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Intermediary structures during membrane fusion as observed by cryo-electron microscopy

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Lipidic phases, containing 'lipidic particles' (dioleoylphosphatidylethanolamine/cholesterol/dioleoylphosphatidylcholine and cardiolipin/dimyristoylphosphatidylcholine in the presence of Ca^{2+}) have been investigated by preparing thin films from a suspension of sonicated vesicles. These thin films were vitrified and observed 'directly' by cryo-electron microscopy in their hydrated form. The thin films show various fusion products and fusion intermediates such as lipidic particles.

Freeze-fracture electron microscopy has revealed intermediate fusion structures in artificial membrane systems. Lipidic particles are found at focal fusion points in lipid mixtures where part of the lipids prefer the transition of the liquid-crystalline lamellar phase (L_α) into the inverted hexagonal (H_{II}) phase (Refs. 1 and 2, for review, see Ref. 3). Based on the morphology of these lipidic particles one can distinguish three types [3]; particles which reflect attachment, particles which reflect joining (inverted micelles) and particles which reflect fission, the formation of an aqueous channel between two compartments that were previously separated by their limiting membranes. A lipid mixture consisting of multilamellar liposomes and exhibiting multiple point fusion will show an isotropic spectral component with ^{31}P-NMR [4] because the lipids can exhibit rapid motional averaging on the time scale of NMR within the 'honeycomb' structure. The existence of such honeycomb structures has been confirmed by freeze substitution of rapidly frozen samples [5]. Elegant work of Siegel et al. (for review, see Ref. 6) confirmed the fusion scheme deduced from freeze-fracture studies. From their analysis of the kinetics of fusion they conclude that the 'inverted micellar intermediates' (IMI) have a very short-life-time compared to the 'interlamellar attachments' (ILA), which reflect the stage of fission, the formation of an aqueous channel subsequent to the fusion of the lipid membranes.

The ultrastructural study of fusion events between lipid membranes is largely based on freeze-fracture replication. The structural resolution of this approach is limited by the grain-size of the heavy metal used for shadowing and the nature of the fracturing process (for review, Ref. 7).

Recently the study of hydrated objects in thin-films by cryo-electron microscopy has made a great stride foreward due to the work of Dubochet et al. [8,9]. The vitrification of thin films allows the direct observation of sonicated phospholipids in their hydrated form [10,11]. The vitrification of thin films in liquid ethane takes 10^{-5} s or less [12] and is therefore potentially fast enough to preserve intermediate structures involved in membrane fusion. The advantage of cryo-electron microscopy is the 'through vision'; in a thin film (< 100 nm) the entire contents are visualized in its projection.

We therefore investigated thin films by cryo-electron microscopy from some phospholipid systems which are well documented for their fusion behaviour with freeze-fracturing [1–3]. The systems chosen were a mixture of dioleoylphosphatidylethanolamine (DOPE) with cholesterol and dioleoylphosphatidylcholine (DOPC) in a molar ratio of 3:2:1 in which fusion is triggered by a rise in temperature and a cardiolipin/phosphatidylcholine (PC) mixture in a molar ratio of 1:1 in which fusion is triggered by the addition of Ca^{2+} ions.

Both mixtures exhibit a complex phase behaviour (cardiolipin/PC [13]: DOPE/cholesterol/DOPC [14])

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in which lamellar systems with lipidic particles and a honeycomb phase or a cubic phase can partly coexist in the course of an experiment. The phospholipids used were obtained from Sigma (DOPE, P0510; DOPC, P1013; cardiolipin, bovine heart C 1649; DMPC, dimyristoylphosphatidylcholine, P0888) whereas cholesterol was obtained from Baker. The mixtures of DOPE/cholesterol/DOPC, respectively, cardiolipin/DMPC

