Surface behavior of anti-evaporative tear film wax esters

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# Master’s Thesis Abstract

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## Abstract

The anterior surface of the eye is covered by a thin tear film, which lubricates and protects the ocular surface as well as provides a smooth optical interface for light to enter the eye. The outermost layer of the tear film is a lipid layer produced mainly by the Meibomian glands. Tear film lipid layer (TFLL) is thought to stabilize the tear film and prevent the ocular surface from drying by retarding evaporation of the aqueous tear fluid. A deficient TFLL leads to increased evaporation and is a common cause of dry eye syndrome. Dry eye syndrome is a disease of the ocular surface that causes discomfort, disturbance of vision and possible damage to the ocular surface. It is one of the most common diagnoses among ophthalmologic patients, with tens of millions of people suffering from moderate-to-severe symptoms worldwide and many more from milder or more periodic symptoms.

The molecular organization responsible for the evaporation retarding properties of TFLL is not well understood. Some lipids, like saturated fatty acids and fatty alcohols are known to be efficient evaporation retarding lipids. However, TFLL consists mostly of wax esters, cholesteryl esters and unsaturated polar lipids, which have not been typically considered to be effective in retarding evaporation. According to recent studies, pure wax ester films do retard evaporation, but only close to their bulk melting temperature.

In this Master’s thesis, the properties of behenyl palmitoleate (BP), a wax ester closely resembling the most abundant wax esters found in tear fluid, were studied at the air-water interface. The aims of the study were to characterize the phase behaviour of BP at the air-water interface and determine the molecular basis of its evaporation retarding properties. Isotherms and isochores were measured, coupled with imaging by Brewster angle microscopy. In addition, the evaporation resistance of BP films were measured.

BP was found to exist in a fluid state that spreads efficiently but does not retard evaporation and a solid state that does not spread but efficiently retards evaporation. Approximately 3 °C below bulk melting temperature, solid and fluid monolayer phases coexist, allowing a solid monolayer to cover the water surface. Furthermore, BP was found to assume an extended conformation in the solid phase, which allows tight packing of the molecules and prevents the permeation of water. Taken together, these results provide a molecular level explanation for the evaporation retarding properties of wax esters several degrees below their bulk melting temperature.

**Keywords**
- Tear film, tear film lipid layer, evaporation, wax ester, monolayer, lipid, Meibomian gland, dry eye, ocular surface, isotherm, isochor, Brewster angle microscope

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CONTENTS

1. ABBREVIATIONS AND SYMBOLS ........................................................................... 6

2. INTRODUCTION ........................................................................................................... 8

3. REVIEW OF THE LITERATURE .................................................................................. 9

   3.1 Ocular surface physiology .................................................................................... 9
       3.1.1 Cornea ............................................................................................................. 9
       3.1.2 Conjunctiva .................................................................................................... 11
       3.1.3 Eyelids ........................................................................................................... 12
       3.1.4 Tear film ....................................................................................................... 13

   3.2 Dry eye syndrome .................................................................................................. 14
       3.2.1 Etiology ......................................................................................................... 14
       3.2.2 Epidemiology ............................................................................................... 16

   3.3 Lipids at the air-water interface ......................................................................... 17
       3.3.1 Polar lipids .................................................................................................... 17
       3.3.2 Non-polar lipids ........................................................................................... 19
       3.3.3 Evaporation resistance .................................................................................. 21

   3.4 Tear film lipid layer ............................................................................................... 22
       3.4.1 Composition ................................................................................................... 22
       3.4.2 Structure and thickness .................................................................................. 23
       3.4.3 Evaporation resistance of TFLL ................................................................. 24
       3.4.4 Models of TFLL ............................................................................................ 24

   3.5 Wax esters ............................................................................................................. 27
       3.5.1 Occurrence and functions in nature ............................................................... 27
       3.5.2 Physical properties ....................................................................................... 28

4. AIMS OF THE STUDY ................................................................................................. 31

5. MATERIALS AND METHODS .................................................................................... 32

   5.1 Experimental setup ............................................................................................... 32
5.1.1 Surface pressure measurement ................................................................. 32
5.1.2 Brewster angle microscopy ................................................................. 32
5.2 Materials ................................................................................................. 33
5.3 Methods .................................................................................................. 34
  5.3.1 Isochor measurements ...................................................................... 34
  5.3.2 Isotherm measurements ................................................................... 34
  5.3.3 Thickness measurements .................................................................. 34
  5.3.4 Evaporation measurements ............................................................. 36
6. RESULTS ..................................................................................................... 39
  6.1 Isochor measurements ......................................................................... 39
  6.2 Isotherm measurements ....................................................................... 40
  6.3 Thickness measurements ..................................................................... 41
  6.4 Evaporation measurements ................................................................. 43
7. DISCUSSION ............................................................................................... 44
  7.1 Approach and experimental methods .................................................. 44
  7.2 Surface behavior of wax esters .............................................................. 46
    7.2.1 Effect of temperature ................................................................... 46
    7.2.2 Conformation ............................................................................... 48
    7.2.3 Evaporation resistance ................................................................. 50
  7.3 Implications for the function of TFLL .................................................... 53
  7.4 Conclusions .......................................................................................... 54
8. ACKNOWLEDGEMENTS .......................................................................... 55
9. REFERENCES ................................................................................................. 56
## 1. ABBREVIATIONS AND SYMBOLS

**Abbreviations:**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ADDE</td>
<td>aqueous deficient dry eye</td>
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<td>BAM</td>
<td>Brewster angle microscope</td>
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<td>BP</td>
<td>Behenyl palmitoleate</td>
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<td>CE</td>
<td>cholesteryl ester</td>
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<td>DES</td>
<td>dry eye syndrome</td>
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<td>EDE</td>
<td>evaporative dry eye</td>
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<td>HP</td>
<td>hexadecyl palmitate</td>
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<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>Immunoglobulin M</td>
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<td>IL-1α</td>
<td>interleukin-1α</td>
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<tr>
<td>IL-1β</td>
<td>interleukin-1β</td>
</tr>
<tr>
<td>IL-8</td>
<td>interleukin-8</td>
</tr>
<tr>
<td>JLWE</td>
<td>jojoba-like wax ester</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>MGD</td>
<td>Meibomian gland dysfunction</td>
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<tr>
<td>MMA</td>
<td>mean molecular area</td>
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<tr>
<td>MMP-9</td>
<td>matrix metalloproteinase-9</td>
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<tr>
<td>OAHFA</td>
<td>(O-Acyl)-omega-hydroxy fatty acid</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<td>SE</td>
<td>standard error</td>
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<td>TFLL</td>
<td>tear film lipid layer</td>
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<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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<td>WE</td>
<td>wax ester</td>
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Symbols:

\( C \) water vapor concentration
\( d \) film thickness
\( E \) evaporation reduction (proportional to pure water surface)
\( F \) force
\( G \) Gibbs energy
\( H \) Enthalpy
\( J \) evaporation rate (per unit area)
\( l \) perimeter
\( n(\text{CH}_2) \) number of \( \text{CH}_2 \)-groups
\( n_i \) refractive index of material \( i \)
\( R \) evaporation resistance
\( R_p \) reflectance (p-polarized light)
\( S \) spreading coefficient
\( S \) Entropy
\( T \) temperature
\( \theta \) contact angle
\( \theta_i \) angle of incidence
\( \pi \) surface pressure
\( \Phi \) area fraction covered by a solid monolayer
\( \gamma \) surface tension
2. INTRODUCTION

The anterior surface of the eye is covered by the tear film, a thin film of fluid, which provides a smooth surface for light to enter the eye, lubricates the ocular surface and provides protection against pathogens and debris. The outermost layer of the tear film is a lipid layer, which is thought to stabilize the tear film and prevent evaporation of the tear fluid.1

Dry eye syndrome (DES) is a disease characterized by disturbances of the tear film that result in discomfort, disturbance of visual function and in severe cases, ocular surface damage2. It is a common condition, with tens of millions of people suffering from moderate-to-severe symptoms worldwide and many more from milder or more periodic symptoms3. Evaporative dry eye is the most common type of DES and it is typically caused by a deficient tear film lipid layer (TFLL) that is unable to prevent the evaporation of aqueous tear fluid2,4. The most commonly used therapy for treating DES is using artificial tears to supplement the tear film5. An ideal treatment would supplement the TFLL and restore its evaporation retarding function, but development of such products is hindered by the fact that TFLL structure and function are still poorly understood.

Certain types of lipids are known to retard evaporation of water when spread to the water surface. Efficient evaporation retarding lipids include saturated fatty alcohols and fatty acids. TFLL consists mostly of unsaturated wax esters (WEs) and long-chain cholesteryl esters (CEs)6–9, and its composition does not match the current view of evaporation retarding lipids. However, recently WEs were found to retard evaporation close to their bulk melting temperature10.

In this Master’s thesis behenyl palmitoleate (BP) was used to model tear film WEs. The surface behavior of BP was studied using Langmuir film methods. Isochors and isotherms were measured coupled with imaging by Brewster angle microscopy (BAM). In addition, evaporation reduction caused by BP films was measured. The results provide a molecular level explanation for the ability of WEs to retard evaporation close to their melting temperature and provide insight on the possible evaporation retarding mechanism of TFLL.
3. REVIEW OF THE LITERATURE

3.1 Ocular surface physiology

The anterior surface of the eye consists of the cornea, the principal optical element of the eye, and the conjunctiva, an epithelium covering the sclera and inside of the eyelids. Ocular surface is lined by the upper and lower eyelids and covered by a thin tear film. A schematic representation of the anatomical components of the ocular surface discussed in this chapter is shown in Figure 1.

3.1.1 Cornea

The cornea is transparent tissue that forms the central part of the ocular surface. Light enters the eye through cornea, and it is the main refractive structure of the eye, with a typical refractive power of 48 diopters. In addition to the refractive function, it also provides tensile strength and protection from external factors. Cornea consists of six layers: epithelium, basement membrane, Bowman’s layer, corneal stroma, Descemet’s membrane and endothelium.

Corneal epithelium is the outermost layer of the cornea and it consists of five to seven layers of stratified, non-keratinized, non-secretory epithelial cells. Innermost cells are basal cells attached to the underlying basement membrane, which consists of structural proteins and proteoglycans like collagen, laminin, heparin sulfate, fibronectin and fibrin. Basal cells undergo mitosis and the daughter cells move toward the surface and become increasingly differentiated, until they degenerate and are sloughed off, resulting in a turnover of the whole epithelium in seven days. Outermost layers of epithelial cells, called superficial cells, are joined by numerous desmosomes and tight junctions, forming an effective semipermeable membrane to the surface of the cornea.

Bowman’s layer is a 12-µm-thick acellular layer consisting of collagen fibers located just beneath the basement membrane. Beneath Bowman’s membrane lies the stroma, which constitutes about 90% of corneal thickness. Stroma consists primarily of extracellular matrix, which contains proteoglycans and lamellar arrangements of collagen running parallel to the corneal surface. By dry weight, 71% of corneal stroma consists of collagen, mainly type I isotype. Flattened keratocytes that maintain the extracellular matrix and nerve axons with their surrounding Schwann cells are found between the collagen bundles.
Figure 1. A schematic representation of the anatomy of ocular surface and the structure of tear film. Different components are not drawn to scale.

