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Colloids and Surfaces B: Biointerfaces 48 (2006) 128–137
COLLOIDS AND SURFACES B
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Adsorption of lysozyme to phospholipid and meibomian lipid monolayer films

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Received 10 October 2005; received in revised form 27 January 2006; accepted 27 January 2006

Abstract

It is believed that a lipid layer forms the outer layer of the pre-ocular tear film and this layer helps maintain tear film stability by lowering its surface tension. Proteins of the aqueous layer of the tear film (beneath the lipid layer) may also contribute to reducing surface tension by adsorbing to, or penetrating the lipid layer. The purpose of this study was to compare the penetration of lysozyme, a tear protein, into films of meibomian lipids and phospholipids held at different surface pressures to determine if lysozyme were part of the surface layer of the tear film. Films of meibomian lipids or phospholipids were spread onto the surface of a buffered aqueous subphase. Films were compressed to particular pressures and lysozyme was injected into the subphase. Changes in surface pressure were monitored to determine adsorption or penetration of lysozyme into the surface film. Lysozyme penetrated a meibomian lipid film at all pressures tested (max = 20 mN/m). It also penetrated phosphatidylglycerol, phosphatidylserine or phosphatidylethanolamine lipid films up to a pressure of 20 mN/m. It was not able to penetrate a phosphatidylcholine film at pressures $\geq$10 mN/m irrespective of the temperature being at 20 or 37 °C. However, it was able to penetrate it at very low pressures (<10 mN/m). Epifluorescence microscopy showed that the protein either adsorbs to or penetrates the lipid layer and the pattern of mixing depended upon the lipid at the surface. These results indicate that lysozyme is present at the surface of the tear film where it contributes to decreasing the surface tension by adsorbing and penetrating the meibomian lipids. Thus it helps to stabilize the tear film.

Keywords: Tear film; Langmuir; Surface pressure; Dry eye; Fluorescence microscopy; Lysozyme

1. Introduction

A model of the pre-ocular tear film, first proposed over 30 years ago, has a lipid layer at the air–tear interface which covers an aqueous layer that is in contact with the ocular surface [1]. The aqueous layer contains salts, more than 60 different proteins (including lysozyme) and mucins [2,3]. The mucins are believed to be in higher concentrations close to the ocular surface. At the ocular surface, some of the mucins are membrane-bound and coat the epithelial cells [3,4]. The lipid layer is a mixture of waxes, cholesterol esters, phospholipids, sphingolipids and fatty acids (known collectively as meibomian lipids) which are secreted by meibomian glands in the eyelids [5,6]. A low surface tension is essential for a functional tear film. The meibomian lipid layer is thought to be responsible for lowering the surface tension of the tear film at the air–water interface [7]. With high surface tensions, there is a tendency to have a clinical condition called "dry-eye" [8,9].

The concept of the air–tear interface comprising only meibomian lipids is now being challenged by the idea that some proteins such as lipocalin, lysozyme and lactoferrin might also be present in this layer [7,10–12]. A mixture of proteins and lipids at the air–liquid interface is similar to models of lung surfactant, which, like tears, also serves to lower surface tension. Lung surfactant is made of phospholipids, cholesterol and surfactant proteins (SPA, SPB, SPC and SPD). SPB and SPC are lipophilic and facilitate rapid insertion of lipids into, and folding of the lipid monolayer as the surface area expands and contracts [13]. How the proteins of tears might interact with the meibomian lipids is not known.

Interactions of proteins with lipids, whether it be for transport, e.g. serum lipoproteins; lipid hydrolysis; metabolism or cell signalling, e.g. receptors and channels, is a rapidly expanding field of research. In general, these proteins have structural domains

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doi:10.1016/j.colsurfb.2006.01.017

that enable them to be recognized as predominantly lipophilic. There is some evidence that what are typically regarded as hydrophilic proteins may also have obscure functions when they associate with lipids. One such protein is lysozyme. This is a major bactericidal protein in tears [14]. It primarily catalyses the hydrolysis of $\beta 1 \rightarrow 4$ glycosidic bonds between *N*-acetylglucosamine and *N*-acetylmuramic acid in bacterial walls. It has also been associated with membrane fusion, particularly at low pH. Although the mechanism for this is not clear, evidence suggests that it might be by an initial association with negatively charged phospholipids, followed by reorganization of both the lipids and the protein [15] allowing penetration of lysozyme into the lipid layer.

Lysozyme has also been extensively studied in terms of its adsorption and structure at liquid–air and liquid–solid interfaces [16–20]. For this reason, and that it is a major tear protein, it was chosen from the other tear proteins for these initial penetration studies. In general, it has been found that higher concentrations in the bulk lead to greater adsorption at the air–liquid interface. Once adsorbed, lysozyme unfolds resulting in a conformational change where β-sheet secondary structure predominates over α-helices which are typical of an aqueous phase protein [20]. Both ionic strength and pH of the subphase also affect adsorption and preferred conformation of lysozyme at the air–liquid interface. At low ionic strengths, maximum adsorption occurs at the isoelectric point of lysozyme (pH 11), whereas at high ionic strengths, the adsorption is independent of pH due to salt screening of the charges on lysozyme [19]. However, the presence of a lipid monolayer at the air–water interface prevents the conformational changes as lysozyme adsorbs beneath the lipid layer. Nevertheless lysozyme is able to penetrate the lipid layer at temperatures above 25 °C [21]. In terms of the tear film, the normal temperature is about 36 °C, pH 6.9, and it has a relatively high ionic strength. The meibomian lipid layer is composed of mixed lipids, predominantly waxes [5,6], but the amphiphatic lipids, particularly the anionic lipids, e.g. phosphatidylserine (PS), are believed to give a more stable tear film compared with neutral lipids, e.g. phosphatidylcholine (PC) [22]. Some of these parameters of tears favour lysozyme penetration, e.g. relatively high temperature, where others inhibit penetration onto the surface, e.g. relatively low pH compared with the isoelectric point of lysozyme, and the presence of a lipid layer. Since penetration of lysozyme is likely to lower the surface tension of the tear film, we have investigated whether penetration is likely and which parameters are important in favouring penetration into the meibomian lipid layer.

