

Discontinuous Unbinding of Lipid Multibilayers

B. Pozo-Navas,¹ V. A. Raghunathan,² J. Katsaras,³ M. Rappolt,¹ K. Lohner,¹ and G. Pabst^{1,*}

¹*Institute of Biophysics and X-Ray Structure Research, Austrian Academy of Sciences, Schmiedlstrasse 6, 8042 Graz, Austria*

²*Raman Research Institute, Bangalore 560 080, India*

³*National Research Council, Steacie Institute for Molecular Sciences, Building 459, Stn. 18, Chalk River, Ontario, Canada K0J 1J0*

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We have observed a discontinuous unbinding transition of lipid bilayer stacks composed of phosphatidylethanolamine and phosphatidylglycerol using x-ray diffraction. The unbinding is reversible and coincides with the main ($L_\beta \rightarrow L_\alpha$) transition of the lipid mixture. Interbilayer interaction potentials deduced from the diffraction data reveal that the bilayers in the L_β phase are only weakly bound. The unbinding transition appears to be driven by an abrupt increase in steric repulsion resulting from increased thermal undulations of the bilayers upon entering the fluid L_α phase.

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Phospholipid bilayers are widely used as mimics of biological membranes [1]. They are also known to display complex physical behaviors, some of which we are only now beginning to understand. One such behavior is the much anticipated unbinding transition of a stack of fluid bilayers [2] which arises, as a function of temperature, from the change in balance between the attractive and repulsive interactions and which in turn, determines the stability of the bilayer stack. In the case of bilayers made up of uncharged lipids the competing interactions at large separations are the steric repulsion due to bilayer undulations [3], and the van der Waals attraction.

At lower temperatures van der Waals attraction dominates and the bilayers are in a bound state, with the bilayer separation being determined by the position of the minimum in the interaction potential. Steric repulsion increases with increasing temperature and at some point may overwhelm the attractive interaction. The minimum in the interaction potential would then vanish and the system would transform from a bound to an unbound state. For a multibilayer system consisting of charged lipids the electrostatic repulsive contribution, described by the Poisson-Boltzmann theory, must also be considered. There have been many theoretical studies of the unbinding transition (reviewed in Ref. [4]) and it is predicted to be continuous in the case of two interacting membranes, with the bilayer separation diverging at the transition. Scaling arguments indicate that the width of the critical region is inversely proportional to the number of bilayers in a stack and, therefore, for a multibilayer system the unbinding transition is expected to appear discontinuous [4].

In spite of the large number of theoretical studies, experimental observations of the unbinding transition have so far been scarce. Mutz and Helfrich [5] reported the loss of positional correlations of bilayers in isolated multilamellar vesicles (MLVs) made of digalactosyldiacylglycerol (DGDG) using optical phase contrast microscopy. More recently, a second observation of an unbinding transition was claimed by Vogel *et al.* [6] using x-ray

reflectivity studies on lipids oriented on a solid substrate. However, their claim was contradicted by Pabst *et al.* [7], who showed that these samples peel off the substrate before they can undergo an unbinding transition. Consequently, Pabst *et al.* concluded that aligned multibilayers are not suitable for studying the unbinding transition.

In this Letter, we report the first observation of an unbinding transition in a dispersion of charged MLVs composed of zwitterionic phosphatidylethanolamines (PEs) and negatively charged phosphatidylglycerols (PGs). We find a discontinuous transition from a bound to an unbound state, coinciding with the main gel (L_β) to liquid crystalline (L_α) transition. Analysis of x-ray diffraction data on pure PG membranes reveals that the unbinding transition is not driven by changes in electrostatic interactions, but is due to steric repulsion caused by undulating bilayers in the L_α phase.

