An atomic force microscope study of thermal behavior of phospholipid monolayers on mica

M. F. Luo[a] and Y. L. Yeh
Department of Physics and Nano-catalyst Centre, National Central University, Jungli 32054, Taiwan

P. L. Chen, C.-H. Nien, and Y. W. Hsueh[b]
Department of Physics, National Central University, Jungli 32054, Taiwan

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We observed by using atomic force microscope (AFM) phospholipid (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) monolayers on mica being annealed and cooled to a selection of temperatures through steps of 2–4 °C/min. The annealed phospholipid monolayers started to disappear at 45–50 °C and disappeared completely above 60–63 °C under AFM observation. The phospholipid monolayers reformed when the samples were cooled below 60 °C and developed from fractal into compact monolayer films with decreasing temperatures. Simultaneously the height of the reformed phospholipid films also increased with decreasing temperatures from 0.4 nm to the value before annealing. The observed thermal features are attributed to a phase-transition process that upon heating to above 45–50 °C, the lipids condensed in the monolayers transform into a low-density expanded phase in which the lipids are invisible to AFM, and the transformation continues and completes at 60–63 °C. The lipid densities of the expanded phase inferred from the dissociated area of the condensed phase are observed to be a function of the temperature. The behavior contrasts with a conventional first-order phase transition commonly seen in the Langmuir films. The temperature-dependent height and shape of the reformed phospholipid films during cooling are argued to arise from the adjustment of the packing and molecular tilting (with respect to the mica surface) of the phospholipids in order to accommodate more condensed phospholipids. © *2006 American Institute of Physics*. [DOI: 10.1063/1.2194539]

I. INTRODUCTION

There have been numerous studies on overall chemical and physical properties and composition of cellular membrane systems. One of the main reasons is that the membrane systems hold the channels for cells to communicate materials with outside world, and knowledge of the mass transport is valuable for obvious medical applications.[1,2] These researches observed the major component of the membranes, lipid molecules, possess remarkable self-assembly properties, that draw tremendous attention from researchers in fields of soft-matter and interface sciences.[3–18] The self-assembly properties allow the lipid molecules to form highly uniform thin films, which can be extensively applied in lithography, surface modification, nanomaterial supports, biomedical materials, as well as cell membrane model studies. For instance, recently electrostatic nanolithography using atomic force microscope (AFM) which combines localized softening by Joule heating and extremely large electric field gradient to manipulate the thin films is developed.[19] However, these applications rely heavily on the physical properties of the thin films to be patterned, particularly, the thermal properties.[16–18] The current work is thus aimed to further our understanding of the thermal properties of the self-assembled lipid thin films, specifically, beginning with the simplest lipid monolayers.

Using AFM we monitored changes in morphology of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) monolayers on mica annealed from room temperature to a selection of temperatures through steps of 2–4 °C/min and cooled down in the same manner. DPPC is a well-studied phosphatidylcholine with two 16-carbon saturated acyl chains. Supported monolayers were prepared by deposition of DPPC vesicles onto mica.[3,11] The annealed DPPC monolayers were found starting to be invisible under AFM from 45 to 50 °C. The size of the invisible region increased with the temperature and the monolayers disappeared completely under AFM observation above 60–63 °C. The phospholipid islands reformed little by little when the sample was cooled below 60 °C and the reformation went on until nearly room temperature. With decreasing temperature the shape of the reformed phospholipid films developed from fractal into compact ones. Simultaneously, the height of the reformed phospholipid films also varied from 0.4 nm to the value before annealing. These observations are rationalized by a phase-transition process that on heating to above 45–50 °C, the lipids in the condensed phase start to evaporate into a low-density expanded phase in which the lipids are mobile and invisible to AFM, and the evaporation continues and completes at 60–63 °C. The behavior contrasts with a conventional first-order liquid condensed (LC)–liquid expanded (LE) phase transition observed in, e.g., the Langmuir films.[7–9] Based on the area measurements of confined expanded phase regions, it appears that the lipid density of the

[a]Electronic mail: mfl28@phy.ncu.edu.tw
[b]Electronic mail: yhsueh@phy.ncu.edu.tw

expanded phase is a function of temperature, with its value increasing from a small value at 45 °C to about one-half of the condensed phase density at 60 °C.

