Research Article

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Preparation of a Dipalmitoylphosphatidylcholine/ Cholesterol Langmuir−Blodgett Monolayer That Suppresses Protein Adsorption

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The adsorption patterns of human serum albumin (HSA) on the monolayers of dipalmitoylphosphatidylcholine (DPPC) and DPPC/cholesterol mixtures were studied by using the tapping mode atomic force microscopy (AFM). The pure DPPC and DPPC/cholesterol monolayers with different packing density were deposited on alkylated substrates by using the Langmuir−Blodgett technique, with the varying concentrations of cholesterol. The structures of solid supported pure DPPC and solid supported DPPC/cholesterol monolayers were analyzed by using AFM, ellipsometry, and cyclic voltammetry. The results indicated that the 10−30 mol % cholesterol mixed monolayers had densely packed and ordered structures, but the 50 mol % cholesterol mixed monolayer had irregular and disordered structures. When the DPPC and DPPC/cholesterol monolayers were exposed to a 0.1 mg/mL solution of HSA, the adsorption patterns of albumin indicated that the pure DPPC and the 50 mol % cholesterol mixed monolayers greatly activated protein adsorption, whereas the 10−30 mol % cholesterol mixed monolayers strongly suppressed protein adsorption due to highly packed phosphocholine groups. Highly packed DPPC molecules in the monolayer were vertically oriented and the protein-resistant neutral phosphocholine groups were in direct contact with proteins, thereby reducing protein adsorption. Therefore, the protein adsorption patterns greatly depended on the condensing effect of cholesterol in the DPPC monolayer.

Introduction

Cholesterol is one of cell membrane constituents and modulates the fluidity of cell membranes.[1,2] It has been known that cholesterol increases the structural order of the phospholipid hydrocarbon chains of cell membranes.[3] In addition, an extensive modeling system of mixed monolayers of phospholipid/cholesterol, such as Langmuir−Blodgett films at the air/water interface, indicated that cholesterol has a pronounced condensing effect.[4−7] On the basis of phospholipid/cholesterol monolayer studies, this condensing effect reduces the number of defects and increases the packing density of the monolayer.

Although the role of cholesterol in the phospholipid monolayer at the air/water interface has been extensively studied, phospholipid/cholesterol monolayers on solid substrates have been seldom investigated. Studies have so far shown that a supported phospholipid monolayer may lose its tightly packed structure during its transfer from the air/liquid interface to the solid support, thereby causing disordered structure and many defects.[8−11]

Protein adsorption on artificial surfaces is an important property to take into consideration when evaluating their biocompatibility.[12,13] Albumin adsorption on artificial surfaces has been extensively studied since it is the most abundant protein in the blood. Albumin has several strong binding sites for fatty acid molecules,[14−16] and recently, it was reported that human serum albumin (HSA) appears to bind more tenaciously to the surfaces covered with less dense, more disordered alkyl chains.[17,18] The interaction between albumin and an alkylated surface is significantly affected by alkyl chain length, density, and ordering of the hydrocarbon chains on the surface. Therefore, the albumin adsorption pattern on different surfaces is a useful model for studying the interaction between proteins and surfaces, such as well-defined Langmuir−Blodgett (LB) films.

The purpose of this study was to engineer the surface of phospholipid monolayers using the LB technique, which could be densely packed by controlling the condensing effect of cholesterol on the phospholipid monolayer. We also tried to find the relationship between the packing density of phospholipid molecules and the protein adsorption patterns.

Experimental Section

Materials. Dipalmitoylphosphatidylcholine (DPPC) and cholesterol (99% pure) were purchased from Sigma Chemical Co.

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(St. Louis, MO). Dichlorodimethylsilane and octadecanethiol (99% pure) were obtained from Aldrich Chemical Co. (Milwaukee, WI). These chemicals were used without further purification. All solvents were analytical grade and purchased from Sigma.

