Helfrich Repulsion and Dynamical Phase Separation of Multicomponent Lipid Bilayers

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Thermal fluctuations of surfactant bilayers in an aqueous solution produce an effective, long-range repulsion that can lead to a continuous unbinding transition. We report on an optical interferometry study of the thermal fluctuations of multicomponent bilayers close to the unbinding transition. We find that, in contrast to the case of single-component bilayers, the thermal fluctuation spectrum of multicomponent bilayers does not agree with a continuous unbinding transition but instead indicates the proximity of an unbinding tricritical point.

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Surfactant bilayers played an important role in the development of soft condensed-matter physics as model systems for the study of the statistical mechanics of interacting surfaces. The successful elucidation in the 1980s of the subtle competition between entropy and enthalpy that rules the interaction between surfactant bilayers in an aqueous environment was a major step forward [1]. Interest in biological membranes, such as the lipid-rich cell surface, has been an important secondary motive for the study of surfactant bilayers.

The "bare" bilayer-bilayer interaction energy per unit area—denoted by $V(h)$—is repulsive for spacings h in the subnanometer range, due to the hydration interaction between the polar (or charged) headgroups of the surfactant molecules, while at larger spacings, attractive van der Waals forces compete with electrostatic repulsion. In the biologically relevant range of salt concentrations, $V(h)$ has a single minimum, the "van der Waals minimum," with a depth $W = -V(h^*)$ of about 10^{-4} to 10^{-5} J/m^2 at an optimal spacing h^* of about 2–3 nm. Thermal fluctuations of the bilayer spacing, known as *undulations,* renormalize $V(h)$ when W is reduced. The classical theory of Lipowsky and Leibler ($\mathrm{LL_I}$) [2] obtained a continuous *unbinding transition* at a critical value W_c (around 10^{-5}–10^{-6} J/m^2 for lipid bilayers), characterized by a divergence of the mean spacing $\langle h\rangle$. If in the unbound phase (i.e., $W < W_c$) two bilayers are maintained at a fixed mean spacing $\langle h\rangle$ by an external constraint, then membrane-membrane "collisions" take place [3] with a typical lateral spacing of the order of [4] $\xi_\parallel \approx \sqrt{(\kappa/k_BT)\langle h\rangle}$. Here, κ is known as the "Helfrich bending energy," a measure of the bending stiffness of the bilayer. If ΔE_c is the *free energy cost per collision* then the total free energy cost per unit area V_{HE} imposed by the constraint can be estimated as [5] $V_{\mathrm{HE}}(\langle h\rangle) \propto \Delta E_c/\xi_\parallel^2$. Both renormalization-group calculations [6] and Monte Carlo simulations [7,8] obtain for this "Helfrich repulsion"

$$V_{\mathrm{HE}}(\langle h\rangle) = c_{fl}\,\frac{(k_BT)^2}{\kappa\langle h\rangle^2}\,, \tag{1}$$

where $c_{fl}(W) \equiv \Delta E_c(W)/k_BT$ is the dimensionless collision energy. It is predicted that for $W > W_c$ this is a *universal number* $c_{fl} \cong 0.116$, independent of both the internal structure of the membrane and the details of the form of the wall potential. Small-angle x-ray diffraction (SAXS) studies [9] of stacked lamellar bilayers confirmed the validity of Eq. (1), albeit with a discrepancy in the value of c_{fl}, and Helfrich repulsion has become a central concept for analyzing the phase behavior of surfactant systems.

More recently, interest has focused on the closed lipid bilayers—known as vesicles or liposomes—that are used in biotechnology applications. These vesicles normally contain *additives* such as low molecular weight *PEG lipid,* a lipopolymer that prevents van der Waals aggregation between bilayer vesicles through steric repulsion [10]. A second important additive is *cholesterol,* which acts as a stabilizing and antifreeze agent [11]. Both lipopolymers and cholesterol are highly soluble in lipid membranes. Since the collision energy is a universal quantity that does not depend on the details of the membrane structure, Eq. (1) should retain its validity for multicomponent systems. However, an earlier SAXS study [12] of stacked bilayers containing the PEG-lipid additive found an unexpected and singular dependence of the Helfrich repulsion on the surface concentration σ of the additive. The current Letter reports on a microscopy study of the applicability of Eq. (1) to bilayers containing PEG-lipid and cholesterol additives.

