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ARTICLE TYPE

Surface relaxations as a tool to distinguish the dynamic interfacial properties of films by normal and diseased meibomian lipids

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The surface properties of human meibomian lipids (MGS), the major constituent of the tear film (TF) lipid layer, are considered of key importance for the TF stability. Therefore the dynamic interfacial properties of films by MGS from normal eyes (nMGS) and eyes with meibomian gland dysfunction (dMGS) were studied using a Langmuir surface balance. The sample's behavior during dynamic area changes was evaluated through the surface pressure-area isotherms and isocycles. The films surface dilatational rheology was probed in the frequency range of 10^{-5}-1 Hz via the stress-relaxation method. Significant difference was found with dMGS showing slow viscous dominated relaxation at 10^{-4}-10^{-3} Hz, while nMGS remained predominantly elastic for the whole range. Cole-Cole plot unveiled two characteristics processes contributing to the relaxations, fast (on the scale of characteristic time τ<5 sec) and slow (τ>100 sec), the latter prevailing in dMGS films. Brewster angle microscopy revealed better spreading of nMGS at the air/water interface while dMGS layers were non-uniform and patchy. The distinctions in the films interfacial properties *in vitro* correlated with the accelerated degradation of meibum layer pattern at the air/tear interface and with the decreased stability of TF *in vivo*. These results and the recent findings on the modest capability of meibum to suppress the evaporation of the aqueous subphase suggest rethinking the role of MGS. A probable key function of meibomian lipids might be to form viscoelastic films capable to oppose dilation of the air/tear interface. The impact of temperature on the meibum surface properties is discussed in terms of possible effects on the film normal structure.

Keywords: human meibum, dry eye syndrome, meibomian gland dysfunction, Langmuir surface balance, stress-relaxation, dilatational rheology

Introduction

Tear Film (TF) consists of a lipid layer at the air/tear interface and of underlying aqueous tear positioned over the corneal epithelium. A primary function of TF is to rapidly reorganize and spread as continuous layer over the ocular surface following the upward movement of the eyelid during blink and to maintain its integrity during the interblink time.[1,2] If TF surface properties are disturbed, it breaks up prematurely, dry spots form over the cornea and dry eye syndrome (DES) develops. DES, a major public health disease affecting between 10 and 30% of the population worldwide, results in eye dryness, visual disturbances, foreign body sensation, and eye pain etc, thus decreasing the people's productivity and quality of life while the compromised integrity of the TF exposes the ocular surface epithelium to the invasion of pathogens and contaminants from the environment.[3,4]

Tear film lipid layer (TFLL) is of key importance for maintaining the low surface tension and the high area-to-volume ratio of the TF and also for ensuring tangentially immobile air/tear interface (the Gibbs-Marangoni effect) opposing the outflow of aqueous tear in an open eye.[5-7] The major component (more than 90 %) of TFLL is the oily substance produced by specialized Meibomian glands located within the upper and lower eyelids. Meibomian lipids represent a complex mixture of more than hundred thousand molecular species[8] consisting predominantly of wax and sterol esters (saturated and unsaturated) and up to 10 % polar lipids (being phospholipids or (O-acyl)-omega-hydroxy fatty acids, OAHFA).[9-11] The highly heterogeneous compositions results in a broad non-cooperative melting transition in the temperature interval of 15 to 35 ºC.[10,12]

According to the DEWS (international dry eye workshop, 2007), DES is classified into two types, aqueous-deficient and evaporative. The major cause of the latter is the meibomian gland dysfunction (MGD) characterized with modifications of meibum composition (increased content of sterol esters, elevated chain melting temperature, decreased levels of OAHFA) and impairment of the secretory glands. The compositional variations in meibomian secretion reportedly correlate with decreased stability of TF at the ocular surface.[10,11]

Thus it is important to study how the surface properties of meibomian gland secretion collected from normals, nMGS, and

from patients with Meibomian gland dysfunction, dMGS, differ from each other which is the goal of the current work. The experiments with films by meibomian lipids, nMGS and dMGS, spread over saline subphase were performed with Langmuir surface balance. Two measurement temperatures were utilized: 25°C and 34°C, the latter considered as the consensus mean temperature of TFLL in office conditions.[13,14] The surface pressure (π)/area (A) dynamic isocycles were recorded and the film structure was monitored with Brewster Angle Microscopy.

After area change is applied to natural lipid/polymer films and then ceased, the heterogeneous films constituents undergo complex reorganizations associated with characteristic low frequency processes until a new equilibrium is reached at the interface. Therefore the rheological dilatational properties of human meibum films were probed by Fourier transform analysis of the surface pressure relaxation transients recorded after application of an abrupt stress in the form of instantaneous little compression deformation.[15,16] The stress-relaxation method allows to obtain the real and imaginary part of the complex dilatational modulus in the broad range of very low frequencies, 10^{-5} Hz, to frequencies as high as 1 Hz and has been successfully applied to variety of biointerfacial systems.[17-20] The surface chemistry properties of meibomian, nMGS and dMGS, films are considered in relation to the clinical observations, already published and our original ones, on the behavior of tear film lipid layer at the air/tear interface and on the stability of the whole TF *in vivo*.

Materials and Methods

Collection of human meibum

Meibum samples were collected from eyes of 7 normal subjects (22-73 years old; mean age 42.7±7.5) and eyes of 7 MGD patients (64-82 years old; mean age 73.5±2.5). The criteria for selection of meibum donors, healthy volunteers and MGD patients, are described in ***Supplement I, point I***. None of the normal healthy volunteers and MGD patients wore contact lenses, or applied eye makeup prior to sample collection. The research was conducted in accordance with the tenets set forth in the Declaration of Helsinki, and prior written informed consent was obtained from all subjects after receiving a detailed explanation of the nature of the study and possible consequences associated with participation in the study.

