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Evidence of the hydration force in gel phase lipid multibilayers

J. Katsaras[1] and K. R. Jeffrey[2]

[1] *Atomic Energy of Canada Limited - Chalk River, Ontario, K0J 1J0, Canada*
[2] *Department of Physics, University of Guelph - Guelph, Ontario, N1G 2W1, Canada*

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Abstract. – We present applied osmotic pressure *vs.* distance curves constructed from high-resolution neutron and X-ray diffraction scattering density profiles of dipalmitoyl phosphatidylcholine (DPPC) multibilayers in the gel $L_{\beta'}$ phase. The data, obtained from three distinct regions of the lipid bilayer, show that at small interbilayer distances the repulsion observed in these bilayers can be accounted for by the so-called "hydration force". Also, a previous observation of a sharp upward break in the pressure-distance curve of highly ordered $L_{c'}$ DPPC bilayers is not the result of steric repulsion but rather of a 2D $\rightarrow$ 3D phase transition. The data are consistent with a recent theoretical model which predicts that the repulsive forces in ordered phases (*e.g.*, $L_{\beta'}$ and $L_{c'}$ phases) are dominated by the hydration force.

Lipid multilayer membrane systems, which consist of lipid bilayers separated by water, are of fundamental importance as they provide us with a good model for biological membranes. Of interest to biology are the intermembrane interactions. The basic forces at small bilayer-bilayer separations (≤ 20 Å) are the van der Waals attractive forces and repulsive forces arising from either direct hydration or from entropic fluctuations [1], [2], all of which probably play an important role in protein assembly, cell-cell interactions and cell fusion.

There are both experimental [1], [3] and theoretical studies [4] which demonstrate, at small intermembrane distances, the presence of a strong repulsive force commonly known as the "hydration force". It is believed that this repulsive interaction between the bilayers is the result of lipid molecules perturbing the water structure near the lipid-water interface [5]. A somewhat different idea is that the hydration force results from a partial charge transfer between the polar headgroups and the water molecules resulting in a sort of "chemical hydration" of the bilayer surface [6].

In recent years, a completely different explanation has been proposed which argues that the repulsive forces between dynamic interfaces, such as lipid bilayers, are primarily due to conformational entropic fluctuations such as membrane undulations, single molecule protrusions and headgroup overlap [2], [7], [8]. Experimentally, it has been demonstrated that entropic

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steric undulatory repulsions dominate intermembrane interactions in a sodium dodecyl sulfate (SDS) fluid membrane system at large intermembrane distances of 38 Å $\leq d \leq$ 163 Å [9]. It has also been argued that in the ordered low-temperature $L_{c'}$ phase, DPPC bilayers exhibit a steric pressure, as a result of headgroup motion, at an interbilayer spacing of about 3 Å [3].

In the last few years a simple model has been introduced which takes into account the competition between the hydration and protrusion forces [4]. Lipowsky and Grotehans, using Monte Carlo simulations and functional renormalization group methods, have developed a hybrid model wherein the repulsion force is dominated by either entropic or hydration forces, depending on whether the system was, respectively, above or below its main gel-to-liquid crystalline phase transition temperature [4].

X-ray diffraction [1], [3], [7], [9] is one of the most common techniques employed in the construction of the pressure-distance curves [1]-[3], [7] used for the determination of the various repulsive forces between bilayers. It is predicted that plotting the log of osmotic pressure *vs.* surface separation should yield very different results for the various predicted types of forces. a) For the hydration force, an exponential dependence described by $P = P_0 e^{-d_x/\lambda}$ is predicted, where P is the applied pressure, P_0 is the applied pressure in the limit $d_x = 0$, where d_x is the separation between adjacent bilayers and λ is the decay length characteristic of the solvent molecule. b) For undulatory steric repulsion of the type predicted by Helfrich [10] and demonstrated at large intermembrane spacings in the SDS fluid membrane system [9], a power law dependence is expected. c) A discontinuity in the pressure-distance curve at distances between 3–5 Å is the signature of headgroup collisions [3], [8] and molecular protrusions. Molecular protrusion and headgroup collision models do predict an exponential dependence over several decades in pressure [2] and only deviate from this dependence when the amphiphilic surfaces are very close to one another.

In this paper, we present data obtained from neutron and X-ray diffraction measurements of DPPC bilayers in the $L_{\beta'}$ phase. Pressure-distance curves constructed, for the first time, from three distinct regions of the lipid bilayer show that this system is in the regime dominated by hydration forces. In addition, using X-ray diffraction data, we make the case that the single piece of evidence for steric repulsion in ordered bilayers [3] is in fact the result of the system experiencing a phase transition from a 2D to a 3D structure.

