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Effects on Interactions of Oppositely Charged Phospholipid Vesicles of Covalent Attachment of Polyethylene Glycol Oligomers to Their Surfaces: Adhesion, Hemifusion, Full Fusion and "Endocytosis"

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Abstract Oppositely charged giant vesicles are known to adhere, hemifuse and fuse, all of which depend upon the nature of surface contacts. To further understand such interactions, vesicles were surface-modified with polyethylene glycol (PEG), a moiety that reduces surface–surface interactions. Positively charged vesicles were composed of *O*-ethyldioleoylphosphocholine (EDOPC), dioleoylphosphatidylcholine (DOPC) and a carbocyanine dye (DiO), with and without DPPE-PEG (dipalmitoylphosphatidylethanolamine-N-PEG MW of the PEG portion = 2000). Negatively charged vesicles were composed of dioleoylphosphatidylglycerol (DOPG), DOPC and a rhodamine B dye (Rh-PE), with as well as without DPPE-PEG (MW 2,000). A microscope-mounted electrophoresis chamber allowed selected pairs of vesicles to be brought into contact while color images were collected at video rates (30 frames/s). Data collection focused on effects of PEG on vesicle interactions as a function of the surface charge density. Relative to PEG-free preparations, vesicles containing DPPE-PEG (1) formed larger contact zones, (2) underwent adhesion and fusion processes more slowly (by two to four times) and (3) at high charge density were less susceptible to rupture upon contact. Unexpectedly, PEG-containing vesicles exhibited engulfment of a smaller by a larger vesicle, a process topologically similar to cellular endocytosis. These observations are interpreted to mean that, although initial surface–surface interactions are weakened by the intervening layer of PEG chains, eventual and strong bilayer–bilayer contact is still possible, evidently because the lipid anchors of these chains can diffuse away from the contact zone.

Keywords Lipid bilayer · Membrane fusion · Polyethylene glycol · Video microscopy · Fluorescence resonance energy transfer

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Introduction

Membrane fusion and fission are essential cell phenomena, involved in exocytosis, endocytosis, membrane trafficking, cell division and a number of other essential cellular processes. Because of the central role of membrane fusion in cell function, the molecular mechanisms of the process have been under intensive investigation for many years. A number of proteins have been identified as being involved in both regulating and driving fusion processes (Lentz et al., 2000; Brunger, 2001; Jahn & Scheller, 2006). In spite of the identification of many protein participants, however, the complexity of the required lipid rearrangements and of the protein machines that drive them is sufficiently great that the mechanism is still very incompletely understood.

Even the lipid reorganizations that must occur during the fusion of bilayers are poorly understood; hence, a variety of model systems have been developed in efforts to understand what kinds of rearrangements must occur at various stages of the fusion process. One such model system we developed previously involves oppositely charged giant vesicles; although extremely simple, it allows identification of all the major phases of lipid rearrangement that are thought to occur in bilayer fusion (Pantazatos &

MacDonald, 1999; Lei & MacDonald, 2003). This system allows observation, by fluorescence microscopy, of adhesion, hemifusion and full fusion of oppositely charged vesicles; and the probabilities of each step can be controlled by changing vesicle surface charge density. Because of the large size of the vesicles (typically ~ 10 μm), individual pairwise interactions are readily observed and video images can be captured with a VCR or a computer.

The basis for hemifusion of oppositely charged vesicles is evidently the reduction in area per molecule as the two contacting monolayers neutralize one another and mutually experience a diminution in electrostatic repulsion. Although documenting such a change in intact bilayers would be difficult, Langmuir trough techniques have recently provided evidence that the head group area in monolayers (especially in the cationic monolayer) is reduced upon charge neutralization (MacDonald et al., 2006). As the head groups in each of the monolayers pack more closely, gaps or rends must form within the monolayers, exposing the hydrophobic cores (this must occur predominantly at the contact zone, where there is no water contact and, hence, no resistance from hydrophobic effect to formation of an opening) so that when these hydrophobic patches come into contact, hemifusion ensues. Fission (full fusion of the hemifused intermediate) is presumably a result of the tension generated in each of the bilayer walls as the vesicles flatten against each other (flattening the surface of a sphere increases the internal pressure and, therefore, tension in the walls). The reader is referred to earlier reports on these interactions for a more detailed description of the hypothesis as well as for clarifying illustrations of the head group condensation (Pantazatos & MacDonald, 1999) and of vector diagrams of the surface forces involved (Lei & MacDonald, 2003).

Although viral fusion peptides typically have a lysine residue at the N terminus, indicating the role of some form of electrostatic interaction (Martin & Ruysschaert, 2000), fusion based on electrostatic interactions of oppositely charged membranes as a whole is impossible for biological membranes. Among other reasons, there are no known biological membranes that are positively charged; however, the structure-modulating elements that characterize electrostatic-based fusion could be provided by membrane proteins (or cations, whose concentrations could be under the control of proteins), as has been described previously (Kozlov & Markin, 1984; MacDonald, 1988; Chanturiya, Scaria & Woodle, 2000; Chanturiya et al., 2002; Lei & MacDonald, 2003). Those proposals involved protein- (or cation-) controlled changes in the lipid molecular area such that hemifusion is analogous to the fusion of vesicles described here. We are not in a position to judge if condensation of the lipid occurs during biological membrane fusion, but we note that the mechanism should be feasible given it is one of the very few mechanisms of membrane fusion based on a well-characterized model system. In any case, a situation of immediate relevance to fusion of anionic and cationic membranes is that which represents a stage in the release of DNA during transfection of DNA by cationic lipids. Since the latter are the same type of cationic lipid used in this study and because cellular membranes are negatively charged, it seems likely that understanding how to optimize fusion of cationic lipid membranes with anionic membranes could lead to improved efficacy of lipid-based transfection; e.g., charge density is known to affect transfection efficacy (Reynier et al., 2004; Safinya et al., 2006).[1]

