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Freeze-Fracture of Lipids and Model Membrane Systems

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ABSTRACT Model membrane systems are used extensively in all aspects of membrane research, and freeze-fracture is the preeminent procedure for directly visualizing local structure in these lipid dispersions. Here we describe in detail the formation of liposomes and how freeze-fracture is routinely employed as a complementary technique to biophysical and biochemical procedures in the characterization of multilamellar vesicles (most commonly known as liposomes) and unilamellar vesicles. Many preparative procedures exist for the formation of multi- and unilamellar vesicles. Examples of each system are given and their properties as well as freeze-fracture morphology are discussed. The detection of lipid-phase transitions is considered, in particular, with emphasis on the application of freeze-fracture to the study of lipid polymorphism. We briefly discuss the fracturing of apolar lipids which do not adopt bilayer structures but which can be stabilized into microemulsions by a phospholipid monolayer. Finally, a critical assessment is made of filipin as a morphological marker for cholesterol domains in the plane of the bilayer.

INTRODUCTION

Freeze-fracture electron microscopy is very useful for the characterization of aqueous dispersions of lipids. This is particularly true for the diacylphospholipids which spontaneously adopt the bilayer organization in excess water to form liposomes or multilamellar vesicles (MLVs). These structures represent excellent models of a biological membrane lipid matrix and are regularly employed in all aspects of membrane research (Hope et al., 1986; Szoka and Papahadjopoulos, 1980). The lack of straightforward chemical fixatives for lipids and the artefacts associated with negative stain techniques have led to the use of freeze-fracture for the direct visualization of hydrated lipid systems.

Many other physical techniques are routinely applied to the study of membrane structure. However, most of these report on the averaged structure in a given sample. For example, X-ray diffraction is the classical technique employed to investigate structures adopted by macromolecular assemblies. However, it is limited in that it requires a lattice composed of many molecules in periodic arrays which then give rise to a diffraction pattern (Gruner et al., 1985a). Several macroscopic structures of lipids are amenable to X-ray analysis, and diffraction patterns from phospholipids organized in multi-bilayer sheets were used to first elucidate bilayer structure. Unfortunately, biological membrane systems rarely lend themselves to this type of analysis because they are not naturally arranged in a repeating lattice. Moreover, many membrane functions studied using liposomal systems cannot be carried out under the conditions of low hydration which force the membranes into such an array. Nuclear magnetic resonance (NMR) has extended our knowledge of the structure-function relationship of membrane lipids because it can be applied to more heterogeneous systems. The NMR spectrum obtained from phosphorus in phospholipid headgroups is sensitive to the macroscopic structure (bilayer, hexagonal, etc.) in which the phospholipid is moving. This has been used to great effect in describing the modulation of lipid structures by many biological effector molecules (Cullis and de Kruijff, 1979; Cullis et al., 1985). Similarly, deuterium can be chemically inserted into lipid molecules and ^{2}H-NMR employed to analyse the motion of labelled lipids (Davis, 1986). Both of these techniques have the advantage of being nonperturbing; the reporting molecule is either natural or modified to a minimal extent. Other physical techniques used to report lipid motion and hence provide structural information, are always limited by often quite extensive molecular modifications such as the insertion of bulky electron spin label or fluorescent groups (Davis, 1986).

The advantages of NMR techniques over X-ray are that a particular type of lipid organization (bilayer or non-bilayer) can be detected even though the macroscopic structure is not arranged in a lattice and mixtures of different structures can be detected under biologically relevant conditions of hydration. However, the reported signal is still derived from an average of all the molecules in a particular structural configuration (Cullis et al., 1985). Therefore freeze-fracture electron microscopy is unique in that it reveals local structure and does not require fixatives. In this review we will describe the use of freeze-fracture as a complementary technique to other physical techniques used to characterize model membrane systems in attempts to elucidate the functional roles of lipids in biological membranes.

