Shape Evolution of Lipid Bilayer Patches Adsorbed on Mica: an Atomic Force Microscopy Study

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The events occurring at various stages of bilayer formation via vesicle rupture were monitored by time lapse atomic force microscopy. We found that when the bilayer patch resulted from a newly adsorbed vesicle comes in contact with a previously formed supported bilayer patch, they fuse on the surface. The shape change as a function of time of the fused bilayer patch points to the line tension as being the driving force behind the lateral reorganization of supported bilayers. From the relaxation times observed, it is clear that the relaxation process is not dominated by the viscous drag but by the interaction between the bilayer and the mica substrate. Our results also help understand the mechanism by which a continuous supported bilayer is formed.

Introduction

Planar supported lipid bilayers (PSB's) are commonly used as model for cell membranes. A substantial body of evidence suggests that a thin layer of water approximately 1−2 nm thick is trapped between the support and the headgroups of the lower leaflet of the bilayer.[1−4] One way to prepare a PSB is via vesicle rupture whereby liposomes self-assemble into a fluid PSB on solid supports.[3−6] It has been asserted that extensive lateral reorganization has to take place in order to obtain a uniform coverage of the substrate.[7,8] Although bilayer spreading was observed and the kinetics well characterized,[7,8] the connection between the vesicle rupture and a uniform coverage of the substrate by the PBS has yet to be made. Indeed, in some earlier works, patches of adsorbed vesicles (see, for instance Figure 13 in Rädler, Strey and Sackmann[7]) were observed to be stationary with time. A large number of techniques has been employed to study the dynamics of lipid bilayers, which includes fluorescence microscopy,[9] reflection interference contrast microscopy,[7] and fluorescence recovery after photobleaching (FRAP).[10] Recently, atomic force microscopy (AFM)[11] has been used to monitor both the surface topography and morphology of supported lipid bilayers.

The major advantage of AFM is that it affords both lateral and vertical resolution in the nanometer regime. While the high resolution in the lateral direction allows one to follow the rupture event of any single vesicle, say, with a typical radius on the order of 50 nm, that in the vertical direction allows for the quantification of the adsorbed film thickness. Moreover, the advent of new imaging modes like the Tapping ModeTM (Digital Instruments, Santa Barbara, CA) and the MAC ModeTM (Molecular Imaging, Tucson, AZ) makes it possible to obtain nondestructive imaging of soft surfaces under aqueous conditions. These new AFM imaging modes are definitely superior over the contact mode, where the contact between the tip and the sample intrinsic in the imaging process of the latter may, in fact, affect the dynamics of the adsorbed bilayer patches.

We have used AFM to monitor the events that take place during bilayer formation in order to get a better understanding of the process. This work differs from previous ones[11,12] in that we are interested in following the evolution of individual bilayer patches. Our goals are to determine whether it is possible to directly visualize the fusion between two independent patches of bilayer and to identify the driving force behind the lateral rearrangement as well as the strength of the interaction with the substrate. To monitor the evolution of individual bilayer patches, we have used a vesicle solution with very low lipid concentration. This allows us to capture and monitor individual vesicle rupture event and the subsequent relaxation process by imaging the same location over time. By so doing, we are able to formulate a pathway whereby a continuous bilayer is formed upon vesicle rupture on solid surfaces. Here, we also report the direct observation of line tension at work in restoring a minimum boundary of a bilayer patch.

Experimental Section

Egg−phosphatidylcholine (egg−PC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). High-grade mica was purchased from Ted Pella, Inc. (Redding, CA), and muscovite mica was purchased from McMasterCarr (Chicago, IL). Pure water (resistivity >18 MΩ cm) was obtained with a MilliQ (Millipore, Bedford, MA) system. Vesicle solutions were made in aqueous solution and then diluted to the desired concentration in a 10−30 mM $MgCl_2$ solution. $MgCl_2$ (crystals) was purchased from Fisher Scientific (<90% purity, certified ACS).

