Surface Properties of Artificial Tear Film Lipid Layers: Effects of Wax Esters

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Purpose. The tear film lipid layer is believed to stabilize the tear film and to retard evaporation. Based on previous simple in vitro studies, the evidence for the latter property is scarce. In this study, we used complex lipid mixtures including various wax esters to study their physical properties and evaporation retarding effect.

Methods. Twelve samples of artificial tear film lipid layer mixtures composed of (L-α)-phosphatidylcholine, cholesterol oleate, and triglycerides were mixed with wax esters. A Langmuir balance was used to analyze the compressibility and rheological properties of these mixtures. In addition, a custom-built system was used for the evaporation studies used at 35°C. Lipid films were imaged with Brewster angle microscopy.

Results. None of the studied lipid mixtures decreased the evaporation rate. All lipid mixtures had similar compression isotherms and viscoelastic properties regardless of the wax ester species or its concentration. The results suggest that the overall properties of these mixtures are independent of individual lipid species and that these films are very cooperative and showed minor variation depending on the wax ester species. Brewster angle microscopy images revealed that the lipid films assembled into multiple layers.

Conclusions. Wax ester–containing lipid mixtures resembling the tear film lipid layer are organized in a layered fashion so that amphiphilic lipids are adjacent to the aqueous phase and the nonpolar lipids are layered on top of these. This organization does not retard evaporation and raises overall questions about the role of lipids in the tear film.

Keywords: wax ester, tear film lipid layer, dry eyes, Langmuir films, rheology

Dry eye syndrome (DES) affects approximately one-tenth of elderly individuals.[1] Evaporative DES is believed to be caused by alterations in the tear film composition, which cause excessive evaporation of the tears. Although vastly studied, its etiology is still partly unknown. The tear film lipid layer (TFLL) is believed to play a major role in retarding the evaporation of tears and, therefore, it would be reasonable to argue that alterations in lipid composition would have a causative relationship to the development of evaporative DES.[2–4]

Tear fluid forms a film-like structure on the surface of the cornea. It serves several functions: it maintains the ocular surface moisture, flushes contaminants and foreign objects from the eye, nourishes the corneal cells and protects them from pathogens, lubricates the lid–cornea interface when blinking and sleeping, and improves optical properties by modifying the refractive index of the cornea. Tear fluid can be roughly divided into three qualitatively distinct layers,[5–7] although the layers are formed gradually. The corneal epithelium is lined with a mucin-rich layer, overlaid by an aqueous layer that is covered by the lipid layer at the air–water interface. The TFLL is composed of polar and nonpolar lipids. Polar or amphipathic lipids such as phospholipids consist of a hydrophilic head group and hydrophobic tail/tails. They are surface active and can form bilayers, monolayers, vesicles, and other structures in an aqueous environment. Phospholipids reduce surface tension in high concentrations. Nonpolar lipids, on the other hand, repel water and, if placed in an aqueous environment, they tend to form droplet-like structures to minimize contact with water.[8] Nonpolar lipids are derived from oily secretions produced by the meibomian glands located at the lid margin. Major meibomian lipids are cholesterol esters (CE), triglycerides (TG), and wax esters (WE).[4,6,9] Few polar lipids have been found from the meibomian glands, and the origin of the polar lipids in the TFLL is still unknown. Phospholipids have been found in tear samples in large quantities,[8,10–13] along with the nonpolar lipids. In our previous studies, we demonstrated the organization of an artificial lipid layer at the air–water interface. Polar phospholipids form a monolayer at the air–water interface, while nonpolar lipids (CE and TG) lie on top of the polar lipid layer.[14–16] It has been proposed that similar layering takes place in the human tear film.[8,17] It has also been demonstrated that polar lipids need to be the major element in a stable lipid layer, and nonpolar lipids further stabilize the film.[15,16]

The TFLL, in combination with ocular surface mucins, facilitates rapid and more uniform spreading of the tear film across the ocular surface by lowering the surface tension of the air–tear interface.[18] Additionally, it has been suggested that the tear film lipids effectively stabilize the tear film by delaying tear film rupture. Therefore, the lipid film prevents dewetting of the ocular surface in the interval between blinks. One factor that may influence the timescale of the rupture is evaporative thinning of the tear film. In order to prevent the dewetting of the ocular surface by evaporation, the TFLL is believed to retard evaporation.[4,19] However, the mechanism, of this potential function is not known. Therefore, it is plausible that alterations

in the TFLL's composition could cause excess evaporation of tears, which could lead to hyperosmolarity of the tear fluid. This hyperosmolarity results in inflammation of the epithelial cells[20] and triggers the pathological cascades leading to evaporative DES. However, in a recent study it was shown that the retardation of evaporation is a property highly specific to certain lipid classes.[21,22] Wax esters have antievaporative properties and are a major component in the TFLL,[22,4] however, their function in tear film is unknown so far. In many plants, WEs retard evaporation through the leaves, and pure WEs are also efficient evaporation retardants in in vitro measurements.[22] Based on this, it seems feasible that WEs in the TFLL would function similarly.