The mobile lipids recondense into the visible condensed phase when the temperature is lowered down. The seemingly temperature-dependent height and shape of the reformed lipid films may result from packing of the lipids. The packing in the reformed films adjust gradually with decreasing temperature to accommodate more condensed phospholipids, which changes the island's shapes and molecular tilting with respect to the surface.

II. EXPERIMENT

A. Atomic force microscope

In this investigation a NT-MDT Solver P47 AFM was employed to image morphology of the phospholipid films. Standard silicon tips with typical force constants of 5.5 and 11.5 N/m were used and the scan line speed was optimized between 1 and 3 Hz with a pixel number of 256×256. All images were recorded in a tapping mode in air with humidity of 50–60 relative humidity (RH).

B. Materials and methods

DPPC from Avanti Polar Lipids (Alabaster, AL) and deionized water were used in all preparation of vesicles. Mica was purchased from Agar Scientific (Essex, UK). Lipids were hydrated using Hepes buffer (50 mM HEPES, 150 mM NaCl, 4 mM EDTA, *p*H=7.4) to a concentration of 1 mg/1000 μl. The mixture was freeze-thaw-vortexed five times between liquid nitrogen temperature and 50 °C, then extruded through one polycarbonate membrane filter (Avanti Polar Lipids) with nominal pore diameters of 100 nm held in an extruder (Avanti Polar Lipids). Extrusions were performed for 50 cycles at 50 °C. After extrusions, the vesicle suspension was placed in a 500 ml glass of 50 °C water overnight to achieve equilibrium. The supported lipid thin films were obtained by depositing the vesicle suspension onto freshly cleaved mica and gently rinsing the sample with 1 ml of Hepes buffer 30 s later. The sample was left at room temperature overnight prior to the AFM experiments.

III. RESULTS AND DISCUSSIONS

Figure 1(a) is an example of the AFM image (3×3 μm^2) of the mica-supported phospholipid thin films at room temperature, in which we see a gap running from the upper-left corner toward lower right. The thin films are compact and uniform, with a circular hole at the upper-right corner and a small second overlayer at the lower-left corner. These features are further manifested in the inset of Fig. 1(a), showing a line profile across the films. The line profile confirms that the first layer is at a height of about 1.5 nm, somewhat shorter than the height of a single phospholipid molecule at about 2.0 nm.[3,8] We believe that the films are phospholipid monolayers with slight molecular tilting, as implied by the smaller height. The second overlayer with a total height larger than 2.5 nm should be a second phospholipid monolayer on top of the first one. The phospholipid head groups in the first monolayer are believed to face the mica substrate, since both mica and head groups are hydrophilic. How the lipid monolayer films are transformed from liposomes is not clear. Whether it is like the known transformation of lipid-bilayer films[11] would be another interesting issue. We speculate that these lipid monolayers are primarily transformed from single-layer micelles, as these films form mostly only when the liposomes are prepared with low lipid density (see Sec. II B sample preparation).

After the films were prepared at room temperature, they were annealed through steps of 2–4 °C/min. The lipid films remained stable until the temperature reached 45 °C. Figure 1(b) shows the sample surface at 50 °C. There are three significant changes in the film morphology compared to Fig. 1(a): (1) the second lipid monolayer at left nearly disappeared under AFM observation, (2) the hole in the film at right became larger, and (3) the gap in the middle also widened. The results suggest that the lipid monolayers are dissociated with increasing temperature and become invisible to AFM or desorb from the substrate. It should be noted that the morphology is stable under a fixed temperature, at least in the time scale of 1–2 h. Figure 1(c) manifests the surface morphology while the films were annealed further to 60 °C. The film at the left survived, while both the second monolayer and the film at the right disappeared under AFM observation. The remaining film sustained even when the sample was heated to 80 °C.

The series of AFM observations show the essential features of the annealed phospholipid films that with increasing temperature the condensed phase gradually disintegrates. This process continues until the films are fully dissociated. Thermal behavior of multilayer films is more complicated. The top and bottom layers may respond in different manners to temperature changes.[17] To reveal fundamental thermal properties of the lipid films, in this report we shall concentrate on monolayers.

