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Biophysical investigations of the structure and function of the tear fluid lipid layer and the effect of ectoine. Part A: Natural meibomian lipid films

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ABSTRACT

The tear fluid lipid layer is the outermost part of the tear film on the ocular surface which protects the eye from inflammations and injuries. We investigated the influence of ectoine on the structural organization of natural meibomian lipid films using surface activity analysis and topographical studies. These films exhibit a continuous pressure–area isotherm without any phase transition. With the addition of ectoine, the isotherm is expanded towards higher area per molecule values suggesting an increased area occupied by the interfacial lipid molecules. The AFM topology scans of natural meibomian lipid films reveal a presence of fiber-like structures. The addition of ectoine causes an appearance of droplet-like structures which are hypothesized to be tri-acyl-glycerols and other hydrophobic components excluded from the lipid film. Further the material properties of the droplet-like structure with respect to the surrounding were determined by using the quantitative imaging mode of the AFM technique. The droplet-like structures were found to be comparatively softer than the surrounding. Based on the observations a preliminary hypothesis is proposed explaining the mechanism of action of ectoine leading to the fluidization of meibomian lipid films. This suggests the possibility of ectoine as a treatment for the dry eye syndrome.

1. Introduction

The human tear film is essential for the protection of the corneal epithelium against drying and inflammation [1]. It mainly comprises the innermost mucin-rich layer, the aqueous fluid containing proteins, metabolites, salts and the outermost lipid layer at the aqueous air interface [2,3]. The tear fluid lipid layer prevents the tear fluid from evaporation and decreases the surface tension at the interface [4]. The structural integrity and stability of this lipid layer is delicately dependent on the lipid composition including polar lipids (mainly phospholipids and free fatty acids), cholesterol esters (and other sterol esters), tri-acyl-glycerides and wax esters [5]. The lipids in the tear fluid lipid layer are assumed to be mainly secreted by the meibomian glands. The tear fluid lipid layer is believed to be a multilamellar film where all the acyl chains of different lipid species are oriented towards the air side. However, the structural organization of the aforementioned lipid species is still ambiguous. It has been suggested that the polar lipids form the lowermost part of the lipid layer and the non-polar lipids accumulate on top of the polar lipid film forming a second layer [6]. Increased evaporation rates of the tear fluid cause drying and inflammation of the ocular surface also known as the dry eye syndrome (DES). The drying of the ocular surface can be caused either by insufficient production of the tear fluid or an increased evaporation rate of the tears through the lipid layer barrier. The latter may be caused by an altered lipid composition of the lipid layer leading to its decreased stability or disruptions and consequently to an increased rate of evaporation [7,8].

The lipid composition of the tear fluid lipid layer from dry eye patients and healthy volunteers has been examined by a plethora of techniques including mass spectrometry and nuclear magnetic resonance technique [7,9–11]. The dry eye patients have been shown to have a decreased polar to neutral lipid ratio [7]. The amount of saturated components increases in the meibomian lipids [12], also known as the meibum thus increasing the melting temperature of the lipids. The meibum obtained from patients with DES had higher lipid order along with increased lipid–lipid interaction as compared to the meibum from healthy volunteers [9,11,13,14]. Hence, it is apparent that one of the factors responsible for premature rupture of the tear film is increased rigidity of the lipid layer as a consequence of altered lipid composition [15]. Consequently, molecules that are able to fluidize the lipid film might prevent the premature film rupture and one of the possible candidates known to fluidize the lipid film is ectoine.

Ectoines are low molecular weight, zwitterionic molecules possessing a strong water binding property. They are synthesized and secreted by

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aerobic chemoheterotrophic and halophilic/halotolerant bacteria as a response to adverse environmental conditions such as extreme temperature, excess dryness and high salinity [16–18]. Besides their function as osmoprotectants these solutes are known to stabilize biomolecule like proteins, nucleic acids and biomembranes. Since they do not interfere with the cellular functions even if they are present in high concentrations within the cytoplasm, they are known as compatible solutes. Recently, the fluidizing effect of ectoine on lipid monolayers and bilayers has been shown using surface activity studies, epi-fluorescence microscopy and atomic force microscopy [19]. Numerous studies attempting to understand the ability of the ectoine to act as biomolecule stabilizers are available and are interpreted by theories including water replacement hypothesis [20], transfer free energy, excluded volume, contact interaction [21–23], preferential exclusion model [24,25] and, recently, direct interaction of the osmolytes with the biomolecules [26]. However, the exact mechanism explaining the protective function of osmolytes still remains unclear.

The present study aims to understand the effect of ectoine on the biophysical properties of the tear fluid lipid layer with respect to the alteration in the structural organization of the film and consequently the fluidity of the film. This article presents the effect of ectoine on the natural meibomian lipid film with the focus on the surface activity behavior, topological studies and rigidity analysis. A continuation of this study is the part B, where an artificial tear fluid lipid layer is prepared from binary and ternary mixtures of the different lipids and the effect of ectoine is studied. Although the major contribution of the lipids in the tear fluid lipid layer comes from the meibomian gland, the composition of the tear lipid is slightly different than the meibomian gland lipids and may contain 12–48% PL compared to 0.006% in meibum [5,27]. Combining both the studies, a model is proposed to explain the effect of ectoine on the tear fluid lipid layer.