Human meibomian lipids were collected from the upper lid margin by applying pressure to the the central one third of upper eyelid with a thumb or a aid of forceps specialized to express meibomian lipids (Yoshitomi's meibomian gland pressure forceps, T.M.I. Co. Ltd., Saitama, Japan) for MGD patients with difficulty in expression of meibomian lipids. The expressed samples were collected at the meibomian orifices at the upper lid margin with a platinum spatula, paying greatest attention to pick up only the expressed meibum, weighed and dissolved in chloroform to a solution with a concentration of 1 mg MGS/ml. Prior to experiments the lipid solutions obtained were kept at -80°C. The composition of meibum was examined by thin layer chromatography and revealed the presence mainly of wax and sterol esters in agreement with previous reports.[21]

Langmuir surface balance experiments

Surface pressure (π)- area (A) isotherms were measured using a computer-controlled Langmuir surface balance (Kibron, Helsinki, Finland) equipped with a μTrough XL (area 225 cm^2, volume 40 ml) by the Wilhelmy wire probe method (instrumental accuracy 0.01 mN/m) as previously described.[22,23]

Human meibum dissolved in chloroform was spread over the air/saline solution (PBS, pH 7.4) interface with a Hamilton microsyringe. Typically 46 μg MGS were deposited on the surface. An acrylic cover was put over the trough to protect the surface from dust and to suppress subphase evaporation. After 15 min to allow chloroform evaporation, film area compression was started using two symmetrically moving barriers. Fast dynamic compression-expansion isocycling of film area was performed with the maximal possible barrier's rate (1.37 cm^2s^{-1}) at which there was no leakage of the film. Ten consecutive cycles were performed with each sample. Normally between the first and third cycle the π(A) curves achieve stationary shape and those π(A) compression isotherms are presented.

Film surface compressional modulus, Cs^{-1}, at a given surface pressure was calculated from π/A compression isotherm using the equation:

$$C_s^{-1} = A_\pi \left(\frac{d\pi}{dA}\right)_T \qquad (1)$$

where A_π is the area at the indicated π. The inflexion points in the π/ Cs^{-1} dependencies indicate the surface pressures at which significant reorganization of the surface film takes place in the course of the film compression.[24]

All isotherms were repeated at least three times; the difference between the repetitions did not exceed two percents. Experiments were performed at two temperatures 25°C and 34°C (the consensus mean temperature of TFLL in an office environment[13,14]). The morphology of the films was observed by Brewster Angle Microscopy using UltraBAM (Accurion GmbH, Germany).

Interfacial dilatational rheology evaluated via the stress-relaxation method

In order to gather information about the viscoelastic properties of meibum films, nMGS and dMGS, the relaxation of surface pressure was monitored after small rapid compression deformation was applied to the surface film. In these experiments, the meibomian film pre-equilibrated with the subphase at 25°C or 34°C was instantaneously, for up to a second, and slightly contracted with a small area perturbation, $\Delta A/A_o \leq 5$ % (where A_o is the initial area of the film, and ΔA is the area change). As a result of the area contraction π rose from its initial value and then relaxed gradually with time until new equilibrium value was established (see the upper panel of Fig. 2). The kinetics of the relaxation of surface pressure was presented in terms of Δπ/t dependence, where $\Delta\pi = \pi(t)-\pi_0$; here π(t) is the momentum value of π after the start of the relaxation and π_0 is the surface pressure of the film before the area stress was applied. For the experiments shown here π_0 = 25 mN/m.

Loglio and coworkers[15,16] showed that dependence of the real, E_R, and imaginary part, E_{IM}, of the complex dilatational elasticity

modulus E*(ν) on frequency, ν, can be obtained via Fourier transformation, F, of the relaxation transients:

$$E^*(\nu) = E_R(\nu) + iE_{IM}(\upsilon) = \frac{F\{d\Delta\pi(t)/dt\}}{F\{dlnA/dt\}} = \frac{i6.18\nu}{\Delta A/A_0}\int_0^\infty \Delta\pi(t)\exp(-i6.18\nu t)\,dt \qquad (2)$$

Here E_R accounts for the elasticity of the surface film, while $E_{IM} = 6.18\,\nu\,\eta_d$ (where η_d is the dilatational viscosity) accounts for the dissipative, viscous properties of the film. The number 6.18 is a brief denotement of the doubled Archimedes constant (2 x 3.14159…).

Once the real and imaginary parts of the dilatational elasticity modulus are obtained it is possible to calculate the tangent of phase angle:

$$\tan\varphi = \frac{E_{IM}}{E_R} \qquad (3)$$

If $E_R > E_{IM}$ then tan φ < 1 and the film is predominantly elastic. On the contrary if $E_R < E_{IM}$ then tan φ > 1 and the film is predominantly viscous.

Results

Dynamic compression isotherms of MGS films

Typical isotherms obtained at dynamic compression of meibomian surface layers at 25°C and 34°C are shown on Fig. 1. It can be seen that the dynamic surface properties of nMGS and dMGS were similar. The increase of temperature shifted the isotherms to lower surface areas and to lower π values and also resulted in formation of brighter, i.e. thicker, meibum films for both specimen.

The structure of the surface films however showed characteristic differences. At 34°C and π < 8 mN/m both, nMGS and dMGS, films contained thicker brighter regions with darker thinner regions interspersed between them. In the case of nMGS at >10 mN/m, these thinner sectors almost extinct and a thick rough film was formed at ≥ 20 mN/m. The surface pressure at which the presence of the dark regions diminished in the nMGS layers corresponded to the inflection point of the π/Cs-1 dependencies in the range of low (≤ 8mN/m) surface pressures as reported in previous studies.[25-29] The meibum films with which the compression π/A isotherms were obtained are typically formed by spreading of 46 μg MGS at the trough surface, i.e. at 4.89 cm^2/μg MGS. The lift off of the surface pressure took place at 4-4.5 cm^2/μg MGS which is in excellent agreement with other studies.[30] Based on the lipidomics data on the type and ratio of the main MGS constituents (wax and sterol esters, tryacylglycerols, OAHFA etc.) it is possible to roughly deduce apparent average molecular weight of meibomian lipid ~700 and molecular area of ~60 $Å^2$.[31] Thus the apparent area per molecule was varied between 58 $Å^2$ and 9.86 $Å^2$, which means that films containing regions of increasing multilayer thickness can be implied in the course of film compression. This is to be expected concerning the non-polar nature of the major MGS species (wax and sterol esters, tryacylglycerols) known to spontaneously form multilayer and not monolayer films at the air/water surface.[32,33]

