °C, at which temperatures NBD-PE quickly lost its asymmetric distribution. Recent observations (Talbot *et al.*, 1997)

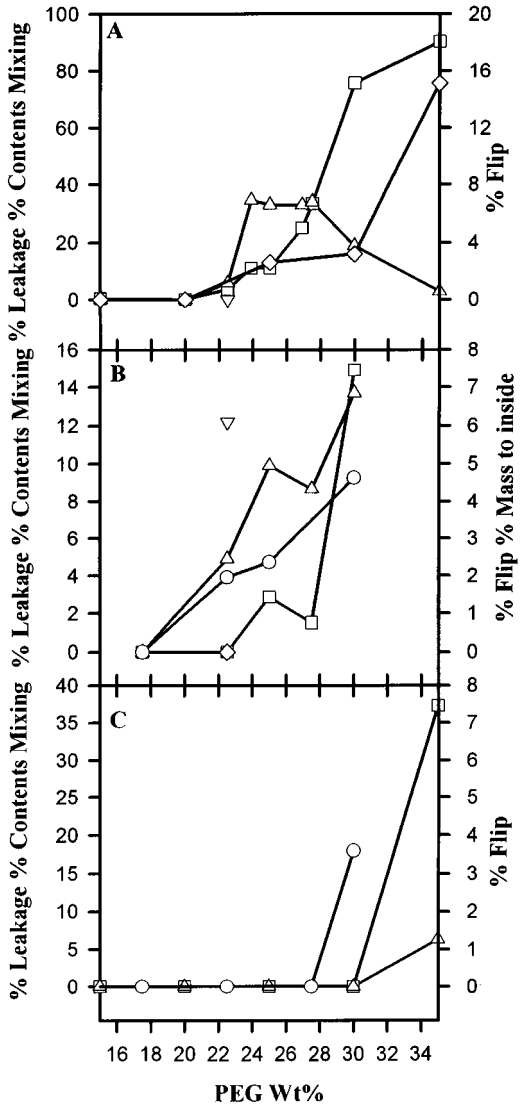

FIGURE 3: PEG-induced leakage, contents mixing, and lipid flip or flop for eggPC vesicles. Data are shown as a function of PEG concentration (wt %) for PEG-induced leakage (squares), contents mixing (triangles), NBD-PE flip (circles), NBD-PS flip (diamonds), and NBD-PS outer to inner leaflet mass transfer (inverted triangles). All measurements were made after incubating vesicles with PEG for 5 min. (A) SUV. (B) SUV prepared in a buffer containing glucose at appropriate concentrations so that addition of vesicles to PEG-containing assay buffer would not produce an osmotic gradient across the vesicle bilayer. (C) LUV. Data were obtained using the Tb/DPA leakage and contents mixing assays.

suggested that highly curved SUV composed of unsaturated lipid mixtures might meet this requirement at temperatures for which NBD-PE asymmetry could be maintained. NBD-PE was incorporated at a total concentration of 0.75 mol % into egg PC SUV and SUV prepared from an 85/15 mixture of DOPC/di$C_{18:3}$PC. In both cases, control experiments showed that spontaneous redistribution of NBD-PE (NBD-PS) at 23 °C was less than 3−5% (0.5%) in the hour required to complete PEG treatment and fusion assays.

PEG-mediated fusion of egg PC SUV is documented in Figure 3A. As can be seen, fusion was substantial and leakage minimal in the presence of 22−28 wt % PEG. PEG also induced NBD-PE flip (circles), but not at any PEG concentration below those needed to induce leakage or fusion. Unfortunately, even the small amount of rupture seen in this system obscures the interpretation of the observed NBD−lipid redistributions. In an attempt to minimize rupture, vesicles were prepared in a buffer containing appropriate concentrations of glucose so as to match the internal buffer osmolality with the osmolality of the external PEG buffer into which vesicles were diluted. Vesicles prepared by this procedure should not experience the flattening osmotic forces experienced by vesicles normally treated by PEG (Burgess *et al.*, 1992). The results obtained with these osmotically balanced SUV (Figure 3B) demonstrated that transmembrane osmotic stress and the resulting shape distortions favor both fusion and rupture, but are not necessary for fusion. Indeed, conditions were found (22.5 wt % PEG) for which no contents leakage but still substantial (5%) contents mixing was observed. Under these conditions, NBD-PE redistributed (flipped) from the inner to the outer leaflet (circles), but NBD-PS did not (diamonds). Nonetheless, fusion in the absence of leakage (at 22.5 wt % PEG) was clearly accompanied by inward movement of lipid mass (inner leaflet contained 39.5% of total lipid before and 45.6% after fusion) as monitored with NBD-PS (inverted triangle). At higher PEG concentrations, contents mixing, NBD-PE flipping, and leakage all increased, as might be expected. That membrane curvature encourages all three processes is clearly documented in Figure 3C, in which we see that uncurved egg PC LUV did not fuse and NBD-PE did not flip at PEG concentrations below 35 and 30 wt %, respectively. It is also worthy of note that a small amount of PEG-induced NBD-PE flipping (circles) could be detected in the highly stressed membranes that exist at high PEG concentrations.