The collagen fibers are 30 nm thick and are spaced 64 nm apart. This close spacing is the basis of corneal transparency, because only fluctuations in refractive index over distances larger than 200 nm cause significant scattering of light. Many pathological conditions disturb the normal tight packing of the collagen fibers leading to opaqueness of the cornea.⁴

Posterior surface of the cornea is covered by endothelium, lying on a basement membrane called Descemet’s membrane. Endothelium consists of a single layer of polygonal cells. Endothelial cells are connected by tight junctions and gap junctions but form a leaky barrier allowing flow of water and solutes between aqueous humor and the stroma in contrast to the corneal epithelium. Endothelial cells are amitotic and their density decreases throughout life. Descemet’s membrane is secreted by the endothelial cells and consists of type IV collagen, laminin and fibronectin, providing physical durability for the endothelium.⁴

Corneal endothelium has an important function in maintaining the water content of the cornea. Corneal stroma has a tendency to take up water due to the charged proteoglycans,
and corneal endothelial cells maintain an endothelial “pump” that removes water from the corneal stroma and prevents corneal swelling caused by hydration. The pump function is likely accomplished by secretion of $\text{HCO}_3^-$ or $\text{Cl}^-$, but the details of this mechanism are still unclear.\textsuperscript{11}

### 3.1.2 Conjunctiva

Conjunctiva is a mucous membrane that lines the anterior globe and inner surfaces of the eyelids. It produces tear fluid mucins, is involved in the resorption of tear fluid and has an important role in the immune defense of the ocular surface. Conjunctiva is composed of two layers: epithelial layer and substantia propria.\textsuperscript{12}

The epithelial layer of the conjunctiva is continuous with the epidermis of the eyelids and corneal epithelium. It consists of two to ten layers of non-keratinized, stratified epithelial cells. Epithelial surface cells are hexagonal and completely covered with microvilli. Many different types of surface epithelial cells can be identified, including goblet cells, other secretory cells and cells involved in resorption of tear fluid.\textsuperscript{12}

Goblet cells constitute 15% of epithelial surface cells in the conjunctiva. They are normally found in the middle and superficial layer of the epithelium, but are typically attached to the basement membrane by a thin cytoplasmic stalk. Goblet cells contain membrane-encircled mucus packets assembled by Golgi apparatus, which are likely secreted in an apocrine manner. Goblet cells are a main source of tear fluid mucus but also non-goblet epithelial cells appear to secrete mucin in a similar fashion to goblet cells.\textsuperscript{12}

Substantia propria is a connective tissue layer that contains a large number of mast cells, lymphocytes, plasma cells and neutrophils. In addition, it contains extracellular antibodies IgG, IgA and IgM, making it a potent anti-infectious structure. Substantia propria can be divided into two parts: a superficial lymphoid layer and a deeper fibrous layer. Lymphoid layer contains lymphocytes aggregated into nodules and fibrous layer consists of thick, collagenous, elastic tissue that contains vessels and nerves.\textsuperscript{12}

Lacrimal glands empty into the superior conjunctival fornix as is seen in Figure 1. They are tubulo-acinar exocrine glands that consist of secretory epithelium, myoepithelial cells, fibroblasts and immune cells. Secretory epithelial cells of the lacrimal glands secrete water into the gland lumen by secreting $\text{K}^+$ and $\text{Cl}^-$, which are followed by water driven by osmosis and $\text{Na}^+$ through the paracellular pathway. They also secrete other aqueous tear
components, like proteins, by exocytosis. Secretory epithelial cells are electrically coupled by gap junctions, allowing them to activate simultaneously. Myoepithelial cells squeeze the secretory products down the tubules and fibroblasts produce the extracellular matrix of the lacrimal gland interstitial regions.\textsuperscript{13}

### 3.1.3 Eyelids

Eyelids serve to maintain the proper position of the globe inside the orbit, offer the eyes physical protection from light or airborne particles and spread the tear film on the ocular surface. From anterior to posterior, the eyelids consist of the skin, orbicularis muscle, levator aponeurosis, tarsal plate and conjunctiva. The eyelids also contain multiple glands including Meibomian glands and accessory lacrimal glands of Krause and Wolfring.\textsuperscript{1}

Orbicularis muscle is a muscle roughly encircling the surface of the eye and its contraction causes the eyelids to close during blinking or blepharospasm. Levator aponeurosis is a group of collagenous fibers that connects the tarsal plate and skin of the eyelids to the levator muscle located superior to the ocular surface. The contraction of the levator muscle elevates the upper lid to open the eye. The tarsal plate is a fibrous plate that serves as the main structural element of the eyelids.\textsuperscript{1}

Meibomian glands are modified sebaceous glands located inside the tarsal plates. They consist of grape-like acinar clusters that empty into a main duct, which opens to the lid margin. Secretory cells of the acini, meibocytes, synthesize meibomian lipids into droplets that are then released in a holocrine manner, destroying the cells. The whole contents of the cells then form meibum, the oily secretion of the Meibomian glands. Secreting acinar cells are replaced by proliferation of basal acinar cells. The production of meibum appears to be controlled by neural innervation and hormonal control by the sex hormones.\textsuperscript{14}

A series of cilia or lashes grow from the margin of the eyelids. They are strong cylindrical hairs growing from a typical follicle, which is innervated by a neural plexus with a very low threshold for tactile sensation. Tactile sensation of the cilia induces reflex blinking to protect the eye. Each cilium is associated with sebaceous gland called gland of Zeis, which produce a sebaceous secretion at base of the lashes. Glands of Moll, modified sweat cells that empty to the base of the lashes are located in the same area.\textsuperscript{1}

Blinking is a high-speed movement of the lids that has both reflex and voluntary origins. During spontaneous blinking, the lower eyelid remains almost stationary and the upper
eyelid completes most of the movement. The eyelids close in a zipperlike motion from lateral to medial side moving the tear fluid towards the lacrimal puncta. The duration of one blink is 300 to 400 ms and average time between blinks is 3 to 4 seconds. Each blink draws fluid from the menisci of the upper and lower eyelid margin and spreads the tear film on the ocular surface. Blinking also has role in the secretion of meibomian lipid, as small aliquots of lipid are delivered to the surface of the tear film with each blink.

3.1.4 Tear film

Tear film is an approximately 4-11 µm thick fluid layer covering the ocular surface. The functions of the tear film are to provide a smooth optical interface for the light that enters the eye, to provide nutrients and oxygen for the avascular cornea, to serve as lubricant for the lids and to provide protection against debris and bacteria. Tear film can be divided into three parts: mucous matrix, aqueous layer and lipid layer (Figure 1).

Mucous matrix, a loose network or gel formed by glycoproteins called mucins is the innermost layer of the tear film. The mucous matrix is not likely a clearly separate layer, but a concentration gradient of mucins extends through the aqueous layer of the tear film. It can be divided into two parts: cell membrane-bound mucins and secreted mucins.

Corneal and conjunctival epithelial cells express membrane-bound mucins MUC1 and MUC16 and conjunctival epithelium also expresses membrane-bound MUC4. Membrane-bound mucins form a glycocalyx that extends up to 500 nm from the plasma membrane of the epithelial cells. A main function of membrane-bound mucins is to increase the hydrophilic character of the epithelial surface, allowing the tear film to efficiently wet the ocular surface. Other functions of the membrane-bound mucins are to prevent the adhesion of facing epithelial surfaces of the eye and eyelids, to act as a protective barrier against foreign molecules and possibly to act as osmosensors.

Secreted mucins originate mainly from the goblet cells of the conjunctiva, which produce gel forming mucin MUC5AC. In addition, conjunctival epithelium expresses soluble MUC7 and lacrimal gland epithelium expresses multiple mucins. Secreted mucins are considered to act as lubricants and clearing agents for allergens and debris as well as possible antimicrobial agents.

Aqueous layer of the tear fluid contains water, electrolytes, proteins and metabolites. Aqueous tears are isotonic with serum and the main electrolytes in tears are Na⁺, K⁺, Cl⁻.
and HCO\textsuperscript{-}. Also lower levels of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} are found. Large numbers of different proteins have been identified in the tear fluid but only lysozyme, lactoferrin, lipocalin and secretory IgA are found in large amounts.\textsuperscript{15} The main lacrimal gland produces 95\% of aqueous layer of the tear film\textsuperscript{1}. The rest is produced by accessory lacrimal glands of Wolfring and Krause\textsuperscript{1}. The aqueous layer forms the bulk of the tear film, providing a smooth optical interface, delivering nutrients to the avascular cornea and acting as lubrication. Dissolved proteins have also been identified to take part in immune defense and wound healing\textsuperscript{20}.

Lipid layer is the outermost layer of the tear film. It consists mainly of the oily secretion produced by the Meibomian glands. Its main functions are to stabilize the tear film and retard evaporation of the tear fluid. The composition and structure of the tear film lipid layer are discussed in detail in chapter 3.4.

### 3.2 Dry eye syndrome

According to the definition by 2007 Report of the International Dry Eye Workshop\textsuperscript{2},

“Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface.”

Dry eye syndrome is one of the most common diagnoses among ophthalmologic patients. Common symptoms of DES are overall dryness, grittiness, burning, ocular fatigue and foreign body sensation\textsuperscript{21}. DES increases the risk of corneal infections and ulcerations, which may endanger visual function\textsuperscript{22}. The following chapters discuss the etiology of DES, which is depicted as a flowchart in Figure 2, and the epidemiology of DES.

### 3.2.1 Etiology

DES can be classified into two categories: aqueous-deficient dry eye (ADDE) and evaporative dry eye (EDE). ADDE is caused by a failure of lacrimal tear secretion. Typical causes of lacrimal failure are autoimmune damage or destruction of the lacrimal glands (Sjögren’s syndrome), age-related changes in tear dynamics or obstruction of the lacrimal gland ducts. When lacrimal secretion is reduced, evaporation of water from the ocular surface leads to hyperosmolarity of tear fluid.\textsuperscript{2}
Evaporative dry eye is caused by excessive loss of water from the ocular surface. EDE can be caused by a defective TFLL, diseases affecting lid structure or dynamics, or environmental factors. Most common cause of evaporative dry eye is Meibomian gland dysfunction (MGD), in which the composition of meibum is abnormal or its delivery to the surface of the tear fluid is prevented. When MGD is severe enough, it is thought to lead to a deficient TFLL and increased tear evaporation. Increased evaporation in the presence of normal tear secretion leads to hyperosmolarity of tear fluid.²

Both in ADDE and EDE the underlying causes lead to hyperosmolarity of the tear fluid, which is a central mechanism in dry eye. Hyperosmolarity initiates an inflammatory cascade involving mitogen-activated protein kinase (MAPK) pathways that stimulate the generation of inflammatory cytokines (interleukins IL-1α, IL-1β and IL-8, tumor necrosis factor TNF-α and matrix metalloproteinase MMP-9)²³⁻²⁵. The inflammatory response leads to apoptotic death of epithelial cells, including goblet cells²⁶. Goblet cell loss leads to reduced levels of gel-formin mucin MUC5AC, disturbing the function of the mucous matrix. Disturbance of the epithelium and the mucous matrix causes instability of the tear film, predisposing it to breakup and local hyperosmolarity². In some cases, tear film instability can also be the primary causative mechanism for dry eye, as in xerophthalmia and allergic eye disease, in which there is a disturbance of ocular surface mucins².

