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Anomalous swelling in phospholipid bilayers is not coupled to the formation of a ripple phase

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Aligned stacks of monomethyl and dimethyl dimyristoyl phosphatidylethanolamine (DMPE) lipid bilayers, like the much studied dimyristoyl PC (DMPC) bilayers, swell anomalously in a critical fashion as the temperature is decreased within the fluid phase towards the main transition temperature, T_M. Unlike DMPC bilayers, both monomethyl and dimethyl DMPE undergo transitions into a gel phase rather than a rippled phase below T_M. Although it is not fully understood why there is anomalous swelling, our present results should facilitate theory by showing that the formation of the phase below T_M is not related to critical phenomena above T_M.

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Lipid molecules are of general interest not only because they are the major structural component of cell membranes but also because they present interesting examples of phase transitions. Phase transitions in lipid bilayers are challenging phenomena for physical theory because there are many different kinds of order parameters that may be involved. In the case of both dimyristoyl PC (DMPC) and dipalmitoyl PC (DPPC) bilayers (see Fig. 1 for chemical structure) the pretransition transforms flat bilayers ($L_{\beta'}$ phase) to rippled bilayers ($P_{\beta'}$ phase) [1–3]. The $L_{\beta'} \rightarrow P_{\beta'}$ transition is particularly intriguing and has over the years attracted the interest of many experimentalists [1–6] and theorists [7–12]. Of the phase transitions occurring in lipid-water systems, the best understood is the main transition at T_M which is driven by the conformational disordering of the hydrocarbon chains of the lipid molecules [13]. However, there are poorly understood aspects even of this transition which will be addressed in this Rapid Communication.

As the temperature is lowered from the conformationally disordered, fluid (L_α) phase, the lamellar repeat spacing increases dramatically in bilayers such as DPPC, DMPC, and DLPC [14–20]. This anomalous swelling, also referred to as ''pseudocritical'' [14] or ''precritical'' [19], seems strikingly similar to the critical behavior that has been suggested for many years in these systems [13]. Although the main transition is ultimately a first order transition, it is quite plausible that there is a critical point lurking nearby in a high dimensional thermodynamic parameter space. However, even though various theories have been devised to explain anomalous swelling [14,16,19] a satisfactory understanding of the important order parameter(s) remains unclear [19]. It is significant that anomalous swelling does not occur in all lipid bilayers, a notable example being dimyristoyl phosphatidylethanolamine (DMPE).

A different puzzle for theory regarding the main transition concerns the participating phases. For lecithins such as DMPC and DPPC, the phase just below T_M is the ripple phase, whereas for phosphatidylethanolamines (PEs) such as DMPE and DPPE the phase below T_M is the flat gel phase [21]. This difference in phase behavior is correlated to a structural difference in the gel phases. The conformationally ordered chains of the PEs are aligned along the normal to the bilayer. This phase is commonly known as a smectic *A* or L_β phase. In contrast, the hydrocarbons for the disaturated lecithins are tilted with respect to the bilayer normal and this latter phase is commonly known as a smectic *C* or $L_{\beta'}$ phase, with refinements due to orientation of the tilt direction [22]. It has been established that the presence of a ripple phase is tightly coupled to having tilted fatty acid chains in the gel phase [23]. However, one should add the caveat that some lipids have a ripple phase without having any gel phase, only a subgel phase [24] which involves, in addition, an ordering of the PC headgroups [25]. While indirectly related to the nature of the main transition, the relation between gel phases

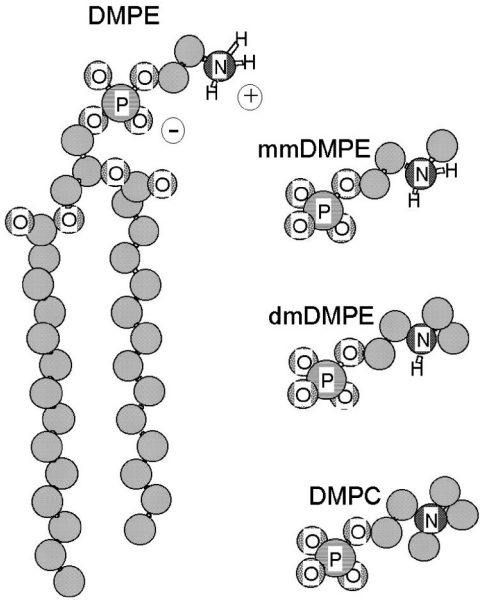

FIG. 1. Schematic of a DMPE molecule and the various methylated forms of the PE headgroup. One methylation creates the mmDMPE lipid while two methylations result in dmDMPE. DMPC has three methyl groups associated with its amide group. The fatty acid chains in all of these lipids are 14 carbons long. In contrast, DPPC and DLPC have hydrocarbon chains that are 16 and 12 carbons long, respectively.

