Critical Swelling of Phospholipid Bilayers

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We reexamine a critical phenomenon of phospholipids in lamellar phases. The question is, Is the anomalous divergence in the repeat spacing near the main transition the result of a divergence of the water layer or of the lipid bilayer? X-ray diffraction of DLPC lamellae was measured in partially dehydrated conditions. Its critical behavior is much more pronounced than the previously studied DMPC. The bilayer thickness was calculated from dehydrated conditions and then extrapolated to full hydration. The results show that the anomalous divergence is primarily due to the water layer expansion. [S0031-9007(97)04513-4]

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There has been a great deal of interest in the physical properties of phospholipid bilayers because of their relevance to biological membranes. One important characteristic of lipid bilayers is the main (phase) transition where "melt," fluidlike hydrocarbon chains discontinuously change to "stiff," extended forms upon cooling. A consensus based on both theoretical and experimental studies is that the main transition is in the vicinity of a critical point [1]. This critical point T_c was estimated to be about 260 K [2,3]. On the other hand, the main transition temperature T_m depends on the chain length of the lipid n_c, the longer the chain, the higher the transition point. Evidence shows that T_m approaches T_c when the chain length $n_c \sim 9-10$ [2]. For $n_c > 10$ this critical point is preempted by the first-order main transition. Nonetheless, some pretransitional critical behaviors are evident. Particularly in the lamellar phase, where roughly parallel lipid bilayers are separated by water layers, the repeat distance D increases anomalously as the temperature lowers toward T_m. What is the cause of the D spacing divergence? Is it the result of a divergence of the water layer thickness or that of the lipid bilayer? On the surface, this seems to be a simple question. However, undulation fluctuations of lipid bilayers make the measurement of the bilayer or the water layer thickness a nontrivial problem. This problem was recently investigated by Mouritsen's [4] and Nagle's [5] groups, but they reached conflicting conclusions: one favored a divergence by the water layers, another by the lipid bilayers.

These two interpretations underline two different mechanisms of critical fluctuations. Let the bilayer thickness be the order parameter of the critical transition. (A more or less equivalent choice for the order parameter is the in-plane cross sectional area per lipid molecule because the area is roughly proportional to the inverse of the thickness.) As the temperature approaches the critical point, increasing fluctuations of the lipid area soften the bilayer and reduce its bending rigidity. Consequently, Helfrich's steric repulsion between bilayers (inversely proportional to the bending rigidity) [6] is enhanced, hence a divergent water layer. Mouritsen's group proposed that this mechanism is the dominant cause of the D spacing divergence [4]. But the experiment by Nagle's group found no evidence of bilayer softening, so they concluded that it is the intrinsic nature of the main transition that lipids gradually extend their chains as the critical point is approached from higher temperatures [5]. The system studied by these two groups is dimyristoyl phosphatidylcholine or DMPC ($n_c = 14$). Its T_m at 297 K is about 37° above T_c. Thus its pretransitional critical phenomena are relatively weak. Another problem of the previous investigations was that their analyses were based on intrinsically low resolution data. They performed diffraction from hyperswollen lamellar phases that produced only a few very broad Bragg peaks. It is difficult to extract structural information (e.g., the bilayer thickness) from such data. Here we reexamine the problem with dilauroyl phosphatidylcholine or DLPC ($n_c = 12$) which has a $T_m = 272$ K just below the ice point, and its pretransitional critical behavior is much more pronounced than DMPC. In order to obtain high resolution data, we performed diffraction experiments in partially dehydrated states. We will then extrapolate the structural analysis to the state of full hydration. The results unambiguously show that the anomalous divergence in D spacing is due mainly to the water layer expansion.

