CRITICAL ROLE OF FLUCTUATIONS IN LIPID BILAYERS

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Fluctuations play several major roles in lipid bilayers and their phase transitions. One role is in the approach to the main phase transition from the biologically relevant fluid (L_α) phase. Another role is in the interpretation of the low angle scattering intensities that yield structural information. Another is in the diffuse scattering at wide angle. The different kinds of fluctuations that contribute to these different phenomena are discussed in this chapter.

1 Introduction

Biology provides a fascinating array of materials for physical study, with complex structure and behavior, including unusual phase transitions. The initial challenge of such materials is to survey the phenomena over multidimensional parameter space where one does not know *a priori* precisely which are the most relevant parameters to reveal the important physics. Then, the challenge matures to providing quantitative theories and obtaining good quantitative data to test those theories. One possible outcome of such studies is to verify that the phenomena can be explained completely by straightforward combination of conventional concepts. A more exciting outcome is to discover that essentially new physics is present. The systems under discussion in this chapter are examples of materials in the latter class.

Lipid bilayers form the structural basis of biomembranes which, in turn, are essential to biology; indeed, one can not even have cells without membranes. While the entire biomembrane is very complex, containing a variety of different lipid molecules and a host of proteins that perform amazingly versatile biochemical functions, even the "simple" lipid bilayer consisting of only one kind of lipid molecule is complex by the standards of what is understood about the statistical mechanics of phases and phase transitions. This chapter will discuss only such "simple complex" membranes, as visualized schematically in Fig. 1.

A most striking experimental result for one component lipid bilayers is a "main" phase transition that is calorimetrically huge (5-10 kcal/mole) on a molar basis. Notationally, as T is increased, this transition takes place into a phase that I prefer to call simply the fluid phase; another name is the L_α phase; in biophysics, it is often just called the liquid-crystalline phase, but one should really distinguish this by the refined name smectic A. (Sometimes

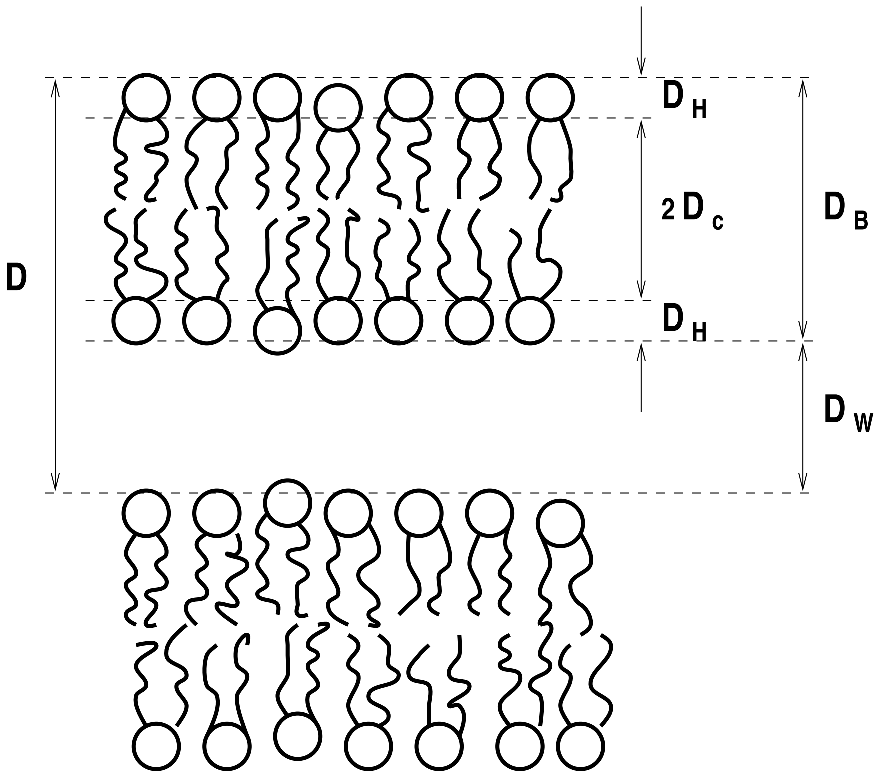

Figure 1: Sketch of two bilayers in the fluid L_α phase. Each circle represents the headgroup of a lipid molecule and the wavy lines represent the conformationally disordered hydrocarbon chains. Shown are the lamellar repeat spacing D, the overall bilayer thickness D_B, the pure water thickness D_W, the hydrocarbon chain thickness $2D_c$, and the headgroup thickness D_H. The D spacing is easily obtained from diffraction measurements; the other Ds are much more difficult to obtain. The head region also contains significant numbers of water molecules. Not all the headgroups are in the same plane, even on the short molecular length scale of this sketch, but the undulation fluctuations are on an even longer length scale.

the complexity of the material is mirrored by the multiplicity and confusion of the names.) The main phase transition involves conformational disordering of hydrocarbon chains [1] and so there is a great deal of similarity between this transition and the melting of the polymer polyethylene. The large transition entropy comes from the many internal conformational degrees of freedom available in long (14-20 methylenes) hydrocarbon chains. The transition enthalpy is due partly to the higher energy of disordered (gauche) conformations but at least half of the transition enthalpy comes from the work required for volume expansion; this is true both for the lipid bilayer main transition and for melting of polyethylene [2]. In addition to the similarities, there are important differences between these two phase transitions. The hydrocarbon chains in the fluid phase of polyethylene are spatially isotropic whereas the chains in the high temperature phase of lipid bilayers are still oriented preferentially perpendicular to the local bilayer plane. Theoretical modeling of the polymer melting transition [3] is sufficiently different that I will now focus on the lipid bilayer main transition. Because the average end-to-end distance of disordered hydrocarbon chains is smaller than for ordered (all-trans) chains and because one end of the chain is attached to a headgroup [a] which is more or less pinned at the aqueous interface (Fig. 1), the bilayer becomes thinner during melting at the transition, even though the lipid volume increases. This essential feature of the main transition is grounded in the polymeric nature of the hydrocarbon chains and the spatial anisotropy of lipid bilayers.

