

Finite-size effects do not reduce the repeat spacing of phospholipid multibilayer stacks on a rigid substrate

T.A. Harroun^{1,a}, M. Koslowsky¹, M.-P. Nieh¹, V.A. Raghunathan², and J. Katsaras^{1,b}

¹ National Research Council, Steacie Institute for Molecular Sciences, Chalk River, Ontario, K0J 1J0, Canada

² Raman Research Institute, Bangalore 560 080, India

Received 20 January 2004 /

Published online: 20 April 2004 – © EDP Sciences / Società Italiana di Fisica / Springer-Verlag 2004

Abstract. Finite-size effects in stacks of phospholipid bilayers, in the fluid L_α phase, are investigated using samples oriented on silicon substrates. Recently in this journal, such effects have been suggested as the probable cause of reduced lamellar repeat spacings in very thin samples made up of a few (<10) bilayers. Our systematic studies on samples of different thicknesses do not support this conclusion. At full hydration all samples are found to have the same repeat spacing, irrespective of their thickness. At lower hydrations, on the other hand, very thin samples, consisting of only a few bilayers, have a slightly *larger* spacing.

PACS. 68.08.Bc Wetting – 87.15.Ya Fluctuations – 87.16.Dg Membranes, bilayers, and vesicles – 87.64.Bx Electron, neutron, and X-ray diffraction and scattering

1 Introduction

Phospholipid bilayers on solid substrates have been of interest for many years, both as model membranes for biophysical studies and as general soft condensed matter [1]. Of late, there has been renewed interest in the nature of bilayer fluctuations, and their effects on the structure and phase behavior of substrate supported systems [2–7].

It was noted early on that lipid bilayer stacks hydrated from water vapor at 100% relative humidity (RH) have a lower repeat spacing (d -spacing) than those hydrated in bulk water, indicating less water uptake between the bilayers in the former case [8–11]. Since the chemical potential of water molecules in these two situations is the same, there was no obvious reason for this discrepancy, which came to be called the vapor pressure paradox (VPP). It was believed to be present only in the L_α phase, since Katsaras *et al.* [12] obtained maximal d -spacing in gel ($L_{\beta'}$) phase dipalmitoyl phosphatidylcholine (DPPC) bilayers hydrated from water vapor. This result was later confirmed by Tristram-Nagle *et al.* [13]. Prompted by the extensive and seemingly consistent experimental evidence, an explanation of VPP in L_α phase lipid bilayers was given by Podgornik and Parsegian [14], who suggested that this difference was due to the quenching of thermal undulations of the bilayers by the surface tension at the bilayer stack interfaces. It is well known that these undulations lead to an entropic interbilayer repulsion [15]. Therefore,

their suppression by the interfaces was expected to reduce this repulsion, resulting in a smaller d -spacing of the stack in the L_α phase, where the bilayers are much more flexible.

VPP was resolved experimentally when it was demonstrated that truly 100% RH samples do indeed swell to the same value as those in bulk water, and that the previous discrepancy was due to the experimental difficulties in maintaining strictly 100% RH [2, 16]. A recent theoretical analysis of this problem by Gao and Golubović [17] has shown that the influence of the interfaces are important only in very thin stacks, containing a few bilayers, and not in thicker stacks as had been expected on the basis of the earlier calculations of Podgornik and Parsegian [14].

Lately, the issue of the VPP has again resurfaced in experimental reports published in this journal [5–7]. These papers have focused on a quantitative analysis of bilayer fluctuations in stacks containing a small number of bilayers, supported on a solid substrate. Of particular interest is the paper of Perino-Gallice *et al.* [5], which uses neutron reflectometry and atomic force microscopy (AFM) to examine the surface coverage and lamellar repeat spacing of very thin stacks, containing about 10 bilayers, of the phospholipid dimyristoyl phosphorylcholine (DMPC) in the L_α phase. They find DMPC stacks to be unstable and to dewet the substrate. They also observe a smaller d -spacing of 52 Å for bilayers at 100% RH, instead of the 62 Å expected for fully hydrated DMPC bilayers at the same temperature. They attribute this difference to the VPP. They also point out that this difference could as well arise from a lower hydration of the sample. However,

^a e-mail: Thad.Harroun@nrc-cnrc.gc.ca

^b e-mail: John.Katsaras@nrc-cnrc.gc.ca

no systematic studies were done to distinguish between these two possible scenarios.

