Interaction of lysozyme amyloid fibrils with Langmuir monolayers

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The capability of amyloid lysozyme fibrils to insert into phospholipid monolayers composed of dimyristoylphosphatidylcholine (DMPC) and its mixtures with dimyristoylphosphatidylglycerol (DMPG) or dimyristoylphosphatidylserine (DMPS) in different molar ratios was studied using the Langmuir monolayer technique. Fibrillar lysozyme was found to possess a greater ability to penetrate the lipid monolayer compared to the native protein. Electrostatic interactions between cationic groups of the protein and negatively charged head groups of the phospholipids were shown to play a significant rôle in the insertion of lysozyme fibrils and monomers into the model membranes. Evaluation of the limiting surface pressure suggests that both lysozyme monomers and fibrils are able to insert into biological membranes.

Keywords: amyloid fibrils, Langmuir monolayers, lysozyme, membrane penetration, monomers

1. INTRODUCTION

The conversion of specific proteins from their soluble native states into highly organized fibrillar aggregates (amyloid fibrils) is a cause of numerous human disorders, such as Alzheimer's and Parkinson's diseases, systemic amyloidosis, type II diabetes, etc. [1, 2]. Fibrillar aggregates associated with different pathologies were found to share common structural properties [3, 4]. Specifically, X-ray diffraction and solid state NMR studies revealed that fibrils are characterized by the presence of a cross-β structure in which β-strands are aligned perpendicular to the long axis of the fibril [5–8]. An increasing interest in protein folding and aggregation has led to the proliferation of both theoretical and experimental studies on the structure and toxicity of amyloids.

Protein fibrillization proceeds via the formation of metastable oligomeric intermediates [9, 10]. A number of studies have shown that prefibrillar aggregates are the primary cytotoxic species, which produce cell dysfunction and death through membrane permeabilization [11–14]. It has been reported that oligomeric aggregates of various disease-related proteins, in contrast to monomers or fibrils, disrupt the integrity of both plasma and intracellular membranes, giving rise to significant elevations of Ca^{2+} concentration [15]. Membrane permeabilization induced by amyloid oligomers was found to initiate increased production of reactive oxygen species [16]. Furthermore, membrane thickness and capacitance measurements revealed that insertion of Aβ oligomers into planar lipid bilayers leads to domain formation and a diminished dielectric barrier to ion transfer [17]. Alterations in the bilayer dielectric properties under the influence of oligomeric amyloid β-peptide was also observed by Kayed et al. [18] and Sokolov et al. [19]. In a recent study on amyloid cytotoxicity, lysozyme oligomeric aggregates were found to cause destabilization and permeabilization of the mitochondrial membrane followed by essential (up to 60%) release of mitochondrial enzymes, whereas interaction of mature fibrils with mitochondria did not result in any damage to the mitochondrial membrane [20].

In spite of significant evidence supporting the notion that soluble prefibrillar oligomers are responsible for cytotoxicity, a growing number of recent reports have demonstrated membranotropic activity of mature amyloid fibrils [21–24]. In particular, a high toxicity of amyloid fibrils of recombinant mammalian prion protein to cultured cells (hippocampal and cerebellar neurons) was observed by Novitskaya and coworkers [21]. Hen egg white lysozyme (HEWL) fibrils were found to initiate extensive aggregation of human erythrocytes and POPC:POPG: cholesterol lipid vesicles [24]. It should be noted that the toxicity mechanisms of amyloids can involve fibril fragmentation leading to the production of shorter fibrillar aggregates, which possess a higher cytotoxic activity comparing to their longer counterparts [25]. Therefore, cell dysfunction in the conformational diseases mentioned above could also be attributed to the mature amyloid fibrils. Interestingly, a number of reports have shown that the extent of cell damage produced by amyloids depends on their specific structural and stability properties [26–28]. Heterogeneity and polymorphism of protein assemblies originating from differences in the destabilizing conditions seem to be the main factors modulating toxic effects of amyloid aggregates [29].

