Understanding the physical properties of meibomian lipid films

Chendur Kumaran Palaniappan

Thesis submitted for the degree of Doctor of Philosophy

Supervisors: Thomas J. Millar, Poonam Mudgil

School of Science and Health
University of Western Sydney
Parramatta Campus, NSW, Australia
June, 2013
Acknowledgements

I would like to thank my supervisor Thomas Millar, for being not only a supervisor but a mentor, who has allowed me to achieve so much. His indomitable patience, support and guidance have been much appreciated.

I would also like to thank my research colleagues. This includes Poonam, Shiwani, Santosh, Martin and Dr (B) Burkardt for all their support.
Dedication

I would like to dedicate this to “my Devi”, Joe Thangathurai and my family, without whom none of this would have been possible.
Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

............................................................

Chendur Kumaran Palaniappan

June, 2013
Table of Contents

List of Tables............................................................................................................................ iv
List of Figures............................................................................................................................. v
Abbreviations............................................................................................................................ ix
Abstract..................................................................................................................................... x

1 Introduction........................................................................................................................ 1

1.1 Overview of the tear film............................................................................................... 2
1.2 Structure of the tear film.............................................................................................. 4
1.3 Components of the tear film......................................................................................... 7
1.3.1 Aqueous-mucin layer of the tear film..................................................................... 7
1.3.2 Aqueous components............................................................................................... 8
1.4 Lipid layer of the tear film........................................................................................... 13
1.4.1 Composition and structure.................................................................................... 13
1.4.2 Function of meibomian lipid layer........................................................................ 21
1.4.3 Surface tension and surface activity of the TPLL................................................... 21

2 Effect of cholesterol, cholesterol esters and free fatty acids on meibomian lipid films... 23

2.1 Introduction.................................................................................................................... 23
2.1.1 Cholesterol and its esters...................................................................................... 24
2.1.2 Fatty acids and phospholipids............................................................................... 26
2.1.2 Background to experimental design and methodology........................................ 28
2.2 Materials and methods............................................................................................... 29
2.3 Collection of human and koala meibomian lipids....................................................... 29
2.4 Surface pressure-area (Π-A) measurements................................................................ 30
2.5 Epifluorescence microscopy of spread lipid films........................................................ 33
2.6 Results............................................................................................................................ 33
2.6.1 Effect of cholesterol on the surface activity of meibomian lipids............................ 33
2.6.2 Epifluorescence microscopy of meibomian lipid films seeded with cholesterol..... 37
2.6.3 Effect of cholesteryl oleate and oleic acid on the surface activity of meibomian lipids......................................................................................................................... 38
2.7 Discussion...................................................................................................................... 41

3 Effect of specific protein and lipid components on meibomian lipid films............... 46

3.1 Introduction.................................................................................................................... 46
3.2 Materials and methods............................................................................................... 48
3.2.1 Purification of lung surfactant................................................................................ 48
3.2.2 Gel electrophoresis of bovine surfactants.............................................................. 49
3.2.3 Western blot analysis of purified proteins.............................................................. 50
3.2.4 Estimating total protein (Bradford Assay).............................................................. 50
3.3 Surface pressure-area (Π-A) measurements................................................................. 50
3.4 Penetration of keratin into meibomian lipid films....................................................... 51
3.5 Results............................................................................................................................ 52
3.5.1  Purification of lung SPs................................................................. 52
3.5.2  Western blot analysis of purified bovine lung surfactant................. 53
3.5.3  Surface activity of β-carotene....................................................... 54
3.5.4  Effect of β-carotene on the surface activity of meibomian lipids........ 55
3.5.5  Effect of bovine lung surfactant on the surface activity of meibomian lipids. 57
3.5.6  Surface activity of keratin at 35°C.................................................. 61
3.5.7  Penetration and effect of keratin on the surface activity of meibomian lipids at 35°C................................................................. 63
3.5.8  Dynamic-cyclic II-A profile of fluorescently tagged human meibomian lipids before and after penetration of different concentrations of keratin at 35°C .... 65

3.6  Discussion......................................................................................... 67

4  Effects of phospholipids and proteins on meibomian lipid films.............. 71

4.1  Introduction....................................................................................... 71
4.2  Methods............................................................................................. 72
  4.2.1  Penetration of lysozyme into meibomian lipid films seeded with 10 mol % cholesterol or oleic acid.......................... 72
  4.2.2  Surface activity measurement of phospholipids............................. 72
  4.2.3  Penetration of phospholipids into meibomian lipid films............... 73
  4.2.4  Pre and post penetration isocycles............................................... 73

4.3  Results............................................................................................... 74
  4.3.1  The effect of cholesterol and oleic acid on the interaction of lysozyme with human meibomian lipids ......................... 74
  4.3.2  Surface activity of phospholipids................................................... 77
  4.3.3  Interaction of phospholipids in an aqueous environment with human meibomian lipids ............................................. 78

4.4  Discussion......................................................................................... 80

5  Viscoelasticity of cholesterol, its esters and β-Carotene............................. 83

5.1  Introduction....................................................................................... 83
5.2  Materials and solutions.................................................................... 84
5.3  Pendant drop method....................................................................... 84
5.4  Results............................................................................................... 87
  5.4.1  Viscoelasticity of meibomian films and lysozyme......................... 87
  5.4.2  Viscoelasticity of meibomian films with and without 1 mol % cholesterol or 1 mol % β-carotene............................ 88
  5.4.3  Viscoelasticity of meibomian films seeded with and without oleic acid or cholesteryl oleate................................. 89
  5.4.4  Viscoelasticity of meibomian films seeded with and without varying molar ratios of cholesterol............................... 91
5.5  Discussion......................................................................................... 92

6  X –Ray Reflectivity of meibomian lipid films......................................... 97

6.1  Introduction....................................................................................... 97
6.2  Materials and methods.................................................................... 98
6.3  Results............................................................................................... 100
6.3.1 Spread films of human meibomian lipids at 20°C................................. 100
6.3.2 Spread films of human meibomian lipids seeded with cholesterol at 20°C.. 103
6.3.3 Spread films of human meibomian lipids seeded with β-carotene at 20°C... 107
6.3.4 Spread films of 1:1 mixture of cholesteryl oleate and stearyl oleate at 20°C.110
6.3.5 Spread films of DPPC at 20°C............................................................... 112

6.4 Discussion........................................................................................................ 114

7 Conclusion............................................................................................................. 117

References........................................................................................................... 120
List of Tables

Table 1.1: Lipid composition of normal human meibum................................................................. 14

Table 6.1: Refined structure of spread meibomian lipid films (50μL, 1mg/mL) at the air-buffer interface as function of surface pressure (Scattering length density of the aqueous subphase: 9.45×10^{−6} Å²) ... 103

Table 6.2: Refined structure of spread meibomian lipid films seeded with 1 mol % cholesterol at the air-buffer interface as function of surface pressure (Scattering length density of the aqueous subphase: 9.45×10^{−6} Å²) ................................................................................................................................. 106

Table 6.3: Refined structure of spread meibomian lipid films seeded with 1 mol % β-carotene at the air-buffer interface as function of surface pressure (Scattering length density of the aqueous subphase: 9.45×10^{−6} Å²) ........................................................................................................................................ 109

Table 6.4: Refined structure of spread 1:1 mixture of cholesteryl and stearoyl oleate (40μL, 0.5mg/mL) at the air-buffer interface as function of surface pressure (Scattering length density of the aqueous subphase: 9.45×10^{−6} Å²) ................................................................................................................................................ 111

Table 6.5: Refined structure of spread DPPC film (40μL, 0.5mg/mL) at the air-buffer interface as function of surface pressure (Scattering length density of the aqueous subphase: 9.45×10^{−6} Å²) ..... 113
List of Figures

Figure 1.1: Traditional view of the tear film, illustrating the lipid, aqueous and mucus layers (Bron et al, 2004). .................................................................................................................................................. 5

Figure 1.2: Tear film model illustrating the homogeneous aqueous-mucin layer and protein lipid mixture at the surface (Millar et al, 2006). .............................................................................................. 6

Figure 2.1: Schema of typical Π-A compression and expansion curves: Π_{max}, maximum surface pressure. A: Rigid film. B: Fluid film. ................................................................. 33

Figure 2.2: Dynamic cyclic Π-A profile of koala and human meibomian lipids seeded with and without 1 mol % cholesterol at 35 °C. ................................................................. 34

Figure 2.3: Dynamic cyclic Π-A profile of human meibomian lipids seeded with and without 5 mol % cholesterol at 35 °C. ................................................................. 35

Figure 2.4: Dynamic cyclic Π-A profile of human meibomian lipids seeded with varying molar ratios of cholesterol at 35 °C. ................................................................. 36

Figure 2.5: Dynamic cyclic Π-A profile of human meibomian lipids seeded with varying molar ratios of cholesterol at 20 °C. ................................................................. 36

Figure 2.6: Fluorescent micrographs of human meibomian lipid films and mixtures with cholesterol at 20 °C. ................................................................. 38

Figure 2.7: Dynamic cyclic Π-A profile of pure cholesteryl oleate films, as well as human meibomian lipids with and without 10 mol % cholesteryl oleate. ................................................................. 39

Figure 2.8: Dynamic cyclic Π-A profile of human meibomian lipids with and without 5 mol % oleic acid. ................................................................. 40

Figure 2.9: Dynamic cyclic Π-A profile of oleic acid films at varying concentrations. ......................... 41

Figure 3.1: Optical density profile of lung surfactant fractions separated using Sephadex LH20 column. ........................................................................................................................................... 52
Figure 3.2: SDS PAGE analysis of lung surfactant fractions purified using hydrophobic affinity-size exclusion column. .................................................................53

Figure 3.3: Western blot analysis of purified bovine lung protein. Circled bands represent positive reaction to anti-SP-B. .................................................................54

Figure 3.4: Dynamic cyclic Π-A profile of β-carotene film at 20°C and 35°C. .................................................................54

Figure 3.5: Dynamic cyclic Π-A profile of human meibomian lipids seeded with and without 1 mol % β-carotene at 20°C. .................................................................56

Figure 3.6: Dynamic cyclic Π-A profile of human meibomian lipids seeded with and without 1 mol % β-carotene at 35°C. .................................................................57

Figure 3.7: Comparative isocycles at 20°C and 35°C of lung SP alone and mixtures of lung SP with meibomian lipids. .................................................................60

Figure 3.8: Micrographs showing the appearance of a mixture of lung SP (2μg) and meibomian lipids (20μg) seeded with a fluorescent marker. .................................................................61

Figure 3.9: Dynamic cyclic Π-A profile of keratin (5μL, 9.2mg/mL) at 35°C. .................................................................62

Figure 3.10: Representation of a typical penetration profile. .................................................................63

Figure 3.11: A series of penetration curves at 35°C showing the penetration of different concentrations of keratin (9.2mg/mL) and control solutions into a human meibomian film (20μL, 1mg/mL) compressed to a predetermined surface pressure of 5mN/m. .................................................................64

Figure 3.12: Dynamic cyclic Π-A profile of human meibomian lipids (20μL, 1mg/mL) before and after penetration of different amounts of keratin (9.2mg/mL) at 35°C. .................................................................66

Figure 4.1: Lysozyme (35μL, 3.2mg/mL) penetration into meibomian lipid films with and without 10 mol % cholesterol or oleic acid. .................................................................75

Figure 4.2: Dynamic isocycle Π-A profiles of meibomian lipid films with and without 10 mol % cholesterol or oleic acid before penetration of lysozyme (35μL, 3.2mg/mL) at 35°C. .................................................................75
Figure 4.3: Dynamic cyclic Π-A profile of meibomian lipid films with and without 10 mol % cholesterol or oleic acid after penetration of lysozyme (35µL, 3.2mg/mL) at 35°C. .......................................................... 76

Figure 4.4: Surface activity measurement of phospholipids at 35°C. .......................................................... 78

Figure 4.5: Penetration of phospholipids (6mL, 0.5mg/mL) into a human meibomian film (20µL, 1mg/mL) compressed to a predetermined surface pressure of 5mN/m at 35°C. .................................................. 79

Figure 4.6: Dynamic cyclic Π-A profile of meibomian lipid film (20 µL, 1mg/mL) before and after penetration of phospholipids (6mL, 0.5mg/mL) at 35°C. ............................................................................... 79

Figure 5.1: A typical drop hanging from the tip of a needle giving the pendant shape. ...................... 86

Figure 5.2: Commercial set up of the pendant drop apparatus. ............................................................ 87

Figure 5.3: Complex viscoelasticity as a function of frequency of human meibomian films, lysozyme and meibomian films coated with lysozyme. .................................................................................................. 88

Figure 5.4: Complex viscoelasticity as a function of frequency of human meibomian films seeded with and without 1 mol % cholesterol or 1 mol % β-carotene. .......................................................... 88

Figure 5.5: Complex viscoelasticity as a function of frequency of lysozyme (LZ) coated human meibomian films seeded with 1 mol % cholesterol or 1 mol % β-carotene. ........................................ 89

Figure 5.6: Complex viscoelasticity as a function of frequency of meibomian films with and without 5 mol % oleic acid. ............ ........................................................................................................ 90

Figure 5.7: Complex viscoelasticity as a function of frequency of meibomian films with and without 10 mol % cholesterol oleate. .................................................................................... 90

Figure 5.8: Complex viscoelasticity as a function of frequency of meibomian films with and without 5 or 10 mol % cholesterol. .......................................................... ...................................................................... 91

Figure 6.1: Dynamic cyclic Π-A profile of human meibomian lipid film (50µL, 1mg/mL). ........... 101

Figure 6.2: X-ray reflectivity profiles of human meibomian lipid films (50µL, 1mg/mL) at the air-buffer interface. ............................................................................................................... 102
**Figure 6.3:** Dynamic cyclic Π-A profile of human meibomian lipid film seeded with 1 mol % cholesterol. .........................................................................................................................................................................................104

**Figure 6.4:** X-ray reflectivity profiles of human meibomian lipid films seeded with 1 mol % cholesterol at the air-buffer interface. .........................................................................................................................................................................................105

