Biochimica et Biophysica Acta 1838 (2014) 2716–2727

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Biophysical investigations of the structure and function of the tear fluid lipid layers and the effect of ectoine. Part B: Artificial lipid films

Mridula Dwivedi [a,b], Marc Brinkkötter [b,1], Rakesh Kumar Harishchandra [b,1,2], Hans-Joachim Galla [b,*]

[a] NRW International Graduate School of Chemistry and Institute of Biochemistry, Germany
[b] Institute of Biochemistry, Westfälische Wilhelms Universität, Wilhelm-Klemm-Str.2, 48149 Münster, Germany

ARTICLE INFO

Article history:
Received 21 January 2014
Received in revised form 2 May 2014
Accepted 5 May 2014
Available online 20 May 2014

Keywords:
Tear fluid lipid layer
Langmuir film balance
Epi-fluorescence microscopy
Atomic force microscopy
Ectoine
Dry eye syndrome

ABSTRACT

The tear fluid lipid layer is present at the outermost part of the tear film which lines the ocular surface and functions to maintain the corneal surface moist by retarding evaporation. Instability in the structure of the tear fluid lipid layer can cause an increased rate of evaporation and thus dry eye syndrome. Ectoine has been previously shown to fluidize lipid monolayers and alter the phase behavior. In the current study we have investigated the effect of ectoine on the artificial tear fluid lipid layer composed of binary and ternary lipid mixtures of dipalmitoyl phosphatidylcholine (DPPC), cholesteryl esters and tri-acyl-glycerols. The focus of our study was mainly the structural and the biophysical aspects of the artificial tear fluid lipid layer using surface activity studies and topology analysis. The presence of ectoine consistently causes an expansion of the pressure–area isotherm indicating increased intermolecular spacing. The topology studies showed the formation of droplet-like structures due to the addition of ectoine only when tri-acyl-glycerol is present in the mixture of DPPC and chol-palmitate, similar to the natural meibomian lipids. Consequently, the hypothesis of an exclusion of tri/di-acyl-glycerol from the meibomian lipid film in the presence of ectoine in the subphase is confirmed. A model describing the effect of ectoine on meibomian lipid films is further presented which may have an application for the use of ectoines in eye drops as a treatment for the dry eye syndrome.

1. Introduction

Tear fluid lipid layer is the outermost layer of the tear film that forms the outer lining of the ocular surface [1]. It reduces the rate of evaporation of the tear fluid thus preventing drying of the corneal epithelium [2]. Further, it is also involved in maintaining a clear optical surface [3] and acting as a protective barrier against the microbes and organic matter such as dust and pollen. As suggested by Holly [1] in 1973, the tear film is depicted as a two-layered structure: polar lipids forming the lower sublayer and the nonpolar lipids forming the upper sublayer that is in contact with the air. This proposition was further elaborated by Shine and McCulley [4]. Each sublayer is assigned a specific role in maintaining the structural integrity of the tear fluid lipid layer. The lower layer comprising the polar lipids acts as a surfactant and the spreading of the tear fluid lipid layer becomes thermodynamically favorable. The composition of the lower lipid layer was proposed to include polar lipids like the phosphatidylcholines, phosphatidylethanolamine, sphingomyelin (SM), ceramides and cerebrosides [5]. Recently, another group of amphiphiles called (O-acyl)-ω-hydroxy fatty acids has been found to play a major role in the polar lipid sublayer [6]. The upper layer consists of the hydrophobic lipids which form a coating and seal the underlying aqueous portion of the tear fluid. This layer hence plays a major role in preventing the evaporation of the tear fluid as lipid films have low water vapor transmissivity, depending on the thickness and the compositions. The tear fluid lipid layer is believed to consist majorly of lipids secreted by the meibomian gland. The most commonly found classes of lipids in the meibomian glands have been reported to be ubiquitous WEs and cholesteryl esters (CEs) [6–8]. However, the composition of the whole tear lipids has been found to be different than the secretions of the meibomian gland. Primarily the differences involve the higher molar ratio of the low molecular weight wax esters (WE) — type species in the human meibum [7–10]. Further, it has been reported that the meibomian lipids have a lower acyl chain ordering thus leading to a lower melting temperature than the whole tear lipids [11]. However, the whole tear samples showed the spectroscopic signal of organic phosphate ester groups as found in phosphatidylcholines and sphingomyelin [12,13]. Wollensak et al. characterized the whole tear lipids and showed the WEs and the CEs accounting for 45% of the total lipid weight. The free fatty acids (FFAs) and the tri-acyl-

* Corresponding author at: Institute of Biochemistry, Westfälische Wilhelms Universität, Wilhelm-Klemm-Str. 2, 48149 Münster, Germany. Tel.: +49 251 8333200; fax: +49 251 8333206.
E-mail address: gallah@uni-muenster.de (H.-J. Galla).
[1] These authors contributed equally.
[2] Current address: Dept. of Chemistry & Biochemistry Worcester Polytechnic Institute (WPI) 60 Prescott Street, Worcester, MA 01605 USA.

http://dx.doi.org/10.1016/j.bbamem.2014.05.007
0005-2736/

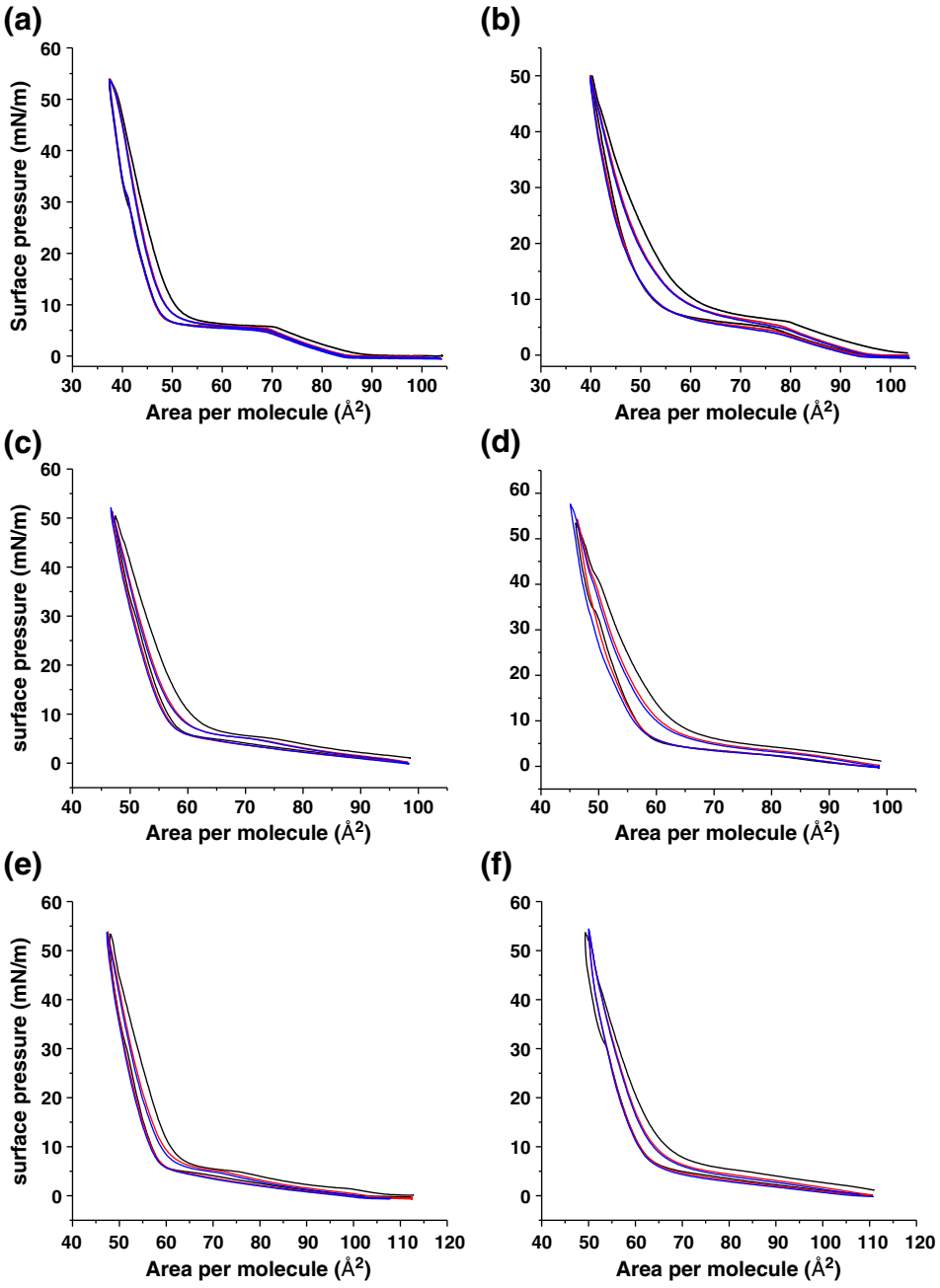

Fig. 1. Cyclic compression–expansion isotherms for (a) DPPC/CP (9:1) on PBS subphase, (b) DPPC/CP(9:1) on 100 mM Ectoine (c) DPPC/DPOG (9:1) on PBS subphase (d) DPPC/DPOG(9:1) on 100 mM Ectoine (e) DPPC/CP/DPOG (8:1:1) on PBS subphase (f) DPPC/CP/DPOG (8:1:1) on 100 mM Ectoine. *Black curve-first cycle*; *red curve-second cycle*; *blue curve-third cycle*. All measurements were performed at 20 °C.

glycerides (TAG) account for less than 15% each and the polar lipids were reported to comprise 15% of the total weight [14]. Further, the presence of polar lipids in the human tears has been shown by Fourier transform infrared spectroscopy [13]. The composition of the tear fluid lipid layer has been established to play an essential role in maintaining the structural integrity of the lipid layer and any alteration can lead to several ocular disorders.