Large unilamellar vesicles were prepared via the freeze−thaw extrusion method. The lipids, dissolved in chloroform, were dried to a thin film under a stream of nitrogen and were left overnight in house vacuum for the solvent to completely evaporate. The lipid film was then hydrated and vortexed. After five freeze−thaw cycles, the suspension was passed through the pores of a polycarbonated membrane (100 nm, Avanti Polar

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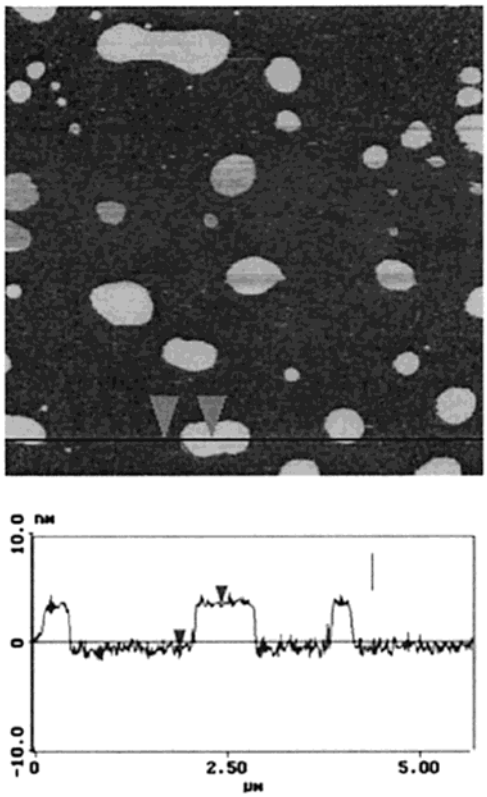

Figure 1. AFM image of phospholipid bilayer patches on a mica substrate. The image was obtained 50 min after the vesicle suspension was put in contact with the substrate. The vesicle concentration was 5 μg/mL in 10 mM $MgCl_2$ solution. The line scan shows that the features in the image have a height of ~4 nm, which corresponds to the thickness of a bilayer.

Lipids Inc.) with the aid of a lipid extruder (Avanti Polar Lipids Inc.). The size distribution of the resulting vesicles was determined by dynamic light scattering using a model PD2000DLS (Precision Detectors, Franklin, MA) instrument and was typically found to be centered around a diameter of ~130 nm with a standard deviation of 28 nm.

The vesicle suspension diluted to a final concentration of 0.5−50 μg/mL was deposited on freshly cleaved mica, mounted on a magnetic stainless steel disk. Imaging of the sample was performed in fluid with a MultiMode Nanoscope IIIA Scanning Probe Microscope (Model MMAFM-2, Digital Instruments) without using an o-ring. Cantilevers with oxide-sharpened silicon nitride tips (Digital Instruments), with a nominal spring constant of 0.32 N/m, were used in tapping mode with a type J scanner. The drive frequency was 8−9 kHz ,and the drive amplitude was between 0.25 and 0.4 V, which corresponds to 5−8 nm. The setpoint was usually around 0.85−0.9 of the free amplitude in our experiments. The sample was imaged continuously for up to 2 h.

Results and Discussion

When the vesicle suspension comes in contact with the mica surface, the vesicles rupture[3−6] and form bilayer patches. Though both the mica substrate and the PC vesicles are slightly negative,[11,13] the divalent ions from the solution partially neutralize the mica surface so that adhesion of the lipid bilayer on the mica substrate becomes possible.[11] Figure 1 shows a typical image of the bilayer patches 50 min after the vesicle suspension was put in contact with the mica surface. As seen from the line section in the image, the height of the features is consistent with the height of a bilayer (~4 nm). The main feature is the existence of large round or elliptical domains with surface areas 3−4 times larger than that of one adsorbed vesicle ($4\pi r^2$). The observation that larger domains have a symmetrical shape is different from previous reports.[11]

