Polymer Enhanced Fusion of Model Sperm Membranes as Induced by Calcium

Wenfeng Guo, Peter J. Photos, and T. Kyle Vanderlick*

Department of Chemical Engineering, Princeton University, Princeton, New Jersey 08544

Vesicle contents-mixing assays were used to study the fusion of a lipid membrane system that was designed to model the sperm plasma membrane. Comparisons were made to a simpler membrane system that was composed of an equimolar mixture of neutral and anionic phospholipids (phosphatidylcholine (POPC) and phosphatidylserine (POPS)). For both membrane systems, fusion was induced by calcium and was significantly enhanced by the presence of poly(ethylene glycol) (PEG) at a low concentration that was insufficient to cause depletion interactions. In particular, the polymer serves to increase the initial burst and subsequent kinetics of calcium-induced fusion. In comparison with the simpler membrane system, the more-complex sperm mimic vesicles are more resistant to fusion, requiring a higher threshold of calcium concentration. This difference in behavior is attributed to the large fraction of cholesterol (30%) in the sperm mimic membranes. We examined the influence of PEG on ion-binding to membranes using a system that was amenable to nuclear magnetic resonance (NMR). The data are consistent with a PEG-induced reduction of the ion residence time on the vesicle surface, suggesting that ion-binding alterations modulate the kinetics of polymer-enhanced vesicle fusion. Our work suggests that it may be possible to use small doses of PEG in conjunction with fusion-altering agents as effective spermicides.

Introduction

Membrane fusion is an essential event in many physiological processes, including viral entry, endocytosis and exocytosis, and most notably fertilization.[1,2] In vivo, such events are triggered by carefully regulated endogenous agents such as calcium.[1−4] To sperm cells, fusion is an especially important process, as they must undergo both intracellular and extracellular membrane fusion at well-defined times to ensure effective fertilization.[5] Therefore, any agent that can change the kinetics of sperm cell fusion may also be useful as a contraceptive.

Various polymers are able to alter the fusogenic abilities of calcium. Most notably, poly(ethylene glycol) (PEG) has been shown to have synergistic effects with calcium in regard to promoting the fusion of vesicles entirely composed of phosphatidylserine.[6] The acceleration of fusion is thought to be due to the significant aggregation of the vesicles by the PEG, poising them for subsequent fusion by calcium binding. Aggregation of vesicles arising from these polymer-induced depletion forces is now fairly well understood[7] and requires sufficient concentrations of PEG (which vary with molecular weight). The anionic polymer dextran sulfate has been shown to affect calcium-induced membrane dynamics at extremely low concentrations, but this is likely due to charge effects.[8] In any case, all these studies use simple and contrived model membranes, and, although useful from a physical chemistry vantage, they may miss physiologically relevant membrane properties.

Recently, we introduced a series of model membranes that can mimic some of the properties of the sperm plasma membrane.[9] These systems are cholesterol-rich, contain 3′-sulfogalactosyl ceramide, which is a commercially available analogue to seminolipid, and contain highly unsaturated ether-linked phospholipids (in contrast to traditional ester-linked lipids) that are abundant in, and unique to, sperm plasma membranes. The combination of these with more-conventional lipids captures, for example, the leakage behavior that has been observed in vesicles made from rabbit sperm lipid extracts as caused by the presence of the surfactant spermicide nonoxynol-9.[9] The presence of cholesterol is of particular note, because it has a 2-fold impact on the membrane. Foremost, the stiffness of the membrane is dramatically increased, imparting resistance to membrane rupture.[10] Second, the significant amount of neutral cholesterol dilutes the charged lipids on the surface, making the overall surface more weakly anionic. It would be expected, then, that such membrane systems might behave differently than simple membrane models that are based on one or two lipid components.

In this paper, we show a rabbit sperm mimic, designated here as RSP, does undergo fusogenesis in the presence of calcium, but at a higher concentration than that observed in a simpler system consisting of an equimolar mixture of phosphatidylcholine (POPC) and phosphatidylserine (POPS) (referenced hereafter as PCPS). Moreover, using only small amounts of PEG8000 (lower than that necessary to cause depletion forces), we are able to abrogate this hardiness and lower the threshold concentration of calcium required to induce fusion. Such a reduction in the threshold concentration has been reported previously by Arnhold and co-workers,[6] albeit at a much higher PEG concentration than used here. In fact, the concentrations of PEG used in this study are well below those reported in the literature to cause, on their own, significant changes to the anionic membrane or the surrounding solution.[11−13] In combination with calcium, however, we show that PEG at low doses not only magnifies the initial burst of fusion and reduces the threshold concentration, but also extends the duration of the fusogenic events by several orders of magnitude.

