Effect of Ca^{2+} on the Morphology of Mixed DPPC−DOPS Supported Phospholipid Bilayers

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The morphology of supported phospholipid bilayers (SPBs) containing mixtures of phospholipids in gel (dipalmitoyl phosphatidylcholine, DPPC) and fluid (dioleoyl phosphatidylserine (DOPS) or -choline (DOPC)) states at room temperature was investigated by atomic force microscopy (AFM). Fluid−gel phase separation was clearly detectable on topography images of mixed SPBs. The presence or absence of Ca^{2+} in the subphase was found to have a dramatic effect on the organization of the gel phase when the fluid phase was composed of pure DOPS: In the absence of Ca^{2+}, large, well-defined DPPC domains were found in both the DPPC/DOPC and DPPC/DOPS mixtures, while in its presence small, isolated DPPC domains were found in the DPPC/DOPS mixture. Ca^{2+} had no effect on the organization of DPPC in DPPC/DOPC mixtures, and its effect was abolished by adding DOPC to DPPC/DOPS mixtures.

Introduction

Supported phospholipid bilayers (SPBs, reviewed in ref 1) occupy a unique position as model systems for investigating problems such as structure−function relationships in cell membranes and protein−lipid interactions. A range of surface-sensitive techniques, such as quartz crystal microbalance,[2] surface plasmon resonance,[3] and atomic force microscopy (AFM),[4−8,17] is available for their characterization. We have recently demonstrated that SPBs are also suitable for the growth of protein two-dimensional (2D) crystals and investigation of crystal growth mechanisms in situ by AFM.[9] SPBs composed of mixtures of phospholipids are especially interesting in this context, because not only were most protein 2D crystals obtained to date grown on monolayers composed of at least two components[10] but also mixing lipids with different phase transition temperatures allows the effect the lipid bilayer properties on crystal growth to be investigated, leading ultimately to a better understanding and control of the crystallization process. Investigating the mixed DPPC/DOPS SPBs by AFM with this goal in mind, we came across intriguing phenomena not reported in the literature.

Experimental Section

Lipids used in this study—dipalmitoyl phosphatidylcholine (DPPC), dioleoyl phosphatidylcholine (DOPC), and dioleoyl phosphatidylserine (DOPS)—were purchased from Avanti Polar Lipids (Alabama, USA). Other chemicals were purchased from Merk (Germany) or Sigma (St. Louis, MO). All glassware used in this study was stored overnight in a mixture of chromic and sulfuric acids and rinsed thoroughly with water prior to use.

Buffers contained the following: (1) 4 mM $CaCl_2$, 150 mM NaCl, 10 mM HEPES, 3 mM NaN_3, pH 7.4; (2) 4 mM EDTA, 150 mM NaCl, 10 mM HEPES, 3mM NaN_3, pH 7.4; (3) 2 mM $CaCl_2$, 150 mM NaCl, 10 mM HEPES, 3 mM NaN_3, pH 7.4. All buffers were prepared in MilliQ water and filtered through a 200 nm syringe filter (Schleicher and Schuell, Germany) prior to use.

Multilamellar vesicles (MLVs) were obtained by mixing appropriate amounts of lipids dissolved in chloroform or chloroform/methanol (2:1 v:v) and evaporating the solvent with argon. After a further 30−40 min of drying in a desiccator connected to a rotary vacuum pump, the lipids were resuspended in an appropriate buffer at 0.5 mg/mL lipid. To produce unilamellar vesicles, the suspension was sonicated under argon for ∼45 min with a bath sonicator (Laboratory Supplies Co. Inc., New York), or with a tip sonicator (Brandson Ultrasonics Corp., Connecticut) (in pulsed mode at 30% duty cycle). Sonication was performed above the transition temperature of the lipid mixture.

Freshly prepared unilamellar vesicles (100 μL) were deposited onto freshly cleaved mica disks (12 mm in diameter, Metafix, Montdidier, France) glued to Teflon adhesive tape ("BYTAC", Norton Performance Plastics Corp., Ohio) coated metal disks using Rapid epoxy glue (according to the procedure described in ref 11), and incubated for 4−8 h at 4 °C. The excess of vesicles was removed by repeatedly exchanging the solution covering the mica disks with an appropriate buffer. DPPC/DOPC samples had to be heated briefly to ∼60 °C to remove liposomes associated with the SPB,[4] while washing with buffer (2) at room temperature was sufficient for the PS-containing preparations. After the

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washing procedures, the sample was installed in the fluid cell of the AFM. The microscope was allowed to thermally equilibrate for a minimum of 30 min before imaging.