2. Materials and methods

2.1. Materials

Lysozyme and the phospholipids were purchased from Sigma Chemical Co., Australia. Lysozyme at a concentration of 3.2 mg/mL in 10 mM phosphate buffered saline (PBS; 0.9% NaCl, pH 7.4) was always made fresh and was used within 30 min of making the solution. The concentration of lysozyme was based on that reported in human tears [2]. However, the working concentration was 40 μg/mL or less because 1 mL was the maximum volume injected into the ∼80 mL subphase. The main phospholipids used were: phosphatidylcholine (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine); phosphatidylglycerol (PG; l-α-phosphatidyl-dl-glycerol (1,2-diacyl-*sn*-glycero-3-phospho-[1-rac-glycerol]; with 32% palmitic acid, 43% linoleic acid); phosphatidylserine (l-α-phosphatidyl-l-serine (1,2-diacyl-*sn*-glycero-3-phospho-l-serine; with 40% stearic acid, 29% oleic acid) and phosphatidylethanolamine (PE; l-α-phosphatidylethanolamine (1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; from bovine brain). In some experiments diarachidoyl phosphatidylcholine (DAPC; 1,2-diarachidoyl-*sn*-glycero-3-phosphocholine) and distearoyl phosphatidylethanolamine (DSPE; 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine) were used. The purity of the phospholipids was at least 99% by TLC. Phospholipid solutions were made up at 0.5 mg/mL in chloroform. Bovine meibomian lipids were obtained from slaughterhouse material. Eyelids were squeezed using forceps and the extracted meibomian lipids were dissolved in chloroform. They were then dried, weighed and reconstituted at 1 mg/mL in chloroform. The water used in all experiments was purified by ion exchange and had a resistance of 18.2 MΩ (Millipore, Milli-Q).

2.2. Penetration of the lipid films by lysozyme

Lipids dissolved in chloroform were applied drop-wise (10 μL for phospholipids and 20 μL for meibomian lipids) from a microsyringe (Hamilton Co., Switzerland) onto an air-10 mM PBS buffer (pH 7.4) interface of a double-barrier Langmuir trough (NIMA, 102 M, Nima Technology Ltd., UK). Surface pressure (Π) was monitored using a Wilhelmy plate (Whatman, Chr 1 filter paper) and Π was set to near 10, 15 or 20 mN/m (Π_{init}) by reducing the surface area. Once the film had been 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_{eq}). Each experiment was repeated at least three times. In some experiments, a low amount of lysozyme was injected into the subphase and allowed to equilibrate into the lipid layer, and then more lysozyme was injected into the subphase to determine if penetration into the air–liquid interface were still possible. The temperature of the trough was maintained at 20 or 37 °C by a water jacket. The trough and pressure transducer were calibrated using stearic acid. The relative humidity in the laboratory ranged from 35 to 55%, but there was no noticeable difference between the same experiments carried out at a different relative humidity. Lipids or lysozyme alone were used as control experiments.

2.3. Fluorescence microscopy and photography of the films

For microscopic studies of surface films and their penetration by lysozyme, a mixture of 1% fluorescently tagged lipid with 99% of the parent unlabelled lipid was used. The fluorescently labelled lipids (Avanti Polar Lipids Inc., OR, USA) were: 1-acyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino dodeca-

noyl]-*sn*-glycero-3-phospho-choline; 1-acyl-2-[12-[(7-nitro-2-1,3-benzoxadiaz-ol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phospho-ethanolamine; 1-acyl-2-[12-[(7-nitro-2-1,3-benz-oxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-[phospho-rac-(1-glycerol)] and 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] dodecanoyl]-*sn*-glycero-3-phospho-l-serine. When meibomian lipids were used, they were doped with 1% labelled PC. Texas red labelled lysozyme was prepared according to the method of Brinkley [23]. The purity was confirmed using SDS-PAGE and it was used at a ratio of 5% labelled to 95% unlabelled lysozyme. The results of the penetration experiments with or without the fluorescent tags were almost identical, which was similar to previous findings [24].

The doped lipid mixtures were spread onto the buffer surface between the barriers in the Langmuir trough and the Texas red doped lysozyme mixture was injected into the subphase outside the barriers as described above. The trough was placed under a Leica epifluorescence microscope equipped with an excitation band pass filter of 450–490 nm, a dichroic mirror with a reflection short pass of 510 nm and a barrier filter with a line pass of 515 nm for labelled lipids (fluorescing green), and an excitation band pass filter of 535–550 nm, dichroic mirror with a reflection short pass of 590 nm and a barrier filter with a band pass of 610–675 nm for Texas red labelled lysozyme (fluorescing red). The filters could be swapped using a manual slide. The monolayer was then observed using a 40× objective giving a total magnification of 400×. Digital images were recorded using an Andor Ixon back illuminated DV887ECS-BV camera at a shutter speed of 0.01 s.

3. Results

3.1. Adsorption of lysozyme to monolayers of phospholipids and meibomian lipids

As soon as the predetermined Π_{init} had been reached with a particular phospholipid or meibomian lipids on the surface, the barriers were stopped and lysozyme was injected into the subphase. The results from application of 50 μL of lysozyme into the subphase with different phospholipids on the surface are compared first. There was an initial fall in Π as the surface film relaxed (Fig. 1). This was followed by an increase in pressure, which indicated adsorption of lysozyme to the surface film (Fig. 1). For meibomian lipids, with Π_{init} = 10 mN/m at 20 °C, the Π–T profiles showed three distinct slopes as lysozyme adsorbed to the film (Fig. 1). These phases during lysozyme adsorption are represented by more continuous transitions in the PG, PS and PE films. The first slope has been interpreted as representing initial adsorption (Π increases as more molecules are added to the same surface area) [16]. The second slope represented a combination of new lysozyme molecules adsorbing to