I was fortunate to discover a simple toy model [b] for the main transition that includes the essential physics and that is isomorphic to a basic statistical mechanical model that can be solved exactly.[5,6,7,8] The underlying statistical mechanical model is the K-model of Kasteleyn, who solved the model by the same method that he developed to re-solve the two-dimensional Ising model. Despite the fact that the same method solves both problems, the nature of the phase transition of the K-model is quite different from the Ising or lattice gas phase transition. The K-model specific heat diverges above the critical point T_K, but is finite and analytic below T_K, whereas the Ising model specific heat symmetrically diverges on both sides. To describe the result that K-model behavior looks critical (2nd order) on one side and first order on the other side, I once called this a $3/2$ order transition [3,7], but I also like calling it a Kasteleyn K-type transition, distinguishing it from an Onsager O-type transition [5]. The

[a] This chapter will primarily simplify the description of the lipid molecule into chains and heads. We have recently discussed more refined partitioning of the lipid molecule ; this is important for comparing our volumetric experiments with molecular dynamics simulations. [4]

[b] Although the model is a toy model in the sense of caricaturizing some physical features, when properly interpreted, it can yield quite reasonable values for a variety of measured quantities, such as changes in area, volume and enthalpy at the first order transition.[8]

K-type critical point is quite different in another crucial aspect - it has no order parameter of the usual sort.[5] The fact that the essential physics of the lipid bilayer system leads to a model whose exact critical behavior is distinctly different from conventional critical behavior is an exciting result. This suggests that if one pays attention to the specifics, then complex systems may teach us something rather than just act as proving grounds for conventional ideas and models.

The actual main phase transition in lipid bilayers is not a critical point, but a first order transition. This is understood in the context of the model by the fact that the critical point is only one point in a phase diagram that includes, most importantly, the lateral surface pressure π as an intensive thermodynamic variable with the conjugate extensive variable being the area A of the bilayer. When volume expansion is included in the model, the critical point is displaced away from the physically accessible value $\pi = 0$, so thermodynamic divergences, that would be encountered when the critical point is hit directly, are not realized. Instead, the toy model predicts that there should be "precritical" or incipient critical behavior on the high temperature side of the main phase transition. Such behavior requires enhanced fluctuations in thickness and area A; it has been proposed that the area fluctuations account for the anomalous increase in permeability of the bilayer to small ions as T approaches T_M from above.[9]

1.1 Precritical Behavior of $D(T)$

Recent neutron scattering[10] and x-ray[11] data have been obtained that strongly indicate precritical behavior on the high temperature side of the main phase transition. [c] Some of our recent data for the lamellar repeat spacing $D(T)$ are shown in Fig. 2. These data show that D increases dramatically as T approaches T_M from above. These data above T_M are robust and reproducible; although the original neutron study[10] obtained substantially different values of D, these were corrected in a later study by the same group[14], and all data show the same curvature, whether the lipid is deuterated DMPC, DMPC or DPPC. Attempts have even been made to obtain critical exponents and the critical temperature T_c from such data.[14,15] However, because the critical point is cut off by the first order transition, the temperature range is less than a decade in $log((T - T_c)/T_c)$; this is too small to extract either critical exponents or T_c reliably.

In contrast to the robustness of the $D(T)$ data above T_M, $D(T)$ data below

[c] Different kinds of data supporting precritical behavior have also been previously reported.[12,13]

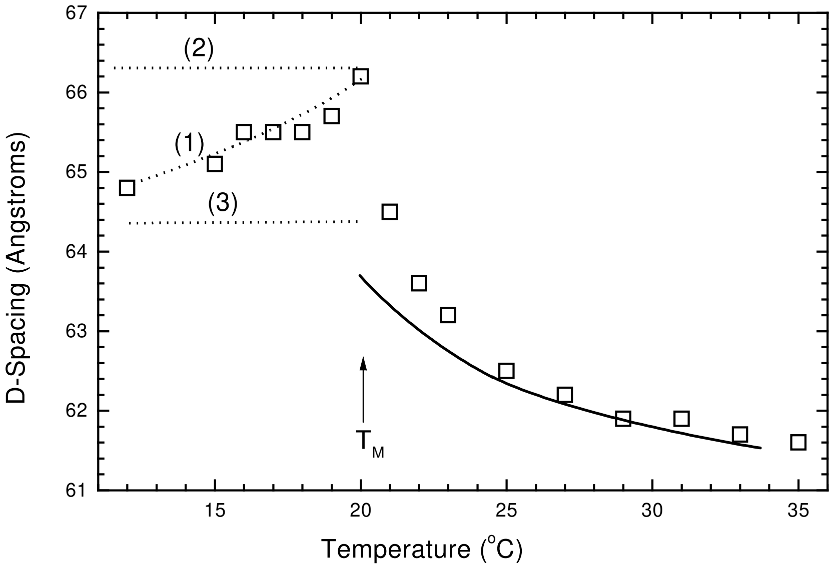

Figure 2: Data (open squares) show $D(T)$ for DMPC with perdeuterated hydrocarbon chains. The rapidly changing slope of $D(T)$ above T_M indicates precritical behavior. The solid line above T_M shows the thickness of the hydrocarbon chain region $2D_C$ as determined by NMR order parameters (courtesy of Dr. K. Gawrisch). The dotted lines below T_M indicate the behavior of $D(T)$ reported in various papers.

T_M are quite uncertain, even when reported by the same laboratory. Line (1) in Fig. 2 shows the temperature dependence of our recent data and the earliest data of Mouritsen's group [10]. Line (2) shows our earlier data [11] and line (3) shows later data from Mouritsen's group [14]. A reason for such differences will be given in the next section. The important point to emphasize here is that whichever line $D(T)$ follows for T below T_M is irrelevant to the interpretation of the $D(T)$ data for T above T_M because the transition is first order into a phase of different symmetry, as will be discussed in the next section.

An explanation for the precritical $D(T)$ behavior for T above T_M can be based on the above theory, namely, bilayers thicken critically due to reduced conformational disorder in the hydrocarbon chains. However, bilayers are complex systems and this turns out to be only part of the cause of this precritical behavior. Before discussing other parts, let us turn to a different aspect of the lipid bilayer problem, namely, the structural detail that is necessary to advance theoretical understanding of the phases.

2 Structure of Lipid Bilayers

Recent efforts of my research group have focussed on obtaining more quantitative determinations of the structure of lipid bilayers. This research involves a number of different lipids, but only the benchmark lipid DPPC will be discussed in this section. A major goal has been to obtain the structure of the biologically relevant fluid (L_α) phase discussed above. This has been attempted many times by a variety of methods (for additional references, see [16,17]), but this problem is a challenging one, due to the fluctuations that occur in this phase, and so we first turn to a description of other thermodynamic phases.