Materials and Methods

Materials. Lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC 16:0−18:1), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine (DPPE 16:0−16:0), 1-alkyl-2-acyl-sn-glycero-3-phosphatidylethanolamine (C16:0−22:6), egg sphingomyelin, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidyl-L-serine (POPS 16:0−18:1), cholesterol, and brain sulfatide were obtained from Avanti Polar Lipids (Birmingham, AL). Sodium chloride (NaCl), 2-[4-(2-hydroxyethylene)-1-piperazinyl]-ethane-

* To whom correspondence should be addressed. Tel.: (609) 258-4891. Fax: (609) 258-0211. E-mail address: vandertk@princeton.edu.

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sulfonic acid (HEPES), and phosphorus standard solution were obtained from Sigma (St. Louis, MO). l-Amino-naphthalene-3,6,8-trisulfonic acid (ANTS) and *N*-*N*′-*p*-xylene-*bis*-pyridinium bromide (DPX) were obtained from Molecular Probes (Eugene, OR). Poly(ethylene glycol) (PEG 8000) from Fisher Scientific was used without further purification. All reagents were used at the highest purity available. Deuterium oxide (D_2O) for the nuclear magnetic resonance (NMR) study was obtained from Sigma−Aldrich. The 2.5-mm micro NMR capillary tube for the Varian probe was purchased from New Era Enterprises, Inc. (Vineland, NJ). Praseodymium(III) chloride ($PrCl_3$, 99.9%) was acquired from Alfa Aesar. All solutions were prepared using Millipore water.

Preparation of Large Unilamellar Vesicles. Large unilamellar vesicles (LUVs) were prepared according to a procedure reported in the literature.[14] Briefly, stock solutions of individual lipids dissolved in chloroform were mixed in a 10-mL flask, to produce the desired molar fraction of the individual phospholipid compositions. For PCPS, an equimolar ratio of POPC and POPS was used. For RSP, the following constituents were assembled: 28% C16:0−22:6, 10% POPC, 10% egg sphingomyelin, 5% POPS, 30% cholesterol, and 5% brain sulfatide. Chloroform solvent was removed via evaporation for >4 h under vacuum in a nitrogen environment to form a transparent thin film on the bottom of the flask. The dry lipid film was suspended into a HEPES-buffer solution that contained either 25 mM ANTS or 90 mM DPX and vortexed for 1 min. The suspension of lipids was frozen in liquid nitrogen and thawed in a water bath at room temperature. The vortexing and freeze−thaw cycle was repeated 5 times to form a multilamellar lipid suspension. The suspension was then extruded 10 times through a 100-nm polycarbonate filter (Nucleopore Co.) to form large unilamellar bilayer vesicles. The vesicles were then separated from unencapsulated ANTS or DPX by column chromatography on a Sephadex G-50 column, using the HEPES-buffer solution as the eluant. HEPES-buffer, which is a nontoxic biological buffer solution, was prepared with 16.66 mM HEPES, 125 mM NaCl. The pH of the buffer solution was adjusted to 7.4 ± 0.1 using sodium hydroxide. The osmolarity of the buffer was adjusted to 265 ± 3 mOsm, as measured with a Fisk Micro Osmometer (Fisk Associates, Burlington, MA). The same HEPES-buffer was used for vesicle preparation, fluorescence assays, and light-scattering measurements, as well as NMR experiments. In all cases, the osmolarity of the added reagents were matched with that of the encapsulated aqueous contents inside the vesicles, using NaCl, if needed.

The lipid content of vesicular solutions was determined using a procedure made available by Avanti Polar Lipids.[15] This assay for total phospholipid content was performed with a Genesys 2 spectrophotometer (Thermo Spectronic Instruments).

