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Letter to the Editor

On the Analysis of Elastic Deformations in Hexagonal Phases

INTRODUCTION

The elastic parameters of lipid mesophases, such as bending modulus and intrinsic curvature, have commonly been obtained from the response of inverted hexagonal (H_{II}) phase to osmotic stress. H_{II} phase consists of a large number of hexagonally packed rods, one of which is shown in cross section in Fig. 1. In the original work on this approach (Rand et al., 1990), in which these rods were assumed to be circular cylinders, only two terms of the free energy of H_{II} phase were accounted for, the elastic bending energy of the H_{II} monolayer and the osmotic energy (normalized per lipid molecule):

$$G_{H_{II}} = \frac{1}{2} K_b A_p \left(\frac{1}{R_p} - \frac{1}{R_{0p}} \right)^2 + \Pi V_w, \quad (1)$$

where K_b is the bending modulus, A_p is the molecular area, and R_p and R_{0p} are the actual local radius of curvature and the intrinsic radius of curvature at the pivotal plane, respectively. Π is the difference in osmotic pressure between the outside and inside of the H_{II} cylinder, and V_w is the volume of water phase per lipid inside the H_{II} cylinder. Since hexagonal phase is normally studied in distilled water in the absence of solutes, the osmolality inside does not change when the cylinder radius changes, i.e., Π is normally constant. The pivotal plane is defined as a surface inside the lipid phase such that both A_p and V_p are constant when the distance between rod axes varies. V_p is the volume per molecule between the Luzzati plane (a plane that divides the lattice into purely water and lipid phases) and the pivotal plane. More rigorous and complex definitions of these planes can be made (Leikin et al., 1996), but the practical definitions of Rand et al. (1990) suffice for this discussion. The minimum of the free energy as a function of only R_p at any given osmotic pressure Π can be resolved analytically and is defined by the simple expression:

$$\Pi R_p^2 = 2K_b \left(\frac{1}{R_p} - \frac{1}{R_{0p}} \right). \quad (2)$$

A plot of ΠR_p^2 versus $1/R_p$ yields a straight line, whose slope gives $2K_b$ and whose intercept defines $1/R_{0p}$. As a confirmation of this expression, experimental data from several studies are reasonably well described by straight lines.

This treatment, however, does not account for the energy associated with the poorly packed regions (''interstices'') between circularly cylindrical rods. Because of this, parameters obtained with this conventional approach should be valid only as long as two assumptions are met: 1), the shape of the rods (i.e., of the water core) is indeed a circular cylinder; and 2), the interstice (i.e., packing frustration) energy does not depend on the size of a hexagonal unit. The first assumption is a rather rough approximation, since the true cross-sectional shape of the water core as reconstructed from x-ray diffraction analysis deviates from a circle by as much as 5% of the average radius (Turner and Gruner, 1992). The second assumption actually contradicts the first one, since larger hexagonal unit sizes would lead to larger interstitial defects and, thus, larger frustration energies. We consider here the consequences of relaxing these assumptions.

To relax these assumptions, one must have a way of calculating the ''interstice'' or ''frustration'' energy. There have been two approaches taken to this problem. In the first, the rods were assumed to be circular in cross section and the ''frustration'' energy was assumed to be proportional to the surface area of the ''hydrophobic interstices'' (called the ''void'', although there is not actually a void) between hexagonally packed circular cylinders (Siegel, 1993, 1999). The proportionality constant was obtained by equating the free energies of the lamellar and hexagonal phases at the temperature of transition between the two phases (Siegel, 1993). In a later approach, the rods were assumed to be hexagons in cross section, and the ''frustration'' free energy was accounted for in terms of the degree of acyl-chain ''tilt'' (away from the monolayer normal) and ''splay'' (generalized form of bending) required to fill this hexagonal cylinder (Hamm and Kozlov, 1998). The ''tilt modulus'' (free energy per unit projection of the acyl chains along a tangent to the monolayer surface in the *X* direction—see Fig. 1) was also determined by equating the free energies of lamellar and hexagonal phases at the phase transition temperature (Hamm and Kozlov, 1998), so it is not surprising that the frustration free energies calculated by the two methods, with similar assumptions of constant geometry, are consonant (Lentz et al., 2002). The former approach makes no assumptions about the molecular mechanisms by which lipid bending frustration is relieved, but, at first glance, is parameterized in a way that might not be extendable to other structures. The latter approach does make an assumption about the molecular mechanism by which bending frustration might be relieved (molecular tilt), and, if that assumption is correct, it might be possible to extend the method to other geometries (e.g., fusion intermediates (Kozlovsky and Kozlov, 2002)). Both methods

