SPECIAL TOPIC — Physical research in liquid crystal

Behavior of lysozyme adsorbed onto biological liquid crystal lipid monolayer at the air/water interface*

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The interaction between proteins and lipids is one of the basic problems of modern biochemistry and biophysics. The purpose of this study is to compare the penetration degree of lysozyme into 1,2-diapalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethano-lamine (DPPE) by analyzing the data of surface pressure–area (π–A) isotherms and surface pressure–time (π–T) curves. Lysozyme can penetrate into both DPPC and DPPE monolayers because of the increase of surface pressure at an initial pressure of 15 mN/m. However, the changes of DPPE are larger than DPPC, indicating stronger interaction of lysozyme with DPPE than DPPC. The reason may be due to the different head groups and phase state of DPPC and DPPE monolayers at the surface pressure of 15 mN/m. Atomic force microscopy reveals that lysozyme was absorbed by DPPC and DPPE monolayers, which leads to self-aggregation and self-assembly, forming irregular multimers and conical multimeric. Through analysis, we think that the process of polymer formation is similar to the aggregation mechanism of amyloid fibers.

Keywords: lysozyme, adsorption curves, liquid crystal monolayers, phase state

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1. Introduction

The biological membrane is a natural barrier that maintains cells and organelles with relatively independent and stable environments and is mainly composed of phospholipids and proteins.[1,2] Phospholipids, mostly liquid crystal and the most abundant lipids in cell membranes,[3,4] are used in a wide range of applications for the detection and characterization of biomolecular interactions, including biological sensing, transmembrane transport, receptor interactions, and cellular signaling.[5–7] Lipids and proteins are the basis of the biological membrane structure and the interaction between them has been widely studied,[8–12] which is one of the basic problems of modern biochemistry and biophysics. For instance, the signal transduction process via the cell membrane is controlled by membrane-associated[13] and soluble proteins. Furthermore, the arrangement of the phospholipid bilayer in the biological membrane and the binding mode of the membrane protein with the membrane protein can also form a polymorphic liquid crystal structure. Spontaneous closure of membrane lipid liquid crystal provides conditions for the generation, survival, and evolution of living cells,[4] while the membrane proteins can be moved in the two-dimensional fluid of the membrane. The liquid crystal state is not only closely related with life, but also plays an important role in the realm of science and technology.[14–16] Over the past few years, the field of disk-shaped (discotic) liquid crystals has grown enormously because of the interesting electro optical properties of the molecules. Liquid crystalline materials used as material interfaces to biomolecular events have been explored.[17–19]

Interaction of proteins with lipids, whether it is for transport, serum lipoproteins, lipid hydrolysis, metabolism or cell signaling, receptors and channels, is a rapidly expanding field of research. In general, these proteins have structural domains that enable them to be recognized as predominantly lipophilic. Some evidences that are typically regarded as hydrophilic proteins may also have obscure functions when they associate with lipids.[20] One such protein is lysozyme. Lysozyme is a globular antibacterial protein, which can be found in the human body, such as in blood and saliva and has antibacterial activity.[21,22] The detection of lysozyme in serum can play an important role in the early diagnosis of leukemia.[23] Eye conjunctivitis can lead to the decrease of the concentration of lysozyme.[20,23] The content of lysozyme in cerebrospinal fluid is a sensitive signal to diagnose inflammation and central nervous system diseases.[24] So far, the mechanism of membrane fusion in the process of lysozyme is not completely understood. Although it has been reported in the literature, this process is related to the interaction of lysozyme and phospho-

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lipids, which may be caused by the penetration of lysozyme into the lipid layer. In addition, the tear film is a liquid layer of a complex, dynamic, and changing structure covering the front surface of the eye, which consists of the ocular surface, lipid layer, and aqueous layer, forming a barrier between the extraocular environment and the corneal conjunctive surface to protect the eye. Lysozyme is one of the most important characteristics of the tear and can be found at the aqueous layer, containing salts, more than 60 different proteins and mucins.[25] A low surface tension is essential for a functional tear film. The lipid layer is thought to be responsible for lowering the surface tension of the tear film at the air–water interface.[11] With high surface tensions, there is a tendency to have a clinical condition called "dry eye". The interaction of lysozyme on the lipid layer can lead to the decrease of the surface tension of the film, but how the proteins of tears interact with the meibomian lipids is not known. Lysozyme is the ideal model as a stable small molecule enzyme to study the interactions between protein and lipid in the air/water interface.