The thermodynamic phase of DPPC that is best characterized is the so-called gel or L'_β phase. The hydrocarbon chains in this phase are basically ordered in their all-trans ground states and lie parallel to each other. This gives rise to distinctive wide-angle scattering patterns (Fig. 3) that yield in-plane structure, such as the mean area/molecule A. The problem of determining the structure of this phase is made interesting and challenging by the tilting of the hydrocarbon chains.[d] Dr. Tristram-Nagle [19] led a group effort that used oriented films to obtain the tilt angle of about 32^o; this is the most difficult datum to obtain, but one that is key for obtaining the gel phase structure. The result for the tilt angle on oriented samples was confirmed by data on powder samples; surprisingly, the powder samples also allowed a fuller determination of structure because the data were more accurate. [20] Combining these x-ray results with polarized attenuated total reflection infrared data even allows estimates for a different order parameter for rotation about the long chain axes.[21] On the other hand, there is still an unresolved dichotomy regarding fluctuations in the gel phase. While the (2,0) wide angle scattering peak is very narrow, corresponding to in-plane correlation lengths of 2900Å, the total diffuse scattering intensity in the wide angle region is even larger than the intensity under the peaks (see Fig. 3). This requires a great deal of fluctuational disorder in the range (4-10 Å) of intermolecular spacings within the bilayer. One hypothesis to account for this dichotomy is that, while the scattering peaks come from well packed chains, the diffuse scattering is primarily due to the disordered head groups which scatter more strongly than the chains due to their higher electron density contrast with the solvent. However, closer analysis suggests that the chains must have considerable disorder as well. [20,21]

As one goes lower in temperature, there is a kinetically very slow transition, called the subtransition, into the subgel phase.[22] It has often been assumed that

[d] The chain tilt θ_t is easily understood [18] as relief of the frustration between packing headgroups with area A and chains (two per lipid) with intrinsic area $2A_c$ such that $A = 2A_c cos\theta_t$.

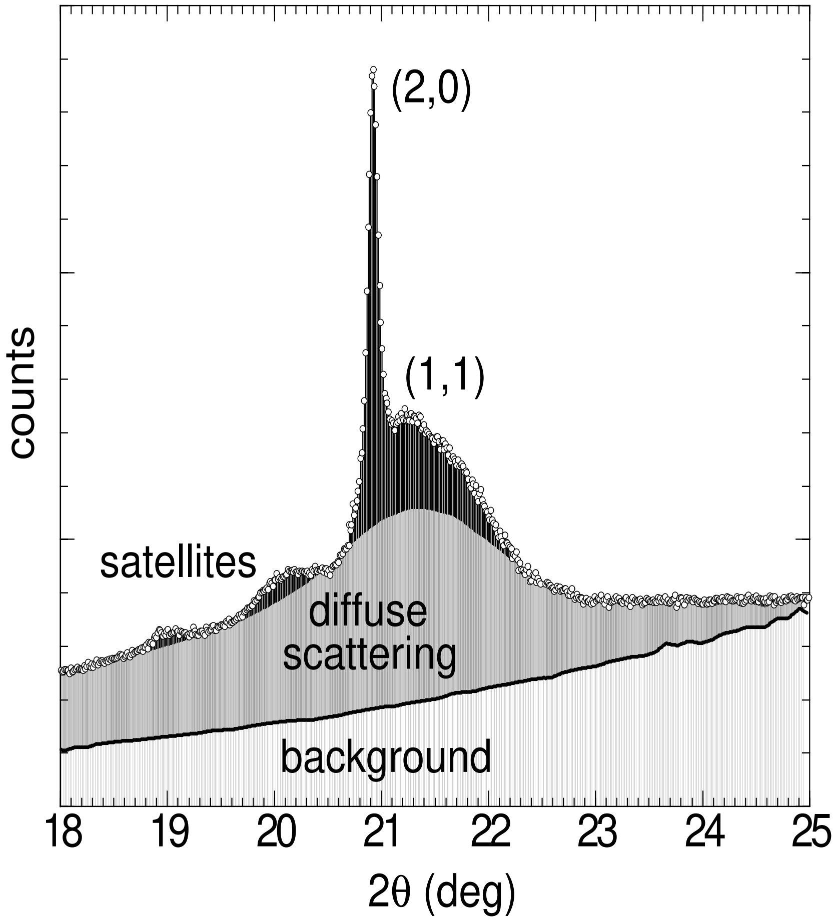

Figure 3: Wide angle x-ray scattering from unoriented samples of gel phase DPPC. The peaks (black shading) are well fit by a model of tilted chains. There remains a large amount of diffuse scattering between the peaks and the background scattering due to water. The (1,1) peak is much broader than the (2,0) peak because the chains are tilted with respect to the bilayer normal. The satellites also come from the (1,1) Bragg rod in q-space.

the distinguishing character of the subgel phase is a different kind of crystalline ordering of the chains, namely, freezing out of rotation about their long axes,[21] which would then give a herring-bone structure like crystalline polyethylene. However, a recent analysis emphasizes, instead, ordering of the headgroups in the subgel phase.[23]

The DPPC phase diagram is further complicated by the appearance of another phase, the ripple phase, between the well-characterized gel phase and the fluid phase. The phase transition between the gel phase and the ripple phase is often called the "pretransition"; I prefer to call this the lower transition in that it occurs below T_M, though not as low as the subtransition. The ripple phase is fascinating because it involves ripples in the plane of the bilayer that are in registry from one bilayer to the next; this phase is also called P'_β. Until recently, details of the structure of this phase have been lacking, despite excellent high resolution x-ray scattering [24], because of the difficulty of phasing the x-ray form factors. This classic problem was recently solved by my former student Wenjun Sun [25] and we now have good profiles of the bilayers showing the highly asymmetric or triangular saw-tooth profile (see Fig. 4). Details of chain packing in the ripple phase bilayer are still lacking, although wide angle scattering indicates that the chains are conformationally well ordered, at least in the major side of each ripple, as in the theory described in the introduction.