Bilayer adhesion, hemifusion and fusion of model membranes depend strongly on the lipid composition (Korlach et al., 1999; Niles, Silvius & Cohen, 1996) and, in the case of oppositely charged membranes, on the surface charge density (controlled according to the ratio of lipid with a net charge to that with no net charge) (Pantazatos & MacDonald, 1999; Garcia et al., 2001). Other types of modification of bilayer surfaces have not been significantly examined with respect to fusion, and one of potential interest is addition of polyethylene glycol (PEG) chains. PEG, which has been widely used to prolong the residence of drug-containing liposomes in the circulation (Klibanov et al., 1990; Mori et al., 1991; Maruyama et al., 1991; Allen et al., 1991a, 1991b), is a hydrophilic polymer that generates depletion forces arising from the tendency of PEG chains to resist confinement. Proteins and polysaccharides protrude from the surface of cell membranes, so the PEGylation of oppositely charged bilayers could constitute a system more closely resembling the interaction of a cationic bilayer (either as a vesicle or as a lipoplex particle) with cell membranes.

Here, we describe how surface-grafted PEG retards the interactions of oppositely charged vesicles. The additional steric barrier of the PEG coat mitigates against the initial close approach of an oppositely charged vesicle, but surprisingly, this can actually increase fusion frequencies by reducing the violence of the initial membrane–membrane association.

[1] This fusion-release step typically occurs within endosomes, the inner monolayer of which is derived from the external monolayer of the plasma membrane. The latter contains only small amounts of anionic phospholipids but does have a significant negative charge due to glycoprotein, with smaller contributions from glycolipids. A larger distance between the bilayer and the charge plane of the proteins which project out from the surface of the bilayer would considerably reduce the linkage of those charges to the bilayer. On the other hand, changes in environmental conditions can lead to changes in exposure of the anionic phosphatidylserine which favor fusion with *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium (DOTAP) lipoplexes, as shown by Stebelska, Wyrozumska & Sikorski (2006).

Materials and Methods

Chemicals

1,2-Dioleoyl-*sn*-glycero-3-ethylphosphocholine (EDOPC), 1,2-dioleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (DOPG), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[lissamine rhodamine B sulfonyl] (Rh-PE) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000 (DPPE-PEG; MW 2,000 for the PEG portion, corresponding to nearly 50 C-C-O- units) were from Avanti (Alabaster, AL). 3,3-Dioctadecyloxacarbocyanine perchlorate (DiO) was from Molecular Probes (Eugene, OR). Routine chemicals were from Sigma (St. Louis, MO). Milli-Q water was used (Millipore, Bedford, MA).

Vesicle Preparation

The procedure for negatively charged giant vesicles was similar to that described previously (Pantazatos & MacDonald, 1999; Lei & MacDonald, 2003); the lipids (typically about 100 μg) DOPC, DOPG, Rh-PE and, if desired, DPPE-PEG2000 were mixed in chloroform in a small glass vial. The solvent was removed under a stream of argon and the residue then placed under high vacuum for 1 h. The dried mixture was hydrated in 0.5 ml 320 mM sucrose at room temperature overnight. A characteristic cloudiness was observed in these samples after several hours of incubation, at which time the vesicles could be used.

Positively charged giant vesicles were produced by mixing EDOPC, DOPC, DiO and, if desired, DPPE-PEG2000 in chloroform and treating as described above for negatively charged vesicles. After overnight hydration, the preparation was put in a −20°C freezer for 1–2 h until completely frozen. The vial was kept without stirring at room temperature for a day or more, after which the vesicles were ready to use.

The presence of DPPE-PEG reduced the yield of giant unilamellar vesicles (GUVs), particularly in the case of positively charged vesicles. We found that DPPE-PEG2000 was the most satisfactory derivative for generating GUVs among the three PEG compounds we tested. This compound has been studied by others (e.g., Visser et al., 2005; Tirosh et al., 1998). The other two we tested were molecular weight (MW) 500 and 5,000 (PEG portion). GUVs were difficult to form with the latter compound, and GUVs prepared with the former did not behave significantly differently from unmodified liposomes. Since inclusion of PEG derivatives reduced the GUV yield, there were limits on the amount that could be incorporated; and even with the PEG2000 compound, it was virtually impossible to generate GUVs at 30 wt%.

It should be recognized that the PEG–lipids do carry a negative charge so that there is some reduction of the cationic charge and some increase in the anionic charge; however, given that the mole percentage, as a consequence of high MW, was only about 3%, it would have a minor effect on the data reported here.