In the early stages of dry eye disease, the osmotic, inflammatory and mechanical stress will result in reflex stimulation of the lacrimal gland, which acts to compensate for the tear fluid hyperosmolarity. However, long-term stimulation of the reflex tearing system caused by chronic dry eye is thought to lead to desensitization of the reflex tearing system and reduction of lacrimal flow, which will aggravate both ADDE and EDE. Therefore the division of DES into two categories is not often absolute but instead features of both types exist.²
Figure 2. A flowchart depicting the progression of dry eye disease. Typical underlying conditions are listed below each type of dry eye disease. MGD, Meibomian gland disorder; MAPK, mitogen-activated protein kinase; IL, interleukin; TNF, tumor necrosis factor; MMP, matrix metalloproteinase. Adapted from the 2007 DEWS report.2

3.2.2 Epidemiology

Infants have a markedly more stable tear film than healthy adults, an effect thought to arise from a thick and stable TFLL.27 Among children the prevalence of dry eye is also low.28,29 The prevalence of dry eye disease generally increases with age, at least among over-50 years-old patients.3 In a Canadian study, an additional peak in prevalence across age was found in 21-30 years age range.28 In over-50-year-old patients, it has been estimated that close to 5% of patients have moderate-to-severe dry eye symptoms and close to 30% milder, more periodic symptoms.3 Both Meibomian glands and lacrimal glands undergo acinar atrophy as a result of aging,30,31 which could explain the increasing prevalence of DES with age. Meibomian gland dropout is associated with increased ocular surface evaporation and likely contributes to the prevalence of DES, but it is not known whether age-related changes of the lacrimal gland lead to decreased aqueous secretion.31 Over 80%
of dry-eye patients also exhibit signs of MGD, making evaporative dry eye the most common type of DES\(^4\).

Risk factors for dry eye disease include female sex, postmenopausal estrogen therapy, low levels of dietary omega-3 fatty acids, low-humidity environment, computer use, contact lens use and refractive surgery\(^3\). Patients with DES have been found to be approximately three times more likely to report problems with everyday activities, especially reading, professional work, computer use, watching television and driving, than those without DES\(^3\). Therefore DES is a significant public health problem, which is gaining importance in countries such as Finland, where the proportion of elderly people in the population is rapidly increasing. In Finland additional challenges are posed by the long winter season, during which the air is cold and dry, which typically exacerbates dry eye symptoms.

### 3.3 Lipids at the air-water interface

This chapter contains a general introduction to the behavior of lipid molecules at the air-water interface, including spreading behavior and evaporation reducing properties of different kinds of lipids. The aim is to provide the reader with a basic understanding of how the structure of lipid molecules is linked to their behavior at the air-water interface.

#### 3.3.1 Polar lipids

Polar or amphiphilic lipids consist of two parts: a hydrophilic part commonly called “head” and a hydrophobic part typically called “tail”. Such molecules are called surfactants, meaning that in contact with water, they prefer the surface of the water in contrast to dissolving evenly. If the hydrophobic part of the molecule is small, they are typically water soluble, but have a preference for the water surface compared to the bulk water. If the hydrophobic part is large enough, they are insoluble in water and form an isolated twodimensional system at the surface of the water, called a Langmuir monolayer. Typical molecules that behave in this manner are fatty acids, fatty esters and phospholipids.\(^32\)

At the air-water interface, polar lipids organize so that their hydrophilic head groups face the water, while the hydrophobic tails are oriented towards air. Typically amphiphilic lipids spread very efficiently at the air-water interface and when the surface area of water per lipid molecule (mean molecular area, MMA) is large, they form a two-dimensional gas phase. In the gas phase the orientational freedom of lipid molecules is large and their hydrophobic tails are moving in a disordered manner due to thermal motion. When surface
area of the monolayer is decreased by compression, neighboring lipid molecules start to affect each other, the hydrophobic tails become gradually more ordered and orient perpendicular to the water surface.32,33

When monolayers of amphiphilic lipids are compressed beyond a certain limit, the molecules no longer fit into a single monolayer and start to collapse into three-dimensional aggregates or multilayers. This collapse can occur into the aqueous phase, as has been observed for phospholipids, which form bilayer folds or vesicles into the subphase33,34. Another mode of collapse is to fold into multilayers on top of the monolayer film, as has been observed for fatty acids35.

In three dimensional systems, the state of the system is often identified by temperature, volume and pressure. In Langmuir monolayers, analogous two-dimensional variables (temperature, area and surface pressure) can be controlled. The area of the monolayer can be altered by compressing with barriers that move along the water surface. Surface pressure $\pi$ in Langmuir monolayers is defined

$$\pi = \gamma_w - \gamma$$

where $\gamma_w$ is the surface tension of a pure air-water interface and $\gamma$ is the surface tension of the air-water interface containing a Langmuir monolayer. In other words, surface pressure represents the ability of the lipid monolayer to lower the surface tension of an air-water interface. Also rheological properties of the monolayer can be defined analogously to three dimensional systems. In monolayers, reciprocal compressibility is commonly used and is defined as36

$$C^{-1} = -a \left( \frac{d\pi}{da} \right)$$

where $a$ is the mean molecular area. Changes in surface pressure or compressibility can often be associated with phase transitions of the monolayer, as is demonstrated in a hypothetical compression isotherm in Figure 3.
Figure 3. A hypothetic compression isotherm. Schematic representations of typical behavior of polar lipids in different phases are shown along the compression isotherm. Hydrophilic headgroups are shown in red and hydrophobic tails in black.

3.3.2 Non-polar lipids

Non-polar lipids or oils have only a weakly polar group or groups and consist mostly of hydrophobic parts. Typical non-polar lipids include hydrocarbons, triglycerides, cholesterol esters (CEs) and wax esters (WEs). They are only marginally surface active and do not readily spread at the air-water interface. Instead they typically form droplets or lenses.

The spreading behavior of non-polar oils on water can be characterized by spreading parameter $S$ that is defined

$$ S = \gamma_{aw} - (\gamma_{ow} + \gamma_{ao}) $$

where $\gamma_{wa}$ is the air-water surface tension, $\gamma_{ow}$ is the oil-water surface tension and $\gamma_{ao}$ is the air-oil surface tension. When $S$ is positive, the energy of the system is lowered when the oil spreads on water, because the sum of air-oil and oil-water surface tensions is smaller than air-water surface tension. This situation is called total wetting. When $S$ is negative, oil does
not spread but forms lenses, which is called partial wetting.\textsuperscript{37} These spreading states are illustrated graphically in Figure 4.

Two different spreading coefficients can be determined, initial spreading coefficient $S_{in}$ and equilibrium spreading coefficient $S_{eq}$. For some oils or oils mixed with surfactants $S_{in}$ is positive and the oil initially spreads and totally wets the water surface. If the oil film is thick enough, it can be considered to display bulk properties with two separate interfaces (air-oil and oil-water), and is called a duplex film. However, according to theoretical considerations and experimental evidence, $S_{eq}$ is always negative. Therefore a duplex film is not stable and will eventually dewet and form droplets.\textsuperscript{37}

In addition to total or partial wetting, more complex wetting states are also possible. One of them is called pseudo-partial wetting, in which equilibrium prevails between a thin wetting film and a non-spreading drop. In this state a small amount of oil will completely wet the surface forming a thin film, typically in the scale of molecular dimensions, similar to polar lipids. If more oil is added than is needed to cover the whole surface with this thin film, excess oil will form droplets.\textsuperscript{38}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Two types of spreading behavior for nonpolar oils on water. Partial wetting occurs when spreading coefficient $S$ is negative, and total wetting occurs when $S$ is positive. $\gamma_{aw}$, air-water surface tension; $\gamma_{ow}$, oil-water surface tension; $\gamma_{ao}$, air-oil surface tension.}
\end{figure}
3.3.3 Evaporation resistance

Retarding evaporation of water by using oily films is an old idea dating back to the days of Benjamin Franklin\(^{39}\). Both monolayers\(^{40}\) and duplex films\(^{37}\) have been studied for this purpose, usually with the goal of preventing water evaporation from open water stores.

Saturated alcohols and fatty acids and their methyl or ethyl esters are typical molecules known to form evaporation retarding monolayers at the air-water interface\(^{40}\). Amphiphiles that have long, saturated, unbranched hydrocarbon chains are potentially effective evaporation retardants\(^{40}\). Substances that contain double bonds or large hydrophilic groups are not typically efficient in retarding evaporation\(^{41}\). Amphiphiles with long, saturated hydrocarbon chains typically form a solid monolayer at high surface pressures\(^{40}\) (See Figure 3). In the solid phase, the hydrocarbon chains are closely packed and the free volume inside the monolayer is very low, preventing the diffusion of water molecules through the monolayer\(^{42}\). Large headgroup or double bonds in the hydrocarbon chain typically prevent such tight packing. The resistance to evaporation has been found to be nonlinearly dependent on the thickness of the monolayer, which indicates that the resistance to evaporation is better modeled by an energy barrier than a diffusive mechanism\(^{40}\). The proportional reduction in water evaporation is highly dependent on the experimental conditions, but the highest reduction in evaporation achieved by using monolayers of long-chain alcohols in non-controlled conditions on the surface of lakes and ponds is approximately 60%\(^{39}\).

In contrast to monolayers, the evaporation resistance of duplex films has been observed to vary linearly with film thickness, indicating that duplex films retard evaporation, because the diffusion of water molecules through the oil layer is slower compared to air\(^{43,44}\). Measuring evaporation reduction of duplex films is more complicated than monolayers due to the fact that they are inherently unstable and tend to rupture into lenses during the time needed to measure changes in evaporation rate. For this reason, duplex film evaporation retardation has typically been studied with oils that contain some amphiphilic spreaders in order to keep the duplex film stable long enough to measure evaporation rates. This complicates the measurements, because the interfacial layer formed at the oil-water interface by the spreader also affects the evaporation rates in similar way as a monolayer at the air-water interface. Gilby and Heymann\(^{44}\) measured evaporation rates through duplex films of paraffin oil combined with several different spreaders and found out that the
spreader used had a strong effect on the evaporation retarding properties of the film. In fact, they concluded that the duplex film typically has to be thicker than 20 µm in order for the diffusive evaporation resistance of the duplex film to be greater than the interfacial resistance of the spreader. Very high evaporation reduction (> 90%) can be achieved with duplex films, but the film thickness typically has to be tens or hundreds of micrometers.\textsuperscript{37,43,44}

3.4 Tear film lipid layer

Tear film lipid layer consists mainly of meibum, which is produced by the Meibomian glands and secreted to the lid margin. A reservoir of meibum is formed to the eyelid margins, from which it spreads to the surface of the tear film. When lids are closed, meibum forms a common reservoir, which also seals the eyelid margins offering protection during prolonged closure. When the eyes are opened, TFLL spreads rapidly upwards to cover the ocular surface. It is not known how meibomian lipid is removed from the ocular surface but it is likely that it gradually flows over to the neighboring skin and lashes. At the lid margin, the functions of TFLL are to make the lid skin hydrophobic and prevent spilling of the aqueous tears, as well as resist contamination of the ocular surface with skin lipids. On the tear film, the main functions are to retard evaporation of the aqueous tear and stabilize the tear film.\textsuperscript{19}

3.4.1 Composition

The composition of TFLL has been studied in two ways, by using samples obtained by collecting meibum directly or by collecting tear fluid samples and extracting the lipids that they contain. The major components of meibum are wax esters (30-50%) and cholesteryl esters (30-50%)\textsuperscript{6-8}. In addition, meibum contains small amounts of triglycerides (2%), free cholesterol (< 2%) and (O-Acyl)-omega-hydroxy fatty acids (3%)\textsuperscript{6,7}. Tear fluid samples contain the same major components as meibum, but in addition they contain polar lipids: phospholipids (6-12%), free cholesterol (6%) and sphingolipids (2%)\textsuperscript{6,7}. It appears that the nonpolar lipids are produced mainly by the Meibomian glands, but the origin of the polar lipids in tear fluid is not known. It is likely that they originate either from the lid margin, possibly from the glands of Moll and Zeis\textsuperscript{45}, or the epithelial cells of the ocular surface. The composition of the tear fluid polar lipids is similar to the composition of mammalian cell plasma membranes\textsuperscript{46}, which might suggest that they originate from the shedding cells of the ocular surface. A large proportion of the tear fluid phospholipids are
lysophospholipids, possibly due to breakdown of phospholipids by phospholipase A2 in tear fluid\textsuperscript{6,47}. It has been suggested that a significant fraction of the polar lipids in tear fluid might be bound to tear fluid proteins like tear lipocalin\textsuperscript{48}. Therefore the polar lipids might not be located at the tear film surface and may not participate in the function of the TFLL, even though they are found in tears.