2. Materials and methods

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 >18 MΩm. The natural meibomian lipids were extracted from the meibomian glands of four healthy volunteers (two in the age group of 20–35 and two in the age group of 50–65) with no apparent dry eye symptoms. The lipids secreted by the meibomian gland were squeezed out and wiped by using a sterile cotton bud. The lipids from the cotton bud were dissolved in chloroform which was then evaporated by using a stream of nitrogen gas. The obtained lipid was weighed and dissolved in chloroform/methanol (1:1, v/v) solution and spread on the subphase for all the experiments. All the experiments were repeated for all the four volunteers to obtain consistent data and the samples were not pooled.

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 and 32 °C. The lipid mixture consisted of the meibomian lipids extracted from the meibomian glands of the healthy volunteers. These lipid mixtures were dissolved in chloroform/methanol solution (1:1, v/v) and spread onto the subphase. After an equilibration time of 10–15 min the lipid films were compressed at a rate of 2.9 cm^2/min. Ectoine was added to the PBS buffer to prepare the subphase solution prior to the experiment.

2.2. Atomic force microscopy

Atomic force microscopy (AFM) images of the Langmuir–Blodgett (LB) films were transferred onto mica sheets [28] under ambient conditions (20 °C and 32 °C) and scanned by 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 by using Quantitative mode imaging using the same cantilever used for topography scanning.

3. Results

3.1. Surface activity studies

The compression of natural meibomian lipids on a PBS subphase at 20 °C displays a gradual increase in the surface pressure without evidencing any phase transition up to 20 mN/m (Fig. 1a). A plateau is formed under further compression demonstrating the high stability of the lipid film to collapse (Fig. 1a). The transition to a plateau region implies the formation of a highly compressible phase or reversible expulsion of the lipid material into the third dimension.

With the addition of ectoine, the isotherm is expanded towards higher area per molecule values implying an increased area occupied by the lipid headgroups (Fig. 1b). A comparatively lower amount of lipid sample was applied for this measurement to obtain zero pressure values, which made it technically unfeasible to reach the plateau region in Fig. 1b. However, the onset of the plateau is still visible. The ectoine effect is concentration dependent (Fig. 1c). In the presence of 500 mM ectoine in the subphase the slope of the curve decreases up to 150 $Å^2$ area per molecule values suggesting a decreased cooperativity between the lipid molecules. The continuous compression expansion cycles of natural meibomian lipids on PBS subphase and ectoine do not show significant differences in their hysteresis (Fig. 1b). At 32 °C, the presence of 100 mM ectoine causes a change in the slope of the isotherm (unlike at 20 °C) along with an expansion towards higher area per molecule values (Fig. 2). The fact that the effect of ectoine is more prominent at higher temperatures suggests that an already fluid lipid film is more sensitive to the ectoine than a rigid film. It should be noted that the area per molecule values for the meibomian lipid mixture is a relative value and is meant for comparing the data between the PBS subphase and the ectoine containing PBS subphase. The value is not absolute due to unavailability of the exact molar concentration of the lipids and hence the isotherms of samples from different volunteers cannot be compared.

3.2. Topographical studies

The lipid film was transferred from the subphase onto a mica sheet at the desired surface pressure using Langmuir Blodgett technique. The dried samples were then scanned under AFM using the tapping mode. Images were processed using JPK data processing software. AFM scans for natural meibomian lipids at low pressure (5 mN/m) show small fiber-like polymorphous structures of about 5 nm height distributed over the entire area (Fig. 3a). Owing to the complex nature of the composition of natural meibomian lipids, it is rather difficult to interpret the contents of these structures. However, we expect that it could be stacks formed by sterol esters, tri-acyl-glycerols and other hydrophobic components. At high surface pressure (20 mN/m) the number of fiber-like structures increases (Fig. 3b). A high resolution scan reveals that these polymorphous structures consist of various height structures indicating the presence of different structural organizations forming more than one phase (Fig. 4). The darker color of these structures in the rigidity scans indicates that they are softer (more fluid,

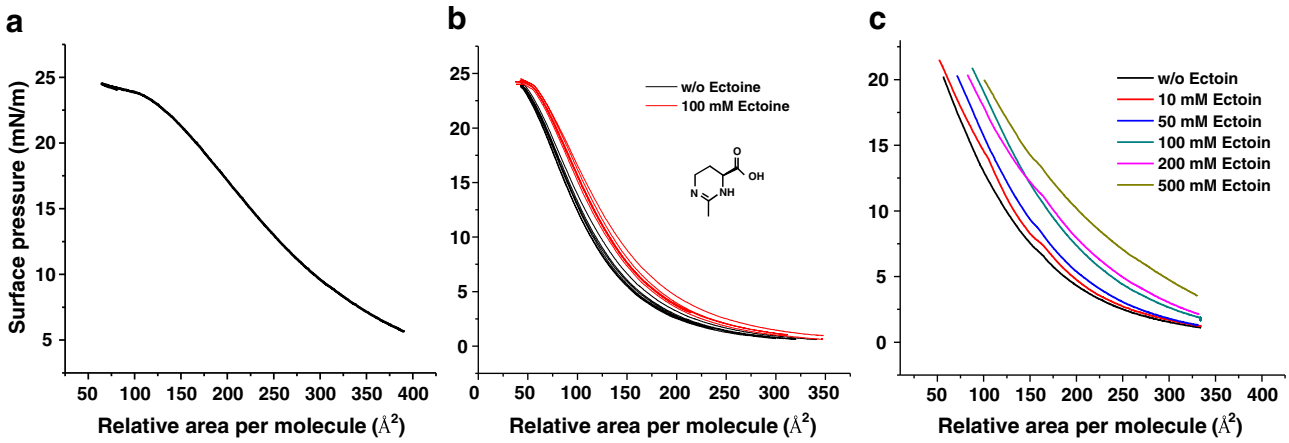

Fig. 1. (a) Surface pressure–area isotherm for natural meibomian lipids on PBS subphase at pH 7.4, (b) cyclic compression expansion cycles for natural meibomian lipids on PBS subphase at pH 7.4 in the presence and absence of ectoine, (c) cyclic compression expansion curves for natural meibomian lipids on PBS subphase in the presence of increasing concentration of ectoine. All measurements were performed at 20 °C.

