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Directly Observed Membrane Fusion Between Oppositely Charged Phospholipid Bilayers

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Abstract. A novel method was developed for the direct examination of pairwise encounters between positively and negatively charged phospholipid bilayer vesicles. Giant bilayer vesicles (unilamellar, 4–20 μm in diameter) prepared from 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine, a new cationic phospholipid derivative, were electrophoretically maneuvered into contact with individual anionic phospholipid vesicles. Fluorescence video microscopy revealed that such vesicles commonly underwent fusion within milliseconds (1 video field) after contact, without leakage. Fusion occurred at constant volume and, since flaccid vesicles were rare, the excess membrane was not available after fusion. Hemifusion (the outer monolayers of each vesicle fused while the inner monolayers remained intact) was inferred from membrane-bound dye transfer and a change in the contact area. Hemifusion was observed as a final stable state and as an intermediate to fusion of vesicles composed of charged phospholipids plus zwitterionic phospholipids. Hemifusion occurred in one of three ways following adhesion: either delayed with an abrupt increase in area of contact, immediately with a gradual increase in area of contact, or with retraction during which adherent vesicles dissociated from a flat contact to a point contact. Phosphatidylethanolamine strongly promoted immediate hemifusion; the resultant hemifused state was stable and seldom underwent complete fusion. Although sometimes single contacts between vesicles led to rupture of both, in other cases, a single vesicle underwent multiple fusion events. Direct observation has unequivocally demonstrated the fusion of two, isolated bilayer-bounded bodies to yield a stable, non-leaky product, as occurs in cells, in the absence of proteins.

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Key words: Hemifusion — Fluorescence microscopy — Cationic lipid — EDOPC

Introduction

Membrane fusion, which is fundamental to a host of eukaryotic cell functions, involves an exceedingly complex series of membrane interactions in which many proteins participate [51, 52, 60]. A prominent strategy for investigating the mechanism of fusion has been to determine the conditions necessary for fusion of simple, purely lipid bilayers and to infer from such conditions what vital functions could be contributed by proteins. The most popular model bilayer systems are those in which anionic phospholipid vesicles are induced to adhere either to one another or to a planar bilayer of similar composition by addition of calcium or other cation [13, 45, 49, 63].

Many imaginative approaches have been taken to dissecting out the details of vesicle-vesicle and vesicle-planar bilayer interactions, as monitored by a number of assays of the merger of membranes and the coalescence of aqueous compartments [63]. Amperometric measurement of ion channels transferred to the planar membrane has been an additional powerful tool for investigating veiscle-planar membrane interactions [16]. A host of investigations have provided abundant evidence for membrane and contents mixing, as well as channel transfer, e.g., [7, 9, 10, 14, 16, 20, 21, 41, 42, 61, 62]. Investigations on related systems, i.e., supported bilayers [26, 36] and vesicles treated with [38] or containing fusogenic agents [59] have also provided evidence for fusion.

Oppositely charged vesicles are beginning to be investigated in bulk vesicle-vesicle and vesicle-planar bilayer fusion systems [2, 4, 18, 24, 67] and in two cases there was evidence for fusion from contents mixing [42, 55]. The cationic amphipaths used in these studies were

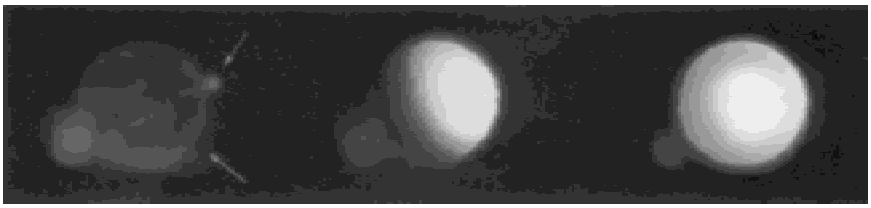

Fig. 1.

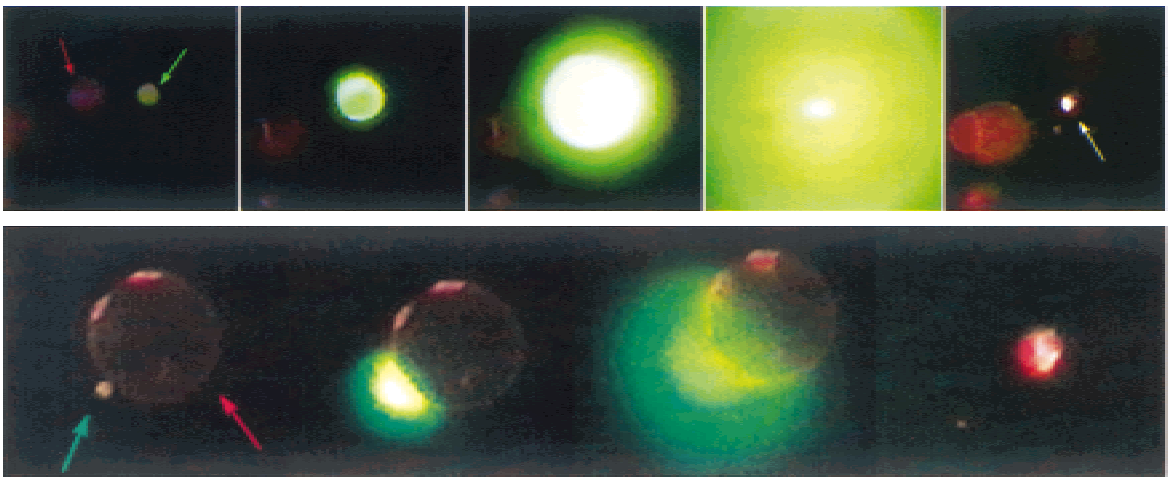

Fig. 2.