X-ray diffraction of aligned lamellae was measured on a conventional diffractometer by θ-2θ scan. DLPC was purchased from Avanti Polar Lipids (Alabaster, AL), and used as delivered. Lamellar phases consisting of large monodomains were prepared on a clean glass slide [7] and were equilibrated inside a temperature/humidity chamber enclosing the goniometer head. The temperature of the glass slide was controlled to ± 0.025 °C. The chamber was connected to a water source whose temperature was adjusted to vary the relative humidity in the immediate vicinity of the lipid sample. A combined thermometer and hygrometer (accuracy 0.1 °C and 0.1% RH, respectively)

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was positioned next to the sample. The measured vapor temperature T_v and relative humidity RH_v were used to calculate the relative humidity for the sample $\mathrm{RH} = \mathrm{RH}_v \times$ (saturated vapor pressure at T_v/saturated vapor pressure at the sample temperature T). The osmotic pressure Π is defined as $\Pi = -(k_B T/v_w)\ln(\mathrm{RH})$, where k_B is the Boltzmann constant and v_w is the volume of a single water molecule. The sample was measured above the main transition, along isothermal lines as a function of Π. The range of Π was limited and varied with temperature (see figures). High Π's were limited by a dehydration induced liquid-to-gel phase transition [8]. The closer the T to T_m, the smaller the high Π limit. Low Π's were limited by practical reasons: (1) Near the full hydration, the sample had a tendency to flow off the substrate and had long equilibration times (>1 day). (2) The RH measurement became difficult and inaccurate. (3) Diffraction peaks became very broad, even the D spacings were difficult to determine. All of these limitations diminished the range of Π as T approached T_m.

For DLPC in low hydrations (high Π) the diffraction pattern consists of eight discernible sharp Bragg peaks. Within the resolution, the peak width was the same in all orders. As Π approaches 0, the increasing distance between bilayers weakens the forces between them. The consequence is an increase in the undulation fluctuations of the bilayers, which damp the higher order diffraction peaks and broaden the remaining ones. Furthermore, the peak broadening increases with the Bragg order. Such damping and broadening also occurred when T approached T_m at constant Π. Figure 1(a) shows the broadening of the second Bragg peak taken at 3.6 °C compared with the same peak taken at 10.4 °C at the same value of Π, as well as at a higher Π. Figure 1(b) shows that the broadening by hydration ($\Pi \to 0$) is much more severe as T approaches T_m. Such temperature-dependent broadening was not seen in DMPC. Zhang *et al.* [5] observed no broadening in DMPC (in excessive water) at 24.3 °C (only 0.3 °C above T_m) compared with the same sample at 33 °C, despite the difference of 3.6 Å in D spacing. This indicates that there are no significant pretransitional anomalous fluctuations in DMPC.

We analyzed all diffraction patterns of DLPC consisting of five or more discernible Bragg peaks, where the peaks are well defined and well separated. The electron density profile of a lipid bilayer consists of two peaks approximately at the positions of the phosphate in the lipid headgroup [Fig. 2(a) inset]. We define the bilayer thickness D_l as the peak-to-peak distance in the profile. Neutron diffraction has shown that water penetrates into the headgroup region [9]; therefore, there is no clear definition for the water layer thickness D_w. We define D_w as $D - D_l$. To obtain the electron density profile, we assume that the q (the momentum transfer) dependence in the diffraction intensity is entirely due to the bilayer

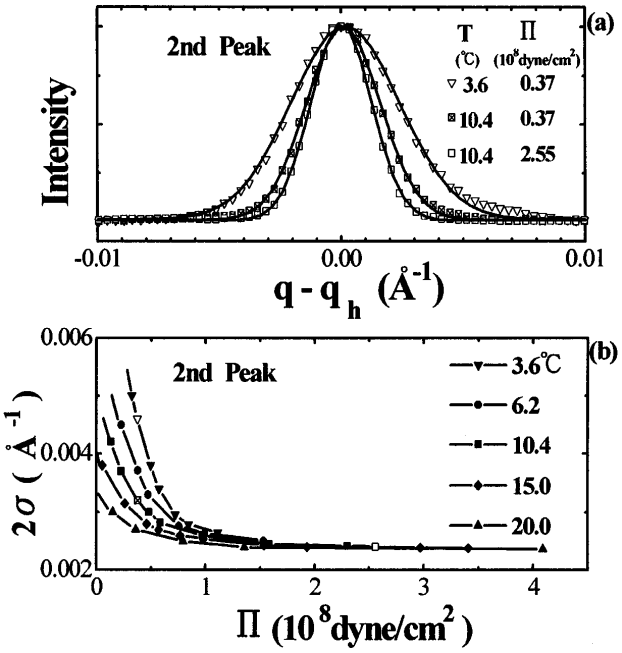

FIG. 1. (a) The line shape of the second Bragg peak at 3.6 and 10.4 °C (normalized to the same height). See the corresponding points in (b)—open symbols. The solid lines are Gaussian fits. (b) The Gaussian whole width 2σ of the second Bragg peak.

