How lipid asymmetry can make vesicles fusion-competent by inhibition of the thermal undulations

P. Devaux, L. Mathivet, S. Cribier, E. Farge

How lipid asymmetry can make vesicles fusion-competent by inhibition of the thermal undulations

P. F. Devaux, L. Mathivet, S. Cribier and E. Farge
Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie 75005 Paris, France

Introduction

The first step in any fusion event involving biological membranes or liposomes is the close contact between the two lipid bilayers. Before the local collapse which gives rise to a single membrane from two membranes, the two phospholipid interfaces have to approach each other. Different interacting forces are involved. Van der Waals forces are attractive and can extend to distance of the order of 10 nm. Electrostatic forces between charged lipid head groups belonging to different membranes are generally repulsive, but the high dielectric constant of water and the screening by ions can minimize this repulsion. Moreover the lateral diffusion of phospholipids enables the charged lipids to slide away from the contact points. The so-called hydration forces are responsible for a potential barrier with a thickness of about 2–3 nm, depending on the nature of the head groups; aminophospholipids, being less hydrated than choline-containing phospholipids, are more favourable for fusion [1, 2]. In addition to these terms, Helfrich's group have shown that the thermal undulations of lipid bilayers correspond to a steric interaction, which can be accounted for by a repulsive force between membranes, with a typical length of interaction of the order of 10 nm [3]. Thus, van der Waals attractive forces and steric repulsions due to thermal undulations are the most important and opposing terms in fusion events and to adhere, membranes have to overcome this steric repulsion.

The thermal undulations correspond to the superposition of surface fluctuations with different wavelengths. The amplitude of these waves gives an indication of the distance of steric repulsion. With a light microscope very long waves with a large amplitude are visible, but typical amplitudes are usually below the resolution of a light microscope.

The thermal undulations can be inhibited in a variety of ways, for example by reducing the temperature or by stretching the membranes. Indeed, phospholipid membranes undulate because the lateral tension is normally approximately zero [4]. Inducing a lateral tension can inhibit the fluctuations and hence permits membrane cohesion. Servuss and Helfrich have shown that cohesion can be 'turned on' by an osmotically-induced lateral tension [5]. Bending the membranes is also a way of reducing the undulations: firstly because very long wavelengths can no longer exist, thus the frequency spectrum is reduced and secondly because bending itself can induce lateral tension.

In this paper we demonstrate that an asymmetrical distribution of phospholipids, which is generally associated with membrane curvature, can favour membrane adhesion, probably by inhibition of the thermal fluctuations.

Theory

In the following we will consider only phospholipid bilayers made of a single phospholipid species, so that the areal asymmetry is associated with a proportional difference in the number of lipids between leaflets: $\Delta N/N_0$, where ΔN is the difference in number of lipids in both layers and N_0 the average number of lipids per leaflet. It has been shown previously that the transition from a unilamellar liposome with a discoid shape, characterized by an average radius R_0, to a liposome made of two connected spheres of approximately the same radius, necessitates a change in lipid asymmetry,

$$\frac{\Delta N_v}{N_0} - \frac{\Delta N_{ini.}}{N_0} = \lambda \left(\frac{h}{R_0}\right) \qquad (1)$$

where ΔN_v and $\Delta N_{ini.}$ are the difference in the number of lipids in both layers in the final state and in the initial state, respectively, h is the thickness of the bilayer and λ a constant equal to 0.83 [6]. This transition from a discoid to two spheres is sometimes called a 'vesiculation', although it does not imply the separation into two independent vesicles. From (1), it can be deduced that the quantity h/R_0 is a scaling factor for the shape changes reduced by a compositional asymmetry. For giant liposomes R_0 is of the order of 10 μm and h is 8 nm, thus the percentage of lipid transfer necessary for a shape change is very small (of the order of 0.1%). For the so-called large unilamellar vesicles (LUV) with a radius of about 45 nm, the percentage of lipid trans-

Abbreviations used: α, areal strain; δS, surface elements; ΔN, difference in number of lipids in both layers; BSA, bovine serum albumin; EPC, egg phosphatidylcholine; h, thickness of bilayer; N_0, average number of lipids per leaflet; R_0, average radius.

fer required for similar shape change is 15% and it would be more than 30% for sonicated vesicles with a radius of 20 nm. Thus in the two latter cases a redistribution of a few per cent of the lipids does not trigger any significant shape change. On the other hand, the lipid asymmetry builds up a lateral tension faster in small vesicles than in giant vesicles.