**Figure 6.5:** Dynamic cyclic Π-A profile of human meibomian lipid film seeded with 1 mol % β-carotene. .........................................................................................................................................................................................107

**Figure 6.6:** X-ray reflectivity profiles of human meibomian lipid films seeded with 1 mol % β-carotene at the air-buffer interface. .........................................................................................................................................................................................108

**Figure 6.7:** Dynamic cyclic Π-A profile of 1:1 mixture of cholesteryl oleate and stearyl oleate (40μL, 0.5mg/mL). .........................................................................................................................................................................................110

**Figure 6.8:** X-ray reflectivity profile of a 1:1 cholesteryl oleate: stearyl oleate film (40μL, 0.5mg/mL) at the air-buffer interface. .........................................................................................................................................................................................111

**Figure 6.9:** X-ray reflectivity profile of spread DPPC film (20μL, 0.5mg/mL) at the air-buffer interface. .........................................................................................................................................................................................112
Abbreviations

$\Pi$  surface pressure
$
\Pi_{\text{init}}$
 initial surface pressure
$
\Pi_{\text{max}}$
 maximum surface pressure
$\Pi_i$
 transition state surface pressure
$\Pi$-$\text{A}$
 surface pressure-area
$\Pi$-$\text{T}$
 surface pressure-time
$\text{Alcs}$
 alcohols
$A_{\text{min}}$
 minimum area
$\text{AT}$
 aqueous tears
$\text{Ch}$
 cholesterol
$\text{DPPC}$
 dipalmitoylphosphatidylcholine
$\text{FFA}$
 free fatty acids
$\text{HC}$
 hydrocarbon
$\text{Ig}$
 immunoglobulin
$\text{MGD}$
 meibomian gland dysfunction
$\text{SE}$
 sterol ester
$\text{SP}$
 surfactant proteins
$\text{SP-A}$
 surfactant protein A
$\text{SP-B}$
 surfactant protein B
$\text{SP-C}$
 surfactant protein C
$\text{SP-D}$
 surfactant protein D
$\text{TFLL}$
 tear film lipid layer
$\text{TG}$
 triglycerol
$\text{WE}$
 wax ester
lung SPs
 lung surfactant proteins
tear SPs
 tear lung surfactant proteins
Abstract

Purpose: Meibomian gland secretions form the major component of the lipid layer of the tear film. The composition of meibomian gland secretions, their interactions and the resulting physicochemical properties of the lipid layer have been shown to be important determinants of tear film stability and pathophysiology of various dry eye diseases. A prevailing view in ocular research is that the components of the tear film are integrated and therefore, if a particular component of the tear film is impaired it will alter the performance of the whole tear film and such changes pre-empt dry eye. Indeed, changes in the levels of various protein and lipid components have been correlated with various dry eye conditions such as blepharitis and Sjögren’s syndrome, but have never been experimentally tested. Therefore, the aim of this project was to evaluate how changes in specific components of meibomian gland secretions affect both the structural and physicochemical properties of meibomian lipid films.

Method: A range of physicochemical techniques including surface pressure measurements using the Langmuir trough, viscoelasticity (pendant drop) and X-ray reflectometry were used to evaluate pure meibomian lipid films and meibomian lipid films seeded with specific lipids including cholesterol, cholesteryl oleate, fatty acids, phospholipids and β-carotene, or proteins including surfactant proteins and keratin, and mixtures of lipids and proteins.

Results: Surface pressure-area (Π-A) profiles of meibomian films seeded with 1 mol % cholesterol, 5 mol % oleic acid and 10 mol % cholesteryl oleate only demonstrated minor differences in hysteresis and maximum Π (Πmax). Fluorescence microscopy and Π-A profiles of films with 10-100 mol % cholesterol indicate that cholesterol displaces meibomian lipids at high Π. Π-A profiles and fluorescence microscopy demonstrated that the addition of 1 mol % β-carotene at 20°C caused a strong decrease in the surface area occupied by meibomian lipids,
but β-carotene was squeezed out at high Π. However, at 35°C the addition of 1 mol% β-carotene had minimal influence on the surface activity of meibomian films.

Monomeric (≈10kDa) and dimeric (18kDa) lung surfactant proteins were successfully isolated from bovine lungs. Western blotting confirmed that it was surfactant protein-B (SP-B). Films of SP-B were extremely surface active, with a $\Pi_{\text{max}} \approx 45\text{mN/m}$. Seeding meibomian films with minimal amounts of SP-B cause a marked increase in $\Pi_{\text{max}}$ but significant hysteresis was not observed. Another protein, keratin, known to be a component of meibomian gland secretions, demonstrated strong surface activity with an $\approx \Pi_{\text{max}}$ of 28mN/m. Keratin was capable of penetrating meibomian films, and Π-A profiles after penetration demonstrated a notable increase in Π and increased hysteresis. Fluorescence microscopy revealed that dark lipid protein complexes formed due to the presence of keratin in the meibomian lipid film. The penetration profile of lysozyme into meibomian films seeded with 10 mol% cholesterol or oleic acid was similar to meibomian films without 10 mol% cholesterol or oleic acid. Π-A profile of phospholipids spread onto the air-buffer interface demonstrated strong surface activity, but minimal hysteresis. The Π at which the phase transition occurred was increased in our experiments at 35°C. Phospholipids injected into the buffered subphase of the Langmuir trough demonstrated nominal penetration into a meibomian lipid film, but Π-A profiles after penetration demonstrated a notable rise in $\Pi_{\text{max}}$. Films of meibomian lipids and lysozyme individually demonstrated gel-like behaviour. However, films coated with lysozyme demonstrated a 2 fold increase in viscoelastic modulus compared to individual meibomian lipid films. The viscoelastic modulus values of meibomian films with and without 1 mol% cholesterol or β-carotene was similar across the frequency range, and the addition of lysozyme to the subphase made the meibomian film more resistant to changes in frequency.

Seeding the meibomian film with 5 mol% oleic acid caused minimal differences in the modulus (less than 10mN/m), but the elasticity of meibomian films was reduced with the addition of 10 mol% cholesterol oleate. Seeding the meibomian film with 5 mol% and 10 mol% cholesterol caused a strong increase in the viscoelasticity of meibomian films, particularly at high frequencies. A three layered model was required to satisfactorily fit X-ray
reflectivity data of spread human meibomian lipid films at a range of surface pressures including the lowest surface pressure of 0.6mN/m. Meibomian films seeded with 1 mol % cholesterol demonstrated the same structural characteristics as unseeded meibomian films. In contrast to unseeded meibomian films and meibum seeded with 1 mol % cholesterol, the addition of 1 mol % β-carotene to meibomian films caused an increase in overall thickness in the film at medium II, before decreasing at high II, the X-ray reflectivity data indicate a phase transition in the system. II-A isocycles of a 1:1 mixture of cholesteryl oleate and stearyl oleate demonstrated very little surface activity, and reflectivity of the compressed mixture showed very little structure and contrast compared to the aqueous subphase. A compressed DPPC monolayer gave the expected two phase model, representing polar head groups adjacent to the air (~9Å) and hydrocarbon tail groups adjacent to the subphase (~15Å).

**Conclusion:** Under *in vitro* controlled conditions, the physicochemical properties of meibomian films are resistant to large changes in lipid composition across a range of lipids. Changes well beyond any physiologically realistic level to known lipid components such as cholesterol, cholesterol esters and wax esters, had little effect. This tolerance is most likely due to the ability of meibomian films to form complex multilayers at low surface pressures, as well as increased thickness and reorganisation at high II. In contrast, even minor changes in concentration of proteins, particularly lung surfactant proteins and keratin could cause considerable changes in II and activity of meibomian films. There is also indisputable evidence from this investigation that proteins from the aqueous strongly interact with the meibomian lipid film and affect both surface tension and viscoelastic properties. Future studies should evaluate newly discovered amphiphilic components of the tear film including (O-acyl)-omega-hydroxy-fatty acids which could have an important biophysical role. Moreover, although this investigation focused on the effect changes in ratios of lipids and proteins have on meibomian films, future research should evaluate the effect decreased lipids and proteins have on the physicochemistry of meibomian films.
1 Introduction

For vision, the eye must have a transparent window and the cornea provides this. To be transparent, it must be avascular and consequently, systems other than the blood have to be able to provide immune protection and nutrition. The pre-corneal tear film protects the cornea from most environmental challenges and is a major source of nutrients. Beyond its biochemical and immunological roles, the tear film must also retain particular physical properties in order to serve its function. It must be optically smooth, maintain transparency, and form a stable film that spreads readily and evenly across the ocular surface (Montés-Micó et al, 2010). Tear film spread, stability and function are based on physicochemical properties such as viscosity and surface tension.

For the layman, the tear film is regarded as a simple salt solution, whereas professionals generally regard it as a three layered structure comprising an innermost mucous layer attached to the corneal epithelium, an overlying aqueous layer and a superficial lipid layer on the outer surface (Fig. 1). For those trying to investigate its physical characteristics, particular attention has to be given to its dynamic nature. For example, Tiffany (1991) was able to show that whole tears were truly a complex fluid because they did not show normal viscosity. Instead the viscosity decreased rapidly under stress which is characteristic of non-Newtonian fluids (Tiffany, 1991). This complexity might be expected when one considers that it is a dynamic film that has to readily collapse during a blink but must remain stable during environmental stressors such as high temperatures and airflow when not blinking. There is strong evidence from biomarkers that the aqueous layer is in constant flux (Khanal and Millar, 2010), whereas the lipid layer appears to be more stable in interferometric observations (Bron et al, 2004). Therefore, it is likely that the molecular composition, interactions and the resulting physicochemical properties within the lipid layer are responsible for the ability of the tear film to resist changes and enable tear stability.
Developing an understanding of the lipid layer provides many challenges. It is dynamic, made from many components, and is a transparent film less than 0.1µm thick (Bron et al, 2004). In order to gain insight into such complex systems, one approach is to develop models and then test them under dynamic conditions similar to those of tear film on the eye. Such strategies also assist in understanding how the lipid layer changes in diseases such as blepharitis and Sjögren’s syndrome, which are causes of dry eye.

The major components of the lipid layer are lipids secreted from meibomian glands in the eyelids. Therefore, analysing them for their lipid composition and how this changes in disease has been the foundation of most structural models of the lipid layer. More recently, our understanding of the lipid layer has been expanded by studies of its physicochemical properties such as surface pressure, viscosity and free energy (Miano et al, 2005; Tiffany, 2006; Leiske et al, 2010). The aim of this thesis is to extend our understanding of the tear film lipid layer (TFLL) by examining the contribution of specific components in maintaining its function and integrity, using a range of physicochemical measurements and techniques including, surface pressure, viscoelasticity and X-ray reflectivity.

1.1 Overview of the tear film

The tear film is a thin layer of fluid that constantly bathes and lubricates the exposed ocular surface that comprises the cornea and conjunctiva. The role and the function of the tear film has been extensively reviewed (Stern et al, 2004; Report of the International Dry Eye Workshop, 2007; Perry, 2008). Briefly, this film is of particular importance to the cornea because unlike other tissues, the cornea lacks a vascular system and therefore, the tear film subserves as a source for nutrients, oxygen, growth factors, immune and antimicrobial proteins, and cells of the immune system. In addition to these biological properties, the tear film also has physicochemical properties that are both important for optical function and maintaining its own integrity. Optically, it forms the air-liquid interface which means it is the prime refractive surface of the eye and it has been shown that a change in the physical and
chemical properties of the tear film leads to loss of image quality and vision (Rieger, 1992; Tutt et al, 2000). In terms of integrity, it has surface tension and viscoelastic properties which enable it to spread evenly across the ocular surface and remain stable. Above all, its integrity must be maintained through the blink cycle and this area has been of recent interest with a focus on understanding the factors that determine visual acuity and stability during a blink cycle (King-Smith et al, 2010). The physicochemical properties are only just being explored and the relationship is unknown, but it is well recognised that the constantly changing nature of the tear film could alter the pathway of light that enters the eye and therefore, may be the most important factor determining the optical quality of the eye (Wang et al, 2009). The tear film is also a lubricant allowing the eyelids to ride over the surface of the eye during a blink without causing damage to the epithelium. Other functions include maintaining a stable pH and suitable osmolarity (Tomlinson et al, 2006).

Although it has always been recognised that the tear film is a multifaceted three dimensional fluid comprising a complex mixture of lipids and proteins (Chen et al, 1997; Seppala et al, 2005), most studies on the tear film have tended to focus on determining the roles of the individual components within the three main layers of the tear film. Besides determining the roles of individual components, a prime reason for studying the tear film has been to understand a clinical condition called dry eye. Dry eye has been classified as aqueous deficient or evaporative, and it is thought that a deficiency in a particular aqueous, mucin or lipid component of the tear film leads to tear film instability and consequently dry eye (Report of the International Dry Eye Workshop, 2007). Examples are the proteins lactoferrin, which has an antibacterial role in the tear film through its iron binding ability (Flanagan and Willcox, 2009), and lysozyme, which destroys bacterial cell walls (Knop and Knop, 2007). In evaluating dry eye, lactoferrin levels in tears have been examined as a possible diagnostic test for dry eye, but a correlation between the lactoferrin concentration and tear break-up time (a measure of dry eye) was weak, showing low sensitivity and specificity scores for dry eye diagnosis (Lucca et al, 1990; Yolton et al, 1991). Indeed, despite numerous individual
components being evaluated as biomarkers for dry eye, a match has not yet been found. This indicates there might be enough built in redundancy within the system to enable considerable tolerance of deficiencies in individual components.

A gradual shift has occurred in studies of the tear film and dry eye, leading to a more holistic approach to understand the interaction of molecules across different layers in the tear film. For example, it has been shown that lactoferrin is able to ionically bind to lysozyme and lipocalin, and this interdependence has been proposed to have a functional role “in formation of the tear film” (Gasymov et al, 1999). The major proteins also appear to have complex interactions with meibomian lipids of the TFLL which determine mechanical and physicochemical properties such as viscoelasticity, surface pressure and evaporation (Gouveia and Tiffany, 2005; Tragoulias et al, 2005; Herok et al, 2009).

