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Phase behaviour of dipalmitoyl phosphatidylcholine (DPPC)-cholesterol membranes

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Abstract

We have determined the phase behaviour of dipalmitoyl phosphatidylcholine (DPPC)-cholesterol mixtures from small angle x-ray diffraction studies of oriented multilayers. A cholesterol induced modulated phase, denoted as P_β, is obtained at intermediate cholesterol concentrations, which is distinct from the ripple ($P_{\beta'}$) phase found in earlier studies on similar systems. We also report some confocal fluorescence microscopy observations on giant unilamellar vesicles (GUVs) made from these mixtures.

1. Introduction

Lipids and cholesterol are essential constituents of plasma membranes. The distribution of cholesterol in these membranes is believed to be inhomogeneous, but the organization of cholesterol even in lipid model membranes is poorly understood in spite of a large number of studies [1]. The phase behaviour of lipid-cholesterol mixtures has been extensively studied using a variety of experimental techniques [2–13]. Differential scanning calorimetry studies show that the main- and pre-transitions of lipid-cholesterol mixtures broaden and the transition temperatures decrease progressively with cholesterol concentration [12]. The structure and phase behaviour of these mixtures have been probed using x-ray diffraction [2, 10, 13], neutron scattering [4], freeze fracture electron microscopy [3] and nuclear magnetic resonance (NMR) [5]. Monolayer studies on these mixtures have suggested the formation of lipid-cholesterol complexes at specific stoichiometric ratios [11].

The present paper describes the phase behaviour of DPPC-cholesterol mixtures deduced from x-ray diffraction data. Almost all of the earlier diffraction studies on this system were carried out using unoriented samples [14], although oriented multilayers are known to provide more information, especially about the in-plane structure of the bilayers [15]. These studies have led to the observation of a novel modulated phase in these mixtures, whose origin is

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presently not clear. We have also carried out some fluorescence microscopy studies on giant unilamellar vesicles (GUVs) made from these mixtures. A partial phase diagram of these mixtures was reported by us earlier [13].

2. Experimental section

Dipalmitoyl phosphatidylcholine (DPPC) was obtained from Fluka and cholesterol from Sigma. They were used as received.

2.1. X-ray diffraction

Appropriate amounts of DPPC and cholesterol were dissolved in chloroform and the solution was deposited on a curved glass substrate. Evaporation of the solvent leaves a thin layer of the lipid-cholesterol mixture on the substrate. Samples were kept overnight in a desiccator under vacuum to remove traces of chloroform. They were then hydrated in a water saturated atmosphere for a couple of days. This results in well oriented samples with the bilayers aligned parallel to the substrate, which were transferred to a sealed chamber for x-ray diffraction experiments. The temperature of the chamber was controlled to an accuracy of $\pm 0.01\,^{\circ}$C using a circulating water bath. The relative humidity was maintained at $98 \pm 2\%$ by keeping a reservoir of distilled water inside the chamber. A thermo-hygrometer (*Testo 610*) was used to monitor the temperature and humidity in the chamber close to the sample.

The incident x-ray beam (wavelength $= 1.54$ Å) from a rotating anode generator (Rigaku Ultra X18) was tangential to the substrate. Diffraction patterns were recorded on an image plate detector (Marresearch). All samples were initially heated above the main transition of DPPC (42 °C) and then cooled down to 5 °C in steps of 5 °C. Diffraction patterns were recorded at each step during cooling. Typical exposure time was $\sim$1 h.

2.2. Fluorescence microscopy

GUVs were prepared using the electroformation method first described by Angelova *et al* [16, 17]. The sample chamber consists of two platinum electrodes across which an electric field (1.5 V, 10 Hz) is applied. Appropriate amounts of DPPC, cholesterol and a fluorescent dye were dissolved in chloroform (0.5 mg ml^{-1}) to prepare a stock solution. About 1 μl of the stock solution was taken out and coated onto each platinum electrode and the solvent was allowed to evaporate. After removing traces of the solvent 500 μl of Millipore water was added to the chamber. The temperature of the chamber was maintained at 23 °C using a circulating water bath. The electric field was turned on for about 1.5 h, and 100 μl of the vesicular solution from the chamber was transferred to a dish for microscopy observations.

