Effect of Substrate Roughness on *D* Spacing Supports Theoretical Resolution of Vapor Pressure Paradox

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ABSTRACT   The lamellar D spacing has been measured for oriented stacks of lecithin bilayers prepared on a variety of solid substrates and hydrated from the vapor. We find that, when the bilayers are in the L_α phase near 100% relative humidity, the D spacing is consistently larger when the substrate is rougher than when it is smooth. The differences become smaller as the relative humidity is decreased to 80% and negligible differences are seen in the $L_{\beta'}$ phase. Our interpretation is that rough substrates frustrate the bilayer stack energetically, thereby increasing the fluctuations, the fluctuational repulsive forces, and the water spacing compared with stacks on smooth surfaces. This interpretation is consistent with and provides experimental support for a recently proposed theoretical resolution of the vapor pressure paradox.

INTRODUCTION

According to elementary thermodynamics the properties of macromolecular or multimolecular assemblies immersed in water (fully hydrated) should be no different than when in saturated water vapor, i.e., 100% relative humidity (RH), because the chemical potential of water is the same in both environments. However, as has been known for a long time, lipids hydrated from saturated vapor often take up less water than when immersed in bulk water (Jendrasiak and Hasty, 1979; Torbet and Wilkins, 1976). This dichotomy has been called the vapor pressure paradox (Rand and Parsegian, 1989). Resolving this paradox is important to enrich our understanding of fundamental forces and how they may influence the application of thermodynamics to biophysical systems. It is also important to evaluate the validity of using oriented samples. Such samples allow more revealing biophysical measurements, but they often require hydration from the vapor, and it has been unclear whether they are equivalent to fully hydrated, biologically relevant, unoriented multilamellar or unilamellar bilayers.

It appears from a recent paper by Podgornik and Parsegian (1997) that the vapor pressure paradox is being resolved theoretically. One key development was the realization that fluctuations, which take the form of undulations in lipid bilayers, create an effective entropic force (Helfrich, 1978) that repels bilayers from each other, thereby resulting in uptake of more water, as indicated by larger lamellar repeat spacing D and larger water spacing D_W. As shown by McIntosh and Simon (1993), this explanation accounts for larger D_W in the L_α phase immersed in bulk water than in gel ($L_{\beta'}$) phase because the gel phase has stiffer bilayers that suppress undulations and therefore weaken the repulsive

forces that compete against the attractive van der Waals forces. This distinction between gel and fluid phases hydrated in bulk water has a counterpart for lipid bilayers hydrated from the vapor. For the gel phase, Tristram-Nagle et al. (1993) studied samples oriented on glass substrates; these samples were hydrated from slightly supersaturated water vapor, and D spacings were obtained that were as large as those obtained for samples immersed in water. However, the same method applied to the L_α phase gave D spacings nearly 10 Å less than for immersed samples.

Podgornik and Parsegian (1997) have proposed that the crucial distinction for understanding the vapor pressure paradox is that hydrating from the vapor involves interfaces that are not present for samples in excess water. The interface between the vapor and the bilayer that is at the surface of the lipid sample imposes a surface tension on that bilayer. As is well known (Helfrich and Servuss, 1984), surface tension suppresses undulational fluctuations of bilayers. The interface between a flat solid substrate and the adjacent bilayer also suppresses fluctuations in that bilayer because there is an energetic preference for the bilayer to maintain a fixed distance from the substrate. Both kinds of interface can be described as having a pinning effect on the adjacent bilayer.

Conventionally, such a pinning effect of interfaces would die off exponentially as one proceeds into the bulk of the sample, and so this would not explain how the D spacing is decreased uniformly throughout samples that are several microns thick. However, L_α phase lipid bilayers are smectic liquid crystals, which are well known not to be conventional, either in the liquid or in the crystalline sense. Instead, these systems have quasi-long-range-order (QLRO); the correlation functions decay very slowly, as power laws (Caillé, 1972; Zhang et al., 1994) rather than exponentially, much like the correlation functions of simple fluids at the critical point. Therefore, pinning a surface of such a sample suppresses fluctuations far into the bulk (Holyst, 1991), and Podgornik and Parsegian (1997) have developed this essential insight into a full-fledged mathematical theory.

