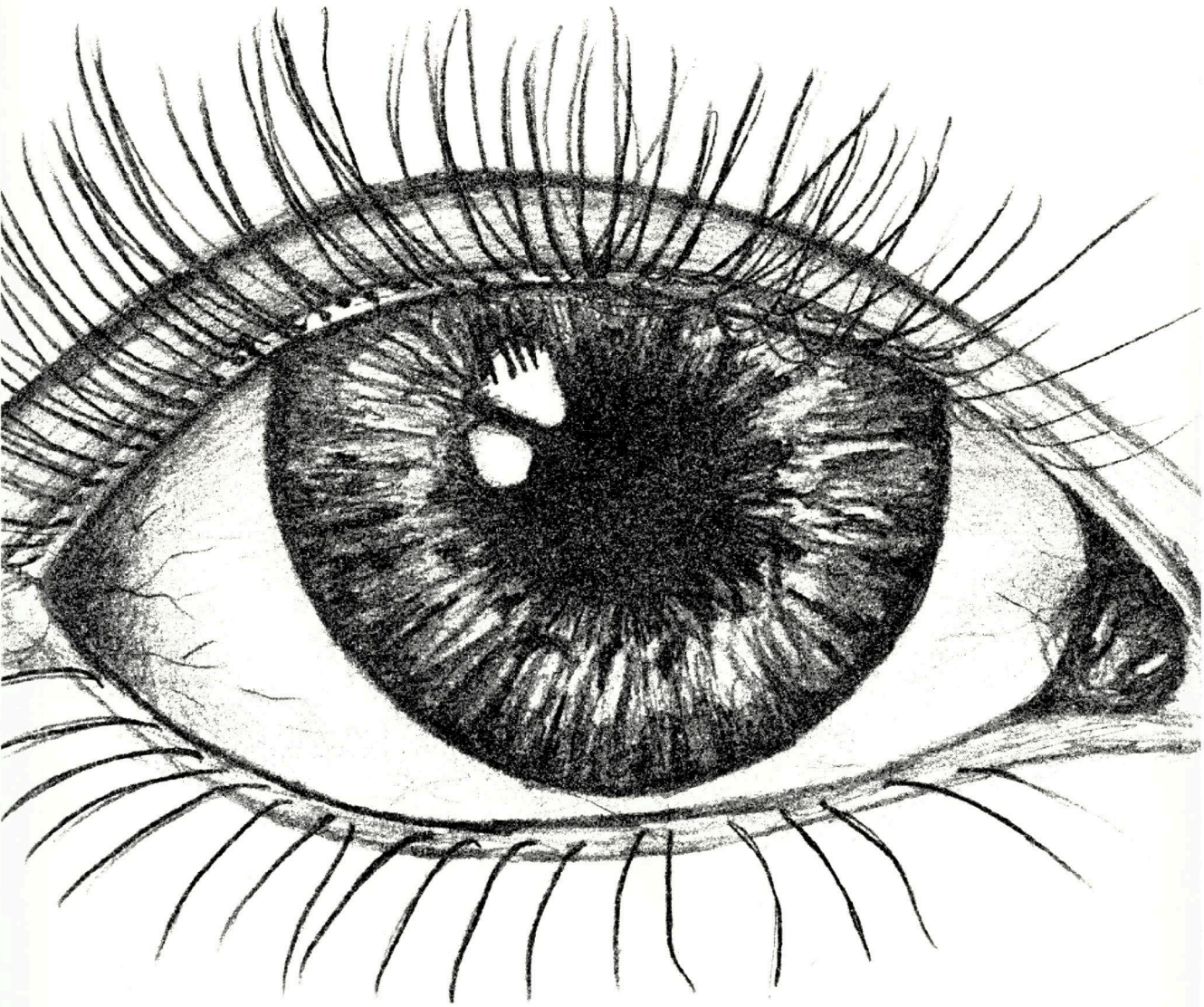
TEAR FILM LIPID LAYER

PIPSA KULOVESI



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Tear film lipid layer

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ACADEMIC DISSERTATION

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This, too, will pass.
Eckhart Tolle

Contents

List of original publications

This thesis is based on the following original publications, which will be referred to in the text by the Roman numerals:

I. Pipsa Kulovesi, Jelena Telenius, Artturi Koivuniemi, Gerald Brezesinski, Antti H. Rantamäki, Tapani Viitala, Esa Puukilainen, Mikko Ritala, Susanne K. Wiedmer, Ilpo Vattulainen and Juha M. Holopainen **2010** Molecular organization of the tear fluid lipid layer. *Biophys J* 99: 2559-2567

II. Pipsa Kulovesi, Jelena Telenius, Artturi Koivuniemi, Gerald Brezesinski, Ilpo Vattulainen and Juha M. Holopainen **2012** The impact of lipid composition on the stability of the tear fluid lipid layer. *Soft Matter* 8: 5826-5834

III. Pipsa Kulovesi, Antti H. Rantamäki and Juha M. Holopainen **2014** Surface properties of artificial tear film lipid layers: Effect of wax esters *Invest Ophth Vis Sci* 55: 4448-4454

Abbreviations

ADDE Aqueous-deficient dry eye
AFM Atomic force microscopy
ATFLL Artificial tear film lipid layer
BAM Brewster angle microscopy
BO Behenyl oleate
CE Cholesterol ester
Cer Ceramide
Chol Cholesterol
CO Cholesterol oleate
DES Dry eye syndrome
DPPC Dipalmitoylphosphatidylcholine
DPPE 1,2-bis(diphenylphophino)ethane
EDE Evaporative dry eye
eggPC Egg-yolk L-alpha phosphatidylcholine
FFA Free fatty acid
GIXD Grazing incidence X-ray diffraction
LA Lauryl oleate
LC Liquid condensed (phase)
LE Liquid expanded (phase)
LI Linolenyl oleate
LL Lignoceryl lignocerate
MGD Meibomian gland dysfunction
OAHFA (O-acyl)-omega-hydroxy fatty acid
PBS Phosphate buffered saline
PC Phosphatidylcholine
PE Phosphatidylethanolamine
PL Phospholipid
POPC 1-palmitoyl-2-oleoyl-sn-glycero-2-phosphocholine
PSD Position-sensitive detector
sIgA Secretory immunoglobulin A
SM Sphingomyelin

TG Triglyceride
TFBUT Tear film break up test
TFLL Tear film lipid layer
TLC Tear lipocalin
TSBA Tear specific prealbumin
WE Wax ester
VMD Visual molecular dynamics

Abstract

The tear film is a thin film with a complex structure that covers the exposed ocular surface. It protects the corneal epithelium from pathogens and helps to keep it moist. It is composed of three intermixed layers. Mucous matrix covers the epithelial cells. It gradually mixes with the aqueous layer that contains mostly water, proteins, and electrolytes. The aqueous film is covered by a thin lipid layer which is composed of polar and nonpolar lipids. Polar lipids, such as phospholipids, are surfactants with polar and nonpolar components. The nonpolar lipids of the tear film lipid layer (TFLL) repel water and are therefore organized on top of the polar lipids. Lipids are thought to prevent the collapse of the tear film onto the ocular surface and to retard evaporation from the tears.

Dry eye syndrome (DES) is one of the most common ophthalmic disorders affecting 10-30% of population at some point in life. It can be caused by a lack of tear production or increased evaporation of the tears. Tear film stability is a key factor in DES as unstable tear film can cause irritation and other dry eye symptoms. A defective TFLL is believed to be the cause of DES, but at the moment there is only a limited understanding of the structure of the TFLL, very little understanding of what might lead to changes in the TFLL, and how these changes link to DES. Therefore it is important to gain better understanding of the structure and dynamic performance of the TFLL.

Several methods were used *in vitro* to study the surface properties and organization of different lipid mixtures. The lipids were chosen to resemble the tear film lipids. Phosphatidylcholine, phosphatidylethanolamine, and free fatty acids were used as the polar lipids and cholesterol oleate, triglycerides, and wax esters were used as the nonpolar lipids. Langmuir film technique was used to examine the behavior of the lipid films during compressions and de-compression. Brewster angle microscopy (BAM) and atomic force microscopy (AFM) were utilized for visualizing the films. Grazing incidence X-ray diffraction was used for surface structure studies. The results of experimental studies were compared with coarse-grained molecular dynamics simulations. Custom built system was used to evaluate the evaporation retarding effect of several lipid mixtures containing wax esters.

Films of single polar lipids or simple mixtures of two or three polar lipids have been studied extensively. However, our understanding of the behavior of complex films involving polar and nonpolar lipids is in its infancy. Although it

is expected that the polar lipids will align at the interface and the nonpolar lipids will integrate with the nonpolar parts of the polar lipids essentially aligning the nonpolar lipids with the air interface, what happens to this arrangement, particularly at high pressures such as would occur during a blink, is unknown. Compression isotherms showed one to two kinks in the compression curve that account for the rearrangement of the lipids in the film for all mixtures studied. Hysteresis was small meaning that these films are very stable and little, if any, solubilization of lipids into the aqueous phase takes place. All lipid films studied were more or less inhomogeneous when viewed with BAM, especially in higher surface pressures. This is most likely caused by the nonpolar lipids aggregating on the lipid film surface. This was also seen in the simulation studies where cholesterol oleate (CO) and triglyceride (TG) formed circular aggregates on top of the polar lipids. Nonpolar lipids stabilized the films under high compression by arranging so that the lipid film could have a lower surface pressure than would be expected for small surface areas. However, an excess of nonpolar lipids caused the fillms to be more inhomogenous and to have less stable structure. Lipid mixtures that contained wax esters did not retard evaporation, which suggests that lipids' function in the tear film may have more to do with maintaining a thin tear film and preventing its collapse rather than preventing evaporation.

