Effects of Lipid Headgroup and Packing Stress on Poly(Ethylene Glycol)-Induced Phospholipid Vesicle Aggregation and Fusion

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ABSTRACT The effect of lipid headgroup and curvature-related acyl packing stress on PEG-induced phospholipid vesicle aggregation and fusion were studied by measuring vesicle and aggregate sizes using the quasi-elastic light scattering and fluorescence energy transfer techniques. The effect of the lipid headgroup was monitored by varying the relative phosphatidylcholine (PC) and phosphatidylethanolamine (PE) contents in the vesicles, and the influence of hydrocarbon chain packing stress was controlled either by the relative amount of PE and PC content in the vesicles, or by the degree of unsaturation of the acyl chains of a series of PEs, e.g., dilinoleoylphosphatidylethanolamine (dilin-PE), lysophosphatidylethanolamine (lyso-PE), and transacylated egg phosphatidylethanolamine (TPE). The PEG threshold for aggregation depends only weakly on the headgroup composition of vesicles. However, in addition to the lipid headgroup, the curvature stress of the monolayer that forms the vesicle walls plays a very important role in fusion. Highly stressed vesicles, i.e., vesicles containing PE with highly unsaturated chains, need less PEG to induce fusion. This finding applies to the fusion of both small unilamellar vesicles and large unilamellar vesicles. The effect of electrostatic charge on vesicle aggregation and fusion were studied by changing the pH of the vesicle suspension media. At pH 9, when PE headgroups are weakly charged, increasing electrostatic repulsion between headgroups on the same bilayer surface reduces curvature stress, whereas increasing electrostatic repulsion between apposing bilayer headgroups hinders intervesicle approach, both of which inhibit aggregation and fusion, as expected.

INTRODUCTION

The study of the effect of poly(ethylene glycol) (PEG) on the aggregation and fusion of phospholipid vesicles has received considerable attention in the past two decades. Successive additions of PEG to a suspension of small unilamellar vesicles (SUVs) or large unilamellar vesicles (LUVs) can cause vesicle aggregation or fusion once the concentration of PEG reaches a certain threshold, as can be monitored by quasi-elastic light scattering, fluorescence spectroscopy, or electron microscopy (Boni et al., 1981; Burgess et al., 1992; Lentz et al., 1992; Viguera et al., 1993; Ohki, 1991). It is believed that depletion of PEG molecules from the bilayer surface, which changes the osmotic pressure (Yamazaki and Ito, 1990; Arnold et al., 1990; Kuhl et al., 1996), is the driving force for vesicle aggregation. Fusion is found to be a result of bilayer destabilization due to extreme dehydration, which creates and amplifies existing defects, followed by the expansion of fusion sites due to vesicle swelling (Hui et al., 1985; Ahkong and Lucy, 1988).

Bilayers of different lipid compositions have different sensitivities to PEG-induced aggregation and fusion (Boni et al., 1981; Burgess et al., 1992; Lentz et al., 1992). There are several factors that contribute to the difference in sensitivity. These include, for instance, the hydrophobicity of the phospholipid headgroups, the phase state of the bilayer, the curvature of the vesicle walls, as well as the "curvature energy" or "bending energy" arising from constraining a monolayer of lipids at a curvature differed from that of their lowest energy packing conformation (spontaneous curvature). Natural membranes usually differ in lipid compositions and hence in curvature energies (Hui and Sen, 1989). Phospholipid vesicles composed of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) offer a simple model for investigating the role of molecular packing stress in the fusion process, because unsaturated PE imposes high curvature stress on the bilayers. In these model lipid vesicles, the curvature stress can be changed by varying the molar ratio of PC and PE, and more precisely, by varying the unsaturation of acyl chains in a series of PEs. In this paper we studied the PEG-induced aggregation and fusion of SUVs and LUVs possessing different headgroups and acyl chain unsaturation.

MATERIALS AND METHODS

Egg phosphatidylcholine (EPC), transacylated egg phosphatidylethanolamine (TPE), dilinoleoylphosphatidylethanolamine (dilin-PE), and egg lysophosphatidylethanolamine (lyso-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). PEG (M_r 8000) was purchased from Fisher Scientific Co. and used without further purification. His (L-histidine)-TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) buffer (Sigma, St. Louis, MO) was prepared in 2 mM concentration at pH 7.4.

