Cholesterol-Induced Modulated Phase in Phospholipid Membranes

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(Received 6 February 2003; published 28 August 2003)

We report the observation of a cholesterol-induced modulated phase (P_β) in dipalmitoyl phosphatidylcholine bilayers. It occurs below the main transition of the lipid at cholesterol concentrations of around 15 to 20 mol % and is distinct from the ripple ($P_{\beta'}$) phase found in between the main and pretransitions at lower cholesterol concentrations. An electron density map of this phase, constructed from x-ray diffraction data from oriented multilayers, shows that the bilayers in this phase have a one-dimensional periodic height modulation with an amplitude of about 2.5 Å. A partial phase diagram of the system deduced from diffraction data is in broad agreement with earlier studies.

DOI: 10.1103/PhysRevLett.91.098102 PACS numbers: 87.16.Dg, 61.10.Eq, 64.75.+g

Cholesterol is an essential constituent of plasma membranes and is known to play a very important role in many membrane functions [1]. The distribution of cholesterol within the membranes is believed to be inhomogeneous, and there is some evidence that cholesterol-rich domains called rafts are present in cell membranes. The organization and function of these membrane rafts are presently far from understood [2]. A mechanism based on the coupling of molecular chirality to the in-plane tilt order, leading to the budding of membrane vesicles, has recently been proposed [3], which is expected to be relevant to the functioning of rafts.

The importance of lipid-cholesterol interactions has led to a large number of studies on the influence of cholesterol on the structure and dynamics of phospholipid bilayers [4–11]. Partial phase diagrams of phosphatidylcholine (PC)-cholesterol bilayers have been constructed using a variety of experimental techniques [6,9,10,12,13]. Phase boundaries have been found at around 5 and 20 mol % cholesterol concentration (x_c) at temperatures below the main transition [8]. For $x_c > 20$ the main transition is absent, and only the cholesterol-rich L_α phase is found even at lower temperatures. Spectroscopic studies indicate a microphase separation of cholesterol-poor and cholesterol-rich regions of the bilayer in the L_α phase for $5 < x_c < 20$ [10,12]. These two are often referred to as the liquid disordered (l_d) and liquid ordered (l_o) phases, since cholesterol is known to stretch the hydrocarbon chains of the lipid molecules [14]. In some systems a macroscopic phase separation has been observed at higher temperatures in the L_α phase [15]. Further, studies on PC monolayers at air-water interface have indicated the formation of lipid-cholesterol complexes at a specific stoichiometric ratio [16].

In this Letter we present results of small angle x-ray diffraction experiments on oriented dipalmitoyl phosphatidylcholine (DPPC)-cholesterol multibilayers, which were motivated by recent theoretical studies on chirality induced budding of vesicles, mentioned earlier [3]. Our main aim was to probe the effect of cholesterol on the in-plane order of the bilayer. Although there have been a large number of diffraction studies on lipid-cholesterol membranes, almost all of them have been carried out on unoriented samples. Oriented multilayers, of course, provide much more information, especially in relation to chain tilt and in-plane order [17]. In the course of these studies we observed a novel modulated phase (denoted as P_β) at intermediate values of x_c. An electron density map of this phase calculated from diffraction data shows that it consists of bilayers with a one-dimensional periodic height modulation as in the ripple ($P_{\beta'}$) phase of pure DPPC. However, these two phases are distinct and differ in many structural details. A partial phase diagram of this system has also been constructed from the diffraction data.

DPPC was obtained from Fluka and cholesterol from Sigma. Samples for x-ray experiments were prepared on curved glass substrates from DPPC-cholesterol solutions in chloroform. After evaporation of the solvent, samples were kept under vacuum to remove traces of chloroform. They were then allowed to hydrate for a day in a water saturated atmosphere. This results in well-aligned samples with bilayers parallel to the substrates. They were transferred to a fully sealed sample chamber, whose temperature could be controlled to within ± 0.05 °C using a circulating water bath. The relative humidity was maintained at 98% ± 2%, by saturating the air inside the sample chamber with water vapor. The incident monochromatic x-ray beam from a rotating anode generator (Rigaku, UltraX18) was tangential to the substrate and the diffraction patterns were collected on an image plate detector (Marresearch). Mixtures with $x_c = 0$, 5, 10, 15, 20, 22, and 33 were studied. Diffraction patterns were collected from 45 to 5 °C at 5 °C intervals from each sample.

DPPC exhibits three lamellar phases at high hydration, consisting of stacks of bilayers separated by water: the L_α phase above the main transition, the gel phase ($L_{\beta'}$) below the pretransition, and the ripple phase ($P_{\beta'}$) in between [18]. Diffraction patterns of pure DPPC obtained by us

are consistent with a main transition at ~42 °C and a pretransition at ~34 °C, in agreement with earlier studies [18].