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Fig. 2. Freeze-fracture electron micrographs of multilamellar dispersion of 1-C18:1$_c$-phosphatidylethanolamine at pH 8.2. Bar = 200 nm (from Tilcock et al., 1988).

and nonequilibrated solute distributions across the lamellae (Gruner et al., 1985b; Hope et al., 1986; Mayer et al., 19851. In most instances it is desirable to maximize the amount of internal aqueous medium that can be trapped during the preparation of MLVs. This volume is normally expressed as volume entrapped per mole lipid and the MLV preparation shown in Figure 1A would typically produce a low trapped volume on the order of 0.5 μl/μmol phospholipid. Differences in solute concentrations between bilayers probably arise during the initial lipid hydration process to form the liposome. For example, the most common technique used to form liposomes is to dry a film of bilayer-forming lipid from organic solvent onto the walls of a suitable vessel, add aqueous buffer and then shake. The surface of the film will first adopt a bilayer configuration in response to the buffer. Water readily permeates through bilayers (Deamer and Bramhall, 1986) and quickly penetrates the lipid film where 30-40 molecules of water per phospholipid are sufficient to orient the remaining bulk lipid into bilayers (Rand, 1981). However, electrolytes and other solutes permeate bilayers much more slowly than water. For example, Na + permeates across a bilayer of phosphatidylcholine ten orders of magnitude more slowly than water (Cullis and Hope, 1985). Therefore, the initial bilayers act as a molecular sieve and tend to exclude solute from internal compartments.

It is possible to overcome these problems by subjecting MLVs to multiple cycles of freezing and thawing (Mayer et al., 1985). The resulting frozen and thawed MLVs (FATMLVs) exhibit a characteristic morphology (Fig. 1C) which differs radically from that depicted in Figure 1A. Cross-fractures now reveal much larger interlamellar spaces with apparent vesiculation of internal membranes. As might be expected the wider aqueous space between bilayers results in a larger trapped volume for FATMLVs (5-10 μl/μmol), which is 10 fold higher than for MLVs and the physical disruption induces an equilibration of solute between the internal aqueous compartments. The mechanism by which vesiculation and increased swelling of MLVs occurs is not clear. However, it is most likely that the formation of ice crystals during the freezing step is important; a FATMLV morphology is not observed if cryoprotectants are present during the freeze-thaw cycles (Fig. 1B).

Lipid composition is also an important factor in determining MLV characteristics. The bilayer separation in MLVs is determined by an equilibrium between attractive van der Waals forces and repulsion due to electrostatic and hydration forces (Rand, 19811. For phosphatidylcholines this equilibrium distance is approx. 2.5 nm, equivalent to about 35 molecules of water per phospholipid head group. This separation can be increased by the incorporation of charged lipids into the bilayer. The effect of the resulting surface charge is to increase electrostatic repulsion between bilayers inducing swelling.

Different hydration protocols can also result in FATMLV-like morphology as determined by freeze-fracture. Most notable are the reverse phase procedures in which lipids are hydrated directly from an organic solvent. Several procedures have been developed whereby hydration occurs as the organic phase is evaporated (Deamer, 1984; Gruner et al., 1985b; Szoka and Papahadjopoulos, 1980). The hydrophilic nature of the emulsion increases as organic solvent evaporates, promoting lipids to aggregate into bilayer structures. Liposomes formed this way normally have

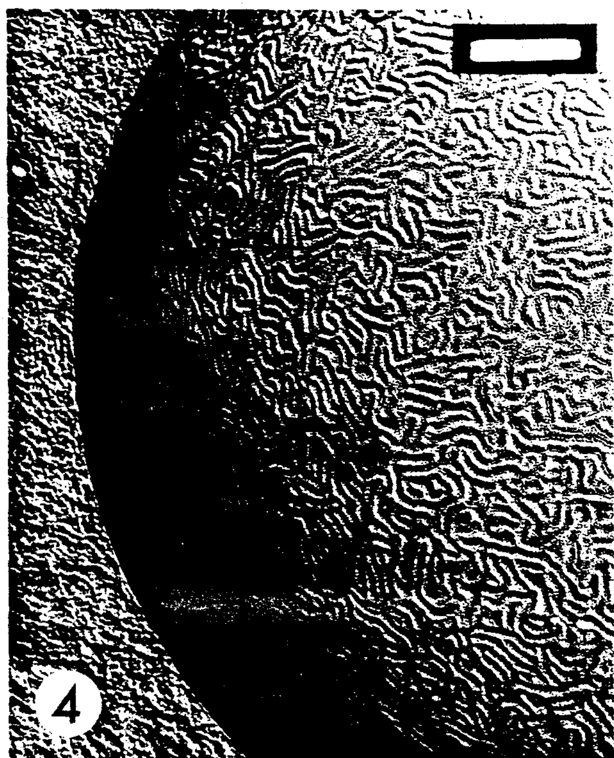


Fig. 4. *The* rippled fracture face observed for some saturated phospholipids in the gel state. Sample was composed of dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol (7:3 mole ratio) and quenched from *room* temperature. Bar represents 100 nm.