We should answer first where the dissociated lipid monolayers at the elevated temperatures have gone. Figure 2(a) shows the AFM image from the sample that had been annealed to above 60 °C, at which the films were dissociated totally, and cooled down to room temperature for 48 h. Two huge nonflattened liposomes are outstanding and the other areas are primarily either mica (*M*) or lipid monolayers (ML), as indicated. From the observation we know that at least a major portion of the dissociated lipid films has reformed. The result allows us to rule out desorption and conclude that the dissociated films are still on the surface but cannot be detected by AFM. That is, they have not physically gone but are only invisible to AFM, probably due to a low density and/or a high mobility. However, the reformed films differ slightly from those before annealing. Inside the reformed films, there are many tiny holes, which are not observed in freshly prepared films as seen Figs. 1(a) and 1(b). Additionally, the height of the reformed films ranges from 0.8 to 2.0 nm, a distribution wider than the typical height before annealing, i.e., 1.5–2.0 nm. The inset, a line profile across a large circle defect, shows that the average thickness of the indicated films is only near 1.0 nm. These differences must arise during the reformation.

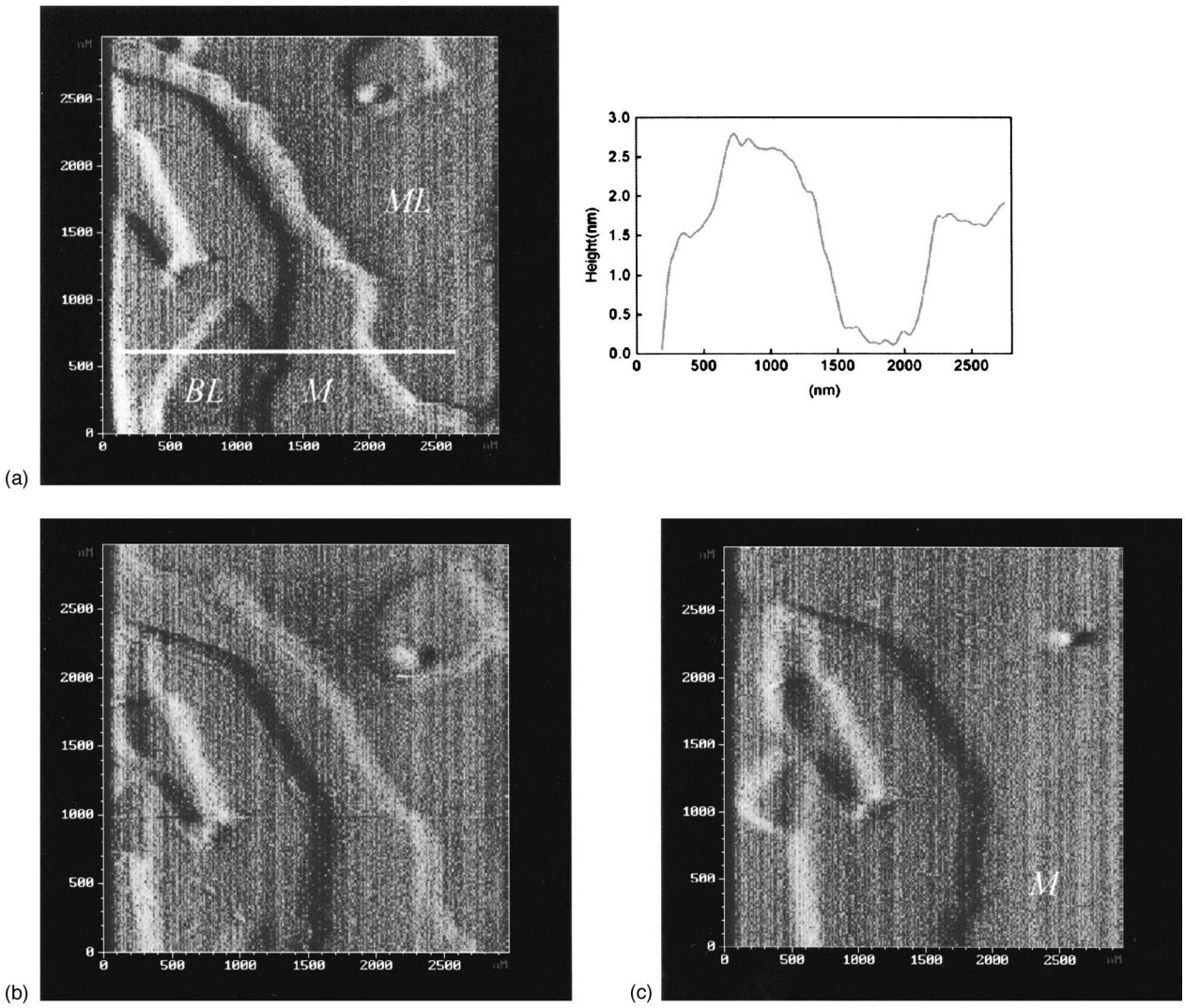

FIG. 1. AFM images (3×3 μm^2) of the mica-supported phospholipid thin films at (a) 25 °C, (b) 50 °C, and (c) 60 °C. The inset shows a line profile across the films, as indicated in (a). Different domains in the figure are indicated by different marks: *M* for mica surface, ML for monolayer, and BL for bilayer.

Figures 2(b)–2(f), recording morphology changes caused by heating and cooling, will illuminate the reformation processes. Figure 2(b) shows the surface at 50 °C. The dissociation of the lipid films already began and, similar to that of the nonannealed films, the process occurred at the film boundaries, evidenced by the enlargement of both large mica regions and small holes inside the films. The nonflattened liposomes were still