less ordered) compared to surrounding materials (Fig. 5). At higher compressed states, where the plateau is formed, the AFM scans show 5 nm height structures which are more flat unlike the polymorphous structures present at low pressures (Fig. 3c).

In the presence of ectoine the AFM scans show droplet-like structures with a height of 300 nm (Figs. 6 and 7). With increasing concentration of ectoine, the lateral size of the droplet-like structures increases (Fig. 6). In the presence of 50 mM ectoine, we observe the onset of the formation of these structures which are comparatively sparse and smaller in size. The appearance of droplet-like structures is observed at all the surface pressures measured (Fig. 7b). At 20 mN/m, droplet-like structures are observed along with the polymorphous structures originally present in the lipid layer without ectoine in the subphase (Fig. 7b). Also, at the plateau region, the presence of droplet-like structure is observed along with the 5 nm flat structures (Fig. 7c). The darker color of these structures in the rigidity scans indicates that they are softer compared to surrounding materials (Fig. 8). These are assumed to be di/tri-acyl-glycerols that are more fluid at room temperature and lack the molecular organization of the polar lipids and cholesteryl esters.

At 32 °C, the effect of ectoine is similar. On PBS subphase the AFM scans show the presence of rounded structures along with polymorphous structures both at low and high pressure (Fig. 9a, c). In the presence of ectoine, we observed the formation of droplet-like structures similar to those obtained at 20 °C (Fig. 9b, d).

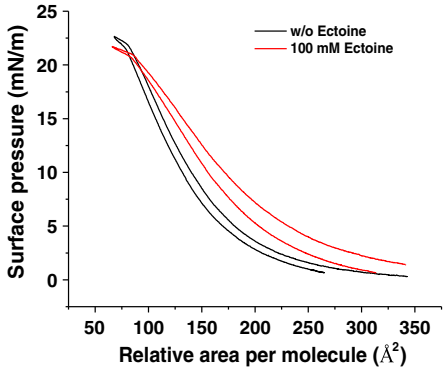

Fig. 2. Surface pressure–area isotherm for natural meibomian lipids on PBS subphase in the presence and absence of 100 mM ectoine at 32 °C.

4. Discussion

The structure of the tear fluid lipid layer has been investigated in numerous studies and a consensus has been reached over the model where the polar lipids form the lowermost interfacial lipid layer and neutral lipids and the hydrophobic components are stored in the polar lipid – air interface. In this study, we have tried to analyze the structure of the tear fluid lipid layer using the natural meibomian lipids and we have studied the effect of ectoine on the functional and the structural aspects of the tear fluid lipid layer. The surface activity studies of the meibomian lipids obtained from different volunteers displayed a gradual increase in surface pressure until it reached a plateau at 20 mN/m.

The topology scans reveal the presence of polymorphous structures at low pressures which increase in number as the pressure is increased. Rigidity scans show that these polymorphous structures consist of mainly softer materials. Due to the highly complex composition of meibomian lipids, it is difficult to be certain about the contents of these structures. However, the increase in the number of these structures with increasing pressure implies that these are probably the hydrophobic component phase separating from the polar lipid interfacial monolayer due to a decreased solubility at high pressures. Similar behavior has been observed in previous simulation studies [29] with artificial tear fluid lipid layers wherein the tri-acyl-glycerides are phase separated first aggregating together to form nanometer scale domains followed by cholesteryl ester aggregation due to decreasing solubility. It has been shown by grazing incidence x-ray diffraction and x-ray diffraction studies that the compression of the meibomian lipid layer does not cause a decrease in the intermolecular lattice spacing. This suggests structural organization of the lipid layer components causing vertical rearrangement [30]. These observations support the assumption that the compression of the meibomian lipid film causes a rearrangement of the hydrophobic components to obtain increasing number of polymorphous structures.

Formation of a plateau in the pressure–area isotherm implies expulsion of the lipid material into the third dimension. The topography scans of the meibomian lipids at the plateau region i.e. surface pressure above 20 mN/m, show 5 nm height flat structures which are absent at lower pressures. We assume this to be the lipid film folding reversibly out of the plane of the film as a response to continuous compression or expulsion of a component of the film, consequently preventing an increase in pressure. Recent studies by Telenius et al. [29] have elucidated the structural properties of artificial tear fluid lipid layers consisting of phospholipids, fatty acids, cholesteryl esters and tri-acyl-glycerides using simulation studies. Their measurements support the formation