Dry eye syndrome or DES is the most commonly occurring ocular disorder which is accompanied by drying of the ocular surface and the inflammation of the corneal epithelium. It mainly causes premature rupture of the tear film thus leading to increased rate of evaporation of the tear fluid. An altered composition of the tear fluid lipid layer can lead to a structurally unstable film thus leading to a dry eye syndrome. McCulley et al. demonstrated that various forms of dry eye can be caused by meibomian gland dysfunction (MGD) [15] whereby a decreased lipid production or production of lipids with higher melt temperature can cause inefficient formation of the lipid layer and increased rate of evaporation. The alteration in the lipid composition in dry eye patients and healthy volunteers has been determined using various techniques. Recently, nuclear magnetic resonance studies have shown the compositional difference between the MGD patients and the normal individuals [16]. It was shown that the meibum obtained from MGD patients had higher lipid order than the normal individual meibum. A higher lipid order and phase transition temperature has also been evidenced from IR studies with the samples of the meibum from donors with meibomian gland dysfunction [17,18]. Additionally,

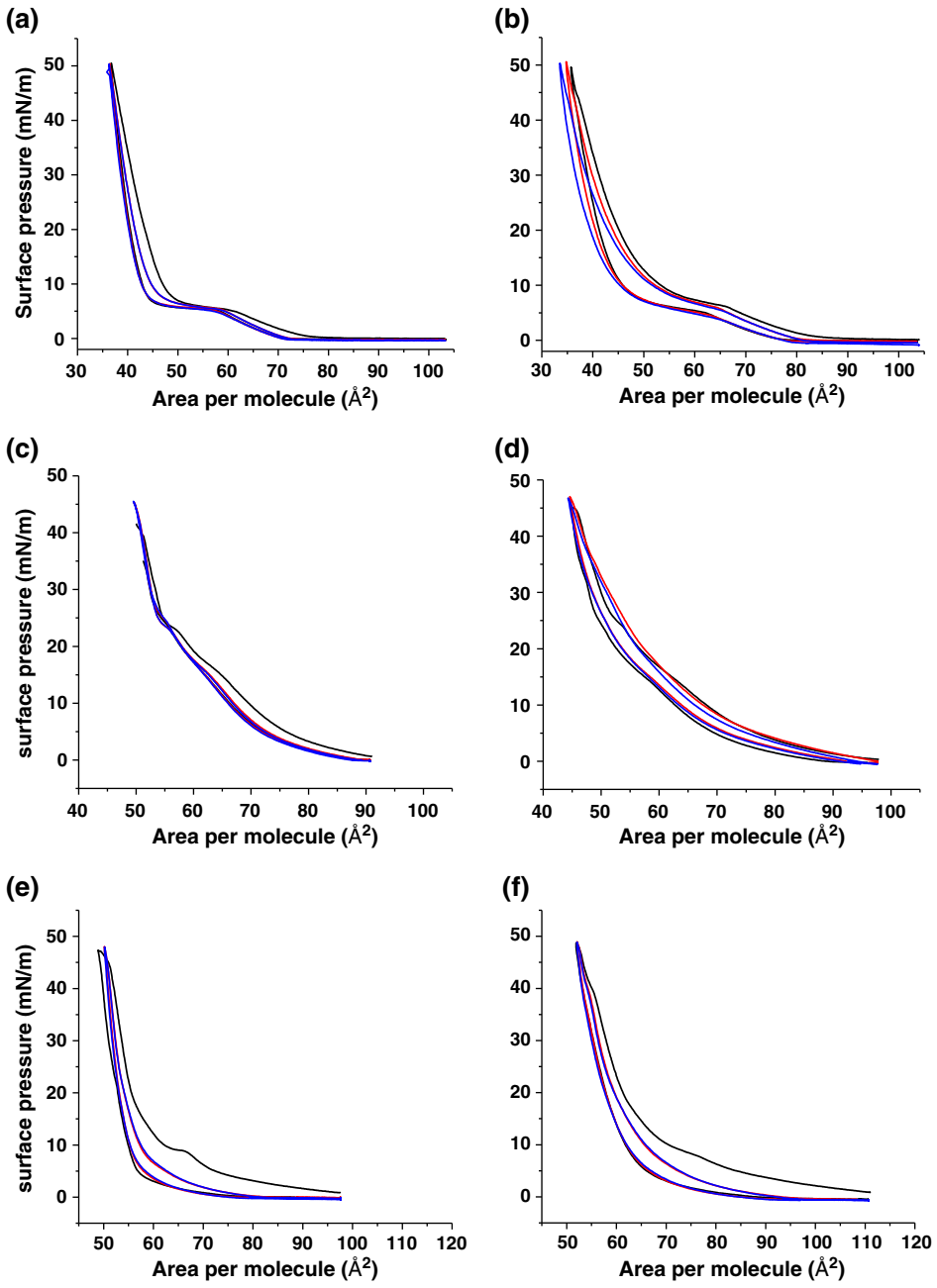

Fig. 2. Cyclic compression–expansion isotherms for (a) DPPC/CP (7:3) on PBS subphase, (b) DPPC/CP (7:3) on 100 mM ectoine (c) DPPC/DPOG(7:3) on PBS subphase (d) DPPC/DPOG (7:3) on 100 mM ectoine (e) DPPC/CP/DPOG (4:3:3) on PBS subphase (f) DPPC/CP/DPOG (4:3:3) on 100 mM ectoine. *Black curve-first cycle*; *red curve-second cycle*; *blue curve-third cycle*. All measurements were performed at 20 °C.

dry eye patients have been shown to have a decreased polar lipid to neutral lipid ratio [19]. The increased ordering between the acyl chains leads to an increased rigidity of the tear fluid lipid layer. Further, decreased polar to neutral lipid ratio might also lead to decreased surfactant efficiency for maintaining the structural integrity and an increased rigidity. Hence, it is reasonable to assume that the tear film instability can be caused due to a rigidified lipid layer with the presence of increased saturation levels of the lipids and decreased polar to neutral lipid ratios. Therefore the solutes which are capable of inducing fluidization of the lipid monolayers may prove to be a potential treatment for the DES. Ectoine has been previously shown to fluidize lipid monolayers in vitro and hence is one such candidate.

Ectoines are low molecular weight organic solutes which are synthesized and secreted by the aerobic chemoheterotrophic and halophilic/halotolerant bacteria against extreme stress conditions like high salinity, high temperature and extreme dryness [20]. These solutes do not interfere with the regular cellular metabolism even at high concentrations and hence the name 'Compatible solutes' [21]. Ectoine has been previously shown to increase the spacing between the lipid headgroups [22]. The mechanism by which ectoine is able to cause this is still elusive however there are some simulation studies attempting to understand this [23]. In this study we have studied the effect of ectoine on the biophysical and structural characteristics of the artificial tear fluid lipid layer composed of binary and ternary mixtures of dipalmitoyl phosphatidylcholine (DPPC), cholesterol ester and tri-acyl-glycerol. Although the major lipid phospholipids present in the tear fluid lipid layer are unsaturated, we have chosen DPPC due to its well-studied phase behavior and cholesteryl palmitate and dipalmitoyl oleoyl glyceride due to their comparable chain length to DPPC. The artificial system has been aimed to be very simple to study the lipid–lipid interactions and does not