Wax esters consist of two hydrocarbon chains (fatty alcohol and fatty acid chain) connected by an ester linkage (see Figure 6). The wax ester species found in meibum typically contain a long, saturated fatty alcohol chain (20-29 carbons) and an unsaturated fatty acid chain, mostly 18 or 16 carbons long with one double bond. In total, only 18\% of wax esters are saturated and the rest are unsaturated, with 90\% of the unsaturated lipids containing an oleate chain (18 carbons, 1 double bond). Additionally, most of the saturated wax esters contain a branched chain, which has a similar effect on the physical properties as unsaturation (For a more detailed discussion on the physical properties of WEs, see chapter 3.5.2).\textsuperscript{8} CEs contain a cholesterol moiety, which is linked to a hydrocarbon chain by an ester linkage. Meibum CEs have been found to contain very long hydrocarbon chains (18 to 34 carbons), 80\% of which are saturated\textsuperscript{9,49}.

### 3.4.2 Structure and thickness

Tear film lipid layer can be observed as colorful interference patterns on the surface of the tear film. In order for this kind of patterns to arise, the thickness of the lipid layer has to be on the order of the wavelength of visible light (390-700 nm). Colored patterns usually only arise when the palpebral fissure is narrowed deliberately, leading to a general consensus that the thickness of TFLL is less than 100 nm\textsuperscript{19}. Recent measurements using interferometry obtained lipid layer thickness values between 15 and 157 nm with a mean value of 42 nm\textsuperscript{50}.

The thickness of the tear film lipid layer is often not uniform, but contains large local differences. Using high-resolution microscopy, the tear film lipid layer can be described as either a uniform or irregular background, in which either thick objects (spots, lenses, islands or snowflakes) or thin objects (lakes or dots) are found. Some shapes like lenses and holes can be linked to dewetting processes that were described in chapter 3.3.2. Others like snowflakes appear similar to lipid domains seen in monolayers, which are created by local segregation of different lipid species. Therefore the different shapes may be created
either by local differences in lipid thickness or lipid composition. However, it is not currently well known, which morphologies are linked to healthy or dry-eye conditions.\textsuperscript{51}

### 3.4.3 Evaporation resistance of TFLL

Evaporation resistance of TFLL has been studied both \textit{in vivo} in humans and rabbits and \textit{in vitro} in model systems using meibum or TFLL components. Pioneering \textit{in vivo} studies using rabbits by Mishima and Maurice\textsuperscript{52} found that TFLL caused a 17-fold reduction in evaporation rate. Later studies in rabbits and humans have reported 4-fold or smaller reduction in evaporation rate caused by the TFLL\textsuperscript{53-56}. The thickness of the lipid layer does not appear to affect the evaporation rate, unless the TFLL is very thin or completely absent\textsuperscript{50,55}. Abnormal morphology is also associated with increased evaporation, even in cases where TFLL is thick\textsuperscript{50,55}. It should be noted that the methodology of \textit{in vivo} studies is variable and it is challenging to design robust experiments, which is shown by the more than 1000-fold differences in measured evaporation rates between different \textit{in vivo} experiments\textsuperscript{57}.

The difficulties of \textit{in vivo} methods make it appealing to attempt measuring this effect more precisely \textit{in vitro}. Interestingly, \textit{in vitro} studies have not been able to show a significant evaporation retarding effect for meibum films with physiological thickness. The maximum evaporation reduction found in these studies is 6-8\%\textsuperscript{37,58,59}. Other studies using model films that simulate TFLL have also failed to find evaporation reducing effects\textsuperscript{37,57,60}. This is surprising, because the experimental conditions of \textit{in vitro} studies are easier to control and therefore a strong effect is expected to be easily detected. Due to the large amount of \textit{in vivo} evidence, however, the general consensus is that TFLL has an evaporation retarding effect. It has been suggested that the \textit{in vitro} experiments might lack some component that is present \textit{in vivo}, which would explain the inability to find this effect \textit{in vitro}\textsuperscript{57}. These findings underline the fact that the basis of the evaporation retarding effect of TFLL is not well understood and this complex effect clearly cannot be accomplished by every oily substance added to the tear film surface.

### 3.4.4 Models of TFLL

The traditional model of the tear film lipid layer by McCulley and Shine\textsuperscript{61} is based mostly on the knowledge about tear fluid lipid composition. It contains two layers: A thin polar lipid layer and a thick non-polar lipid layer. The polar lipid layer is located to the water
interface and consists of phospholipids, fatty acids, cerebrosides and some triglycerides. The function of the polar phase is to aid the spreading of the non-polar lipid layer on the water surface. The non-polar layer consists of wax esters, cholesteryl esters, triglycerides and hydrocarbons. Its function is to control the evaporation of water from the ocular surface and act as a lipid reservoir.

King-Smith et al\(^{62}\) recently proposed a new model of the TFLL. It also contains a polar phase and a non-polar phase in a similar manner to the model of McCulley and Shine, but in addition to compositional information, it aims to explain the need of the lipid layer to simultaneously retard evaporation and withstand the structural disruption caused by blinking. In this “multilamellar sandwich model” the non-polar phase is formed by multiple lamellae of interdigitated cholesteryl esters and wax esters. The middle portion of these lamellae would consist of tightly packed saturated hydrocarbon chains, which are expected to retard evaporation. The outer edges of the lamellae would consist of unsaturated hydrocarbon chains or cholesterol moieties. Separate lamellae would then not be strongly attached to each other allowing lamellae to slip between each other or generate additional folds during compression caused by blinking.

Another model for the TFLL is a duplex layer model that has been proposed by Rosenfeld et al\(^{63}\). It postulates that TFLL is a duplex layer that shows bulk characteristics with distinct air-lipid and lipid-water interfaces. In this model the bulk is formed by a continuous liquid of lipids that has viscoelastic and shear-thinning properties, instead of highly ordered structures proposed by the other models. Lamellar lipid crystals are suspended in the liquid phase in random orientation, and are thought to confer viscoelastic properties to the TFLL. Increased viscoelasticity caused by lamellar ordering is thought to prevent the film from dewetting. Polar lipids are thought to concentrate to the oil-water interface and cause local ordering of lipids close to the air-water interface.
Figure 5. Three models of tear film lipid layer structure: the traditional two-layered model by McCulley and Shine, the multilamellar sandwich model by King-Smith et al. and the duplex layer model by Rosenfeld et al. Red, polar headgroups; blue, ester or glycerol groups of nonpolar lipids; black, hydrocarbon tails; yellow, continuous fluid phase of lipid; ring structures, cholesterol. See chapter 3.4.4 for details. Modified from McCulley and Shine\textsuperscript{61}, King-Smith et al.\textsuperscript{62} and Rosenfeld et al.\textsuperscript{63}

The differences in recently proposed models highlight a fundamental issue concerning TFLL that is still under debate. It is whether the function of the tear film lipid layer is based on its bulk properties or interfacial properties. The idea of a bulk-like film is based on the intuitive assumption that a thicker, around 100 nm-thick film of meibum would be more efficient in retarding evaporation than a thin film consisting of only a monolayer or several lipid layers\textsuperscript{63}. However, three factors indicate that this is not necessarily the case. First, according to the \textit{in vivo} experiments, evaporation rate is not dependent on the thickness of the TFLL, unless the thickness is very small (< 30 nm) or TFLL appears to be completely absent\textsuperscript{50,55}. Second, 100 nm-thick duplex-films do not significantly retard evaporation\textsuperscript{37}. Third, duplex layers typically also contain a thin interfacial layer at the air-oil interface, which may have significant evaporation retarding properties\textsuperscript{44}. Interfacial lipid films like monolayers at the air-water interface can have strong evaporation retarding properties. Even though TFLL appears to be thicker than necessary to form a thin interfacial layer, it is possible that the excess lipid simply acts as a reservoir to ensure...
complete coverage of the ocular surface or fulfills other TFLL functions than evaporation retardation. It appears evident that the proper function of TFLL is not based solely on the amount of lipid but instead a specific organization of lipids, either in the interfacial layer or the whole lipid film. This makes biophysical studies that aim to characterize the organization of these lipids and the underlying molecular interactions especially important.

3.5 Wax esters

Wax esters are esters of fatty acids and fatty alcohols, and therefore their structure consists of two hydrocarbons chains linked by an ester moiety (see Fig. 6). As was discussed in chapter 3.4.1, wax esters are a major component of meibum and therefore also TFLL. Compared to common biological lipids like phospholipids, fatty acids or triglycerides, relatively few studies have concentrated on wax esters. The following chapters provide review of the literature on WEs, including their occurrence and role in nature as well as physical properties, both in bulk and at the air-water interface.

3.5.1 Occurrence and functions in nature

Many marine animals have been found to utilize wax esters. Marine copepods, which live in cold waters, store lipids into wax esters to store energy and can accumulate up to 75% of their body weight as wax esters, in order to survive the long winters. Some bacteria, like acinetobacteria also use WEs to store energy. Marine animals also use was esters to control their buoyancy, and vertically migrating animals or animals living below sea level typically contain high levels of wax esters. Large amounts of WEs are also found in the melon of many toothed whales, which use it to modulate their vocalizations during communication and echolocation.

In birds, the preen oil secreted by the uropygial glands contains mostly wax esters. Birds typically transfer the preen oil from the uropygial gland to their body, feathers and the skin of the legs by using their beak. It can also be applied to the surface of the eggs during breeding season. The functions of the preen oil are likely to differ between bird species, but typical functions are believed to be to maintain the integrity of the feathers and the skin, to provide a waterproofing effect for aquatic birds and to provide protection against feather and eggshell microorganisms.

Wax esters are also utilized among the insects. Epicuticle is the outermost, waxy layer of insects, which in most insects consists mainly of hydrocarbons. In some insects, however,
most of the epicuticular lipids are wax esters. The function of these lipids is to produce a waterproofing effect and prevent evaporation and dessication of insects, or in the case of aquatic insects, prevent the imbibition of water. It has been observed that the melting temperature of the epicuticular lipids coincides with the critical temperature, at which the water loss from the insects increases rapidly. Wax esters are also used for other purposes by insects. For example, beeswax used by bees to build nests consists mostly of wax esters. Wax esters are also a major component of the wax produced by female scale insects, which are immobile plant parasites that use a thick wax layer to protect themselves.