The Langmuir technique has been useful to determine the mechanism of action of antimicrobial and membrane lytic peptides in cell membranes. Advantages of using a Langmuir monolayer include the possible fine control over the composition and packing of the membrane being mimicked. In this paper, we have used Langmuir–Blodgett (LB) technology and atomic force microscopy (AFM) to investigate the biological mechanism of the interaction between lysozyme and lipid monolayer and the thermodynamic changes in the adsorption process. Zwitterionic lipid DPPC and DPPE have been used to analyze different interaction degrees between LZM and them.

2. Materials and methods

2.1. Materials

Lysozyme (LZM) from chicken egg white, crystallized, was supplied from Sigma (purity 95%) and stored according to the supplier information. The protein spreading solution was a mixture of concentration of 3.2 mg/mL in 10 mM phosphate buffered saline (PBS; 0.9% NaCl, pH 7.4), and the PBS was always made fresh and was used within 30 min of making the solution. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) from bovine heart was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Spreading solutions of DPPC and DPPE were prepared in chloroform/methanol 3:1 (v/v) mixture at a concentration of 1 mg/mL. The ultra pure water used as subphase was distilled and purified with a Millipore purification system (electrical resistivity ≥ 18.2 MΩ·cm).

2.2. Langmuir–Blodgett films

Surface pressure–area (π–A) isotherms were obtained by means of a computer-controlled commercial device (Minitrough, KSV, Helsinki, Finland). Symmetric compression was achieved with two moving barriers at a constant rate of 10 mm/min. The surface pressure (π) of the monolayer was measured using the Wilhelmy method, using a Wilhelmy platinum plate, with an accuracy of 0.1 mN/m and a dynamic surface pressure range 0–150 mN/m. The value was monitored with a Wilhelmy article probe hanging from a high-precision microbalance connected to a computer, and is defined as[26]

$$\pi = \gamma_0 - \gamma, \tag{1}$$

where γ_0 is the surface tension of the air–water interface and γ is the surface tension in the presence of a mixed monolayer compressed to varying packing densities. Monolayers have been formed by spreading an aliquot of lipid solution on 10 mM phosphate buffered saline (PBS; 0.9% NaCl, pH 7.4). Different concentrations of DPPC were first spread on the subphase surface. Then certain volumes of the LZM solutions were injected into the subphase using Hamilton micro syringes. Compression was started 1 h after injection which allowed for chloroform evaporation and LZM unfolding. The isotherms for DPPC and DPPE were then recorded from online measurements and plotted by compressing the barriers at a forward rate of 10 mm/min. The trough and barriers were thoroughly cleaned with absolute ethyl alcohol and ultra pure water between each separate isotherm. All experiments were conducted at 23 ± 1 °C.

2.3. Penetration of the lipid films by lysozyme

In order to keep the area of the monolayer, pressure which changes with time has been recorded so as to obtain π–T isotherms when the lipid monolayer is compressed to a certain surface pressure. Surface pressure was set to near 10, 15, or 20 mN/m (π_0) by reducing the surface area. Once the film was set to near the desired pressure, the area was kept constant, lysozyme was injected into the subphase outside the barriers, and π was monitored until it became constant. This was deemed to be the equilibrium pressure (π_e). Compression of the DPPC monolayer occurred until the target surface pressure of 15 mN/m was reached. Monolayers have been transferred to the surface of mica substrate (transfer ratio is 1) with a method of vertical pulling transferring. Each experiment was repeated at least three times. The behavior of protein molecules on the surface of the lipid monolayer and the rearrangement of the structure can be obtained by[20]

$$\ln\left(\frac{\pi_e - \pi}{\pi_e - \pi_o}\right) = -\frac{t}{\tau}, \tag{2}$$

where τ is the relaxation time, π_e, π_o, π are the surface pressures of interaction between protein and lipid at equilibrium times, $t = 0$, and $t = t$, respectively. The relaxation time τ can be determined by fitting the π–T curves.