Preparation of LB Monolayer. A commercial NIMA trough (type 611, Warwick Science Park, Inc., Conventry, U.K.) was used for the preparation of LB monolayers and the measurement of surface pressure. Both DPPC and cholesterol were mixed in chloroform at molar percentages of 0−100% of cholesterol and the solution was spread on the ultrapure water surface at 20 °C. After time for solvent evaporation was allowed, the monolayer was compressed to a surface pressure of 15 mN/m. Then the monolayer was transferred onto a silanized silicon substrate by using the vertical dipping method. The silicon oxide surface was silanized with dichlorodimethylsilane, according to the established procedure.[19]

AFM Measurement. Tapping mode AFM images were obtained in air at room temperature using an Autoprobe CP system (Park Scientific Inc., Sunnyvale, CA). Tapping mode AFM images were made by using noncontact silicon ultralevers (Park) with a resonance frequency of 60−140 kHz and a spring constant of 20 N/m at a scan rate of 1 Hz using a small size piezoscanner (5 μm, Park). Tapping mode AFM images were analyzed by using PSI Proscan Software (Park). Images presented were obtained after several repetitions of the samples, which always revealed similar features with regard to the depth of holes and the root-mean-square (rms) roughness.

Ellipsometry Measurement. Single-wavelength ellipsometry was performed using an automatic ellipsometer (S2000, Roudolph Research) with a He−Ne laser light source ($\lambda = 632.8$ nm) at an angle of incidence of 70°. The alkylated substrates were measured prior to transferring LB monolayers, and the collected average values of the refractive index were used in measuring the thickness of supported monolayers on alkylated substrates.

Cyclic Voltammetry Measurement. For the reproducible voltammograms of solid supported monolayers, gold was deposited onto chromium-coated silicon substrates (2 cm × 2 cm) to a thickness of 2000 Å. Then alkylation was performed with 1 mM ethanol solution of octadecanethiol for several days at room temperature.[20] After this modification, pure DPPC and DPPC/cholesterol monolayers were transferred onto thiol-treated gold substrates with the same condition of silanized silicon substrates. The supported monolayers on thiol-treated gold substrates were used as a working electrode. The electrochemical cell had a three-electrode system consisting of a Ag/AgCl (3 M NaCl, BAS) electrode, a platinum wire which served as a reference, and counter electrodes.[20,21] The body of the cell was a polyethylene cylinder which was positioned on top of the planner working electrode and a flattened gold wire lead. The exposed working electrode area was 0.32 cm^2 and the volume of the cell was approximately 0.6 mL. Cyclic voltammetry was performed with a solution of 1 mM $K_3Fe(CN)_6$ in 1 M KNO_3 by using a poteintiostat/galvanostat system (model-263, EG&G, PAR, Princeton, NJ).

Protein Adsorption. Human serum albumin (99%, Sigma, essentially fatty acid and globulin free) was used as received. Phosphate-buffered saline (PBS, pH = 7.4, ion strength 0.15) was used to prepare solutions of desired albumin concentration, ranging over 0.001, 0.01, and 0.1 mg/mL. Protein adsorption experiments were performed in a closed adsorption cell (Falcon tube, 15 mL). Supported monolayers on alkylated silicon substrates (1 cm × 1 cm) were placed vertically in the adsorption cell. Then, the adsorption cell was filled with PBS and left alone for 1 h to hydrate the supported monolayers. After the hydration process, the cell was quickly refilled with 5 mL of albumin solution of a desired albumin concentration. Adsorption was then allowed to take place for 10 min. After that time, the samples were withdrawn from the HSA solution, rinsed with PBS with slight agitation to remove the weakly adsorbed protein, and finally washed with distilled water to remove residual buffer salts. In particular, these experiments were carefully performed to prevent additional adhesion of proteins at the solution/air interface. After all experiments were finished, the samples were dried under nitrogen gas. Then the samples were immediately characterized by atomic force microscopy (AFM) measurements.

For the tapping mode AFM experiments, we minimized the undesirable interaction between the AFM tip and the adsorbed protein layer by maintaining the force constant and as low as possible. The amount of adsorbed protein layer on supported LB monolayer was determined by performing the bearing analysis (PSI Proscan, Park).