Vesicle Size Measurements. Dynamic light scattering measurements were performed to determine vesicle size using a BI-200SM laser light scattering goniometer that was equipped with a solid-state laser with a wavelength of $\lambda = 532.5$ nm (Brookhaven, Inc.). All measurements were performed in triplicate at 24 °C at a scattering angle of 90°. Vesicle size was derived by the software of the instrument, using a second-order correlation function of cumulant fit.[16]

Contents-Mixing Assays. Membrane fusion was studied by a vesicle contents-mixing assay, based on the collision quenching of the fluorophore of ANTS by its quencher DPX.[3] Vesicles encapsulated with 25 mM ANTS in HEPES-buffer solution and vesicles with 90 mM DPX in HEPES-buffer solution were mixed at a 1:1 molar ratio. The total lipid concentration was 75 μM, as determined by phosphorus assay. The membrane fusion was determined by monitoring the fluorescence signal, which decreases due to the contents mixing of ANTS with DPX. Because ANTS in the vesicles is not self-quenching, the mere leakage of ANTS does not result in a change in fluorescence intensity.[17] In addition, the minute concentration of DPX in solution is not enough to quench any ANTS in solution. Upon the addition of fusogenic agents, the decay in fluorescence was measured by a Hitachi model F-4500 fluorescence spectrophotometer in time scan mode. The samples were excited at a wavelength of $\lambda = 353$ nm, and an emission maximum was read at 519 nm and performed in triplicate.

^{31}P NMR Analysis. ^{31}P NMR spectra were obtained and processed on a Varian Unity 500 NMR spectrometer (Varian, Inc.) operating at a frequency of 202.48 MHz at 24 °C. To provide the necessary magnetic locking reagent required for NMR measurements, a 0.5-mm micro NMR capillary tube containing D_2O was coaxially inserted into a 5-mm regular NMR tube that contained the vesicle sample. The spectra were recorded with a ^{31}P pulse length of 14 ms (corresponding to a 90° flip angle) and a relaxation delay of 1 s, with broadband ^{1}H decoupling. Measurements were performed both in the presence and absence of $PrCl_3$ shift marker (at 10 mM). A total of 19 200 transients were accumulated for samples with shift marker $PrCl_3$ and 4800 transients for samples without $PrCl_3$ to achieve the same signal-to-noise ratio for all the measurements. A standard phosphoric acid (H_3PO_4) solution was used as a reference (0 ppm) of the theoretical chemical shift. Each sample contained 70 μM POPC lipid in HEPES-buffer solution as described above. All spectra were treated with automatic baseline correction. ^{31}P NMR peak areas were analyzed by computer integration using Varian software VNMR 6.1B. All measurements were performed in duplicate.

Results

Comparison of the Influences of PEG and/or Ca^{2+} on the Contents-Mixing of Vesicles in the PCPS System. To examine the influence of PEG 8000 and/or Ca^{2+} on the process of vesicle fusion, we apply contents-mixing assays with a dye-and-quencher system.[3] Vesicles that contain either dye (ANTS at 25 mM) or its respective quencher (DPX at 90 mM) are formed. When two or more vesicles fuse together, the encapsulants mix; if the vesicles had initially consisted of at least one ANTS-containing vesicle and one DPX-containing vesicle, then the newly fused vesicle is nonfluorescent. Therefore, any decay in fluorescence represents either some fraction of the extent of fusion (note that ANTS-with-ANTS fusion and DPX-with-DPX fusion do not affect the total fluorescence) or dye bleaching. Here, we report the normalized fluorescence intensity F/F_o, where F_o is the fluorescence intensity before adding any fusogens. Incubations with PEG were started by injecting small volumes of 15 wt % PEG (to keep an osmolarity of 265 ± 3 mOsm) into 1 mL of the liposome suspension and gently mixing.

In the control experiment (data denoted by asterisks (*) in Figure 1A), ANTS- and DPX-containing vesicles were mixed in the presence of only buffer solution, and a slight decrease of fluorescence intensity was observed, which may be due to either the spontaneous collisions of vesicles or, more likely, dye bleaching. Similarly, the extent of fusion upon addition of 1.5 wt % PEG alone was very small and almost as insignificant as that of vesicle solution containing buffer only (data represented by open squares (□) in Figure 1A). This is consistent with a lack of depletion interactions at this small polymer concentration.