A lipid film that contains both polar and nonpolar lipids is a good model for the tear film lipid layer. Polar lipids allow the lipids to spread evenly on the surface whereas the nonpolar lipids provide the film with higher surface pressure tolerance and prevent the collapse of the film. Lipids are co-operative in a manner that allows small changes in constitution not to have a major effect on the bulk properties, however larger changes in the composition can lead to instability. Lipids did not retard evaporation which leads to questioning the role of lipids in the tear film more as stabilizing agents rather than evaporation retardants.

1 Introduction

The tear film covers the cornea and conjunctiva, providing nutrients and moisture to the corneal cells and protecting it from the external environment. It also provides the cornea with a smooth surface, which is important because the tear film, specifically the lipid layer, is the first refractive interface for light on its way to the retina. Alterations in the TFLL can cause changes in refraction. The tear film is described as a three-layered interlaced structure, meaning that although the tear film can be divided into three distinct layers, the layers are gradiental instead of sharp. Mucins, highly glycosylared proteins, create an easily wettable layer covering the corneal epithelium, which is covered by the aqueous layer consisting of electrolytes and several different proteins. At the air-water interface, lipids are organized as a thin film that is thought to retard evaporation. The tear film lipid layer is composed of several different lipid species, both polar and nonpolar. Polar lipids are amphipathic, meaning that they have both polar and nonpolar parts, as is with phospholipids, the most abundant polar lipid in the TFLL.[1] This causes them to organize at the air-water interface with their polar head groups pointing towards the water and nonpolar tails pointing towards the air. The hydrophobic tails integrate with the nonpolar lipids effectively binding a thick layer of the nonpolar lipids at the air-water interface of the TFLL. Nonpolar lipids, such as triglycerides, repel water and therefore often form 3-dimensional structures in aqueous environment to minimize their contact with water and therefore it is energetically favourable for them to be interlaced with the nonpolar tails of the polar lipids. Although the TFLL is most likely to have this general structure, the vast variety of individual polar and nonpolar species in meibum means that the details of its organization are unknown, and in particular how its structure changes or resists change during a blink cycle.

Typically in the physical chemistry domain, lipid films are studied as monolayers and with one or two species. In order to model the TFLL, much more complex mixtures are required. One strategy has been to study meibomian films and test the effects of seeding them with different molecules to determine their tolerance to changes in composition. These are wery complex experiments and difficult to interpret because the exact composition of meibum is unknown. A different approach and the focus of this thesis, is to model the TFLL using a mixture of a small sample of individual lipid species that

represent some of the classes of lipids found in meibum and whole tears. This approach also provides a platform for modelling how the TFLL may prevent evaporation, which is a function that is commonly attributed to the TFLL. However, it should be noted that to date, the *in vitro* experimental evidence supporting that the TFLL is the reason for the film resisting evaporation is lacking.

Therefore, the purpose of this thesis was to create a simplified model of the TFLL to provide a basis for understanding how different lipid classes affect these films with respect to their: ability to retard evaporation; surface physical chemistry; rheology; and molecular organization. The techniques used for these studies included Langmuir through experiments which were complemented with simulation studies to gain information of the films at molecular level as well.

2 Literature review

2.1 The structure of the eye

The eye is a multilayered complex structure. Figure 1 shows the main anatomical features of the eye. The cornea is the outermost part of the eye, followed by the aqueous humor, iris, lens, vitreous chamber, retina, choroid, and sclera. The cornea is the transparent tissue covering the iris and pupil and it is curved with approximately 6 mm radius. The cornea continues with the sclera, a white and fibrous layer that gives the eye its shape and has a radius of about 12 mm. The aqueous humor is filled with clear fluid and is located at two compartments: the anterior chamber between the cornea and the iris and the posterior chamber between the iris and the lens. The colored iris regulates the size of the pupil. The lens is held up by transparent fibers called zonule. The vitreous chamber is filled with a clear gel-like fluid forming the largest volume of the eye. The light sensory cells, rod and cone cells, are located in the retina.

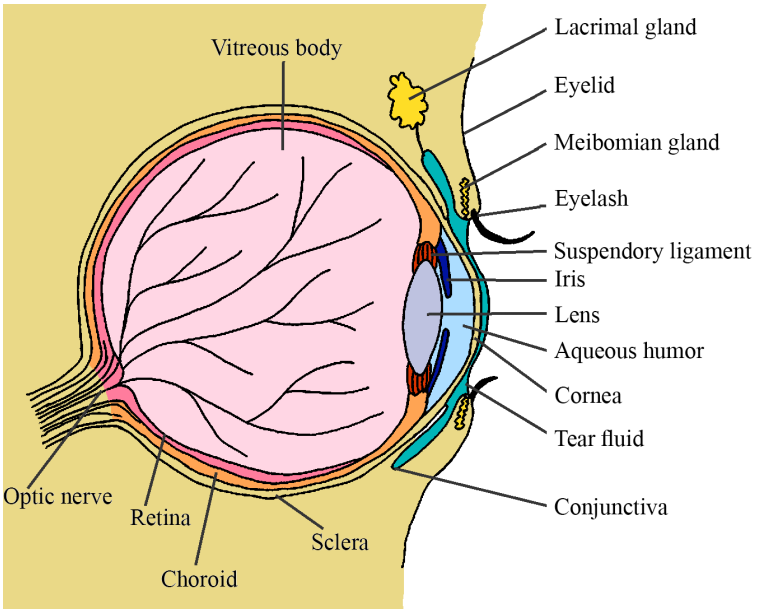

Figure 1: The structure of the eye.

2.1.1 Corneal structure

The human cornea is approximately 520 µm thick from the center and 650 µm at the periphery.[2] A schematic view of the corneal structure is represented in Figure 2. Superficial to the cornea lays the tear film which will be discussed in section 2.2. The cornea is a transparent structure that is composed of five layers: corneal epithelium, Bowman's layer, corneal stroma, Descemet's layer and the corneal endothelium.

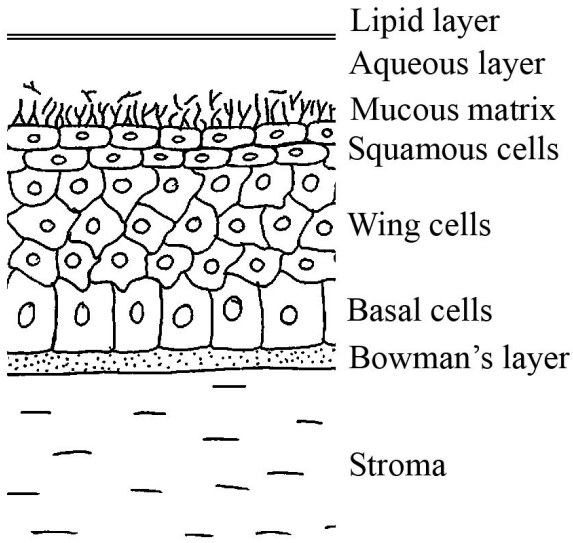

Figure 2: The structure of the tear film and corneal epithelial cells. Not drawn in scale.

The epithelium is comprised of 5-7 cell layers. It covers the anterior surface of the cornea. Epithelial cells are divided into three different cell types; i. Outer surface cells or squamous cells are flat cells, typically approximately 4-6 µm thick with 40-60 µm diameter. They function as a barrier between the tear fluid and the epithelial cells. The outer surface cell layer is constructed of microvilli that makes the surface area much larger and aids with oxygen absorbance.[3] ii. Wing cells are the intermediate cells and they are composed of a cytoskeletal network of intermediate filaments and microtubules. They also consist of free glycogen granules and mitochondria. iii. Basal cells are approximately 18-20 µm high and 8-10 µm wide cells with many filaments, glycogen granules, and scattered ribosomes. The basal cells are the only cells in the epithelia that go through mitosis with a turnaround of approximately 7 days.[3]

The Bowman's layer is a smooth layer (8-12 µm thick) that is made of

collagen type I fibers and keratocytes that are embedded into a matrix of proteoglycan. Its task is to help cornea maintain its shape and to separate the epithelium from stroma. It does not regenerate after injury.[3]

The corneal stroma is a thick layer composed of parallel collagen fibers. It comprises ∼90% of the thickness of the cornea. It is made up of approximately 300-500 layers, each 1.5-2.5 µm thick and is composed mainly of type I collagen fibrils. Some keratocytes also exist in the stroma (comprising 3-5% of the total volume of the stroma) and their task is to maintain the stromal matrix. The extracellular matrix maintains the separation of adjacent collagen fibrils at ∼60 nm. There is little variation in the size and spacing of the collagen fibers which accounts for good optical properties and the clarity of the cornea.

The Descemet's membrane is a thin layer composed mainly of collagen type IV fibrils which are less rigid than collagen type I fibrils. This layer is approximately 5-20 µm thick. The Descemet's membrane is secreted by the endothelium and it consists of an anterior banded layer and a posterior non-banded layer. The Descemet's membrane has been shown to increase in thickness during life from 2 to 10 µm.[4] A separate layer between the corneal stroma and the Descemet's membrane has been suggested to exist by Dua et al.[5] This layer is a thin well-defined acellular layer in the pre-Descemet's cornea.

The corneal endothelium is a monolayer of cells rich in mitochondria. They regulate fluid and solute transport and thus provide means to regulate the nutrition and hydration of the cornea. The endothelial cells do not regenerate, but instead they enlargen and flatten in order to compensate for dead cells.[6] The endothelial cells are the most metabolically active cells in the cornea.