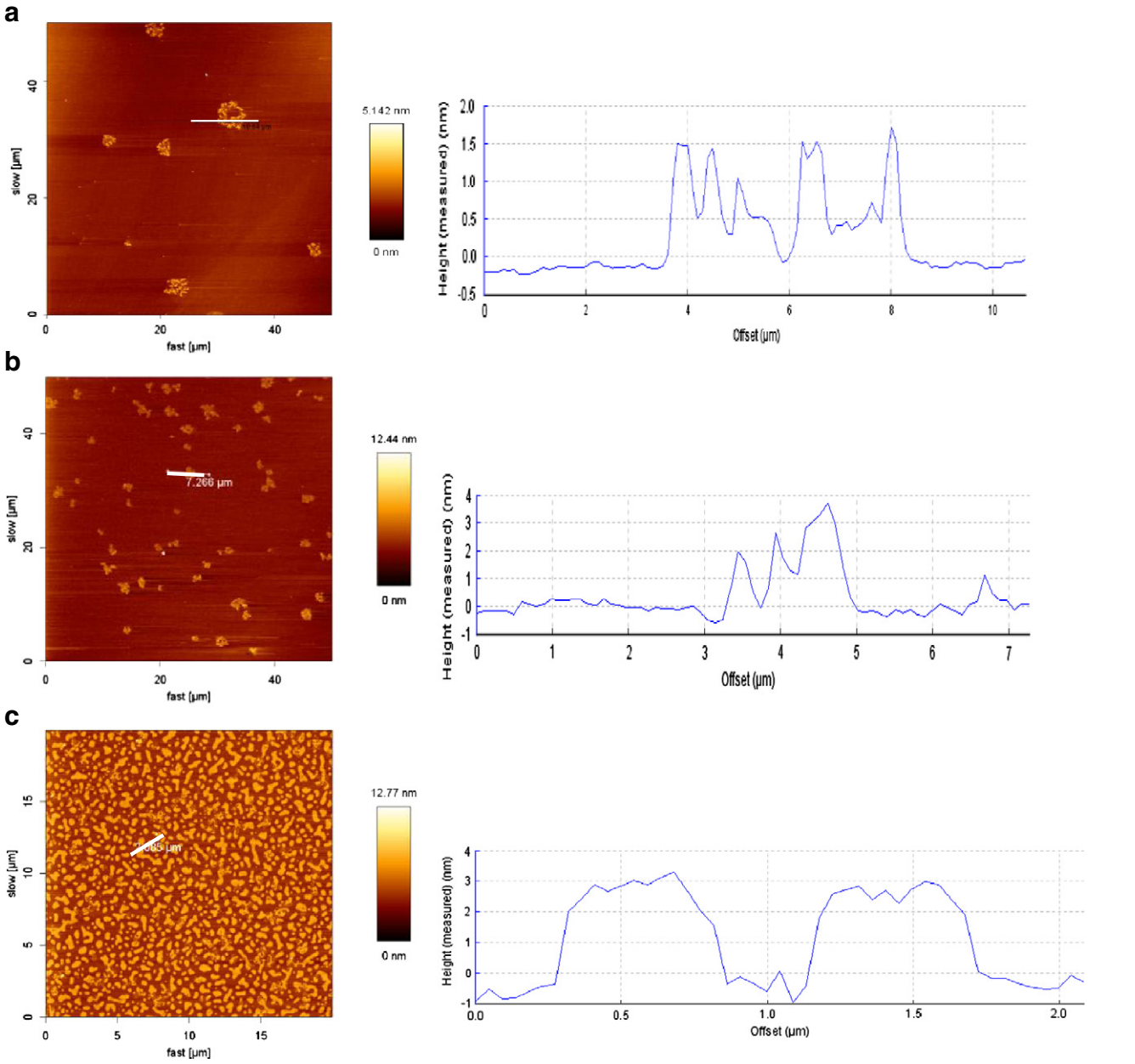

Fig. 3. AFM scans for natural meibomian lipids on PBS at pH 7.4 at surface pressure (a) 5 mN/m, (b) 20 mN/m, (c) 23.5 mN/m. The AFM scans are accompanied by height analysis graphics along the white bar at its right. All transfers were performed at 20 °C.

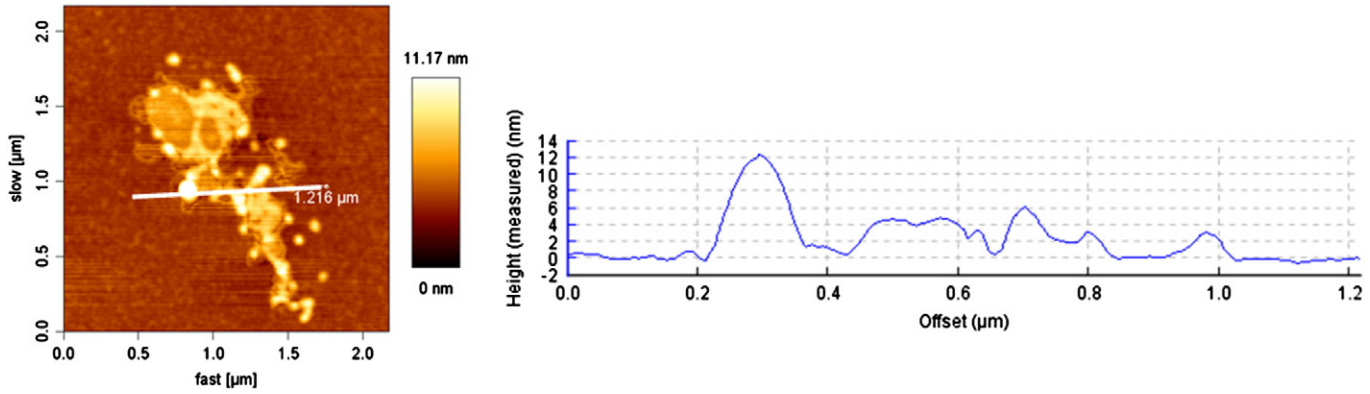

Fig. 4. High resolution scan of polymorphous structures obtained in the AFM scans of the natural meibomian lipids (Fig. 3b) transferred from PBS subphase at 20 °C.

