Effect of Physical Parameters on the Main Phase Transition of Supported Lipid Bilayers

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ABSTRACT Supported lipid bilayers composed of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) were assembled by the vesicle fusion technique on mica and studied by temperature-controlled atomic force microscopy. The role of different physical parameters on the main phase transition was elucidated. Both mixed (POPE/POPG 3:1) and pure POPE bilayers were studied. By increasing the ionic strength of the solution and the incubation temperature, a shift from a decoupled phase transition of the two leaflets, to a coupled transition, with domains in register, was obtained. The observed behavior points to a modulation of the substrate/bilayer and interleaflet coupling induced by the environment and preparation conditions of supported lipid bilayers. The results are discussed in view of the role of different interactions in the system. The influence of the substrate on the lipid bilayers, in terms of interleaflet coupling, can also help us in understanding the possible effect that submembrane elements like the cytoskeleton might have on the structure and dynamics of biomembranes.

INTRODUCTION

Supported lipid bilayers (SLBs) are continuously gaining importance as model systems to study fundamental processes of the biological membrane and as building blocks in biotechnological applications such as biosensors (1–3). First introduced by Tamm and McConnell (4) and McConnell et al. (5), SLBs can be easily prepared by the vesicle fusion technique or the Langmuir Blodgett/Langmuir Schaefer technique on a variety of substrates including glass, quartz, mica, and many metal oxide surfaces (4,6–8). One of the advantages of this model system relative to other well-established and convenient models such as liposomes or black lipid membranes lies in the benefit of a resultant robust structure, which can be studied by many different surface-sensitive techniques (e.g., ellipsometry, waveguide spectroscopies, x-ray and neutron reflectivity, quartz crystal microbalance, scanning probe techniques, etc.) (9–13). SLBs also enable the simultaneous study of bilayer structure and function, and of the bilayer interaction with membrane proteins. Moreover, SLBs enable our reproducing biologically relevant situations like the compositional asymmetry of the membranes (14). Indeed, it is well known that biological membranes present a different lipid composition between the inner leaflet, in which phosphatidylserine and phosphatidylethanolamine are the most abundant lipid species, and the outer leaflet, where phosphatidylcholine preferentially resides (15,16). Compositional asymmetry in SLBs can be reproduced by preparing the bilayers by the Langmuir-Blodgett and Langmuir-Schaefer techniques and it can be studied by spectroscopy and microscopy techniques (17,18). Other developed and closely related model systems are tethered polymer cushioned lipid bilayers (2).

The structure of SLBs obtained either by the vesicle fusion procedure or the Langmuir-Blodgett/Langmuir Schaefer technique includes a 0.5–2-nm thick trapped water layer between the lipids and the support (19–21). This layer can act as a lubricant for the lipids, allowing them to laterally diffuse in the plane of the membrane.

In general, lipid bilayers display a reversible phase transition between a solid-ordered (s_o) and a liquid disordered (l_d) phase. The transition is accompanied by changes in lipid chains (ordered or disordered) and lattice order (solid or liquid). This transition depends on parameters such as temperature, pH, or ionic strength. Sterols induce a third phase, the so-called liquid-ordered phase, with a loss in lattice ordering as for the l_d phase, but a higher lipid order as for the s_o phase. This kind of phase is likely to appear in biological membranes, where it is referred to as a lipid raft (22). Melting from the s_o to the l_d phase involves an increase in lipid bilayer area and a bilayer thickness decrease.

Many studies on solid supported lipid membranes have dealt with lateral compositional and conformational heterogeneity of lipid bilayers. Great effort has been devoted to the raft domain formation in mixtures of lipids comprising sphingolipid and cholesterol. Clear evidence of the coexistence of liquid immiscible phases has been obtained by many techniques (23,24). The implementation of temperature-controlled atomic force microscopy (AFM) allowed us to image, with high lateral resolution, the main phase transition of supported lipid bilayers, both in the case of single lipid component and lipid mixtures (25–31). The phase transition is characterized by variations in bilayer thickness, which can be easily tracked by AFM. The behavior of temperature-induced phase transitions, as observed by

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AFM, displayed some features that raised some doubts on the equivalence of the SLB model system with liposomes (28,32). In particular, in some cases a clear decoupling in the behavior of the two membrane leaflets has been observed at the main phase transition. Two separate transitions, at variance with what is observed in liposomes, where the two leaflets act together and domain formation is transmembrane symmetric (33), have been observed. The two transitions have been attributed to the two leaflets undergoing separated phase transitions at different temperatures. This behavior has been attributed to the presence of the solid substrate, which might somehow modify the behavior of the lipid leaflet nearer to the support (proximal leaflet). The transition occurring at higher temperature has been assigned to the proximal leaflet. The transition occurring at lower temperature has been attributed to the lipid leaflet facing the bulk aqueous phase (distal leaflet), which is less influenced by the support. The lower temperature transition takes place in a temperature range similar to that of liposomes with the same lipid composition.

From a biological point of view, it should be considered that in biological membranes the two leaflets differ in composition. Domain-forming lipids are usually found in the outer leaflet. The extent to which domains formed in the outer leaflet of a biological membrane can induce the formation of domains in register in the inner leaflet, is not clear. SLBs, allowing for the presence of asymmetric lipid composition, can help in the study of this lipid signaling mechanism across the bilayer. Thus, the use of supported lipid membranes appears to deal with a model system different from other well-studied models in some aspects, but characterized by features that might be of biological relevance. Moreover, SLBs may reproduce conditions similar to the lipids in membranes in contact with the cytoskeleton. Hence, rather than being regarded as a drawback of the model, the presence of the substrate could be regarded as a feature that can provide further information on the principles ruling the behavior of biological membranes.

To the best of our knowledge, no systematic study has been performed on the influence that different sample preparation conditions can have on the bilayer behavior, including interleaflet coupling. Therefore, it is difficult to compare experiments performed in different laboratories where the SLB preparation conditions can be even slightly different. In this work, we used temperature-controlled AFM to study the effect that different preparation conditions have on the main phase transition of SLBs. We concentrated on bilayers composed of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) and -phosphatidylglycerol (POPG), which represent a good model for bacterial membranes (34). We have found that it is possible to tune the preparation procedure in order to progress from a decoupled behavior of the two leaflets to a situation in which the two leaflets undergo, simultaneously, the main phase transition with domains in register. The results were obtained by mainly tuning two parameters: ionic strength and deposition temperature of the vesicle solution. We have considered the physical interactions between the lipid bilayers and the solid support, which might explain the different observed behaviors. Finally, we have discussed the possibilities that SLBs open in the AFM study of protein-lipid bilayer interactions, particularly with respect to the distribution of the proteins as influenced by lateral heterogeneity of the lipid bilayer.