For samples used in the X-ray diffraction studies, the procedure for their preparation is outlined in ref. [11]. The samples used in the neutron studies were prepared as follows: A concentrated solution of lipid and methanol was placed on a silicon wafer. After initial evaporation of the methanol, the sample was placed under vacuum for $\approx$ 24 h to remove any traces of the solvent. Once dry, a 0.2 M *p*H 7.0 phosphate buffer was pipetted onto the dry sample and left to saturate the sample for 0.5 h after which the excess was removed from the top of the sample. Once this process was complete, the sample was annealed at 60 °C and $\approx$ 100% relative humidity (RH). The resulting samples were highly aligned having a mosaic spread of $\approx 0.5°$. Data for $L_{\beta'}$ phase bilayers were collected at a temperature of 20 °C.

Figure 1 shows 1D *a*) X-ray and *b*) neutron scattering density profiles of $L_{\beta'}$ DPPC multibilayers having a repeat spacing (*d*-spacing) of 57.2 Å along with *c*) a model of the unit cell contents. The angle θ made by the hydrocarbon chains with the bilayer normal (fig. 1 *c*)) is known to vary in DPPC bilayers as a function of hydration from $\approx 22°$ at 0% RH to $\approx 32°$ at RHs approaching 100% [12]. The distance measurements of the pressure *vs.* distance curves presented in fig. 2 were obtained from such 1D scattering profiles (fig. 1 *a*) and *b*)).

Since model membrane systems are for the most part centrosymmetric structures, changing the *d*-spacing allows for the determination of the structure factors (*e.g.*, [12], [13]) necessary for the reconstruction of the 1D scattering density profiles (fig. 1). Using this methodology, electron density profiles were solved to $\approx$ 4.5 Å resolution (*d*-spacing/# of reflections) while

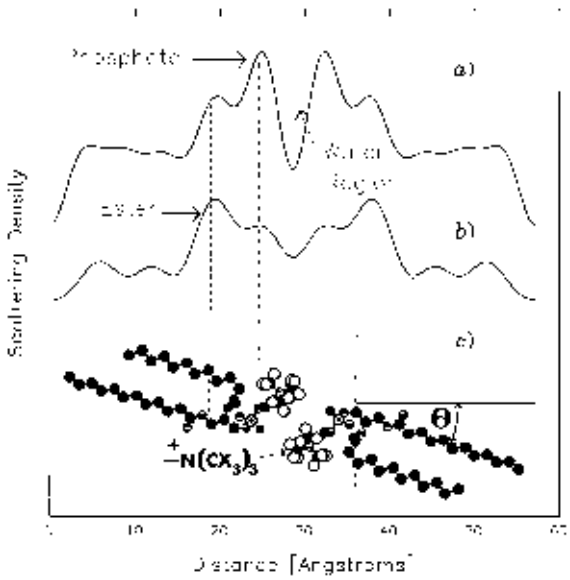

Fig. 1. – 1D *a*) X-ray and *b*) neutron scattering density profiles of DPPC multibilayers having a repeat spacing of 57.2 Å. *c*) The positions of the phosphate (PO_4^-), ester (—COO—) and *tert*-methylamine [$—^+N(CX_3)_3$] groups of the lipid molecule are shown with respect to the 1D profiles. The X in the CX_3 group represents either a deuteron or a proton. The deuterons or protons on the *tert*-methylamine group are shown as open circles. In DPPC, the hydrocarbon chains are tilted at an angle θ with the bilayer normal that varies as a function of hydration.

neutron scattering profiles were calculated to a resolution of about 7.0 Å.

