Biochimica et Biophysica Acta 1859 (2017) 319–330

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

# The effect of repeated lateral compression and expansions mimicking blinking on selected tear film polar lipid monofilms☆

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ARTICLE INFO

*Article history:*
Received 31 July 2016
Received in revised form 17 November 2016
Accepted 15 December 2016
Available online 16 December 2016

*Keywords:*
Tear film polar lipid
Langmuir monolayer
Compression expansion cycles
Brewster angle microscopy
Blinking

ABSTRACT

The tear film lipid layer is formed on the anterior surface of the eye, functioning as a barrier to excess evaporation and foreign particles, while also providing stability to the tear film. The lipid layer is organized into a polar lipid layer consisting of phospholipids, ceramides, and free fatty acids that act as a surfactant to a non-polar multilayer of wax and cholesterol esters. Due to shear forces from eye movement and the compression and expansion of blinking, the tear lipids are under constant stress. However, tear film is able to resist immediate rupture and remains intact over multiple blinks. This work aimed to better understand the lateral organization of selected tear film polar lipids. The polar lipid biomimetic studied here consisted of dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidylethanolamine (DPPE), palmitoyl glucosylceramide (PGC), and palmitoyl sphingomyelin (PSM). Surface pressure-area isocycles mimicked blinking and films were visualized by Brewster angle microscopy (BAM). All lipid systems formed relatively reversible films as indicated by limited hysteresis. However, pure DPPC and PSM films experienced greater changes in lipid packing upon compression and expansion compared to pure PGC and DPPE. This suggests that the driving force behind maintaining the lateral organization of the polar lipids from tear film may be the hydrogen bonding propensities of the head groups. Additionally, isocycles of films containing DPPC, DPPE, and PGC mixtures exhibited evidence for reversible multilayer formation or folding. This was supported by 3D analysis of structures that formed during compression but reintegrated back into the bulk lipid film during expansion near the *in vitro* tear film surface pressure of the open eye. Therefore, the polar lipids of tear film may be directly involved in preventing film rupture during a blink.



## 1. Introduction

Tear film is a protective coating that lines the outermost region of the corneal epithelium and is essential for maintaining healthy eyes [1]. It functions by preventing excess evaporation, acting as a barrier to dust and foreign particles, combating bacterial infections, lubricating the eyelids, and maintaining a proper optical surface for vision [2–4]. These varied functions can be attributed to three semi-distinct layers of tear film. Adjacent to the cornea lies the mucosal matrix which is composed primarily of highly glycosylated proteins known as mucins that act as a barrier to bacterial infection [5]. Moving outwards, the middle aqueous phase contains many soluble antimicrobial proteins along with electrolytes and metabolites [6]. Lastly, the anterior lipid layer is composed of both an outermost non-polar lipid multilayer and a polar lipid layer intersecting the aqueous and hydrophobic phases [7]. The non-polar section primarily contains long chain cholesterol and wax esters [8,9] while the polar lipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), glucosylceramide (GC), lyso-PCs, PE plasmalogens, and (O-acyl)-omega hydroxy fatty acids [10–13]. The distinctly unique lipid layers allow for localized specific functions. For instance, the nature of the non-polar lipids makes them ideal for preventing water evaporation and spillover from the aqueous subphase [14]. The polar lipid fraction stabilizes the entire tear film structure by acting as a surfactant for the non-polar lipids, enabling rapid and even spreading of cholesterol and wax esters across an aqueous surface [15,16].