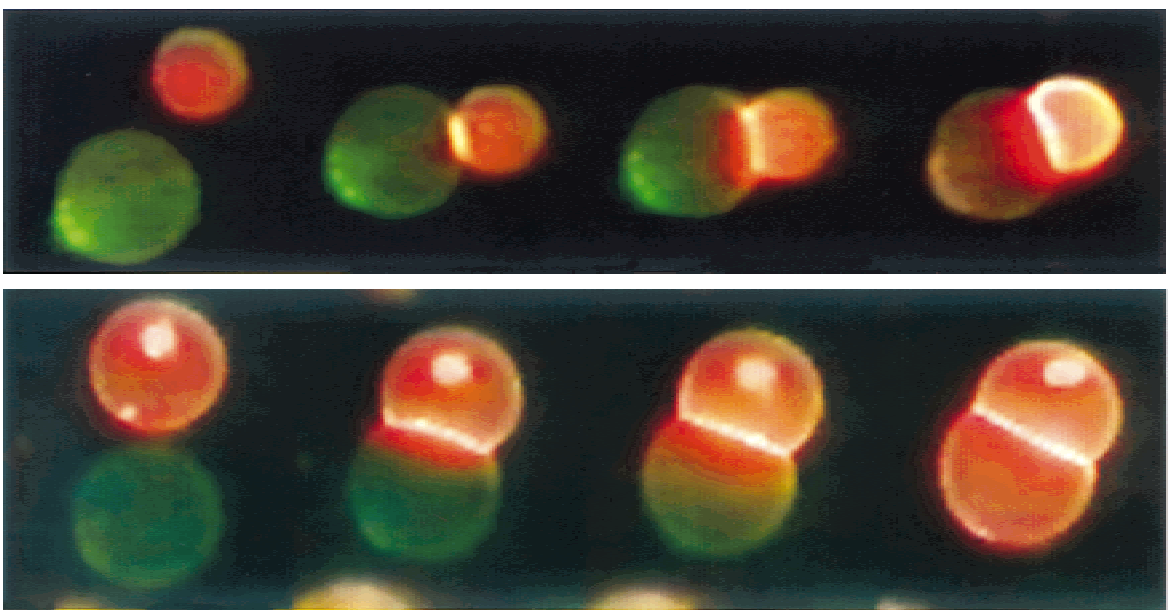

Fig. 3.

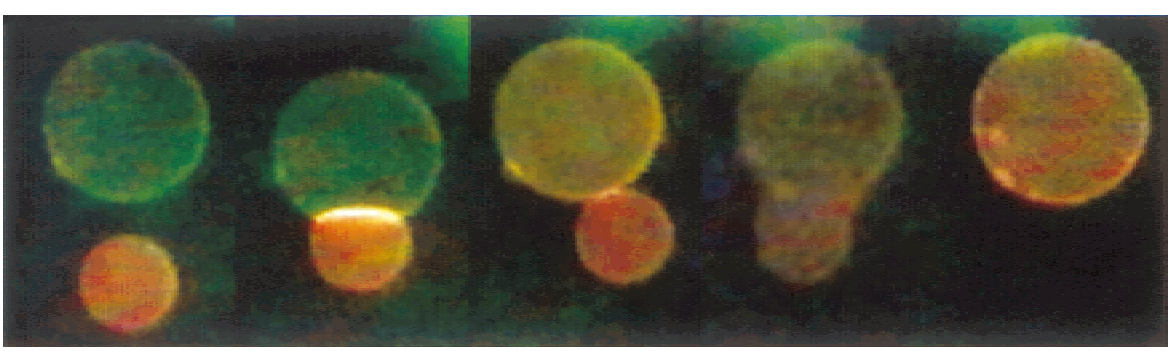

Fig. 4.

Fig. 1. Contents mixing between a cationic vesicle of PC+/Rh-PE (98:2) and an anionic vesicle of PG/Rh-PE (98:2) containing 80 mM calcein. Upon contact, the anionic vesicle (upper arrow, $d \approx 2$ μm) fused (<30 msec) and released its contents (calcein) into the cationic vesicle (lower arrow, d ≈ 14 μm). Dilution of the self-quenched calcein in the larger volume of the cationic vesicle was observed as a dramatic increase in green fluorescence which occurred over the course of 90 msec. The fused product remained stable for as long as it was observed (tens of sec). Medium: 320 mM sucrose.

Fig. 2. Contents leakage, rupture and collapse of cationic vesicles of PC+/Rh-PE (98:2) and anionic vesicles of PG/Rh-PE (98:2) containing 80mM calcein. (*A*) Anionic (green arrow) and cationic (red arrow) vesicles fuse (2nd image). The fused product ruptures, releasing calcein into the external medium (3rd and 4th images). The final product is the residual lipid particle (white arrow, 5th image). (*B*) After contact, the anionic vesicle ruptured outside the contact region and released its contents externally (2nd and 3rd images). As the residue of the ruptured anionic vesicle contacted the cationic vesicle, the latter also ruptured and collapsed into a lipid particle (4th image) of unknown structure. Medium: 320 mM sucrose.

Fig. 3. "Delayed hemifusion" and "immediate hemifusion" of oppositely charged vesicles. (*A*) "Delayed hemifusion." Upon contact, the cationic (green) and anionic (red) vesicles adhered (2nd image). After adherence for 600 msec, they rapidly hemifused as indicated by the abrupt increase in contact area and onset of color flow to the opposite vesicle (3rd and 4th images). Vesicles were PC+/PC/DOC (25:73:2) and PG/PC/Rh-PE (25:65:10). The third image is an average of the first four frames subsequent to the abrupt increase in contact area. The fourth image was taken after transfer of dye which lasted approximately two seconds. Similar morphological characteristics were observed in ionic and nonionic solution. Medium: 100 mM NaCl, 7.5 mM Tris, pH 7.5. (*B*) "Immediate hemifusion." Color flow began immediately after adherence (2nd image) during which the contact zone increased, leading to the formation of a stable hemifused vesicle pair (3rd and 4th images). Dye transfer was complete in approximately two seconds. Vesicles were PC+/PC/PE/DOC (38:30:30:2) and PG/PC/PE/Rh-PE (30/30/30/10). Medium 320 mM sucrose, 100 μM EDTA.

