Quantitative Analysis of Holes in Supported Bilayers Providing the Adsorption Energy of Surfactants on Solid Substrate

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We investigated the topography of mixed bilayers consisting of a first monolayer of DMPE (dimyristoylphosphatidylethanolamine) and of a second monolayer of DOPC (dioleoylphosphatidylcholine) that were Langmuir–Blodgett deposited on mica. Using transfer ratio measurements and tapping mode atomic force microscopy experiments, we show that the subnanometric holes in the bilayers result from the desorption of lipids of the first monolayer during the transfer of the second monolayer. We present a new simple technique based on the quantitative analysis of these holes that allows determination of the adsorption energy of amphiphilic molecules on solid surfaces. This technique is valid for relatively low adsorption energies in the range 1 to 10 $k_B T$.

Introduction

Supported lipid bilayers were commonly considered as model membranes.[1] In this respect, Langmuir-Blodgett bilayers have been used for a long time in many force measurements and were described as perfectly smooth films.[1–4] These last years, atomic force microscopy (AFM) experiments allowed imaging of these bilayers, but most of the results concentrated on the molecular scale description of the bilayers.[5–7] On a larger scale, the existence of holes in these films has been observed for several years.[7,8] Different surfactants (lipids, fatty acids) and different types of substrates (hydrophilic or hydrophobic) were used. The depth of these holes that most of the authors measured usually corresponds to the thickness of the bilayer, which suggests that the first monolayer peels off during the deposition of the second layer. Up to now, only qualitative observations were reported and, to our knowledge, no quantitative analysis has been published to explain the origin of the holes. This lack of quantitative analysis may hinder a correct interpretation of some force measurements for which a precise understanding of the surface arrangement of the molecules is required.

In this paper, we have combined transfer ratio and AFM measurements on mixed bilayers consisting of a first monolayer of DMPE (dimyristoylphosphatidylethanolamine) and of a second monolayer of DOPC (dioleoilphosphatidylcholine). We were able to demonstrate that, as suspected before, the holes in these supported bilayers originate in the desorption of molecules of the first monolayer during the second transfer. This effect is shown to be related to the balance between the adsorption energy of the molecules on the solid substrate and their energy at the air–water interface. As the surface pressure of the monolayer at the liquid interface is controlled, the knowledge of the number of molecules peeling off provides directly the adsorption energies of amphiphilic molecules on solid substrates.

Experimental Section

Langmuir-Blodgett Deposition. We prepared asymmetric bilayers consisting of a first monolayer of DMPE and of a second monolayer of DOPC. This system is commonly used in surface force apparatus (SFA) experiments.[9] DMPE and DOPC were purchased from Avanti Polar Lipids Inc., dissolved in chloroform, and stored under argon at −20 °C. The layers were Langmuir-Blodgett deposited on freshly cleaved mica translated vertically in the home-made trough[10] (15 × 25 cm²). The size of the mica was ∼1 × 1 cm² for the AFM experiments and 1.5 × 8 cm² for the transfer ratio measurements. A schematic representation of the samples is given in Figure 1. For the DMPE layer, the substrate was raised from the trough. In all the experiments described in this paper, the surface pressure and deposition velocity of the substrate were, respectively, 42 mN/m corresponding to the solid phase[11]) and 200 μm/s. The DOPC layer was then transferred during the vertical dipping of the substrate into the trough, resulting in an hydrophilic bilayer. This transfer was performed at different deposition velocities (3 and 200 μm/s) and surface pressures (Π_{DOPC}) between 2 and 40 mN/m. The DOPC is in a liquid phase for this pressure range (data not shown). To prevent any dewetting problem, the samples always remained immersed in highly pure water.

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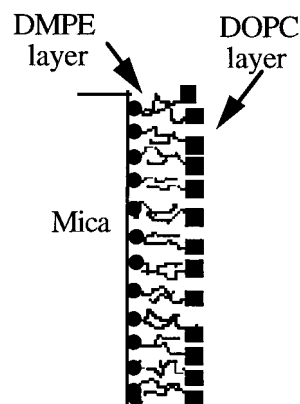
Figure 1. Schema of the deposited bilayer on the mica.

