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Tear Lipids Interfacial Rheology: Effect of Lysozyme and Lens Care Solutions

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Abstract

Purpose—To evaluate the interfacial properties of *ex vivo* tear lipid multilayers with controlled and varying thickness. The influence of lysozyme and surfactant-containing multi-purpose lens care solutions (MPS) on interfacial rheology of lipids and mixed lipid-protein films were studied.

Methods—Lipids were extracted from lotrafilcon A lenses worn continuously for 1 month. Interfacial properties of the lipids without and with lysozyme in the aqueous phase were examined using tensiometry and step-strain relaxation. Lipid-lysozyme multilayers were exposed to either diluted Optifree Express (OFX) or Optifree Replenish (OFR) for 30 min, and then MPS was displaced from the bulk phase. Surface tension and rheological parameters before and after MPS exposure were measured and compared.

Results—Thick lipid films exerted high surface pressure at the air-aqueous interface, 50 ± 2 mN/m, with little inter- and intra-subject variability. The rheological storage modulus (E_∞ ; 25.3 ± 2 mN/m) and relaxation time (τ ; 87 ± 25 s) were similar among subjects. Neither lysozyme nor MPS changed the surface tension of the lipid multilayers. Lysozyme adsorbed irreversibly onto multilayers without changing E_∞ , but increased τ 2.5 times. Exposure of mixed multilayers to OFX reduced E_∞ to less than a half of its original value (13 ± 4.5 mN/m; $p < 0.001$); whereas after OFR exposure, small but statistically significant changes in E_∞ were found (21 ± 3.0 mN/m; $p = 0.0044$). MPS-treated mixed films relaxed much slower than an untreated one, τ increased 4.5 times after OFX and 7 times after OFR exposure.

Conclusions—Thick multilayers of the *ex vivo* tear lipids have exhibited surface tension 15 ± 1 mN/m lower than reported in the literature for meibomian lipids. The lipid-lysozyme interaction altered the interfacial rheology of the *ex vivo* lipids. OFX and OFR changed rheological properties of the mixed films to different extents.

Keywords

ex vivo human tear lipids; tear proteins; soft lenses; lens care solution; lens induced dryness

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An evenly distributed tear film sustains a proper degree of hydration of the cornea, contributing to overall ocular health and comfort.¹⁻¹¹ Specifically, the tear lipid layer plays an integral role in maintaining ocular surface homeostasis by facilitating the spreading of the aqueous layer over the cornea after a blink.^{1,2,6,8,10} The interactions between the lipid layer and tear fluid constituents (e.g., proteins) are essential for maintaining tear film stability.¹⁻¹¹ The human tear film is a complex biological system and its composition must be delicately balanced in order to maintain its optimal functions.^{1-4,7,10} Unfortunately, this intricate balance of essential components can be easily disrupted by many factors, including contact lenses and lens care solutions. For example, many multipurpose solutions (MPS) contain surface-active ingredients (i.e., surfactants) added to enhance their cleaning efficiency and lens-surface wettability. Some surfactants may destabilize the superficial lipid layer of the tear film by lowering the oil-water interfacial tension to nearly zero, which leads to emulsification of the lipid layer of the tear film.¹ Therefore, these surfactants introduced into an eye upon lens insertion can weaken already susceptible pre-lens tear film.^{1,12}

The stability and spreading ability of thin liquid films in general, tear films included, are governed by the balance of the interfacial forces acting at a three-phase contact line, namely, the tensions at liquid-air, liquid-solid and solid-air interfaces.^{1,13} Therefore, evaluation of the equilibrium surface tension/pressure at the air-aqueous interface is important for understanding tear film stability and spreading behavior.^{1,3-5,9-11} Studies involving tear lipids confront a great challenge because only tiny amounts of the material can be collected harmlessly from a single human eye. As a result,⁶ most studies have focused on model or animal meibomian lipid monolayers (one-molecule thick) and their equilibrium surface pressures have been investigated employing the Langmuir trough technique. However, recent findings show that human tear lipids form multilayers, not monolayers, and are of a 20-molecule thickness at minimum.¹⁴⁻¹⁷ Several investigations of tear lipids, carried out in the presence of model proteins (e.g., lysozyme, mucin and lactoferrin), have shown that proteins change the surface pressure of lipid monolayers and can penetrate into Meibomian lipid monolayers.¹⁹⁻²³ As all the above-mentioned studies have emphasized, the interaction between tear proteins and lipids can affect the equilibrium surface tension of the lipid layers, thus suggesting that a similar mechanism could impact tear film stability *in vivo*. Human tear film stability, however, is likely dependent not only on the equilibrium surface tension, but also on the mechanical properties of films operating under dynamic conditions and high stresses exerted by the eyelids in a blinking cycle.^{24,25} Recent clinical studies have shown that *in vivo* relaxation of tear lipid layers after a blink can be described as a viscoelastic process.²⁵ Unfortunately, the information regarding interfacial viscoelastic properties of lipid films is scarce.²⁴ Clearly, in order to advance the understanding of human tear lipid film dynamics and the role of proteins in the mechanical properties of complex tear films, a new experimental approach was needed to create thick tear lipid films at an air-aqueous interface and then to investigate the interfacial properties of these multilayered films under dynamic conditions that mimic human eye conditions more realistically.