Our experiments were carried out on deflated, giant unilamellar vesicles of the 1,2-Dielaidoyl-sn-Glycerol-3-Phosphocholine (DEPC) lipid containing various amounts of the PEG lipid (1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-[Polyethylene glycol 2000]) and

cholesterol [13]. The Flory radius R_g of the PEG lipids—about 3.8 nm—is comparable to the position of the minimum h^* of $V(h)$. The vesicles first were prepared by swelling the lipid material in a 170 mM sucrose solution in the presence of an electric field. To prevent suppression of undulation fluctuations by osmotic tension, the vesicles were solvated in a solution of 100 mM NaCl, 10 mM HEPES at pH 7.2, with sufficient external osmotic pressure to deflate them. The vesicle suspension was placed in contact with a flat substrate with the vesicles sinking to the substrate under the action of the gravitational force. The strength of the van der Waals interaction between bilayer and substrate was controlled by incubating the substrate (cleaned glass cover slips) with a thin, nonadhesive, protein passivation film (Blotting Grade Blocker Non-Fat Dry Milk, BioRad, CA) of adjustable width. The width of the film was increased to the point that the bilayer was just in the unbound phase, with an estimated value for W of about 1.3×10^{-6} J/m^2.

To examine the collisions between the bilayer and the substrate, we sampled the bilayer displacement field $h(\vec{r}, t)$ as a function of time and lateral position, using the reflection interference contrast microscopy (RICM) method [14]. This technique allows reconstruction of the real-space profile $h(\vec{r}, t)$ by an inverse cosine transformation of the interference pattern generated by light reflected from the substrate-buffer interface and the buffer-membrane interface. The RICM method permits sensitive measurement of spacing *differences*, with a typical vertical resolution of 5 nm, while the absolute value of the spacing can be determined only with a resolution of 15 nm. The closed measuring chamber was mounted on an inverted Axiomat microscope (Zeiss, Germany), equipped with an antiflex objective (Plan Neofluar, 63 $\times$ /1.25 Oil, Zeiss). The interferograms were observed with a Peltier-cooled 10 bit CCD camera (C4880-85, Hamamatsu, Japan), and the digitized images were directly stored, using real time imaging software developed by one of the authors [15].

Figure 1(a) shows three RICM images of a DEPC bilayer with 50 mol% cholesterol, taken with a time interval of 12 s. The characteristic length scale Δx of the "leopard-skin" texture is of the order of a few microns. Comparison of successive images provides an indication of the temporal variation (see framed area). Figure 1(b) shows a time series of the spacing fluctuations of $h(\vec{r}, t)$, averaged over an area of 8×10^{-15} m^2, recorded at 100 Hz. The power spectrum (not shown) has a zero frequency "peak anomaly" with a width $\Delta\omega = 2\pi/\Delta t$ of the order of 0.1 Hz. This peak anomaly is shown in Fig. 1(b) as the slow switching between two mean values about 30 nm apart, with more rapid fluctuations taking place in the two "substates." Comparison of the typical size of the spacing fluctuations (about 30–50 nm) with the typical value of h^* in the bound state (2–3 nm) demonstrates that the bilayer indeed is in the unbound state. It must be noted that a single minimum in the measured times series of $h(\vec{r}, t)$ may consist of repeated