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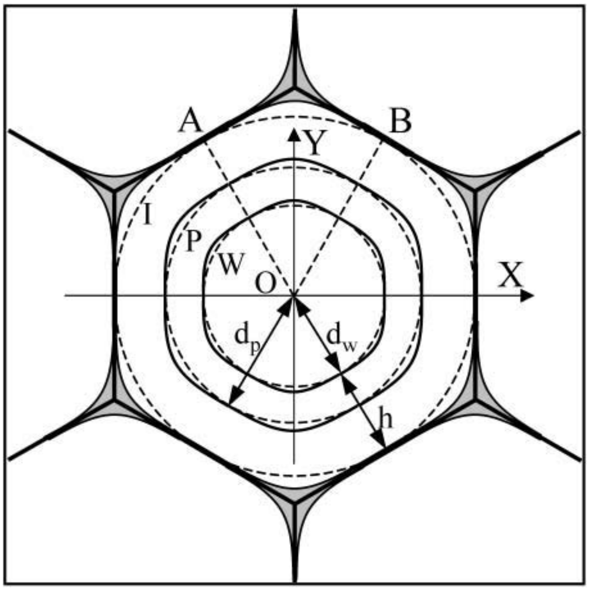

FIGURE 1 Cross section of the H_{II} phase. The cross-sectional structure of hexagonally packed cylinders (delimited by *thick solid lines*) in H_{II} phase is illustrated. Labeled are the Luzzati (*W*) and pivotal (*P*) planes and the distances from the center to these two planes along the interaxial distance (d_w and d_p). The imaginary hydrophobic plane (*I*) is a constant distance, *h*, from the Luzzati plane, leading to the hydrophobic interstices, which are shaded gray. The locations of these planes in the conventionally assumed circular cylindrical geometry is illustrated by dashed boundaries, whereas the solid boundaries illustrate the more complex planes predicted by our calculations.

assume fixed cross-sectional rod geometries (circular and hexagonal), although the actual cross-sectional geometry is probably intermediate to these (Harper et al., 2001; Rappolt et al., 2003). The assumption of fixed geometries probably leads to overestimation of the free energy of nonlamellar (Markin and Albanesi, 2002) or fusion intermediate (May, 2002) structures. In this letter and the accompanying article on fusion intermediates (Malinin and Lentz, 2004), we adopt the simpler (''interstice'') approach, and parameterize the hydrophobic interstice energy as proportional to the volume of an imaginary ''void'' volume (V_v) (shaded area in Fig. 1) defined by the outermost curved surfaces shown in Fig. 1 and described below. We also relax the assumption of a fixed geometry by insisting that the H_{II} structure be at a free energy minimum consistent with the geometric assumptions of our model. Here we ask whether this simple model can account for the variation of H_{II} lattice size with osmotic stress (Leikin et al., 1996). We have found that: 1), the ''imaginary void'' approach fully accounts for the experimental osmotic stress-strain data; 2), fitting these data provides an alternative and more accurate approach to obtaining the free energy per unit void volume; 3), the minimal free energy structure is intermediate between cylindrical and hexagonal in cross section; and 4), this structure must vary with osmotic stress to explain the observed variation of hexagonal phase unit cell size and water content.