form factor. In other words, the damping effect in the structure factor is ignored. While this assumption involves errors, it has negligible effect on the peak-to-peak distance. This is seen as follows [10]. Suppose that the diffraction amplitude A_h of order h is modified by a Debye–Waller-type factor $\exp[-(\varepsilon/2)(2\pi h/D)^2]$, where ε is a constant indicating the strength of damping. Then the electron density profile ρ along the bilayer normal z is $\rho(z,\varepsilon) = \sum_h A_h \exp[-(\varepsilon/2)(2\pi h/D)^2]\cos(2\pi hz/D)$. The peak positions $\pm z_p$ are the solution of $(\partial\rho/\partial z)_{z=z_p} = \partial\rho/\partial z_p = 0$ as a function of ε. It is straightforward to show that the fluctuation correction for the peak-to-peak distance D_l is given by $\delta D_l = 2\delta z_p = -[(\partial^3\rho/\partial z_p^3)/(\partial^2\rho/\partial z_p^2)]\delta\varepsilon$. Since the peaks of the profile are in general approximately symmetric, $\partial^3\rho/\partial z_p^3 \sim 0$, the damping due to fluctuations does not affect the peak-to-peak distance D_l. Nagle *et al.* [11] have shown by an actual data analysis that, even when the damping and broadening effects in the structure factor are severe, neglecting the structure factor has little effect on the peak-to-peak distance.

The phases of the diffraction amplitudes were determined by the swelling method [Fig. 2(b) inset] [12,13]. Otherwise the data reduction procedure is straightforward [13,14]. Let the electron density profile of a bilayer be $\rho(z)$. Then the unnormalized diffraction amplitudes are Fourier transformed to obtain $\rho' = b\rho + c$, i.e., an unnormalized electron density profile with two unknown constants b and c. The peak-to-peak distance is a solution of $\partial\rho/\partial z = 0$ which is the same as $\partial\rho'/\partial z = 0$. Hence