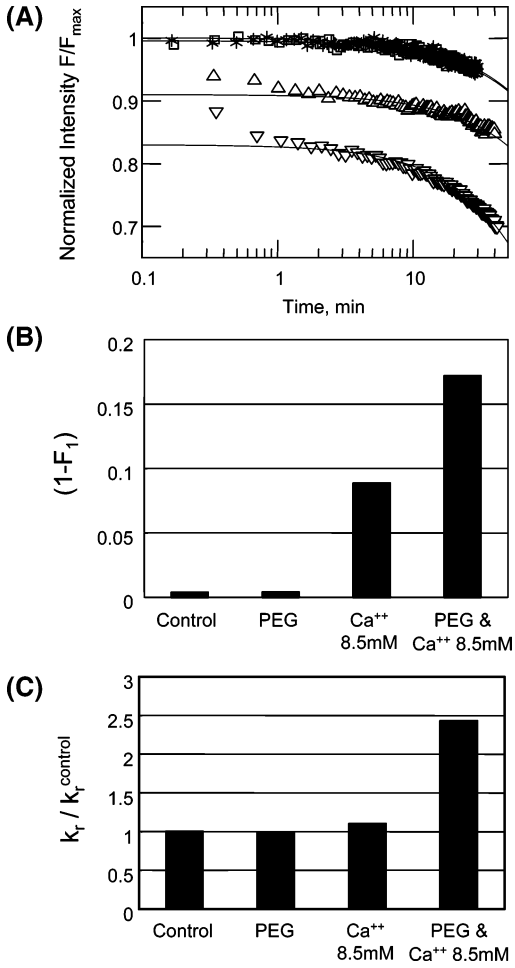

Figure 1. Influences of poly(ethylene glycol) (PEG) on calcium-induced fusion of PCPS vesicles. (A) Fluorescence data showing two-part decay: the first, rapid decrease at times of <2 min, where only fusion occurs; and a second, long-term decay, where bleaching and fusion occur. The curve fits represent data fitted for times >2 min and are for $F = F_o \exp(-kt)$. (Legend: (*) buffer only, (□) PEG (1.5 w/v%) only, (△) Ca^{2+} (8.5 mM) only, and (▽) Ca^{2+} and PEG.) (B) Intensity of the initial decrease in fluorescence in the first two minutes after adding fusogen. In the absence of calcium, little or no decrease is observed. The addition of PEG significantly enhances calcium's ability to induce fusion. (C) Normalized rate constant for subsequent decay. In the control where no calcium is added, it is assumed that only bleaching is occurring. Therefore, further fusion only occurs in the presence of PEG.

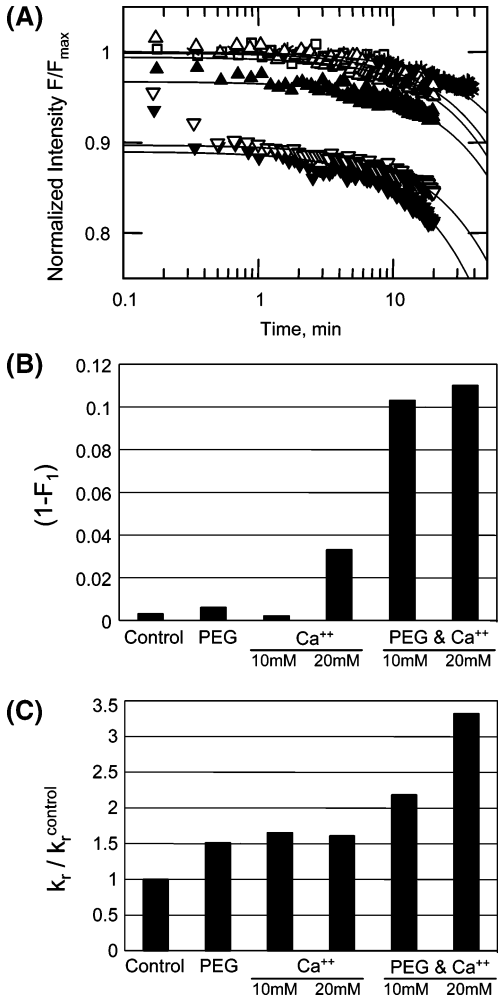

Figure 2. Influence of PEG on the calcium-induced fusion of RSP vesicles. (A) Raw fluorescence data. (Legend: (*) buffer only, (□) PEG (1.5 w/v%) only, (△) Ca^{2+} (10 mM) only, (▽) Ca^{2+} (10 mM) and PEG, (▲) Ca^{2+} (20 mM) only, and (▼) Ca^{2+} (20 mM) and PEG.) (B) Intensity of the initial decrease in fluorescence in the first two minutes after adding fusogen. Note that the lower concentration of calcium is below the threshold required to induce fusion; the addition of PEG significantly reduces this threshold and allows fusion to occur. (C) Normalized rate constant for subsequent decay. The control, PEG-only, and both calcium-only rates are assumed to be within statistical error. Again, any discernible rate is observed only in the presence of PEG.