RESULTS

The central assumption of our model is that an imaginary plane (plane *I* in Fig. 1), which is located at a monolayer thickness, *h*, from the Luzzati plane, delimits the region of efficient lamellar-like lipid packing in the H_{II} structure. This distance *h* was assumed to be uniform throughout the structure and was obtained by insisting that the volume fraction of water calculated according to our model (Eqs. 5–7 below) matched the experimental volume fraction of water, which is given as a function of the lattice dimension in the experimental data presented in Fig. 2 *b* of Leikin et al. (1996). The value of *h* obtained in this way varied from 1.60 to 1.63 nm as osmotic pressure varied from 0 to 1.5 kT/nm^3 ($\sim 10^{7.8}$ dynes/cm^2 or 63 atm). The assumption of a uniform lamellar structure leads to a space between the imaginary plane and the actual edge of the hexagonal cell, which we call the ''void''. As stated, we estimate the void energy (also referred to as frustration or interstice energy) as proportional to an imaginary ''void'' volume, though we acknowledge that this space is actually filled with acyl chains resulting in distortions in lipid packing elsewhere in the H_{II} phase structure. Previous treatments of the frustration energy have assumed proportionality to the ''void'' surface (Markin and Albanesi, 2002; Siegel, 1993). Our reasons for assuming proportionality to volume are discussed elsewhere (Malinin and Lentz, 2004).

Next, we relax the assumption that the water core has a circular cylindrical shape. To do so, we allow the pivotal plane to bulge outward from its circular location in a sector *AOB* defined as shown in Fig. 1. We define the *Y* coordinate of the pivotal plane as a function of the *X* coordinate by defining the extent of the bulge above the normally assumed circular cylindrical surface, $\delta(x)$, as $y = \sqrt{d_p^2 - x^2} + \delta(x)$, where d_p is the distance from the axis of a rod to the pivotal plane in the interaxial direction (see Fig. 1). Since $\delta(x)$ is a symmetrical function around $x = 0$, then a polynomial series presentation of $\delta(x)$ should have only the terms of even orders. We used this series up to the sixth-order term, and applied boundary conditions, $\delta(0) = \delta_o$; $\delta(d_p/2) = \delta'(d_p/2) = 0$, to obtain the polynomial, $\delta(x) = \delta_o(1 - 4x^2/d_p^2)^2(1 + 4cx^2/d_p^2)$, where δ_o is the maximal deviation from circular cylindrical geometry and *c* is the last undetermined coefficient. This coefficient, as defined by requiring that the bending free energy per molecule be minimized, was ~ -0.05 for $\delta_o < 0.1$. To simplify calculations, we truncated this polynomial by assuming $c = 0$ to a form that was still accurate within 1% of δ_o. Thus, we used the following function to define the shape of the bulged hexagonal rods in cross section:

$$y = \sqrt{d_p^2 - x^2} + \delta_o\left(1 - 4x^2/d_p^2\right)^2. \quad (3)$$

Third, we assume that the pivotal plane is positioned at a constant distance h_1 from the Luzzati plane, thus allowing for a small extent of lipid compressibility. The distance h_1 would be defined as $R_p - R_w$ if we assumed circular geometry, where R_p and R_w are the radii of cylinders at the pivotal and Luzzati planes, respectively. In this case, R_p and R_w are constant and defined experimentally in terms of the circular geometry of the model (Leikin et al., 1996). However, in our approach, we do not assume a fixed, circular geometry, and R_p and R_w can vary along the perimeter of the cross section of the pivotal and Luzzati planes. Thus, we must define h_1 as the difference between apparent average R_p and R_w values ($h_1 = R_p^a - R_w^a$) in order that our values for h_1, R_p^a, and R_w^a remain consistent with the experimentally determined quantities used by Leikin et al. (1996):

$$R_w^a = d_{hex}\sqrt{\frac{2\phi_w}{\pi\sqrt{3}}}; \quad R_p^a = R_w^a\sqrt{1 + \frac{1-\phi_w}{\phi_w}\frac{V_p}{V_l}}, \tag{4}$$

where d_{hex} is the measured first-order Bragg spacing of the hexagonal phase, ϕ_w is the measured volume fraction of water, and V_l is the experimental volume of a lipid molecule. The pivotal plane defined by this method led to the calculated average molecular volume between the Luzzati and pivotal planes being equal to the experimental V_p to within an accuracy of ~0.1%. Knowing h_1, we determine $d_p = d_{hex}/\sqrt{3} - h_2$, where $h_2 = h - h_1$. Then, the aqueous volume, the void volume, and the total H_{II} phase volumes per lipid molecule are:

