J. Membrane Biol. 194, 129–139 (2003)
DOI: 10.1007/s00232-003-2031-y

The Journal of
Membrane
Biology

Bilayer Mixing, Fusion, and Lysis Following the Interaction of Populations of Cationic and Anionic Phospholipid Bilayer Vesicles

D.P. Pantazatos, S.P. Pantazatos, R.C. MacDonald
Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL 60208-3500, USA

Received: 17 June 2002 / Revised: 18 April 2003

Abstract. Cationic, *O*-alkylphosphatidylcholines, recently developed as DNA transfection agents, form bilayers indistinguishable from those of natural phospholipids and undergo fusion with anionic bilayers. Membrane merging (lipid mixing), contents release, and contents mixing between populations of positive vesicles containing *O*-ethylphosphatidylcholine (EDOPC) and negative vesicles containing dioleolylphosphatidylglycerol (DOPG) have been determined with standard fluorometric vesicle-population assays. Surface-charge densities were varied from zero to full charge. All interactions depended critically on surface-charge density, as expected from the adhesion-condensation mechanism. Membrane mixing ranged from zero to 100%, with significant mixing (>10 $<70\%$) occurring between cationic vesicles that were fully charged and anionic vesicles that had fractional surface charges as low as 0.1. Such mixing with membranes as weakly charged as cell membranes should be relevant to transfection with cationic lipids. Unexpectedly, lipid mixing was higher at high than at low ionic strength when one lipid dispersion was prepared from EDOPC plus DOPG (in different proportions), especially when the other vesicles were of EDOPC; this may somehow be a consequence of the ability of the former mixture to assume non-lamellar phases. Leakage of aqueous contents was also a strong function of charge, with fully charged vesicles releasing essentially all of their contents less than 1 min after mixing. EDOPC was more active in this regard than was DOPG, which probably reflects stronger intermolecular interactions of DOPG. Fusion, as measured by contents mixing, exhibited maximal values of 10% at intermediate surface charge. Reduced fusion at higher charge is attributed to multiple vesicle interactions leading to rupture. The existence of previously published data on individual interactions of vesicles of the same composition made it possible for the first time to compare pairwise with population interactions, confirming the likelihood of population studies to overestimate rupture and hemifusion and underestimate true vesicle fusion.

Key words: Cationic lipid — EDOPC — Hemifusion

Introduction

Cellular membrane fusion is complex and difficult to study, but because the process presumably requires that the lipid bilayer acquire some kind of discontinuity followed by a resealing, it has been reasonable to conclude that some fundamental aspects of fusion could be revealed by model studies on lipid bilayers. Thus, several model fusion systems were developed, of which most prominent were those involving populations of natural anionic lipids treated with cations of various types, in particular the phosphatidylserine/calcium ion system [32]. To quantify the extent of fusion, a number of fluorescent probe-based membrane-mixing assays and contents-mixing assays were developed [5, 7, 13, 17, 37].

Contents-mixing assays (in which a fluorescence signal is generated when the contents of one set of vesicles mix with the contents of a second set) have led to the conclusion that significant proportions of vesicles of certain compositions may undergo fusion [40]. Relative to the extent of fusion, a much greater extent of membrane mixing (lipids of the two sets of vesicles combine, leading to a change in the signal from fluorophores in the bilayers) could often be demonstrated, but these were almost invariably associated with extensive lysis. A problem with fusion

Correspondence to: R.C. MacDonald, email: macd@northwestern.edu

assays that could not be resolved, however, was the difficulty in assessing whether the fusion so measured followed the biological pattern, namely the contact of two membranes followed by their merging and combination of the internal aqueous volumes [5]. Membrane mixing assays, although easy to apply and commonly used, do not distinguish between complete rupture followed by merging of the vesicle remnants and vesicle-vesicle fusion without contents loss [13]. Although clearly more reliable in many situations, contents-mixing assays frequently suffer from a lack of information on the extent of aggregation of the vesicles and on how much of the observed signal might be due to their lysis within the potentially protected confines of the aggregate where the marker reaction could be isolated from the external quenchers that are normally included to eliminate any signal that might arise as consequence of lysis [16].

To avoid possible ambiguities of assays based on populations of vesicles, some investigators sought to study fusion of single vesicles large enough to be seen in the light microscope; however, catching vesicles in the process of fusion was problematic [15]. Individual spherical bilayers were also examined, but these membranes contain solvent and it is not clear what effect it has [4].

With the development of cationic versions of phospholipids (in particular, *O*-ethylphosphatidylcholine-EDOPC—a derivative of the zwitterionic phosphatidylcholine in which the negative charge has been eliminated by ethylation) that are able to form giant vesicles, the investigation of their possible fusion with anionic vesicles became practical [23]. Individual interactions could be studied because pairs of oppositely-charged vesicles can be readily manipulated in electric field gradients. Examination of this system by video fluorescence microscopy revealed large extents of both fusion and hemifusion (merging of external monolayers with internal volumes remaining separate) [11, 29].

Although the finding of fusion of simple bilayers having opposite electrostatic charge was of considerable interest with respect to bilayer fusion theory, it acquired additional significance because of the widespread use of cationic lipids in gene delivery, an area that has experienced explosive development in recent years [14]. Cationic lipids[1] are typically synthetic compounds that are physically similar to but chemically different from natural polar lipids, having a positively charged headgroup attached in most cases to two long hydrophobic chains. Cationic lipids form complexes with DNA (lipoplexes) that are taken up by cells and, because some of that DNA makes its way to the nucleus, these compounds have been widely used in nonviral gene therapy applications [9, 10, 21, 24].

Since interaction of lipoplexes with cells is likely to involve at least some contact between oppositely charged membranes [2, 43, 46], population assays have been used to study interactions of oppositely charged membranes [3, 36]. Similarly relevant to lipoplex uptake were the findings of fusion of anionic vesicles to a positive planar bilayer [1], and adhesion of oppositely-charged vesicles in which one vesicle was held with a micropipette [26, 27]. These studies have involved cationic amphipaths (most commonly, DOTAP) at surface-charge fractions between 0.15–0.5 in combination with phospholipids because some cationic amphipaths do not form large or stable vesicles on their own. Contents-mixing measurements between various compositions of cationic and anionic vesicles have been attempted as well, but such investigations have been hindered by the tendency of cationic lipids to aggregate or fuse in the presence of the common anionic aqueous-phase dyes.

Membrane fusion could also be involved in the actual formation of lipoplexes from vesicles and DNA [12, 18] and, indeed, addition of DNA or anionic polyelectrolytes to cationic amphipath dispersions leads to considerable membrane mixing [6, 18, 19, 30, 45].

