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The Effect of Phospholipids on Tear Film Lipid Layer Surface Activity

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Purpose. We illustrate the importance of small quantities ($<$10 mol%) of polar phospholipids on the surface-active behavior of meibum-like lipid compositions.

Methods. Artificial meibum-like lipid mixture containing cholesteryl and wax esters was mixed with differing amounts of phosphatidylcholine (PC). The surface activity of these mixtures was investigated at the air–water interface by recording surface pressure created by the lipid layer as a function of molecular area at 37°C. The PC proportion in the mixtures was 0, 2.5, 5, 7.5, or 10 mol%, and the remaining proportion in the mixture was 50:50 (mol/mol) of cholesteryl oleate (CO) and behenyl oleate (BO). Also, the effect of temperature was investigated.

Results. The surface activity of the mixtures increased in a very predictable and consistent fashion as a function of the PC proportion. The lipid mixture containing only CO and BO showed miniscule surface activity. However, already 2.5% PC mixed with the nonpolar CO and BO generated considerable increase in surface pressure. At small surface areas, the behavior of 7.5% and 10% PC compositions started to approach that of a pure PC monolayer. The temperature did not have a considerable impact on the surface-active behavior of the PC-containing compositions.

Conclusions. The polar phospholipids have a considerable effect on the surface-active properties of artificial tear film lipid layer (TFLL) compositions. Surprisingly, this takes place already at very low and physiologically relevant PC proportions. The effect is more dependent on the actual amount of the phospholipids at the air–tear interface than on the relative amount of these lipids in TFLL.

Keywords: dry eye, ocular surface, Langmuir films, lipids, surfactants

The tear film lipid layer (TFLL) covering the aqueous tear film comprises mainly nonpolar lipids such as cholesteryl esters (CEs)[1] and wax esters (WEs),[2] but also smaller amounts of polar lipids such as phospholipids (PLs).[3–9] The estimated amount of PLs in tears is on the order of 5 to 20 mol% of all lipids.[10] Despite their small proportion relative to nonpolar lipids, PLs seem to have an essential impact on the function of the TFLL and, consequently, on the health of the ocular surface.[11]

Cholesteryl esters and WEs are classified as nonpolar since they lack the large highly hydrophilic head group of polar lipids. Polar lipids in aqueous environment orient in such fashion that the contact of water with the hydrophobic parts of the molecule, the acyl chains, is minimized, whereas the head group remains in contact with water. Therefore, they typically pack into three-dimensional aggregates such as liposomes and vesicles. This hydrophobic effect is the basis for the formation of cells and cell organelles (see Ref. 12 for a more detailed description).

At the biological air–water interface, such as TFLL and lung surfactant, PLs form a layer of lipids, so that the hydrophobic acyl chains are pointing toward the air, whereas the head group is submerged in the water.[13] When lateral pressure is applied to such a layer, for instance, due to blinking or breathing, a lateral tension is formed in the layer. This lateral tension (surface pressure) is opposite to the surface tension created by the air–water interface, and the surface tension is therefore relieved.

Phospholipids can accommodate differing molecular areas depending on the lateral pressure they are experiencing. At large molecular areas there is more space for the movement of the chains, but when the area becomes restricted, the chains pack into smaller space and their movements become more limited. Another crucial property of PLs, which separates them from nonpolar lipids, is the local charges of the head group, which create a strong repulsion between the adjacent molecules. These counteracting properties of the lipid film provide elasticity: Lipids pack tightly when the surface pressure is high, therefore hindering thermal motion of the acyl chains. When the pressure is released, lipids spread back because of electrostatic repulsion, and they occupy the larger area they were accommodating before application of the lateral pressure. Therefore, adaptation to the changing lateral pressure through spatial reorganization of the acyl chains, and in contrast, the repulsion between the head groups, allow PLs to remain as a monolayer in a wide variety of conditions. Accordingly, the same number of molecules can entirely cover a smaller or larger air–water interface.

