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Effects of model tear proteins and topical ophthalmic formulations on evaporation inhibition and biophysical property of model tear lipid nanofilm *in vitro*

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ABSTRACT

Hypothesis: Biophysical property and water evaporation retardation through a lipid nanofilm can be altered by model tear protein and topical ophthalmic formulation.
Experiment: Evaporation rate and dynamic surface pressure were measured using a sessile drop technique. Water evaporations from 5 individual protein solutions, their mixture, and 6 ophthalmic formulations were quantified. Biophysical property and evaporation through model lipid nanofilms spread on model electrolyte solutions, tear protein solutions, and ophthalmic formulations were assessed.
Findings: Model lipid nanofilms spread on electrolyte solution reduced evaporative fluxes by 43–60%. Evaporative fluxes from individual protein solutions without lipids were 3–19% lower than from electrolytes solution. Evaporative fluxes through lipid nanofilms were decreased by the presence of albumin or lactoferrin in solutions but increased by lysozyme and mucin.
Evaporative fluxes from ophthalmic formulations were 10–43% lower than from electrolyte solution. Evaporations through lipid nanofilms spread on formulations were higher than through lipids on electrolyte solution. Model lipid nanofilms spread on Diquas appeared more rigid than on electrolyte solution but showed softening when spread on Refresh Mega-3.
Some proteins and ophthalmic formulations altered model lipid nanofilms evaporative barriers. Ophthalmic formulation induced changes in biophysical property of model lipid nanofilms *in vitro* may suggest possible tear film destabilization *in vivo*.

1. Introduction

A stable tear film uniformly distributed over the ocular surface is vitally important for vision and ocular surface health [4,6,14,15,35]. Human tear film is an intricate multi-layered biological colloid system, which has complex and immensely variable compositions. The aqueous part of human tears contains over 1500 proteins, enzymes, and other biologically active ingredients, and lysozyme, lactoferrin, lipocalin, albumin and mucin are the most abundant proteins [44]. Human tear lipids are mixtures of over 300 non-polar and polar organic compounds, which form a transparent and a structured gel-like nanofilm (30-120 nm-thick) spread as the outmost layer at the air-tear aqueous interface. [McCulley and Shine, 2001]. In healthy eyes, aqueous film composition and biophysical property are naturally attuned in response to the constantly changing environment thus maintaining an optimal protective function and tear film stability. Fig. 1 illustrates a schematic structure of human tear film, introduced earlier elsewhere [39].

This hypothetical structure is constructed of four sub-layers: a 2–5 μm tear aqueous layer, a 30–100 nm lipid film separated from tear aqueous layer by a phospholipid monolayer, and the outmost lipid-air interface that is coated by an inverted phospholipid bilayer. This tear film structure emerged from our previous interfacial property studies of human tear lipid nanofilm. This representation departs from the classical three-layer model of human tear film structure [22]. It introduces the new concept of an inverted phospholipids bilayer self-assembled at the air-lipid interface. This hypothetical feature of tear lipid layer was recently supported by the PM-IRRAS, FTIR, and ellipsometry experimental results, which provide strong experimental evidence that

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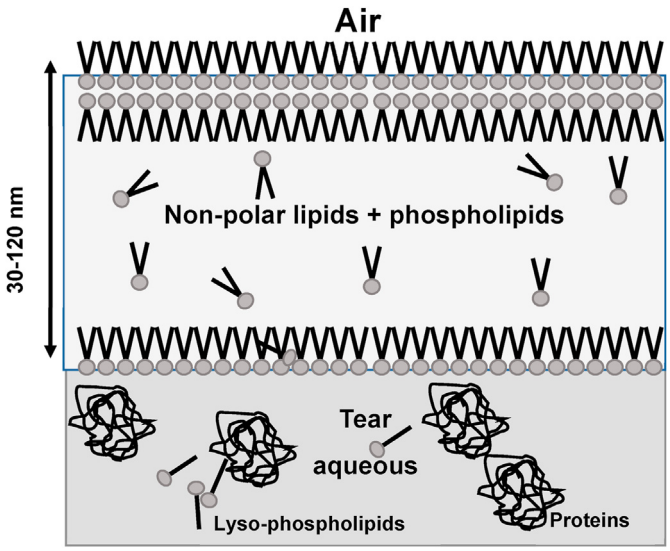

Fig. 1. Tear film structure schematic [39].

phospholipids are present at the outmost surface of human tear lipid film [11].

Over the last several decades, dry eye disorder has become widespread with global prevalence ~ 12% and is recognized as a major clinical problem [30]. Dry eye patients experience a range of symptoms, frequently accompanied by visual disturbance. However, the biophysical mechanisms triggering tear instability remain elusive.

Tear aqueous film thinning, eventually triggering tear film breakup, occurs through different pathways. Evaporation-driven thinning [16,17,31,32,42] and meniscus-driven hydrodynamic thinning [23,37] are considered the most crucial mechanisms. Evaporation of human tears *in vivo* has been extensively studied. It was reported that healthy human tear evaporation rate is 5–10 times slower than water [7,12,31,42,43]. The reduced evaporation from human tears is generally attributed to the ability of tear lipid nanofilms to inhibit aqueous evaporation [1]. Evaporation rates of dry eye patients were approximately 2–3 times higher than those of healthy eyes.[21]. Nevertheless, numerous investigations of water evaporation through model lipid films or reconstituted meibum lipid films *in vitro* have shown either very modest (i.e., no more than ~25% reduction) [29,33,34,38,41], or no water evaporation retardation [3,13,26]. Many of these studies explored only the monolayers of meibum lipids or phospholipids [25,27,28]. These monolayers were less than 2 nm thick and were studied at low surface pressures, not exceeding ~ 35 mN/m, with irregular coverage of aqueous surface. The human tear lipid films are not monolayers but 15–50 times thicker nanofilms with structures and interfacial properties significantly more complex than that of the monolayers. It is obvious that lipid monolayers are not exhibiting the interfacial behaviors properly replicating the behaviors of 30–120 nm self-assembled tear lipid nanofilms. Thus, the multilayered tear lipid with thickness comparable with typical ocular lipid layer thickness should be used for tear evaporation studies *in vitro*. The data regarding the effect of proteins on water evaporation through the lipid films are limited to a single publication, reporting the evaporation through human meibum mixed with glycoprotein (bovine submaxillary mucin). It was shown that bovine mucin made evaporation through these lipid films faster than evaporation through the films on water [3]. Recently, we reported that it is possible to accurately quantify evaporation retardation through lipid nanofilm *in vitro* [40]. It has been shown that model and human tear lipid nanofilms reduce evaporation from aqueous microdroplets (8–10 μl). Several crucial requirements for such experiments have been established: a) lipid nanofilm of 60–100 nm thick; (b) lipid nanofilm equilibrated and aged for 24 h or longer; and (c) model lipid nanofilm containing polar phospholipids necessary for evaporation reduction. It has been reported that biophysical dynamic interfacial properties of model lipid nanofilms are strongly dependent on their compositions and that only model lipid compositions closely mimic dynamic interfacial behavior of human tear lipid film can significantly reduce water evaporation [20,40].

There are many artificial tears and dry eye ophthalmic formulations that can be purchased without prescription, over the counter (OTC). These formulations are widely advertised for alleviating dry eye symptoms and stabilizing tear films. Some formulations are aqueous solutions containing water soluble polymers like carboxymethyl cellulose, hyaluronic acid, and synthetic polymers. There are also eye drops formulated as emulsions comprising mineral oil or plant-derived oils like castor or flax seed oil. These formulations contain surface-active emulsifiers, emulsion stabilizers or polymers. No quantitative information has been reported regarding the rates of evaporation from these formulations *in vitro* or *in vivo*.