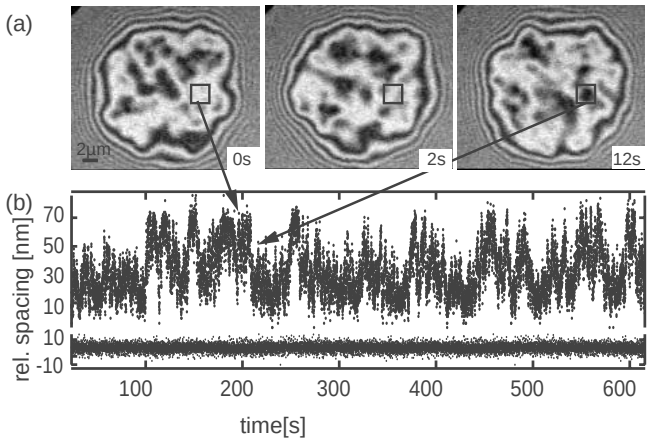

FIG. 1. (a) RICM interferograms of a giant DEPC (50 mol% cholesterol) vesicle at three consecutive times. The grey scale pattern is changing in time due to fluctuations of the substrate-membrane distance (cf. framed area). (b) Time series of the spacing between substrate and membrane obtained by an inverse cosine transformation of the intensity of the RICM interferogram of the framed area of Fig. 2(a) (upper trace). The lower trace is the signal noise level.

collisions that are not time resolved. Very similar time series were obtained at different substrate locations, with no evidence for substrate heterogeneity.

To compare with theory, we determined the probability distribution $P(h)$ obtained from time series measured at various locations, each with about 60 000 data points. The resulting sequence of distributions is shown in Fig. 2, plotted as a function of the PEG-lipid and cholesterol concentrations. Leibler and Lipowsky [16] (LL_{II}) theoretically analyzed the fluctuations of membranes in the unbound phase around a given mean spacing $\langle h \rangle$ (imposed by a linear confining potential) finding that the fluctuations $\phi(\vec{r}, t) = h(\vec{r}, t) - \langle h \rangle$ around the mean should be governed by a Gaussian Hamiltonian:

$$H([\phi]) = \tfrac{1}{2}\kappa \int d^2r\, [(\nabla^2\phi)^2 + 4\xi_\parallel^{-4}\phi^2]. \qquad (2)$$

Here,

$$\xi_\parallel = \frac{8}{C_\parallel}\sqrt{\frac{2\kappa}{k_B T}}\,\langle h \rangle$$

plays the role of the in-plane correlation length with $C_\parallel \approx 5.83$ a universal amplitude ratio [17]. The probability distribution $P(h)$ that results from Eq. (2) is a single-peak Gaussian with an rms width $w = \langle \phi^2 \rangle^{1/2}$ (or "roughness") proportional to the mean spacing: $\langle w \rangle = \langle h \rangle / C_\perp$, where $C_\perp = \sqrt{5}$ is another universal amplitude ratio [17].

The left panel in the first row of Fig. 2 shows the average of the measured probability distribution for a single-component DEPC bilayer. $P(h)$ can indeed be fitted by a single Gaussian of rms width 25 $\pm$ 6 nm and mean spacing 50 $\pm$ 15 nm. The in-plane correlation length $\xi_\parallel$ equals 540 $\pm$ 50 nm assuming $\kappa = 25 k_B T$ for DEPC. If we compute the mean spacing $\langle h \rangle$ by equating the gravitational force on the vesicle $F_g = g\Delta\rho V_V$ with the total