Nonpolar lipids may also form films at the air–water interface. For instance, pure WEs are known to form monolayer-like lipid films at the air–water interface at temperatures above their melting points. In addition to the extended orientation of the molecules adopted in bulk lipid, the molecules are able to adapt a V-shape orientation at the air–

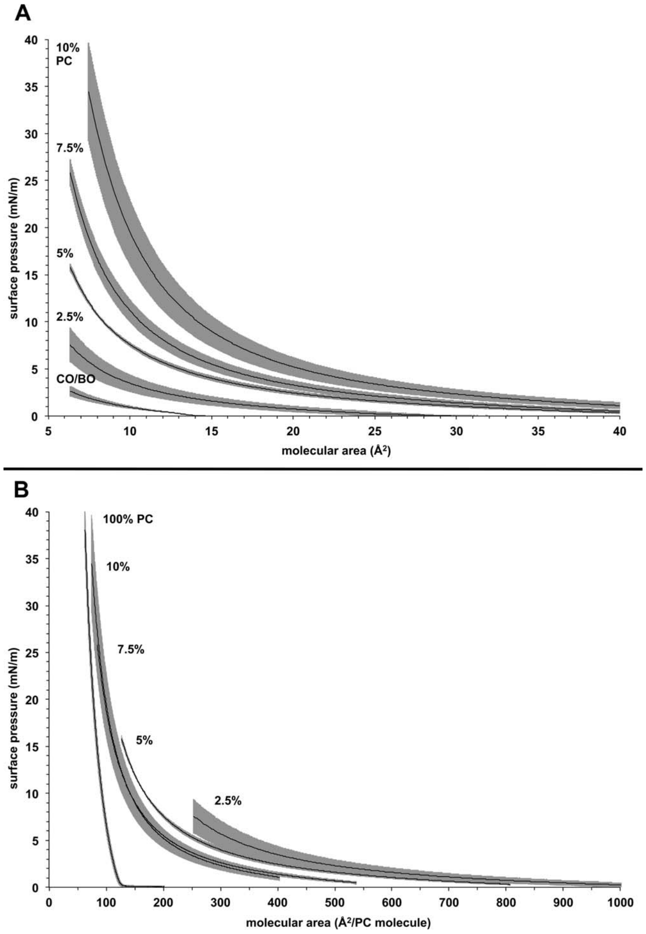

Figure 1. The effect of the PC proportion in PC/CO/BO mixtures. (**A**) The isotherms of TFLL-like model compositions containing 0, 2.5, 5, 7.5, and 10 mol% PC. An equimolar amount of each composition was applied to the air–water interface. The *black line* represents the mean value, and the *gray areas* represent the standard deviation defined based on three independent measurements. Target temperature 37°C during the measurement (average temperature during the measurement 36.9 ± 0.3°C, min 35.8°C, max 37.7°C). (**B**) The isotherms of TFLL-like model compositions containing 2.5, 5, 7.5, 10, and 100 mol% PC presented relative to the amount of PC at the interface.

water interface in a similar fashion as PLs.[14] In such cases the ester bond is submerged in water, and the acyl and alkoxy chains point toward the air. However, neither of these possible orientations seems to withstand lateral tension; moreover, the molecules aggregate into rafts or three-dimensional aggregates in order to minimize contact with water. Because these lipids cannot adapt to increasing lateral pressure and are missing the inter-headgroup repulsion required for spreading, they are missing the elastic properties of PL layers. Additionally, due to the phase transition behavior of WEs, their interfacial behavior may differ greatly depending on the temperature of the surroundings.[15]

Because nonpolar lipids are not able to provide coverage of the entire tear film and lack the elasticity to respond to changing surface pressure, polar lipids in the TFLL are a necessity. However, the (relative) amount of polar lipids found in the tear fluid is very low; in meibum the amount is even lower. The low relative amount of PLs may, however, be explained by the duplex film structure of the TFLL. In the duplex film there is an organized layer of lipids at the air–water interface and a less organized but evenly spread bulk lipid layer on top of the organized interfacial layer (see a more detailed description of duplex films in Ref. 16). In such a lipid layer, polar lipids would be located at the tear film interface and the major part of nonpolar lipids in the unorganized overlaying layer. This structure would allow very low proportions of PLs in the TFLL.