In our previous studies,[21,22] we investigated the evaporation-retarding effect of either very simple lipid monolayers, such as phosphatidylcholine (PC) and WEs, or certain more complex lipid mixtures. However, these complex mixtures contained only one type of WE (behenyl oleate [BO]). Additionally, the dynamic properties of WE-containing artificial lipid mixtures have not been studied before. In this study, we aim to explore the effect of WEs on TFLL stability and to investigate the evaporation retarding effect of WE-containing lipid layers. This will provide further insights into the characteristics of the TFLL and the progression of pathological events in the development of DES.

Materials and Methods

Materials

Egg PC, behenyloleate (BO 22:0/18:1), and behenyl alcohol were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA); cholesterol oleate (CO) was obtained from Fluka (Buchs, Switzerland); triglyceride mixture (20% of each: tricaprin, tricaprylin, trilaurin, tripalmitin, and trimyristin) was obtained from Supelco (Bellemonte, PA, USA). Lignocerylignocerate (LL 24:0/24:0), linolenyloleate (LI 18:3/18:1), and lauryl oleate (LA 12:0/18:1) were obtained from Nu-Check-Prep (Elysian, MN, USA).

Lipid Mixtures

A total of 12 mixtures consisting of egg PC, CO, TG, and WE (BO, LL, LA, or LI) were prepared. Three different concentrations of WE were used. Mixtures with 20% of WE also contained 40% of PC, 20% of CO, and 20% of TG; mixtures with 30% WE had also 36% PC, 18% CO, and 18% TG; and mixtures with 40% WE contained 28% PC, 16% CO, and 16% TG. All lipid mixtures were dissolved in chloroform and had a total concentration of 1 mM for compression isotherms or 10 mM for evaporation studies. Behenyl alcohol (10 mM) was used as a positive control in the evaporation studies.

Evaporation Studies

Evaporation studies were performed using a custom-built system founded on a Langmuir trough (KSV Instruments, Helsinki, Finland) enclosed in a poly (methyl methacrylate) cabinet, as described previously.[21] Briefly, 150 mL of PBS was applied on the preheated (average temperature 35.3 ± 0.5°C) trough (area ~273 cm^2). The surface was covered with 50 μL (10 mM in chloroform) of the lipid solution. After the first 10 minutes, the cabinet was closed. The buffer was allowed to evaporate through the lipid film for 90 minutes, and the remaining buffer was then collected and weighed. The evaporation rate was determined as micrometers per minute.

Airflow of 1.7 L/s through the cabinet was employed in order to minimize variation in humidity during the measurement. The relative humidity inside the cabinet varied daily (from 9.6%–23.8%) and therefore all measurements were performed in triplicate on different days. The overall relative humidity inside the device cabinet could not be controlled due to the semiclosed nature of the system, but the airflow was employed to remove humid air from the cabinet. This has proven to diminish the effect of the changes in humidity.[21,22] The local humidity just above the air–water interface is the determining factor for the rate of evaporation.[19] Most likely in our system the convection-generating airflow in the cabinet keeps the conditions somewhat constant at the immediate vicinity of the interface, and, therefore, the mass transfer of water molecules into the surrounding atmosphere remains approximately constant despite the changing overall humidity conditions.

A PBS solution was used as a negative control. Behenyl alcohol was used as a positive control to test the accuracy of the method. Student's *t*-test was used to compare the results with the control. *P* values less than 0.05 were considered significant.

Compression Isotherms

A Langmuir minitrough (KSV Instruments) equipped with two moving barriers and a platinum plate for surface pressure measurements was used to record the compression isotherms. The temperature of the subphase was controlled with a thermostat (Lauda Eco E4, Köninghofen, Germany) and measured with a thermometer inside the trough. The preheated pool (21°C or 35°C) received an application of 180 mL of 10 mM PBS (NaCl [0.140 M], KCl [0.00027 M], pH 7.4), and 34 μL of the lipid solution was spread to the air–water interface. The solvent was allowed to evaporate for 10 minutes before compressions. Compressions were performed at a speed of 10 mm/min to a surface pressure of 38 to 40 mN/m and then relaxed at the same rate. The compression-relaxation cycle was repeated five times. The fifth cycle is shown in the results. Each sample was measured twice.

Reciprocal compressibilities (C_s^{-1}) were then calculated from the compression isotherm data using:

$$C_s^{-1} = -A_\pi (dA/d\pi)_\pi, \quad (1)$$

where A_π is the area per molecule at the designated surface pressure.[23] The data was smoothed by 8-point adjacent averaging.

Brewster-angle microscopy images (MicroBAM; KSV Nima, Espoo, Finland) were captured during the first compression at 21°C. The resolution of the MicroBAM was 12 μm.