In this paper, we examine whether the effect seen by Perino-Gallice *et al.* is in fact a technical issue in achieving 100% RH, or whether surface effects on very thin bilayer stacks are indeed significant. Using neutron diffraction, we have measured the d -spacing of DMPC under conditions of 84, 93 and 100% RH. We find no difference in the d -spacing at 100% RH of samples with approximately 7, 15, or 2000 bilayers. At lower hydrations, the two thicker samples have identical spacings, but the sample with 7 bilayers exhibits a slightly larger spacing. These results show that the influence of the substrate is indeed important but only in stacks containing a few bilayers, as expected theoretically [17]. Further, the spacing of the thinnest sample appears to be determined by the interplay between the suppression of bilayer undulations, imposed by the substrate, and a repulsive van der Waals interaction between the substrate/lipid and lipid/vapor interfaces.

2 Materials and methods

1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and used without further purification. A silicon substrate of dimensions $6.0 \times 2.5 \times 0.1$ cm was cleaned in an ultrasonic bath for 30 minutes in a solution of detergent, followed by a 30 minute rinse in ultra pure water ($18.3 \text{ M}\Omega \text{ cm}$). The lipid was dissolved in chloroform, and an appropriate amount of the solution was sprayed onto the substrate with an artist's airbrush. Residual solvent was removed by placing the sample in a vacuum for 2 hours. The average number of bilayers (N) in the lipid layer was estimated from the amount of DMPC deposited and the area of the substrate, assuming an area per lipid of 50 \AA . The values of N for the three samples studied are approximately 7, 15 and 2000. These values were found to be in good agreement with the multibilayer stack thickness obtained directly from the neutron scattering experiments, using the FWHM of the first-order Bragg reflection (please see the discussion).

For neutron diffraction, samples were suspended upright in an air-tight aluminum cannister (Fig. 1). The hydration of the samples was set by controlling the relative humidity in the aluminum can. For humidities less than 100% RH, saturated salt solutions in D_2O of either KCl ($\sim 84\%$ RH) or KNO_3 ($\sim 93\%$ RH) [18] were placed at the bottom of the can. The sample can was affixed onto a copper base which was connected to a circulating water bath. A thermocouple at the top of the can measured the sample temperature, which was maintained to an accuracy of $\pm 0.2 \text{ K}$. Thermal gradients were kept to a minimum by shielding the aluminum can containing the sample with an evacuated thermal jacket (Fig. 1).

The techniques for achieving 100% RH at the sample have been described elsewhere [2,16]. The important point is the placement of a porous sponge vertically along one side of the cannister, held in place by a stiff metal mesh. The increased evaporative surface area provided to

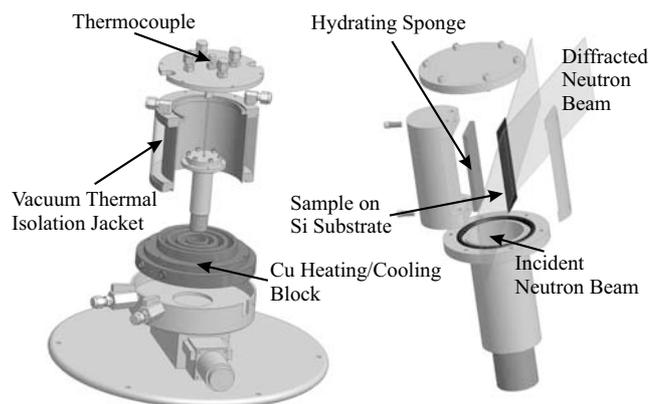


Fig. 1. Sample cell suitable for neutron diffraction and capable of achieving 100% RH. The sample assembly consists of a single-crystal silicon substrate on which the sample is aligned, a hydrating sponge in close proximity to the sample ($\sim 5 \text{ mm}$), and an aluminum block that holds the two in the appropriate geometry. Temperature gradients are minimized by isolating the sample from the outside environment using a thermal jacket and a massive aluminum block.

water by the sponge near the surface of the sample is essential for maintaining maximal hydration. The sponge was placed on the distal side of the sample can, such that neutron diffraction was from the back-side of the silicon substrate, and the sponge did not interfere with the signal. Equilibration of the thin samples took, at most, only a few minutes, whereas the thick sample took several hours. Equilibrium was ascertained by two or more successive scans that exhibited no peak movement.

Data were taken on the N5 spectrometer located at the NRU reactor (Chalk River Laboratories, Canada). The neutron wavelength was an unfiltered 2.37 \AA obtained from the (002) reflection of a pyrolytic-graphite monochromator. The positions of the Bragg peaks were determined by fitting a Gaussian, and an exponential background over the entire q range of data collected.