The lateral tension of a liposome which is not under osmotic stress and which has a large radius is very small. An expansion or contraction of the surface area, δS, associated with the areal strain,

$$\alpha = \frac{\delta S}{S_0},$$

can generate a tension τ. When considering the inhibition of the undulations, the relationship between τ and α has been established by Helfrich and Servuss; it is not a simple linear relation [3]. The value of α must be approx. 10^{-3} or larger to inhibit the undulations partially.

The asymmetrical insertion (or depletion) of molecules is equivalent to the addition (or depletion) of a surface element δS^* from one monolayer. We have shown that, because of the coupling between the two elastic monolayers, the actual surface element incorporated (or subtracted) is $\delta S = 1/2\ \delta S^*$ [7], so that

$$\alpha = \frac{\delta S^*}{2S}. \qquad (2)$$

The transfer of a surface element from one monolayer to the other can be decomposed into two steps: (1) its depletion from one monolayer, which builds up a tension τ; (2) its insertion into the opposite monolayer, creating an opposite tension which cancels out the tension created by (1). However if the membrane has an average local radius of curvature R_0, the relative surface area taken from one side is different from the relative surface area added to the other side. It has been shown [6] that the areal strain α is related to the lipid asymmetry by the following relation

$$\alpha \cong \frac{h}{R_0}\,\frac{\Delta N}{N_0}. \qquad (3)$$

In this equation h/R_0 appears also as a scaling factor. For giant liposomes, $h/R_0 \cong 4 \times 10^{-3}$ and $\alpha \cong 5 \times 10^{-6}$, a value too small to inhibit the thermal undulation [4]. On the other hand for large unilamellar vesicles, a lipid translocation of a few per cent generates a tension sufficient to block the undulations.

The Experimental section gives a few examples of inhibition of thermal undulation accompanied by vesicle aggregation.

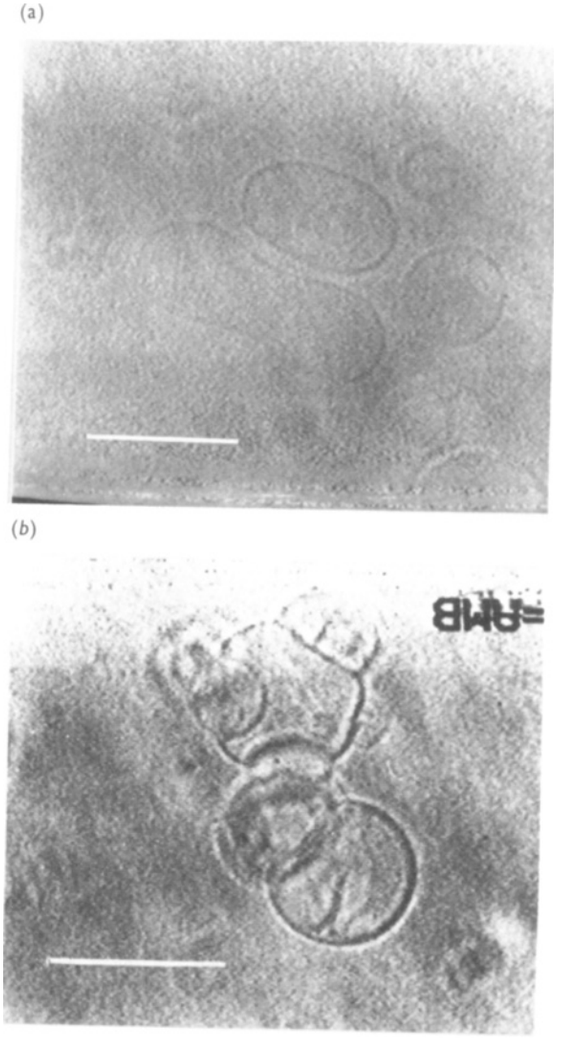
Experimental section

Giant liposomes of egg phosphatidylcholine (EPC) with a diameter in the range 10–50 μm were obtained by two different procedures. The first procedure was described in [8] and involves incubating a lipid film overnight in water, at 45°C, under an argon atmosphere. This technique gives an inhomogeneous population of liposomes, some of which are quasi-spherical, others elongated with a