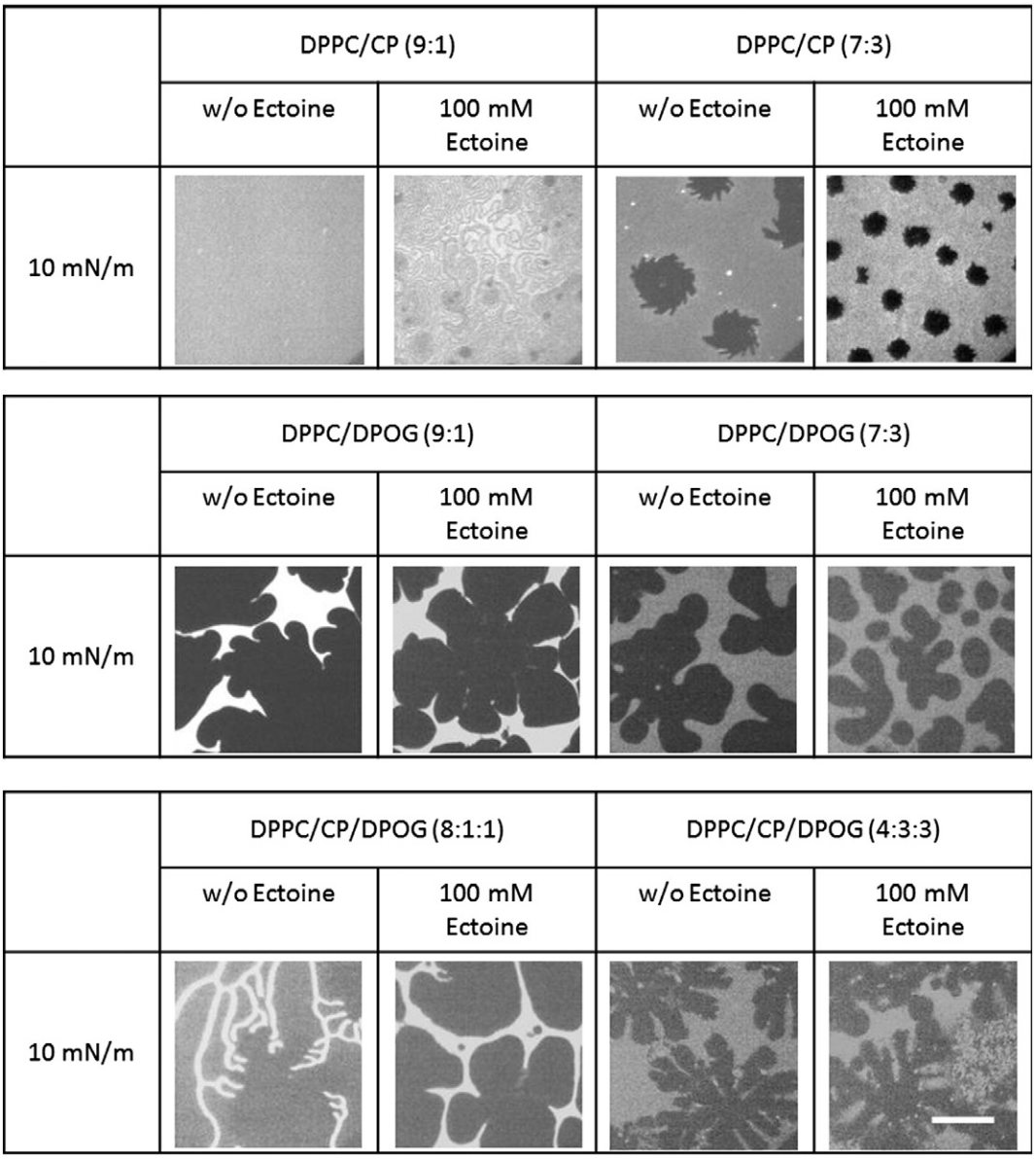

Fig. 3. Epi-fluorescence images for artificial tear fluid lipid layer composed of DPPC, CP and DPOG. The lipid mixtures were doped with 0.5 mol% BODIPY-PC which preferentially partitions into the liquid expanded (LE) phase. The images were taken by compressing and stopping the barrier at the desired surface pressure. All measurements were done at 20 °C. Scale bar is 50 μm.

take into account the part played by proteins in the tear fluid lipid layer. The surface activity studies, the domain morphology and the topographical studies have been used to understand the influence of ectoine and a hypothesis is proposed to explain the mechanism of action of ectoine.

2. Materials and methods

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from the Avanti Polar Lipids Inc. (Alabaster, AL). Cholesteryl-3-palmitate (CP) and 1,3-dipalmitoyl-2-oleoylglycerol (DPOG) was purchased from Sigma-Aldrich (Steinheim, Germany). 2-(4, 4-difluoro-5-methyl-4-bora-3a, 4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoylsn-glycero-3-phosphocholine (β-BODIPY®500/510C_{12}-HPC, BODIPY-PC) was obtained from Molecular Probes (Eugene, OR). Chloroform and methanol were high pressure liquid chromatography grade and purchased from Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany), respectively. Ectoine ((S)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid) was provided by Bitop AG (Witten, Germany). Water was purified and deionized by a multi-cartridge system (Sartorius, Goettingen, Germany) and had a resistivity of >18 M Ω m.

2.1. Surface pressure–area isotherms

All the film balance experiments were performed on an analytical Wilhelmy film balance (Riegler and Kirstein, Mainz, Germany) with an operational area of 144 cm^2. All surface pressure measurements were done on PBS at pH 7.4 as buffered subphase with and without ectoine at 20 °C. The lipid mixture was consisted of DPPC, CP and dipalmitoyl oleoyl glycerol at various compositions. These lipid mixtures dissolved in chloroform/methanol solution (1:1, v/v) were spread onto the subphase. After an equilibration time of 10–15 min the monolayers were compressed at a rate of 2.9 cm^2/min.

2.2. Video enhanced epi-fluorescence microscopy

Domain structures of lipid mixtures were doped with 0.5 mol% BODIPY-PC and visualized by means of an epi-fluorescence microscope

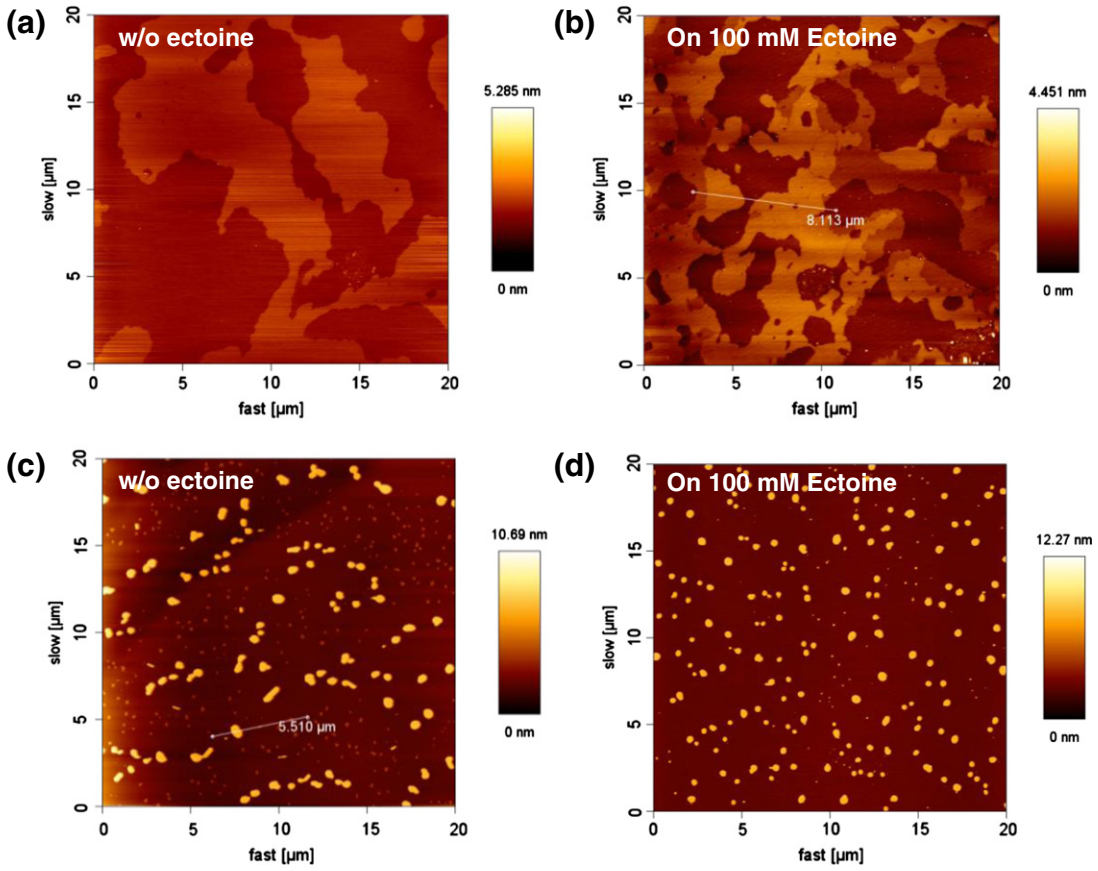

Fig. 4. AFM topography scans for DPPC/CP (9:1) at surface pressures (a), (b) 6 mN/m and (c), (d) 45 mN/m transferred from PBS buffer with and without ectoine. All measurements were done at 20 °C.

(Olympus STM5-MJS, Olympus, Hamburg, Germany) equipped with xy-stage and connected to a CCD camera (Hamamatsu, Herrsching, Germany). The images were captured by stopping the barrier at desired surface pressures.

2.3. Atomic force microscopy

Atomic force microscopy (AFM) images of the Langmuir–Blodgett (LB) films were transferred onto mica sheets [24] under ambient conditions (20 °C) and scanned using NanoWizard III (JPK Instruments, Berlin, Germany). Silicon nitride tips (Budget Sensors, Sofia, Bulgaria) with a spring constant of 40 N/m and a resonance frequency of 300 ± 100 kHz were used. In this study the intermittent-contact mode was used. The images were obtained at 512 × 512 pixel resolution and analyzed with JPK data processing software. The rigidity scans were obtained using Quantitative mode imaging using the same cantilever used for topography scanning.

3. Results

In this study, we used different combinations of the phospholipid (DPPC), the cholesterol ester cholesterol-palmitate (CP) and the triacyl-glycerol dipalmitoyl-oleoyl-glycerol (DPOG) to investigate the effect of ectoine on artificial tear fluid lipid layer and its individual components. We have used Langmuir film balance to study the surface activity of various lipid mixtures in the presence and absence of ectoine. The topographical studies are performed using atomic force microscopy and the height structures are analyzed to establish a model for the natural tear fluid lipid layer. Finally, comparing the data sets from natural and artificial meibomian lipid layer we present a model for the observed effects of ectoine on the lipid film.