Fig. 4. "Hemifusion with retraction" followed by fusion. Cationic, (PC+/PC/DOC, (25:65:10) and anionic (PG/PC/Rh-PE, (25:60:15) vesicles adhered and formed a transiently stable contact zone (2nd image). Over the course of approximately 30 sec the fluorescent dye from the anionic vesicle ($d \approx 10$ μm) was transferred to the cationic vesicle ($d \approx 17$ μm) and the adhesion zone shrank to a point contact (3rd image). After 35 sec, fusion occurred and was completed within 8 video frames. Fusion occurred at constant volume with the excess surface area taken up by the highly fluorescent surface lipid particles. The 4th image is a single processed frame showing breakage of the point contact and joining of the two internal compartments. Medium: 320 mM sucrose, 100 μM EDTA.

physically similar to lipids but chemically unrelated to phospholipids.

Given the large number of studies that have reported positive results with such a variety of assays, it is likely that isolated lipid bilayers of some compositions are capable of fusing with each other under some conditions. It should be recognized, however, that with very few exceptions [29, 43, 50] vesicle fusion has not been quantified directly and conclusions represent inferences from the experimental data, such as fluorescence signals indicating mixing of membranes or vesicle contents [19, 27] or electrical currents from channels in planar target membranes [15, 16]. The vesicle-vesicle system is popular and convenient, but probably because of their generally indirect nature, the results of different assays are not always congruent [17, 56]. It has also been suggested that bulk measurements of vesicle-vesicle interactions, in which adhesion is initiated by addition of an external agent and substantial vesicle aggregation is typical, are not suitable for revealing the sequence of events leading to fusion of two lipid bilayers [19, 30, 49]. Nevertheless, this approach has provided much useful information on the responses of many lipids under many conditions. The vesicle-planar bilayer model system focuses on single interactions and hence offers higher temporal resolution and potentially higher spatial resolution than the vesicle-vesicle system, but is technically more demanding, gives apparent low yields of fusion, and is less well defined in terms of composition of the interacting species. In particular, it requires osmotic stress to induce fusion and the channel-forming peptides used to generate that stress may influence the course of bilayer interactions [15, 47, 64, 65], so that this system may not be entirely representative of simple phospholipid bilayers. In addition, the solvents needed to form the planar membrane may affect bilayer-bilayer interactions [11, 47]. An alternative approach for detailed examination of fusion, freeze-fracture electron microscopy, while capable of singling out pairwise vesicle interactions [6, 28, 50], is a static method that is not suited to examining the unfolding of a particular interaction. There has thus been a need for a simple method that can provide unambiguous, simultaneous information on membrane and contents interactions with high spatial and temporal resolution.

To provide a direct method of investigating model membrane fusion, a procedure has been developed for the observation of real-time interactions between individual vesicle pairs and used to study fusion between oppositely charged bilayers. It has allowed us to monitor the fusion process in considerable detail. For this investigation, we used a new lipid, 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (PC+), synthesized from dioleoylphosphatidylcholine (PC) by ethylating the unsubstituted phosphate oxygen [3]. Alkylation eliminates the negative charge on the zwitterionic precursor by replacing it with an ethyl group, leaving as the only charge on the molecule that of the trimethylammonium cation. PC+ forms lipid bilayers in aqueous phases that are indistinguishable from those of biological phospholipids, either by light microscopy, as described here, or by X-ray diffraction (the bilayer is normal based on the elec-

tron density distribution; R.C. MacDonald, T.J. McIntosh et al., *submitted*). Since vesicles of these lipids migrate in an electric field in the direction opposite to that of natural anionic phospholipid vesicles, it is possible to select and bring individual positive and negative vesicles into contact electrophoretically. By capturing the consequences of such interactions by video fluorescence microscopy, we have been able to visualize features of bilayer vesicle interactions not observed heretofore. This communication describes the procedure and reports on interactions of vesicles of different compositions.

The use of cationic phospholipids to deliver a small molecule to the cytoplasm and to transfect DNA into the nucleus of cultured cells, both processes that may involve membrane fusion, has already been reported [3, 5, 48].

Materials and Methods

Reagents

PC+ was either obtained as the chloride salt from Avanti Polar Lipids (Alabaster, AL), or synthesized as the trifluormethanesulfonate salt by reacting 3 equivalents of ethyl trifluoromethanesulfonate (Aldrich) with 1 equivalent of dioleoylphosphatidylcholine in chloroform at room temperature for several hours (MacDonald et al., *in preparation*). In the latter case, purification was by silica gel chromatography, the product eluting in chloroform:methanol (9:1). The two salt forms appeared to give equivalent behavior with respect to interactions with anionic vesicles. All other lipids were from Avanti.

Vesicle Preparation

Contents Mixing Assay [1, 30]

Chloroform solutions of lipids were evaporated under argon gas and placed under oil pump vacuum for 1 hr. PC+ (50 μg) was hydrated in 500 μl of 320 mM sucrose. PG (phosphatidylglycerol; 0.5 mg) was hydrated in 200 μl of 80 mM calcein pH 7.3. To eliminate external calcein, the anionic vesicles were eluted from a Sepharose CL-4B column with 320 mM sucrose, which is approximately isotonic with the internal calcein solution.

Membrane Mixing Assay [57]

Fifty micrograms of total lipid were mixed at the appropriate molar ratios in chloroform solution. The bulk of the solvent was blown off with argon and the sample was then placed under oil pump vacuum. Cationic and anionic vesicles were labeled with 2% N-N′-dioctadecyloxacarbocyanine (DOC; Eastman Kodak) and 10% N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (Rh-PE; Avanti), respectively. Lipids were slowly hydrated in 320 mM sucrose and dispersed by gentle swirling.

Electrophoresis Cell/Video Microscopy

A coverslip was attached to and supported from a microscope slide with 0.0035″ spacers at each corner of the coverslip. Stainless steel or platinum wires were fastened along each side of the cover slip and opposite pairs were connected to the outputs of two 0–25 volt, variable output, DC power supplies through polarity-reversing switches. The microscope slide was mounted on the stage of an upright fluorescence microscope equipped with a color video camera. The volume between the slide and coverslip was filled with the appropriate solution (usually 320 mM sucrose) and 10 μl each of the cationic and anionic vesicle suspensions were introduced under the coverslip. Pairs of oppositely charged vesicles were selected at the moving vesicle front where no previous interactions had taken place and carefully brought into contact by manipulation of the voltage between the two sets of electrodes. The voltage was turned off after vesicle-vesicle contact and frequently, before, allowing vesicle contact to occur by Brownian motion. Wideband fluorescein filters in the microscope were used for studies with all dyes described here. Images were continuously recorded on videotape, from which selected sequences were transferred to a computer where color and contrast adjustments were made using Photoshop 4 software. Single frames were processed for images in Figs. 1 and 2 while 4 adjacent frames were averaged for images in Figs. 3 and 5.