Many cornea-specific dysfunctions exist and affect different layers of the cornea.The epithelium can be affected by the dry eye disease (DES) and physical injuries. Reis-Bückler's and Thiel-Behnke dystrophies as well as several others affect the Bowman's layer or the basement membrane.[7] Granular dystrophy, macular dystrophy and lattice dystrophy all affect the stroma. In stromal dystrophies an abnormal substance is accumulated within the kerotocytes or collagen fibrils.[8] Fuchs dystrophy affects the endothelial cells and in advanced cases results in loss of vision. It causes endothelial cell density to reduce and therefore changes the endothelial morphology.[9] Other dystrophies affecting the endothelial cells are posterior polymorphous corneal dystrophy and congenital hereditary endothelial dystrophy 1 and 2.[10]

2.2 Tear fluid

The tear fluid has many functions. It acts as the lubricator for the conjunctiva, cornea, and the eyelids. It maintains a smooth surface for light refraction, which is important because tears are the first barrier for the light on its way to the retina. Tear fluid also supplies nutrients to the anterior cornea. The posterior cornea is supplied by the aqueous humor. Also the immune responses in posterior cornea come from the aqueous humor. Through the process of blinking, foreign materials trapped in the tear film are mechanically removed from the ocular surface. Tear film protects the ocular surface by responding to stress situations such as bright light, low temperature, physical injury, chemicals, and pathogens.[11] Tear fluid enters the corneal surface from lacrimal glands, Meibomian glands and conjunctiva.[12] It is distributed to the surface of the eye and mixed by blinking and lost by outflow from tear ducts and evaporation.[12]

The tear fluid is commonly described as a 3-layered structure, but it can also be considered as a gradiental fluid. The tear film is approximately 3 μm thick and consists of the following layers.[13] i. Adjacent to the corneal epithelia is the mucous area with several different mucins. ii. On top of that lays the aqueous layer that contains ions and proteins. iii. At the air-water interface lays the lipid layer which contains various lipids. A more detailed description of the components of the tear fluid is provided in the following sections.

2.2.1 Mucous matrix

The mucous matrix is the layer adjacent to the epithelial cells. It consists of many different types of mucins. Mucins are large glycosylated proteins that often form gels. Mucins are hydrophilic and 50-80% of their mass consists of O-linked glycans. Tear fluid mucins consist of both membrane-associated mucins and secreted mucins.[14,15] Mucins MUC1, MUC4, and MUC16 are membrane associated mucins found in tear fluid, whereas MUC5AC is the major secreted type mucin found in the tear fluid although MUC2 exists at low levels in the tear fluid as well.[14,16] Mucins function as the wetting agents in the tear fluid by providing a surface and properties for the water to be on.[15] One of the functions of mucins has been said to be a barrier for pathogens by offering adhesion points for microorganisms, thus protecting the epithelium from the direct interaction with the contaminants.[16]

2.2.2 Aqueous layer

The aqueous layer of the tear film consists of water, electrolytes, carbohydrates, peptides, and proteins.[17] It also contains phospholipids bound to

proteins,[18] and it is also speculated that it may contain lipid micelles. Tear fluid has high protein concentration, with a total protein concentration as high as ∼10 mg/mL.[19] It includes over a thousand different proteins such as lipocalin, lysozyme, lactoferrin, secretory immunoglobulin A (sIgA), albumin, and lipophilin.[20,21] Most tear fluid proteins are secreted by the lacrimal glands (see Figure 1) but some serum proteins are present in the tear fluid as well as some proteins secreted by the epithelial cells.[22] Although most proteins in tear film are hydrophilic, there are also hydrophobic surfactant-associated proteins found in tears. These proteins, SP-B and SP-C are tightly bound to phospholipids and play important role in maintaining the surface tension-lowering properties. They can also be found in lungs where their role as pulmonary surfactants is important.[23]

Lysozyme is one of the most common tear proteins, constituting approximately 20-40% of the total tear protein.[11] The lysozyme concentration in tears is higher than it is in any other body fluid. Lysozyme is also found for example in saliva and nasal mucus. Normal tear lysozyme levels vary between 0.6-2.6 mg/mL. Lysozyme is the most alkaline protein in the tears, with an approximate size of 14 kDa. It is a single chain polypeptide with 129 amino acid residues. It is an enzyme that catalyses the lysis of bacterial cell walls, particularly Gram negative bacteria. It has been proposed to stabilize the tear film by decreasing the surface tension of the tears by adsorbing and penetrating the meibomian lipids.[24]

Lactoferrin is another alkaline antibacterial protein found in tears. It can also act as a scavenger for free radicals.[11] Lactoferrin concentration has been measured to be above 1.6 ng/mL in tears.[11] It is an iron-binding protein and its structure is closely related to serum transferrin but it is mostly found in other secretions such as milk. It can bind to other proteins such as lysozyme and synergize with them allowing the iron saturated form to be active as well as the non-saturated form. It also has antiviral properties.[25] It is believed to regulate inflammation at the ocular surface.[26]

Tear lipocalin (TLC) is another protein found abundantly in tears. It is an acidic protein that can bind and transport hydrophobic molecules such as fatty acid ligands.[11] It can also bind to other proteins such as lactoferrin and lysozyme.[27] Lipocalin has many functions in tears. It has been proposed that TLC would scavenge harmful lipids from the cornea and conjunctiva, and from the tear film.[28] Other roles for TLC such as viscosity control, anti-inflammatory, antibacterial, and antimicrobial functions have been proposed as well.[27]

2.2.3 Lipid layer

A thin lipid layer lays on top of the aqueous layer. The tear film lipid layer thickness has been measured to be between 15-157 nm with a mean of 42 nm.[12] The variation between the values is due to different measuring techniques, and its thickness varies between individuals and during blink cycles. Due to their hydrophobicity the lipids are organized at the air-water interface. Tear fluid lipids consist of both polar and nonpolar lipids. Most lipids in the TFLL are derived from the meibomian glands that are located in the upper and lower eyelids, and their orifices are located in the lid margin.[29]

Lipid composition in the tear fluid has been vastly studied, but because of the large number of different lipid types in low concentrations, it is still under discussion. There are basically two different ways to study the lipid composition in the tears: the actual tear fluid can be studied by obtaining the tears from the lower meniscus of the eye. Another way is to study the meibum which is the secretion from the meibomian glands taken from the lid margin. The most common lipids in both tear and meibum samples are cholesterol esters and wax esters.[30] Analysis of whole tears has snown large variation between individuals in the TFLL composition even among normal subjects but significant differences was seen with patients that suffer from dry eye syndrome or blepharitis.[31]

2.2.3.1 Tear film lipids

Tear film lipids can be studied from the tear samples or directly from the meibum. The meibum can be collected using a spatula and pressing the eyelids to express meibum. With this method large amount of meibum can be gained. Micro capillary pipettes are used for collecting tears as well as the meibum. Paper strips such as Schirmer strips are used for tear collection by placing them over the edge of the lower eyelid. The lipids are collected from the strips by extraction. Paper strips or swabs can also be used for meibum sampling.

The constitution of tear film lipids varies somewhat from the meibum constitution. There are more polar lipids (mainly phospholipids) in the tear fluid samples than in the meibum samples. A reason for this is still under debate. Table 1 presents the most common lipid species found in the meibum and tear fluid samples. As can be seen in the table 1, WE and CE are the most common lipids in both tear fluid and meibum samples detected in these studies.[1,30–32] Rantamäki et al. showed them only using thin layer chromatography and therefore offered no quantitative analysis of these nonpolar lipids. Therefore the percentage values of this study also vary greatly from other studies but if the missing WE and CE were taken into account the ratios would be similar to the other studies. OAHFAs have been the major

polar lipids class detected in meibum and represent ∼4% of the total lipids. In whole tears, the major polar lipid class are the phospholipids representing ∼10% of the total lipids. It is not known, but theoretically possible that both surfactant lipid classes contribute to the TFLL.[33]

Table 1: Lipid species found in tear fluid (Brown 2013, Rantamäki 2011, Lam 2014) and meibum (Pucker 2012 and Brown 2013) as percentage from all detected lipid species.

Lipid	Brown 2013[30]	Rantamäki 2011[1]	Lam 2014[32]	Pucker 2012 meibum[31]	Brown 2013 meibum[30]
CE	39	+	44.82	0-65	44
WE	43	+	35.21	25-68	52
TG	2.1	6	2.84	0-9	1.5
OAHFA	4.4		2.52		3.1
PC	12	69	2.96	0-14.8	0.006
PE		19	1.99		
SM		3.3	1.50		
Cer		3.4	0.26		
FFA				0-10.4	
Chol			5.94		

2.2.3.2 Meibum

The meibum is an oily secretion that is produced by the meibomian glands, located in parallel rows at the lid margin. The meibum contains a mixture of various lipids but also salts, and other compounds.[34] Lipid types found in meibum include WEs, cholesterol esters, di- and triacylglycerols, (O-acyl)-ω-hydroxy fatty acids (OAHFA), free cholesterol, free fatty acids, and phospholipids.[34] Majority of the Meibomian lipids are nonpolar, but ∼4% of total lipids found in meibum consists of OAHFA, an amphipathic molecule reported to be an important part of the TFLL acting as a surfactant among with the phopsholipids in the TFLL.[33]

The meibomian glands secrete meibum to the ocular surface. They are modified sebaceous glands, located at the upper and lower eyelids.[35] There are 30-40 glands in the upper eyelid and 20-30 in the lower eyelid.[36] The glands excrete meibum constantly, while blinking aids in the spreading of the lipids. The melting range of meibum is between 22.5-45°C.[31] The corneal temperature is approximately 32°C, but environmental factors, such as wind and temperature, might affect the corneal temperature and therefore could also change the properties of the lipids, fluidity as an example.[37]

Figure 3 shows a cross-section of the lid rim and meibomian glands adapted from Sappey 1853.[38] The meibomian glands are vertical straight channels and they have 10-40 lateral ducts in their sides that lead to single or composite acini.[39]