Figure 1

Suspension of EPC liposomes seen by phase contrast microscopy with an inverted optical microscope (Leitz)

The bars correspond to 20 μm. (*a*) Before addition of BSA; (*b*) after addition of BSA.



discoid shape. The unilamellarity can be verified by the micropipette technique of Kwok and Evans [9]. In the second procedure, the lipid film is submitted to very low frequency electric field for approx. 2 h, using the technique described by Angelova et al. [10]. The latter technique generates unilamellar spherical liposomes with a rather homogeneous size distribution. The liposomes were used either immediately or after several days of incubation at 4°C. This storage period was accompanied by partial lipid degradation with formation of lysophosphatidylcholine and with some lipid peroxidation. Addition of fatty acid-free bovine serum albumin (BSA) had no effect on freshly-prepared liposomes, but generated important modifications in the 'aged' liposomes, as indicated below.

Discoid liposomes

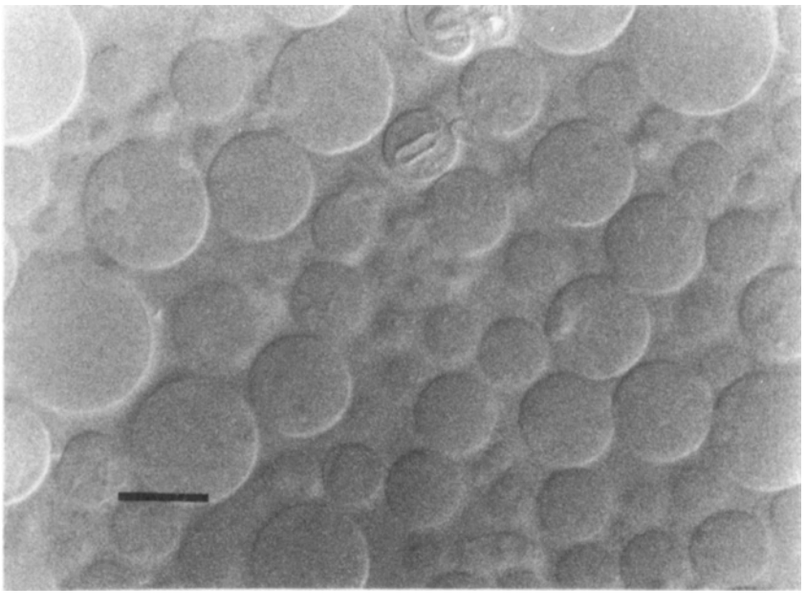
We will first review briefly previous experiments from this laboratory [8]. Lysophosphatidylcholine was injected through a micropipette in the vicinity of a non-spherical giant liposome. This liposome underwent a sequence of shape change from discoid to two connected spheres (see Figure 5 (a)–(c) in [8]), followed rapidly by the adhesion of the two spheres over a large fraction of their surface. The same result was obtained recently with the charged molecule lysophosphatidylglycerol (data not shown). We interpret these observations by assuming that the addition of the lyso-compound with a micropipette, which injects a large quantity of molecules onto the outer monolayer of the liposome, creates a surface tension large enough to block the undulations. The two vesicles composing the giant liposome then attract each other by van der Waals forces and adhere, blocking any further evolution. By contrast lipid reorientation by the pH gradient technique of Hope et al. [11], which we carried out with EPC liposomes containing 1% egg phosphatidylglycerol, gave rise to complicated shapes with many vesicles, but with no adhesion of vesicles as seen in Figure 2 of reference [8]. In the latter case, the fraction of lipid which is reoriented is too small to induce a significant surface tension.

Figure 1*a* shows several giant liposomes with a discoid shape. These liposomes were stored for several days and then BSA was injected through a micropipette in the vicinity of these liposomes. The same liposomes, are shown in Fig. 1*b* and are now aggregated. BSA, by depleting the outer monolayer of lyso-compounds and oxidized lipids, not only triggers shape changes [8], but also, by inducing lipid asymmetry, generates a surface tension that

Figure 2

Spherical unilamellar liposomes obtained by submitting a lipid film of EPC to a low-frequency electric field for 2 h

The bar corresponds to 20 μm. Observation was with a Normarsky contrast interdifferential (Zeiss inverted microscope).



blocks the thermal undulations. Note that the vesicles become 'fusion competent', but do not fuse.