An alternative hypothesis of a pathological pathway in amyloid-related diseases has been proposed by Engel

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and coworkers [30]. According to their observations, oligomers or fibrils of human islet amyloid polypeptide associated with type 2 diabetes are not the primary agents responsible for cell damage, but membrane disruption is caused by the process of fibril growth in the lipid matrix. Aggregated proteins were found to possess exposed hydrophobic surfaces [31], which may account for bilayer insertion of polypeptide chains, leading to membrane damage and cell dysfunction.

Thus, the particular structure of toxic protein aggregates and the exact mechanisms of membrane permeabilization remain controversial. Elucidating the molecular-level mechanisms of amyloid toxicity is important for the development of effective therapeutic agents. In the present work we focused our efforts on examining the interactions between lysozyme amyloid fibrils and model lipid membranes using the Langmuir monolayer technique. Lysozyme fibrillization is associated with non-neuropathic systemic amyloidosis [9]. Chicken egg white lysozyme, which is structurally homologous to human lysozyme, has been used as a model to study the initial step of interaction between lysozyme fibrils and membranes. Langmuir monolayer methodology is often employed for studying peptide or protein adsorption and insertion into model membranes [32, 33]. This rather simple technique allows the insertion kinetics of proteins into a lipid film to be monitored and the affinity of proteins for monolayers of various compositions to be characterized quantitatively. The ability of native lysozyme to adsorb and penetrate into phospholipid monolayers has been previously reported as predominantly due to electrostatic interactions [34, 35]. A great number of Langmuir monolayer experiments have been carried out to shed light on amyloid–lipid interactions. Particularly, the insertion of amyloid precursor protein and amyloid β-peptide (Aβ) into cholesterol-rich monolayers was demonstrated by Lahdo [36] and Ji et al. [37], respectively. A study of the surface behaviour of amyloidogenic peptides revealed the high affinity of Aβ and human prion protein for monolayers containing sphingolipids [38]. Furthermore, islet amyloid polypeptide (IAPP) exhibited strong penetration into negatively charged monolayers in monomeric form, in contrast to fibrillar aggregates of IAPP [39]. Along with its ability to adsorb on anionic and zwitterionic monolayers, the Aβ peptide was found to undergo lipid-induced conformational changes [40].

From this wealth of results we can conclude that the Langmuir monolayer technique is useful for tracing molecular details of the interactions between amyloid proteins and model lipid membranes. In the present study, we have examined the capability of fibrillar lysozyme to insert into lipid monolayers composed of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and its mixtures with 1,2-dimyristoyl-sn-glycero-3-phospho-rac-(1-glycerol) (DMPG) or 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (DMPS) in different molar ratios (10, 20 and 40 mol% DMPG or DMPS). The use of negatively charged phospholipids was dictated by the aim to gain further insights into the rôle of electrostatics in the lysozyme–lipid interactions.

The interaction of fibrillar lysozyme with model lipid membranes was investigated using the Langmuir monolayer technique. Protein insertion into the phospholipid monolayer was inferred to be the cause of an increase in the surface pressure of the film. The penetration ability of lysozyme fibrils was compared to that of the monomeric protein. The monolayer experiments were performed with lipid monolayers composed of DMPC and its mixtures with DMPG or DMPS in various molar ratios (10, 20 and 40 mol% DMPG or DMPS) at several initial surface pressures (π_0).

2. MATERIALS AND METHODS

2.1. *Materials*

The lipids (DMPC, DMPG and DMPS) were purchased from Avanti Polar Lipids (Alabaster, Alabama) in powder form. Chicken egg white lysozyme was obtained from Sigma (St. Louis, Missouri).

2.2. *Preparation of lysozyme fibrils*

The reaction of lysozyme fibrillization was initiated by protein incubation in 10 mM glycine buffer (pH = 2.2) at 60 °C during 8 days.

2.3. *Transmission electron microscopy (TEM)*

5 μL lysozyme samples (558 μM) were adsorbed on carbon-coated copper 200 mesh grids. After 1–2 min, excess fluid was drawn out using a paper filter, and a drop of 1% uranyl acetate was added. After the next 2 min, excess stain was removed and the samples were washed with deionized water, and viewed with a transmission electron microscope (Tecnai 12 BioTWIN; FEI Company, USA).