robust. There were no changes in the liposomes in the temperature range under investigation. At 60 °C [Fig. 2(c)] no films were visible. There was a slight thermal drift in the observation scope of AFM, but fortunately it did not cause obvious loss of information. When the sample temperature was cooled down in Fig. 2(d) to 46 °C, we observed that the lipids reappeared and aggregated into fractal islands. Further lowering the temperature down to room temperature, more lipids came into view and large compact lipid films formed, with a number of small holes, as in Fig. 2(e). Keeping the sample at room temperature for 48 h, we noticed that the holes in the films merged into a few large ones [see Fig. 2(f)], which are already quite similar to those freshly prepared samples (see Fig. 1). Although the result has suggested that the lipids in the films are not rigid but slightly mobile at room temperature, they are still quite static in short observation time, since the images at fixed temperatures remain nearly the same for 1–2 h.

The series of observations is rationalized by a scenario that the lipids in the films upon increasing temperature to above 45 °C dissolve into a low-density expanded phase in which the lipids are invisible to AFM due to its lower density and high mobility. At 60 °C the dissociation is completed. When the temperature is lowered down, these mobile lipids aggregate into fractal monolayer films. At this stage, the packing is not optimized and the lipid molecules either lie on the substrate or tilt with large angles. Upon further lowering the temperature, more mobile lipids are incorporated into the films so the fractal films evolve into compact ones. Simultaneously, the molecular tilting also changes in order to accommodate more lipids. We shall demonstrate the tilting change later by the measured height of the reformed films. The packing is optimized tens of hours later, during which the small holes merged into the large holes, as shown Fig. 2(f), to reduce the interfacial energy. The evidences for the packing not optimized will be provided in the following paragraphs.