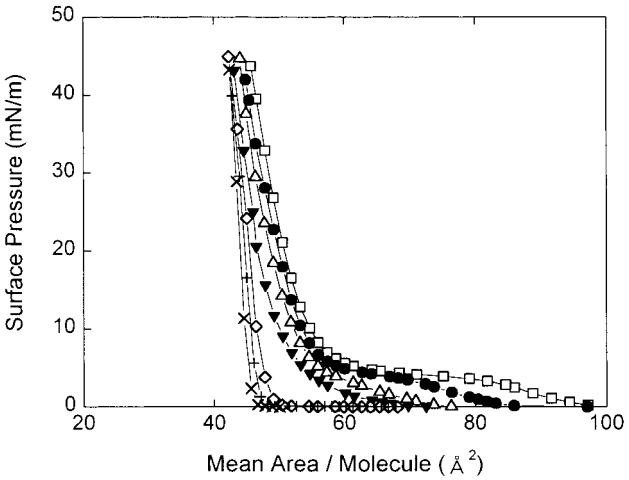

Figure 1. Surface pressure−area isotherm of DPPC and cholesterol mixed monolayers. Mole fractions of DPPC/cholesterol: 100/0 (□), 90/10 (●), 80/20 (△), 70/30 (▼), 50/50 (◇), 33/67 (+), 0/100 (×). The monolayer was compressed at 30 cm^2/min at 20 °C.

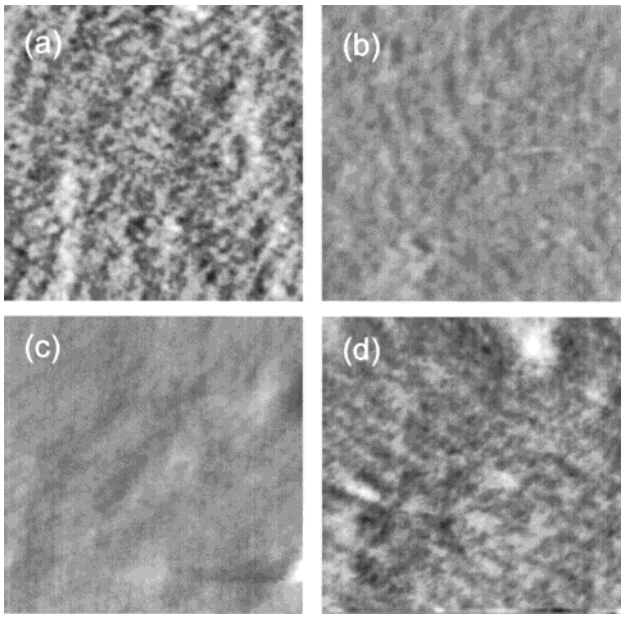

Figure 2. Tapping mode AFM images (1 μm × 1 μm) of transferred LB films of (a) DPPC, (b) 10 mol % cholesterol, (c) 30 mol % cholesterol, and (d) 50 mol % cholesterol mixed monolayers.

Table 1. The Root Mean Square Roughness and Thickness of Supported LB Films

monolayer	roughness (Å)	thickness (Å)
DPPC	6.04	22.3 ± 3.64
10 mol % cholesterol	2.54	27.5 ± 3.31
30 mol % cholesterol	2.20	33.5 ± 3.64
50 mol % cholesterol	5.16	27.3 ± 2.36

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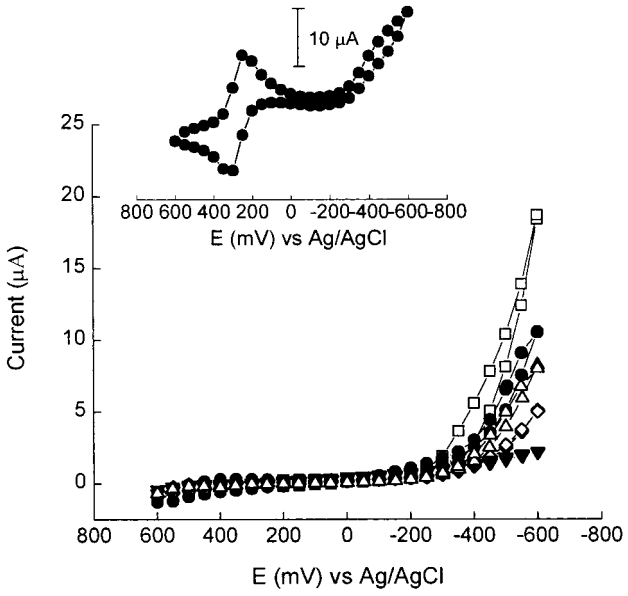