Annexin V binding was performed by injecting ~10 μg of the protein dissolved in buffer (3) into the fluid cell of the AFM.

AFM observation was performed using a Nanoscope IIIa-MultiMode AFM (Digital Instruments, CA) equipped with a "J" (120 μm) scanner. The contact mode fluid cell (Digital Instruments) was washed extensively with ultrapure water, 95% ethanol and again with water before each experiment. O-rings, washed in 1% Helmanex solution (GMBH, Germany) overnight and sonicated 3 times in freshwater were used in experiments where exchange of buffers was required.[12]

Images were recorded in the constant force mode using oxide-sharpened silicon nitride tips mounted on cantilevers with a nominal force constant of 0.06 N/m, at scanning rates of 8−15 Hz.[13] The scan angle was 90°. The force was kept at the lowest possible value by continuously adjusting the set point during imaging. Images were flattened and plane-fitted as required.

Results

Continuous SPBs were successfully prepared from mixtures of DPPC/DOPC, DOPC/DOPS, and DPPC/DOPS containing up to 80% PS by weight. Tendency toward multilayer formation was observed in phospholipid mixtures containing gel-phase phospholipids (data not shown). Washing the PS-containing SPBs with EDTA-containing buffer resulted in clean, single SPBs. Heating was required to achieve the same in the case of DPPC/DOPC preparations.[4]

The gel and the fluid phases (e.g., DPPC and DOPS or DOPC, respectively) could be distinguished on the topography images due to ~1 nm difference in height between them (Figures 1 and 2). [4,5,7] In the case of DPPC/DOPS SPBs with 50−80% DPPC, in the absence of Ca^{2+}, DPPC was found to be organized in large domains (Figure 1a). Similar organization was observed with the DPPC/DOPC SPBs (not shown) and has previously been reported by others for DPPC/DOPC[7] and DPPC/POPC mixtures.[4] In the presence of Ca^{2+} the organization of DPPC in the DPPC/DOPS mixture changed dramatically, and small DPPC domains were observed instead (Figure 1b). The shape and size of the small domains were found to vary from experiment to experiment, presumably depending on whether the sample did or did not reach equilibrium. The two most representative cases are presented in Figure 1b and Figure 2. The changes induced by Ca^{2+} were reversible and were repeated up to four times by cycling the buffers through the fluid cell of the AFM (not shown).

Ca^{2+} had no effect on either the organization of DPPC in DOPC or that of DPPC in the ternary mixture with DOPC and DOPS (3:3:1 ratio by weight), which behaved like the binary DPPC/DOPC mixture (results not shown).

No DPPC domains could be observed in the 1:4 DPPC/DOPS mixture.

Annexin V,[14] a soluble protein that binds to negatively charged phospholipids in the presence of Ca^{2+}, was used to aid in the identification of the DOPS component of the SPBs and to obtain some information concerning their organization. It was shown earlier that annexin V crystallizes on 4:1 DOPC/DOPS SPBs in the "*p*6" crystal form.[9] In contrast, it did not crystallize on the 4:1 or 1:1 DPPC/DOPS SPBs. Instead, 2D close-packed "aggregates" were formed in the areas surrounding the DPPC domains (Figure 1c). A different crystal form was observed on 1:4 DPPC/DOPS (Figure 1d).[15] On the other hand, the "*p*6" crystal form was found on the ternary mixture of DPPC/DOPC/DOPS, where DPPC domains remained devoid of annexin V (Figure 1e). This latter result confirms that in the ternary mixture, DPPC is excluded from the fluid phase which behaves like the DOPC/DOPS mixture at this ratio.

The process by which large DPPC domains are transformed into small islands (presumed to be composed predominantly of DPPC) is captured in Figure 2. The large DPPC domains are eroded at the interface between the two phases, giving rise to the small domains, which spread across the available area and ultimately cover the whole surface. Channels of DOPS-rich phase are seen to separate individual domains (Figure 2d and Figure 1b).

Discussion

Fluid−gel phase separation in phospholipid mixtures is a relatively well-investigated phenomenon.[16] The immiscibility of the two phases stems from the interactions between the acyl chains in the fluid vs the gel phase. The solid and fluid phases can be distinguished on topography AFM images due to the differences in the size of the molecules, the mechanical properties of the two phases, and, in the case of charged vs zwitterionic phospholipids, surface charge density.[4−7,17] The difference of ~1 nm between the height of the solid (DPPC) phase and the fluid (DOPC/DOPS) phase observed in this study compares well with the values found previously by AFM for the DPPC/DOPC system,[7] and the DPPC/POPC one,[5] but differs significantly from the value reported for the DSPE/DOPE system.[17]

In the case of DPPC/DOPC mixtures, the gel phase (DPPC) was found to be organized in large, well-defined domains (similar to those shown in Figure 1a). Replacing the zwitterionic PC headgroup with the negatively charged PS one does not change the nature of the headgroup−headgroup interaction (repulsion),[18] but only increases its magnitude. Thus, the same morphology is observed for DPPC domains in mixtures with DOPS in the presence of EDTA (Figure 1a) as in mixtures with DOPC.