3 Results

Figure 2 shows the d -spacings of three stacks with different numbers of DMPC bilayers supported on a silicon substrate. Each data point is the average of at least two independent samples. Data for 100% and 93% RH were collected at $30 \text{ }^\circ\text{C}$, while the 84% RH data were collected at $35 \text{ }^\circ\text{C}$, in order to ensure that all samples were in the L_α phase [19]. At 84% and 93% RH, there is little difference between the d -spacings of the 2000 and 15 bilayer samples. The value of $\sim 52 \text{ \AA}$ at 93% RH is equal to that reported by Perino-Gallice *et al.* [5], and is indicated by the lower dashed line in Figure 2. However, the spacing of the very thin, 7 bilayer sample was consistently larger at both 84% and 93% RH.

Increasing the relative humidity to 100% results in all of the samples, regardless of the number of bilayers in the stack, exhibiting the same 62 \AA d -spacing, which is exactly

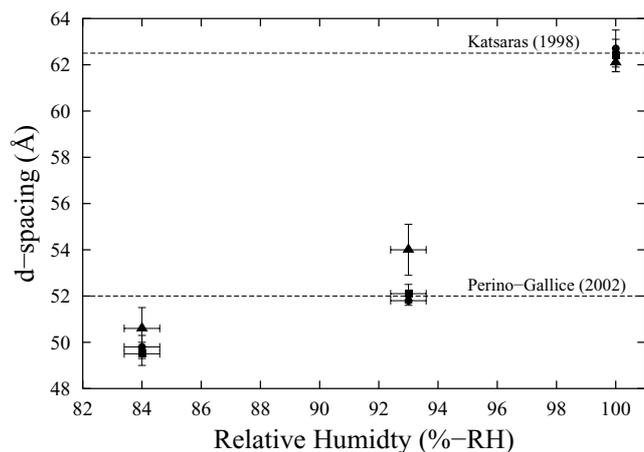


Fig. 2. The d -spacing of DMPC measured as a function of hydration and number of bilayers. The number of bilayers for a given sample were determined to be approximately 2000 (■), 15 (●), 7 (▲). As a function of increasing RH the d -spacing increases from a low of ~ 50 Å at 84% RH, to 62 Å at 100% RH. The dashed lines indicate the values reported by Katsaras [2] and Perino-Gallice *et al.* [5]. The temperatures chosen to carry out the experiments were such that the DMPC bilayers were in the L_α phase.

the value reported by Katsaras [2]. The value of 62 Å occurred whether or not the sample was initially dry or had previously been hydrated using one of the salt solutions. Generally, the samples were found to be stable at 100% RH with the intensity of the Bragg peaks remaining constant over many hours. However, after 24 h or so, the peak intensity would diminish as condensation on the sample's surface washed off the lipid from the silicon substrate.

The stability and quality of the samples can be seen in the rocking curves of the first Bragg peak. Rocking curves are obtained by placing the detector at the Bragg condition ($2 * \theta_{\text{Bragg}}$) and scanning the sample over a range of θ . They are a measure of the sample's alignment and were taken only after the samples had equilibrated. This was determined when the Bragg peaks were no longer changing with time, as seen by repeated $\theta - 2\theta$ scans (a mode where the incident and reflected neutrons make equal angles to the sample surface). Figure 3 shows typical rocking curves for both the thick, 7 and 15 bilayer samples, at low and high hydrations. There are no features beyond the Bragg reflection, in the $\pm 20^\circ$ θ range measured. At $\theta = 0$ and $2 * \theta_{\text{Bragg}}$, the sample is aligned parallel to the incident and diffracted beams, respectively, and in the case of the thick samples the signal decreases due to the maximal absorption by the sample. The width of the base of the peak increases with hydration, due to increasing sample mosaicity, which can be seen most clearly in the data from the thick sample.

4 Discussion

The results presented above, once again demonstrate that the vapor pressure paradox is essentially a technical issue.