3.1. Surface activity studies

As seen from Fig. 1 and 2, the isotherms of DPPC/CP (9:1), DPPC/CP (7:3) and DPPC/DPOG (9:1) are quite similar to that of pure DPPC [25] with the phase transition coexistence region at a surface pressure of 4–7 mN/m. In the presence of 100 mM ectoine the isotherms are expanded toward higher area per molecule values and the phase coexistence region is decreased. This implies that the area occupied by the lipid headgroups increases. The increased slope of the liquid condensed region implies weakened interactions between the interfacial lipid molecules. The continuous compression–expansion cycles do not show significant effects of ectoine on the hysteresis. The isotherm of DPPC/DPOG (7:3) is drastically different from that of pure DPPC implying immense structural reorganization due to the addition of the given concentration of non-polar lipid like tri-acyl-glycerol (Fig. 2). The presence of ectoine in the subphase causes an expansion of the isotherm toward higher area per molecule values as previously observed in other lipid mixtures (Fig. 2 b, d, and f). The continuous compression–expansion cycle shows the change in hysteresis of the lipid film in the presence of ectoine.

The isotherms of DPPC/CP/DPOG (8:1:1) are also similar to those of pure DPPC with a phase transition region at around 5 mN/m (Fig. 1). Similar to the previous isotherms, the effect of ectoine can be seen in the expansion of the isotherm toward higher area per molecule values and increased slope of the liquid compressed phase. The compression re-expansion cycles do not show significant changes in the hysteresis

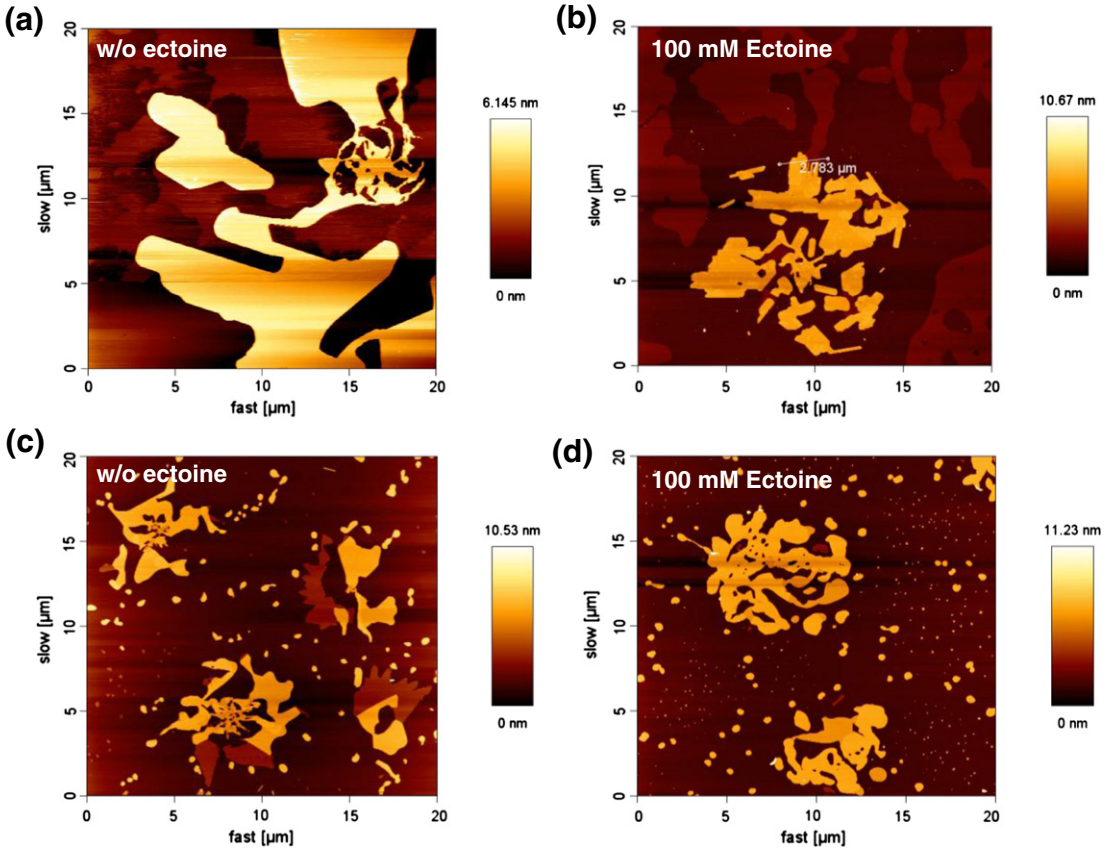

Fig. 5. AFM topography scans for DPPC/CP (7:3) at surface pressures (a), (b) 6 mN/m and (c), (d) 45 mN/m transferred from PBS subphase with and without ectoine. All measurements were done at 20 °C.

behavior due to the presence of ectoine. Similarly, the isotherms of DPPC/CP/DPOG (4:3:3) lipid mixtures are expanded toward higher area per molecule in the presence of 100 mM Ectoine (Fig. 2).

In the presence of ectoine the expansion of the isotherms toward higher area per molecule values is observed consistently in various lipid mixtures. This demonstrates that in all cases investigated the area occupied by the lipid head groups increases in the presence of ectoine. Similar effect of ectoine was observed with natural meibomian lipids.

3.2. Epi-fluorescence microscopy studies

The effect of ectoine on the properties of a lipid monolayer like phase separation and domain morphology was studied using epi-fluorescence microscopy. A lipid monolayer forms phase separated domains upon compression depending on the surface pressure. The domain morphology is studied using epi-fluorescence microscopy by doping the lipid monolayer with BODIPY-PC dye which is known to preferentially partition into the liquid expanded (LE) phase. Hence the liquid condensed (LC) phase appears as dark domains.

The fluorescence images for different lipid mixtures in the absence and presence of ectoine are shown in Fig. 3. For the convenience of the readers, only the data at 10 mN/m surface pressure are presented here. For a DPPC/CP (7:3) mixture, the darker domains (or liquid condensed phase) mainly appear as very fine fibrils which are visible under close inspection. With the addition of ectoine, the size of the fibrils increases and they become less dense. For DPPC/CP (7:3) mixtures, the fine fibrils seem to be emanating out of the darker domains which can be identified as the center of nucleation. In the presence of ectoine, the darker domains are smaller in size and the size of the fibrils increases. Since the darker domains appear at higher concentration of CP, we expect that these domains are mainly enriched in CP. A similar effect of ectoine is observed for DPPC/DPOG mixtures. For DPPC/DPOG (9:1) mixtures, we observe large domains of liquid condensed phase which are reduced in size in the presence of ectoine. Also for DPPC/DPOG (7:3) mixtures, the liquid condensed domains appearing at 10 mN/m surface pressure are reduced in size in the presence of 100 mM Ectoine. For the ternary mixtures, i.e. DPPC/CP/DPOG (8:1:1) and DPPC/CP/DPOG (4:3:3), the size of the liquid condensed domains decreases and consequently the density of the domains increases with the addition of ectoine, which is consistent with the previously observed lipid mixtures.

The equilibrium shapes and sizes of the isotropic co-existing liquid phases in a monolayer have been rationalized using mainly two competing factors-*i*) the line tension present between the adjacent domains (λ) and *ii*) the difference in the dipole densities in these phases (μ) [26–28]. Line tension arises due to the hydrophobic mismatch between the coexisting phases hence it favors the formation of large circular domains. The dipole density difference or the long range dipolar forces favor the formation of small non-circular domains. These forces compete with each other to assign the shape and size of the domains based on the ratio λ/μ^2 which is proportional to R_{eq}, the equilibrium radius. If the actual radius of the domains is smaller than R_{eq} the domains formed are circular and in the case where it is greater than R_{eq} the domains formed are non-circular and extended. With the increasing surface pressure this ratio decreases thus forming large non-circular domains. As we observed for various lipid mixtures, the domain size decreases with the addition of ectoine. Also, the domains appear more circular which implies increased values of R_{eq}, which could be due to either decreased μ or increased λ. The formation of small domains hints toward the decreased value of μ, the dipole density difference. The dipole density difference is dependent upon many factors as mentioned