The difference in symmetry between the ripple phase and the fluid phase dictates that the transition should be first order. One would then generally expect that $D(T)$ would be discontinuous at T_M, as in curve (3) in Fig. 2 or even with a jump of opposite sign, but continuous $D(T)$ is also not disallowed. The reason for the disparate $D(T)$ behavior below T_M shown in Fig. 2 and discussed in Sec. 1.1 is due to two factors. First, there are many x-ray peaks, indexed by (K,L) in the low angle region, where K indexes the ripple in-plane repeat spacing, instead of just easily indexed (0,L) lamellar peaks. The (K,L) peaks are impossible to resolve at full hydration and low instrumental resolution, so that the only peak consists of a composite of many (K,L) reflections. Using Bragg's law on this composite peak therefore does not give the true D. Second, there is some lability in the ripple phase structure that depends upon hydration and sample preparation. This can shift the relative intensities of the (K,L) peaks and thereby shift the apparent lamellar peak position; this gives artifactual D spacings when such composite peaks are interpreted as (0,L) peaks.

Let us now turn back to the fluid (L_α) phase structure. Compared to the lower temperature phases, there is less scattering information to obtain structure because the wide angle scattering is completely diffuse, consistent with having conformationally disordered hydrocarbon chains and disordered

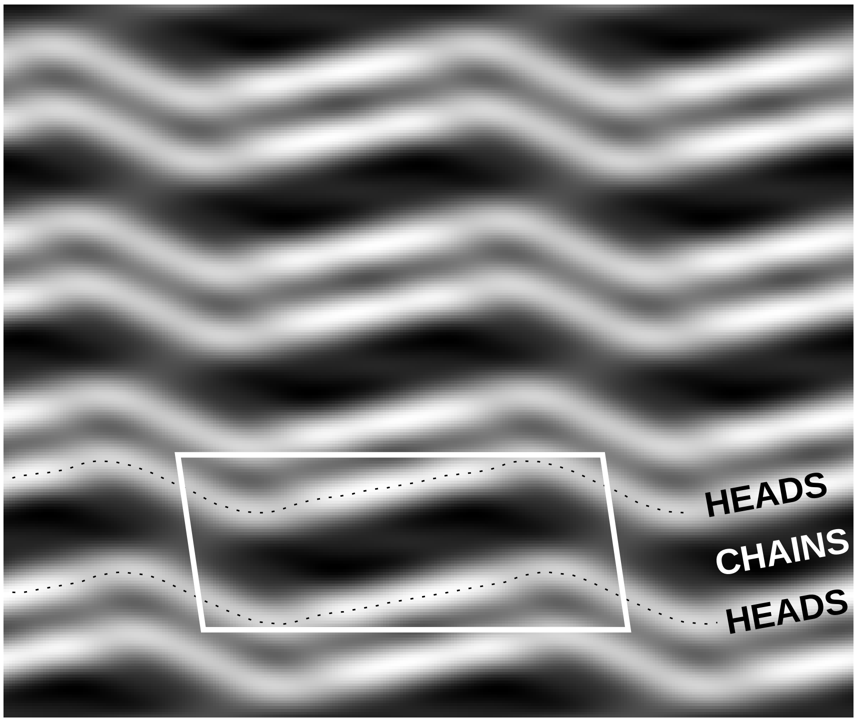

Figure 4: Grey scale electron density profile of the ripple phase where white shows the most electron dense headgroup region and black shows the least electron dense hydrocarbon region. The white box shows the unit cell with ripple repeat length 141.7$\mathring{A}$. The black dotted lines show the loci of maximum electron density.

heads. That is, the bilayer is a two dimensional fluid. Nevertheless, the bilayers pack as a smectic liquid crystal, so there are very sharp and well defined low angle scattering peaks corresponding to the lamellar repeat D-spacing. With four or more orders of low angle scattering, one can obtain reasonably reliable electron density profiles of low spatial resolution that enable one to obtain the quantitative change in thickness of the bilayer when compared to the well-determined gel phase. From this change, from structural results for the gel phase [e] , from volume measurements [26], and using some simple geometrical relations, we have obtained the average structure of the fluid phase. [17]

A major problem in implementing the preceding procedure is that fully hydrated DPPC bilayers have only two observable orders of diffraction. It is possible to obtain more orders of diffraction by partially drying the sample. This, however, raises the spectre that partial dehydration might change the fully hydrated bilayer structure that we want to measure. This danger is emphasized by an alternative method for obtaining structure, the so-called Luzzati method, from which it was concluded that the structure does change upon partial dehydration. This conclusion was false for the following reason. In that method the D-spacing is measured as a function of total water concentration and it is assumed that all the water goes between the bilayers. However, that assumption is incorrect in powder samples because there are highly disordered regions that are water-rich but do not diffract x-rays. [16,27] Therefore, that method overestimates how much water goes between the bilayers and the overestimate becomes worse as more water is added. This systematic error in that method was first established for gel phase DPPC [19] where we had enough data to test it thoroughly. However, this did not logically allow one to draw the conclusion for fluid phase DPPC that there was no change in structure upon partial dehydration.

A different line of evidence also argued for change in structure with partial dehydration. If the structure does not change, then the continuous Fourier transform $F(q)$ of the excess electron density normal to the bilayer

$$F(q) = \int_{-D/2}^{D/2} [\rho(z) - \rho_{water})e^{iqz} dz, \tag{1}$$

must not change. The continuous Fourier transform is easily obtained from the

[e] It is worth emphasizing, when questions of relevance in research arise, that, although the gel phase is not directly biologically relevant, effort to establish its structure was essential to provide a base from which to bootstrap to obtain the structure of the biologically relevant fluid phase.

amplitudes[f] of the observed orders of diffraction using the sampling theorem

$$F(q) = \sum_h F(2\pi h/D) sinc[(qD/2) - \pi h]. \qquad (2)$$

Straightforward measurement of the intensities of the observed peaks gave Fourier transforms that definitely varied with hydration level, especially when the bilayers were close to full hydration.[17] The flaw in this line of evidence is that this method treats the scattering data as if the bilayers were crystals and does not take into account the critical nature of the fluctuations that are intrinsic to smectic liquid crystals.