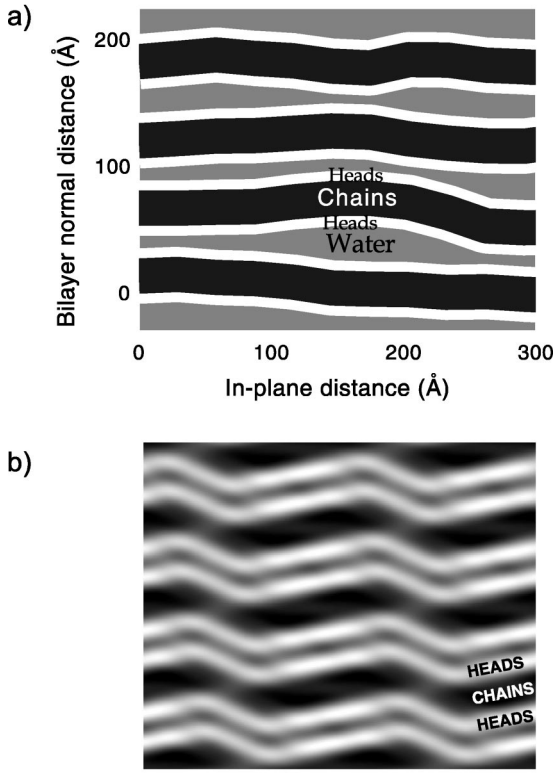

FIG. 2. Comparison of (a) simulated fluctuating fluid phase (L_α) bilayers [34] and (b) electron density map of rippled ($P_{\beta'}$) DMPC bilayers [5] having a ripple wavelength of 141.7 Å.

and the existence of a ripple phase is more closely related to the pretransition and the subtransition ($L_{\beta'} \rightarrow L_{c'}$).

Returning to the main transition, the possibility that anomalous swelling in the fluid phase is associated with a transition into a ripple phase has been strongly suggested [26]. This suggestion may be reinforced by comparing the structure of the ripple phase with the fluctuations that occur in the fluid phase. Although the bilayers in the fluid phase are on average, flat, at any moment they exhibit appreciable undulation fluctuations shown by the simulation in Fig. 2(a) [34], whose experimental root mean square average is about 9 Å for fully hydrated DPPC bilayers at a temperature 9 °C above T_M [27]. Because there are considerable amounts of water between the bilayers, the interbilayer interactions are small enough that the fluctuations between neighboring bilayers are weakly correlated, as expected for a fluid phase. One interesting theory of the anomalous swelling effect is that the bilayers become even more flexible near T_M, causing the fluctuations and the water space to increase even further [14,18]. At T_M the hydrocarbon chains ''freeze'' and water is expelled, bringing neighboring bilayers closer together and strengthening the interactions between them. Undulations might then become correlated into the solidlike pattern of the ripple phase shown in Fig. 2(b), which has a ripple amplitude of 19 Å. On the other hand, it has been suggested that the nature of the low temperature phase should have no effect on anomalous swelling and is a property of the L_α phase, alone [16,19]. In this paper we address these different views experimentally. We use a sequence of

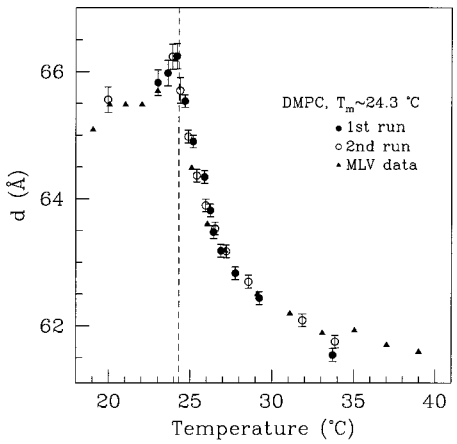

FIG. 3. D as a function of T for cooling runs ($L_\alpha \rightarrow P_{\beta'}$) of DMPC multibilayers. The circles, both open and closed, represent neutron data from aligned, fully hydrated bilayers, while the triangles are x-ray diffraction data from Ref. [19] using perdeuterated lipids which experience a T_M at $\approx$20 °C. T_M for the multilamellar vesicle (MLV) or so-called powder data was thus shifted upwards by 4 °C to allow for a comparison between the two data sets.

lipids that differ only in the degree of methylation of the choline head group (see Fig. 1). The specially synthesized monomethyl DMPE (mmDMPE) and dimethyl DMPE (dmDMPE) lipids give results which unambiguously show that anomalous swelling in lipid bilayers is not coupled to the formation of a ripple phase.