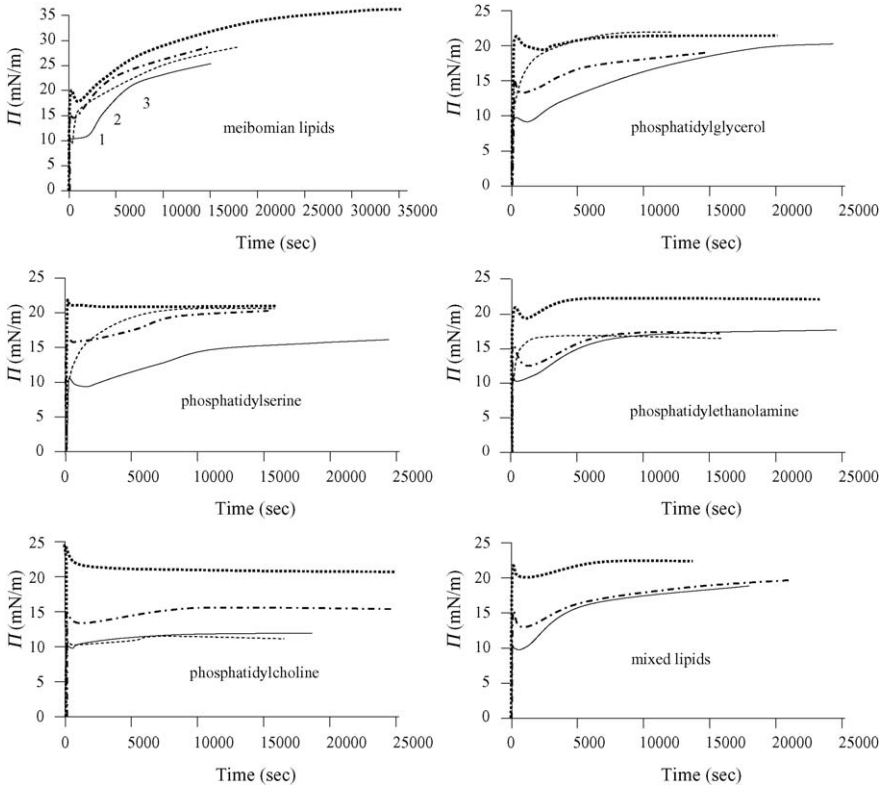

Fig. 1. Π–T curves for penetration of 50 μL of lysozyme at 20 °C into a film of different lipids set at various Π_{init}: Π_{init} = 10 mN/m (—), 15 mN/m (. _ . _) and 20 mN/m (.....). For meibomian lipids with Π_{init} = 10 mN/m, three distinct phases of penetration (1–3) can be seen and are less apparent for the other lipids. For all lipids except DPPC, as Π_{init} increased, penetration still occurred, but the initial rate of penetration decreased illustrated by the decrease in slope at equivalent position to 1. Increasing the temperature to 37 °C for Π_{init} = 10 mN/m (- - - -) sharply increased the rate of penetration of lysozyme into all lipid layers except for phosphatidylcholine. At 37 °C, the degree of penetration was unchanged as illustrated by the pressure asymptote.

the surface, and unfolding/rearranging of lysozyme molecules that had reached the surface [16]. The third slope was due solely to the unfolding/rearranging of lysozyme molecules on the surface [16]. Here, rearranging includes rearranging of lipid and water molecules as well as lysozyme molecules. Temporal overlap of these phases was thought to cause the smoother curves seen with the other lipids. For meibomian lipids, as Π_{init} increased, there was a slight decrease in the rate of adsorption (decreased slope) but there was no apparent inhibition of adsorption as there was when films of phospholipids were on the surface. For films of PG or PS, adsorption was virtually eliminated in films held at $\Pi_{init} = 20$ mN/m, and for PE it was about 16 mN/m. At 37 °C, diffusion of lysozyme through the subphase and adsorption to the lipid film was much faster, but the final Π was very similar to that obtained at 20 °C. Π–T curves for DPPC were quite different from the other phospholipids (Fig. 1) because there was either no adsorption or only very minor adsorption for $\Pi_{init} \geq 10$ mN/m. Raising the temperature to 37 °C had no effect. However, adsorption into a mixture of phospholipids that included DPPC (PG, PS, PE and DPPC; 1:1:1:1) gave similar results to the other phospholipids, but the rate of adsorption, particularly at $\Pi_{init} = 10$ mN/m, was faster (Fig. 1). A marked difference between meibomian lipids and the phospholipids or a mixture of the phospholipids was that when $\Pi_{init} = 20$ mN/m, lysozyme adsorption into a meibomian lipid film continued to increase the pressure up to 35 mN/m, whereas for the other lipid films there was hardly any pressure increase.

To confirm that lysozyme at the concentration used above could exert a higher pressure and was being prevented from doing so by the presence of DPPC on the surface, some experiments were carried out where Π was simultaneously monitored both outside (no DPPC on the surface) and between the barriers (DPPC on the surface). The total surface areas inside and outside the barriers were the same. In all cases, a higher Π at equilibrium was reached outside the barrier (DPPC absent), than between the barriers.

Since DPPC forms a liquid condensed and liquid expanded phase at a $\Pi \sim 5$ mN/m [24] (Fig. 2), further investigations were carried out to determine if phase separation, or the presence of a liquid condensed phase could account for the ability of DPPC to prevent adsorption of lysozyme at low surface pressures. Π–A isocycles of a DPPC film show a transition phase at $\Pi \sim 5$ mN/m which is indicative of the formation of a liquid condensed/liquid expanded phase. The limiting area of lecithins in a monolayer, has been found to be around 44 Å^2/molecule (Fig. 2B) [25]. The existence of this transition phase is dependent upon pH, temperature, and the length and saturation of the acyl chains [26], and under the conditions used in these experiments, it was not present in Π–A isocycles of other lipids (Fig. 2A). Therefore, it is not clear at what Π these films form liquid condensed phases. However, based on their Π–A isocycles, it is likely to be at $\Pi \sim 20$ mN/m, which was also the pressure at which lysozyme was excluded from adsorption. The idea that a condensed phase is primarily responsible for preventing adsorption can be tested by increasing the length of the hydrocarbon chains in the fatty acids. It has been shown previously that there is an increase in coherence with increasing chain length and this favours a condensed state [27]. Therefore by using DAPC films (C20 rather than C16 for DPPC), it would be expected that lysozyme adsorption would be prevented at lower Π than for DPPC films. This is because DAPC forms a predominantly condensed state at pressures below 5 mN/m (Fig. 2B) [25]. However, this was not the case. The results for DAPC (not shown) were almost identical to those shown for PE as shown in Fig. 1. Adsorption still occurred with $\Pi_{init} = 10$ mN/m to give a $\Pi_{eq} = 16$ mN/m, but adsorption was strongly inhibited at $\Pi_{init} = 15$ mN/m.