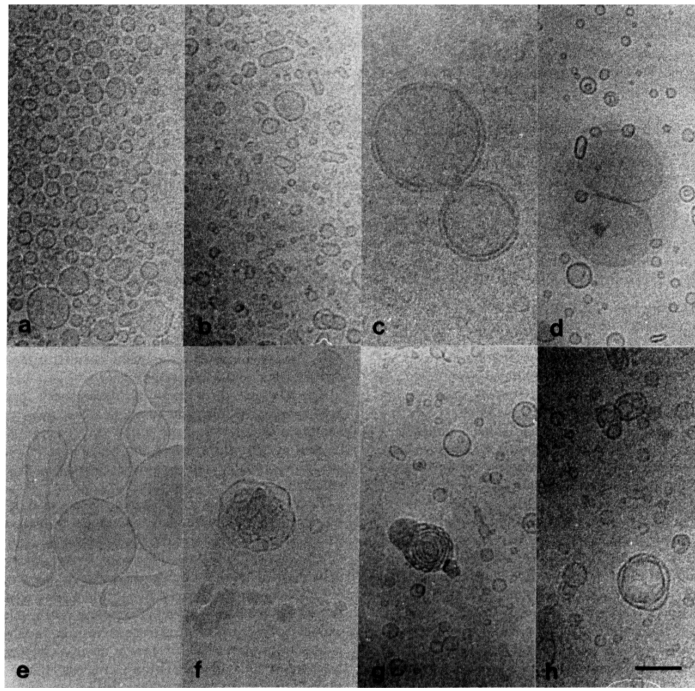

Fig. 1. Cryo-electron microscopy of vitrified thin films prepared from cardiolipin/DMPC (a, b, c, d, g, h) and DOPE/cholesterol/DOPC (e, f). Thin aqueous films were prepared on specimen grids (without supporting film), vitrified in ethane and observed at 90 K (see text for details). (a) Sonicated vesicles (cardiolipin/DMPC, without Ca^{2+}) in a thin film. The rim of the film (right) is devoid of vesicles, whereas unilamellar vesicles are found in a thicker part of the film. Occasionally vesicles are found with two concentric bilayers. Sonicated vesicles prepared by DOPE/cholesterol/DOPC at 0 °C have the same appearance in a thin film (data not shown). (b) Same preparation as (a) showing rod shaped vesicles. This shape might have been induced during sonication. (c) Cardiolipin/DMPC film prepared in the presence of 2 mM Ca^{2+} (Ca^{2+} concentration may rise during film formation, see text). Attachment between vesicles with concentric membranes. Note the discontinuity in the concentric arrangement. (d) Cardiolipin/DMPC film prepared in the presence of 4 mM Ca^{2+}. Adhesion between vesicles is apparent giving the impression of particles at the attachment site. (e) DOPE/cholesterol/DOPC film prepared from a suspension of sonicated vesicles. During formation of the thin film the temperature was allowed to rise (0–4 °C of the bulk suspension, room temperature around the film). Various forms of attachment can be observed between vesicles. The size of the vesicles indicates that they are the result of several ('leaky' or 'non-leaky') fusion rounds. (f) Same preparation as (e), 'honeycomb' structure. (g) Cardiolipin/DMPC film prepared in the presence of 3 mM Ca^{2+}. Multiple contacts between concentric membranes are present giving an almost honeycomb appearance. (h) Cardiolipin/DMPC film prepared in the presence of 2 mM Ca^{2+}. Note the contact between the concentric membranes. Bar represents 100 nm.

were prepared after evaporating the solvents of the stock solutions under a stream of dry nitrogen, redissolving the mixture in methanol, which was again evaporated under dry nitrogen and finally adding Tris-HCl buffer (10 mM (pH 7.4), 150 mM NaCl) to obtain a final concentration of lipids of 12 mM. The suspensions were sonicated at intermittent intervals (at 0°C for DOPE/cholesterol/DOPC) until a 'clear' suspension was obtained. A thin aqueous film was formed by dipping and withdrawing a specimen grid (700 mesh hexagonal, without supporting film), from the suspension. The grid was blotted with filter paper and in the grid the thin film forms as a 'soap film' between the grid bars. These films have the tendency to thin spontaneously as the monolayers formed at the air/water interface come to attract each other [11]. Thin films are vitrified within one second after their formation by plunging the grid into ethane cooled to its melting point by liquid N_2 [8]. The vitrified specimens are mounted at 77 K in the cryoholder (Philips PW 6599/00, line resolution 0.9 nm at 90 K) and transferred to the microscope (Philips CM 12). During transfer and observation the cryoholder remained cold (below 90 K) and precautions were taken to prevent contamination of the specimens [15].