In this conceptual study we aimed at showing how even very low (10 mol% and below) proportions of PLs can have a considerable effect on the surface-active properties of TFLL-like lipid compositions. This study addresses two critical unanswered questions. First, it provides experimental evidence for a crucial role of PLs in maintaining surface rheologic properties, and secondly, it provides a rationale for improving and maintaining lipid spreading on the ocular surface. Importantly, both of these properties are maintained even with very low levels of PLs resembling those in the tear film in vivo.

METHODS

Egg yolk L-alpha phosphatidylcholine (PC), egg yolk L-alpha-lysophosphatidylcholine (LPC), cholesteryl oleate (CO), behenyl oleate (BO), and HPLC-grade chloroform were purchased from Sigma-Aldrich (Darmstadt, Germany).

Isotherms were recorded using a KSV Minitrough (KSV, Espoo, Finland) Langmuir balance instrument. The temperature of the trough was controlled with an external water-circulating water bath. The Langmuir balance was enclosed in an acrylic plastic cabinet. A flow of ozone-free air through the cabinet was utilized to minimize the effect of ozone on the WEs and CEs. Preceding every measurement, the cabinet door was kept closed for 15 minutes in order to remove most of the ozone in the cabinet using the airflow.

In each experiment, an equimolar amount of lipid (10 μL 10 mM chloroform solution) was applied on the air-PBS-buffer (air–water) interface, and the lipids were allowed to spread for an additional 15 minutes. The isotherms were recorded utilizing symmetrical compression at a rate of 10 mm/min (15×10^{16} Å²/min). Three independent isotherms were recorded for each composition.

RESULTS

Figure 1A illustrates the isotherms recorded at a target temperature of 37°C for mixtures of PC/CO/BO, namely, in compositions of 10/45/45 (designated as 10%), 7.5/46.25/46.25 (7.5%), 5/47.5/47.5 (5%), and 2.5/48.75/48.75 (2.5%) mol/mol and CO/BO in 50/50 mol/mol. A gradual and very predictable increase in the surface pressure and the liftoff area was detected as PC proportion was increased. Figure 1B presents the data relative to the amount of PC in the samples. Additionally, an isotherm for pure PC was added as a reference. Presented in this manner the data emphasize more clearly the impact of PC in the mixtures, as discussed later in the paper. Figure 2 illustrates the identical measurements made for LPC. A similar effect of a gradual increase in the surface activity and the liftoff area was detected as a function of LPC proportion.

Figure 3 illustrates the effect of temperature on the 5% and the CO/BO compositions at target temperatures of 27°C and 46°C. The isotherms for the 5% and the CO/BO compositions measured at 37°C are shown as a reference. For 5% composition at target temperature of 27°C the maximum surface pressure is very close to the maximum surface pressure reached at target temperature of 37°C. However, the liftoff at 27°C takes place at a considerably later phase than at 37°C. A kink is observed in the isotherm at ~18 Å² and the overall slope of the isotherm is steeper, suggesting that the lipid layer is less compressible. In contrast, the CO/BO composition at