2.4. *Monolayer measurements*

Changes in the surface pressure of the phospholipid monolayer, induced by fibrillar or native lysozyme, were measured with a DeltaPi-4 tensiometer (Kibron, Finland). A Teflon trough was filled with 1.3 mL of filtered 10 mM Hepes buffer (pH 7.4). The sub-phase was continuously stirred with a magnetic bar during the measurement. A monomolecular lipid film was formed by carefully spreading the lipid solution (100 μM in chloroform) with a Hamilton syringe on the buffer surface to the desired initial surface pressure π_0 (5–25 mN/m). After the lipid

monolayer was stable, 25 μL of fibrillar or native lysozyme (8.9 μM) was injected into the sub-phase. The surface pressure was monitored until a stable value was reached, usually within 15 min. For each sample, the increase of surface pressure $\Delta\pi$ was determined as a function of π_0. Linear fitting of $\Delta\pi$ *versus* π_0 yielded a straight line with negative slope, which intersects the *x*-axis at the limiting surface pressure. This parameter is defined as the highest surface pressure of a monolayer below which a protein can insert; its value is used as a quantitative characteristic of the ability of a protein to penetrate into membranes of particular compositions.

3. RESULTS AND DISCUSSION

The formation of lysozyme amyloid fibrils under the acid denaturing conditions employed was confirmed by transmission electron microscopy. As shown in Fig. 1, mature lysozyme fibrils exhibited a typical structure. They were characterized as long, filamentous, straight and partly twisted species with diameter of 15–20 nm and a length of more than 1 μm.

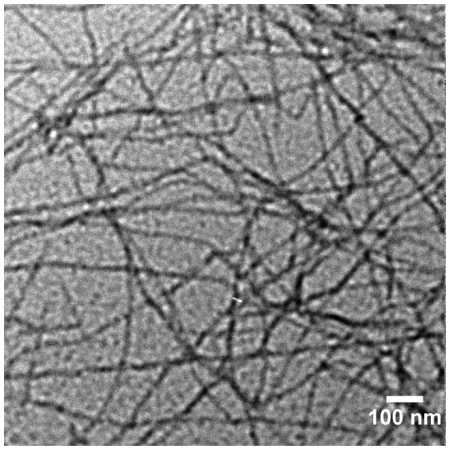

Figure 1. Transmission electron micrograph of amyloid lysozyme fibrils.

Model systems of DMPC, DMPG and DMPS have been widely explored throughout the last decades. Particularly, recent work examined interfacial properties of antimicrobial and membrane-lytic peptides and their interactions with lipid membranes [41], phospholipid-induced fibrillization [42], investigation of the effects of Ca^{2+} on mixed monolayers [43], lipid activation of protein kinase [44] and membrane–ion interaction [45]. Complete miscibility of DMPS in DMPC bilayer was reported [45]. According to high resolution magic angle spinning (MAS) NMR data, DMPC and DMPG are comiscible lipids [46]. Surface pressure isotherms obtained for these systems [43, 47] demonstrated the phase transition of DMPG, preserved even in a 50:50 mixture with DMPC.

Injection of both monomeric and amyloid lysozyme into the subphase resulted in the immediate rise of the surface pressure for all the systems studied. The kinetics of the penetration process into DMPC monolayers is shown in Fig. 2. Similar curves were obtained for the other monolayers (data not shown). These results indicate fast diffusion and incorporation of protein into the lipid films. The presence of a plateau in the penetration kinetics suggests that approximately 6–8 min after addition of the native or fibrillar lysozyme the monolayer becomes saturated with protein.