The fluctuations that are important for x-ray and neutron scattering in the low angle region are long wavelength undulations of the bilayers, described with a bending modulus K_c, and compression modes perpendicular to the bilayer planes, described by a modulus B.[28] This combination of fluctuations gives power law correlation functions, similar to correlation functions at a critical point.[g] Unlike crystals, the mean square displacement of bilayers diverges logarithmically with the size of the system; this is the well-known quasi-long-range-order (QLRO) in soft condensed matter physics. As shown by Caille[29], this has a profound effect on the low angle lamellar scattering peaks, changing them from Bragg peaks (essentially delta functions or Gaussians) to peaks with long power law tails (see Fig. 5). For powder samples the tails of the low angle peaks decay[30,31] as $|q - q_h|^{-1+\eta_h}$, where $q_h = 2\pi h/D$ and

$$\eta_h = \frac{q_h^2 kT}{8\pi\sqrt{K_c B}}. \qquad (3)$$

The long power law tails have important consequences for measurements of the total scattering intensity $I(h)$ because large fractions of the total scattering appear outside the sharp central peaks. Not knowing this and drawing reasonable baselines leads to substantial underestimation of $I(h)$ (see Fig. 5). This would not be a problem for obtaining electron density profiles if it affected all orders h uniformly. However, one sees from Eq. 3 that η_h grows quadratically with h through the q_h^2 factor. This has the effect, not only of making the tails longer, but also of increasing their amplitudes, so that the error in $I(h)$ grows rapidly with h. Indeed, when η_h becomes of order unity, the scattering peaks are smeared out and become unobservable. These effects qualitatively account for the misleading conclusion in the preceding paragraph.

[f] Phasing x-ray reflections is well established for planar thermodynamic phases.

[g] Note that this critical behavior applies to the entire fluid phase, not just near the transition. One may also note that the toy theoretical model described earlier has power law correlation functions at all temperatures above the transition[5], but the physical quantities that fluctuate are quite different.

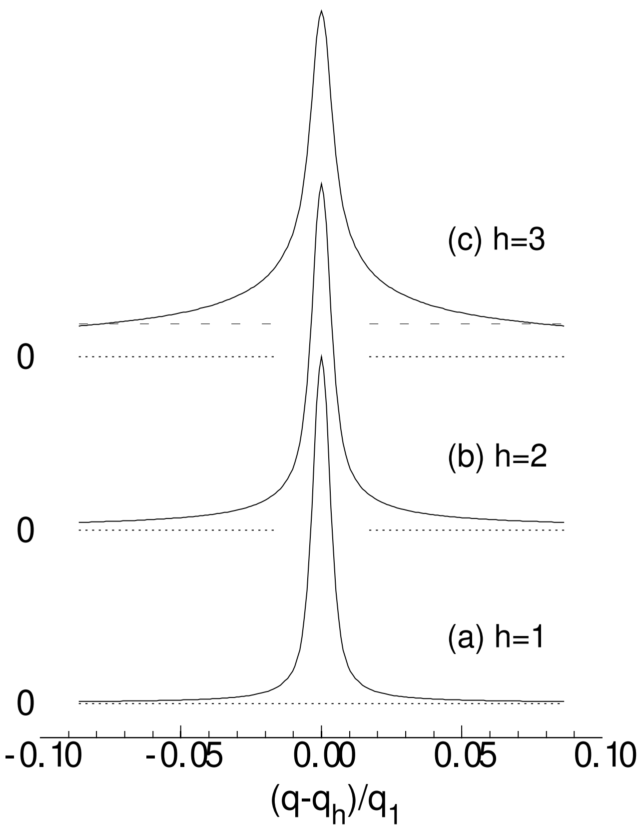

Figure 5: Theoretical scattering from smectic fluid phase for the first three orders h when $\eta_1 = 0.05$. Peak heights are normalized to the same value. The dotted lines show the true baselines. The dashed line for $h = 3$ shows a plausible baseline if one did not know about the long power law tails due to fluctuations. The error in measured intensity would then be the area under the dashed line plus the area under the tails that extend to ± 0.5 on the relative q axis.

My former student, Ruitian Zhang, carried the analysis of Caille further [30] so that we could make quantitative corrections to the apparent $I(h)$. Technically, this involved a number of improvements. Caille had taken the upper cutoff on a q-space integral for the pair correlation functions to infinity in order to obtain a closed form solution; Zhang kept the proper cutoff and still obtained a closed form solution, efficient for non-linear least squares fitting of data. Previously, the finite size effect was incorporated in a too simple way that made it difficult to fit the central scattering peaks; Zhang's improvement gives better fits to the central peaks as well as the power law tails. [32] Finally, Zhang derived the powder scattering formula rigorously. Our experimental procedure is to measure $I(q)$ for values of $|q - q_h|/q_1$ within 0.1 where the signal is safely above background and within which the bilayer form factor does not change significantly. Then, the data analysis program allows us effectively to extrapolate the experimental $I(q)$ scattering so as to obtain the total scattering $I(h)$.

Obtaining true $I(q)$ data obviously requires sufficient instrumental resolution so that scattering peak shapes report intrinsic sample properties and not extrinsic instrumentation artifacts. The central peaks are quite sharp (see Fig. 6) with full widths in $|q - q_1|$ of order $0.01q_1$ ($q_1 = 2\pi/D \approx 0.1\text{Å}^{-1}$), so our resolution was chosen to be of order $0.002q_1$. To obtain reasonable counting rates required use of high intensity synchrotron x-rays. Our data are well fit by Zhang's analysis[32]. This confirms earlier work that proved the existence of Caille power law tails. [31,33] It also showed that Zhang's refinements improve the fits compared to the classical Caille theory. [32]

The data and Zhang's analysis show that there is no detectable change in bilayer structure upon partial dehydration down to about 98% relative humidity at which about half the water is removed. This opened the door to meaningful analysis of bilayer structure at reasonable spatial resolution (h=4 orders). Detailed results for bilayer structure, such as the area A, have been reported for DPPC at 50^oC in the fluid phase. [17] The area/lipid $A = 63\text{Å}^2$, the hydrocarbon chain thickness $2D_C = 29\text{Å}$, the bilayer thickness shown as D_B in Fig. 1 is 45Å and the number of water molecules between the bilayers is 29/lipid for our sample with the fullest hydration. Such results will be of value to a variety of researchers, not least to molecular dynamics simulators, who either need to know A to set the dimensions of their simulation system, and/or who need to have data with which to test the results of simulations, which still have a number of untested options. The point to emphasize here is that our work has required careful attention to fluctuations in a complex system which is critical in the sense of having quasi-long-range-order with power law decay of correlation functions.