Oscillating Barrier Studies

The oscillating barrier method is described in detail elsewhere.[14] In short, the lipid layer is exposed to short dilatational pulses induced with Langmuir trough barriers, and the minute changes in surface pressure are recorded. The method is used for studying the dilatational viscoelasticity of the films at differing oscillation frequencies. The surface dilatational modulus determined based on the measurement is divided into two components: E_d (dilatational elasticity) and E_v (dilatational viscosity). These components quantitatively describe the viscoelastic properties of the film. Oscillations were performed at 35°C after two compression-expansion cycles. Eight oscillation cycles were performed in each of the following frequencies: 200, 150, 100, 50, 25, 12.5, and 6.3 MHz. All measurements were done in duplicate.

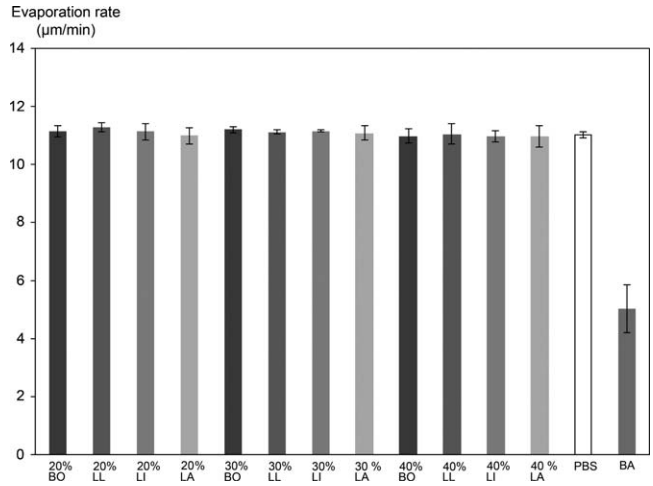

FIGURE 1. Evaporation rates for the lipid mixtures containing 20%, 30%, or 40% of each WE (BO, LL, LI, or LA). All mixtures also contained PC, CO, and TG, as explained in the Materials and Methods section. Plain PBS was used as a zero-control and behenyl alcohol (BA) was used as a positive control. None of the evaporation rates varied significantly from the zero-control. The *P* value for the BA was less than 0.006. Experiment procedures are explained in the Methods section.

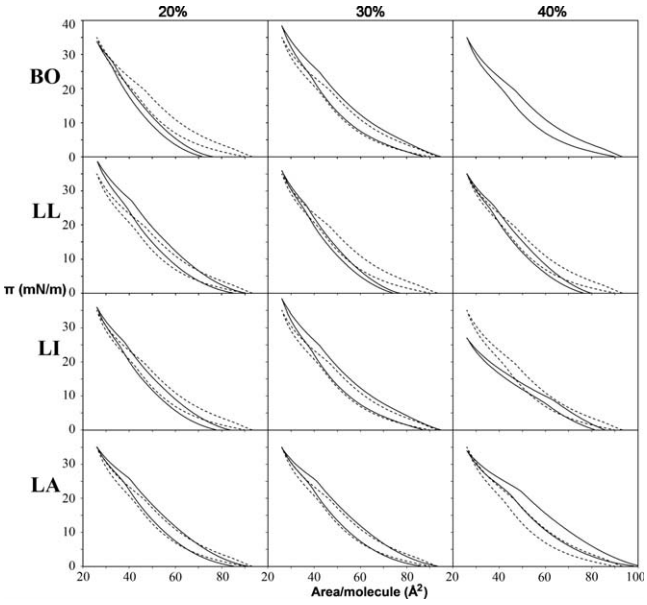

FIGURE 2. Representative compression-relaxation isotherms for all lipid films according to their WE content. A mixture with 40% of BO was used as a comparison for all samples (*broken line*). The *first row* represents films with BO, the *second row* is films with LL, the *third* is LI, and the *fourth* is LA. All lipid films also contained PC, CO, and TG, as explained in the Materials and Methods section. All measurements were performed at 35°C to 36°C; each compression-relaxation cycle was performed four times before the recorded cycle shown in the figure.

RESULTS

Evaporation

The results from the evaporation studies are shown in Figure 1. None of the lipid films retarded evaporation significantly. Their evaporation rates were between 10.96 and 11.29 μm/min, which is not different from the evaporation rate of pure PBS (11.06 μm/min, $P > 0.11$). Although initial relative humidity and temperature values varied daily from 21.0°C to 22.6°C and 6.7% to 24.0%, they did not have a significant effect on the results. Initial surface pressure of the film was 43.0 ± 1.4 mN/m for all mixtures. Behenyl alcohol, previously shown to retard evaporation,[21] was used as a positive control and decreased the evaporation rate to 5.1 μm/min, which was significantly lower than the evaporation rate for any other lipid mixture ($P < 0.006$).