1,2-dipalmitoylphosphatidylethanolamine (DPPE), 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE), 1,2-dipalmitoylphosphatidylglycerol (DPPG, Na-salt) and 1-palmitoyl-2-oleoyl-phosphatidyl-glycerol (POPG, Na-salt) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. 5 wt% multilamellar liposomal dispersions were obtained by hydrating previously prepared dry lipid films of appropriate PE/PG composition in a 20 mM Na-phosphate buffer containing additional amounts of NaCl. The pH of the solvent was adjusted to 7.4 to ensure complete dissociation of the PG headgroups [8]. The PE/PG molar ratio was kept below 9 in order to eliminate any possibility of macroscopic phase separation [9].

X-ray diffraction patterns were recorded simultaneously in the small and wide-angle regions using a SWAX camera (Hecus-MBraun, Graz, Austria) mounted on a sealed-tube generator (Seifert, Ahrensburg, Germany) operating at 2 kW. Cu K_α radiation ($\lambda = 1.542 \text{ \AA}$) was selected using a Ni filter. Samples were taken up in 1 mm diameter quartz capillaries and the temperature was controlled using a programmable Peltier unit. Samples were equilibrated for 10 min at each temperature prior to

measurement, and the exposure time was set to 50 min. With the above-mentioned experimental setup repeat spacings of up to 1500 Å are discernible.

Figure 1 shows the diffraction patterns of a POPE/POPG (87:13 mol/mol) mixture in a buffer containing 130 mM NaCl at three temperatures. At 20 °C [Fig. 1(A)] the quasi-Bragg peaks are clearly observable indicating that the bilayers are in a bound state. However, the peaks are very broad. The large peak width, which is characteristic of a system with a short scattering correlation length, is an indication that the bilayers are weakly bound. Further, the large lamellar periodicity, d , of 125 Å shows that the bilayers are in a highly swollen state. The swelling is, however, not limited by the amount of water in the system, since the lipid concentration is only 5 wt %. Hence, the interbilayer interaction potential has a minimum at the corresponding bilayer separation. Upon heating to 30 °C the quasi-Bragg peaks disappear and the system exhibits pure diffuse scattering due to the bilayer form factor. This indicates that the interbilayer positional correlations are completely lost [Fig. 1(B)]. However, upon cooling back to 20 °C the system returns to the bound state [Fig. 1(C)]. The diffraction pattern in Fig. 1(C) is practically indistinguishable from that in Fig. 1(A), demonstrating that the unbinding transition is fully reversible.

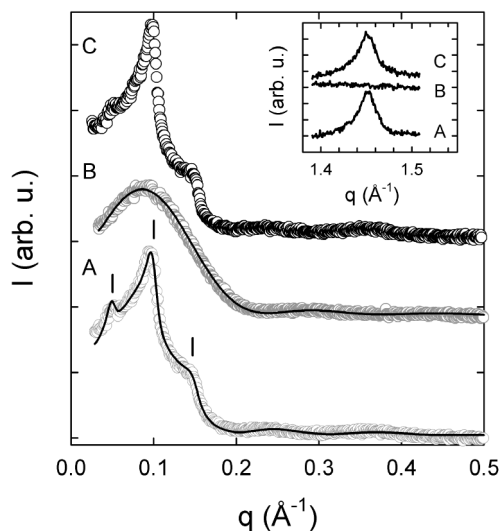


FIG. 1. Unbinding transition upon heating through the main transition ($T_M = 23.4$ °C) of a POPE/POPG (87:13 mol/mol) mixture in 20 mM Na-phosphate buffer ($pH = 7.4$) containing 130 mM NaCl. The scattered intensity is plotted on a linear relative scale. Diffraction pattern (A), taken at 20 °C, corresponds to a weakly bound multibilayer system. For clarity the position of the quasi-Bragg peaks are indicated by the dashes. Pattern (B) shows the system at 30 °C, where interbilayer positional correlations are lost. The system returns to its loosely bound state after cooling back to 20 °C (C). Solid lines show full q -range fits (see text). The corresponding wide-angle diffraction patterns showing the in-plane order of the hydrocarbon chains are given in the inset.