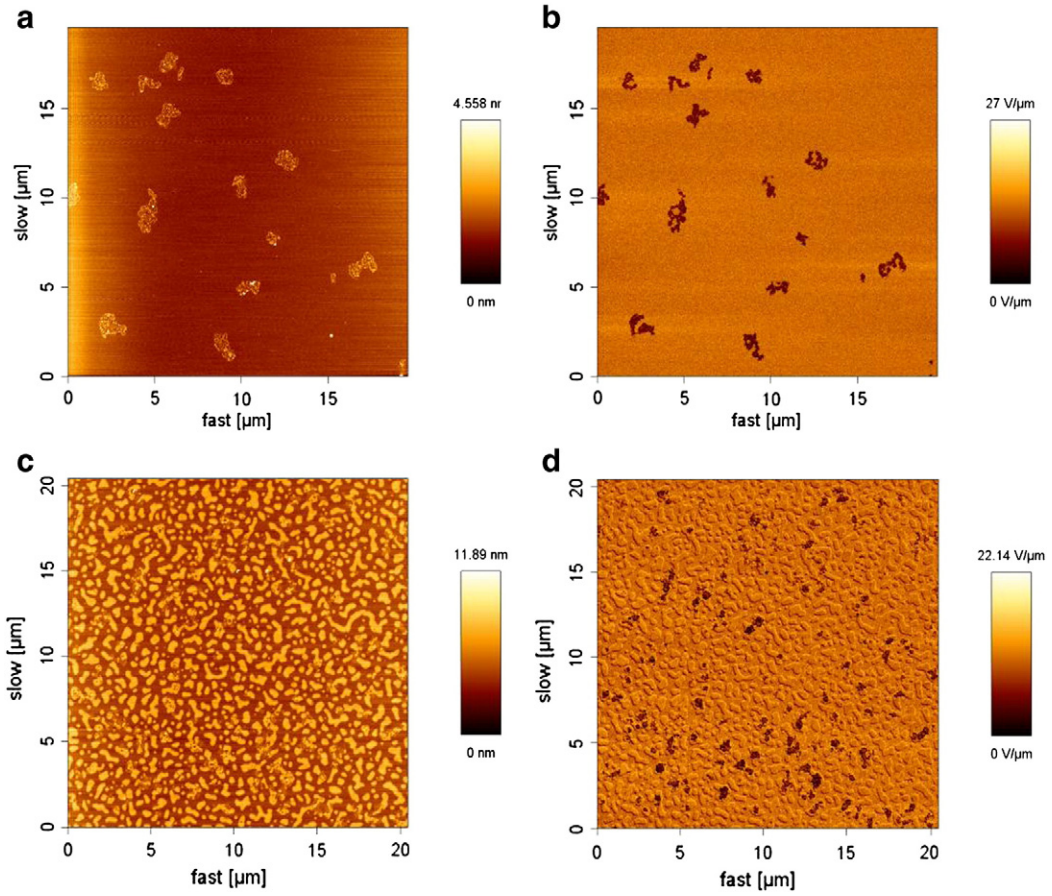

Fig. 5. AFM topography (left) and corresponding rigidity scans (at its right) for natural meibomian lipids transferred at surface pressure values (a), (b) 20 mN/m and (c), (d) 24 mN/m from PBS subphase at pH 7.4 at 20 °C.