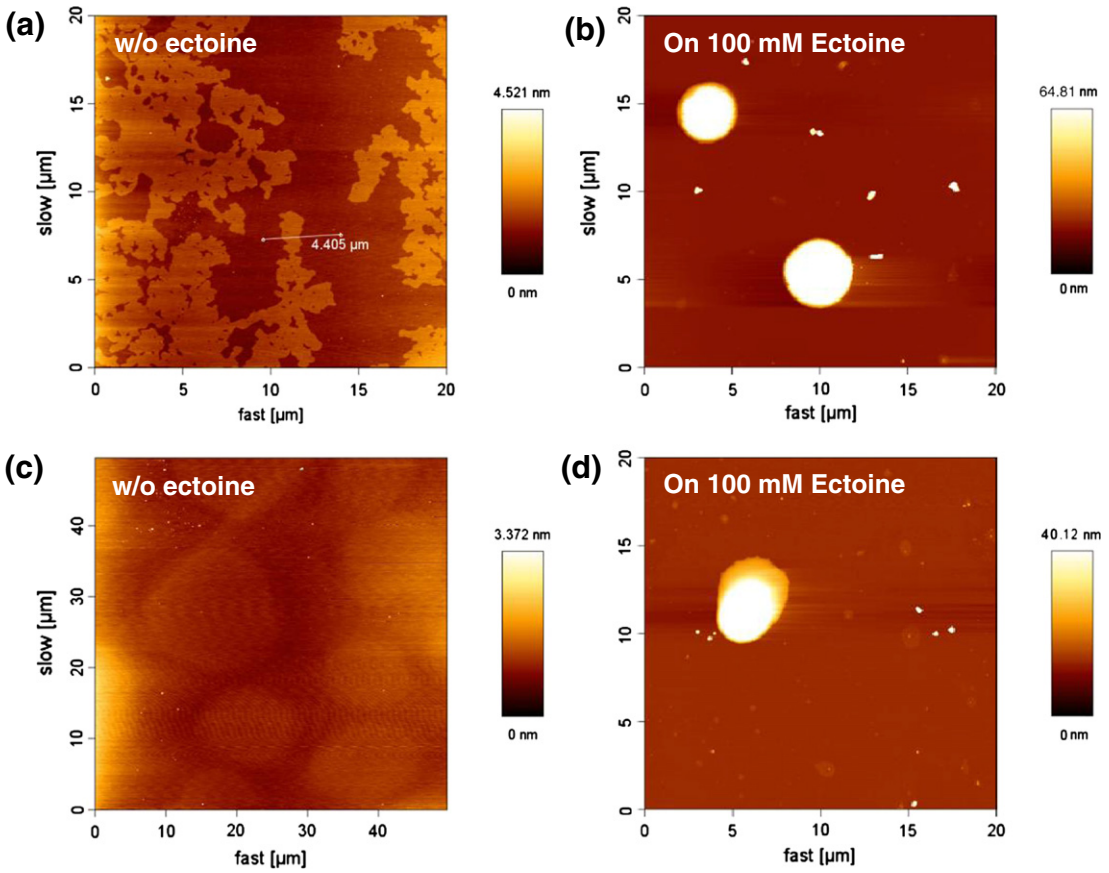

Fig. 6. AFM topography scans for DPPC/DPOG (9:1) at surface pressures (a), (b) 6 mN/m and (c), (d) 45 mN/m transferred from PBS buffer with and without ectoine. All measurements were done at 20 °C.

earlier. One of the factors is the lipid packing density which is expected to decrease with the addition of ectoine. We assume that this decrease in the lipid packing density is responsible for the decrease in the dipole density difference which consequently leads to the formation of smaller domains. It is also possible that the presence of ectoine affects both the line tension and the dipole density differences; however the effect on the dipole density difference is more prominent.

3.3. Topographical studies

The structural organization of the different lipid molecules in the lipid mixtures with and without ectoine was analyzed using atomic force microscopy at different compressed states. The AFM scans for DPPC/CP (9:1) on a PBS subphase at low pressure show the presence of two phases with 0.36 ± 0.16 nm height difference (Fig. 4a). In the presence of ectoine, the domain size decreases and three different phases appear with 0.73 ± 0.23 nm height differences (Fig. 4b). At high pressure, we observe arranged dotted structures which are 4.96 ± 0.33 nm in height (Fig. 4c). In the presence of ectoine, the dotted structures (with height of 4.85 ± 0.5 nm) are smaller in size and more dispersed, furthermore implying decreasing dipole density differences between various phases (Fig. 4d). The simulation studies for PC/CE (9:1) [29] as already mentioned in part A of this study (Dwivedi et al. 2014 in press) indicate that at low pressure the cholesterol ester molecules are located between PC molecules and the ester bond is directed toward the water phase. At higher pressure, the ester bond of CE is flipped upwards and the CE molecules are excluded from the aqueous–polar lipids interface to organize into the air–polar lipid interface. This is consistent with our observation in the AFM studies where at low pressure, phase separated domains of only 0.36 ± 0.16 nm in height are observed. However, at higher pressure, flat 4.96 ± 0.33 nm high structures are observed which could be cholesterol esters excluded from the PC monolayer to form interdigitated stacks. The interdigitated cholesterol esters would induce another phase in the interfacial PC monolayer. This phase is also affected by the presence of ectoine. The ratio of line tension and dipole density difference between the phases are altered which could affect the domain size.

For DPPC/CP (7:3), the AFM scans at low pressure show the presence of flat 4.47 ± 1.03 nm high structures which are larger in size than previously observed with 10 mol% cholesterol esters (Fig. 5a). This supports the previous assumption that the 4.96 ± 0.33 nm height structures observed consist mainly of cholesterol esters and probably consists of interdigitated multilayer stacks. Increased concentration of cholesterol esters perhaps causes their exclusion at low pressures unlike for 10 mol% cholesterol esters. At high pressure, the AFM scans are in accordance with the epi-fluorescence images wherein we observed liquid condensed domains along with fibrils emanating from the domains. The AFM scans show 4.92 ± 0.33 nm height domain structures surrounded by aligned dotted structures (Fig. 5c) which are thought to be distorted fibrils as seen in epi-fluorescence images. In the presence of ectoine the domains become more round and comparatively smaller (Fig. 5b, d). This is similar to the previous observations of the epi-fluorescence microscopy studies, where decreased domain size was justified by an increased λ/μ^2 ratio. This again demonstrates decreased packing order of the lipid molecules in the lipid film due to the presence of ectoine.

The AFM scans for DPPC/DPOG (9:1) mixtures show 0.56 ± 0.07 nm height phase domains at low pressure (Fig. 6a). It is rather difficult to predict the major content of the domains since the area per molecule value is high and the aggregation of the DPOG molecules might not be expected yet. At high pressure, highly compact phase domains are observed with a height difference of 0.315 ± 0.06 nm (Fig. 6c). The DPOG is expected to be enriched in the thicker domains since they

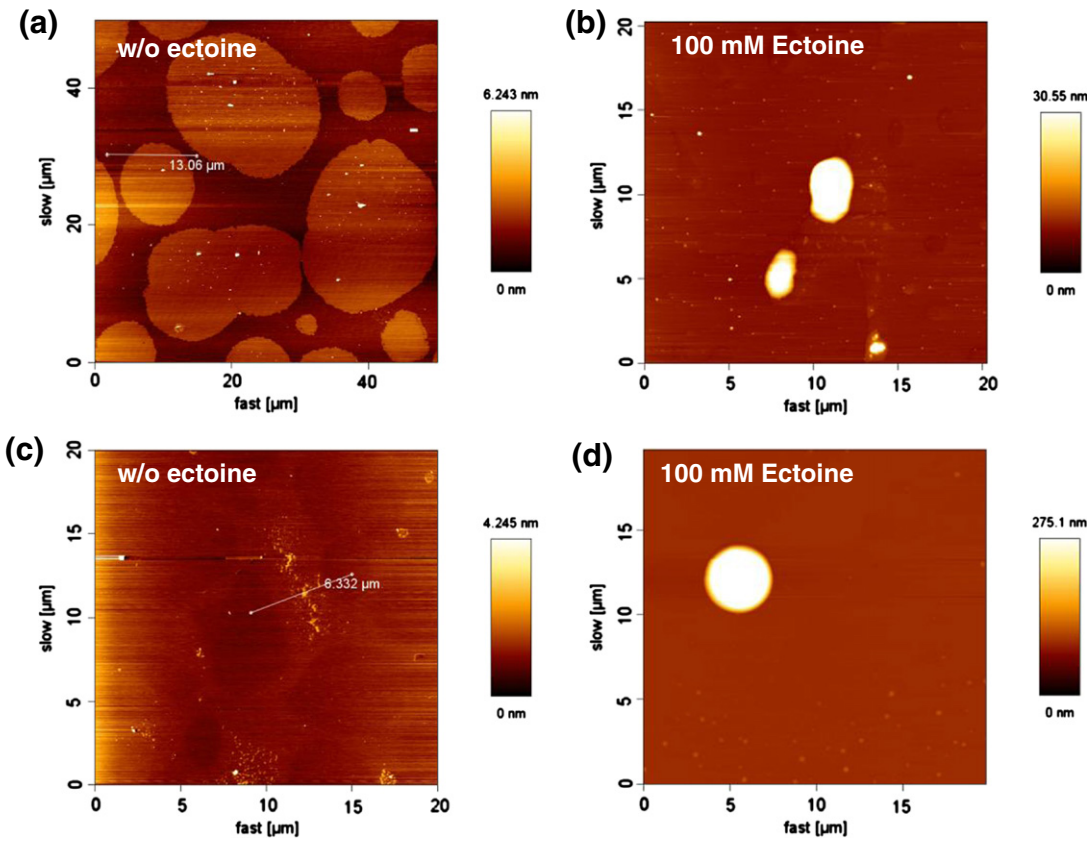

Fig. 7. AFM topography scans for DPPC/DPOG (7:3) at surface pressures (a), (b) 6 mN/m and (c), (d) 45 mN/m transferred from PBS buffer with and without ectoine. All measurements were done at 20 °C.

are, most likely, more hydrophobic. With the addition of ectoine at both low and high pressure, we observe a formation of droplet-like structures (Fig. 6b, d) similar to those observed with natural meibomian lipids (Dwivedi et al. 2014 in press). The height of these droplet-like structures ranged from 70 nm to 600 nm.