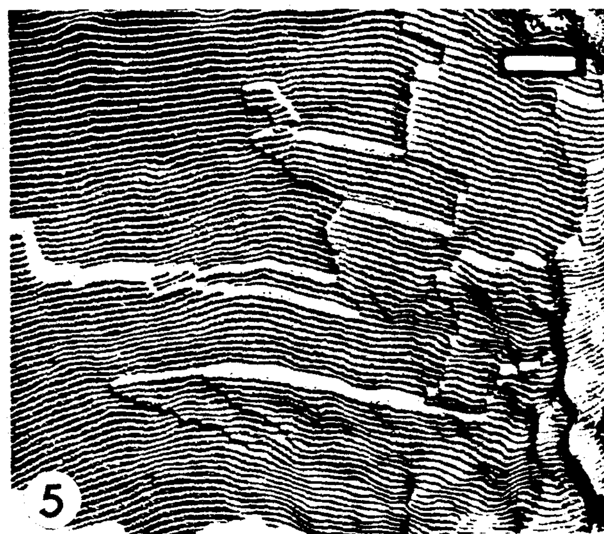


Fig. 5. The hexagonal H_{II} phase formed by unsaturated phosphatidylethanolamine. Bar represents 100 nm.

to the temperature at which the acyl chain moieties of the lipid molecule melt. Sharp transitions that occur over a small temperature range are observed for pure lipid mixtures with well defined acyl chain *species.* Biological membrane lipids generally exhibit broad transitions between the gel and liquid-crystalline states due to heterogeneity in the acyl chain composition. For this reason freeze-fracture techniques have mostly been applied to the gel-liquid crystalline phase behaviour of pure lipid systems and their mixtures. For example, a saturated, short chain phospholipid such as dimyristoylphosphatidylcholine (DMPC) exhibits a sharp transition at T_c = 23°C. Liposomes prepared from DMPC and quenched from temperatures below Tc resulted in a banded fracture plane whereas quenching from above Tc gave rise to the familiar smooth fracture face normally associated with lipid bilayers (Luna and McConnell, 1978; Verkleij et al., 1972; Ververgaert et al., 1972). Similarly, for dipalmitoylphosphatidylcholine (DPPC), T_c = 41°C a banded pattern is also observed upon freezing from below 41°C. However, in order to trap this lipid in the liquid-crystalline state, quenching from 60°C using ultrarapid cryofixation techniques is necessary (Menco, 1986; Ververgaert et al., 1973). The rippled freeze-fracture pattern is shown in Figure 4 and has been characterized as an intermediate gel state common to phosphatidylcholines (and phosphatidylglycerols that exhibit homogeneous acyl chain compositions (Houslay and Stanley, 1982; Silvius, 1982). It is called the P_{β} phase in which the fatty acid moieties are tilted with respect to the bilayer normal. These kinds of freeze-fracture patterns are not observed in biological membranes because the heterogeneity of lipid species and unsaturation gives rise to very broad phase transitions between the liquid-crystalline and gel state. Moreover, most biological membranes contain cholesterol which inhibits the ability of phospholipids to crystallize into the gel state (Demel and de Kruijff, 1976). The presence of cholesterol also prevents the formation of the rippled phase described above (Verkleij et al., 1972).

The transition of pure lipid species from the viscous gel state to the fluid liquid-crystalline state has received much attention because such a transition might regulate membrane-mediated processes. However, in eucaryotic membranes there is no evidence for the presence of gel-state lipid at physiological temperatures, and it is unlikely that modulation of membrane function occurs by formation of local crystalline domains.

