Effect of lysozyme subphase and insertion on several lipid films

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Abstract

The influence of proteins on lipid monolayers is a subject of biological interest. In this work the influence of lysozyme on lipid films of stearic acid, oleic acid, cholesterol, DPPC and POPC has been studied. The Langmuir monolayer technique, using surface pressure–area isotherms and elastic modulus plots, as well as protein insertion experiments in lipid monolayers, have been used. Results indicate that lysozyme affects the lipid monolayer formation, the elastic modulus and, sometimes, the physical state of the monolayer. This influence is more important till moderate surface pressures. At high surface pressures and near the physiological value of lateral pressure of 33 mN/m, there is expulsion of lysozyme out of the monolayer. This expulsion is more important for stearic acid, DPPC and POPC. The lower value of maximum insertion pressure is for stearic acid and the higher one is for oleic acid. The relation between the initial and the increased surface pressure in insertion experiments has been analysed.

Introduction

Fatty acids, cholesterol and phospholipids have been studied with the Langmuir technique because of their biological interest and they form ordered and compact monolayers. Interactions of proteins with lipid membranes and films are of great interest for biological systems as cellular membranes, tear films [1], and mimicking them for technological applications. Studies on surface properties of protein solutions are described elsewhere [2–11]. Similarly, some studies of the influence of proteins on lipid monolayers are those of references [12–35]. The interpretation of protein influence and interactions with lipid monolayers has been studied in [5,36–40].

Only some of these cited works have studied lysozyme [9,11] or the influence of lysozyme on lipid films [17,18,26,27]. On the other hand, the influence of lysozyme on liposomes are those of references [41–43]. Biological functions of lysozyme have been suggested to be determined by the lipid binding properties of this protein [41]. Several contributions have been proposed for that, including electrostatic and hydrophobic ones. Lysozyme is a tear film protein, with bacteriostatic functions among others, and its influence on Meibomian lipid films has been studied by Miano et al. [1] and Mudgil et al. [34], but Meibomian lipid films from tears are a complex mixture of lipids.

In this work the binding properties and insertion of lysozyme on lipid layers will be studied using the monolayer technique and analysed using the method proposed in reference [37]. The lipids selected are neutral, slightly ionised or zwitterionic, in order to discard neat charge contributions. The specific systems here proposed, that is the influence of lysozyme on lipid films of stearic acid, oleic acid, cholesterol, DPPC and POPC, which are different in nature (fatty acids, sterol, phospholipids), have not been studied previously. Some of them present an unsaturation on the fatty acid or in the phospholipid hydrocarbon chains, which permits to see the influence of unsaturations on the effect of lysozyme. The Langmuir monolayer technique using P–A isotherms and compressibility plots, as well as protein insertion experiments in lipid monolayers, have been used since this technique permits the control of the surface pressure.

Materials and methods

Materials

Oleic acid (OA) was provided by Fluka and palmitoyloleoylphosphatidylcholine (POPC) was purchased from Avanti Polar Lipids. Stearic acid (SA), dipalmitoylphosphatidylcholine (DPPC), cholesterol (CHOL) and lysozyme (LYS) from chicken egg white were provided by Sigma-Aldrich. Chloroform of analytical grade from Sigma-Aldrich were used in lipid solution preparation. Water was ultrapure MilliQ® (18.2 MΩ·cm).

Techniques

Monolayer formation: Langmuir monolayer formation was carried on a trough (Nima Technology, Cambridge, UK) model 1232D1D2 equipped with two movable barriers. The surface pressure was measured using paper Whatman® held by a Wilhelmy balance connected to a microelectronic system registering the surface pressure (II). The paper sheet was always cleaned and conditioned in the subphase prior measurements. In one case, the subphase used in these experiments was MilliQ® quality water. In other case, the subphase used was a solution containing LYS (at a concentration of 0.1 g·L–1). Prior to the subphase addition, the trough was cleaned twice with chloroform and once with MilliQ® quality water. Residual impurities were cleaned.

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from the air-liquid interface by surface suctioning. Solutions of the lipids were prepared using chloroform (at concentrations of 0.5 mg·mL⁻¹) and an aliquot of 100 µL was spread at the air-liquid interface using a high precision Hamilton microsyringe. Barrier closing rates were fixed at 50 cm²·min⁻¹. Surface pressure-area isotherms were recorded, in one case, for the LYS subphase without lipid addition, and in the other case recording was carried out adding the lipid solution drop by drop to the subphase, waiting 15 minutes for perfect spreading and solvent evaporation. X-axis of the isotherm plot represents the area per molecule. Experiments were conducted at 22 ± 1°C.