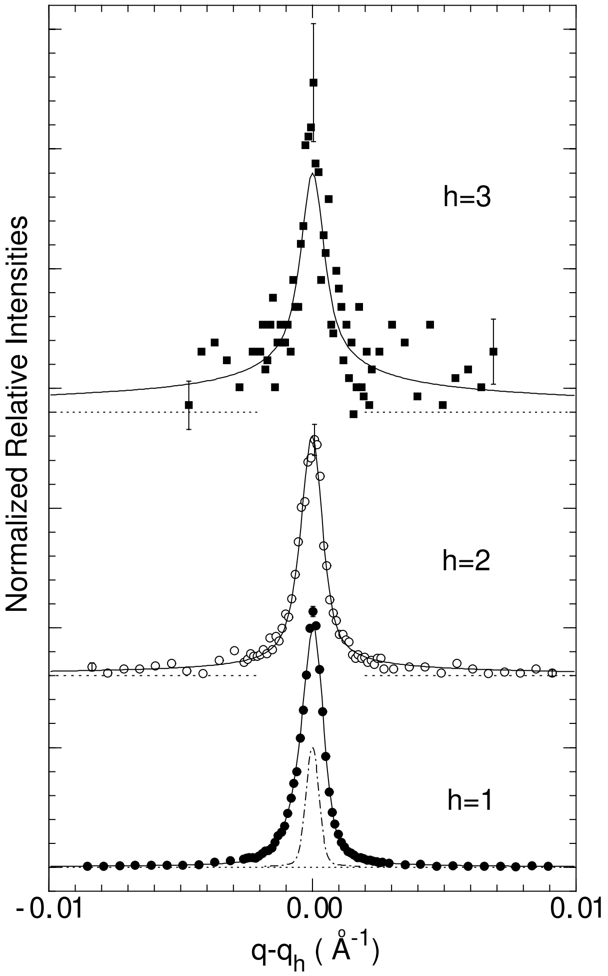

Figure 6: Synchrotron x-ray scattering from DPPC under osmotic stress with $D = 58.2 \AA$. The fits to the theory are shown by solid lines. The instrumental resolution is shown by the dash-dot line. The background is shown by dotted lines. For visibility, data for the different orders h are shifted vertically and normalized to the same peak height. For each order error bars are shown for one point in the central peak and for one point in each tail.

2.1 *Precritical Behavior of $D(T)$ (Continued)*

As promised in subsection 1.1, we now return to the apparent precritical behavior as T approaches T_M from above. This behavior occurs in the lipid DPPC discussed above, but the effect is larger for the lipid DMPC, so this lipid will be the focus of this topic.[h]

An interesting proposal for the precritical behavior was advanced[10] that we will call Model I. It differs substantially from the theoretical Model presented in the first section which we will henceforth call Model II. Model I supposes that the water spacing D_W increases anomalously near T_M whereas Model II supposes that the bilayer thickness D_B accounts for the anomalous increase in $D = D_W + D_B$. Model I and Model II share the assumption that there is an incipient critical point that is cut off by a first order transition at T_M. Model I assumes that there are critical fluctuations within each bilayer that bring about a softening of the membrane which would reduce the bending modulus K_c. Model II also has similar intrabilayer critical fluctuations but says nothing directly about K_c, so we depart from Model II at this point. Smaller K_c would increase the undulational fluctuations discussed in the preceding section. This would increase the Helfrich undulational force[35] (see Section 3) which would drive the membranes further apart, thereby increasing the water spacing D_W.

Since the Caille η_1 parameter in Eq. 3 depends on the inverse square root of K_c, we thought that a careful study of the temperature dependence would allow us to test Model I by observing changes in η_1. To our surprise, $\eta_1(T)$ was nearly constant as T approaches T_M[11] as can be seen from the new data in Fig. 7.[34] We emphasize that our experimental method does see changes in η_1 in other contexts. As seen in Fig. 7, there is a systematic increase in η_1 as temperature is increased well above T_M. Even more dramatic is that η_1 decreases by a factor of 6 with increasing osmotic pressure[17]. So the main question is, how much change should one see in η_1 in order to account for the anomalous increase in $D(T)$? The answer we first gave[11] was at least a factor of 2, which should have been easy to detect with the data in Fig. 7. However, the answer to this question is more subtle than we first thought. To answer it properly requires a discussion of another major thrust of our current research, namely, the elucidation of interactions between bilayers.

[h]The chemical difference between DPPC and DMPC is small; DPPC has 16 carbons in each hydrocarbon chain and DMPC has 14, but they both have the same glyceryl-phosphatidylcholine headgroup.

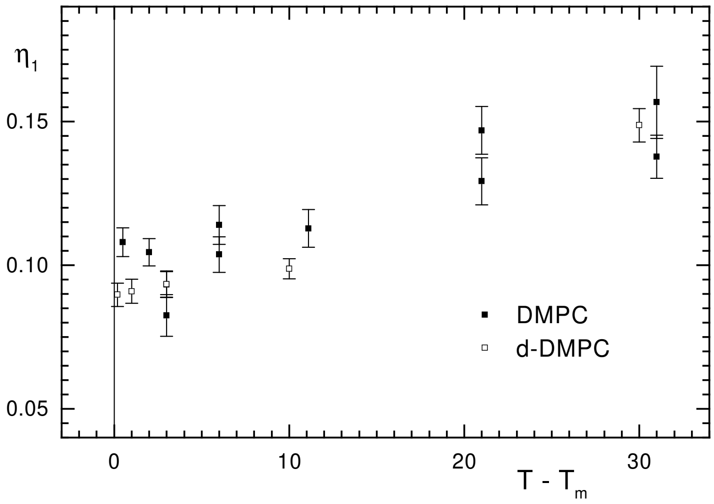

Figure 7: η_1 for fully hydrated DMPC and chain deuterated d-DMPC. η_1 was obtained by simultaneously fitting orders $h = 1$ and $h = 2$ with $\eta_2 = 4\eta_1$.