Electrophoresis Control Chamber

Vesicles were manipulated electrophoretically by imposing an electric field along two orthogonal channels in a chamber mounted on the stage of the microscope, as described previously (Pantazatos & MacDonald, 1999; Lei & MacDonald, 2003).

The standard procedure was to apply a voltage (15–30 *V*) between the electrode pairs so as to bring selected large, oppositely charged vesicle pairs close to each other. When they were a few microns apart, the electric field was turned off and the vesicles were allowed to contact one another by Brownian motion. We preferred to have unilamellar vesicles for the obvious reason that the outcomes of their interactions should be most readily interpretable. Although we sometimes took data on vesicles that might have more than one bilayer or that contained internal vesicles, it was generally not difficult to identify vesicles that were unilamellar (or at least very likely so) because of their uniform sphericity and low intensity of fluorescence (intensity is proportional to the number of layers and one can almost invariably identify a number of vesicles with minimum fluorescence). Of course, bilayer–bilayer repulsion of surfaces with a significant net charge strongly favors separation of lamellae, especially in the low–ionic strength solutions, so the proportion of vesicles that are multilayered in the preparations we used was far less than is normally the case in, say, neutral vesicles of phosphatidylcholine (PC).

Fluorescence Video Microscopy and Video Analysis

Interactions of vesicles were monitored with an inverted fluorescence microscope (Diaphot-TMD; Nikon, Tokyo, Japan) equipped with a plan neofluor 100x oil objective lens and a 100 W mercury lamp. Filters were as follows: exciter, 455 nm with 40 nm bandpass; dichroic, 505 nm longpass; emitter, 510 nm longpass (all from Omega Optical, Brattleboro, VT).

This filter combination provided good visibility of DiO-labeled vesicles. Rhodamine-labeled vesicles were dim but

sufficiently visible to be selected for interaction with the oppositely charged cationic DiO-labeled vesicles. Although the consequence of this choice of probes and filters was unequal intensities of emission of the separate vesicles, its advantage was in providing information on vesicle–vesicle interactions through energy transfer from DiO to rhodamine. Thus, contacting bilayers appeared yellow, whereas mixed membranes (after fusion) appeared red. The yellow is presumed to be the result of partial energy transfer such that the green DiO emission is reduced while the rhodamine emission is increased so that the two intensities become comparable and the perceived color of the contact is yellow. Membrane mixing occurs when fusion brings the two fluorophores together in the same membrane. Under such circumstances, the energy transfer is very high (as can be demonstrated in a conventional fluorometer with vesicles having mock fusion compositions), with the DiO becoming almost completely quenched and the emitted light—effectively just from rhodamine—being red.

Events of interest were captured with a color video camera (SHC-710; Samsung, Seoul, Korea) on the microscope and recorded on videotape for subsequent transfer to a computer. The digital images were transferred to the computer with a video capture card (Studio DC10 Plus; Pinnacle Systems, San Jose, CA). Adobe Photoshop (Adobe Systems, Mountain View, CA) was used to adjust the brightness and contrast of the images to improve visibility.

Results

Vesicles without PEG

Before describing the interactions of GUVs with PEG on their surface, we first describe the interactions of vesicles without PEG in order to better highlight the effects of the surface modification. For illustration (Fig. 1), we have chosen vesicles with moderately high surface charge density (31% charged lipid) because these exhibit the complete course of interaction events, namely approach, contact, hemifusion and full fusion of oppositely charged membranes. With excitation of the positively charged vesicle labeled with DiO at 455 nm through the 40-nm bandpass filter, it is outlined in bright green (first two images of Fig. 1). The negatively charged vesicle, which is to the right of the positively charged vesicle and is labeled with rhodamine, fluoresces red but is considerably less bright (excitation peak 555 nm). When the two vesicles contacted (Fig. 1c), a flat contact zone formed, the area of which increased for several frames (each frame = 33 ms, until Fig. 1f). The contact zone became yellow and relatively bright due to the close association of the two surfaces, which allowed some energy transfer to the rhodamine so that the green and red intensities are more closely the same and give the appearance of a single yellow line. The brightness of the vesicle wall in regions of the bilayer outside the contact zone remains about the same from Figure 1c through e; however, beginning with Figure 1f, orange color begins to appear in the green positively charged vesicle, and by Figure 1g, about 1.5 s later, the negatively charged vesicle has become brighter red. This red color is the result of much more extensive energy transfer and indicates that interdiffusion of the two probes between vesicles has occurred. Because it generally occurs abruptly, this changing of the color of each vesicle is presumed to be the result of hemifusion, in which the outer monolayers of the two vesicles become continuous. Since the fluorophores are seen to interdiffuse, so must also the charged lipids, with the result that the net charge on each vesicle is reduced. As the charge is diminished, adhesion is also reduced and the adhesion zone shrinks. The contact zone gets smaller from Figure 1f to h but then, between h and i, the vesicles fuse and do so in considerably less than the 33 ms of one video frame. From Figure 1i to j, the fusion product rounds up and the red color due to extensive mixing of DiO and rhodamine has almost covered the surface.