MATERIALS AND METHODS

Sample preparation

The lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Stock solutions (in $CHCl_3$) were mixed to obtain the desired molar lipid ratios. Then the chloroform was evaporated under a flow of nitrogen while heating the sample in a water bath at 50°C. Thereafter, the sample was kept under vacuum (10^{-2} mbar) for at least 4 h to remove the remaining chloroform molecules. Afterwards, the lipids were rehydrated in a buffer solution of 450 mM KCl, 25 mM HEPES at a pH of 7. The sample was stirred at ~30°C for 1 h. During this time, the sample was vortexed at least two times. At the end, a lipid suspension with a lipid concentration of 0.25 mg/mL was obtained.

Preparation of supported lipid bilayers

The supported lipid bilayers were prepared by the vesicle fusion technique. The lipid suspension was sonicated for 30 s in an ultrasonic bath to get small unilamellar vesicles (SUVs). Then we equilibrated the vesicle solution at the temperature of interest together with the sample holder consisting of a freshly cleaved muscovite mica sheet glued to a Teflon disk attached to a metal holder. After that, we added 70 μL of our lipid suspension on the mica. The lipid suspension was incubated for 15 min and then rinsed abundantly with the 450 mM KCl, 25 mM HEPES, pH 7 buffer solution. The solution was then exchanged for the imaging solution by extensive rinsing. The imaging solutions were either pure water, or 10- or 150-mM KCl water solutions. The pH of all these imaging solutions was 5.6. Then the mica support with the formed lipid bilayer was mounted on the temperature-controlled stage of the AFM.

Details about AFM imaging, image analysis, and differential scanning calorimetry (DSC) measurements are reported in the Supporting Material.

RESULTS

POPE/POPG (3:1) lipid bilayers in pure water

Vesicle fusion on mica performed at 23°C produced almost continuous lipid bilayers. AFM imaging of the bilayers at 35°C did not show any heterogeneities (see Fig. 1 *A*). The presence of a few defects (holes) in the lipid bilayer allowed us to measure the height of the bilayer with respect to the mica substrate. This resulted in a figure of (3.5 ± 0.2) nm.

We then studied the behavior of the supported bilayer while decreasing the temperature by steps of 1.4°C. For each step, a series of AFM images was acquired until the system reached an equilibrium state. Fig. 1 reports a series of images from a temperature of 34.5°C to a temperature of 9.5°C. Lipid domains approximately (0.7 ± 0.2) nm higher than the surrounding lipids started to appear at 30.4°C (Fig. 1 *B*). Upon further cooling, they grew (Fig. 1 *C*) until they almost entirely covered the imaged area (Fig. 1 *D*).

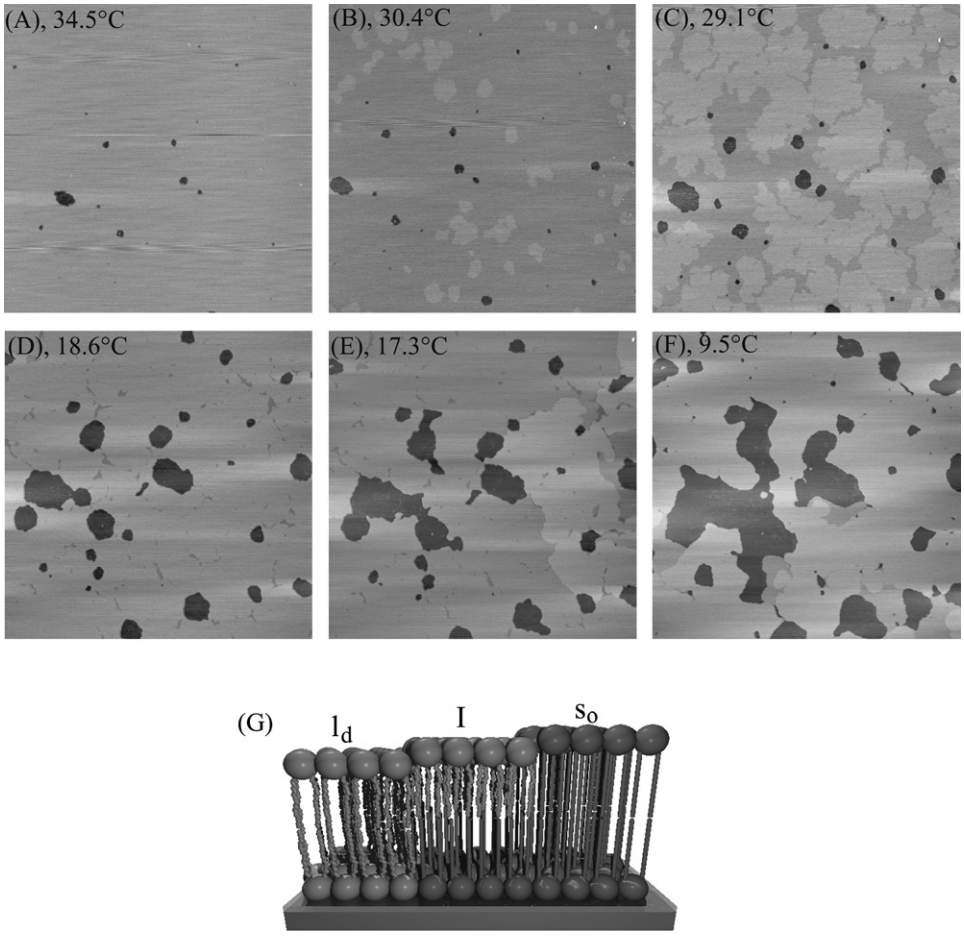

FIGURE 1 A temperature-controlled AFM experiment was performed on a supported lipid membrane of POPE/POPG 3:1 hydrated in pure water. (*A–F*) Representative height images (10 μm × 10 μm) of the lateral membrane organization at different temperatures (from 34.5°C to 9.5°C). The lighter the color, the thicker the membrane. The evolution of lipid domains and holes upon cooling is visible. Two phase transitions ascribable to independent phase transitions of the two leaflets composing the bilayer are clearly observed. A sketch of the general behavior is given in panel *G*, where the three different bilayer phases are shown. In our AFM measurements, we have found that step sizes are the same for the two transitions (0.7 ± 0.2) nm.