In this paper we report observations that support this resolution of the vapor pressure paradox. The initial observation was serendipitous and was motivated by the desire to prepare better oriented bilayer samples to obtain more diffraction orders in the fluid phase. When we used an atomically flat silicon substrate, which indeed does give a better oriented sample, we also found that the D spacing was 1–2 Å smaller than when we used less perfect glass substrates. An explanation of why there might be such a substrate-dependent difference between D spacings of nominally identical bilayers is shown in Fig. 1. This explanation involves the additional concept of frustration for bilayers adjacent to rough surfaces. A bilayer next to a smooth flat substrate (Fig. 1 *A*) or next to a vapor phase can, by adopting a flat configuration, minimize two energies, the energy of interaction with the substrate and the intrinsic bending energy. In contrast, a bilayer next to a rough substrate (Fig. 1 *B*) is frustrated because it cannot simultaneously minimize both energies. Compared with a nonfrustrated bilayer next to a smooth substrate, the frustrated ground state energy is not as deep and the configurational phase space that has energy within kT of the ground state is larger when nonharmonic forces are considered. Therefore, one would expect larger fluctuations of the surface bilayer. This weakens the pinning effect of rough surfaces, which would then result in increased D spacings.

MATERIALS AND METHODS

Lipids

Synthetic lecithins, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC),
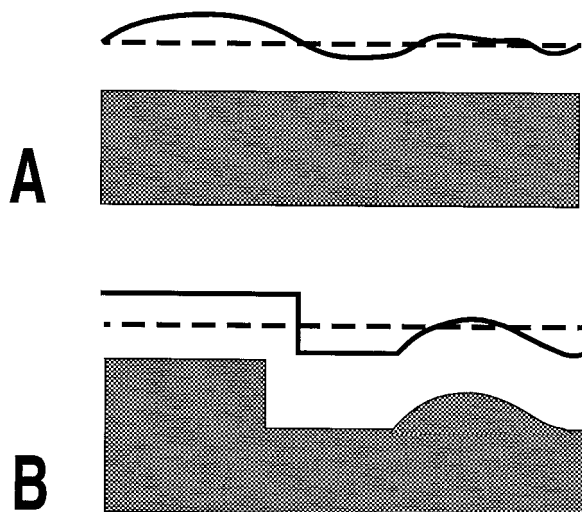

FIGURE 1   Substrates are shown as hatched areas. The centers of bilayers are shown by lines. (*A*) Flat substrate with a flat bilayer (- - -) and a fluctuating bilayer (——). The flat bilayer minimizes both its bending energy and the energy of interaction with the substrate. (*B*) Nonflat substrate with a flat bilayer (- - -) that minimizes the bending energy and a bilayer that follows the substrate contour (——) that minimizes the energy of interaction with the substrate.

were purchased from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. Thin layer chromatography of the pure lipids using chloroform/methanol/7 N NH$_4$OH (46:18:3, v/v) revealed only a single spot when stained with a molybdic acid stain.

Substrates

Several substrates were used for lipid deposition: thick (1/8-inch) silicon wafers (Semiconductor Processing, Boston, MA), flint glass coverslips (70 μm thick; Biophysica Technologies, Baltimore, MD), smooth glass microscope slides (Corning Glass Works, Corning, NY), frosted glass microscope slides (Erie Scientific Co., Portsmouth, NH), and both smooth and frosted glass microscope slides that had been treated for 3 days with a Piranha etch solution (70 ml of 30% H$_2$O$_2$ and 30 ml of H$_2$SO$_4$) (Seul and Sammon, 1990). Microscope slides were cleaned either 1) with swabbing and rinsing with HPLC-grade chloroform or 2) with a two-step acid cleaning procedure: 30 min chromerge acid bath followed by copious rinsing with Barnstead nanopure water and then 30 min in 37% hydrochloric acid followed by copious rinsing with nanopure water and air drying. Both methods of cleaning yielded similar results.