The wide-angle diffraction patterns, corresponding to the in-plane packing of the hydrocarbon chains (Fig. 1, inset), confirm that the system is in the L_β phase at 20 °C (A) and (C) and in the fluid L_α phase at 30 °C (B). The transition from the gel to the fluid phase was also detected by differential scanning calorimetry (DSC) using a VP calorimeter (MicroCal, Northampton, MA). DSC measurements show a single, sharp asymmetric transition at $T_M = 23.4$ °C with a half-width of 1.6 °C and a transition enthalpy of $\Delta H = 6.9$ kcal/mol.

Diffraction patterns collected at 1 °C intervals demonstrate that the unbinding transition occurs abruptly and in step with the main transition. Thus the unbinding temperature, T_U , coincides with the main transition temperature, T_M . This fact is further supported by diffraction experiments at different POPE/POPG compositions (POPG molar ratio between 0.13 and 0.5). Increasing the PG content decreases both T_M and T_U .

The unbinding transition occurs only over a limited range of salt concentrations. Figure 2 shows diffraction patterns of POPE/POPG bilayers at 0 and 800 mM NaCl. In the absence of NaCl the diffraction patterns indicate that both gel and fluid bilayers are in the unbound state. In this case the unscreened electrostatic double-layer repulsion seems to result in a repulsive interbilayer potential for all values of bilayer separation. On the other hand, the addition of 800 mM NaCl gives rise to quasi-Bragg peaks for both phases, as the screening of the electrostatic repulsion by the salt makes the attractive van der Waals interaction dominant.

We have analyzed the data presented in Fig. 1 in more detail by fitting them with those data calculated from

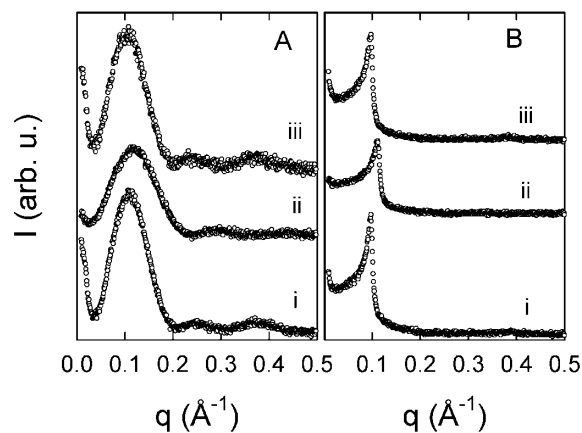


FIG. 2. Diffraction patterns of POPE/POPG (87:13 mol/mol) bilayers in a 20 mM Na-phosphate buffer ($pH = 7.4$) in the absence of NaCl (a) and 800 mM NaCl (b). (i)–(iii) correspond to 20 °C, 30 °C, and 20 °C, on cooling, respectively. At 20 °C the bilayers are in the L_β phase and in the L_α phase at 30 °C. Note that no quasi-Bragg peaks are seen in either of the two phases in the salt-free case (a), whereas, they are seen for both phases at 800 mM NaCl (b). The width of the peaks in the latter case is limited by the instrumental resolution, indicative of very long correlation lengths.

a model over the entire range of the scattering vector, q [10]. The scattered intensity is given by $I(q) = S(q)|F(q)|^2/q^2 + N_u|F(q)|^2/q^2$, where $S(q)$ is the structure factor and $F(q)$ the form factor of the bilayers. $F(q)$ is given by the Fourier transform of the bilayer electron density profile, which is described by two Gaussians, representing the headgroups, of width σ_H centered at $z = \pm z_H$, and a third Gaussian at the bilayer center ($z = 0$) representing the terminal methyl groups [10]. The last term in the model is due to diffuse scattering from uncorrelated bilayers and is not generally taken into consideration. However, phospholipid samples may well contain a certain amount of positionally uncorrelated bilayers such as unilamellar vesicles (ULVs), which can give rise to diffuse scattering. This applies especially to the case of the weakly bound systems described in the present study. According to paracrystalline theory [11] $S(q) = N + 2 \sum_{k=1}^{N-1} (N-k) \cos(kqd) \times \exp(-kq^2\Delta/2)$, where N is the number of bilayers per scattering domain and Δ denotes the mean square fluctuations of the bilayers. This theory neglects thermal undulations of the bilayers, but is applicable in the present case due to the high bilayer bending rigidity in the gel phase [12].