The simple semi quantitative model of Wilke and Maggio,[34] relates the intensity, I, of the film regions in the BAM image with its thickness, d ($\mathbf{d_i/d_j = [I_i/I_j]^{1/2}}$, where the subscripts **i** and **j** denote the different images). Thus by comparing the MGS images with BAM micrographs (obtained at identical experimental conditions) of typical monolayer lipid, dipalmitoylphosphatidylcholine, it is possible to estimate that the thinner dark sectors in the expanded meibum films were of monolayer thickness while the bright regions were 3-8 monomolecular layers thick. These evaluations agree well with previously published data on the structure of meibomian films at the air/water interface.[35] The results concur with a study of Petrov et al.[36] showing that for films by synthetic meibum replica the lift off area is as low as 50 $Å^2$ and that from the very onset of π the synthetic and bovine MGS layers consist of coexisting monolayer and multilayer thick patches with the content of the latter increasing in the course of compression.

In the case of dMGS the lipid films maintained inhomogeneous structure with thinner regions present in the layers even in the higher (> 8 mN/m) surface pressure range. Still the π/Cs^{-1} dependence also showed an inflexion at π< 8 mN/m, similarly to nMGS films. This suggests that the alterations in the shape of the π/Cs^{-1} curve could be due to changes in the molecular packing density of the ply of polar lipids interfacing the aqueous subphase, rather than due to amendment in the organization of the bulk mass of non-polar lipids layered over the polar lipid sheet (see discussion and **Fig. 6** for details on the probable structure of meibomian surface films).

The π/A-isotherm reversibility of the meibum films was also measured; significant thermal hysteresis was observed with nMGS and dMGS showing similar behavior at both temperatures (See ***Supplement I, point II***).

Dilatational rheology of MGS films characterized by the method of stress/relaxation

Experiments were performed at 10 mN/m and 25 mN/m. At both surface pressures analogous effects were observed. The samples from normals were predominantly elastic for the whole frequency range, while for MGD specimens the contribution of the dissipative modulus became predominant at the low frequencies. Further only the data obtained at 25 mN/m are presented. This surface pressure was chosen due to two reasons: (i) to compare our data with previous rheology studies of meibum conducted at identical π values[37] and (ii) as X-ray diffraction studies indicate that at π > 18 mN/m the MGS films does not contain monolayer patches, but entirely adopt multilayer structure characteristic for TFLL *in vivo*.[38]

Typical Δπ/t relaxation transients for films by nMGS and dMGS at 34°C are shown on Fig. 2. It can be seen that compared to nMGS the π of dMGS films relaxed faster and to a lower surface pressure value. Such behavior indicates more dissipative, viscous properties of dMGS in regard to nMGS. This is clearly notable after the Fourier transform of the relaxation curves (left panels of Fig. 3), when it can be seen that in the range of 10^{-4}-10^{-3} Hz dMGS turned viscous, while for nMGS the contribution of elasticity prevailed for the entire frequency scale.

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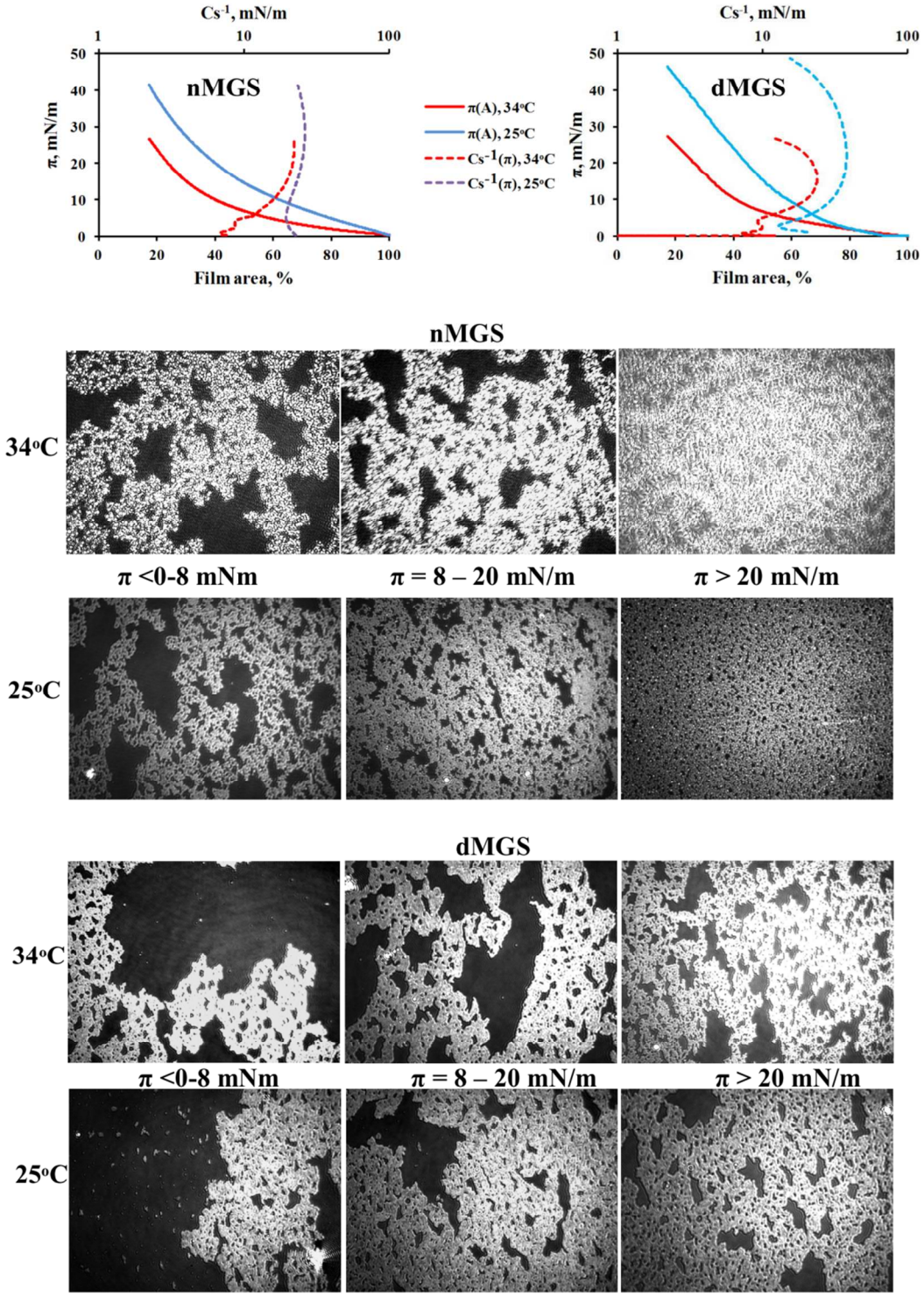