Mixtures of lipids with different PE and PC ratios in chloroform were dried in a rotary evaporator for about 1 h for SUV experiments, or dried first by passing a stream of N_2 over the mixtures before placing them in a vacuum for LUV experiments. Further drying made no difference in the final outcome. The dried lipids were then dispersed in aqueous buffer (2 mM His, 2 mM TES, pH 7.4). All transfers were done under nitrogen. A vortex mixer was used to disperse the lipids at room temperature into

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multilamellar vesicles (MLVs). SUVs were obtained by sonicating MLV suspensions under nitrogen in a bath sonicator (Laboratory Supplies Co.) for up to 2 h until the suspension became clear. No degradation product was found by thin-layer chromatography after sonication. LUVs were produced by the extrusion method through 0.2-μm polycarbonate filters (Millipore, Bedford, MA), as described previously (Sen et al., 1991).

Quasi-elastic light scattering (QELS) was performed on a model 370 submicron particle sizer (Nicomp Particle Sizing Systems, Santa Barbara, CA). An argon ion laser with a maximum CW output of 2 W was used as the light source. The photon counts were always adjusted to ~300 kHz. The apparatus was calibrated by measuring monodispersed polystyrene latex spheres of known sizes. To distinguish fusion and aggregation, we assume that aggregation was reversible upon dilution, whereas fusion was irreversible. Therefore, the criterion for fusion was the increase in vesicle size from the original suspension after the PEG treatment and a 50-fold dilution. The threshold PEG concentration was defined as the range of concentration within which the size of the particles started to increase significantly from the initial value. In a typical SUV aggregation experiment, 0.2 ml of SUV suspension, at a concentration of 5 mM, was mixed with 0.8 ml of buffer or PEG solution of desired concentration in a tube to make 1 mM the final concentration of the lipid. After PEG treatment, the tube containing vesicles and PEG was placed in the particle sizer to be measured for aggregation. A small aliquot of treated vesicles was diluted at least 50-fold with aqueous buffer and measured for fusion in a cuvette. For LUV aggregation experiments, 20 μl of vesicles (4 mg/ml lipid) was mixed with 700 μl PEG solution of the desired PEG concentration in a tube for direct size measurement. For the LUV fusion experiments, an aliquot of vesicles less than 40 μl (4 mg/ml lipid) was mixed with 40 μl of PEG solution to reach a desired final PEG concentration, incubated for 2 min at room temperature, and then diluted with 4 ml of aqueous buffer and measured in a cuvette. The volume-weighted mean vesicle diameter was taken for the initial suspension as well as after PEG solutions were added, and diluted in the case for fusion. Each measurement usually took a few minutes to reach the steady state. All experiments were performed at 24°C. The viscosity and the index of refraction for water were taken from *The Handbook of Chemistry and Physics* (Weast, 1988–1989), and those for PEG 8000 solutions of various concentrations were measured with a viscometer (Kuhl et al., 1996).

Vesicle fusion was also assayed by the fluorescence energy transfer (FET) method. Because the decrease in the energy transfer efficiency indicates the extent of dilution of fluorescence labels upon fusion between the labeled and unlabeled vesicles, the vesicle fusion yield can be measured (Li and Hui, 1997). In labeled vesicles, 1 mol% each of (*N*-(7-nitrobenzoyl-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine) NBD-PE and (*N*-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine) Rh-PE were incorporated into dilin-PE/Egg-PC or pure EPC vesicles. The energy transfer efficiency, i_t/i, of these vesicles was 3.5, where i and i_t were the NBD fluorescence intensity (Ex = 450 nm, Em = 533 nm) before and after the addition of 0.05% Triton 100-X. In the fusion assay, 10 μl of labeled (1 mg/ml lipid) and 20 μl of unlabeled (4 mg/ml lipid) vesicles were mixed at room temperature with 10 μl of PEG solution to reach the desired PEG concentration. Two minutes later, the mixture was quickly diluted 100-fold. The NBD fluorescent intensity was measured before and after Triton 100-X addition.