Although the ultimate goal of most model systems involving population assays for fusion has been to assess what would happen in encounters of single pairs of membranes as they occur in the cell, the characteristics of those assays precluded such conclusions and therefore it had not been possible to compare the outcomes of pairwise encounters with the results of population assays. That situation changed when it became possible to form unilamellar vesicles of cationic phospholipids like EDOPC in the absence of a neutral lipid and, as a result, to demonstrate vesicle-vesicle fusion in pairwise encounters with anionic vesicles [11, 29]. We have therefore taken advantage of this opportunity to carry out population assays with the same kinds of lipids and to compare these results with those obtained earlier on single vesicles.

In addition to illuminating the differences between the assays, we have gained insight into why and in which direction each of the assays is likely to be biased. Furthermore, because it is now possible to vary the charge of vesicles both by dilution with a neutral lipid and by partial neutralization with the oppositely-charged lipid, new information about partially charged vesicles has arisen. These results should also be useful in allowing more critical evaluation of population studies in interactions related to gene transfer mediated by cationic amphipaths. Among others, the effects of ionic strength we observed may mean that the high ionic strength of cells could even facilitate interactions of cationic lipid/DNA complexes with cell membranes.

[1]Because they are not natural products, they are properly termed cationic "lipoids" or cationic amphiphiles.

Materials and Methods

Materials

1,2 Dioleoyl-*sn*-glycero-3-ethylphosphocholine (*O*-ethyldioleoylphosphatidylcholine, EDOPC) was either a gift from Gary Ashley, synthesized in our laboratory as the trifluoromethane sulfonate salt, or, alternatively, obtained from Avanti Polar Lipids (Alabaster, AL) as the chloride salt. 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol, sodium salt (DOPG), l,2-dioleoyl-*sn*-glycerol-3-phosphocholine (DOPC), N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (RhPE) and N-(7-nitrobenzo-2-oxa-l,3-diazole-4-yl)-phosphatidylethanolamine (NBDPE) were purchased from Avanti. Triton X-100 was from the Aldrich Chemical Company. Q-Sepharose and Sephadex were purchased from the Sigma Chemical Company. All solutions were prepared with water from a Millipore Milli-Q Water System.

Lipid Mixing Assays and Energy Transfer Efficiency Calculations

Chloroform solutions of lipids and probes were mixed at the appropriate molar ratios to give a total of 50 μg of lipid. The bulk of the solvent was removed under argon gas and the lipid was placed under high vacuum for a minimum of 15 minutes. The lipids were then hydrated in 200 mM sucrose to a final concentration of 1 mM. All vesicle preparations were vortexed vigorously and bath-sonicated for 10 seconds. Although it is well known that highly charged anionic vesicles disperse readily in low ionic-strength media, size characterization of vesicles containing cationic phospholipid derivatives has not been previously reported. These vesicles were therefore sized by dynamic light scattering. A Brookhaven Instruments model BI90 was used and the data were analyzed with the software provided with the instrument. Vesicles of DOPC, EDOPC and their mixtures were all in the size range of 100–200 nm, sizes that, for low ionic-strength dispersions, indicate unilamellar or paucilamellar vesicles. Mixtures of EDOPC and DOPG having net positive charges of up to 0.6 net charge fraction were heterogeneous and too large to accurately measure by this technique. Addition of NaCl to 100 mM had no evident effect on the sizes of these mixtures. Examination using the phase microscope verified their heterogeneity and revealed refractile aggregates, large numbers of small particles too small to resolve, and some giant vesicles. EDOPC and DOPG mixtures with 0.8 net charge fraction were in the same size range as the EDOPC-DOPC vesicles.

The lipid mixing assay was based on fluorescence energy transfer [23, 37]. Labeled vesicles included 0.005 mole fraction of RhPE and NBDPE. Fluorescence was monitored before and after the addition of 25 μg of unlabeled vesicles in a 200 μl reaction volume of either 200 mM sucrose or 100 mM NaCl, 5 mM Tris, pH 7.5 solution with 25 μg of labeled vesicles. An AlphaScan fluorometer (PTI, Princeton, NJ) was used with a bandpass of 4 nm and with excitation at 460 nm and emission at 534 nm. Dispersions were mixed and read after 45 seconds, which was determined to be ample time for the completion of all fluorescence changes.

To construct the transfer-efficiency standard curve, DOPG, EDOPC, DOPC and probes were mixed in chloroform prior to preparation of liposomes for each of the compositions studied (fractional cationic or anionic charge was 0, 0.2, 0.4, 0.6, 0.8 and 1.0) so as to generate lipid mixtures corresponding to complete (100%) lipid mixing of the various vesicle compositions examined. The energy transfer was then measured for each of these compositions and the data used to construct a standard curve corresponding to complete (100%) lipid mixing. Calculation of the energy-transfer efficiency (*ET*) was based on the expression $ET = F_R/F_N$, where F_R and F_N are the peak fluorescence values of RhPE and NBDPE. Percent lipid mixing was calculated with the equation % Lipid Mix $= (ET_i - ET_o)/(ET_i - ET_{st})$. The initial ($ET_i$) and observed ($ET_o$) transfer efficiencies represent energy transfer values before and after addition of unlabeled vesicles, and ET_{st} is the standard curve value.

Vesicle Fusion and Leakage Assays

Contents mixing assays were based on the cobalt calcein-EDTA system previously described [17, 28]. The maximum fluorescence, corresponding to 100% fusion, was obtained by solubilizing the vesicles in the presence of a final concentration of 1% Triton in 100 mM NaCl, 10 mM EDTA, pH 7.5. (Although EDTA does interact enough with cationic lipids so that vesicles will not form in the presence of 10 mM EDTA, the addition of EDTA to that concentration had no detectable effect on vesicles preformed in 100 mM NaCl.) Contents-mixing values were subtracted from the contents-mixing + leakage values to obtain vesicle-leakage values. The percentages were obtained by dividing the fluorescence values of the contents-mixing and leakage curves by the maximum fluorescence value and multiplying by 100. Cobalt-calcein was encapsulated in the cationic vesicles and EDTA encapsulated in the anionic vesicles because the reverse procedure led to aggregation of the cationic vesicles by EDTA. Thus, in order to obtain an initial fluorescence signal, the anionic vesicles were added to the cationic vesicles (reverse order relative to the lipid-mixing experiments.) For the contents-mixing assay, the external phase contained a low concentration of $CoCl_2$ to prevent dissociation of cobalt-calcein, which would generate a concomitant fluorescence signal from ruptured vesicles. Based on our earlier work with this assay, this concentration of cobalt ion was considerably below that which caused interaction of anionic vesicles with themselves. Dispersions were mixed and read after 45 seconds.