Upon further temperature decrease, the lipid bilayer appeared stable until new domains started to appear at 17.3°C (Fig. 1 *E*). Again the height of the newly developed domains was (0.7 ± 0.2) nm with respect to the surrounding lipids. Continuing the temperature sweep, the new domains grew and extended almost all over the imaged area at a final temperature of 9.5°C. Along with the nucleation and growth of the domains, holes in the lipid bilayer expanded with decreasing the temperature. Their expansion was more pronounced during the transition at the lower temperature. A similar behavior has already been observed for supported lipid bilayers (28–30). This has been generally interpreted as due to two independent thermally induced phase transitions of the two leaflets composing the bilayer. The observed images can be interpreted based on the scheme in Fig. 1 *G*. (Note that throughout the article, we will use, for the lipid monolayer, the same well-established nomenclature of the lipid bilayer relative to the adopted phases.) Starting from a bilayer in which both leaflets are in the liquid-disordered phase, we found that by decreasing the temperature, the proximal leaflet undergoes a phase transition to the solid-ordered phase. When this higher-temperature transition is over, the transition of the distal layer to the solid-ordered phase occurs. The same measured height of the growing domains confirms this interpretation. In reference to the various phases of the lipid bilayer, we use the terms liquid-disordered phase (both leaflets are in the l_d configuration), intermediate (one leaflet is in the l_d, the other one in the s_o configuration), and solid-ordered (both leaflets are in the s_o configuration) phase.

Fig. 2 *A* reports the fractional occupancy on the lipid bilayer for each of the three phases observed in the AFM experiment. It is clear from the graph that the two transitions were well separated. The fact that the intermediate phase reached a fractional occupancy almost equal to one ensured that the transition of the second leaflet started only when the first leaflet had completed its transition. Considering separately the transition of the two leaflets in Fig. 2, *B* and *C*, reveals that the lower temperature one was steeper than the higher temperature one, probably due to a higher cooperativity. The phase transition for a two-state process can be described by a van 't Hoff analysis. Such an approach has already been used to quantify AFM measurements on SLBs (28). Analyzing the two transitions according to a van 't Hoff interpretation allows for a quantitative comparison. The equilibrium constant K for the l_d-to-s_o phase transition is given by the fraction of the leaflet in the solid-ordered phase divided by the fraction in the liquid-disordered phase. In this case we can introduce the integrated form of the van 't Hoff equation (35),

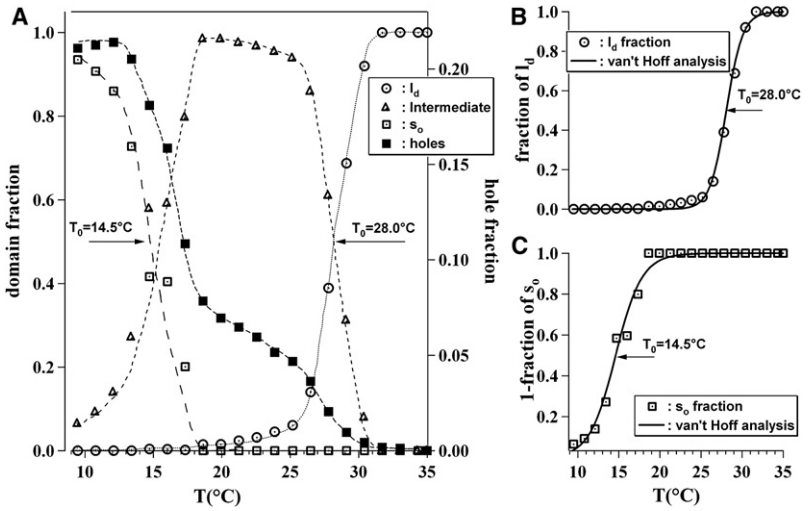

FIGURE 2 In panel *A*, the fractional occupancy of the three bilayer phases in the experiment of Fig. 1 (l_d fraction (*open circles*), s_o fraction (*open squares*), and *I* fraction (*open triangle*)) is shown as a function of temperature. In addition, the development of the fractional occupancy of holes in the lipid bilayer (*solid squares*) is reported. The given lines are included to guide the eyes. We also performed a van 't Hoff analysis of the two transitions. These are separately added in panels *B* and *C*. In both graphs, the original data is depicted by the markers and the fit is indicated by the solid lines. The arrows in all three panels depict the transition temperature obtained.

$$\ln K = \frac{\Delta H_{vH}}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right), \quad (1)$$

where ΔH_{vH} is the van 't Hoff enthalpy and T_0 the transition temperature (the temperature for $K = 1$). In Fig. 2 we have plotted the fractional occupancy of the phases with respect to the total membrane area. Thus, our data has to be fitted with the expression $1/(1 + K)$. Performing this operation, we obtained the following figures for the two transitions: $\Delta H_{vH} = 761$ kJ mol^{-1} and $T_0 = 28°$C for the proximal leaflet, and $\Delta H_{vH} = 431$ kJ mol^{-1} and $T_0 = 14°$C for the distal leaflet. The average size in numbers of molecules of the cooperativity unit is given as $N = \Delta H_{vH}/\Delta H_{DSC}$ (35). This number can also be seen as the number of lipids in an intrinsic domain. Assuming that each of the two leaflets contributed equally to the overall thermodynamic enthalpy determined by DSC (21 kJ mol^{-1}), a cooperative unit of 36 is obtained for the distal leaflet and 20 for the proximal leaflet. The comparison between the cooperative unit obtained for SLB and the value obtained for liposomes of the same lipid composition should be considered with caution due to the possible differences in the thermodynamic parameters of the two model systems (36).

In Fig. 2 *A*, the fractional occupancy of the holes, as a function of temperature, is additionally shown. The two transitions behave in a different way as far as the area decrease of the lipid bilayer is concerned. A more pronounced variation occurred during the distal leaflet transition, with respect to the proximal leaflet one (15% vs. 5%). This behavior again suggests that the two transitions are due to two physically or chemically different systems.

POPE/POPG (3:1) in different ionic strength solutions

The most relevant interaction between the substrate and the lipid bilayer is the electrostatic interaction between the charged mica surface and the lipid headgroups. Based on this consideration, we investigated the behavior of lipid bilayers of the same compositions in solutions with different ionic strengths. It was suggested that by increasing the ionic strength of the solution, the connected Debye length decrease should lower the electrostatic interaction between the substrate and the lipid bilayer. Hence, a coupled transition of the two leaflets as is usually observed in liposomes (28) should be observable. We performed two series of measurements according to the same procedure as above, but with two different ionic strengths (10 mM and 150 mM KCl).