Figure 1 is a typical π–T adsorption curve. The first area represents the initial adsorption. π increase indicates that more

molecules are adsorbed to the same interface membrane. The second regions indicate that there is a new protein molecule adsorbed to the interface, the combination, expansion, and rearrangement. The third region is due to the expansion and rearrangement of the protein molecules on the interface, and gradually reaches the saturation stable state.[27]

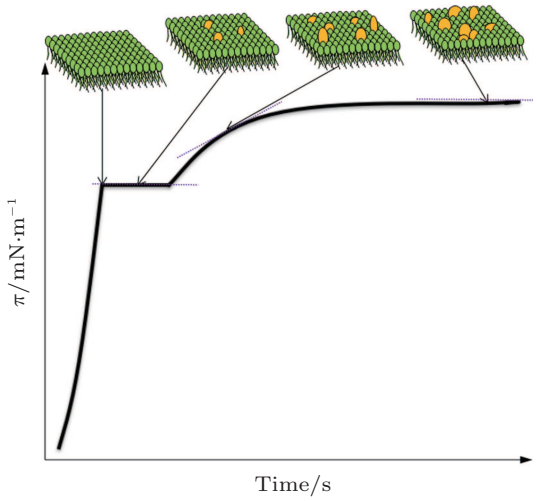


Fig. 1. (color online) Ideal π–T curve of lysozyme interaction with lipid layer.

2.4. Atomic force microscopy observation

AFM images were acquired in air at room temperature using a SPM-9500-J3 AFM (Shimadzu Corporation, Japan), which provided both a topographical image and a phase contrast one. The sample imaging has been carried out in the contact mode through a Micro-V-shaped Cantilever probe (Olympus Corporation, Japan) with cantilever spring constant about 0.06 N/m. A 55 μm × 55 μm tube-type piezoelectric scanner and a Si_3N_4 probe were used and images in height mode were collected simultaneously with 512 × 512 points with scan rate of 1.0 Hz per line.

3. Results

3.1. Surface pressure–area (π–A) isotherm

DPPC and DPPE are components of biological membranes, which have different head groups.[28] In this paper, we change the concentration of lysozyme to study the different effects of protein on DPPC and DPPE monolayer.

The surface pressure–area isotherms for a series of mixtures of LZM with (a) DPPC and (b) DPPE at temperature of 23 ± 1 °C are shown in Fig. 2. The DPPC isotherm displayed the characteristic plateau attributed to the first-order liquid expanded (LE) to liquid condensed (LC) phase transition at 4–7 mN/m,[20,27] which is consistent with the reported literature.[5] The subsequent sharp increase of surface pressure corresponds to the formation of a compact LC phase, and with further compression, a solid phase is formed.[29] Figure 1(a) shows that, even at very low concentration, the presence of LZM molecules strongly affects the shape of curves. As the LZM concentration increases, the curves are gradually shifted toward higher area values, and the typical plateau shown by the DPPC isotherm gradually disappears. This demonstrates that LZM molecules were adsorbed onto the DPPC monolayer and strongly affect the arrangement. Lysozyme increases the orderliness of the DPPC monolayer and reduces the lipid membrane surface tension,[28] the lateral movement of the monolayer is influenced by LZM, which further influences the phase transformation process. For lysozyme and DPPE systems, as shown in Fig. 1(b), with the increase of subphase concentration of lysozyme, the π–A curves of the monolayer are similarly shifted toward higher area values. This indicates that LZM was inserted into DPPE molecules by adsorption on membrane lipid monolayers to change the π–A curve shape.