Spherical liposomes

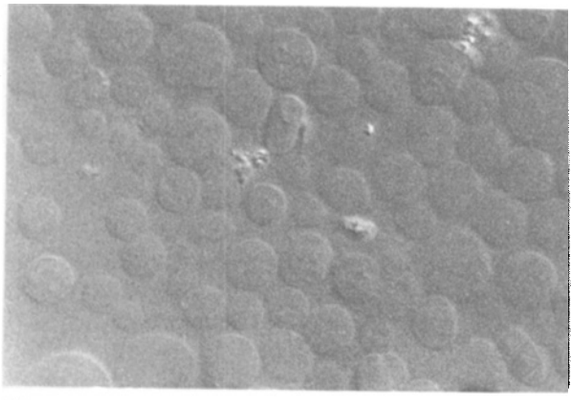
Figure 2 shows a populaton of spherical EPC liposomes obtained by the electric-field technique (the second procedure described previously). It is not known why this technique generates unilamellar liposomes that are so perfectly spherical. BSA added to these liposomes when freshly-prepared had no effect. After several days, the undulations became clearly visible as indicated by the less spherical pattern of the vesicles in Figure 3*a*. At this stage, BSA provokes aggregation of the vesicles to form typical honeycomb regions (Figure 3*b*). Again the most probable interpretation is that a fraction of the lipids is degraded during the storage, so that BSA extracts degraded lipids from the outer monolayer selectively and creates an imbalance between the inner and outer monolayers.

Figure 3

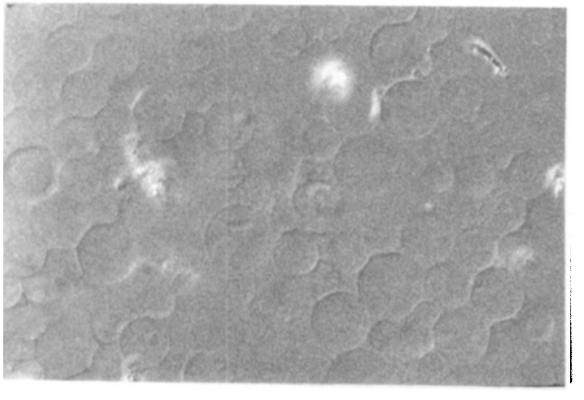
The same population of liposomes as in Figure 2, after 5 days storage (*a*) before and (*b*) after addition of BSA

Observation was with a Normarsky contrast interdifferential (Zeiss inverted microscope)

(*a*)



(*b*)



Discussion

The results presented above show that the selective addition or removal of lipids from one monolayer can be accompanied, not only by shape changes, but also by vesicle adhesion. In fact in the case of spherical vesicles the shape changes are rarely observable. In the experiments reported here, only lipid insertion or lipid depletion efficiently created a tension. The redistribution of lipids from one monolayer to the other did not cause adhesion. We have explained in the Theory section why lipid reorientation in giant liposomes with a typical diameter of 10–30 μm should not be expected to lead to adhesion. Indeed equation (3) reveals that a scaling factor is involved, so that the redistribution of phospholipids necessary to allow membrane adhesion varies with the size of the vesicle.

With vesicles the size of a cell (several μm), phospholipid redistribution driven, for example, by a lipid pump such as the aminophospholipid translocase [12] triggers essentially shape changes. However with organelles the size of endosomes or granules (about 100 nm) the aminophospholipid translocase is more likely to induce or maintain a surface tension than to trigger shape changes. In turn, surface tension will inhibit surface undulations making these small vesicles fusion competent.

The work described here was supported by grants from the Centre National de la Recherche Scientifique (UA526), the Institut National de la Santé et de la Recherche Medicale (CRE 90 0104), the Université Paris VII and the Direction des Recherches, Etudes et Techniques of the Délégation Générale pour l'Armement (91.079); L.M. holds a fellowship from the Délégation Générale pour l'Armement.