Fig. 1. Upper row: Dependencies of (i) surface pressure, π, on film area, A, and of (ii) film compressional modulus, Cs^{-1}, on π for nMGS and dMGS at 25°C and 34°C. Note that the decrease of temperature resulted in shift of the isotherms to lower surface pressures and to higher surface areas for both, nMGS and dMGS. At π ≥ 10 mN/m the compression isotherms at 25°C and 34°C remained almost parallel to each other with π/A curves displaces to 1.5-1.7 times lower film areas. **BAM micrographs**: Representative images (720 x 400 μm) of nMGS and dMGS layers at the denoted temperatures and surface pressure ranges. The intensity of the images taken at > 8mN/m is attenuated for better visual perception.

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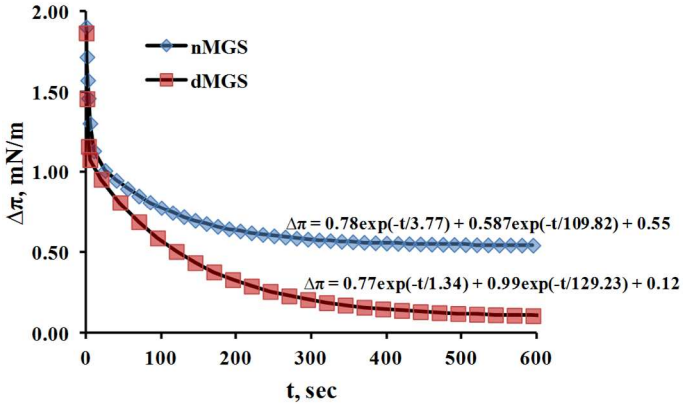

Fig.2. Typical surface pressure relaxation transients for nMGS and dMGS films at 34°C obtained after rapid small film compression. The surface pressure term Δπ represents the difference between the momentum surface pressure π(t) after the area change is stopped and π starts to relax and the initial film pressure π_0 = 25 mN/m prior the area contraction to be applied. The solid lines represent best fit curves ($R^2 > 0.98$) with the corresponding equations denoted on the plot. The dependence of the real and imaginary part of the complex dilatational modulus on frequency obtained via the Fourier transformation of the relaxation transients are shown on the right panels of Fig.3.

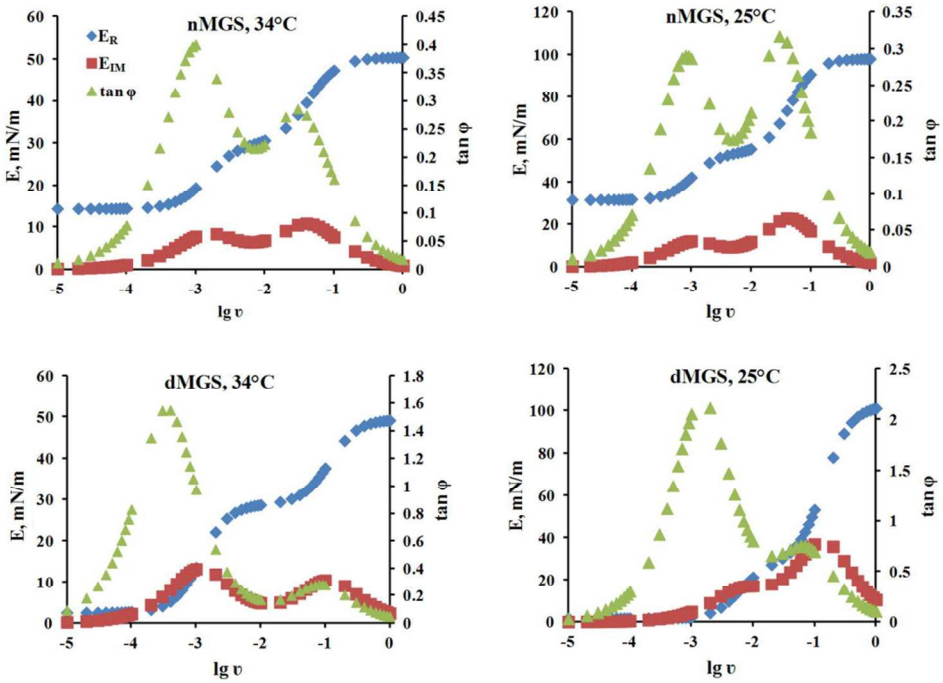

Fig.3. Typical dependences of the real and imaginary part of the complex dilatational modulus on frequency for nMGS and dMGS films at 25°C and 34°C obtained after Fourier transformation of surface pressure relaxation transients. The data for all studied samples, seven nMGS and seven dMGS specimens, are provided in ***Supplement II***. Note that for nMGS films for the entire frequency scale tan φ < 1, i.e. the samples are predominantly elastic, while for dMGS layers for the frequency range of 10^{-3}-10^{-4} Hz tan φ > 1, i.e. the samples became dominated by viscosity.

As can be seen by the typical curves at the right panels of Fig. 3 analogous trend was observed at 25°C (the data for the dilatational rheology of all samples at 25°C and 34°C are provided in ***Supplement II***).

For the range 10^{-2}-1 Hz, both, nMGS and dMGS, were predominantly elastic. For both types of lipid films at these high frequencies the elasticity at 25°C is higher than the one at 34°C which is in agreement with previous studies.[37] These results correlate with the data for the broad chain melting peak of MGS, from 17 to 35°C,[12] which suggest that the decrease of temperature results in increased content of gel fraction within the lipid film, and thus to formation of more rigid and elastic layers.