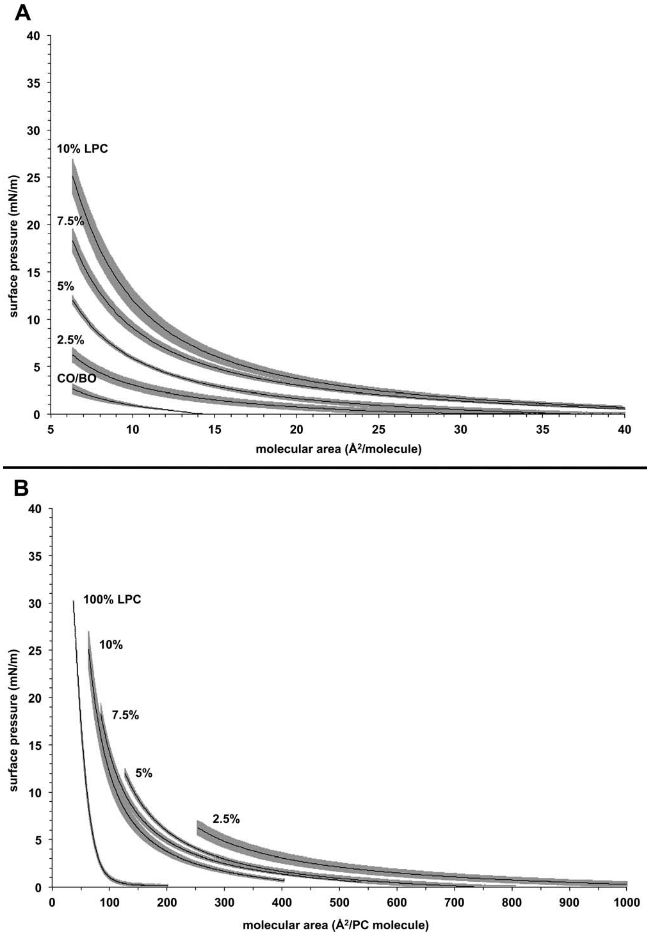

FIGURE 2. The effect of the LPC proportion in LPC/CO/BO mixtures. (**A**) The isotherms of TFLL-like model compositions containing 0, 2.5, 5, 7.5, and 10 mol% LPC. An equimolar amount of each composition was applied to the air–water interface. Target temperature 37°C during the measurement (average temperature during the measurement 36.4 ± 0.4°C, min 35.1°C, max 37.4°C). (**B**) The isotherms of TFLL-like model compositions containing 2.5, 5, 7.5, 10, and 100 mol% of LPC presented relative to the amount of LPC at the interface.

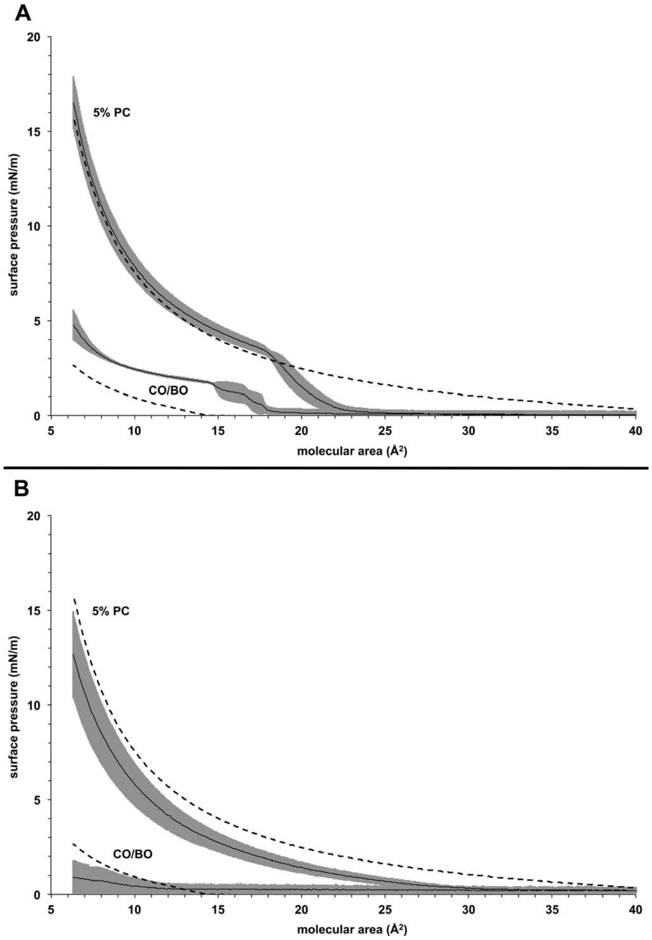

FIGURE 3. The effect of temperature on the behavior of the 5% PC and CO/BO compositions. (**A**) 27°C (average temperature during the measurement 27.3 ± 0.6°C, min 25.1°C, max 28.2°C). The isotherms recorded for 5% PC and CO/BO composition at 37°C are included as a reference (*dashed line*; error bars have been omitted for clarity). (**B**) 46°C (average temperature during the measurement 46.0 ± 0.6°C, min 44.4 °C, max 47.3°C).