GUVs were prepared with DPPC-cholesterol mixtures containing 0.2 mol% of rhodamine dihexadecyl phosphatidylcholine (Rho DHPE). A He–Ne laser of wavelength 543 nm was used to excite the Rho DHPE. Fluorescence images of the GUVs were taken using a Zeiss confocal laser scanning microscope with a 40× water immersion objective having a numerical aperture of 1.2.

3. Results

3.1. X-ray diffraction

The phase behaviour of the mixtures at cholesterol concentrations (X_c) varying from 0 to 50 mol% in steps of 5 mol% have been examined. DPPC shows three lamellar phases at high

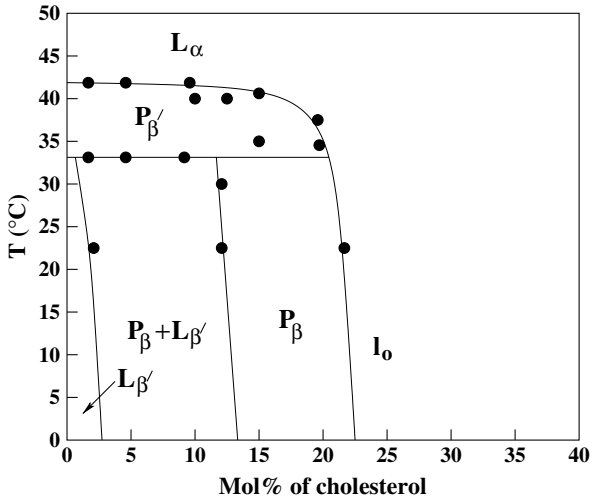


Figure 1. Phase diagram of DPPC-cholesterol mixtures. L_α: fluid phase; $P_{\beta'}$: ripple phase; $L_{\beta'}$: gel phase; P_β: cholesterol induced modulated phase; l_o: L_α phase rich in cholesterol (liquid ordered phase).

hydration (relative humidity $\sim 98 \pm 2\%$). The fluid phase (L_α) occurs above 42 °C, the ripple phase ($P_{\beta'}$) from 42 to 33 °C and the gel phase ($L_{\beta'}$) below 33 °C. The diffraction patterns from the L_α phase consist of a few lamellar reflections along q_z in the small angle region, z being the direction of the bilayer normal. Below 42 °C (main or chain melting transition temperature T_M) additional reflections (satellites) with $q_\perp \neq 0$ are seen, which arise from the modulation of bilayers in the ripple phase. Diffraction patterns obtained from the gel phase of DPPC for $T < 33$ °C (pre-transition temperature T_P) consist of a large number of lamellar reflections in the small angle region and two sharp wide angle reflections from the hydrocarbon chain lattice.

The phase diagram of the mixtures deduced from the diffraction data is shown in figure 1. For $5 < X_c < 20$, we observe the $P_{\beta'}$ phase below L_α. In the corresponding diffraction patterns the satellite reflections due to the rippling of the bilayers are not distinct, but are smeared out. This may be due to an increase in the ripple wavelength with increasing X_c, which would bring these reflections closer [3, 4]. Below pre-transition, the $L_{\beta'}$ phase is found to coexist with the P_β phase which is characterized by rectangular lattice (figure 2). A pure $L_{\beta'}$ phase was not observed even at the lowest temperature studied (5 °C). At $X_c = 15$ and 20, a pure P_β phase was detected at temperatures below T_P (figure 3), and this phase continues down to 5 °C. For $X_c \geqslant 22$, only the L_α phase is found throughout the temperature range studied.