In contrast with most previous studies that investigated lipid monolayers at equilibrium, we have developed an experimental technique allowing assessment of both the equilibrium and the dynamic interfacial properties of lipid multilayers with accurately controlled and varying

thickness. This was achieved by using sessile bubble tensiometry, in which the air-aqueous interface area was 15-20 mm², approximately 300 times smaller than that in a typical Langmuir trough, and a few micrograms of *ex vivo* human tear lipids were sufficient to deposit thick films. With this method, we investigated the equilibrium surface tension and dynamic mechanical properties of thick lipid films by studying relaxation after instantaneous expansion and compression (i.e., dilatational interfacial rheology), mimicking human blink cycles. We also examined how the interaction of lipid multilayers with the model tear protein lysozyme influenced their mechanical properties. Lysozyme was chosen not only because it is the most abundant protein in human tears,^{26,27} but also because it has recently been shown that interaction between model lipids and lysozyme alters the mechanical properties of model lipid films.²⁵ In addition to examining the effect of lysozyme on the dynamic properties of lipid multilayers, this novel experimental approach provided information regarding the possible mechanisms involved in clinically observed human tear film destabilization and related discomfort induced by surfactant-containing MPS during contact lens wear.¹²

Materials and Methods

Distilled and deionized water from a MilliQ filter system (Millipore Co., Bedford, WA, resistivity greater than 18 MΩ cm) was used for solutions preparation. The aqueous phase in all experiments was buffered saline solution composed of 5 g/L NaCl with 4 g/L of sea salts (Sigma, Milwaukee, WI 53233, USA) added to supplement the aqueous phase with other ions found in human tears, (e.g., K⁺, Ca²⁺ and Mg²⁺, phosphate and bicarbonate), which are essential for maintenance of the corneal epithelial surface.²⁸ The pH of the solution was adjusted to 7.3 by addition of small amount (e.g., 0.5-0.8 ml) of 250 M KH₂PO₄.

The protein studied was hen egg-white lysozyme from Sigma-Aldrich Co. (USA, Milwaukee, WI 53233), used without further purification. Lysozyme has a relatively low molecular weight of 14 kDa; its iso-electric point is 11, therefore the lysozyme net charge was positive under experimental conditions.

Lipids Extraction Procedure

Tear lipids were extracted from lotrafilcon A (Focus Night & Day [Pre-Aqua formulation]; CIBA®) contact lenses that had been worn by 25 neophyte human subjects. Each participant first went through a period of adaptation to the day-wear modality with lotrafilcon A lenses, followed by one month of continuous wear. All subjects were recruited from the campus of the University of California at Berkeley and were free from ocular disease or any ocular abnormality that contraindicated contact lens wear. A thorough explanation of the study protocols was given to each prospective subject, and informed consent was obtained thereafter. This study observed the tenets of the Declaration of Helsinki and was approved by the University of California, Berkeley Committee for Protection of Human Subjects.

Lotrafilcon A lenses were chosen for this study because they are FDA approved for continuous overnight wear, and because these lenses were reported by the manufacturer (CIBA) to be free of any surface-active modifiers; the blister packaging solution has a surface tension of 71.5 mN/m, which is close to pure water, as confirmed by our

measurements. Furthermore, it has been shown that lotrafilcon A lenses worn for 30 days of continuous wear accumulated up to 640 μg of tear lipids per lens.²⁹ Taking all these factors into consideration, we concluded that lotrafilcon A lenses worn for 30 days would be a suitable source of tear lipids in an amount sufficient for the interfacial properties studies employing sessile bubble tensiometry.

The worn lenses were collected by research optometrists wearing powder-free gloves. The lenses were rinsed in deionized distilled water and blotted with filter paper. Each lens was placed in a separate 20-ml glass scintillation bottle containing 4 ml of solvent. The solvent used for extraction consisted of 5 parts of toluene, and 1 part of isopropyl alcohol (5:1 v/v); both from Sigma, spectroscopic grade. Toluene is widely used for lipid extraction from tissues, and systematic studies of the solubility of lipids in a toluene-ethanol mixture have shown that this combination has superior lipid-extraction properties compared with chloroform-methanol.³⁰ The lenses immersed in solvent were subjected to 30-min sonication in a water-filled bath. The solvent with extracted lipids was then transferred into smaller (5 ml) clean glass vials and evaporated under vacuum. Special care was taken to avoid contact of solvent and lipid extracts with human skin, any plastic parts, or gloves. All glassware was cleaned with saturated KOH solution in ethanol, then copiously rinsed first with distilled, then deionized water and dried under vacuum prior to use.