Binding of Ca^{2+} to PS was found, surprisingly, to change the morphology of the gel phase (Figures 1 and 2). The phases are still immiscible, but DPPC is now organized in small domains (Figure 1b, Figure 2), indicating that the interfacial energy between the two phases has changed.

The domains of the gel phase are held together by a repulsion between the gel and the surrounding fluid (DOPC or DOPS) phases. Depending on the extent of this repulsion, the large domains may become unstable. The subsequent discussion is devoted to the possible role of DOPS−Ca^{2+} interaction in this process.

In studies on multilayered systems, Ca^{2+} has been found to increase the gel-to-fluid transition temperature of PS-containing phospholipids, including DOPS (−11 to ~120 °C).[19,20] However, DOPS monolayers were found to remain in liquid-expanded phase in the presence as well as in the absence of Ca^{2+}.[21,22] The results presented here are concerned with a single bilayer, which is expected to behave similarly to a monolayer rather than a multilayered system. We therefore consider it unlikely that an ionotropic phase transition in DOPS is the cause of the

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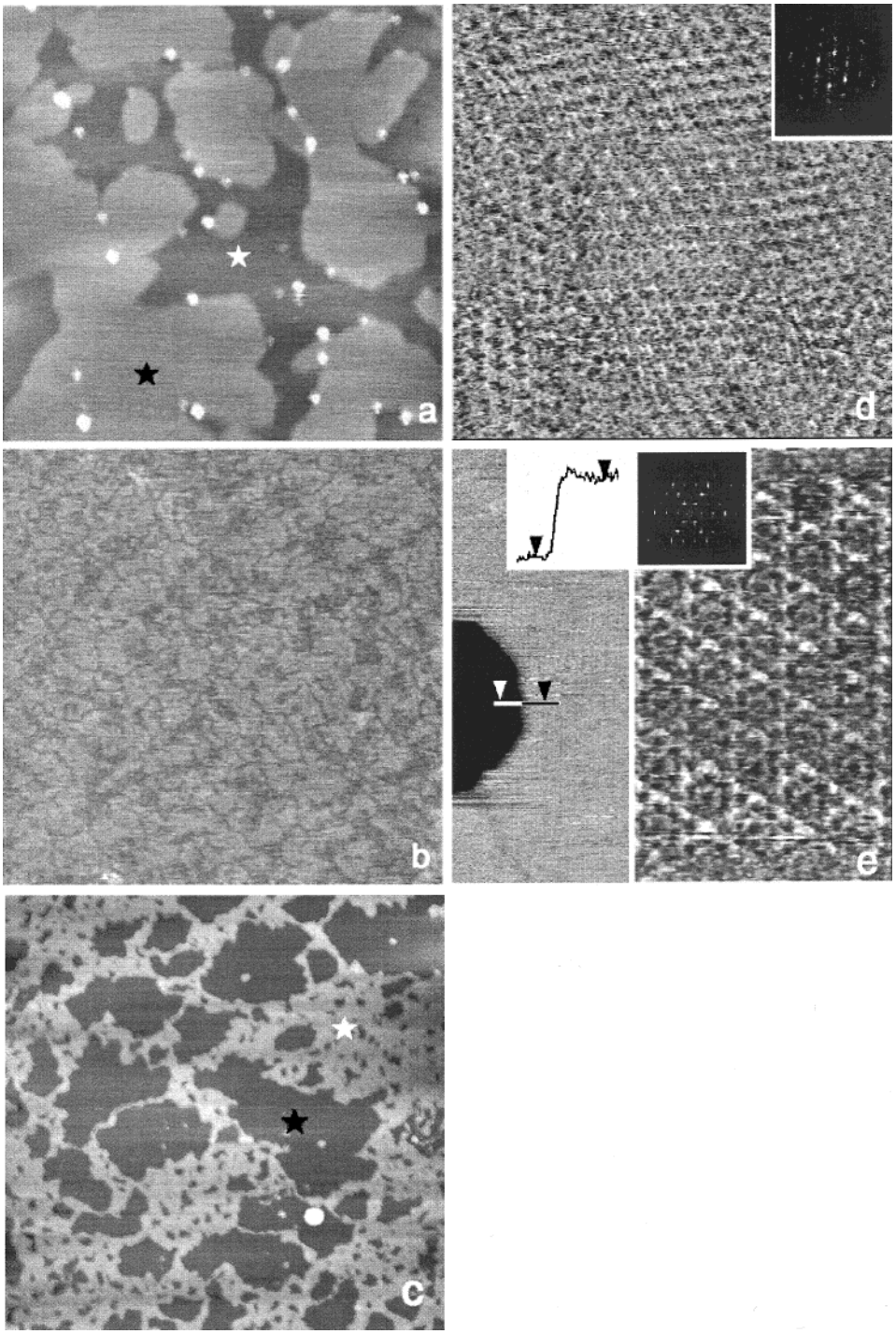