The distances between adjacent *tert*-methylamine groups ($—^+N[CX_3]_3$) as a function of applied pressure (full circles) are presented in fig. 2 *a*). They were obtained by fitting the difference neutron scattering profiles ($[—^+N[CD_3]_3] - [—^+N[CH_3]_3]$) with two pairs of Gaussians. As a function of hydration, the probability of headgroup overlap decreases from a maximum probability [14] of 0.75 at 0% RH to a Prob_{max} of ≈ 0 for multibilayers at a RH of approximately 100%. Regardless of the high probability of headgroup collisions in the low-hydration regimes, the forces measured show an exponentiality over 4 decades in pressure. It is also interesting to note that at $\approx$ 100% RH the *tert*-methylamine group exhibits a bimodal distribution (not shown) not present for bilayers at 0% RH. The existence of this bimodal distribution was recently confirmed using a molecular-dynamics simulation of an $L_{\beta'}$ DPPC bilayer [15]. The datum at 5.0×10^5 dyn/cm^2 (full square) was obtained in the following manner: The adjacent phosphate and ester distances for excess water DPPC bilayers having a *d*-spacing of 64.0 Å were measured to be $\approx$ 19.0 and 28.2 Å, respectively [16]. At $P = 5.0 \times 10^5$ dyn/cm^2, the *d*-spacing for DPPC bilayers is 63.2 Å [3]. Since, to a first approximation, the change in *d*-spacing can be solely attributed to a decrease in the water region [12], then the phosphate and ester separations for bilayers under 5.0×10^5 dyn/cm^2 of applied pressure are estimated to be 18.2 and 27.4 Å, respectively. The separation for adjacent *tert*-methylamine groups at the above-mentioned pressure was also assigned a value of 18.2 Å. In fig. 2 *b*) and *c*) the pressure-distance plots were constructed using the separations between adjacent phosphate (fig. 2 *b*)) and ester (fig. 2 *c*)) groups obtained from 1D electron density (open squares) and neutron scattering (full circles) profiles. As in fig. 2 *a*), the plots show an exponential dependence. However, we believe that

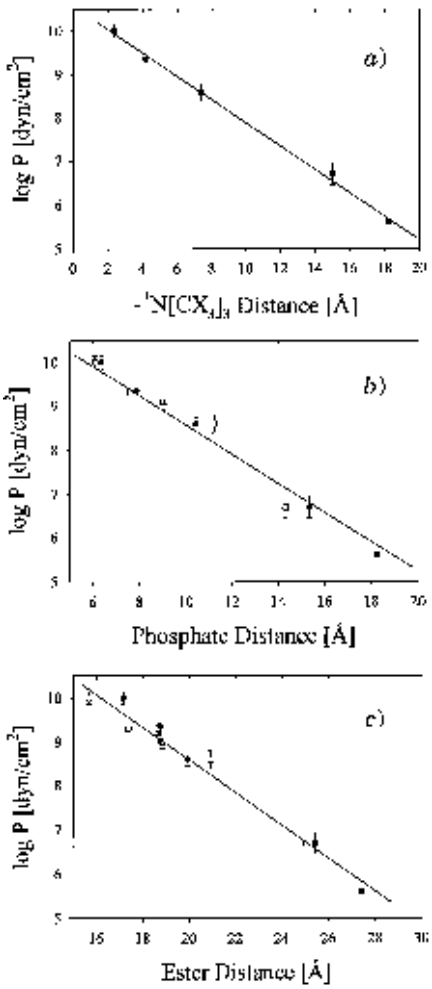

Fig. 2. – Logarithm of applied pressure ($\log P$) *vs.* distance between various groups along the bilayer. Separations between adjacent *a*) *tert*-methylamine, *b*) phosphate and *c*) ester groups obtained using a variety of RHs and resulting in decay lengths (λ's) of 1.63, 1.18 and 1.14 Å, respectively. The distances represented by full circles were obtained from neutron measurements, by open squares from X-ray diffraction measurements and the full square from an X-ray datum presented in ref. [16].

simply showing that the force has the form of an exponential dependence is not sufficient proof for the presence of the hydration force since, as we mentioned above, some steric forces are expected to show exponentiality over a large range in applied pressure.

The adsorption isotherm of DPPC at 22.0 °C shows that at percent RHs of 20, 45, 75 and 100 the amounts of adsorbed water molecules per lipid molecule are 1.2, 2.0, 3.5 and 9.0, respectively [17]. Using an area of 19.5 $Å^2$/hydrocarbon chain, a volume of 30 $Å^3$ for a molecule of water and hydrocarbon chain tilt angles (θ's) of 22, 23, 28 and 32° for DPPC at the above-mentioned humidities [12], then direct hydration alone would result in separations of 1.7, 2.8, 4.7 and 11.7 Å for RHs of 20, 45, 75 and 100%, respectively. These results are in very good agreement with the average separations of 1.6, 3.1, 4.5 and 9.5 Å calculated using the data