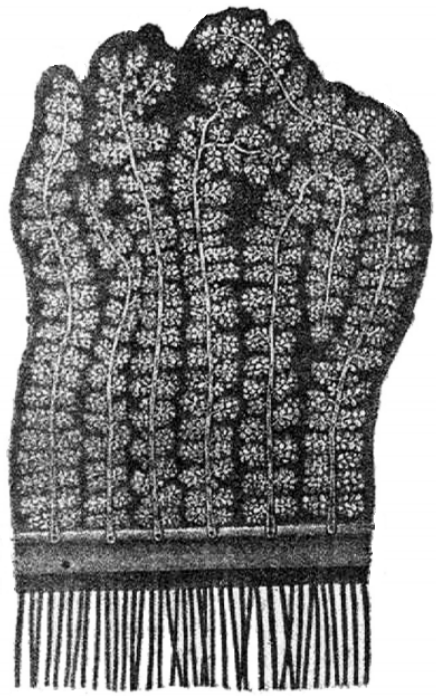

Figure 3: The structure of Meibomian glands at the lid margin. Adapted from Sappey 1853[38]

2.3 Tear fluid properties

2.3.1 Viscosity

Viscosity is an important property of the tear film because tear fluid must stay in its place under duress. Mucins, as described in section 2.2.1, are able to produce solutions with high viscosity, but they do not account for the overall viscosity of the tears due to their low amount in tears.[40] Tear fluid is a non-Newtonian fluid meaning that its flow properties differ from Newtonian fluids. Newtonian fluids are fluids that have a linear relationship between viscosity and shear stress, meaning that their viscosity remains constant when force is applied to them, for example when mixing. Water is a common example of a Newtonian fluid, it flows the same way whether a force is applied to it

or not. Non-Newtonian liquids are either shear-thinning or shear thickening. Tear fluid is a shear-thinning fluid meaning that when force is placed upon it, it gets thinner.

When the eye is open or its movements are slow, a shear-thinning tear fluid has high viscosity which is important to prevent it from drainage and film break-up.[41] On the other hand, when the eye is closed and the shear rate is high, the shear-thinning properties cause the viscosity to lower which prevents the damage to the epithelial surface which could be caused by highly viscous fluid.

2.3.2 Surface tension

Surface tension is a measure of interfacial forces in the surface of a liquid. It is caused by intermolecular forces between molecules. These forces are directed towards the bulk phase which causes the surface molecules to be drawn towards the interior as the surface seeks the minimum area. Van der Waals forces are an example of these interfacial forces. They are induced by attractive interactions between nonpolar molecules, caused by the presence of dipoles in the molecules. Surface tension can be reduced by the use of surfactants, which are surface active molecules. Tear fluid has relatively low surface tension, between 35-40 mN/m, compared to water 72.8 mN/m at 20°C.[42,43] This surface tension is caused by the high amount of surface active molecules. Proteins were suggested as a major factor affecting the surface tension to be low.[44] However, tear film surface tension is relatively complex quality to measure, as it is caused by multiple factors and their interactions in tears, including lipids, proteins including mucins. Measuring the tear film surface tension often requires pooled samples, which limits its usage in measuring individual variations. Nevertheless, some indication that surface tension measurement of tear samples might be a useful clinical tool for determining dry eye comes from studies of Tiffany et al 1989 who showed that tears from dry eye patients had a surface tension ($\sim$50mN/m) that was$\sim$6mN/m higher than those from normal subjects.[45] It should be noted that the technique used meant that these measurements were likely to represent the surface tension of the aqueous, not the aqueous covered by a lipid layer.

2.3.3 Tear film thickness

The tear film is estimated to be between 3-10 μm thick for humans.[46] There are many methods for measuring tear film thickness and the results vary between the methods used. Tear film thickness depends on the phase of the blink and also the measuring location. Most useful area to measure the tear film thickness is the central cornea. Quite recently Werkmeister et al

used ultrahigh-resolution optical coherence tomography to measure tear film thickness with great reproducibility and got an average of 4.79 $\pm$ 0.88 μm for the central tear film thickness.[47]

2.3.4 Blinking

Blinking is necessary for ocular health. It protects the eye by sweeping irritants away from the surface of the eye towards the lacrimal ducts. It aids in spreading the tears and therefore has an important role in corneal hydration. When the eyes are closed, tear fluid is contained in the conjunctival sac and when the eyes are opened the tear fluid is re-distributed between the conjunctival sac, pre-ocular tear film and menisci.[37] Blink duration and speed varies and depends on the health of the eye. On average, a blink lasts approximately 300 ms and adults blink in average 12 times per minute.[37,48] Blinking speed has been measured to be 250 mm/s when closing eyes and 160 mm/s when opening the eyes.[48] Tear film lipid layer spreading speed has been measured to be between 3.49-9.83 mm/s and the lipid film stabilizes approximately 1 s after a blink.[37] Interestingly, the lipid layer of the tear film is maintained same after a series of blinks with only small mixing. This phenomena was detected by studying the interference patterns of the TFLL which was preserved over several blinks and then abruptly the pattern changed completely when extensive mixing happened between the lipid film and marginal reservoir.[37] Therefore it has been suggested that the TFLL is compressed when the lids are closed and reformed when the lids are opened. The lipid film has been shown to spread upwards over the aqueous surface when the upper lid rises.[37]

2.4 The properties of lipids

A definition of a lipid is a natural organic molecule that is soluble in nonpolar organic solutions such as chloroform or ether but is very poorly soluble in water. Lipids vary greatly in their structure and properties. Most common lipids found in nature are fatty acids, mono-/di-/triglycerides, waxes, sterols, and phospholipids. Lipids have many biological functions. They form cellular membranes. Triglycerides form the major component of fat stores in both animals and plants. Lipids also function as signaling molecules and are an important part of cell signaling pathway. Vitamins A, D, K, and E are lipids that are essential for many bodily functions.

Lipids are divided into nonpolar and polar molecules. Nonpolar lipids are hydrophobic. Polar lipids on the other hand often have a polar group and nonpolar group/groups. They are amphipathic molecules as they have both polar and nonpolar qualities. Amphipathic molecules can form different

structures in water or can act as surfactants. They can also form a monolayer at the air-water interface.

2.4.1 Lipids at the air-water interface

Lipids cannot be studied as individual molecules since they always interact with nearby molecules and form complexes such as micelles, vesicles, films etc. The properties of lipid monolayers have been studied for over a century. Lord Rayleigh showed already in the 19th century that when oily substances were spread on top of water, they formed a monolayer.[49] The behavior of lipids at the air-water interface depends on the type of the lipid. Nonpolar lipids tend to form droplets when they are placed in aqueous environment which is demonstrated in Figure 4 B. Because they are hydrophobic, they try to minimize their contact with water molecules, and therefore tend to pack together. Polar lipids on the other hand organize in a manner that their polar head groups are adjacent to the water and nonpolar tail/tails point towards the air. This process often produces a monolayer of polar lipids but also multilayered structures are possible. Figures 4 A-C show sketches of polar, nonpolar and mixed lipids at the air-water interface.

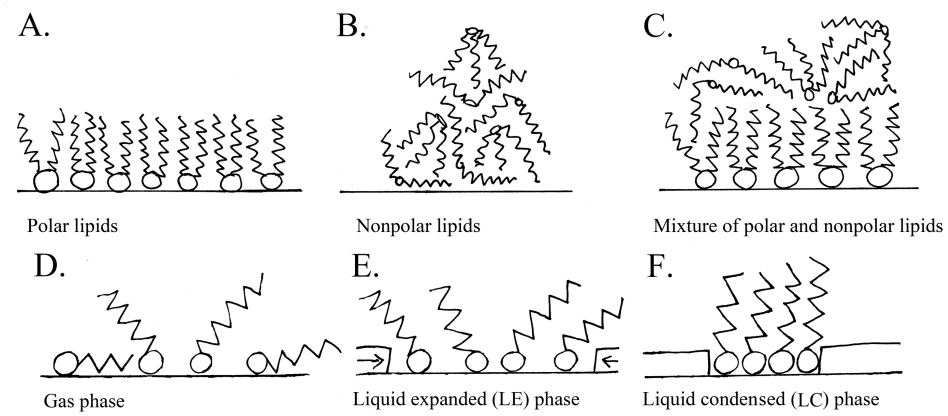

Figure 4: A schematic view of lipids at the air water interface. A. Polar lipids B. Nonpolar lipids C. A mixture of polar and nonpolar lipids. D. Gas phase E. Liquid expanded phase F. Liquid-condensed phase.

Lipid organization in aqueous solutions depends on many factors. Molecules tend to organize into 3D-aggregates in a way that the free energy of the system is minimal. This means that for example triglycerides, which are highly hydrophobic molecules, pack into circular, unstable, and rather large aggregates to minimize their contact with water molecules. Phospholipids, on the other hand, have large head groups relative to the acyl chains, and therefore

will form planar bilayers with their polar head groups adjacent to the water, whereas their acyl chains are shielded by the head groups to minimize the exposure to water. In lipid mixtures, the more hydrophobic molecules are embedded deeper into the hydrophobic core of the 3D-aggregates whereas the hydrophilic molecules form an umbrella-like shield to these molecules.