Leaves of terrestrial plants are covered by a thin waxy layer called the cuticle, which consists of a hydrophobic polymer called cutin and wax that typically consists of hydrocarbons, alcohols, ketones, esters and acids. In some plants, like the carnauba palm, most of the cuticular wax consists of wax esters. Main functions of cuticular wax are to control water loss, keep the plant surface clean and mediate interactions with insects and pathogens. In some plants, like jojoba, wax esters constitute most of the energy storage reserves of seeds.

In humans and most mammals, wax esters are only produced by the sebaceous glands of the skin and specialized sebaceous glands like the Meibomian glands. In humans and rats, 25% of sebum weight consists of wax esters, but for example in mice and rabbits the amounts of WEs are lower, which likely reflects the different functions sebum has in these animals. In mice and likely in aquatic mammals, sebum has a waterproofing effect. The functions of sebum in humans are not completely understood but they are thought to include photoprotection, antimicrobial activity, delivery of antioxidants to the skin and regulating inflammatory events.

3.5.2 Physical properties

As was described in the previous chapter, WEs appear to have many different roles in different species, but two functions, energy storage and waterproofing appear to be the most characteristic for wax esters. Wax esters are clearly well-suited molecules for storing energy, because they have a very high energy density, up to 15% higher than that of triglycerides. In addition, WEs are almost as hydrophobic as alkanes, which makes them efficient water-repellent materials and suitable also for retarding evaporation.
The bulk properties of wax esters have been mainly studied by measuring melting temperatures and X-ray diffraction. Bulk melting temperature of wax esters increases with increasing chain length and decreases with increased unsaturation or branching$^{10,79,80}$. Increase with chain length reflects the increased van der Waals-interactions between hydrocarbon chains, which stabilize the solid crystal. Decrease with unsaturation and branching reflects frustrated packing of unsaturated or branched hydrocarbon chains, which destabilize the solid crystal. Melting temperatures of saturated wax esters are approximately 15 °C lower compared to alkanes of the corresponding length$^{80,81}$, which implies that the ester moiety also slightly disturbs the tight packing of the hydrocarbon chains. Crystal structure has been determined using X-ray diffraction for both completely saturated wax esters and jojoba-like wax esters (JLWE), which have a single double bond in both hydrocarbon chains. Both form bulk crystals, in which the molecules are completely extended and area per molecule is close to the cross sectional area of a single hydrocarbon chain (18.8 Å$^2$)$^{82-84}$. Long crystal spacing values are also close to those of alkanes of corresponding length, but some wax esters form tilted crystals$^{82,83}$. So although unsaturation decreases the melting temperature of wax esters, it does not appear to have a strong effect to the solid crystal structure.

Several authors have attempted to study wax esters at the air-water interface using Langmuir film-techniques. The pioneering studies by Adam$^95$ using esters with variable alkoxy chains showed that the ester group is anchored to the water and both the hydrocarbon chains orient away from the water. If the alkoxy chain is short (4 carbons or less), it can be oriented towards the water with compression, but this did not appear possible for esters, in which both hydrocarbon chains were long. He observed that hexadecyl palmitate (HP), a wax ester with 16 carbons in both hydrocarbon chains, formed unstable solid films which collapse rapidly, and assumed that both hydrocarbon chains of HP also orient towards the air but pack tightly to form a solid film. He also observed that the film of HP expanded when heated to 50 °C, which is 3.5 °C below the bulk melting temperature of HP, but paid no further attention to this phenomenon.

Later studies have elucidated the behavior of multiple different wax esters. Wax esters with long, saturated chains typically form solid, unstable films that do not spread again after compression and appear to form solid rafts floating on the water surface$^{10,86}$. Unsaturated wax esters form fluid films, in which the hydrocarbon chains are disordered and the molecules collapse and leave the surface at rather high surface areas when compressed$^{86,87}$. 


However, wax esters have generally been considered to act in a similar way to polar lipids at the air-water interface, with the ester moiety acting as the polar head group, but with a tendency to collapse and form three dimensional aggregates.

Recently it has been discovered that WEs retard evaporation by up to 50% \textit{in vitro}^{10,60}. However, they only appear to have this effect close to their bulk melting temperature\textsuperscript{10} and the effect is lost if more than 10\% of phospholipids are mixed with the WEs\textsuperscript{60}. The evaporation retarding effect was suggested to originate from the formation of a specific condensed phase, but the nature of this phase has remained unclear\textsuperscript{10}. As was discussed above, WEs have been thought to behave differently in bulk and at the air-water interface, preferring an extended conformation in bulk and a V-shaped hairpin conformation at the air-water interface. However, the fact that the evaporation retarding condensed phase only appears to form close to the bulk melting temperature suggests that WEs might exhibit bulk-like behavior also at the air-water interface.
4. AIMS OF THE STUDY

This study has three principal aims, all of which seek to elucidate the possible mechanism, by which TFLL retards evaporation and stabilizes the tear film:

(1) To determine how changes in temperature affect the spreading and phase behavior of wax esters at the air-water interface.

(2) To elucidate what kind of conformation wax esters adopt at the air-water interface in the different phases.

(3) To determine how the phase behavior affects the evaporation retarding properties of wax esters and explain the molecular level mechanism behind this effect.
5. MATERIALS AND METHODS

5.1 Experimental setup

The experimental setup was based on a Langmuir trough (KSV Mini trough, Helsinki, Finland) equipped with a platinum Wilhelmy plate for measuring surface pressure, a temperature sensor for measuring subphase temperature and a Brewster angle microscope (BAM) (KSV NIMA microBAM, Helsinki, Finland) for visualization of the lipid films. The trough was connected to a thermostat (Lauda ECO E4, Lauda, Germany). The measurement apparatus was closed in a plastic enclosure and air from the laboratory pressurized air system was supplied to the enclosure in order to maintain a constant airflow. Airflow was measured using an airflow meter (Testo 410-1, Lenzkirch, Germany). Relative humidity and temperature inside the enclosure were measured using a digital hygrometer (Testo 608-H1, Lenzkirch, Germany).

5.1.1 Surface pressure measurement

Surface pressure was measured using a Wilhelmy plate, a thin platinum plate hanging from a microbalance that measures the weight of the plate. The plate is lowered to the water, perpendicular to the water surface and a meniscus wets the platinum plate. Water surface tension is an inward force parallel to the water surface, which causes the meniscus to pull the plate downwards, which can be measured as an apparently increased weight by the microbalance. Surface tension is then be calculated using the Wilhelmy equation

\[ \gamma = \frac{F}{l \cdot \cos \theta} \]  

where \( \gamma \) is the surface tension, \( F \) is the force exerted on the plate by the surface tension, \( l \) is the perimeter of the plate and \( \theta \) is the contact angle between the water and the plate. Because of the high surface energy of platinum, the plate is completely wetted and contact angle is practically zero (\(< 1^\circ\))\textsuperscript{88}. Measured surface tension can then be used to calculate surface pressure as described in chapter 3.3.1.

5.1.2 Brewster angle microscopy

According to Fresnel equations, the reflectance for p-polarized light at an interface of two materials is
\[
R_p = \left[ \frac{n_1 \sqrt{1 - \left( \frac{n_1}{n_2} \sin \theta_i \right)^2} - n_2 \cos \theta_i}{n_1 \sqrt{1 - \left( \frac{n_1}{n_2} \sin \theta_i \right)^2} + n_2 \cos \theta_i} \right]^2
\]  

(5)

where \( n_1 \) and \( n_2 \) are the refractive indices of the two materials and \( \theta_i \) is the angle of incidence.

In Brewster angle microscopy, a p-polarized laser beam is directed towards the water surface at Brewster angle (~53°) and the reflection is recorded using a detector. At this angle, according to equation (5), reflectance is zero. Therefore p-polarized light is perfectly transmitted through the air-water interface with no reflection. Adding a layer of lipid to the air-water interface changes the local refractive index of the interface and results in a low intensity reflection from the surface. As a result, an image is formed on the detector, in which intensity is determined by the molecular size and packing density of the lipid molecules on the surface. Image intensity is increased when either the thickness of the lipid film increases or packing density of the lipid molecules increases.

### 5.2 Materials

Behenyl palmitoleate (BP) (see Figure 6 for structure) was obtained from Nu-Chek-Prep (Elysian, MN). 1-Octadecanol, 1-Docosanol, 1-Hexacosanol and 1-Triacontanol were obtained from Sigma-Aldrich (St. Louis, MO). All lipids were dissolved in chloroform and stored in -20 °C. Samples were discarded after 3 measurements from the same sample in order to minimize deterioration of unsaturated lipids. PBS buffer was used as the subphase in all experiments.

![Figure 6. Behenyl palmitoleate. Behenyl palmitoleate consists mostly of hydrocarbon, with a saturated 22-carbon chain and an unsaturated 16-carbon chain connected with an ester linkage. The ester linkage gives behenyl palmitoleate a slightly polar character.](image)
5.3 Methods

5.3.1 Isochor measurements

BP was spread to the air-buffer interface of the Langmuir trough in 1 mM chloroform solution at 20 °C to a mean molecular area of 30 Å². Trough temperature was increased at a rate of 2 °C/min to 42 °C and surface pressure was measured using a platinum Wilhelmy plate. The film was imaged using a KSV NIMA microBAM at 1 °C intervals. BAM aperture was kept closed while images were not being recorded in order to avoid heating the film with the laser beam. Measurements were performed twice to ensure repeatability of the measurement.

5.3.2 Isotherm measurements

BP was spread to the air-buffer interface of the Langmuir trough in 1 mM chloroform solution. Mean molecular area was reduced at a rate of 10 (Å²/molecule)/min by compressing with two symmetric barriers. Surface pressure was measured by using a Wilhelmy plate and BAM images were recorded during the compression isotherm to observe the morphology of the film. Trough temperature was maintained within 36 ± 1 °C during the experiments. Reciprocal compressibility was calculated from the surface pressure data using equation (2) and smoothed using adjacent averaging with a 5 data point window. Each measurement was performed three times to ensure repeatability of the results.

5.3.3 Thickness measurements

Five BAM images were captured from each film at different exposure times between 0.1 – 125 ms. Average image intensity was measured from each image using ImageJ-software. A linear fit to the exposure time-image intensity data was used to obtain values for image intensity per unit time (Figure 7A). In order to obtain reflectance values from the intensity data, following calibration procedure was used: aqueous solutions of 0, 9, 20, 30, 50, 60 and 90 g/l NaCl were prepared and the intensity per unit time was measured from each solution using the BAM. The addition of salt increases the refractive index of water, causing reflectance to increase, according to equation (5). Using the refractive index data and equation (5), a calibration curve connecting the measured intensity per unit time and film reflectance was obtained (Figure 7B). A power function was fitted to the data using...
least-squares fit and used to convert BAM intensity measurements to reflectance in further experiments.