The effect of PEG on SUV prepared from an 85/15 mixture of DOPC/di$C_{18:3}$PC is recorded in Figure 4A. Again, no PEG-induced NBD-PE flipping was observed for PEG concentrations below those that induced rupture or fusion. This membrane system was remarkable in that there was a substantial range of PEG concentrations (15−20 wt %) for which vesicle rupture was undetectable but fusion, as indicated by contents mixing, was substantial. Although NBD-PE inner to outer leaflet redistribution was not as extensive as for egg PC SUV, it did occur (circles) and increased in parallel with contents mixing (triangles) throughout the PEG concentration range of interest (Figure 4A). Just as we observed for egg PC SUV, the behavior of the NBD-PS probe did not parallel that of NBD-PE. At 17.5 wt % PEG, where contents mixing reached nearly 50% of an ideal round of fusion, NBD-PE reported 2% flip while NBD-PS recorded 0% flip. Both probes showed the same change in outer leaflet to total probe ratio, *i.e.*, the same net inward lipid transfer, upon treatment with PEG: the hexagons in Figure 4A record the values obtained using NBD-PE at several PEG concentrations; the inverted triangle at 17.5 wt % PEG records this value determined using NBD-PS. This net movement of lipid inward (inner leaflet contained 42% of total probe before fusion and 46% after) increased as fusion increased with increasing PEG concentrations (hexagons in Figure 4A).

The behavior of less highly curved LUV prepared from pure DOPC is recorded in Figure 4B to contrast with the SUV behavior recorded in Figure 4A. The LUV showed no sign of fusion up to PEG concentrations as high as 35 wt %, but ruptured at PEG concentrations (20 wt %) comparable to those causing fusion (15 wt %) and rupture (25 wt %) in the more highly curved SUV system. The importance to fusion of curvature-induced stress has been noted previously

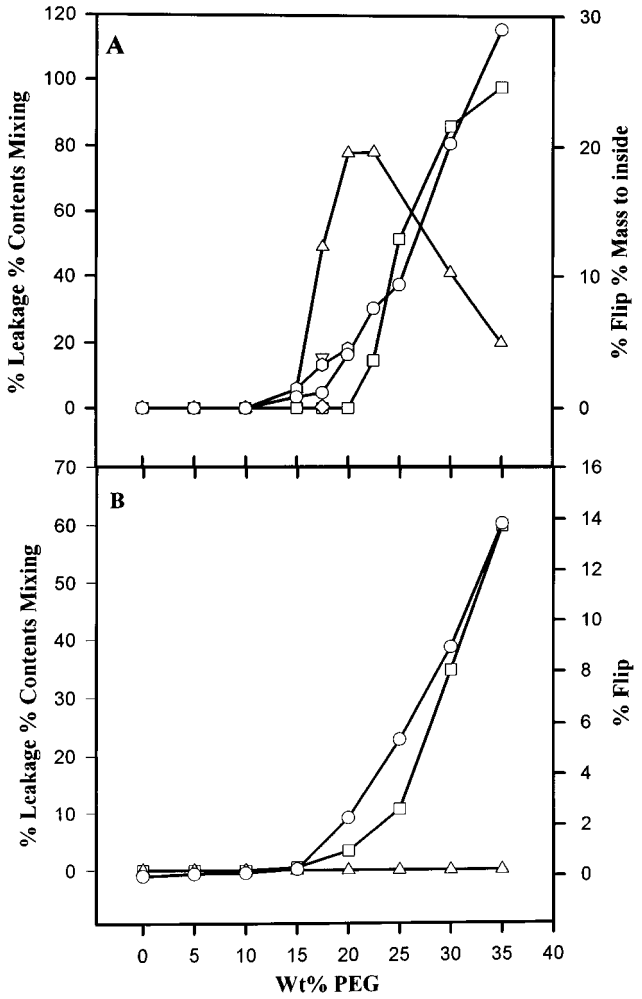

FIGURE 4: PEG-induced leakage, contents mixing, and NBD-lipid flip for (A) DOPC/$DC_{18:3}$PC SUV and (B) DOPC LUV. Data are shown as a function of PEG concentration (wt %) for leakage (squares), contents mixing (triangles), NBD-PE flip (circles), NBD-PS flip (diamonds), NBD-PE outer to inner leaflet mass transfer (hexagons), and NBD-PS outer to inner leaflet mass transfer (inverted triangles). All measurements were made after incubating vesicles with PEG for 5 min. Data in frame A were obtained using the Tb/DPA contents mixing and the ANTS/DPX contents leakage assays, while those in frame B were obtained using the Tb/DPA contents mixing and leakage assays.

