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Modulated phase of phospholipids with a two-dimensional square lattice

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We report an observation of a modulated phase of phospholipids in which intrabilayer density modulations form an in-plane square lattice. Similar to the well-known ripple (P'_β) phase, this phase can be induced by either dehydration or cooling from the liquid crystalline (L_α) phase. However, further lowering of hydration or temperature induces either an untilted straight-chain gel phase (L_β), or the tilted L'_β phase after a brief appearance of the P'_β phase. The structural characteristics of this phase support the notion that the coupling between variations in local chain tilt and bilayer shape plays an important role in the formation of modulated phases.

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Under hydration, phospholipids are lyotropic liquid crystals with rich phase behaviors that are driven by both temperature and hydration (e.g., [1]). For phospholipids with saturated hydrocarbon chains, most of the phases found in the temperature (T) versus the relative humidity (RH) phase diagram (see Fig. 1) are structurally well established [2,3]. The phase at high T and high RH (designated as L_α) is known as the liquid crystalline phase; in each bilayer, chains are disordered and two-dimensionally fluid-like. A main transition upon decreasing T and/or RH leads to a more ordered phase with straight chains, also known as the gel phase. The commonly seen gel phase (L'_β) is analogous to the lamellar hexatic phases of liquid crystals in which chains are tilted with respect to the layer normal. Some phospholipids also form a gel phase with untilted chains (L_β), i.e., chains oriented along the layer normal.

In addition to these laterally uniform multilamellar phases, hydrated phospholipids are known to form phases with periodic in-plane modulations in the membrane shape (see review by MacKintosh [4]). The best-known example is the ripple phase (P'_β), which often interposes between L_α and the tilted L'_β, and consists of the stacking of bilayers that wrinkle into asymmetric sawtooth-like profiles (e.g., [5]). There has been a great interest in the origin of the ripple phase and a number of competing theoretical models exist [4]. In particular, the model by Chen *et al.* [6,7] (hereafter referred to as the CLM model), based on the coupling between chain tilt and membrane shape, is unique in that it not only reproduced the ripple phase, which is one-dimensionally modulated, but also predicted a more exotic two-dimensional (2D) modulated phase with a square in-plane lattice. The actual observation of structures that are reminiscent of this square phase has been reported in a freeze-fractured electron microscopy study in which both structural modulations with egg carton-like square symmetry and ripplelike stripes were observed in cellular membranes and in model membranes of extracted lipids [8]. However, their extremely rare occurrence has so far prevented detailed experimental studies of 2D modulated phases that would provide further insights into the origin of modulated phases in general.

Here, we report an x-ray diffraction observation of a square modulated phase in multilamellar films of *pure* phospholipids with saturated chains. For ditridecanoyl-phosphatidyl-choline (DTPC; 13:0 hydrocarbon chains), this phase is found within a narrow RH range (Fig. 1) between L_β and L_α at high T and between P'_β/L'_β and L_α at low T. The observed topology of the phase diagram and the lateral non-