When a monolayer of polar lipids is spread on top of water, their alignment depends on the area available for them. In gas phase the lipids can move freely and their acyl chains can point to any direction. Loosely arranged lipids are in liquid expanded phase (LE) where the acyl chains have room for moving, but a slight compression is forcing them to be more organized than in the gas phase. When the film is compressed up to a certain point (phase transition site) the film undergoes a phase transition where the acyl chains re-arrange themselves and a kink can be observed in the compression isotherm. This phase where the acyl chains are pointed upwards is called liquid-condensed phase (LC). Figures 4 D-E illustrate the gas phase, LE and LC phases using a simple polar lipid surface. The phase behavior gets more complicated when different lipid species are mixed.[50]

2.4.2 Evaporation retarding lipids

Tear film lipids are believed to retard the evaporation of water from the tears. Although this is generally regarded as a fact, there is little evidence to prove this. *In vivo* studies on rabbits and human have shown relatively large evaporation retardation, but in these studies it is difficult to determine what is the effect of lipids compared to other substances in the tear fluid.[51,52] Evaporation retardation of 6-8% has been measured for meibum samples in physiological temperature *in vitro* which is much less than *in vivo* studies have shown.[53,54] Fatty acids retard evaporation and up to 60% decrease in evaporation has been seen using long chain alcohols, hexadecanol, and octadecanol.[55] Wind and impurities of the film disrupt the evaporation retarding effect.

There are two main characters for evaporation retarding lipids. First, the lipid's hydrocarbon chains should be saturated.[56] Saturated chains can pack tighter than the unsaturated chains, and therefore prevent water molecules from going through the film more efficiently. Second character is the length of the hydrocarbon chain, lipids with longer chains retard evaporation more than shorter chained ones.[57] Archer and La Mer discovered that evaporation retardation is an exponential function of chain length.[57]

A well-ordered structure is required for a thin film to be able to retard evaporation. This kind of structure of tightly packed molecules is an effective barrier to water permeation.[55,58,59] *In vitro* studies performed with WEs have shown that they are able to retard evaporation only at or near their

melting points.[58] At the melting temperature WEs are able to form a well-ordered uniform layer on the water surface, and therefore they are able to retard evaporation.[59] Mixing other lipids with WEs has shown to disrupt the evaporation retardation effect which can be explained by the fact that mixed lipid films are not as ordered as pure WE films can be, which causes the water to permeate the film easily.

2.5 The physiological role of the tear film lipid layer

Lipids have been shown to have multiple roles in the tear film, for instance:

1. They prevent the tears from overflowing and work as a dam to the tears. This is caused by the elasticity of the tear fluid.

2. They decrease the friction of the lid-ocular surface interface. Tear film lipids are more viscous than water and therefore they guide the outflow of tears through the naso-lacrimal duct.

3. They produce a smooth layer on top of the tear film and therefore improve the refraction of the light.

4. They enable the formation of a thin tear film.

5. They retard evaporation from the water phase.[51]

All these physiological functions are caused by the unique features that lipids have.

2.6 Dry eye syndrome

Dry eye syndrome (DES) is a multifactorial disease that has varying forms. It compromises the patients' quality of life, not only because of the symptoms, but because the treatment often requires the patient to apply eye drops frequently, which can be inconvenient. It may be somewhat difficult to diagnose because there is no single clinical test to find out whether a person has DES or not. It has also been reported that less than 60% of the patients with DES are symptomatic.[12] The symptoms may also vary between the eyes of the patients and the measuring time. This makes it rather difficult to diagnose DES in some cases. The most common clinical symptoms of DES are the instability of the tear film which leads to rapid breakup between blinks, the composition of tears differs from normal and tear osmolarity is elevated.[12]

Tear film stability can be measured with a tear film breakup test (TFBUT). It measures the time that is required for a random break to appear in the tear film after a blink. Tear breakup does not normally happen between blinks

in healthy individuals but DES patients may have a tear breakup time of 2-3 seconds or less. As tear film begins to break up there is a spike in tear osmolarity which further instabilizes and dries the tear film.[12]

The prevalence of the DES is very high, approximately 5-30% of the people aged 50 or over.[60] Dry eye syndrome is divided into aqueous deficient dry eye (ADDE) and evaporative dry eye (EDE) although a combination of these two subtypes is also possible. Aqueous deficient dry eye is caused by reduced lacrimal secretion meaning that an inadequate amount of tears is produced by the lacrimal glands. It is found to be the major reason for DES in 10% of the DES patients. More common DES type is EDE where tear evaporation is increased from normal. There are intrinsic ways and extrinsic ways for the evaporation to be increased. Intrinsic ways include insufficient Meibomian oil delivery, often regarded as Meibomian gland dysfunction (MGD), low blinking rates, and low blinking speed. Meibomian gland dysfunction is the most common cause of DES. It is associated with a decrease of TFBUT, lid margin abnormalities, and corneal/conjunctival staining and increase of dry eye symptoms (itching, burning, and grittiness).[61] It also causes the meibum constitution to change. Meibum becomes more viscous and has more cholesterol esters when compared to normal.[31] Extrinsic factors affecting the evaporation rate are contact lens wear, topical use of anesthetics and preservatives and vitamin A deficiency among others.[12]

There are several factors that contribute to DES; age, gender, low humidity conditions, sex hormones, computer use, and contact lens wear for example.[60,62] Dry eye syndrome is more prevalent in females, which may be partly contributed to the use of hormone replacement therapy. However, older people have higher prevalence of DES than younger ones which is due to decreased androgen production. There are also many environmental and lifestyle factors that affect the prevalence of DES. High room temperature and low humidity can cause DES. Many people have symptoms when traveling in the aircraft where the humidity can be less than 20%. Wind and air-conditioning, that causes air convection, can also contribute to the DES. Wearing contact lenses is also a common cause for DES. Some autoimmune diseases, such as Sjögren's syndrome, which affects the lacrimal and salivary glands, can cause DES as well. Refractive- and cataract surgeries, as well as glaucoma therapies, have been shown to cause DES in some patients.[12]

Lipids are believed to retard evaporation from the tears as discussed in section 2.4.2. Therefore, their role in DES, especially in EDE, may be significant. It has been shown that patients that suffer from DES can have variations in their tear film lipid profile. The amount of polar lipids was decreased in blepharitis patients which has been suggested to cause TFLL instability.[63] Dry eye patients also had less saturated fatty acids than healthy subjects which may also cause instability in the tear film as the lipids with

unsaturated chains are unable to form tightly packed structures.[64]

Dry eye syndrome is often treated with eye drops. They must be applied frequently, and can cause further problems such as alterations in the permeability of the ocular surface epithelium. Benzalkonium chloride, a common preservative, is known to be irritative and to cause oxidative damage to corneal and conjunctival epithelial cells and to affect the behavior of the TFLL.[65] Jee and co-workers studied the difference between preserved and preservative-free eye drops and found out that the preservative-free drops were more effective in decreasing ocular inflammation and in increasing antioxidant contents.[66]

2.6.1 Blepharitis

Blepharitis is one of the most common ophthalmic disorders. Blepharitis, as well as DES described in the previous section, is a multifactorial disease with varying forms of severity and type. Generally, it can be described as a chronic inflammation of the eyelid. Blepharitis can be divided into two subcategories: anterior and posterior. Anterior blepharitis is often caused by bacterial overgrowth or activity of sebaceous glands or both. There typically is excessive colonization of normal lid bacteria and inflammation with symptoms such as burning of the eyelids, eyelid irritation, crusting and stickiness but it can also occur asymptomatically.[67] Posterior blepharitis is almost always caused by meibomian gland dysfunction. The most common clinical features in posterior blepharitis/MGD are changes in meibomian gland secretions, an obstruction of the meibomian glands, a deficiency of aqueous tears and excess lipid secretion.[68]

3 Aims of the study

The main aim of this thesis was to provide insight into the structure and function of tear film lipid layer by developing an *in vitro* model using various lipid mixtures. The goals were:

1. To provide information of the structure of the tear film lipid layer using artificial lipid mixtures.
2. To determine the effect of polar and nonpolar lipids on the properties of the tear film lipid layer.
3. Evaluate the evaporation retarding effect of various lipid mixtures that contain wax esters.

4 Methods

4.1 Compression isotherms

Compression isotherms were measured using a Langmuir Mini Through (KSV Nima, Helsinki, Finland). Phosphate buffered saline (PBS: 0.140 M NaCl, 0.0027 M KCl, pH 7.4) was used as a subphase in all experiments. The surface pressure was measured with 20 mm paper probe (studies I & II) or a platinum plate (study III.). Sample was placed on the surface using a Hamilton syringe and the solvent was allowed to evaporate for 10 minutes before starting the compressions. Compression cycles were performed at 21°C (I, II & III.) and also at 35°C (III.). For the 35°C experiments, the temperature was controlled with a thermostat (Lauda Eco E4, Köninghofen, Germany) and measured with a thermometer placed in the subphase. Compression rate was 10 mm/min and the film was compressed up to a surface pressure of ∼40 mN/m and then relaxed back with the same rate.

Equation 1 can be used to obtain isothermal compressibilities (C_s) from the molecular area vs. surface pressure data.[69] A surface area per molecule (A) at the surface pressure (π).

$$C_s = -\frac{1}{A} \times \left(\frac{dA}{d\pi}\right)_\pi \tag{1}$$

The data was analyzed further using the reciprocal of isothermal compressibilities (C_s^{-1}).

$$C_s^{-1} = -A \times \frac{dA}{d\pi} \tag{2}$$

The data gained from these measurements was smoothed using 8 point adjacent averaging.

4.2 Oscillating barrier studies

Oscillating barrier studies were performed with Langmuir film balance system. The film was first compressed to a surface pressure of 30 mN/m. Then the film was subjected to small periodical compressions using the barriers as the oscillator. Frequency (f) varied between 6.7 to 200 mHz and amplitude ($\Delta A/A_\pi$) was kept constant at 1% of the surface area. Surface pressure (π) was monitored. The surface dilatational modulus (E) was obtained from

equation 3 where E_d is the dilatational elasticity and E_v is the surface dilatational viscosity.

$$E = -\frac{d\pi}{d\ln A} = E_d + iE_v \tag{3}$$

The tangent of the loss angle (θ) can be calculated with equation 4.

$$tan\theta = \frac{E_v}{E_d} \tag{4}$$

For purely elastic films the loss angle tangent is zero.