1.2 Structure of the tear film

Despite the various functions of the tear film being well established, its actual structure is still an enigma. Models have been useful in developing a conceptual view of the tear film, and to form a basis on which to test hypotheses. The tear film has traditionally been described as a three layered structure comprising an innermost mucous layer attached to the corneal epithelium, an overlying aqueous layer and a superficial lipid layer (Bron et al, 2004). It was proposed that: the mucins in the mucous layer (thickness of approximately 20-30nm) (Proust et al, 1984) were secreted by the epithelial goblet cells of the ocular surface, the corneal and conjunctival epithelial cells (Holly and Lemp, 1977; Johnson and Murphy, 2004); the aqueous component of the tear film (thickness of approximately 7μm), made up of electrolytes, water, proteins and glycoproteins, was secreted by the lacrimal and its accessory glands; and the lipid component of the tear film (0.1μm thickness) was derived exclusively from meibomian gland secretions that included waxes, cholesterol esters, triglycerides and fatty acids (Holly and Lemp, 1977; Ohashi et al, 2006).
Figure 1.1: Traditional view of the tear film, illustrating the lipid, aqueous and mucus layers (Bron et al, 2004).

More recently, researchers have proposed a two layered model of the tear film that indicates a homogeneous integration of the aqueous and mucous layers, as opposed to a distinct mucous layer (Gipson, 2004). The original concept of the air-interface layer comprising only lipids has also been re-evaluated to include major tear proteins such as lipocalin, lysozyme and lactoferrin. Current models of the tear film regard the aqueous and mucous layers as a single homogenous mixture, with an increasing gradient concentration of mucins from the air-liquid interface to the corneal epithelium, and a mixture of lipids, denatured proteins and mucins at the surface (Millar et al, 2006; Fig. 2).
Figure 1.2: Tear film model illustrating the homogeneous aqueous-mucin layer and protein lipid mixture at the surface (Millar et al, 2006).
1.3 Components of the tear film

In order to consider the possible interactions between the different components of the tear film, it is important to appreciate the current understanding of the components making up the tear film and the glands that secrete them.

1.3.1 Aqueous-mucin layer of the tear film

1.3.2 Mucins

Mucins are a family of large glycoproteins that are categorized as either transmembrane (MUC1, MUC4, MUC16) or secretory (MUC5AC, MUC5B) (Guzman-Aranguez and Argüeso, 2010). Transmembrane mucins are glycoproteins secreted by the corneal and conjunctival cells. These mucins anchor to the apical surface of corneal epithelial cells and facilitate formation of the glycocalyx on the ocular surface. The glycocalyx acts as a barrier against pathogens and as a surfactant. Its wettability allows the hydrophilic aqueous layer of tear film to bind to the cornea providing lubrication to the apical surface of the epithelial cells during each blink. Transmembrane mucins have also been implicated in intracellular signalling cascades, resulting in the regulation of epithelial cell growth (Gipson et al, 2004; Johnson and Murphy, 2004; Paulsen et al, 2004).

Secretory mucins are secreted by goblet cells, the lacrimal gland, corneal cells and conjunctival cells. Mucins are the most abundant protein component in tears. It appears that secretory mucins form polymers responsible for viscoelastic and non-Newtonian properties of tear film. These physical properties prevent damage to the ocular surface caused by friction and shearing forces generated during movement of eyelids (Johnson and Murphy, 2004). Although mucins were initially only considered for their role at the ocular surface and the aqueous layer, it was proposed as early as 1971 that secretory mucins are important surfactants that reduce surface tension and promote tear stability through interactions with meibomian lipids at the air-liquid interface (Holly and Lemp, 1971; Holly, 1973a). This idea
has been further developed by our group and it is now being accepted that ocular mucins are not just limited to the lipid-aqueous interface, but present at the air-liquid interface and contribute to the physicochemical stability of both the tear film lipid layer (TFLL) and tear film (Millar et al, 2006).

1.3.3 **Aqueous components**

The aqueous contains not only water and mucins mentioned above, but also electrolytes, peptide growth factors, vitamins, various soluble proteins that have antimicrobial and immunological functions that protect and nourish epithelial cells of the cornea, and lipids (Schechter et al, 2010). The aqueous component is primarily secreted from the lacrimal gland and accessory lacrimal glands which are under hormonal and neuronal regulation (Krause and Wolfring). The cornea and the conjunctiva also contribute electrolytes (Rolando and Zierhut, 2001; Johnson and Murphy, 2004) to this layer. The regulatory processes that determine secretion of mucus and lacrimal components are neuronal and hormonal. Activation of the efferent parasympathetic and sympathetic nerves that surround the lacrimal gland acini has been shown to activate receptors on the acinar cells of the lacrimal gland, and consequently leads to secretion of lacrimal components (Dartt, 2001). Hormonal control of the lacrimal gland is also well recognised, and a range of hormones including androgens, estrogens and progesterone have been shown to have a role, but androgens appear to have the most prominent effect in secretion (Wickham et al, 2000; Report of the International Dry Eye Workshop, 2007; Sullivan et al, 2009).

The lacrimal gland secretes over 60 different proteins, including immune response polypeptides, and their profile varies with conditions such as age, tear flow rate, eye closure and stimulation by the conjunctiva (Schechter et al, 2010). Of these, tear lipocalin, lysozyme, and lactoferrin are the major protein components of the tear film. These proteins are secreted from the lacrimal gland and distributed into the aqueous layer through the lacrimal gland duct (Ohashi et al, 2006).
1.3.3.1 Tear lipocalin

Tear lipocalin belongs to a superfamily of proteins known as calycins (Dartt, 2011). Tear lipocalin, also known as Lcn1 or von Ebner’s gland protein, is secreted by the lacrimal gland and composes 15-30% of tear protein by mass (1.2mg/mL) (Redl, 2000). Tear lipocalins have a lipophilic pocket which enables them to bind hydrophobic molecules, including fatty acids, glycolipids and phospholipids (Breustedt et al, 2005). The interaction of tear lipocalins with lipids may have a role in the protection and physiology of the eye. Tear lipocalin may bind to harmful lipids and remove these from the corneal surface thus preventing hydrophobic spots forming on the cornea. This allows the tear film to lubricate the ocular surface and prevent microbial infection of the exposed corneal surface (Redl, 2000). Tear lipocalin may also affect the surface pressure of the tear film by its interaction with lipids both within the aqueous layer and at the surface (air-liquid interface) (Gasymov et al, 2005; Millar et al, 2009; Nagyova and Tiffany, 1999).

1.3.3.2 Lysozyme

Lysozyme constitutes about 20-40% of tear proteins, with levels ranging between 0.6-2.6mg/mL (Ohashi et al, 2006). It is also found in saliva and nasal mucosa, but is most abundant in tears. Its antibacterial role appears to be due to its ability to break down bacterial cell walls through enzymatic digestion, mediated by the presence of antibodies and complement (Ohashi et al, 2006). Although early research reported no other known function for lysozyme other than its immunological role (Walcott, 1998; Sack et al, 2001), recent studies indicate that lysozyme has surface activity and is an important contributor to tear film stability by lowering the surface tension at the air liquid interface (Pandit et al, 1999; Miano et al, 2005; Mudgil et al, 2006). Research by Glasgow’s group has also demonstrated that lysozyme has the ability to interact with tear lipocalin. The consequence of such an interaction is unknown. It may be antimicrobial or have a role in tear lipocalin’s lipid transfer activity. It may also have physicochemical consequences by influencing protein-lipid
interactions within the aqueous phase and at the aqueous-lipid interface (Gasymov et al, 1999).

1.3.3.3 Lactoferrin

Lactoferrin comprises around 25% of tear proteins (Kijlstra et al, 1983) and is regarded as an iron binding protein that inhibits the growth of bacteria by depriving them of iron. Despite being regarded as an iron binding protein, the biological role of lactoferrin is still not well understood. There is evidence that it could be a multifactorial protein with antimicrobial and anti-inflammatory properties (Caccavo et al, 2002). It appears to interact and enhance the activity of other proteins in the tear film, and is also thought to be surface active. As a result of its surface activity, it may contribute to surface tension of the tear film at the air-liquid interface. Its interaction with proteins, such as tear lipocalin, could also contribute to the non-Newtonian property of tear film (Selinger et al, 1979; Gasymov et al, 1999; Miano et al, 2005).

1.3.3.4 Immunoglobulins and miscellaneous proteins

Secretory immunoglobulin A (IgA) is the most abundant immunoglobulin in tears, with levels ranging around 20-30mg/mL (Selinger et al, 1979; Ohashi et al, 2006). It is similar to IgA in saliva, trachea and bronchial mucous, but structurally different from serum IgA. IgA in tears is produced by plasma cells in the lacrimal gland and combines with a secretory component produced by acinar cells before being secreted by the lacrimal gland. It forms a key role in the defence system of the eye by neutralizing viruses and inhibiting the adherence of bacteria to epithelial surfaces. The immune complexes containing the microorganisms are then washed away by the tear film (Selinger et al, 1979). IgG and IgE are also present, but in low concentrations (Selinger et al, 1979; Johnson and Murphy, 2004).

A small proportion of proteins such as serum albumin and transferrin are also found in tears, and originate from the conjunctiva through leakage of plasma from capillaries (Johnson and Murphy, 2004).
1.3.3.5 Surfactant proteins: SP-A, SP-B, SP-C and SP-D

Surfactant proteins\(^1\), a relatively recent discovery in the tear film (Bräuer et al, 2007a; Bräuer et al, 2007b; Bräuer and Paulsen, 2008), have strengthened the idea that tear proteins interact with meibomian lipids and form part of the TFLL. Surfactant proteins in the tear film are very similar to those in the lung and it is likely that their interactions with the TFLL stabilise the tear film by lowering surface tension. The idea is supported by the hydrophobic nature of these proteins and their ability to bind lipids. SP-B and SP-C in the pulmonary system have been shown to interact with lipids to reduce surface tension of lung surfactant and promote its stability (Orgieg et al, 2010). It appears that tear SP-B and SP-C would have a similar role in stabilising the tear film during the blink cycle by either acting as an interfacial anchor between the lipid and aqueous component or forming an integral part of the TFLL. The interaction of these proteins within the TFLL could not only alter surface tension and viscoelastic properties of the TFLL and tear film, but also determine the structural arrangement of meibomian lipids within the TFLL.

SP-A and SP-D are known to have antimicrobial functions through their opsonising effect on microbes due to their ability to bind both microbes and macrophages (van Iwaarden et al, 1990; Orgieg et al, 2010). While SP-A and SP-D have immunological functions, they potentially could have a biophysical effect on the tear film through interactions with lipids. There is evidence that this occurs in lung surfactant where SP-A and SP-D have been shown to influence the structure and function of lipids (Kingma and Whitsett, 2006), and SP-A bound to surfactant lipids facilitates the role of SP-B in lowering the surface tension (Orgieg et al, 2010). Similar studies to understand their influence on surface tension, viscoelasticity of the tear film, as well as structural modelling of these surfactants in a lipid-aqueous environment are needed (Bräuer and Paulsen, 2008).

\(^1\)For the sake of clarity, lung surfactant proteins in the rest of this thesis will be referred to as lung-SPs and tear lung surfactant proteins as tear-SPs.
1.3.3.6 Growth factors and vitamins

Growth factors including lacritin, EGF and transforming growth factor, as well as vitamins are involved in epithelial differentiation and immune response (Johnson and Murphy, 2004; Ohashi et al, 2006; Wang et al, 2006; McKown et al, 2009). They also appear to have a regulatory role in healing of wounds associated with the cornea (Johnson and Murphy, 2004; Ohashi et al, 2006). Due to the avascular nature of the cornea, most of these are present in the tear film. In particular, Vitamin A is involved with the maintenance of the epithelium (Johnson and Murphy, 2004), and is of interest in terms of this thesis because of its lipid solubility and possibility of interacting with the TFLL. It can also exist in the form of retinoic acid, retinol or retinal, and is important for the health of goblet cells. Vitamin A deficiency can elicit a reduction in goblet cells and mucins, which causes dysfunction of the ocular surface or tear film, and thereby dry eye disease (Rolando and Zierhut, 2001). Vitamin A has also been effectively used in treatment of dry eye by various researchers (Murphy et al, 1996; Kim et al, 2009).

1.3.3.7 Electrolytes

Electrolytes present in tear film are responsible for its osmolarity and are similar to those found in the blood: magnesium, sodium, chloride, and potassium ions. A notable difference is that the potassium ion concentration is much higher in tears compared with blood (Van Haeringen, 1981). Increased electrolyte levels have been implicated in dry eye syndrome and activate inflammation which damages the ocular surface (Gilbard, 1994; Tomlinson et al, 2006). Electrolytes also act as a buffer, and changes to pH are important to molecular structure, particularly with respect to proteins. For example, tear lipocalin has been shown to have different lipid binding affinities depending upon pH. Release of lipids from lipocalin to the lipid layer could be affected by pH and this in turn would affect surface tension of the tear film (Gasymov et al, 2004).
1.3.3.8 Lipids in the aqueous layer

Lipids have been found in the aqueous part of the tear film. Their origin, secretion and conformation i.e. whether they form micelles or whether they interact with the tear film lipid layer (TFLL) is still unknown. It is unlikely that they originate from the meibomian glands because analysis indicates that lipids in aqueous tears (AT) significantly differ from those in the meibomian gland secretions (Borchman et al, 2007). Quantitatively, larger quantities of polar components such as cholesterol (7%) and phospholipids (16%) have been identified in AT samples, compared with meibomian lipids (cholesterol 1%; phospholipids 0-7%) (Borchman et al, 2007; Butovich, 2008; Dean and Glasgow, 2012). Structurally, it has been proposed that lipids in the aqueous layer contain fewer double bonds and hydrocarbons than those in the TFLL, and also contain OH groups that are unique to AT lipids (Borchman et al, 2007).

Since the role of lipids in AT have only been investigated recently, their origin, secretion and conformation is still unknown. However, the knowledge of the presence of lipids in AT has renewed interest in their relationship with the tear film lipid layer. For example, Butovich’s group have in vitro examined the interaction of fatty acids in an aqueous environment with meibomian films (Uchiyama et al, 2007; Arciniega et al, 2011). Further studies to evaluate the contribution of aqueous lipids in tear film physiology, physicochemistry and stability are required.