The smooth and frosted glass microscope slides were characterized using atomic force microscopy (AFM) with an Autoprobe-Contact AFM from Park Scientific Instruments (Sunnyvale, CA) using the ProScan Image Processing software. The images were not deglitched or filtered.

Bilayer deposition

Lipids were deposited onto the flat solid substrates by evaporation from an organic phase. Two organic solvents were used: a chloroform/methanol mixture (3:1, v/v) (Tristram-Nagle et al., 1993) and isopropanol (Seul and Sammon, 1990). All chemicals were HPLC grade from Sigma-Aldrich, Milwaukee, WI. Lipid (5, 10, or 20 mg) was applied in 300 μl of solvent to a 3-in^2 substrate resulting in calculated thicknesses of 700, 1400, or 2800 bilayers. Evaporation of chloroform/methanol took place in a glove box by first rocking the sample until all liquid disappeared and then leaving the sample in the glove box for 1 day and then in room air for at least 1 day. Truncation of the room air drying procedure resulted in significant chemical degradation of the lipids upon X-irradiation. When the samples were sufficiently dried, only a small amount of lysolecithin (0.1–1%) was detected by thin layer chromatography after 2–3 days of equilibration at various humidities at 30–50°C. Evaporation of isopropanol took place uniformly in room air and was not aided by rocking the substrate. Using polarized microscopy, bilayers on smooth glass appeared dark with holes and terraces as is common for well oriented samples (Tristram-Nagle et al., 1993), and no evidence for MLVs was seen. With isopropanol, deposition was limited to 5 mg of lipid as additional lipid resulted in a ring of unoriented lipid near the edge of the substrate. Naked frosted glass had a patchwork appearance, indicating an uneven surface; this pattern persisted when lipid was deposited on frosted glass.

X-ray diffraction

The main x-ray source was a Rigaku microfocus sealed tube, typically run at 1.4 kW. A graphite monochromator selected CuK$_\alpha$ radiation ($\lambda = 1.542$ Å) and defined a beam with angular resolution $\delta(2\theta) = 0.14°$ full width at half-maximum (FWHM) in the horizontal direction in the scattering plane, with a horizontal dimension of 0.75 mm and with a vertical dimension defined by slits to be 4 mm. A Bicron NaI scintillation detector was placed 43 cm in the horizontal direction from the sample. Diffraction peaks were observed using θ–2θ scans. The D-spacings were obtained from the low-angle third- or fourth-order lamellar peak to minimize slit smear. The position of the diffraction peaks could be determined to within an accuracy in D of 0.1 Å by fitting Gaussians to the data points, so differences in D values are accurate to 0.2 Å. The degree of orientation of the bilayers on the substrate (mosaic spread) was determined by holding the detector to the 2θ of a diffraction peak and then rocking the angle θ of the sample. The

FWHM of a Gaussian fit to the rocking data is the mosaic spread. Because the sample blocks the incoming beam at $\theta = 0$ angle and the diffracted beam at $\theta = 2\theta_h$, this method is limited to mosaic spreads less than $2\theta_h$ for the hth order.

Alternative x-ray detection employed a phosphor imager (Molecular Dynamics, Sunnyvale, CA) placed 25 cm from the sample; data analysis was performed using ImageQuant Software after scanning the phosphor imager. Mosaic spread was determined by a Gaussian fit to the intensity data versus angle along the diffraction arcs; this method was not limited to small mosaic spread.

Two substrates with nominally identical lipid samples were mounted back-to-back and placed vertically near the top of a cylindrical sample container (diameter = 6 cm; height = 12 cm) with thin (1.5-μm) Mylar windows (DuPont, Wilmington, DE). A Rotronics HT225R humidity/temperature sensor (Huntington, NY) was placed just beneath the samples. In the bottom of the chamber was a stainless steel cup containing Barnstead nanopure water or various ultrapure salt solutions (Sigma-Aldrich, Milwaukee, WI) to establish the humidity (O'Brien, 1948; Hasegawa, 1986). The temperature of the chamber was controlled by a Lake Shore Cryotronics (Westerville, OH) model DRC 91C temperature controller connected to six heating strips (Minco Products, Minneapolis, MN) attached to the chamber. Variations in sample temperature were estimated to be within 0.5°C of the reported temperatures. Samples were allowed to equilibrate typically 1–2 days after changes in temperature or humidity. Many of the reported D data were duplicates obtained at intervals of several hours.