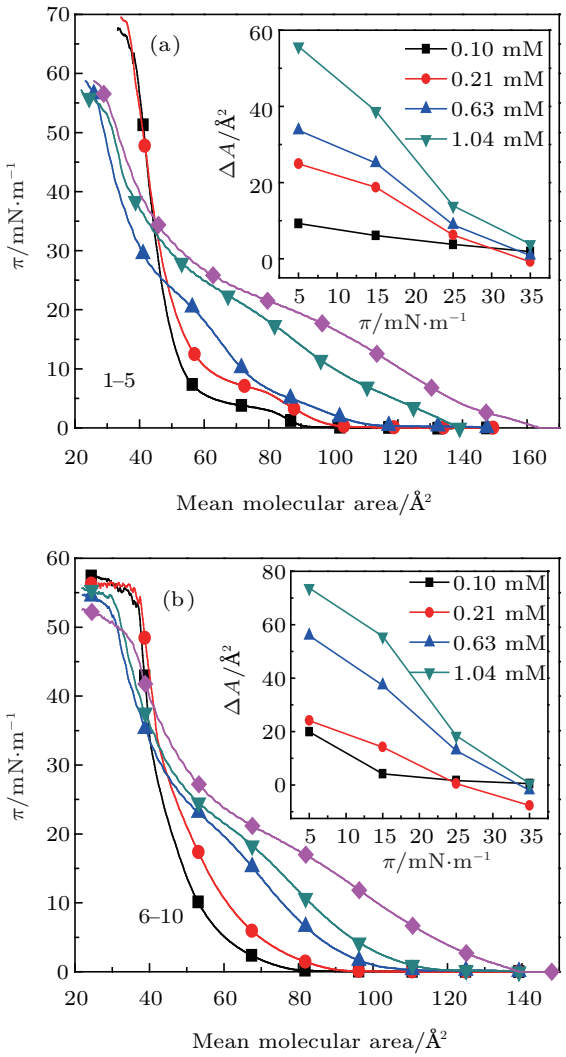


Fig. 2. (color online) π-area isotherms of different subphase concentration of lysozyme interaction with (a) DPPC and (b) DPPE monolayer. (i) Pure DPPC; (ii), (vii) 0.10 mM; (iii), (viii) 0.21 mM; (iv), (ix) 0.63 mM; (v), (x) 1.04 mM; (vi) pure DPPE. $\Delta A = A_\xi - A_0$, where A_0 stands for mean molecular area of pure lipid monolayer and A_ξ is the mean molecular area of the mixed monolayer.

In order to study the adsorption process of different subphase concentrations of lysozyme into the lipid monolayer, under different pressures, we calculate the change of mean molecular area of the two kinds of system, denoted as ΔA. When the concentration of lysozyme is 0.10 mM, the surface pressure is 5 mN/m, ΔA of DPPC and DPPE is 19.92 Å^2 and 9.25 Å^2. Variation of DPPC was significantly higher than that of DPPE. This is because when the pressure is 5 mN/m, DPPC is in LE–LC phase coexistence, and protein molecules are easily embedded into the membrane. When the surface pressure is 5 mN/m, 15 mN/m, and 25 mN/m, the curves variation of DPPE (ΔA) is greater than the change of DPPC monolayer in the amount of LZM (0.21 mM), which indicates that the effects of lysozyme molecules on DPPE is larger and there are more lysozyme molecules adsorbed on the DPPE monolayer. The reason may be that DPPE molecules are arranged loosely below the pressure of 25 mN/m, so lysozyme is more easily embedded into the monolayer on the surface. On the contrary, at the same surface pressure, the curves variation of DPPC (ΔA) is greater than the change of DPPE monolayer in the amount of LZM (0.63 mM and 1.04 mM). However, the corresponding (ΔA) of the DPPC and DPPE were negative when the pressure is 35 mN/m. This may be induced by the squeezing out of LZM from the lipid monolayer. When adding a certain amount of lysozyme, the ΔA of two systems increases with the decrease of surface pressure. The experimental results showed that the low concentration of lysozyme in subphase solution tends to adsorb or insert into the DPPE monolayer and the high concentration of lysozyme solution is more likely to be adsorbed or inserted into the DPPC monolayer. It could be expected that the interactions of the head groups could influence penetration. DPPC and DPPE are different in size and orientation of the head groups. More space is required for the larger and more hydrated PC head group than for the PE head group.

3.2. Interfacial properties of mixed films

The elastic compressibility (C_s^{-1}) is a useful parameter for assessing elastic properties of Langmuir monolayers, which was calculated from the π–A isotherm using Eq. (1).[30–33] The result is presented in Fig. 3. A is the area in the trough, and π is the surface pressure.

$$C_S^{-1} = -A\left(\frac{\partial \pi}{\partial A}\right)_T. \tag{3}$$

In general, a lower C_s^{-1} value means an increase in the monolayer compressibility.