The dependence of the tangent of the phase angle, tan φ, on frequency (Fig. 3 and ***Supplement II***) revealed that at high, ≥ 10^{-2} Hz, frequencies for all samples tan φ < 1. However at frequencies lower than 3x10^{-3} Hz nMGS remained predominantly elastic (i.e.

tan φ < 1), while for dMGS the imaginary modulus raised and became comparable and higher than the real modulus (tan φ > 1).

The number of peaks observed in the so called Cole-Cole plot, i.e. the graph of E_R vs. E_{IM}, indicates the number of relaxation processes contributing to the relaxation of π.[39] Fig. 4 depicts the E_R vs. E_{IM} plot of the results from the FT spectra of the typical relaxations at 34°C at Fig. 2 and 3.

For each of the two films, nMGS and dMGS, two peaks emerged in the Cole-Cole plot, i.e. two major processes take place in the course of the relaxation. Similar pattern is observed at 34°C (the typical temperature of TFLL) for all studied samples, healthy and MGD (data not shown). As described in detail by Tschoegl[40] the theory of linear viscoelasticity suggests that in such case the Δπ/t relaxation transients can be described as a sum of two decaying exponents and a plateau:

$$\Delta\pi(t) = A_1 exp\left(-\frac{t}{\tau_1}\right) + A_2 \exp\left(-\frac{t}{\tau_2}\right) + \Delta\pi_\infty \qquad (4)$$

where Δπ(t) is the value of Δπ in any moment t; τ_1 and τ_2 are relaxation times for rapid and slow processes which take part in the total relaxation process; A_1 and A_2 are constants which reflect the contribution of the fast relaxation time and of the slow relaxation time to the total relaxation process respectively; $\Delta\pi_\infty$ is the equilibrium value of Δπ reached at the end of the relaxation.

After small instantaneous compression of the film is performed in order to establish a new equilibrium some molecular reorientation, adsorption/desorption, re-spreading, and structural rearrangement processes are necessary, which are not completed instantaneously. All processes on the scale of the short relaxation time, τ_1, can be described mainly by elasticity, while the slower processes, on the scale of the long relaxation time τ_2, by viscosity; the plateau value of $\Delta\pi_\infty$ is a measure of the equilibrium elasticity (i.e. the "pure" elasticity, characterizing the energy stored within the film).[15,16]

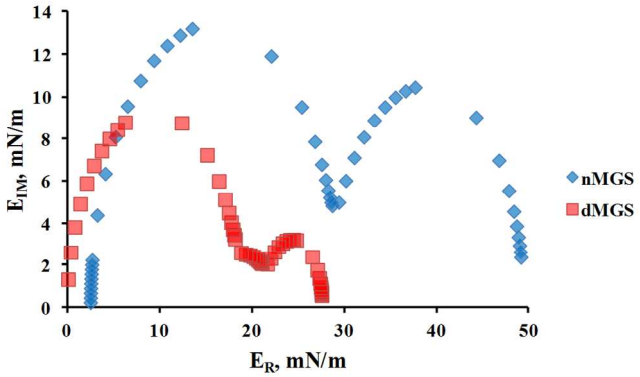

Fig. 4. Cole-Cole plot of the dependence of the imaginary part on the real part of the complex dilatational modulus for the typical nMGS and dMGS relaxation transients shown at Fig. 2. For each of the two films, nMGS and dMGS, two peaks emerged in the Cole-Cole plot, i.e. two major processes take place in the course of the relaxation.

Thus the ratio $\mathbf{[A_1 exp(-t/\ \tau_1) + \Delta\pi_\infty]/\Delta\pi(t)}$ provides a primary semi-quantitative measure of the contribution of elasticity to Δπ(t), while $\mathbf{A_2 exp(-t/\ \tau_2)/\Delta\pi(t)}$ – of the impact of the slow viscous-dominated process. The dependencies shown in Fig. 5 are obtained using the typical Δπ/t curves and the fitting equations in Fig.2.

It can be seen that for nMGS the contribution of $\mathbf{[A_1 exp(-t/\ \tau_1) + \Delta\pi_\infty]}$ to $\mathbf{\Delta\pi(t)}$ increased with time and prevailed over the contribution of $\mathbf{A_2 exp(-t/\ \tau_2)}$ for the entire time scale. In contrast for dMGS during the first 300 sec of the relaxation the impact of the predominantly dissipative, viscous process characterized by the term $\mathbf{A_2 exp(-t/\ \tau_2)}$ was prevalent for the value of $\mathbf{\Delta\pi(t)}$.

Discussion

Films by nMGS and dMGS at dynamic isocycling

The compression isotherms of both, nMGS and dMGS, exhibited significant similarities. At 34°C during compression both types of MGS increased π to 28-30 mN/m at maximal compression and the π/C_s^{-1} dependencies displayed inflexion points in the π range < 8 mN/m.

UltraBAM images show that even in the low surface pressure region, 0-8 mN/m, the meibomian lipids did not form monolayers, but heterogeneous surface films consisting of thin patches (most probably of monomolecular thickness) and of thick lipid aggregates. As discussed in previous studies, ours and others, such behavior is to be expected concerning the composition of meibum, comprised of less than 10% polar lipids of still disputable nature (OAHFA or phospholipids[9-11]) localized on the interface with water and more than 90% non-polar lipids (primarily wax and sterol esters), which distribute over the polar lipids and form thick aggregates.

At π > 8 mN/m the amount of thinner dark regions significantly diminished in the nMGS layers and thick rough film of multilayer thickness was formed as also reported by Kaercher et al.[35]

However the dMGS layers maintained a heterogeneous structure for the whole range of surface pressures. Such behavior correlates with the interferometric observations for the heterogeneous structure of TFLL in case of MGD at the ocular surface *in vivo*[41] and with the findings on modified composition of dMGS, in particular the decrease in the level of OAHFA, the major class of polar lipids, which might result in impaired spreading of the diseased meibum at the air/water interface.

