Biochimica et Biophysica Acta 820 (1985) 315–318
Elsevier

BBA 70249

BBA Report

Cryo-electron microscopy of artificial biological membranes

Jean Lepault, Franc Pattus and Nadine Martin

European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg (F.R.G.)

(Received July 9th, 1985)

Key words: Membrane structure; Liposome; Electron microscopy; Cryo-electron microscopy; Electron diffraction

Vitrified synthetic phosphatidylcholine liposome suspensions were studied by cryo-electron microscopy. The bilayer structure is resolved on vitrified liposome images. The packing of the aliphatic chains of the lipid within vitrified liposomes can be determined by the analysis of electron diffraction patterns. Images and electron diffraction patterns show that the structure of vitrified liposomes is related to the structure that liposomes have before vitrification. In fact, vitrified liposomes have a different structure, depending whether they are maintained before cooling at a temperature higher or lower than that corresponding to the 'melting' of the hydrocarbon chain of the lipids. Below the melting temperature, liposomes are formed by small domains.

The structure and the polymorphism of lipid-water phases have been extensively studied by X-ray diffraction [1–3] and by freeze-fracturing electron microscopy [4]. This latter technique is, however, limited in resolution and by freezing-induced perturbations [4]. Recently it has been shown that biological object suspensions can be imaged by cryo-electron microscopy [5,6]. The technique involved the following operations: (i) forming a thin layer of suspension; (ii) cooling it into a vitreous state; (iii) transferring it into the electron microscope without rewarming above the devitrification temperature ($T_d \simeq 140$ K) and (iv) observing it below T_d and with an electron dose low enough to preserve the structure of the specimen. Cryo-electron microscopy of vitrified viruses [6,7] and membrane proteins [8] offers possibilities for high-resolution observation that compare favourably with any other electron microscopical method. To assess the advantages of cryo-electron microscopy for the study of biological membranes we investigated vitrified synthetic phosphatidylcholine liposome suspensions.

Vitrified synthetic phosphatidylcholine liposome images are characterized by two concentric lines separated by approx. 40 Å (Fig. 1). The tri-lamellar structure of frozen membranes has already been reported [9]. It is reasonable to interpret the two high density domains as arising from the polar heads of the lipid constituting the bilayer. Depending upon the temperature at which the liposome suspension is maintained before vitrification, the overall structure of the bilayer is different. Fig. 1a shows vitrified 1,2-dimirystoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) vesicles which were maintained at 40°C before vitrification. They do not show any spherical symmetry. However, the separation between the polar head domains of the bilayer is constant all around them. In contrast, vitrified DMPC vesicles maintained at 4°C before vitrification have a spherical shape and the separation between the polar head domains of the bilayer varies along the periphery of the vesicle (Fig. 1b). Phospholipids undergo a temperature-dependent, reversible, order-disorder transition associated with the 'melting' of the hydrocarbon chain region of the lipid bilayer. This transition depends upon the fatty acid chain length and the