As can be seen from Fig. 3(a), with the increase of the content of lysozyme in the subphase, the maximum elastic modulus of DPPC monolayer gradually decreased. The maximum elastic modulus of pure DPPC is 245 mN/m and the maximum elastic modulus at 0.10 mM is about 175 mN/m. The maximum value of elastic modulus decreases rapidly when the subphase concentration is greater than 0.10 mM. The subphase concentration is 0.63 mM and 1.04 mM, and the maximum value of two lipids with the same elastic modulus is 51 mN/m. For the DPPE system, as shown in Fig. 3(b), the concentration of lysozyme is 0 mM, 0.10 mM, 0.21 mM, 0.63 mM, 1.04 mM, corresponding to elastic modulus maximum value of 232 mN/m, 181 mN/m, 74 mN/m, 65 mN/m, 57 mN/m. We can conclude that with the increase of the content of lysozyme in subphase, the maximum elastic modulus decreases. The elastic modulus can characterize monolayer compressibility. These phenomena indicate that the compressibility of the pure lipid is smaller than that of the mixed monolayer film. A certain amount of the lysozyme increases the monolayer compressibility and this may be due to lysozyme embedded into the lipid membrane, stabilizing it.

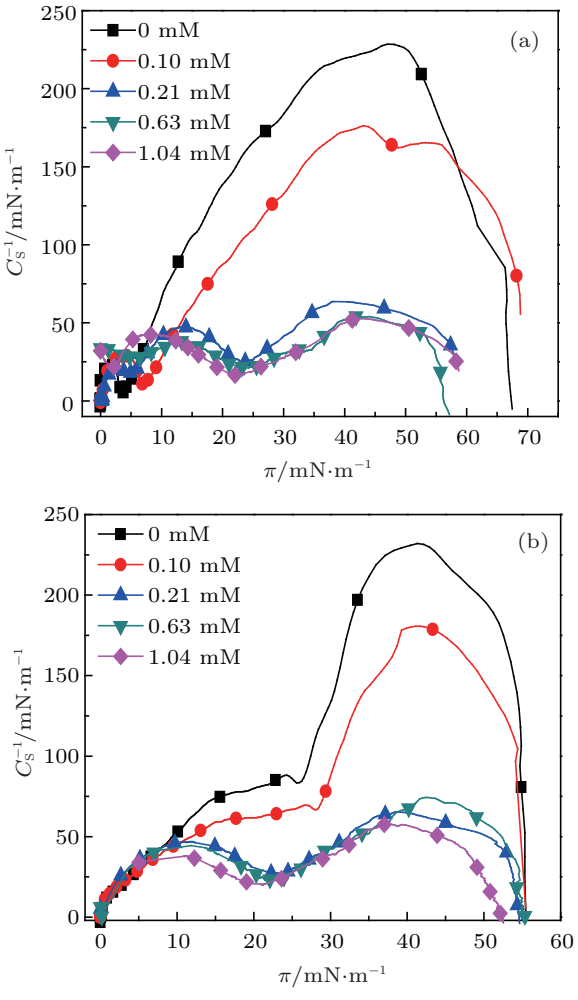


Fig. 3. (color online) C_s^{-1} calculated from π–A isotherm of different subphase concentrations of lysozyme interaction with (a) DPPC and (b) DPPE monolayers.

3.3. Penetration kinetics of the lipid films with lysozyme

As soon as the predetermined π_o had been reached with particular phospholipids on the surface, the barriers were stopped and a certain amount of lysozyme was injected into

the subphase, forming a concentration of 0.10 mM, 0.21 mM, 0.63 mM, 1.04 mM of the subphase solution. Figure 4 shows the π–T curve of LZM adsorption onto phospholipid when initial pressure is 15 mN/m. We can see that interaction of the lysozyme molecule with lipid undergoes three important stages: initial adsorption, adsorption rearrangement and equilibrium state expansion and rearrangement.[13] In addition, with the increase of concentration of lysozyme, the equilibrium pressure of DPPC showed increasing trend, suggesting that with the increase of LZM, more and more LZM are adsorbed or inserted into the monolayer. In contrast, for the DPPE system, the equilibrium pressure decreases with the increase of the amount after the addition of LZM. This may be induced by the squeezing out of LZM from the DPPE monolayer due to the stronger interaction. This is consistent with the π–A isotherms analysis results.

