Pressure Dependence of Aggregation State of (DMPC/Cholesterol) Mixed Monolayer Based on AFM Observation

Yasunobu Tagami,[1] Takashi Matsufuji,[1] Hajime Ikigai,[2] Takayuki Narita,[1] and Yushi Oishi*[1]

[1]Department of Chemistry and Applied Chemistry, Saga University, 1 Honjo, Saga 840-8502

[2]Department of Chemistry and Biochemistry, Suzuka National College of Technology, Shiroko, Suzuka 510-0294

Received October 8, 2008; E-mail: oishiy@cc.saga-u.ac.jp

We investigated the aggregation state of a mixed monolayer of 1,2-dimyristoyl-*sn*-glycero-3-phospholine and cholesterol at different surface pressures by AFM. AFM images revealed a nanoscopically separated structure despite a macroscopic homogenization of the monolayer under compression.

The study of multicomponent lipid membranes is of current interest in developing novel devices such as bio-reactors,[1] a surface-based catalysis,[2] and membrane chips,[3] and in understanding the behavior of biological systems.[4] Mixed membranes of phospholipid and cholesterol, the main skeletal components of cell membranes, have attracted much attention because this system forms microdomains, called "lipid rafts," within which cellular signaling and trafficking is mediated.[5,6] It is not yet clear how the phospholipid–cholesterol system forms the microdomains to interact at a molecular level with individual molecules such as proteins on the cell membrane surface. Phospholipid–cholesterol mixed monolayers prepared at an air–water interface can be used as a simplified cell membrane model; the structure of these monolayers has been extensively investigated.[7–31] This method is experimentally useful for readily changing chemical and physical parameters. In particular, the molecular aggregation state in the multicomponent systems is strongly dependent on surface pressure and temperature.[7–9]

Atomic force microscopy (AFM) allows us to detect the small height differences that distinguish microdomains and coexisting phases in various multicomponent systems.[10–16] Yuan and Johnston, using an AFM, have reported the presence of submicrometer-size domains and matrix phases with different heights on the phospholipid–cholesterol monolayer transferred onto a mica plate by the Langmuir–Blodgett (LB) technique.[15,16] However, previous studies have made little mention of the effects of surface pressure on the phase diagram of the multicomponent system and on the thickness of the monolayer, which may lead to improved understanding of the phospholipid–cholesterol mixed monolayer.

In this study, we investigated, using morphological and height information from AFM, the phase behavior and aggregation state of dimyristoyl phosphatidylcholine–cholesterol (DMPC/Chol) mixed monolayers that were prepared at different surface pressures by the LB technique.

1,2-Dimyristoyl-*sn*-glycero-3-phospholine (DMPC, Avanti Polar Lipids, Inc.) and cholesterol (Chol, Sigma Chemical Co.) mixtures with molar ratios 70/30 and 80/20 were dissolved in chloroform at a concentration of 1.0×10^{-3} mol dm^{-3}.[32] The subphase temperature, T_{sp}, was adjusted to 296 ± 0.5 K. Room temperature was adjusted to the same temperature as T_{sp} using an air-conditioner. After standing for 30 min, the monolayer was compressed to surface pressures of 0, 1, 3, 6, 9, and 14 mN m^{-1}. The monolayer was transferred onto a mica plate (Okabe Mica) by the horizontal drawing-up method.[17] Topographic images of the monolayer were taken with an AFM (SPA 300, Seiko Instruments, Japan) in air at 296 K. Images were recorded at a scan rate of 2 Hz in the "constant-force" mode. The monolayer thickness was evaluated from hole depth after piercing a hole with an area of 100×100 nm^2 through the monolayer with the AFM probe at an applied force of 28 nN using an AFM image with a scan area of 1×1 μm^2.

Figure 1 shows the π–A isotherm of a DMPC/Chol mixed monolayer (molar ratio 70/30) on the water subphase at a T_{sp} of 296 K. The surface pressure on the π–A isotherm gradually increased with a decrease in surface area without any appearance of a plateau region. The molecular-occupied area of 0.66 nm^2 at the rising point of surface pressure for the mixed monolayer was smaller than that of 0.96 nm^2 for the monolayer consisting only of DMPC.[18] This may be due to the condensation effect of cholesterol on DMPC molecules in the monolayer, as is well known for lipid/cholesterol systems.[19,20]

Figures 2a–2f show the AFM images of the DMPC/Chol mixed monolayer transferred at surface pressures of 0, 1, 3, 6, 9, and 14 mN m^{-1}, respectively. The image at 0 mN m^{-1} (Figure 2a) exhibited larger indefinite domains with dimensions of about 4 μm, smaller domains with dimensions about 100 nm, and a matrix region. These domains and matrix region have been assigned to the regions of the Chol-rich phase and the DMPC-rich phase, respectively, on the basis of fluores-