**Insertion experiments**

The insertion of LYS into lipid monolayers was measured using a NIMA Langmuir Film Balance equipped with a Wilhelmy plate (Nima Technology, Coventry) and a small Teflon trough that was rinsed with chloroform and distilled water before use. All experiments were performed at 22 ± 1°C.

For these experiments, a lipid stock solution was prepared and added drop wise on the water subphase until the desired lipid pressure was achieved. After 15 min, when the equilibrium of the lipid monolayer was reached, a LYS solution was injected into the water subphase to attain a concentration of 0.1 g·L⁻¹. The surface pressure changes were monitored as a function of time during 15 min. For a blank experiment the same procedure was followed but without using lipid.

**Results and discussion**

**Surface lysozyme behaviour**

In this section the surface behaviour of LYS is reported.

At first the surface tension of the subphase with 0.1 g·L⁻¹ (6.94 10⁻⁶ M) LYS solution was measured, obtaining a value of 71.0 mN/m. This value is only slightly lower than that of water (72.6 mN/m at 22°C). Also, a value of pH=5.0 was measured for this solution, which is only slightly more acidic than water (pH=6.0). A fresh LYS solution of 0.1 g·L⁻¹ have shown a value of zero for the surface pressure when recording an isotherm (Figure 1). A similar behaviour was observed for asparaginase [44] on its aqueous subphase, during compression.

**Influence of lysozyme over lipid monolayer isotherms.**

The presence of LYS in the subphase and its influence on lipid monolayers has been investigated. The lipids used are DPPC, POPC, oleic acid (OA), stearic acid (SA) and cholesterol (CHOL).

Figures 2A-6A show the surface pressure-area per molecule isotherm for these lipids in water and in 0.1 g·L⁻¹ LYS aqueous subphase. Figures 2B-6B show the calculated elastic modulus, 

\[
\gamma = -A\left(\frac{d\pi}{d\lambda}\right)
\]

(1)

Figure 2A shows the surface pressure-area isotherms for DPPC in the absence and in the presence of LYS in the subphase. Figure 2B shows the elastic modulus obtained from these isotherms. A notable influence of LYS on DPPC monolayer behaviour is observed at low and moderate surface pressures. The lift off area increases in respect to DPPC, then a first inflection at around 8 mN/m, usual for DPPC, occurs and a second inflection is observed at around 20 mN/m. After the second inflection the isotherm in presence of LYS gets closer to that in its absence, and the collapse surface pressure is practically the same, with only a small increase in the collapse area. In addition, the elastic modulus shows a decrease in presence of LYS in respect to what happens in its absence after the first inflection, with a phase change from a LE state to another LE state at the first inflection, according to the 

\[
C_{\gamma}^{-1} \text{ values, that we are going to indicate as LE1 to LE2, and a phase change from LE2 to LC at the second inflection. DPPC in the absence of LYS shows the well stablised phase change from LE to LC at the inflection point around 8 mN/m.}
\]

Fainerman et al. [47] studied another protein system and proposed that 

\[
\beta\text{-lactoglobulin penetration into a DPPC monolayer induces a first-}
\]

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order phase transition in the fluid-like monolayer, and after the phase transition point, condensed domains similar to those of pure DPPC are formed. These authors also indicate that the adsorbed protein induces the condensation of the pure DPPC compound: At the beginning, aggregation to condensed phase structures does not occur, induced by the penetrated species, and that the condensed phase consists only of the insoluble monolayer component.

Figure 3A shows the surface pressure-area isotherms for POPC in the absence and in the presence of LYS in the subphase. Figure 3B shows the elastic modulus obtained from these isotherms. It is observed a notable influence of LYS on POPC monolayer behaviour at low and moderate surface pressures. The lift off area increases and an inflection is observed at around 20 mN/m. After the inflection, the isotherm in presence of LYS gets closer to that in its absence and the collapse point is practically the same. In addition, the elastic modulus shows a decrease in presence of LYS in respect to what happens in its absence, especially after the inflection, with a phase change from LE1 to LE2. POPC in the absence of LYS does not show a phase change.