AFM scans for DPPC/DPOG (7:3) mixtures show approximately 1.5 ± 0.36 nm high phase separated domains (Fig. 7a). Due to the high concentration of DPOG and hence the low solubility of DPOG in DPPC monolayer, it is possible, that the DPOG molecules are excluded from the DPPC monolayer to form these phase separated domains. In the presence of ectoine, we consistently observe the formation of droplet-like structures (Fig. 7b). At high surface pressure, AFM scans for DPPC/DPOG (7:3) mixtures show compactly arranged domains with height differences of about 0.34 ± 0.06 nm (Fig. 7c). Along with these, we also find 2–3 nm high structures in between the domains. These structures are expected to be DPOG molecules aggregated due to its low solubility in the DPPC monolayer. In the presence of ectoine, again we find the formation of droplet-like structures which are several hundred nanometers in height (Fig. 7d). It is worth noticing that this formation of droplet-like structures is not evidenced for DPPC/CP mixtures. This implies that the more hydrophobic components like DPOG are mainly excluded from the DPPC monolayer in the presence of ectoine thus forming droplets.

AFM scans for DPPC/CP/DPOG (8:1:1) show 0.6 ± 0.12 nm height phase separated domains along with 5 nm high flat structures at low pressure (Fig. 8a). At high pressure, flat 5.15 ± 0.17 nm high structures appear to be more defined implying organized molecular arrangements (Fig. 8c). These 5.15 ± 0.17 nm height structures are similar to those previously observed in DPPC/CP mixtures and possibly are formed by the intercalation of the CP molecules into the DPPC monolayer. This intercalation of molecules can be expected to be a multilayered structure, hence conferring a height of about 5 nm. In the presence of 100 mM ectoine, we again observe the formation of droplet-like structures (Fig. 8b, d). As previously studied with different mixtures of DPPC, CP and DPOG, we observed, that the more hydrophobic component is more likely to be excluded from the DPPC monolayer. Hence we conclude that the droplet-like structures mainly consist of the more hydrophobic component which is DPOG in this case.

For the lipid mixture DPPC/CP/DPOG (4:3:3), AFM scans, at low pressure, exhibit well organized branched domains with height up to 1.704 ± 0.2 nm (Fig. 9a). Along with those we can also observe around high 0.5 nm phase separated domains. At high pressure we see flat 5.25 ± 0.26 nm high structures as previously observed in DPPC/CP/DPOG (8:1:1) (Fig. 9c). As discussed before, these structures are thought to be CP molecules forming organized multilayered structures. With the addition of ectoine, we observe the several hundred nanometer high droplet-like structures distributed throughout at both low and high pressure values (Fig. 9b, d). In addition, we also observe 5 nm height structures.

To further understand the material property of the droplet-like structures obtained in the presence of ectoine, rigidity scans were performed. The force curves obtained using AFM can be used for calculating the rigidity of the sample at specific points. Additionally, quantitative imaging mode widens the scope by allowing force scan measurements at all the points in the scan region. To analyze the material property of the droplet-like structures obtained in the presence of ectoine, we performed a rigidity scan measurement. The topology and the corresponding rigidity scan for a droplet-like structure obtained in DPPC/CP/DPOG (8:1:1) film are presented in Fig. 10. The droplet-like structure appears darker as compared to the surrounding scan region implying its non-rigid nature. Hence, it consists of less rigid components which are similar to the rigidity scans for the natural meibomian lipids in the presence of ectoine (Part A of this study (Dwivedi et al. 2014 in press)).

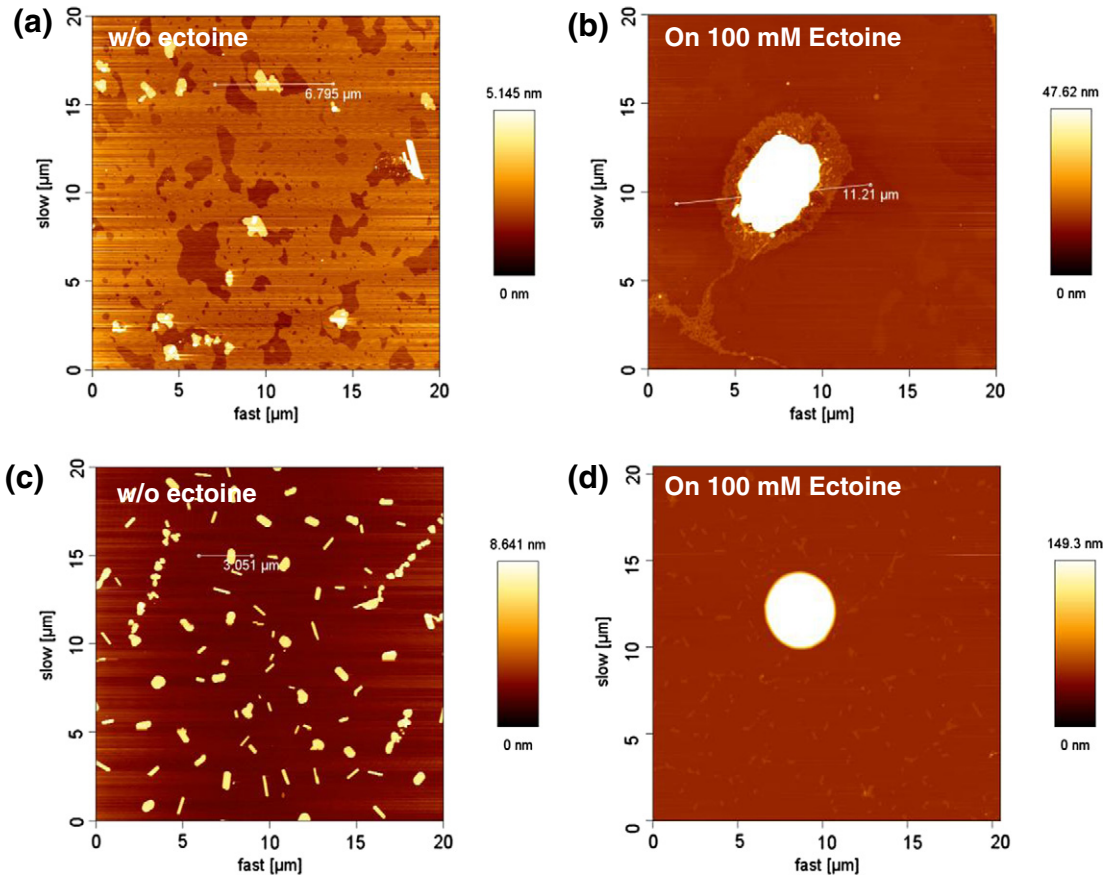

Fig. 8. AFM topography scans for DPPC/CP/DPOG (8:1:1) at surface pressures (a), (b) 6 mN/m and (c), (d) 45 mN/m transferred from PBS buffer with and without ectoine. All measurements were done at 20 °C.

4. Discussion

In part A of this study (Dwivedi et al. 2014 in press), the effect of ectoine was studied on the meibomian lipid layer using surface activity and topographical studies. Further, the structural aspects of the tear fluid lipid layer comprising the meibomian lipids at different compressed states were investigated. It was found that at low pressures polymorphous structures appeared which increased in number on further compression. These were hypothesized to be the hydrophobic components of the meibomian lipid layer which were excluded from the lipid film on compression. Further, at higher compression states, the lipid film is reversibly folded out of the plane of the lipid layer and the increase in surface pressure becomes gradual. In the presence ectoine, the surface pressure–area isotherms exhibited an increased intermolecular distance thus inducing a 'fluidizing' effect. The topographical studies showed the appearance of the droplet-like structures at all compressed states. These drop-like structures were hypothesized to comprise mainly the hydrophobic components which are excluded out of the film due to the increased intermolecular distance in the lowermost polar lipid layer and consequently decreased the hydrophobicity of the film.

To confirm the hypothesis established in the part A of this paper (Dwivedi et al. 2014 in press), with respect to the structural organization of the tear fluid lipid layer and the influence of ectoine on its different components, artificial tear fluid lipid layers consisting of binary and ternary lipid mixtures were studied. The presence of ectoine in the subphase consistently causes the surface pressure–area isotherm for the different DPPC, CP and DPOG mixtures to expand to higher area per molecule values, as previously observed with the natural meibomian lipids. The epi-fluorescence microscopy experiments with artificial tear fluid lipid layers revealed that the size of the liquid condensed domain decreases and that they become more circular with the addition of ectoine. This is in accordance with the surface activity studies supporting the observation that the interfacial lipid packing order decreases in the presence of ectoine. Similarly, the topography scans for DPPC/CP mixtures show a decreased domain size verifying a decreased lipid packing order in concordance with the surface activity and epi-fluorescence microscopy studies. The topography scans of DPPC/CP mixtures show 5 nm height flat structures which are expected to be the CP molecules interdigitated between the polar lipids. The height of 5 nm suggests that these structures may also be multi-stacked forming flat structures. The presence of ectoine in the subphase causes alteration in domain morphology as mentioned earlier. However, the topography scans for lipid mixtures with DPOG consistently show the appearance of droplet-like structures in the presence of ectoine. This proves that the hydrophobic components like DPOG are preferentially excluded from the lipid film in the presence of ectoine (Fig. 11). Additionally the quantitative imaging has enabled us to distinguish the material properties of these droplet-like structures from the surrounding scan region. It could be shown that these droplet like structures indeed consisted of softer non rigid components of the lipid layer.