In this study, we applied sessile drop technique to examine the effects of proteins and topical ophthalmic formulations on tear evaporation and biophysical property of model tear lipids, which were multilayered nanofilms with compositions that better replicated the intricate biological tear lipid films. Considering the extensive complexity of human tear aqueous and lipid phases, we choose a model lipid system that closely mimics the human tear lipid self-assembled nanofilms *in vivo*.

2. Methods

An automatic tensiometer (Ramé-Hart Instrument Co., USA) controlled by DropImage Advanced program, v.2.2, was used for collecting interfacial tension/pressure and evaporation data. A sessile drop with constant and precise (±0.1 μl) drop volume control was used to quantify the water evaporation rates at constant temperature of 36 °C and relative humidity of 75%. Drops (volume 8–10 μl, areas 18–20 mm^2) of the aqueous solutions or ophthalmic formulations were formed and kept in a sealed optical cell for 24–48 h. The details of the setup and procedures are described elsewhere [39,40]. Fig. 2 shows the sketches of the sessile drops of different aqueous phases and lipid nanofilms spread atop the aqueous drops.

In previous publications it was shown that during the aging the evaporation fluxes through lipid nanofilms and their biophysical properties slowly changed and reached steady state within 20–24 h [39,40]. The gradual development of the nanofilms is likely due to slow diffusion of phospholipids from the lipid film bulk, resulted in their adsorption and development of specific multilayered structures shown in Fig. 1.

Evaporative flux $F_{ev,}$ i.e., evaporation rate per unit area, was calculated as:

$$F_{ev} = (\Delta V(t)/\Delta t)/A_{dr}), \quad (1)$$

where V(t) is the volume of the model tear electrolytes solution (MTE) inside a drop-volume controlling a mini-pump syringe at time t. The volume of the electrolyte solution inside the syringe decreased during measurements to maintain drop volume constant. A_{dr} is the drop surface area and was constantly measured during the experiment with an accuracy of 0.1 mm^2. The initial drop area varied for different aqueous phases and in the presence of lipid nanofilms due to surface tensions differences. The drop surface area to drop volume ratio (A_d/V_d) was kept constant (within an interval of 2.22–2.26) for all evaporation runs [40]. Every set of measurements was repeated at least 3 times. An absolute value of F_{ev} from the surface of electrolyte solution was 1.42 x10 $^{-4}$ μl/mm^2 s (standard deviation (SD) = 0.04 x10 $^{-4}$ μl/mm^2 s). The electrolyte solution measurements were repeated regularly prior to assessing each model system. This value was used as a baseline value to calculate relative evaporative flux,

$$F_R = (F_x/F_{MTE}) \text{ x } 100, \% \quad (2)$$

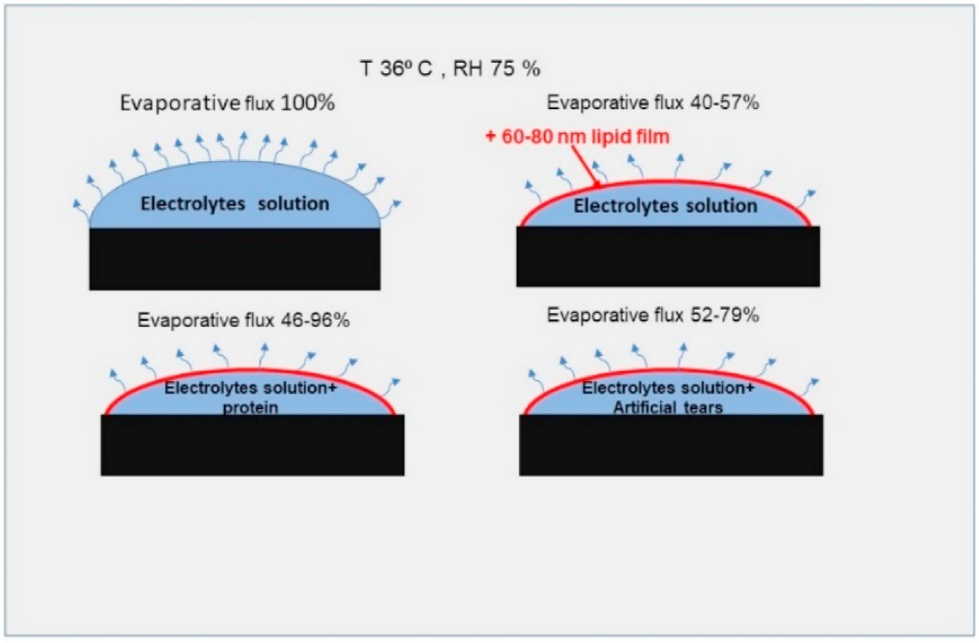

Fig. 2. Schematic drawings of sessile drops of different aqueous phases and lipids nanofilms spread atop of the drops.

Table 1
Model tear proteins.

Name	Molecular Mass [kD]	Concentration [mg/ml]
Human lysozyme	14.7	2.0
Human serum albumin	66.5	0.35
Human lactoferrin	76.165	1.0
Bovine milk β-Lactoglobulin	18.4	1.0
Bovine submaxillary mucin	400–4000	0.08
Model tear electrolytes (MTE)	NaCl, Sea Salts, 0.9 wt % total, pH 7.3–7.4, Osmolality 290–310 mOs	

where F_x is the value of the evaporative flux for the system, and F_{MTE} is a baseline evaporative flux for electrolyte solution, calculated according to Equation (1).

Measurements of dynamic surface tension/pressure were performed under the same conditions. The sessile drops of all solutions and ophthalmic formulations without or with lipid nanofilms were slowly expanded from their original volume of 8–10 μl to 34–40 μl using a precision J-KEM (J-KEM Scientific, USA) pump with a flow rate of 8 μl/min. The 70–80 nm nanofilms were deposited on a freshly formed drop surface of the electrolyte solution, each protein and their mixture solutions, and each ophthalmic formulation. 10 sec after the expansion, the drops were contracted to their original volumes. These cycles were repeated 5 times for each sessile drop, with 15–20 min of rest between runs. The thickness of lipid films was calculated using the values of deposited volume of lipids solution (concentration 1 mg/ml), multiplied by average density of dry lipids 870 kg/m^3 and divided by drop surface area.

2.1. Materials

Human proteins, model lipids and other chemicals were purchased from Sigma-Aldrich (USA) and used as received. Polar lipids were purchased from Avanti Polar Lipids, USA. All proteins were dissolved in the

Table 2
Ophthalmic formulations and pure oils used in the formulations.

