Investigation of Polymer-Cushioned Phospholipid Bilayers in the Solid Phase by Atomic Force Microscopy

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The preparation and the structure of polymer-cushioned phospholipid bilayers (PCPBs) in the solid phase through vesicle fusion was studied by atomic force microscopy. It was found that the anionic phospholipid α-L-dipalmitoylphosphatidic acid could form a good PCPB on the polyethylenimine surface but not on that of poly(dimethyldiallyammonium chloride), indicating that the attractive electrostatic force alone cannot guarantee the fusion of the vesicles. The zwitterionic phospholipid α-L-dipalmitoylphosphatidylcholine could not form a good PCPB on either polymer surface. It was found that the PCPB rearranged to an alternate monolayer and trilayer structure while it was exposed to air and turned back to a bilayer structure when it was dipped into water again. Various factors affecting the PCPB formation were discussed. The preparation of such bilayers through the LB transfer method was also investigated.

Introduction

One of the goals of the studies of polymer-cushioned phospholipid bilayers (PCPBs) is to model biomembranes.[1] People often choose lipids in the fluid state to prepare this kind of bilayer.[2−6] As a surface specific technique with a high spatial resolution, atomic force microscopy (AFM) can provide topological information, so it can be a useful technique to study the structure of PCPBs.[7] However, it is not easy to image the fluid-state phospholipid bilayer by AFM.[8,9] On the other hand, the PCPB in the solid phase is more stable and also has great potential in applications such as biosensors. It is expected that AFM study on this kind of PCPB should be feasible and of significance.

There are several preparation methods for supported phospholipid bilayers. Covalently attaching the polymer chains to the phospholipid headgroups can prepare a stable enough bilayer,[10−12] although it is not a generally applicable method. Successive Langmuir−Blodgett (LB) monolayer transfer on the surface of a polymer film is an alternative way to prepare such structures.[13,14] The advantage of LB transfer is the ease of controlling the components and the state of each layer.[1] However, it is not easy to incorporate the transmembrane protein to further construct a biosensor.[15] Sometimes, the LB transfer method cannot result in the expected structure.[4] Vesicle fusion is another method for the formation of the supported bilayers,[3,4,16−18] and it is the most convenient preparation method.

To further probe the results of PCPB preparation and to characterize the PCPB structure with the AFM technique, we studied different PCPBs from the vesicle fusion of the anionic phospholipid α-L-dipalmitoylphosphatidic acid (DPPA) and zwitterionic phospholipid α-L-dipalmitoylphosphatidylcholine (DPPC) adsorbed onto the polycation polyethylenimine (PEI) or poly(dimethyldiallylammonium chloride) (PDMDAAC) surface. The T_m values of DPPC and DPPA are 42 and 66 °C, respectively.[19] At room temperature, the PCPBs made of them should be in the solid phase. Because the properties of the solid-state phospholipids differ from those of the fluid phospholipids, some of our experiment results are different from the findings in similar systems in the literature.[3,4]

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Experimental Section

DPPA was purchased from Sigma (St. Louis, MO) as a monosodium salt. DPPC was purchased from ACROS (Geel, Belgium). PEI (Mw = 50 000) was purchased from Sigma as a 50% w/w water solution. They were used without further purification. PDMDAAC (Mw = 69 000) was synthesized following the procedure described in the literature.[20] Other reagents were analytical grade and used without further purification. Deionized water was used except in the LB film deposition where the deionized water was further treated by an EASY pure RF system (Barnster Co.).

Solutions of 2.0 mg mL^{-1} PDMDAAC in 10 mmol L^{-1} NaCl and 100 ppm PEI in water were used in the adsorption of the polymer on the mica surface. The vesicle solutions of DPPC and DPPA were prepared by sonication in deionized water for 30 min of the corresponding pure compound, which was obtained from the removal of the organic solvent ($CHCl_3$ or $CHCl_3/CH_3OH$) from their solutions. The size of the resulting vesicles was found to be 40−50 nm by dynamic light scattering. All the adsorption solutions were neutral except for the PEI solution whose pH was about 10. The substrates for the adsorption were freshly cleaved mica or polymer-modified mica. If not specified, the adsorption time was 30 min for PEI, DPPA, and DPPC and 1 h for PDMDAAC, and the adsorption was carried out at room temperature, which is lower than the phase transition temperature of both phospholipids.[19] No salt was used in the vesicle adsorption procedure unless specified. After each adsorption, the samples were washed by deionized water six times and then dried in the air for further studies. For AFM imaging under the solution, they were kept under the water.