The height of the lipid films changes significantly during

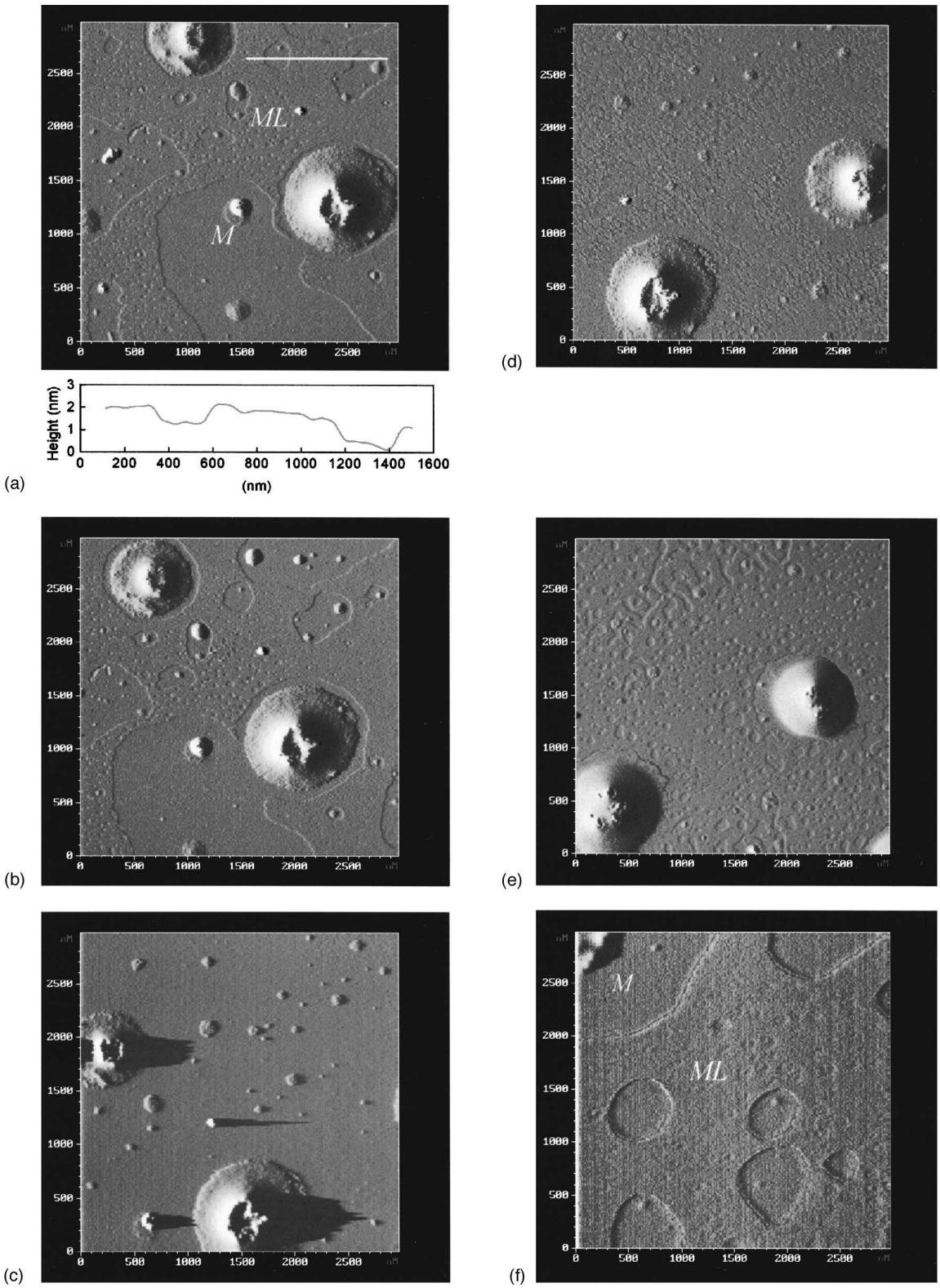

FIG. 2. AFM images (3×3 μm^2) of the lipid thin films at (a) 25 °C, (b) 50 °C, (c) 60 °C, (d) 46 °C, (e) 25 °C, and (f) 25 °C, 48 h later. The inset shows a line profile across the films, as indicated in (a). The mica surface and the monolayer film are indicated by *M* and ML, respectively. The series of AFM images recorded morphology changes after heating and cooling to illuminate the reformation processes of the lipid thin films.

lipid condensation. Figure 3 plots the height of the reformed films as a function of temperature. The error bars indicate standard deviations of the height distributions at a temperature and the dot line is drawn to guide the eyes. The height increases evidently with decreasing temperature and at room temperature reaches the height before annealing. We note that the height of the reformed films at initial stage is around 0.4 nm, resembling the size of the head group of phospholipid molecule,[3,8] which implies that the chainlike lipid molecules nearly lied down on mica at the initial stage of the

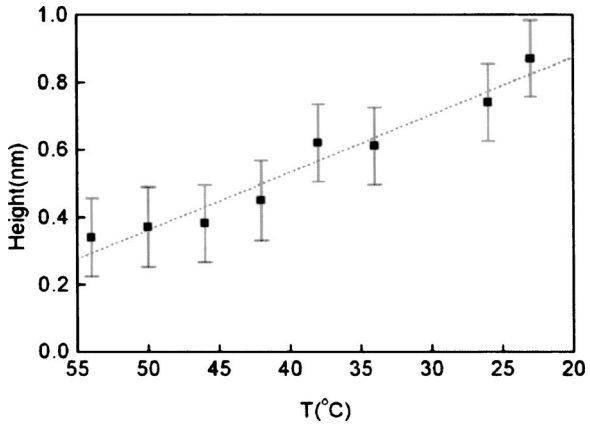

FIG. 3. Measured film heights at different temperatures during the thin film reformation. The error bars indicate standard deviation of the height distribution and the dot line is drawn to guide the eyes.