Figure 4A shows the surface pressure-area isotherms for OA in the absence and in the presence of LYS in the subphase. Figure 4B shows the elastic modulus obtained from these isotherms. It is observed a notable influence of LYS on OA monolayer behaviour at low and moderate surface pressures. The lift off area increases considerably and an inflection is observed at around 23 mN/m. After the inflection, the isotherm in presence of LYS gets closer to that in its absence but with still an important area increase, and the collapse surface pressure and area are bigger in respect to those of OA in absence of LYS. In addition, the elastic modulus shows a notable decrease in presence of LYS, and at the inflection a phase change from LE1 to LE2 is observed. OA in absence of LYS does not shows a phase change, remaining in a LE state at all surface pressures.

Figure 5A shows the surface pressure-area isotherms for SA in the absence and in the presence of LYS in the subphase. Figure 5B shows the elastic modulus obtained from these isotherms. It is observed a notable influence of LYS on SA monolayer behaviour at low and moderate surface pressures. The lift off area increases considerably and an inflection is observed at around 23 mN/m. After the inflection, the isotherm in presence of LYS gets closer to that in its absence, and practically superpose. The value of the surface pressure at the inflection is practically the same as that usual of SA, and the collapse point is practically the same. In addition, the elastic modulus shows a decrease in presence of LYS. Meanwhile SA shows a phase change from LC to S at the inflection in the absence of LYS, a phase change from LE to LC and from LC to S is observed in the presence of LYS. This second phase change, at around 46 mN/m, is visible in the elastic modulus plot more clearly than in the isotherm.

Figure 6A shows the surface pressure-area isotherms for CHOL in the absence and in the presence of LYS in the subphase. Figure 6B shows the elastic modulus obtained from these isotherms. It is observed a notable influence of LYS on CHOL monolayer behaviour at low and moderate surface pressures. The lift off area increases and an inflection is observed at around 23 mN/m. After the inflection, the isotherm in presence of LYS gets closer to that in its absence and practically coincides, and the collapse point is practically the same. In addition, the elastic modulus shows a notable decrease in presence of LYS, in respect to what happens in its absence, with a phase change from LC to S at the inflection. CHOL in absence of LYS shows the well stablished S state at all surface pressures.
In order to see the affinity towards lipids, the ability of LYS to insert into monolayers of the lipids studied here was monitored by measuring variations in surface pressure, ΔΠ. For that different initial surface pressures, Πᵢ, were used and protein was injected in order to obtain a 0.1 g·L⁻¹ concentration in the subphase. The general observed trend is that, greater the Πᵢ, lower the ΔΠ. That is, at a closer packing of the lipid at higher initial surface pressures corresponds a lower degree of incorporation of the protein into the monolayer (Figure 7).

The maximum insertion pressure (MIP), that is, the surface pressure above which the protein does not penetrate into the monolayer, was obtained by extrapolating the plot ΔΠ vs Πᵢ to ΔΠ = 0 mN·m⁻¹ [37]. The MIP of proteins in lipid monolayers has been shown to be useful in characterizing protein adsorption and lipid specificity. Table 1 shows these values. It is noteworthy that the MIP values obtained for POPC and OA are larger than the membrane lateral pressure, which has been estimated to be in the range of 30-35 mN/m.

The sequence on MIP, in the ascending order of values, is SA, DPPC, CHOL, POPC, and OA. That means that OA allows the insertion till higher surface pressures, meanwhile SA allows insertion till the lowest surface pressure values. The values of MIP for DPPC, SA and CHOL are closer to the values of the inflection point observed in the isotherms in presence of LYS. This observation coincides with the fact that the isotherm becomes close to that in absence of LYS. It means that at this surface pressure, around 20-24 mN/m, the expulsion of LYS should occur from the monolayer while compressing. A value of 20 mN/m reported by Mudgil et al. [34] for DPPC is close to the value of MIP found by us.

For POPC the value of MIP32.6 is higher than that of the inflection point but is close to the surface pressure where isotherms become closer. For OA the high value of MIP OA the high value of MIP44.6 is in 44.6 is in accordance with that observed in the isotherm, where the influence of LYS occurs even at high surface pressures. Miano et al. [1] reported that several proteins (lysozyme, lactoferrin, albumin, slgA, B-lactoglobulin, tear lipocalin) were excluded from the interface formed by Meibomian lipids, obtained from tear films, at a surface pressure above 30 mN/m. Meibomian lipids are a complex mixture and only the MIP of POPC agrees with the later reported value, indicating that POPC could be a good model for Meibomian lipids.