The deposition of LB films was performed on a British NIMA LB 622 trough with the subphase of pure water (pH = 5.6). The subphase temperature was controlled by a HAAKE system (Mell-Technik GmbH a Co., Germany) at 20 ± 1 °C. A 1.0 mg mL^{-1} chloroform solution of DPPC and a 3:1 $CHCl_3/CH_3OH$ (v/v) solution of DPPA were spread on the subphase by drops. After the evaporation of the organic solvent, the surface was compressed at a speed of 4.3 $Å^2$ $molecule^{-1}$ min^{-1}. The monolayer was vertically transferred onto the polymer-modified mica at a constant surface pressure of 30 mN m^{-1}. The second monolayer was transferred with the same condition. The transfer ratio was 1.0 for all the monolayer transfer except the transfer of the second monolayer of DPPC, in which the transfer ratio was low.

AFM measurements were performed at room temperature on a Nanoscope IIIa (Digital Instruments, Santa Barbara, CA) equipped with a bioscope G scanner (90 μm). The scanner was calibrated by a 10 μm standard grid with 180 nm deep etch pits. The surface at the air−solid interface was imaged with tapping mode, using silicon cantilevers with resonance frequencies of 260−340 kHz. When the thickness of the membrane could not be measured from the natural defects, contact mode imaging was applied to create the defects by the AFM tip by scanning a 1 × 1 μm^2 region with a high load (∼100 nN) and a high scan speed (∼122 Hz). The measurements in the aqueous solution were carried out with contact mode in a homemade fluid cell using Si_3N_4 cantilevers. Most imaging under the water was carried out in a ∼40 mmol L^{-1} $MgCl_2$ solution to reduce the electrostatic repulsion between the tip and the surface.[21]

All of the static water contact angles were measured with the JJC-II goniometer (Changchun No.5 Optical Instruments, China). For each sample, the contact angle was acquired by the average of over three measurements.

Results and Analysis

Vesicle Fusion to Prepare PCPB. To study the membrane structure by AFM, the substrate should be a flat surface. Therefore, it is important to know the morphology of the surface after the modification of the mica by the polycations. Figure 1a shows a typical AFM image of the PEI film adsorbed on the mica surface. It can be seen from the image that the polymer covered all the mica surface and formed a flat film. Large aggregates are hardly seen. Figure 1b shows the image acquired with contact mode, after the removal of the film in the central

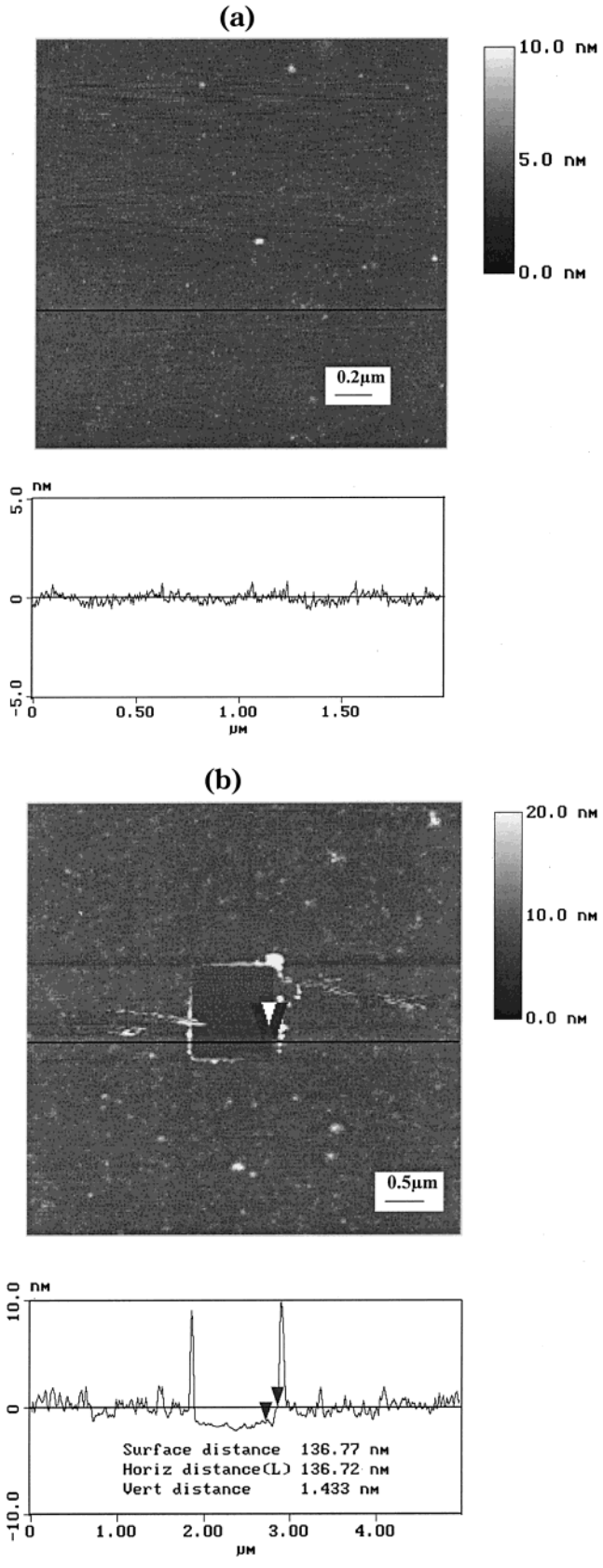

Figure 1. Surface morphology of dry PEI layer adsorbed on the mica: (a) tapping mode image and (b) contact mode image. The square defect was created by AFM tip scanning with a high load and a high scan speed.