1.4 Lipid layer of the tear film

1.4.1 Composition and structure
At this stage the structure of the TFLL is unclear. It is evident that the major constituents are meibomian lipids secreted from the meibomian glands located in the tarsal plate of the eyelids (Abelson et al, 2005). The meibomian glands and their secretion have been the subject of a
A major review published in 2011 (Report of the International Workshop on Meibomian Gland Dysfunction, 2011), and so only aspects significant to this thesis will be outlined here. The meibomian glands have been classified as large sebaceous glands lacking hair follicles. However, the lipids secreted by the meibomian glands (Table 1) are vastly different to lipids secreted from other sebaceous glands: ceramides, free cholesterol and phospholipids are major components in sebum, but minor constituents of meibum, which has shown to be a complex mixture of long chain wax and cholesteryl esters, triacylglycerols, diesters, omega-acyl hydroxyl fatty acids (OAHFA) and long chain fatty acids (Smith and Thiboutot, 2008; Feingold, 2007; Butovich et al, 2007; Butovich, 2009).

**Table 1.1:** Lipid composition of normal human meibum

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HC</td>
<td>—</td>
<td>26–38</td>
<td>—</td>
<td>1</td>
<td>—</td>
<td>1–38</td>
</tr>
<tr>
<td>WE</td>
<td>64a</td>
<td>13–23</td>
<td>35</td>
<td>68</td>
<td>51</td>
<td>13–68</td>
</tr>
<tr>
<td>SE</td>
<td>Seea</td>
<td>8–34</td>
<td>30</td>
<td>16</td>
<td>39</td>
<td>8–39</td>
</tr>
<tr>
<td>Diesters</td>
<td>8</td>
<td>—</td>
<td>8</td>
<td>—</td>
<td>2</td>
<td>2–18</td>
</tr>
<tr>
<td>TG</td>
<td>2</td>
<td>11–43</td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>2–43</td>
</tr>
<tr>
<td>Alcs</td>
<td>5</td>
<td>0–2</td>
<td>2</td>
<td>—</td>
<td>—</td>
<td>0–5</td>
</tr>
<tr>
<td>FFA</td>
<td>10</td>
<td>0–24</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>3 0–24</td>
</tr>
<tr>
<td>Ch</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1</td>
<td>0–1</td>
</tr>
<tr>
<td>Polar</td>
<td>—</td>
<td>0–5</td>
<td>16</td>
<td>4</td>
<td>—</td>
<td>0–16</td>
</tr>
</tbody>
</table>

HC hydrocarbon, WE wax ester, SE sterol ester, TG triglycerol, Alcs alcohols, FFA free fatty acids, Ch cholesterol, Polar polar lipids. *Composite of WE and SE (Butovich et al, 2008).

The best evidence suggests that meibomian lipids are released onto the ocular surface as a result of simple mechanics. The lipids, which have a melting point range 19-32°C (Bron et al, 2004), are squeezed out from the meibomian gland orifices located in the lid margin as pressure is put on the tarsal plates during blinking (Abelson et al, 2005). The control and the process of meibomian gland production is still not clear. Small nerve terminals in the basal...
region of the gland have been observed (neuron-specific enolase, tyrosine hydroxylase, neuropeptide, vasoactive intestinal polypeptide, calcitonin gene related peptide, substance P), but there is no evidence of actual synapses (Chung et al, 1996). There is also strong evidence that the glands contain androgen, oestrogen and progesterone receptor mRNA, and proteins, which indicate hormonal control of lipid production (Wickham et al, 2000). A recent study using cell cultures of meibomian gland epithelial cells has indicated that the parasympathetic neurotransmitters, vasoactive intestinal peptide and muscarinic acetylcholine, control the function of meibomian gland epithelial cells and may exert a role in the production and secretion of meibomian lipids (Kam and Sullivan, 2011).

An important question is whether or not proteins are secreted along with the meibomian lipids and hence contribute to the TFLL. Using very sensitive techniques (microcapillary reverse phase chromatography and nano-electrospray mass spectrometry), over 90 proteins including lysozyme and lactoferrin have been identified in secretions collected from meibomian glands. Keratins were markedly the most predominant proteins (Tsai et al, 2006; Thangavelu et al, 2010). Due to the sensitivity of the technique, some of the proteins detected could have come from contamination by the ocular surface during collection. However, the abundance of keratins and the fact that the meibomian lipids are squeezed out through keratinised epithelial ducts means that keratin is a part of the meibomian secretion. Therefore, it is of interest how keratin might affect the structure and physical properties of the TFLL, if at all.

Observations of the TFLL in situ using various interferometric techniques have also provided important insights into TFLL dynamics (Yokoi et al, 1996; King-Smith et al, 2010). By analysing interference patterns created by light reflected from the surface lipid layer, scientists have been able to extract information about the thickness and fluidity of the lipid layer and have used these parameters to distinguish normals from dry eye patients. A grey colour pattern with homogenous distribution has been associated with normals, whereas a more coloured pattern with non uniform distribution has been correlated with dry eye and a
thickened lipid layer (Yokoi and Komuro, 2004). Rheological analysis of interference images revealed that the TFLL is a non-Newtonian viscoelastic film, of which the viscosity is dependant on stress (shear dependant viscosity) and shear thinning (a fluid that becomes thinner when stress is applied) (Yokoi et al, 2008). The studies have also demonstrated that unlike the aqueous layer, the lipid layer is stable for multiple blinks and is extremely resilient to change (Goto and Tseng, 2003). However, interferometry is limited to the microscopic level, and how the individual components contribute to changes in lipid thickness, viscoelasticity, or its structure (such as in disease states) is unknown.

McCulley and Shine developed extremely insightful models of the lipid layer at the atomic scale to promote an understanding of how the individual components organise to form a structurally functional film. They depicted the meibomian lipid layer as a two layered/phase\(^2\) structure; a thin lower polar phase adjacent to the aqueous layer of tear film constituting the polar components sphingolipids and phospholipids, and upper layers at the air interface constituting the non-polar wax and cholesteryl esters (McCulley and Shine, 1997). The two phases of the TFLL are thought to subserve different roles.

The upper non-polar layers of lipids constitute the bulk of the TFLL and form a thick blanket over the aqueous layer of the tear film. This outer lipid blanket has been associated with preventing evaporative loss; a role influenced by the thickness and composition of the TFLL (Tiffany, 1985; Bron et al, 2004; Mathers, 2004). In particular, McCulley and Shine, through their experimental and clinical studies, have indicated that cholesterol and cholesteryl esters, along with fatty acids, could be important determinants of the physicochemical properties of the TFLL (Shine and McCulley, 1991; Shine and McCulley, 1996; McCulley and Shine, 1997).

\(^2\) In physical chemistry the term layer is used to mean a phase e.g. a polar and a non-polar phase would be called two layers even if they were from the one molecule such as phospholipids. In this thesis phase will be used to indicate different phases and layer will be used to convey the meaning of different molecules at different levels in the film.
In their model, the lower polar phase is made from phosphatidylcholine, phosphatidylethanolamine, ceramides and sphingomyelin and fatty acids arranged in one or two layers at the aqueous interface. The non-polar phase by itself is thermodynamically unstable and the lipids in the polar phase provide a stabilising interface between the aqueous layer of tear film and the overlying non-polar rich lipid blanket, by orientating the polar lipids vertically, with the hydrophobic tails immersed in the non-polar phase and polar head groups are exposed to the aqueous layer of the tear film. The polar lipids are important surfactants that enable spread of the tear film, but it has been proposed that they also act as a “structural matrix” for the non-polar lipids (McCulley and Shine, 1997; Shine and McCulley, 2003). The nature of polar lipids in the TFLL has been a particularly contentious issue (Wojtowicz et al, 2009). While some researchers report the presence of phospholipids (Shine and McCulley, 1998; McCulley and Shine, 1997; Saville et al, 2011), others have reported very small amounts or no phospholipids in the TFLL (Ehlers N, 1965; Butovich et al, 2007; Chen et al, 2010). This subject is important for modelling and understanding the TFLL, as phospholipids are considered key surfactants which could influence the functional integrity of the TFLL (McCulley and Shine, 1997; Shine and McCulley, 2003). More recent work indicates that a different group of amphiphilic lipids, i.e. (O-acyl)-omega-hydroxy-fatty acids maybe responsible for the role previously attributed to phospholipids (Butovich, 2009).

Whilst there is a general belief that the lipid layer is governed by the constituents of meibomian gland secretions, meibum secreted onto the tear film has the opportunity to interact with other tear film components of the aqueous phase. Thereby, the final composition and structure of the TFLL varies from its original secretion. Recent models of the TFLL include aqueous proteins (Fig. 1.2).

It is logical that proteins could have surface activity and lower the surface tension of an aqueous medium, as the diffusion of the proteins from the subphase to the air interface causes
them to unfold and denature at the surface; the conformational change means that the proteins are at a lower free energy on the surface (do not re-enter the subphase) and form a protein film at the air-liquid interface (Graham and Phillips, 1979). However, when meibomian lipids are located at the air interface (as in the case of the tear film), the proteins compete with the lipids for space, unfold at the surface and are trapped in the lipid layer. The evidence for this has come from research that evaluated surface tension changes of meibomian lipid films when ocular mucins and major tear proteins including lysozyme, lactoferrin, and tear lysozyme were added to the subphase (Tiffany et al, 1996; Tiffany and Nagyova, 1999; Millar et al, 2009; Tragoulias et al, 2005). A strong basis for the concept of proteins being a part of the TFLL also comes from the detection of lipid binding proteins and tear-SPs. The extremely hydrophobic nature of tear-SP-B and tear-SP-C (discussed earlier) makes them strong contenders for a biophysical role in the TFLL (Bräuer et al, 2007b; Bräuer and Paulsen, 2008).

The original model of the TFLL proposed by McCulley and Shine formed an excellent basis for understanding its structure, and with further contribution from other researchers, the model has now evolved to be a mosaic structure constituting a mixture of lipids, denatured proteins and mucins (Millar et al, 2006). This model is historically analogous to cell membrane models, which were considered to be static lipid systems (Boesze-Battaglia and Schimmel, 1997), but have now evolved to a stage where they are considered mosaic heterogeneous structures, containing domains of lipids and proteins, that are dynamic and interactive (Singer and Nicholson, 1972; Boesze-Battaglia and Schimmel, 1997). For example, changes in the concentration of cholesterol domains have been shown to disrupt the function of proteins in cell membranes (Singer and Nicholson, 1972; Simons and Ikonen, 2000). By analogy, it is therefore possible that biophysical changes in the composition or organisation of lipids, e.g. changes in cholesterol or the levels of its ester analogs in the TFLL, can affect the interaction of proteins from the aqueous layer.
A basic problem associated with modelling and understanding the role of components in the lipid layer is that its composition is unknown. At the time of this thesis there was good agreement on the major non-polar constituents of the TFLL. In terms of the polar lipids, there was still considerable conjecture over their specific nature and contribution to TFLL functionality. Discrepancies are likely due to sample collection technique, analytical method employed and contamination (Wojtowicz et al, 2009). To overcome the problem of not knowing the exact composition, one approach is to deliberately increase known components and evaluate their effects on function and integrity of meibomian lipid films, while another is to take individual major constituents of meibomian lipid films or mixtures of these and compare their properties with meibomian lipid films to glean information about their contributions and roles. Based on this strategy, this thesis will examine the effect of various components of the TFLL on its structure, taking into consideration that some components of the TFLL could come from meibomian gland secretions, ductal cells, or from the aqueous.

1.4.1.1 Compositional changes in disease state

An understanding of how meibomian gland secretions are altered in disease states is crucial to identify and evaluate components that could be important in the pathophysiology of ocular diseases. For example, there is important evidence from McCulley and Shine’s work that alterations in the secretion of phosphatidylcholine and sphingomyelin, as well as changes in lipid composition involving unsaturated fatty acids, free cholesterol, wax and cholesterol esters are associated with ocular diseases such as keratoconjunctivitis and blepharitis (Shine and McCulley, 1991; Shine and McCulley, 1993; McCulley and Shine, 1997; Shine and McCulley, 1996; Shine and McCulley, 1998).

Obstructive meibomian gland dysfunction is one of the most common forms of meibomian gland dysfunction (MGD) in which the meibomian gland is inflamed and its function is impaired. MGD causes a change in the composition/secretion and viscosity of the meibum (Knop et al, 2011). Secretions from MGD patients reveal lower levels of cholesterol, and
triglycerides when compared with normals (Mathers and Lane, 1998). Meibomian lipid profiles of patients with meibomian seborrhoea revealed an increase in oleic acid (monounsaturated fatty acid), and although the exact role of fatty acids is unclear, it was thought that it could alter the melting point of lipids and have some contribution to the disease process (Shine and McCulley, 2000). Joffre et al, have found higher levels of unsaturated fatty acids and lower levels of saturated fatty acids in patients with MGD (Joffre et al, 2008). Their group has hypothesised that increased levels of unsaturated fatty acids could in fact improve fluidity and stability of the lipid layer to counteract the low tear volume in these patients. Unsaturated fatty acids/branched chain fatty acids have a lower melting point than saturated fatty acids, and therefore increased levels of unsaturated fatty acids could increase the viscosity of lipids and potentially also inhibit evaporation (Joffre et al, 2008; Joffre et al, 2009). However, the effects of unsaturated fatty acids such as oleic acid on the physicochemical properties of meibum are yet to be tested.