The lipid layer of tear film is in constant flux from the shear forces of eye movement to the compression and expansion associated with blinking. Following a blink, the tear lipids spread upwards along a surface tension gradient lagging briefly behind the upper eye lid [17]. The lipid organization during respreading was initially hypothesized by Holly who suggested that there were two different fluid lipid phases, a non-polar layer flowing over a polar layer [15]. Coarse grained molecular dynamics simulations further expanded this idea and included free fatty acids acting as mediators between the polar and non-polar lipids

*Abbreviations:* BAM, Brewster angle microscopy; CB, Cerebroside; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; $L_C$, liquid condensed; $L_E$, liquid expanded; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PGC, D-glucosyl-β-1,1′ *N*-palmitoyl-D-erythro-sphingosine; PSM, N-palmitoyl-D-erythro-sphinogsylphosphorylcholine; Rv, isotherm reversability; SM, sphingomyelin.

☆ Dedication: This paper is dedicated to Prof. Fritz Paltauf on the occasion of his 80th birthday.

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http://dx.doi.org/10.1016/j.bbamem.2016.12.010
0005-2736/

[18]. As the eye lids come together during the compression of a blink the lipid layer folds to prevent tear film rupture [16,19]. The lipid folds that protrude out towards the air are thought to primarily contain non-polar lipids, resulting in reduced free energy at high pressures [16]. Similar folding behaviour was observed for extracts exclusively consisting of non-polar lipids [20]. However, every 20 to 50 s or after approximately five blinks the tear film breaks [21,22]. In pathological cases, such as individuals suffering from Dry Eye Syndrome (DES), the tear break up time can be under 10 s [21].

The aim of this work is to better understand the influence of blinking on the lateral organization of the selected tear film polar lipids. These lipids were investigated exclusively because as noted above, research on the molecular organization during blinking has either focused on mixtures of the polar and non-polar lipids [18,23,24] or on the non-polar lipids alone [20,25]. In these studies, surface pressure-area isocycles were recorded for the selected polar lipid species on a buffered subphase mimicking the middle aqueous layer of tear film. The repetitive cycling of compression and expansion isotherms provides a useful approximation of the blinking process. Furthermore, five cycles were performed to elucidate possible lipid packing changes that may occur over multiple blinks. To visualize possible lateral reorganization Brewster angle microscopy (BAM) was used in conjunction with the isocycles. These measurements were performed on a biomimetic model system based on tear film polar lipid compositions found within the literature, comprised of 3 parts 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 3 parts D-glucosyl-β-1,1′ N-palmitoyl-D-erythro-sphingosine (PGC), 2 parts 1,2-dipalmitoyl-*sn*-glycero-3-phospho-ethanolamine (DPPE), and 2 parts N-palmitoyl-D-erythro-sphingosylphosphocholine (PSM) [10,12,13,26]. Individual lipids were investigated first and followed by increasingly complex models including binary, ternary, and quaternary mixtures of the model polar lipids.

## 2. Materials and methods

### 2.1. Lipids and reagents

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), D-glucosyl-β-1,1′ N-palmitoyl-D-erythro-sphingosine (PGC), and N-palmitoyl-D-erythro-sphinogsylphosphorylcholine (PSM) were purchased from Avanti Polar Lipids (Alabaster, AL). NaCl, KCl, $Na_2HPO_4 \cdot 7H_2O$, and $KH_2PO_4$ were purchased from Sigma-Aldrich (Oakville, ON). Lipids were weighed using a Sartorius MC 5 Microbalance (Göttingen, Germany). Stock lipid solutions were dissolved in a 7:3 (*v*/v) mixture of chloroform:methanol to a final concentration of 1.0 mg/mL. Lipid mixtures were prepared from these stock solutions into the binary, ternary, and quaternary models according to the ratio of 3DPPC:2DPPE:3PGC:2PSM (mol/mol). These samples were dried down under argon and stored at −20 °C until further use, where the samples would then be re-solvated in 7:3 chloroform:methanol to a final concentration of 1.0 mg/mL. HPLC grade chloroform and methanol were used as solvents. The water used in the buffer was purified by using a Synergy 185 Millipore with Simpak2 purifying system (Billerica, MA) to a final resistivity of 18.2 MΩ.