condensation. This is also a strong indication that the lipids in the mobile phase (invisible to AFM) also lie on mica. With decreasing temperature the lipids change orientation from lying to titling and eventually reach the titling angle before annealing. The molecular titling is in line with the packing and island shapes. The lipids with large titling angles would pack poorly and thus more likely form fractal islands. With decreasing temperature efficient packing with smaller tilting is achieved to accommodate more lipids, forming compact films. Since the height was measured based on the height difference between the films and "the void regions,"[20] one might concern that the invisible, mobile lipids in the void regions cause elevation[9] and therefore the measured changes of the film's height are actually variations of the density of the invisible lipids with temperature. We cannot rule out this elevation effect entirely, but upon heating the measured height of the holes' edges (relative to the void areas) remained the same from the room temperature to any temperatures at which the films are dissociated partially. Furthermore, using AFM tips with two different force constants makes negligible difference. These observations confirm that the invisible lipids do not cause apparent elevation. Accordingly, we trust that the height changes of the films are due to the changes of lipid's orientation during condensation.

The packing of the reformed films can be illustrated by observing the evolution of confined void areas. Figures 4(a)–4(f) focus on changes of the holes in the lipid films being annealed and cooled. Figure 4(a) shows the surface morphology at 45 °C, where the lipid films just began to evaporate. The holes as marked by arrows in the figures have enlarged at 56 °C in Fig. 4(b). Decreasing the temperature we found that the invisible lipids reappeared and aggregated mostly at edges of the existing films. When the temperature was down to 46 °C [Fig. 4(c)], the sizes of the indicated void areas were apparently reduced in comparison with those at 56 °C and some fractal islands already developed along the edge of the existing films. It is surprising when the sample was cooled down to below 46 °C, such as 38 °C shown in Fig. 4(d), that the fractal islands continued to grow and occupied most of the void areas. The result implies that below 46 °C there were still lipids in the invisible phase. This contrasts with the observation from freshly prepared samples being annealed up, where there were no invisible lipids below 46 °C since nearly no lipid films are dissociated. The irreversibility could result from the newly reformed films having worse packing and weaker bonding between the lipids.

Such reformed islands transit to the invisible phase at lower temperatures. The worse packing is evident through two features: first, the reformed films occupy larger surface areas than those before dissociation; second, when the films were annealed again, the newly reformed films disappeared first, as presented by Figs. 4(e) and 4(f). The initial packing is poor because of the low lipid mobility in the condensed phase. Figure 2(f) has shown that the optimized packing might take tens of hours. Once the optimization is done, the thermal features demonstrated in the series of Figs. 2 and 4 can be reproduced.