Figure 1. Effect of Ca^{2+} on the organization of DPPC/DOPS SPBs. (a) 4:1 (w:w) DPPC/DOPS SPB in the absence of Ca^{2+} (buffer 2). The black star indicates the 1 ± 0.2 nm higher DPPC domains. The white star corresponds to the lower height level of DOPS. Scan size: 2.8 μm. *Z*-scale (black-to-white): 5 nm. (b) Same as in (a), in the presence of Ca^{2+} (buffer 1). Scan size: 1 μm. *Z*-scale: 2.5 nm. The states depicted in both (a) and (b) were stable for up to 10 h. (c) Annexin V (white star) bound to the SPB shown in (b). The height of annexin V over the DPPC domains (black star) is 1.5 ± 0.2 nm. Scan size: 3 μm. *Z*-scale: 15 nm. (d) Annexin V crystallizes on 1:4 (w:w) DPPC/DOPS SPB. The crystal form is different from the one reported earlier.[9] Scan size: 200 nm. *Z*-scale: 2.5. nm. Inset: Fourier transform of an area within the image indicates a hexagonal lattice with a lattice constant of 9.8 ± 0.5 nm.[12] (e) Annexin V crystals found on DPPC/DOPC/DOPS (3:3:1 by weight) SPB. Left: The DPPC domain (appears black, indicated with the white marker) remains devoid of annexin V, as in (c). The height of annexin V over the DPPC domain, as measured between the two markers (inset), was found to be 1.8 ± 0.2 nm. Image size: 0.8×2 μm. *Z*-scale: 5 nm. Right: An area within the left image was scanned at a higher magnification. The crystal form ("*p*6") is indistinguishable from the one reported earlier.[9] Image size: 115×198 nm. *Z*-scale: 3 nm. Inset: Fourier transform of the image indicates a hexagonal lattice with lattice constants of 27 ± 2 nm.[12]

change in domain morphology.[23] The ability of DPPC/DOPS (1:4 mixture) to support crystallization of annexin V (Figure 1d) is indicative of efficient diffusion in the DOPS phase of the SPB and corroborates the above interpretation.

Other effects Ca^{2+} exerts on DOPS include the change in the surface area (SA) per molecule—from 0.675 to 0.625 nm^2—upon Ca^{2+} binding,[21,22] possibly a partial or complete neutralization of negative charge on the PS headgroup as well as its dehydration and consequent conformational