### 2.2. Surface pressure area isocycles

Isocycles were performed on a 20 cm × 5 cm Teflon Langmuir trough (Biolin Scientific, Solna). Prior to the isotherm experiments, the trough and a 50 μL Hamilton syringe (Hamilton Company, NV) were cleaned with organic solvents. 20 μL of the lipid sample was added drop-wise onto a subphase of 20 mM phosphate buffered saline (PBS) salts. After a period of 10 min to allow for solvent evaporation, the lipid films were compressed at room temperature at a rate of 96 $cm^2$/min until surface pressures reached 10 mN/m prior to film collapse. The lipids selected have transition temperatures above the surface temperature of the eye (approximately 34 °C) and thus room temperature was sufficient to access the different lipid phases through manipulation of the surface pressure. Collapse of the 15 model systems was determined from previous surface pressure – area isotherms [27]. Films were expanded at a rate of 96 $cm^2$/min until the surface pressure was equal to zero mN/m. Compression and expansion were repeated four more times for a total of five isocycles. For each system, the isocycles were completed in at least triplicate ($n \geq 3$). Measurements were obtained *via* the Wilhelmy Plate technique [28] in which a thin section of filter paper was suspended from a surface pressure sensor (Biolin Scientific, Solna). Forces acting on the plate were then recorded using the KSV Nima software (Biolin Scientific, Solna).

#### 2.2.1. Isocycle analysis

The ability of the film to resist deformation is related to the degree of hysteresis exhibited during the isocycle. Hysteresis describes a system where the internal state depends not only on its current environment but also on its past. Within the context of compression and expansion isocycles, if hysteresis is present the shape of a given cycle will be different between expansion and compression. The hysteresis was quantified by integrating the compression and expansion isotherms and then calculating the isotherm reversibility (Rv) [29]:

$$\mathrm{Rv}(\%) = 100\% \frac{\int \Pi dA \; expansion}{\int \Pi \; dA compression.}$$

where Π is the surface pressure (mN/m), and A is the molecular area ($Å^2$/molecule). Highly reversible films exhibit less hysteresis due to their ability to rapidly restore their lateral organization during expansion. In terms of the Rv value, the larger the percentage the smaller the hysteresis, which therefore equates to a more reversible film.

### 2.3. Brewster angle microscopy

Brewster angle microscopy (BAM) was used for direct visualization of the lateral architecture of lipid monolayers in real time. BAM takes advantage of the principle that p-polarized light incident on a transparent subphase is not reflected at the Brewster angle (approximately 53° for water). Introduction of a monomolecular film onto the subphase surface changes the refractive index, thereby reflecting the p-polarized light into the microscope and allowing the images to be captured by a camera [30]. The Langmuir trough used in the BAM experiments was obtained from Accurion (BAM 601 model, Göttingen, Germany). This Teflon trough has an area of 950 $cm^2$, with a subphase volume of 400 mL. The surface pressure was measured using a 20 mm Wilhelmy Plate and a Nima model PS4 surface pressure sensor (Biolin Scientific, Solna). Both the trough and the microscope were positioned on a Halcyonics anti-vibration system, from Accurion (Göttingen, Germany). Images were obtained using the EP3 View software, version 2.30 also from Accurion (Göttingen, Germany). Each image taken was a compilation of 60 frames with a lateral resolution of 1 μm. The resulting image had a size of 218 μm × 271 μm. All experiments were conducted at room temperature.

## 3. Results

### 3.1. Isocycles of single lipids

The first and fifth compression-expansion isocycles of the single lipid films are presented in Fig. 1. Generally the expansion isotherms followed the shape of the compression isotherms albeit shifted to smaller molecular areas, indicating slight hysteresis. However, DPPE exhibited a shoulder in the 1st expansion trace (Fig. 1-B arrow). Additionally, the hysteresis appeared less for choline containing (Fig. 1-A,D) than non-choline lipids (Fig. 1-B,C). This was confirmed by isotherm