RESULTS

Suspensions of sonicated SUVs or extruded LUVs were clear and showed a uniform vesicle size of ~30 nm for SUVs and 200 nm for LUVs. The sizes are fairly consistent for different lipid components. As PEG 8000 was introduced, samples became cloudy when the PEG concentration exceeded certain threshold values. This is consistent with the turbidity measurements of Boni et al. (1981). Measurement by quasi-elastic light scattering revealed a drastic increase in particle size, indicating aggregation and/or fusion.

Fig. 1 shows the results of the average vesicle size as a function of PEG concentration for four different EPC/TPE molar ratios. It can be seen from Fig. 1 *a* that aggregation occurs above 4 wt% PEG for pure EPC vesicles. As the PE concentration in the vesicles increases, the threshold of PEG concentration needed for aggregation remains constant until TPE reaches 75%, when the threshold becomes higher. Even at 8% PEG, the size of aggregates containing 75% TPE is still smaller than those aggregates formed by SUV of lower PE contents at 6% PEG.

The thresholds for fusion are considerably higher than those for aggregation (Fig. 1 *b*). More interestingly, PEG threshold concentrations for fusion are very sensitive to the TPE/EPC ratio, and decrease as the amount of TPE increases, as shown in Fig. 1 *b*.

To isolate the effect of curvature stress from that of lipid headgroups on the fusion and aggregation processes, EPC was mixed with a series of PEs with different unsaturated acyl chains in an experiment to keep the headgroup effect constant and curvature stress varied. Fig. 2 *a* shows the PEG threshold concentrations for aggregations of SUVs of PE/EPC as functions of PE mole percentage, for dilin-PE, TPE, and a mixture (0.8 × TPE + 0.2 × LysoPE), of decreasing order of acyl chain unsaturation (and number of acyl

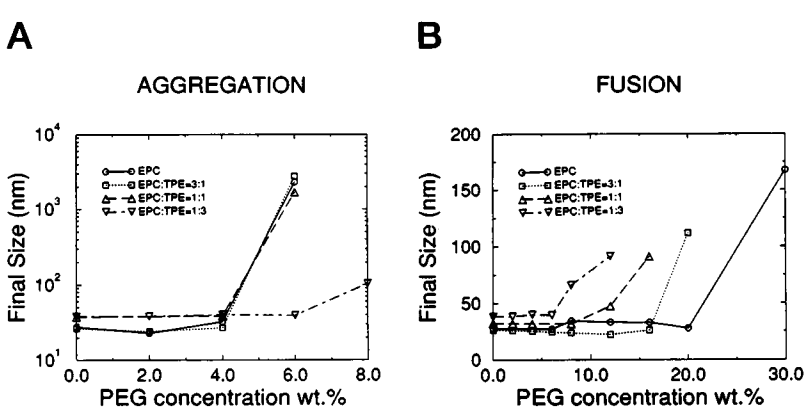

FIGURE 1 The final average particle size due to (*a*) aggregation of EPC/TPE SUVs in PEG solutions of given concentrations and (*b*) fusion of EPC/TPE SUVs measured after 50-fold dilution of PEG solutions of given concentrations.

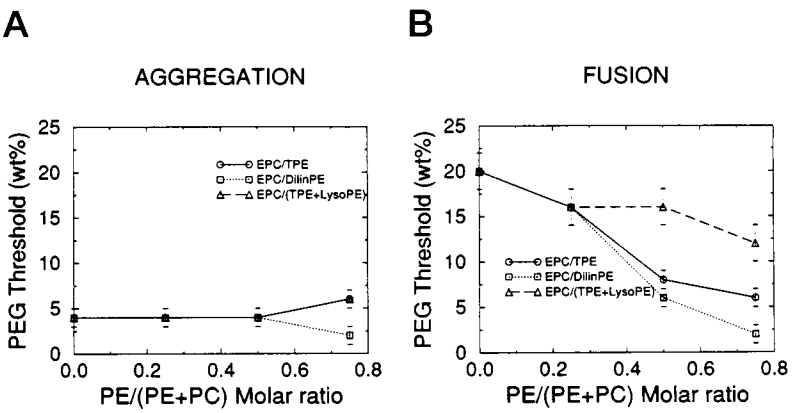

FIGURE 2 The threshold concentrations of PEG 8000 needed for (*a*) aggregation and (*b*) fusion of different kinds of PE and EPC SUVs.