Name, Manufacturer (Country), Physical state	Active ingredients	Inactive ingredients	Preservatives
Purcell, NOF Corporation (Japan), Aqueous Solution	2-methacryloyloxyethyl methacrylic acid, butyl phosphorylcholine (lipidure), hypromellose	Water, sodium chloride	Unknown
Thealoz, Duo Laboratories Thea (France), Aqueous solution	Trehaloze, Sodium Hyaluronate	Sodium Chloride, Trometamol, Hydrochloric Acid, Water	None
Diquas, Santen (Japan), Aqueous solution	Diquafosol sodium	Dibasic sodium phosphate hydrate, disodium edetate hydrate, sodium chloride, potassium chloride, dilute hydrochloric acid, and sodium hydroxide, pH 7.2–7.8	Chlorhexidine gluconate
Retain PM, OcuSoft (USA), Ointment	Mineral oil (20%), white petrolatum (80%)	None	None
Soothe® XP, Bausch + Lomb (USA),Emulsion	Light mineral oil, mineral oil	Boric acid, edetate disodium, octoxynol-40, polysorbate 80, water, sodium borate.	Polyquaternium-1
Refresh Optive Mega-3, Allergan (USA), Emulsion	Carboxymethylcellulose, sodium salt, Glycerine; Polysorbate 80.	Boric acid; butylated hydroxyl toluene; carbomer copolymer type A; castor oil; erythritol; flaxseed oil; levocarnitine; polyoxyl 40 stearate; water; trehalose; hydrochloric acid; sodium hydroxide	None
Castor Oil, Sigma Liquid	Pure grade castor oil		
Flaxseed oil, Sigma Liquid	Pure grade flaxseed oil		

Table 3
Relative evaporative fluxes from the drop surface of individual and mixture of model proteins without and with model tear lipid films, and maximum surface pressure. (MTE = Model tear electrolytes; SD = standard deviation).

Protein	Mean relative evaporative flux without lipid film, %	SD, %	Mean relative evaporative flux with lipid film, %	SD, %	Mean maximum dynamic surface pressure, with lipid films, mN/m	SD, mN/m
Human Lysozyme	97.0	2.4	96.0	2	49.2	0.5
Bovine Mucin	91.0	3.1	86.0	3	48.6	0.2
Human Lactoferrin	84.0	2.1	49.0	2	53.9	0.6
Human serum albumin	83.0	5.2	47.0	4	54.7	0.4
Bovine β-Lactoglobulin	82.0	6.3	62.0	3	50.1	0.5
5 component protein mixture	81.0	4.1	59.0	1.5	52.4	0.8
MTE solution	100	2.8	57.0	1.7	54.4	0.5

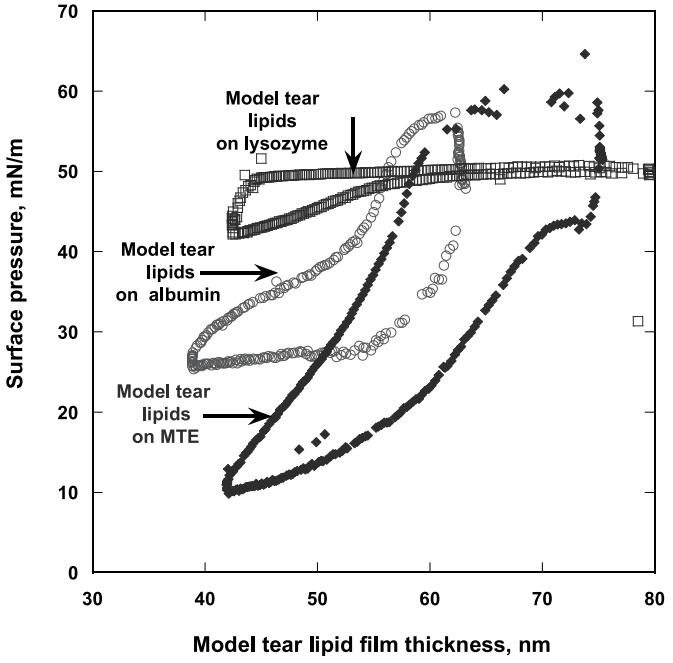

Fig. 3. Comparison of dynamic interfacial pressure iso-cycles for model tear lipids alone on MTE, and the same model tear lipid films deposited on MTE with human lysozyme and human serum albumin solutions. (MTE = Model Tear Electrolyte). The arrows point to the compression branches of iso-cycles. The maximum surface pressure of lipid film on lysozyme decreased by 5–7 mN/m but was not changed by human serum albumin.

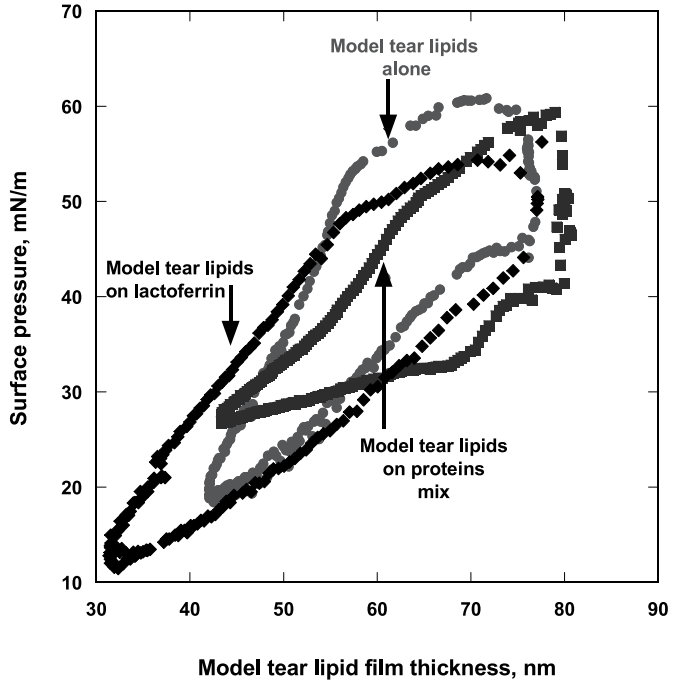

Fig. 4. Dynamic interfacial pressure iso-cycles for model tear lipid nanofilm on MTE, on lactoferrin, and model tear proteins mixture solutions. (MTE = Model Tear Electrolyte). The arrows point to the compression branches of iso-cycles. The maximum surface pressure of lipid film on lactoferrin decreased by 3–5 mN/m but was not changed by protein mixture.

model tear electrolytes solution. The model tear proteins were chosen as representatives of the most abundant classes of proteins found in human tears. The solutions were prepared with protein concentrations within typical for human tears concentration range of corresponding protein. The mixture of 5 proteins contained 4.43 mg/ml proteins total, and the concentration of each protein was the same as listed in Table 1 for their individual solutions. Most of the selected ophthalmic formulations were purchased from Walgreens Pharmacy while Diquas was purchased over the Internet and Purcell was from NOF Corporation. Model tear proteins and ophthalmic formulations are listed in Tables 1 and 2, respectively. The choice of the lipids for the model lipid mixtures was based on the previously reported human tear lipid compositions [2]. Specifically, human tear lipids contain 43 ± 4 wt % wax esters, 39 ± 3 wt % Cholesteryl esters, 12 ± 7 wt % polar lipids (mostly phosphatidylcholines and sphingomyelins), 4.4 ± 0.8 wt % OAHFA, and ~2 wt % triacyl glycerides. Our model lipid mixtures included the most common and abundant components found in both polar and non-polar parts of human tear. [5]. The non-polar part of these mixtures contained 1:1 mixture of cholesteryl oleate and a mixture of behenyl oleate and palmitoyl oleate (2:1), plus 0.03 parts glyceryl tripalmitate (C16-TAG). Polar lipids were represented by di-stearoyl phosphatidyl choline (18:0 DSPS), di-palmitoyl phosphatidyl choline (16:0 DPPC), palmitoyl-oleyl phosphatidyl choline (16:0–18:1 POPC) and chicken egg sphingomyelins. Total concentrations of polar lipids were 15% and 18% wt. Model lipid mixtures, oil-soluble Retain PM ointment, castor oils, and flaxseed oils (chemically pure ingredients of Refresh Optive Mega-3 ophthalmic emulsion) were dissolved in toluene - iso-propanol (5:1, v/v) solvent in a concentration of 1 mg/ml. A mixed solution (1 mg/ml total) of oil-soluble ointment Retain PM and model tear lipids was prepared by blending equal volumes of the corresponding solutions. 1.4–1.6 μl of model lipid mixture were deposited on electrolyte solution drops, on model tear protein solutions, and on ophthalmic formulations using 2-μl Hamilton syringe.