The comparison between the fractional area occupancy of the intermediate phase in the 150 mM KCl case and in pure water is shown in Fig. S1 of the Supporting Material. Even if the two transitions in 150 mM KCl were closer to each other than in the pure water case, they were still separated. For the proximal and distal leaflet we obtained, from a van 't Hoff analysis, $T_0 = 23.6°$C and $T_0 = 18.3°$C, respectively. In both measurements the intermediate phase reached a fractional area near one. Moreover, in the 10 mM case, the separation and positions of the two transitions appeared very similar to the 150 mM case. This means that, if the ionic strength is able to modify the behavior of a solid supported lipid bilayer, a saturation level is already attained at 10 mM KCl. It should be stressed that from a DSC analysis of liposome phase transition, negligible differences were observed between the 10 mM and 150 mM KCl concentrations on the same lipid system (data not shown).

POPE in different ionic strength solutions

When studying solid supported lipid bilayers composed of a lipid mixture, the possibility of a compositional asymmetry of the two leaflets has to be considered. The presence of the substrate may induce a preferential distribution of one of the lipid species in one particular leaflet. This is mostly true in the system at issue, where one of the two lipids is negatively charged at pH 5.6 (POPG) and the other one is zwitterionic (POPE). The lipid flip-flop mechanism is slow in a formed

bilayer. This does not apply while a solid supported lipid bilayer is being formed on a support by the vesicle fusion technique. Moreover, our flat lipid bilayers were formed starting from SUVs for which an asymmetric distribution and packing difference of lipids between the inner and outer leaflet are possible (37). A chemical asymmetry of the two leaflets may position the two layers in different regions of the phase diagram for the POPE/POPG lipid mixture and it can shift the corresponding transition temperatures. It should be noted that the abundance or the lack of one of the two lipid species in one of the two leaflets is not necessarily related to the lack or abundance of the same lipid species in the opposite leaflet. In fact, a continuous lipid exchange between liposomes in solution and the bilayer on the surface can occur during the supported lipid bilayer formation (38,39).

Nevertheless, we partially tested the role of possible compositional asymmetries on the independent behavior of the two leaflets as far as the phase transition is concerned. To that aim we studied the behavior of a lipid bilayer composed of only POPE. Once formed on the mica surface, the bilayer was studied in pure water as above. Interestingly, even in this case, two separate transitions were found after decreasing the temperature: one at 31°C and the other one at 22°C. Even in the case in which the measurement was performed in 150 mM KCl, two transitions separated by 5°C were detected (data not shown).

The observed behavior illustrates that, even if a lipid compositional asymmetry is present, it is not the only cause of an independent behavior of the two leaflets.

Moreover, we studied also the behavior of a POPE SLB incubated directly in pure water. We wanted to check that the solution exchange did not leave the system in a situation different from the effective bulk ionic strength. In this case, we obtained for the bilayer, imaged in pure water, the same behavior as in the case when the bilayer was incubated in the high ionic strength solution and then the solution was exchanged for pure water. The same was obtained also for the other ionic strengths. A little difference was observed in some cases in the temperature of each transition, but the coupling or uncoupling behavior of the leaflets was maintained independently from the ionic strength of the incubation solution.

POPE/POPG (3:1) and POPE: effect of the incubation temperature

In a next step we tried to vary the preparation temperature of the SLB when the mica surface was exposed to the liposomes. The previous preparations were obtained at a temperature of 23°C, which is below the transition temperature of the proximal leaflets in all the studied cases. A decoupled melting behavior was also found at incubation temperatures below 23°C. In a following experiment the vesicle fusion procedure was performed at 27°C in 150 mM KCl. We started with a temperature of 35°C at which the bilayer was completely in the liquid disordered phase. When we decreased the temperature, domains of the intermediate phase appeared and grew. Before completing the transition, some of the already formed intermediate domains immediately transformed into transbilayer symmetric solid-ordered domains (Fig. 3). This behavior implies that the distal leaflet undergoes a rapid phase transition completely in register with the preformed solid domains of the proximal leaflet.

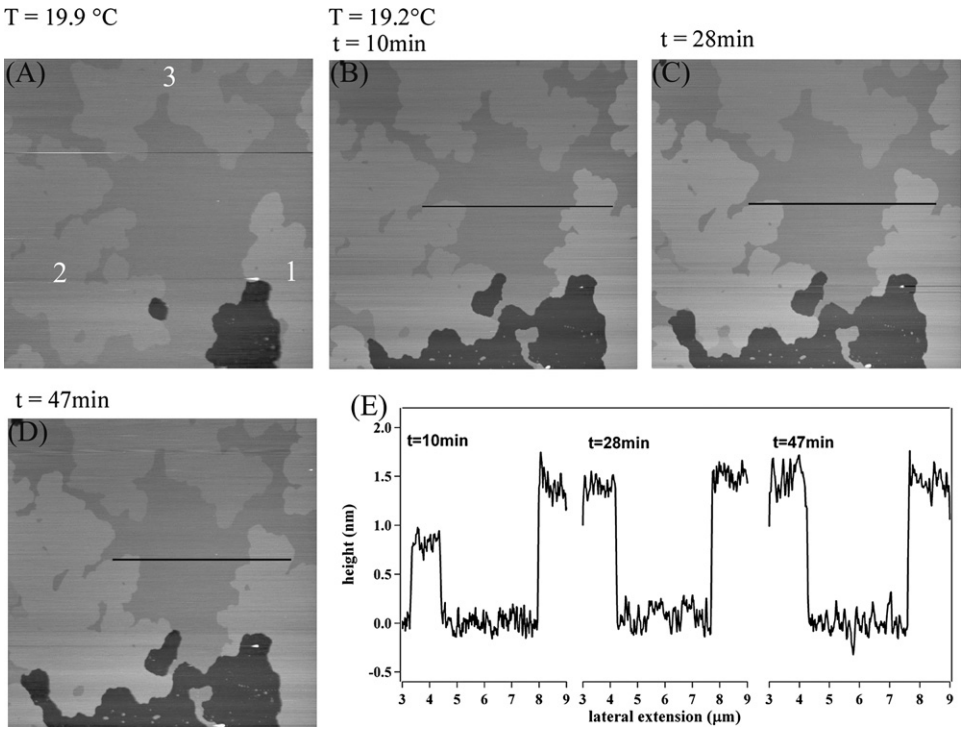

FIGURE 3 Sequence of images (10 μm × 10 μm) of a POPE/POPG 3:1 bilayer assembled at a temperature of ~27°C. An intermediate phase developed starting at a temperature of 27°C and grew upon further cooling. (*A*) Image of the bilayer at 19.9°C. (*B–D*) Time evolution of the domains after a decrease of the temperature to 19.2°C. The domains expanded and the second leaflet started to change phase in-register. At first, domain 1 became solid-ordered (*B*), then was followed by domain 2 (*C*). Domain 3 stayed in the intermediate phase. The profiles along the solid lines in images *B–D* are shown in panel *E*.