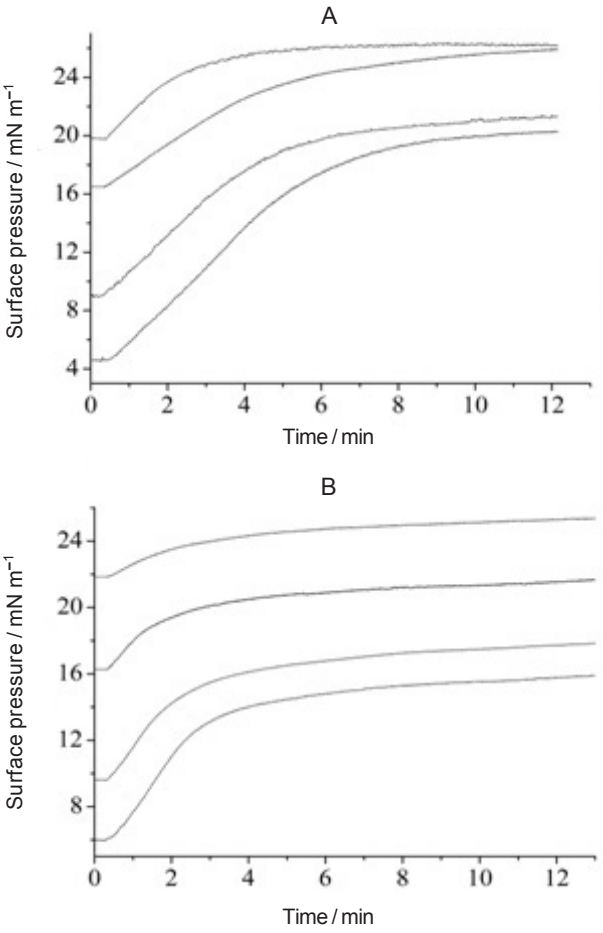

Figure 2. Penetration kinetics of fibrillar (A) and native (B) lysozyme into DMPC monolayers. The different curves were obtained with different initial surface pressures π_0. The subphase protein concentration for all samples was 0.17 μM.

The extent of surface pressure increase was found to depend on monolayer composition. The graphical relationship between the absolute increase in surface pressure ($\Delta\pi$) and the initial monolayer pressure (π_0) is shown in Fig. 3, and Fig. 4 reflects the protein affinity for lipid monolayers [42]. The higher the value of $\Delta\pi$ the more pronounced is protein insertion into the lipid monolayer. In order to characterize quantitatively the capability of lysozyme fibrils to penetrate into lipid films of various compositions we have determined the maximum

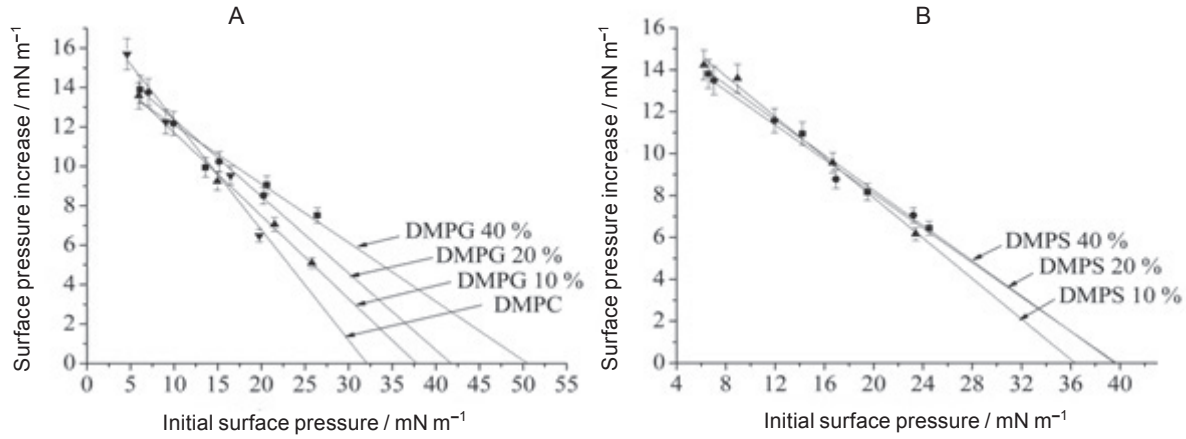

Figure 3. Surface pressure increase $\Delta\pi$ under the influence of fibrillar lysozyme (0.17 μM in the subphase) as a function of initial surface pressure π_0. A, DMPC:DMPG monolayers; B, DMPC:DMPS monolayers.