chains), and therefore of decreasing curvature stress. A salient feature of these results is that the PEG threshold values are not sensitive to the PE percentage and remain nearly the same for all three kinds of lipid vesicles over a wide range of PE concentrations. The only difference was observed at the highest PE concentration (75%), where the PEG threshold concentration for dilin-PE/EPC vesicles is slightly lower, and those for TPE/EPC and (TPE + Lyso-PE)/EPC vesicles are slightly higher.

Remarkably, the threshold PEG concentrations needed for fusion decrease with increasing PE percentage and differ significantly for the three different kinds of PEs. The differences become even more noticeable when the PE concentration increases (Fig. 2 *b*). The slopes of decrease of PEG fusion thresholds with increasing PE content are in the order dilin-PE > TPE > (0.8 × TPE + 0.2 × lyso-PE), in the same order as decreasing curvature stress.

To show that this curvature stress effect on fusion and aggregation is not biased by the curvature and the resultant asymmetrical lipid distribution among the outer and inner leaflets of small vesicles, we repeated some of the experiment shown in Fig. 2, *a* and *b*, with LUV. As shown in Fig. 3 *a*, the PEG threshold for aggregation is weakly lipid composition dependent, i.e., the higher the PE percentage, the higher the PEG threshold for the aggregation of (EPC/dilin-PE). The increase is limited to 0.5–2% PEG. This is not too different from the SUV experiments that show little effect of lipid composition on aggregation, until 75% PE is reached, when the threshold increases from 4% to 6% PEG (Fig. 2 *a*).

The PEG thresholds needed for LUV fusion follow the same trend as that for SUV. For LUV, the fusion threshold decreases from 20% PEG for (50% TPE + 25% lyso-PE)/25% EPC to ~1% PEG for 75% dilin-PE/25% EPC, with increasing PE acyl chain unsaturation (Fig. 3 *b*). This is compared to the SUV fusion threshold decrease: from 12% PEG for (60% TPE + 15% lyso-PE)/25% EPC to 2% PEG for 75% dilin-PE/25% EPC. Both threshold decreases are in the order dilin-PE > TPE > (TPE + lyso-PE), which is the same order as decreasing curvature stress.

So far in this paper, fusion is defined as a significant irreversible size increase after dilution (Figs. 1 *b* and 3 *b*). This criterion may not distinguish slow disaggregation from fusion. To verify that this irreversible size increase truly represents a new continuity forming between membranes of fusing vesicles, we compare results using both the size and the fluorescence energy transfer (FET) criteria. As shown in Fig. 3 *b*, judging by the size criterion, LUVs of EPC/dilin-PE fuse at the PEG threshold concentration of 5%, whereas LUVs of pure EPC do not fuse up to 35% PEG. The same result is reproduced in Fig. 3 *c*, using the FET method. This gives us confidence that the size criterion is a reliable indication of fusion for our experiments.

The effect of pH on the aggregation and fusion of TPE/EPC SUV was also studied. The pK_a of PE is ~9.5, so that at high pH, PE headgroups are charged. The results are shown in Fig. 4, *a* and *b*. For vesicle aggregation and fusion, the threshold dependence on PE content is the same whether the SUVs are suspended in media at pH 7.4 or at pH 9, but the threshold values are always higher for PE-containing vesicles suspended in pH 9 buffer. In other words, lower PEG concentrations are needed for aggregation or fusion at pH 7.4 than at pH 9, especially for vesicles containing a higher percentage of PE. Aggregation and fusion of pure PC vesicles are not sensitive to pH.