Results

Contents Mixing

We first examined fusion by monitoring the transfer of a water-soluble dye from the interior of one vesicle to the interior of another. Anionic vesicles of dioleoylphosphatidylglycerol (PG) were loaded with calcein, a hydrophilic dye which is intensely fluorescent in dilute solution but is strongly self-quenched at the encapsulated concentration [1, 30, 31]. A second set of giant PC+ vesicles contained isotonic sucrose. Individual vesicles of each type were chosen from the population visible in a given field under the fluorescence microscope, maneuvered into close proximity, and observed as random motion brought them into contact. The cationic vesicles chosen for these experiments had diameters between 5 and 20 μm. Vesicles that were obviously not unilamellar using minimum brightness of the membrane as the criterion were not chosen except to investigate their distinctive behavior. When cationic vesicles with multiple membranes were mistakenly chosen, that circumstance revealed itself upon fusion as a failure of the calcein to fill the entire cationic vesicle. These instances — much less than 10% of the total — were not tallied. The anionic vesicles were usually smaller ($d \leq 2$ μm), having been formed in a higher ionic strength solution, and because of internal fluorescence (even though highly quenched), could not necessarily be identified as unilamellar, *a priori*. The presence of anionic vesicles that were multilamellar or contained multiple internal vesicles was often revealed by the behavior of the internal vesicles as they became injected into the larger cationic vesicle upon fusion (*see below*).

Fusion or leakage into the external medium from the anionic vesicle became visible as a dramatic burst of fluorescence as self quenching of the calcein was re-

lieved by dilution. Figure 1 illustrates a common result with vesicles of these lipid compositions, namely, vesicle fusion without leakage. Fusion occurred in less than one video frame (30 msec) and calcein became uniformly distributed from the anionic to the giant cationic vesicle in less than 90 msec without detectable leakage into the external medium. The product giant vesicle remained stable as long as we could observe it before the calcein was bleached (tens of seconds); 27 of the 55 interactions we examined led to this type of stable fusion without leakage and all occurred fast relative to our time resolution; 28 events involved some calcein leakage, the majority of which were accompanied by rupture during or shortly after fusion.

Under some conditions, fusion was far more probable than rupture and most striking when a large cationic vesicle fused with an anionic vesicle containing many small vesicles. When this occurred, the primary fusion process injected numerous small vesicles into the large vesicle, the inner surface of which fused with the small vesicles such that calcein was released into the external medium. This release of calcein was seen as a sudden, small, green flare of fluorescence emanating from a point on the surface of the large vesicle which remained visible for about two video frames. In one instance, 20 such "exocytotic" events were counted. "Exocytotic" fusion was also observed between unilamellar and multilamellar vesicles.

Loss of vesicle integrity occurred in a variety of ways, in particular (from most to least common): fusion of vesicles followed by leakage without vesicle rupture, contact leading to rupture of one vesicle only, and contact leading to rupture of both vesicles. When both vesicles ruptured, they seldom did so simultaneously. Figure 2*A* illustrates fusion followed by rupture. An example of rupture without fusion of an anionic vesicle induced by contact with a cationic vesicle is shown in Fig. 2*B*. In this case, the cationic vesicle also subsequently ruptured. Such observations make it clear that rupture, which would be manifested as contents leakage in a bulk population assay, is not necessarily concurrent with fusion, contrary to what has sometimes been concluded. However, leakage from intact fused vesicles — identified by rapid loss of fluorescence — sometimes followed fusion. It was unclear in those cases whether leakage occurred throug pre-existing defects or through pathways created during fusion. In either case, it would appear that overall vesicle stability is compatible with leak paths large enough to allow the loss of a molecule the size of calcein in a few seconds. This observation is consistent with the finding that giant vesicles can be perforated electrically, and the pores so induced can exist for seconds and then reseal without vesicle rupture [66]. Rupture invariably led to collapse of a vesicle or vesicle pair into a small particle of residual lipid (*see* last panel of Fig. 2). An abortive fusion process in which only one vesicle collapses probably accounts for the fact that bright particles were frequently seen at the surfaces of both positively and negatively charged vesicles. Particles that are at least superficially similar to the residual particles we saw have been observed during spontaneous breakage of phosphatidylcholine vesicles [34].

Membrane-Membrane Interactions

To obtain a more detailed understanding of vesicle-vesicle interactions, the dynamics of membrane intermixing were examined by including fluorescent amphipaths in the vesicle membranes. Cationic vesicles were labeled with 2 % DOC. Anionic vesicles were labeled with Rh-PE at a higher concentration — 10 % — for self-quenching. Since both vesicles were membrane labeled for this set of experiments, the selection of oppositely charged pairs of unilamellar vesicles was fairly easy and, as in the case of cationic vesicles for the contents mixing experiments, we avoided those that were not obviously unilamellar.

We found that vesicles underwent both fusion and hemifusion,[1] as well as rupture, in proportions that depended markedly upon the amount of charged lipid included in the vesicles. At >70 % net charged component (the percent cationic lipid in positive vesicles equaled the percent anionic lipid in negative vesicles), fusion occurred immediately upon contact. The fused product was almost always spherical and in most cases exhibited lipid particles on its inner or outer surface. The latter presumably consisted of the lipid in excess of that necessary to maintain the surface to volume ratio of the fused product (two spheres of volume 1× have about 20 % greater area than a sphere of volume 2×). Occasionally, tubelike invaginations, as well as evaginations, appeared during or immediately after fusion. Excess membrane was frequently seen at the edge of the contact zone, and it may be that the opening in the partition to give full fusion (fusion pore) occurs preferentially near the junction of the two vesicle membranes and less commonly near the center of the contact zone.