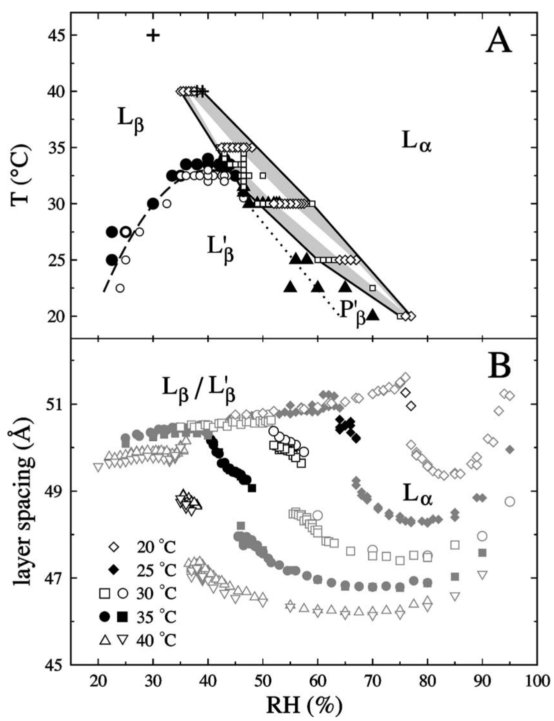

FIG. 1. (a) The phase diagram for DTPC. The solid lines bracket the range over which the square phase was observed, identified by either distinct layer spacing (◇) or satellite peaks (□). The gray area represents coexistence of the square phase with a neighboring phase, based on layer spacing measurements. The dashed boundaries are based on the observations of ripple satellite peaks and/or the characteristics of chain-packing peaks. The 1D modulated phase is located at higher T (+). (b) Layer spacing as a function of RH at various T, based on the fourth-order layering peaks, with the data points from the square phase in black and other phases in gray. All data points were reproduced upon raising and lowering the RH. For each given T, L_α appears at high RH and the layer spacing increases rapidly with RH as the lipid film takes up water. Lowering the RH induces a phase transition from L_α into a more ordered structure.

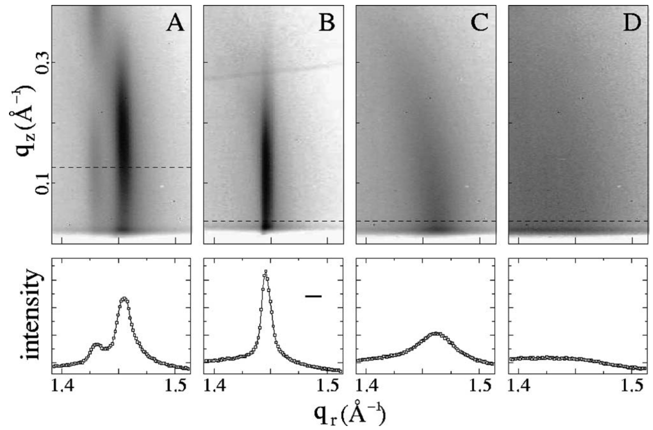

FIG. 2. The chain-packing peaks and their q_r profiles observed in (a) L'_β (30 °C, 48%); (b) L_β (43 °C, 31%); (c) square (30 °C, 54%); and (d) L_α (30 °C, 61%) phases of DTPC. The measurements were made with $\lambda = 1.626$ Å, an incident angle of 1°, the beam footprint of 10 mm on the sample, and a sample-to-detector distance of 1 m, corresponding to an in-plane q resolution of ~0.014 Å^{-1} [indicated by the bar in (b)]. The dashed lines indicate the q_z position of the q_r profiles.

uniformity of the chain tilt in the square modulated phase are consistent with the CLM model.

We studied the structure of highly aligned (mosaic spread <0.015°) DTPC multiple bilayers in the form of both substrate-supported films and freestanding films [14] at various T and hydration. We controlled sample hydration by adjusting the temperature of a water reservoir and therefore the RH in the sample chamber [9]. The x-ray measurements were carried out on beamlines X21 (operated at $\lambda = 0.743$ Å) and X16B ($\lambda = 1.626$ Å) at the National Synchrotron Light Source (NSLS). For each (T,RH) setting, we measured the layer spacing with a θ–2θ scan and collected the off-specular small- and wide-angle diffraction pattern using a 13.3 cm MarCCD detector in glancing-incidence reflection geometry. Since the samples are well aligned, diffraction peaks arising from layer stacking, in-plane electron density modulation, and chain packing can be easily distinguished from each other. With q_z and $q_r = (q_x^2 + q_y^2)^{1/2}$ denoting the components of the wave-vector transfer normal to and parallel to the layers, respectively, the chain-packing peak(s) typically occur at $q_r \sim 1.4$ Å^{-1}; whereas the possible in-plane modulation structure manifests as satellite peaks around the layering peaks at ($q_r = 0, q_z = 2n\pi/d$), where d is the bilayer repeat distance and n is an integer. The use of the CCD detector minimized the exposure time required for each diffraction pattern, and thus prolonged the lifetime of each sample. For high q-resolution measurements, we increased the distance between the sample and the detector and/or reduced the beam footprint on the sample in the direction of x-ray incidence.