Figure 3. Cyclic voltammograms of $K_3Fe(CN)_6$ in 1 M KCl at a octadecanethiol monolayer (□), pure DPPC (●), 10 mol % cholesterol (△), 30 mol % cholesterol (▼), and 50 mol % cholesterol (◇) mixed monolayers. Inset: cyclic voltammogram of a bare gold electrode. Scan rate 10 mV/s.

Results and Discussion

DPPC/Cholesterol Monolayer at the Air/Water Interface. The surface pressure−area isotherms of pure DPPC and DPPC/cholesterol mixtures were studied before depositing the monolayers on the alkylated substrate at 20 ± 0.1 °C (Figure 1). The isotherms of the pure DPPC showed a transition from the liquid expanded (LE) phase to the liquid condensed (LC) phase between the surface of 4 and 15 mN. As the cholesterol concentration in the DPPC monolayer increased, the LE−LC phase transition became less clear and the DPPC isotherm was shifted to the left, indicating the condensing effect of cholesterol in the phospholipid monolayer. When cholesterol concentration was above 30 mol %, the DPPC isotherms showed a steep isotherm, indicating an ordered cholesterol-like isotherm.[7]

The extent of the condensing effect was readily determined by plotting the mean molecular area as a function of cholesterol concentration at the transferring surface pressure of 15 mN/m (data not shown). This transferring surface pressure was determined by optimizing the transfer efficiency of the monolayers and the condensing effect of cholesterol. At this surface pressure, there was a negative deviation from the ideal mixing line for DPPC and cholesterol, which was consistent with the condensing effect. The condensing effect was maximized at 30 mol % cholesterol. On the other hand, the condensing effect decreased when cholesterol concentration was above 30 mol %. These results suggest that cholesterol molecules are readily mixed with DPPC molecules at a low cholesterol concentration (<30 mol %), whereas cholesterol molecules are not readily mixed with DPPC molecules (>50mol %). It means that the segregated or unmixed cholesterol