LIPID POLYMORPHISMS

The bilayer configuration is not the preferred macromolecular organization for all hydrated membrane lipids. A large proportion either adopt the hexagonal H_{II} phase upon hydration or induce the formation of the Hn phase in mixed lipid systems. The hexagonal H_{II} phase is characterized by a hydrocarbon matrix penetrated by hexagonally packed aqueous cylinders, with diameters of about 2 nm, towards which the hydrophilic head groups are onented (Cullis and de Kruijff, 1979). This structure gives rise to an immediately recognizable fracture plane (Fig. 5) consisting of

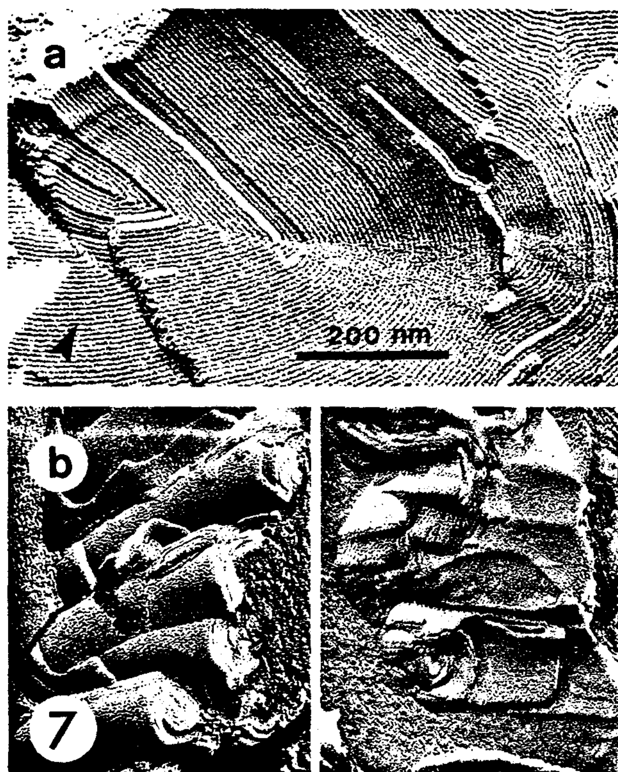
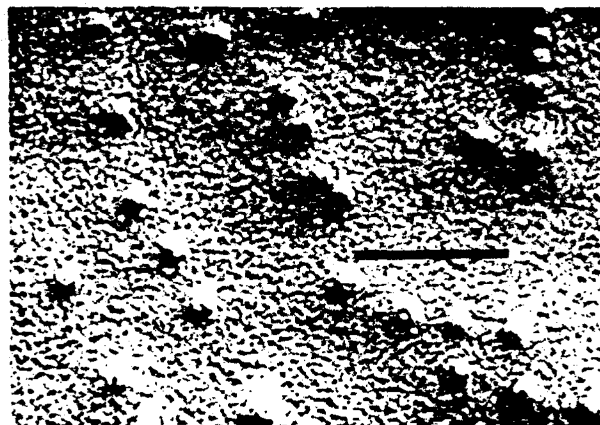


Fig. 7. Freeze-fracture electron micrographs of domains within a mixture of dioleoylphosphatidylethanolamine and dioleoylphosphatidylserine (1:1) in the presence of Ca^{2+}. Panel a shows H_{II} structure and panel b (left and right) cochleate structures adopted by phosphatidylserine in the presence of Ca^{2+} (from Tilcock et al., 1984).