Dry lipid extracts were stored in a freezer at -20°C . Extracted samples were re-dissolved in 200 μl of the same solvent prior to use. Fifteen unworn blank lotrafilcon A lenses identical to the lenses distributed to the subjects (from the same lot number) were subjected to exactly the same procedures. The reconstituted extracts from blank unworn lenses were tested for dry residues using ellipsometry and for surface activity using tensiometry in exactly the same manner as with the lipid extracts. The dry residues from all blank lenses were less than 0.5 nm in thickness, practically indistinguishable from the dry residues left after evaporation of an equal amount of pure solvent. For all blank extract samples the surface tension was 71.6 ± 0.8 mN/m and it remained constant for 24 hours after deposition and solvent washout. The blank experiments confirmed that no dissolution or degradation of the lotrafilcon A lenses occurred during treatment with solvent and that the lenses did not contain any surface-active ingredients, which would contaminate the collected lipid extracts.

Lipid Film Thickness Measurement

An SE-400 Ellipsometer (Sentec Instruments GmbH, Germany) was used to measure the thickness of the lipid films. Five drops of Millipore water, 10 μl each in volume, were placed on a silicon wafer with a 2-nm thick layer of native SiO_2 . A 1- μl drop of the reconstituted lipid extract was then deposited and spread over the surface of each water droplet. The silicon wafers were kept in covered Petri dishes to reduce the evaporation rate and thus to get relatively uniform lipid film thickness. After slow complete evaporation of both solvent and aqueous drops, the thickness of the lipid films left on the silicon wafer surface after water evaporation was measured with an ellipsometer across the entire film area (total number of measurements was 50-75 points) and averaged. The variations in thickness for each film did not exceed 15 %. The mean thickness of the dry remnants left by pure solvent drops evaporated from the water droplet surface was used as a baseline. The average

thickness of the lipid films varied from 8 to 25 nm between extract samples. Based on the results of the ellipsometric measurements, we calculated the amount of the lipid-extract solution necessary to build the interfacial layer around an air bubble of known surface area with a thickness of 8-12 nm and a surface tension of 25 ± 1.2 mN/m.

To deposit a multilayer with varying thickness up to 30 nm, the lipid extracts collected from 6 worn lenses were pooled together, and then reconstituted in 200 μ l of solvent.

Tensiometry

There are several methods available to measure surface tension. A Wilhelmy plate is used in the Langmuir trough technique for tension measurements at a planar air-water interface^{6,20,21}, whereas automatic axisymmetric drop/bubble shape analysis is used for tension determination at the curved surfaces of pendant drop^{19,32} or sessile bubble^{31,33,34}. Miano *et al.* used the pendant drop technique to investigate the interaction of model tear proteins with monolayers of bovine meibomian lipids.¹⁹ Others have employed the Langmuir trough with a Wilhelmy plate to examine the physical properties of lipids.^{18,20-22}

In this study, the sessile bubble configuration was employed. A small air bubble was formed at the underside of a straight hydrophobic capillary vertically immersed into an aqueous phase and lipids were deposited on the surface of this bubble. A similar method was employed in earlier studies investigating surface activity and protein-lipid interactions in pulmonary surfactant systems.^{31,32} The major advantage of the sessile bubble in comparison with the widely used Langmuir trough coupled with a Wilhelmy plate is that the surface area of the sessile bubble can be very small (*i.e.*, 15 ± 2 mm² in this study). Thus, a few micrograms of lipids were sufficient to form multilayers up to 30 nm thick. The advantage of the sessile bubble configuration over the pendant drop was faster equilibration of the interface due to continuous stirring of the aqueous phase and larger volume-to-area ratio.^{33,34} Another advantage was high stability of the bubbles when compared with the pendant drop, especially at low surface tension. In addition, this method eliminated the influence of aqueous phase evaporation. Lastly, the sessile bubble technique was suitable for studies of the interfacial dilatational rheology by the step-strain relaxation method.³³⁻³⁷

The sessile bubble setup was augmented with a bulk phase exchange option allowing displacement (wash-out) of one aqueous phase (for instance, MPS solution) in the measuring cell with another fluid such as a pure buffer solution while maintaining the interface intact during displacement.³⁶ This “washout” option was used to validate the irreversible character of lysozyme binding to the reconstituted *ex vivo* lipids and to evaluate the effect of MPS on the lipid multilayer's interfacial properties.