The phase diagram shown in Fig. 1(a) is based on the data collected from DTPC films over a wide range of RH and T. The results of layer spacing measurements are summarized in Fig. 1(b), and representative chain-packing peaks are shown in Fig. 2. At lower T, DTPC behaves like other longer-chain lipids (e.g., dimyristoyl-phsphatidyl-choline (DMPC) 14:0 hydrocarbon chains, [2]) and displays the L_α, P'_β, and L'_β phases. At higher T, the P'_β phase is not observed, and the lipid chains in the low-RH ordered phase become perpendicular to the lipid layers (L_β). The L_α phase can be identified by its characteristic diffuse chain-packing peak [Fig. 2(d)] and distinguished from the gel phases by the abrupt d-spacing change across the main transition. The two gel phases differ in that the chain-packing peaks split and are lifted to nonzero q_z for the tilted L'_β [Fig. 2(a)], whereas those for the untilted L_β remain degenerate and centered at $q_z = 0$ [Fig. 2(b)]. The P'_β phase is identified by the appearance of satellite peaks that form a cross pattern centered at the layering Bragg peaks (e.g. [10]).

In addition to these known phases, a new phase appeared in narrow RH ranges between L_α and the more ordered lamellar phases. The layer spacing in this phase is distinct from and intermediate between that of L_α and L'_β/L_β [Fig. 1(b)]. Although a similar intermediate d spacing was observed previously for freestanding films of DMPC at 40 °C [3], the corresponding structure was neither elucidated nor speculated.

The most visible signature of this new phase is the appearence of satellite peaks in the off-specular diffraction patterns [Fig. 3(a)] around each layering peak at the same q_z and small q_r, indicating that in-plane ordering had developed. In order to resolve these tightly spaced satellites, we performed high resolution measurements at a selected set of (T,RH). We used a beam size of 0.1 mm in the direction parallel to the lipid layers, comparable to the full width at half maximum (FWHM) of the CCD detector point-spread function. With the CCD detector at 1.8 m away from the sample, the instrumental q_r resolution was ~0.0007 Å^{-1} at $\lambda = 0.743$ Å. These measurements clearly show that the positions of these satellite peaks correspond to an in-plane square lattice [Fig. 3(b)]. Remarkably, the square lattice constant depends strongly on T and RH and spans a range from ~200 Å at 20 °C to ~600 Å at 40 °C [Fig. 4(a)]. When plotted as a function of average layer spacing [Fig. 4(b)], these data sets appear to fall on a single curve, with a d-spacing reduction of only ~2.3 Å resulting in a monotonic, threefold increase in the square periodicity. The lattice constants are significantly larger than both the nearest-neighbor chain-chain distance (~5 Å) and the length of the chains (a few nanometers), indicating that some sort of 2D electron density modulation must be present within each layer. Furthermore, the fact that these satellites are observed around the layering peaks, at $q_z \neq 0$, indicates that the square-lattice modulation is correlated from one bilayer to the next.

This square phase also shows a unique chain-packing peak [Fig. 2(c)]. A comparison of peak widths (in q_r) clearly indicates that the chain arrangement in the square phase displays *positional* order that is intermediate between that of L_α and L'_β/L_β. On the other hand, the most intense spots in Figs. 2(a) and 2(c) (both at 30 °C) occur at nearly the same $q = (q_r^2 + q_z^2)^{1/2}$ (~1.465 Å^{-1}), implying that at least part of the square phase exhibits as dense *local* packing as in L'_β and thus consists of all-*trans* chains. The *orientational* order of chains in the square phase, characterized by a diffuse peak profile along an arc of fixed q [Fig. 2(c)], is also poorer than