Results

Lipid Mixing

An extensive group of experiments was done to characterize lipid mixing between oppositely charged membranes as a function of surface charge densities on one set of vesicles, the other set of vesicles being fully charged, both positively as well as negatively. The charge on the variable-charge set of vesicles was varied from zero to fully charged in two ways: In one case, different proportions of a charged lipid—either EDOPC (positive) or DOPG (negative)—were combined with the zwitterionic lipid DOPC (no net charge) and formed into vesicles; in this case, the lipid with the net charge varied from none to all of the lipid, generating vesicles ranging from uncharged to fully charged. In the other case, different proportions of positive and negative lipids were combined and formed into vesicles. In this case, the vesicles with zero net charge contained equal amounts of positive and negative lipid, whereas increasing net charge corresponded to an increasing amount of one of the charged lipids at the expense of the other. Thus, four different types of oppositely charged vesicles were investigated. Each of these was tested for lipid mixing at both low ionic strength (sucrose solution) and high ionic strength (0.1 M NaCl, 5 mM Tris), so a total of 8

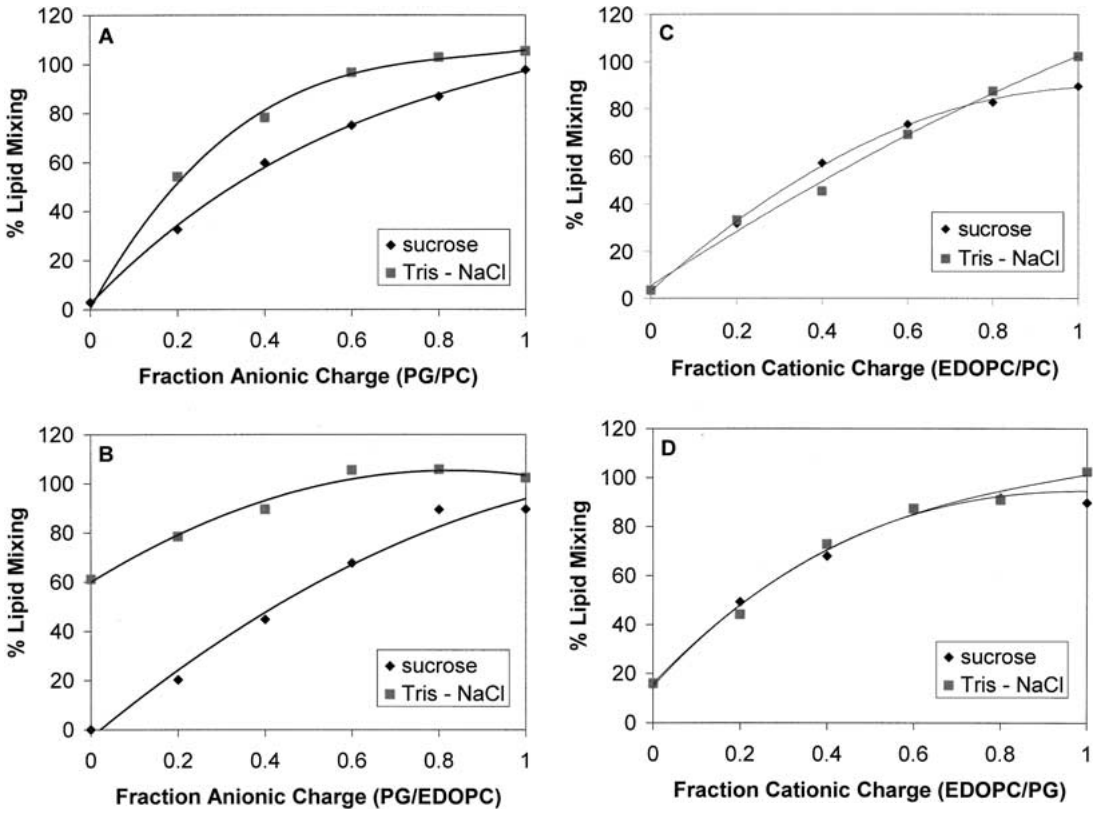

Fig. 1. Interaction of positively and negatively charged phospholipid vesicles: Membrane mixing. (*A*) EDOPC vesicles were added to DOPG/DOPC vesicles (fractional anionic charge ranging from 0–1.0). (*B*) EDOPC vesicles were added to DOPG/EDOPC vesicles (net fractional anionic charge ranging from 0–1.0). (*C*) EDOPC/DOPC vesicles (fractional cationic charge ranging from 0–1.0) were added to DOPG vesicles. (*D*) EDOPC/DOP vesicles (net fractional cationic charge ranging from 0–1.0) were added to DOPG vesicles. Experiments were done in both 200 mM sucrose and 100 mM NaCl/Tris buffer (pH 7.5) solutions. Negatively charged vesicles contained NBD and rhodamine probes at 0.5 mole%. Measurement of fluorescence resonance energy transfer allowed estimation of the extent of membrane mixing, which is plotted in the figure. Net fractional charge is the number of moles of net electrostatic charge divided by the total moles of lipid. Measurements were made 45 s subsequent to mixing of the vesicles.

experimental conditions was examined. Other procedures were the same for all groups of experiments; anionic vesicles contained the fluorescent probes and cationic vesicles were added to them.

Positive EDOPC Vesicles Mixed with Vesicles Having Fractional Negative Charge

When the positive vesicles were fully charged and the negative vesicles partially charged, lipid mixing (Fig. 1*A* and 1*B*) increased smoothly to essentially 100%, with increasing anionic charge. At low ionic strength, lipid mixing did not depend on how the anionic surface charge density was varied (*compare diamonds* in 1*A* and 1*B*). As will be seen, this lipid mixing follows much the same pattern as that in the case in which the charge on the positive vesicles was varied and the negative vesicles were fully charged (Fig. 1*C* and 1*D*). Although these patterns of mixing were similar at low ionic strength, mixing of fully positive vesicles with negative vesicles of fractional charge was very different when the ionic strength was high. When the charge of the negative vesicles was varied by increasing DOPG relative to the neutral DOPC, a difference was seen at intermediate charge densities where there was greater lipid mixing at high than at low ionic strength (*compare squares* with *triangles* in Fig. 1*A*). When the charge of the negative vesicles was varied by increasing the ratio of DOPG to the positive EDOPC, an unusual effect occurred in that even at net zero charge there was a very substantial amount—nearly 60%—of lipid mixing (Fig. 1*B*, *squares*). Larger lipid mixing at high ionic strength relative to that at low ionic strength is unexpected because, the higher the concentration of electrolyte, the weaker the electrostatic interactions between oppositely charged vesicles. This unusual behavior is considered in the Discussion.