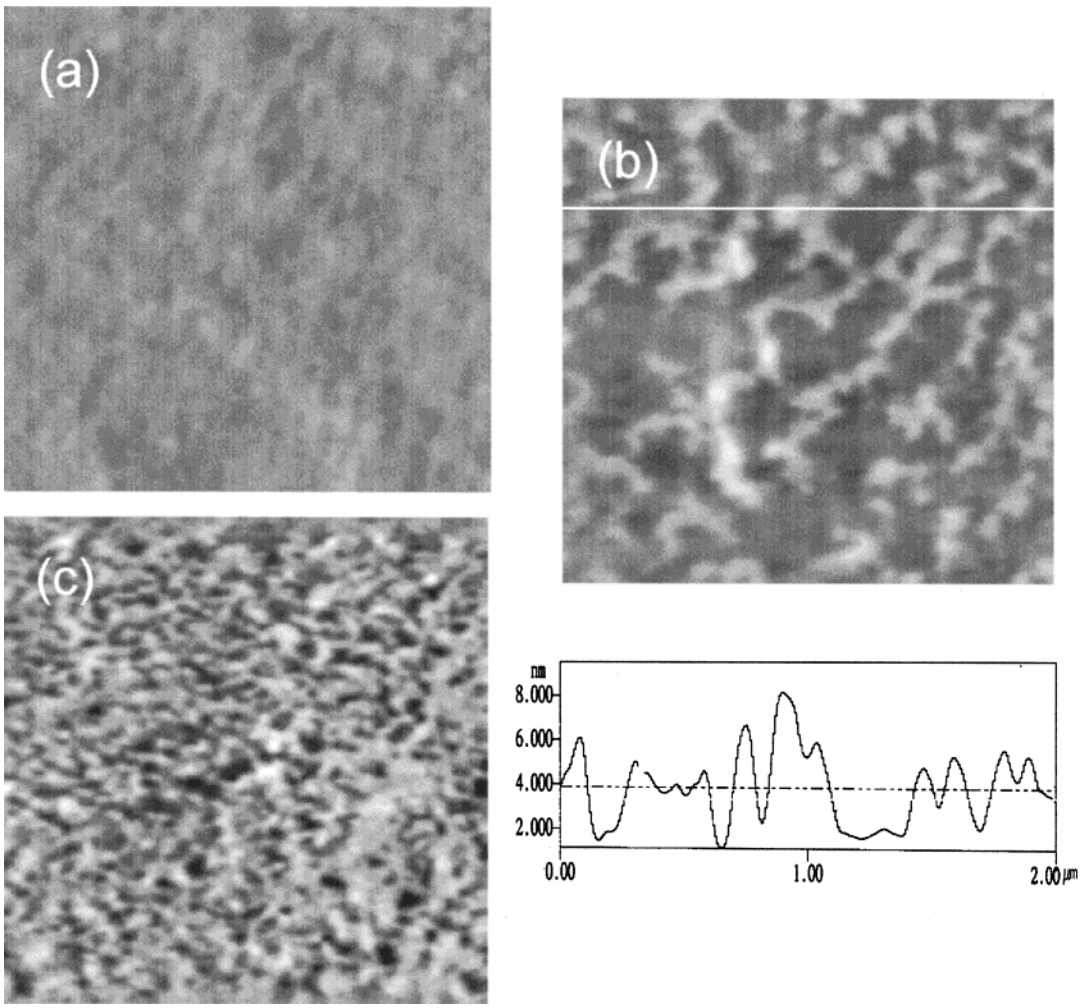

Figure 4. Tapping mode AFM images (2 μm × 2 μm) of pure DPPC monolayer adsorbed at different protein concentrations: (a) 0.001, (b) 0.01, and (c) 0.1 mg/mL in PBS.

molecules do not exhibit a condensing effect on the monolayer.

Characterizations of Supported LB Films. After DPPC/cholesterol monolayers containing 0−50 mol % of cholesterol were deposited on alkylated silicon substrate, the surface morphology was characterized using the tapping mode AFM. Figure 2 shows the tapping mode AFM images (1 μm × 1 μm) of supported monolayers in air. The pure DPPC monolayer had a rough surface with the rms roughness of 6 Å and an average height of 23 Å from the monolayer surface to the hole bottom (Figure 2a). This average height of the monolayer is less than the fully extended molecular length of DPPC (34 Å), indicating the disordered and tilted orientation of DPPC. We also observed that there were many holes and the surface was uneven since the DPPC monolayer was deposited on an alkylated substrate as a losening structure.

The surface of the 10 mol % cholesterol mixed monolayer was more smooth than the DPPC monolayer, with a rms roughness of less than 2.5 Å. Its average height increased to 29 Å (Figure 2b), but some holes and uneven regions still existed on the monolayer surface. As the content of cholesterol was increased up to 30 mol % in the monolayer, the monolayer surface became regular and smooth, and the average height also increased to 30 Å, with a rms roughness of only 2.2 Å (Figure 2c).

However, the 50 mol % cholesterol mixed monolayer showed rough and disordered surface where the rms roughness increased up to 5.1 Å and the average height decreased to 27 Å (Figure 2d). The height difference of uneven regions in the monolayer was 10−20 Å, indicating the molecular length difference of DPPC and cholesterol. This result suggests that excess cholesterol, which is unmixed with DPPC molecules, eventually forms a disordered cholesterol-rich region.