The GUVs of Figure 1 contained 31 wt% of positively or negatively charged lipid. Typically, vesicles in the range of about 25–50% charge exhibit the stages of interaction shown in Figure 1, namely adhesion, hemifusion and fusion. With a higher charge density, especially >60%, the hemifusion stage may be too short to capture with a video camera. In addition, the interactions are more violent and the probability of rupture is considerably increased. At 80% net charged component, most interactions lead to

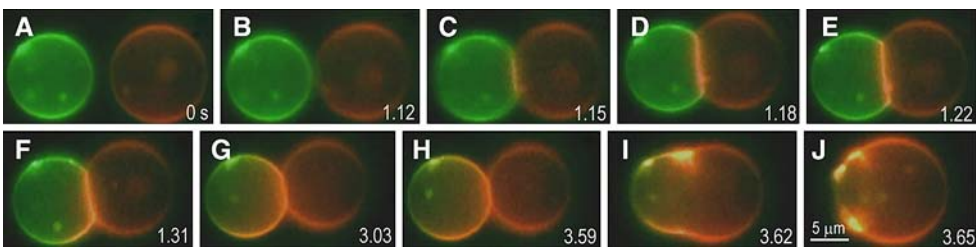

Fig. 1 A pair of oppositely charged vesicles undergoing contact, adhesion, hemifusion and full fusion. Negatively charged vesicle (*red*): 65 wt% DOPC, 31% DOPG, 4% Rh-PE. Positively charged vesicle (*green*): 65% DOPC, 31% EDOPC, 4% DiO

Table 1 Distribution of various outcomes of vesicle interactions with and without PEG[a]

% Charged component[b]	10%		20%		50%		80%	
	No PEG	With PEG[c]	No PEG	With PEG[c]	No PEG	With PEG[c]	No PEG	With PEG[c]
Adhesion	0	4	2	11	0	4	0	0
Hemifusion	9	12	55	33	14	16	4	8
Full fusion	0	0	21	14	31	42	26	31
Rupture	0	1	4	7	49	13	67	38
Engulfment	0	8	1	30	2	20	3	23
Null event	91	75	17	7	4	5	0	0

[a] For each charge ratio, 100 events were tabulated

[b] Positively charged vesicle composition EDOPC, DOPC, DiO; negatively charged vesicle composition DOPG, DOPC, Rh-PE, both with and without DPPE-PEG, as indicated

[c] With PEG, Cationic and anionic vesicles both contained 10 wt% DPPE-PEG2000. This corresponded to about 3 mole% negative charge, which reduces somewhat the charge on the cationic vesicles and increases it on the anionic vesicles relative to the charge percentage shown at the top of the table

immediate rupture, leaving only a very small bright fluorescent particle (although fusion may well have occurred in the meantime). At lower charge density, in the range of 10–25% charge, hemifusion commonly occurs, but it often does not lead to full fusion. At the lower end of this range of charge density, adhesion may be the final stable state; i.e., the vesicles flatten against each other, but there is no visible change in appearance and the colors remain separate for as long as they can be observed (bleaching limits observation to about 100 s). At low charge, <10%, adhesion may not even occur or, if it does, it is so weak that there is no flattening of the contacting vesicles.

A tabulation of the frequencies of the different outcomes that were observed is given in Table 1; it will be considered in detail below, along with comparable data for PEG-containing vesicles.

Vesicles with PEG on Their Surfaces

Giant vesicle formation

Inclusion of PEG in the lipid mixture had an influence on GUV formation, considerably more so for positively charged than for negatively charged vesicles, in which case the effect was relatively minor. Even in the absence of PEG, the yield of positively charged GUVs is generally lower than that of negatively charged GUVs, however; and this may be why the effect of PEG is more obvious.

Vesicle–vesicle interactions

Interaction of oppositely charged vesicles containing PEG but having a moderate (20%) percentage of charge commonly led to shape distortions of interacting vesicles. The vesicles of Figure 2 contain internal vesicles and, although it is not clear how those internal compartments affect the properties of the vesicle as a whole, it is nevertheless clear that the PEG-containing bilayers exhibit a slow, time-dependent tendency to increase their contact surfaces. Furthermore, because there are no color changes involving loss of green intensity and increase in red intensity, it is likely that this adhesion process occurs without hemifusion (or any exchange of fluorophores), even out to a minute, at which point the DiO has become extensively photobleached.

As with unmodified vesicles (Fig. 1), full fusion of PEG-modified vesicles is more probable at higher surface charge density, but there are some distinct differences in the behavior of the two types of vesicle. These differences may be seen by comparing Figure 3, which shows fusion of PEG-modified vesicles, with Figure 1. The differences seen here, as well as in many other instances (Table 1), are as follows: (1) the diffusion of the Rh-PE fluorescent probe from the negatively charged vesicle (red) to the positively charged vesicle (green) was slower in the presence than in the absence DPPE-PEG, (2) the PEG-modified vesicles did not retract from each of prior to under going full fusion and (3) most of the time the contact zone between PEG-containing vesicle surfaces was curved rather than flat as in the case of unmodified vesicles.