A Ramé-Hart tensiometer (Ramé-Hart Instrument Co., Netcong, NJ, USA) with DropImage Advanced (v.2.2) software and automated dispensing system was used for real time surface tension data acquisition. Figure 1 displays the schematics of the experimental setup. A magnified image (1 μ m = 10 pixels) of a bubble was displayed on the computer screen. A more detailed description of this experimental setup is available elsewhere.^{33,34}

First, 18-20 ml of aqueous phase was placed into an optical glass cell (Hellma, USA, NY, 11803). The pre-calculated volume (typically in the range 1-2 μl) of lipid extract was manually deposited onto the air bubble surface ($15 \pm 2 \text{ mm}^2$) from underneath using a 10 μl high-precision glass syringe (Hamilton, NV) to produce the lipid film of desired thickness. The aqueous phase in a cell was stirred to provide uniform lipid distribution at the air-aqueous interface and to accelerate solvent dissolution into the aqueous phase. The aqueous phase was then displaced with pure buffered saline to remove all traces of organic solvents. After this first washout, the lipid-coated bubble was left to rest for 17-24 hours (overnight) without stirring. For elasticity studies lipid films with constant initial surface tension $25 \pm 1 \text{ mN/m}$ were deposited; the working concentration of the lipid extracts, assuming lipid density $0.9 \text{ } \mu\text{g}/\mu\text{m}^3$, was $0.8\text{--}1.5 \text{ } \mu\text{g}/\mu\text{m}^3$; resulting lipid film thickness at the air-liquid interface was 8-12 nm. After the lipid film was aged overnight, the model tear protein lysozyme solution, 40-50 mg in 2 ml of buffered saline, was injected into a cell and stirred for additional 16-19 hours. The working concentration of lysozyme in the cell was 2-2.5 mg/ml, which was within the concentration range found in normal human tears.^{26,27} Interfacial tension was monitored during these experimental procedures. After lysozyme-lipid film equilibration, the rheological parameters of the mixed layers were determined.

Interfacial Rheology

The transient step-strain relaxation technique was used to investigate the interfacial dilatational rheological properties of the individual and mixed lysozyme-lipid layers.³³⁻³⁷ An air-aqueous interface, previously equilibrated with a surface-active substance (lipids, protein, lipid-protein complex or MPS), was instantaneously expanded or contracted, then held constant while the interfacial stress (tension) was measured. The amplitude of the interfacial area change (ΔA) was less than 10% of the initial surface area (A_0). After the area perturbation, the interfacial tension was measured for 1 hour during which it relaxed toward the equilibrium value, decreasing after expansion and increasing after contraction. This time was sufficient for the tension to reach almost constant value. The transient elasticity $E(t)$ and its decay with time were then determined as follows:

$$E(t) = A_0 \Delta \gamma(t) / \Delta A \quad [1]$$

where $\gamma(t)$ is the change in surface tension induced by an area change. Single-exponential decay (equation 2) was fit (KaleidaGraph, version 4.03, software, Least Squares method, 100 iterations using all 1860 data points collected during an experimental run) to the experimentally observed transient elasticity, and the viscoelastic parameters of the interfacial layer were estimated according to the Maxwell model³⁵⁻³⁷

$$E(t) = E_\infty + A_1 \exp(-t/\tau_r) \quad [2]$$

where τ_r represents the characteristic time of viscoelastic relaxation, E_∞ is the elasticity at time approaching infinity, or storage modulus, and A_1 is the amplitude of response to the initial surface perturbation. The response of each interfacial film to the area expansion and contraction was measured 10 times.

At this initial exploratory stage of the project all the experiments were performed at ambient temperature, $22^{\circ} \pm 1^{\circ} \text{C}$.

Results

Surface Tension Measurements

Surface tension of the reconstituted *ex vivo* lipid films was first studied as a function of film thickness. Figure 2 shows that the surface tension of the lipid layers was reduced from 32 to 22.5 mN/m as the layer thickness increased from 2 to 15 nm and did not change with further thickening of the lipid film.

Figure 3 shows the surface tension history at the air-saline interface after injection of lysozyme with continuous stirring of the aqueous phase; the working concentration of lysozyme in the cell in this experiment was 2 mg/ml. The surface tension decreased from 72.5 to 53 mN/m during the first hour, then continued to descend slowly for over 16 hours. After 16-24 hours of interface aging, the final tension was 50 ± 1 mN/m. This experiment was repeated for lysozyme concentrations ranging from 2.0 to 2.5 mg/ml and the results (not shown here) suggested that the final surface tension was independent of the concentration. This observation was in agreement with data reported in the literature.^{4,5}

In these experiments, after 16 hours of equilibration the aqueous lysozyme solution in the cell was displaced with buffered saline. The washout provided complete protein removal from the bulk phase inside the cell.^{31,32} The surface tension did not change after washout and remained 50 ± 1 mN/m, thus indicating an irreversible character of the protein binding to the air-water interface.

Figure 4 illustrates the surface tension history of the mixed lipid-protein layer. An arrow indicates the moment when lysozyme was injected into the cell. Surface tension decreased by 1.5 mN/m immediately after lysozyme injection, then increased back to 26 mN/m and remained constant for another 10 hours of measurements.