Fully hydrated aligned bilayers [mosaic $<1°$, full width at half maximum (FWHM)] immersed in water were prepared on silicon substrates as previously described [28]. Lipids used in the present experiments were obtained from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Calorimetric scans of all lipid-water mixtures exhibited transition temperatures in excellent agreement with published results [21,29,30]. Neutron diffraction experiments were carried out at Chalk River's NRU reactor using the N5 (for DMPC) and the C5 (all other lipids) triple-axis spectrometers. Neutrons of wavelength 2.37 Å were selected using the (002) reflection of a pyrolytic-graphite monochromator (mosaic of $\simeq 0.4°$) and a graphite filter was used to eliminate neutrons from higher-order reflections. The instrumental resolution for the C5 spectrometer was calculated and experimentally verified to be 0.004 $Å^{-1}$ (ΔQ,FWHM) while that of the N5 spectrometer was 0.008 $Å^{-1}$ (ΔQ, FWHM). Temperature stability, using a PolyScience (Niles, IL) water circulator, was ± 0.05 °C.

Figure 3 shows the temperature dependence of the repeat spacing d of aligned, fully hydrated DMPC multibilayers. Above T_M, the rapidly changing slope of $d(T)$ shows the anomalous swelling that has been repeatedly observed in unaligned, powder samples of many lipid multibilayers [14–17,19,26,31]. One aim of this figure is to show that the properties d and T_M of aligned samples are identical to their unaligned counterparts when care is taken to assure full hydration [28] so that not even a vestigial vapor pressure paradox remains [32]. This is further evidence that the interlamellar forces and fluctuating conditions are the same for

both fully hydrated aligned and powder preparations.

Figure 4 shows how d varies with temperature for fully hydrated aligned multibilayers of (a) dmDMPE, (b) mmDMPE, and (c) DMPE. For all three samples, T_M was found to be in excellent agreement with values previously reported using differential scanning calorimetry [21,30]. The difference between DMPC and dmDMPE of just one CH_3 group in the phosphorylcholine headgroup (Fig. 1) results in slightly different values of d, when comparing L_α phase bilayers, and very different values of T_M [Figs. 3 and 4(a)]. Despite these differences dmDMPE bilayers exhibit anomalous swelling [Fig. 4(a)]. Removal of another methyl from DMPC results in differences in d and T_M between DMPC (Fig. 3) and mmDMPE [Fig. 4(b)] that are more pronounced than in the case of dmDMPE. Figure 4(b) shows that mmDMPE bilayers also exhibit a nonlinear increase in d as T_M is approached from above. The removal of all three CH_3 groups from the phosphorylcholine headgroup of DMPC to form DMPE (Fig. 1) results in considerably different values of both d and T_M [Fig. 4(c)]. The most striking difference, however, is that there is no anomalous swelling since d only increases linearly with T near T_M [Fig. 4(c)].

Clearly, dmDMPE and mmDMPE exhibit anomalous swelling, though to a somewhat lesser extent as methyl groups are successively removed from DMPC. It should be emphasized that anomalous swelling does not mean that there is a maximum in d at T_M. Although such a maximum was previously considered to be an intrinsic feature [14,18], data supporting such a maximum have been controversial because of difficulties in obtaining reliable and reproducible d values in the ripple phase [19,33]. A recent study of the dependence of anomalous swelling on hydrocarbon chain length further substantiates the view that a maximum in d at T_M is not tied to the fundamental phenomenon of anomalous swelling [31].

A diffraction study of unaligned powder samples strongly indicated that there is no ripple phase in either dmDMPE or mmDMPE [21]. Because low resolution powder diffraction is sometimes difficult to interpret clearly, we examined the mmDMPE and dmDMPE phases below T_M at the Cornell High Energy Synchrotron Source using fully hydrated, aligned samples and a charge-coupled-device detector as previously described [3]. Ripple phases in such aligned samples have striking and unambiguous diffraction patterns [3], which in the case of both mmDMPE and dmDMPE bilayers below T_M, were absent.