DISCUSSION

There are many forces controlling membrane aggregation and fusion. For aggregation to occur, apposing membranes must be attracted to each other, through van der Waals, electrostatic, depletion/osmotic, or molecular/ionic bridging, against the thermal motion that tends to keep individual vesicles apart. The integrity of the partner membranes is not affected by aggregation. The mechanism through which PEG induces bilayer aggregation is now known to be a phenomenon related to colloidal destabilization by polymer depletion (Napper, 1983; Evans and Needham, 1987; Arnold et al., 1990; Kuhl et al., 1996). PEG of molecular weights between 1000 and 20,000 depletes from the bilayer surface because of steric hindrance. The thickness of the depletion layer is typically on the order of the Flory diameter of PEG, e.g., ~45 Å for PEG8000. As two vesicles

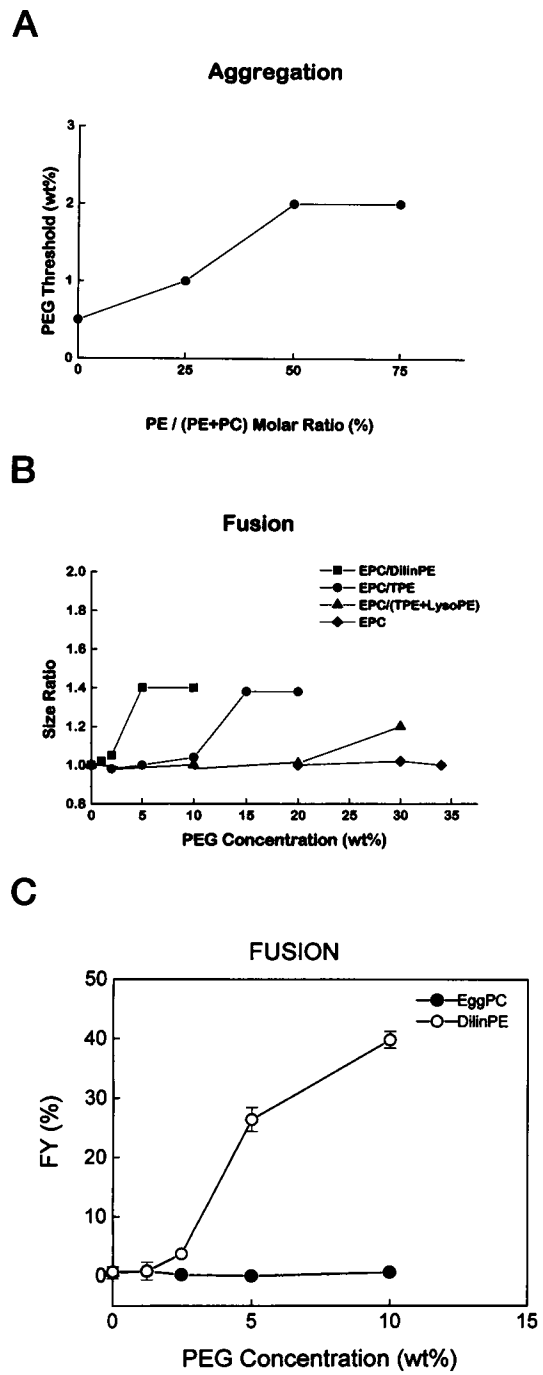

FIGURE 3 (*a*) Threshold concentrations of PEG 8000 needed for the aggregation of dilin-PE/EPC LUVs. (*b*) Final average particle size due to fusion of LUV of 25% of EPC and 75% of different types of PE, after dilution of PEG 8000 solutions of given concentrations. ■, Dilin-PE; ●, TPE; ▲, 2 × TPE + 1 × LysoPE. (*c*) PEG-induced LUV fusion assayed by the NBD-PE/Rh-PE fluorescence energy transfer method. ○, Fusion of labeled and unlabeled PE LUV vesicles (75% dilin-PE + 25% egg-PC); ●, fusion of labeled and unlabeled PC LUV vesicles (100% egg-PC).