The decrease of the temperature (from 34°C to 25°C) resulted in a shift of the isotherms to higher surface pressures both for nMGS and dMGS, with the effect increasing with the degree of film compression, i.e. the same value of π was achieved at lower surface area at 25°C than at 34°C. Also the film thickness increased as can be judged by the raised intensity of the BAM micrographs. Similar data are obtained *in vitro* by independent researchers[25,26,38] and are still unexplained. The clinical observations also reveal that after a goggle-type chamber is placed over the eyes of the examined subject and the temperature of the air in contact with the ocular surface is increased, the thicker TFLL is formed at the air/tear surface.[42] The result is just the opposite to the expected for lipid monolayers (for which cooling normally results in condensation when it is necessary to compress the film more in order to achieve certain π), but correlates well with the data on the structure of the meibomian layers.

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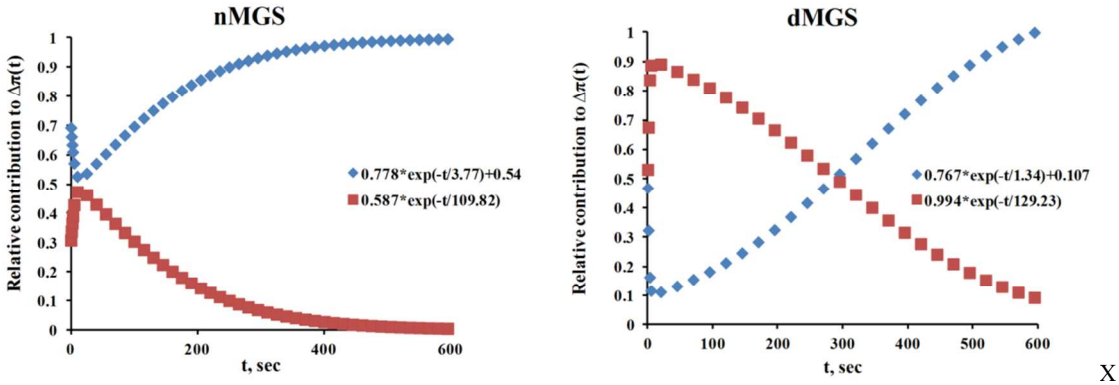

Fig. 5. Relative contribution of the different terms in Eq. (4) to the momentum value of Δπ(t) (see main text for details). For nMGS the contribution of $[A_1\exp(-t/\tau_1) + \Delta\pi_\infty]$ to Δπ(t) increased with time and prevailed over the contribution of $A_2\exp(-t/\tau_2)$ for the entire time scale. For dMGS during the first 300 sec of the relaxation the impact of the predominantly dissipative, viscous process characterized by the term $A_2\exp(-t/\tau_2)$ was prevalent for the value of Δπ(t).

Meibum forms thick films and at π ≥ 18 mN/m it is assumed[38] that the films are entirely composed of rough layers (with thickness corresponding to 3-8 stacked monomolecular layers) and no monolayer patches present. Thus particularly at higher surface pressures the polar lipids are not located at the air/water surface, but on the interface between the aqueous subphase and the thick oily cap on top. At 34°C most of the nonpolar lipid constituents are in melted state and the oily layer can be considered as lipophilic fluid with some crystallites dispersed within.[37] In such systems the oily strata can act as a solvent, as a reservoir, for the polar lipids,[32,33] which must adsorb from its vicinity to the aqueous interface and also can distribute between the interface and the oily cap in the course of film area changes. If we refer to the basic Gibbs equation for adsorption:

$$\Gamma = \frac{1}{RT}\frac{d\pi}{d\ln C_s} \qquad (5)$$

one will see that the amount of lipid adsorbed on the interface is inversely proportional to the temperature. Thus the decrease of temperature from 34 to 25°C must increase the surface concentration of polar lipids Γ roughly 1.4 times. This estimation agrees well with the shift of film areas (i.e. a measure of 1/Γ) to 1.5 - 1.7 times higher values at equivalent surface pressures (see Fig. 1) for both, nMGS and dMGS, at the π range > 10 mN/m (i.e. in the region where the multilayer thick structures become the major constituent of the meibomian film) with the π/A curves at 34°C and 25°C remaining almost parallel to each other until the end of the film compression. The suggestion for increased partitioning of polar and other lipid compounds in the oily cap at higher temperature, and for increased adsorption of polar lipids to the aqueous interface when the temperature is decreased is also supported by the elevated thickness of MGS films formed at 34°C observed with UltraBAM. The capability of the oily nebula to accommodate polar lipids within, might explain the high isotherm reversibility (the low π/A hysteresis) and the non-collapsibility of meibomian films at dynamic area cycling reported previously[25-29] as in such case polar lipids are prevented from tight compression at the interface and from squeeze out in the aqueous subphase. Likely *in vivo* consequences are that the meibum layer can maintain its performance at the air/tear surface for prolonged times and there is no need for the meibomian glands to constantly deliver new lipids at the interface. These assumptions agree well with the very slow TFLL turnover rate *in vivo* where full exchange of TFLL takes more than eight hours.[43] Although the interpretation of the changes in the π/A curves at 25°C and 34°C is in good agreement with the expected temperature-induced alterations in the solubility of polar lipids in the bulk oily cap and in their adsorption at the aqueous surface as predicted by the Gibbs equation, other factors such as specific interactions between the polar and nonpolar lipids (acyl chain matching, etc.), changes in the structure and consistence of the oily layer (from liquid-like nebula at 34°C to a more crystalline substance with decreased solvent properties at 25°C) etc, can and definitively also contribute to the observed effects.

A characteristic distinction between nMGS and dMGS layers is that the "diseased" meibum films displayed more heterogeneous structure containing thinner dark regions for the entire surface pressure range. Thus it can be expected that although nMGS and dMGS showed similar properties at dynamic compression certain differences can be expected if their rheological properties are examined at a broad frequency scale.

Dilatational rheology properties of nMGS and dMGS

FT analysis of the relaxation transients of nMGS and dMGS layers reveals characteristic similarities and distinctions in the rheology properties of the two specimens.