3. Results and discussion

3.1. Impact of model tear protein

The majority of the publications regarding the evaporation of protein

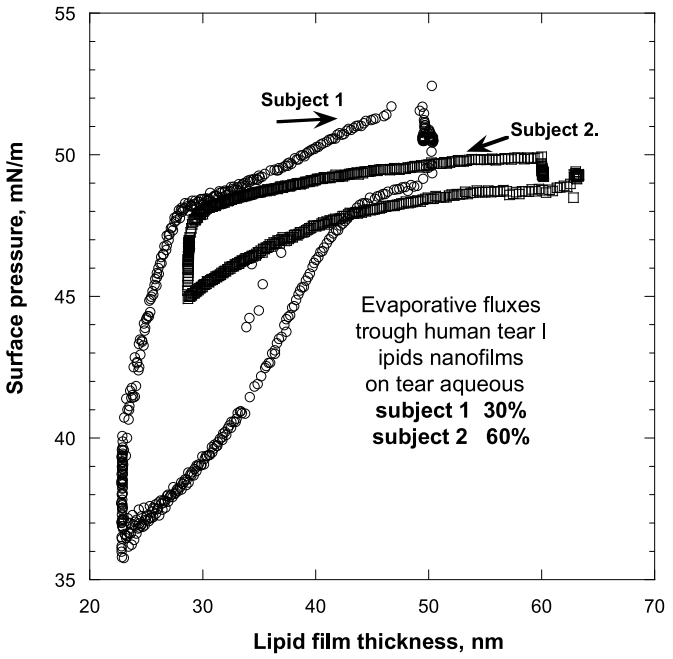

Fig. 5. Dynamic interfacial pressure iso-cycles for human tear lipid nanofilms deposited on human tear aqueous. Both subjects are young white females. The arrows point to the compression branches of iso-cycles. The maximum surface pressure of lipid nanofilms for subject 1normal tears was higher than for dry eye subject 2 by 2–3 mN/m.

solutions refer to the processes occurring during purification, desiccation, and crystallization, often under vacuum or with air flow, heating and active stirring. Of which, there is only a small number of publications reporting the water evaporation rates from aqueous protein solutions under conditions similar to the eye (i.e., 35 °C and moderate humidity). [35 8,19,24.

Previous studies of dynamic interfacial properties of human tear lipids and their interactions with model tear proteins [39,40] have shown that interfacial behaviors and rheological biophysical characteristics of human tear lipid films are altered by the model proteins. Thus, we conducted the experiments using individual protein solutions with the concentrations listed in Table 1, without and with model lipid nanofilms deposited on them. These measurements allowed to estimate the impact of each protein on evaporative fluxes through lipid nanofilms. Then the measurements were performed with 5-protein mixture (the ingredients and their concentrations are listed in Table 1) as an aqueous phase.

The most significant reductions in relative evaporative fluxes, from 1.42 μl/mm^2/s, down to 1.2–1.15 μl/mm^2/s, or by 16–19% relative to baseline evaporative flux of electrolyte solution, was observed with individual lactoglobulin, lactoferrin, human serum albumin and 5-component model protein solutions without lipid films. These results agree with the data reported in Ref. [8] for evaporation reduction observed for human serum albumin and lactoglobulin individual solutions. The smallest reductions of relative evaporative fluxes were observed for individual lysozyme and mucin solutions. The relative evaporative fluxes for model tear protein solutions without and with the lipid films deposited on them, are summarized in Table 3.

The model tear lipid nanofilms containing 15 wt% of polar lipids deposited on electrolyte solution drops and aged for 24 h reduced evaporative fluxes down to 8.05×10^{-5} μl/mm^2/s, or 57 ± 2% of electrolyte solution (baseline, 100%). These model tear lipid nanofilms spread on top of human albumin and lactoferrin solutions had relative evaporative fluxes lower than lipid nanofilms on electrolytes solution by 8–11%. As shown in Table 3, the relative evaporative fluxes through the model tear lipid nanofilms spread on lysozyme, lactoglobulin and mucin solutions were higher than through tear lipid nanofilms on electrolyte solution. The results for model lipid nanofilms spread on bovine mucin solution agreed with previously published data [3] where these authors reported that evaporations through human meibum films in the presence of mucin was higher than through meibum films on water. Interestingly, the evaporative flux through model tear lipid nanofilms spread on the 5-component protein solution was practically the same as evaporative fluxes through lipid nanofilms on electrolyte solution.

Table 4
Relative evaporative fluxes through model tear lipid nanofilms deposited on ophthalmic formulations. (MTE = Model tear electrolytes; SD = standard deviation).

System	Mean relative evaporative flux without model lipids, %	SD, %	Mean relative evaporative flux with model lipid film, %	SD, %
Thealoz Duo	90	3	79	3
Purcell	80	3	69	3
Soother XP	68	4	64	4
Diquas	60	3	63	4
Retain PM	57	3	56	5
Refresh Mega-3	57	3	52	4
Flax seed oil on MTE	–		55	3
Castor oil on MTE	–		66	3
MTE solution	100		40	4

Dynamic interfacial behaviours of model tear lipid nanofilms deposited on lysozyme or human albumin solutions (Fig. 3) were noticeably different from that of lipid nanofilms on electrolyte solution. The top branch of each curve corresponds to the drop contraction leading to the lipid-film thickening while the lower branch corresponds to the drop expansion leading to the lipid-film thinning. During lipid nanofilm compression on the electrolyte solution, the maximum surface pressure of 51.2 mN/m was reached at the thickness of ~ 60 nm, typical for healthy human tear lipid films [37 9]. The hysteresis, the difference of dynamic surface pressure between expansion and contraction branches at the same film thickness at a maximum surface pressure region, was ~ 10 mN/m. In general, a thin fluid film with a higher surface pressure delivers greater film stability. The maximum surface pressure of the model lipid nanofilms on human albumin solution was shifted to a region of thinner films of 45–55 nm as compared to 60–70 nm for lipid nanofilms on electrolyte solution. The hysteresis between expansion and compression branches slightly increased to 12–14 mN/m, compared to 10 mN/m for nanofilms on electrolyte solution. These changes suggest strengthening of intermolecular bonds in the lipid nanofilms due to interactions with human albumin. As shown in Table 3, these shifts in maximum surface pressure correlate with the increased evaporative resistance of the model film lipids on human albumin solution. In contrast, the reduction of the maximum surface pressure by ~5 mN/m was observed on human lysozyme solution. The hysteresis at the maximum surface pressure completely disappeared. The most likely interactions between the model lipids and lysozyme caused a phase transition from visco-elastic semi-solid state to liquid state. The solid-liquid phase transition in the model tear lipid nanofilms significantly reduced evaporative resistance of lipid nanofilms (see Fig. 3).