The results of the surface tension measurements during the injection, 30-minute equilibration and then washout of OFX are presented as a function of time in Figure 5. In this set of experiments, an air bubble was formed in 19 ml of buffered saline with a surface tension of 72.5 mN/m. A few minutes later, as indicated by the arrow in Figure 5, 1 ml of OFX was injected into the cell with continuous stirring for 30 min. The surface tension dropped to approximately 40 mN/m almost instantaneously upon OFX injection. Continuous stirring and buffer flow through the cell caused the scatter (± 1 mN/m during washout) of the surface-tension data during the washout period shown in Figure 5. Prior to washout, stirring was stopped for 2-3 minutes and the surface tension at the air-OFX (1:20 dilute) interface was found to be 36.5 ± 0.5 mN/m. This value was in agreement with that reported by Dalton et al. for undiluted OFX.³⁸ Stirring was then resumed and 500 ml of buffered saline was pumped through the cell. The final tension at the air-saline interface increased by 3 mN/m after OFX was washed out from the bulk phase, but still remained low at 39.5 ± 0.5 mN/m. Similar behavior was observed upon injection and washout of OFR. The surface tension for OFR (1:20 dilute) was 35.2 ± 0.5 mN/m, which then increased to 38.5 ± 0.5

mN/m after washout. The surface tension at the air-aqueous interface after a complete washout of both OFX and OFR from a bulk solution remained much lower than 72.5 mN/m, which is typical for a pristine air-water interface. These observations suggested that both MPSs used in this study contained some irreversibly-adsorbing components, which remained attached to the interface after bulk-surfactant washout.

Figure 6 illustrates the surface tension history of the mixed lipid-protein layer that was exposed to OFX for 30 minutes then rinsed with buffered saline. Injection of OFX into the cell caused a slight decrease (~ 1.5 mN/m) of the mixed film surface tension. After displacement of the aqueous phase with pure buffered saline, the surface tension returned to its original value of 26.1 ± 0.3 mN/m. The same trend was observed upon injection of OFR followed by washout with buffered saline. Unchanged surface tensions indicated that MPS did not displace, solubilize, or destroy the pre-deposited *ex vivo* lipids during the 30-minute exposure. However, these observations do not provide sufficient information regarding specific interactions between MPS and the mixed lipid-protein films.

To acquire more information in relation to the interactions between MPS and lipid-protein complexes, we employed the interfacial step-strain relaxation method to study in detail the rheological properties of the layers formed by each of these substances alone, and of the mixed interfacial layers. Rheological parameters are often used to characterize the interfacial properties of thin films under dynamic conditions and are relevant for many practical applications where the interfacial film stability is involved.³⁴⁻³⁷

Interfacial Elasticity Measurements

The interfacial viscoelastic behavior of the individual layers formed by lysozyme and the reconstituted *ex vivo* lipids was studied. Figure 7 shows the interfacial elasticity history of the layer formed by lysozyme (bulk concentration 2.5 mg/ml), before (Figure 7a) and after (Figure 7b) washout of protein from the bulk solutions, which served as control experiments for further studies of MPS's influence on the interfacial elasticity of mixed lipid-protein films.

Figure 7a shows that prior to washout (i.e., in the presence of lysozyme in a bulk solution), the responses of the interfacial protein layer to expansion and contraction of surface area completely coincide. The single-exponential decay model (Eq. 2) was fit to the experimental data and rheological parameters were thus estimated. E_{∞} was found to be 25.6 ± 0.5 mN/m and τ was 460 ± 20 s for both expansion and contraction of the protein interfacial layers.

As seen in Figure 7b, protein washout from the bulk phase changed the viscoelastic response of the interfacial layer, and for post-wash dilatations, some hysteresis arose between the expansion and contraction steps. E_{∞} increased after washout, becoming 34.5 ± 0.5 mN/m and 33.2 ± 0.5 mN/m for expansion and contraction, respectively. The post-wash relaxation time τ also increased from 460 to 760 ± 10 s and 630 ± 15 s for expansion and contraction, respectively.

Figure 8 depicts typical transient elasticity vs time curves for the interfacial layers formed by reconstituted *ex vivo* lipids (curve 1), for the same lipids after injection and equilibration of

this layer with lysozyme (curve 2), and after washout of the mixed layer (curve 3). Elastic response of the lipid layer alone was independent of whether expansion or contraction was applied, i.e., no hysteresis was observed. E_{∞} of the *ex vivo* lipid layers was close to that of the lysozyme, 25.3 ± 2.5 mN/m; τ for the lipid multilayers was 90 ± 25 s, 6-7 times shorter than that of the lysozyme layers. For the mixed *ex vivo* lipid-lysozyme interfacial films, E_{∞} was found to be 24.8 ± 3.7 mN/m; it did not change upon lysozyme adsorption at the lipid-aqueous interface. This result is as expected, as both of the components, lipids and lysozyme, have very close values of E_{∞} . However, the relaxation time τ for the mixed lipid-protein films was 250 ± 35 s, 2.7 times higher than for the lipids alone and 1.8 times lower than for lysozyme alone. In the control run (curve 3), where a lipid-protein mixed film was washed with buffered saline without exposure to MPS, E_{∞} increased slightly by 2 ± 1.2 mN/m, after protein removal from the bulk phase.