Negative DOPG Vesicles Mixed with Vesicles Having Fractional Positive Charge

The extent of lipid mixing for the situation in which the fractional anionic charge varied and the positive

vesicles were fully charged is shown in Figs. 1*C* and 1*D*. In both cases, lipid mixing increased with increasing charge density. It made little difference whether the charge on the positive vesicles was increased by incorporating more EDOPC relative to DOPC (Fig. 1*C*) or relative to DOPG (Fig. 1*D*), although the latter vesicles were somewhat more fusogenic (by about 1/4 to 1/2) at low and intermediate charge densities than were the former. In both cases, ionic strength had little effect, which is in marked contrast to the situations of Figs, 1*A* and 1*B*, where high ionic strength led to much higher lipid mixing. There was a modest difference between Fig. 1*C* and Fig. 1*D* in that, in the latter case (positive charge varied according to the EDOPC/DOPG ratio), there was detectable mixing when there was no net charge on those vesicles. This effect, seen at both high and low ionic strength, is reminiscent of that of Fig. 1*B* for high ionic strength only, but much smaller.

Macroscopic and Microscopic Appearance of Vesicle Interactions

Other, qualitative, observations made it clear that the magnitude of net surface charge density is the most important property in determining vesicle interactions. Both the turbidity of the mixed solutions and the extent of lipid mixing were affected by membrane surface charge density. At high charge proportions (>0.6) dispersions of cationic and anionic vesicles mixed in 200 mM sucrose were seen to be somewhat turbid due to vesicle aggregation. In electrolyte solutions, mixing produced small aggregates that were visible under the microscope in the case of vesicles that contained 1.0 or 0.8 fractional charge, while an increase in turbidity without visible aggregation was observed at 0.4 and 0.6 fractional charge (fractional charge varied by changing the ratio of charged lipid to DOPC). These precipitates are most likely due to vesicles that have collapsed and formed large lipid particles of the type previously observed under the fluorescence microscope from ruptured cationic and anionic giant vesicles [29]. Sonicated, fluorescent-labeled vesicles were also examined under the fluorescence microscope before and after mixing with sonicated, unlabeled vesicles. Labeled vesicles appeared as small bright specks in solution, whereas after addition of unlabeled vesicles, larger spherical vesicles could be seen, evidently due to vesicle fusion.

Vesicle Rupture as Determined from Contents Release

Because membrane mixing assays only indicate the amount of merging of bilayers, which gives little indication as to what proportion of the vesicles remains intact, it is important to separately establish the extent to which vesicle integrity is preserved during interactions potent enough to produce a significant extent of membrane mixing. We therefore determined what fraction of the internal volume of the vesicles was retained [16, 23].

We had previously shown that cationic vesicles composed entirely of EDOPC were able to encapsulate cobalt-calcein and fuse with anionic vesicles composed of DOPG and containing 10 mM EDTA [23]. Here we describe the further characterization of this cationic lipid at four different charge fractions (1.0, 0.75, 0.50, 0.25). To measure the extent of leakage, we used the cobalt-calcein assay [17]. The quenched chelate, cobalt-calcein, was encapsulated in cationic vesicles, while EDTA was included in the outer aqueous phase so that rupture led to disassociation of the chelate and the restoration of calcein fluorescence. This assay was performed in two ways: l) by mixing positive and negative vesicles with the same surface charge density but of opposite polarity (homologous mixing); 2) by mixing vesicles of varying charge density with oppositely charged vesicles consisting of 100% charged lipid (heterologous mixing). It should be noted that the variable-charge vesicles for these experiments only included lipid compositions in which the charged component was diluted with DOPC, and not when it was also neutralized with the oppositely charged lipid as in the lipid mixing experiments of the previous section.

Homologous (same fractional charge on both sets of vesicles) mixing of 1.0, 0.75, 0.5, and 0.25 fractional charged EDOPC/DOPC and DOPG/DOPC vesicles in the presence of 10 mM EDTA resulted in approximately 80%, 27%, 10%, and 2.5% vesicle leakage, respectively (Fig. 2*A*). Heterologous (full charge on one set of vesicles, variable charge on the other set) mixing gave different results that were dependent on the surface-charge density of the vesicles. Increasing the cationic fractional charge density to 1.0 and mixing with 0.75, 0.5, and 0.25 anionic charge compositions produced significantly greater leakage than did homologous (same fractional charge on both sets) mixing (Fig. 2*B*). Increasing the fractional anionic charge density to 1.0 and mixing with 0.75, 0.5, and 0.25 cationic charge compositions had no significant effect on leakage compared to homologous mixing; i.e., *C* of Fig. 2 is similar to *A*, whereas *B* differs significantly from *A*.

Vesicle Fusion as Estimated from Contents-Mixing

Although lipid-mixing measurements give an overall estimate of membrane mixing, they are subject to bias and errors that may depend upon the environment of the fluorescent probes in the bilayer membrane. Such experiments also provide no information on the extent of true vesicle fusion, which, in population as-

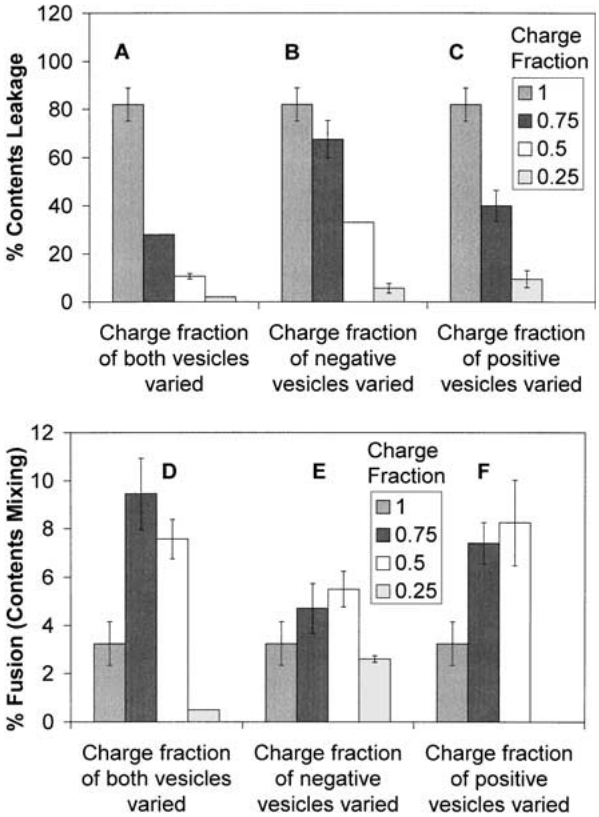

Fig. 2. Contents-leakage and contents-mixing measurements of interactions of positively and negatively charged vesicles. In experiments *A* and *D*, DOPG vesicles were added to EDOPC vesicles and DOPG/DOPC vesicles were added to EDOPC/DOPC vesicles (fractional cationic and anionic charge for the oppositely charged vesicles were equal and are indicated for each in the key). For the experiments *B* and *E*, EDOPC vesicles were added to DOPG/DOPC (fractional anionic charge indicated by key). For experiments *C* and *F*, DOPG vesicles were added to EDOPC/DOPC vesicles (fractional cationic charge indicated by key). Cationic vesicles contained 1 mM $CoCl_2$-calcein, 10 mM Tris, 85 mM NaCl, pH 7.5. Anionic vesicles contained 2% rhodamine-PE for visualization during column elution and either 100 mM NaCl, 0.3 mM $CoCl_2$ (contents leakage, A–C) or 100 mM NaCl, 10 mM EDTA pH 7.5 (contents mixing, D–F). Contents-leakage and contents-mixing measurements were carried out in 100 mM NaCl, 10 mM EDTA, and 100 mM NaCl, 0.3 mM $CoCl_2$, respectively. Charge fraction is the mole fraction of EDOPC or DOPG in the vesicles. Measurements were made 45 s subsequent to mixing of the vesicles.