Minor positive alterations in dynamic interfacial behaviors were also observed for tear lipid nanofilms deposited on lactoferrin solutions (see Fig. 4). Adsorption of lactoferrin onto lipid nanofilms shifted maximum surface pressure region to the thinner lipid films. However, it caused a reduction in the maximum surface pressure and narrowed the hysteresis between expansion and contraction branches, as seen in Fig. 4. Surprisingly, the 5-component mixture of model tear proteins only slightly altered the model lipid nanofilm interfacial dynamic properties and shifted the maximum surface pressure region toward thicker films than

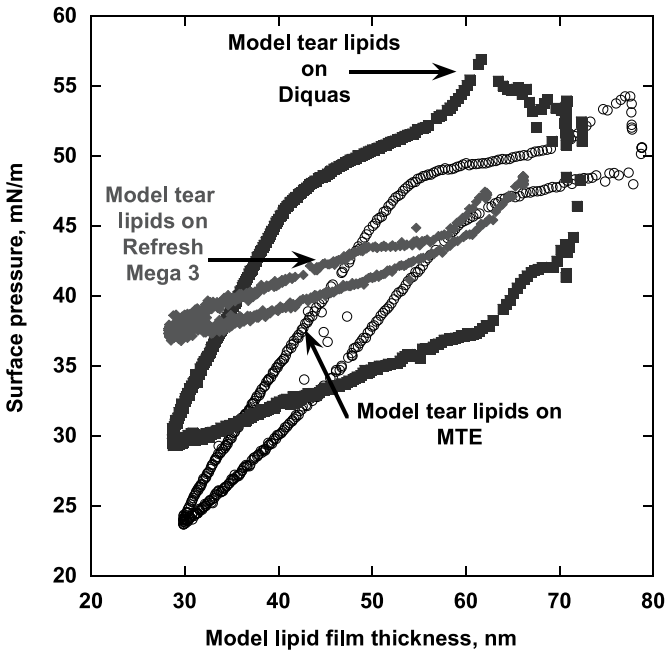

Fig. 6. Dynamic interfacial pressure vs lipid-film thickness iso-cycles for model tear lipids on MTE, on Refresh Optive Mega3, and on Diquas. (MTE = Model Tear Electrolyte). The arrows point to the compression branches of iso-cycles. The maximum surface pressure of lipid film on Refresh decreased by 3–5 mN/m. Diquas solidified lipid nanofilm and increased maximum surface pressure.

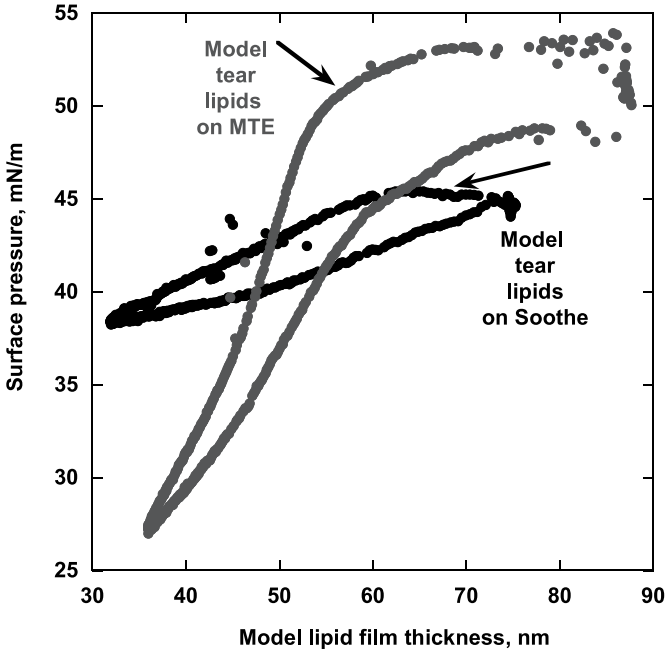

Fig. 7. Dynamic interfacial pressure vs lipid-film thickness iso-cycles for model tear lipids on MTE, and on Soothe. (MTE = Model Tear Electrolyte). The arrows point to the compression branches of iso-cycles. The maximum surface pressure of lipid film on Soother decreased by 8–9 mN/m.

that of model tear lipids alone. The interactions of the model lipid nanofilms with model 5-protein mixture did not noticeably influence evaporation retardation. Apparently, the negative effects on evaporative resistance and interfacial rheological properties of model lipid nanofilms caused by lysozyme, mucin and lactoglobulin were counterbalanced by the positive impacts of human albumin and lactoferrin.

It is important to note that mucin extracted from bovine saliva, and lactoglobulin extracted from bovine milk do not exactly mimic natural human tear proteins in their chemical structures, compositions, and interfacial properties. Therefore, it would be unwise to draw definitive conclusions that human tear aqueous containing over 1500 ingredients will produce the same effects as oversimplified 5-component model protein mixture. However, our approach to the problem of exploring the evaporation *in vitro* provides new insights concerning the possible impacts of some proteins in human tear stability *in vivo*. Effects of the individual proteins on evaporation through model tear lipid films of different composition were also examined. The composition of our 2nd model lipid system containing 18% of phospholipids the evaporative flux down to 5.6×10^{-5} μl/mm^2/s, or 40 ± 4% compared with 57 ± 3% of the previously mentioned model lipid system with the composition containing only 15% of phospholipids. For similar model lipid mixtures [18], it was also found that the higher ratio of polar to neutral nonpolar lipids corresponds to improved model lipid film stability. The evaporative fluxes through nanofilms containing 18% of polar lipids deposited on proteins solutions were 3–6% lower than through these nanofilms on electrolyte solution. These observations suggest that the composition of model tear lipid mixtures has stronger impact on evaporation reduction than the presence of model proteins in subphase.

We are currently conducting a pilot study of evaporation through reconstituted human tear lipid films deposited on human tear aqueous films. Both lipid and aqueous samples are collected from the same single individual. The data show that evaporative fluxes through reconstituted human lipid nanofilms deposited on human tear aqueous are 5–15% lower than the evaporative flux through the same human lipids on electrolyte solution. Fig. 5 illustrates the dynamic interfacial pressure iso-cycle for human lipid nanofilms on tear aqueous for two subjects, (1) subject with healthy stable tear films and (2) subject with mild dry eye symptoms. This plot also shows the values of evaporative fluxes measured *in vitro* using the same sessile drop technique. The shape of iso-cycles for healthy and dry-eye lipids are distinctly different. The healthy lipids exert maximum surface pressure of 51.3 mN/m, whereas the maximally compressed dry-eye lipids exerted lower surface pressure of 49.8 mN/m. Hysteresis between compression and expansion at the maximum pressure was 2.8 mN/m and 1.7 mN/m for healthy and dry-eye lipid nanofilms, respectively. At the same time, evaporative flux through dry-eye lipid nanofilms spread on its natural tear aqueous phase was twice as high as that through healthy tear lipid nanofilms on its corresponding tear aqueous. It must be revealed that these results are an example for this pair of subjects, and the values for evaporative fluxes through healthy and dry-eye lipids *in vitro* may vary significantly between subjects due to the general eye health, gender, or race. Nevertheless, the data reported on Fig. 5 confirm that the results obtained for our model tear lipid systems agree with the results obtained for the reconstituted human tear lipid films *in vitro*.