At the highest membrane surface charge densities ($\geq$ 70 % charged component), hemifusion was the end result of about 17 % of the encounters (*see* Table). If hemifusion occurred during the events leading to full fusion, it was very short-lived, for we were unable to record any fusion intermediates at such charge densities. In the majority of cases, the speed of the process also

[1] Hemifusion, unlike fusion, was not directly visible, but was inferred from an abrupt onset of dye transfer between vesicles and an increase in contact area. These observations are described more fully below.

Table. Percentages of various outcomes of vesicle interactions composed of PC+/PC/DOC and PG/PC/Rh-PE at the indicated compositions

% Charged component[a]	% Fusion	% Hemifusion	% Rupture
100	42 ± 9	15 ± 7	46 ± 9
70	38 ± 10	16 ± 10	47 ± 4
40	37 ± 2	51 ± 6[c]	12 ± 4
20	23 ± 9	64 ± 12[d]	5 ± 1
6[b]	0	<1	0
40 + 30% PE	16 ± 2	83 ± 3[e]	1 ± 1

[a] For each composition, the number of sampled populations (n) and total number of sampled vesicle interactions (t) was: 100%, $n = 3$, $t = 122$; 70%, $n = 3$, $t = 300$; 40%, $n = 3$, $t = 332$; 20%, $n = 2$, $t = 211$, 40% +30% PE, $n = 2$, $t = 110$. Uncertainties are expressed as standard deviations of the average values of the n sets of sampled populations. The total number of vesicle-vesicle interactions examined was 1075.

[b] Interactions involved contact but there was no stable adhesion. Vesicle charge due to the average composition of both probes.

[c] About 75–80% of these were delayed hemifusion.

[d] Approximately evenly divided between delayed hemifusion and hemifusion with retraction.

[e] All represented immediate hemifusion.

Cationic and anionic vesicles contained 2% DOC and 10% Rh-PE, respectively. Medium: 320 mM sucrose.

precluded our recording the extent of flattening of the vesicles when they came into contact.

As the proportion of charged component in the vesicle membranes was reduced, the interactions between vesicles changed quantitatively and qualitatively. Whereas hemifusion was the least common outcome observed between vesicles composed only of charged lipid, hemifusion became most common when a zwitterionic lipid (phosphatidylcholine or phosphatidylethanolamine) constituted half or more of the total lipid (Table). Hemifusion among vesicles of lower surface charge density was easily studied because it was not only common but also relatively long-lived. We observed three distinctly different modes of hemifusion; (i) delayed hemifusion, (ii) immediate hemifusion and (iii) hemifusion with retraction, depending upon the lipid composition of the vesicles.

Modes of Hemifusion

"Delayed hemifusion" occurred hundreds of milliseconds after contact. An example involving a PC+/PC/DOC (25:73:2) vesicle and a PG/PC/Rh-PE (25/65/10) vesicle is shown in Figure 3*A*. For reference, the entire process of fusion is shown diagrammatically in Fig. 5, in which IV represents the hemifused state. In the first step, an adhesion zone formed. This is a flat interface which, in Fig. 3*A* (second image), is visible as a yellow region due to overlap of the red (Rh-PE) fluorescence of the anionic vesicle and the green (DOC) fluorescence of the cationic vesicle. After 600 msec, the contact zone between the two vesicles abruptly increased in area and rhodamine began to diffuse from the anionic membrane to the cationic membrane (third image, Fig. 3*A*). Subsequently, because the loss of dye relieved self-quenching in the anionic rhodamine-labeled vesicle, its fluorescence grew brighter (fourth image, Fig. 3*A*). The increased adhesion and onset of fluorescent probe diffusion evidently signal the beginning of hemifusion, in which each of the opposed monolayers (outer monolayers of the vesicles in the contact zone) separately pulls apart laterally and then reseals with the other outer monolayer, allowing the inner monolayer of each vesicle to become opposed across the contact zone. The resealing allows dye to diffuse, via the outer monolayers, from one vesicle membrane to the other (this process occurs between III and IV of Fig. 5). Increased flattening of the vesicles and the corresponding abrupt dilation of the contact zone is presumably due to the effect of the vesicle tension operating on the contact zone as a portion of it is being reduced from two bilayers to one; not only will the tension stretch one bilayer more than two, but also, the inner monolayer is released from the compression imposed upon it by the condensation that we postulate occurs in the contact zone upon mutual neutralization of the oppositely charged adhering surfaces. This behavior is considered more fully in the Discussion.

As the charge density of the vesicles was reduced to about 20 % charge component, the distribution of outcomes continued to change (Table). Hemifusion was followed by fusion less frequently (32 %) and by rupture infrequently (5 %) and thus constituted a relatively stable final state.

The second mode of hemifusion, "immediate hemifusion" was distinguished by the diffusion of membrane dye from one vesicle to the other upon contact (within the 30 msec time resolution) and occurred when significant amounts of dioleoylphosphatidylethanolamine (PE) were included in the vesicles (Table, last row). As shown in Fig. 3*B* for vesicles of 40 % net charged component and 30 % PE, diffusion of dye from the anionic to the cationic vesicle began essentially simultaneously with formation of the adhesion zone, the area of which increased gradually, not abruptly as in the absence of PE (compare Fig. 3*A*). Immediate hemifusion involved an expansion of the contact area very shortly after contact and a near-simultaneous commencement of dye transfer from one vesicle to the other. We intrepret this as corresponding to the transition from stage II to stage IV of Fig. 5. Although we cannot, of course, distinguish adhesion of two bilayers from hemifusion on the basis of optical resolution, if what we observed had been just adhesion, we would not expect the transfer of dye to be