The explanation for the existence of such domains (larger area than that of an individual vesicle but still retaining a symmetrically rounder shape) is due to the fusion of ruptured vesicles on the mica surface, accompanied by a relaxation process driven by the bilayer line tension. The relaxation process renders the fused bilayer patch the final symmetrical shape. We believe that the difference in the observed domain shape here and those reported by Egawa and Furusawa[11] arises mainly from the difference in the vesicle concentration used. With the low concentrations used in our experiments, the vesicle adsorption rate is small so that a patch of adsorbed bilayer can stay undisturbed (i.e., without having the addition of lipid molecules to the patch via the rupture of another vesicle) for an extended period of time, allowing the original asymmetrical shape to relax to the more symmetrical one observed. By continuously imaging the substrate during the adsorption process, we were able to directly observe rupture, fusion, and relaxation events. Figure 2 shows images of the same spot on the mica substrate taken over time; the time $t = 0$ in all figure captions refers to the start of the imaging process. In Figure 2a, egg−PC bilayer patches of various sizes were adsorbed on the mica surface. Seven minutes later, a scan over the same area showed a newly adsorbed vesicle (see arrow) fusing with a preexisting bilayer patch (see Figure 2b). After an additional 7 min the larger patch exhibited a decrease in its perimeter while maintaining its surface area. This series of images allows us to capture the fusion event of two adsorbed bilayer patches and the subsequent relaxation to a less extended configuration to minimize the interfacial boundary of the resulting bilayer patch.

As our data clearly shows, the first stage of the interaction between the vesicles and the mica surface results in the adsorption of isolated bilayer patches on the mica. How, then, do subsequent vesicle rupture events lead to the formation of a continuous uniform bilayer sheet at the substrate surface? To understand the mechanism involved, we have followed rupture and fusion events in the vicinity of large bilayer patches. Figure 3a shows a few adsorbed bilayer patches with the arrow pointing to the interstitial space between two such patches. Either the adsorption of a new vesicle at the interstitial space or simply the lateral movement of the vesicle in the right (we cannot rule out either possibility, as the uncertainty in the measurement of surface area occupied by lipids in the initial and final stage is on the same order of magnitude as the area of a newly adsorbed vesicle) created a neck-like structure connecting the two patches (Figure 3b), and eventually, the smaller bilayer patch was incorporated into the larger one via a relaxation process (Figure 3c). The driving force for this relaxation process is line tension, λ, which is the two-dimensional equivalent of surface tension in three-dimensional systems. Line tension, defined as the work done to increase the perimeter of the membrane with a unit length, is an important material property, as it determines the resistance of the membrane to rupture, pore formation. Edges of bilayer patches are energetically costly due to the additional energy required to curve the lipid molecules in order to prevent the hydrocarbon chains from exposing to the aqueous environment.

The dynamics of bilayer relaxation can provide useful information on the magnitude of the line tension or the strength of the interaction between the bilayer and the substrate.[14,15]