In the high frequency range (see Fig. 3 and ***Supplement II***) both types of meibum showed predominantly elastic properties

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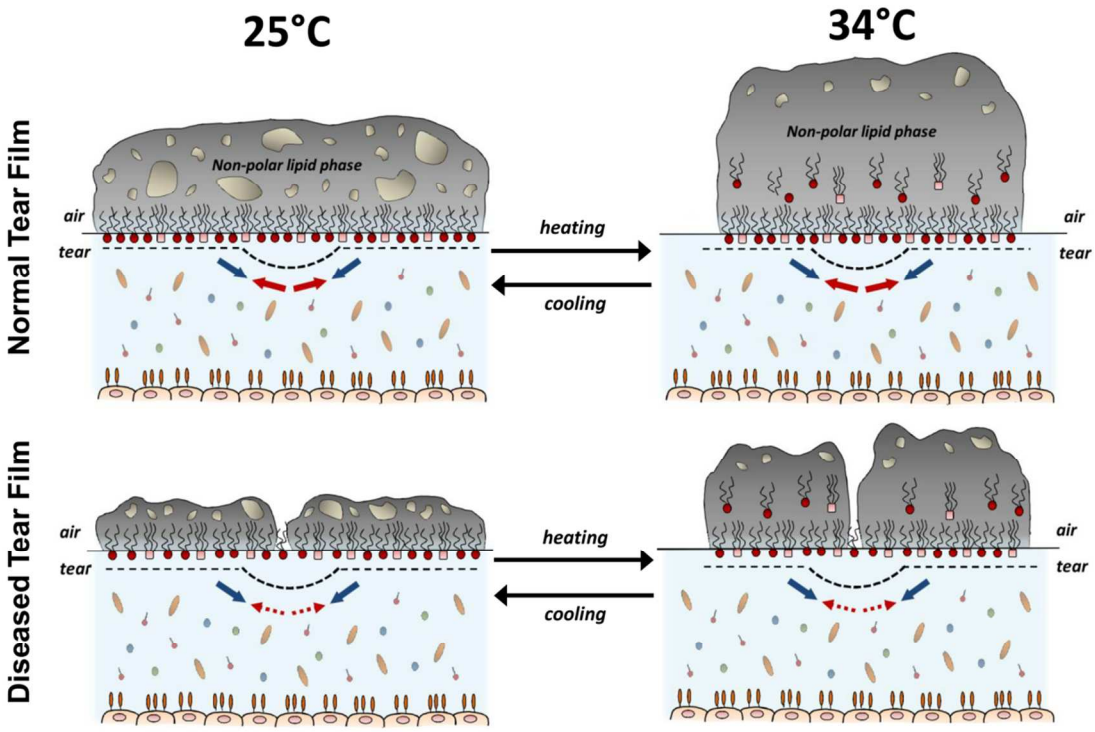

Fig. 6. Simplified scheme of nMGS and dMGS films at 25°C and 34°C at high (> 20 mN/m) surface pressure spread over subphase of aqueous tear (a composite saline solution of secretory mucin, proteins and some polar lipids) and underlying corneal epithelium cells. In order dewetting event to take place the air/tear interface must bend towards the cornea (presented as dashed line and with blue arrows) which dilates the air/tear surface. This process is opposed amongst others by the elasticity of the lipid film (presented as red arrows) which counteracts to the expansion of the air/tear interface. Thus the more elastic nMGS films can be expected to be more efficient in maintaining of TF stability than the more viscous dMGS layers. The effect of temperature on the structure of meibomian films is also presented. Meibum forms films of multilayer thickness with polar lipids located at the aqueous surface and an oily "cap" of non-polar lipids on top. At 34°C most of the nonpolar lipid constituents are melted and the oily layer can be considered as a lipophilic fluid with some crystallites dispersed within. In such systems the oily strata can act as a solvent, as a reservoir, for the polar lipids. Changes in temperature will alter the distribution of the polar lipids between the film/subphase interface and the oily cap with heating favoring increased solubility of polar lipids in the oily strata. Such hypothesis is in good agreement with the shift of film areas to 1.5 - 1.7 times higher values at equivalent surface pressures (see Fig. 1) for both, nMGS and dMGS, at the π range > 10 mN/m (i.e. in the region where the multilayer thick structures become the major constituent of the meibomian film) and with the temperature induced increase of BAM images intensity. Apart from the expected temperature-induced alterations in polar lipids distribution, other factors such as specific interactions between the polar and nonpolar lipids (acyl chain matching, etc.), changes in the structure and consistence of the oily layer (from liquid-like nebula at 34°C to a more crystalline substance with decreased solvent properties at 25°C), effects of aqueous tear compounds etc, also contribute to the observed effects. The figure is not drawn to scale.

(tan φ < 1). However in the low frequency range, 10^{-4}–10^{-3} Hz, nMGS remained mostly elastic while dMGS turned viscous (tan φ > 1).

These differences in the viscoelastic properties of dMGS compared to nMGS correspond well to the data for the compositional variations between the "normal" and "diseased" meibum. dMGS has more sterol esters and increased order of the acyl chains which results in higher melting temperature and increased bulk viscosity.[10] Also in dMGS the content of OAHFA, currently considered as the most likely polar lipid compound in meibum, is decreased.[11] It is assumed that the polar lipids act as a spreading agent for meibum; they form a monomolecular film at the water surface, on top of which lay over the bulk part of the meibomian film, i.e. hydrophobic lipids with very limited surfactant potency (mainly wax and sterol esters, and triacyl glycerols). Therefore the insufficiency of polar lipids might impair the distribution of meibum constituents at the interface and to result in formation of more heterogeneous, patchy and discontinuous layers. Such interpretation is consistent with the UltraBAM observations. The major points presented up to here in the Discussion are briefly depicted and summarized in Fig. 6.