1. Rand, R. P. and Parsegian, V. A. (1989) Biochim. Biophys. Acta **988**, 351–376
2. Herrmann, A., Clague, M. J., Puri, A., Morris, S. J., Blumenthal, R. and Grimaldi, S. (1990) Biochemistry **29**, 4054–4058
3. Helfrich, W. and Servuss, R.-M. (1984) Nuovo Cimento Soc. Ital. Fis. D: **3**, 137–151
4. Evans, E. and Rawicz, W. (1990) Phys. Rev. Lett. **64**, 2094–2097
5. Servuss, R.-M. and Helfrich, W. (1989) J. Phys. (Paris) **50**, 809–827
6. Farge, E. and Devaux, P. F. (1993) J. Phys. Chem., in the press

7. Farge, E., Bitbol, M. and Devaux, P. F. (1990) Eur. Biophys. J. **19**, 69–72
8. Farge, E. and Devaux, P. F. (1992) Biophys. J. **61**, 347–357
9. Kwock, R. and Evans, E. (1981) Biophys. J. **35**, 637–652
10. Angelova, M. I., Soléau, S., Méléard, P., Faucon, J. F. and Bothorel, P. (1993) Prog. Colloid Polym. Sci., in the press
11. Hope, M. J., Redelmeier, T. E., Wong, K. F., Rodrigueza, W. and Cullis, P. R. (1989) Biochemistry **28**, 4181–4187
12. Devaux, P. F. (1991) Biochemistry **30**, 1163–1173

Received 16 December 1992

Loss of phospholipid asymmetry in cell fusion

Jack A. Lucy
Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London, NW3 2PF, U.K.

Hydrated bilayers of phospholipid normally repel each other strongly, as a consequence of hydration repulsion arising from solvation of the polar head groups. When Ca^{2+} ions are added to phosphatidylserine, however, the surface water that gives rise to the repulsive forces is completely displaced by the bound Ca^{2+} and the phospholipid bilayers achieve molecular contact [1, 2]. Ca^{2+} ions are much more effective than other cations (for example, Mg^{2+}) in inducing membrane fusion in model membrane systems based on phosphatidylserine [3] because the corresponding complex of phosphatidylserine with Mg^{2+} does not lose its layer of hydration [4].

In common with most cell types, erythrocytes do not exhibit spontaneous cell-fusion reactions. This would seem to be consistent with the topological distribution of phosphatidylserine in their plasma membranes, since the phospholipids in the outer leaflet of the human-erythrocyte plasma membrane are primarily phosphatidylcholine and sphingomyelin and the inner leaflet contains the acidic phospholipids [5]. It is therefore important to know if there is a requirement for phosphatidylserine to be on the outside surfaces of cells for cell fusion to occur and, in my laboratory, the human erythrocyte has been employed in work on this question. We have investigated whether changes in phospholipid asymmetry occur in human erythrocytes that are induced to fuse artifically by two different protocols: (1) osmotic swelling in the presence of Ca^{2+} and (2) exposure of the cells to electrical-breakdown pulses in the presence of several different divalent cations.

Abbreviation used: PEG, poly(ethylene glycol).

Exposure of phosphatidylserine is associated with osmotically-induced cell fusion

In our initial work we investigated the fusion of human erythrocytes that occurs when monolayers of the cells are induced to swell in the presence of 10 mM Ca^{2+} by the entry of small molecules such as malonamide or small poly(ethylene glycol)s, for example, PEG 400. Under these conditions a proportion of the cells fuse before they lyse, whereas in the absence of Ca^{2+}, lysis occurs without any cell fusion [6]. Exposure of phosphatidylserine on the outside of platelets [7, 8] and sickled erythrocytes [9] yields procoagulant surfaces that facilitate the conversion of prothrombin into thrombin. The catalytic potential of such surfaces has been considered to provide a semi-quantitative measure of the exposed phosphatidylserine [7, 8]. Using this assay we found that the cell fusion which accompanied cell swelling induced by the entry of small permeant molecules in the presence of Ca^{2+} was associated with the exposure of phosphatidylserine in the outer leaflet of the plasma membrane of erythrocytes [10].

It has been observed now that the fusion of monolayers of human erythrocytes which accompanies cell swelling induced by the entry of malonamide or of PEG 300 is preceded by hemi-fusion (that is, membrane fusion without cytoplasmic fusion). This was demonstrated by the rapid movement of a fluorescent membrane probe (carbocyanine) from unlabelled to labelled cells [11]. Unlike complete cell fusion, hemi-fusion occurred both in the presence and in the absence of added Ca^{2+}, but was inhibited by the addition of 10 mM EGTA. We also found that hemi-fusion (and hence complete fusion) in these experiments was prevented strikingly by pre-incubation of the cell