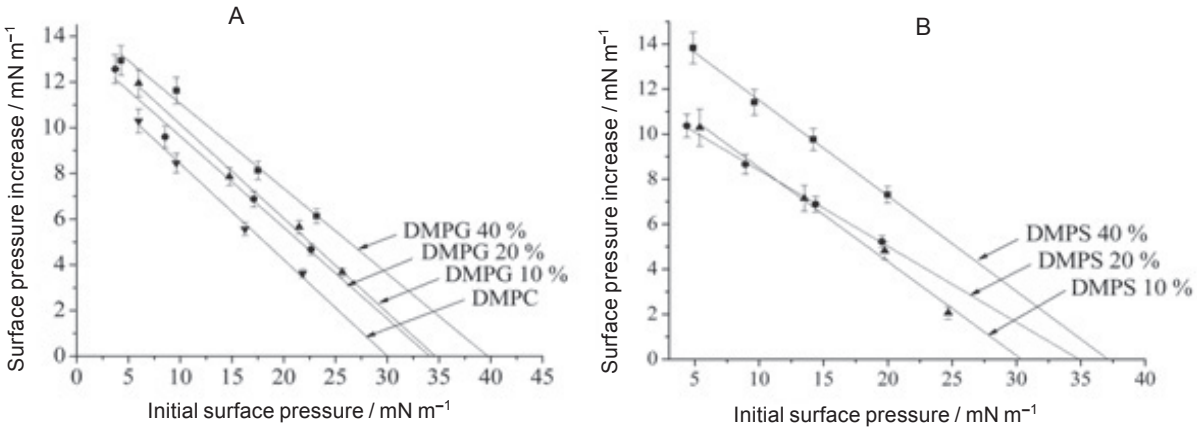

Figure 4. Surface pressure increase $\Delta\pi$ under the influence of native lysozyme (0.17 μM in the subphase) as a function of initial surface pressure π_0. A, DMPC:DMPG monolayers; B, DMPC:DMPS monolayers.

surface pressure at which the protein can still cause a surface pressure increase (limiting surface pressure).

Figs 3 and 4 also illustrate that the dependence of $\Delta\pi$ on π_0 is approximately linear. The slopes of the lines point to the extent of adsorption and incorporation of protein into the phospholipid monolayers: a steep slope exemplifies high sensitivity of monolayer binding to the surface pressure. The extrapolated intercepts with the *x*-axis correspond to the limiting surface pressures, greater values of which indicate higher ability of the protein to penetrate into the monolayer [42]. Values of the limiting pressure for the various model systems are given in Table 1.

As is evident from the data, both fibrillar and native lysozyme easily insert into phospholipid films containing 40 mol% DMPG: limiting pressures for fibrils and monomers were found to be 50 and 40 mN/m, respectively. Decreasing the molar fraction of DMPG resulted in the reduction of the limiting surface pressure. In pure DMPC monolayers this parameter attained values slightly exceeding 30 mN/m for both fibrillar and native protein. A similar, but less pronounced, dependence of penetration efficiency on the molar fraction of anionic lipid was observed for DMPS-containing monolayers: for DMPC:DMPS (40 mol%) the monolayer limiting pressure was 40 mN/m for lysozyme fibrils and 37 mN/m for monomeric protein. Taking into account that the packing density of lipids in biological membranes corresponds to a surface pressure ~ 30 mN/m [48, 49], the results obtained suggest that both lysozyme fibrils and monomers are able to insert into membranes under physiological conditions; fibrillar lysozyme was found to possess a greater ability to penetrate into the lipid monolayer compared to the native protein (Table 1).

Table 1. Limiting surface pressure for lysozyme fibrils and monomers in lipid monolayers of various composition.