It could also be expected that the interactions of the head groups could influence penetration. It has been shown that lecithins (PC) form more expanded films than phosphatidylethanolamines due to differences in the size and orientation of the head groups [25]. More space is required for the larger and more hydrated choline head group than for the ethanolamine head group. An indication of the possible role of the head group can be achieved by using DSPE and comparing it with PE, DPPC and DSPE (C18 acyl chains). Similar to DAPC, it does not demonstrate an expanded form in an isocycle (Fig. 2B) and therefore it should block adsorption at a very low pressure similar to DPPC. For films of DSPE adsorption still occurred with $\Pi_{init} = 12$ mN/m to give a $\Pi_{eq} = 18$ mN/m, but

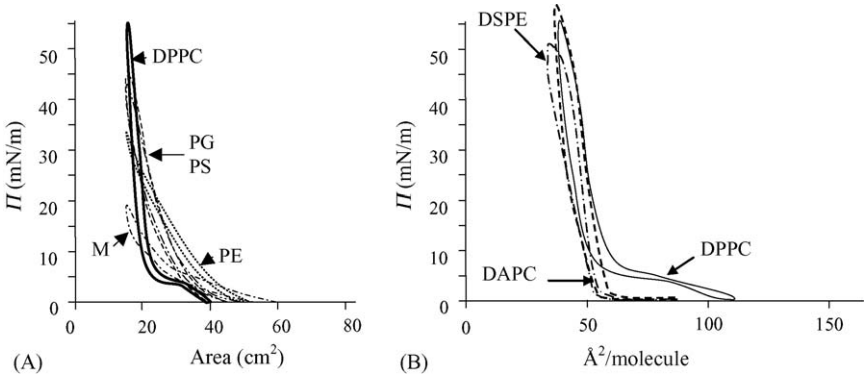

Fig. 2. (A) Comparative isocycles obtained for different phospholipids and meibomian lipids (M). PG and PS exhibit very similar shaped curves whereas PE and M have curves that are less steep and although they have a larger area when Π was first detected, their Π_{max} was lower than that for PG, PS and DPPC. DPPC (solid line) is the only lipid which has a transition region around $\Pi \sim 5$ mN/m. (B) Shows the effect of increasing acyl chain length. The longer chains found in DAPC and DSPE show only the condensed phase even at very low Π.

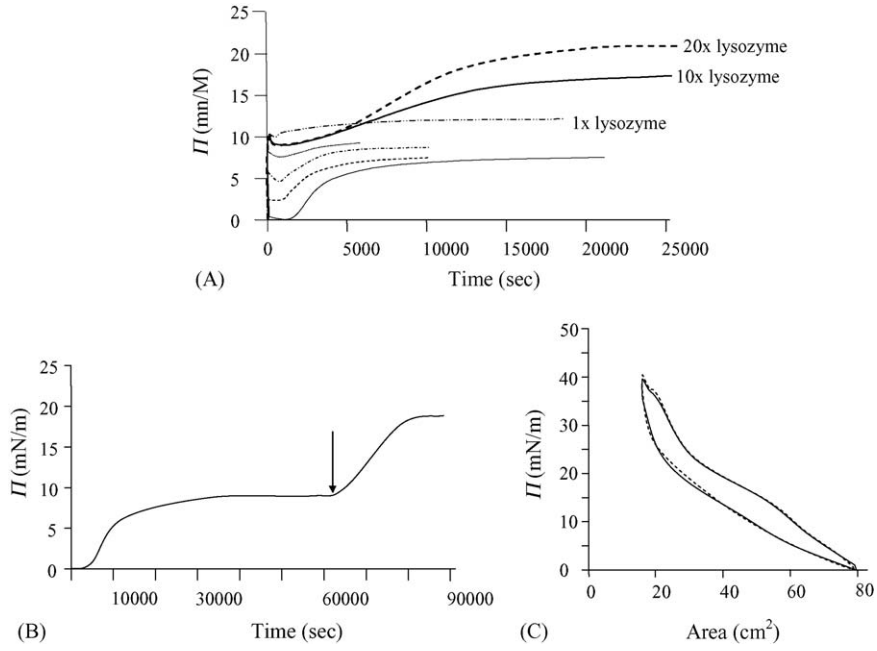

Fig. 3. (A) A series of curves showing the penetration of 50 μL of lysozyme into a DPPC film with Π_{init} set to 0, 2, 5, 8 or 10 mN/m (0–10). With Π_{init} = 10 mN/m higher amounts of lysozyme (10× lysozyme = 500 μL and 20× lysozyme = 1000 μL) applied to the subphase increase Π_{eq}. (B) After 50 μL of lysozyme was allowed to penetrate into a DPPC film with Π_{init} set to 0 and allowed to reach equilibrium, a further 950 μL added to the subphase (arrow), showed that lysozyme was able to still penetrate the film. (C) Equilibrium isocycles virtually overlapped irrespective of whether it was the equilibrium when 50 μL of lysozyme was allowed to penetrate into a DPPC film with Π_{init} set to 0 (unbroken line), or the final equilibrium of the experiment shown in B (broken line).

adsorption was strongly inhibited at Π_{init} = 16 mN/m (data not shown).

Since the concentration of lysozyme used to test penetration was about 1600 times less than that found in tears, it was of interest to determine if increasing the concentration of lysozyme in the subphase resulted in increased adsorption. If Π_{init} were set at 10 mN/m, there was no change in pressure when 50 μL of lysozyme solution was injected into the subphase, but there was a substantial change in pressure if higher volumes were applied. The equilibrium pressure attained was concentration dependent with 1000 μL giving a Π_{eq} of ∼20 mN/m (Fig. 3A).