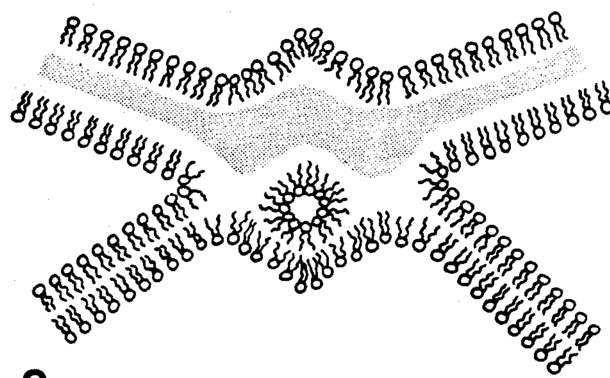


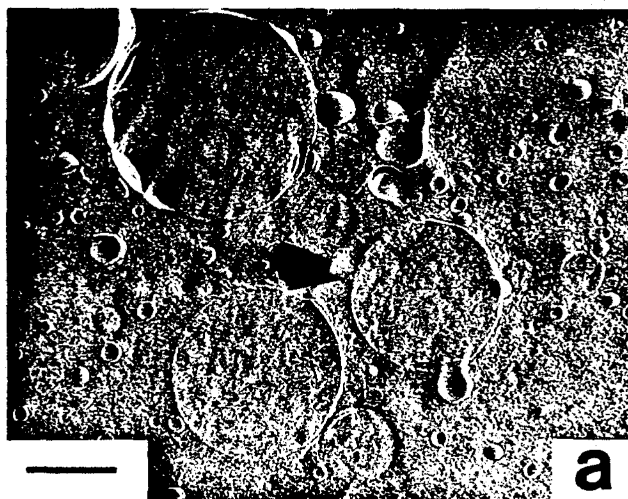
Fig. 8. A schematic representation of the proposed fracture plane around an inverted micelle formed at the contact point between two bilayers. The corresponding lipidic particles observed by freeze-fracture electron microscopy are shown in the micrograph of a mixture of soya phosphatidylionositol(7.5 mol%) with soya phosphatidylethanolamine. The bar represents 100 nm.

phospholipids can modulate the polymorphism of lipid mixtures. Phosphatidylinositol and phosphatidylglycerol promote the bilayer when mixed with unsaturated PE, but this capacity is diminished in the presence of Ca^{2+}. On the other hand, cardiolipin and phosphatidic acid both are able to undergo bilayer to hexagonal H_{II} transitions in the presence of Ca^{2+} (Cullis et al., 1985; Tilcock, 1986). Nonphospholipids also exhibit polymorphic phase behaviour. The major polar lipids of plant photosynthetic membranes are mono- and digalactosyldiglycerides. Similar to PE's, monogalactosyldiglycerides can adopt either lamellar or hexagonal H_{II} phase dependent upon temperature and acyl chain unsaturation (Sen et al., 1981, 1982; Sprague and Staehelin, 1984; Tilcock, 1986; Wieslander et al., 1981).

The morphology of arrays of hexagonal Hn tubes visualized by freeze-fracture is quite similar for the different lipids and lipid mixtures. Repeat distances vary from 4-15 nm; tubes containing at least one lipid component that would prefer to adopt the lamellar phase in isolation usually have a larger diameter than tubes composed of the non-bilayer lipid alone (Verkleij, 1984). In the gel state the preferred organization for phospholipids is the bilayer. Consequently, there is a tendency for non-bilayer lipid systems to revert to a bilayer organization during the freezing process. The temperature of the **lamellar-H_{II}** transition (T_{BH}) is dependent upon acyl composition and normally occurs at temperatures higher than or concomitant with Tc (Cullis et al., 1985). Freeze-fracture of pure PE systems, known to be in the Hn phase, often give rise to fracture planes of bilayers or mixtures of bilayer and H_{II} phase lipid (Verkleij, 1984). Dioleoylphosphatidylethanolamine, for example, exhibits a T_{BH} of 10°C (Cullis and de Kruijff, 1979). In order to visualize this lipid in hexagonal arrays by freeze-fracture, ultrarapid freezing procedures are required (van Venetie et al., 1981).

Lipidic particles

As indicated in the previous section, the lamellar to hexagonal H_{II} phase transition can be induced in pure lipids and lipid mixtures in a variety of ways. Using ^{31}P-NMR techniques a characteristic spectral feature can often be observed during the transition: a narrow peak arising from phospholipids which experience isotropic motional averaging (Cullis et al., 1985). There are a number of lipid structures that give rise to this