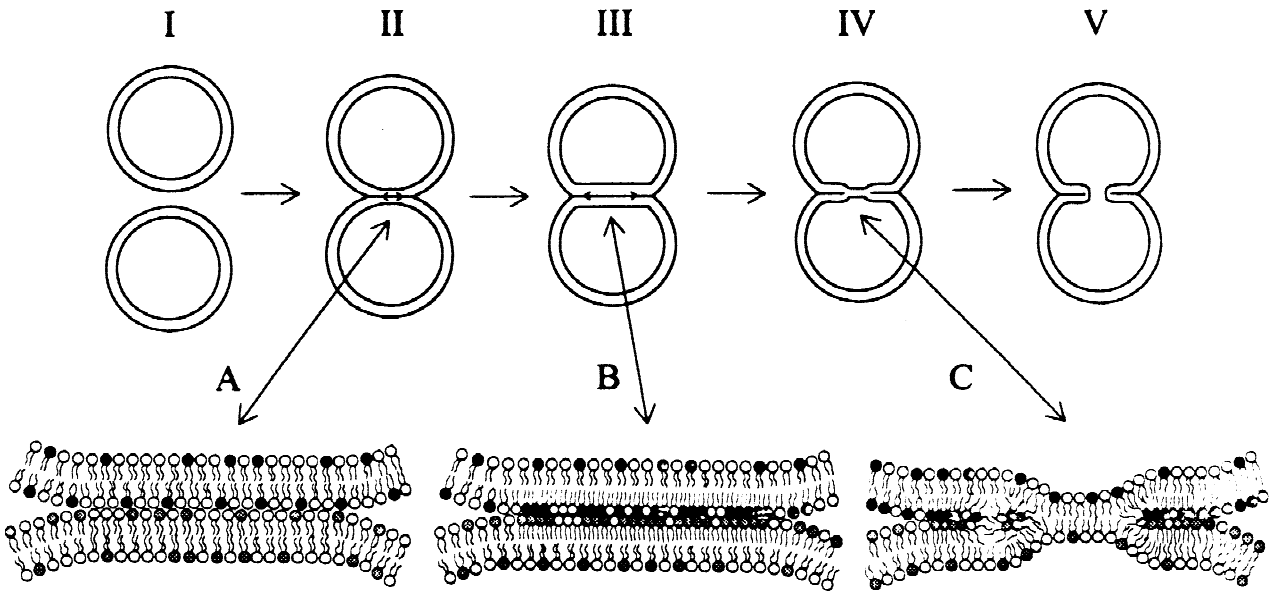

Fig. 5. Hypothesized interactions of oppositely charged lipid vesicles. Upon adhesion (drawing II), vesicles flatten against each other. Flattening requires an increase in the vesicle surface area, so that the membrane of each vesicle is stretched slightly, generating a tension in the vesicle bilayers. The tension so generated will oppose further adhesion and flattening; equilibrium will be attained when the component of tension in the bilayers that is normal to the adhesion zone is equal and opposite to the adhesion force (stage III). Charge neutralization and accumulation (details *A* and *B*) in the contact zone reduces repulsion within each of the contacting outer monolayers. A differential tension between vesicle inner and outer monolayers and perhaps also a tendency for nonlamellar phase formation causes the two outer monolayers to rend (hemifusion; stage IV, detail *C*). This leaves a weakened partition — the double bilayer becomes a single bilayer in some areas (here shown in the center) — which breaks under the combined tension of both vesicle membranes, leading to fusion (stage V). White circles represent neutral lipid (e.g., PC or PE) head groups and black and gray circles represent charged head groups. If the proportion of charged lipid in each vesicle is low, accumulation of charged lipids in the contact zone could be delayed and then the entire process would be slowed. In such cases it is also possible that charged lipids are exchanged between adherent vesicles (via contacting outer monolayers) prior to hemifusion, in which case vesicle-vesicle adhesion may be reduced to the point where tension in the bilayers is insufficient to rend the partition. Then hemifusion is a stable final state. If vesicles contain *only* charged lipids, a situation resembling detail *B* (white circles would be absent) would be established almost immediately after contact and subsequent events (IV and V) would occur very quickly.

so fast, nor would we expect the contact zone to expand after the initial contact. The properties of PE that presumably contribute to its stimulation of hemifusion are its low hydration and high intrinsic negative curvature (*see* Discussion and [25]).

The third mode of hemifusion, "hemifusion with retraction," was common when vesicles contained 10–40 % charged lipid in combination with PC. As shown in Fig. 4, vesicles initially formed a large contact zone, but over the course of 30 sec, retracted, occasionally to almost a point contact, with a slow transfer of rhodamine from the anionic to the cationic vesicle. The point-connected vesicles (3rd image, Fig. 4) must have shared outer monolayers (otherwise they would separate), indicating hemifusion occurred at some stage in this adhesion-retraction process. About 20 % of the hemifused vesicle pairs of this type progressed to full fusion. An important advantage of continuous observation of interacting vesicles is the potential for recognizing this kind of process; if one observed both point contacts and plane contacts by, e.g., electron microscopy, it might be difficult to avoid the erroneous interpretation that the former preceded the latter.

Discussion

By monitoring pairwise encounters of lipid bilayer vesicles under conditions of high resolution in the fluorescence microscope, we have identified several pathways open to interacting vesicles. Vesicles with a high charge density undergo fusion almost upon contact, whereas vesicles with lower charge densities undergo hemifusion which precedes contents mixing, if it occurs at all, by seconds. Hemifusion occurred by one of three different time-dependent processes, depending upon the charge content of the vesicles. Given this variety of different interactions for a relatively small selection of different phospholipid bilayers, it is evident that direct visualization is essential if the details of bilayer vesicle fusion are to be elucidated.