With the addition of Ca^{2+} ions, a two-stage fluorescence decay was observed: an initial rapid decrease, followed by a slower, gradual decay. Upon injection of Ca^{2+} in either the absence (Figure 1A, upright open triangles (△)) or presence of PEG (Figure 1A, inverted open triangles (▽)), a 10%−20% decrease in fluorescence occurred within the first 1 min. This decrease is completely absent in the controls, and it is almost doubled by the presence of PEG. After this initial period, the rate of fluorescence decay dramatically decreases. To systematically determine the kinetics from the data, fluorescent intensities after the first two minutes of the experiment are fit to the curve $F/F_{max} = F_1 \exp(-k_r t)$, from which $(1 - F_1)$ is reported as the extent of the initial decrease (Figure 1B) and k_r the subsequent rate of decay (Figure 1C). As is clearly seen, the rate of fluorescence decrease is markedly changed (by 2.5 times) only when both PEG and Ca^{2+} are present.

Comparison of the Influences of PEG and/or Ca^{2+} on the Contents-Mixing of Vesicles in the RSP System. Figure 2 shows the contents-mixing assays of the RSP system. As in the PCPS system, we observed no significant fusion of vesicles in a buffer solution or upon the addition of PEG 8000 to a concentration of 1.5 wt % (Figure 2A; asterisks (*) represent buffer data and squares (□) represent PEG-only data). With only PEG present, the initial burst is nonexistent (Figure 2B), as in the case of the PCPS system. Although a slightly more significant subsequent decay rate is observed (Figure 2C), we presume this rate to be within experimental error.

When calcium is added to RSP at a concentration of 10 mM (data represented by open triangles (△) in Figure 2A), no difference in fusogenic behavior is observed, relative to the PEG-only system. That is, no initial decrease was observed, and the subsequent decay rate was only marginally greater than the control system and was similar to the PEG-only system. At 20 mM Ca^{2+} (data represented by open inverted triangles (▽) in Figure 2A), the behavior of the RSP then becomes similar to that observed in the PCPS system at 10 mM Ca^{2+}; i.e., displaying an initial decrease within the first minute. As previously observed, the rate of the decay after this decrease is within error of the buffer-only rate (see Figure 2C). Thus, in

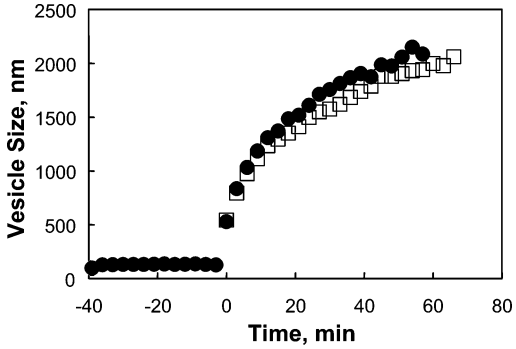

Figure 3. Dynamic light scattering (DLS) measurements of vesicles undergoing fusion. Vesicle size is determined during the fusogenesis experiment. Vesicles are incubated for 40 min prior to the addition of calcium at $t = 0$ min, during which only a modest increase in size occurs. In the presence of PEG, both (●) PCPS and (□) RSP increase in size continually over the course of the experiment, suggesting that fusion continues after the initial burst.

the absence of PEG, RSP requires more calcium to induce fusion than does PCPS.

When PEG is preincubated with the vesicles, both the extent of the initial decrease and the later decay rate are dramatically increased (represented by solid triangles (▲,▼) in Figure 2A), as observed previously with the PSPC vesicles. At 10 mM Ca^{2+}, we now observe fusion, in stark contrast to the PEG-free system. Unlike the behavior in the absence of PEG, the initial drop does not change significantly with increasing amounts of calcium (see Figure 2B), but the rate of subsequent decay increases substantially (see Figure 2C).