Additional x-ray diffraction was carried out at the Cornell High Energy Synchrotron Source (CHESS), using a silicon monochromator to select x-rays with $\lambda = 1.2147$ Å. An in-plane resolution of 0.002° FWHM in 2θ was achieved using a silicon analyzer crystal for selecting the scattered radiation. A DMPC sample was mounted horizontally, and the temperature was held constant at 30°C.

RESULTS AND DISCUSSION

The initial observation, mentioned in the Introduction, was that D was $\sim 1-2$ Å smaller when an atomically flat Si substrate was used than when an ordinary glass substrate was used. The interpretation of this observation was obscured by two criticisms. The first criticism is a technical one, but quite important to minimize random errors. It is quite difficult to maintain accurate relative humidities near 100% so that comparison of results from one sample, after equilibration with a vapor phase, with results from another sample, usually obtained at least a day later, is subject to poorly controllable environmental conditions that introduce much randomness in the results. We overcame this problem by mounting simultaneously two samples back to back in our closed sample holder so that both samples have the same thermal and humidity history. Simple 180° rotation of the sample container allowed x-ray diffraction from either sample. The control experiment was to mount two identical substrates with the same lipid; the resulting D spacings were identical to within our accuracy of 0.2 Å. The second criticism with interpreting different results for Si versus glass substrates is that the physical flatness effect may be minor compared with other differences in the substrate. The obvious chemical differences might seem more important. However, chemical differences would also be a surface interaction with only the first bilayer, so invoking this cause would also support the theoretical resolution of the vapor pressure paradox. In addition, there might be differences in the long-range van der Waals interactions with the different

substrates that could give different values of D, so we focused on pairs of substrates of the same material. Our first pairs were to use the rough back side of the Si crystal as well as the smooth side. This combination gave D larger for the rough side by ~ 2 Å near 100% RH.

Most of our data were taken with glass substrates with different roughness. We obtained some results with glass deliberately roughened in our lab by mechanical means, but the roughnesses of such substrates were hard to reproduce, so we focused on comparing smooth glass substrates with commercially available frosted glass, which is produced by sandblasting smooth glass slides. We characterized the roughness of these substrates using AFM as shown in Fig. 2. Although Fig. 2 A appears rougher than Fig. 2 B, Fig. 2 A is the smooth glass slide and Fig. 2 B is the frosted slide. The apparent visual contradiction is due to a higher total gray scale (570 Å) in the case of the frosted glass than with
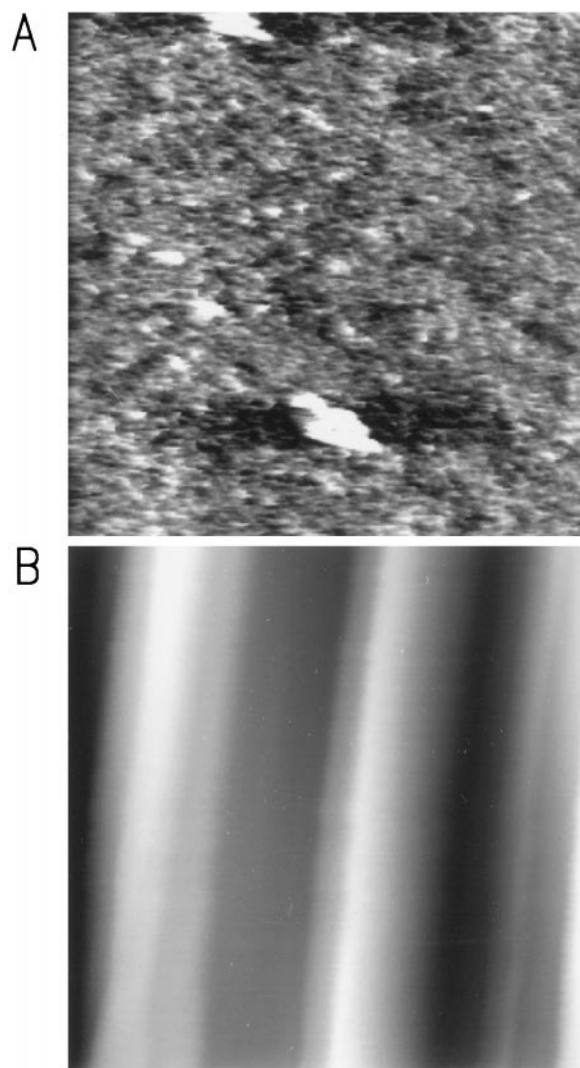