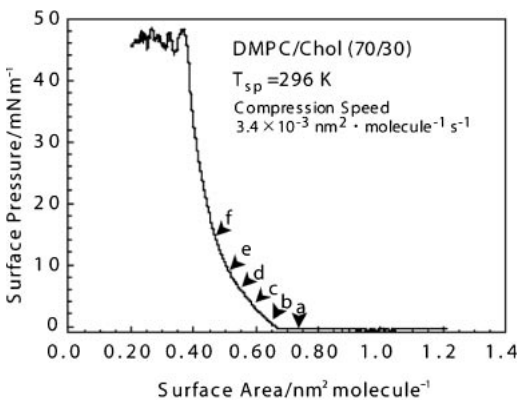

Figure 1. π–A isotherm of a DMPC/Chol mixed monolayer with a molar ratio 70/30 at T_{sp} of 296 K. Alphabets on the isotherm showed the figure's alphabet of AFM images in Figure 2.

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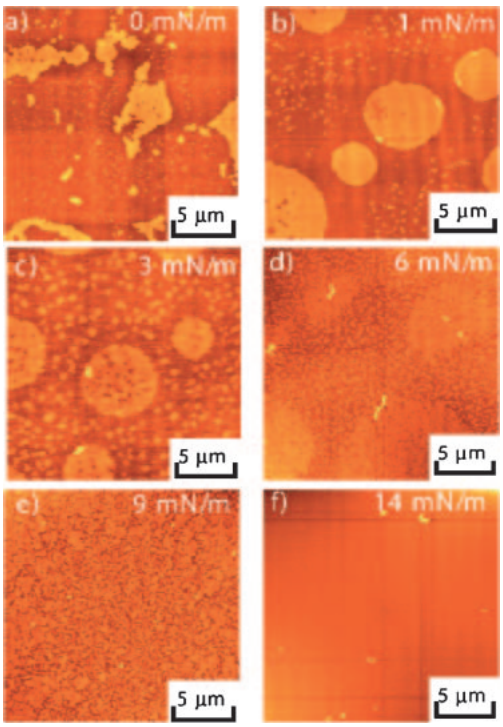

Figure 2. AFM images of the DMPC/Chol mixed monolayer at surface pressures of 0, 1, 3, 6, 9, and 14 mN m^{-1}. Alphabets on the AFM images correspond to that on the π–A isotherm in Figure 1.

cence microscopic observations.[22] At 1 mN m^{-1}, larger circular domains with a diameter of about 4 μm were observed instead of the indefinite domains obtained at 0 mN m^{-1}. Voids with a diameter of about 0.5 μm appeared in the smooth and flat domains. By compressing the monolayer using a pressure of 3 mN m^{-1}, the number and size of the voids increased in the larger circular domains. The domains dispersed into the surrounding matrix at 6 mN m^{-1}, resulting in a marbled pattern with a particle size of about 200 nm at 9 mN m^{-1}. The marbled pattern, however, disappeared at 14 mN m^{-1}, resulting in a smooth and flat morphology without the contrast of domain and matrix regions in a scan area of 20 × 20 μm^2. These results obtained from the AFM images qualitatively agree with those of previous studies using fluorescence microscopic observations[21–23] and theoretical calculations.[24,25] According to these studies, the DMPC/Chol system in a monolayer on the water subphase exhibits the phase behavior of UCSP (upper critical solution pressure) type, showing that the phase mixing would be enhanced at higher surface pressures.[19,20,31] Hence, the destruction and fusion of domains into the matrix from 3 to 14 mN m^{-1} suggests the phase miscibility between the cholesterol-rich phase and the DMPC-rich phase. The shape difference between indefinite and circular domains at 0 and 1 mN m^{-1}, respectively, can also be explained by the schematic phase diagrams based on the theory for the DMPC/Chol binary mixture[22,27] as follows: At the critical fraction for miscibility of (DMPC/Chol) monolayer, a stripe phase appears with an increase in surface pressure before becoming a homogeneous-single phase.[23,25] The molar ratio 70/30 in this study, however, deviates from that of 73/27 at the critical point.[23,25] Near the

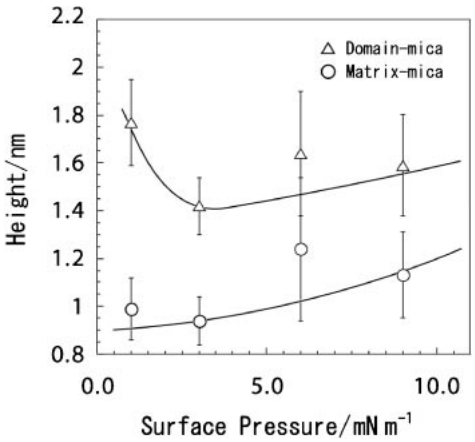

Figure 3. Thickness of domain and matrix regions in the DMPC/Chol mixed monolayer as a function of surface pressure with a molar ratio 70/30.

critical fraction, stripe and hexagonal phases appear sequentially with the increase in surface pressure.[22,27] In the hexagonal shape, an isotropic force applied to the domains by compression rounds the corners. Also, in the stripe phase, melting induced by thermal fluctuations mediates breaking and rejoining of the stripes.[28,29] Hence, it seems that the indefinite shape at 0 mN m^{-1} and the circular shape at 1 mN m^{-1} correspond to the stripe phase and the hexagonal phase, respectively.