Compressions and Compressibilities

All lipid films were compressed up to a surface pressure of 40 mN/m and then relaxed back to 0 mN/m. This procedure was repeated five times for each mixture at 35 ± 1°C. Representative compression isotherms for the fifth cycle are shown in Figure 2. Mixture containing 40% BO was used as a comparison for all the samples because this composition closely resembles the physiological TFLL, according to recent studies on human tear fluid and meibum.[22,24] All lipid layers showed some hysteresis, but the extent of the hysteresis was dependent on the WE species. Behenyloleate showed very little hysteresis at 20% concentration, but slightly larger hysteresis at higher concentrations. In contrast, the hysteresis in the LL layer decreased in 30% and 40% concentrations. Linolenyloleate showed somewhat more hysteresis at 30% concentration than in other concentrations, whereas LA showed similar hysteresis in all concentrations. All mixtures could be compressed to a very low area/molecule of approximately 30 Å²/molecule. The compression isotherms showed very little variation when they were compared with the one with 40% BO (Fig. 2). All mixtures had rather monotonous rise in surface pressure versus molecular area isotherms, with one kink at smaller area/molecule (40–45 Å²/molecule). The main finding is that although there is a somewhat large variation in the structure of the WE and also a large variation in the lipid composition, the isotherms are almost identical.

Reciprocal compressibilities (C_s^{-1}) versus area/molecule are shown in Figure 3. A mixture with 40% BO was used as a comparison for all the other samples. Compressibility values were relatively low for all mixtures: under 40 mN/m. All mixtures showed a sharp dip in their C_s^{-1}-values but the location of the dip varied between the mixtures.

Rheological Parameters

Representative dilatational elasticity (E_d) and dilatational viscosity (E_v) values measured at 35°C are shown in Figure 4. All lipid films showed similar behavior: at lower frequencies ($f < 100$–150 MHz) the films were more elastic and at higher frequencies they were more viscous.

Brewster Angle Microscopy

Brewster angle microscopy images were captured at 21°C during the first compression cycle at 35 mN/m (Fig. 5). Lipid layers with less WE also showed fewer condensed areas. This was observed for all the samples. Wax esters that were more liquid had much fewer condensed areas, if any (Fig. 5, LI and LA). Both LI and LA formed uniform-looking films that seemed condensed only at the highest concentration of WE. Behenyloleate formed stripe-like regions across the surface in all concentrations, which was clearest at a concentration of 40%. Lignoceryllignocerate was the most condensed of the mixtures forming island-like condensed areas throughout the surface in all concentrations.

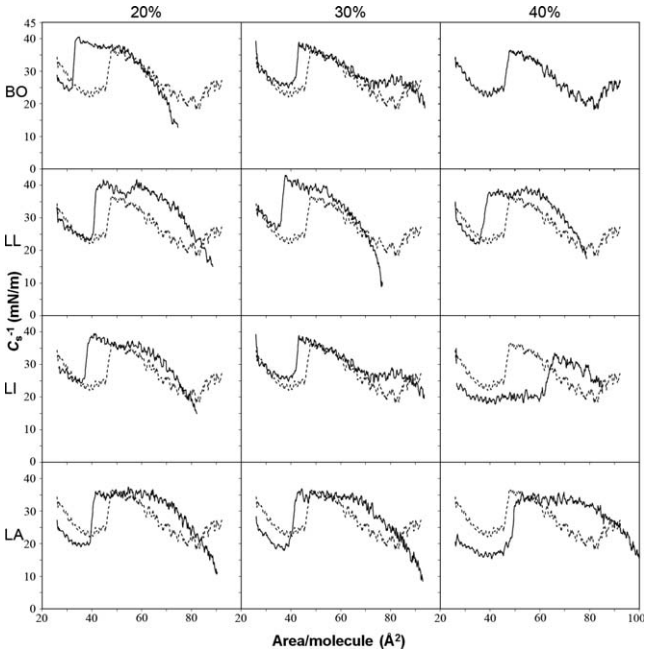

FIGURE 3. The reciprocal compressibilities (C_s^{-1}) versus mean molecular area for all lipid mixtures according to their WE content. The mixture with 40% of BO was used as a comparison for all samples (*broken line*). The *first row* is films with BO, the *second row* is films with LL, the *third* is LI, and the *fourth* is LA. All lipid films also contained PC, CO, and TG, as explained in the Materials and Methods section. All measurements were performed at 35°C to 36°C; each compression-relaxation cycle was performed four times before the recorded cycle shown in the figure.

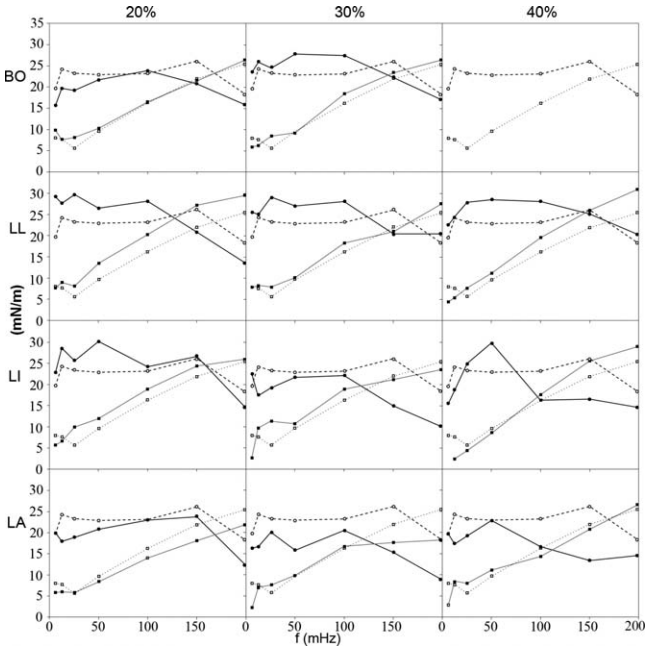

FIGURE 4. Rheological parameters E_d (dilatational elasticity) and E_v (dilatational viscocity). Lipid mixture with 40% of BO was used as a comparison for all other mixtures. ○ E_d –40% BO; ● E_d; □ E_v 40% BO; ■ E_v.