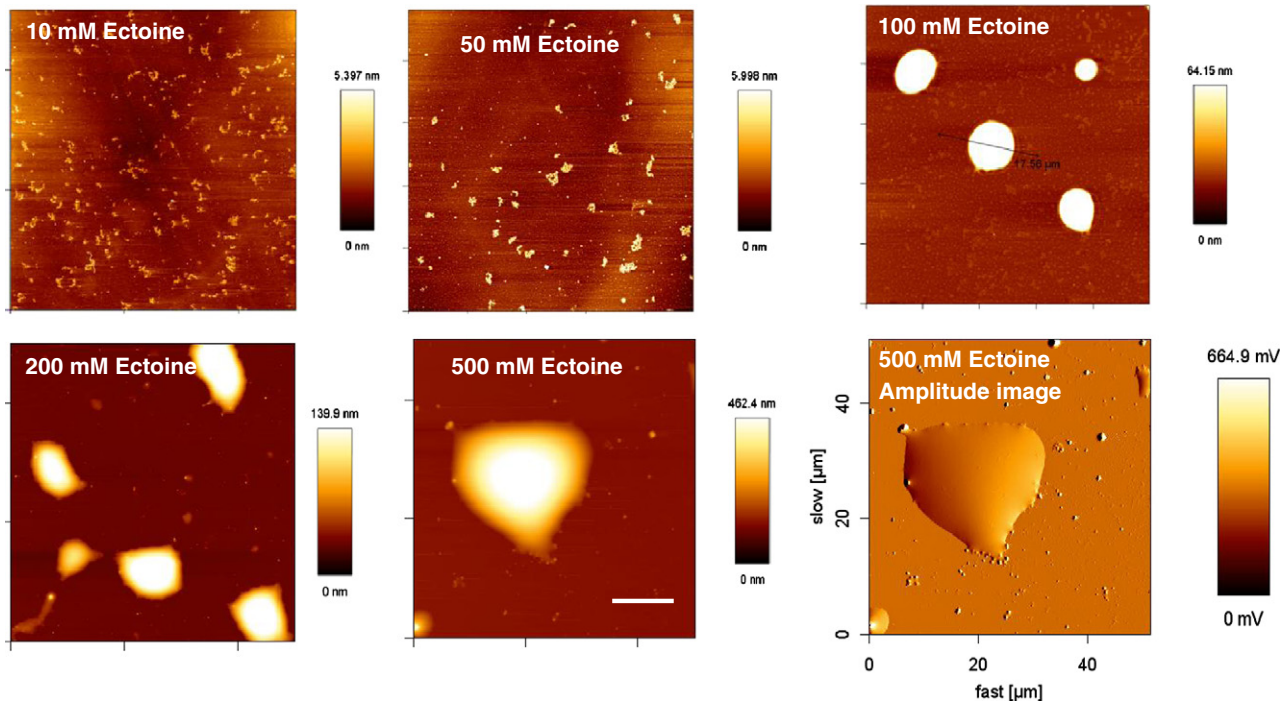

Fig. 6. AFM topography scans for natural meibomian lipids transferred from PBS subphase in the presence of varying concentrations of ectoine. The topography scans are accompanied with amplitude image showing the droplet-like structure. Scale bar is 10 µm. All transfers were performed at 20 °C.

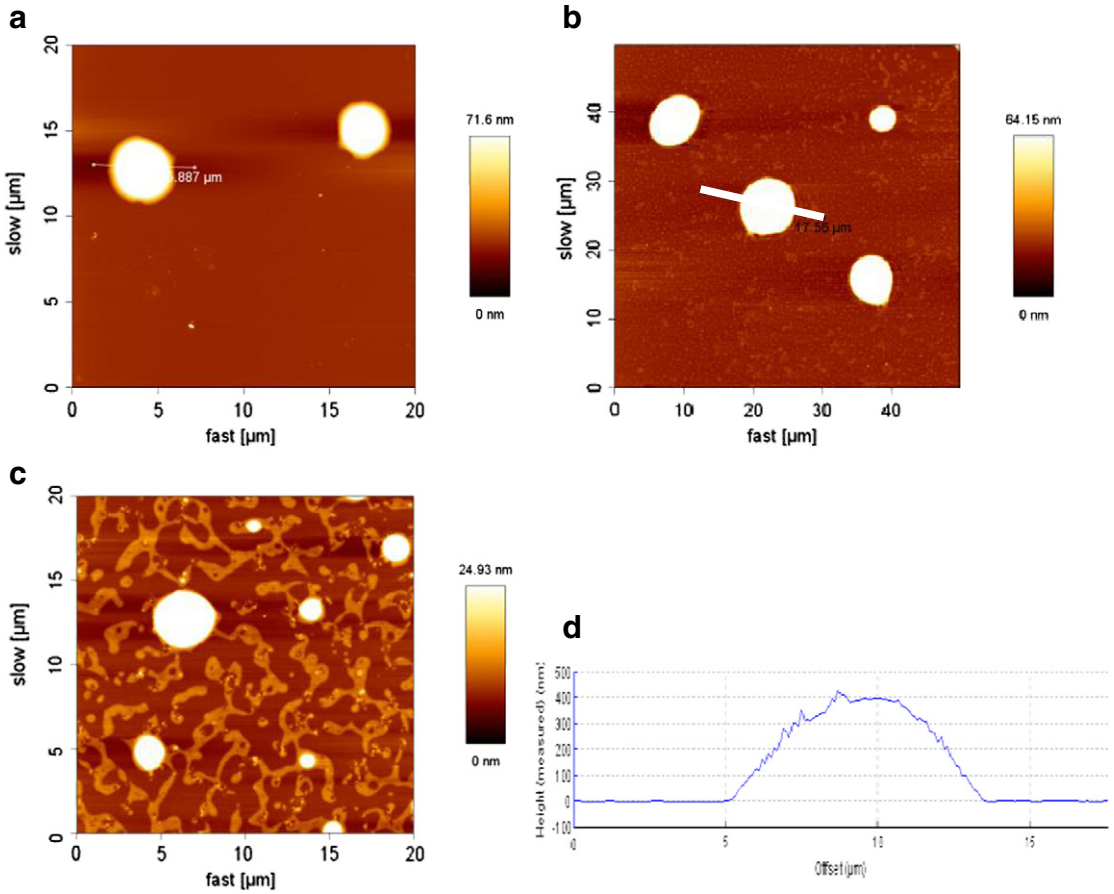

Fig. 7. AFM topography scans for natural meibomian lipids transferred from PBS subphase in the presence of 100 mM ectoine at 20 °C at surface pressures (a) 5 mN/m, (b) 20 mN/m and (c) 23.5 mN/m. (d) represents the height analysis graphics for (*b*).

of curvature in the lipid film, stabilized due to the presence of cholesterol esters and other hydrophobic components. Therefore, the reversible folding of the lipid film at higher compressed states is a plausible explanation for the formation of a plateau at higher surface pressures (Fig. 10). Consequently, at higher compressed states, the compressibility of the meibomian lipid film increases tremendously preventing the lipid film from collapse.