Proposed Mechanism of Fusion

Based on our observations and elementary membrane electrostatics, a mechanism for fusion between oppositely charged bilayers can be proposed. In the first stage, adhesion due to electrostatic attraction leads to mutual flattening of the vesicles. As shown by others, such an interaction must lead to an increase in the vesicle membrane tension [23, 46]. If fusion is to ensue, the second stage must generate a weakened partition, otherwise, strong adhesion will lead to lysis and weak adhesion will lead to stable vesicle pairs. When vesicles are composed mostly of charged lipids, nearly complete electrostatic neutralization of all molecules in each contacting monolayer must occur as soon as the two surfaces adhere (and water is extruded). Neutralization drastically reduces intermolecular repulsion in those monolayers, and creates a tendency for the contacting monolayers to undergo a reduction in area per molecule.[2] Some of this contraction of the external monolayer will be resisted by compression of the inner monolayer, with the result that a differential tension will arise in the bilayer. Thus, the whole bilayer will be under tension (due to adhesion and flattening), but more of the tension will reside in the outer than in the inner monolayer (because of neutralization and condensation of the outer monolayer). There is a limit to which the outer monolayer can condense relative to the inner monolayer, and a significant decrease in area per molecule can only come about if at some point the outer monolayer becomes discontinuous, i.e., one or more openings are created (shown as just one large central opening in Fig. 5). This can occur readily if openings in each of the contacting outer monolayers form opposite one another, for in that case, the opening is filled in by the acyl chains of the inner monolayers, a process that can occur at a rather low energy cost (some energy is needed for formation of the highly curved edges of the opening of the outside monolayer and for slight bending of the inner monolayers). Rough calculation on the basis of membrane electrostatics [8] indicates that neutralization of a fully charged monolayer will generate a force even greater than the lysis tension of a bilayer (several mN/m; [40]). Since the formation of an opening that is filled as it opens is, of course, the process of hemifusion (transition from stages III to IV of Fig. 5), the neutralization of two charged surfaces should easily provide enough energy for hemifusion to occur. As soon as hemifusion begins, the components of the tension (parallel to the plane of the contact zone) in both vesicle membranes (present because the two vesicles are still largely flattened against one another) becomes applied to the single remaining bilayer in the partition, which then rends, leading to full fusion. If the adhesion energy is low, then there will be insufficient flattening of the vesicles to generate a net tension large enough to rend the monolayer portion of the contact zone and the hemifused state becomes indefinitely stable.

When the vesicle membranes contain neutral phosphatidylcholine in addition to charged lipid, the surface charge density in the contacting monolayers is reduced such that there is insufficient differential tension at the contact zone to induce hemifusion (Fig. 5, detail *A* and Fig. 3*A*, 2nd image). We further hypothesize that, in time, electrostatic interactions will cause anionic and cationic components to accumulate in the contact zone (Fig. 5, detail *B* of stage III). When the concentrations reach a threshold value, hemifusion can occur (Fig. 5, IV-*C* and Fig. 3*A*, 3rd image) and may be followed by full fusion if there is sufficient bilayer tension in the vesicles. Fusion is expected to occur shortly after hemifusion; otherwise, exchange between the outer monolayers will reduce adhesion and hence also vesicle tension, diminishing the driving force for full fusion and thereby stabilizing the hemifused state (Fig. 3*A* and *B*, 4th images).

If significant concentrations of PE are present, vesicles undergo immediate hemifusion even at rather low net charge densities. Two well-known characteristics of PE may contribute to the events observed. First, PE is less well hydrated than PC, so the thickness of the water layer between membranes is inversely related to the PE content [46]. PE has been observed to increase lipid mixing between membranes [20], presumably due in part to closer adherence of the bilayers [63]. Second, PE tends to stabilize regions of high negative curvature [21, 53], which occur at the edges of the hemifusion opening in the external monolayers in stage IV of Fig. 5, so that PE could facilitate the formation of the openings in the apposed monolayers by stabilizing their edges (Fig. 5, detail *C* of stage IV).

An exception to the general scheme persented above is illustrated by Fig. 4; we believe that in this mode of fusion, retraction is a consequence of the charged lipids becoming sufficiently concentrated in the contact point that the contact point becomes unstable even in the absence of significant bilayer tension. It seems significant that this behavior was observed only when a large proportion of uncharged lipid was found in both membranes. Under those conditions, the uncharged lipid in the con-

[2] Preliminary measurements of PC+, PG, and their mixtures in monolayers at the air-water interface revealed that the average area/molecule of a 1:1 mixture of PC+ and PG was significantly (nearly 10% at 30 mN/m) smaller than the mean area of these molecules. This is presumably due to an electrostatic effect (rather than steric) in which neutralization of positive by negative charge eliminated internal repulsion and induced monolayer condensation. These results indicate that a similar condensation could occur upon apposition of the external monolayers of positive and negative vesicles (R.C. MacDonald, H.L. Brockman, et al., *in preparation*).

tact zone is not electrostatically linked to molecules in the apposing monolayer and is free to diffuse out of the contact zone. If it does, then the charged lipid becomes more concentrated, with the result that the contact zone should become more compact, as a halo of uncharged lipid diffuses away radially from the contact zone. In effect, the driving force for diffusion of the uncharged lipid out of the contact zone is membrane tension due to adhesion; that tension can be relieved and thus lead to a lower free energy if the neutral lipid leaves the contact zone, so long as the original attractive electrostatic interactions between oppositely charged lipids in the adhering membranes remain. Although this explanation appears quite consistent with what is known about liquid crystalline bilayers and elementary electrostatics, considerable additional experimentation is needed to test it. If correct, it would mean that fusion can occur when a threshold concentration of cationic and anionic lipid is exceeded, even in the absence of an adhesion-induced tension.

This monolayer contraction model for fusion of oppositely charged bilayers has features in common with several others in which increased negative curvature of the constituent lipids promotes fusion [12, 28, 37, 49, 53, 54, 58], but it differs from previous models in that it identifies the mechanical effect of electrostatic charge neutralization as the proximate cause of hemifusion. In this regard the model is related to our earlier proposal for bilayer-bilayer fusion [39] and to the adhesion-condensation mechanism proposed even earlier by Kozlov and Markin, both of which contain the essential element wherein condensation of the outer monolayer promotes hemifusion [33]. These mechanisms were originally proposed to explain fusion in the calcium-PS model system. They assume the condensation occurs because calcium ion, bridging the two PS surfaces, induces gel phase formation in the contact zone. Since such a disorder $\rightarrow$ order transition leads to a reduction in area per molecule, a monolayer condensation can be inferred to also occur. We had, in fact, measured such a condensation in a PS monolayer at the air-water interface when the subphase was treated with calcium ion [39]; probably a phase transition was responsible for the observed monolayer contraction, although some effect would also be expected from simple charge neutralization. The analysis of Kozlov and Markin was purely theoretical, but since what we believe is essential to initiate hemifusion is the contraction of the external monolayers, much of their analysis carries over directly to the situation described here. Particularly relevant is their quantitative analysis of the energy of forming a hole in the outer monolayer [33].

Support for a monolayer condensation-triggered mechanism also comes from studies on the hemifusion of bilayers supported on mica using the surface force apparatus [26]. In that case, hemifusion was found to be promoted by influences that tended to lead to a lateral parting of the head groups on contacting monolayers, in particular, depletion, mechanical stress, or a phase transition. These authors thus also emphasize the importance of moving the head groups aside to allow contact of the hydrophobic tails of the trans monolayers, although in addition, they ascribe an attractive force to the hydrophobic patches [26].