Figure 9 depicts an example of the transient elasticity of a post-wash OFX interfacial layer at the air-water interface, without pre-adsorbed lipids or protein. One can see from this plot that the interfacial rheological behavior of post-wash OFX was distinctly different from that of the lipids, protein or the mixed lipid-protein layer. The OFX layer exhibited a 3-fold lower E_{∞} than lysozyme or lipids. For both post-wash OFX and OFR layers, E_{∞} was 8.3 ± 0.5 mN/m. In contrast, τ was 950 ± 50 s for the post-wash MPS layers – these layers relaxed 2 times slower than protein and 9.5 times slower than the lipids. MPS formed relatively weak, viscous layers relaxing slowly after expansion or contraction of the interface.

Figure 10 shows the elasticity history of the mixed lipid-protein layers first exposed to either OFX or OFR for 30 min, then washed with buffered saline. MPS induced noticeable changes in the viscoelastic behavior of the mixed lipid-protein layers. After exposure to OFX, the post-wash E_{∞} of the mixed layers was reduced from approximately 25 ± 2 to 13 ± 4.5 mN/m ($p < 0.001$, pair-t test), almost equal to the value observed for post-wash OFX alone. The τ of the films treated with OFX increased from 250 to 450 ± 30 s ($p < 0.001$, pair-t test), suggesting that some of OFX remained adsorbed onto the initial pre-deposited lipid-protein layer.

Exposure of the mixed lipid-protein films to OFR produced a weaker effect on their viscoelastic behavior. The E_{∞} of the mixed layers was reduced from 25.6 mN/m to 21 ± 3.0 mN/m after 30-min exposure to OFR ($p = 0.004$, pair-t test). In contrast, τ increased almost three-fold to 730 ± 55 s ($p < 0.001$, pair-t test). Once again, these results suggest that the surface-active components of MPS partially displaced the lysozyme and became irreversibly attached to the pre-deposited lipid-protein layer.

Discussion

Previous investigations of human tear constituents and whole tear surface properties such as surface tension have shown that the tear lipid layer plays significant role in tear film surface tension reduction and tear film stability.^{4-6,10,11} McCulley and Shine^{15,16} have developed a composition-based model for the thick tear lipid layer, proposing a complex stratified multilayered structure. According to this model, polar amphiphilic lipids (mostly phospholipids comprising 6-16 percent of the total lipids) with surfactant characteristics

serve as a structure upon which the stability of tear lipid film is largely dependent, although most of the tear lipid film consists of non-polar components (84-94%). The thickness of the tear lipid layer is one of its most extensively studied properties, and the estimates range from 13 to 100 nm depending on the method used for measurements.^{14,17} These measurements were consistent with the thick multilayer model of McCulley and Shine.^{15,16} The thickness of a dipalmitoyl phosphatidyl choline (DPPC, a phospholipid which is the major constituent of pulmonary surfactant and is most commonly used as a model for tear lipids) monolayer was in the range 2 – 2.5 nm as determined by neutron reflectivity measurements.²³ Even at the lowest estimated thickness of 13 nm, the tear lipid film is at least 6 times thicker than a monolayer; therefore, monolayers of meibomian lipid previously investigated may not be an adequate model of the human tear lipid layer. In a subsequent investigation, Shine *et al.* conducted a detailed study of polar lipids in human meibomian secretion and suggested that the polar lipid layer was most likely 1 to 3 molecules thick and served as a surfactant between the aqueous tear and the nonpolar part of the lipid layer.³⁹ Recently, Butovich *et al.* summarized the most current data regarding the structure and chemical composition of meibomian and tear lipids.^{17,18} The authors proposed that tear lipids form a thick blanket, with a minimum thickness of 20 molecules, over the aqueous layer of the tear film.

The interfacial pressure of meibomian lipid monolayers has been examined in a number of papers.^{6,19-23} In these publications, lipid monolayers were chosen to model the tear lipid layer and to investigate in greater detail the interaction of lipid monolayers with model tear proteins. Tragoulias *et al.* found that model tear proteins possessed significant surface activity; from these results the authors concluded that tear proteins were the major contributors to the surface pressure of a tear film.²⁰ Several studies¹⁹⁻²¹ have explored monolayers of meibomian lipids at relatively low surface pressures corresponding to low surface coverage by polar lipids. Under these conditions, proteins were able to penetrate into the lipid monolayer and reduce the surface pressure.¹⁹