$$V_w = A_p\left(6\int_{-d_p/2}^{d_p/2}\left(y - \frac{h_1}{\sqrt{1+y'^2}}\right)\left(1 - \frac{h_1}{R_p}\right)dx - \frac{3\sqrt{3}}{2}(d_p - h_1)^2\right)\Bigg/\left(6\int_{-d_p/2}^{d_p/2}\sqrt{1+y'^2}dx\right) \tag{5}$$

$$V_v = A_p\left(\frac{7\sqrt{3}}{2}(d_p + h_2)^2 - 6\int_{-d_p/2}^{d_p/2}\left(y + \frac{h_2}{\sqrt{1+y'^2}}\right) \times \left(1 + \frac{h_2}{R_p}\right)dx\right)\Bigg/\left(6\int_{-d_p/2}^{d_p/2}\sqrt{1+y'^2}dx\right) \tag{6}$$

$$V_t = A_p\left(\frac{2}{\sqrt{3}}d_{hex}^2\right)\Bigg/\left(6\int_{-d_p/2}^{d_p/2}\sqrt{1+y'^2}dx\right), \tag{7}$$

where $R_p = (1 + y'^2)^{3/2}/y''$ is the local radius of curvature at the pivotal plane, and y' and y'' are the first and second derivatives of y. From this, the apparent monolayer thickness h was chosen so that the calculated $\phi_w^{cal} = V_w/V_t =$ the experimental $\phi_w = -0.0084d_{hex}^2 + 0.1827d_{hex} - 0.5265$, a function obtained from the experimental data presented in Fig. 2 *b* of Leikin et al. (1996). Now, the total free energy per lipid molecule can be expressed as:

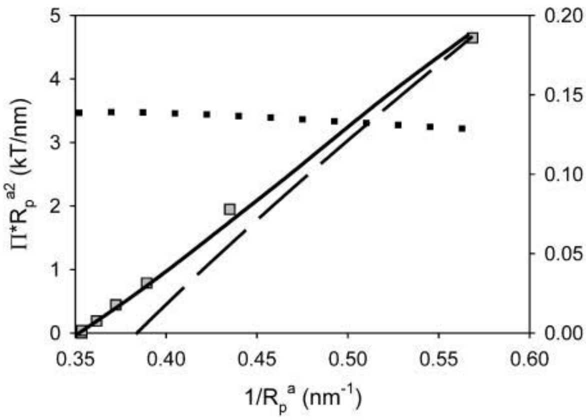

FIGURE 2 Fitting the stress-strain relationship experimental data. The data (*squares*) are taken from Leikin et al. (1996). The solid line was obtained from the model allowing H_{II} cylinder deformation and using parameters $1/R_{0p} = -.32$ nm^{-1}, $K_b = 11$ kT, and $K_v = 2.0$ kT/nm^3. Using the same parameters but assuming a fixed circular geometry yielded the dashed line. The dotted line shows the value of the molecular void volume (*right axis*) as a function of the apparent monolayer curvature ($1/R_p^a$) that increases with applied osmotic stress.

$$g = A_p\frac{K_b}{2}\int_{-d_p/2}^{d_p/2}\left(\frac{1}{R_p} - \frac{1}{R_{0p}}\right)^2\sqrt{1+y'^2}dx\Bigg/\int_{-d_p/2}^{d_p/2}\sqrt{1+y'^2}dx + K_vV_v + \Pi V_w, \tag{8}$$

where K_v is a proportionality coefficient representing the free energy of a unit of void volume.