Numerous studies have attempted to elucidate the structure of the tear fluid lipid layers using different techniques and reached a basic understanding that the film is a multilayered structure. The polar lipids are thought to form the surfactant layer and the cholesterol esters and the other hydrophobic components are thought to form the upper layer acting as evaporation deterrent. The important role of cholesterol esters in defining the structure of the tear fluid lipid layer has been acknowledged by many studies where the idea is based on the fact that cholesterol esters have a tendency to form crystalline structures in a spread

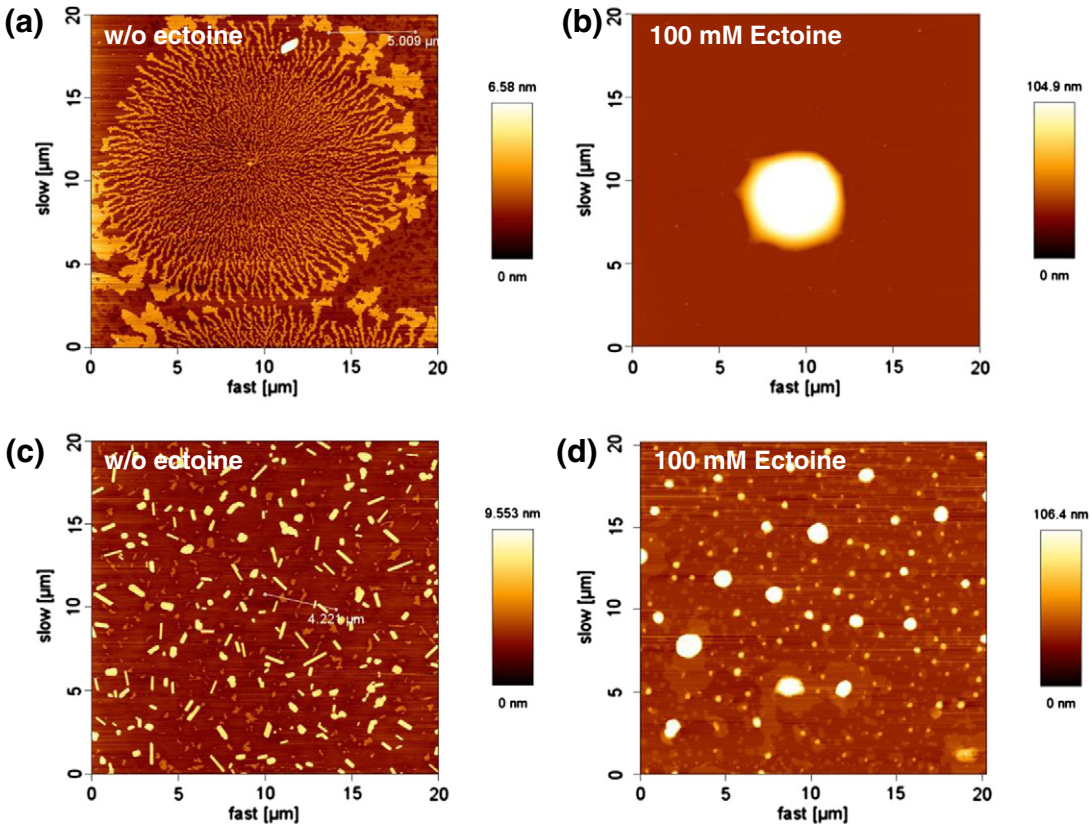

Fig. 9. AFM topography scans for DPPC/CP/DPOG (4:3:3) at surface pressures (a), (b) 6 mN/m and (c), (d) 45 mN/m transferred from PBS buffer with and without ectoine. All measurements were done at 20 °C.

film. Recent studies by Millar and King-Smith [30] favored the duplex nature of the meibomian lipid film as first suggested by Holly [1] and further elaborated by McCulley and Shine [4].

Based on our observations and the previous literature elucidating the structural aspects of tear fluid lipid layer, we consider the model shown in Fig. 11 for understanding the influence of ectoine on the molecular organization of the tear fluid lipid layer. The polar lipids, as previously established, form the aqueous–lipid interfacial layer which is mainly responsible for the spreading properties of the lipid film. The sterol esters and structurally similar lipids interdigitate into the polar lipid layer with the acyl chains facing to the air side thus forming multilayered stacks. These multilayered stacks of neutral lipids form a hydrophobic platform which accommodates the more hydrophobic components of the lipid film like tri-acyl-glycerides. These hydrophobic components thus form the lipid–air interface and are crucial for decreasing the rate of evaporation of the tear fluid. A decrease in the polar lipids to neutral lipids ratio causes condensation of the interfacial polar lipids thus making it comparatively rigid and hence more susceptible to rupture. It should be noted that this is a very simplistic model which only aims at understanding the lipid–lipid interaction and does not take into account the role played by the proteins in maintaining the biophysical function of the lipid layer. In the presence of ectoine, the interfacial polar lipid packing density decreases, thus decreasing the rigidity of the film. In the presence of high concentration of ectoine (100 mM), the decreased lipid packing density decreases the hydrophobic platform available to the hydrophobic lipids. This consequently leads to the aggregation of the hydrophobic components like tri-acyl-glycerides to form lipid droplets on the lipid film. It should be noted that the appearance of the droplet-like structures

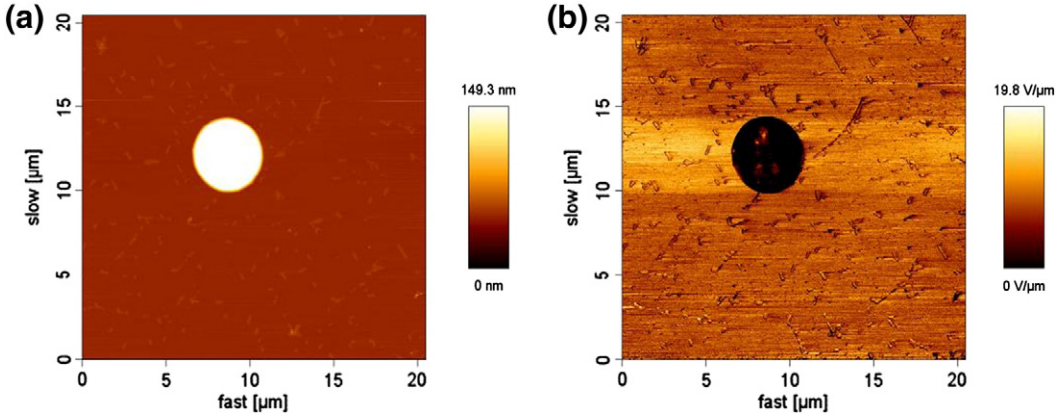

Fig. 10. AFM scans of DPPC/ CP/DPOG (8:1:1) lipid film in the presence of 100 mM ectoine in the subphase. (a) Topology scan (b) corresponding rigidity scan of the droplet-like structure. The rigidity scan shows that the droplet-like structures appear darker as compared to the surrounding indicating its non-rigid nature. All measurements were performed at 20 °C.

is only observed at higher concentrations of ectoine whereas at lower concentrations only the isotherms are shifted toward higher area per molecule. This implies that formation of droplet-like structures is an extreme effect of the fluidization of the film caused by ectoine. Hence, in relevant doses, the fluidizing effect of ectoine may have an application in eye drops to prevent the dry eye syndrome.

5. Conclusions

The presence of ectoine had been shown to cause an increased fluidization of the meibomian lipid film. In the present study, the effect of ectoine was studied on the artificial tear fluid lipid layer using surface activity, domain morphology and atomic force microscopic studies. The presence of ectoine consistently caused an increase in the pressure–area isotherms implying increased fluidity. The topology scans evidenced the formation of droplet-like structures in the presence of ectoine only when a nonpolar component (tri-acyl-glycerol in this case) was present with DPPC and cholesteryl ester. This result was similar to that obtained in the case of meibomian lipids. Hence, the hypothesis that these droplet-like structures consists of the hydrophobic component of the lipid film was confirmed. An inference based on these observations was developed stating that the presence of ectoine in the subphase causes an increase in the intermolecular spacing and hence decreased hydrophobicity of the film. This consequently leads to the expulsion of the hydrophobic component to from droplet-like structures. Further, the