As a first order approximation, reflectance of an interface containing a thin lipid film can be written:

\[ R_p \propto d^2 \]  \hspace{1cm} (6)

where \( d \) is the thickness of the lipid layer. Therefore film thickness was calculated using the following equation:

\[ d = A \sqrt{R_p - R_{bg}} \]  \hspace{1cm} (7)

where \( A \) is a constant and \( R_{bg} \) is the reflectance of a pure buffer interface. A series of linear alcohols (1-Octadecanol, 1-Docosanol, 1-Hexacosanol and 1-Triacontanol) with chain lengths of 18, 22, 26 and 30 carbons were used to determine values for the constants of equation (7) (Figure 7C). Alcohols were spread to the air-buffer interface of the Langmuir trough in 5 mM chloroform solution and compressed to a mean molecular area of 18 Å². After compression BAM images were captured and average intensity per unit time was measured for each film. Theoretical thickness for the alcohol films in all-trans conformation was calculated according to Tanford:

\[ d_T = 1.5Å + 1.265 \times n(CH_2) \]  \hspace{1cm} (8)

where \( n(CH_2) \) is the number of CH₂ groups in the chain. Measured reflectance of the linear alcohols was fit to the theoretical thickness calculated by equation (8) to obtain values of 11.6 ± 0.4 \times 10^3 Å (mean ± SE) for \( A \) and 1.3 ± 0.2 \times 10^{-6} for \( R_{bg} \). After calibration, equation (7) was used to determine the thickness of BP film compressed to mean molecular areas of 18 Å² (solid) and 45 Å² (fluid).
Figure 7. Calibration procedure of the Brewster angle microscope for thickness measurements. (A) Average image intensity as a function of exposure time. Intensity per unit time was determined from the slope of the data points. Error bars have been omitted for clarity. (B) Calibration curve between intensity per unit time (± SE) and reflectance. The data points have been obtained by measuring the reflected intensity from NaCl solutions of different concentrations. (C) Determination of constants for equation (7). Reflectance (± SE) was determined from intensity-per-unit-time data measured for linear alcohols using the calibration curve shown in B. Theoretical thickness was calculated for the alcohol films using equation (8).

5.3.4 Evaporation measurements

Langmuir trough was pre-heated to approximately 37 °C and 150 grams of PBS buffer was added to the trough. BP was added to the buffer surface in chloroform solution. Plastic enclosure was kept open for 10 minutes to allow for the chloroform to evaporate. Then the enclosure was closed and water was allowed to evaporate for another 80 minutes, after which the buffer was collected and weighed. During the experiment, trough temperature was maintained within 37 ± 1 °C using a thermostat and airflow in the enclosure was constant at 1.7 ± 0.1 L/s. The change in mass of the buffer was converted into volume change using a density of 1000 kg/m³ for the buffer. In addition, evaporation rate was
measured daily from a pure PBS buffer surface, either at 35 °C or at 37 °C, in order to take the humidity variation into account.

In non-vacuum conditions, evaporation rate \( J \) from a water surface is\(^{37} \)

\[
J = \frac{C_{\text{sat}} - C_{\infty}}{R_k + R_e}
\]  

where \( C_{\text{sat}} \) is the concentration of saturated water vapor at the water surface, in equilibrium with the liquid phase, and \( C_{\infty} \) is the concentration of water vapor far from the interface. \( R_k \) is the resistance for kinetic escape from the liquid phase to the thin layer of water vapor adjacent to the interface. \( R_e \) is the environmental resistance for mass transfer by convection and diffusion from the thin vapor layer to the surrounding atmosphere. In case of a pure water interface with no lipid film, the resistance for kinetic escape is negligible and evaporation rate from a pure water surface becomes

\[
J_w = \frac{C_{\text{sat}} - C_{\infty}}{R_e} = -\frac{C_{\text{sat}}^\infty}{R_e} H_R + \frac{C_{\text{sat}}}{R_e}
\]  

where \( C_{\text{sat}}^\infty \) is the theoretical saturated water vapor concentration far from the interface and \( H_R \) is relative humidity. Temperature far from the surface remained at room temperature (22 ± 1 °C) during the experiments. At this temperature saturated water vapor concentration \( C_{\text{sat}}^\infty \) is 19 ± 2 g/m\(^3\). Figure 8 shows the evaporation rates measured from a PBS buffer surface at constant temperatures of 35 °C and 37 °C. A least squares method was used to simultaneously fit two linear curves to the data, requiring the same slope for both curves but allowing separate intercepts, because \( C_{\text{sat}} \) depends on the temperature of the surface. From the slope of the linear fit, \( R_e \) was determined to be 1.9 ± 0.3 s/cm, according to equation (10). According to the intercepts, saturated water vapor concentration at the surface was 43 ± 6 g/m\(^3\) at 37 °C and 38 ± 5 g/m\(^3\) at 35 °C, as expected in these temperatures. Using these constants, evaporation reduction \( E \) was then calculated as

\[
E = 1 - \frac{J}{J_w} = 1 - \frac{J R_e}{C_{\text{sat}} - C_{\text{sat}}^\infty H_R}
\]  

where \( J \) is the evaporation rate measured from a PBS buffer surface covered by a lipid film and \( H_R \) is the relative humidity above the surface.
Figure 8. Evaporation rate from a pure PBS buffer surface as a function of relative humidity above the surface. Error bars represent the average humidity variation during the experiments.
6. RESULTS

6.1 Isochor measurements

Figure 9 shows a representative isochor measurement of BP at a mean molecular area of 30Å² with the associated BAM images along the isochor. At 20 °C, BP did not appear to spread and formed two dimensional solid domains and three dimensional aggregates (Fig. 9a). Surface pressure was also zero at low temperatures because the isolated solid domains floating on water surface do not transmit any lateral force to the surface pressure probe. As temperature is increased, a fluid monolayer was observed to spread around the solid domains at 34.5 °C with BAM (Fig. 9b). As the fluid monolayer spread to cover the whole water surface (Fig. 9c), surface pressure started to increase. Solid monolayer domains were observed to coexist with the fluid monolayer in BAM images (Fig. 9d). When temperature was increased beyond 38 °C, the bulk melting temperature of BP, solid monolayer domains were seen to melt into bright, mobile droplets with BAM (Fig. 9e) and surface pressure reached a plateau of 2.3 mN/m.
Figure 9. Isochor of behenyl palmitoleate at a mean molecular area of 30 Å². Representative Brewster angle microscope images are shown along the isochor. Note that the exposure time of images b and c is longer than, a, d and e. Scale bar is 500µm. (a) black: water, gray: solid monolayer, white: aggregates; (b-c) black: water, gray: fluid monolayer, white: solid monolayer; (d-e) black: fluid monolayer, gray: solid monolayer, white: aggregates

6.2 Isotherm measurements

A representative compression isotherm of BP with the associated BAM images at 36 ºC is shown in Figure 10A. The reciprocal compressibility calculated from the same data is shown in Figure 10B. At a MMA of 65 Å², surface pressure and reciprocal compressibility were zero and a fluid monolayer of BP was observed with BAM to coexist with a monolayer gas phase (Fig. 10Ai). Upon compression, an increasing fraction of the surface was covered by the fluid monolayer until it completely covered the surface at a MMA of 50 Å² (Fig. 10Aii). At this point the surface pressure started to increase and reciprocal compressibility was increased to 2.5 mN/m. During compression solid domains were observed to form with BAM (Fig. 10Aiii). At 18 Å²/molecule, the whole film was covered with the solid monolayer (Fig. 10Aiv). This was accompanied with sharp increase in surface pressure and a 30 mN/m peak in reciprocal compressibility. With further
compression, the surface pressure reached a plateau at 9 mN/m and the film was observed to collapse into folds with BAM (Fig. 10Av).

**6.3 Thickness measurements**

The reflectance values determined from BAM image intensity data and the according thickness values obtained by using equation (7) are presented in Table 1. Taking into account the measurement error, all determined thickness values agree with the theoretical thickness calculated using equation (8). The thicknesses of a solid and fluid BP monolayers were determined to be 42 ± 6 Å and 9 ± 4 Å, respectively. It is likely that the thickness determined for the fluid monolayer is an underestimate, because the packing density of the fluid BP monolayer is expected to be lower than a solid fatty alcohol monolayer. The lower packing density leads to lower reflectance, and therefore underestimation of the thickness.

**Table 1.** Film thicknesses determined from BAM image intensity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Reflectance × 10⁻⁶</th>
<th>Determined thickness (Å)</th>
<th>Theoretical thickness (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Octadecanol</td>
<td>4.4 ± 1.1</td>
<td>20 ± 4</td>
<td>21.6 ± 0.2</td>
</tr>
<tr>
<td>1-Docosanol</td>
<td>6.6 ± 1.6</td>
<td>27 ± 4</td>
<td>26.7 ± 0.2</td>
</tr>
<tr>
<td>1-Hexacosanol</td>
<td>9.9 ± 2.4</td>
<td>34 ± 5</td>
<td>31.8 ± 0.3</td>
</tr>
<tr>
<td>1-Triacontanol</td>
<td>11.5 ± 2.7</td>
<td>37 ± 5</td>
<td>36.8 ± 0.3</td>
</tr>
<tr>
<td>Behenyl palmitoleate (solid)</td>
<td>14.3 ± 3.4</td>
<td>42 ± 6</td>
<td>-</td>
</tr>
<tr>
<td>Behenyl palmitoleate (fluid)</td>
<td>2.0 ± 0.5</td>
<td>9 ± 4</td>
<td>-</td>
</tr>
</tbody>
</table>

Reflectance and thickness are presented as value ± SE. Theoretical thickness in all-trans conformation is calculated according to Tanford⁹².
Figure 10. (A) Isotherm of behenyl palmitoleate at 36 °C with representative Brewster angle microscope images along the isotherm. Note that the exposure time of images i and ii is longer than iii-v.
(i-ii) black: water, gray: fluid monolayer; (iii-v) black: fluid monolayer, gray: solid monolayer, white: aggregates. Scale bar is 500µm. (B) Reciprocal compressibility of behenyl palmitoleate.
6.4 Evaporation measurements

Evaporation reduction capacity of BP as a function of MMA is shown in Figure 11. At 90 Å²/molecule, BP film had no measurable evaporation reducing effect. At 45 Å², corresponding to the fluid phase, BP caused a small but a measurable reduction of approximately 3%. When the packing density of the film was increased, corresponding to the appearance of the solid phase as seen in Figure 10Aiii, evaporation reduction increased, until a constant value of approximately 56% was reached at 9 Å². After this point further increase in the amount of lipid caused no increase in evaporation reduction.

Figure 11. Evaporation reduction (mean ± SD) by behenyl palmitoleate at 37 °C. The line depicts evaporation reduction predicted by equation (20) (see chapter 7.2.3 of Discussion for further details).
7. DISCUSSION

7.1 Approach and experimental methods

Tear film has been studied using *in vivo* in humans and animals\(^{23,27,50-55,93}\), *in vitro* using model eye or surface chemistry models\(^{10,37,57,59,60,87}\) and *in silico* using computer simulations and mathematical models\(^{94-96}\). Each of these approaches has its own strengths and weaknesses and is best suited for certain kinds of studies. An advantage of *in vivo* methods is that they correspond to the physiological conditions and are often fairly directly applicable to medical practice. The weaknesses of *in vivo* methods are that the system under study is often complicated and there is often overlapping effects and compensatory systems, which make interpretation challenging. In addition, *in vivo* methods are often limited by practical and ethical considerations. *In vitro* methods typically offer wider possibilities for measurement and manipulation of the system under study. They can often reproduce the most important aspects of the tear film considering the study question, but do not completely correspond to the physiological system. *In silico* methods can often offer data on time scales and size scales that are difficult to reach experimentally, but they rely heavily on the models and assumptions used and therefore may produce biased or inaccurate results.