3.2. Impact of topical ophthalmic formulation

The model lipid nanofilms used for this part of the study containied18 wt% of polar lipids. This model lipid system deposited on electrolytes solution reduced evaporative fluxes down to 5.6×10^{-5} μl/mm^2/s, or 40% (SD 6%). We studied 6 ophthalmic formulations – three were aqueous solutions, two were aqueous emulsions and one was an ointment (Retain PM). Aqueous ophthalmic formulations are used as an eye drops and the volume of a drop varies from 20 to 40 μl. This volume is significantly larger than the volume of human tear aqueous, 3–8 μl. Aqueous ophthalmic formulations are expected to mix with tear aqueous upon drop instillation and increase the aqueous phase volume. After the eye drop instillation, the tear lipid layer is quickly restored after a few blinks. We studied ophthalmic formulations without adding any proteins to minimize confounding effects. The ointment is also expected to mix with the lipid part of tear film after application on the ocular surface, thus we tested the ointment as individual films and as a 1:1 mixture with our

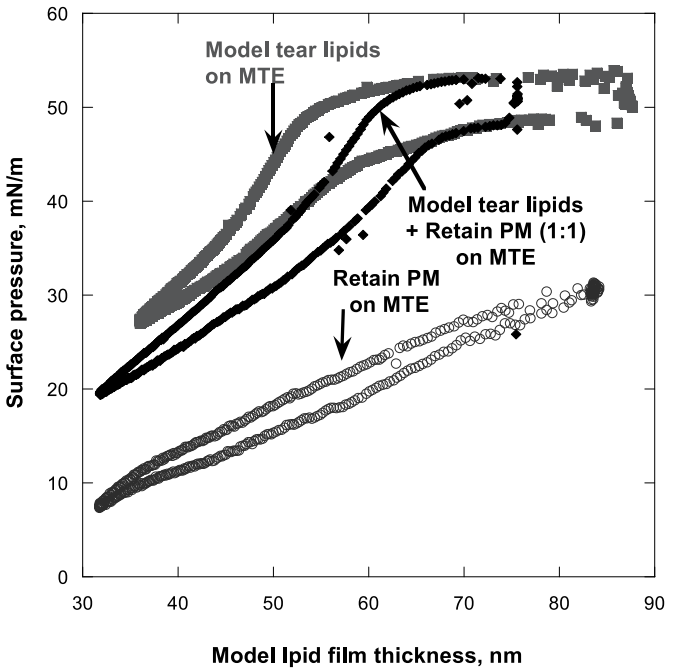

Fig. 8. Dynamic interfacial pressure vs lipid-film thickness iso-cycles for model tear lipids, Retain PM film on MTE, and 1:1 mixture of Retain PM and model tear lipids on MTE. (MTE = Model Tear Electrolyte). The arrows point to the compression branches of iso-cycles. The maximum surface pressure of model lipids + Retain PM film remained close to the model lipid nanofilm however it was attained for ~ 15 nm thicker nanofilm.

model lipid system.

Table 4 summarizes the evaporative fluxes for the selected topical ophthalmic formulations without and with the model lipid nanofilms spread on them, as well as for the 75–80 nm nanofilms of Retain PM, castor oil, and flaxseed oil spread on electrolyte solution. The ophthalmic formulations without lipid nanofilms, castor oil and flaxseed oil films had shown some reduction of evaporative fluxes compared to electrolyte solution. However, none of them had reached the level of evaporation inhibition achieved by our 2nd model tear lipid nanofilms spread on electrolyte solution. Among the formulations tested, pure flaxseed oil films and Refresh Mega-3 containing both flaxseed and castor oils had the best evaporative flux reduction. Thealoz Duo and Purcell exhibited the highest evaporative fluxes. As seen from this table, the relative evaporative fluxes trough the model lipid nanofilms deposited on over-the-counter formulations were 12%–39% higher than through the model lipid nanofilms on electrolyte solution. This observation suggests that the ophthalmic formulations designed to alleviate dry eye symptoms may not achieve the same level of evaporative inhibition as our model tear lipids and may inadvertently increase evaporation *in vivo* for eyes that do not have compromised tear lipid films.

Next, the results from the study of dynamic interfacial pressure as a function of the nanofilm thickness showed that lipid nanofilms deposited on Diquas, Refresh Mega-3 and Soothe formulations exhibited dynamic interfacial behaviors strikingly different from the model tear lipid nanofilms on electrolyte solution. Figs. 6 and 7 depict the surface pressure on lipid film thickness dependences for tear lipid nanofilms deposited on electrolyte solution and on these ophthalmic formulations.

The arrows point to the compression branches of iso-cycles. The maximum surface pressure of lipid film on Refresh decreased by 3–5 mN/m. Diquas solidified lipid nanofilm and increased maximum surface pressure.

Interactions of model tear lipids with Diquas ingredients made tear lipid nanofilms more rigid. The hysteresis between the expansion and contraction branches of the iso-cycles substantially increased, suggesting that lipid nanofilms became brittle rather than visco-elastic and apparently collapsed at high surface pressure, similar to condensed monolayers. Refresh Mega-3 ingredients produced the opposite effect because the lipid nanofilms became more fluid, with a lower maximum surface pressure and a smaller hysteresis. Refresh Mega-3 formulation contains several surface-active ingredients (e.g., Polysorbat 80, (Tween-80), and Polyoxyl 40 stearate) as emulsifiers. It is likely that interactions of the model lipids with these emulsifiers caused softening of model lipid nanofilms, with reduced hysteresis and maximum surface pressure decreased by ~ 3–5 mN/m (Fig. 6).

The most substantial alterations of dynamic interfacial behavior were caused by the interactions between the model lipid nanofilms and Soothe ingredients. These interactions caused partial displacement of phospholipids from the lipid film resulting in prominent distortion of the iso-cycle shape, as seen in Fig. 7. The changes of iso-cycle shape indicate that the originally visco-elastic gel-like nanofilm was transformed into a viscous liquid film with low elasticity at a higher film thickness. It should be noted that the maximum surface pressure for lipid nanofilms at the full compression was reduced by 8–9 mN/m. This reduction was even stronger than that was observed in the presence of Refresh Mega-3. Soothe® XP formulation is produced as emulsion, which contains emulsifying additives. These additives, such as octoxynol-40 and polysorbate 80, are surfactants with significant surface activity. The negative effect of Soothe on the model lipid nanofilms biophysical behavior is likely the results of the interaction between the polar lipids and these surface-active emulsifiers.

The question regarding how mineral oils and other petrochemicals such as petrolatum, used in over-the-counter formulations, affect tear lipid nanofilms biophysical properties remains unclear. To clarify, we tested the biophysical properties of nanofilms formed by Retain PM ointment, containing only mineral oil and white petrolatum without any emulsifiers. We also tested this ointment mixture with the model tear lipids. As seen in Fig. 8, 75 nm nanofilms of Retain PM on electrolyte solution has low surface pressure and behaves like viscous liquid film without elasticity. The addition of Retain PM to model tear lipid did not cause significant changes in the shape of nanofilm iso-cycle compared with the model tear lipid nanofilm on electrolyte solution. The maximum surface pressure at the full compression remained the same as it was for model lipid nanofilms on electrolyte solution.

However, the expansion-contraction iso-cycle of these mixed nanofilms, Retain ointment and model lipids, was shifted to the right, toward thicker film region, as compared with original model tear lipids iso-cycle. This shift is associated with a decreased total concentration of polar lipids in the mixed film due to the dilution of model lipids by petrochemical components of Retain PM. These alterations correlate to the noticeable increase by ~ 15% in evaporative flux through the mixed films (i.e., model lipids + Retain PM) as compared to model tear lipid nanofilms on electrolyte solution.