The partial phase diagram shown in Fig. 1 was deduced from the x-ray data. The L_α and $L_{\beta'}$ phases were identified, respectively, by the absence and presence of sharp reflections in the wide angle region from the chain lattice. The $P_{\beta'}$ and P_β phases were identified by the presence of additional "satellite" reflections due to the correlated rippling of the bilayers. The main and pretransition temperatures are found to be not significantly affected by cholesterol for $x_c < 15$. Earlier studies have found the wavelength of the modulations in the $P_{\beta'}$ phase to increase with x_c and to diverge near $x_c \sim 20$ [5,9]. In the present studies we were not able to get good diffraction patterns in this phase, with well-resolved satellites arising from the rippling of the bilayers. This is probably a consequence of the decrease in the positional correlations of the ripples in the presence of cholesterol [8]. However, we could unambiguously identify this phase from the smearing of the lamellar peaks due to unresolved satellites. The small angle region of the diffraction patterns shows a coexistence of two phases for $5 < x_c < 10$; the wide angle region is similar to that of the $L_{\beta'}$ phase of pure DPPC. One of the coexisting phases is a lamellar phase with a periodicity of ~60 Å, which is comparable to that of the $L_{\beta'}$ phase of pure DPPC. Hence it can be identified as the cholesterol-poor $L_{\beta'}$ phase. The tilt angle of the chains in this phase is $30° \pm 2°$, as in the $L_{\beta'}$ phase of DPPC. The diffraction pattern of the coexisting cholesterol-rich phase corresponds to a primitive rectangular unit cell with lattice parameters, $a \sim 65$ Å and $b \sim 70$ Å; b is in the plane of the substrate and a normal to it. a is found to be insensitive to the temperature and x_c, whereas b decreases slightly both on decreasing the temperature and on increasing x_c. The relative amount of this (P_β) phase increases with x_c and at $x_c = 15$ the $L_{\beta'}$ phase is absent. Figure 2 shows the small angle region of the diffraction pattern obtained from the P_β phase. The wide angle chain reflection is very close to the equatorial plane ($q_z = 0$), indicating that the chains are not tilted (Fig. 3). At $x_c = 15$ and 20 the L_α phase goes into the P_β phase at about 40 °C on cooling. Both main and pretransitions disappear at higher x_c: the pretransition at ~15 and the main transition at ~20. For $x_c > 20$ the L_α phase continues down to the lowest temperature studied.

In many ways Fig. 1 is similar to the phase diagram of dimyristoyl phosphatidylcholine-cholesterol bilayers obtained earlier from small angle neutron scattering studies on unoriented samples [9]. However the P_β phase was not observed at lower temperatures in that study, instead a ripple phase with a modulation periodicity of about 400 Å was found. The origin of this discrepancy is presently unclear. The positions of the two phase boundaries near 5 and 22 are comparable to those in the phase diagram obtained by Vist and Davis from NMR and calorimetric studies [10]. However, this concentration range does not correspond to the coexistence of the $L_{\beta'}$ and the cholesterol-rich L_α phases, as proposed in Ref. [10]. The coexistence of two phases for $3 < x_c < 10$ has been observed in earlier diffraction experiments [13]. But it was not possible to determine the modulated nature of the cholesterol-rich phase, probably since unoriented samples were used in that study. Spectroscopic experiments have found evidence for phase separation of cholesterol-rich and cholesterol-poor regions of the bilayer in the L_α phase [10,12]. Similar behavior has also been seen recently in giant unilamellar vesicles made of lipid mixtures containing cholesterol [19]. The diffraction technique used here is sensitive only to a macroscopic phase separation leading to the formation of two L_α phases with different periodicities. We do not find any evidence for such a trend, consistent with the results of earlier scattering studies [8,9,13,15]

An electron density map of the P_β phase has been constructed from the diffraction data shown in Fig. 2. Phasing of the reflections was done using a modeling procedure used in the context of the $P_{\beta'}$ phase [20].

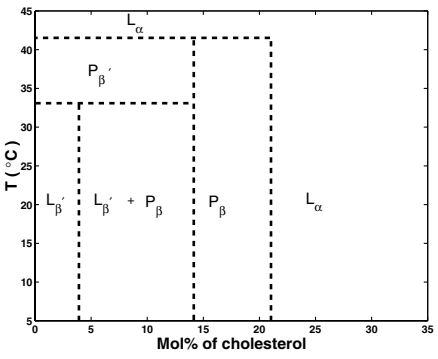

FIG. 1. Partial phase diagram of DPPC-cholesterol mixtures obtained from the diffraction data. P_β is the modulated phase induced by cholesterol. The precise locations of the phase boundaries have not been determined in this study.