Although the expanded phase is invisible to AFM, we can still derive some of its main characteristics. First, as we have observed many lipid films evaporating into a confined geometry [see, for example, the enlargement of the hole in Figs. 1(a) and 1(b)], we can, by tracing the amount of evaporation, calculate the lipid densities inside the holes, i.e., the lipid density of the expanded phase. From different samples the sizes of the holes in the monolayers at different evaluated temperatures were measured. The fractional hole area change $\delta(T)=[A(T)-A_0]/A_0$, with $A(T)$ the area of a hole as a function of the temperature T and A_0 the area at room temperature, increases with temperature. Above 45 °C, $\delta(T)$ increases almost linearly with the temperature until 63 °C, where the lipid films are dissociated completely. If ρ is the density of the condensed phase, the lipid density inside the hole is given directly by $D(T)=\delta(T)\rho$, shown in Fig. 5. To estimate $D(T)$, we use a value of ρ at $2/\mathrm{nm}^2$ (50 $\mathrm{Å}^2$/molecule), the density of DPPC Langmuir monolayers in liquid condensed phase, because our lipid monolayers resemble such phase in aspects of height, lipid mobility, and humidity.[2,3,7–9] Different symbols in the plot denote the results obtained from different holes, and the dash line is drawn to guide the eyes. We should note that these data were collected from the annealed-up samples that were either freshly prepared or packing optimized, so they should not be compared with the data from the films whose packing is not optimized yet, such as those in Figs. 4(c)–4(f). Figure 5 shows that $\delta(T)$ is a well-defined function of the temperature. In our observations the holes were stable at least at a time scale of 1–2 h at each temperature. Therefore, we conclude that upon heating to above 45 °C, instead of going through a first-order transition, the lipid films evaporate gradually into a low-density phase. Just before the lipid films are dissociated entirely, the density of the mobile lipids reach a maximum around $1/\mathrm{nm}^2$ (100 $\mathrm{Å}^2$/molecule), nearly half of the original condensed films. Referring to the lipid density in the DPPC isotherm diagram, this density is comparable to that of the low-density expanded liquid phase of the DPPC Langmuir monolayers.[7–9] The reason is still not clear why the lipid monolayers on the mica surface go through this board dissociation process, rather than a phase transition as commonly seen in the Langmuir films at the air-water interface