The model we suggest also has a feature in common with the popular "stalk-pore" model [12, 32], namely that molecules with intrinsic positive curvature, like lysophosphatidylcholine, should inhibit fusion when present externally [10]. Although we have not tested the effects of this molecule, any molecule that inserts into the vesicle's external monolayers should oppose their contraction and hence inhibit hemifusion. Similarly, such a molecule is predicted to stimulate hemifusion (and probably also full fusion) when present on the inside of the vesicles, since partitioning into the internal monolayer would expand the external monolayer and cause stretching of the external monolayer.

Sphericity of Fusion Products

Because the membrane area of two spheres considerably exceeds that of a single sphere with the same volume, the product of fusion of two spherical vesicles should be "deflated bags." With only a few exceptions, fused vesicles formed spheres. We measured the radii of the pre- and postfusion vesicles and verified that the process occurred at constant volume. This means that there must have been membrane left over after fusion. Where did that extra membrane go? One possibility was suggested by the sometime observation of tubelike invaginations and, less frequently, evaginations, immediately after fusion. Bending is favored whenever the monolayer area on one side of a bilayer is slightly different from that of the other side, and the resultant induced curvature can readily lead to in- or evagination of vesicles [22]. There are several possibilities whereby such an uncompensated difference in areas of internal and external monolayers of fusing vesicles could arise, given that whenever charge neutralization occurs, the area per molecule is expected to change. Some form of charge neutralization occurs upon adhesion, hemifusion (mixing of external monolayers) and fusion (mixing of inner monolayers). An alternative explanation is that the adhesion zone never comes apart, and when the fusion pore widens to encompass the entire contact zone, the bilayers in the contact zone simply reorganize into some form of bulk lipid phase (possibly, in part, a cubic phase, *see below*). It may be that what in some cases appeared to be invagination was simply this rearrangement of the contact zone lipid. In any case, we frequently saw bright spots that appeared on vesicles just after fusion; these seem to represent lipid

from the membrane that becomes in excess upon fusion. With higher time and spatial resolution, it should be possible to determine the path of disappearance of the excess membrane.

Potential Roles for Proteins in Biological Membrane Fusion

Although extrapolations to biological membrane fusion from simple systems — particularly from a protein-free model system to a biological membrane where proteins clearly have critical functions — must be made with caution, one of the aims of studying lipid bilayers is to understand more about biological membranes. Thus, cautious speculation is appropriate when it can provide a new perspective on the natural process. Indeed, our model does suggest a novel possibility for biological fusion. The fundamental requirement for a mechanism such as we propose is a condensation of the external monolayers of the fusing membranes. Any *differential* increase in surface tension of the external monolayers relative to that of the internal monolayers could produce such an outcome. Electrostatic neutralization as described here, seems unlikely to operate in most cases of cellular membrane fusion — even in the case of exocytosis, where the calcium concentration is transiently elevated, it probably does not become high enough to neutralize anionic membrane surfaces — yet, more subtle mechanisms are possible in the cell. One such possibility is a conformational change that causes proteins in external monolayers of the fusing membranes to withdraw from those monolayers. For small vesicles, such a mechanism could operate anywhere on the surface, even in areas of membrane distant from the contact zone, since the tension so induced on the external monolayer would still be transmitted to the zone of contact. This hypothetical mechanism, although very speculative, constitutes a new paradigm for fusion in that the usual suggestions for roles of membrane proteins postulate insertion, not extraction, as the trigger for fusion.

A second potential role for protein in the fusion process is dramatically highlighted by our observations of the need for protection against the consequences of membrane rupture. Since the collapse of a fusing membrane generates a lipid array that itself promotes membrane rupture, to the extent that our system provides a guide, bilayer collapse could be harmful to cells. What structure is formed by a collapsed bilayer resulting from abortive fusion is not known in general, but in the case of interaction of oppositely charged phospholipid bilayers described here, it seems very likely that it contains lipid in a cubic phase. We draw this conclusion because thin section and freeze fracture electron microscopy of equimolar mixtures of EDOPC and DOPG revealed regular square or hexagonal arrays (R.C. MacDonald, D.P. Pantazatos and Y.S. Tarahovsky, *in preparation*), that correspond to one of the bicontinuous cubic lipid phases [35]. Others have suggested structures related to cubic phases[3] as an intermediate stage in membrane fusion [28, 53, 58], although from our point of view, the cubic phase is likely to appear after, not during fusion. That is, association of cubic phases with fusion conditions is likely to be a consequence of the requirement for head group condensation. Given head group condensation, the participating molecules could acquire an intrinsic negative curvature sufficient to cause conversion to a cubic phase. Although there is currently little evidence either that cubic phases are involved in or that they are a product of cellular membrane fusion, nonetheless, the destabilization process that leads to bilayer fusion in the cell must be controlled and some protein(s) may be presumed to play such a role.

A predicted third role for protein is in promoting full fusion, by eliminating the single bilayer septum that remains after hemifusion. In the case of oppositely charged vesicles, the septum is evidently pulled opened by the tension in the vesicles due to their adhesion. Since this mechanism is probably not available to cells, even though there is clear electron microscopic evidence for a hemifused intermediate [44], there must be an alternative mechanism. One obvious possibility is that proteins in the *trans* monolayers (*inside* monolayers in terms of vesicle-vesicle fusion) of fusing bilayers could by mutual recognition, generate an opening or pore in the septum that could grow into the fully fused state.

A corollary to the above speculation concerning roles for proteins in membrane fusion is the use of the system described here to assay the putative function of isolated proteins for fusion activity. Because the steps of fusion can now be directly observed, it may be possible to assess the point of action of membrane proteins (or peptides obtained therefrom) in the fusion process.

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[3] Some caution needs to be exercised in describing cubic phases because there are several types and the actual structures of many of them are incompletely understood [35]. Given that caveat, the high degrees of curvature associated with all cubic phases do seem suited to the membrane contortions required for bilayer fusion, whatever the specific molecular details may be.

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