27°C generates 1 to 2 mN/m higher surface pressure than at 37°C when the maximum values are compared. The liftoff takes place at around 18 Å^2 whereas at 37°C the liftoff is at 13 to 14 Å^2.

The 5% lipid layer is slightly less surface active at target temperature of 46°C than at 37°C based on the maximum surface pressure. However, the effect of temperature on surface activity seems to be very small. The CO/BO layer shows only very minimal surface activity at 46°C.

DISCUSSION

In this study the effect of egg yolk PC and LPC on artificial meibum composition was investigated. We used a 50/50 (mol/mol) lipid mixture of CO and BO as the artificial meibum composition. This clearly is a simplification of natural meibum. However, natural meibum could not be used since the surface pressure produced by lipid molecules is not only dependent on the properties of the lipids, but also dependent on the molar amount of lipids (number of molecules) at the interface. For “fair comparison,” an equimolar amount, that is, the same number of molecules of each lipid mixture, was applied on the interface. This would not be possible with natural meibum, since the exact composition of this complex mixture is not known in the required detail. However, such a simplified lipid composition serves the purpose of showcasing the rheologic effect of polar lipids emphasized in this study. Moreover, the exact relative amounts of the lipids are not as important as the phase behavior of the lipids, and therefore more effort was put into the selection of proper lipid model compounds. As known from our previous studies, the bulk melting temperatures of the WEs greatly affect the spreading of these lipids and finally the orientation of the molecules at the air–water interface.[14,15] Behenyl oleate was chosen since it represents well the WE species present in meibum.[2,17,18] In addition, the interfacial behavior of such WEs is well known from previous studies. Since the long-chain CEs are abundant in meibum, CO was chosen as the representative CE.[1]

The artificial meibum was mixed with differing proportions of natural unsaturated PLs. The chosen model for the TFLL PLs was PCs and LPCs extracted from egg yolk. This mixture of PCs represents well the PLs in TFLL, since both of these lipid mixtures contain a large proportion of unsaturated PLs.[3–9] The proportions of PC and LPC in the test compositions were 2.5, 5, 7.5, and 10 mol% so that the remaining proportion was divided 50/50 for CO and BO. These PL proportions were selected in order to simply illustrate the impact of small

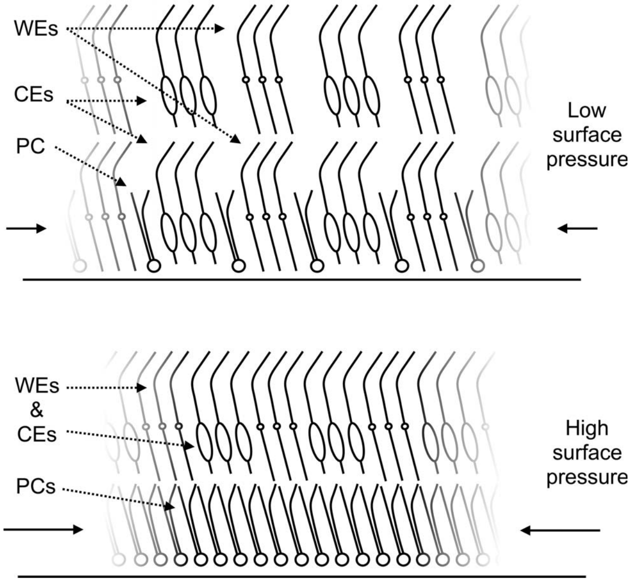

FIGURE 4. "Squeeze out" of nonpolar lipids at high surface pressures. At low surface pressures polar PCs and nonpolar CEs and WEs are mixed at air–water interface. CEs and WEs potentially form partial overlaying layers. At higher pressures nonpolar lipids are squeezed out from the mixed interfacial layer and the interface remains covered mainly with phospholipids. Additional CE/WE layers are formed (not shown). Since the detailed behavior of nonpolar CEs and WEs in mixed lipid layers is yet unknown, the presented organization and orientation of these lipids do not necessarily represent the true situation. *Solid arrows* designate the lateral pressure. PC, phosphatidylcholine; CE, cholesteryl ester; WE, wax ester.