The ellipsometry characterization showed that the thickness of DPPC/cholesterol monolayer was very consistent with that of the AFM measurements. The thickness of the pure DPPC monolayer was 22 Å. As the cholesterol concentration in the monolayer increased up to 30 mol %, the average thickness was increased. In particular, the thickness of 30 mol % cholesterol mixed monolayer was about 33 Å, which is very consistent with the length of DPPC. It indicates that DPPC molecules are vertically oriented and densely packed with each other on solid substrates. As predicted from the tapping mode AFM images, the average thickness of 50 mol % cholesterol mixed monolayer decreased to 27 Å. The thickness and the rms roughness of supported monolayers were summarized in Table 1.

For further characterization of supported monolayers, we studied the electrochemical properties of supported DPPC/cholesterol monolayers on thiol-treated gold substrates. Plant et al. reported that the densely packed phospholipid monolayer on gold electrode acts as an insulator to electron transfer.[20] Thus, we could evaluate the packing density of supported DPPC/cholesterol monolayers by using cyclic voltammetry. Figure 3 shows cyclic voltammograms at a series of monolayers on thiol-treated gold electrodes. The voltammogram at a bare gold electrode showed a typical redox peak for $Fe(CN)_6^{3-}$. This result showed that the octadecanethiol monolayer reduced oxidation and reduction of the ion. The series of supported DPPC and DPPC/cholesterol monolayers on thiol-treated gold electrodes further reduced the amount of faradic current compared to the thiol-modified gold electrode, since the deposited LB monolayer formed an even more effective barrier to electron transfer.

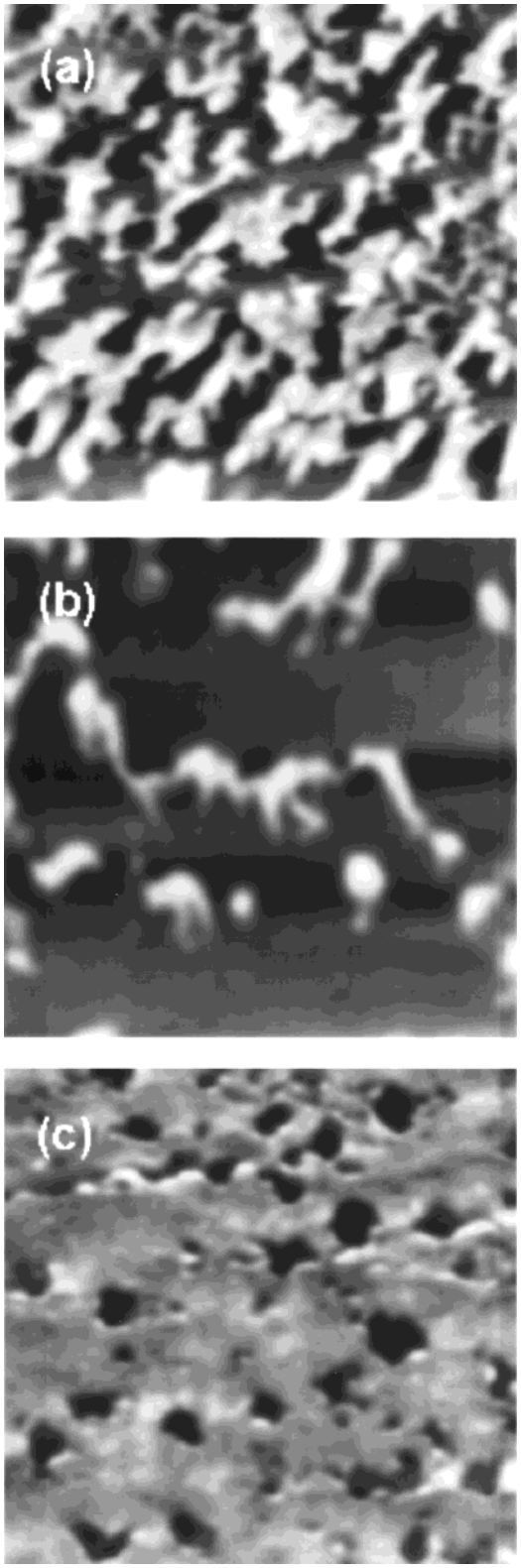

Figure 5. Tapping mode AFM images (2 μm × 2 μm) of transferred LB films with albumin layers adsorbed at the concentration of 0.1 mg/mL: (a) 10 mol % cholesterol, (b) 30 mol % cholesterol, and (c) 50 mol % cholesterol mixed monolayers.