3 Interbilayer Interactions

Interactions between bilayers composed of uncharged lipids like DMPC and DPPC are currently supposed to consist of three main terms. The first is an attractive van der Waals interaction E_W and the second is the mysterious repulsive hydration force E_H which together give an interaction energy

$$E_W + E_H = (-H/12\pi)D_W^{-2} + P_H \Lambda e^{-D_W/\Lambda}. \tag{4}$$

The third term, first elucidated by Helfrich,[35] takes into account the effective repulsion required by the loss of undulational entropy as membrane separation decreases. Helfrich showed that the resulting free energy decreases as D_W^{-2} when the only other forces are hard-wall or steric. In this hard confinement case, the smectic system will only have a finite equilibrium D-spacing if an external pressure, such as osmotic pressure, is applied. When there are other more gradual forces, such as those in Eq. 4, one has soft confinement in contrast to the hard confinement solved by Helfrich. Also, if the attractive van der Waals force is large enough, the system will have a finite D-spacing even with no applied osmotic pressure. This is certainly the case for DPPC and DMPC, since the low angle scattering exhibit such sharp and well-defined lamellar peaks.[i]

The Helfrich theory of hard confinement was non-trivial. A quantitative theory of soft confinement is even more difficult because the equivalent of the Helfrich force depends upon the subtle interplay of the undulational modes and the soft interactions. The best theory of soft confinement, in my opinion, is due to Podgornik and Parsegian[37], which will be abbreviated as the P^2 theory. The P^2 theory approximates the true potential by an effective potential given by

$$W_0 = \frac{K_c}{2}\int (\nabla^2 z(r))^2 d^2r + \frac{B}{2}\int z^2(r) d^2r, \tag{5}$$

where r gives the in-plane coordinates perpendicular to the z direction which is along the normal of the average bilayer position. This effective potential is essentially the same as in the standard liquid crystal theory.[28] The first term in Eq. 5 accounts for the energy of membrane bending with modulus K_c. The second term approximates the actual interbilayer potentials in Eq. 4 with an effective harmonic potential, where B is a model parameter. B looks like a bulk modulus for the compression modes perpendicular to the stack of bilayers, but it is not the same as the thermodynamic bulk modulus that comes from a second derivative of the total free energy. Since B is a parametrization of all the soft confinement forces in Eq. 4, it depends upon D_W. The P^2 theory uses the Feynman inequality to calculate $B(D_W)$ as well as other important quantities, such as the osmotic pressure $P(D_W)$ required to maintain the system with spacings D_W and D. Most importantly for our experimental x-ray studies, this theory predicts the mean square fluctuation σ^2 in D_W, which is directly related to η_1 by

$$\sigma^2 = \eta_1 D^2/4\pi. \tag{6}$$

The ratio $\mu = (\sigma/D_W)^2$ is also a central quantity. It is constant for hard confinement, with numerical values given as $1/6$[35], 0.183[37] and $1/4$[38]. However, our data in Fig. 8 show that μ varies considerably with osmotic pressure and with values smaller than $1/6$; this is direct evidence that a theory of soft confinement is required for lipid bilayers.

The traditional way to determine interbilayer interactions experimentally is to measure the osmotic stress P as a function of D or D_W. Measuring η_1, *i.e.*, σ^2, as a function of D_W opens an additional experimental window on these interactions. One of the projects being worked on by Horia Petrache and Nikolai Gouliaev in my group is to combine these measurements with theory

[i] It may be noteworthy, however, that D spacings for fully hydrated samples vary much more from sample to sample than experimental resolution, suggesting that the balance of forces is rather delicate.

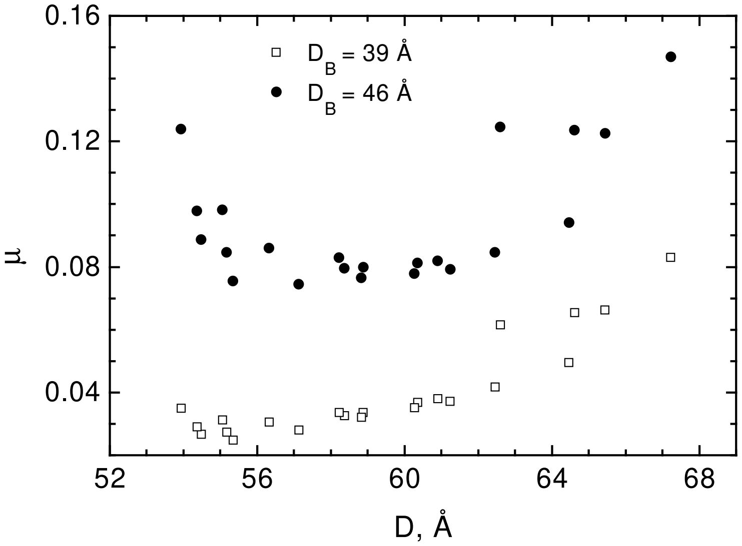

Figure 8: $\mu = (\sigma/D_W)^2$ as D is decreased by applying osmotic pressure. Results are shown for two interpretations of the effective bilayer thicknesses $D_B = D - D_W$.

to obtain better estimates of the basic parameters in Eq. 4, as well as to test the functional forms of the various interaction energies in the soft confinement regime. Our current efforts indicate that the P^2 theory gives qualitatively correct trends, but that it is quantitatively inaccurate.[36] This is not too surprising, because, while the P^2 theory is elegant, it is approximate. Nikolai Gouliaev is engaged in a project to provide quantitatively more accurate predictions of P and η_1 from the mesoscopic model in Eq. 4.

3.1 Precritical Behavior of $D(T)$ (Continued)

The theory in this section has led us to make an improvement in our previous estimate [11] for predicted changes in η_1 as T approaches T_M. An essential aspect of this improvement was to realize, as emphasized above, that the B that appears in Eq. 5, in the Caille η_1 in Eq. 3, and in the P^2 theory, is a bare modulus that is not quantitatively equal to the thermodynamic modulus. We have therefore repeated our calculation using the P^2 theory and now find that for Model I the expected change in η_1 to account for the full change of $4\mathring{A}$ in D is about 50% instead of the previous 100%.

4 Precritical Behavior of $D(T)$ (Concluded)

Some of the ingredients in this intriguing topic have been assembled in previous subsections. Let us now add a few more ingredients en route to some tentative conclusions. Our x-ray data showed that the bilayer form factors $F(q_h)$ change as a function of T and that these changes require that the bilayer thickens as T approaches T_M, as required by Model II.[11] It was not possible, however, to verify that all the change in the D spacing is accounted for by thickening in the bilayers because only two orders of diffraction could be observed. Therefore, while this proved that Model II is valid, it did not exclude a mixed model with anomalous increase in the water spacing D_W as well.