Monolayer composition	Limiting pressure / mN m^{-1}	
	Fibrillar lysozyme	Monomeric lysozyme
Pure DMPC	32.1 ± 2.5	30.0 ± 0.9
10 mol% DMPG	37.8 ± 1.1	34.1 ± 1.0
20 mol% DMPG	41.8 ± 1.9	34.1 ± 2.0
40 mol% DMPG	50.6 ± 7.0	39.7 ± 1.8
10 mol% DMPS	36.4 ± 1.3	30.4 ± 1.0
20 mol% DMPS	39.7 ± 2.4	34.9 ± 0.6
40 mol% DMPS	39.6 ± 1.3	37.1 ± 1.1

This property of amyloid aggregates is under continuous debate. Using the Langmuir monolayer technique, it was shown that the fibrillar form of human islet amyloid polypeptide is *not* able to insert into a lipid film, whereas monomers can easily incorporate themselves into membranes [39]. In support of this idea is the recent study of the membrane interactions of Aβ peptides in multiple assembly states; soluble Aβ oligomers, in contrast to the monomers, showed themselves incapable of inserting into membranes [50]. Furthermore, penetration of Aβ monomers was followed by their rapid assembly into channel-like oligomers within the membrane.

In contrast, a number of recent works provide evidence for membranotropic activity of fibrillar aggregates. In particular, while examining the membrane effects of amyloid HEWL, it was found that lysozyme fibrils, in contrast to monomeric or nonfibrillar species, disrupt both neutral and negatively charged liposomes [51]. Nevertheless, despite experimental evidence for the toxic nature of mature amyloid fibrils, the question concerning the insertion ability of fibrillar aggregates still remains open. In this context, the fact of penetration of lysozyme fibrils into phospholipid monolayers established in the present study may be of value toward explaining the toxic effects of disease-associated lysozyme aggregates.

Our results clearly indicate that electrostatic interactions between cationic groups of the protein and negatively charged headgroups of phospholipids play a significant rôle in the insertion of lysozyme fibrils and monomers into model membranes. Indeed, the importance of electrostatic interactions for insertion of positively charged amyloidogenic proteins or peptides in monomeric form is not surprising and has been previously reported [39, 50, 52]. Electrostatic interactions favour protein crowding on the membrane surface, thereby increasing the penetration probability. In addition, a significant finding from the present study is that mature amyloid fibrils of lysozyme effectively incorporate into anionic monolayers. Our results are in agreement with the reported ability of lysozyme fibrils to induce higher leakage in DOPG:DOPC (1:1) liposomes than in pure DOPC vesicles [51]. The enhanced disruption effect of the fibrillar protein was attributed to the increase of charge density through fibril formation. On the other hand, both fibrillar and native lysozyme were found to penetrate into a DMPC monolayer at surface pressures below 32 and 30 mN/m, respectively. These findings suggest the occurrence of hydrophobic interactions, plausibly arising from the enhanced exposure of hydrophobic surfaces in amyloid fibrils. The increase in hydrophobicity of lysozyme during its fibrillization has been inferred in a number of studies [22, 24, 31]. Together, the above rationales allow us to propose that both electrostatic and hydrophobic interactions are involved in the insertion of lysozyme fibrils into lipid films.

We have also examined the dependence of the insertion process on protein concentration by performing measurements at four concentrations of fibrillar lysozyme. Fig. 5 shows the penetration kinetics of fibrillar aggregates into DMPC:DMPG (40 mol%) and DMPC:DMPS (40 mol%) monolayers at different protein concentrations. Analogous insertion profiles were observed for the other monolayers (data not shown). The penetration rate was found to be strongly dependent on protein concentration. Accordingly, at the lowest lysozyme concentration (0.08 μM), the time needed to complete the penetration was about 18 min. Increasing lysozyme concentration led to a speeding up of the penetration process; for instance, at a concentration of 0.56 μM the monolayer was saturated in 3 min after injection. These results revealed that the rate of penetration is limited by diffusion of lysozyme fibrils in the subphase. The higher the protein concentration, the larger the amount of protein aggregates reaching the air–water interface and eventually inserting into the monolayer.