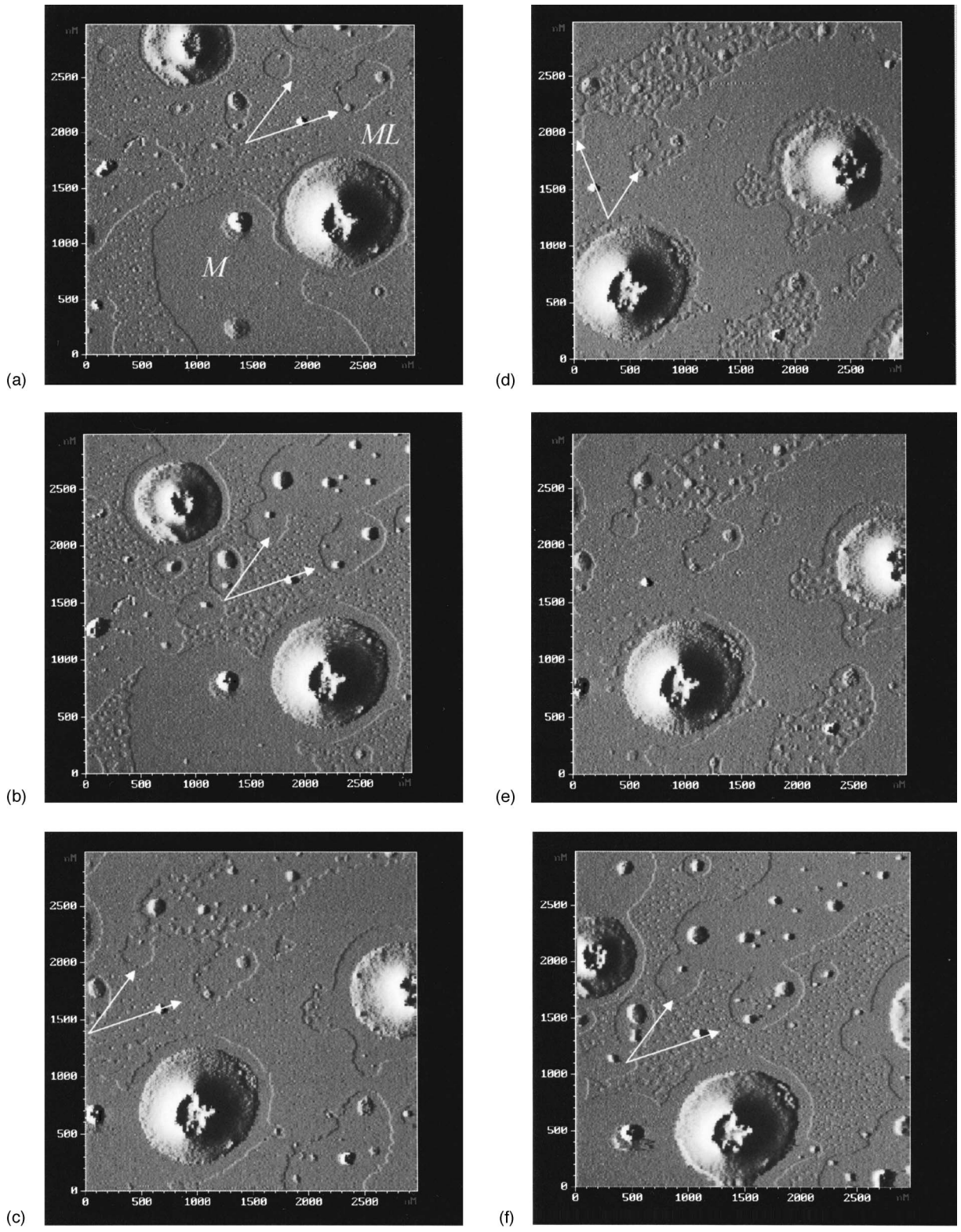

FIG. 4. AFM images (3×3 μm^2) of the lipid thin films at (a) 45 °C, (b) 56 °C, (c) 46 °C, (d) 38 °C, (e) 46 °C, and (f) 57 °C. The series of images focus on changes of the holes in the lipid films being annealed and cooled. The mica surface and the monolayer film are indicated by *M* and ML, respectively. The arrows in the figure point out the holes as discussed in the text.

or usual adsorbate-surface systems.[7–9,16–18,21] The interaction between the lipid molecules and mica substrate should play a significant role.

As discussed above we infer that the lipids in the expanded phase are nearly lying down on the mica surface, because the first reformed films from these invisible lipids have an average height consistent with such orientations. This may be the reason why the system exhibits a broad transition, as the lipid-mica interaction would be different in the condensed phase and liquid phase due to different molecular orientations. Furthermore, different molecular orientations also result in different lipid-lipid interactions. A

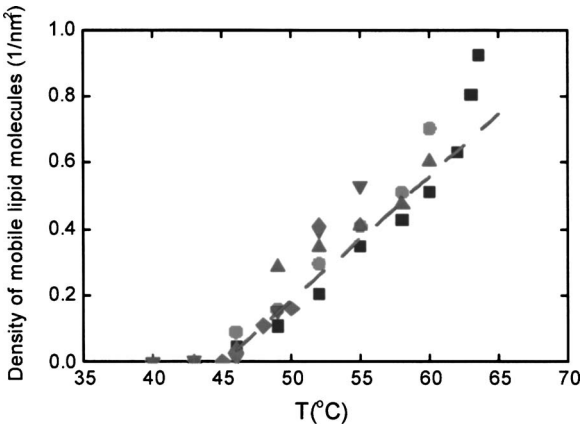

FIG. 5. Plot of the densities of mobile lipid molecules confined in the holes at different temperatures. Different marks in the plot denote the results obtained from different holes and samples, and the dash line is drawn to guide the eyes.

simple short-range attractive interaction between lipids along only leads to a regular condensed-gas phase transition, as we see in our molecular dynamics simulations. According to the simulations, we anticipate a weaker interaction and a longer favorable interdistance in the expanded phase. These characteristics are in agreement with the molecular orientations.

IV. CONCLUSION

We observed by using AFM morphologies of DPPC lipid monolayers on mica annealed from room temperature to a selection of temperatures through steps of 2–4 °C/min and cooled down in the same manner. The sizes of the annealed monolayer films were dependent on the temperature. The films started to shrink at about 45–50 °C and disappeared entirely under AFM observation above 60–63 °C. The lipid monolayers reformed little by little when the sample was cooled below 60 °C and developed from fractal into compact films with decreasing temperature until nearly room temperature. Simultaneously, the height of the reformed lipid films also varied with decreasing temperature, from 0.4 nm to the value before annealing. The observed features suggest that the lipids in the monolayers evaporated from the condensed phase into a low-density expanded phase upon heating of the sample during a range of temperature (~15 °C), rather then transforming via a first-order phase transition. The lipids in the expanded phase recondense into the visible condensed phase when temperatures are lowered down. The seemingly temperature-dependent height and shape of the reformed lipid films may result from packing of the lipids. The packing of the lipids in the newly reformed films adjusts gradually to accommodate more condensed lipids with decreasing temperatures, which changes the island's shapes and lipids' tilting with respect to the mica surface.

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