Global minimization of the molecular free energy of H_{II} phase was performed with Mathematica 4.1 (Wolfram Research, Champaign, IL). To perform calculations, we used published material parameters for DOPE ($A_p = 0.65$ nm^2, $V_p = 0.375$ nm^2, $V_l = 1.235$ nm^3) (Leikin et al., 1996). We determined the best fit of our model to published stress-strain data ($\Pi^*R_p^2$ versus $1/R_p$ from Fig. 5 in Leikin et al. (1996)) to establish the values of the mechanical parameters R_{0p} (intrinsic curvature), K_b (bending modulus), and K_v (void energy coefficient). This fit is shown in Fig. 2. To obtain this fit, we minimized the total free energy (Eq. 8) with respect to d_{hex} and δ_o, and adjusted h as mentioned earlier at each value of the osmotic pressure, with assigned initial values of K_b, R_{0p}, and K_v. Using d_{hex} found in this way, we obtained R_p^a values according to Eq. 4 for each value of Π. These R_p^a values were then used to plot the calculated curve of $\Pi^*R_p^{a2}$ versus $1/R_p^a$. Values of K_b and R_{0p} were then varied and the procedure repeated to minimize the sum of squared residuals between calculated and experimental stress-strain curves. This was initially carried out for an assumed value of K_v, and then the optimized values of K_b and R_{0p} were used to obtain a new estimate of K_v by Siegel's approach (Siegel, 1993). In this, the energy of hexagonal phase is equated to the energy of a plain monolayer at the lamellar/hexagonal phase transition temperature ($T_H \sim 3.3°C$ (Toombes et al., 2002)). We used an estimate of −0.015 nm/K for the dependence of R_{0p} on temperature (Kozlov et al., 1994) to determine that $R_{0p}(T_H)$ is ~8% larger than R_{0p} determined from our fitting (room temperature). This new estimate of K_v was then used to obtain new best-fit values of K_b and R_{0p}. This iterative procedure quickly converged to give estimates of all three energy parameters: $1/R_{0p} = -.32\ \text{nm}^{-1}$, $K_b = 11$ kT, and $K_v = 2.0\ \text{kT/nm}^3$. K_b is nearly identical to, although the intrinsic curvature is somewhat less than, the values one would obtain from the same data by the classical approach ($-.35\ \text{nm}^{-1}$ and 10.8 kT). The ''void'' modulus, K_v, is the same as the estimate (2.1 kT/nm^3) we (Malinin and Lentz, 2004) obtained using Leikin's published estimate of the frustration energy (Leikin et al., 1996). It is interesting that the $1/R_{0p}$ and K_b parameters are so close to the classical ones. This probably results from compensating effects resulting from our relaxation of two conventional assumptions. First, instead of ignoring it (Rand et al., 1990), we accounted explicitly for the frustration or ''void'' energy. Second, instead of assuming a fixed geometry (either circular or hexagonal), we allowed geometries intermediate between these two extreme shapes so as to minimize the free energy at each osmotic pressure, thereby reducing the void volume. A significant result of relaxing this latter assumption is it distributes unfavorable free energy between the ''void'' and bending contributions (0.035 kT), probably accounting for the lower total frustration energy found from our analysis (0.278 kT) as compared to the classical analysis that assumes a rigid geometry (0.352 kT) (Kozlov et al., 1994), and which assumes that bending does not contribute to the free energy.

The resulting plot of $\Pi^* R_p^{a2}$ versus $1/R_p^a$ as presented in Fig. 2 is very close to, but not exactly, linear. By comparison, the plot calculated using the same values of R_{0p}, K_b, and K_v, but with a fixed circular cylindrical geometry, deviates more significantly from a straight line and has the wrong slope (*dashed line*). Thus, a change in H_{II} structure is essential, in our formulation, to obtain the experimental response to osmotic stress. This has not been previously noted, and, in fact, the earlier treatment of these data assumed a fixed hexagonal cross section (Leikin et al., 1996). As the applied osmotic pressure increases from 0 to 1.5 kT/nm^3 (~$10^{7.8}$ dynes/cm^2 or 63 atm), the shape of the H_{II} cylinder changes, with δ_o decreasing from ~0.061 nm to ~0.011 nm. Absent this change in shape, the ''void'' volume per molecule would decrease dramatically as the osmotic pressure increased (the hexagonal dimension decreased). With this change in shape, the void volume per molecule (V_v) decreased only slightly from 0.139 to 0.128 nm^3 with decreasing H_{II} cylinder size, as can be seen from the plot of V_v versus $1/R_p^o$, (*dotted line* in Fig. 2). It is worth noting that the calculated radii of the water cylinder in the interaxial and interstitial directions at $\Pi = 0$ differ from circular by only ~3%, which is in good agreement with the ~5% distortion from circularity seen by x-ray diffraction (Turner and Gruner, 1992).