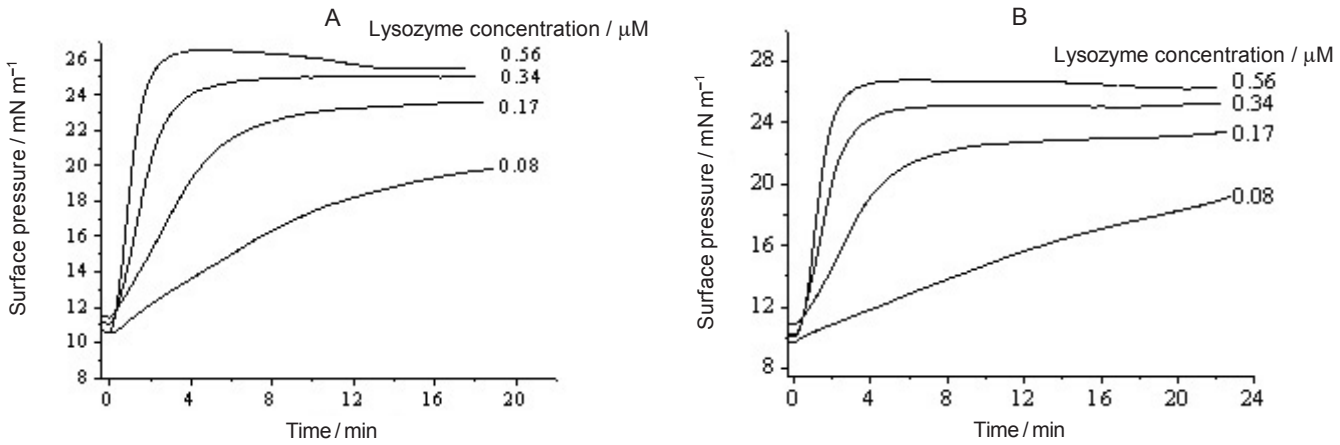

Figure 5. Surface pressure profiles after injection of lysozyme fibrils into DMPC:DMPG (40%) (A) and DMPC:DMPS (40%) (B) monolayers.

A particular feature of the curves obtained at high lysozyme concentration is the presence of a maximum followed by a smooth decrease of the surface pressure; this could be a consequence of polypeptide structural reorganization or a change in bound protein orientation as was suggested in [41]. These changes lead to area contraction and diminution of the surface pressure to the equilibrium value. A phase transition of the lipids may result in a similar effect. In our case it might be a temporary overpressure caused by the fast kinetics of lysozyme insertion.

The extent of monolayer penetration, traced as a surface pressure increase, was enhanced with increasing lysozyme concentration (Fig. 6). In the case of fibrillar protein the $\Delta\pi$ dependence on lysozyme concentration was not linear and the monolayer seemed to become saturated at a certain protein concentration. On the contrary, monomeric lysozyme caused saturation of DMPC:DMPG (40 mol%) and DMPC:DMPS (40 mol%) monolayers at concentrations as low as 0.08 μM (Fig. 7). Further addition of native protein into the sub-phase led to an insignificant increase in surface pressure. The lysozyme concentration at which the maximum insertion is observed could be considered as an additional characteristic of penetration ability. Taken together, the above results imply that fibrillar aggregates of lysozyme insert into phospholipid monolayers more efficiently than their monomeric counterpart.