amounts of PLs on the behavior of meibum-like lipid compositions. Yet, based on a recent review, the relative amounts seem to be on the correct order of magnitude when compared to in vivo human tear fluid.[10]

The isotherms measured at 37°C for pure meibum-like compositions suggest practically no surface activity when the degradation of the BO and CO induced by ambient ozone is prevented (Fig. 1). In contrast, when the ozonolysis of the lipids is not prevented, WEs degrade gradually into surface-active compounds[19] and show high surface activity.[15] Based on our preliminary studies, the same applies to CO (data not shown). However, degradation products have not been found in TFLL in vivo.[19] Such degradation in most likely prevented by antioxidants in the TFLL.

With small additions of PC, surface activity of the lipid mixtures increases in a very consistent and predictable manner at 37°C (Fig. 1A). Figure 1B illustrates the same data relative to the amount of PC. In this graph the isotherm of pure egg PC is based on true molecular area, whereas the molecular areas for the mixtures are presented relative to the number of PC molecules in each mixture. Therefore, if the isotherm of a mixture deviates from the isotherm of the pure PC layer, there are also molecules other than PC on the interface. At larger molecular areas (low surface pressure) the isotherms for the 7.5% and 10% PC layers show that the lipids at the interface are a mixture of PC and nonpolar lipids as suggested by the large deviation from the pure PC isotherm (Fig. 1B). The same also applies to the other compositions independent of molecular area. Instead, at small areas (high surface pressure), the isotherm of the 7.5% mixture starts to approach that of pure egg PC. With the 10% mixture the effect is even more evident. This means that the lipids at the air–water interface are mainly PC molecules, and COs and BOs have been "squeezed out" to the overlaying layer—behavior already illustrated by our previous study.[20] This phenomenon is clarified also in Figure 4.

The effect of the PC on the surface activity of the mixtures is evident in all of the PC-containing compositions. It seems feasible that at low surface pressures, nonpolar lipids mix with polar lipids at the air–tear interface to form a thin, smooth, and more uniform lipid layer. When pressure increases, nonpolar lipids squeeze out of the interface and form a heterogeneous duplex layer. This reversible reaccommodation of lipids allows maintenance and stability of the TFLL.

Since natural TFLL also seems to contain large amounts of LPC,[6–9] the same experiments were performed using egg yolk LPC as the PL. The effect of LPC proved to be identical to that of PC with the exception that the same number of LPC molecules induced lower surface pressure. This was expected because of the lower surface area taken up by these molecules due to the missing acyl chain. However, this result well illustrates the importance of the local charges of the head group on the surface-active properties of the surfactant, since LPC molecules are still very surface active despite the lack of the other acyl chain.

We also tested the effect of the temperature using the lipid mixture containing 5% PC. Based on our experience, the behavior of the nonpolar lipids, such as spreading at the air–water interface and the compressibility of the lipid, is greatly affected by the temperature. The bulk melting temperatures of BO and CO are 38°C[15] and 44°C to 47°C.[21] respectively. In addition to 37°C, the experiments were performed at the temperatures of 27°C and 46°C.

At 27°C, both nonpolar lipids are well below their melting temperatures; therefore they are solid-like and, as pure lipids, poorly spreadable at the air–water interface. At 37°C, BO is close to its melting transition and shows fluidic properties, whereas CO is still in solid state. At 46°C, BO is in molten state and very fluidic and CO close to the melting transition, showing also some fluidic behavior. Despite these differences in the physical state of the lipids, the isotherms recorded for the 5% PC compositions at 27°C, 37°C, and 46°C are almost identical at low molecular areas, that is, below 18 Å^2 (Fig. 3). This observation suggests that the physical state of the nonpolar lipids plays a minor role in the surface-active behavior of the compositions. Particularly on small molecular areas, nonpolar lipids are most likely squeezed out from the lipid layer and therefore have a very small impact on the dilatational properties of the lipid layer.