An *in vitro* model based on a Langmuir trough system was used in this study. It contains an interface between water and air as is found at the ocular surface, but has slight differences, a major one being that the water layer in the trough is approximately 5 mm thick, about 1000 times thicker than the tear film. The PBS buffer used as a subphase contains a similar electrolyte composition as tear fluid but lacks the tear fluid mucins and proteins. The thickness and presence of mucin and proteins affect the rheological properties of the tear fluid\(^{97}\), but are likely to have a minor effect on the tear film lipid layer. Many tear fluid proteins are also surface active and it has been suggested that they interact with the lipid layer\(^{98}\). This possibility is not taken into account in the current experimental model.

Multiple studies have used similar Langmuir trough-models to study the properties of meibum collected from live specimens\(^{99-102}\). The strength of these studies is that the used lipid layers are likely close in composition to TFLL *in vivo*. A major disadvantage in Langmuir film studies using biological samples is that the composition or amount of lipid inserted to the water surface is not well known. For this reason, only crude estimations of
the film properties can be made. In addition, as was discussed in chapter 3.4.1, practically no polar lipids are found in meibum and the origin of polar lipids in tear fluid is not known. Therefore films formed using meibum alone might lack a crucial component of the TFLL. In this study, known amounts of pure BP were used, which makes the interpretation of the results simpler and makes it possible to understand the behavior of the film on a molecular level. A disadvantage of this approach is that even though BP closely resembles the most abundant wax esters found in meibum\(^8\), other components of TFLL are missing and therefore the results are not directly applicable to the function of TFLL \textit{in vivo}. However, by understanding the behavior of individual TFLL components it is possible to move into more complex mixtures of lipids and possibly also incorporate different tear fluid proteins into the model, but still retain the molecular level understanding of the film behavior.

Measuring surface pressure to study wax esters is not without problems. As has been noted previously\(^10,86\), the concentration and amount of WEs added to the air-water interface affect the results and compression isotherms are not typically very reproducible as WEs tend to form aggregates instead of spreading evenly. Three measures were taken to avoid these difficulties. First, BP was spread from a chloroform solution with a low (1 mM) BP concentration in order to minimize the initial aggregation. Second, single isotherms were measured in contrast to multiple compression-expansion cycles in order to minimize the formation of aggregates by compression. Third, surface pressure measurements were always accompanied with BAM imaging to help the interpretation of surface pressure changes.

BAM has been used for determining the thickness of lipid films by multiple authors\(^91,103,104\). However, it is more commonly used for general visualization purposes, and using BAM for thickness measurements requires additional calibration steps and has certain limitations. In this study the BAM was calibrated by using subphases with different salt concentrations (Fig. 7B), as demonstrated by Rosetti et al.\(^104\), in order to take the nonlinear response of the detector into account. The main limitation of using BAM for film thickness measurements is that some assumptions concerning the optical properties of the film are required. If all measured films have a similar structure, assuming their optical properties to be equal is not a major issue. The main goal of the thickness measurements of this study was to estimate the thickness of BP in the solid phase. Therefore BP was compared to linear, saturated fatty alcohols in the solid phase, which are likely to have
similar optical properties. As can be seen from the error margins of the results (Table 1), this method is not ideal for precise thickness measurements but is well-suited for the crude estimation necessary for the discussion below.

7.2 Surface behavior of wax esters

7.2.1 Effect of temperature

Isochor experiments reveal interesting spreading behavior close to the bulk melting temperature of BP (38 °C). When BP is spread from solvent at low temperatures, it forms solid monolayer domains and three dimensional aggregates, which appear to float on the water surface. This kind of behavior is typically observed for WEs with long, saturated hydrocarbon chains that have high melting temperatures\(^{10,86}\). Similar behavior is observed for long-chain (> 30 carbons) alkanes, which also form mono- and multilayers with perfect crystal symmetry when spread form solvent on the water surface\(^{105}\). Similar to alkanes, these monolayer domains do not spread and appear to be unstable, aggregating and exposing the water surface. At 34.5 °C, well below the bulk melting temperature, the solid domains appear to melt into a fluid monolayer that begins to spread around the solid monolayer domains and cover the water surface. The melting stops, however, when water surface is completely covered by the fluid monolayer and the remaining solid domains coexist with the fluid monolayer.

This behavior can be rationalized with simple thermodynamic arguments. In bulk material, change in Gibbs energy between the solid and liquid states is

\[
\Delta G_b = \Delta H_b - T \Delta S_b
\]  

(12)

where \(\Delta H_b\) is the enthalpy change, \(T\) is temperature and \(\Delta S_b\) is the entropy change. The solid state is favored by the enthalpy, which originates from the cohesive interactions between the closely packed BP molecules. The liquid state is favored by increased entropy associated with the increased degrees of freedom in the liquid state. As temperature increases, the entropic contribution to change in Gibbs energy increases and begins to favor the liquid state. At the melting point the change in Gibbs energy is zero and entropy and enthalpy change are related by

\[
\Delta S_b = \frac{\Delta H_b}{T_b}
\]  

(13)
where $T_b$ is the bulk melting temperature. BP has a slight polar character and at the air-water interface the molecules in liquid state reduce the air-water surface tension as seen in Figure 9. The reduction of water surface tension brings an additional component to the change in Gibbs energy for melting at the air-water interface, which becomes

$$\Delta G_s = \Delta H_s - T(\Delta S_s) - \pi a$$  \hspace{1cm} (14)$$

where $\pi$ is surface pressure increase caused by the spreading of the fluid monolayer and $a$ is the mean molecular area in the fluid monolayer. The reduction of surface tension by the BP molecules in the liquid state therefore favors melting at the air-water surface. Assuming that the entropy and enthalpy change are approximately equal in bulk and at the air-water surface ($\Delta H_s \approx \Delta H_b$ and $\Delta S_s \approx \Delta S_b$), surface melting temperature can be calculated by

$$T_s = T_b \left(1 - \frac{\pi a}{\Delta H} \right)$$  \hspace{1cm} (15)$$

From figures 9 and 10 it can be seen that the surface pressure increase caused by the spreading of the liquid monolayer is 2.3 mN/m and the mean molecular area is approximately 50 Å$^2$. Melting enthalpy has been determined to be in the range of 100-180 $\times$ $10^{-21}$ J/molecule for unsaturated wax esters$^{83,106}$. Using these values the surface melting temperature predicted by equation (15) is 34-36 °C, in close agreement with the observed behavior. As the water surface is completely covered by the fluid monolayer, no further reduction in water surface tension occurs and the additional driving force for melting is removed. Therefore the remaining solid domains do not melt, until the temperature is increased above the bulk melting temperature, as is seen in Figure 9. Even though the experimental evidence seems to agree with the theoretical description, it is worthwhile to consider whether the assumptions made above are justified. As can be seen from Figure 9, the remaining solid domains melt at the bulk melting temperature, implying that the conformation in the solid domains is similar to bulk conformation, in which case the enthalpy of melting should be equal in bulk and at the water surface. It is more difficult to say whether the entropy of melting is also equal in bulk material and at the surface. However, according to the studies on surface freezing in alkanes, the surface melting entropy is approximately equal to bulk melting entropy$^{107}$, so this assumption is also likely to be accurate enough for the estimation used.

In conclusion, three different temperature ranges with different behavior can be identified for BP (see Figure 12): At temperatures below 34-35 °C, BP does not spread and forms solid aggregates. In the 35-38 °C temperature range, BP spreads to the air-water interface
as a fluid monolayer, but if more molecules is added than is required to cover the whole surface, the excess will remain as solid domains. At temperatures higher than 38 °C, BP is in a pseudo-partial wetting state described in chapter 3.3.2 with a fluid monolayer covering the surface and excess lipids as fluid droplets as seen in Figure 9c.

Figure 12. A schematic representation of the behavior of behenyl palmitoleate at the-air water interface in different temperatures. Lines depict the hydrocarbon chains and circles depict the ester linkages. Blue arrows represent the evaporation rate of water through different phases of the lipid film.

7.2.2 Conformation

Isotherm experiments made at 36 °C (Fig. 10), in the temperature range where fluid and solid monolayer phases coexist, and BAM thickness measurements (Table 1) give valuable information about the rough molecular conformation assumed by BP at the air-water interface. As can be seen in Figure 10, the MMA in the fluid monolayer appears to be 50 Å². It is likely that in the fluid phase, BP assumes a previously suggested V-shape conformation (see Figure 12), in which the slightly polar ester linkage is oriented towards the water and the hydrocarbon chains point towards the air in a disordered manner. The disorder of the hydrocarbon chains would explain the very low reflectance measured with BAM. This conformation would also explain the small increase in surface pressure caused by the fluid monolayer. As the film is compressed, the fluid monolayer appears to undergo a transition to the solid state and if the film is expanded, the solid domains melt to cover the whole surface with the fluid monolayer (Fig. 10Aiii).

According to the isotherm measurements, MMA of BP in the solid phase appears to be approximately 18 Å², which is close to the cross-sectional area of a hydrocarbon chain in an all-trans conformation (18.8 Å²). This implies that BP assumes a completely extended
conformation in the solid monolayer (see Figure 12). Crystallographic studies have revealed that saturated wax esters and unsaturated Jojoba-like wax esters both assume an extended conformation in the bulk solid state\textsuperscript{82,83}. The length of a 38-carbons-long saturated wax ester is $51 \pm 3 \text{ Å}$ in a rectangular cell\textsuperscript{82} and the length of 38-carbons-long Jojoba-like wax esters is $43 \text{ Å}$ or $48 \text{ Å}$ depending on crystal structure\textsuperscript{83}. The thickness of a solid BP monolayer was determined by BAM to be $42 \pm 6 \text{ Å}$, which also suggests that BP resides in a completely extended conformation. Bulk-like extended conformation would also explain why the melting of solid monolayer domains coincides with the bulk melting temperature. This behavior is similar to long chain alkanes, which have been found to form solid, extended monolayers on water or SiO\textsubscript{2} surfaces\textsuperscript{105,108}.

In the 35-38 °C temperature range these two conformations coexist. When MMA is larger than $50 \text{ Å}^2$, all the molecules adopt the fluid V-shape conformation driven by the water surface tension. If MMA is smaller than $50 \text{ Å}^2$, all the molecules will not fit to the air-water interface in the V-shape conformation and excess molecules will adopt the bulk-like extended conformation that is stabilized by the maximal cohesive interactions between adjacent hydrocarbon chains. When MMA is smaller than $18 \text{ Å}^2$, all the molecules will adopt the solid, extended conformation.