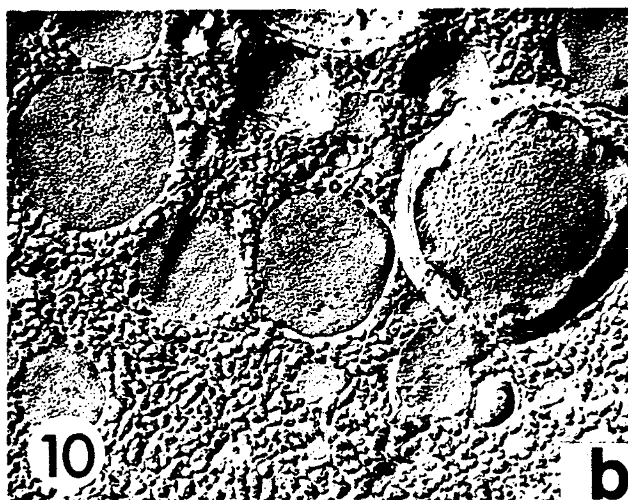


Fig. 10. Freeze-fracture electron micrographs of a aonicated mixture of egg phosphatidylcholine/cholesteroUcholesteryl linoleate/triolein (1:0.5:1:2.5 molar ratio). Sample a was shadowed immediately alter fracturing, and b was etched for 2 min at 183 K, $10^{-6}-10^{-7}$ torr before shadowing. In a, the solid arrow indicates a model lipoprotein particle with a smooth cross-fracture face while the arrowhead shows the presence of a bilayer vesicle. The bar represents 200 nm (from Wong et al., 1987).

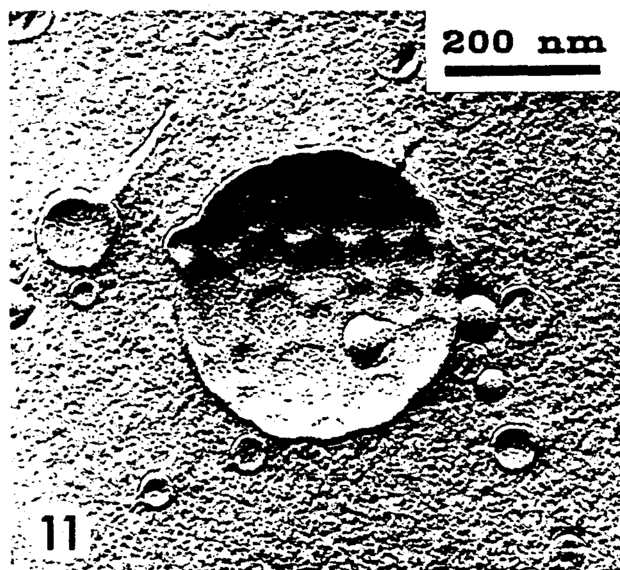


Fig. 11. Freeze-fracture electron micrograph of a "golf-ball" like fracture plane occasionally observed in sonicated dispersions of the same lipid mixture described for Figure 10.

"golf-ball" like fracture planes are observed (Fig. 11) in which large lipid structures appear to be blebbing off vesicles or neutral lipid particles (Wong et al., unpublished observation).

VISUALIZATION OF LIPID IN BIOLOGICAL MEMBRANES

It is clear that isolated membrane lipids adopt a variety of different phases and structures which are identifiable by freeze-fracture. However, under normal physiological conditions biological membranes only reveal a bilayer fracture plane. There is strong evidence that the abundance of membrane lipids that can adopt transitory or long-lived nonbilayer structures are involved in membrane mediated events such as fusion, but these departures from bilayer structure have yet to be detected in natural membranes.

Lipid markers such as the polyene antibiotic filipin have been used extensively to locate what has been claimed to be cholesterol-rich domains within the plane of the bilayer of biological membranes (see Severs and Bobenek, 1983; Bolard, 1986, for reviews). However, it is clear that many researchers now believe that filipin-induced morphologies must be interpreted with caution (Behnke et al., 1984b; Bolard, 1986; Miller, 1984; Severs and Bobenek, 1983). Very few freeze-fracture studies have been performed using protein-free, model membrane systems and filipin. Those that have been published clearly demonstrate that the 25-nm bilayer bulges which are the characteristic of filipin-sterol aggregates are themselves accumulated into large aggregates which contrasts with the even, random distributions often observed in biological membranes (Behnke et al., 1984a; Miller, 1984). The difference suggests that membrane components other than lipid are influencing the morphology. It has been argued that the degree of localization reported in the literature is not compatible with the known physical properties of the membrane (Miller, 1984). The lateral diffusion rate of cholesterol compared to the length of time filipin would take to diffuse into the bilayer and complex with the sterol means that the best resolution of localization of cholesterol is over 4 μm. Moreover, a lipid-cholesterol or protein-cholesterol interaction has not been identified in any liquid crystalline membrane system with a lipid composition comparable to biological membranes that could be envisaged to restrict the lateral diffusability of cholesterol to the extent required to form discrete domains. The available evidence suggests

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