All our experiments were performed at room temperature.

Transfer Ratio. The difference between the total area of the DOPC monolayer before and after transfer (ΔA) at the air/water interface was recorded. The ratio between ΔA and the area of the mica (A) will be referred as the "transfer ratio" and denoted R ($R = \Delta A/A$). A value for R of 1 reflects that the monolayer is properly transferred on the substrate and that no desorption of molecules occurs during the transfer.

Atomic Force Microscopy. The topography of the surface of the bilayers was observed with a Nanoscope III (Digital Instruments) AFM setup. The images were obtained in tapping mode in water to prevent any damage of the bilayer structure. The sample had to remain immersed under water after lipid deposition, so the mica substrate was transferred under water and taped on the center of a Teflon disc with a small groove on its periphery. This simple system allowed a large water drop to be maintained over the mica when the sample was installed on the scan head. The commercial tapping liquid cell was then placed over the sample. We used a medium scan head (15×15 μm^2) and commercially available silicon nitride tips on a cantilever with a spring constant (k) of 0.56 N/m, oscillating at a frequency of 90 kHz. The images were obtained with a scan rate of 2 Hz. Prior to every experiment, we exposed the tips for 30 min to an ozone flux to oxidize any contaminant. The first image was usually obtained between 30 min and 1 h after the deposition. For the AFM experiments, the DOPC layer was deposited at a velocity of 200 μm/s (similar to classical SFA experiments and to some previous AFM experiments[3,6,12]). We insured that the force plot was correct and not modified before and after every experiment to check that the tip had not been contaminated during the scans.

Results and Discussion

Desorption of the DMPE Layer. The DMPE monolayer in the air is smooth and defect free, as observed with AFM (see Figure 2a). This result is confirmed by the transfer ratio ($R = 1.04 \pm 0.03$), which indicates that the monolayer was slightly denser on the mica than at the air/water interface. If moved through the monolayer-free air/water interface at 3 μm/s, the DMPE monolayer is then covered with holes. The proportion of the surface covered by holes (x) is very high $x = 38 \pm 4\%$ (see Figure 2b)], confirming that the DMPE molecules desorb at least in the extreme case where there are no DOPC molecules ($\Pi_{DOPC} = 0$ mN/m) at the air/water interface.

For DOPC, the deposition ratio decreased with both the surface pressure of the monolayer and the transfer velocity (see Table 1). The transfer ratio (R) is commonly considered as direct evidence of the good quality of a transfer. In fact, this ratio reflects the balance between molecules desorbing from the substrate and molecules of
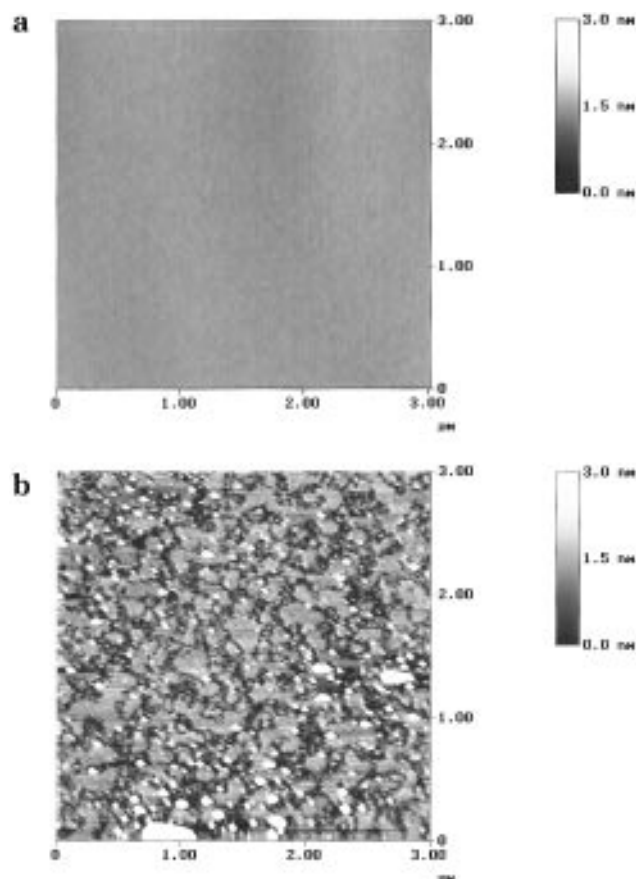
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Figure 2. (a) AFM image (tapping mode in air) of a DMPE monolayer deposited on mica at 42 mN/m. (b) AFM image (tapping mode in water) of the same DMPE monolayer after it was passed through the air/water interface. The coexistence of holes, monolayers, and multilayers (white spots) is similar to the results described in ref 4.