It was also important to know if lysozyme could adsorb to the surface once an apparently stable lysozyme/DPPC film had formed. For these experiments, DPPC was applied to the surface with Π_{init} = 0 mN/m, 50 μL of lysozyme solution was injected into the subphase, and the pressure was allowed to reach equi-

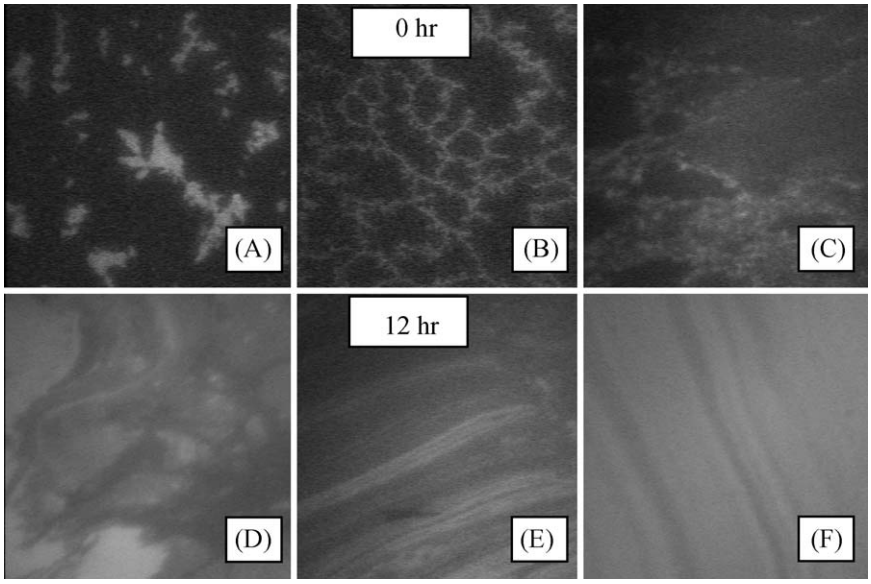

Fig. 4. Micrographs showing the appearance of Texas red labelled lysozyme:lysozyme (5%:95%) applied to the air–buffer interface just after application (A–C), 12 h later (D and E) and 12 h after applying it into the subphase (F). A and B were both when Π = 0: A was when the surface area was 80 cm^2 and B when the surface area was 40 cm^2. C was at Π = 24 mN/m (maximum compression), D was at Π = 10 mN/m, E was at Π = 24 mN/m and F at Π = 16 mN/m.

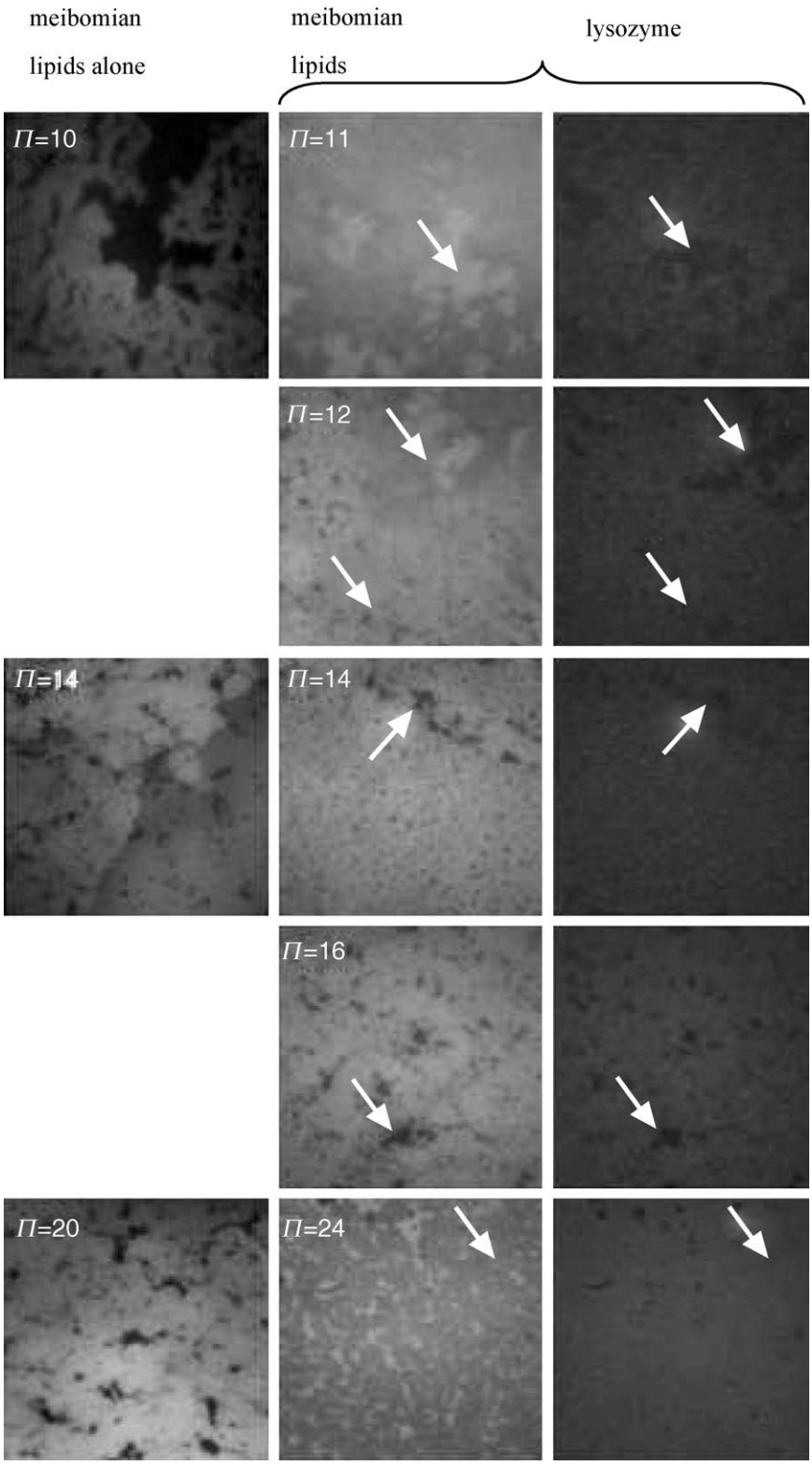

Fig. 5. Micrographs showing a pure meibomian lipid film (left column) at different pressures, and the effects of lysozyme penetration on a meibomian lipid film set at $\Pi_{init} = 10$ mN/m (middle and right column). Middle and right columns are matched fields showing the lipids fluorescence (middle column) and lysozyme fluorescence (right column) at different pressures as penetration progressed. Penetration caused dispersion of lipids at all pressures and initially there were lipid rich zones that excluded lysozyme (arrows). These then disappeared as the pressure increased and corresponding dark zones appeared in both films, e.g. both zone types can be seen at $\Pi = 12$ mN/m (arrows). At the highest pressure there were bright lipid zones distributed throughout the film which had no corresponding regions in the lysozyme film.