says, is best estimated by measuring contents-mixing between vesicles. We have hence also made use of a contents-mixing assay to monitor vesicle interactions of cationic and anionic vesicles.

The vesicle aqueous contents mixing assay we used is based on the cobalt-calcein system described above for measurement of leakage [17]. The concentrations used were as given [23]. EDTA was encapsulated in the anionic vesicles and cobalt-calcein was encapsulated in the cationic vesicles. Upon fusion and mixing of internal compartments, EDTA in one vesicle chelates the cobalt ion from the cobalt-calcein of the other vesicle, releasing the calcein, which thereupon fluoresces. A low concentration of cobalt is included in the external solution so that any vesicle interactions that lead to rupture will not give a fluorescent signal. That any signal due to rupture was indeed negligible was verified by lysing the vesicles with detergent at the end of the fusion experiment.

Intermixing of aqueous contents was modest at best (~10%), but displayed very clear dependence on charge composition. Homologous mixing, in which both sets of vesicles have the same fractional charge, produced a maximum fusion of 8–11% at a 0.75 fractional charge (Fig. 2*D*). Considerably less fusion (2–4%) was seen with vesicles having fractional charges of 1.0, whereas vesicles containing 0.5 fraction of charged component were only slightly less fusogenic than those with 0.75 fraction of charged component. At 0.25 charge fraction, there was practically no fusion. The decrease in fusion of the fully charged vesicles is consistent with the near-complete loss of contents described in the previous section; since leakage of fully charged vesicles was over 80%, significant vesicle fusion in which the fusion product remained intact would not be possible. Of course, leakage could compete with fusion or occur subsequent to fusion; in both cases the effect would be to reduce the amount of measured fusion. Maximum fusion occurred with vesicles of 0.75 fractional charge. The amount of lysis that occurred at this charge density was a more modest 28%.

Fusion of partially charged vesicles with vesicles of 100% opposite charge (heterogeneous mixing) was also examined. Fully charged vesicles, either of EDOPC or of DOPG, were mixed with vesicles of the opposite charge having charge fractions of 0.25, 0.5, and 0.75 (the remainder being DOPC). For both directions of mixing, a maximum at 0.5 charge fraction was found (Figs. 2*E* & *F*). Significantly more fusion was seen when the fully charged vesicles were of DOPG than when they were of EDOPC. This is consistent with the lysis pattern in that, when the fully charged partner was cationic, rupture was more extensive than when the fully charged partner was anionic, that is, a large degree of lysis simply subtracts from the extent of inner compartment fusion.

Contents Leakage and Contents Mixing at Vesicle Mixing Ratio of 2:1

Additional experiments, the results of which are not sufficiently unique to describe in detail, were undertaken to determine whether percent fusion and leakage were dependent on the ratio of cationic to anionic vesicles. EDOPC/DOPC vesicles of charge fractions 1.0, 0.75, and 0.5 were mixed with DOPG/DOPC vesicles of charge fractions, 1.0 0.75, and 0.25, respectively, in a lipid molar ratio of 1:2 (cationic vesicles to anionic vesicles). The extents of fusion for

each combination were similar to the results obtained when 1:1 ratios were used. Likewise, mixtures of EDOPC vesicles with DOPG vesicles and EDOPC/DOPC (0.25 charge fraction) with DOPG/DOPC (0.25 charge fraction) resulted in similar leakage percentages at 1:1 or 1:2 lipid molar ratios.

Discussion

This study of interactions of oppositely charged vesicles represents one of the most complete characterizations of such vesicles by population assays and the first involving a lipid for which a comparison between population assays and individual vesicle-vesicle interaction assays can be made. It is clear that the population assays do reflect the behavior of individual vesicles, although there are likely considerable distortions due to differences between single and multiple vesicle interactions. The various measures of interaction, namely lipid mixing, contents release and contents mixing, can generally be rationalized on the basis of the adhesion-condensation mechanism of bilayer fusion [20, 22, 29], although some interesting and unexpected effects of ionic strength were seen. Thus, in addition to adding to our understanding of basic bilayer fusion, these studies have also provided some new indication of unusual behavior of bilayers containing mixtures of charged lipids. Furthermore, examination of interaction of highly charged cationic bilayers with more weakly charged anionic bilayers can clarify events likely to occur when cationic lipids are used to transfect DNA into cells.

Lipid Mixing

Membrane mixing increased with increasing surface charge under all conditions examined (Figs. 1*A*–1*D*). This is to be expected, since the fluorophores used are essentially non-exchangeable and, when both probes are initially in the same membrane, changes in energy transfer cannot come about unless there is lipid mixing; lipid mixing, in turn, cannot come about in the absence of membrane contact. We cannot, of course, distinguish between mixing caused by lipid exchange between intact membranes and mixing due to hemifusion or full fusion, but given the short times of incubation (45 seconds in our case), the initial drop in energy transfer is probably not due to exchange but rather to hemifusion. This is particularly true at high charge density, in which case lipid mixing was essentially complete, since under those conditions, hemifusion and complete fusion followed by rupture would be likely. Such an interpretation is consistent with our observations on pairwise interactions between vesicles [29].

Although it was expected that lipid exchange would increase as surface charge was increased, the effects of ionic strength were surprising. Since charge-charge interactions are screened more effectively the higher the electrolyte concentration [25], we expected stronger bilayer-bilayer interactions at low than at high ionic strength and hence also more lipid mixing at low than at high ionic strength. Such behavior was not observed. When the negative vesicles were fully charged (Figs. 1*C* and 1*D*), mixing was the same at high and low ionic strength, whereas when the negative vesicles were partially charged (Figs. 1*A* and 1*B*), mixing was greater at high ionic strength. Moreover, DOPG/EDOPC vesicles with a zero net charge underwent lipid mixing with EDOPC vesicles at high but not at low ionic strength (Fig. 1*A*, first *square*). To a much smaller extent, this behavior was seen at both high and low ionic strength when the variable charge was on the positive vesicles (Fig. 1*D*, first *square* and *diamond*). The fact that there was a smaller effect of ionic strength on the mixing of anionic vesicles with vesicles consisting of anionic plus cationic lipids than there was when cationic vesicles were mixed with such mixed lipid vesicles, may reflect the generally higher fusogenic activity of the cationic vesicles.