In the present study, we focused on the interfacial properties of relatively thick *ex vivo* human lipid multilayers. We believe that the behaviors of thick lipid films provide a more realistic model of the human tear lipid layer than the lipid monolayers studied previously. The results shown in Figure 2 clearly indicate that surface tension strongly depended on *ex vivo* lipid layer thickness. The thinnest lipid film we studied was a 2 nm thick monolayer. The surface tension of this layer was close to 32 mN/m, corresponding to the surface pressure of 40 mN/m reported by other researchers.¹⁹⁻²¹ However, as the thickness of the deposited lipid layers increased, the surface tension decreased until it reached a constant value of 22 ± 1 mN/m at a lipid layer thickness of 15 nm or greater. These results suggested that the saturation, or coverage, of the lipid-aqueous interface with polar lipids increased in concert with the total *ex vivo* lipid film thickness. At a total lipid thickness between 15 and 25 nm, when a completely saturated, densely packed polar lipid layer was formed, the films exhibited a very high surface pressure of ~ 50 mN/m, which is in agreement with the values found for condensed phospholipid layers and lung surfactants.^{40,41} The important implication for lipid film dynamic behavior *in vivo* arises from these observations. The curve in Fig. 2 can be dissected into two linear parts: one for thin films between 2 and 10 nm, and the second for films thicker than 20 nm. The first part of the linear fit gives a

surface tension gradient, $d\gamma/dh = 1.0$ (mN/m)/nm, whereas for the second part the tension gradient is 30 times lower, 0.033 (mN/m)/nm. The tension gradients are known to be the driving force for the mass transfer along a liquid-air interface, which causes flows in liquid films. This phenomenon is known as the Marangoni effect. In thick lipid films, small tension gradients lead to sluggish flows and slow film thickness rearrangement. Therefore, clinically observed interference patterns²⁵ persist in the case of a thick lipid film. However, in thinner lipid films, a slightest decrease in the film thickness gives rise to high surface tension gradients, resulting in strong Marangoni flows, which lead to thickness re-equilibration.

As shown in Figure 4, lysozyme injected into the aqueous phase surrounding an *ex vivo* lipid film did not significantly change the surface tension at the air-aqueous interface even after long-time equilibration with continuous stirring. This observation was consistent with the results reported by Miano *et al.* for a mixed DPPC-lactoferrin layer at an initial DPPC surface pressure of 35 mN/m or higher.²³ In that study, neutron reflection measurements were performed on a DPPC-lactoferrin layer, and it was found that at high surface pressures, protein adsorption into the lipid layer was very low. The results of our surface tension measurements led us to the conclusion that lysozyme, like lactoferrin, could not penetrate into or displace the condensed, thick layer of reconstituted *ex vivo* lipids. The evidence of lipid-lysozyme interaction and formation of an interfacial lipid-lysozyme complex in the present study was obtained using an interfacial relaxation technique in combination with a washout option.

We found significant differences between the interfacial rheological parameters of the *ex vivo* lipid layers alone and of the mixed lipid-lysozyme layers. These differences persisted even after complete protein removal from the bulk phase, indicating that lysozyme was irreversibly bound to the lipid layer. At high surface pressures, the presence of lysozyme in the mixed interfacial layers was undetectable by surface tension measurements. However, it manifested itself by an approximately two-fold increase in mixed film relaxation time when compared with that of a layer formed by *ex vivo* lipids alone. The interfacial rheological properties of the reconstituted *ex vivo* lipids in the thick mixed layers were much more sensitive indicators of their interaction with protein than were surface tension or surface pressure. Changes in interfacial rheological parameters after protein washout were most likely caused by the removal of loosely-bound protein molecules from the adsorbed interfacial layer, leading to increased interfacial viscosity.

The adsorption of lysozyme into the *ex vivo* lipid layers was likely driven by electrostatic interactions. Lysozyme has an iso-electric point of 11 and a net positive charge at pH 7.3. Polar tear lipids are known to contain a significant amount of the species with negatively charged head groups, such as anionic phospholipids and fatty acids.^{15,17} These anionic head groups are likely to interact with the positively charged groups of the lysozyme molecules, forming complexes through an ion-pairing mechanism.

Lysozyme is one of the most abundant proteins in human tears.^{26,27} We hypothesize that the binding of lysozyme molecules to anionic lipid head groups may play a very important role in maintaining tear film integrity. Lysozyme and other tear proteins, coupled with polar

lipids in the tear lipid film, may provide additional tethering and stronger linkage between the hydrophobic lipid layer and the underlying tear aqueous.