FIGURE 2 AFM characterization of substrates: (*A*) Smooth glass slide, total gray scale from 0 to 23 Å. (*B*) Frosted glass slide, total gray scale from 0 to 570 Å. White indicates the highest features. Slides were scanned horizontally on a 1-μm $\times$ 1-μm square.

the smooth glass (23 Å) and also to the small area scanned. When scanned over a 20×20 μm square, the frosted slide appeared more isotropic and rougher. For the smooth glass slide, the root mean square (RMS) roughness was 6.2 Å overall, with an average feature height of 15–20 Å. For the frosted glass slide, the RMS roughness was 146 Å overall. Even in smaller regions, the RMS roughness of the frosted glass was greater than for the smooth glass; for example, it was 30 Å along the top of the white ridges in Fig. 2 *B*. The ridges themselves were ~400 Å high. One additional surface treatment was to submit both the frosted and smooth glass slides to a 3-day Piranha etch treatment. This treatment caused the smooth slide to behave as a frosted slide and increased water uptake by DPPC on the frosted slide by 2 Å at 97% RH and 45°C. Most of the comparisons, however, were between untreated smooth and frosted glass slides.

As expected, the bilayers were better oriented on the smoother substrates. Rocking scans showed that the mosaic spread of the bilayers was less than 0.2° on the smooth silicon substrate and ~0.3–1° on the smooth glass substrate. The mosaic spread on the frosted glass substrate was too large to be determined by rocking scans. Using the PhosphorImager, we determined the mosaic spread on the rough glass substrates to be ~13° and on the smooth glass substrates ~7°. No isotropic rings were observed, indicating lack of a completely unoriented sample.

The diffraction peaks in samples measured at moderate resolution ($\delta(2\theta) = 0.14$° FWHM) had widths that were resolution limited when scanned in the usual longitudinal direction using θ–2θ scans as shown in Fig. 3. This is a typical result that shows that the samples are consistent with having a single, uniform D spacing. Technically, it would be possible for the samples to have a distribution of D spacings provided that the relative deviation $\delta D/D$ is less than the half-width of our instrumental resolution function relative to the peak angle. Using a fourth-order peak shows that δD must be less than 0.7 Å. To reduce this upper bound, one sample of DMPC on a smooth glass substrate was examined at high resolution at CHESS. All lamellar peaks were fully resolved and the third-order peak had a width of 0.007° FWHM as shown in Fig. 3. This would require that any distribution of D spacings would have a range less than 0.04 Å, considerably less than the measured differences in D spacings between smooth and rough substrates. The additional result that the high-resolution peak widths did not increase with diffraction order is also strong evidence that the observed widths are not due to a distribution of D spacings. This result of uniform D spacing in the stack strongly supports the basic theory of Podgornik and Parsegian (1997).