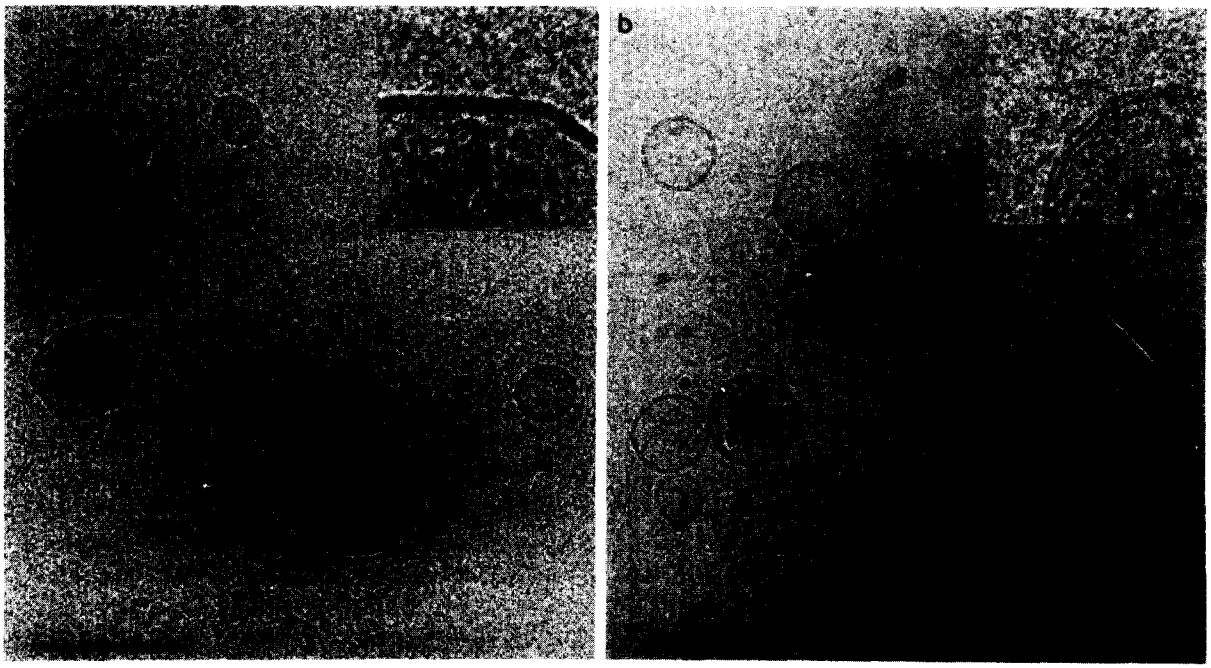

Fig. 1. DMPC liposomes embedded in vitreous ice. The phospholipid vesicles were prepared as follows. A suspension of phospholipid (1 to 10 mg/ml) in 10 mM Tris-acetate buffer (pH 7.4), 0.1 M NaCl, was sonicated and then mixed with some neutral detergent polydisperse octyloligooxyethylene (octyl POE) (1 to 3% final concentration) until optical clarity. The solution was then extensively dialysed against the detergent-free buffer at a temperature at least 10 K higher than the melting temperature of the phospholipid. Vitrified liposome suspensions (lipid concentration approx. 5 mg/ml) were prepared as described previously [6]. A drop of the suspension was deposited on a glow-discharged grid coated with a perforated carbon film. The grid was mounted on a guillotine-like frame and the suspension excess blotted with a filter paper. Then the frame was released and the grid plunged into liquid nitrogen cooled liquid propane. The grid was transferred from liquid propane to the chamber of the Reichert FC4 cryomicrotome in liquid nitrogen. The cold nitrogen gas flow inside the microtome chamber prevents ice contamination of the specimen during the transfer. In the cryomicrotome cooled at its lowest temperature (about −160°C) the grid was mounted in the specimen holder and transferred into a Zeiss EM 10A microscope equipped with a low temperature stage via the transfer device as recommended by the manufacturers. (a) The DMPC liposome suspension was maintained at a temperature (40°C) higher than the lipid melting temperature (24°C). (b) The suspension was maintained before vitrification at a temperature (4°c) lower than the lipid melting temperature. The bar in (a) and (b) represents 2000 Å.

extent of saturation, the degree of hydration, and the nature of the polar head. For DMPC liposomes the transition temperature is 24°C [10]. Therefore, the difference between the liposome structure shown in Figs. 1a and 1b might be attributed to the difference between the liposome structure above and below the hydrocarbon chain melting temperature. The liposomes shown in Fig. 1b are multi-faceted; they are constituted by small domains. The size of these domains varies from approx. 100 to 300 Å. The maximal separation between the polar head domains of the bilayer is equal to 40 ± 5 Å. An identical value is found when liposomes are maintained above their melting temperature (insert Fig. 1a). Liposomes of 1,2-dilauroyl-*sn*-glycero-3-phosphatidylcholine (DLPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) have melting temperatures of 0, 41 and −22°C, respectively [10,11]. Vitrified liposomes composed of DLPC, DPPC and DOPC and maintained at 20°C before freezing, have images (not shown) similar to those shown in Figs. 1a, 1b and 1a, respectively. Therefore, vitrified liposomes have a characteristic appearance according to whether they are main-

tained before freezing at a temperature above or below their melting temperature. However, in all cases, the maximal separation between the polar heads of the bilayer is measured to be 40 ± 5 Å. Despite differences in hydrocarbon chain length and conformation, liposomes display a constant separation between the polar head domains of the bilayer. Two reasons explain this observation. Firstly an electron micrograph represents a projection of the object. The positions of the polar head domains arising from 'spherical' vesicles are spread towards the center of the vesicle. Secondly, unstained vitrified biological objects have to be visualized with optimum phase contrast [7,12]. This requires underfocussing [13] which also results in the spreading of the image of polar head domains. The separation between the high density lines is then related but not equal to the bilayer thickness.