Size Determination of Vesicles Using Light Scattering. RSP and PCPS vesicles are formed with a diameter of ∼100 nm, and, upon preincubation with 1.5 wt % PEG 8000 for 40 min, the vesicle size increases rapidly from ∼100 nm to ∼130 nm within the first 5 min and remains the same for the remaining preincubation period (Figure 3). Upon the addition of Ca^{2+} to a final concentration of 8.5 mM, preincubated PCPS vesicles (data represented by solid circles (●) in Figure 3) continually increase in size for more than 60 min to over 2000 nm in diameter. Interestingly, the RSP system at 10 mM Ca^{2+} (data represented by open squares (□) in Figure 3) shows the same behavior.

Parenthetically, we note that the data in Figure 3 include not only fused vesicles, but also aggregates that have likely formed. For example, upon 5× dilution of the system with buffer, the Ca^{2+} ion and PEG concentrations are dramatically reduced, and the PCPS aggregate size decreases to ∼400 nm, with broad polydispersity (data not shown).

Influence of PEG on the Lipid Membrane Monolayers of POPC Vesicles, as Determined by ^{31}P NMR. To further examine the role PEG can have on the interactions of cations with membranes, we use the technique of ^{31}P NMR on vesicles in the presence of Pr^{3+} ions. Briefly, the ^{31}P NMR spectrum of a homogeneous lipid membrane consists of a single peak, because both leaflets of the vesicle bilayer are identical. When praseodymium chloride ($PrCl_3$), which is a paramagnetic shift marker, is added to a pure POPC vesicle solution, Pr^{3+} ions can interact with the phosphorus in the lipid headgroups of the outer monolayer. This causes the spectrum of the ^{31}P in the outer leaflet to shift downfield by ∼20 ppm. Consequently, the two leaflets of vesicle bilayers become spectrally differentiated and provides a way to distinguish the effects of PEG on each of the outer and inner membrane monolayers.[18,19]

We first examine the effects of PEG alone on the bilayer. In the absence of the shift marker, the leaflets of the membrane

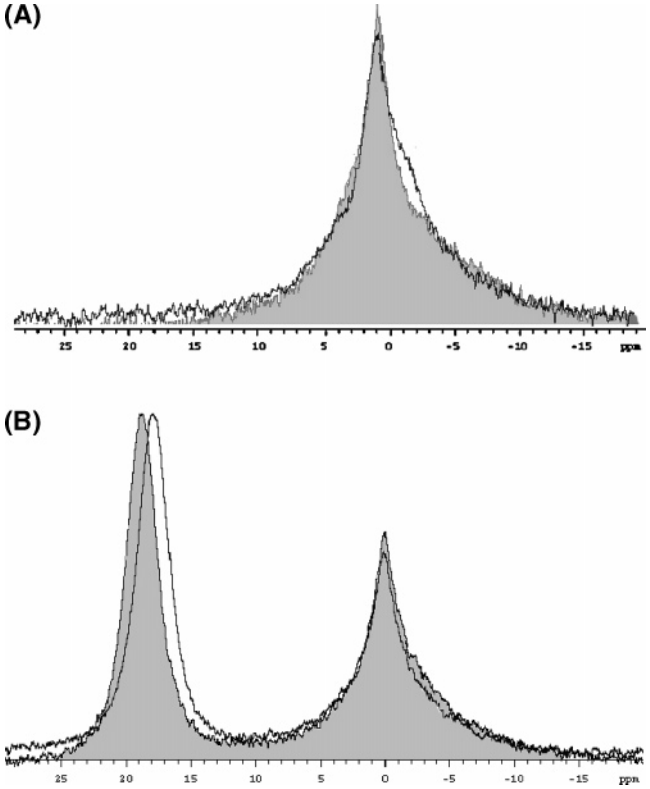

Figure 4. ^{31}P NMR data for praseodymium interactions with the membrane surface. (A) The gray area shows that, in the absence of praseodymium ions, the phosphorus headgroups in both the inner and outer leaflet have identical spectra and therefore the membrane appears as a single peak. The black features show that, when PEG is added, the spectrum remains a single peak but a small but noticeable shoulder is present, which suggests that some alteration in the membrane has occurred. (B) In the presence of Pr^{3+} ions, however, the inner and outer leaflets are split, with the outer leaflet downshifted. Without PEG (lower spectrum), the outer leaflet peak is shifted downfield further than when PEG is present (upper spectrum), which is a result of a shorter residence time of the Pr^{3+} ion over individual phosphorus headgroups.