The solid lines in Fig. 1 show the best fits of the model to the data, taking into consideration the effects due to domain size distribution and instrumental resolution. The diffraction pattern corresponding to the unbound system was fitted to $I(q) = |F(q)|^2/q^2$. As can be seen from the figure, the model is in very good agreement with the experimental data ($\chi^2 = 0.99$). The correlation length in the gel phase obtained from the fit is 680 Å, which corresponds roughly to five bilayer repeat units. This is significantly lower than the limit of 3500 Å imposed by our current instrumental resolution and attained by the 800 mM NaCl samples [Fig. 2(b), (i),(iii)]. The much shorter correlation length suggests that the gel phase bilayers are weakly bound at 130 mM NaCl.

The observed results can be better understood by considering, in detail, the various forces. At large values of the bilayer separation z , the competing forces are the van der Waals attraction, with an energy per unit area given by $f_{\text{vdW}}(z) \approx -H/(12\pi)[1/z^2 - 2/(d_B + z)^2 + 1/(2d_B + z)^2]$, and the electrostatic double-layer repulsion, which for low surface charge density is given by $f_e(z) \approx [2\sigma^2\lambda_{\text{DH}}/(\epsilon_0\epsilon_r)]\exp(-z/\lambda_{\text{DH}})$ [13]. Here, H is the Hamaker constant, d_B the membrane thickness, σ the surface charge density, λ_{DH} the Debye-Hückel screening length, ϵ_0 the permittivity of vacuum, and ϵ_r the relative permittivity of the solvent (~ 80). In the present case $\sigma = -4.8 \times 10^{-22} \text{ C}/\text{Å}^2$ and $\lambda_{\text{DH}} = 6.7 \text{ Å}$ at 130 mM NaCl. σ has been estimated by assuming that the PG headgroups are uniformly distributed in the bilayer, which is supported by DSC experiments [8].

The bilayer thickness $d_B = [2(z_H + 2\sigma_H)]$ [14] and the water layer thickness $d_W (= d - d_B)$ can be estimated from the values of the model parameters obtained from

the fits. For gel phase POPE/POPG bilayers $d_B = 58.0 \pm 2 \text{ Å}$ and $d_W = 67.0 \pm 2 \text{ Å}$. The Hamaker constant can be estimated by requiring that the minimum of the interaction potential, $f = f_{\text{vdW}} + f_e$, is at the observed value of d_W . Using the above-mentioned parameter values, H turns out to be $2.36 \times 10^{-14} \text{ erg}$ and compares favorably with values reported for other gel phase systems [15]. The resulting interaction potential is shown in Fig. 3(B). We have also calculated the interaction potential for the strongly bound system at 800 mM NaCl, using the above value of H and $\lambda_{\text{DH}} = 3.3 \text{ Å}$ [Fig. 3(A)]. The minimum in this case is at a higher value than the observed bilayer separation of $\sim 6.0 \text{ Å}$ [16]. This may be due to the existence of an additional short-range attractive interaction in PE bilayers [17]. We have also presented the interaction potential for the salt-free case in Fig. 3, where the system is unbound at all temperatures; in this case $\lambda_{\text{DH}} = 11.1 \text{ Å}$. A comparison of curves (A) and (B) shows that the adhesion energy is about an order of magnitude lower at 130 mM NaCl. Thus, any additional repulsive force can easily cause the minimum in the potential to disappear, in this case, leading to the unbinding of the membrane stack. There are two possibilities which can account for the unbinding scenario [18]. One is an increase, at the main transition, of the electrostatic double-layer repulsion interaction, and the other is steric repulsion due to bilayer undulations, which is significant only in L_α phase bilayers [3].