According to a *in vivo* study [10,36], dry eye formulations containing mineral oil increased human tear lipid film thickness 15 min after instillation. However, it was not indicated whether this increase in lipid film thickness led to better tear film stability *in vivo*. In a clinical study [17] of Soothe eye drops *in vivo,* it was stated that an increase in lipid layer thickness at 15 min after instillation did not produce significant change in evaporation-driven tear film thinning rate, that is consistent with our findings. Recently, we have shown that an increase of model lipid film thickness above 60 nm *in vitro* did not significantly enhance evaporation retardation [40].

These observations suggest that long-term use of some topical ophthalmic formulations containing significant amounts of surface-active emulsifiers, such as Refresh Mega-3, Retain MGD, and Soothe, may cause tear film instability attributable to adversely altered biophysical properties of human tear lipid nanofilms *in vivo*.

4. Conclusions

The novel micro-volumetric method was used to quantify evaporation rates *in vitro*. For the first time, it was empirically verified that model tear lipid nanofilms, some of model tear protein solutions and dry eye artificial tear formulations alone can reduce water evaporative fluxes. It was demonstrated that the combinations of model tear lipid nanofilms with some of the proteins, such as human serum albumin and lactoferrin, enhance evaporation inhibition by lipid nanofilms. However, human lysozyme and bovine mucin increased evaporative fluxes through model tear lipid nanofilms. 5-component model tear proteins mixture induced only minor changes in model tear lipid biophysical behaviors and evaporative fluxes.

Finally, none of the studied ophthalmic formulations enhanced evaporative resistance of model tear lipid nanofilms. Diquas, Refresh Optive Mega3 and Soothe significantly altered biophysical properties of model tear lipid nanofilms and reduced evaporative resistance of model tear lipid nanofilms. These changes may potentially lead to human tear film destabilization and premature tear-film breakup *in vivo*.