The synchrotron data also allowed a study of the hydration kinetics. Fig. 4 shows that the third-order peak of the dry sample continuously decreased as the third-order peak of the hydrated sample grew. The two peaks were distinct at all times during the hydration. It is also interesting that the peak for the hydrated sample initially had the same width (0.02°) as for the dry sample, and this width gradually decreased with time to (0.007°) as the D spacing gradually increased from its initial value of 51.0 Å to its final value of 51.4 Å.
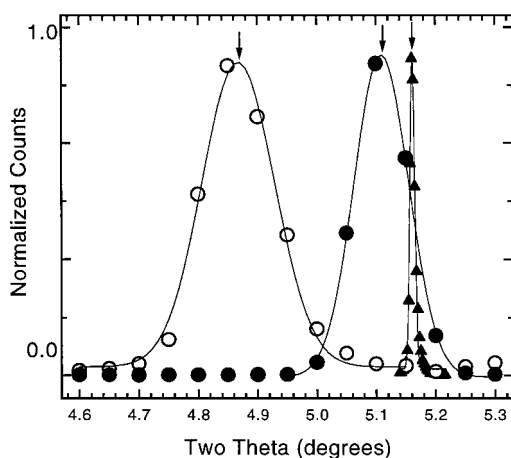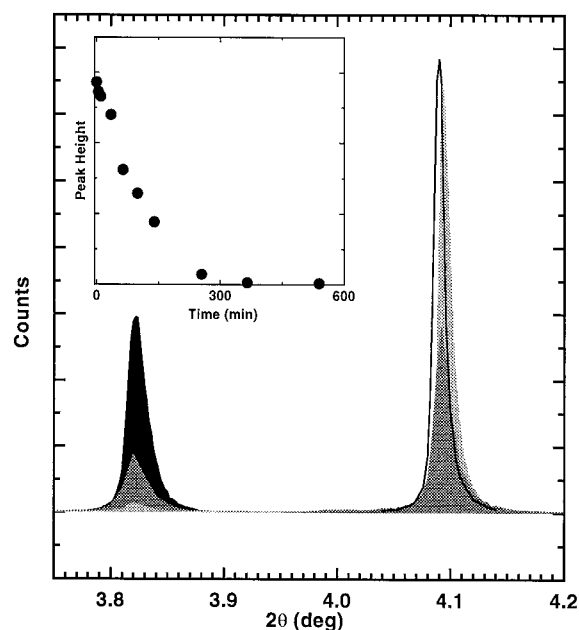

FIGURE 3   Typical third-order peaks for DMPC. Open symbols indicate frosted glass substrate and filled symbols indicate smooth glass substrate. ○ and ●, moderate resolution data taken for a pair of samples in the hydration chamber at the same time; ▲, high-resolution CHESS data taken at a different time and with 2θ adjusted to $\lambda = 1.5418$ Å. The peaks obtained at moderate resolution are resolution limited, but the high-resolution peak is fully resolved. The vertical arrows indicate the peak positions. Estimated errors of ± 0.01° in the peak positions correspond to ± 0.1 Å in D.

FIGURE 4   Hydration kinetics of the third-order peak of DMPC at $T = 30$°C. When dry, the sample has a third-order peak near 3.8°, and at a nominal 100% RH, the sample has a third-order peak near 4.1°. Peaks for four different times (0, 2, 4, and 13 h) after establishing 100% RH are shown with different gray levels (black, dark gray, light gray, and white, respectively). The inset shows the time dependence of the height of the peak near 3.8° that corresponds to the dry sample.

Our D spacing data for smooth glass/frosted glass pairs are shown in Fig. 5. To show data for both DMPC and DPPC on the same figure, all D spacings for DPPC have been decreased by 3.2 Å to account for the effective length of 0.8 Å for each of the four additional methylenes in DPPC (Nagle, 1993). The humidity is higher for pairs of samples with the larger values of D_{rough}, as one would expect. Because osmotic pressure is very sensitive to differences of relative humidity from 100%, and because such humidity differences are difficult to measure accurately near 100% RH, plots of D spacings versus RH contain much scatter. The idea behind the plot in Fig. 5 is that our best rank ordering of the actual relative humidities is D_{rough}. The vertical axis of Fig. 5 then shows that D_{rough} can be as much as 3–4 Å larger than D_{smooth} near 100% RH, well above the measurement error of 0.2 Å. Just as important for testing the main ideas behind the vapor pressure paradox is the result that the difference ΔD becomes smaller as the RH is reduced, as shown in Fig. 5 by the decrease in ΔD as D_{rough} decreases. The reason for this is that, as the water space is decreased with increasing osmotic pressure, the hydration force becomes the dominant force determining the D spacing. As the hydration force does not depend upon fluctuations, the difference in D spacing between samples on rough and smooth surfaces should become negligible as confirmed by the data shown in Fig. 5.