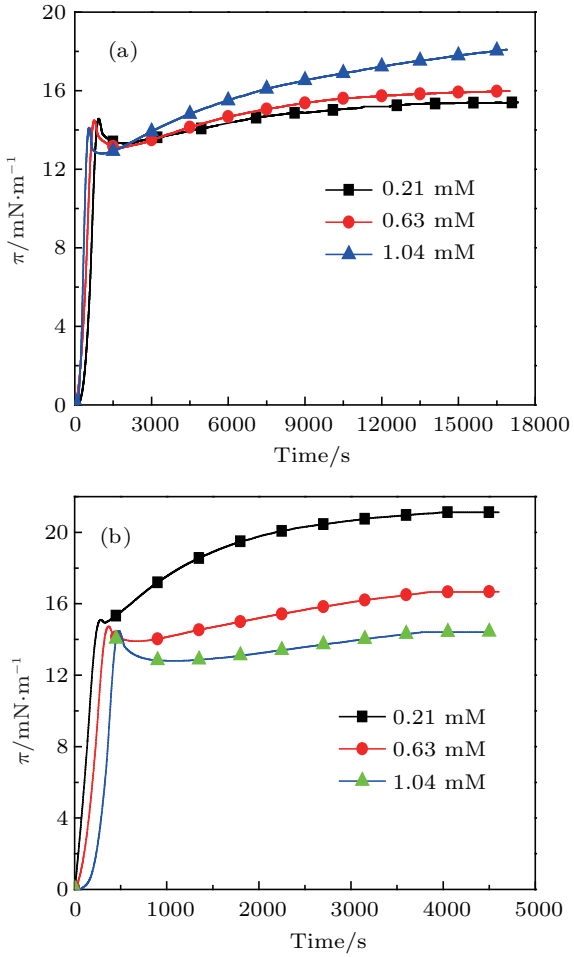


Fig. 4. (color online) π–T curves of different subphase concentration of lysozyme interaction with (a) DPPC and (b) DPPE monolayer at the initial surface pressure of 15 mN/m.

Parameters are obtained according to the π–T curve in order to study the ability of lysozyme molecules better embedded in lipid monolayer, as shown in Tables 1 and 2. When the concentration of lysozyme was 0.21 mM, the surface pressure DPPC and DPPE adsorption curve in 2500 s and 500 s, respectively, changes, showing that from now on, lysozyme begins to adsorb on the lipid monolayer,[34] interpreted as representing initial adsorption. With the increase of time, the surface pressure increases continuously, until 89951 s and 2593 s, to reach the equilibrium stage, the equilibrium pressure is 15.4 mN/m and 21.1 mN/m, respectively. This shows that more lysozyme molecules are embedded into DPPE monolayer at this concentration. In addition, the equilibrium time of interaction between lysozyme and DPPE is less than the time of the interaction with DPPC. The shapes of all the curves are very similar to that with the increase of adsorption time, in which the slope of adsorption curves at first increase and then decrease. That is in the process of lysozyme molecules with constant in a steady stream of adsorption on the membrane, until the pressure reached a relatively steady state (equilibrium pressure), protein at the interface of self-assembling and arranging.[5] The interaction of protein and lipid is mainly through physical adsorption, namely van der Waals forces. Protein and lipid interactions generally do not form chemical bonds. Physical adsorption is reversible, so we can finally see, protein did not have unlimited adsorption on the membrane, but eventually reached a dynamic equilibrium.

Table 1. Parameter values of π–T curves of LZM/DPPC.

Concentration of lysozyme/mM	Parameters		
	T/s	π_o/mN·m^{-1}	π_e/mN·m^{-1}
0.21	89951	15.0	15.4
0.63	11494	15.0	16.0
1.04	17185	15.0	18.1

Table 2. Parameter values of π–T curves of LZM/DPPE.