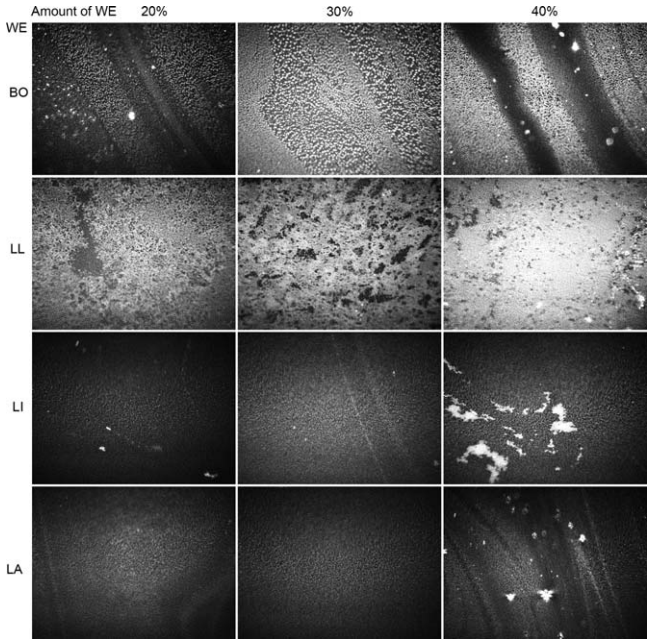

FIGURE 5. Brewster angle microscopy (BAM) images at 21°C. Images are taken from the first compression of the film at 35 mN/m surface pressure. The image size is 3600 × 4000 μm and the resolution is 12 μm.

DISCUSSION

Tear-film lipids are believed to retard evaporation from the tear film, and thus help to provide a moist surface for the corneal epithelial cells. In evaporative DES, the lacrimal glands produce a sufficient amount of tears, but the composition of the tear film is abnormal, causing the water to evaporate faster than normal and making the tears hyperosmolar. This hyperosmolarity induces an inflammatory response in the corneal epithelial cells.[2,20] Lipids are thought to play a central role in DES by maintaining a low evaporation rate. Wax esters are a major component in the TFLL, and they have been found to retard evaporation in previous studies.[21,22] They constitute 25% to 68% of the total meibomian lipid concentration[4] and can also be found in other natural systems, such as plants and skin, where their main function is to prevent evaporation.[25–28] It would logically follow that WEs are the component in the TFLL that causes the thin lipid layer to retard evaporation. In this study, we examined the contribution of WEs to the antievaporative effect of the TFLL. The lipid mixtures chosen for this study resemble the composition of those in tear films.[8,10–13,29,30]

In our previous study,[22] we examined the evaporation retarding effect of a range of WEs with differing lengths of hydrocarbon chains and differing degree of saturation. The evaporation retarding effect was highly dependent on the bulk melting temperature of the WEs. Based on this study we selected four of these WEs with differing melting points: one that is molten at 35°C (LA), one that is approximately at melting temperature and known to retard evaporation at 35°C (BO), and one that is in solid phase at 35°C (LL). The fourth lipid, LI, was selected due to its high degree of saturation. We investigated the potential effect of these differing WEs in the total behavior of the lipid mixture. We selected a wide variety of WEs, not only the ones that are present in the tear fluid. Egg PC was selected as the tear film phospholipid model compound, since it is mainly a mixture of unsaturated natural phospholipids,[8,10,29,30] which form fluid lipid layers at

physiological temperatures. A lipid layer composed of purely saturated lipid, such as dipalmitoyl phosphatidylcholine, would have very different behavior, since such layers may experience phase transition to condensed phase. Based on the TFLL phospholipid composition, such behavior is not expected in the TFLL in vivo.

In vivo studies have shown that tear evaporation rates in humans vary from 0.0012 to 3.8 μm/min. These rates are significantly lower than those measured for PBS in vitro at 34°C approximately 8.0 μm/min,[31] or for the approximately 11 μm/min measured in the current study at 35°C. In vitro studies with meibum have resulted in lower retardation of evaporation (6%–8% reduction in evaporation rate).[19,32] Our previous results showed reduction of evaporation by WE layers to be approximately 50%.[22] Surprisingly, none of the mixtures in the current study retarded evaporation to a measurable level. This is most likely due to the excess of phospholipids in the film, which act as solubilizers for the WEs and disrupt the high ordering of WEs. Alternative explanation for the loss of evaporation retarding effect could be phase separation of WEs causing the lipid film to spread unevenly.[15] This was also illustrated in a previous study where BO was shown to retard evaporation, but when it was mixed with other lipids it lost its evaporation-retarding effect.[21] The packing behavior of the entire lipid film seems to have more importance to retardation of evaporation than the amount or thickness of the WEs. Specifically, WEs in mixtures with other lipids are unable to form tightly packed, condensed layers on the air-buffer interface.