The most dramatic effect of PEGylation was the frequent occurrence of a large vesicle engulfing a small vesicle, a process that topologically bears considerable similarity to cellular endocytosis. Although engulfment was seen among interactions of vesicles that did not contain PEG, it was rare, whereas among PEG-containing vesicles it was commonplace. Figure 4 shows several examples of such processes that differ in their final outcomes. The upper panel shows a case where the smaller vesicle virtually instantly (less than one frame) entered the large vesicle. The latter evidently leaked contents in the

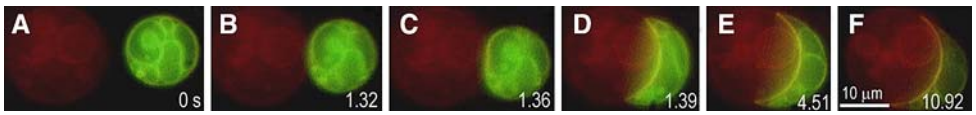

Fig. 2 Adhesion of oppositely charged, PEG-containing vesicles. Negatively charged vesicle (*red*): 66% DOPC, 20% DOPG, 10% DOPE-PEG2000, 4% Rh-PE. positively charged vesicle (*green*): 20% EDOPC, 10% DPPE-PEG2000, 66% DOPC, 4% DiO

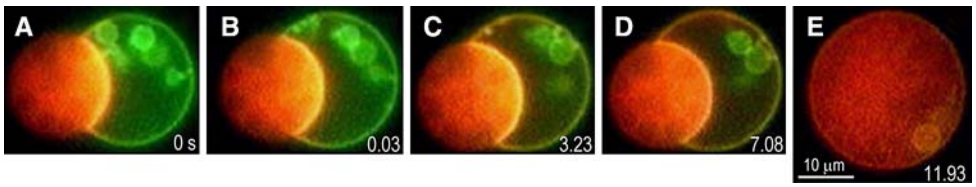

Fig. 3 Visualization of oppositely charged vesicles undergoing hemifusion and full fusion. Negatively charged vesicle (*red*): 60% DOPC, 26% DOPG, 10% DPPE-PEG2000, 4% Rh-PE. Positively charged vesicle (*green*): 60% DOPC, 26% EDOPC, 10% DPPE-PEG2000, 4% DiO

process since its volume decreased after the small vesicle entered. Finally, the two vesicles ruptured, perhaps as result of the rend that occurred during engulfment or possibly because of a preexisting defect in the large vesicle.

The middle panel of Figure 4 shows a small vesicle that entered a large vesicle slowly. Here, following engulfment, the small vesicle fused with the large vesicle while inside the latter. Although the fusion process is too fast to be captured with the camera we used for the experiments described here (it can be faster—possibly much faster—than 10 ms; Lei & MacDonald, 2003), it is clear that fusion has occurred because the large vesicle has increased in area. The lower panel of Figure 4 shows a situation that appears to involve engulfment followed by "regurgitation"; i.e., a small vesicle entered a large vesicle but then was ejected. As described under "Discussion," the actual event shown here must be more complex than engulfment and then its reverse. In any case, the final step was still fusion, for as Figure 4i–j shows, the large vesicle became larger simultaneously with the disappearance of the small vesicle (again, fusion processes are usually much too fast to be captured by a video camera).

An interesting case of multiple engulfment is shown in Figure 5. Here, four separate cationic vesicles are seen to be engulfed by a single, but considerably larger, anionic GUV. Judging from the yellow color of the negatively charged vesicle and small internal vesicles already present in the first frame, the GUV had already taken up a number of small cationic vesicles. There is little indication that any interactions beyond engulfment occurred.

Comparison of Vesicle–Vesicle Interactions in the Presence and Absence of PEG-Modified Surfaces

As the proportion of net charged component in the vesicle membrane was reduced, the interactions between vesicles changed quantitatively and qualitatively; moreover, the presence or absence of PEG influenced the outcomes of those interactions. The various interactions we observed are summarized in Table 1.

As revealed in the table, at a given charge ratio, the proportions of adhesion, hemifusion, full fusion and rupture were affected by PEG content. Adhesion and hemifusion were the least common consequences of interactions of all oppositely charged vesicles, but these were also proportionately affected most strongly by PEG. Significant effects not disclosed by the table had to do with the time courses of all events (see below).

In addition to the differences in the distribution of outcomes as shown in Table 1, there were some other distinctive effects of PEG. Because engulfment was more common in the presence than the absence of surfaces covered with PEG, curved contact zones were the rule (although they were transient because engulfment often followed adhesion). In contrast, in the absence of PEG, hemifusion or hemifusion followed by fusion was more common and these processes typically proceeded from flat contact zones.

Another obvious difference between the two sets of vesicles that is not reflected in the table is the relative slowness of interactions of PEG-containing vesicles. For vesicles lacking PEG, a flat contact zone formed within a few video frames and commonly hemifusion ensued, as was indicated by interdiffusion of the fluorescent probes. It appeared that DiO diffused into the negatively charged vesicle faster than Rh-PE diffused in the opposite direction, although we cannot know whether this is real or merely the perception of effects of energy transfer on the color intensities. In the case of the vesicles containing DPPE-PEG, the time for transition from adhesion to hemifusion, when it did occur, was four to eight frames, corresponding to a process two to four times slower than in the absence of PEG.