Figure 3 shows the surface pressure dependence of thickness at domain and matrix regions of the DMPC/Chol mixed monolayer with the molar ratio 70/30. In the matrix region, the averaged value of thickness gradually increased from about 0.8 to 1.2 nm with an increase in surface pressure in a range of 0–9 mN m^{-1}. This is probably because compression causes the orientational ordering or conformational ordering of the alkyl chain of the DMPC molecule, resulting in a decrease in the area per molecule. In contrast, in the domain region, the maximum thickness of 1.8 nm at 0 mN m^{-1} decreased to the minimum thickness of 1.4 nm at 3 mN m^{-1}; the thickness then increased slightly with increasing surface pressure from 3 to 9 mN m^{-1}. It has been proposed[19,20] that Chol causes the conformational ordering (large fraction of trans conformation) of the alkyl chain of the surrounding DMPC molecules. Consequently, the domain that is assigned to the Chol-rich phase[18,30,31] would be thicker than that of the matrix region that is assigned to the Chol-poor phase. As mentioned previously, the phase behavior of this system is of the UCSP type. Therefore, the fraction of Chol in the domain decreases by an increase in surface pressure from 0 to 3 mN m^{-1}, resulting in a decrease of domain thickness. However, above 3 mN m^{-1}, the thickness in the domain gradually increases owing to the orientational or conformational ordering of the alkyl chain of DMPC molecule in the same manner as in the matrix region.

Figure 4 shows a magnified image (1 × 1 μm^2) of the center region in Figure 2f (20 × 20 μm^2) of the DMPC/Chol mixed monolayer at 14 mN m^{-1}. Figure 2f exhibited a smooth and flat morphology of the monolayer surface, except for some aggregates. This indicates a homogeneous phase of the monolayer, which agrees with many theoretical[21–23] and experimental[24,25] results. However, the magnified image (Figure 4) showed a marble pattern consisting of lower and higher regions

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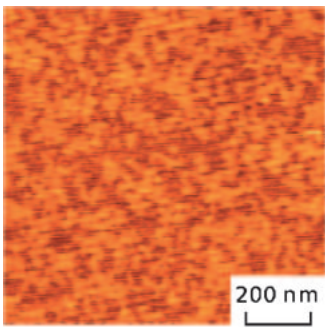

Figure 4. AFM image ($1 \times 1\,\mu m^2$) of the DMPC/Chol mixed monolayer with a molar ratio 70/30 at a surface pressure of $14\,mN\,m^{-1}$.

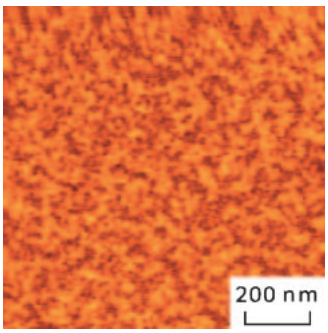

Figure 5. AFM image ($1 \times 1\,\mu m^2$) of the DMPC/Chol mixed monolayer with a molar ratio 80/20 at a surface pressure of $25\,mN\,m^{-1}$.

with thicknesses of 1.2 and 1.6 nm, respectively, and with a transverse size of 20 nm. A similar morphology of nanoscopic separation was also observed, under a different condition. Figure 5 shows the AFM image ($1 \times 1\,\mu m^2$) of the DMPC/Chol mixed monolayer with a molar ratio 80/20 at a surface pressure of $25\,mN\,m^{-1}$. This monolayer was assigned to a mixing state,[24,25] and here also, the AFM image with a scan area of $20 \times 20\,\mu m^2$ exhibited a macroscopically homogeneous morphology. However, a nanoscopically separated morphology was observed in the AFM image with a scan area of $1 \times 1\,\mu m^2$ (Figure 5). The results in Figures 4 and 5 indicate that the molecules in a monolayer do not always mix at a molecular level under the state of mixing indicated by the phase diagram. The thermodynamic separation or mixing is a phase phenomenon, that is, a macroscopic phenomenon characteristic of a huge number of molecules. Hence, it is possible for a relatively small number of molecules to separate nanoscopically owing to a concentration fluctuation even above the binodal line.[21–23] In any case, however the reason for the formation of nanoscopically separated structures remains unclear at present.

In conclusion, surface compression causes 1) an orientational ordering of the alkyl chain of DMPC, and 2) a change in concentration of DMPC and Chol in the domain and matrix regions based on the USCP type phase behavior. This results in a change of aggregation state by means of phase separation and mixing. DMPC and Chol molecules in the mixed monolayer nanoscopically separate at higher surface pressures, although the monolayer exhibited a homogeneous morphology at the microscopic level.

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