4.3 Evaporation studies

Evaporation studies were performed using a custom-built system based on a Langmuir balance (KSV Nima, Helsinki, Finland). The pool (273 cm^3) was placed in a cabinet that protected the trough from wind and contaminants. Airflow of 1.7 L/s was used through the cabinet to minimize humidity variation daily. The temperature of the pool was controlled with a thermostat (Lauda Eco E4, Köningshofen, Germany) and the temperature in the subphase was measured with a thermometer connected to the Langmuir balance unit. A preheated pool was filled with a pre-measured amount of buffer and the lipid mixture was carefully placed on top of the buffer. The solvent was allowed to evaporate for 10 minutes and after that the cabin door was closed and the water was allowed to evaporate through the film for 80 minutes more. After the total of 90 minutes, the buffer was collected and the amount left after evaporation was measured. Evaporation rate calculations were based on the difference between the buffer amounts before and after the evaporation.

4.4 Brewster angle microscopy

A KSV NIMA MicroBAM was used to examine the organization of the lipids at the air-water interface. Brewster angle microscopy (BAM) is based on p-polarized light that is angled towards the water at a certain angle, Brewster angle (for water 53°). No reflection takes place with a blanc surface, but when there is a thin film on top of the water, light is reflected to the detector. Varying densities can then be detected according to the amount of light at the detector. All Brewster angle measurements were performed at 21°C in this study.

4.5 Grazing incidence X-ray diffraction

Grazing incidence X-ray diffraction (GIXD) was used for investigating the lateral structures of lipid monolayers at the air-water interface. GIXD mea-

surements were performed at BW1 beamline in HASYLAB (DESY, Hamburg, Germany). BW1 uses a monochromatic X-ray beam (λ = 1,304 Å) that strikes the water surface at a grazing incidence angle $\alpha_i = 0.85\alpha_c$ (α_c = 0.13°) and illuminates approximately 2× 50 mm^2 of the surface. Thermostated (20°C) Langmuir trough was placed into a sealed container that was filled with helium. Wilhelmy plate was used to monitor lateral pressure and one movable barrier was used to compress the film to the wanted surface pressure. During the experiment, the trough was laterally moved to avoid the sample to be damaged by the X-ray beam. Diffraction was measured with a linear position-sensitive detector (PSD) (OEM-100-M, Braun, Garching, Germany) that was rotated to scan the in-plane Q_{xy} component values of the scattering vector. PSD vertical channels measured the out-of-plane component, Q_z of the scattering vector between 0.0 and 0.9 Å^{-1}.

4.6 Atomic force microscopy

A multimode V with Nanoscope V controller (Veeco Instruments, Santa Barbara, CA) was used for atomic force microscopy (AFM) studies. A phosphorus-doped silicon probe (RTESP) (Veeco Instruments) was used for measuring the samples in tapping mode, in air. Langmuir-Blodgett technique was used to transfer the lipid films from the air-water interface to the freshly cleaved mica. Before the transfer, the lipid film on the Langmuir trough was compressed and relaxed twice and then set to the transfer surface pressure. The mica substrate was elevated through the air-water interface at a rate of 2 mm/min while the surface pressure was kept constant. Several scans were made from different parts of the samples to check the uniformity of the surface. All films were scanned within 24 hours of transfer.

4.7 Molecular dynamics simulations

Molecular dynamics simulations were used to gain information of the lipids organization at the air-water interface in molecular level. All the simulation studies and their analysis was performed by Jelena Telenius. Lipid mixtures that were used in the molecular dynamic simulations were similar as the ones in the *in vitro* studies. The molecules were chosen to be POPC or DPPC as PC, DPPE as PE, CO, trioleate as TG and palmitic acid and oleate acid were chosen as FFA for the studies I and II. The systems were described using the coarse-grained representation in the terms of the Martini model.[70,71] The simulations were carried out with the use of GROMACS software package (Ver. 4.0.2.). Simulation temperature was maintained at 305 K (∼32°C) with the Berendsen temperature coupling using the time constant of 0.3

ps.[72,73] GROMACS analysis tools (Ver. 4.0.4.) was used for the calculations from the simulation data for radial distribution functions, mass densities, order parameters and other physical observables. Visual molecular dynamics (VMD) software was used to visualize the simulation results.

5 Results

5.1 Compression isotherms

In study I a mixture of eggPC, TG, CO, and free FA was used as a model for the tear film lipid layer (here called the artificial tear film lipid layer (ATFLL)) and it was compared with an eggPC lipid film. Although this mixture does not represent all of the classes of molecules known to be present in meibum, they were chosen in part because of their commercial availability and hence consistency, and also to keep the model system relatively simple. By doing this, the methods for exploring these lipid films could be thoroughly explored and the data obtained would provide a platform for further modelling. The compression isotherms as well as the compressibility curves are shown in Figure 1. The compression of the pure eggPC film showed a monotonous increase in the surface pressure-area curve. Minimal hysteresis was observed as well, but no sign of phase transitions or layering could be seen. On the other hand with ATFLL, a kink was observed at ∼18 mN/m which indicates that a phase transition or possibly some folding/re-arranging occurs in the film. There was also slightly more hysteresis seen for the ATFLL than for the pure eggPC film. Both films were compressed to a surface pressure of >40 mN/m and no film collapse occured.

The possible phase transition, folding, or other re-arrangement of the film was more clearly seen in the compressibility curve plotted on figure 1.The $C_S{}^{-1}$ values were lower for the ATFLL monolayers than for the eggPC films. Low $C_S{}^{-1}$ values indicate the film to be in the liquid-expanded phase with typical values between 10 and 50 mN/m. Liquid condensed phase would have values above 100 mN/m.[74]

Study II focused on finding differences between lipid mixtures with polar and nonpolar components. The compression isotherms of two samples and pure eggPC are shown in Figure 2. Mixture 1 contained 40% of PC, 30% of CO and 30% of TG and it resembled the highly nonpolar mixture whereas mixture 2 contained 40% of PC, 40% of PE and 20% of TG and represented the highly polar mixture. Mixture 2 had very similar compression behaviour as pure eggPC and showed no sign of phase transitions or re-organizing whereas mixture 1 clearly had 2 distinct kinks at the compression isotherm, shown in Fig. 2.

More complex lipid mixtures were introduced in the study III by adding

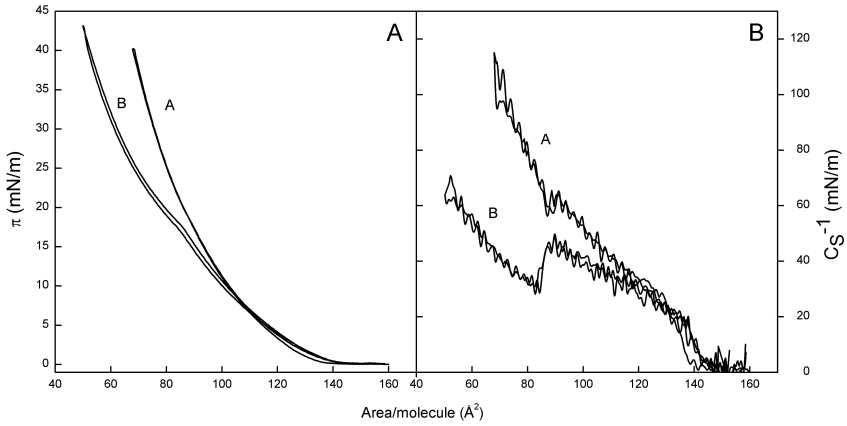

Figure 1: Compression isotherms (A) and compressibility curves (B) for study I. A = pure eggPC films, B = ATFLL films (PC:FFA:CO:TG 6:2:1:1 mol%)

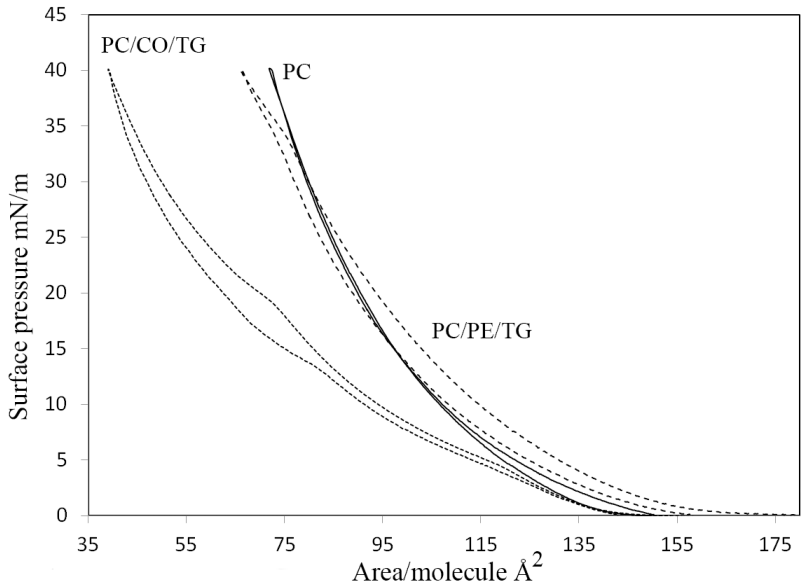

Figure 2: Compression isotherms for study II. Pure eggPC, PC:CO:TG 4:3:3 mol%, and PC:PE:TG 4:4:2 mol%

different wax esters in with PC, CO and TG. The wax esters that were used in this study were behenyloleate (BO 22:0/18:1), lignoceryllignocerate (LL

24:0/24:0), linolenyloleate (LI 18:3/18:1), and lauryl oleate (LA 12:0/18:1). The compression isotherms of all these WE-mixtures behaved very similarly. All the mixtures had one kink at the compression curve which was also seen in the compressibility curves. See Figure 2, p. 4450 in the original article III.