The phenomenon cannot be ascribed to lipid flip-flop, because a domain area decrease is expected if this were the case (31). Upon a further decrease in temperature, the solid-ordered domains grew much faster than the intermediate ones. Additionally, the other intermediate domains transformed to the solid-ordered phase with an in-register transition. This behavior is shown in Fig. 3, in which images on the same sample area at 19.9°C (Fig. 3 *A*) and 19.2°C (Fig. 3, *B–D*) are reported. The sequence from Fig. 3, *B–D*, shows that the transbilayer symmetric solid-ordered domains do not reach equilibrium on the timescale of an hour. This is at variance with what happens for domains present just in one leaflet. Moreover, single leaflet domains at 19.2°C are not stable, and they can transform to transbilayer symmetric domains in register (e.g., domain 2 in Fig. 3, *B* and *C*). Domain 3 remained in the intermediate phase. The transitions of domain 1 and 2 are also depicted in Fig. 3 *E* by plotting the evolution of the height sections (the one on the *left side* corresponding to 3 *B* and then progressing to the *right*) along the solid lines of the images in Fig. 3, *B–D*. The corresponding heights increments, with respect to the lipid l_d phase, were ~0.7 nm and 1.4 nm for the intermediate and the solid-ordered phase, respectively.

By assembling the supported lipid bilayer at 33°C in 150 mM KCl, a different behavior was observed, as shown in Fig. 4. At a temperature of 25.5°C, domains in the solid-ordered phase with a height of 1.4 nm above the surrounding lipid bilayer appeared (Fig. 4, *A* and *B*). The height of the domains suggested that both leaflets have undergone a phase transition in complete register. The fact that the transition starts at 25.5°C (the highest of the two transition temperatures reported before) implies that the proximal leaflet transition was able to induce a transition in the other leaflet as well. It is important to stress that the same experiments with lipid bilayers, both POPE/POPG 3:1 and then POPE only, prepared at higher temperatures of 35°C performed in pure water, led to two separate transitions. These transitions were equal to the transitions observed in lower temperature preparations. The kinetics of this double transition is completely different from those of uncoupled leaflets (40). In fact, soon after the transition started, a new equilibrium state was not reached within 4–5 h. The different kinetics observed in our systems is currently under investigation.

When the SLBs were directly incubated in pure water and imaged in pure water, instead of exchanging the solution after the assembling of the bilayer, we obtained two independent transitions also at an incubation temperature of 33°C.

DISCUSSION

In this work we elucidated the role of two physical parameters on the main phase transition of SLBs composed by POPE/POPG (3:1) and pure POPE: the ionic strength of the solution and the incubation temperature for the assembling of the bilayer from SUVs by the vesicle fusion technique. The lipid bilayer/substrate interactions play a fundamental role for

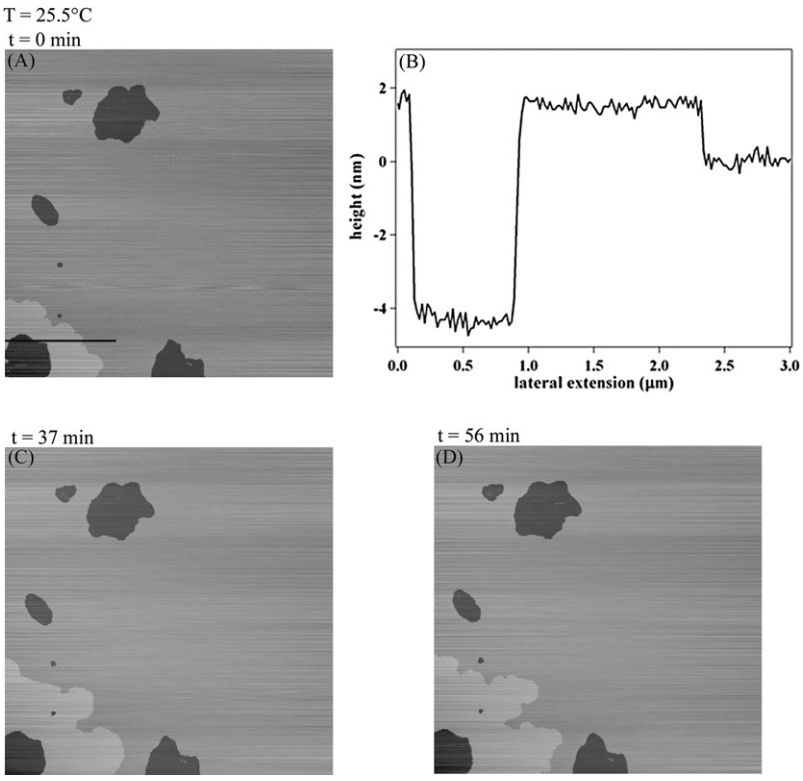

FIGURE 4 Sequence of images (10 μm × 10 μm) of a supported lipid bilayer of POPE/POPG 3:1 in 150 mM KCl assembled at a temperature of 33°C. In this cooling experiment, the first domain formation process was observed at a temperature of 25.5°C (*A*). The section analysis in panel *B* along the solid line in panel *A* clearly shows that the developed domain corresponds to the two leaflets in the solid-ordered phase in-register. The images taken at $t = 37$ min (*C*) and $t = 56$ min (*D*) at a constant temperature of 25.5°C demonstrate that a slow kinetics for the domain evolution is present.

determining the SLB behavior at the main phase transition and a detailed description of these interactions is provided in the Supporting Material.