3.2. Fluorescence microscopy

Fluorescence microscopy studies were carried out on GUVs composed of DPPC at different cholesterol concentrations (0, 5, 10, 15, 20, 25 and 30 mol%). The electroformation of GUVs produced mainly unilamellar vesicles, whose diameter varied from 10 to 100 μm.

For $5 < X_c < 10$ most of the smaller GUVs with diameter $<\sim 20$ μm are spherical in shape and show dark irregular domains on a bright background indicating the co-existence of two phases (figure 4(a)). Partitioning of the dye into the two phases suggests that these dark domains are in the gel phase, since rhodamine DHPE is known to prefer the fluid phase.

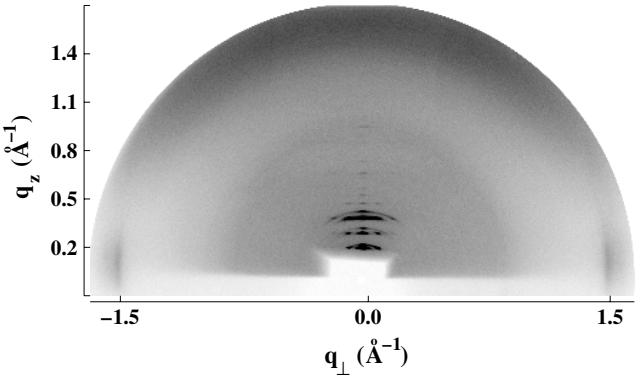


Figure 2. Diffraction pattern of a mixture containing 12.5 mol% cholesterol at 10 °C and a relative humidity of 98%. The figure shows the coexistence of P_β and gel phases.

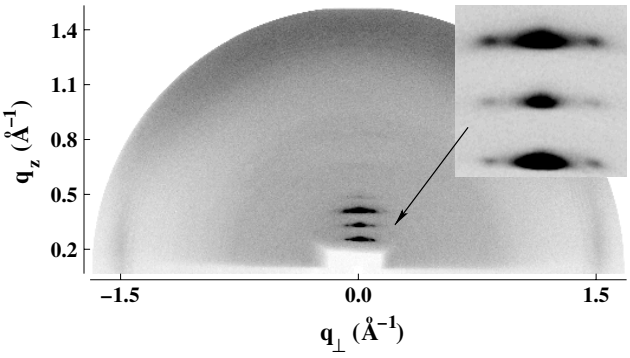
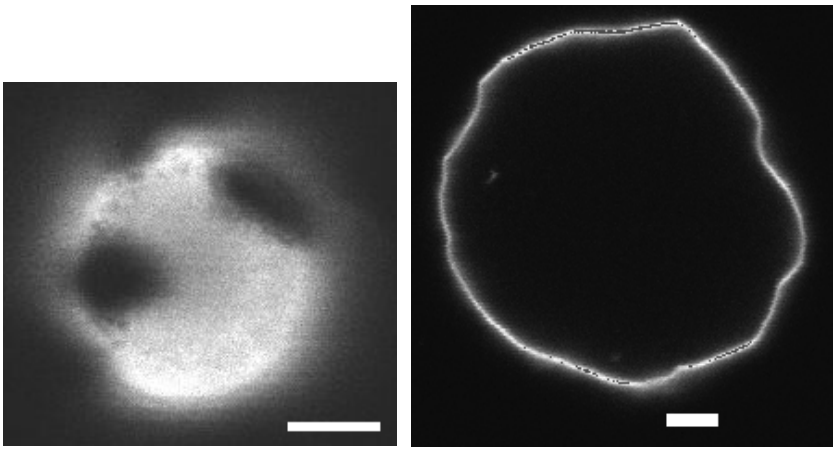


Figure 3. Diffraction pattern of the P_β phase in a mixture with 12.5 mol% cholesterol at 24 °C and a relative humidity of 98%. The small angle region of the diffraction pattern is shown in the inset.