Concentration of lysozyme/mM	Parameters		
	T/s	π_o/mN·m^{-1}	π_e/mN·m^{-1}
0.21	2593	15.0	21.1
0.63	3257	15.0	16.7
1.04	3562	15.0	14.4

3.4. AFM observation

When the lysozyme concentration was 0.21 mM, the lysozyme molecules were adsorbed onto the DPPC lipid monolayer (see Fig. 5(b)). With the increase of concentration of lysozyme, the aggregation of lysozyme molecules is increasingly large. When the concentration was 0.63 mM, clear lipid membranes can be seen and membrane arrangement is still more regular (Fig. 5(c)). This shows that the subphase of lysozyme molecules adsorbed on the surface of the DPPC and a small part of the lysozyme molecules embedded into the lipid monolayer, and with the increase in the amount of lysozyme, lysozyme molecules on the surface of the membrane were self-assembled. Lysozyme has little effect on the

structure of the lipid monolayer. For the DPPE monolayer, when the lysozyme concentration is 0.21 mM, the same as for DPPC, lysozyme is evenly dispersed in the phospholipid. However, density is higher compared to the DPPC, as shown in Fig. 5(f). With the increase of the amount of lysozyme, lysozyme aggregation and its lipid molecules around the center to attract to the surrounding, eventually causes the DPPE film morphology to have a greater impact. LZM interaction with DPPC and DPPE is obviously different. This may be possibly due to the different head groups. The head group of DPPC and DPPE are similar in charge and differ in size and orientation of the head groups. More space is required for the larger and more PC head group than for the PE head group.[20,26] Previous studies have shown that as lysozyme adsorbs to a PC film, it causes a stretching of the O–P–O diester bond, but at the same time lowers the chain-disorder transition temperature by a few degrees. Penetration is difficult once adsorption has occurred because even though there are polar interactions between lysozyme and the acidic phospholipids, it is excluded from interacting with the hydrophobic portion of the lipid below the chain transition temperature.[20] Our experiments were all carried out below the transition temperature for PC (~ 41 °C). We suggest this may cause a stretching of the O–P–O diester bond. Then penetration is more difficult for lysozyme to adsorb to the DPPC than DPPE monolayer. In addition, with surface pressure at 15 mN/m, DPPC is in the liquid condensed phase and molecules are arranged closely whereas DPPE is in liquid expanded phase and molecules are arranged loosely, so lysozyme is easily embedded into the lipid membrane, and the membrane LZM molecular quantity increases.

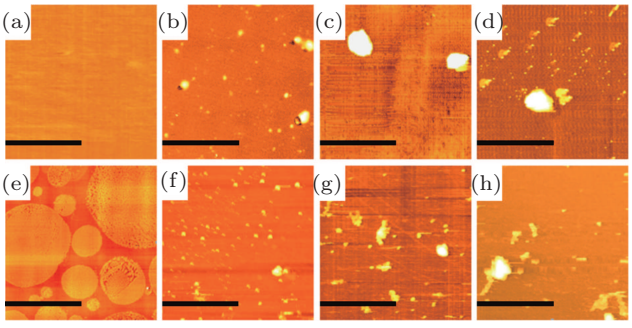


Fig. 5. (color online) The AFM images of DPPC and DPPE monolayer at different concentrations LZM amount : (a) DPPC; (e) DPPE; (b), (f) 0.21 mM; (c), (g) 0.63 mM; (d), (h) 1.04 mM; scale bar: 5 μm.

A more detailed observation of the effect of lysozyme on the DPPE monolayer is shown in Fig. 7. There are a large number of lysozyme molecules adsorbed on the DPPE monolayer, varying in size and shape. In this paper, the information of the diameter and height of lysozyme are statistical. The results show that the height and diameter of lysozyme are in the range of 3.7 nm–13.9 nm and 47.5 nm–66.8 nm respectively. The three-dimensional structure of the lysozyme molecule is nearly elliptical, and its size is 4.5 nm×3 nm×3 nm, which was obtained by x-ray analysis method in 1965.[34] Through observation and analysis, lysozyme forms a cone polymer after adsorption on a layer. Lysozyme not only changed the conformation of lipid monolayer, but also self-assembly. This is consistent with the π–T isotherms analysis results.