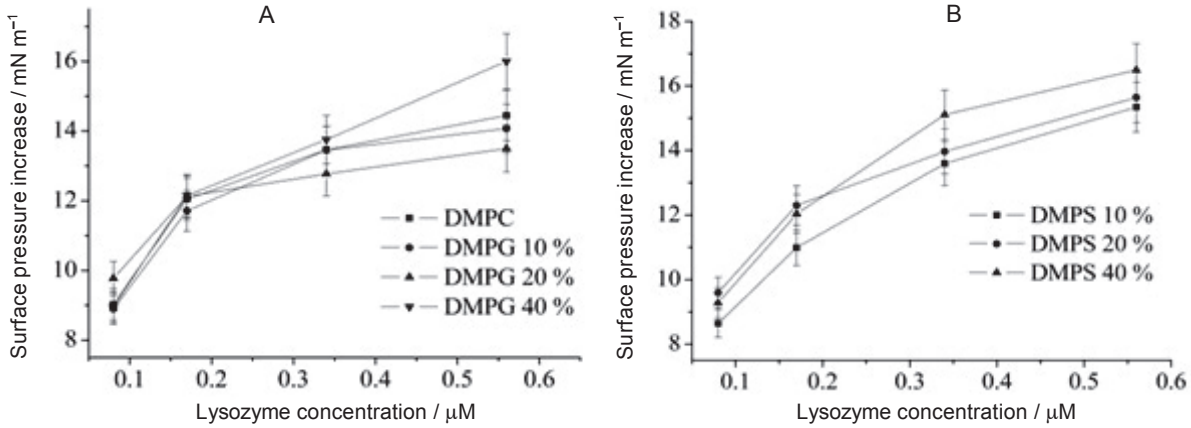

Figure 6. Increase of surface pressure at varying concentration of fibrillar lysozyme. A, DMPC:DMPG ; B, DMPC:DMPS.

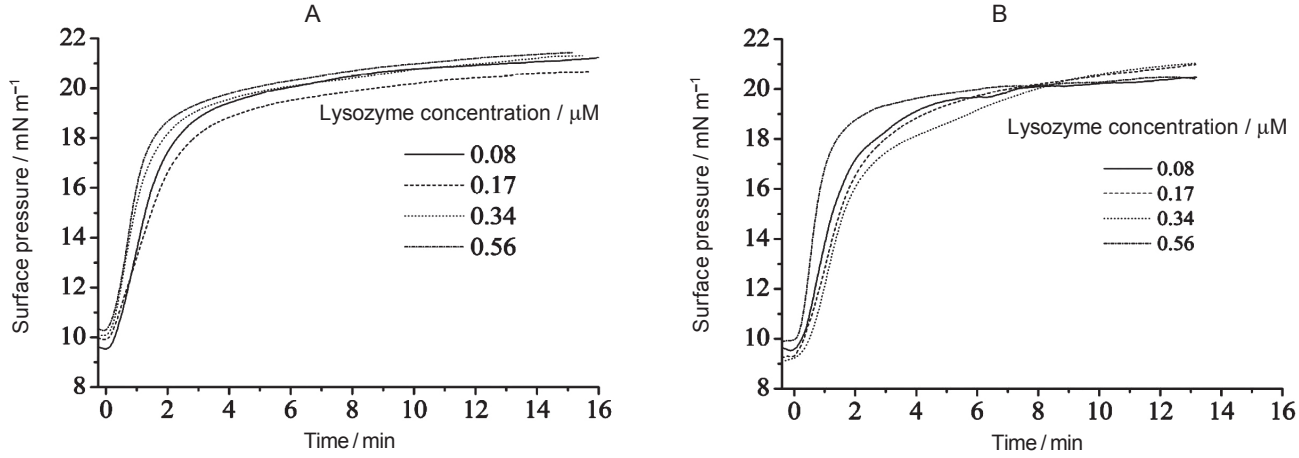

Figure 7. Surface pressure profiles after injection of native lysozyme into DMPC:DMPG (40%) (A) and DMPC:DMPS (40%) (B) monolayers.

4. CONCLUSIONS

To summarize, the present study sheds light on the molecular details of interactions between amyloid aggregates of lysozyme and model lipid membranes. Using the Langmuir monolayer technique we have quantitatively characterized the capability of fibrillar and native lysozyme to insert into phospholipid monolayers composed of zwitterionic DMPC and its mixtures with the anionic lipids DMPG or DMPS. Mature amyloid fibrils exhibited a higher ability to penetrate the lipid film in comparison with lysozyme monomers as was inferred from the values of limiting surface pressure. A significantly

lower limiting pressure for monolayers composed of zwitterionic lipids compared to the negatively charged monolayers implied that electrostatic interactions are a predominant factor in the insertion of lysozyme fibrils into phospholipid monolayers. Increasing protein concentration increased the rate and extent of penetration of fibrillar lysozyme; in the case of the monomers the insertion process was not markedly affected by increased lysozyme concentration. The results obtained allow us to conclude that fibrillization enhances the membranotropic activity of this protein.

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