Effect of the ionic strength on the main phase transition

When we performed temperature-controlled AFM imaging in pure water on POPE/POPG 3:1 or pure POPE lipid bilayers prepared at 23°C, the two leaflets of the bilayer presented two decoupled phase transitions. This behavior has already been observed in AFM studies of other planar SLBs (27–30) and in DSC studies of mica-supported DPPC bilayers (41). Besides affecting the coupling of the two transitions, the presence of a substrate generally resulted in a shift to higher temperature of one of the two transitions and a broadening of the transition widths due to a reduced cooperativity. Our results clearly showed that this characteristic behavior does not pertain only to lipid mixtures, but also to single component lipid bilayers. A lipid compositional asymmetry between the two leaflets or different demixing properties compared to vesicles cannot be considered the only reasons to explain what is observed. The lower temperature transition cannot be associated to a subgel phase or to a ripple phase. In fact, in the first case, a gel/gel transition would not lead to the height difference we observed between the two phases. The second possibility, the transition from a ripple to gel phase, can be neglected because high-resolution imaging was not able to identify the typical height modulations generated by the ripple phase and already observed by AFM (42). The higher temperature transition has been attributed to the proximal leaflet (28). In the case of POPE/POPG 3:1, it started at ~30°C, with a considerable shift to higher temperature if compared to what is obtained by DSC on vesicles of the same lipid mixture (22°C) (see Fig. S2). The lower-temperature transition, attributed to the distal monolayer, initiated itself at a temperature near to the transition temperature obtained by DSC. Based on these results, the proximal leaflet seems to be strongly influenced by the substrate whereas the distal leaflet behaves similar to an unsupported bilayer (28). In comparing the two solid-ordered to liquid-disordered transitions, we note their differences in the cooperativity units. A smaller unit was found for the proximal leaflet than for the distal one. Moreover, it seems that domains that develop in the distal layer are influenced by the presence of domain boundaries in the proximal leaflet. A difference is also observed in the variation of the fractional occupancy of holes in the bilayers during the transitions. Neglecting possible rearrangements of lipid molecules between the two leaflets at the hole interfaces during the transitions, the higher temperature transition resulted in an area-per-lipid reduction of 5%. If the formation of holes in nonscanned areas can be neglected, this feature is significantly lower than that of 20–25% observed for unsupported lipid bilayers (43) or of supported lipid bilayers that are not laterally confined (44). The observed discrepancy may suggest that a residual stress is present in the lipid bilayer. This is at variance with vesicles, where the transition occurs at constant pressure but variable surface area. Variations in the lateral pressure have already been interpreted as a cause for a variation of the transition temperature with respect to vesicles and for an increased transition width (30). The lower temperature transition is characterized by a fractional increase of the hole area of 15–20%. The obtained feature is similar to what is expected for a not-supported bilayer, and it is in favor of a distal leaflet not influenced by the presence of the substrate.

When the supported lipid bilayers were prepared at 23°C, by increasing the ionic strength of the solution to 10 mM KCl or 150 mM KCl, we still observed two decoupled transitions; however, the temperature difference between the two was reduced to 5°C, irrespective of the two ionic strengths. It is important to stress that almost no difference between 10 mM KCl and 150 mM KCl was observed in DSC analysis. The lower temperature transition took place in the same temperature range as in the case observed by DSC on vesicles. This becomes evident from the superposition of the temperature-dependent evolutions of the enthalpy obtained by DSC and the fractional occupancy of the solid-ordered phase obtained by AFM (see the Supporting Material). Even if it is not clear whether the ions distribute in the region between the bilayer and the substrate, as would be the case in the absence of the bilayer, a high ionic strength decreases the Debye length and also screens the substrate surface charge more efficiently. As a consequence, the bilayer/substrate equilibrium distance is altered, changing their viscous coupling. On the other side, if the electric field produced by the substrate has a role in determining a compositional asymmetry in the two leaflets, the use of electrolytes decreases this effect (45). It is important to stress that we used monovalent electrolytes, because it has been found that divalent ions such as Ca^{2+} can induce a more accentuated effect on the lipid distribution. Their presence favors the presence of negatively charged phospholipids in the proximal leaflet for a negatively charged substrate like mica (46).

Our results clearly show that, even if the SLBs are assembled in a high ionic strength solution, by changing the bulk imaging solution to different ionic strengths we affected the lipid bilayer behavior differently. This means that, even if the first layer of the ions in contact with the mica may remain unaltered by changing the bulk ionic concentration (47), the bulk ionic concentration has an effect on the proximal leaflet. The bulk electrolyte concentration modifies the structure of the water layer between the solid substrate and the lipid bilayer. Moreover, the experiment performed by incubating a POPE bilayer directly in pure water resulted in the same general behavior as in the case where the bilayer was incubated at high ionic strength and the solution was exchanged for pure water.

Effect of the incubation temperature

The results of this work show that by increasing the incubation temperature, the bilayer moves from a decoupled behavior of the two leaflets to a coupled one with in-register domains. This trend is obtained only in the presence of electrolytes in the bulk solution. The main results are summarized in Table S1 of the Supporting Material. The incubation temperature has an effect on the phase behavior of the SUVs during the SLB formation. In particular, upon increasing the temperature, the area per lipid is increased, with the higher increment being across the main phase transition. Consequently, the lipid density deposited on the surface is affected by the incubation temperature, and hence also the lateral pressure in the leaflets is affected. The sample prepared at 27°C displayed a behavior initially similar to that of samples prepared at 23°C or below. However, as the imaging temperature decreases, some solid-ordered domains initially present in only one leaflet instantaneously transform into transbilayer solid-ordered domains in complete register (symmetric domains). Upon a small decrease in temperature, the domains in-register start growing without reaching equilibrium on the timescale of some hours, and other single leaflet domains transform to transbilayer solid-ordered domains. The appearance of domains in the proximal leaflet started at 26°C, which means at about the same temperature obtained for incubations at lower temperature. A possible explanation for this behavior could be that the increased incubation temperature does not have an effect on the behavior of the proximal leaflet, but somehow influences the distal leaflet in a way that favors the interleaflet coupling. The interleaflet coupling is at the moment a subject of extensive investigations, both theoretically and experimentally (48), but its mechanism is not fully understood, especially when there are no molecules able to transversally diffuse like cholesterol. It has been recently demonstrated that unsupported lipid bilayers in the presence of cholesterol present a strong interleaflet coupling, which means that each leaflet is able to induce or suppress phase separation in the other one (49). Even if interdigitation between chains is expected to play a minor role in the presence of cholesterol or in the case of chains of equal length, it might be that, in the present case, it is relevant to induce a coupling effect between the two layers. Further, it is to be considered that, in our case, the bilayer midplane is modified by the single leaflet domain formation (see Fig. 1 *G*). Actually, in a theoretical study Wagner et al. (50) demonstrated that in lipid membranes with cholesterol, a low coupling between the monolayers can result in domains out of register. The incubation temperature can modify the lipid density in the distal layer so to favor interdigitation and to increase the interleaflet coupling. Otherwise, a reduced density of the distal layer could influence the mobility of the lipids and allow a coupled growth of domains. The effect is even more evident when the sample is prepared at higher temperature. Only a direct transition to solid-ordered domains in-register between the two leaflets appears, starting at a temperature of 25.5°C. In both cases, the kinetics of domain evolution is very long, as shown in Fig. 4.