Table 1. Evolution of the Transfer Ratios of DOPC Monolayer from the Air/Water Interface onto DMPE-Coated (42 mN/m) Mica at Deposition Speeds of 200 μm/s (R_{fast}) and 3 μm/s (R_{slow})

Π_{DOPC} (mN/m)	R_{fast}	R_{slow}
40	0.94 ± 0.05	0.92 ± 0.05
25	–	0.71 ± 0.05
15	0.74 ± 0.05	0.42 ± 0.05
2	0.41 ± 0.05	-0.04 ± 0.05

the monolayer transferred onto the substrate. The fact that R is always <1 (see Table 1) is not proof for a low quality transfer but could be equally well interpreted as desorption from the substrate of some DMPE molecules. Moreover, the decrease of R with Π_{DOPC} indicates that the number of DMPE molecules desorbed from the mica increases when Π_{DOPC} decreases. Eventually, $R = -0.04$ (see Table 1), which is evidence of a very large desorption from the substrate at low surface pressure.

Bilayer-Deep Holes. The AFM images clearly exhibit holes in the bilayers (see Figure 3) at every transfer pressure. The proportion and size of the holes depend on the deposition pressure (see Table 2 and Figure 3). These defects are very stable; that is, their shape and position do not change over at least 30 min. Besides, we never observed any growth of these holes over a few hours. For the high-pressure transfer, the depth of the holes cannot be obtained with a good accuracy because their lateral size is of the order of the size of the tip radius ($\approx$30 nm). For the low pressures (15 and 2 mN/m), the holes are larger (up to 500 nm); their typical sizes are, respectively, 100 and 250 nm. A depth of 3.2 ± 0.6 nm could be
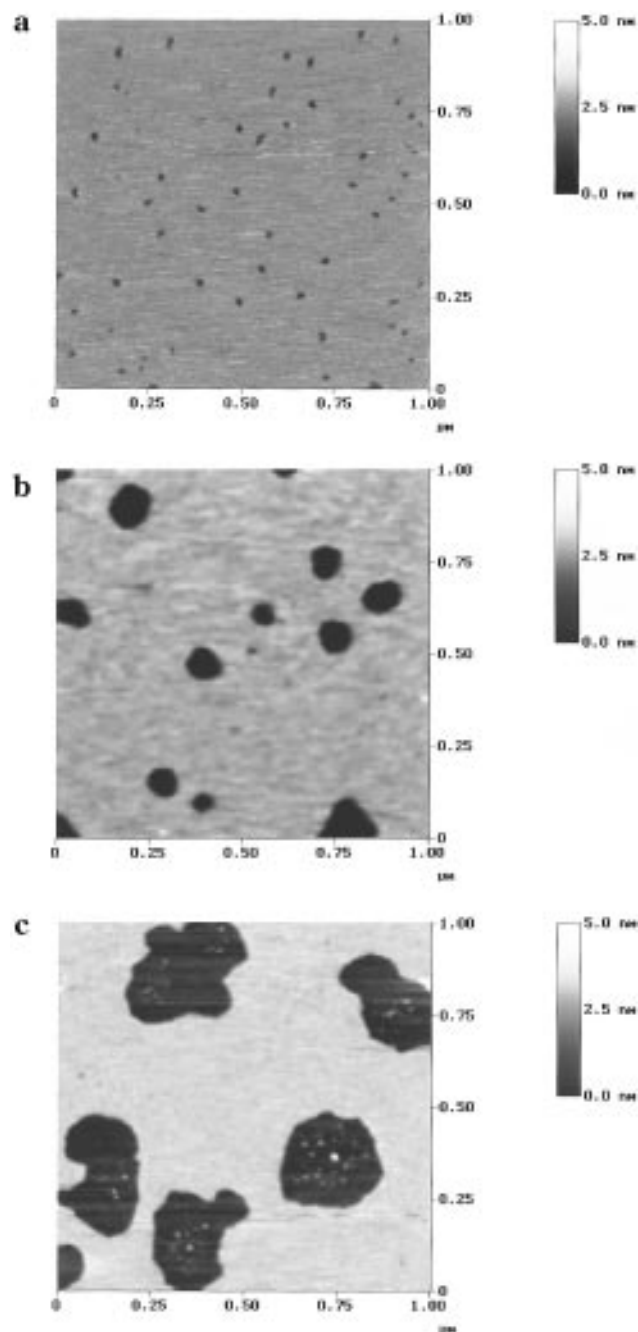
Figure 3. AFM images in tapping mode of lipid bilayers deposited on mica surface. The first monolayer is made of DMPE (42 mN/m), and the second monolayer is made of DOPC deposited at (a) 40, (b) 15, and (c) 2 mN/m.