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region by the AFM tip. The measured thickness of the film is 1.4 ± 0.4 nm (average of 40 measurements). The PDMDAAC surface is also found to be flat and complete. The measured thickness of the PDMDAAC film is 0.9 ± 0.3 nm (average of 37 measurements).

The surface properties undergo great change after the modification of the freshly cleaved mica by polymers. Zwitterionic phospholipids can easily form bilayers on highly hydrophilic surfaces such as glass,[22] quartz,[23] and freshly cleaved mica through vesicle adsorption.[17,18,24] We prepared the supported DPPC bilayer on mica shown in Figure 2a by adsorbing 0.1 mg mL^{-1} DPPC vesicle solution in the presence of 10 mmol L^{-1} $MgCl_2$ for about 10 h. However, the DPPC bilayer cannot be formed through the adsorption of the vesicle on the PDMDAAC-coated surface at different conditions (carried out in deionized water, 20 mM NaCl, or 10 mM $MgCl_2$). A typical result is shown in Figure 2b. No bilayer can be seen in this AFM image, and the adsorbed vesicles remained intact on the polymer surface.

We found it difficult to perform AFM imaging of PCPBs under the solution. The tip was easily contaminated, resulting in the lower stability during the imaging process. Sometimes, the contamination of the AFM tip could cause serious artifacts. To find an appropriate system, we first studied the air−solid surfaces after the removal of water. Although the membrane would have some structural rearrangement, the results from the air−solid surface could give us some hints about the structure in the solution.[25]

The surface contact angle measurement can serve as a quick and easy indication of the amount of the adsorbed phospholipid because the phospholipid tends to arrange in the way that the nonpolar tails point outward to the air and form a hydrophobic surface. The more phospholipid adsorbed, the more hydrophobic the surface may be. Table 1 is the contact angles of various surfaces after the adsorption of polymers and subsequent phospholipids. The most noticeable phenomenon was, after the mica adsorbed PEI and DPPA, that it became very hydrophobic. Its contact angle amounted to 94°. This indicated that the surface was composed of well-packed alkyl chains. For the surfaces formed through the adsorption of other phospholipid−polymer combinations, the contact angles indicated that the orderly packing of the phospholipid was not likely.

Figure 3a shows the AFM image of the DPPA membrane on the PEI surface at the air−solid interface. An ordered membrane structure is evident. The thickness of the islands is 5.9 ± 0.4 nm (average of 60 measurements), which is coincident with the thickness of a phospholipid bilayer.[17,24] The membrane structures in different sites were similar except for the difference in the surface coverage. The average coverage of the islands is about 60%. Because this surface is very hydrophobic, it is not possible that the membrane with bilayer thickness had the structure like the bilayer under the water, in which the surface groups are the polar headgroups. We removed the membrane along with the underlying PEI layer by creating a square defect similar to that in Figure 1b. It was found that the islands were sitting on a film with a thickness of 3.6 ± 0.3 nm (average of 24 measurements). Considering the thickness of the PEI layer (1.4 nm), there