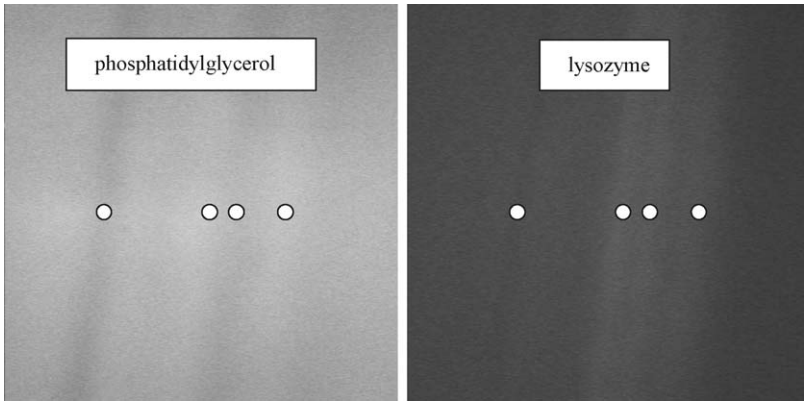

Fig. 6. Micrographs showing penetration of lysozyme into a PG film set at $\Pi_{init} = 10$ mN/m after 4 h. The same region of the film is shown using different filters; left, PG fluorescence; right, lysozyme fluorescence. The PG film (left) is relatively amorphous with some striping. Lysozyme penetration shows a similar pattern (right). The dots are the same points on the surface. In some cases the dark and bright stripes correspond in both images. In other regions the bright stripe in lysozyme corresponds with a dark region in the PG film. Note also that the right hand side of this figure where there is strong staining with PG but little fluorescence with lysozyme.

librium. A further 950 μL of lysozyme was injected into the subphase. The result was that the lysozyme/PC film was not a barrier and there was an increase in pressure to a new Π_{eq} of 20 mN/m (Fig. 3B). Unexpectedly, if isocycles were performed at the end of either the first equilibrium or the second equilibrium, then they were almost identical, despite there presumably being more protein in the surface film after application of additional protein (Fig. 3C). Note that isocycles were not performed at the first equilibrium in experiments where a higher concentration of lysozyme was to be added to the subphase.

3.2. Fluorescence microscopy of surface films

To provide a base, the microscopic appearance of pure films was examined initially, and then compared with films at different stages of adsorption. For lysozyme, there was a difference when it was applied to the surface as compared to when it was applied into the subphase. Lysozyme applied to the surface did not immediately disappear into the subphase but formed clumps, which gradually coalesced as the surface area of the trough was reduced and Π increased (Fig. 4). With time, the clumping disappeared and lysozyme was more evenly dispersed and formed a striped patterning on the surface at maximum Π (24 mN/m) (Fig. 4E). When lysozyme was injected into the subphase and the surface was examined between the barriers, it also showed a dispersed striped patterning (Fig. 4F).

Meibomian lipids formed a film with dark irregular zones at low Π. These gradually became much smaller as Π increased. At the highest pressure obtainable with meibomian lipids ($\Pi = 20$ mN/m), the dark zones were smaller and grey rather than

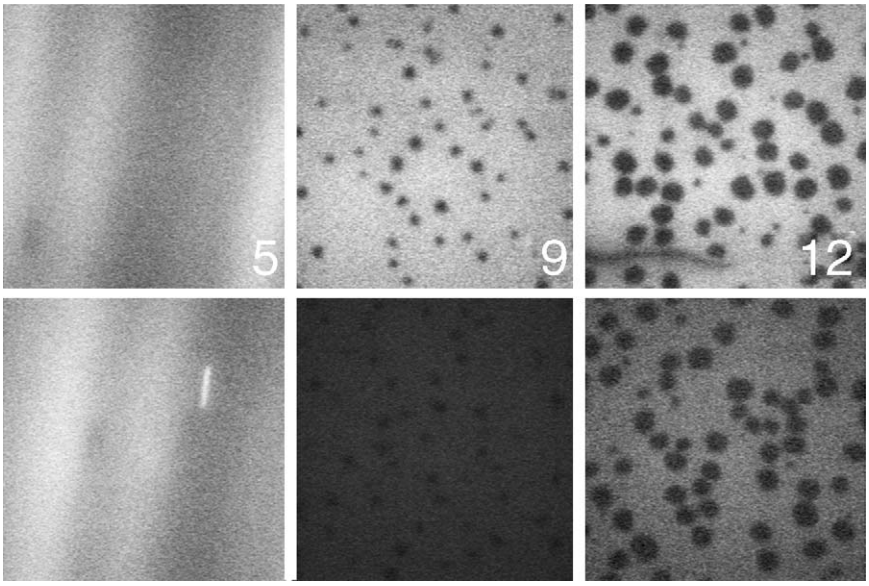

Fig. 7. Micrograph showing penetration of lysozyme into a DPPC film originally set with $\Pi_{init} = 0$ mN/m. The top row shows DPPC fluorescence and the bottom row lysozyme fluorescence. The numbers represent the pressure of the film at the time of the micrograph. Initially there was a striped pattern which gradually gave way to small liquid condensed (dark) regions that gradually increased in size.

black (Fig. 5). When lysozyme was injected into the subphase, the meibomian lipid layer had a much more dispersed appearance at all pressures. At the beginning of adsorption, there were areas which showed both red (lysozyme) and green (lipids) fluorescence and areas with more intense green fluorescence (high lipid density) devoid of red fluorescence. Since the Texas red fluorescence is seen only when the fluorophore is sticking out from the surface, this indicated that lysozyme had actually penetrated the lipid layer except in some regions of higher lipid density. As Π increased due to penetration, some dark regions appeared which were deficient of both lipid and lysozyme fluorescence. These dark regions became more pronounced as the pressure increased and the fluorescence outside these areas was very even. At Π_{eq} (24 mN/m), lysozyme fluorescence appeared to be evenly dispersed throughout the film whereas the lipids had more intense patches. The dark regions became small circular areas in both the lipid and lysozyme views.