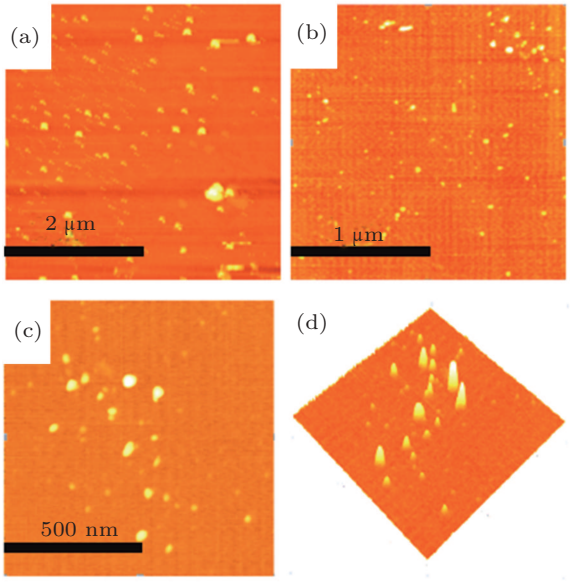


Fig. 6. (color online) The AFM images of DPPE monolayer at 0.21 mM of LZM concentrations. Scanning range: (a) 5 μm×5 μm; (b) 2.5 μm×2.5 μm; (c) 1.25 μm×1.25 μm; (d) three-dimensional graph of panel (c).

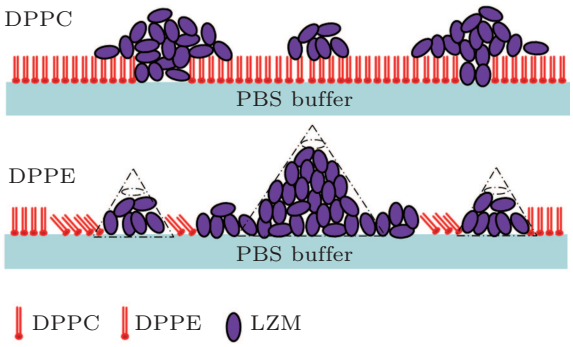


Fig. 7. (color online) Interaction mechanism model of lysozyme molecule with DPPC and DPPE monolayer.

Through the analysis of the lysozyme molecule aggregation mechanism may be related to amyloid fibril nucleation stage. Similar models suggest that the proteins and peptides monomer under certain conditions form the core fiber, and the fiber core and the monomer binding, Ever fount monomer and nuclear binding form fibrils further integrated fiber.[36] This model consists of two stages: nucleation stage and elongation stage.[37] The extension of the protein fiber can be interrupted and then form a new kernel, so as to shorten the formation time of skin amyloid fibers. In this process, lysozyme molecules are able to aggregate into its nucleus, inevitably accompanied by thermodynamic changes of the molecular aggregation of lysozyme. This may be induced by lipid,[38] but

when lysozyme molecules are adsorbed to the lipids, lipid arrangement because of protein insertion into it. Previous literature reported that lysozyme can form amyloid fibrils under certain limiting conditions.[36,38] The formation of lysozyme molecules was similar to the first stage of amyloid fibrils. Therefore, the lysozyme molecules can form oligomers under the induction of lipids.

Possible interfacial structural changes of the mixed lysozyme/DPPC and lysozyme/DPPE films on compression are schematically depicted in Fig. 7. For DPPC at the surface pressure of 15 mN/m, the monolayer was in the liquid condensed phase, lipid molecules were arranged closely, a small amount of lysozyme molecules embedded into the membrane, and lysozyme molecules will form irregular multimers. However, DPPE at this surface pressure which is in liquid expanded phase, has molecules arranged loosely, so lysozyme molecules easily embedded into the lipid membrane, and the number of LZM molecules on lipid membranes increased, forming conical multimeric.

4. Conclusions

In this paper, the interaction between lysozyme and lipid monolayer was studied by observing the morphology and analyzing the thermodynamic isotherm. The results show that lysozyme with DPPE is stronger than DPPC, which may be due to the different bonding capacity of head group. For DPPC at the certain surface pressure of 15 mN/m, the monolayer is in the liquid condensed phase, lipid molecules are arranged closely, a small amount of lysozyme molecules embedded into the membrane, and lysozyme molecules will form irregular multimers. However, DPPE at this surface pressure which is in liquid expanded phase, has molecules arranged loosely, lysozyme molecules easily embedded into the lipid membrane, and the number of LZM molecules on lipid membranes increased, forming conical multimeric. Penetration of lysozyme into the lipid layer leads to a decrease of surface tension and the membrane fusion process accompanied by the antibacterial activity may be related to the interaction of lysozyme and phospholipids. In addition, the aggregation mechanism of lysozyme aggregates is similar to amyloid fibrils.

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