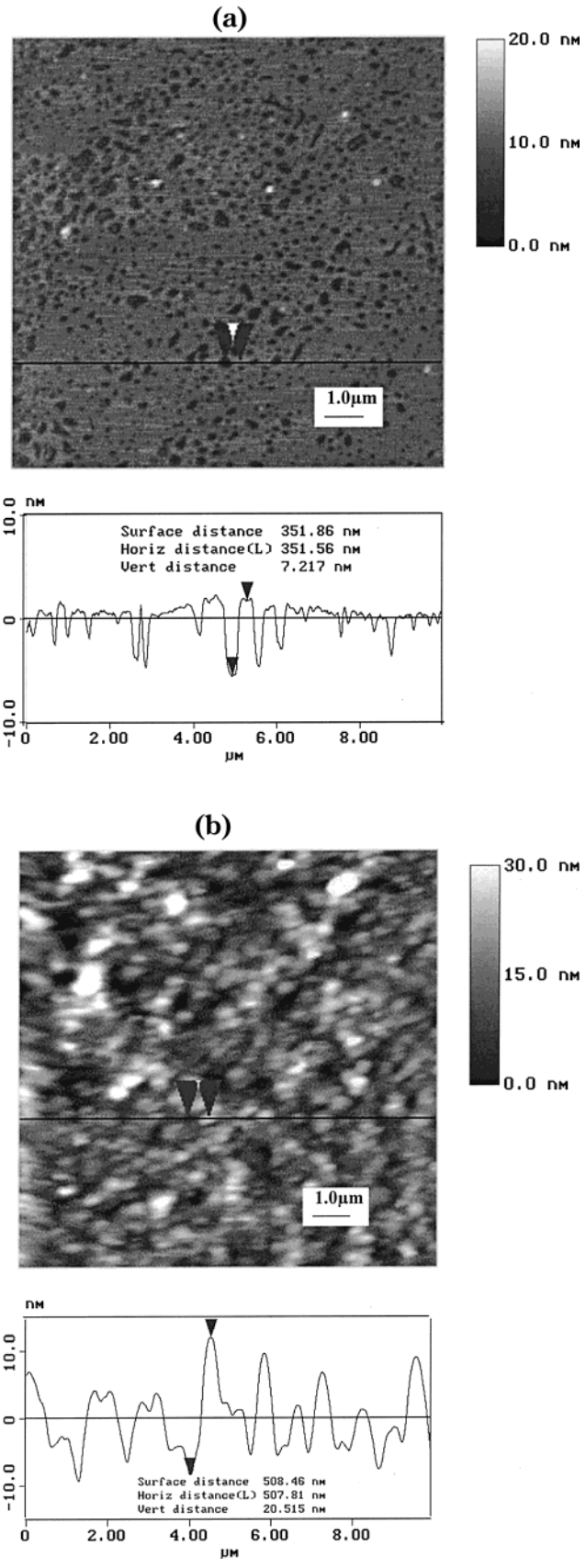

Figure 2. AFM images of the surface prepared by DPPC vesicle adsorption on mica and polymer: (a) the DPPC bilayer supported on a mica surface by the adsorption of DPPC vesicles in a 10 mmol L^{-1} $MgCl_2$ solution and (b) the complex structure formed after the DPPC vesicle adsorption on PDMDAAC in a 10 mmol L^{-1} $MgCl_2$ solution under low temperature for 15 h.

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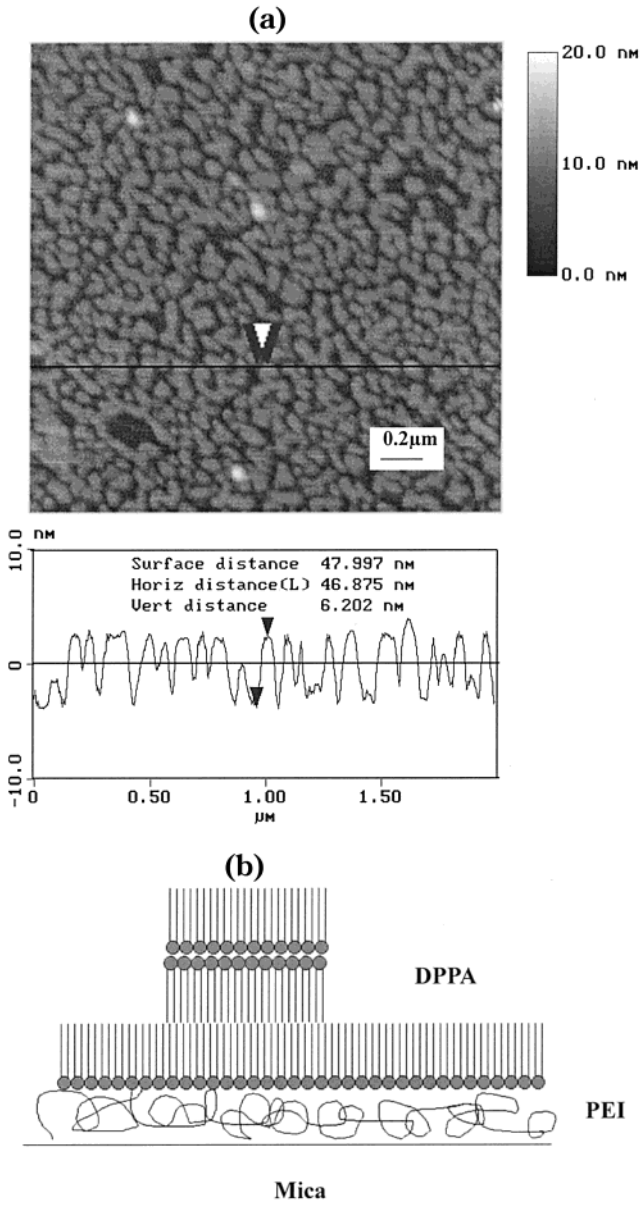

Figure 3. (a) The surface formed by DPPA adsorption on the PEI surface at the air−solid interface. (b) Schematic structure of the DPPA/PEI membrane at the air−solid interface.