To compare our results to those obtained by X-ray diffraction, we studied vitrified concentrated liposome suspensions by electron diffraction. The large angle X-ray diffraction pattern is characteristic of the hydrocarbon chain conformation [1,2]. The hydrocarbon chains have a liquid-like organization or α packing type above the 'melting' temperature [1]. The X-ray diffraction pattern is characterized by a broad band at $(4.6\ \text{Å})^{-1}$. Below the melting temperature, the chains are stiff, parallel and either oriented at right angles (β conformation type) or tilted with respect to the membrane (β' conformation type) [1]. Lipids in the β conformation have an X-ray diffraction pattern which consists of a sharp and intense ring at $(4.2\ \text{Å})^{-1}$ and two sharp rings at $(4.2\ \text{Å}/\sqrt{3})^{-1}$ and $(4.2\ \text{Å}/2)^{-1}$. X-ray diffraction patterns of lipids in the β' conformation consist of a sharp and strong ring at $(4.2\ \text{Å})^{-1}$ and a weak ring at approx. $(4.0\ \text{Å})^{-1}$.

Electron diffraction patterns of vitrified DMPC liposomes, maintained at 4°C before freezing, are characterized by a sharp ring and a strong ring at $(4.2\ \text{Å})^{-1}$ and a sharp weak ring at approx. $(3.6\ \text{Å})^{-1}$ (Fig. 2a). In this study, the electron diffraction patterns have been calibrated with the two broad rings arising from vitreous water (Fig. 2d). These rings are located at $(3.7\ \text{Å})^{-1}$ and $(2.1\ \text{Å})^{-1}$ [14]. Due to the width of these rings, errors up to 1% cannot be discounted. DMPC liposomes have their lipid hydrocarbon chains in a β' conformation at 4°C [3]; the hydrocarbon chains have a similar or identical conformation in the vitrified state. The same results are obtained with vitrified DPPC liposomes when they are maintained below their melting temperature before freezing. Therefore, vitrification does not affect the packing of the lipid hydrocarbon chains having a β' conformation.

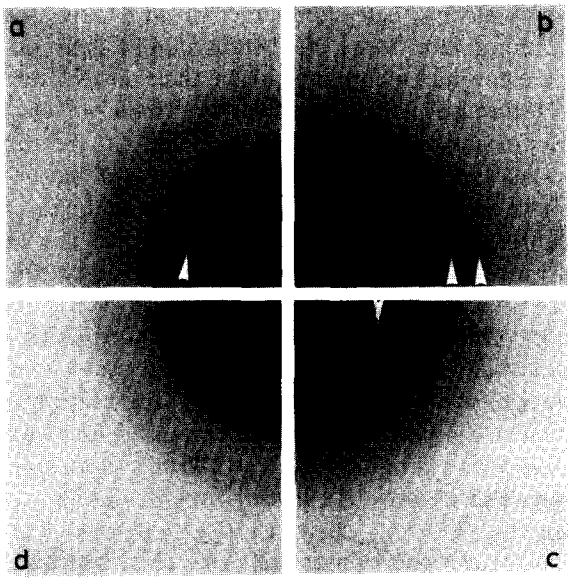

Fig. 2. Electron diffraction patterns of liposomes embedded in vitreous ice. The lipid concentration was about 20 mg/ml. (a) Vitrified DMPC liposome suspension maintained before vitrification at a temperature lower than the lipid melting temperature. The strong ring is located at $(4.2\ \text{Å})^{-1}$. The arrow indicates a weak ring at $(3.6\ \text{Å})^{-1}$. Note the absence of ring at $(4.2\ \text{Å}/\sqrt{3})^{-1}$. (b) Vitrified DMPC suspension maintained before vitrification at a temperature higher than the lipid melting temperature. The strong ring is located at $(4.2\ \text{Å})^{-1}$. The arrows indicate weak rings at $(4.2\ \text{Å}/\sqrt{3})^{-1}$ and $(4.2\ \text{Å}/2)^{-1}$. (c) Vitrified DOPC liposomes maintained before vitrification above their melting temperature. The head peak indicated by an arrow is located at approx. $(4.4\ \text{Å})^{-1}$. (d) Vitrified water. The diffraction pattern of vitreous water is visible on the previous pattern and is characterized by a broad ring at $(3.7\ \text{Å})^{-1}$ and $(2.1\ \text{Å})^{-1}$.