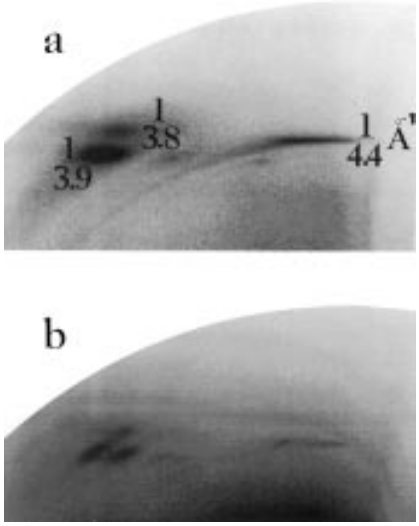

Fig. 3. – X-ray diffraction patterns at 7 °C of hydrocarbon chains from *a*) aligned $L_{c'}$ DPPC multibilayers (*d*-spacing = 56.4 Å) forming a 2D molecular lattice with no out-of-plane correlations (*i.e.* correlation length < repeat spacing) and *b*) the formation 3D DPPC multibilayers (*d*-spacing = 55.7 Å) resulting in the breakup of the hydrocarbon chain reflections as a result of having an out-of-plane correlation length greater than the repeat spacing of the multibilayer stacks.

presented in fig. 2 for the various regions of the lipid molecule. In fact, the separations from adjacent *tert*-methylamine groups (fig. 2 *a*)) obtained from neutron experiments yielded results which most closely agreed with the expected data (1.8, 5.0 and 12.6 Å, for RHs of 20, 75 and 100%, respectively). The small discrepancy between the calculated and measured separations at 100% RH may be the result of an increased presence of steric repulsion forces due to the bilayers becoming more disordered.

One of the principal pieces of evidence for the existence of the steric repulsion force in DPPC bilayers is the data of McIntosh and Simon [3]. Using osmotically stressed $L_{c'}$ DPPC multibilayers, it was shown that there was a sharp upward break in the pressure-distance curve at a bilayer separation of about 3 Å, indicative of headgroup overlap repulsions. It was recently shown that the $L_{\beta'} \rightarrow L_{c'}$ phase transition can be regarded as a disorder-order transition in which the $L_{c'}$ phase results in the formation of a molecular lattice which is positionally correlated across the bilayer [11]. However, there are no out-of-plane correlations making the $L_{c'}$ phase a highly ordered 2D phase [11]. How, therefore, can steric repulsions at interbilayer distances of 5 Å or less play a minor role in the $L_{\beta'}$ phase, a phase in which the headgroups exhibit a large degree of positional disorder [18], and yet dominate at similar interbilayer distances in a phase ($L_{c'}$ phase) where it is known that these same headgroups are ordered on a 2D lattice [11]?

In fig. 3 we present hydrocarbon chain reflections from aligned $L_{c'}$ DPPC multibilayers. The reflections in fig. 3 *a*) are from 2D DPPC bilayers having a *d*-spacing of 56.4 Å and exhibiting no out-of-plane correlations [11], [12]. The *d*-spacing for such 2D bilayers can easily be altered by changes in the percent RH, similar to the way we altered the *d*-spacing in the $L_{\beta'}$ DPPC multibilayers. However, one of the great advantages of $L_{\beta'}$ DPPC multibilayers at 20 °C is that all the phases exhibited over a range of hydrations (0% to 100% RH) are 2D phases with no out-of-plane correlations [12], [19]. This is not the case for $L_{c'}$ DPPC bilayers. Figure 3 *b*)

shows hydrocarbon chain reflections that have broken up into smaller bars indicative of the formation of a 3D structure. In addition, the relative intensities between the two $L_{c'}$ bilayers (fig. 3) have changed considerably (*e.g.*, 1/4.4 Å^{-1} *vs.* 1/3.9 Å^{-1}). McIntosh and Simon [3] show five RHs in which the d-spacing of $L_{c'}$ DPPC multibilayers ($\approx$ 54 Å) remains unaltered resulting in a sharp upward break in the pressure-distance plot. This can easily be explained in terms of a 3D phase which once fully formed will not undergo changes in d-spacing as a function of hydration. *This break is not the result of steric repulsion*! As such, if we take 56.4 Å $L_{c'}$ DPPC bilayers to represent the lower d-spacing limit in which these bilayers exist as 2D structures, we observe that for the pressure-distance plots presented in ref. [3] the force can have the form of an exponential.

In conclusion, we have presented evidence showing that the dominant repulsive force in gel phase bilayers is the result of direct hydration. Pressure-distance plots derived from three different regions of the $L_{\beta'}$ bilayer show an exponential dependence with the changes in d-spacing corresponding closely to the changes in hydration predicted by adsorption isotherms. This is direct evidence for the dominance of the hydration force at these intermembrane separations. Also, data were presented showing that highly ordered $L_{c'}$ DPPC bilayers, at very small interbilayer separations ($\leq$ 3 Å), are capable of forming a 3D phase causing a sharp upward break in the pressure-distance curve. This result thus negates any evidence for steric repulsion at distances $\leq$ 3 Å in the $L_{c'}$ phase of DPPC.

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