Table 1. Contact Angles of the Surfaces Formed by the Adsorption of Different Substances

surface	contact angle (deg)
mica	3
mica + DPPC	39
mica + DPPA	26
mica + PDMDAAC	44
mica + PEI	76
mica + PDMDAAC + DPPC	14
mica + PDMDAAC + DPPA	47
mica + PEI + DPPC	8
mica + PEI + DPPA	**94**

is about 2 nm remaining, corresponding to a phospholipid monolayer. On the basis of these observations, we propose that the surface has an alternate monolayer and trilayer structure, as shown in Figure 3b. This structure is rearranged from the DPPA bilayer when it is moved from the aqueous solution to the atmospheric environment.[26]

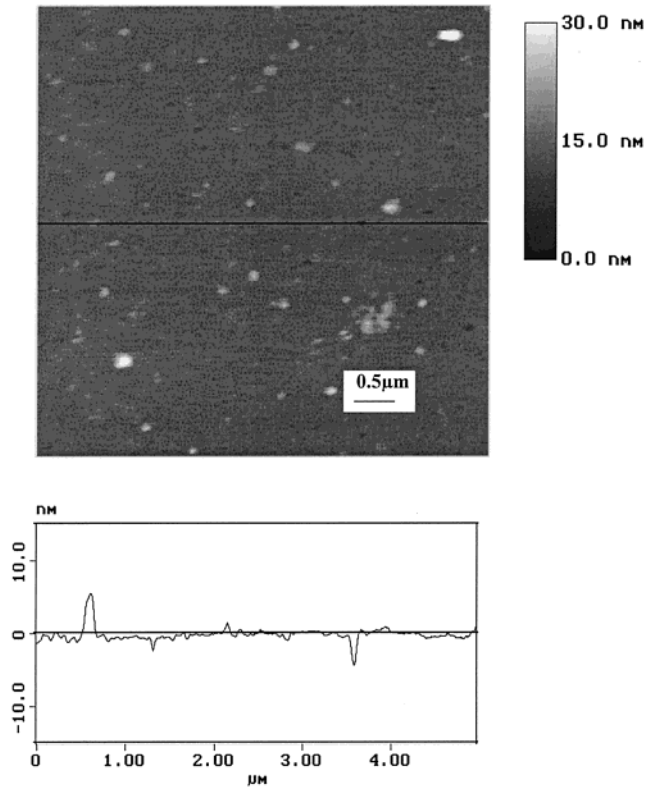

Figure 4. AFM image of the PEI-cushioned DPPA bilayer in 40 mmol L^{-1} $MgCl_2$.

The monolayer was not seen in the image because it had covered all the PEI surfaces.

The surfaces of other phospholipid−polymer combinations on mica were also imaged at the air−solid interface. According to the AFM investigation, they were not uniform surfaces. The surface morphology varied at different places in the sample. No ordered structure could be found. The amount of the adsorbed phospholipid in these samples was actually very small, as indicated from the creation of possible defects by the AFM tip.

From the contact angle and AFM studies of the adsorption of different phospholipids and polymers at the air−solid interface, we could conclude that only the adsorption of DPPA−PEI is likely to form a PCPB under the water. Because the membrane structure under the water can be different from that in the air,[27,28] we turned back to perform the observation under the water.

The PEI surface under the water was found to be flat by AFM imaging. Because of the water swelling, the highly hydrated polycation layer can be several times thicker than that in the quasi-dry state.[4−6] We tried to measure the thickness of the hydrated polymer layer by the AFM tip scratching. However, no defect could be created. The failure to create defects from the PEI surface may come from the pliable and fluid nature of the hydrated PEI molecules. Except for some pinholes and vesicles or additional bilayer adhered, the adsorbed DPPA molecules were seen assembling to a good bilayer, as shown in Figure 4. The surface coverage of the bilayer was over 98%. For the phospholipid bilayers in the fluid state, defects cannot be created by the AFM tip.[8,9] Because the DPPA bilayer is in the gel state at room temperature, there exist natural defects, and it is possible to create defects in the DPPA

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bilayer. Measured from the edge of the natural or tip-created defects, the bilayer thickness was about 6 nm. The cushioned bilayer can also be formed through the hydration of the dried DPPA monolayer and trilayer on the PEI (discussed above) but with less uniformity. Much care had to be taken in order to image the DPPA bilayer of PCPB under the solution. Sometimes, the adsorbed DPPA bilayer was easy to remove by the AFM tip, when the normal imaging force (about 1 nN) was applied.