Vitrified DMPC liposomes maintained above their melting temperature (40°C) before freezing have an electron diffraction pattern which is characterized by a sharp and intense ring at $(4.2\ \text{Å})^{-1}$ and two weak rings at $(4.2\ \text{Å}/\sqrt{3})^{-1}$ and $(4.2\ \text{Å}/2)^{-1}$ (Fig. 2b). The same results have been observed with DLPC. This pattern is typical for

lipids having their aliphatic chain in a β conformation. Since X-ray diffraction studies show that the liposomes maintained above their melting temperature have their hydrocarbon chains in an α conformation [3], we must conclude that freezing induces a rearrangement of the lipid hydrocarbon chain from a liquid-like α organization to the β conformation. It could be argued that the rearrangement is due to some artifacts arising during the preparation of a thin suspension layer. We did not consider this hypothesis because X-ray diffraction data of frozen lipid-water model systems are in agreement with our results [4]. The rearrangement is minimized when the carbon chain is unsaturated. For example, DOPC liposomes maintained above the melting temperature before freezing, have an electron diffraction which consists of a broad ring at $(4.4\ \text{Å})^{-1}$ (Fig. 2c). Lipid hydrocarbon chains having a liquid-like organization rearrange into a more ordered organization during vitrification. The double bond present in the hydrocarbon chain of DOPC introduces an asymmetry which minimizes this ordering during vitrification.

Liposome structures above and below their melting temperature can be differentiated by cryo-electron microscopy. Liposomes maintained below their melting temperature are similar, as far as the hydrocarbon chain packing is concerned, in the vitrified and the liquid state. Images such as Fig. 1b can be considered as 'true images' of a lipid bilayer maintained below its melting temperature. From these images, it can be concluded that liposomes below their melting temperature are faceted. X-ray diffraction studies only provided support for this conclusion [3]. This study, then, illustrates the possible complementarity between X-ray diffraction and electron microscopy. Liposomes having their lipid hydrocarbon chain in a liquid-like organization undergo a rearrangement during vitrification. Structural information concerning such liposomes cannot be directly extrapolated from the vitrified to the liquid state. However, as hydrocarbon chains in an α conformation do not undergo an α-β' transition during vitrification, vitrified liposomes can be considered as being in structural continuity with the aqueous liquid state but at a lower temperature. These results show that the study of the vitrified state is a reasonable model for the study of membranes in their native aqueous environment.

The close relationship of liposome structure in the liquid and vitrified states makes cryo-electron microscopy a powerful technique to investigate biological membranes. Such investigations can be performed not only on systems at equilibrium, but also on dynamic systems. In fact, it is reasonable to hope that by controlling the time between the perturbation of a system and its vitrification it will be possible to study kinetic structural rearrangements by cryo-electron microscopy.

We are grateful to Drs. A. Tardieu, T. Gulik-Krzywicki, J. Tessie and J.-F. Tocanne for helpful discussions during the course of this study.

References

1 Tardieu, A., Luzzati, V. and Roman, F.C. (1973) J. Mol. Biol. 75, 711–733
2 Janiak, M.J., Small, D.M. and Shipley, G.G. (1979) J. Biol. Chem. 254, 6068–6078
3 Blaurock, A.E. and Gamble, R.C. (1979) J. Membrane Biol. 50, 187–204
4 Gulik-Krzywicki, T. and Costello, M.J. (1978) J. Microsc. 112, 103–113
5 Lepault, J., Booy, F.P. and Dubochet, J. (1983) J. Microsc. 129, 89–102
6 Adrian, M., Dubochet, J., Lepault, J. and McDowall, A.W. (1984) Nature 308, 32–36
7 Lepault, J. and Leonard, K. (1985) J. Mol. Biol. 182, 431–411
8 Brisson, A. and Unwin, P.N.T. (1985) Nature 315, 474–477
9 Taylor, K.A., Grano, D.A. and Chiu, W. (1976) in Proceedings 34th Annual Meeting EMSA (Bailey, G.W., ed.), pp. 136–137, Claibor's Publishing Division, Baton Rouge
10 Chapman, D., Williams, R.M. and Ladbrooke, B.D. (1967) Chem. Phys. Lipids 1, 445–475
11 Lee, A.G. (1977) Biochim. Biophys. Acta 472, 237–281
12 Lepault, J. and Pitt, T. (1984) EMBO J. 3, 101–105
13 Erickson, H.P. and Klug, A. (1971) Phil. Trans. Roy. Soc. Lond. B261, 105–118
14 Dubochet, J., Lepault, J., Freeman, R., Berriman, J.A. and Homo, J.-Cl. (1982) J. Microsc. 128, 219–237