The behavior represented by zero fractional charge in Fig. 1*B* (*squares*) is most puzzling. By X-ray diffraction, this composition is largely in the inverted hexagonal phase at high lipid concentration at high ionic strength [38] and both microscopic observation and dynamic light scattering revealed mixtures of many different sizes, and apparently, kinds, of particles. Although X-ray diffraction has not been done on such mixtures at low ionic strength, by light scattering and under the light microscope, this mixture was not markedly different in high than in low ionic strength. Although such examination did not suggest why this composition should be so strongly affected by ionic strength, given that this mixture (as well as the mixtures with higher net charge—except for >0.8 fractional net charge) is capable of residence in a non-lamellar phase, it is possible that some form of phase separation could affect fusion, and furthermore, that phase behavior could, in turn, be influenced by ionic strength. In this respect, it is of particular interest that Stamatatos et al., who used the same probe combinations to measure lipid mixing between oppositely charged vesicles, but DOTAP as the cationic amphipath, also reported that increasing ionic strength increased fusion, particularly when the neutral component was PE [36]. They suggested that fluctuations of surface geometry [34], which could depend significantly on the electrostatic properties of the surfaces, might play an important role. The effect was so large in their case that vesicle interactions were not observed at low ionic strength. It seems clear that cationic lipid mixtures, especially with anionic lipids, can exhibit unusual behavior that cannot now be explained on the basis of information currently

available and that additional research into such intriguing behavior should be very rewarding.

Membrane Fusion as Estimated from Contents Release

It is certainly possible that the simple adhesion of two vesicles can rupture one or both of them [33]. The only requirement is that the adhesion be strong enough that the resultant flattening stretch the bilayers beyond their lysis tension [8, 29]. Our previous observations on interactions of pairs of oppositely charged giant vesicles suggest, however, that lysis can involve more than simple stress on adherent membranes and that rupture can occur after an initial fusion event, perhaps one in which there has been incomplete resealing after fusion [29]. It then becomes a matter of semantics as to whether such processes are deemed fusion. "Membrane fusion" is probably an acceptable description, whereas "vesicle fusion" frequently implies more than is justified on the basis of the experimental data.

Beyond the issue of the initiation process that eventually leads to rupture, the knowledge of the extent of rupture is important in assessing the meaning of the extent of fusion in the same system. Specifically, contents mixing was maximal between 0.5 and 0.75 charge fraction. The fact that increased fusion was not seen with fully charged vesicles is surely due to the large increase in rupture between 0.75 charge fraction and full charge. It cannot be known, at least with present methods, whether the contents of the two vesicles communicated before the vesicles ruptured; as discussed below, it seems likely that multiple vesicle interactions frequently contribute to rupture.

Comparison of *B* and *C* of Fig. 2 shows that, except when leakage may be close to maximal, there is more (≥2–4×) leakage when the fully charged vesicles are positive, the other set having fractional surface charge. This indicates that the EDOPC bilayer is more prone to interactions leading to leakage than is the DOPG bilayer. This difference may be attributable to its lack of polar group-polar group interactions. It cannot hydrogen-bond as can DOPG and there are no opposite charge interactions like in DOPC. Both EDOPC (quaternary ammonium) and DOPG [39] must possess essentially one electronic charge per molecule.

Vesicle Fusion as Estimated from Contents Mixing

The largest extent of contents mixing (10%) was seen when both sets of vesicles had fractional charges of 0.75 (Fig. 2*D*), although the mixing measured for DOPG vesicles with 0.5 charge fraction EDOPC/DOPC vesicles (Fig. 2*F*) was not statistically different. In the latter situation (heterogeneous mixing), it appears that, because of the greater lability of the cationic membrane, a 0.5 charge fraction is adequate and the reduced charge-charge interaction can be compensated for by increasing the charge fraction of the anionic vesicles to 1.0 (entirely DOPG). Probably in both of these cases, rupture may have placed a limit on fusion, since increasing the charge fraction to the next level (0.75 to 1.0 for both or from 0.5 to 0.75 for the positive vesicles) led to rupture of almost all of the population.

We cannot determine from these experiments whether those vesicles that lost their contents did so because of i) immediate rupture on contact, ii) because of an initial fusion step that turned into rupture or iii) as consequence of multiple vesicle interactions that led to destabilization. Similar questions have been discussed with respect to the PS/Ca^{2+} system [42]. Our previous studies of oppositely charged membrane interactions allowed us to assess these different possibilities by measuring the extent of fusion, hemifusion and rupture by direct observation of individual pairwise interactions between oppositely charged vesicles [11, 29]. Fusion occurred in more than 40% of the interactions between vesicles containing only charged components and rupture upon contact occurred with similar frequency. At lower surface-charge densities, hemifusion occurred in well over half of the encounters and both fusion and rupture diminished dramatically. In those studies, we observed numerous cases where subsequent interactions of fused vesicles resulted in leakage due to vesicle collapse, a situation that may resemble interactions among whole vesicle populations as occurs in the present fluorometer-based population assay.

A model fusion system consisting of oppositely charged membranes has the apparent advantage that multiple interactions tend to be reduced, because with each interaction, the charge and hence probability of further interaction is reduced. In particular, it would seem that rupture due to multiple interactions of vesicles would be considerably lower than for the PS/Ca^{2+} system, in which very large aggregates are possible [17]. Nevertheless, higher extents of fusion (to 25% or so) have been reported for the PS system [31, 41]. If the latter percentages are not inflated by fusion events that occur within aggregates, then possibly the cationic-anionic vesicle system may not be so self-correcting relative to rupture as expected. One feature of mixtures of oppositely charged lipids that may mitigate such an advantage, is that these mixtures of lipids tend to form non-lamellar phases [38], a feature mentioned above with respect to enhanced fusion of EDOPC-DOPG mixtures at high ionic strength. We previously reported the presence of regions containing both lipids (evident from energy

transfer) that appeared on fused vesicles and in collapsed "wads" when vesicles ruptured. These bodies, which evidently consist of non-lamellar phases (inverted hexagonal phase when cationic and anionic charges are equal and cubic phases when the charge ratio is 2:1), were observed to interact with intact vesicles and initiate their rupture. It may be that this phenomenon introduces an element of instability that is particularly prominent when interactions of bulk populations are examined. On the other hand, these same kinds of phase transformations may be of benefit in releasing DNA when cationic lipids are used to deliver DNA to cells, for at least one publication shows an electron micrograph of what is almost certainly cubic phase lipid in cells into which DNA had been transfected with cationic lipid [44]. It should perhaps also be acknowledged that the possibility of formation of a cubic phase could mean that the final product of some fusion conditions would be a cubic phase in which the original vesicle contents could be trapped and, in some cases, mix. Such a phenomenon could account for the fact that contents leakage is always somewhat less than 100%, even at the most extreme conditions of charge (Fig. 2*A–C*).