(Lentz *et al.*, 1987, 1992; Talbot *et al.*, 1997). We note, however, that NBD-PE flip (circles) accompanied rupture of this vesicle system, even in the absence of fusion.

DISCUSSION

Inner to Outer Leaflet Lipid Redistribution Accompanies PEG-Mediated Membrane Stress but Is Not Directly Related to Fusion. The purpose of this paper has been to examine the relationship between PEG-mediated fusion of phospholipid vesicles and the transbilayer redistribution of lipid in these vesicles. It is worth noting that our mass redistribution and inner to outer leaflet lipid movement assays detect fundamentally different processes. The former is meant to monitor the net movement of lipid from the outer leaflet to the inner leaflet. This net lipid movement is required when highly curved SUV fuse to form less highly curved unilamellar vesicles. This assay does not reflect a loss of probe asymmetry because vesicles are not made asymmetric in probe composition before treatment with PEG. Our inner to outer lipid movement assay, on the other hand, does

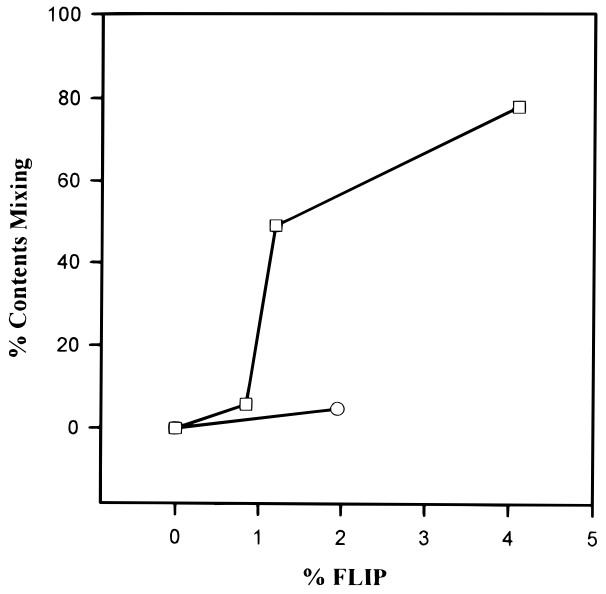

FIGURE 5: Relationship between fusion and flip assays. Contents mixing for osmotically balanced egg PC SUV (circles) and DOPC/$diC_{18:3}$PC SUV (squares) as a function of the NBD-PE transbilayer redistribution observed at fusing PEG concentrations for which rupture was not observed.

measure the loss of probe transbilayer asymmetry associated with PEG treatment. This assay necessarily reports transbilayer lipid exchange (*i.e.*, "flip-flop", since outward moving lipid is expected to be replaced by inward moving lipid) diminished by any net outer to inner leaflet mass redistribution that is forced by the fusion process.

Intimate intermembrane contact appears necessary for fusion (Portis *et al.*, 1979; Burgess *et al.*, 1992). Mg^{2+}-induced formation of a tight dehydrated complex between phosphatidylglycerol-containing membranes has been shown to mediate flip-flop of lipid in the absence of fusion (Lentz *et al.*, 1989), even though lipid redistribution is normally very slow in pure lipid vesicles (Shaw *et al.*, 1977). It might be expected that the close contact that results in mixing of lipids between apposed membrane leaflets (Lee & Lentz, 1997b) might also be sufficient to bring about transbilayer redistribution of lipid. However, efforts to detect a correlation between outward lipid flip and either Ca^{2+}-induced fusion of phosphatidylserine vesicles (Hoekstra & Martin, 1982; Balch & Sleight, 1993) or PEG-induced fusion of phosphatidylcholine vesicles (Balch & Sleight, 1993) have been inconclusive, with a clear correlation seen for some systems and little or no correlation seen for others.

A possible reason for the ambiguous relationship between fusion and outward lipid flip is that PEG-mediated fusion often occurs at PEG concentrations that also bring about bilayer rupture (Massenburg & Lentz, 1993). Our results clearly demonstrate that rupture is accompanied by enhanced NBD-PE flip (Figure 3 or 4). Indeed, flip can occur under conditions of extreme dehydration and membrane stress, even in the absence of fusion (Figure 3C; and Lentz *et al.*, 1989). Our results also show that NBD-PE flip also accompanies fusion in the absence of vesicle rupture (Figures 3A,B, Figure 4A). The correlation between NBD-PE flip and fusion is summarized in Figure 5 in which we have plotted contents mixing observed between DOPC/$diC_{18:3}$PC SUV (squares) or between egg PC SUV (circles) as a function of the NBD-PE flip observed at PEG concentrations for which rupture

does not occur. It seems from this figure that the correlation between NBD-PE flipping and contents mixing is stronger for egg PC as compared to DOPC/di$C_{18:3}$PC SUV. If NBD-PE flip were an inherent feature of the fusion mechanism, we would expect a similar correlation in all fusing systems. This argues that the NBD-PE flip seen at non-rupture-inducing concentrations of PEG may reflect the membrane stress imposed by high PEG concentrations rather than being a structural consequence of the fusion process.