The adsorption of DPPA vesicles on the mica surface was also studied, and no bilayer could be found under the aqueous solution. This is consistent with the reports in the literature.[3,8] Because the surface electric charge was positive after the mica was modified by PEI, the force to maintain the bilayer on the surface was mainly the electrostatic interaction.

Preparation of PCPB by LB Transfer. We also examined the LB transfer method to produce PCPBs. First, the transferred monolayer on the polymer was investigated. The DPPA LB monolayer deposited on the PEI surface was found to be a defect-free surface by AFM. The contact angle of this surface was 93°. In the LB monolayer of DPPC deposited on PEI or PDMDAAC surfaces, a lot of defects were found by AFM imaging. The contact angles of both LB films were very small (less than 20°), indicating that the monolayer was not stable subject to water. The monolayer would be washed off or underwent reorganization to a bilayer structure.[12,27,28] The above result showed that the DPPA monolayer on the polycations was more stable than that of DPPC.[12]

DPPA bilayers could be formed on both the PEI and PDMDAAC layers through the successive LB monolayer transfer. Figure 5a is the AFM image of the DPPA LB bilayer on PEI in solution. The DPPA LB bilayers on both polymers were fully covered. Figure 5b shows the image of the DPPA LB monolayer on the PEI surface under the aqueous solution. Obviously, the monolayer had self-organized into a bilayer.[27,28] The DPPA monolayer was not stable in the water even though a strong electrostatic attraction existed between the anionic phospholipid and polycation layer. Under the aqueous solution, the membranes produced by successive LB transfer of DPPC monolayers on the PEI and PDMDAAC were also imaged. No bilayer structure could be found.

DPPA/PDMDAAC System. Like PEI, PDMDAAC is also a polycation. There is also electrostatic attraction between the anionic phospholipid and the PDMDAAC layer. One would expect that a good bilayer should be formed by the adsorption of DPPA vesicles on the PDMDAAC surface, but both the contact angle measurement and the AFM investigation at the air−solid interface showed that a DPPA bilayer could not be formed through vesicle adsorption on PDMDAAC.

We found that the addition of NaCl in the solution could improve the adsorption of the DPPA vesicles. The contact angles of the surfaces, formed by the adsorption of 200 μL of 0.1 mg mL^{-1} DPPA along with 100 μL of 2, 20, and 200 mmol L^{-1} NaCl solution, were 61°, 74°, and 71°, respectively (the adsorption without NaCl resulted in a contact angle of 47°). From the AFM imaging of these surfaces at the air−solid interface, the amount of the adsorbed phospholipid also increased with the addition of the NaCl concentration.

Parts a and b of Figure 6 are the AFM images of the surfaces obtained by DPPA vesicles adsorbed on PDMDAAC with and without the presence of NaCl, respectively. In Figure 6a, only adhered DPPA vesicles were seen, and no bilayer could be found. When NaCl solution was used during the vesicle adsorption, in addition to seeing more adsorbed vesicles, small patches of DPPA bilayer could also be found, indicating that some of the vesicles were fused by the surface. The electrolyte here might play a