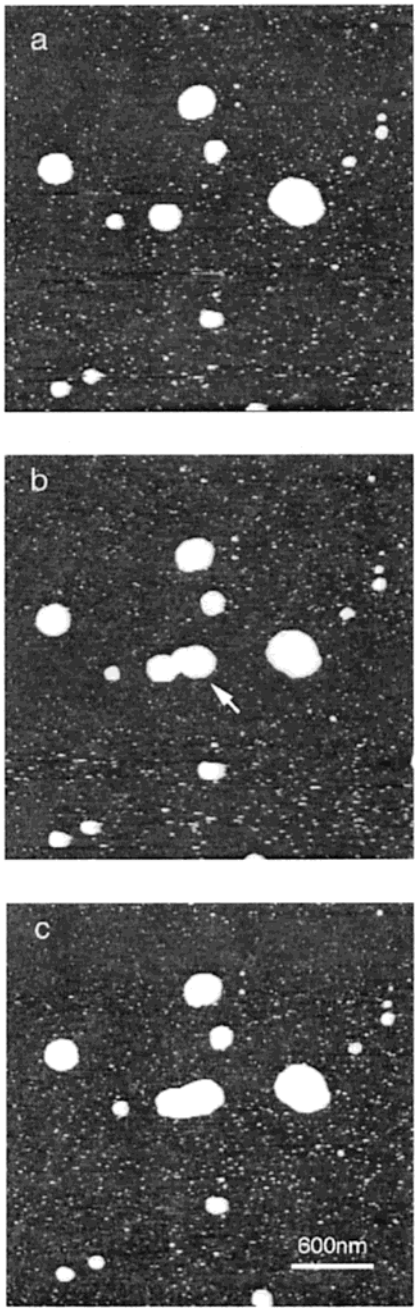

Figure 2. AFM images capturing the dynamics of bilayer patch fusion on a mica surface. Images were taken at (a) $t = 0$, which signifies the start of the imaging process, (b) $t = 7$ min, and (c) $t = 13$ min. The arrow in panel b points to a newly adsorbed vesicle. The image is 3 μm in size, zoomed-in from an 8 μm scan.

Previous studies suggested that the bilayer−substrate interaction is a purely viscous one for atomically flat surfaces such as mica.[7] In this case, the characteristic time for the relaxation process would be $\tau = \eta S^{3/2}/h\lambda$,[16] where η is the viscosity of the buffer, S is the surface area of the bilayer patch undergoing the relaxation process, h is the height of the water layer between the bilayer and the substrate, and λ is the line tension. Writing $\lambda \approx \eta S^{3/2}/h\tau$ and using the values $\eta = 1.1 \times 10^{-3}$ kg m^{-1} s^{-1}, $S^{1/2} = 100$ nm, $h = 1$ nm,[1−4] and $\tau = 1200$ s (20 min), one gets a value of $\lambda \approx 0.91 \times 10^{-18}$ N, which is well below the value of line tension of a pore in a free membrane, previously measured by Zhelev and Needham.[17] As a matter of fact, the 10^{-18} N value is also well below what one would expect using the "kT criterion":[18] $kT/l \cong 10^{-10}$ N at room temperature, where k is Boltzmann' s constant, T is the room temperature, and $l =$ 10 Å is the characteristic length (distance) between molecules in the bilayer. If the line tension were smaller than 10^{-10} N, thermal fluctuations alone would not allow such relaxation to take place. The fact that relaxation to a rounder shape takes place after the fusion of two bilayer patches points to a gross underestimation for the value of line tension when the formula above is used; obviously, other forms of dissipation must exist to account for the discrepancy in the value of line tension, as well as the extremely long relaxation times observed. We have also noticed that the relaxation times depend on the quality of the mica substrate. High-grade mica from Ted Pella gave the best results (shorter relaxation times), whereas the bilayer patches adsorbed on muscovite mica from McMaster Carr had a larger range of relaxation behaviors, with the patches being immobilized in some instances. Our results clearly indicate that the interaction with the substrate contributes a great deal to the dissipation force experienced by the bilayer patches. Furthermore, we have also observed larger domains relaxing slower than smaller ones (data not shown), but a clear functional dependence of the relaxation time on domain size could not be extracted. The interaction between the adsorbed bilayer and the substrate, as well as the increase of τ with patch size, can account for the fact that no shape relaxation for micrometer size patches has been observed in the time scales on the order of minutes or hours.[7]

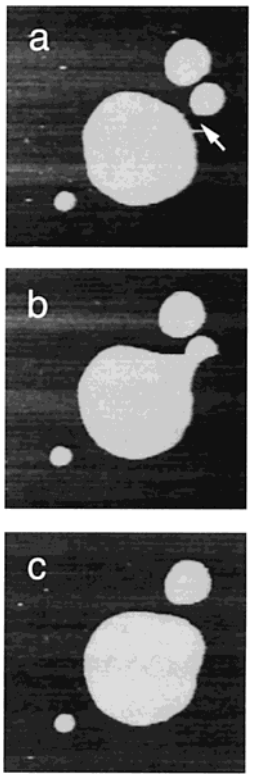

Figure 3. AFM images capturing the lateral reorganization and shape relaxation of adsorbed bilayer patches on a mica surface. Images were taken at (a) $t = 0$, which signifies the start of the imaging process, (b) $t = 4$ min, and (c) $t = 9$ min. The images are 1.8 μm in size, digitally zoomed-in from an 8 μm scan.