Phospholipids form uniform layers at the water interface, but are unable to retard evaporation due to their fluidity, resulting from lateral diffusion and wobbling motion, and therefore high free volume of the lipid layer. In the current study the mixtures contained 28% to 40% of PC. Phospholipids primarily cover the air–water interface and the nonpolar lipids are layered as a secondary layer on top of the interfacial phospholipid layer. The most significant difference introduced by the smaller proportion of phospholipids is that the overlaying nonpolar phase is thicker. However, as shown in this study, moderate changes in the phospholipid or WE composition do not significantly affect the surface properties of such layers. Increasing thickness in nanoscale lipid layers is a rather inefficient method to retard evaporation since the evaporation retarding-effect of such lipid layers is mainly induced by highly ordered molecules located at the air–water interface, not by the overlaying layers. The evaporation resistance of lipid layers seems to be dominated by the interfacial lipid monolayer, when the thickness of the layer is less than 1 μm.[33] Also, based on our very recent results, increasing WE layer thickness beyond monolayer does not increase the evaporation retarding effect of the layer.[34] The data reviewed by Cerretani et al.[19] also suggest that lipid films, such as duplex layers, need to be significantly thicker than 100 nm in order to have practical impact on the evaporation rate. Altogether, lipid layer compositions based on the latest lipidome studies[11–13] would most likely have very similar interfacial behavior as suggested in this study.

Compression isotherms (Fig. 2) were very similar for all the lipid mixtures, which suggest that the quantity or chemical structure of the WE does not determine the behavior of the film. This suggests that even relatively large changes in film composition do not have a large effect on its properties. This is due to the cohesive organization of the lipid film, the high cooperatively of the lipids, and the high elasticity of the lipid layer. These findings are well in line with in vitro meibum studies that suggest that the behavior of the meibum at the air–water interface is unaffected by changes in nonpolar lipid composition. Only large (nonphysiologic) amounts of free cholesterol seemed to have an effect on the film properties. Oleic acid, WE palmityl oleate, and WE-like (O-acyl)-ω-hydroxy fatty acids (OAHFA) did not significantly affect the meibum behavior.[35,36] Our previous studies have shown how nonpolar lipids are layered on top of polar phospholipids and stabilize the film.[14–16] This study provided further evidence of the layered structure, since all the lipid mixtures could be compressed to a very small molecular area, which means that the lipids were not organized in a monolayer but formed a multilayered structure on top of the PBS.

Reciprocal compressibilities presented as a function of molecular area (Fig. 3) provide more information on phase transitions and the behavior of the film. All mixtures in this study behaved similarly and had one clear dip in their compressibility curve. It is most likely that at the dip point, a three-dimensional (3D) reorganization occurs in the film, but we are unable to provide proof of exactly what happens in the film with the methods used in this study. We assume that this behavior is caused by the WEs and nonpolar lipids reorganizing into 3D aggregates,[14–16,37–39] or alternatively, by a large conformational change of the WEs, that is, from a hairpin to an extended conformation.[34] Reciprocal compressibility values were below 40 mN/m for all samples, which suggests that the films have high interfacial elasticity.[23,40,41] This is supported by the interfacial elasticity measurements (Fig. 4).

Even though the compression isotherms and compressibilities as well as the elastic properties were very similar for all the lipid mixtures, a clear difference could be seen in the BAM images (Fig. 5). The BAM experiments were performed at 21°C, since at 35°C the temperature gradient above the air–water interface caused convection of air, which decreased the resolution of the images. The change in temperature might have had a minor effect on the appearance of the BO-containing lipid layers, since the BO is close to its melting point at 35°C.[22] However, it is unlikely that this would have had any effect on the conclusions made based on the BAM images. The WEs that we used had varying melting temperatures, 38°C for BO, 79°C for LL, and less than 22°C for LI and LA. This difference in melting point temperatures was clearly seen in BAM images where at 21°C the liquid LI and LA did not form condensed regions and even at the highest concentration LI only showed some small condensed clusters. On the other hand, BO and LL formed several condensed areas throughout the film. For BO, these condensed areas formed stripe-like structures with increasing amounts of condensed stripes. Lignoceryllignocerate had island-like clusters of more condensed areas throughout the film. What is surprising is that even when taking this into account, the rheological properties are practically identical for all of the lipid mixtures. Altogether, at small molecular areas, nonpolar lipids will form a secondary nonpolar phase overlaying the polar lipid monolayer at the air–water interface. Therefore, somewhat independent of the nonpolar lipid composition, the dilatational properties of this multilayer, such as compressibility, viscosity, and elasticity, are defined almost entirely according to the polar monolayer at the air–water interface, since the nonpolar lipids are squeezed out from this layer. This explains why the measured multilayer behavior is virtually unaffected by changes in the nonpolar lipid composition, and why these changes can only be observed in BAM images as differing layer formation induced by nonpolar lipids.