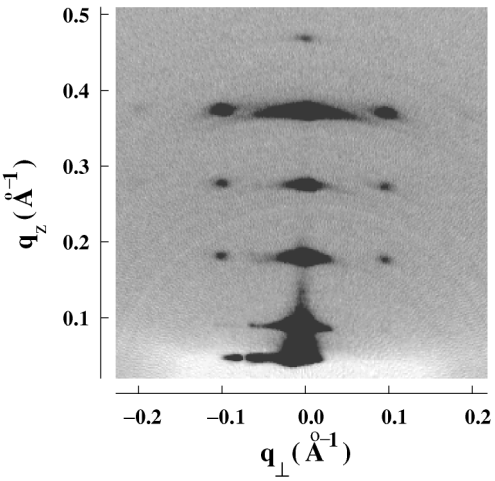

FIG. 2. The small angle diffraction pattern of the P_β phase at $x_c = 15$ and $T = 6$ °C.

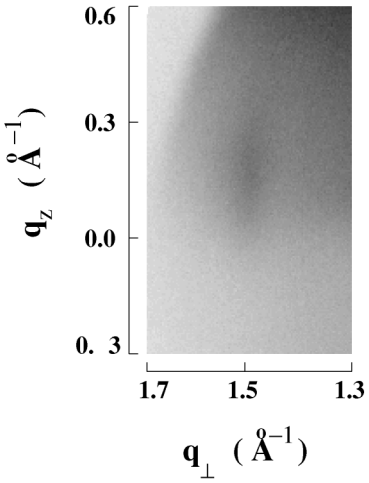

FIG. 3. The wide angle chain reflection in the P_β phase at $T = 10\,^\circ$C. The scattered intensity is negligible for $q_z < 0$ due to absorption by the substrate.

Because of the rectangular symmetry (h k) and (h $\bar{k}$), reflections overlap. Therefore, their intensities could not be independently obtained and we have taken them to be equal. We have assumed the bilayers in this phase to have a one-dimensional height modulation as in the $P_{\beta'}$ phase, since we have no *a priori* information about their shape. Another possibility is a modulation of the bilayer thickness; however, this would tend to form a centered rectangular lattice instead of the observed primitive rectangular. The resulting electron density map is presented in Fig. 4. It clearly shows a stack of modulated bilayers separated by water. The quality of the map is very good, indicating that the model used to phase the reflections is essentially correct. The height modulations are asymmetric (arms of unequal length), with a wavelength of ~70 Å. The bilayer thickness estimated from the map is about 50 Å and the amplitude of the height modulation is about 2.5 Å. In contrast, the amplitude of the ripples in the $P_{\beta'}$ phase is typically ~10 Å and their wavelength is of the order of 150 Å [20–22]. An electron rich band is clearly seen in Fig. 4 at a distance of about 10 Å from the bilayer center, in addition to the one corresponding to the head group region. The position of the former is very close to that of a secondary peak due to cholesterol seen in the electron density profiles of DPPC-cholesterol bilayers [4]. The bilayer thickness is also in good agreement with earlier reports [4].

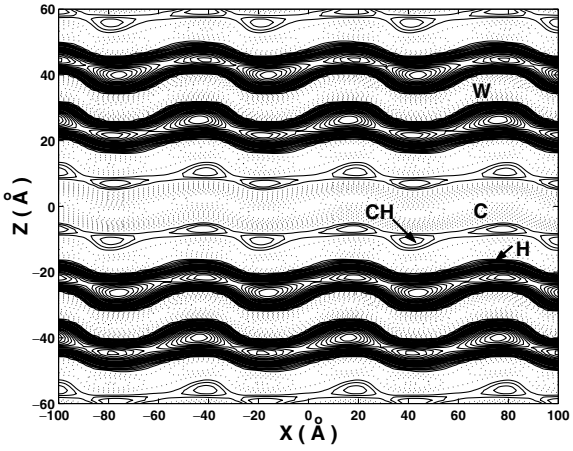

FIG. 4. Electron density map of the P_β phase of DPPC-cholesterol bilayers calculated from the diffraction data of Fig. 2. The solid (dotted) contours correspond to the electron rich (poor) parts of the bilayer. H, W, and C denote the head group, water, and chain regions, respectively. CH denotes the electron rich band in the bilayer due to the presence of cholesterol.