Microscopic examination of fluorophore doped films for PG, PS or PE at all $\Pi > 0$ showed a very even fluorescence without any substructural details (not shown). When lysozyme was injected into the subphase with the lipid film at $\Pi_{init} = 10$ mN/m, as the pressure increased (representing penetration) an amorphous red fluorescence of the protein could be detected which indicated that the protein was distributing evenly (mixed) with the lipid film. In addition to this general appearance, there were occasional areas where weak striping appeared in the film and

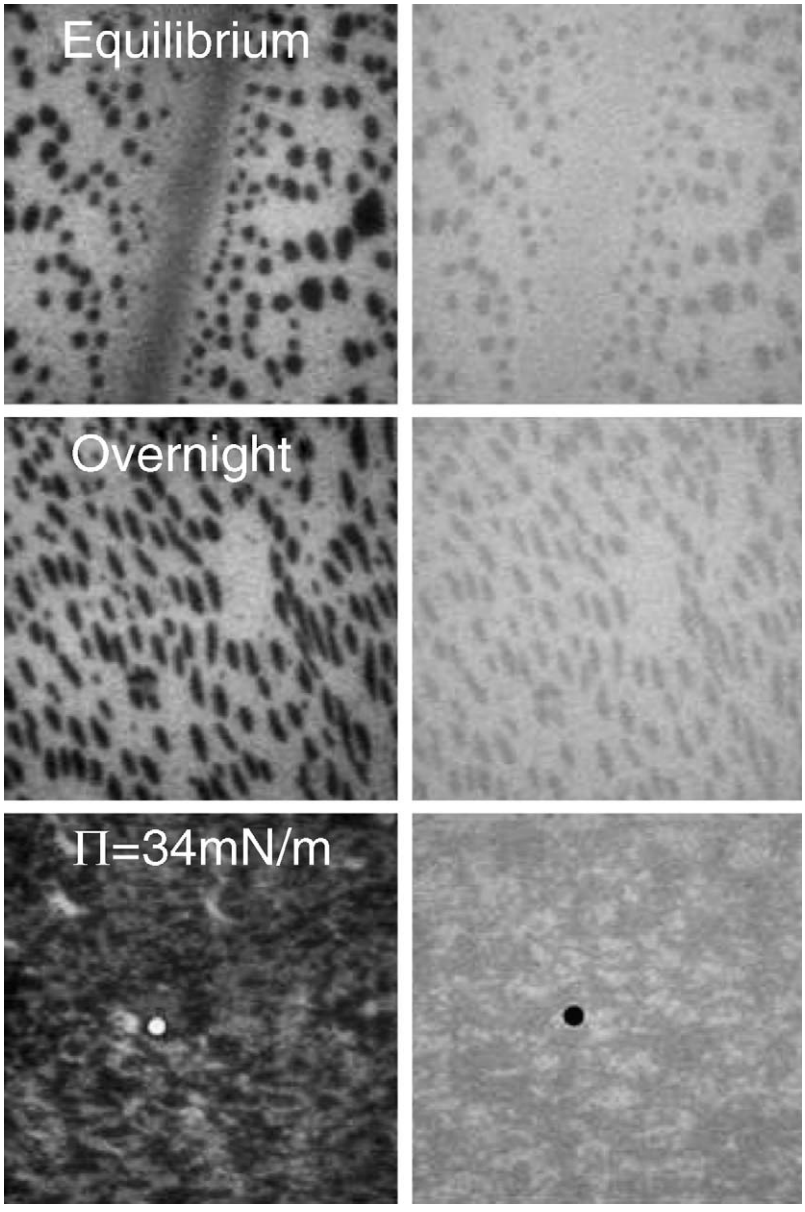

Fig. 8. Micrographs showing a DPPC film after penetration with 10× lysozyme which was originally set with $\Pi_{init} = 0$ mN/m. Left column shows DPPC fluorescence and the right column lysozyme fluorescence. The first row shows the appearance of the film once it had reached equilibrium (15 mN/m), the middle row after a further 12 h and the bottom row the same film which had been further compressed to 34 mN/m. The film is very stable in all cases (dots in bottom row show the same location), and liquid condensed areas are generally devoid of protein. However, the dark striped region in the first row has protein, and in the bottom row at high pressure, the overall organization of liquid condensed and liquid expanded regions is much disrupted.

on casual inspection lysozyme appeared to have complementary striping to the lipids. However, careful examination showed that every combination occurred, i.e. regions of high lysozyme concentration corresponding with lipid poor domains; regions rich in lipids and poor in proteins; regions poor in both lipids and proteins and regions rich in both proteins and lipids. This patterning was seen for all three: PS, PG and PE, and an example is shown in Fig. 6 for PG. The striping was observed at all pressures and there was no qualitative indication that it increased as the pressure increased.

This appearance differed markedly from films of PC. As previously reported [28], DPPC formed films with a pattern of liquid condensed domains within liquid expanded domains. Injection of lysozyme into the subphase with DPPC film at $\Pi_{\text{init}} = 0$, showed a gradual change from a striped even film to the appearance of small liquid condensed regions that gradually increased in size (Fig. 7). If a low volume of lysozyme (50 μL or 1×) were applied to the subphase then the film at equilibrium, although stable in structure was moving very rapidly and it was difficult to photograph the same field of view with the different filter sets. However, if 10× or 20× lysozyme were added to the subphase, the film became very stable at equilibrium (Fig. 8), and the liquid condensed regions excluded lysozyme, i.e. lysozyme penetrated the liquid expanded phases of DPPC. This is consistent with previous findings [29]. Under these conditions if the film were further compressed, the liquid condensed regions began to lose their discrete structure at $\Pi \sim 20$ mN/m.