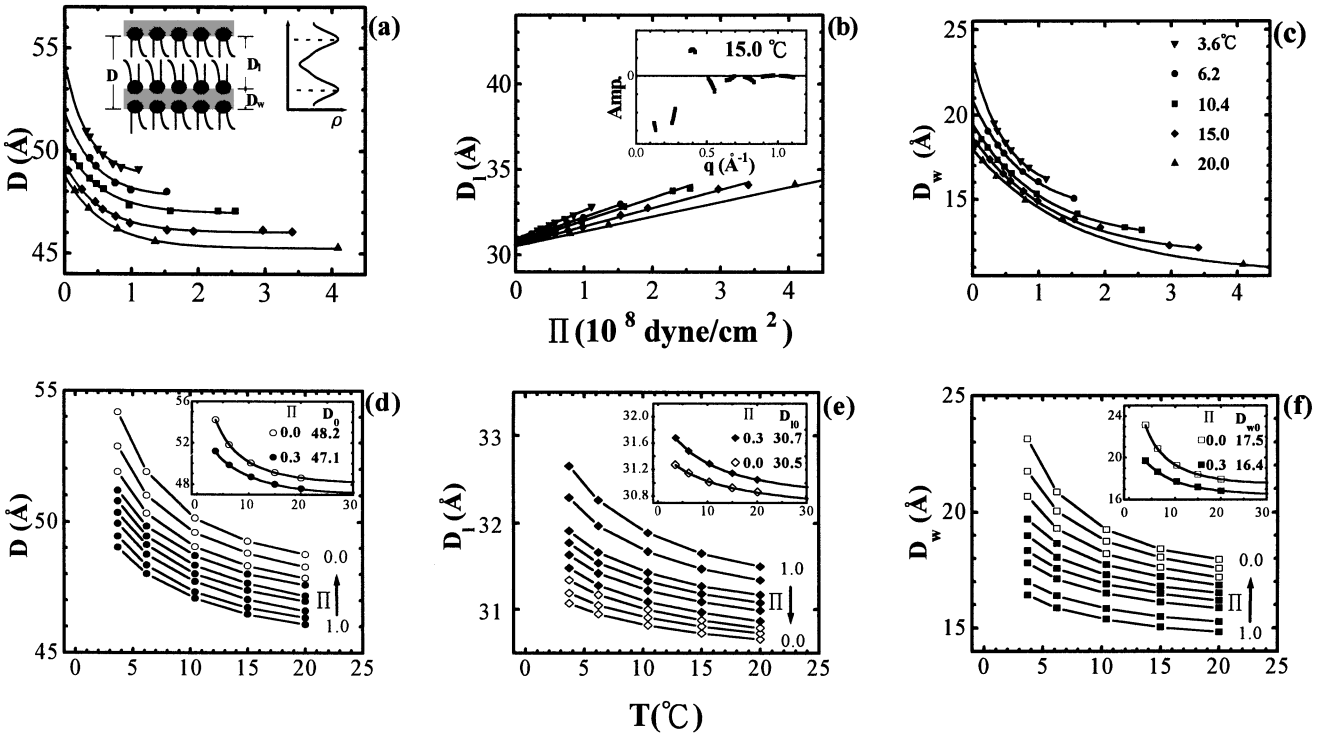

FIG. 2. (a) D spacing of DLPC as a function of Π at five different T's [symbols in (c)]. (b) D_l is calculated from the electron density profiles. (c) D_w is $D - D_l$ of (a) and (b). The solid curves for D, D_l, and D_w are four-order polynomial fits to the isotherms. (d)–(f) D, D_l, D_w of (a)–(c) plotted as functions of T for nine Π values: $(1.0, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.0) \times 10^8$ dyn/cm^2. The open symbols are the extrapolated values as shown by the solid lines in (a)–(c). The filled symbols are the data points. The insets: (a) schematic of one and one-half lipid bilayers. The black dots represent phosphorylcholine headgroups, each connects with two acyl chains. The shaded areas represent water. A schematic electron density profile ρ is plotted along the coordinate z normal to the plane of the bilayer. D, D_l, and D_w are defined as shown. (b) Shows an example of the phasing diagram (the swelling method). (d)–(f) Show multiple-exponential fits to obtain the high temperature plateaus D_o, $D_{\rm lo}$, and $D_{\rm wo}$ for $\Pi = 0.3$ and 0 (10^8 dyn/cm^2).

D_l can be determined from the unnormalized diffraction amplitudes. Figures 2(a)–2(c) show five isotherms for D, D_w, and D_l. For each temperature the data were fit by a four-order polynomial and extrapolated to $\Pi = 0$. This procedure is valid, provided there is no singularity at $\Pi = 0$. From these we constructed iso-Π curves as a function of temperature [Figs. 2(d)–2(f)]. Note that D and D_w have parallel Π dependence, that is, both increase as Π decreases, whereas the Π dependence of D_l is opposite, i.e., it decreases slightly with Π. However, all three quantities appear to diverge as T approaches T_m, although the degree of divergence for D_l is much less than D and D_w (note the scale of D_l was magnified). To make a quantitative analysis of these divergent behaviors, we first used multiple-exponential fits to estimate the high temperature plateaus D_o, $D_{\rm wo}$, and $D_{\rm lo}$ for D, D_w, and D_l, respectively [Figs. 2(d)–2(f) insets]. We then computed the ratios $r_w = (D_w - D_{\rm wo})/(D - D_o)$ and $r_l = (D_l - D_{\rm lo})/(D - D_o)$ for the lowest measured Π ($\sim 0.3 \times 10^8$ dyn/cm^2), as well as the extrapolated values at $\Pi = 0$ (Fig. 3). It is quite obvious that even at $\Pi > 0$ the anomalous divergence in D is due primarily to the anomalous divergence of the water layer [Fig. 3(a)]. As T approaches T_m at full hydration ($\Pi = 0$) the increase in D is completely dominated by the swelling of the water layer [Fig. 3(b)].