approach each other in the suspension within the thickness of their depletion layer, the intervesicle space is devoid of PEG, causing an osmotic pressure gradient to develop from that of the bulk medium, thus leading to vesicle aggregation (Kuhl et al., 1996). This may occur at a relatively low polymer concentration that is sufficient to create an osmotic gradient for stabilizing aggregation. The colloidal instability due to polymer depletion is mainly a property of the polymer, and less a property of the vesicle surface, which do not alter significantly during the aggregation process (Napper, 1983; Evans and Needham, 1987). Our findings, that the aggregation thresholds of vesicles of various PC/PE mixtures occur at ~5% PEG for SUVs and 1–2% PEG for LUVs, and are relatively constant with changing PC/PE ratios, agree with this prediction. The lowering of the aggregation threshold for the 75% dilin-PE sample (Fig. 2 *a*) may indicate that fusion has occurred in this highly stressed sample (see later discussion), the fusion threshold of which is only 2% PEG (Fig. 2 *b*). The fact that LUV (and SUV with 75% TPE or TPE + lyso-PE) aggregation thresholds increase slightly with increasing PE percentage is probably due to the contribution of decreasing flexibility with decreasing PC content. Being able to form hydrogen bonds between headgroups, PE bilayers are more rigid than PC bilayers (Boni et al., 1984b). Bilayer rigidity works against the distortion of vesicles upon contact, limits the contact zone area in the aggregate, and impedes aggregation.

Fusion is a more involved process. An interruption in vesicle wall continuity is needed to allow for the formation of a new continuous membrane and the mixing of vesicle contents in fusion. A form of transient destabilization of the bilayer is necessary for fusion to occur. This means that before fusion of vesicles, the activation energy barrier associated with the disruption or coalescence of bilayers must be overcome. The dehydration defect-causing property of PEG helps to overcome this energy barrier (Boni et al., 1984b; Hui and Boni, 1991). This energy barrier is lower if the "ground state" of prefusion lipid packing energy, including the curvature stress, is higher (Hui and Sen, 1989). Therefore, it is expected that the curvature energy relative to the fusion energy barrier plays a crucial role in the occurrence and kinetics of the fusion process (Siegel, 1993).

Because of the large difference between the equivalent cross sections of headgroups and hydrocarbon chains in their respective polar and nonpolar environments, unsaturated PE molecules are energetically more favorable to be packed in a monolayer of high curvature (Hui and Sen, 1989). When unsaturated PEs are constrained to bilayers of lower curvatures, they are under a curvature stress. The more unsaturated their acyl chains, the higher the curvature energy they have to pay to stay in a low curvature bilayer. The curvature of the inner wall of SUV (~1/100Å) is still considerably lower than the spontaneous curvature of most unsaturated PE (~1/8Å). In mixed lipid bilayers, the curvature stresses of the components are additive (Marsh, 1996). In our vesicles of mixed EPC/PE, the curvature stress may be ranked in the order of the unsaturation (and the number of acyl chains per headgroup) of the PE component, i.e., dilin-PE (18:2;18:2) > TPE (mostly 16:0;18:1) > {TPE + lyso-PE (16:0)}. This is indeed the finding of our experiments. By using a series of PE with decreasing unsatura-

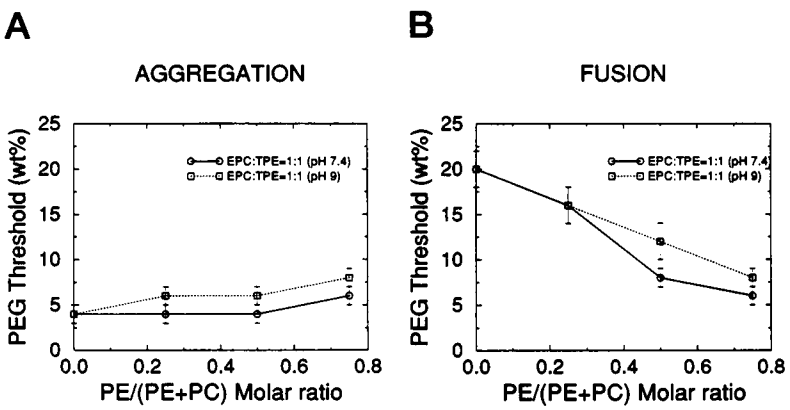

FIGURE 4 Threshold concentrations of PEG 8000 needed for (*a*) aggregation and (*b*) fusion at pH 7.4 (○) and pH 9 (□) for EPC/TPE vesicles.

tion, and by mixing single-chain lyso-PE with TPE, we reduce the curvature stress stepwise. The fusion thresholds increase with decreasing curvature stress, as expected.