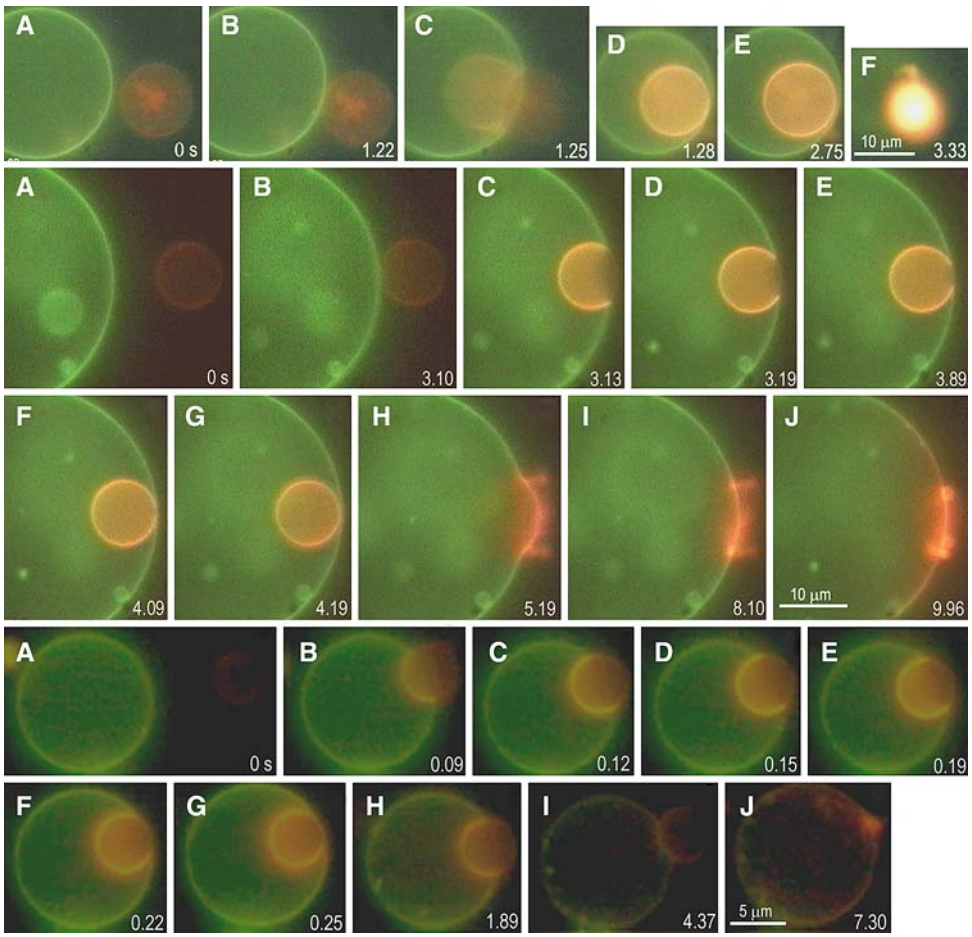

Fig. 4 Engulfment in vesicle–vesicle interactions. *Upper panel* As the small vesicle entered the large vesicle (*b–e*), the large vesicle became smaller, indicating a loss of aqueous contents. During entry, the intensity of the small vesicle increased and its color changed from red to yellow, a result of weak energy transfer from the large vesicle bilayer to that of the small vesicle and indicating that the surfaces of the two vesicles have come into relatively close contact. Negatively charged vesicle (*red*): 56% DOPC, 30% DOPG, 10% DPPE-PEG2000, 4% Rh-PE. Positively charged vesicle (*green*): 56% DOPC, 30% EDOPC, 10% DPPE-PEG2000, 4% DiO. *Middle panel* A large vesicle engulfs a considerably smaller vesicle. After the small vesicle becomes entirely enclosed, fusion occurs (*h*), indicated by the reddish bulge on the continuous membrane and by the diffusion of red fluorescence along the surface of the large vesicle (*h*(*j*). The latter becomes slightly larger (*i, j*), but excess membrane apparently evaginates, seen as a surface roughing. Negatively charged vesicle (*red*): 66% DOPC, 20% DOPG, 10% DPPE-PEG2000, 4% Rh-PE. Positively charged vesicle (*green*): 66% DOPC, 20% EDOPC, 10% DPPE-PEG2000, 4% DiO. *Lower panel* Engulfment that is initially similar to that of the other panels but with a different outcome. In this case, the small vesicle enters and then more slowly exits (*h, i*) the large vesicle. Although almost completely extruded, the small vesicle retains a significant area of contact and a few seconds later fuses with the large vesicle (*i*→*j*). Negatively charged vesicle (*red*): 61% DOPC, 25% DOPG, 10% DPPE-PEG2000, 4% Rh-PE. Positively charged vesicle (*green*): 61% DOPC, 25% EDOPC, 10% DPPE-PEG2000, 4% DiO

Discussion

Effects of PEG on the Interactions of Oppositely Charged Vesicles

Three factors may be presumed to influence interactions of vesicles containing PEG relative to those lacking PEG. First, the diffusion rate of charged lipid in the membrane can be expected to be reduced. Suppose the lateral diffusion rate constant of lipid molecules of a vesicle is 4×10^{-8} cm^2/s (Jacobson, Ishihara & Inman, 1987). For a 10-μm-diameter vesicle, according to $t=<r^2>/4D$ (random walk theory for two dimensions), the diffusion of molecules over the surface takes on the order of a minute. This value is basically consistent with our experimental results that when hemifusion occurred between EDOPC and DOPG vesicles, the rhodamine of the cationic vesicle took some tens of seconds to cover the cationic vesicle. (As seen in Figure 1, the rhodamine had not diffused to the far side of the vesicle—some green color remained—by the time full fusion occurred at about 4 s and, in Figure 3, where larger vesicles are involved, there is green color visible at over 7 s, the last hemifusion image shown.) Such a relatively high diffusion rate in the unmodified vesicles would allow rapid accumulation—after vesicle contact—of additional oppositely charged lipid in the contact zone. This could (indeed,