As mentioned in the Introduction, we were previously able to obtain D spacings of oriented gel-phase DPPC on glass substrates that were equal to the D of fully hydrated unoriented samples. However, we were not able to achieve such large D for L_α phase bilayers, and this is consistent with the picture that gel-phase bilayers do not have significant fluctuations. Another necessary control was to examine samples in the gel phase on the same rough and smooth substrates used in the L_α phase studies. As expected, there were negligible differences in D spacings between frosted and smooth glass substrates when in the gel phase near 100% RH.

Samples on solid substrates also have an interface with the vapor, and so a natural question is how the D spacing is determined by this pair of interfaces. As the correlations are long range, we hypothesize that the surface that has the stronger pinning effect should be the dominant one in determining the D spacing, which is consistent with the way that two surfaces compete in a theory of wetting (Li and Kardar, 1990). The fact that the roughness of the solid substrate makes a difference in the D spacing suggests that the solid substrate has the stronger pinning effect, at least for the smooth substrate. This hypothesis is supported by older experiments on free-standing films that have two vapor interfaces and no solid interface (Smith et al., 1987). Fresh free-standing films of DMPC near 100% RH were reported to thin rapidly from $D = 60$ Å to $D = 54$ Å. The latter value is larger than the largest value of D_{smooth} of ~52 Å for DMPC in Fig. 5; this is consistent with the hypothesis that the pinning effect of a smooth glass surface is greater than for a vapor interface. The hypothesis also suggests that the upper limit to D_{rough} should be 54 Å; the fact that we achieved $D_{rough} = 56$ Å may be that our RH was higher than for the free-standing films.

The new theoretical consideration that we use to interpret these data involves the concept of frustration, as previewed in the Introduction and in Fig. 1. The usual way to treat bilayers and smectic liquid crystals is to use harmonic potentials. For a rough substrate with profile $z_s(r)$, where r is the in-plane coordinate (x,y), the energy per unit area $E(r)$ of the adjacent bilayer with coordinates $z(r)$ is

$$E(r) = [B(z - z_s - a)^2 + K_c(\nabla^2 z)^2]/2, \qquad (1)$$

where the interaction with the substrate is given by the first term with harmonic coefficient B and with mean water spacing a between the bilayer and the wall, and the second term gives the bending energy governed by the modulus K_c (Holyst, 1991). Fourier analysis of $E(r)$ gives the energy of each q mode as

$$E(q) = (B + K_c q^4)(u(q) - v(q))^2/2 + c(q), \qquad (2)$$

where $u(q)$ is the amplitude of the q-mode of the bilayer, $c(q)$ is independent of u, and

$$v(q) = Bu_s(q)/[B + K_c q^4], \qquad (3)$$

where $u_s(q)$ is the Fourier component of the substrate profile. According to this analysis, the effect of a rough substrate is merely to displace the mean value of $u(q)$ to $v(q)$, but it keeps the same effective harmonic modulus $B + K_c q^4$ in Eq. 2. Therefore, the volume of phase space is independent of the substrate profile, and so this analysis does not predict frustration due to rough substrates. One missing ingredient in this traditional analysis is anharmonicity. Be-
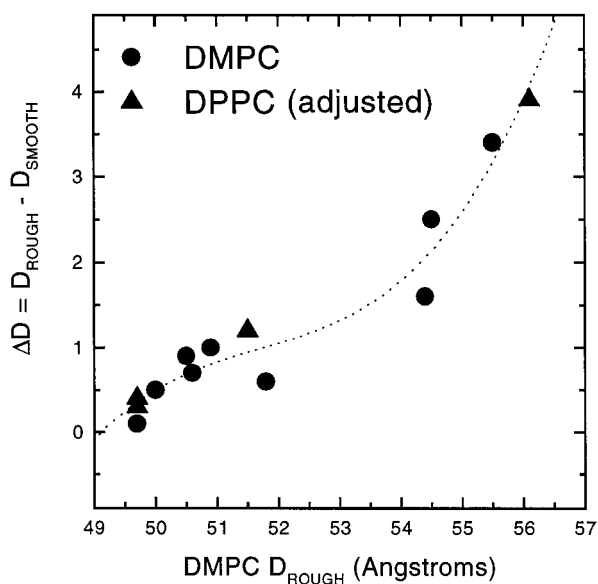