There have been a variety of factors that have been linked between lipid composition of the normal TFLL and dry eye. Changes in composition could also be due to hydrolysis of lipids by microbes colonising the eye e.g. patients with blepharitis and meibomian seborrhoea tended to contain a higher number of coagulase negative bacteria that hydrolyse cholesterol oleate (when compared with normals). This would increase the concentration of components such as cholesterol, free fatty acids and fatty alcohols and affect the physicochemical properties or melting point of meibum (Dougherty and McCulley, 1986a; Dougherty and McCulley, 1986b). Hormonal changes have also been nominated as a mechanism for changes in lipid composition. It has been shown that patients with an androgen deficiency have a decrease in triglycerides, wax and cholesterol esters, but an increase in relative free cholesterol (~1-1.5 % increase) in their meibomian lipids. It was proposed that the increased cholesterol could alter the viscosity of meibum and lead to an obstruction of the meibomian glands (Krenzer et al, 2000; Sullivan et al, 2000). Despite the conjectures about these changes to the physicochemical properties of meibum, it has not been experimentally tested. How
compositional changes manifest in altered viscosity, high surface tension or influence lipid-protein interactions will be the subject of this thesis.

1.4.2 Function of meibomian lipid layer
The primary function associated with the meibomian lipid layer has been to provide a smooth surface for the refraction of light for the cornea and to inhibit evaporation of AT. The latter is a significant concept, as dry eye is classified as either evaporative or aqueous deficient (Nicholls, 2011). Various methodologies have been employed to evaluate the role of meibomian lipids in evaporation and it is not surprising that there have been notable variances in results. Controversially, some groups have even found that meibomian lipids alone do not prevent evaporation (Brown and Dervichian, 1969), but evaporation rate is governed by interaction of lipids with other components such as proteins and mucins (Herok et al, 2009). However, scientists have been quick to question the methodology of such results, and there continues to be a steadfast belief that meibomian lipids in vivo reduce evaporation (Bron et al, 2004).

1.4.3 Surface tension and surface activity of the TFLL
The functions of the TFLL are underpinned by physicochemical properties such as surface tension that allow the TFLL to spread over the aqueous phase and to maintain its structural integrity under enormous stresses of a blink cycle (Holly, 1973a; Holly, 1973b). Surface tension encompasses various aspects of the tear film, both at the air-liquid interface and the corneal epithelium. The wettability of solid surfaces such as the corneal epithelium is dictated by surface tension i.e. the tear film is able to lubricate the ocular surface because its surface tension is lower than that of the corneal epithelium. Surface tension is also responsible for the thickness of the tear film and the distribution of tears between compartments (Holly, 1977). It has been suggested that a low surface tension in tears is important for tear film stability (Wong et al, 1996).
With respect to the TFLL, seminal investigations of the surface activity of the TFLL were carried out by Holly in the early 1970’s, and he proposed that the sum of the surface and interfacial tension between the TFLL and aqueous phase has to be lower than the surface tension of the aqueous phase for the TFLL to be able to form and spread (Holly, 1973b). In essence, a higher surface tension of the air-aqueous interface allows meibomian lipids to spread over the aqueous layer to form the tear film, whereas the lower surface tension of a newly formed lipid layer is vital to tear stability (Holly, 1973b). Holly further suggested that the low surface tension and surface activity of meibomian lipids are influenced by non-specific interactions between meibomian lipids and mucins in the tear film (Holly, 1973b).

More recently, Tiffany and our group have demonstrated the role of other major tear protein-lipid interactions as a basis for the low surface tension and stability of tears (Tiffany and Nagyova, 1999; Mudgil et al, 2006; Millar et al, 2009).

Knowledge of which components of the tear film are responsible for surface tension, the nature of their contribution, and what effect changes in composition have on surface tension is of great importance for tear film stability and consequently conditions such as dry eye. Therefore, the aim of this study is to evaluate how major and specific tear film components affect both the structural and physicochemical properties of meibomian gland secretions to correlate compositional changes in meibum with its integrity and stability.

---

3 Surface pressure is the difference in the surface tension before and after the film was spread onto the subphase. Higher surface pressure corresponds to lower surface tension and more surface activity.
2 Effect of cholesterol, cholesterol esters and free fatty acidson meibomian lipid films

2.1 Introduction

One of the important features of the TFLL is that it forms an interfacial layer at the air-liquid interface. Langmuir developed a technique for examining such films by measuring changes in their surface pressures (Langmuir and Schaefer, 1939; Langmuir and Waugh, 1940). His techniques have been widely used to investigate simple lipid films and to evaluate the behaviour of biological lipid models. The field of study has had a long and important history (Möhwald, 1990) with most of the initial studies being carried out on well defined systems that were typically films of a single molecule type, such as a particular fatty acid or phospholipid, or films of defined binary mixtures using pure components such as a defined phospholipid - e.g. dipalmitoylphosphatidylcholine (DPPC) and cholesterol. Over the last decade, the field has grown extensively, particularly to study artificially synthesised amphipathic molecules e.g. polymers. Many of these have been developed to be used for encapsulating other molecules such as in drug delivery (Mu and Seow, 2006) and as emulsifiers for paints (Mehta et al, 2006). There has also been great interest in using these techniques to understand the surface behaviour of naturally occurring biological molecules such as proteins and self-assembled biological films such as lung surfactant and cell membranes (Edidin, 2003; Zasadzinski et al, 2010). The TFLL falls into this category of a self-assembling biological film and is most comparable to lung surfactant, except for a fundamental difference: it is much more complex in its composition and the physicochemical contribution of major components is unclear. However, although the TFLL is complex, there is information about the composition of meibomian lipids, which form the major component of the TFLL. A strategy that has been employed in binary systems to evaluate the contributions of the individual components in mixed films will be used here to study films of meibomian lipids: to deliberately vary the ratios of the individual components.
2.1 Cholesterol and its esters

Changes in the levels of cholesterol, its esters and fatty acids have been correlated with poor stability and functionality of the TFLL in patients with MGD (Shine and McCulley, 1991). These findings indicate that the levels of cholesterol and cholesterol esters, or ratios of free cholesterol and cholesterol esters to other lipid components affect the physical properties of the TFLL. Although cholesterol is qualitatively a minor component of the normal TFLL (approximately 1-2%) (Butovich et al, 2008; Joffre et al, 2008), it is a surfactant through its hydroxyl group at 3’ carbon and therefore might be a key molecule at the aqueous-lipid interface. An understanding of its role in other lipid systems may indicate its putative functions within the TFLL. Literature about cholesterol in cell membranes has been made difficult to interpret by usage of common terms such as stabilizing, condensing, and fluidity, which have not been defined scientifically. For example, it has been proposed that complexes formed through cholesterol-lipid interactions lead to the “condensing effect of cholesterol” by which the surface area occupied by lipids is decreased as a result of interactions with cholesterol, thereby reducing permeability of molecules, “increasing fluidity” and promoting a “mechanically stable” lipid bilayer (Simons and Ikonen, 2000; Yuan and Johnston, 2002). A possible explanation for the use of mixed terminology might come from experiments on vesicle membranes where it has been shown that cholesterol affects phase separation. Phase separation means that particular lipid molecules in small areas of the membrane condense from a very fluid non-organised mixture (liquid disordered) into a very ordered arrangement (liquid ordered) giving that region gel like characteristics. This means that the membrane becomes more fluid in some regions and more gel-like in others and this characteristic appears to be enhanced in the presence of cholesterol. For example, in a vesicle made from a mixture of sphingomyelin and DPPC, the molecules exist in a liquid disordered array until cholesterol is added. In the presence of cholesterol, the sphingomyelin molecules separate to form a liquid condensed phase and so do the DPPC molecules making gel like phases within
the fluid mixture of sphingomyelin and DPPC. Another interesting aspect of these studies was
that this phase separation was temperature dependent with cholesterol enhancing the phase
separation at 37°C, but having little effect at 23°C (Ahmed et al, 1997).

The effect of cholesterol on a lipid system will vary based on the composition of nearby lipid
species. Cholesterol will generally stiffen disordered lipids (e.g. lipids containing many
unsaturated hydrocarbon chains), but fluidise ordered lipids (lipids containing many saturated
hydrocarbon chains) and lower their phase separation temperature (Lippert and Peticolas,
1971). When in a mixture of ordered and disordered lipid pools, cholesterol is likely to
associate with the disordered lipids causing them to become stiffer/rigid (de Kruff et al, 1974;
Verkleij et al, 1974; Lee, 1976; van Dijck et al, 1976). It is well recognised since the 1970’s
that cholesterol has a particular affinity for sphingomyelins over other phospholipids (Demel
et al, 1977; Yeagle et al, 1977; Calhoun and Shipley, 1979). In cell membranes, this
interaction is particularly important in the formation of lipid microdomains known as rafts.
Cholesterol, sphingolipids and caveolae proteins organise into ordered and segregated
domains (clusters) known as rafts that serve as platforms to perform important membrane
trafficking and cellular functions. Cholesterol acts as the important “glue” in the formation of
sphingolipid complexes, and modulates the partitioning and activity of proteins within the raft
complex (Verkade and Simons, 1997; Simons and Toomre, 2000).

In the eye, rafts have been identified in membranes of human lenses. It is suggested that
recruitment and enrichment of cholesterol by specific membrane proteins (such as caveolin-1)
is important in the formation of ordered lipid domains and raft complexes (Rujoi et al, 2003).
These rafts have not been detected in cataractous human lenses and thus may be important in
the transparency of the human lens (Rujoi et al, 2003).

Cholesterols also form crystals based on ratios of sphingomyelin and dihydrosphingomyelins
(phospholipids) (Epand, 2003), and specific interactions with these phospholipids (Kuikka et
The crystals have an important role in lens membrane properties, and thereby ocular function (Epand, 2003).

The functional importance of cholesterol in vision is also supported by clinical and animal studies. The ocular lens is a unique tissue, having the highest cholesterol to phospholipid ratios - as high as 10:1 (Borchman et al, 1989). Any alterations to the levels of cholesterol are believed to lead to membrane derangement and ineffective lens function. Inhibition of cholesterol synthesis is associated with cataract formation, because cholesterol could influence changes in lens proteins and membrane permeability, but the specific mechanism of cholesterol induced cataracts is unclear (Cenedella, 1996).

Clues to the possible roles of cholesterol esters in liquid films come from studies on lung surfactant. A study to better understand lung surfactant showed that different ratios of cholesterol palmitate, an ester of cholesterol, were more miscible, than cholesterol alone, with a film of a lipid based extract from bovine lung lavage (Panda et al, 2007). It was then theorised that this occurred due to the more hydrophobic nature of cholesterol palmitate allowing it to mix more freely and interdigitate with the lipids and proteins of the bovine lung extract (Panda et al, 2007). It therefore makes sense to adapt similar strategies to investigate the interaction of meibomian lipids with cholesterol and cholesterol esters. Whilst changes in particular lipid components could have a detrimental influence, it is also likely that such changes could be beneficial to counteract tear instability in patients.

2.1.1 Fatty acids and phospholipids
Fatty acids and phospholipids are amphipathic, so they can both act as surfactants: fatty acids interact with water through their carboxyl group and phospholipids through their phosphate polar head group. The acyl chains in these molecules are also important to their arrangements in films. Saturated chains are easily ordered and so readily pack into liquid condensed phases, whereas the more cis unsaturated the acyl chains, then the more difficult it is to pack the
molecules into ordered arrays. This means that with unsaturated chains, the films are more liquid disordered (Putzel and Schick, 2008). The predominant fatty acid obtained from meibomian lipids is oleic acid, which has a single cis double bond. Changes in the profile of fatty acids, particularly oleic acid, have been correlated with various disorders related to meibomian gland dysfunction (Shine and McCulley, 2000). These theories have not been tested experimentally, but the concept that fatty acids may play a role in surfactant stability and pathophysiology of disease has been supported in pulmonary surfactant research. Fatty acid profiles have been found to vary in surfactant secretions of patients with cystic fibrosis (Meyer et al, 2000).

It has also been proposed that increases in oleic acid or other fatty acids could increase the surface tension and promote alveolar collapse and contribute to lung disease in patients with cystic fibrosis (Griese et al, 1997).

Studying surface activity of phospholipids, particularly DPPC, also has the advantage of being supported by a wide literature in physical chemistry. Phospholipids are the most abundant/major lipid class in lipid systems including cell membranes and pulmonary films, and are a primary surface tension lowering component; therefore, they (DPPC in particular) have been widely used in research that examines lipid-lipid interactions and lipid formulations. Many earlier studies with phospholipids have used them in defined binary systems, and while these have provided information about the physicochemical properties of a particular lipid, they do not provide insight into the role of a component within complex lipid systems, such as in lung surfactant, cell membranes and human meibum. These systems consist of at least eight different lipid classes, resulting in significant diversity in physical properties such as charge, hydrophobicity, lipid structure and degree of saturation e.g. oleic acid has a different melting point from a saturated fatty acid such as palmitic acid. This emerging concept, in which classes of biomolecules are considered together, is known as “lipid polymorphism”, has an important role in current pulmonary and cell membrane
research. Lipid polymorphic phase behaviour has been shown to cause changes in lipid geometry and spreading of liposomes. The changes have a role in design of effective pulmonary synthetic surfactants, and more importantly, affect the function of native surfactant (Perkins et al, 1996).

In this study, we have taken this novel approach and conducted an investigation to evaluate the role of cholesterol, its esters and oleic acid on the structure and dynamics of meibum in vitro, using a Langmuir trough to measure surface pressure as an indicator of the interaction of lipids and stability of the films formed by the lipids spread at an air aqueous interface. In particular, changes to the dynamics of meibomian lipid films caused by alteration of the lipid composition will be investigated.

2.1.2 Background to experimental design and methodology

Analysis of surface pressure/tension in tears have been measured through the contact angle method, capillary tube measurement or a Langmuir film balance, which comprises a Langmuir trough with moveable barriers and a Wilhelmy plate for monitoring pressure changes. Contact angle measurement of surface tension involves placing a droplet of tears on a defined surface and is evaluated by measuring the angle formed by liquid-solid interfaces i.e. the contact angle is a function of surface tension (Kwok and Neumann, 1999). Capillary tube measurement has the advantage of requiring only small volumes of tears which is useful when considering the low yield of tears sampled from patients (only 1μL of tears is required). Tears are collected in a glass capillary tube and an electronic manometer measures the surface tension of tears based on the change in appearance of the liquid meniscus under different air pressures (Tiffany and Nagyova, 1999).