An earlier fluorometric study with another cationic-anionic pair (DOTAP/PE and PS/PE) found maximum contents mixing of approximately 10% and lipid mixing of 50% [35]. These results are quite similar to ours and indicate that extensive rupture may be a characteristic of fusion of whole populations driven by electrostatic charge interactions. We note in passing that such phenomena might be circumvented by using a very high mixing ratio, such as 10:1 so that the entire population would have the same charge (sign, not magnitude) when vesicle interactions cease.

The Physical Basis of Fusion, Lipid Mixing and Rupture among Oppositely Charged Bilayer Vesicles

Why should oppositely charged lipid bilayers fuse when they come into contact? The adhesion-condensation mechanism [20, 22] appears to explain hemifusion and fusion of oppositely charged bilayers even better than that of the system for which it was proposed, namely anionic bilayers brought into contact by means of cations [29]. More details are given in [29], but enough of the hypothesis will be given here to illustrate how it relates to the current observations. It is clear that when two vesicles adhere, by whatever mechanism, the flattening at the junction zone stretches each bilayer [8]. This could cause some fusion if random rends were to occur opposite each other in the adhering bilayers of the contact zone, but such events should be rather rare. Instead, it is likely that the mutual neutralization of each of the contacting monolayers reduces repulsion of the head groups of the same type so that they condense, which process would lead to rends in those monolayers, allowing the tails of the two monolayers on the inside surfaces of the vesicles to flow into contact through the openings generated in the contacting monolayers. In other words, contraction of the contacting surfaces leads to surface fissures and hence automatically generates hemifused vesicles. If the tension in the bilayers due to the vesicle-vesicle flattening is large enough, then because the tension in both vesicles acts on the single bilayer of the hemifused junction, it will exceed the lysis tension of the latter and lead to full fusion. Thus, the adhesion-condensation mechanism predicts increasing lipid mixing (at least by hemifusion) as well as increased fusion (at least initially) as the charge density on the vesicles in increased. If the bilayer tension is large (very high surface charge density), it can be that the contact zone is pulled open before full hemifusion has occurred, so that the contents of one or both vesicles are released to the external phase and not into the other vesicle.

Conclusions

1. Vesicles of the cationic phospholipid derivative ethylphosphatidylcholine interact with those of phosphatidylglycerol to:

a. Undergo lipid mixing, which ranges from 0% to 100% as the fractional surface charge increases from 0 to 1.0.
b. Undergo rupture that follows a similar dependence on fractional surface charge as lipid mixing.
c. Undergo content mixing that has an optimum at about 0.5 fractional surface charge, being lower at lower charge due to lack of interaction and lower at higher charge due to excessive rupture, in part as a consequence of multiple interactions.

2. Lipid mixing can be significant between highly charged cationic vesicles and weakly charged anionic vesicles, especially at high ionic strength, conditions that resemble those obtaining during transfection of DNA into cells by cationic lipids.
3. At high but not at low ionic strength, DOPG/EDOPC (1:1) dispersions with no net charge undergo lipid mixing with cationic vesicles; surface heterogeneity and/or phase polymorphism may account for the unusual behavior of DOPG/EDOPC mixtures.

This research was supported by National Institute of Health grants GM57305 and GM52329. We are very grateful to Rumiana Koynova for carrying out the light-scattering measurement to characterize vesicle sizes. We would also like to acknowledge Reviewer #1 for several suggestions that led to an improved manuscript.