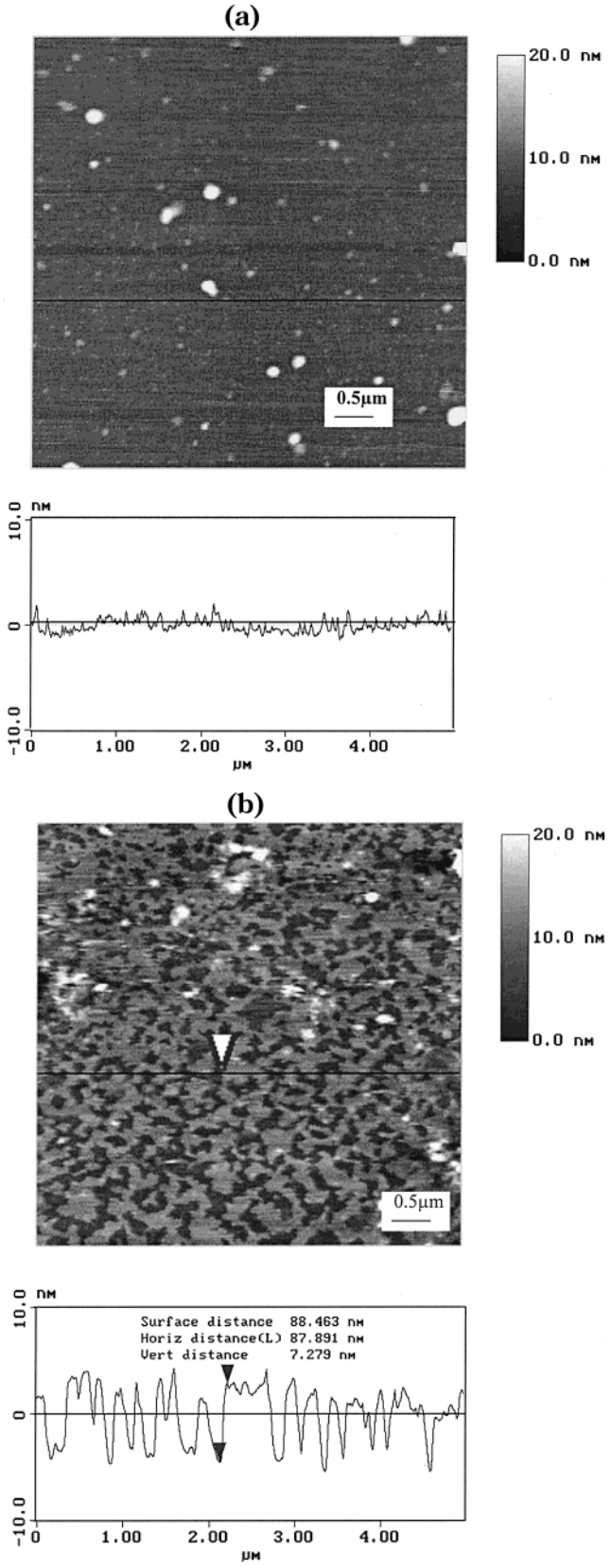

Figure 5. AFM images of polymer-cushioned DDPA LB bilayers under the aqueous solution (15 mmol L^{-1} phosphate buffer, pH = 7.4). (a) DPPA LB bilayer deposited on a PEI surface. (b) Partially covered cushioned bilayer formed after the hydration of the DPPA LB monolayer on the PEI.

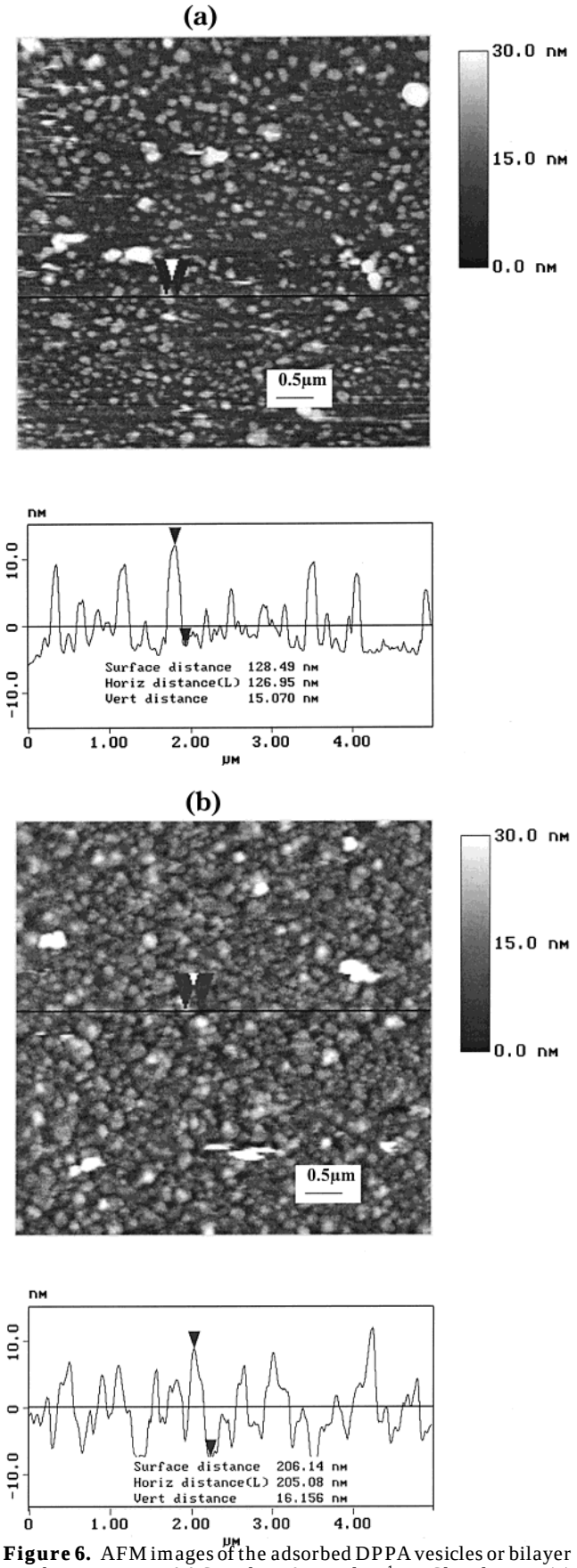

Figure 6. AFM images of the adsorbed DPPA vesicles or bilayer patches on PDMDAAC under 40 mmol L^{-1} $MgCl_2$ solution. (a) DPPA vesicle adsorption without NaCl; the adsorbed DPPA existed as vesicles. (b) Vesicle adsorption in the presence of NaCl with a final concentration of 70 mmol L^{-1} NaCl. More vesicles were adsorbed, and some were fused into bilayer patches.