However, the above methods are fixed systems that measure only a single surface tension measurement at a particular concentration. They do not allow evaluation of movement of lipids and proteins onto the surface, removal from the surface at different surface pressures, the elastic properties of meibomian lipid films and the effects of repeated compression, or the
expansion cycles on films in response to various lipids or proteins. A Langmuir trough with moveable barriers overcomes this problem. Thin lipid films spread on an aqueous subphase of a Langmuir trough have been well established as model systems for cell membrane and pulmonary lipid systems. In addition, the microscopic structure of the films can be observed and correlated with the surface pressure. Other variables such as pH, temperature and salt concentrations of the subphase can also be controlled.

The nature of the minor components in meibum and hence their contributions to surface pressure in the film are unknown. A mechanism to overcome this is to compare the physicochemical properties of human meibum with animal meibum. The composition of a number of different animal meibomian lipids including koala, possum, rabbit and wallaby, have been studied. From these it appears that the overall composition of koala and human meibomian lipids is similar, with differences being mainly in the minor components (Butovich and Millar, 2009). Therefore, the effects of changes in cholesterol on koala and human meibomian lipid systems has been compared to gain some insight into how minor compositional differences influence physicochemical behaviour, and how this may contribute to the functionality of these lipid systems.

2.2 Materials and methods
Cholesterol (Sigma Aldrich, Australia; Batch #045K5311), cholesteryl oleate (Sigma Aldrich, Australia; Batch #045K5311098K5016) and oleic acid (Nu-Chek Prep, U.S.A; Batch #U-46A-110-R) were minimum 99% pure and therefore not purified further. Chloroform was used as the solvent and was of HPLC grade (Labscan).

2.3 Collection of human and koala meibomian lipids
Koala eyelids were obtained from a veterinary clinic. The eyelids were squeezed using forceps and the extracted lipids dissolved in chloroform. They were dried, weighed and reconstituted to a concentration of 1mg/mL. Local ethics permission was obtained for
collecting human meibomian lipids and the Tenets of the Declaration of Helsinki were adhered to. Human meibomian lipids were gently squeezed out of the tarsal plates of the subject's lower eyelids by applying pressure to the eyelids using sterile cotton swabs on either side of the lid. The secreted lipids were harvested using a sterile stainless steel spatula and dissolved in HPLC grade chloroform. The meibomian lipids were dried through vacuum concentration and centrifugation, and then reconstituted in chloroform to give a final concentration of 1mg/mL. They were stored at -20°C until used. Multiple collections were carried out on a single male subject to provide consistency in the experiments. The subject was devoid of any external signs or symptoms of dry eye disease.

2.4 Surface pressure-area (Π-A) measurements

Mixtures of specific lipids (cholesterol, cholesteryl oleate and oleic acid) and meibomian lipids were prepared by mixing appropriate volumes in solutions of chloroform. Human or koala meibum was mixed with specific lipids in the mole fraction range of 0 to 100 mol % assuming an average molecular weight of 350 for meibomian lipids. The total number of molecules was kept constant: 2.63 x 10^{16} for mixtures in the range of 10-100 mol % cholesterol, 3.44 x 10^{16} for 5 mol % cholesterol, 4.30 x 10^{16} for cholesterol 1 mol % mixtures, 3.44 x 10^{16} for human meibum/oleic acid mixtures and 3.23 x 10^{16} for human meibum/cholesterol oleate mixtures. Prior to conducting surface pressure-area (Π-A) measurements, the trough was thoroughly cleaned using HPLC grade chloroform (Labscan Australia). Fresh artificial buffer (NaCl: 6.63g/L, KCl: 1.72g/L, NaHCO₃: 1.38g/L, CaCl₂ 2H₂O: 0.15g/L, NaH₂PO₄·H₂O: 0.10g/L and MOPS: 4.18g/L dissolved in MilliQ water; Mirejovsky et al, 1991) was added to a ribbon barrier Langmuir trough (Nima Technology Ltd, UK; Working surface area 20cm²-200cm²) or double barrier minitrough (Nima Technology Ltd, UK; Working surface area 15cm²-80cm²). The surface of the buffer

---

4 A molecular weight of 350 was a conservative estimate based on data and research at the time (Petrov et al, 2007; Joffre et al, 2008). Towards the end of my thesis, a more up to date molar weight currently is thought to be in the range of 550-700. The concentration of seeded lipids (cholesterol, cholesteryl oleate, oleic acid) would be lower when using a molecular weight of 700 (compared to 350), but this did not change the outcome of our findings.
(subphase) was cleaned by compressing the barriers to the minimum A ($A_{\text{min}}$) and the surface between the barriers was swept clean of contaminating surfactants using a teflon tube connected to a vacuum pump. This was repeated until there was less than 0.2 mN/m change in $\Pi$ when the barriers were closed to $A_{\text{min}}$.

Lipid solutions were spread drop-wise from a microsyringe onto the cleaned surface (air-buffer interface) of the subphase. Ten minutes was allowed after sample injection before dynamic $\Pi$-A profiles of lipid films at the air-buffer interface of the Langmuir trough were collected using software supplied by NIMA Technology. $\Pi$ was monitored using a paper plate connected to a Wilhelmy balance. Compression and expansion cycles (isocycles, Fig. 2.1) were conducted over an A of 200 cm$^2$-20 cm$^2$ (ribbon barrier trough), or over an A of 80 cm$^2$-15 cm$^2$ (mini trough) at a barrier rate of 15 cm$^2$/min. The artificial tear buffer was kept at either 20$^\circ$C or 35$^\circ$C by a water jacket connected to an external thermostat controlled water bath. A plastic cover was placed over the trough to minimize air currents and contamination of the trough. Although the humidity of the laboratory did vary between experiments, the conditions did not seem to cause a visible difference in results. Experiments were repeated on a minimum of two different occasions for each mixture to verify reproducibility.

Typical $\Pi$-A profiles of rigid and fluid lipid films (Fig. 2.1) are shown to assist in the interpretation of dynamic measurements of protein and lipid films applied to the air-buffer interface. An increase in $\Pi$ occurs when molecules at the surface are pushed together and interact with each other (compression), and a decrease in $\Pi$ occurs when the molecules are pulled apart (expansion). Compression and expansion of a film results in an isocycle showing hysteresis, whereby the compression part of the isocycle differs from the expansion cycle. The hysteresis and obvious slope changes in the isocycle are indicative of the extent of molecular reorganisation that occurs at the surface. A rigid film would be expected to demonstrate a sharp rise in $\Pi$, particularly towards maximum surface pressure ($\Pi_{\text{max}}$) and
smaller area. A fluid film would be expected to show a gradual increase in Π when
compressed; even at Π_{\text{max}}, and this is consistent with molecules taking up less space compared
to a rigid film. Our group has recently detailed the phase behaviour of meibomian lipids at
different Π in a Langmuir trough (Millar, 2013) and some of the general concepts also apply
in other lipid films. Meibomian lipids when first spread, will form a duplex film, with
surfactant lipids (omega hydroxyacyl or phospholipids) forming an interfacial layer with the
subphase, and hydrophobic lipids including cholesterol and wax esters forming a liquid
crystal layer on top. With further compression of the barriers and decrease in surface area, the
surfactant lipids interact with each other and the cholesterol and wax esters begin to
reorganise on the top layer. This is represented in Figure 2.1A in the starting part of the
compression cycle before the transition surface pressure (Π_t). Further compression forces the
liquid crystal lipids on the outer layer to interact, resulting in a sharp rise in Π. With
expansion of the film, the liquid crystals break up into islands, resulting in a sharp decrease in
Π.

The meibomian film when heated is more fluid, and the liquid crystal islands form liquid
lenses that merge with each other and readily interact with the surfactant lipids at the bottom
The different molecules are evenly dispersed throughout the film and because of the fluidity,
the rate of increase in Π, Π_{\text{max}} and hysteresis are lower than a rigid film (Fig 2.1B).
Figure 2.1: Schema of typical Π-A compression and expansion curves: Π_{max}, maximum surface pressure. A: Rigid film. B: Fluid film

2.5 Epifluorescence microscopy of spread lipid films
Films of meibomian lipids and its mixtures were fluorescently tagged with 0.5% NBD-phosphatidylcholine (1-acyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phospho-choline) and microscopic changes to the structure of the films were observed at 400x magnification using an Andor DV887 Inverted CCD camera.

2.6 Results

2.6.1 Effect of cholesterol on the surface activity of meibomian lipids
At 35°C, Π-A profiles of human and koala meibomian lipids were comparable and showed a gradual increase in Π with little hysteresis. Meibomian lipid profiles did not collapse under high compression and were reversible. The addition of cholesterol at 1 mol % had little effect on the Π-A profile of either koala or human meibomian lipids. Π_{max} was unchanged in koala lipid films and there was less than 2mN/m difference in human meibomian films seeded with 1 mol % cholesterol, which is within the variance of Π seen with unseeded meibomian lipid experiments. Transition states or phase transitions were not observed with the addition of
cholesterol, indicating that significant reorganisation of molecules at the air-water interface did not occur (Fig. 2.2).

![Graph](image_url)

**Figure 2.2:** Dynamic cyclic Π-A profile of koala and human meibomian lipids seeded with and without 1 mol % cholesterol at 35 °C.

Interestingly, the addition of cholesterol at levels comparable to disease states, or even much higher such as 5 mol % and 10 mol % did not change the overall Π-A profile and caused no apparent phase changes, and the film retained its unique meibomian lipid characteristics of being non-collapsible and reversible. The addition of 5 mol % cholesterol caused a 3mN/m difference in Π_{max}, but take off was comparable with meibomian films without 5 mol % cholesterol. Take off was at a greater area and there was a 6mN/m increase in Π_{max} in human meibomian films seeded with 10 mol % cholesterol. This indicated that the molecules were occupying more area. However, the film retained its unique meibomian lipid characteristics of being non-collapsible and reversible (Fig. 2.3, Fig. 2.4A).

Meibomian lipid films seeded with excessive amounts of cholesterol (50-70 mol % cholesterol) revealed clear phase transitions at 10mN/m that was more pronounced with progressive increases in cholesterol concentration. The sharp increase in Π from Π_{t} indicates that the cholesterol has not mixed with meibum and cholesterol is dominant at the air-liquid interface at high Π. Therefore at high Π, meibomian lipid films seeded with excessive
amounts of cholesterol are likely to be stiff and elastic films (Fig. 2.4A). This idea is supported by the almost identical collapse pressures (40mN/m) of meibomian lipid films seeded with 30 mol % cholesterol and pure cholesterol films (Fig. 2.4B). Pure cholesterol films contrasted to meibomian lipid films in that they were extremely elastic and irreversibly collapsed. Their elastic nature is evidenced by an extremely sharp rise in Π over a very limited surface area of around 120 cm$^2$ (Figure 2.4B). It is also important to observe that meibomian films have a late take-off compared with pure films of cholesterol due to their fluid nature and ability to orientate and form multilayers. Cholesterol on the other hand is a rigid molecule that forms monolayers and therefore, has an earlier take off pressure due to each molecule occupying more area compared with meibomian lipids.

**Figure 2.3:** Dynamic cyclic Π-A profile of human meibomian lipids seeded with and without 5 mol % cholesterol at 35°C.
Figure 2.4: Dynamic cyclic Π-A profile of human meibomian lipids seeded with varying molar ratios of cholesterol at 35°C. A) Π-A profile of cholesterol seeded human meibomian lipids before collapse. B) Π-A profile of cholesterol seeded human meibomian lipids after collapse.

The effect of cholesterol on meibomian lipids was also investigated at 20°C (below the melting point of meibum). Pure and seeded meibomian films demonstrated higher Π_{max} and slightly increased hysteresis on expansion, which could be attributed to the quasi-solid nature of meibum at this temperature (Butovich et al, 2010; Mudgil and Millar, 2010). However, the general influence of cholesterol on the surface activity of meibum was comparable to 35°C (Fig 2.5).

Figure 2.5: Dynamic cyclic Π-A profile of human meibomian lipids seeded with varying molar ratios of cholesterol at 20°C. A) Π-A profile of human meibomian lipids with and without 1 mol % cholesterol. B) Dynamic cyclic Π-A profile of human meibomian lipids seeded with varying levels of cholesterol.
2.6.2 Epifluorescence microscopy of meibomian lipid films seeded with cholesterol

Changes to meibomian lipid films were observed microscopically by seeding films with NBD-phosphatidylcholine. The absence in fluorescence or presence of dark regions could indicate that the area is depleted in lipids, or that meibomian lipids have condensed together to squeeze out NBD-phosphatidylcholine.

Microscopically, pure meibomian lipid films were an amorphous mass, and there were no obvious differences between 20°C and 35°C, other than a fast moving film at 35°C due to thermodynamics. While the films initially appeared amorphous, with dark regions of lipids, these regions tended to coalesce as pressure increased (Fig. 2.6A). The addition of 10 mol % cholesterol had minimal influence on the appearance of meibomian films. The films still retained their amorphous appearance at low pressures, with mixtures of regions with more intense and less intense fluorescence. The dark regions appeared darker in the 10 mol % cholesterol seeded films (Fig. 2.6B). In contrast, meibomian films with 50 mol % cholesterol and pure cholesterol films, revealed obvious clumping and numerous small crystalline domains (Fig. 2.6C, Fig. 2.6D).
2.6.3 Effect of cholesteryl oleate and oleic acid on the surface activity of meibomian lipids

The addition of cholesteryl oleate did not have a notable effect on the surface activity of human meibomian films. Meibomian films seeded with 10 mol % cholesteryl oleate demonstrated a minor increase in $\Pi$ and an earlier take off, but there were no phase changes and the profile of meibum was still conserved. The hysteresis (gap between the compression and expansion cycle) was also only slightly increased, which indicates that there was no significant reorganisation of lipids due to the influence of cholesteryl oleate. Pure cholesteryl oleate films however, did reveal transition states. A phase transition was observed around 4mN/m. At $\Pi$ below 4mN/m, the profile seemed characteristic of the cholesterol portion of the molecule, indicating a vertical orientation of the ester group in the subphase. However, at higher $\Pi$ (4-18mN/m), the very gradual increase in $\Pi$ and liquid expanded characteristic of the profile indicated the influence of the ester component. This second phase was similar to
meibomian film profiles, and the ability of the film to resist increases in $\Pi$ under high compressions indicated that the film was forming multilayers. Cholesteryl oleate, under the tested conditions did not collapse at all, and at medium and higher $\Pi$ showed similar profiles to meibum. This is unlike other pure components such as cholesterol which collapsed under high compression and formed monolayers (Fig. 2.7).