5.2 Evaporation studies

Evaporation studies were performed in study III where the effect of wax esters was examined. The evaporation retardation effect of several mixtures was studied but none of these mixtures retarded evaporation significantly. The evaporation rate was measured to be 10.96-11.29 μm/min which does not differ from the measurements from the blank interface (11.06 μm/min). Figure 1 in original article III p. 4450 shows the evaporation rate of all the mixtures studied.

5.3 Rheological studies

The rheological properties of the lipid films were studied using the oscillating barrier method that was used to gain information of surface dilatational rheological properties such as surface dilatational moduli (E), its elastic (E_d) and viscous (E_v) components. In study I it was shown that at lower frequencies eggPC monolayers present elastic behaviour and at higher frequencies (f $>$ 25 mHz) they behave viscoelastically. Artificial tear film lipid layer behaviour was more complex than pure eggPC. These films had a lower value of E at all frequencies and are therefore viscoelastic throughout the frequency range studied.

In study II, similar behaviour was observed. In addition the lipid films with an excess of nonpolar lipids had lower values of the rheological parameters than pure eggPC and other mixtures studied.

5.4 Grazing incidence X-ray diffraction

Grazing incidence X-ray diffraction was used to detect the monolayer homogeneity and lipid packing behaviour. Bragg peaks were not detected for pure eggPC films because of the fluidity of the films which causes them not to show long range ordering. In study I, the ATFLL mixture showed a clear Bragg peak and a weak hint of a halo. The halo could only be observed in the precense of CO, which means that CO had a condensing effect on the film. Cholesterol oleate concentration was only 10% which caused the halo to be weak. The mixture only showed one Bragg peak which leads into a conclusion that the condensed part of the monolayer exhibiting ordering

should be a homogeneous mixture. The Bragg peak arrises from the mixture of FFA and/or TG. More mixtures were investigated in study II with similar results. Mixtures that contained CO had a halo that is caused by formation of liquid-ordered domains within the monolayer. Because of a rather large unit cell area (29 $Å^2$) the lipid chains are still in a fluid state. No halo was observed for samples that did not contain CO.

5.5 Brewster angle microscopy

Brewster angle microscopy images showed varying degree of inhomogeneous lipid layers for all lipid mixtures studied. Pure eggPC however showed homogeneous BAM images. This means that all mixtures studied have more or less multilayered structures. The differences between similarly behaving films could best be seen in study III, where WEs were studied. Although their surface properties and rheological properties remained similar for each different WE and for all concentrations their BAM images showed relatively large variations, seen in Figure 3.

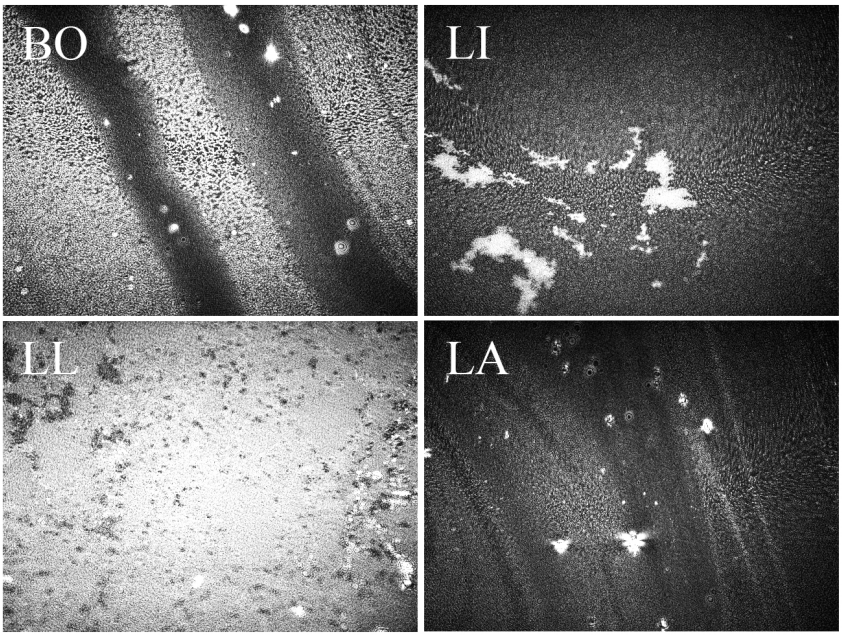

Figure 3: Brewster angle microscopy for 40% WE containing mixtures from study III, taken at 30 mN/m. BO-Behenyl oleate, LL -Lignoceryl lignocerate, LI - Linolenyl oleate, LA - Lauryl oleate. All mixtures also contained PC, CO, and TG.

5.6 Atomic force microscopy

Atomic force microscopy (AFM) was used in study I to complement the BAM results and to give insight into the three-dimensional organization of the lipid films. The lipids were transferred to mica substrates from the air-water interface at surface pressures of 20 and 30 mN/m. EggPC films were relatively homogeneous in both surface pressures and they showed no evidence of multilayered structures. On the other hand, ATFLL films showed clear multilayered aggregates on the surface of the lipid membrane as well as inhomogenous areas. This complements the inhomogenous areas seen in the BAM images.

5.7 Simulations

Simulation studies were performed using a coarse-grained model that is based on Martini model 2.[71] The surface pressure in the simulations ranged from 15-50 mN/m. The structure of the lipid layer was highly dependent on the surface pressure. In study I a mixture of PC, FFA, TG, and CO (55:16:9:8) was used as a model of tear film lipid layer. PC and FFA were located at the air-water interface with the choline group of the PC deepest into the water phase. PC and FFA showed no substancial difference between different surface pressures. CO and TG on the other hand clustered together in hemispherelike structures in higher surface pressures, see Fig 4. This cluster of lipids was constructed on top of the PC/FFA layer.

In study II, several different lipid mixtures were studied. Pure PC layer folded towards the water phase in higher surface pressures before collapse. The mixture of PC/CO (9:1) formed a monolayer where COs were located between PCs and the ester bond regions of the CO were exposed to the water phase. Decreasing the area per molecule caused some CO molecules to be excluded from the water-lipid interface but no phase separation could be observed. Comparing PC/CO systems with PC/TG it was seen that TG is removed from the PC-water interface in lower surface pressures than CO. The system with PC/CO/TG (4:3:3) formed a planar lipid layer in lower surface pressures but formed island-like clusters in higher surface pressures. It was also seen that when the ratio between the polar lipids and nonpolar lipids was low, the lipids were not able to cover the entire air-water interface. In these occasions, the nonpolar lipids formed a separate phase surrounded by PC molecules in tube-like structures.

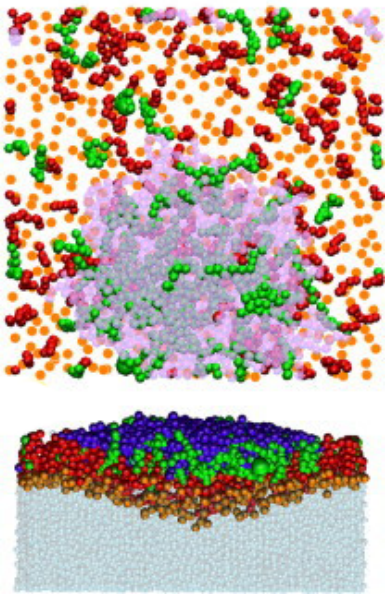

Figure 4: A snapshot of a coarse grained simulation in study I, illustrating the CO and TG clusters on top of the PC/FFA film taken at molecular area of 42.1 $Å^2$. Orange - PC headgroups, pink/blue - TG, green - CO, red - FFA (For PC only the headgroup is shown, whole molecule is shown for all other lipids)

6 Discussion

The motivation behind this thesis was to determine if a number of techniques could be combined to develop models of the TFLL, so that initially simple models could be built to help determine critical parameters that might influence the performance of the TFLL. These lipid mixtures resemble the TFLL and the aim was to understand the physiological role of lipids in the tear film. Relatively simple lipid mixtures composed of the most common lipid classes in the tears and meibum were used to gain information about how different individual lipid species affect the film properties.

These studies started with phospholipids based on the traditional views of the TFLL and some earlier studies on whole tears, and the commercial availability of these lipids, which enables control over one of the variables. By contrast using meibum will have variability from sample to sample. These studies perform as a platform for further modeling using more complex lipid mixtures. In study I a mixture of PC, CO, TG, and FFA was used and it was compared with pure eggPC films. Study II focused on simple mixtures of two to four components per mixture, aiming to understand the effect that nonpolar lipids have on the film. The main focus of study II was on the effect that individual lipid species have on the film and whether changing the polar lipid PC to PE would have an effect on the film properties or not. In study III the mixtures were somewhat more complex by an addition of WEs. The aim was to find out how WEs with different melting points affect the film properties and also to test the evaporation retardation effect that TFLL has been claimed to have. The results showed that these lipid mixtures are a good starting point for modeling the TFLL. These films showed the dynamic behavior of the lipid films that can be compressed and decompressed to even rather high surface pressures, which is an important property of the TFLL. We also provided a platform for studying such lipid films *in silico* with our simulation studies that complimented the experimental results.