DAPC and DSPE films were similar in that they had no regular domain patterning as seen for DPPC, but there was large condensed islands interspersed with liquid expanded regions. These films were very mobile (not shown). After lysozyme had been injected into the subphase, most notable in both films was that they became very immobile. Switching filters to observe the protein showed no red fluorescence in the DAPC film which indicated that lysozyme had adsorbed to the surface, but not penetrated it. For the DSPE film the red fluorescence of the protein could be seen at the surface. In some regions where there was relatively bright lipid fluorescence (liquid expanded phases) the protein was excluded, in some areas both protein and lipid occurred and in still other areas not showing lipid fluorescence (possibly liquid condensed) protein was present.

4. Discussion

The main purpose of these experiments was to gain a better understanding of the air–liquid interface of the pre-ocular tear film through reduction modelling, i.e. using simple models of some of the individual components of the tear film such as individual phospholipids and lysozyme. These experiments have given us further insight into the likely nature of the surface of the tear film.

Combining adsorption experiments with microscopy has shown that lysozyme, at concentrations much lower than that in the tear film (~1600× less concentrated), can readily adsorb to, and penetrate a meibomian lipid film, even at a relatively high pressure $\Pi = 20$ mN/m and at a temperature lower than the physiological temperature (20 °C versus 37 °C). Under normal conditions where the typical maximum surface pressure of whole tears is ~24 mN/m, the temperature is normally close to 37 °C, and lysozyme concentration is much higher, the expectation would be that penetration would be increased.

From experiments using pure lipids, it was clear that only very special conditions prevented lysozyme penetration. In part, this seemed to be associated with formation of a very condensed pure lipid film. For example, lysozyme penetration was prevented at $\Pi \sim 20$ mN/m for films of either PG or PS. Based on calculations of the area occupied by an aqueous dispersion of phospholipids (68 Å^2) it has been estimated that a monolayer with the phospholipids occupying this area would have a pressure of 23 mN/m [30]. Basically, this means that they would be tightly packed at 20 mN/m and hence penetration would be difficult as observed in our experiments. It implies that for penetration to occur there must be space available on the surface. However, previously published work by Kimelberg and Papahadjopoulos [30] differs from our results because they showed lysozyme penetration of PS films held at pressures up to about 38 mN/m, but are similar to our data where they have shown penetration of PS by lysozyme but not PC monolayers [31]. However, having a closely packed film alone was not enough to exclude lysozyme penetration as was indicated from the experiments using DSPE.

An interesting contrast was found between DSPE and DAPC. In both cases, Π–A isocycles were very similar and indicated close packing of the molecules at low Π. The fluorescence microscopy experiments showed that although lysozyme was able to adsorb to both layers (the films became very immobile), it only penetrated the DSPE layer (protein fluorophore was visible at the surface). This may be due to the different head groups. The polar head group of PC and PE are similar in charge and differ in that PC has a quaternary amine, and PE a primary amine. 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, i.e. 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 [21]. Above this temperature it should interact. Since our experiments were all carried out below the transition temperature for PC (~41 °C), then limited penetration as we observed, is consistent with previous data. However, if penetration were simply due to the interaction at the O–P–O diester bond, then it would be expected that all phospholipids would have similar penetration properties, and that even if an allowance were made for charge, then at least PE should have similar behaviour to DPPC, but this was not the case.

Particularly enigmatic is that once a stable lysozyme DPPC film has formed, then additional lysozyme added to the subphase was able to penetrate the film (increased Π and increased lysozyme fluorescence) (Fig. 3). It is clear that it was not simply the pressure that was preventing more lysozyme from penetrating, because low concentrations of lysozyme was able to penetrate films of other lipids held at above 10 mN/m. Also peculiar was that the isocycles of the mixed film after penetration of DPPC by 1× lysozyme virtually overlapped the isocycles when

20× lysozyme was used (Fig. 3C). This is strange because in the case of 20× lysozyme there were more molecules on the surface (indicated by the higher surface pressure at equilibrium; Fig. 3B).

By comparing all of our data on the different lipids leads us to conclude that DPPC behaves in an exceptional way. Hence, it should not be treated as a representative phospholipid as it has been in many previous studies. In addition, how the quaternary amine encourages adsorption of lysozyme but prevents its penetration needs further investigation.

It was interesting from a clinical perspective that PS and PG films were most easily penetrated by lysozyme. It has been previously shown that anionic phospholipids increase the stability of the tear film [22], although the exact reason for this is not known. Our data suggest that it could be due to these lipids having an enabling effect on proteins penetrating the lipid layer and that when proteins are present, the surface film becomes much more gel like (stable). Lysozyme has a net positive charge at pH 7.4, and hence its increased ability to penetrate anionic lipid films could have an ionic component. The negatively charged phospholipids would be at a lower free energy in the presence of the positively charged lysozyme. Such ionic interactions between the lipids and the proteins could also contribute to increased stability of the tear film.

Given these observations, and that meibomian lipids are a mixture and also include some unsaturated fatty acids, it is unlikely to form close packing, it is most probable that lysozyme is present at the surface of the tear film, and not simply in the aqueous phase as current models suggests [3,7,32]. Assuming that lysozyme is present at the surface, then it is of interest to speculate on its possible role. Lysozyme denatures at the surface [33] and hence it is unlikely to have an anti-bacterial role at the surface. By the fluorescent micrographs, lysozyme appears to be acting as a dispersant of the meibomian lipids. This would mean that the surface of the tear film would be more akin to lung surfactant [13] rather than a mixed lipid layer. It is notable that the proteins involved in lung surfactant, surfactant proteins B and C, are strongly hydrophobic rather than hydrophilic like lysozyme. However, the tears also contain lipocalins that are hydrophobic in nature, and it would be of interest to examine their penetration into the surface film of meibomian lipids.

5. Conclusion

Taken together these data indicate that lysozyme is one of the important surface-active proteins present in the tear film and it has ability to move from the bulk solution to the surface and absorb to and penetrate the lipid layer present at the surface. At the surface it contributes to the decrease in surface tension like the lipids and helps in the stabilization of the meibomian lipid layer and the tear film.

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