It is interesting to reflect back to the results obtained by the early studies on hexadecyl palmitate (HP) and evaluate if they can be explained by the behavior pattern observed in this study. The observation that in room temperature HP (melting temperature 53.5 °C) forms unstable films, from which isotherms could not be properly measured\textsuperscript{85,109}, is explained by the fact that wax esters do not spread so far below their melting temperature and tend to aggregate into lipid islands. Adam\textsuperscript{85} observed that solid HP films expand at 50 °C, corresponding to the surface melting temperature of HP, which also fits the behavior described in this study. Alexander and Schulman\textsuperscript{109} measured the surface potential of a HP film at room temperature and obtained values of either 0 mV or 200-300 mV, apparently randomly. They had no way of visualizing the lateral organization of the film, but with current knowledge this finding is easy to explain. Surface potential probe only measures the potential at small spot on the surface, and depending on whether this spot would happen to contain an island of lipid or pure water surface, it would give different values of surface potential. Due to the calibration, pure water surface is expected to result in 0 mV surface potential and 200-300 mV is likely measured from a solid domain of HP. Assuming a MMA of $18 \text{ Å}^2$ in the solid state, 200-300mV would translate to a
perpendicular dipole moment of 100-150 mD, a value that would be expected if the carbonyl moiety was oriented horizontally\textsuperscript{110}, as expected in the extended conformation. In conclusion, all the early findings concerning the surface behavior of HP can be explained in detail by the behavior pattern described in this study, which casts doubt on the original conclusion that WE\textsubscript{s} would adopt a hairpin conformation in the solid state.

7.2.3 Evaporation resistance

The three temperature ranges described in chapter 7.2.1 explain in more detail the previous finding that WE\textsubscript{s} only retard evaporation close to their melting temperature\textsuperscript{10}. The evaporation retardation caused by BP in different temperatures is shown schematically in Figure 12. At low temperatures, WE\textsubscript{s} do not spread on the water surface and a large proportion of the water surface remains uncovered, causing no effect on the evaporation rate. At high temperatures, the wax esters spread, but in the fluid V-shape conformation. Due to the fluidity and large free volume of the monolayer, it only has a very small effect on the evaporation rate, as has been observed for fluid monolayers of phospholipids or fatty alcohols\textsuperscript{41,60}. In the temperature range between the surface melting temperature $T_\text{s}$ and bulk melting temperature $T_\text{b}$, WE\textsubscript{s} appear to have evaporation retarding properties. In the following paragraph, a theoretical model to explain the evaporation reducing effect is derived in a similar manner to Gilby\textsuperscript{44} and Cerretani\textsuperscript{37}.

In the temperature range between $T_\text{s}$ and $T_\text{b}$, solid and fluid monolayer phases coexist as described in chapters 7.2.1 and 7.2.2. The fraction covered by the solid phase can be expressed as

$$\phi = \frac{a_2(a - a_1)}{a(a_2 - a_1)} \quad (16)$$

where $a$ is the total mean molecular area and $a_1$ and $a_2$ are the mean molecular areas of the fluid and solid phases, respectively. By combining equations (9), (10) and (11) from chapter 5.3.4, evaporation reduction $E$ can be expressed as

$$E = 1 - \frac{J}{J_w} = \frac{R_k}{R_k + R_e} \quad (17)$$

It is expected that the addition of a monolayer of lipid on the water surface will decrease evaporation by increasing the resistance for the kinetic escape from the water phase, $R_k$. Assuming that the evaporation resistances of both the solid and fluid monolayer are constant, the total evaporation resistance of the monolayer film containing both phases can
be expressed, analogous to parallel electrical resistances, as a function of the solid phase coverage as

\[ \frac{1}{R_k} = \Phi \frac{1}{R_{k,2}} + (1 - \Phi) \frac{1}{R_{k,1}} \]

(18)

where \( R_{k,1} \) and \( R_{k,2} \) are the evaporation resistances of the fluid and solid monolayer phases, respectively. By combining equations (17) and (18), evaporation reduction is then expressed as a function of solid phase coverage as

\[ E = \left[ \Phi \frac{R_e}{R_{k,2}} + (1 - \Phi) \frac{R_e}{R_{k,1}} + 1 \right]^{-1} \]

(19)

Finally, by combining equations (16) and (19), evaporation reduction can be expressed as a function of total MMA as

\[ E = \left[ \frac{a_2(a - a_1)}{a(a_2 - a_1)} \left( \frac{R_e}{R_{k,2}} - \frac{R_e}{R_{k,1}} \right) + \frac{R_e}{R_{k,1}} + 1 \right]^{-1} \]

(20)

According to equation (19), when \( \Phi = 0 \) or \( \Phi = 1 \) i.e. the surface is completely covered with either a fluid or a solid monolayer, evaporation resistance becomes

\[ R_{k,i} = \frac{R_e E_i}{1 - E_i} \]

(21)

where \( E_i \) is the evaporation reduction caused by a completely fluid (i=1) or solid (i=2) monolayer. As shown in Figure 11, evaporation reduction caused by BP reaches a plateau value when the amount of lipid is increased, as the whole surface is covered with a solid monolayer. Using equation (21), this plateau can be used to determine the value of 2.7 ± 0.9 s/cm for \( R_{k,2} \). As seen from Figure 10, fluid monolayer completely covers the surface at a MMA of 50Å². Therefore the evaporation reduction value measured at 45Å² can be used to determine a value of 0.1 ± 0.1 s/cm for \( R_{k,1} \). When the MMA and evaporation resistances of the fluid and solid monolayer phases have been obtained, the evaporation reduction as a function of MMA can be predicted by equation (20), as shown by the continuous line in Figure 11. Experimental results agree well with the theoretical description, except for values close to 18Å². At least two possible explanations for this exist. First, BP spreads rather slowly and it is possible that when the amount of BP added to the surface is close to the minimum amount needed to cover the whole surface, BP only
reaches full coverage after a certain equilibration time. Before full coverage is reached, evaporation is higher leading to a lower value of $E$. Second, rather small imperfections in film coverage would lead to a large decrease in $E$. As predicted by equation (19), a decrease in coverage from 100% to 98% would lead to a reduction in $E$ from 59% to 41%. Both of these effects are likely to be eliminated when ample amounts of BP are added to the surface (MMA < 9 Å$^2$). In conclusion, these results show that a fluid monolayer of BP has a minor, physiologically insignificant effect on the evaporation rate and a solid monolayer of BP has a large effect, comparable to the effects measured for TFLL in vivo. However, decreasing the MMA to 4.5 Å$^2$, corresponding to four solid layers of BP, does not increase the evaporation retarding effect, likely because additional lipid does not appear to form ordered layers but forms aggregates visible even to the naked eye.

As mentioned in chapter 3.3.3, the relative decrease in evaporation rate depends highly on the experimental conditions. This is mainly because the environmental evaporation resistance for mass transfer depends on conditions like wind speed. In vacuum or high wind speed conditions, the evaporation reduction caused by lipids is more pronounced, because mass transfer resistance is smaller. In contrast, in stagnant air conditions, the measured evaporation reduction is smaller, because the mass transfer resistance is large. Therefore the most sensitive measurements for evaporation reduction caused by lipid films are accomplished by minimizing mass transfer resistance. In our experiments a slow airflow, comparable to light wind, was used in the evaporation chamber. In physiological conditions, the ocular surface is not typically subjected to high wind speeds. Therefore it should be possible to measure any physiologically relevant levels of evaporation reduction using this experimental system. The evaporation resistance of a solid BP monolayer (2.7 s/cm) is close to the resistance values measured for saturated fatty alcohols and fatty acids with ~20 carbons$^{40,111}$, slightly less than expected considering the thickness of the monolayer alone. This could be explained by slower spreading of the BP film, which may lead to incomplete coverage of the surface, or by less efficient packing of BP due to the ester moiety and a double bond. However, evaporation resistance of a solid BP monolayer can be considered physiologically significant, since it is large enough to have significant impact also under stagnant air or low wind speed conditions.
7.3 Implications for the function of TFLL

Meibum melting temperature has been reported to vary in the range of 20-40 °C\textsuperscript{16,112}, with a typical melting temperature of 33-34 °C\textsuperscript{100,113,114}. The temperature of the inner eyelid is approximately 36 °C\textsuperscript{115}, close to body temperature and slightly higher than meibum melting temperature. Therefore healthy meibum will be completely or almost completely fluid inside the Meibomian glands\textsuperscript{116} and will be able to flow from the glands to the ocular surface when the glands are compressed by the blinking action of the lids. Ocular surface temperature is 32-34 °C\textsuperscript{113,117-119}, meaning that meibum will cool down several degrees when it spreads to the ocular surface. It has been suggested that stiffening due to cooling at the ocular surface could explain the remarkable stability of TFLL that is observed between blinks \emph{in vivo}\textsuperscript{19}. In fact, recent studies have recognized that TFLL is likely in a partially crystalline state at the ocular surface\textsuperscript{63,106,116}. This crystalline ordering is likely needed for the proper function of TFLL, since if it was only important that meibum stays liquid in order to flow from the Meibomian glands, there would be no reason for the melting temperature to match the ocular surface temperature.

The anti-evaporative mechanism of wax esters determined in this study would explain the need for matching the melting temperature of meibum with ocular surface temperature. As was described in the previous chapters, a few degrees below the bulk melting temperature, WE films spread at the air-water interface, driven by the surface tension. Surface tension has typically been considered to be the driving force for the spreading of TFLL\textsuperscript{93}. However, due to the cohesive interactions between hydrocarbon chains, WEs finally adopt an extended, bulk-like conformation that effectively retards evaporation, as long as there is an ample supply of WE. Therefore, at the right temperature range TFLL could have both fast spreading properties and efficient evaporation retarding properties. Abnormally high melting temperature would prevent the TFLL from spreading and covering the ocular surface, exposing it to evaporation. On the other hand, abnormally low melting temperature would make the TFLL too fluid to prevent evaporation.

It is clear, however, that the scheme described above for the function of TFLL is a simplification, based on measurements conducted using a simple WE molecule. In reality, TFLL is formed by a complex mixture of lipids and also tear fluid proteins may play a role in the function of TFLL. These results suggest that the evaporation retarding function can be accomplished by a structured interfacial layer and the thickness of TFLL is of secondary
importance. The evaporation retarding layer is likely formed by an organized layer or several layers of WEs and CEs, as suggested previously by King-Smith et al. Tear fluid proteins potentially adsorb to the interface between the aqueous and lipid layers might have a role in stabilizing this interfacial layer. Polar lipids of the tear fluid lipid layer likely have a similar role and possibly improve the spreading of the nonpolar lipids. Due to its apparently complex nature, the proper function of TFLL is likely susceptible to alterations in the quality and proportions of its components. Therefore, treatment options for dry eye aimed to act on the lipid layer should then be aimed to correct the proper composition and organization of TFLL, instead of simply adding some oil to the surface of the tear film, a fact that has been noted also in clinical studies 50.

7.4 Conclusions

In this Master’s thesis, surface behavior of BP, a wax ester closely resembling the most abundant WEs in found in meibum, was studied. The results show that above its bulk melting temperature, BP forms a fluid monolayer and below its bulk melting temperature a solid monolayer. Within approximately 3 °C below its bulk melting temperature the solid and fluid monolayer phases coexist and their relative proportions depend on the MMA of the film. Due to this coexistence, BP films are able to spread rapidly driven by the water surface tension and cover the water surface, but finally form a solid monolayer that prevents evaporation. In addition, it was shown that BP assumes an extended conformation in the solid phase, which allows tight packing of the molecules and explains the ability of this phase to retard evaporation. In total these results provide a molecular level explanation for the previously discovered evaporation retarding properties of WEs. The results cannot be directly applied to the physiological TFLL due to the fact that TFLL contains also large amounts of other lipid species and there may be interaction between tear fluid proteins and the lipid layer. However, understanding the behavior of individual components of TFLL is necessary in order to explain the relationship between the structure and function of TFLL in all its complexity.
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