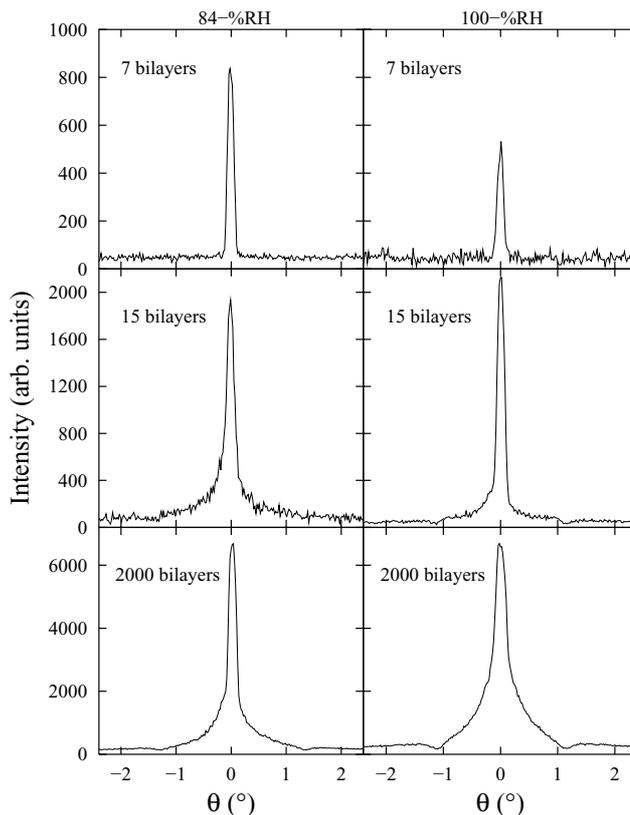


Fig. 3. Rocking curves of the first-order reflection, at 84% and 100% RH. The peak widths increase with both hydration and number of bilayers. The small dips in intensity at around $\pm 1^\circ$ are due to absorption by the sample, of the incident and diffracted beams, respectively. In the case of thin samples, the dips, for obvious reasons, are greatly reduced.

Achieving strictly 100% RH at the sample is a difficult problem when designing a sample cell. From Figure 2 we see that the d -spacing is very sensitive to the RH. Temperature and humidity gradients are always present in any sample cell. The largest gradient is encountered as one moves away from the source of water vapor. In our case, even placing the samples such that they faced away from the sponge caused a marked decrease in the d -spacing. Some lowering of these gradients might be brought about by mixing the air inside the cell. However, a much greater reduction is achieved simply by holding the wet sponge near the face of the sample. This allows the presence of a large evaporative surface for the water source, as close to the lipid as possible.

Since the d -spacing at full hydration is the same regardless of the value of N , one may question whether there are domains of build-up in thin samples, where the number of bilayers is much larger. There are two reasons to believe that the samples are in fact very thin. First, the amount of sample applied was quite small, $\sim 2.8 \mu\text{g}$ per monolayer. By applying the lipid in chloroform with broad, even strokes of the air brush, the solvent mist instantly evaporates on contact, eliminating any pooling of solvent that might deposit more lipid in one area. This technique

applies a reasonably even film across the surface, which cannot be seen by the eye in the case of 7 bilayers. Furthermore, because of over-spray, not all of the lipid makes it onto the substrate, so the sample thickness is invariably overestimated. Secondly, the FWHM of the diffraction peaks can be used to estimate the sample thickness l . The Bragg peak of the thickest sample is found to be resolution limited. The thickness of the other two samples can be estimated from the observed peak widths. Taking the instrumental resolution function and the observed Bragg peak to be Gaussians of width δq_i and δq_o , respectively, the inherent width of the peak $\delta q_s (= 2\pi/l)$ can be estimated using the relation, $\delta q_o^2 = \delta q_i^2 + \delta q_s^2$. We calculated the instrumental resolution of the N5 spectrometer in our configuration to be 0.012 \AA^{-1} . This yields values of 13 ± 2 and 8 ± 2 for the number of bilayers in the two thin samples. Note that these are in good agreement with the corresponding values of 15 and 7 estimated from the amount of lipid deposited on the substrate.

Although the d -spacing at 100% RH is insensitive to the thickness of the sample, the data show the possibility of a novel surface effect at lower hydrations, which leads to a larger d -spacing of the 7 bilayer stack. One possible reason for this behavior is the presence of a repulsive van der Waals interaction between the two interfaces of the lipid layer, which would tend to thicken the layer by taking in more water, resulting in a higher d -spacing. The van der Waals interaction is repulsive in this case, since the dielectric properties of the lipid layer is intermediate between those of the silicon substrate and the vapour [5,20]. As this force is proportional to l^{-3} , it can at best only be expected to have an effect in the thinnest of samples. However, our estimates show that this effect is too small to explain the observed behaviour even in these samples. A more plausible explanation of the increased d -spacing observed in thin samples at less than full hydration is given by the recent theory of Gao and Golubović [17]. We are presently carrying out experiments that will address this question.

In conclusion, neutron diffraction experiments on lipid bilayer stacks oriented on silicon substrates rule out the possibility of VPP in multibilayer stacks comprised of a few bilayers, which had been invoked to account for some recent data on similar systems. Further, our data

at $< 100\%$ RH indicate the existence of novel finite-size effects, which lead to an increase in the repeat spacings of very thin stacks.

The authors would like to thank Asha Suppiah and Stephen Chan for their help with these experiments.

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