To conclude, this study clearly showed that different complex lipid mixtures with WE did not retard evaporation. Although certain WEs have been shown to retard evaporation when applied alone at the air–water interface, they lost this property when mixed with other lipids. This is caused by phase separation and differences in the organization of the lipids in the film. When WEs are alone they need to be at the

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surface of the water where they are able to form uniform layers. On the other hand, when applied with other lipids, such as polar lipids that prefer to stay next to the water phase, WEs are most likely organized on top of the polar lipids and are therefore unable to form a uniform layer that would retard evaporation. Since it is not possible to study artificial lipid layers that would contain exactly the same WEs as the in vivo layers, we selected suitable model compounds. Using a range of structurally differing WEs, we discovered that the quality of the WEs does not considerably affect the surface properties of the entire lipid layer. Therefore, based on our results, it is very likely that the impact of the TFLL WE pool, even though more complex than in this study, would also have very minor impact on the dilatational surface properties of the in vivo layer. However, in vivo branched or hydroxylated WEs may have specific roles the physiology of the TFLL such as in aiding spreading of the oily layer. Another interesting observation was the cooperativity of these films: they all shared similar compression isotherms as well as rheological properties. This is important when it comes to physiological changes in the TFLL. Small or even larger changes do not have a major effect on the properties of the film, which raises questions about the exact role of lipids in the pathogenesis of DES.

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References

1. Moss SE, Klein R, Klein BE. Long-term incidence of dry eye in an older population. *Optom Vis Sci*. 2008;85:668–674.
2. Report of the international dry eye workshop (DEWS). *Ocul Surf*. 2007;5:65–204.
3. Green-Church KB, Butovich I, Willcox M, et al. The international workshop on Meibomian gland dysfunction: report of the subcommittee on tear film lipids and lipid-protein interactions in health and disease. *Invest Ophthalmol Vis Sci*. 2011;52:1979–1993.
4. Pucker AD, Nichols JJ. Analysis of meibum and tear lipids. *Ocul Surf*. 2012;10:230–250.
5. Camera E, Ludovici M, Galante M, Sinagra JL, Picardo M. Comprehensive analysis of the major lipid classes in sebum by rapid resolution high-performance liquid chromatography and electrospray mass spectrometry. *J Lipid Res*. 2010;51:3377–3388.
6. Butovich IA, Arciniega JC, Wojtowicz JC. Meibomian lipid films and the impact of temperature. *Invest Ophthalmol Vis Sci*. 2010;51:5508–5518.
7. Chen HB, Yamabayashi S, Ou B, Tanaka Y, Ohno S, Tsukahara S. Structure and composition of rat precorneal tear film. A study by an in vivo cryofixation. *Invest Ophthalmol Vis Sci*. 1997;38:381–387.
8. Rantamäki AH, Telenius J, Vattulainen I, Holopainen JM. Lessons from the biophysics of interfaces: lung surfactant and tear fluid. *Prog Retin Eye Res*. 2011;30:204–215.
9. Wojtowicz JC, Butovich IA, McCulley JP. Historical brief on composition of human meibum lipids. *Ocul Surf*. 2009;7:145–153.
10. Dean AW, Glasgow BJ. Mass spectrometric identification of phospholipids in human tears and tear lipocalin. *Invest Ophthalmol Vis Sci*. 2012;53:1773–1782.
11. Lam SM, Tong L, Duan X, Petznick A, Wenk MR, Shui G. Extensive characterization of human tear fluid collected using different techniques unravels the presence of novel lipid amphiphiles. *J Lipid Res*. 2014;55:289–298.
12. Lam SM, Tong L, Reux B, et al. Lipidomic analysis of human tear fluid reveals structure-specific lipid alterations in dry eye syndrome. *J Lipid Res*. 2014;55:299–306.
13. Brown SHJ, Kunnen CME, Duchoslav E, et al. A comparison of patient matched meibum and tear lipidomes. *Invest Ophthalmol Vis Sci*. 2013;54:7417–7423.
14. Kulovesi P, Telenius J, Koivuniemi A, et al. Molecular organization of the tear film lipid layer. *Biophys J*. 2010;99:2559–2567.
15. Kulovesi P, Telenius J, Koivuniemi A, Brezesinski G, Vattulainen I, Holopainen JM. The impact of lipid composition on the stability of the tear fluid lipid layer. *Soft Matter*. 2012;8:5826–5834.
16. Telenius J, Koivuniemi A, Kulovesi P, Holopainen JM, Vattulainen I. Role of neutral lipids in the tear fluid lipid layer: coarse-grained simulation study. *Langmuir*. 2012;28:17092–17100.
17. McCulley JP, Shine WE. A compositional based model for tear film lipid layer. *Trans Am Ophthalmol Soc*. 1997;95:79–88.
18. Sweeney DF, Millar TJ, Raju SR. Tear film stability: a review. *Exp Eye Res*. 2013;117:28–38.
19. Cerretani CF, Ho NH, Radke CJ. Water-evaporation reduction by duplex films: application to the human tear film. *Adv Colloid Interface Sci*. 2013;197-198:33–57.
20. Robciuc A, Rantamäki AH, Jauhiainen M, Holopainen JM. Lipid-modifying enzymes in human tear fluid and corneal epithelial stress response. *Invest Ophthalmol Vis Sci*. 2014;55:16–24.
21. Rantamäki AH, Javanainen M, Vattulainen I, Holopainen JM. Do lipids retard the evaporation of the tear fluid? *Invest Ophthalmol Vis Sci*. 2012;53:6442–6447.
22. Rantamäki AH, Wiedmer SK, Holopainen JM. Melting points—the key to the anti-evaporative effect of the tear film wax esters. *Invest Ophthalmol Vis Sci*. 2013;54:5211–5217.
23. Brockman HL, Jones CM, Schwebke CJ, Smaby JM, Jarvis DE. Application of a microcomputer-controlled film balance system to collection and analysis of data from mixed monolayers. *J Colloid Interface Sci*. 1980;78:19890502–1980512.
24. Butovich IA. Tear film lipids. *Exp Eye Res*. 2013;117:4–27.
25. Grncarevic M, Radler F. The effect of wax components on cuticular transpiration—model experiments. *Planta (Berl)*. 1967;75:23–27.
26. Mack Correa MC, Mao G, Saad P, Flach CR, Mendelsohn R, Walters RM. Molecular interactions of plant oil components with stratum corneum lipids correlate with clinical measures of skin barrier function. *Exp Dermatol*. 2014;23:39–44.
27. Lampe MA, Williams ML, Elias PM. Human epidermis lipids: characterization and modulations during differentiation. *J Lipid Res*. 1983;24:131–140.
28. Friberg SE, Kayali I, Beckerman W, Rhein LD, Simion AJ. Water permeation of reaggregated stratum corneum with model lipids. *J Invest Dermatol*. 1990;94:377–380.
29. Saville JT, Zhao Z, Willcox MD, Blanksby SJ, Mitchell TW. Detection and quantification of tear phospholipids and cholesterol in contact lens deposits: the effect of contact lens material and lens care solution. *Invest Ophthalmol Vis Sci*. 2010;51:2843–2851.
30. Saville JT, Zhao Z, Willcox MD, Ariyavidana MA, Blanksby SJ, Mitchell TW. Identification of phospholipids in human meibum by nano-electrospray ionization tandem mass spectrometry. *Exp Eye Res*. 2011;92:238–240.