Table 2. Fraction of the Surface Covered by Holes in the DMPE/DOPC Bilayer Surface at Various DOPC Deposition Pressures (Π_{DOPC}) Obtained with AFM Experiments (x_{AFM}) and By Transfer Ratio Measurements from Equation 1 (x_R)

Π_{DOPC} (mN/m)	x_{AFM} (%)	x_R (%)
40	4 ± 3	3 ± 3
15	10 ± 3	12 ± 3
2	19 ± 3	21 ± 3
0	38 ± 4	—

measured. The thickness of the DMPE monolayer on a silicon substrate is 2.4 nm as measured by ellipsometry. The thickness of a DOPC monolayer is ~2.0 nm in the dense phase at $\Pi_{DOPC} = 40$ mN/m.[13] Consequently, as the thickness of this monolayer at lower surface pressure is <2.0 nm, the defects are extending over a bilayer. At high pressures, the holes have probably the same physical origin and, consequently, should also be one bilayer thick.

Moreover, monolayer-deep holes would not be stable for two reasons. Firstly, hydrophobic chains of the DMPE would be in contact with water. Secondly, we performed fluorescence recovery after photobleaching (FRAP)[14] experiments to estimate the in-plane diffusion coefficient (*D*) of the lipids in the transferred layers. The DMPE or the DOPC lipids were mixed with 5% of their fluorescent NBD analogs (synthesized at the Laboratoire de Physico-Chemie Moléculaire des Membranes Biologiques—Paris[15]). The lipids of the outer monolayer diffuse rapidly, and *D* is between 0.1 and 1 μm²/s, slightly smaller than the diffusion coefficient of phosphatidylcholine (PC) in oriented multilayers.[16] This result indicates that monolayer-deep holes would spontaneously close up in 1 s at the most, and could not be observed on several consecutive AFM images. At the contrary, bilayer-deep holes are stable because no noticeable diffusion of the lipids of the first monolayer on the mica was observed with FRAP experiments over measurable time scales (30 min). This result explains the immobility and shape invariance of the holes.

Holes Origin. The two previous results, desorption of the DMPE and bilayer-deep holes, suggest the following scenario for the arrangement of the bilayers on the mica. When the first DMPE monolayer is dipped in through the DOPC monolayer, some DMPE lipids desorb from the mica to the air/water interface. The DOPC molecules cover only the hydrophobic surface of the remaining DMPE chains, leading to the coexistence of bare mica and lipid bilayers in contact with the water. In addition, if we assume that the molecular area of the DOPC is unchanged after deposition and that holes are due exclusively to DMPE desorption, the proportion of bare mica (i.e., the proportion of holes; *x*) should be related to the transfer ratio *R* by eq 1:

$$x = \frac{1 - R}{1 + \dfrac{a_w}{a_m}} \tag{1}$$

where a_m is the molecular area of DMPE on the mica (0.41 nm²/molecule) and a_w is the molecular area of DMPE at the DOPC transfer pressure of Π_{DOPC}. The term a_w can easily be obtained from the pressure isotherm of the DMPE monolayer. The error bars on *R*, a_w, and a_m are, respectively, 0.05, 0.02 nm², and 0.02 nm², as deduced from statistical analysis. Therefore, the precision on *x* is greater than 0.03.