As mentioned above, the bilayer thickness is regarded as the order parameter for the main transition [3]. In classical models (phenomenologically described by a Landau theory), the order parameter undergoes pretransitional fluctuations, but its average magnitude remains constant above the critical point. Only the susceptibilities diverge. The weak divergence of the order parameter D_l shown in Fig. 2(e) is not describable by classical models. It must be an intrinsic characteristic of the lipid main transition, as suggested by Zhang *et al.* [5]. Their model predicts a chain extension as the transition is approached from higher temperatures.

On the other hand, the concurrence of the anomalous divergence of the water layer and the broadening of the diffraction peaks is a clear indication of bilayer softening as T lowered toward T_m, as proposed by Hønger *et al.* [4]. Lipowsky and Leibler [15] predicted that a continuous variation of a bilayer property can lead to a critical unbinding transition. It was first pointed out by Goldstein and Leibler [3] that thermal fluctuations of the order parameter may cause this new critical phenomenon.

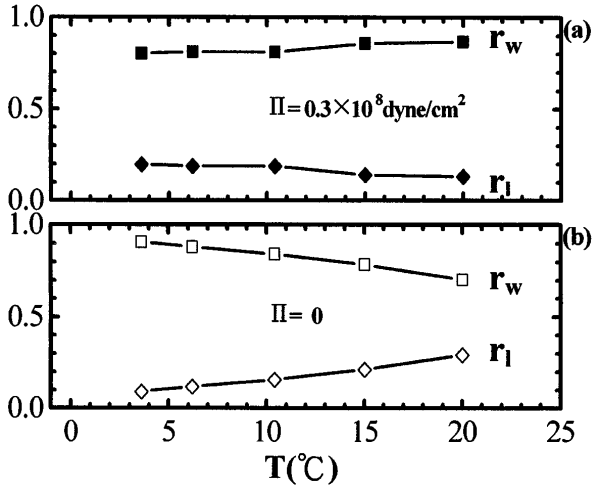

FIG. 3. The ratios $r_w = (D_w - D_{wo})/(D - D_o)$ and $r_l = (D_l - D_{lo})/(D - D_o)$ for (a) the lowest measured Π ($\sim 0.3 \times 10^8$ dyn/cm^2) and (b) for the extrapolated values at $\Pi = 0$. The ratios represent the contributions of water and lipid bilayer to $(D - D_o)$, the anomalous increase in D.

According to the theory of Lipowsky and Leibler [15], Lemmich *et al.* [4] suggested the anomalous divergence in D (in full hydration) be described by $D - D_o \approx (T - T^*)^{-\psi}$, where T^* is a new critical point for the unbinding transition (not to be confused with the T_c discussed above). However, with such a small range of value for D, the fitting value of ψ is very sensitive to the choice of D_o and T^*. With slight variations in D_o and T^*, the data [Fig. 2(d), $\Pi = 0$] fit a wide range of ψ almost equally well [16]. Thus the data are not capable of proving (or disproving) Lipowsky and Leibler's prediction $\psi = 1.0$.

Besides resolving the aforementioned controversy, another purpose of this Letter is to introduce the method of extrapolation from dehydration, for the determination of bilayer (and water layer) thickness. Experiments measuring the changes of these thicknesses with various parameters, such as the osmotic pressure or protein concentrations, are crucial to the studies of membrane-membrane interactions, e.g., [17], and of the energetics of membrane bilayers including membrane-protein interactions, e.g., [10]. The frequently used NMR method [18] and gravimetric method [19] have recently been critically reviewed by Nagle. The dehydration method has been successfully applied to a number of membrane-protein systems [10,20]. Here we show that it can even be used in the relatively difficult pretransitional regions of phospholipids.

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