It is important to stress that the same behavior of a coupled transition directly to solid-ordered domains in-register between the two leaflets is observed for pure POPE bilayers when they are deposited at a temperature of 33°C and imaged in 10 mM or 150 mM KCl (data not shown).

In the Supporting Material, both the dynamical heterogeneity between the two leaflets and the possibility of asymmetric lipid distribution is discussed with respect to their possible influence on the SLB behavior. Moreover, the results obtained by changing the pH and buffer relative to the main phase transition of the SLB are presented in the Supporting Material.

CONCLUSIONS

This work analyzed some of the physical parameters that may influence the behavior of solid supported lipid bilayers as far as their main phase transition is concerned. The results point to a SLB model system as a structure in which the presence of the substrate can play a major role especially on the properties of the proximal leaflet. The SLB model system has been questioned because an independent and uncoupled behavior of the two leaflets has been observed for the main phase transition, at variance with what is observed for other lipid systems such as liposomes (33). Here we demonstrated that it is possible to tune the preparation conditions of SLBs to reproduce a coupled behavior of the two leaflets for a pure or mixed bilayer. This is accomplished by using electrolytes in the solution and by incubating the substrate with a solution at a temperature higher than that of the main phase transition of the lipids in liposomes. In the context of this study, for the SLBs, two interactions have to be considered: the interaction of the bilayer, mainly the proximal leaflet, with the support and the interleaflet coupling. The preparation temperature modifies the physical properties of one or both leaflets so that the interleaflet coupling is strengthened. This may happen via a variation in the lipid density in the leaflets that may have a consequence on interdigitation possibilities and in the lateral tension. At the moment, it is not possible to state whether this is the same mechanism by which the same type of domain is observed in liposomes (33).

A general observation is that when transbilayer symmetric domains are observed, they tend to grow with a slow kinetics and nanometric scale domains are not stable—at variance with the case in which they are present only in one leaflet. However, in biological membranes, functional domains are on the nanoscale dimension (51). This means that the study of supported lipid bilayers can have a biological relevance for understanding the behavior of membranes. Hence, the substrate simulates the role that submembrane elements

such as the cytoskeleton can have on the cell membrane. This includes both the compositional asymmetry and the reduced mobility of the lipids. Moreover, the possibility of tuning the behavior of SLBs opens the way to the study of membrane proteins in this model system. In particular, protein interactions with the lipids and their distribution relative to lateral heterogeneity of the lipid bilayer induced by temperature, pH, or other physical parameters, can be studied (52).

SUPPORTING MATERIAL

Four figures and two tables are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)-01221-1.

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REFERENCES

1. Sackmann, E. 1996. Supported membranes: scientific and practical applications. *Science*. 271:43–48.
2. Tanaka, M., and E. Sackmann. 2005. Polymer-supported membranes as models of the cell surface. *Nature*. 437:656–663.
3. Castellana, E. T., and P. S. Cremer. 2006. Solid supported lipid bilayers: from biophysical studies to sensor design. *Surf. Sci. Rep.* 61:429–444.
4. Tamm, L. K., and H. M. McConnell. 1985. Supported phospholipid bilayers. *Biophys. J.* 47:105–113.
5. McConnell, H. M., T. H. Watts, R. M. Weis, and A. A. Brian. 1986. Supported planar membranes in studies of cell-cell recognition in the immune system. *Biochim. Biophys. Acta*. 864:95–106.
6. Brian, A. A., and H. M. McConnell. 1984. Allogeneic stimulation of cytotoxic T cells by supported planar membranes. *Proc. Natl. Acad. Sci. USA*. 81:6159–6163.
7. Richter, R. P. A. M., and A. Brisson. 2003. Pathways of lipid vesicle deposition on solid surfaces: a combined QCM-D and AFM study. *Biophys. J.* 85:3035–3047.
8. Wong, J. Y., J. Majewski, M. Seitz, C. K. Park, J. Israelachvili, et al. 1999. Polymer-cushioned bilayers. I. A structural study of various preparation methods using neutron reflectometry. *Biophys. J.* 77:1445–1457.
9. Parthasarathy, R., and J. T. Groves. 2004. Optical techniques for imaging membrane topography. *Cell Biochem. Biophys.* 41:391–414.
10. Miller, C. E., J. Majewski, T. Gog, and T. L. Kuhl. 2005. Characterization of biological thin films at the solid-liquid interface by x-ray reflectivity. *Phys. Rev. Lett.* 94:238104.
11. Keller, C. A., and B. Kasemo. 1998. Surface specific kinetics of lipid vesicle adsorption measured with a quartz crystal microbalance. *Biophys. J.* 75:1397–1402.
12. Linseisen, F. M., M. Hetzer, T. Brumm, and T. M. Bayerl. 1997. Differences in the physical properties of lipid monolayers and bilayers on a spherical solid support. *Biophys. J.* 72:1659–1667.
13. Johnston, L. J. 2007. Nanoscale imaging of domains in supported lipid membranes. *Langmuir*. 23:5886–5895.
14. Kiessling, V., C. Wan, and L. K. Tamm. 2008. Domain coupling in asymmetric lipid bilayers. *Biochim. Biophys. Acta*. 1788:64–71.
15. Devaux, P. F., and R. Morris. 2004. Transmembrane asymmetry and lateral domains in biological membranes. *Traffic*. 5:241–246.
16. Daleke, D. L. 2003. Regulation of transbilayer plasma membrane phospholipid asymmetry. *J. Lipid Res.* 44:233–242.
17. Liu, J., and J. C. Conboy. 2007. Asymmetric distribution of lipids in a phase segregated phospholipid bilayer observed by sum-frequency vibrational spectroscopy. *J. Phys. Chem. C*. 111:8988–8999.
18. Crane, J. M., V. Kiessling, and L. K. Tamm. 2005. Measuring lipid asymmetry in planar supported bilayers by fluorescence interference contrast microscopy. *Langmuir*. 21:1377–1388.
19. Bayerl, T. M., and M. Bloom. 1990. Physical properties of single phospholipid bilayers adsorbed to micro glass beads. A new vesicular model system studied by deuterium nuclear magnetic resonance. *Biophys. J.* 58:357–362.
20. Johnson, S. J., T. M. Bayerl, D. C. McDermott, G. W. Adam, A. R. Rennie, et al. 1991. Structure of an adsorbed dimyristoylphosphatidylcholine bilayer measured with specular reflection of neutrons. *Biophys. J.* 59:289–294.
21. Kim, J., G. Kim, and P. S. Cremer. 2001. Investigations of water structure at the solid/liquid interface in the presence of supported lipid bilayers by vibrational sum frequency spectroscopy. *Langmuir*. 17: 7255–7260.
22. Brown, D., and E. London. 1998. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* 14:111–136.
23. Garg, S., J. Rühe, K. Lüdtke, R. Jordan, and C. A. Naumann. 2007. Domain registration in raft-mimicking lipid mixtures studied using polymer-tethered lipid bilayers. *Biophys. J.* 92:1263–1270.
24. Kiessling, V., J. M. Crane, and L. K. Tamm. 2006. Transbilayer effects of raft-like lipid domains in asymmetric planar bilayers measured by single molecule tracking. *Biophys. J.* 91:3313–3326.
25. Connell, S. D., and D. A. Smith. 2006. The atomic force microscope as a tool for studying phase separation in lipid membranes. *Mol. Membr. Biol.* 23:17–28.
26. Tokumasu, F., A. J. Jin, and J. A. Dvorak. 2002. Lipid membrane phase behavior elucidated in real time by controlled environment atomic force microscopy. *J. Electron Microsc. (Tokyo).* 51:1–9.
27. Leonenko, Z. V., E. Finot, H. Ma, T. E. S. Dahms, and D. T. Cramb. 2004. Investigation of temperature-induced phase transitions in DOPC and DPPC phospholipid bilayers using temperature-controlled scanning force microscopy. *Biophys. J.* 86:3783–3793.
28. Keller, D., N. B. Larsen, I. M. Moller, and O. G. Mouritsen. 2005. Decoupled phase transitions and grain-boundary melting in supported phospholipid bilayers. *Phys. Rev. Lett.* 94:25701.
29. Feng, Z. V., T. A. Spurlin, and A. A. Gewirth. 2005. Direct visualization of asymmetric behavior in supported lipid bilayers at the gel-fluid phase transition. *Biophys. J.* 88:2154–2164.
30. Charrier, A., and F. Thibaudau. 2005. Main phase transitions in supported lipid single-bilayer. *Biophys. J.* 89:1094–1101.
31. Lin, W.-C., C. D. Blanchette, T. V. Ratto, and M. L. Longo. 2006. Lipid asymmetry in DLPC/DSPC-supported lipid bilayers: a combined AFM and fluorescence microscopy study. *Biophys. J.* 90:228–237.
32. Stottrup, B., S. Veatch, and S. Keller. 2004. Nonequilibrium behavior in supported lipid membranes containing cholesterol. *Biophys. J.* 86: 2942–2950.
33. Bagatolli, L. A., and E. Gratton. 2000. A correlation between lipid domain shape and binary phospholipid mixture composition in free-standing bilayers: a two-photon fluorescence microscopy study. *Biophys. J.* 79:434–447.
34. Pozo Navas, B., K. Lohner, G. Deutsch, E. Sevcsik, K. A. Riske, et al. 2005. Composition dependence of vesicle morphology and mixing properties in a bacterial model membrane system. *Biochim. Biophys. Acta*. 1716:40–48.
35. Mason, J. T. 1998. Investigation of phase transitions in bilayer membranes. *Methods Enzymol.* 295:468–494.
36. Tokumasu, F., A. J. Jin, G. W. Feigenson, and J. A. Dvorak. 2003. Atomic force microscopy of nanometric liposome adsorption and nanoscopic membrane domain formation. *Ultramicroscopy*. 97:217–227.