At 46°C, the surface pressure generated by the CO/BO compositions is somewhat lower than at 27°C and 37°C. This may be explained by the physical state of the nonpolar lipids. The higher surface activity of the CO/BO at 27°C and 37°C is possibly caused by the "more" solid-like appearance of the lipids (it needs to be remembered that the wax-like lipids have a broad melting range and may have several differing solid states). At solid state the lipid rafts are floating on the surface, and very little spreading takes place. When these lipid rafts pack against each other at low molecular areas, low lateral tension forms between these somewhat rigid rafts and this tension is recorded as a surface pressure. However, the WEs below their melting point are not necessarily hard solids, but possibly candle-like waxy materials; therefore, reorganization and compressibility are also possible. Due to this compressibility, the surface pressure increases gradually (surface pressure of a stiff solid layer would increase in a very steep fashion). In the molten state at 46°C, in contrast, the surface activity of the CO/BO mixture is very low since no organized structures can be maintained. Despite the more efficient spreading of the molten state molecules, they seem to lose their orientation under lateral pressure. Such molecules pack into unorganized aggregates under lateral pressure.

Importantly, the high surface activity of the PC-containing compositions does not change with temperature. This is of importance regarding the physiological TFLL, since the lipid layer needs to maintain its function in cold and warm environments.

In the preliminary experiments of this study we experimented with similar lipid compositions but smaller amounts of the lipid at the interface. No surface activity was observed in those experiments. This, combined with the results presented here, clearly points out the erroneous way of thinking regarding the proportions of the PLs in the TFLL. The composition studies, moreover, concentrate on the relative amounts of the lipids in either meibum samples or the TFLL extracts. More important regarding the surface activity of the PLs is the actual, not the relative, amount of lipids at the interface. Even with very a large proportion of PLs, no surface activity is observed if the total amount of the lipid is too low to form a lipid layer that would cover entirely the air–tear interface. The surface-dilatational properties of the TFLL, such as surface activity, are dependent on the lipids that are at the air–tear interface, not on the lipid bulk at the overlaying layers.[22] Therefore, the surface activity of the TFLL is dependent only on the actual amount of surface-active polar lipids at the interface, and independent of the proportions of polar and nonpolar lipids.

What is the impact of this study on the present knowledge about TFLL? As shown recently, the surprisingly high (apparent) surface activity of meibum,[23] wax esters,[15,23] and OAHFAs[23] is very likely due to the rapid ozonolysis[19] of such lipids at the air–water interface. As expected based on their structure, such lipids show miniscule surface activity when the degradation of the lipids is prevented. Additionally, there has been a discrepancy on the presence and the relative amount of PLs in the TFLL, and it has been questioned if very small amounts of such lipids could have any practical impact on the behavior of the TFLL. Therefore, this study illustrates, in a straightforward fashion, that under 10%, proportions of PLs can have a crucial impact on dilatational properties of meibum-like compositions as long as the total amount of the polar lipids is high enough to cover the interface. Also, the changes in temperature do not have a considerable impact on the surface-active performance of PC-containing compositions.

The present study did not attempt to provide a new TFLL model, but clarifies how recent models, such as the duplex film, could work in light of the known TFLL lipid composition. However, it needs to be remembered that in vivo, TFLL is a highly complex system of lipids and proteins. The present conceptual study clearly illustrates the potential effect of PLs without considering interactions between lipids and proteins. The function of PLs may be highly affected by lipid-binding proteins such as lipocalin,[24–26] and PL transfer protein.[27,28] However, more investigations need to be performed regarding the lipid–protein interactions before the impact of the proteins on TFLL behavior can be explained in more detail.

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