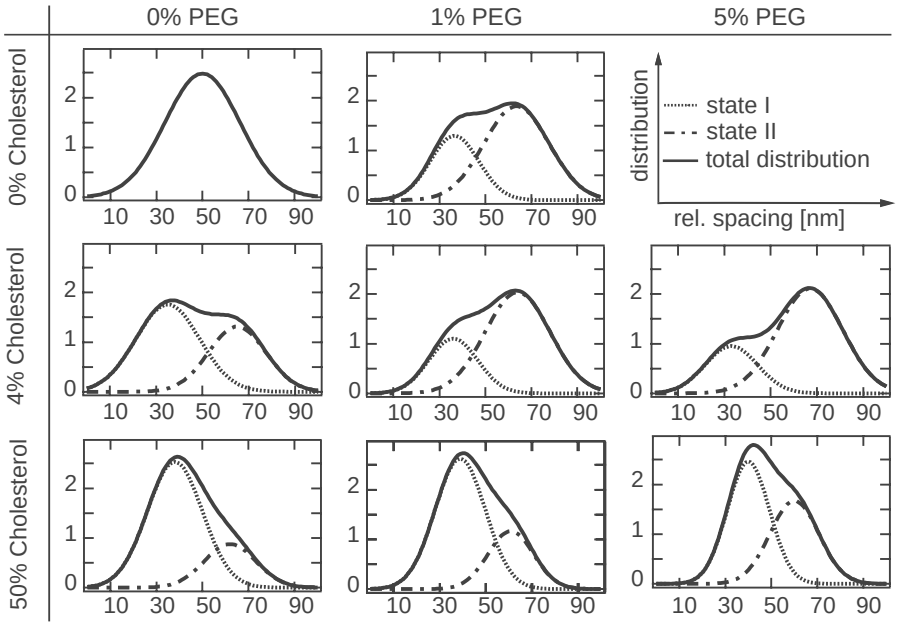

FIG. 2. Fitted probability distributions $P(h)$ of the substrate-membrane spacing for various values of the PEG-lipid concentration (0 mol %, 1 mol %, and 5 mol % PEG) and the cholesterol concentration (0 mol %, 4 mol %, and 50 mol %) in a DEPC bilayer.

Helfrich force $-A_c[dV_{\mathrm{HE}}(h)/dh]|_{h=\langle h\rangle}$, where g is the gravitational acceleration, $\Delta\rho$ is the density contrast of the vesicle interior, V_V is the vesicle volume, and A_c is the contact area, we obtain $\langle h\rangle = 65 \pm 5$ nm. This is consistent both with the measured value and the theoretically predicted ratio $1/\sqrt{5}$ of the width and the mean spacing. The measured fluctuation spectrum for a single-component lipid bilayer thus appears to agree well with the theoretical predictions.

In Fig. 3 we show the $P(h)$ for a bilayer containing 50 mol % cholesterol and it is clear that the measured distribution cannot possibly be fitted by a Gaussian. In fact, *none of the probability distributions associated with the multicomponent systems could be fitted by a single Gaussian*. The measured distributions could, however, be fitted by the sum of *two* Gaussians:

$$P(h) \propto \frac{W_{\mathrm{I}}}{w_{\mathrm{I}}} \exp^{-(1/2)[(h-h_{\mathrm{I}})^2/w_{\mathrm{I}}^2]} + \frac{W_{\mathrm{II}}}{w_{\mathrm{II}}} \exp^{-(1/2)[(h-h_{\mathrm{II}})^2/w_{\mathrm{II}}^2]} \quad (3)$$

where the fitting parameters $h_{\mathrm{I,II}}$ and $w_{\mathrm{I,II}}$ correspond to, respectively, the mean spacings and rms widths of the two states, while the $W_{\mathrm{I,II}}$ are the respective statistical weights. The two peaks, denoted by I and II, can be identified with both the black-and-white regions in Fig. 1(a) and the two substates in the time series Fig. 1(b). As shown in Fig. 2, the peak positions $h_{\mathrm{I,II}}$ or I and II always straddle the mean spacing of the single-component bilayer and are relatively independent of composition, while the $w_{\mathrm{I,II}}$ also do not vary significantly and remain comparable to the width of $P(h)$ for the single-component bilayer (about 25 nm). The *weight ratio* $W_{\mathrm{I}}/W_{\mathrm{II}}$ of states I and II is, however, very sensitive to composition. Consider first the case of fixed cholesterol content (4 mol %, second row of Fig. 2) and varying lipopolymer fraction. The relative weight $W_{\mathrm{I}}/W_{\mathrm{II}}$ of the first peak is reduced to 0.24 of its initial value by a 5 mol % increase in lipopolymer fraction. This indicates that there exists distinct lipopolymer-poor (I) and lipopolymer-rich (II) states, such that increasing the lipopolymer fraction increases the relative weight of state II over state I. For fixed PEG-lipid content and varying cholesterol content (cf. columns of Fig. 2), we observe a similar scenario, except that the weight ratio $W_{\mathrm{I}}/W_{\mathrm{II}}$ dramatically *increases* with cholesterol content, so it is state I that is enriched in cholesterol. The different effect of the two additives is physically reasonable since lipopolymers