FIGURE 5 Differences $D_{rough} - D_{smooth}$ for frosted and smooth glass substrates versus D_{rough} for DMPC and $D_{rough} - 3.2$ Å for DPPC in the L_α phase. Equilibration times were typically 1–2 days.

cause of the strong and relatively short-range hydration force, it is energetically more difficult for a bilayer to approach the substrate to a distance $D_W/2$ than it is to move away to a distance $3D_W/2$. As the bending energy penalizes the bilayer for tracking the substrate too closely, the mean distance of the bilayer from a rough substrate will be greater than its mean distance D_W from a smooth substrate. Fluctuations will be greater at larger distances because there is more room to fluctuate and also because the effective forces are smaller there. This discussion emphasizes that anharmonicity is an essential basis for the theoretical suggestion that a bilayer is less pinned when adjacent to a rough substrate than to a smooth substrate. One may note that Li and Kardar (1990) have shown theoretically that anharmonicity is also necessary for the mathematically similar problem of enhancing the thickness of a wetting layer by surface roughness.

A complete analysis of frustration should consider a stack of bilayers. Although this is an unsolved problem, the qualitative effect is clear. The mean values $v(q)$ would not propagate to all bilayers in the stack because the energy of the many bilayers not next to the wall would be much higher than if their $v(q)$ were zero; in particular, the energy of the bilayer next to the vapor interface would be minimized by $v(q) = 0$. This source of frustration, namely, that $v(q)$ cannot be constant throughout the stack, will also enhance fluctuations when the substrate is rough.

We suggest that the vapor pressure paradox is being resolved. The data reported here are consistent with the main theoretical ideas of Podgornik and Parsegian (1997) when the additional concept of frustration for rough substrates is added. Whereas the Podgornik and Parsegian theory shows why a substrate should suppress fluctuations, the new concept of frustration explains why rough substrates increase fluctuations compared with smooth substrates.

This resolution of the vapor pressure paradox validates the use of oriented bilayers on solid substrates hydrated from the vapor near 100% RH as good models for biophysical studies of bilayer structure. The differences in D spacing with fully hydrated, unoriented samples are due to smectic fluctuations that produce the entropic repulsive force of Helfrich (1978). As is well known (Caillé, 1972; Zhang et al., 1994), these fluctuations suppress the higher-order x-ray scattering peaks, resulting in low spatial resolution of structure determinations of fully hydrated samples. These fluctuation forces are weak compared with the forces between lipids in the same bilayer that maintain internal bilayer structure, so their presence or absence does not affect that structure, but they are comparable to the other inter-bilayer forces, such as the weak van der Waals interaction, so D spacing is affected. This picture of the relative strengths of intra-bilayer and inter-bilayer interactions is consistent with direct structure determination, which shows that bilayer structure in the L_α phase does not change significantly with application of osmotic pressure up to 24 atmospheres (Nagle et al., 1996) and it is consistent with measured area compressibility moduli (Koenig et al., 1997).

Therefore, one can obtain valid bilayer structure with application of modest osmotic pressure that acts uniformly on all bilayers. The especially pleasing aspect of oriented samples on solid substrates is that only the bilayer at the edge of the sample is subject to a different force, but this still suppresses the fluctuations that degrade the x-ray structure determination. In addition, the Lorentz factor for oriented samples allows detection of more diffraction orders. Therefore, it appears that the only loss in studying oriented samples is the loss of fluctuations, which might play a role in measurements of bilayer dynamics. However, this loss is actually an advantage when comparing with molecular dynamics simulations, which also do not see these long-wavelength fluctuations (Feller and Pastor, 1996).

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