With the addition of ectoine into the subphase, the surface activity of the meibomian lipid film is altered whereby the isotherms are expanded towards higher area per molecule values in a concentration dependent manner. This implies that the area occupied by the lipid headgroups on the subphase increases. At higher temperatures this effect is even more prominent. The topology scans for meibomian lipid films evidence the appearance of the droplet-like structures, much larger than the polymorphous structures, with the addition of ectoine in a concentration dependent manner. The rigidity scans show that these structures consist of softer materials. We expect these structures to be the hydrophobic component of the meibomian lipids like triacyl-glycerides excluded from the tear fluid lipid layer. Further, the meibomian lipid extract used in this study might well be accompanied by the tear fluid proteins which are highly surface active. These proteins must play a role in the biophysical characteristics of the lipid layer and hence it would be interesting to study the effect of ectoine on the artificial lipid layer in the presence of these. However we will be considering

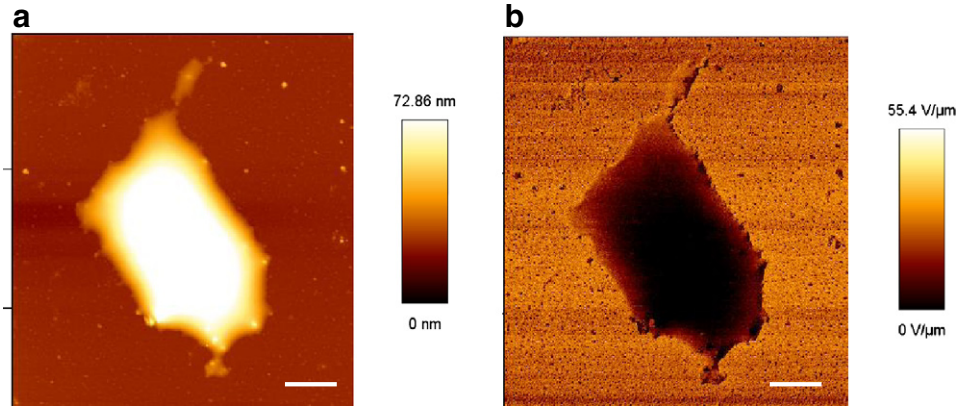

Fig. 8. AFM scans for natural meibomian lipids transferred from PBS subphase in the presence of 200 mM Ectoine. (a) Topography scans of droplet-like structures, (b) rigidity scans for droplet-like structure. Scale bar is 2 µm.

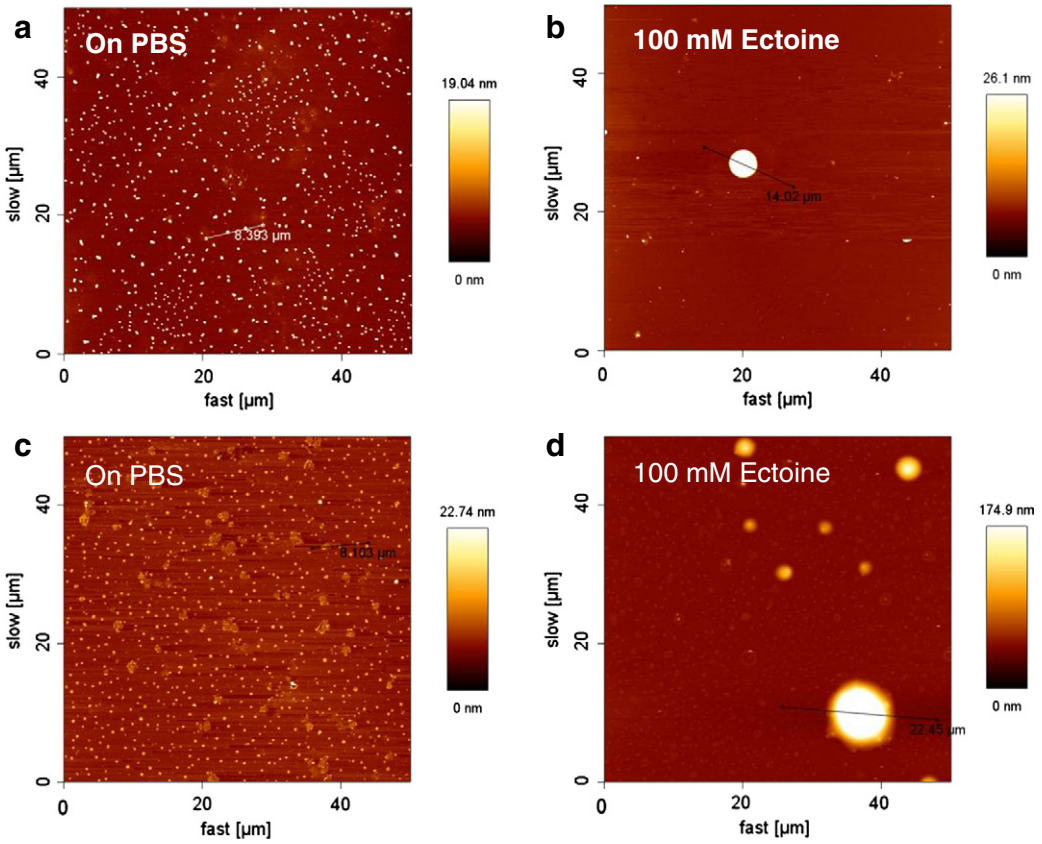

Fig. 9. AFM topography scans for natural meibomian lipids transferred from PBS subphase in the presence and absence of 100 mM Ectoine at surface pressures (a), (b) 5 mN/m and (c), (d) 20 mN/m. All transfers were performed at 32 °C.