This is also supported by the fact that the domains have irregular shape and do not coalesce on colliding. Interestingly, larger GUVs with diameter $>\sim$30 μm do not show domains and have non-spherical shapes (figure 4(b)). These also exhibit thermal shape fluctuations, unlike the smaller spherical GUVs. At higher X_c ($>$10) GUVs are mainly spherical in shape and the fluorescence intensity distribution on the surface of the GUVs is found to be uniform. These GUVs do not show significant thermal shape fluctuations.

4. Discussion

Different phases of DPPC-cholesterol mixtures were identified from their characteristic diffraction patterns described earlier. The tilt of the hydrocarbon chains in the gel phase was determined from the positions of the wide angle reflections to be 30° [18]. The P_β phase that appears below pre-transition is distinct from the ripple phase found in earlier studies on similar systems [4, 3]. The structure of the P_β phase is presented in [13]. The wide angle reflection on the equatorial plane ($q_\perp = 0$) in the P_β phase indicates zero tilt of the chains from the bilayer normal. It is interesting to note that unlike in the ripple phase the wavelength of the modulation ($\sim$70 Å) in the P_β phase is comparable to the lamellar spacing ($\sim$65 Å), and increases with increasing temperature at a given X_c but decreases with increasing X_c at a given



(a) (b)

Figure 4. Fluorescence micrographs of DPPC GUVs containing 5 mol% cholesterol at 23 °C. Scale bars on the images represent 5 μm. The smaller almost spherical GUV in (a) contains domains and does not exhibit significant thermal shape fluctuations. The larger non-spherical GUV in (b) does not contain domains and exhibits thermal shape fluctuations.

temperature. For $X_c \geqslant 22$, only the L_α phase exists over the whole temperature range. This cholesterol-rich phase has been referred to as the liquid ordered phase (l_o) in the literature, and many spectroscopic studies have indicated a coexistence between it and a cholesterol poor L_α phase, called the liquid disordered phase (l_d), at lower X_c [5]. However, there is no evidence for such a coexistence from this or any of the earlier diffraction studies. The width of the wide angle reflection in the l_o phase indicates that it has a longer in-plane positional correlation length than that found typically in the L_α phase, but smaller than that in the $L_{\beta'}$ phase of pure DPPC [13]. The large number of lamellar reflections in the diffraction patterns of the l_o phase can be attributed to the increase in the bilayer rigidity in the presence of cholesterol [13].

The phase separation below the pre-transition found from x-ray diffraction is also seen with fluorescence microscopy in GUVs. Confocal fluorescence micrographs of GUVs show gel phase domains coexisting with a more fluid phase for $5 < X_c < 10$. From the phase diagram the latter phase can be identified as the P_β phase. Domains have been observed earlier with equimolar mixtures of DPPC, dioleoyl phosphatidylcholine (DOPC) and cholesterol [19]. But to date, they have not been reported in binary mixtures of DPPC and cholesterol. There is an important difference in the nature of the domains in the two systems; in the ternary mixtures they are in the fluid phase, whereas in the binary mixtures they are in the gel phase. The observation of shape fluctuations of the GUV is surprising since cholesterol is known to increase the bending rigidity of lipid bilayers. The occurrence of a non-spherical GUV (figure 4(b)) in the absence of any phase separation is also very intriguing, since the formation of such structures is normally associated with the presence of domains with sufficiently different membrane properties. The situation is further complicated by the fact that not all vesicles at the same X_c show these modifications. Further work is under way to understand these aspects better.

5. Conclusion

We have investigated the phase behaviour of oriented stacks of DPPC-cholesterol membranes by x-ray diffraction techniques. The phase diagram obtained from these studies is in good

agreement with earlier studies, but for the observation of a modulated phase (P_β), which has not been observed before. The origin of this phase, which occurs at intermediate cholesterol concentrations, is presently not clear. We have also observed phase separation in giant unilamellar vesicles and the formation of non-spherical vesicles with fluorescence microscopy.

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