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31. Borchman D, Foulks GN, Yappert MC, Mathews J, Leake K, Bell J. Factors affecting evaporation rates of tear film components measured in vitro. *Eye Contact Lens*. 2009;35:32–37.
32. Herok GH, Mudgil P, Millar TJ. The effect of Meibomian lipids and tear proteins on evaporation rate under controlled in vitro conditions. *Curr Eye Res*. 2009;34:589–597.
33. Gilby AR, Heymann E. The rate of evaporation of water through duplex films. *Aust J Sci Res*. 1948;1:197–212.
34. Paananen RO, Rantamäki AH, Holopainen JM. The anti-evaporative mechanism of wax esters—implications for the function of tear fluid. *Langmuir*. 2014;30:5897–5902.
35. Raju SR, Palaniappan CK, Ketelson HA, Dawis JW, Millar TJ. Inteerfacial dilatational viscoelasticity of human meibomian lipid films. *Curr Eye Res*. 2013;38:817–824.
36. Schuett BS, Millar TJ. An investigation of the likely role of (O-acyl) omega-hydroxy fatty acids in meibomian lipid films using (O-oleyl) omega-hydroxy palmitic acid as a model. *Exp Eye Res*. 2013;115C:57–64.
37. Leiske DL, Leiske CI, Leiske DR, et al. Temperature-induced transitions in the structure and interfacial rheology of human meibum. *Biophys J*. 2012;102:369–376.
38. Millar TJ. A mechanism to explain the behavior of spread films of meibomian lipids. *Curr Eye Res*. 2013;38:220–223.
39. King-Smith PE, Nichols JJ, Braun RJ, Nichols KK. High resolution microscopy of the lipid layer of the tear film. *Ocul Surf*. 2011;9:197–211.
40. Smaby JM, Kulkarni VS, Momsen M, Brown RE. The interfacial elastic packing interactions of galactosylceramides, sphingomyelins, and phosphatidylcholines. *Biophys J*. 1996;70:868–877.
41. Holopainen JM, Brockman HL, Brown RE, Kinnunen PK. Interfacial interactions of ceramide with dimyristoylphosphatidylcholine: impact of the N-acyl chain. *Biophys J*. 2001;80:765–775.

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