That PEG-induced NBD-PE outward redistribution reflects membrane stress rather than a mechanistic relationship between flip and fusion is supported by the behavior of NBD-PS. While NBD-PE redistributed significantly during the time course of our experiments, NBD-PS showed no measurable outward movement (Figure 2). Since NBD-PS shows a greatly diminished tendency toward transbilayer movement relative to NBD-PE, it might be expected not to redistribute as readily in response to the stress of dehydration and shape distortion expected at high PEG concentration. Indeed, in both systems studied, NBD-PS showed no transbilayer flip at fusing concentrations of PEG. If transbilayer lipid exchange were structurally and mechanistically related to fusion, NBD-PS should redistribute just as NBD-PE does. Since it does not, we conclude that such a relationship does not exist for PEG-mediated vesicle fusion.

There is one caveat associated with this conclusion. NBD-PS, because of its charge, may not have redistributed like other lipids. For instance, it might be excluded by charge repulsion from the point of contact between bilayers. While there is no way to exclude this possibility, the combination of different NBD-PE and NBD-PS behavior and different NBD-PE behavior for egg PC and DOPC/di$C_{18:3}$PC SUV argues strongly in support of our conclusion that transbilayer flip-flop is not inherent to PEG-mediated fusion.

Net Inward Movement of Lipid during PEG-Mediated Fusion Probably Reflects Vesicle Size Increase and Curvature Decrease. PEG-mediated fusion transforms highly curved, small, unilamellar vesicles into larger, less highly curved unilamellar vesicles (Lentz *et al.*, 1992; Massenburg & Lentz, 1993). The ratio of outer to inner leaflet lipid is thought to be large in small, highly curved vesicles (approaching 2) and is known also to approach 1 with increasing vesicle diameter (Sheetz & Chan, 1972). Once two such small vesicles fuse, they must retain their high curvature and look like locally annealed billiard balls unless net mass transfer of lipid from the outer to the inner membrane leaflets occurs. Such mass redistribution does occur in a probe-independent fashion for the two systems studied here (Figures 3 and 4). This means that SUV that fuse in the presence of PEG relax to a less highly curved state through a process that involves mass redistribution across the bilayer. If we assume that both unfused and fused vesicles are spherical and we use the simple geometric arguments of Sheets & Chan (1972), we can predict from observed outer/inner probe ratios (1.37 → 1.15; see results) that DOPC/di$C_{18:3}$PC SUV of roughly 370 Å diameter increase to LUV of roughly 740 Å after fusion. Quasi-elastic light scattering measurements find an increase from 450 Å to 950 Å (Lee & Lentz, 1997b). Given that quasi-elastic light scattering overestimates vesicle diameters relative to other methods, this is remarkably good agreement and suggests that fusion products do "round up" to form roughly unilamellar spherical vesicles. It is not known at what point during the fusion process that such transbilayer mass redistribution and rounding up might occur. One can imagine that it might occur during spreading of the initial fusion pore to form a continuous aqueous compartment within the fused membranes. Alternatively, it may occur during the slow maturation of the initial fusion intermediate to a later intermediate (Lee & Lentz, 1997b). Distinguishing between these two possibilities will require studies of the kinetics of transbilayer lipid redistribution during PEG-mediated fusion. Whatever be the case, our results show that inward net redistribution of lipid is an intrinsic part of the mechanism of PEG-mediated fusion of highly curved membranes.

Interleaflet Lipid Exchange during PEG-Mediated Fusion. We have seen that outward lipid movement does not accompany PEG-mediated SUV fusion, but that inward net lipid redistribution does. This latter lipid movement is a directed process that presumably occurs in response to the change in membrane curvature that accompanies fusion. The question remains as to whether interleaflet lipid exchange (transbilayer randomization or flip-flop) accompanies fusion. Our observation of net inward lipid movement cannot address this issue, but our failure to observe externally exposed inner leaflet NBD-PS does. If some intermediate in the fusion process were to allow or catalyze interleaflet scrambling of lipid, we would had seen some exposure of internally located NBD-PS. We conclude that the molecular structures formed during PEG-mediated fusion of SUV do not facilitate transbilayer lipid exchange.

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