References

1. Anzai, K., Masumi, M., Kawasaki, K., Kirino, Y. 1993. Frequent fusion of liposomes to a positively charged planar bilayer without calcium ions. *J. Biochem.* **114:**487–491
2. Ashley, G.W., Shida, M.M., Qiu, R., Lahiri, M.K., Levisay, P.C., Jones, R.D., Baker, K.A., MacDonald, R.C. 1996. Phosphatidylcholinium compounds: A new class of phospholipids with transfection activity and unusual physical properties. *Biophys. J.* **70:**A88
3. Bailey, A.L., Cullis, P.R. 1997. Membrane fusion with cationic liposomes: effects of target membrane lipid composition. *Biochemistry* **36:**1628–1634
4. Breisblatt, W., Ohki, S. 1976. Fusion in phospholipid spherical membranes. II. Effect of cholesterol, divalent ions and pH. *J. Membrane Biol.* **29:**127–146
5. Düzgüneş, N., Allen, T.M., Fedor, J., Papahadjopoulos, D. 1987. Lipid mixing during membrane aggregation and fusion: why fusion assays disagree. *Biochemistry* **26:**8435–8442
6. Düzgüneş, N., Goldstein, J.A., Friend, D.S., Felgner, P.L. 1989. Fusion of liposomes containing a novel cationic lipid, N-[2,3-(Dioleyloxy)propyl]-N,N,N-trimethylammonium: induction by multivalent anions and asymmetric fusion with acidic phospholipid vesicles. *Biochemistry* **28:**9179–9184
7. Düzgüneş, N., Wilschut, J. 1993. Fusion assays monitoring intermixing of aqueous contents. *Methods Enzymol* **220:**3–14
8. Evans, E.A., Parsegian, V.A. 1983. Energetics of membrane deformation and adhesion in cell and vesicle aggregation. *Ann. N. Y. Acad. Sci.* **416:**13–33
9. Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., Danielsen, M. 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci.* USA **84:**7413–7417
10. Gao, X., Huang, L. 1991. A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem. Biophys. Res. Commun.* **179:**280–285
11. Garcia, R.A., Pantazatos, S.P., Pantazatos, D.P., MacDonald, R.C. 2001. Cholesterol stabilizes hemifused phospholipid bilayer vesicles. *Biochim. Biophys. Acta* **1511:**264–270
12. Gershon, H., Ghirlando, R., Guttman, S.B., Minsky, A. 1993. Mode of formation and structural features of DNA-cationic liposome complexes used for transfection. *Biochemistry* **32:**7143–7151
13. Hoekstra, D., Düzgüneş, N. 1993. Lipid mixing assays to determine fusion in liposome systems. *Methods Enzymol* **220:**15–32
14. Huang, L., Hung, M.-C., Wagner, E. 1999. Nonviral Vectors for Gene Therapy. Academic Press, San Diego
15. Kachar, B., Fuller, N., Rand, R.P. 1986. Morphological responses to calcium-induced interaction of phosphatidylserine-containing vesicles. *Biophys. J.* **50:**779–788
16. Kendall, D.A., MacDonald, R.C. 1983. Characterization of a fluorescence assay to monitor changes in the aqueous volume of lipid vesicles. *Anal. Biochem.* **134:**26–33
17. Kendall, D.A., MacDonald, R.C. 1982. A fluorescence assay to monitor vesicle fusion and lysis. *J. Biol. Chem.* **257:**13892–13895
18. Kennedy, M.T., Pozharski, E.V., Rakhmanova, V.A., MacDonald, R.C. 2000. Factors governing the assembly of cationic phospholipid-DNA complexes. *Biophys. J.* **78:**1620–1633
19. Keren-Zur, M., Beigel, M., Loyter, A. 1989. Induction of fusion in aggregated and nonaggregated liposomes bearing cationic detergents. *Biochim. Biophys. Acta* **983:**253–258
20. Kozlov, M.M., Markin, V.S. 1984. On the theory of membrane fusion. The adhesion-condensation mechanism. *Gen. Physiol. Biophys.* **5:**379–402
21. Leventis, R., Silvius, J.R. 1990. Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochim. Biophys. Acta* **1023:**124–132
22. MacDonald, R.C. 1988. Mechanisms of membrane fusion in acidic lipid-cation systems. *In:*Molecular Mechanisms of Membrane Fusion. Ohki, S., Doyle, D., Flanagan, T.D., Hui, S.W., and Mayhew, E., editors, pp. 101–112. Plenum Press, N.Y.
23. MacDonald, R.C., Ashley, G.W., Shida, M.M., Rakhmanova, V.A., Tarahovsky, Y.S., Pantazatos, D.P., Kennedy, M.T., Pozharski, E.V., Baker, K.A., Jones, R.D., Rosenzweig, H.S., Choi, K.L., Qiu, R., McIntosh, T.J. 1999. Physical and biological properties of cationic triesters of phosphatidylcholine. *Biophys. J.* **77:**2612–2629
24. MacDonald, R.C., Rakhmanova, V.A., Choi, K.L., Rosenzweig, H.S., Lahiri, M.K. 1999. O-Ethylphosphatidylcholine: A metabolizable cationic phospholipid which is a serum-compatible DNA transfection Agent. *J. Pharm. Sci.* **88:**896–904
25. McLaughlin, S. 1977. Electrostatic potentials at membrane-solution interfaces. *Curr. Top. Membr. Transp.* **9:**71–144
26. Menger, F.M., Keiper, J.S. 1997. Electrostatic layering of giant vesicles. *Angew. Chem., Int. Ed. Eng.* **36:**2489–2491
27. Menger, F.M., Keiper, J.S., Lee, S.J. 1997. Adhesion of giant liposomes as observed by light microscopy. *Langmuir* **13:**4614–4620
28. Oku, N., Scheerer, J.F., MacDonald, R.C. 1982. Preparation of giant liposomes. *Biochim. Biophys. Acta.* **692:**384–388
29. Pantazatos, D.P., MacDonald, R.C. 1999. Directly observed membrane fusion between oppositely charged phospholipid bilayers. *J. Membrane. Biol* **170:**27–38
30. Pantazatos, S.P., MacDonald, R.C. 2003. Real-time observation of lipoplex formation and interaction with anionic bilayer vesicles. *J. Membrane Biol.* **191:**1–12
31. Papahadjopoulos, D., Nir, S., Düzgüneş, N. 1990. Molecular mechanisms of calcium-induced membrane fusion. *J. Bioenerg. Biomembr.* **22:**157–179
32. Papahadjopoulos, D., Vail, W.J., Pangborn, W.A., Poste, G. 1976. Studies on membrane fusion. II. Induction of fusion in pure phospholipid membranes by calcium ions and other divalent metals. *Biochim. Biophys. Acta.* **448:**265–283
33. Parsegian, V.A., Rand, R.P. 1983. Membrane interaction and deformation. *Ann. N. Y. Acad. Sci.* **416:**1–12
34. Siegel, D.P. 1984. Inverted micellar structures in bilayer membranes. Formation rates and half-lives. *Biophys. J.* **45:**399–420
35. Silvius, J.R., Leventis, R., Brown, P.M., Zuckermann, M. 1987. Novel fluorescent phospholipids for assays of lipid mixing between membranes. *Biochemistry* **26:**4279–4287
36. Stamatatos, L., Leventis, R., Zuckermann, M.J., Silvius, J.R. 1988. Interactions of cationic lipid vesicles with negatively charged phospholipid vesicles and biological membranes. *Biochemistry* **27:**3917–3925
37. Struck, D.K., Hoekstra, D., Pagano, R.E. 1981. Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* **20:**4093–4099
38. Tarahovsky, Y.S., Arsenault, A.L., MacDonald, R.C., McIntosh, T.J., Epand, R.M. 2000. Electrostatic control of phospholipid polymorphism. *Biophys. J.* **79:**3193–3200
39. Watts, A., Harlos, K., Maschke, W., Marsh, D. 1978. Control of structure and fluidity of phosphatidylglycerol bilayers by pH titration. *Biochim. Biophys. Acta* **510:**63–74
40. Wilschut, J., Düzgüneş, N., Hong, K., Hoekstra, D., Papahadjopoulos, D. 1983. Retention of aqueous contents during divalent cation-induced fusion of phospholipid vesicles. *Biochim. Biophys. Acta* **734:**309–318

41. Wilschut, J., Hoekstra, D. 1986. Membrane fusion: lipid vesicles as a model system. *Chem. Phys. Lipids* **40:**145–166
42. Wilschut, J., Papahadjopoulos, D. 1979. Ca^{2+}-induced fusion of phospholipid vesicles monitored by mixing of aqueous contents. *Nature* **281:**690–692
43. Xu, Y., Szoka, F.C., Jr. 1996. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* **35:**5616–5623
44. Zabner, J., Fasbender, A.J., Moninger, T., Poellinger, K.A., Welsh, M.J. 1995. Cellular and molecular barriers to gene transfer by a cationic lipid. *J. Biol. Chem.* **270:**18997–19007
45. Zschornig, O., Arnold, K., Richter, W., Ohki, S. 1992. Dextran sulfate-dependent fusion of liposomes containing cationic stearylamine. *Chem. Phys. Lipids* **63:**15–22
46. Zuhorn, I.S., Hoekstra, D. 2002. On the mechanism of cationic amphiphile-mediated transfection. To fuse or not to fuse: is that the question? *J. Membrane Biol.* **189:**167–179