Although a reduction in the electrostatic repulsion is expected above the main transition due, for example, to the larger area/headgroup in L_α bilayers leading to a reduction in σ , one cannot offhand rule out the possibility that electrostatic repulsion is the cause for the observed unbinding transition. We have therefore reanalyzed the diffraction data obtained from pure DPPG dispersions as a function of temperature and NaCl concentration (in

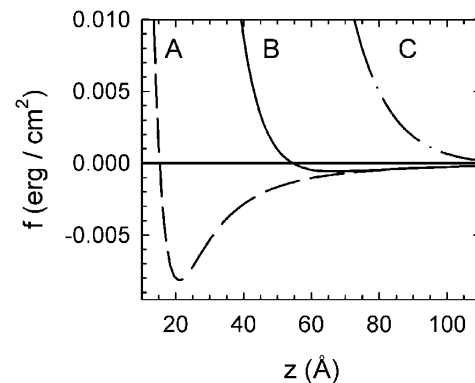


FIG. 3. The total interaction potential as a function of bilayer separation z for PE/PG bilayers at different NaCl concentrations: 800 mM (A), 130 mM (B), and 0 mM (C). The Hamaker constant H was determined by the requirement that the minimum in (B) corresponds to the observed bilayer separation. There are no other unknown parameters in the expressions for the potential.

the range 0 to 1.0 M) [19] for any indications of an unbinding transition [20]. Going across the main transition no unbinding is observed in the pure DPPG system for any salt concentration. Below 150 mM NaCl DPPG forms ULVs both below and above the main transition, whereas, at and above 150 mM NaCl MLVs are found at all temperatures. We can therefore exclude electrostatic repulsion as the cause for unbinding, since any possible changes to this force across the main transition would have been much more pronounced in the pure PG bilayers owing to their much higher charge density compared to the PE/PG mixture.

Steric repulsion is then the only possible cause of the observed unbinding transition. The occurrence of a bound state at $T < T_M$ is consistent with negligible steric repulsion due to the higher rigidity of the bilayers in the L_β phase. However, the main phase transition is accompanied by a drastic reduction (by almost an order of magnitude) in the bending rigidity [12], resulting in significant thermal undulations of the bilayers. The concomitant buildup in steric repulsion can lead to the unbinding of the stack. The fact that undulations do not cause the unbinding of the pure DPPG system can be understood in terms of the higher rigidity of charged bilayers [21].

Mutz and Helfrich [5] noted that T_U of DGDG bilayers varied strongly from sample to sample and also within the same sample. They attributed this variation of T_U to changes in the bending rigidity. One way to explain this variability in T_U is that the tension in the bilayer is a function of MLV size. Therefore, for a typical MLV sample exhibiting a high degree of polydispersity the unbinding may not occur at a well-defined temperature. In the case of the system under study, the electrostatic repulsion has from the outset resulted in weakly interacting gel phase bilayers. Thus, the sudden decrease in the membrane rigidity at the main phase transition is sufficient to unbind all the MLVs simultaneously. The present unbinding scenario is, therefore, somewhat different from the one theoretically studied [4], where no structural phase transition is involved. Nevertheless, in both scenarios the cause of the unbinding transition is steric repulsion due to bilayer fluctuations.

We have presented direct experimental evidence, using x-ray diffraction, for the much theorized unbinding transition in bilayer stacks made up of a mixture of lipids. In the gel phase these bilayers are weakly bound exhibiting a short scattering correlation length. Upon going through the main transition the L_α bilayers lose all positional correlations. We have ruled out the possibility of electrostatic double-layer repulsion accounting for this unbinding and conclude that the unbinding is a direct result of steric repulsion caused by enhanced thermal undulations of the bilayers in the L_α phase.

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*To whom correspondence should be addressed.

Email address: Georg.Pabst@oeaw.ac.at

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