To illustrate how the interaction with the substrate can affect substantially the behavior of the supported bilayer and to demonstrate the final stage of the formation of a continuous bilayer, we show a pore closure event in Figure 4. The concentration of the vesicle suspension used in this case was 25 μg/mL. This process is slow, with the time scale in the minute regime, as opposed to the millisecond regime observed in free bilayers.[17]

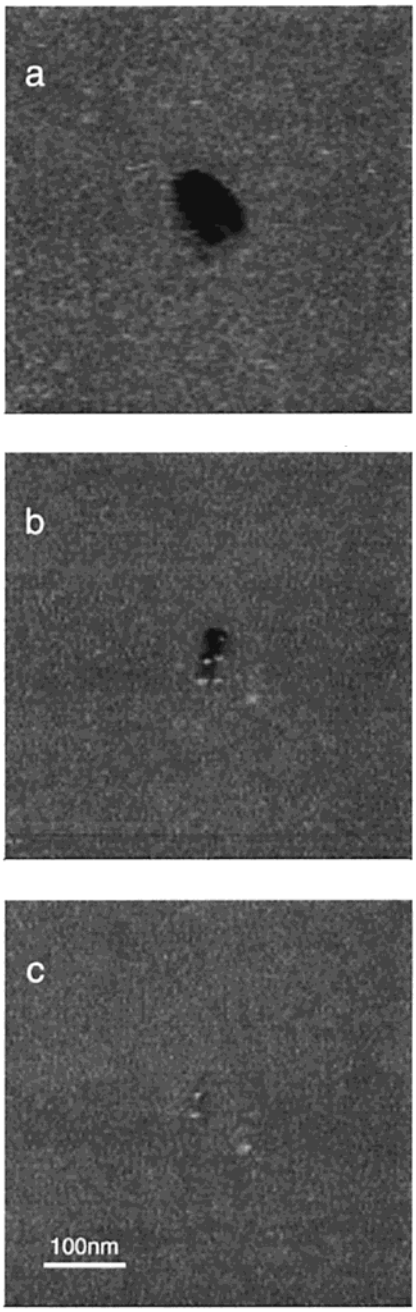

Figure 4. Pore closure (or hole healing) during the final stages of PSB formation. Images were taken at (a) $t = 0$, which signifies the start of the imaging process, (b) $t = 19$ min, and (c) $t = 28$ min. The concentration of the vesicle suspension was 25 μg/mL. The images are 500 nm in size, digitally zoomed-in from a 2μm scan.

Conclusions

We have monitored the lateral rearrangement of supported bilayer patches immediately after their formation via vesicle rupture. Asymmetrical bilayer patches undergo a shape relaxation process toward a rounder shape. The bilayer line tension is identified to be the main driving force for this lateral reorganization. The long time scale for such relaxation processes indicates the presence of dissipative forces other than the purely viscous drag considered. It has been found, for instance, that confinement can dramatically change the viscosity of a liquid, giving rise to an effective viscosity orders of magnitude higher than that of the bulk.[19] Moreover, it should not be surprising that the roughness of the substrate could play a role in slowing the shape relaxation process. During the last stage of bilayer formation, we have observed the healing of holes in the PSB. We conclude that a PSB retains many of the properties of a free membrane, such as line tension, but the interaction with the substrate can dramatically change the time scale of fundamental processes (such as shape relaxation and pore closure events presented in this paper). Such an increase in time scale in the case of a PSB may prove useful in capturing events via surface sensitive imaging techniques, events that may otherwise be too fast to capture in a free-standing membrane. Our observations regarding lateral rearrangement of bilayer patches at the early stage of bilayer formation and hole healing event at the late stage help us understand how a uniform bilayer coverage on the substrate is achieved during the PSB formation via vesicle rupture.

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