appear identical (see Figure 4A, shaded spectrum), and when PEG is added to the system, only a slight perturbation is noticed (see Figure 4A, unshaded spectrum). POPC both with and without PEG has only one ^{31}P NMR peak appearing at 0 ppm, with a relative integral of peak area of 100. The slight broadening in the peak for the sample of POPC with PEG indicates a small effect of PEG on the bilayer. However, this impact is not large enough to either split the peaks of each leaflet or shift the total membrane peak.

The addition of a Pr^{3+} shift marker to a final concentration of 10 mM causes each leaflet to appear as a separate peak (see Figure 4B, shaded spectrum). The position of this peak represents the molar averaged position of Pr−P complexes (whose peak would appear at ∼100 ppm) and lone phosphorus nuclei (whose peak would coincide with the inner leaflet at 0 ppm).[20] Therefore, we can use this technique to explore the residence time of multivalent cations on the membrane surface.[20] Thus, the ^{31}P NMR spectrum of the outer leaflet appears as a single peak at ∼19 ppm, downshifted from the inner leaflet at 0 ppm. The area integrals under the two split peaks are 50/50, indicating that the relative number of POPC molecules in the outer monolayer is equal to that in the inner monolayer of the vesicles.

The unshaded spectrum in Figure 4B shows the ^{31}P NMR spectra of vesicles with a 10 mM $PrCl_3$ shift marker in the presence of PEG. The peak associated with the outer leaflet has shifted from 18.71 ppm without PEG upfield to 17.82 ppm

in the presence of 1.45 wt % of PEG. This is only slightly shifted further upfield to 17.80 ppm at 2.90 wt % PEG (data not shown).

Discussion

Despite its complex makeup, RSP vesicles still undergo fusion in the presence of calcium. A significant fraction of POPS and POPC is in the RSP membrane, and, therefore, the same generic behavior is to be expected. The data suggest that the effect of Ca^{2+} on vesicle aggregation, without the presence of a co-fusogen, occurs entirely within the first minutes upon addition of the ions, after which the fluorescence intensity decrease returns to the same rate as that without the additive (which is most likely due to bleaching). The rapid change in intensity, followed by a virtually absent decrease afterward, suggests that, in both vesicle systems, fusion occurs only in these first few minutes.

However, our results show that the additional components found in RSP vesicles impart a resistance to fusion, and vesicles do not fuse at calcium concentrations (10 mM) greater than that at which PCPS vesicles do (8.5 mM). The threshold concentration is thought to be a result of the ion binding energies to the membrane surface[21] and, therefore, is highly dependent on the vesicle surface makeup. For pure PS vesicles, this threshold value is ~2 mM, although smaller values have been reported.[21] The addition of uncharged constituents to the membrane should have a dilution effect on the surface charge: the membrane is less anionic and, therefore, cationic binding is weakened. Changes other than ion binding can also have a role. The addition of small hydrophobic molecules (for example, fluorinated alkanes) can shift the threshold concentration higher.[22] This effect is reported to be due to an increased resistance to membrane tension—which also is believed to have a role in fusion thresholds[23]—via the increasing of the membrane core hydrophobicity and expansion of the bilayer thickness.[24] Cholesterol, then, should have a similar effect on membrane fusion behavior. More generally, cholesterol is known to have a marked effect on many membrane physical properties, such as fluidity.[25,26] All things considered, the presence of ~30% cholesterol in the RSP membrane is likely the most significant cause for the observed shift in the threshold concentration, which we report here to be in the range of 10−20 mM calcium. However, studies into the roles of the other unique lipids in the RSP membrane are still underway.