The Langmuir film techniques and other experimental methods used in this study are excellent methods for investigating the properties of lipid films. What must be taken into account when interpreting the results of these studies is that these methods do not offer information of the individual lipid species behavior at the molecular level. To complement the experimental studies and shed light on the nanoscale features of the TFLL, molecular level simulations were used. Simulations can be performed in atomic level,

where each atom is its own particle or at a coarse grained model. Atomic level simulations are very precise and give very detailed information but it is often unnecessary to simulate in such detailed way, and it is more expensive and time-consuming than the coarse grained model. In coarse grained simulations, multiple atoms are represented as one particle. This allows larger scales and faster simulations. Simulation studies have their limitations and comparisons between simulation studies and experimental studies must be carefully performed. Simulation results are not directly comparable with the experimental results because of the scale difference between these two studies. The most distinct differences are the time scale and the size. Simulations were performed for 500-1100 ns, or until the equilibrium of the film. This is much shorter time than the experimental studies took. Another clear scale difference is the amount of molecules. Simulation studies consisted of 792 molecules in limited space whereas experimental studies were performed in much larger scale.

6.1 The structure of the tear film lipid layer

Tear film lipid composition has an important role in the function of the tear film lipid layer and in the tear film itself.[1] The main function of the TFLL is to reduce the surface tension of the tears and also possibly to retard evaporation. The structure of the TFLL is classically seen as a two-layered structure with polar lipids at the air-water interface and hydrophobic nonpolar lipids making up the bulk of the lipid layer on top of the polar lipids. Polar lipids provide a good means for the lipids to spread uniformly and rapidly on top of the tear film whereas nonpolar lipids are thought to account for the evaporation retarding effect of the tear film.

Compared to the classical, a rather static view of the TFLL, this thesis suggests that TFLL has a very dynamic structure. When expanded it has a monolayer-like structure with all lipids evenly spread throughout the surface but when placed under compression two distinct layers could be detected. We looked for the dynamic changes that occur during a blink cycle by modelling and investigating the films at different surface pressures.

Brewster angle microscopy of WE-containing ATFLL films showed different patterns depending on the WE used. Films containing low-melting point WE appeared fluid, while condensed areas were apparent in films with high-melting point WE. The amount of condensed regions appeared to increase with increasing wax ester content. BAM images of meibum films have also shown either fluid or condensed appearance depending on the study and experimental conditions.[75–77] Although the ATFLL films have similarities in appearance to meibum films, it is currently difficult to determine, which characteristics correspond to the TFLL in physiological conditions. Atomic

force microscopy studies of meibum showed similar aggregation of lipids into layered structures as our ATFLL films showed, however further comparison or conclusions are impossible to make.[78,79]

This thesis project also showed that mixtures containing more than 30% of nonpolar lipids showed a distinct phase transition in their compression isotherms regardless of the exact composition of the mixture. This phase transition could be explained by either a formation of condensed monolayer or by the rearrangement of the film into a multi-layered structure. Because the surface pressure and surface area appeared to be dependent more on the total amount of nonpolar lipids in the film rather than the composition of the nonpolar lipids, it is more likely that the nonpolar lipids are forming three dimensional structures rather than a condensed phase in higher surface pressures. This view was also supported by the simulation studies where nonpolar lipids formed hemisphere-like structures on top of the polar lipids at high surface pressures. This kind of tolerance to changes in lipid composition has also been seen with *in vitro* meibum films. Mixing meibomian lipids with waxes did not alter the surface pressure-area isocycle profiles much, meaning that meibomian lipids are tolerant to even rather large changes in their composition.[80] Adding phospholipids to meibum films caused them to achieve higher surface pressures than pure meibum films.[77] OAHFA had the similar effect on meibum films as phospholipids, suggesting that they might act as surfactants in the TFLL among with the phospholipids.[33]

The rheological properties of the film, elasticity and viscosity, were very similar in all mixtures studied, which leads to the conclusion that they are mainly dependent on the polar component of the film. Only phospholipids were used in these mixtures as polar components and therefore the films only differed by their nonpolar lipid fractions. Nonpolar lipid composition had no effect on the rheological properties which was caused by the rather high surface pressures that were used while conducting the oscillating barrier measurements, being 30 mN/m. At these surface pressures the nonpolar lipids had left the surface and being on top of the polar lipids they did not contribute to the rheological behavior of the films. Because of this stabilizing effect, the nonpolar lipids may have physiologically important properties in the tear film, such as affecting the tear film break-up time. An increase in the amount of nonpolar lipids may not cause a major effect in the films lateral dilatational properties as long as there is enough polar lipids to allow for the evenly spreading TFLL. Nonpolar lipids on the other hand, affected the films behavior by stabilizing them and allowing for compression into smaller surface areas in low surface pressures because of the layered structure that the films took when compressed. Because of this stabilizing effect, the nonpolar lipids may have physiologically important properties in the tear film, such as affecting the tear film break-up time.

Dilatational elasticity and viscosity values measured for the ATFLL were of the same order with those measured for human meibomian lipid films.[81] However, whereas the rheological properties of meibomian films show only weak frequency-dependence,[81] the viscosity of ATFLL films increases with increasing frequency, similar to pure phospholipid films.[82] This is likely caused by the larger fraction of polar lipids present in the ATFLL compared to pure meibum. In this respect the ATFLL might be a better representation of the TFLL, since tear film has been shown to contain a significantly larger fraction of polar lipids comed to pure meibum.[30]

6.2 The evaporation retardation of the tear film lipid layer

The tear film lipid layer is thought to retard evaporation from the tear film. This is based on several *in vivo* experiments that have shown the evaporation to be multiple orders of magnitude slower from the ocular surface than from the air-water interface *in vitro*.[83] The weakness of *in vivo* experiments is that they cannot show the effect that only lipids have for the retardation of evaporation because the evaporation could not be measured without the lipid layer and therefore the effect could be caused by other substances in the tears as well.

The evaporation retardation effect of the TFLL has not been widely investigated *in vitro* and the mechanism of it is still unclear. However, the properties that define the evaporation-retardation effect in one-lipid-species monolayers are well established.[84] The evaporation retardation of thin fatty acid and fatty alcohol films have shown to retard evaporation up to 60%.[85,86] These films are almost incompressable and if compressed their surface pressure rises rapidly because the molecules arrange themselves in highly condensed lipid layers already at low molecular areas. This is because their head groups are small and there are only low intermolecular forces between them. No major reorganization occurs in the film upon compression. This kind of films would not be optimal for the tear film lipids because they need to be able to withstand compression and expansion during blinking without undergoing irreversible collapse. One mechanism proposed for combining evaporation retardation with compression resistance is the "multilamellar sandwich model" by King-Smith and co-workers,[56] which proposes that the TFLL forms reversible folds under compression. However, this model still awaits experimental support.

The diffusion of water through a monolayer is controlled by van der Waals interaction between the hydrocarbon chains. The evaporation can be retarded when there is little free volume between the molecules. Decrease in

molecular area causes the surface pressure to rise whereas the hydrocarbon chain tilt decreases and therefore the molecules pack tighter and monolayer becomes thicker and more ordered. This has been shown to increase the evaporation retarding effect.[55] A sum of individual resistances has been shown to be the total evaporation retardation when both of the individual molecules retard evaporation.[86] However, when one ore more of the mixed substances do not have evaporation retarding properties, the evaporation retarding effect is lost already in mixtures that contain more than 10% of other substances.[58] This was seen with WEs, a major component of tear film lipids. Certain wax esters retarded evaporation near or at their melting point area but when mixed with other lipids that do not retard evaporation, this property was lost. Another recent study showed that a specific temperature-dependent order in WEs is required in order for them to retard evaporation.[59] It was also shown that the thickness of the lipid layer had no effect on the evaporation retarding effect in the range below 1 micron. This leads to the conclusion that the individual lipid properties and ordering at the air-water interface are the defining factors of the evaporation retarding effect.

To conclude, based on this thesis project and other studies investigating similar lipid mixtures,[58,87] TFLL does not retard evaporation which is also in agreement with latest *in vitro* studies that have shown meibum to only retard evaporation by 6-8% in physiological temperature.[53,54] Films that resemble TFLL in structure and composition differ drastically from the films that are capable of restricting evaporation leading into a conclusion that their role in the tear film must be reconsidered.

7 Summary and Conclusions

This study focused on the tear film lipid layer properties using artificial lipid mixtures that resemble the tear film lipid layer. In study I a mixture of PC, CO, TG, and FFA was used as an artificial tear film lipid layer and it was compared with a film that only contained a polar PC. A clear difference between the surface properties of these films was detected. The ATFLL film was more compressable and showed a distinct kink in the compression curve where the film rearranged itself. Simulation studies showed how the nonpolar CO and TG formed spherical clusters on top of the polar lipids when the molecular area was low. Study II showed how nonpolar lipids stabilize the lipid film by allowing compressions to smaller molecular areas, mainly because of the reorganization of nonpolar lipids on top of the polar lipids in the film. Even though study III used more complex lipid mixtures by an addition of WEs, their main surface properties remained similar to the films in studies I and II. This cooperative behavior supports the role of polar lipids as the component determining the surface behavior of the films whereas the nonpolar lipids stabilize the film and allow them to be more adaptable to changes in surface pressure.

The task of TFLL is still under debate, although it has been regarded to be the evaporation barrier, hindering the movement of water molecules out from the tear fluid. This, however has been questioned in this thesis project. Although some WEs have been shown to retard the evaporation of water, this effect is lost when different lipid species are mixed, as was done in this study where several WE species were mixed with PC, CO, and TG. No evaporation retarding effect was detected for any mixtures studied. Evaporation retarding lipid films must be very tightly packed but this is not possible for TFLL-like films because the tear film lipids must also be suitable for the environment they are in where they must adapt to fast changes in surface pressure and must also be fluid. This cannot be achieved by the films that have been shown to retard evaporation as these films are rather stiff and cannot be compressed.

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