At least two factors are to be considered for the reduction of fusion thresholds with increasing PE/PC ratios. Having a larger and more hydrophilic headgroup, EPC has a low spontaneous curvature and therefore contributes a negligible curvature stress to the mixed bilayer (Hui and Sen, 1989). All three PE components in the PE/PC vesicles we used have higher spontaneous curvature than EPC. Incorporating a higher PE percentage in the PC/PE mixture invariably increases the curvature stress of the sample, thereby favoring defect formation and fusion. In addition, the interbilayer water spacing between PEs is considerably less than that between PCs (Boni et al., 1984b; Fenske et al., 1990), thereby facilitating the PEG-induced dehydration process, a step necessary for PEG-induced fusion. Both factors contribute to the lowering of fusion thresholds for samples with increasing PE percentages. The relative contributions of these two factors may be estimated by comparing the extent of threshold lowering by changing unsaturation alone or by changing (TPE + LysoPE)/PC ratio alone (Fig. 2 *b*). The latter seem to be of less importance. This deduction agrees with that of Burgess et al. (1992), based on x-ray diffraction and fluorescence data.

In general, we found that LUVs of pure EPC do not fuse in PEG solutions up to 35% PEG, but fusion occurs at lower PEG concentrations as PE is added, in agreement with that reported by Burgess et al. (1992) for DOPC/dilauroyl-PE LUVs. On the other hand, SUVs of pure EPC do fuse in PEG, as reported earlier (Boni et al., 1984a). It is likely that defects in bilayers, be they related to lipid mixing, vesicle surface curvature, or lipid packing due to curvature stress, facilitate PEG-induced fusion (Boni et al., 1984b; Burgess et al., 1992). This study, using PEs of different levels of unsaturation, highlights the importance of curvature stress.

Another concern is the possibility of lateral separation of PC/PE components, especially in the vicinity of fusion sites. There is no evidence that these lipid mixtures are phase separated before the fusion event. Previous studies of dilauroyl-PE/DOPC (Burgess et al., 1992), DOPE/DOPC (Rand et al., 1990), plant PE/EPC (Hui et al., 1981) and dilin-PE/POPC (Boni and Hui, 1983) did not indicate any immiscibility of components. Whether the fusion event draws a particular component to the fusion site is debatable. Judging from the gradual decrease of fusion thresholds with increasing PE content, it seems more likely that the fusion site is dominated by the general mixture rather then by a particular phase-segregated component, PE. In the latter case, a jump in the threshold values may be indicated at any given immiscible phase boundaries.

An additional consideration is the electrostatic repulsion of apposing bilayers in PEG-induced aggregation and fusion (Boni et al., 1984a). The electrostatic properties of vesicles can be altered by changing the buffer pH through titration of the basic amine of PE. The pK_a of the TPE amine group is 9.5 (Marsh, 1990). At pH 7.4, both EPC and TPE are zwitterions, each having a negatively charged phosphate and a positively charged amine group. When the pH is raised to 9, a large portion of TPE becomes negatively charged. Because of the strong electrostatic repulsion between charged vesicles, we found that aggregation was hindered at the higher pH, as shown in Fig. 4 *a*. The hindrance of fusion (Fig. 4 *b*) is more likely because of a different reason, because aggregation and fusion in our system are not directly related. The charge of PE at high pH could increase the repulsion between headgroups on the same bilayer, expanding the effective headgroup size and lowering the curvature stress. Thus bilayers containing a high percentage of PE are stabilized at higher pH. This change is also manifested in a higher hexagonal phase transition temperature (Sen et al., 1991). The reduction in bilayer curvature stress at higher pH may therefore be responsible for the increase in fusion thresholds.

In conclusion, by changing the relative concentration and acyl chain unsaturation of PE in PE/PC vesicles, we may manipulate the curvature stress of the vesicles. We found that the threshold concentrations for PEG-induced aggregation depend only weakly on the composition of the lipids, as expected from the theory of colloidal destabilization in polymer solutions. Curvature stress plays an important role

in the threshold concentration for fusion, as expected from the energy of bilayer destabilization required for fusion.

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