In control experiments, e.g. DOPE/cholesterol/DOPC at 0–4°C, cardiolipin/DMPC in the absence of Ca^{2+}, small unilamellar vesicles were observed in their vitrified form by cryo-electron microscopy at 90 K (Fig. 1a). The majority of these vesicles has a diameter between 20 and 30 nm and larger vesicles as well as double layered vesicles are occasionally found. During thinning of the film the vesicles may become concentrated in 'thicker' parts of the thin film whereas a thin rim remains devoid of vesicles as a result of the zippering effect of the approaching monolayers at the air/water interface during the formation of the thin film [11]. Fusion was induced by either raising the temperature (DOPE/cholesterol/DOPC) or by adding Ca^{2+} (to a final concentration 2–4 mM in the cardiolipin/DMPC mixture). In both systems a variety of membrane structures is visible (Fig. 1). Fusion is evident because the average vesicle diameter increases in size. On basis of the morphology it is not possible to decide whether the large vesicles are the result of a 'clean' fusion event (mixing of lipids and of the aqueous contents of vesicles without leakage) or the result of a form of 'leaky-fusion'. Intermediary fusion features are visible between two vesicles but also between concentric bilayers in multilamellar vesicles. Even multiple point fusion products are apparent (Figs. 1 f–g) which are most likely the so-called "honeycomb" structures which give rise to isotropic signals by NMR. With regard to the fusion intermediates several focal contact points can be observed where the membranes come in close contact (Figs. 1c–g). At high magnification (stereo-pair, Fig. 2) the contact or fusion point becomes obvious as a particle having a diameter of approx. 10 nm. Similar diameters have been reported for lipidic particles when this lipid system was studied by freeze-fracturing [3]. The cryo-electron microscopic images of vitrified films prepared from phospholipid suspensions during fusion between vesicles show a bewildering variety of membrane structures. More complex structures, e.g., aggregates of large vesicles, tubular structures, are not shown because these structures are most probably the result of later events in the course of an $L_\alpha \rightarrow H_{II}$ phase transition (Frederik, Stuart, Verkleij, manuscript in preparation). The observation of early fusion products; the lipidic particles [3] or inverted micellar intermediates

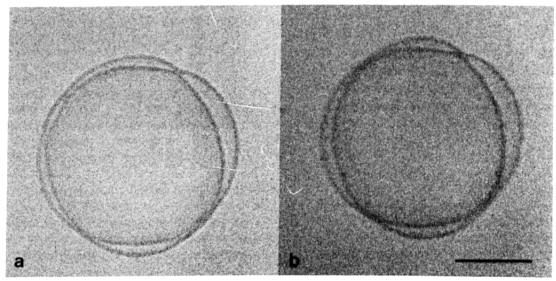

Fig. 2. Cryo-electron microscopy of a vitrified thin film prepared from a DOPE/cholesterol/DOPC suspension of sonicated vesicles. During formation of the thin film the temperature was allowed to rise (0–4°C of the bulk suspension, room temperature around the thin film). The stereo-pair (+6° and −6° tilt) shows the contact between two concentric membranes and gives the strong impression of a round particle (approx. 10 nm diameter) at the contact area.

[6], in the membrane systems thusfar investigated by cryo-electron microscopy remains restricted to a few examples. This may be due to the short life-time of these structure especially in the fusion of small unilamellar vesicles (see Ref. 6 for discussion). The stereo-pair of the DOPE/cholesterol/DOPC system is, however, strongly suggestive for the presence of a particle at the contact area. The presence of a transmembrane channel, an open connection between interior and exterior of a vesicle can as yet not be identified with certainty in the cryo-electron microscopic images presented in this report. We tend to assume that the lipidic particle shown in the stereo-pair (Fig. 2) is a closed structure whereas an open channel might be present in some of the other images (e.g., Fig. 1h).