37. Israelachvili, J. N., D. J. Mitchell, and B. W. Ninham. 1977. Theory of self-assembly of lipid bilayers and vesicles. *Biochim. Biophys. Acta.* 470:185–201.

38. Reinl, H. M., and T. M. Bayerl. 1994. Lipid transfer between small unilamellar vesicles and single bilayers on a solid support: self-assembly of supported bilayers with asymmetric lipid distribution. *Biochemistry.* 33:14091–14099.

39. Wacklin, H. P., and R. K. Thomas. 2007. Spontaneous formation of asymmetric lipid bilayers by adsorption of vesicles. *Langmuir.* 23:7644–7651.

40. Blanchette, C. D., C. A. Orme, T. V. Ratto, and M. L. Longo. 2008. Quantifying growth of symmetric and asymmetric lipid bilayer domains. *Langmuir.* 24:1219–1224.

41. Yang, J., and J. Appleyard. 2000. The main phase transition of mica-supported phosphatidylcholine membranes. *J. Phys. Chem. B.* 104: 8097–8100.

42. Kaasgaard, T., C. Leidy, J. H. Crowe, O. G. Mouritsen, and K. Jorgensen. 2003. Temperature-controlled structure and kinetics of ripple phases in one- and two-component supported lipid bilayers. *Biophys. J.* 85:350–360.

43. Nagle, J. F., R. Zhang, S. Tristram-Nagle, W. Sun, H. I. Petrache, et al. 2006. X-ray structure determination of fully hydrated L_α phase dipalmitoylphosphatidylcholine bilayers. *Biophys. J.* 70:1419–1431.

44. Schuy, S., and A. Janshoff. 2006. Thermal expansion of microstructured DMPC bilayers quantified by temperature controlled atomic force microscopy. *ChemPhysChem.* 7:1207–1210.

45. Käsbauer, M., M. Junglas, and T. M. Bayerl. 1999. Effect of cationic lipids in the formation of asymmetries in supported bilayer. *Biophys. J.* 76:2600–2605.

46. Richter, R. P., N. Maury, and A. R. Brisson. 2005. On the effect of the solid support on the interleaflet distribution of lipids in supported lipid bilayers. *Langmuir.* 21:299–304.

47. White, R. J., B. Zhang, S. Daniel, J. M. Tang, E. N. Ervin, et al. 2006. Ionic conductivity of the aqueous layer separating a lipid bilayer membrane and a glass support. *Langmuir.* 22:10777–10783.

48. Collins, M. D. 2008. Interleaflet coupling mechanisms in bilayers of lipids and cholesterol. *Biophys. J.* 94:L32–L34.

49. Collins, M. D., and S. L. Keller. 2007. Tuning lipid compositions to induce domain formation across asymmetric bilayers. *Proc. Natl. Acad. Sci. USA.* 105:124–128.

50. Wagner, A. J., S. Loew, and S. May. 2007. Influence of monolayer-monolayer coupling on the phase behavior of a fluid lipid bilayer. *Biophys. J.* 93:4268–4277.

51. Jacobson, K., O. G. Mouritsen, and R. G. W. Anderson. 2007. Lipid rafts: at a crossroad between cell biology and physics. *Nat. Cell Biol.* 9:7–14.

52. Alessandrini, A., and P. Facci. 2005. AFM: a versatile tool in biophysics. *Meas. Sci. Technol.* 16:R65–R92.