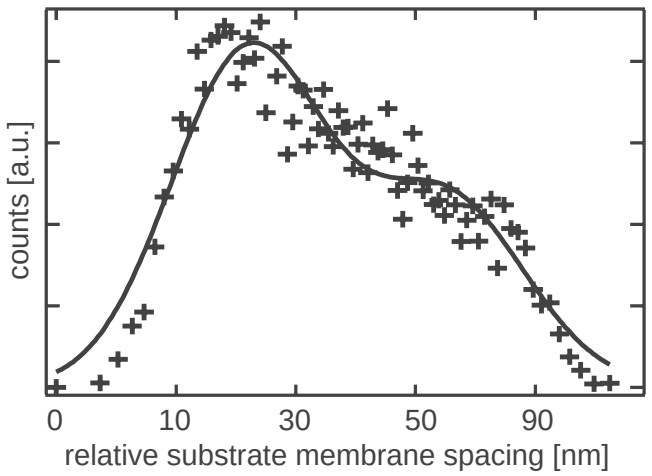

FIG. 3. A typical probability distribution $P(h)$ of the substrate-membrane spacing obtained from time series of a DEPC bilayer with 50 mol % cholesterol and 0 mol % PEG. The markers are the data points and the line is a fit to the data using Eq. (3).

act as *repellers* that favor a state with a larger spacing whereas the increased stiffness of a cholesterol-rich section makes thermal fluctuations energetically more costly and hence favors a state with a smaller spacing.

These observations raise two fundamental issues. First, they indicate that Helfrich collisions induce a form of phase segregation even for highly soluble additives [18]. A theoretical study of Helfrich repulsion in multicomponent membranes by Netz and Pincus [19] indeed predicted that, over an intermediate range of P values, the coupling between *membrane curvature* and the concentration σ of foreign inclusions leads to lateral phase separation, even at extremely low concentrations. The phase segregation reported here is actually only *dynamic* in nature: the statistical properties of the times series $h(\vec{r}, t)$ do not depend on position, so there is no true "macroscopic" phase separation.

The second—and more challenging—issue is the appearance of a double-peak structure in $P(h)$. Because of the universal nature of the collision energy, the predicted single-peak distribution of $\mathrm{LL_{II}}$ should apply to multicomponent bilayers provided the bilayer is homogeneous at sufficiently large length and time scales. In fact, the double-peak distribution could not be understood within the classical framework *even in the presence of lateral phase separation*. The reason is that the dependence of the mean spacing on bilayer composition enters Eq. (1) only through an effective, renormalized bending energy $\kappa(\sigma)$, where σ is the surface concentration of the additive. For a symmetric lipopolymer layer with radius of gyration R_g it is predicted [20] that $\kappa(\sigma) \cong \kappa + k_B T \sigma R_g^2$ (for σR_g^2 less than 1). Assuming lateral phase separation of σ, one finds that for $\kappa = 25 k_B T$ the difference in peak positions is much too small to explain the positions of the two peaks observed in the probability distribution.

Two theoretical studies of the unbinding transition, based, respectively, on the renormalization-group method [21] and the Landau free energy expansion [22], indicated that a continuous unbinding transition is not the only possibility: the phase diagram may contain a line of *first-order transitions* ending at a tricritical point. The observation of two large correlation lengths—the parallel correlation length $\xi_\parallel$ and the characteristic length scale Δx of the dynamical phase separation—appears consistent with tricritical behavior, as is the observation of multiple time scales in the time series. If the Landau theory of Ref. [22] is extended by including the additive concentration as a second degree of freedom that is coupled to the bilayer concentration, one finds that this indeed generically favors the first-order transition scenario.

In summary, we have found that the unbinding transition of multicomponent lipid bilayers cannot be described by the classical $\mathrm{LL_{I,II}}$ theory of a continuous unbinding transition. Instead, we observed a dynamical form of phase separation, both in terms of the bilayer spacing and the composition, possibly governed by a tricritical point. Confirmation of this interpretation would require a more extended experimental study—since two thermodynamic parameters must be independently varied to fully investigate a tricritical point—as well as more theoretical insight in the nature of spacing fluctuations near an unbinding tricritical point.

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