The result that anomalous swelling and rippled bilayer formation are uncorrelated agrees with the view [16] that assumed that the thermodynamic behavior of the two phases involved in the main transition are essentially unrelated, as in a classical first order transition. There are two Gibbs functions that are analytically unrelated, except in the fact that they intersect. Of course, first order transitions are often terminated by critical points. Then, as the critical point is approached the two phases become increasingly alike and the Gibbs free energies become strongly correlated. The conclusion that the main transition in lipid bilayers lies close to a critical point and the comparison made in Fig. 2 suggests that the hypothesis that anomalous swelling could be related to

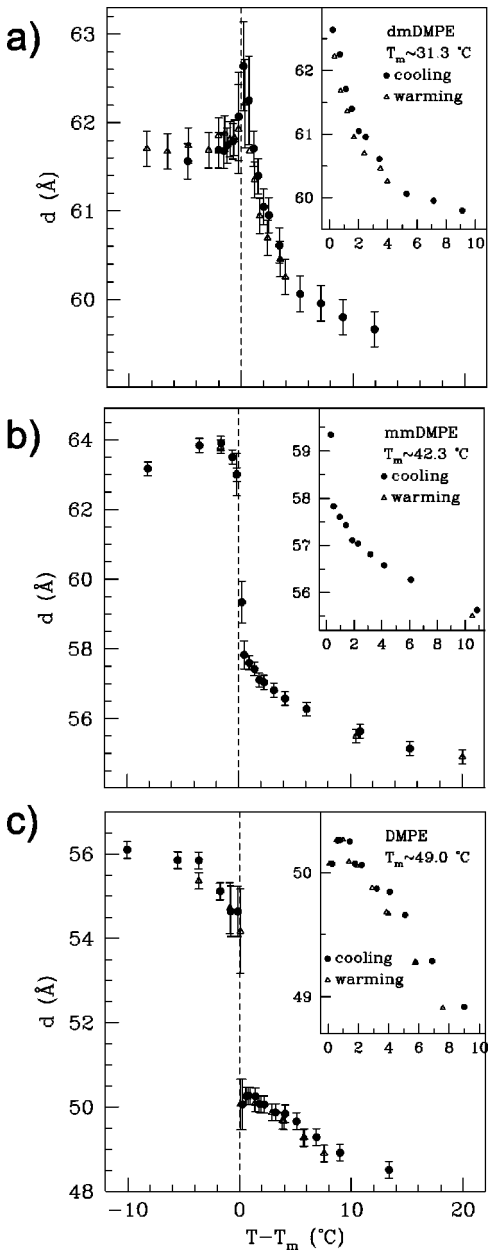

FIG. 4. $D(T)$ data on both cooling (●) and warming (△) for (a) dmDMPE, (b) mmDMPE, and (c) DMPE bilayers. From the data there is no evidence of hysteresis between the cooling and warming runs. Insets highlight the anomalous swelling region between T_M and 10 °C above T_M.

formation of a ripple phase was a reasonable one. However, the existence of anomalous swelling and the absence of the ripple phase in both mmDMPE and dmDMPE bilayers disprove this hypothesis.

In conclusion, the anomalous swelling exhibited by DMPC, dmDMPE, and mmDMPE bilayers appears to be related to an, as yet, unobtainable critical point that is a continuation of the fluid phase Gibbs free energy surface. The main transition is first order because the free energy of either the ripple phase or the gel phase is lower below T_M. Criticality of the anomalous swelling type is not seen in these low temperature phases, suggesting that they cannot be analyti-

cally continued to the imputed critical point. The existence or nonexistence of a ripple phase for bilayers of a particular lipid is then determined entirely by the competition between the ripple phase and the gel (or subgel) phase. We have shown that anomalous swelling occurs independently of whether or not the system has a ripple phase. The converse question, whether the formation of the ripple phase below T_M requires anomalous swelling in the fluid phase, is still open experimentally, since we know of no counterexamples. However, the apparent independence of these two phases suggests that anomalous swelling in a fluid phase bilayer is not essential for a transition into a ripple phase. We therefore suggest that future theoretical development need not be concerned with the arduous task of modeling details of both aspects of the main transition simultaneously but may divide these problems into more manageable pieces.

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