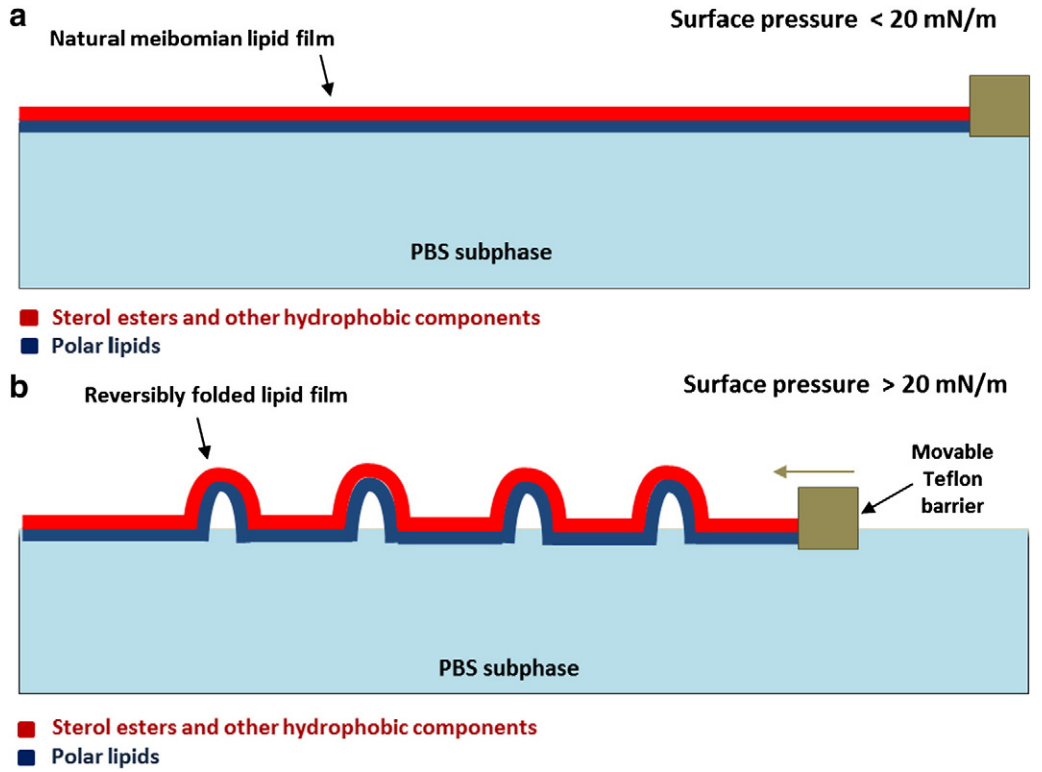

Fig. 10. Schematic representation of the natural meibomian lipid film at (a) surface pressure value below 20 mN/m and (b) surface pressure value above 20 mN/m showing reversibly folded lipid film. The blue layer represents the polar components of the meibomian lipids and the red layer represents the sterol esters and the hydrophobic components of the film.

only the lipid components in Part B of this study and could accommodate the protein aspect in the later studies.

The previously theorized structural organization of the tear fluid lipid layer consists of an interfacial polar lipid layer which provides a hydrophobic platform for the more hydrophobic components of the lipid layer. With the addition of ectoine, the area occupied by the interfacial polar lipid headgroups increases thus decreasing the packing order in the interfacial polar lipids. This consequently prevents the formation of a suitable hydrophobic platform thus excluding the hydrophobic components from the lipid film to form large lipid droplets.

The lipidomics studies of the meibomian glands and tear fluid lipid layer for healthy volunteers and dry eye patients have been performed by many groups using numerous different techniques [31–33]. It has been shown that in many cases the dry eye patients have decreased polar lipid to neutral lipid ratio [7]. Neutral lipids like cholesterol esters are known to have a condensing effect on the polar lipid monolayer by interdigitating between the polar lipid molecules. Additionally, it has also been shown that the packing order of the lipids is much higher for dry eye patients than healthy volunteers [9,34] implying increased rigidity of the lipid film and lower film stability leading to the breaking of tear film. In such cases, an increase in the area occupied by the interfacial polar lipid headgroups due to addition of ectoine might facilitate the fluidization of the tear fluid lipid layer thus increasing the stability of the film.

It is worth noticing that the studies were performed at a varied range of concentrations of ectoine beginning from 10 mM to 500 mM, and that the droplet like structures was only observed at very high concentrations of ectoine. The high concentration studies helped us in understanding the influence of ectoine at molecular level and the effect is still present at low concentrations though not in an exaggerated form as at high concentrations.

5. Conclusion

In the present study, we were able to show the surface activity behavior of the meibomian lipid film and compare it to the topological data. Polymorphous fiber-like structures were visible in the topology scans below 20 mN/m surface pressure. As the pressure increased above 20 mN/m, the pressure–area isotherm displayed the formation of a plateau which was accompanied by the appearance of flat structures in the topology scans. These were hypothesized to be the lipid film reversibly folding out of the plane thus preventing an increase in surface pressure. With the addition of ectoine, the surface pressure–area isotherm showed an expansion indicating fluidization of the monolayer. The topology scans showed appearance of droplet-like structures at all surface pressures. These are assumed to be the hydrophobic components of the film which is excluded out due to increased hydrophilicity. Consequently, the fluidization effect of ectoine has been studied and its potential treatment for the dry eye syndrome has been suggested.

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