Figure 2.7: Dynamic cyclic $\Pi$-A profile of pure cholesteryl oleate films, as well as human meibomian lipids with and without 10 mol % cholesteryl oleate.

Pathological levels of oleic acid do not seem to have a major effect on the surface activity of meibomian lipids. Seeding a meibomian film with approximately 5 mol % oleic acid (Joffre et al., 2008) resulted in identical $\Pi_{\text{max}}$, and a slightly earlier take-off, indicating that the film was more expanded due to the influence of oleic acid. However, the film still retained its unique non-collapsible nature and other characteristic features of meibomian lipid films (Fig. 2.8). In contrast, pure oleic acid, reached a $\Pi_{\text{max}}$ and collapse $\Pi$ of close to 50mN/m on our ribbon enclosed barrier trough. The collapsible nature of the film was evidenced by the late take-off area of the film for the 2$^{\text{nd}}$ and 3$^{\text{rd}}$ isocycle. The profile of oleic acid on the first isocycle suggests that oleic acid at concentrations equivalent to meibomian lipid films undergo
significant reorganisation under repeat compression-expansion isocycles or move off the surface as micelles. Transition states were identified at 25mN/m, 30mN/m and 35mN/m, but these were less pronounced on repeat compression-expansion cycles. As the molecules were forced to interact (under compression), it is likely that their orientation tilts (between 25-30mN/m) from a horizontal orientation at low $\Pi$ up to (20mN/m), to a more vertical orientation at higher $\Pi$ (30mN/m) (Fig. 2.9A). During this tilting of the molecules (Durbin et al, 1994; Peterson et al, 1998), the rate of $\Pi$ change decreases, hence the flattening of the curve. Since the above phenomena could have been influenced by high concentration of molecules, the profile of pure oleic acid at lower volume was also tested to understand its behaviour (75% less concentration/volume than molar equivalent of meibum). Oleic acid at a lower volume demonstrated a profile with very little hysteresis and no changes in transition state. The very sharp rise in $\Pi$ and late take off indicates that the film is likely to be quite elastic. The lack of change in hysteresis over repeated isocycles also indicated that there is very little reorganisation of lipids and they remain oriented in a fixed position despite expansion and compression of the film (Fig. 2.9B).

**Figure 2.8:** Dynamic cyclic $\Pi$-A profile of human meibomian lipids with and without 5 mol % oleic acid.
Figure 2.9: Dynamic cyclic Π-A profile of oleic acid films at varying concentrations. A) Π-A profile of oleic acid film at 100 mol %. B) Π-A profile of oleic acid at 25% less volume than molar equivalent to meibum. 1st and equilibrium isocycles overlapped.

2.7 Discussion

The results of our investigations were surprising because they indicated that meibomian lipid films were very tolerant to relatively large changes in lipid composition. This is against the general consensus in ocular research that small changes in lipid composition could lead to destabilisation of the lipid layer, loss of functionality and consequently symptoms of dry eye disease (Dougherty and McCulley, 1986a; Dougherty and McCulley, 1986b; Shine and McCulley, 1991; Shine and McCulley, 1993).

Clinical and model studies, particularly by McCulley and Shine, have shown that changes in the levels of cholesterol and its esters could have a role in the aetiology of blepharitis (Shine and McCulley, 1991; Shine and McCulley, 1993). Numerous ideas to correlate changes in composition with the disease process were proposed, including that; increased cholesterol or its esters could affect the relative ratios of wax esters, triglycerides and fatty acids; increases in cholesterol may be due to hydrolysis of cholesterol esters by microorganisms; or that fatty alcohols could contribute to the increase in cholesterols (Dougherty and McCulley, 1986a; Dougherty and McCulley, 1986b).
In essence, it was believed that specific quantitative changes in meibomian lipid composition could be directly, or indirectly associated with the pathophysiology of blepharitis. However, there was no direct evidence or understanding of how changes in lipid components affect the stability of the lipid layer.

Recently, Butovich’s group have demonstrated that ceramides, and cholesterol, when present in large quantities in meibum, decrease the stability and elasticity of meibomian lipid films (Arcinega et al, 2010; Uchiyama et al, 2007; Uchiyama et al, 2010). However, the relatively large quantities of cholesterol and ceramides used in the experiments do not reflect the relatively minor variances in lipid ratios in meibum of patients with dry eye. Therefore, it is unlikely that pathological changes in ratios of lipids within meibum can reach a threshold high enough to cause instability of the meibum in vivo. It should also be noted that Butovich’s group had previously reported that oleic acid has a detrimental effect on the stability of meibomian films (Uchiyama et al, 2007), but a more recent study by the group has identified that the effects of oleic acid on meibomian films are minor regardless of concentration, provided that the physiological concentrations of calcium (cation) are used in the subphase (Arciniega et al, 2011). The latter is consistent with the findings here.

Cholesterol when seeded at excessive concentrations was not miscible in meibomian lipids, and clear delineation between the cholesterol and meibomian lipid phases were observed. The influences of cholesterol, cholesteryl oleate and oleic acid were particularly surprising, noting that when using a molecular weight of 350 for human meibum, the molar ratio of these lipids used in our experiments is higher than if a molecular weight of 700 was assumed for human meibum (e.g. concentration of cholesterol with assumed meibum MW of 350 is double that of meibum at 700). Yet despite the increased concentration of seeded lipids, their effect on surface pressure and dynamic profile of meibomian films was minor (unless seeded in excessive concentrations). The difference in estimation of molecular weight of meibum did not affect the outcome of our findings.
Surface pressure measurements were supported by fluorescence microscopy, which revealed that the addition of 10 mol % cholesterol did not significantly alter the structural morphology of meibomian films. Our results indicate that the surface activity of meibomian lipid films is extremely resistant to changes in composition. We believe that this could be due to the extremely complex composition and unique biophysical characteristics of meibum.

Meibomian gland secretions consist of over hundred (if not greater) lipid species (Tiffany, 1987; Butovich et al, 2008) and numerous proteins (Tsai et al, 2006; Thangavelu et al, 2010). The findings here suggest that a complex interaction between these constituents allows meibomian films to avoid collapse and maintain integrity by forming reversible multilayers during compression and expansion. The likelihood that there is a continuous transfer of molecules (including seeded cholesterol, its esters and oleic acid) through multiple layers during compression and expansion is supported by lack of obvious differences in phase or IT-A profiles in meibomian films seeded with different lipids. A transfer of molecules and refolding of different layers could allow the meibomian film to absorb and resist any surface pressure changes when there is an increase in the concentration of particular lipid components. This concept of multilayer formation and reorganisation of different lipid components in the TFLL is supported by both the data presented here (particularly under the high compression of the ribbon trough) and also by other researchers (Bron et al, 2004; Petrov et al, 2007; Mudgil and Millar, 2010).

McCulley and Shine’s concept that changes in lipid composition could have a notable effect on meibomian characteristics such as surface tension and hysteresis were initially based on studies of monolayer films of pure components (McCulley and Shine, 2001) which behave very differently to meibomian films. Pure components, such as phospholipids, are poor models for meibomian lipid films as they form monolayers that collapse under high pressures. Moreover, the model proposed by McCulley and Shine does not account for the fact that proteins and other lipids in meibum could interact with each other during the compression and expansion cycles to selectively squeeze out different components into the bulk or onto the
surface to form mosaic multilayered films. In such a case, the meibomian film could have an inbuilt tolerance in terms of surface pressure to changes in the ratio of lipids, as seen in our results.

This idea is supported by recent work in the pulmonary field. Studies on pulmonary surfactant have shown that cholesterol is able to induce ordered packing of surfactant lipid films (i.e. reduce the area occupied by lipids) and interact with the acyl chains of phospholipids to reduce phase transitions and increase fluidity of phospholipid systems (Simons and Ikonen, 2000; Yuan and Johnston, 2002). These studies were based on experiments using pure components or binary mixtures such as phospholipids, and did not take into account the effect that non-polar/non-phospholipids could have on the function of surfactant films. However, some recent studies that better emulate natural pulmonary surfactant systems have shown that the previously attributed functions of cholesterol are eliminated when proteins and other complex lipids are used in the model under dynamic conditions. It led the authors to propose that cholesterol does not have a role in the surface activity of pulmonary surfactant films (Diemel et al, 2002). Pulmonary surfactants like meibomian films, are also able to withstand high surface pressures during breathing due to their ability to form multilayers (Wang et al, 2005; Lee, 2008; Mao et al, 2008). In particular, it is believed that it is the presence of particular proteins within lung surfactant that facilitates the formation of multilayers (Wang et al, 2005). These unique biophysical characteristics could contribute to their resistance to changes in particular components.

The counter-intuitive concept that changes in individual lipid composition may not have an effect on particular aspects of its functionality is not just restricted to physicochemical studies or pulmonary systems. Increased levels of branched fatty acids had been correlated with blepharitis patients with meibomian gland dysfunction. However, Joffre et al (2009) have discovered that increases in branched chain fatty acids in the tears of blepharitis patients does not affect the conjunctival cell changes that occur in blepharitis (Joffre et al, 2009).
The authors proposed that increases in branched chain fatty acids may be a beneficial and adaptive response of the body to increase the stability of the lipid layer in blepharitis patients with meibomian gland dysfunction (Joffre et al, 2009).

Extrapolating these results directly to the behaviour of meibum in vivo, should be treated with a degree of caution. Cholesterols are known to have a marked effect on the behaviour of phospholipids, and while it has been shown that phospholipids are extremely minor components if present at all in meibum (Butovich et al, 2008), phospholipids from the aqueous layer could interact with specific non-polar components such as cholesterol, and influence the surface activity of meibomian lipids (aqueous phospholipid-meibum interaction investigated in chapter 6).
3 Effect of specific protein and lipid components on meibomian lipid films

3.1 Introduction

Given that the surface activity of meibomian lipid films showed little change when the ratios of well known lipid components or breakdown products were altered to comparable levels expected in disease states and levels well above these, it is possible that less prominent components affect the surface activity of meibum. An area of interest has been the carotenoids such as β-carotene (precursor to Vitamin A). There have been numerous reports on retinoids and their benefits for ocular health (Ubels, 1986; Nelson, 1999; Gipson, 2007; Chen et al, 2009). They are well known as antioxidants (Snodderly, 1995), and formulations containing Vitamin A have been found to be efficacious in the treatment of dry eye (Holly, 1993; Kim et al, 2009).

It has recently been discovered that β-carotene/Vitamin A decreases with age in human meibum, and has been highlighted as a component that could be important in maintaining the stability of the lipid layer (Oshima et al, 2009). Although discovered in very minor microgram quantities, it could be an important determinant of the TFLL’s structural and dynamic properties due its heavily conjugated double bond system which could affect the fluidity of meibum through its interactions with lipids and proteins of the TFLL. It could also modify TFLL biophysical properties through van der Waals interactions with hydrocarbon chains of lipids. This idea is supported by work in lipid membrane systems where carotene has been shown to alter the fluidity and arrangement of lipids (Strzalka and Gruszecki, 1994; Gruszecki and Strzalka, 2005).

Besides lipids, changes in protein levels in the tear film, or even changes in some of the ~90 different proteins that have been identified in the meibomian lipid secretion itself (Tsai et al,
2006), offer a mechanism for affecting the TFLL. This idea is supported by previous work from our group and others (Nagyova and Tiffany, 1999; Tragoulias et al, 2005; Millar et al, 2009), demonstrating that protein-lipid interactions are important in lowering surface tension and promoting tear stability. Furthermore, in meibomian gland dysfunction (MGD), studies have shown that meibomian lipid secretions contain more proteins than normal (Borchman et al, 2010). Keratin is a major hydrophobic protein found in meibomian secretions and originates from the shedding of keratinised epithelial cells that line the ducts of the meibomian glands (Knop and Knop, 2009). This is important in the pathogenesis of obstructive MGD, as hyperkeratinisation of the meibomian gland epithelium leads to obstruction of the meibomian gland ducts, and the shedding of these epithelial cells into the duct can cause up to a 10% increase in keratin in MGD patients compared with normals (Ong et al, 1991). Furthermore, preliminary analysis of tear samples of keratoconus subjects by Pannebaker et al (2010) has revealed the presence of keratin proteins, and the possibility of interactions between differentially expressed keratins in the aqueous component and lipids of the TFLL under these conditions can also not be discounted. It is therefore hypothesised that interactions between keratin and meibomian lipids could have important consequences for the structure of meibomian lipid films.

Another protein class in small amounts in the tear film is tear SPs (see Chapter 1). Their site of activity, as well as contribution within the tear film is still unknown. Interactions between SPs and lipids lower the surface tension of lung surfactant in the pulmonary system and these proteins could have a similar role in the tear film (Orgieg et al, 2010). The hydrophobic nature of lung SPs, particularly SP-B and SP-C (Bräuer et al, 2007a; Bräuer et al, 2007b; Bräuer and Paulsen 2008), suggests that they could stabilise the TFLL by either interacting with it from the aqueous phase or be actually embedded within the TFLL itself.

Whilst β-carotene, keratin and tear SPs might be minor components of the TFLL, this does not exclude them from being important structural determinants that influence its biophysical
functions. Hence, their influence on the surface tension/pressure of meibomian lipid films is investigated in this study.

3.2 Materials and methods

β-carotene (Batch #068K2561) and keratin from human epidermis (Batch #029K1252) were purchased from Sigma-Aldrich, Australia. Note: Keratin in this form is denatured and water soluble.