Considering the physical states of the lipids in the monolayer, it is seen that LYS shows higher MIP for LE states (POPC, OA). These results indicate that LYS insertion is easier in monolayer films of those lipids that have unsaturated acyl chains, meanwhile the insertion is more difficult in those lipids that form more compact monolayers, for which the MIP is around 20-24 mN/m. This value is similar to other published values with different proteins and phospholipids [33,37]. A value of 20.8 mN/m was observed for the MIP of rhodopsin in DDDA-PC monolayers [33], and a similar value was observed for retinitis pigmentosa 2 with DDHA-PC monolayers [37]. Nevertheless, other values were observed for other protein-phospholipid systems [37,48].

Calvez et al. [37] proposed the eq. 2 to analyse the binding, insertion and interaction of proteins in lipid monolayers.

\[ Π_I = aΠ_i + b \]  

(2)

Analysing the eq. 2, that it is equivalent to \( ΔΠ = (a-1)Π_i + b \), where \( Π_i \) is the initial surface pressure and \( Π_I \) the surface pressure after insertion, the values of \( a \) and \( b \) are obtained (Table 1). Value of \( a = 0 \), being called the synergy factor, corresponds to favourable conditions for protein monolayer binding [37]. Value of \( b \) is the extrapolation of \( ΔΠ \) at zero.
surface pressure. The higher value of the parameter a corresponds to DPPC, followed by POPC, CHOL, OA and SA. According to the value of a, the binding of LYS is more favourable for DPPC. On contrary, values of the parameter b increase in the sequence DPPC, POPC, CHOL, SA and OA. Phospholipids present values of a higher than those of fatty acids or CHOL, and the contrary occurs with the values of b. Thus, even DPPC and POPC allows the insertion, the effect on the increase of $\Pi$ is low. The highest effect is shown by OA.

The parameter b must be described as the trend of the monolayer of modifying the protein surface activity [37]. When $b$ is larger than the protein surface tension, protein would insert within the lipid acyl chains. (This happens for SA, OA and CHOL, but no for DPPC and POPC). For low values of $b$, it can be postulated that protein would not be inserted more deeply than the polar headgroup of the lipid monolayer. In contrast when $b$ is high, protein would insert within the lipid acyl chain.

**Global Discussion**

Different behaviours have been observed about the influence of LYS on lipid monolayers depending on the lipid, but the isothem shape in presence of LYS shows more differences with respect to that in the absence of LYS at low compressions for all lipids. This could indicate that at the first stages of compression, this one is more dependent on the protein. The pKa values for SA and OA have been reported to be 10.15 and 9.85, respectively [49], or also 9.0 for SA [49], even lower values were reported elderly (5.63 and 6.22 for SA and OA [50]). These values indicate that at the working pH of 5.0 these acids are present basically in the neutral form, but we have observed a noticeable influence of LYS on the fatty acids, OA and SA, at low compressions. As has been reported that the attractive electrostatic interaction seems to favour the insertion of LYS in the fatty acid monolayer [41] it is plausible to propose that in presence of lysozyme (isoelectric point $IP=10.7$ [51]), fatty acids deprotonated more easily acquiring negative charge and then interacting electrostatically with the positive charges of lysozyme. CHOL has no charge and zwitterion PCs have no net charge. Miller et al. [40] says that the presence of only small amounts of ionic surfactants can significantly modify the structure of adsorbed proteins. With increasing amounts of ionic surfactants, an opposite effect is reached as due to hydrophobic interactions and the protein can be displaced from the interface due to competitive adsorption. In the presence of non-ionic surfactants, the adsorption layer is mainly formed by competitive adsorption and the only interaction is of hydrophobic nature. From a certain surfactant concentration, the interface is covered almost exclusively by the non-ionic surfactant.

When compressing SA, which forms compact monolayers, LYS is practically expelled at high surface pressures (the difference in area between isotherms in absence and in presence of LYS is very small, as well as the difference in the collapse). This expulsion is gradual with the phase change of SA. On the other hand, OA does not form monolayers as compact as SA and thus the expulsion of LYS is not so important (the difference in area between isotherms in absence and in presence of LYS is significant even at high surface pressures, and the collapse surface pressure increases in presence of LYS). And in the case of CHOL, it even forms compact monolayers, the insertion of LYS is notable, the difference in area between isotherms in absence and in presence of LYS is significant at low surface pressures but insignificant at high surface pressures, and the collapse surface pressure doesn’t change. Thus, CHOL shows a behaviour more similar to SA than to OA. This could be due to the degree of compactness shown by SA and CHOL.