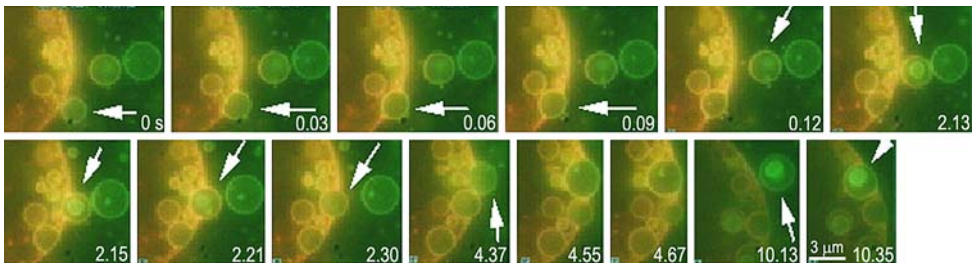

Fig. 5 Small vesicles sequentially enter a large vesicle. The negatively charged vesicle (*yellow*) was 71% DOPC, 15% DOPG, 10% DPPE-PEG2000 and 4% Rh-PE; the positively charged vesicles (*green*) were 71% DOPC, 15% EDOPC, 10% DPPE-PEG2000 and 4% DiO. Close contact but absence of hemifusion is indicated by the persistence of yellow color of the positively charged vesicles after being engulfed

should) occur because ion pair formation between oppositely charged lipid molecules would lower the chemical potential of the lipids in the contact zone and (initially, at least) create a free energy gradient favoring replacement of uncharged PC in the contact zone by charged lipid. The resulting increased electrostatic interactions may produce a mechanical effect large enough to initiate hemifusion.

The presence of lipid molecules with head groups modified through attachment of polymer chains such as PEG has the effect of slowing diffusion in the plane of the membrane. Noppl-Simson & Needham (1996) found that 2 mol% PEG750 reduced the rate of surface diffusion of avidin by a factor of about five. Although a protein will be affected more than a lipid, at the concentrations used here, the PEG molecules are in the "brush" regime and experience considerable interactions with each other (Marsh, Bartucci & Sportelli, 2003). Since the lipid portions of DPPE-PEG will be restricted, so too will be the other lipids in the same monolayer (Soong & Macdonald, 2005). We therefore conclude that PEG in the vesicles will also slow the migration of charged lipids to the contact zone and delay its destabilization.

In addition, and perhaps more importantly, extension of the chains of DPPE-PEG from the bilayer surface will prevent close contact (Marsh et al., 2003) of the oppositely charged membranes and reduce their electrostatic interaction. Yoshida et al. (1999) calculated that 10 mole% DPPE-PEG2000 covers about 90% of the surface of a bilayer, with PEG coverage being approximately proportional to the PEG molar concentration. According to scaling theory (De Gennes, 1980), PEG chains of the MW used here would extend approximately 5 nm from the vesicle surface (Woodle & Lasic, 1992). Therefore, the separation between two oppositely charged adherent vesicles will be larger and the electrostatic force will be smaller than that between vesicles lacking PEG.

Third, because of the resistance to compression of the PEG chains, PEG-lipids must cause some head group expansion and, thus, affect the hydrophilic and hydrophobic balance within the bilayer. Although small, the consequence of such a PEG-driven looser packing is sufficient to be detectable as a reduced chain melting transition of bilayers (Marsh et al., 2003). This effect increases the force needed to bend bilayers, and since bending is normally considered to be required for membrane fusion, a PEG coating is expected to mitigate against bilayer fusion.

These factors appear to be able to account for many of the differences between vesicles that contain PEG and those that do not. Given that the primary influences of PEG chains are those enumerated above, fusion of the cationic and anionic bilayers could still occur, albeit more slowly. In that case, electrostatic-based adhesion between PEG-containing vesicles is presumed to occur but weakly because of the intervention of the PEG chains. The tendency would then be for the two surfaces to come closer together and simultaneously favor diffusion of the PEG-lipid away from the site of close contact, leading to a larger area of even closer contact. Also, there could be some release of the PEG–lipid into the aqueous phase, either spontaneously or encouraged by compression as the contact zone widens. In any case, if the bilayers come into actual contact with exclusion of much of the intervening water, then fusion could ensue through the mechanism for oppositely charged bilayers lacking PEG described elsewhere (Pantazatos & MacDonald, 1999; Lei & MacDonald, 2003) and outlined above (see "Introduction").