CRediT authorship contribution statement

Meng C. Lin: Supervision, Project administration, Funding acquisition, Conceptualization, Methodology, Writing. **Tatyana F. Svitova:** Conceptualization, Methodology, Investigation, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] A.J. Bron, J.M. Tiffany, S.M. Gouveia, N. Yokoi, L.W. Voon, Experimental Eye Research. Functional Aspects of the Tear Film Lipid Layer, vol. 78, 2000, pp. 347–360, https://doi.org/10.1016/j.exer.2003.09.019.
[2] S.H.J. Brown, C.M.E. Kunnen, E. Duchoslav, N.K. Dolla, M.J. Kelso, E.B. Papas, P.L. de la Jara, M.D.P. Willcox, S.J. Blanksby, T.W. Mitchell, A comparison of patient matched meibum and tear lipidomes, Invest. Opthamol Vi.s Sci. 54 (2013) 7417–7424, https://doi.org/10.1167/iovs.13-12916.
[3] C. Cerretani, N.H. Ho, C.J. Radke, Adv. Colloid and Interface Sci. Water-Evaporation Reduction by Duplex Films: Application to the Human Tear Film, vols. 197–198, 2013, pp. 33–57, https://doi.org/10.1016/j.cis.2013.03.007.
[4] J.P. Craig, A. Tomlinson, Optom. Vis. Sci. Importance of the Lipid Layer in the Human Tear Film Stability and Evaporation, vol. 74, 1997, pp. 8–13, https://doi.org/10.1097/00006324-199701000-00014.
[5] A.W. Dean, B.J. Glasgow, Mass spectrometric identification of phospholipids in human tears and tear lipocalin, Invest. Opthamol. Vis. Sci. 53 (2012) 1773–1782, https://doi.org/10.1167/iovs.11-9419.
[6] P.N. Dilly, Structure and functions of tear film, in: D.A. Sullivan (Ed.), Advances in Experimental Medicine and Biology; Lacrimal Gland, Tear Film, and Dry Eye Syndromes: Basic Science and Clinical Relevance, vol. 350, 1994, pp. 239–247. New York.
[7] T.J. Dursch, W. Li, B. Taraz, M.C. Lin, C.J. Radke, Optom. Vis. Sci. Tear-Film Evaporation Rate from Simultaneous Ocular-Surface Temperature and Tear-Breakup Area, vol. 95, 2018, pp. 5–12, https://doi.org/10.1097/OPX.0000000000001156, 1.
[8] V.B. Fainerman, A.V. Makievski, J. Krägel, A. Javadi, R. Miller, Studies of the rate of water evaporation through adsorption layers using drop shape analysis tensiometry, J. Colloid Interface Sci. 308 (2007) 249–253, https://doi.org/10.1016/j.jcis.2006.12.073.
[9] D. Finis, N. Pischel, S.M.D. Schrader, G. Geerling, Cornea. Evaluation of lipid layer thickness measurement of the tear film as a diagnostic tool for meibomian gland, Dysfunction 32 (12) (2013) 1549–1553, https://doi.org/10.1097/ICO.0b013e3182a7f3e1.
[10] J.S. Fogt, M.J. Kowalski, P.E. King-Smith, A.T. Epitropolous, A.J. Hendershot, C. Lembach, J.P. Maszczak, L.A. Jones-Jordan, Barr J. Clin. Ophthalmol. Tear lipid layer thickness with eye drops in meibomian gland dysfunction 10 (2016) 2237–2243, https://doi.org/10.2147/OPTH.S120158.
[11] B. Glasgow, J. Invest. Ophthalmol. Vis. Sci. Evidence for phospholipids on the surface of human tears 61 (2020) 14–19, https://doi.org/10.1167/iovs.61.14.19.
[12] M. Guillon, C. Maissa, CLAE. Tear Film Evaporation: Effect of Age and Gender, vol. 33, 2010, pp. 171–175, https://doi.org/10.1016/j.clae.2010.03.002.
[13] G.H. Herok, P. Mudgil, T.J. Millar, Current Eye Research. The Effect of Meibomian Lipids and Tear Proteins on Evaporation Rate under Controlled *in Vitro* Conditions, vol. 34, 2009, pp. 589–597, https://doi.org/10.1080/02713680902972366.
[14] F. Holly, M.A. Lemp, Exp. Eye. Res. Formation and rupture of the tear film 15 (1973) 515–525, https://doi.org/10.1016/0039-6257(77)90087-X.
[15] F. Holly, J. Trans. Ophthal. Soc. Physical-chemistry of the normal and disordered tear film 104 (1985) 374–380.
[16] S.H. Kimball, P.E. King-Smith, J. Nichols, J Invest. Ophthalmol. Vis. Sci. Evidence for the major contribution of evaporation to tear film thinning between blinks 51 (2010) 6294–6297, https://doi.org/10.1167/iovs.09-4772.
[17] P.E. King-Smith, E.A. Hinel, J.J. Nichols, J Invest. Ophthalmol. Vis. Sci. Application of a novel interferometric method to investigate the relation between lipid layer thickness and tear film thinning 51 (5) (2010) 2418–2423, https://doi.org/10.1167/iovs.09-4387.
[18] P. Kulovesi, J. Telenius, A. Koivuniemi, G. Brezesinski, I. Vattulainen, Soft Matter, The impact of lipid composition on the stability of the tear fluid lipid layer 21 (8) (2012) 5826–5834, https://doi.org/10.1039/c2sm25210d.
[19] K.J. Laylin, D.J.O. Berry, Science. Evaporation Enhancement by Protein Films 140 (1963) 312–314, 3564, https://www.jstor.org/stable/1710416.
[20] M.C. Lin, T.F. Svitova, Bicontinuous Microemulsions and Methods of Use Thereof, 2017. WO/2017/147470 US20190038555.
[21] W.D. Mathers, T.E. Daley, Ophthalmology. Tear Flow and Evaporation 103 (4) (1996) 664–669, https://doi.org/10.1016/s0161-6420(96)30637-4.
[22] J.P. McCulley, W.E. Shine, Bioscience Reports. The lipid Layer: The outer Surface of the Ocular Surface Tear Film 21 (4) (2001) 407–418, https://doi.org/10.1023/A:1017987608937.
[23] J.E. McDonald, S. Brubaker, Am. J. Ophthalmol. Meniscus-induced thinning of tear films 72 (1971) 139–145, https://doi.org/10.1016/0002-9393(71)91604-7.
[24] F. Miano, M. Calcara, F. Giuliano, T.J. Millar, V.J. Enea, Phys.: Condens. Matter. Effect of Meibomian Lipid Layer on Evaporation of Tears, vol. 16, 2004, pp. S2461–S2467, https://doi.org/10.1088/0953-8984/16/26/019.
[25] F. Miano, X. Zhao, J.R. Lu, J. Penfold, J. Biophys, Coadsorption of human milk lactoferrin into the dipalmitoylglycerophosphatidylcholine phospholipid monolayer spread at the air/water interface 92 (2007) 1254–1262, https://doi.org/10.1529/biophysj.105.078592.
[26] T.J. Millar, B.S. Schuett, Experimental Eye Research. The Real Reason for Having a Meibomian Lipid Layer Covering the Outer Surface of Tear Film, vol. 137, 2015, pp. 125–138, https://doi.org/10.1016/j.exer.2015.05.002.
[27] T.J. Millar, S.T. Tragoulias, P.J. Anderton, M.S. Ball, F. Miano, G.R. Dennis, P. Mudgil, Cornea. The surface activity of purified ocular mucin at the air-liquid interface and interactions with meibomian lipids 25 (1) (2006) 91–100, https://doi.org/10.1097/01.ico.0000164779.87795.3c.
[28] P. Mudgil, M. Torres, T.J. Millar, Coll. & Surf. B. Adsorption of Lysozyme to Phospholipid and Meibomian Lipid Monolayer Film, vol. 48, 2006, pp. 128–137, https://doi.org/10.1016/j.colsurfb.2006.01.017.
[29] R.O. Paananen, M. Javanainen, J.M. Holopainen, I.J. Vattulainen, Phys. Chem. Let Crystalline Wax Esters Regulate the Evaporation Resistance of Tear Film Lipid Layers Associated with Dry Eye Syndrome 10 (2019) 3893–3898, https://doi.org/10.1021/acs.jpclett.9b01187.
[30] E.B. Papas, Ophthalmic Physiol Opt. The Global Prevalence of Dry Eye Disease: A Bayesian View, 2021, pp. 1–13, https://doi.org/10.1111/opo.12888, 00.
[31] C.-C. Peng, C. Cerretani, R.J. Braun, C.J. Radke, (a) Adv Colloid and Interface Sci.. Evaporation-driven instability of precorneal tear film 206 (2014a) 250–264, https://doi.org/10.1016/j.cis.2013.06.001.
[32] C.-C. Peng, C. Cerretani, Y. Li, S. Bowers, S. Shahsavarani, M.C. Lin, C.J. Radke, Flow Evaporimeter to Assess Evaporative Resistance of Human Tear-Film Lipid Layer, vol. 53, 2014, pp. 18130–18139, https://doi.org/10.1021/ie5030497 (b) , Ind. Eng. Chem. Res.
[33] A.H. Rantamäki, M. Javanainen, I. Vattulainen, J.M. Holopainen, Invest. Ophthalmol. Vis. Sci. Do lipids retard the evaporation of the tear fluid? 53 (2012) 6442–6447, https://doi.org/10.1167/iovs.12-10487.
[34] A.H. Rantamäki, S.K. Wiedmer, J.M. Holopainen, Invest. Ophthalmol. Vis. Sci. Melting points—the key to the anti-evaporative effect of the tear film wax esters 54 (2013) 5211–5217, https://doi.org/10.1167/iovs.13-12408.
[35] M. Rolando, M. Zierhut, S. Barabino, Ocular Immunology and Inflammation. Should We Reconsider the Classification of Patients with Dry Eye Disease?, 2019, https://doi.org/10.1080/09273948.2019.1682618.
[36] R.C. Scaffidi, D.R. Korb, Eye Contact Lens. Comparison of the Efficacy of Two Lipid Emulsion Eyedrops in Increasing Tear Film Lipid Layer Thickness, vol. 33, 2007, pp. 38–44, https://doi.org/10.1097/01.icl.0000247638.50568.c0, 1.
[37] A. Sharma, S. Tiwari, R. Khanna, J.M. Tiffany, in: Sullivan, et al. (Eds.), In Lacrimal Gland, Tear Film, and Dry Eye Syndromes 2. Hydrodynamics of Meniscus-Induced Thinning of the Tear Film, Plenum Press, New York, 1998, pp. 425–431.
[38] S.M. Sledge, H. Khimji, D. Borchman, A.L. Oliver, H. Michael, E.K. Dennis, D. Gerlach, R. Bhola, E. Stephen, The Ocular Surface. Evaporation and Hydrocarbon Chain Conformation of Surface Lipid Films, vol. 14, 2016, pp. 447–459, https://doi.org/10.1016/j.jtos.2016.06.002, 4.
[39] T. Svitova, M. Lin, Adv. In Colloid and Interface Science. Dynamic Interfacial Properties of Human Tear-Lipid Films and Their Interactions with Model-Tear

Proteins *in Vitro*, vol. 23, 2016, pp. 4–24, https://doi.org/10.1016/j.cis.2015.12.009.

[40] T.F. Svitova, M.C. Lin, Colloids and Surfaces B, Evaporation retardation by model tear-lipid films: role of film aging, Colloids Surf. B Biointerfaces 197 (2021), 11392, https://doi.org/10.1016/j.colsurfb.2020.111392. In this issue.

[41] T. Viitaja, J. Moilanen, K.J. Johanna Svedström, F.S. Ekholm, R.O. Paananen, *Nano Letters*. Tear Film Lipid Layer Structure: Self-Assembly of O -Acyl-ω-Hydroxy Fatty Acids and Wax Esters into Evaporation-Resistant Monolayers, vol. 21, 2021, pp. 7676–7683, https://doi.org/10.1021/acs.nanolett.1c02475.

[42] J. Winebrake, O.J. Drinkwater, A.R. Brissette, C.E. Starr, EyeNet Magazine. The TFOS Dry Eye Workshop II: Key Updates, 2017. https://www.aao.org/eyenet/article/the-tfos-dry-eye-workshop-ii.

[43] S. Wong, P.J. Murthy, L. Jones, Cont. Lens and Anterior Eye. Tear Evaporation Rates: what Does the Literature Tell Us?, vol. 41, 2018, pp. 297–306, https://doi.org/10.1016/j.clae.2017.12.003, 3.

[44] L. Zhou, S.Z. Zhao, S.K. Koh, L. Chen, C. Vaz, V. Tanavde, X.R. Li, R.W.J. Beuerman, Proteomics.. In-Depth Analysis of the Human Tear Proteome, vol. 75, 2012, pp. 3877–3885, https://doi.org/10.1016/j.jprot.2012.04.053.