The term *x* can also be measured from the AFM images by applying a threshold before processing them. The error bars (see Table 2) were deduced from the precision on the threshold and from the statistical analysis over several images and samples.

For the same deposition velocity (200 μm/s), the *x* values calculated from the transfer ratio measurements agree perfectly with the ones deduced from AFM (see Table 2). The same comparison has also been performed with pure bilayers (DOPC/DOPC and DMPE/DMPE, not shown). The agreement is again excellent between the two techniques.

This agreement shows unambiguously that the holes are created in the bilayers by the peeling off of the DMPE molecules during the transfer of the second monolayer.

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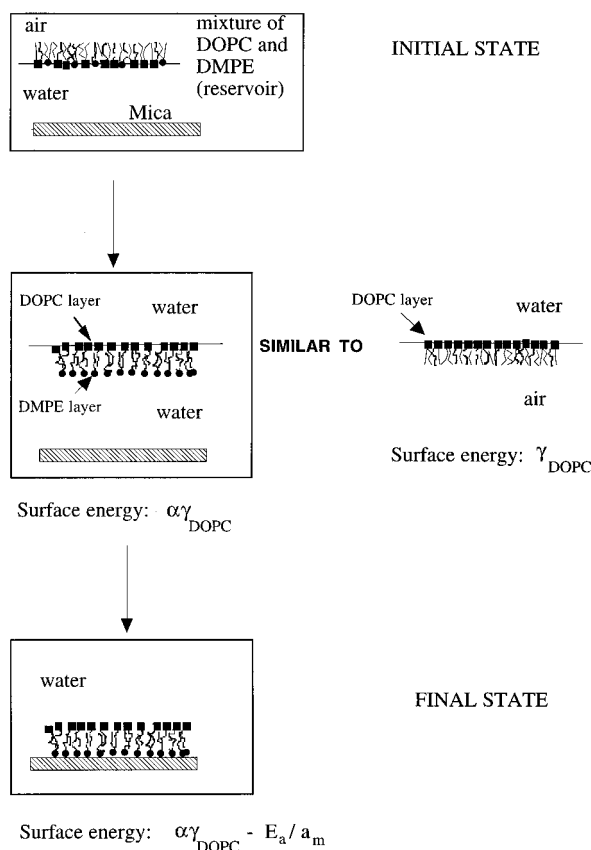(16) Devaux, P. F.; McConnell, H. J. M. *Eur. Biophys. J.* **1972**, *94*, 4475.

Figure 4. Description of the ΔE term, which is the the free-energy difference between the bilayer phase adsorbed on the mica and the bare mica with the lipids in the reservoir at the air/water interface, for a reference area a_m (a_m is the DMPE molecular area). Only surface terms are used. ΔE is obtained through an intermediate state (a DOPC/DMPE bilayer in water). The relative surface energy of this intermediate state can be estimated to be $\alpha\gamma_{DOPC}$ by analogy with the air/water interface.

Adsorption Energy of DMPE on Mica. The hole density is related to the transfer velocity. The diffusion of DMPE lipids in the monolayer at the contact line air/water/mica might limit the desorption of lipids and explain the transfer velocity dependence. The diffusion coefficient of lipids at the air/water interface is of the order of a few $\mu m^2/s$, so the lipids diffuse over a few microns per second. Therefore, at slow velocity (3 $\mu m/s$), it can be assumed that equilibrium is achieved. For the calculation of the proportion of holes x, the simplest approach is to describe the system as two phases in equilibrium : (1) the bilayer phase adsorbed on the mica, and (2) the bare mica with the lipids in the reservoir at the air/water interface. The ratio ρ between the total surface covered by holes and the total bilayer surface ($\rho = x/1 - x$) is given by eq 2:

$$\rho = \exp(-\Delta E/k_B T) \tag{2}$$

where ΔE is the free-energy difference between both phases for a reference area a_m. The term ΔE represents the energy cost to detach the DMPE polar head from the mica (i.e. the adsorption energy per DMPE molecule E_a) minus the interfacial tension of DOPC on DMPE (see Figure 4). This last term is the surface energy required to create a mixed DOPC/DMPE bilayer from the corresponding lipids in the reservoir (see Figure 4). In the absence of exact model or of experimental data, we can make the analogy between the DMPE/water interface and the air/water interface and assume that the DOPC/DMPE interfacial tension is proportional to the surface tension
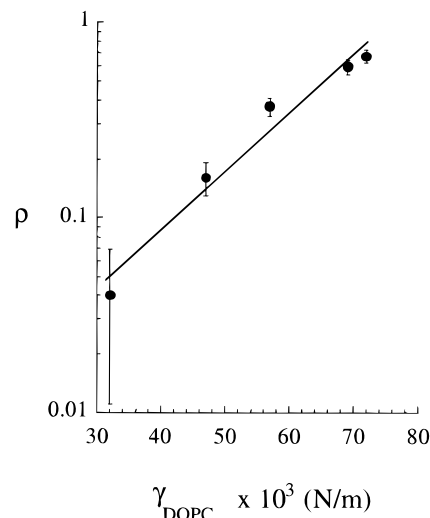

Figure 5. Ratio (ρ) between the surface covered by holes and the surface covered by the bilayer (●) as a function of the surface tension of the DOPC monolayer at the air/water interface (γ_{DOPC} = 72 mN/m − Π_{DOPC}). The straight line represents the best fit deduced from eq 2. The correlation coefficient is 0.979.

of DOPC at the air/water interface γ_{DOPC} (γ_{DOPC} = 72 mN/m − Π_{DOPC}) during the transfer of this molecule. Therefore, for the area a_m, ΔE is given by eq 3:

$$\Delta E = E_a - a_m\alpha\gamma_{DOPC} \tag{3}$$

The two unknowns are the proportionality factor α and E_a. The terms a_m and γ_{DOPC} are directly deduced from the compression isotherm, and α will depend on the DMPE deposition pressure, which is constant in our experiments.

This simple model fits well the transfer ratio data at 3 $\mu m/s$ (see Figure 5). The deduced DMPE/mica adsorption energy is 5.2 ± 0.5 $k_B T$, and $\alpha = 0.7$. These values validate *a posteriori* our assumptions because the observed desorption reflects a moderate energy E_a. Moreover, the interfacial tension between the DMPE chains and water is > 25 mN/m[17] and is weaker than the pure water surface tension of 72 mN/m.[18] Therefore, α was expected to be in the range 0.35 − 1.

The sensitivity of the transfer ratio technique to x does not exceed 3%. We have estimated the strongest adsorption energy accessible with this simple technique to be 10 $k_B T$.

Conclusion

As a conclusion, we have shown that the large scale defects observed in the DOPC/DMPE bilayers on mica are bilayer-deep holes. The holes originate from the balance between the relatively low lipid/substrate adsorption energy (few $k_B T$) and the pressure in the second monolayer at the air/water interface. The size of the holes decreases as the deposition pressure increases and the proportion of the holes may easily be deduced from the transfer ratio. This phenomenon is probably common in any supported bilayer system and has been observed previously by different groups but never been explained. Consequently, in the case of asymmetric bilayer, the second bilayer is never perfectly pure; that is, some lipids from the inner monolayer are present in the outer one (this is very relevant for SFA experiments). The propor-

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tion of contaminant is hard to determine. Some bare mica is exposed to the water, possibly generating double-layer repulsion in force measurements. This phenomenon has been previously observed but not understood.[13] We have used the simplest model for analyzing our experimental results. Because our free energy contains only surface terms, we are unable to predict the size and the shapes of the holes.

Additionally, we propose a novel simple technique for measuring the adsorption energy of amphiphilic molecules on solid substrate. This technique could be widely used with numerous systems, possibly even with copolymers, as long as they can form bilayers. For typical lipids, this technique is appropriate in the range $1-10$ $k_B T$.