The P_β phase reported here has not been seen in earlier freeze fracture and diffraction studies of lipid-cholesterol membranes [5,6,9,13]. One possible reason for not resolving this structure in freeze fracture studies could be the very low amplitude of the ripples. Since the platinum deposition is usually done at an angle of 45°, such low amplitude ripples will not lead to a shadowing effect. Most of the x-ray and neutron scattering studies have been carried out on unoriented samples. This could lead to the overlapping of different reflections in the diffraction pattern and make the identification of the modulated phase difficult.

Profiles of the wide angle chain reflections in the different phases of DPPC-cholesterol bilayers are shown in Fig. 5. The two reflections in the $L_{\beta'}$ phase of DPPC result from a chain tilt towards nearest neighbor [23]. The on-axis reflection is much weaker because of absorption by the substrate. The width of these reflections is inversely proportional to the correlation length, ξ, of the chain ordering in the plane of the bilayer. It is interesting to note that ξ is longer normal to the tilt direction, as seen in some lipid monolayers [24]. The width is not very different in the P_β phase, indicating a high degree of in-plane order. As to be expected, the width is much larger in the fluid L_α phase. However, the width of the chain reflection

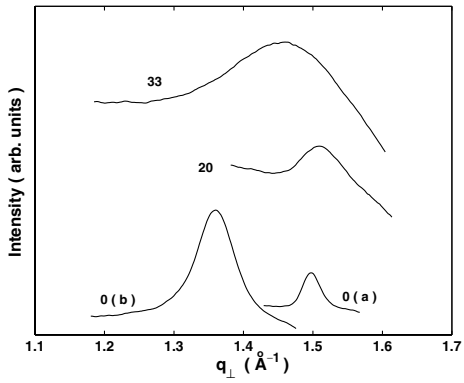

FIG. 5. Profiles of the wide angle chain reflection in the different phases exhibited by the mixtures. The numbers against the curves indicate x_c. (a) and (b) refer to the on-axis ($q_z = 0$) and off-axis reflections in the $L_{\beta'}$ phase of DPPC, respectively. $x_c = 20$ corresponds to the P_β phase, and $x_c = 33$ to the L_α phase.

along q_z in the cholesterol-rich L_α phase is not very large (data not shown), due to the stretching of the chains in the presence of cholesterol [4].

Although P_β and $P_{\beta'}$ phases consist of rippled bilayers, their structures are different in many respects. The oblique unit cell of the $P_{\beta'}$ phase is a consequence of different bilayer thicknesses in the two arms of the ripple, which can at least partly be accounted for by a chain tilt along the ripple wave vector [22]. On the other hand, there is no evidence for a chain tilt along this direction in the P_β phase, and the bilayer thicknesses in the two arms are comparable. This emphasizes the observation made in Ref. [22] that it is not sufficient to consider the bilayer profile alone in determining the symmetry of the unit cell; one has to consider the bilayer thickness in the two arms as well. Thus Ref. [22] presents an example of a symmetric ripple profile leading to an oblique lattice due to different thicknesses of the two arms; here we have an asymmetric profile with equally thick arms giving rise to a rectangular lattice.

It is interesting to note that modulated bilayers similar to those in the P_β phase have been predicted by a Landau theory of phase transitions in bilayers [25]. In chiral systems it envisages the formation of rippled bilayers with an asymmetric height profile and a chain tilt normal to the ripple wave vector, which is oscillatory along this direction. We cannot conclusively rule out such tilt oscillations in the P_β phase if they are of small amplitude. The relatively large lamellar periodicities of the cholesterol-rich phase coexisting with the $L_{\beta'}$ phase for $3 < x_c < 10$, reported in Ref. [13], clearly show that the bilayers in the P_β phase are rather flexible. The occurrence of such a modulated phase, which has not been seen in other lipid systems, might be a consequence of the enhanced bilayer flexibility due to the presence of cholesterol in this phase.

The formation of the P_β phase at intermediate x_c is rather intriguing. We suspect that the miscibility gap between the $L_{\beta'}$ and P_β phases is a consequence of the nonzero tilt in the former. As is well known, the chain tilt arises from the larger cross-sectional area of the head group compared to that of the chains. Therefore, if the tilt can take only values close to 30° and 0° (as observed experimentally), it is conceivable that a well-defined amount of cholesterol has to be incorporated into the bilayer to remove the tilt. A rough estimate based on the areas of the different moieties gives $x_c \approx 20$ in the untilted phase. However, this argument does not give any clue as to why the bilayers become rippled in this phase. Further work is needed to understand this behavior.

We thank Madan Rao, Sarasij Ray Chaudhuri, and Yashodhan Hatwalne for helpful discussions.

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