In the case of DPPC the expulsion of LYS is practically total at medium surface pressures (the difference in area between isotherms in absence and in presence of LYS is practically null, and the collapse surface pressure is the same). In the case of POPC there is also an expulsion practically total at medium surface pressures (the difference in area between isotherms in absence and in presence of LYS is also practically null, and the collapse surface pressure is also practically the same). Thus, even DPPC and POPC differs in one unsaturation and that POPC forms monolayers less compact than DPPC (due to the unsaturation present in one of the hydrocarbon chains), no much differences were observed for LYS influence in the isotherms. But the insertion experiments indicate more difficulty for LYS to insert in DPPC than in POPC, which is in agreement with the tendency of DPPC to form more compact monolayers than POPC, due to the presence of an unsaturation in the later. A lower lysozyme insertion for DPPC in respect to other phospholipids was also observed by Mudgil et al. [34], where PE, PS and PG phospholipids with unsaturations in the acyl chains were used. Moreover, PS and PG are anionic phospholipids.

DPPC and POPC are zwitterion phospholipids, and at a surface pressure around 33 mN/m, close to the physiologic value of natural membranes, the effect of lysozyme on the phospholipid monolayer is practically null. Gorbenko et al. [41] studied the binding of lysozyme to phospholipid bilayers and found an influence of the content of anionic phospholipids on the binding of lysozyme. In our study, no anionic phospholipids were used, but ionisable anionic fatty acids, and these fatty acids are those that show a higher influence.

Comparing all the lipids at high surface pressures, SA and DPPC show similar behaviour due to the absence of unsaturations in the hydrocarbon chains, while OA and POPC show differences even the presence of an unsaturation. This fact could be explained as POPC also have a saturated chain that allows to form more compact monolayers in respect to OA. CHOL presents a specific behaviour which is more similar to that of SA and phospholipids than to that of OA.

![Figure 7. Surface pressure increase ($\Delta\Pi$) in front of the lipid initial surface pressure ($\Pi$), caused by LYS insertion on monolayers of several lipids: DPPC (black), POPC (blue), SA (grey), CHOL (red), OA (yellow).](image-url)

| Lipid  | DPPC | POPC | OA  | SA  | CHOL |
|--------|------|------|-----|-----|------|
| MIP    | 22.6 | 32.6 | 44.6| 19.8| 24.0 |
| a      | 0.947| 0.914| 0.529| 0.417| 0.766|
| b      | 1.20 | 2.79 | 21.01| 11.55| 5.61 |
| State in absence of LYS | LE-LC | LE | LE | LC-S | S |
| State in presence of LYS | LE1-LE2-LC | LE1-LE2 | LE1-LE2 | LE-LE2-C-S | LC-S |
It is also seen that different sequences are obtained depending on the parameter if \(a\), \(b\) or \(M\) IP are used. The sequence, in the ascending order of values, is SA, DPPC, CHOL, POPC and OA for MIP; SA, OA, CHOL, POPC and DPPC for the parameter \(a\); DPPC, POPC, CHOL, SA and OA for the parameter \(b\). MIP is more related to compactness and physical state of the monolayer, and condensed monolayers provide lower MIP. The parameter \(a\) is higher for phospholipids, meanwhile the parameter \(b\) is lower for them, and thus the interaction of lysozyme is more favourable with the headgroups of PCs. As DPPC forms condensed monolayers, thus the insertion between acyl chains is not favoured, but for POPC the presence of the double bond makes the monolayer more fluid and the insertion of lysozyme is more favourable, even according to the parameter \(b\) this insertion is near the headgroups. For SA and CHOL, that form condensed monolayers, the insertion is only possible at low surface pressures, and according to the parameter \(b\) this insertion takes place within the acyl chains. OA presents a double bond in the acyl chain and forms the most fluid monolayer, and consequently the MIP is the highest and the parameter \(b\) is also the highest, indicating the facility of lysozyme to insert within the acyl chains of OA.

Conclusions

According to surface pressure-area isotherms and elastic modulus plots, it is observed that lysozyme affects the lipid monolayer formation. This influence is more important till moderate surface pressures, but at high surface pressures there is expulsion of lysozyme out of the monolayer. This expulsion is more important for stearic acid, DPPC, POPC and cholesterol, meanwhile for oleic acid the isotherms indicate that a residual content of lysozyme remains in the monolayer. The sequence on Maximum Insertion Pressure (MIP) in the ascending order of values, is SA, DPPC, CHOL, POPC and OA. Even DPPC and POPC allows the insertion, the effect on the increase of \(H\) is low, and it can be postulated that in this case protein would not be inserted more deeply than the polar headgroup of the phospholipid monolayer. For CHOL and especially for SA and OA this insertion takes place between the acyl chains.

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