Starting with Mishima and Maurice[44] many classic references accept that the role of TFLL *in vivo* is to suppress the evaporation of the underlying aqueous tear. However recent studies suggest that MGS and TFLL delay the aqueous evaporation merely with up to 10 %.[45] This means that the role of the tear lipids needs to be revisited and that most probably the compositional distinctions between nMGS and dMGS are not of crucial importance for the water holding property of the tear film. Instead these differences in the constituents building the "normal" and the "diseased"

meibum lead to significant divergence in the rheology properties of the materials, with nMGS remaining elastic for the whole frequency range of 1-10^{-5} Hz, while dMGS showed dissipative characteristics at 10^{-4}-10^{-3} Hz, and a $\Delta\pi/t$ transient dominated by the slow (on the scale of τ_2 = 129 sec) viscous-like process for the first 300 sec of the relaxation. It is well known that the viscoelastic properties of the surface layers are of crucial importance for the stability of wetting films over solid support.[46] The predominantly elastic surface layers are capable to resist the inward bending (directed to the solid support) of the air/film interface necessary for dewetting to take place while predominantly viscous layers are not. Thus although dMGS spreads rapidly over the air/tear surface after the upward movement of the eyelid at blink, it can be expected that in the subsequent interblink interval the lipid layer will not be able to maintain sufficient level of surface elasticity and to stabilize the tear film. The results correlate with the reported findings by Goto and Tseng[41] *in vivo*. In this work interferometry observations of the spreading behavior of TFLL at the air/tear surface revealed that immediately after eye opening dMGS spread at the aqueous interface with a rate similar to the one of nMGS; however after the initial spreading event was completed dMGS needed a 10 times longer period (3.54 vs. 0.35 sec) until stable film pattern was established and the TF lifetime decreased to less than 7 sec (being > 15 sec in normal eyes). Such prolonged period for the establishment of stable surface morphology is to be expected for viscous (i.e. with strong dissipative processes within) films. In the case of the meibum donors, healthy volunteers and MGD patients, involved in the current study it was not possible to reliably measure TFLL upward spreading rate *in vivo* due to the irregular movement of the TFLL in the eyes of the patients. Instead we performed image cross-correlation analysis between the stationary interblink patterns of the lipid layer of healthy and diseased people as recently described.[7] It can be assumed that elastic lipid films will maintain relatively stable surface pattern between consecutive blinks, while for viscous-like lipids there will be quick degradation of the TFLL morphology due to energy dissipation. This hypothesis was confirmed by the clinical data showing high stability of TFLL appearance and durable (≥ 15 sec) TF in normals and prompt decomposition of the lipid layer morphology in MGD patients (see ***Supplement I, point III***) accompanied with a rapid breakup of the TF (within 5 sec after eye opening). The good agreement between the *in vitro* results showing significant difference between the rheology properties of nMGS and dMGS, and the TF properties and stability *in vivo,* support the assumption for the importance of the meibum viscoelasticity for the normal function of the tear film.

Conclusion

dMGS displayed characteristic similarities and distinctions of the viscoelastic properties compared with nMGS. Both types of lipid specimens were predominantly elastic at high frequencies (> 2.5 x 10^{-2} Hz), but in the region of 10^{-4}-10^{-3} Hz dMGS became predominantly viscous while nMGS maintained primarily elastic properties. These differences of the viscoelastic properties of dMGS compared to nMGS might decrease dMGS capabilities to oppose invagination of the air/tear interface towards the cornea, a step considered critical for a dewetting event,[46] and to maintain the integrity of the TF in the interblink time. In view of the recent findings[45] that contrary to the older views[44] meibomian lipids have only modest capability to suppress the evaporation of the aqueous tear the results of the current work suggest the need to rethink the role of the tear film lipid layer. It might turn out that the major function of the tear lipids is to maintain elastic layer capable to oppose stretching of the air/water interface (involved in film thinning and in dewetting event) and in this case the alterations in the viscoelastic properties of meibomian lipids might be of key importance for the stability of the tear film. The results might provide a timely hindsight on the role of the meibomian lipids for the structure and function of the human tear film and open the possibility for smart knowledge based chemical design of new lipid materials for ophthalmic applications.

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† Electronic Supplementary Information (ESI) available at http://pubs.rsc.org/en/Journals/JournalIssues/SM#!recentarticles&all

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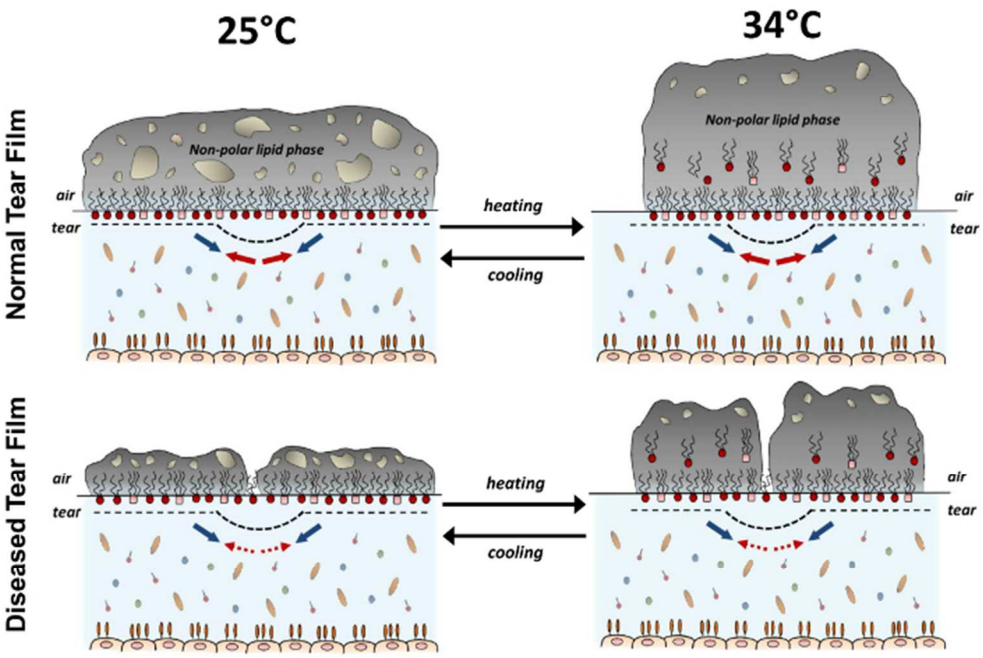

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