Engulfment in Vesicle Interactions

Although engulfment was sometimes observed during interactions of oppositely charged vesicles lacking PEG, it was definitely much more prevalent with vesicles containing PEG (Table 1). The driving force for the process is presumably a result of the adhesion energy of the two membrane surfaces. Such a wrapping around of the small vesicle by membrane of the large vesicle would mean that the inner vesicle is actually—over most of its surface—composed of two bilayers. The fact that the ring of

internalized material is usually bright yellow (whether the engulfed vesicle is negative or positive) means that the membranes usually remain separate, for if hemifusion occurred, the DiO-labeled bilayer would become quenched and the rhodamine-labeled bilayer would exhibit sensitized fluorescence and become brighter red. The fact that the engulfed membrane is yellow, as is the contact zone between two adhering vesicles that have similar diameters—a result of emission of both red and green light in both cases—means that the green fluorescence that remains must not be greatly different from the intensity of the red light emitted after energy transfer. Because energy transfer changes very rapidly with distance, only a narrow set of distances would permit such a situation. Nevertheless, such conditions could apply in the case of apposed membranes because the separation between fluorophores in a given membrane is considerable, corresponding to the thickness of the bilayer (nearly 4 nm). Thus, it may be that the outer monolayers of the contacting bilayers participate in extensive energy transfer whereas the inner monolayers, being much farther apart (by twice the thickness of a bilayer, or more than 6 nm), do not.

In order for the smaller vesicle to be engulfed, the membrane of the larger vesicle must either stretch or transiently tear. We have not systematically examined the probability of engulfment as a function of vesicle size differences but, at least in a number of cases, it is consistent with the amount bilayers can stretch. For example, in the case of the two vesicles in the middle panel of Figure 4, the diameter of the large vesicle is about 40 μm and that of the small vesicle is about 8 μm, so the area ratio is about $S_S/S_L = (8/40)^2 = 0.04$, where S_s/S_L is the ratio of the area of the small vesicle to that of the large vesicle. This corresponds to a stretch in the larger vesicle membrane of 4% for it to wrap around the small vesicle. Although there are substantial variations as a function of bilayer composition, 4% is in the range that bilayers can stretch without breaking (Needham & Nunn, 1990). It is also possible that the larger vesicle tears and then reseals, transiently releasing a sufficient volume of contents to allow it to fully engulf the smaller vesicle. Vesicles made in the same way as those described here are in fact capable of forming large, transient pores, as described elsewhere (Tenchov & MacDonald, 2005), at least when subjected to expansion under the influence of osmotic pressure.

The most obvious question is why PEG-modified vesicles exhibited engulfment so much more frequently than did vesicles lacking the surface coating. One likely factor is that the PEG coat interferes with fusion more than will adhere so that the vesicles are trapped in an intermediate state that, in the case of vesicles of dissimilar diameters, represents the smaller being engulfed by the larger. It could also be that the tendency of the PEG clouds to expand allows the membranes to dilate more under application of tension than do bilayers composed only of phospholipids. If tearing and release of contents plays a role in engulfment, then the implication would be that PEGylation facilitates transient lysis since, if anything, addition of PEG to oppositely charged bilayer surfaces must reduce their electrostatic interactions.

Some engulfed vesicles do eventually undergo fusion, as shown in the middle series of images of Figure 4. We cannot determine whether this is due to the slow accumulation of charged lipids at some point (presumably regions where, due to statistical variations, the space between contacting surfaces is somewhat thinner than average) that, in turn, leads to exclusion of the PEG-PE and thus to additional recruitment of charged pairs in a regenerative fashion. We are also unable to rationalize the tendency of engulfment to occur more commonly when vesicles were prepared in sucrose solution than in water (G. Lei, unpublished data).

The last panel of Figure 4 shows a case where the engulfed small vesicle was released from the large vesicle. Although perhaps an alternative possibility has escaped us, we cannot explain this process on the basis of single pairs of bilayers interacting and suspect that, in fact, such a process is the result of an interaction with a small vesicle that has a double wall. In that case, if the outer bilayer fused with the large vesicle, then the innermost small vesicle would be released as shown in images g through i (although it might rebind after the fused membranes exchange lipids sufficiently that the whole surface of the large vesicle becomes uniformly charged again).

Implications

Under physiological conditions, the cell membrane charge density ranges from −0.03 to −0.24 e/nm^2 (Chou, Jaric & Siggia, 1997). In our experiments, a vesicle with a 10% net charge has a surface charge density of about 0.2 e/nm^2. Thus, the lower charge densities for the negatively charged membranes in the experiments described here were in the range of those of cell membranes. Of course, there is no reason at present to believe that electrostatic interactions of the type described here play any role in normal cellular fusion processes, but they are clearly of paramount importance in transfection of DNA into cells with lipoplexes, a technique that is one of the most common and useful in molecular biology. Lipoplexes, complexes of cationic lipid with DNA in which lipid is normally in excess over DNA, probably have charge densities near or above the upper limit of those of the cationic vesicles used here. The anionic glycolipids and some glycoproteins on cell surface membranes are typically mobile and, like the PEG chains in the in vitro studies described here, would need to diffuse away to allow contact

and fusion of lipoplex bilayers with cell membranes (e.g., endosomal membranes). The present studies are thus likely to be very relevant to fusion processes that occur in lipoplex interactions with cells.

In addition to relevance to transfection of DNA, the data presented here show that PEG–lipid conjugates can be used to modulate membrane fusion. The ability to control such activities could, among other benefits, be of value in developing programmable fusogenic liposomes for intracellular drug delivery.

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