To summarize, our model for the free energy of closed, curved vesicles, which assumes that the frustration energy is proportional to the ''void'' volume (Malinin and Lentz, 2004), is applicable to the H_{II} phase and is consistent with the roughly linear stress-strain relationship observed for this phase. The feature of our model that is needed to correctly account for the stress-stain data is relaxation of the assumption of a fixed cross-sectional geometry, which is allowed to adjust so as to minimize the free energy of the total structure. Analysis of H_{II} phase stress-strain data by this method provides an alternative, and perhaps improved, method for obtaining material parameters for the H_{II} phase.

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REFERENCES

Hamm, M., and M. M. Kozlov. 1998. Tilt model of inverted amphiphilic mesophases. *Eur. Phys. J. B.* 6:519–528.

Harper, P. E., D. A. Mannock, R. N. Lewis, R. N. McElhaney, and S. M. Gruner. 2001. X-ray diffraction structures of some phosphatidylethanolamine lamellar and inverted hexagonal phases. *Biophys. J.* 81:2693–2706.

Kozlov, M. M., S. Leikin, and R. P. Rand. 1994. Bending, hydration and interstitial energies quantitatively account for the hexagonal-lamellar-hexagonal reentrant phase-transition in dioleoylphosphatidylethanolamine. *Biophys. J.* 67:1603–1611.

Kozlovsky, Y., and M. M. Kozlov. 2002. Stalk model of membrane fusion: solution of energy crisis. *Biophys. J.* 82:882–895.

Leikin, S., M. M. Kozlov, N. L. Fuller, and R. P. Rand. 1996. Measured effects of diacylglycerol on structural and elastic properties of phospholipid membranes. *Biophys. J.* 71:2623–2632.

Lentz, B. R., D. P. Siegel, and V. Malinin. 2002. Filling potholes on the path to fusion pores. *Biophys. J.* 82:555–557.

Malinin, V., and B. R. Lentz. 2004. Energetics of vesicle fusion intermediates: comparison of calculations with observed effects of osmotic and curvature stresses. *Biophys. J.* 86:2951–2964.

Markin, V. S., and J. P. Albanesi. 2002. Membrane fusion: stalk model revisited. *Biophys. J.* 82:693–712.

May, S. 2002. Structure and energy of fusion stalks: the role of membrane edges. *Biophys. J.* 83:2969–2980.

Rand, R. P., N. L. Fuller, S. M. Gruner, and V. A. Parsegian. 1990. Membrane curvature, lipid segregation, and structural transitions for phospholipids under dual-solvent stress. *Biochemistry.* 29:76–87.

Rappolt, M., A. Hickel, F. Bringezu, and K. Lohner. 2003. Mechanism of the lamellar/inverse hexagonal phase transition examined by high resolution x-ray diffraction. *Biophys. J.* 84:3111–3122.

Siegel, D. P. 1993. Energetics of intermediates in membrane fusion: comparison of stalk and inverted micellar intermediate mechanisms. *Biophys. J.* 65:2124–2140.

Siegel, D. P. 1999. The modified stalk mechanism of lamellar/inverted phase transitions and its implications for membrane fusion. *Biophys. J.* 76:291–313.

Toombes, G. E., A. C. Finnefrock, M. W. Tate, and S. M. Gruner. 2002. Determination of L(α)-H_{II} phase transition temperature for 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine. *Biophys. J.* 82:2504–2510.

Turner, D. C., and S. M. Gruner. 1992. X-ray diffraction reconstruction of the inverted hexagonal (H_{II}) phase in lipid-water systems. *Biochemistry.* 31:1340–1355.

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