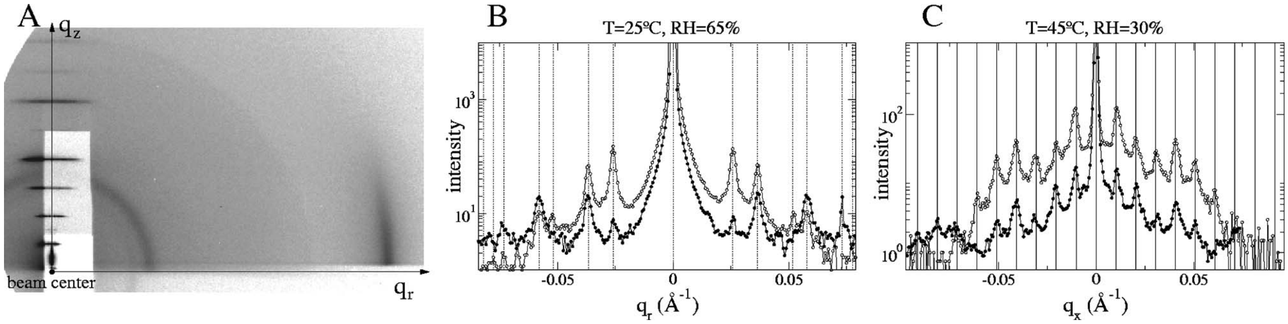

FIG. 3. The DTPC satallite peaks (streaks) located at the same q_z as each layering peak in the glancing-incidence small- and wide-angle diffraction pattern [(a), the ring between the third- and fourth-order layer peak is due to the kapton window on the sample chamber] are resolved in the high-resolution measurements. Representative in-plane (fixed q_z) diffraction profiles as a function of q_r/q_x, about the second- (open circles) and third-order (filled circles) layering peaks are shown for the square phase (b), and the high temperature 1D modulated phase (c). The peak positions were indexed to a square lattice (dotted grid lines) and a one-dimensional lattice (solid grid lines), respectively.

in L'_β/L_β, for which rodlike q_z profiles are observed [Figs. 2(a) and 2(b)]. This suggests that local chain tilt is not fixed but may vary continuously over the unit cell of the square lattice.

The 2D square modulated phase persists up to ~40 °C and is replaced by a one-dimensional (1D) modulated phase at higher T [Fig. 3(c)], which also vanishes above 50 °C. Unlike the stable 1D modulated P'_β at low T, this high-T phase shows satellite peaks at the same q_z as that of the associated layering peak, a feature that is shared also by the *symmetric* ripple phase, which was reported to be metastable [10]. However, its in-plane lattice constant is ~620 Å, compared to the typical ripple periodicity in P'_β in the range of 130–160 Å [10,11], and that of the metastable symmetric ripple phase at ~260 Å [10]. Given that the combination of raising T and lowering the RH leads to a larger lattice constant in the square phase, this 1D modulated phase at high T may be related to the metastable ripple phase at low T as a reentrant phase.

The square phase reported here is different from the modulated crystalline-B phase with square lattices found in thermotropic liquid crystals [12]. In this latter phase, the liquid crystal molecules actually form a three-dimensional (3D) crystal. In contrast, in the square phase of DTPC, the in-plane *molecular* arrangements in neighboring layers remain uncorrelated with each other. This is clearly shown by the chain-packing peak in Fig. 2(c). Had there been significant interbilayer correlations in chain packing, this peak would have displayed intensity modulation in q_z at a periodicity identical to that of the layer peaks. This phase is also different from the L_δ phase reported in [1], where the lipid *chains* pack in a square lattice.

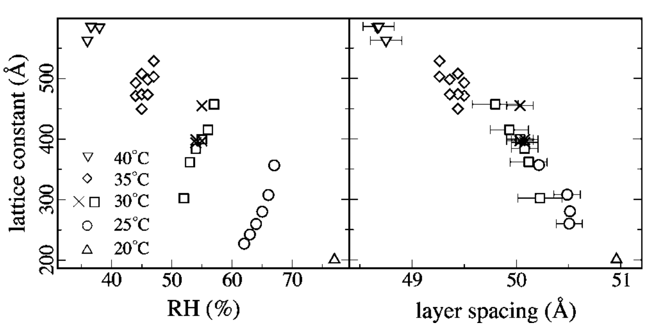

FIG. 4. The DTPC square lattice constant at five different T, plotted against environmental RH (left) and average layer spacing (right). Freestanding film data are shown as ×.