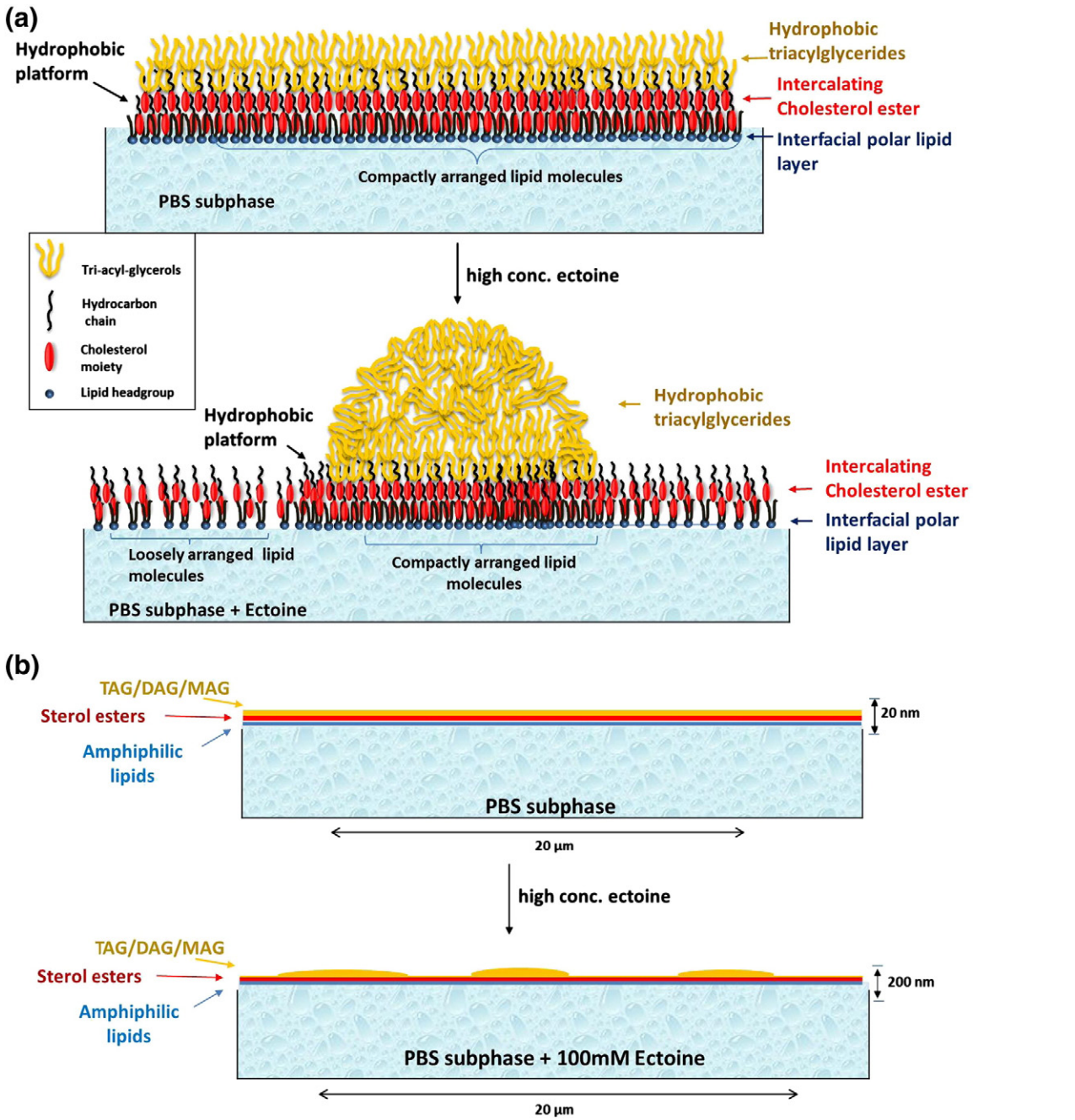

Fig. 11. (a) Molecular model explaining the effect of high concentration ectoine on the tear fluid lipid layer. (b) Macroscopic model explaining the effect of high concentration ectoine on the tear fluid lipid layer.

fluidizing effect of ectoine has been suggested to be of application in the treatment of the dry eye syndrome.

Acknowledgement

This work is funded by NRW International Graduate School of Chemistry by granting a PhD stipend to MD. We acknowledge and appreciate the collaboration with Bitop GmbH, Witten and the support given by Ursapharm Arzneimittel GmbH, Saarbrücken for this project. Especially valuable discussions with Dr. Bilstein from Bitop AG are highly acknowledged.

References

[1] F.J. Holly, Formation and rupture of tear film, Exp. Eye Res. 15 (1973) 515–525.
[2] J.P. Craig, A. Tomlinson, Importance of the lipid layer in human tear film stability and evaporation, Optom. Vis. Sci. 74 (1997) 8–13.
[3] L.N. Thibos, X. Hong, A. Bradley, C. Begley, Deterioration of retinal image quality due to breakup of the corneal tear film, Investig. Ophthalmol. Vis. Sci. 40 (1999) S544.
[4] J.P. McCulley, W.E. Shine, The lipid layer: the outer surface of the ocular surface tear film, Biosci. Rep. 21 (2001) 407–418.
[5] W.E. Shine, J.R. McCulley, Polar lipids in human meibomian gland secretions, Curr. Eye Res. 26 (2003) 89–94.
[6] I.A. Butovich, J.C. Wojtowicz, M. Molai, Human tear film and meibum. Very long chain wax esters and (O-acyl)-omega-hydroxy fatty acids of meibum, J. Lipid Res. 50 (2009) 2471–2485.
[7] I.A. Butovich, E. Uchiyama, J.P. McCulley, Lipids of human meibum: mass-spectrometric analysis and structural elucidation, J. Lipid Res. 48 (2007) 2220–2235.
[8] I.A. Butovich, E. Uchiyama, M.A. Di Pascuale, J.P. McCulley, Liquid chromatography–mass spectrometric analysis of lipids present in human meibomian gland secretions, Lipids 42 (2007) 765–776.
[9] B.D. Sullivan, J.E. Evans, M.R. Dana, D.A. Sullivan, Impact of androgen deficiency on the lipid profiles in human meibomian gland secretions, Adv. Exp. Med. Biol. 506 (2002) 449–458.
[10] B.D. Sullivan, J.E. Evans, M.R. Dana, D.A. Sullivan, Influence of aging on the polar and neutral lipid profiles in human meibomian gland secretions, Arch. Ophthalmol. Chic. 124 (2006) 1286–1292.
[11] G.N. Foulks, D. Borchman, M. Yappert, S.H. Kim, J.W. Mckay, Topical azithromycin therapy for meibomian gland dysfunction: clinical response and lipid alterations, Cornea 29 (2010) 781–788.
[12] D. Borchman, G.N. Foulks, M.C. Yappert, D.V. Ho, Temperature-induced conformational changes in human tear lipids hydrocarbon chains, Biopolymers 87 (2007) 124–133.
[13] D. Borchman, G.N. Foulks, M.C. Yappert, D.X. Tang, D.V. Ho, Spectroscopic evaluation of human tear lipids, Chem. Phys. Lipids 147 (2007) 87–102.
[14] G. Wollensak, E. Mur, A. Mayr, G. Baier, W. Gottinger, G. Stoffler, Effective methods for the investigation of human tear film proteins and lipids, Graefes Arch. Clin. Exp. 228 (1990) 78–82.
[15] J.P. McCulley, W.E. Shine, Meibomian gland and tear film lipids: structure, function and control, Adv. Exp. Med. Biol. 506 (2002) 373–378.
[16] D. Borchman, G.N. Foulks, M.C. Yappert, S.E. Milliner, Differences in human meibum lipid composition with meibomian gland dysfunction using NMR and principal component analysis, Investig. Ophthalmol. Vis. Sci. 53 (2012) 337–347.
[17] G.N. Foulks, D. Borchman, Meibomian gland dysfunction: the past, present, and future, Eye Contact Lens 36 (2010) 249–253.
[18] D. Borchman, G.N. Foulks, M.C. Yappert, J. Bell, E. Wells, S. Neravetla, V. Greenstone, Human meibum lipid conformation and thermodynamic changes with meibomian-gland dysfunction, Investig. Ophthalmol. Vis. Sci. 52 (2011) 3805–3817.
[19] W.E. Shine, J.P. McCulley, Meibomian gland secretion polar lipids associated with chronic blepharitis disease groups, Investig. Ophthalmol. Vis. Sci. 37 (1996) 3926.
[20] E.A. Galinski, H.P. Pfeiffer, H.G. Truper, 1,4,5,6-Tetrahydro-2-methyl-4-pyrimidine-carboxylic acid — a novel cyclic amino-acid from halophilic phototrophic bacteria of the genus Ectothiorhodospira, Eur. J. Biochem. 149 (1985) 135–139.
[21] K. Lippert, E.A. Galinski, Enzyme stabilization by ectoine-type compatible solutes — protection against heating, freezing and drying, Appl. Microbiol. Biotechnol. 37 (1992) 61–65.
[22] R.K. Harishchandra, A.K. Sachan, A. Kerth, G. Lentzen, T. Neuhaus, H.J. Galla, Compatible solutes: ectoine and hydroxyectoine improve functional nanostructures in artificial lung surfactants, Biochim. Biophys. Acta 1808 (2011) 2830–2840.
[23] J. Smiatek, R.K. Harishchandra, O. Rubner, H.J. Galla, A. Heuer, Properties of compatible solutes in aqueous solution, Biophys. Chem. 160 (2012) 62–68.
[24] S. Krol, M. Ross, M. Sieber, S. Kunneke, H.J. Galla, A. Janshoff, Formation of three-dimensional protein–lipid aggregates in monolayer films induced by surfactant protein B, Biophys. J. 79 (2000) 904–918.
[25] C.W. McConlogue, T.K. Vanderlick, A close look at domain formation in DPPC monolayers, Langmuir 13 (1997) 7158–7164.
[26] D.J. Benvegnu, H.M. Mcconnell, Line tension between liquid domains in lipid monolayers, J. Phys. Chem. 96 (1992) 6820–6824.
[27] D.J. Benvegnu, H.M. Mcconnell, Surface dipole densities in lipid monolayers, J. Phys. Chem. 97 (1993) 6686–6691.
[28] H.M. McConnell, Structures and transitions in lipid monolayers at the air–water interface, Annu. Rev. Phys. Chem. 42 (1991) 171–195.
[29] J. Telenius, A. Koivuniemi, P. Kulovesi, J.M. Holopainen, I. Vattulainen, Role of neutral lipids in tear fluid lipid layer: coarse-grained simulation study, Langmuir 28 (2012) 17092–17100.
[30] T.J. Millar, P.E. King-Smith, Analysis of comparison of human meibomian lipid films and mixtures with cholesteryl esters in vitro films using high resolution color microscopy, Investig. Ophthalmol. Vis. Sci. 53 (2012) 4710–4719.