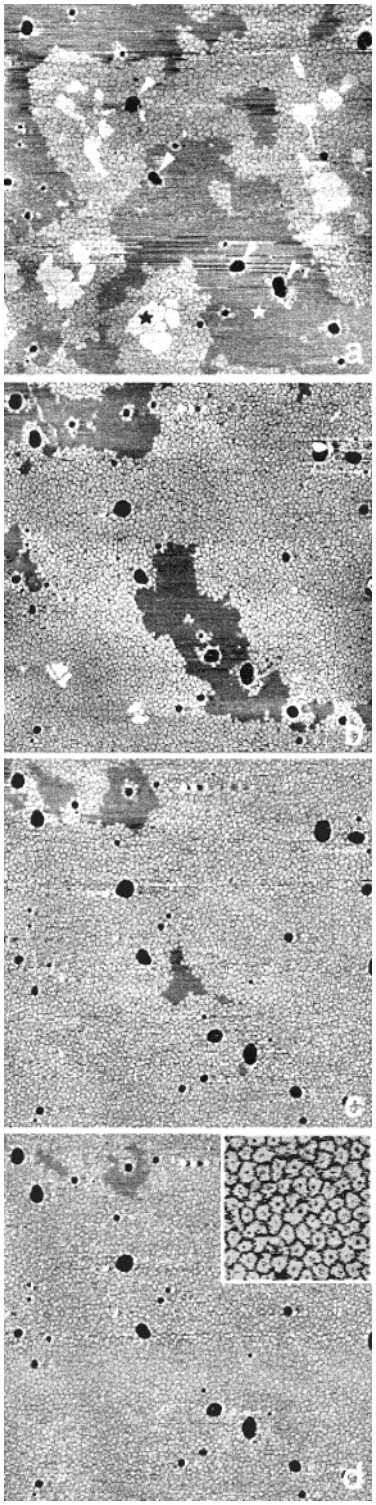

Figure 2. The time course of the large DPPC domains breaking up into the small ones followed in situ. 4:1 DPPC:DOPS SPB was washed with EDTA-containing buffer (2), which was then replaced in the fluid cell of the AFM with buffer (1) containing 2 mM Ca^{2+}. (a) 0 min after onset of imaging, (b) 17 min, (c) 25 min, (d) 28 min. Scan size (*Z*-scale): (a), 6.7 μm (5 nm); (b)−(d), 8 μm (3 nm). Large DPPC domains (black star) are seen to have partially dissolved at their edges, giving rise to the small domains. The process continues until the whole field is covered with them. The difference in height between the large and the small domains was found to be ~0.3 ± 0.2 nm. Defects in the bilayer, some of which are indicated with white arrowheads in (a), can be used as markers to follow the changes occurring on the surface. The 1 × 1 μm (*Z*-scale: 5 nm) inset in (d) shows an enlarged view of the small domains, some of which are seen to contain a central hole.

changes[20] (observed on multilayered systems). Finally, the thickness of the DOPS bilayer may increase due to the decrease in the SA per molecule.[24] Indeed, FTIR studies of PS-containing phospholipids in multilamellar vesicle suspensions indicate that the *sn*-2 chain is pushed toward the lipid−water interface upon Ca^{2+} binding, enough for hydrogen bonds involving the ester groups to be formed.[20] Any one of the effects of Ca^{2+} discussed above can, in principle, be responsible for changing the balance between the intraphase and the interphase repulsive interactions, in the fashion described below.

A decrease in the SA per molecule of DOPS will result in a decrease in the lateral pressure of the fluid phase. Charge neutralization will decrease the magnitude of the headgroup−headgroup repulsion in the fluid phase.[25] An increase in the thickness of the DOPS component upon Ca^{2+} binding will reduce the mismatch between the thickness of DPPC and of DOPS. Further studies are required to elucidate which of the above mechanisms is responsible for the observed effect. As it is evident from Figure 2, the change in the domain morphology occurs over some time. It would therefore be interesting to study the variation of the surface pressure in mixed monolayers of DPPC/DOPS at the air−water interface as a function of Ca^{2+}. Such measurements can, in principle, answer the question of whether the change in lateral pressure is, in fact, responsible for the observed change in the domain morphology.

The effect of Ca^{2+} is abolished by the addition of DOPC to DPPC/DOPS mixture. Although we cannot provide a clear explanation of this phenomenon, the differences between the fluid phase composed of only DOPS vs that composed of the DOPC/DOPS mixture are reflected in the type of organization of annexin V on them. While annexin V crystallizes on the ternary mixture in the "*p*6" crystal form (Figure 1e), indistinguishable from that found on the binary mixture of DOPC and DOPS (3:1 PC to PS ratio), it does not do so on binary mixtures of DPPC and DOPS (Figure 1c,d) at a similar ratio of fluid-to-solid phase lipids.

Conclusions

The effect of Ca^{2+} on the organization of DPPC in binary mixtures with DOPS or DOPC and in ternary mixtures with both diunsaturated phospholipids was investigated by contact mode AFM. In all systems, DPPC was found to be organized in segregated domains, identifiable on the topography images due to the height difference relative to the fluid-phase phospholipids. The morphology of these domains was found to depend on the presence of Ca^{2+} in the subphase in the DPPC/DOPS system, but not in DPPC/DOPC or DPPC/DOPC/DOPS mixtures. Few large, well-defined DPPC domains were found in the two latter systems and in the former one in the absence of Ca^{2+}, while many small, isolated DPPC domains were found in the presence of Ca^{2+} in the former system.

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(25) There is evidence that Ca^{2+} can bridge two adjacent PS molecules,[26] each of which normally carries a dipole and a negative charge. Thus instead of a strong (charge + dipole)−(charge + dipole) repulsion, one obtains a weak quadrupole−quadrupole interaction. (The interaction between the latter is weaker than the dipole−dipole interaction present in PC.)

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