role of reducing the electrostatic repulsion between the polycations, so that the structure of the polymer in the interface of water and polymer could change to ease the vesicle fusion process.[4]

Discussion

We found that both polymers we used formed flat films after the adsorption on the mica surface. The thickness of the polymer layer (~1 nm) at the air−mica interface was far less than that observed by neutron reflectivity (~5 nm).[4,6] The compression of the polymer layer by the AFM tip should not create such a large difference. The swelling of the polymer under the water may account for this difference. According to the neutron refractivity studies, dimyristoylphosphatidylcholine (DMPC) vesicles can fuse on a dry PEI surface and form a good bilayer.[4] All our experiment results showed that a DPPC bilayer could not be formed by vesicle adsorption on dry PEI. The different states of DPPC and DMPC at room temperature may account for the difference. When the surface changed from mica to PEI, the surface became less hydrophilic. Hence, the electrostatic interaction between the surface and the zwitterionic headgroups is greatly reduced.[4,6] This surface−vesicle interaction can induce the rupture[29,30] of DMPC vesicles. The osmotic stress on the vesicles is also important for the rupture of vesicles, as demonstrated by the recent study on the tethered bilayers,[7] but both surface−vesicle electrostatic interaction and osmotic stress are still not strong enough to cause the rupture of DPPC vesicles. However, in the DPPA/PEI system, the strong electrostatic attraction[3] between the surface and DPPA makes the fusion of the vesicles occur.

We proposed that the DPPA bilayer on PEI would reorganize into an alternate monolayer and trilayer structure after the surface is exposed to air. The X-ray scattering study in the literature[3] indicated that only a monolayer remained after the removal of the PEI-cushioned dioleolylphosphatidic acid (DOPA) bilayer from water. Again, different states of DPPA and DOPA can explain this distinction. Stronger hydrophobic interaction in solid-state DPPA helped to keep the phospholipid on the surface, resulting in monolayer−trilayer structure. In contrast, the interaction between the tails in the fluid DOPA bilayer cannot preserve the upper lipid layer, so only the underlayer was maintained. For the same reason, the LB monolayer of DPPA on the PEI surface reconstructs to a partially covered PEI-cushioned bilayer, whereas the PEI-cushioned DMPC monolayer keeps its structure under the water.[4]

At first sight, the adsorption of DPPA vesicles on the PDMDAAC surface should have a result similar to that in the DPPA/PEI system because in both cases strong electrostatic attraction exists. This reasoning was proved to be incorrect by our experiments. Through the AFM imaging of the LB bilayer, it can be found that the PDMDAAC-cushioned DPPA bilayer was stable once being formed. The failure to prepare this bilayer by vesicle adsorption might come from a dynamic factor. The neutron scattering studies showed that DMPC vesicles could form a bilayer on a dry PEI surface but formed complex surface aggregates on a hydrated PEI surface.[4,6] The PDMDAAC molecule has a rigid chain bearing a high positive charge density.[31] At the polymer−water interface, the molecules tend to take a stretch conformation because of the

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electrostatic repulsion of the polymer chains. Many polymer chains will extend into the solution[32] when the PDMDAAC surface is exposed to water. The extended polymer chains can prevent the DPPA vesicles from fusion, similar to the steric protection of the lipopolymer vesicle.[33,34] For the PEI surface, on the other hand, there are not so many chains stretching into the solution because the amino groups in PEI are not fully protonated and there is strong hydrogen bonding between the PEI chains. Thus, the steric protection for DPPA vesicles is not important in the DPPA/PEI system. Another factor could be the difference in charge density of PEI and PDMDAAC.[35] After the addition of the electrolyte (NaCl), the electrostatic shielding reduces the repulsion between the polymer chains, so the number of the chains protruding into the solution is also reduced. The fusion of DPPA vesicles would be easier.

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Conclusion

We have used the AFM technique to determine the structure of PCPBs in the solid phase under water as well as in the atmospheric environment. From the AFM and contact angle studies, the PEI-cushioned DPPA bilayer is formed through vesicle fusion and LB transfer driven by strong electrostatic attraction. Such a bilayer undergoes a reconstruction to form an alternate monolayer and trilayer structure upon the removal of water. Because the interaction between polycations and the headgroups of the zwitterionic DPPC is weak, the PCPB does not form through either vesicle fusion or LB transfer methods. From the experiment on DPPA/PDMDAAC, it is found that the electrostatic attraction alone cannot guarantee the fusion of the vesicles.

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