An interesting observation was that the insulating efficiency of supported monolayers depended on the concentration of cholesterol in the DPPC monolayer. The insulating efficiency of supported monolayers was maximum when the cholesterol concentration was 30 mol %. However, the amount of faradic current increased in the

50 mol % cholesterol mixed monolayer due to undesired excess cholesterol molecules. Therefore, we confirmed that the condensing effect of cholesterol at the air/water interface leads to form a densely packed DPPC monolayer on solid; however, excess cholesterol caused additional disordered structures in the monolayer.

Protein Adsorption. Figure 4 shows the tapping mode AFM images of the adsorbed albumin layer on the DPPC monolayer from solutions with the protein concentration of 0.001, 0.01, and 0.1 mg/mL, respectively. These concentrations were chosen to determine the albumin adsorption isotherm where protein adsorption reaches saturation.

At a low protein concentration of 0.001 mg/mL (Figure 4a), the adsorbed protein was not shown. And partially adsorbed proteins existed on the DPPC monolayer at the protein concentration of 0.01 mg/mL (Figure 4b). The average thickness of adsorbed proteins, indicated by the line profile in Figure 4b, was approximately 4 nm, and some proteins were aggregated with each other. The adsorbed proteins were randomly distributed, and they formed protein patches on the surface. The surface coverage of protein layer was measured by using the bearing analysis of the tapping mode AFM images. The surface coverage of adsorbed proteins was about 42%, indicating a partial surface coverage of proteins. At the protein concentration of 0.1 mg/mL, the AFM image (Figure 4c) revealed that the surface was fully covered with proteins with some holes. The surface coverage of protein layer was above 98%. Thus, the protein adsorption on the DPPC monolayer reached saturation at 0.1 mg/mL protein concentration.

Figure 5 shows the AFM images of adsorbed protein layers on the DPPC/cholesterol monolayers. At first glance, there seems to be fewer proteins adsorbed on the 10 mol % cholesterol mixed monolayer compared to the pure DPPC monolayer (Figure 5a). The thickness of the protein layer was close to 4 nm, and the surface coverage by the protein layer was about 62%. In particular, 30 mol % cholesterol mixed monolayer showed only small amounts of adsorbed proteins (Figure 5b). The surface coverage of the protein layer was only 28%, and the protein thickness was about 4 nm. However, 50 mol % cholesterol mixed monolayer greatly activated the protein adsorption on the surface. The surface coverage of dense protein layer with the thickness of 5 nm was above 87% (Figure 5c).

The reduction of protein adsorption on the DPPC monolayer showed the same tendency with the condensing effect of cholesterol on DPPC monolayer. Highly packed DPPC molecules in the monolayer were vertically oriented and the protein-resistant neutral phosphocholine groups were in direct contact with proteins, thereby reducing the protein adsorption. On the other hand, cholesterol-rich regions produced strong protein binding sites because cholesterol molecules were directly exposed to proteins. Therefore, the protein adsorption patterns greatly depended on the condensing effect of cholesterol in the DPPC monolayer.

Conclusion

In this study, we have prepared monolayers of DPPC/cholesterol mixtures on solid support by using the LB technique. This design strategy can be used to engineer a supported DPPC monolayer, which can be packed densely and orderly assembled by controlling the cholesterol concentration in the DPPC monolayer. The DPPC/cholesterol monolayer containing 30 mol % cholesterol showed the most smooth and vertically ordered structures. These results have clearly demonstrated that cholesterol densely packs DPPC molecules, thereby reducing many defects in the monolayer, and that the 30 mol % cholesterol greatly suppressed the albumin adsorption by its highly packed phosphatidylcholine groups. These protein adsorption patterns on the DPPC/cholesterol mixed monolayer directly confirmed the interaction between protein and densely packed phosphocholine groups. The DPPC/cholesterol mixed monolayer can serve as a useful model for the development of new biosensors and blood-compatible materials.

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