In this paper we have restricted ourselves to the description of the initial stages of fusion between artificial membranes. The study of the initial stages in membrane fusion requires a high resolution, both spatial and on a time scale. Vitrification of aqueous solutions is a fast process ($< 10^{-5}$ s) and enables the visualization of transient structures with a short life-time. The thin film in which the membrane structures are captured enables a through-vision by cryo-electron microscopy. The thin film and the dynamics of its formation also set restrictions to high resolution ultrastructural studies. Membrane vesicles must fit in the thickness of the film, a thickness which must be less than 50 nm when optimum resolution is required although films of 150 nm can still be vitrified. The resolution in the micrographs depends largely on phase contrast which is a function of the defocus setting of the objective lens (see, for example, Ref. 9). We used a setting of 3–6 μm under focus in our micrographs to record structures with spacings between 3 and 10 nm with optimum contrast. During formation of a thin film ('life-time' one second between blotting and vitrification) the air/water interfaces will attract each other and give the film a biconcave shape. This results in the sorting by size of material within the film and thinning may also result in a decreased water content in thin areas of the film [11]. In the case of the cardiolipin/DMPC systems it can be argued that the calcium concentration has increased considerably in thin parts of the film (concomitant with the phospholipid concentration which rises with a factor of more than 60 [11]). During formation of thin films sorting of material by size and concentrating of solutes will be established along a gradient; going from the thin central part to the thicker peripheral part is going from a concentrated solution with no or small particles to a dilute solution with larger particles. These restrictions of thin film formation have to be taken into consideration when thin films are used for cryo-electron microscopy, an approach which seems to be ideally suited for the study of dynamic events such as membrane fusion on basis of lipid–lipid or lipid–protein interactions.

References

1 Verkleij, A.J., Mombers, C., Gerritsen, W.J., Leunissen-Bijvelt, L. and Cullis, P.R. (1979) Biochim. Biophys. Acta 555, 358–361.
2 Verkleij, A.J., Van Echteld, C.J.A., Gerritsen, W.J., Cullis, P.R. and De Kruijff, B. (1980) Biochim. Biophys. Acta 600, 620–624.
3 Verkleij, A.J. (1981) Biochim. Biophys. Acta 779, 43–63.
4 Cullis, P.R., De Kruijff, B., Hope, M., Verkleij, A.J., Nayar, R., Farren, S.B., Tilcock, C., Madden, T.D. and Bally, M.B. (1984) in Membrane Fluidity in Biology (Aloia, V., ed.), Chapter 2, Vol. 1, pp. 39–81, Academic Press, New York.
5 Verkleij, A.J., Humbel, B., Studer, D. and Müller, M. (1985) Biochim. Biophys. Acta 812, 591–594.
6 Siegel, D.P. (1987) in Cell Fusion (Sowers, A.E., ed.), Chapter 9, pp. 181–207, Plenum Press, New York-London.
7 Zingsheim, H.P. (1972) Biochim. Biophys. Acta 265, 339–366.
8 Dubochet, J., Lepault, J., Freeman, R., Berriman, J.A. and Homo, J.-C. (1982) J. Microsc. (Oxford), 128, 219–237.
9 Dubochet, J., Adrian, M., Chang, J.-J., Lepault, J. and McDowall, A.W. (1987) in Cryotechniques in Biological Electron Microscopy (Steinbrecht, R.A. and Zierold, K., eds.), Chapter 5, pp. 114–131, Springer Verlag, Berlin-Heidelberg.
10 Lepault, J., Pattus, F. and Martin, N. (1985) Biochim. Biophys. Acta 820, 315–318.
11 Frederik, P.M., Stuart, M.C.A., Bomans, P.H.H. and Busing, W.M. (1989) J. Microsc., in press.
12 Bachmann, L. and Mayer, E. (1987) in Cryotechniques in Biological Electron Microscopy (Steinbrecht, R.A. and Zierold, K., eds.), Chapter 1, pp. 3–34, Springer Verlag, Berlin-Heidelberg.
13 De Kruijff, B., Verkleij, A.J., Leunissen-Bijvelt, J., Van Echteld, C.J.A., Mile, J. and Rijnbout, H. (1982) Biochim. Biophys. Acta 693, 1–12.
14 Cullis, P.R., Van Dijck, P.W.M., De Kruijff, B. and De Gier, J. (1978) Biochim. Biophys. Acta 513, 21–30.
15 Frederik, P.M. and Busing, W.M. (1986) J. Microsc. 144, 215–221.