Another ingredient comes from NMR order parameters. Order parameters have been used for many years to obtain the effective length of the hydrocarbon chains along the bilayer normal.[39] This length is then conventionally doubled to obtain the thickness $2D_C$ of the hydrocarbon portion of the bilayer. However, because the chains do not necessarily terminate in the center of the bilayer, another formula for $2D_C$ has been advanced[39]. Recent very careful measurements as a function of T have been performed by Dr. Klaus Gawrisch at NIH. The results (to be published) are sketched in Fig. 2, where, to faciliate comparison with the D spacing, a constant of about 36$\mathring{A}$ has been added to account for the water spacing D_W and the thickness $2D_H$ of the lipid headgroups. The other NMR formula[39] requires a different constant, but the resulting T dependence is virtually identical. From this we conclude that the T dependence of $2D_C$ matches that of the D spacing for T greater than $T_M + 3^oC$ regardless of the type of analysis of the NMR data. The match is considerably better than the match between $2D_C$ and the hydrocarbon thickness as determined by interpretation of low resolution small angle neutron scattering (SANS) data[14,15]. Although it was intimated that the latter disagreement may have been due to the interpretation of the NMR data[14], we will discuss later why the SANS result is more likely to be suspect. Finally, Fig. 2 shows that there is a divergence between the T dependence of D and $2D_C$ between T_M and $T_M + 3^oC$. This divergence implies that Model II can only explain an increase in D of about 2$\mathring{A}$ from $T_M + 10^oC$ to T_M and leaves unexplained the additional 2$\mathring{A}$ increase in D between T_M and $T_M + 3^oC$.

We now ask whether Model I can account for this smaller increase of 2$\mathring{A}$ in D near T_M. The predicted increase in η_1 would now only be about 25% from the P^2 theory. This should still be experimentally detectable in Fig. 7, but perhaps the expected increase is not so large as predicted by the P^2 theory, which is not quantitatively accurate for the other properties we measure. At the present time, therefore, a mixed Model I and II should not be ruled out, although it is not positively supported.

Other models are also conceivable. We have considered the possibility that parameters involved in the hydration force or the van der Waals force might change, thereby changing D_W while only changing η_1 within experimental error through their effect on B. Although the end effect, namely, of increasing D_W, is the same as for Model I, the underlying theory is different so I shall call this Model III. As T approaches T_M the membrane thickens and becomes denser; both effects would tend to increase the van der Waals interaction and therefore decrease D_W rather than increase it. A possible change in the right direction is that interfacial surface area per lipid decreases; this would make the interface more like the gel phase and recent data [41] suggest that the hydration force is larger for the gel phase.

Another possible resolution of the dilemma, that the thickness of the hydrocarbon portion of the bilayer $2D_C$ does not change enough and that D_W may not change at all, follows from the realization that the D spacing is really composed of three regions, water D_W, chains $2D_c$ and headgroups $2D_H$, so

$$D = D_W + 2D_C + 2D_H. \tag{7}$$

The dilemma would be resolved if the thickness of each headgroup region, D_H, grows larger by about $1\mathring{A}$ as T approaches T_M. Since the thickness of the headgroup region (which includes the glyceryl backbone and the fatty acid carbonyls) is about $10\mathring{A}$, a $1\mathring{A}$ change could easily be accommodated by fairly subtle conformational changes in the headgroup region. Since the bilayer thickness $D_B = 2D_C + 2D_H$ includes both chains and heads, this resolution is technically still Model II. However, the underlying ideas are sufficiently different that I will call this Model IV. Dr. Gawrisch has performed two additional NMR measurements specifically to test Model IV. First, deuterium NMR quadrupole splittings were observed for DMPC with the two methylenes in the headgroup region deuterated to examine whether the orientation of the headgroup changed anomalously near T_M. These data indicate no anomalous change. Second, the anisotropy of the chemical shift of the carbonyl on the first hydrocarbon chain was measured. These data qualitatively support Model IV in that there is an increase with upward curvature in the magnitude of the chemical shift near T_M. Quantitative interpretation of these chemical shift data is complicated, but we have difficulty justifying an increase in $2D_H$ larger than $0.6\mathring{A}$.

SANS data [14,15] have also been used to address changes in the structure of the layers within the D spacing. Results have only been published [14,15] for $2D_C$ and for the sum $D_A = D_W + 2D_H$. An anomalous increase in D_A was interpreted as evidence for Model I, but this could equally well be taken as evidence for Model IV, since the anomalous increase in D_A could be due to an anomalous increase in either D_W or D_H.[j] However, we have some reservations regarding the interpretation of the SANS data. The model fitted to the SANS data required at least 8 free parameters to fit three poorly resolved peaks. Therefore, peak shapes and not just peak positions and amplitudes must be necessary to provide enough information to obtain all the model parameters. The SANS resolution was at best 0.18 in $\delta\lambda/\lambda$, more than 100 times poorer than the resolution of our x-ray scattering data and about 20 times broader than the intrinsic central scattering peaks. It is therefore impossible, using SANS, to resolve the intrinsic scattering line shapes. Interpretation of the SANS data also employed convolution of a broad resolution function with a theoretical line shape derived from paracrystalline theory. It was argued [14,15] that extending paracrystalline theory to allow for three different fluctuating thicknesses within a single D spacing is more important than allowing for undulations[k], which all paracrystalline theories omit. However, without undulations no paracrystalline theory can produce long power law tails in the scattering peaks. Earlier work [33] showed quite clearly that scattering from lipid bilayers has long power law tails and our recent work [32] specifically confirmed that paracrystalline theory does not give the correct line shape to fit fully resolved peak shapes.

Whatever the final conclusion regarding the anomalous increase in D as T approaches T_M, it now appears that there are at least two different structural responses of lecithin bilayers to temperature. Understanding the details of these complex responses may increase our understanding of the variety of ways that membranes can accommodate the environmental requirements of the different integral membrane proteins that perform biochemical functions.

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[j] Indeed, in a recent preprint [40] it is D_H and not D_W that has the anomalous increase.

[k] The argument for employing paracrystalline theory [14,15] is that it does not require the common assumption that fluctuations affecting the form factors (i.e., fluctuations in the structure of single bilayers) are statistically independent of fluctuations affecting the structure factor (i.e., fluctuations in the positions of the bilayers relative to each other). However, as was noted earlier (see the appendix to [30]) the common assumption is a good one because the spatial fluctuations affecting the structure factor are long range extending over many D spacings, so that the corresponding forces are very small and would therefore not be coupled to fluctuations in bilayer structure. In addition, our recent work [17] shows that even the large changes in D_W induced by an osmotic pressure results in negligible changes in the bilayer form factors. All these results follow from the fact that the intrabilayer forces affecting the structure of a single bilayer are large compared to the interbilayer forces.

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