The resistance of membranes to calcium-induced fusion can be dramatically reduced by adding PEG to the system, at concentrations where the polymer is not fusogenic. Indeed, the presence of PEG alone had no significant effect on either the first-minute rapid decrease or the subsequent rate of fluorescent decay of both vesicle systems. This is as expected; at the low concentration used here, the PEG should, in theory, have little effect on the vesicle membrane. Viguera et al. reported that PEG-induced aggregation of phosphatidylcholine vesicles occurs only above a critical polymer concentration (5% in their study) and that a substantially higher level of PEG is required to induced fusion (23−27 wt %).[27]

Arnhold and co-workers previously reported that the addition of PEG at moderately high concentrations (10 w/v%), in conjunction with millimolar levels of calcium, can not only increase the extent of the initial burst of fusion, but also lower the threshold concentration required to onset fusion events in both small unilamellar vesicles (SUVs) and LUVs.[6] Here, we not only see the increased susceptibility to fusion that has been observed previously, but we also note a long-term extension of fusion events continuing well into the subsequent hour. Only when both PEG and calcium are present do we observe any additional decay in fluorescence beyond that of the control during this time. PEG, then, extends the capabilities of calcium to induce fusion both within the first few minutes (by increasing the initial decrease) and in the subsequent time scale (by increasing the fusogenesis rate) in both RSP and PCPS vesicles.

After the initial burst of fusion, in the absence of PEG, it seems that the calcium cannot escape the membrane surface. However, when PEG is present, the subsequent decay rate is significantly larger than the background rate—i.e., fusion is still occurring—and the continued increase in vesicle size from the DLS data supports that observation. Furthermore, this subsequent rate is dependent on the initial calcium concentration, implicating some calcium-mediated event. Therefore, we suspect that PEG is allowing the release of calcium into the system, which, in turn, allows the continuation of fusogenesis.

While the cause of this synergistic effect between PEG and the Ca^{2+} ion is not clear, Arnhold et al. have argued that PEG induces aggregation in the vesicles, in turn, making them more susceptible to Ca^{2+}-induced fusion.[6] A key difference between our study and that done previously, however, is that our concentration of the PEG is significantly lower. The concentrations required to generate the aggregation of phospholipid vesicles are typically >5 wt %, regardless of the lipid composition used.[28] Furthermore, our DLS data reports only a minor increase in particle size upon the addition of PEG alone to the vesicles, which suggests that pre-aggregation at our PEG concentration is limited.

Although it is possible that PEG may be associating directly with the ion instead of the vesicle, ion−nonpolar-molecule interactions are extremely weak and, therefore, unlikely to be culpable. We instead propose that PEG is associating with the membrane, thereby changing the ion binding rates and, therefore, fusion dynamics. This occurs in both the initial burst, where membrane packing disruption can cause Ca^{2+} ions to bind faster, and in subsequent times, where calcium is allowed to escape from the membrane surface.

Because cation binding is believed to be the cause of calcium-induced fusion, whose kinetics seem to be significantly altered by the presence of PEG, we examined the role of PEG on ion-binding using a model that was amenable to NMR analysis.[20] In short, the data are consistent with a PEG-induced reduction of the ion residence time on the vesicle surface. Gawrisch and Arnold conducted similar NMR experiments, but, again, at much higher PEG concentrations.[29] They noted that the outer membrane peak shifted downward, not upward, with increasing PEG concentration. Although these data initially may seem contradictory to our results, other work by that group suggests that the dielectric constant dependence of PEG−water solutions, with respect to polymer concentration, differs in direction at low and high concentrations.[13] This hints that PEG may be effecting a shortening of the ion lifetime by changing the dielectric constant of the solution, although this connection is tenuous at best. Nonetheless, when PEG is present, the off-rate of the cations is increased.

Conclusions

These studies suggest that PEG can interact with a bilayer surface in a manner that alters its ability to fully attract and capture ions from solution. Specifically, the presence of small amounts of PEG (below that causing depletion forces) augments calcium-based fusion in both the initial burst and subsequent kinetics, as well as a reduction in the threshold concentration required to induce fusion. Furthermore, this synergism occurs

at concentrations that are far more pharmacologically attainable than the high doses reported elsewhere. This may be especially important when unique membrane blends, whose multicomponent makeup may serve to increase the membrane's resistance to fusion, are involved. For example, we find that the more-complex RSP membrane is more resistant to fusion than the simpler PCPS membrane system; this difference in behavior is likely a result of the large amount of cholesterol included in the RSP membrane. From a contraceptive perspective, then, it may be possible to use small doses of PEG in conjunction with fusion-altering agents as novel spermicides.

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