MPS, with its relatively high concentration (0.1 % by weight) of Tetronic T1304 and other surface-active ingredients reduced the surface tension at the air-aqueous interface by approximately 32-34 mN/m. Our previous studies of Pluronics (non-ionic surface-active polyethylene-polypropylene block-copolymers) adsorption at air-water and water-mineral oil interfaces have shown that these polymers bind irreversibly to the interfaces.³³ At the water-oil interface, the polymeric surfactants were found to compete successfully with a synthetic double-tailed surfactant Aerosol OT (AOT, *bis*-ethyl-hexyl sulfosuccinate, sodium salt), whose structure resembles that of natural phospholipids.³⁴ Tetronic T1304 also belongs to the family of nonionic surface-active polyethylene-polypropylene block-copolymers. Several experiments conducted with aqueous solution containing only 0.1 % wt. of Tetronic T1304 have revealed that, like Pluronics, Tetronic binds irreversibly to the air-aqueous interface. We hypothesize that in MPS, Tetronic T1304 is responsible for an irreversible reduction of surface tension at the air-solution interface. Moreover, interfacial rheology studies conducted with mixed lipid-lysozyme layers exposed to MPS (Figure 10) have shown that both OFX and OFR alter the rheological characteristics of mixed lipid-lysozyme films. The changes caused by OFX were found to be significant and persistent, whereas OFR produced milder effects. Besides Tetronic T1304 and other components present in both OFX and OFR, OFX contains an unspecified amount of AMP 95, amino propanol, which is a polar organic solvent, while OFR contains nonanoyl ethylenediaminetriacetic acid, which is a surface-active fatty acid. The differences in formulations between OFX and OFR, slight as they may be, were the major reason for the differences in MPS performance. It is conceivable that MPS cytotoxicity⁴² might be associated with high surface activity of some ingredients and their interaction with the phospholipids on corneal epithelium cells.

The results of the present study show that MPS surface tension (see Figure 5) was at least 10 mN/m lower than that of lysozyme (Figure 4), suggesting that MPS contains ingredients that are more surface active than lysozyme. The interfacial layers became more plastic after treatment with OFX (Figure 10), indicating that OFX partially displaced protein from the mixed lipid-protein interfacial film. The difference in the responses of lipid films to exposure to OFX and OFR suggests a new explanation for documented differences in their cytotoxicities.⁴² It seems likely that OFX ingredients interacting with phospholipids comprising cell walls might compromise the cell-wall protective function by making the lipid membranes more permeable to potentially cytotoxic preservatives. The milder disruptive effect of OFR on lipid-protein mixed films correlates with its lower cytotoxicity level.

Tear films are complex, multilayered biological colloid systems. The composition of the tears is delicately balanced and regulated by ocular tissues to maintain optimal function under the high stress exerted by the eyelids during blinking. It is reasonable to assume that the interfacial rheological properties of healthy tear films are also optimally adjusted to provide timely and full recovery after very rapid compression (closed eye) followed by expansion (open eye). Soaps and shampoos contain highly concentrated surfactants that are

much more surface active than tear proteins. These substances are able to solubilize and destroy tear lipid films,¹ causing the familiar, extremely unpleasant sensation when soap or shampoo comes into contact with the ocular surface. As we have shown above, MPS containing some highly surface-active ingredients was able to displace lysozyme from mixed *ex vivo* lipid-protein layers, leading to alterations of lipid-protein film interfacial rheological characteristics. In an eye, the delicate natural balance of tear film interfacial properties might become compromised by MPS leaching from a lens. There are indications in the literature that highly porous soft pHEMA-MAA lenses (Acuvue 2), immersed in solution overnight, were able to accumulate up to 36 µg/lens of Tetronic and then release this surfactant into a buffered saline.^{43,44} A similar process may occur when a contact lens wearer inserts such a lens on an eye after overnight storage in MPS. The surface active components trapped inside the lens matrix may be slowly released into the eye, interacting with the mixed tear lipid-protein films and displacing proteins from this layer. Such displacement might produce undesirable alterations of tear film interfacial rheological properties, reduced tear film stability and discomfort after several hours of lens wear. These findings help to explain the reduced tear film thickness and discomfort reported by patients who used OFX.¹² It should be emphasized that in clinical practice, special measures must be taken to find a proper combination of lens material and lens care solution that will be the safest, most suitable, and comfortable for each patient, especially for those with compromised tear film stability and short tear break-up time.

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Figure 1.
Schematics of sessile bubble tensiometer with bulk phase exchange.

Figure 2.
The surface tension of the reconstituted ex vivo lipid films as a function of the film thickness.

Figure 3.
Lysozyme (2 mg/ml) surface tension history for 20 hours.

Figure 4.
Surface tension history of the lipid-lysozyme mixed film; stirring continued for 16 hours.

Figure 5.
Surface tension upon OFX injection followed by 30-min equilibration and washout.

Figure 6.
Surface tension history during loading and washout of OFX.

Figure 7. Lysozyme transient elasticity as a function of time before (a) and after (b) washout with buffered saline (post-wash).

Figure 8. Transient elasticity $E(t)$ history for the lipids and mixed lipids-protein layers. Solid lines represent the exponential fits (Eq. 2) of the experimental points.

Figure 9.

Transient elasticity $E(t)$ history for post-wash OFX interfacial layer. Solid line represents the exponential fit (Eq. 2) of the experimental points.

Figure 10. Transient post-wash elasticity $E(t)$ of mixed lipids-protein layers after exposure to OFX and OFR.