Unlike some of reported ripple phases (e.g., the *symmetric* ripple phase [10]), the square modulated phase appears to be stable. We have observed the square lattice repeatedly and reproducibly in DTPC and DLPC (data not shown), regardless of the direction in which T and RH were changed. For DTPC, the square satillites were clearly seen in both substrate-supported and freestanding films, indicating that this phase is a property of the bulk sample. The square lattice was also stable over an extended period of time (>12 h). On the other hand, it is also worth noting that, though repeatedly reproduced, close to the boundaries (solid lines) in Fig. 1(a), the diffraction patterns from the square phase are often accompanied by that of the neighboring phase; the P'_β satellite peaks at the square-L'_β boundary also appear much weaker than those observed at lower T. This could be because the surface layers of the lipid film behave differently than the bulk of the sample. A more systematic study of film thickness dependence is being planned.

Among the existing theoretical models that dealt with modulated lipid phases, only the CLM model predicted both the ripple and square modulated phases, both of which appear in the phase diagram of DTPC. This model emphasizes the coupling between *variations* in the local chain tilt and the shape of membranes as a determinant factor in the formation of modulated phases [6,7]. This is consistent with our experimental data, which suggest both the presence and nonuniformity of chain tilt in the square phase. The predicted phase behavior of modulated phases was presented as a phase diagram (Fig. 9 of [6]) of temperature versus a reduced elastic constant, whose explicit dependence on lipid temperature and/or hydration is not known. A direct comparison to our results is therefore difficult, although it does seem plausible that higher hydration would lead to less rigid membranes. It should be noted that the CLM model is based on chain tilt and does not account for possible chain disordering or melting that accompanies the transition to L_α. Moreover, the model predicts a second-order transition between L'_β and the zero-tilt ($m=0$ in [6]) phase. Since the transition between L_α and L'_β is known to be first order, the $m=0$ phase in their phase diagram could be the zero-tilt gel phase L_β instead of L_α, while the real L_α should appear at higher T. If this interpretation is correct, the similarity between the theoretical phase diagram and Fig. 1(a) is striking, and the point $(T,\text{RH}) \sim (32\ °\text{C}, 45\%)$ where the modulated phases meet L_β and L'_β corresponds to the Lifshitz point.

The CLM model does not explain some of the features in our results. Firstly, the phase transitions between the square modulated phase and L_β appears to be first order, as evidenced by the discrete change in layer spacing across the phase transition, while the CLM model predicts a second-order transition between the square and zero-tilt phases. Secondly, the CLM model may be too simplistic to account for the apparent monotonic increase of square lattice constant with decreasing average layer spacing [Fig. 4(b)]. The average layer spacing, whose decomposition into lipid and water sublayers is yet to be determined for the square phase, is influenced by chain conformation and the hydration interaction between adjacent bilayers, which are not included in the CLM model. Investigations into how these two characteristic lengths are related, in particular the role of interbilayer interaction (e.g., [13]), may provide further insights into the understanding of the square modulated phase.

In summary, we have presented strong evidence that DTPC films exhibit a modulated phase where a 2D intrabilayer electron density modulation exists and forms a well-defined square lattice. In this phase, (i) the average layer spacing and lateral positional order are distinct from, and intermediate between, L_α and L_β/L'_β; (ii) at least a fraction of the chains are all-*trans* and their *local* packing density is close to that in the neighboring L_β/L'_β; and (iii) local chain tilts exist and their magnitudes vary laterally. Though the exact nature of the modulation, the details of the chain-packing structure, and the possible correlations between them remain yet to be uncovered, our data agree with the general features of the square modulated phase predicted by the CLM model.

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[14] The lipid was purchased from Avanti Polar Lipids (Alabaster, AL) and used as delivered. Substrate-supported samples ($\sim$0.4 mg per sample) were prepared on clean 10×20 mm^2 Si substrates following the method described in [9]. Freestanding films were prepared by spreading a paste of lipid-water mixture at RH$>$90% on an $\sim 4 \times 16$ mm^2 aperture on an aluminum base.