3.2.1 Purification of lung surfactant

Bovine lung SPs were used as substitutes for human tear SPs due to human tear SPs being unavailable in amounts that allow experimentation. Bovine SP-B and SP-C were purified by combining organic solvent extraction with size-hydrophobic affinity chromatography (Folch et al, 1957; Beers et al, 1992).\textsuperscript{5}

A bovine pluck was obtained from a local slaughterhouse and the lungs lavaged with approximately 10L of 50mM sodiumphosphate buffer (pH7.4). The proteins were protected by processing the lavage at 4°C. The lavage was centrifuged at 600 x g for 10 min to remove cell contaminants and then surfactant proteins were precipitated by centrifugation of the supernatant at 19,500 x g for 35min. One sixth of the precipitate was dissolved in 10mL of purified water and then extracted 3 times with 20mL diisopropylether:butanol (3:2) and freeze-dried. The freeze-dried sample was dissolved in 2mL of purified water and extracted with 40mL of a chloroform-methanol (2:1) mixture. The chloroform phase was removed and stored, while the water phase and the interfacial fluff was re-extracted twice with 15mL chloroform:methanol:water (86:14:1) according to Folch et al. (1957). The chloroform phases were pooled and the chloroform removed in a rotary-evaporator. The dried precipitate was dissolved in 300µL chloroform:methanol:HCl 1N (1:1:0.008) and applied to a 30 x 1cm Sephadex LH-20 (GE Healthcare) column that had been equilibrated with the same solvent.

\textsuperscript{5}Dr. Burkardt Schütte from our laboratory assisted in the extraction and purification of the bovine SPs.
mixture. The column was resolved using the chloroform:methanol:HCl 1N using a flow-rate of 0.5mL/min and 1mL fractions were collected.

3.2.2 Gel electrophoresis of bovine surfactants
The size and purity of the surfactant proteins in each fraction from the column above were determined via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) run under reducing and non-reducing conditions. In order to meet two different conditions (~pH6.8 and high concentration of SDS) while preparing the sample for application to the gel, a mixture of sample buffer and stacking gel buffer was used. One hundred microlitres of the purified lung SP fractions were dried under nitrogen and diluted in 10µL 3 x SDS-PAGE sample buffer and 10µL 4 x SDS-PAGE stacking gel buffer (0.5 M Tris/HCl pH6.8, 0.4 % SDS). The sample was centrifuged (14,000 x g, 5min, room temperature) and split. Five microlitres of purified water was added to one part (non-reducing sample) and 5µL 0.3 M dithiothreitol (reducing sample) to the other. Samples were heated at 94°C for 5 minutes before loading onto 15% polyacrylamide gels with a 4.5% stacking gel (Bio-Rad Australia). Precision Plus Kaleidoscope standards (Bio-Rad Australia) were used as molecular weight markers. Electrophoresis was performed at an initial voltage of 100V for 30 minutes, and then increased to a constant voltage of 200V for 1 hour using a Tris-glycine running buffer.

Following electrophoresis, gels were silver stained as per the protocol of Nesterenko et al (1994). Gels were fixed for 5 minutes in a solution containing 50% (v/v) acetone, 1.5 % (v/v) acetic acid and 0.05% (v/v) formalin. The gel was rinsed for 5 minutes with purified water, then pre-treated in a 50% (v/v) acetone solution for 5 minutes. This was followed by further pre-treatment in a 0.016% (w/v) sodium thiosulfate solution. After rinsing in purified water for 5 minutes, the gel was then impregnated using a solution containing 0.41% (w/v) silver nitrate and 1% (v/v) formalin. Finally, the gels were rinsed with purified water and developed in a solution containing 2% (w/v) sodium carbonate, 0.005% sodium thiosulfate and 0.05% (v/v) formalin. The reaction was stopped using 1% (v/v) acetic acid. Note, the sensitivity of silver stain is around 1-5ng.
3.2.3 Western blot analysis of purified proteins

Western blot analysis was performed using a mini electrophoresis transfer system from Amersham Biosciences. 15% SDS PAGE gels were used to separate proteins. Separated gel proteins were transferred to nitrocellulose membranes (0.22µm pore size) in transfer buffer (12mM Tris, 96mM glycine, 20% v/v methanol and 0.02% SDS) using a Semidryblot Roth system. The blot procedure is performed at 0.8mA per cm$^2$ of gel. The nitrocellulose membranes were incubated in TBST (66mM Tris base, 137mM NaCl, 2.7mM KCl and 0.1% Tween 20, pH 7.4) with 5% milk powder for 1 hour at room temperature to block non-specific binding of proteins. Following incubation, the membrane blots were probed with 1:500 dilution of rabbit anti-bovine SP-B antibody (primary antibody, Abcam) overnight at 4 °C. After washing three times in TBST (15 minute intervals), membrane blots were probed with 1:1000 dilution of goat anti-rabbit IgG conjugated to HRP (secondary antibody, Cell Signalling Technology) for 2 hours at room temperature. The membranes were washed three times in TBST as before (15 minute intervals) and the immunoreactive protein bands were visualised using Immobilon Western HRP substrate. Precision Plus Protein Standards (Bio-Rad) were used as molecular weights.\(^6\)

3.2.4 Estimating total protein (Bradford Assay)

The concentration of purified SP was estimated using a Bradford protein assay kit and bovine serum albumin as the standard. Filtered ion exchange ultrapure water was the diluent. The bovine SP sample (1µL) was incubated with Bradford reagent (200µL) and the absorbance was measured using an Amersham Biosciences spectrophotometer at 595nm (factor 0.0781).

3.3 Surface pressure-area (Π-A) measurements

Mixtures of β-carotene and human meibomian lipids were prepared by mixing appropriate volumes in solutions of chloroform, in the mole fraction range of 0 to 1. The total number of molecules was kept constant at 3.44 x 10$^{16}$.

\(^6\)This technique was conducted with the assistance of Martin Schicht at University of Halle (Germany) as part of a collaborative project.
Experiments were conducted at 20ºC and 35ºC for surface activity measurements of β-carotene, purified SPs and human meibomian lipids (1mg/mL) (to enable comparison with cell and pulmonary literature). Purified SPs were mixed with human meibum by maintaining a final volume of 20µL, with different volumes of meibum and lung surfactant.

Keratin experiments were conducted at 35ºC. As controls, the solvents used to dissolve keratin (8M urea, 0.1M mercaptoethanol and 0.05M Tris) were tested for surface activity and for their possible effects on meibomian lipid films. Π-A measurements and epifluorescence microscopy of spread lipid films were conducted as described in Chapter 2.

3.4 Penetration of keratin into meibomian lipid films

Human meibomian lipids (20µL, 1mg/mL) were spread over the buffered subphase in a Langmuir trough with a surface area of 80 cm². A minimum of three isocycles were conducted prior to compression of barriers to the target pressure. These expansion-compression cycles were conducted to allow organisation (aging) of the meibomian lipid film and to evaluate the surface activity of meibomian lipids prior to the injection of proteins. The barriers were then closed until the target pressure of 5mN/m was met. Keratin (9.2mg/mL) was injected into the subphase, in the region of the barriers, but outside the barriers. The adsorption and penetration of keratin into the meibomian lipid layer was monitored over a 3-4 hour period. Isocycles were also conducted after the absorption of keratin, to evaluate the change in surface activity of meibomian lipids after keratin absorption/penetration. These experiments were carried out at 35ºC. Assuming even dissolution, the final concentration of keratin in the 80mL subphase was 0.115µg/mL concentration for each µL of keratin sample injected. The actual concentration of keratin in meibum has not been reported and therefore the amounts of keratin used in these experiments was a best guess based on a report of total protein in meibum of ~0.24µg/mL per sample collected using a glass capillary tube (Thangavelu et al, 2010), and that keratin is <10% of the protein concentration in normals (Ong et al, 1991).
3.5 Results

3.5.1 Purification of lung SPs
Optical density profiles of fractions obtained from the hydrophobic affinity-size exclusion column revealed that lung SPs were likely to be present in peak fractions 4 and 5, as well as in fractions on the shoulder of the peak i.e. fractions 6-8 (Fig. 3.1).

![Optical density profile of lung surfactant fractions separated using Sephadex LH20 column.](image)

**Figure 3.1:** Optical density profile of lung surfactant fractions separated using Sephadex LH20 column.

Analysis of fractions eluted from chromatography using Sephadex LH20 by SDS-PAGE techniques under non-reducing conditions revealed a 15kDa and 25kDa band from fraction 4 (Fig. 3.2A, Lane 2) which corresponded to the molecular weights of monomeric and dimeric forms of SP-B (Kang et al, 1996; Bräuer et al, 2007b). A very faint third band, approximately 10kDa was also observed. This corresponded to the reported molecular weight of SP-C (Weaver and Whitsett 1991; Kang et al, 1996). A smear pattern was observed in fraction 6 (Lane 3, Fig. 3.2A), which was believed to be lipids that were separated from lung surfactants. Protein bands were not observed in fraction 2 (Lane 1, Fig. 3.2A) or fractions 8-16 (Lane 4-8, Fig. 3.2A). Under reducing conditions faint bands were present at approximately 10kDa and another at lower than 10kDa (Lane 2, Fig 3.2B). These corresponded to the reported molecular weight of SP-B and/or SP-C (Bräuer et al, 2007b).
3.5.2 Western blot analysis of purified bovine lung surfactant

Western blot demonstrated cross reactivity (positive reaction) of commercial antibodies (anti SP-B) to our purified bovine SP sample (Fig. 3.3, circled in red). The lane containing our purified SP, showed reactive bands approximately 10kDa and 18kDa in size, which indicate monomeric and dimeric forms of SP-B (Fig. 3.3).

![Figure 3.2: SDS PAGE analysis of lung surfactant fractions purified using hydrophobic affinity-size exclusion column. A) SDS 15% polyacrylamide gel of purified lung surfactant fractions separated under non-reducing conditions and stained with silver nitrate. Lane M, protein standard markers (Precision Plus Protein Kaleidoscope standard); Lane 1, fraction 2; Lane 2, fraction 4; Lane 3, fraction 6, Lane 4, fraction 8; Lane 5, fraction 10; Lane 6, fraction 12; Lane 7, fraction 14; Lane 8, fraction 16, Lane 9, fraction 18. B) SDS 15% polyacrylamide gel of purified lung surfactant fractions separated under reducing conditions and stained with silver nitrate. B) Lane M, protein standard markers (Precision Plus Protein Kaleidoscope standard); Lane 1, fraction 2; Lane 2, fraction 4. Circled bands represent SPs.]

On the basis of these techniques, it was determined that fraction 4 comprised SP-B. The concentration of the purified bovine SP-B was approximately 0.39μg/μl (0.39mg/mL), and was used for subsequent surface activity experiments.
Figure 3.3: Western blot analysis of purified bovine lung protein. Circled bands represent positive reaction to anti-SP-B.

3.5.3 Surface activity of β-carotene

Π-A profiles of β-carotene showed a late take-off (~60 cm$^2$) and a rapid increase in Π till Π$_{\text{max}}$ of 36mN/m (Fig. 3.4). There was little hysteresis upon expansion. The results indicate that the film is likely to be elastic and rigid.

Figure 3.4: Dynamic cyclic Π-A profile of β-carotene film at 20°C and 35°C.
3.5.4 Effect of β-carotene on the surface activity of meibomian lipids

3.5.4.1 Effect at 20°C

Meibomian lipid films at 20°C showed notable hysteresis on expansion and \( \Pi_{\text{max}} \) of 23mN/m (Fig. 3.5). Transition/phase states (\( \Pi_t \)) were observed at 3mN/m, 8mN/m and 10mN/m. The meibomian film is in a liquid expanded phase upon initial compression, as evidenced by resistance to increase in \( \Pi \). However, the transition state leads to a liquid condensed phase in the film at higher \( \Pi \). During expansion, there is a sharp drop in \( \Pi \) and significant hysteresis, indicating that once the molecules have interacted, it is difficult to pull them apart (Fig. 3.5).

Dynamic \( \Pi-A \) isocycles showed that the addition of as little as 1 mol % β-carotene had a strong condensing effect on the surface activity of meibomian lipids as indicated by a decrease in area for the same \( \Pi \). Take-off area was less (~60cm\(^2\) vs 68cm\(^2\)) and \( \Pi \) was less at higher area (at 35cm\(^2\), \( \Pi = 4\)mN/m with 1 mol% β-carotene and 8mN/m without), but \( \Pi_{\text{max}} \) was unchanged. For \( A < 20\)cm\(^2\), there was little difference in \( \Pi \) between meibomian films and those seeded with β-carotene (Fig. 3.5).

Meibomian lipid films were amorphous in appearance at low \( \Pi \), interspersed with small dark domains. As \( \Pi \) increased, these dark regions merged together to form large grey islands. Films with β-carotene at low \( \Pi \), were overall low in fluorescence intensity with darker stringy regions surrounding the brighter regions. At high \( \Pi \), the darker stringy regions became much darker and then disappeared from the film - possibly being pushed off the surface which is consistent with the \( \Pi-A \) profiles (Fig. 3.5).
**Figure 3.5**: Dynamic cyclic $\Pi$-A profile of human meibomian lipids seeded with and without 1 mol % $\beta$-carotene at 20°C. Micrographs show appearance of the film at different pressures. Scale bar represents 50µm.

### 3.5.4.2 Effect at 35°C

$\Pi$-A profiles of human meibomian lipid films, showed a constant increase in $\Pi$ with little hysteresis at 35°C (Fig. 3.6) compared with 20°C (Fig. 3.5). $\Pi_{\text{max}}$ at 35°C was less than $\Pi_{\text{max}}$ at 20°C indicating that the rearrangement/behaviour of molecules is different at 35°C compared with 20°C. There was no change in sequential $\Pi$-A curves indicating that the molecular arrangement quickly stabilised in films at 35°C.

Interestingly, the addition of 1 mol % $\beta$-carotene had minimal influence on the surface activity of meibomian films. The condensing effect observed at 20°C was not seen at 35°C. No transition states or change in take off was noticed, but the difference in $\Pi_{\text{max}}$ was $\sim$3mN/m.
Microscopically, pure meibomian lipid films and those seeded with 1 mol % β-carotene at 35°C were similar to films at 20°C except for increased mobility. With the addition of β-carotene, large black domains (not seen with pure meibum) were observed, but these did not seem to affect the fluidity of the meibomian film (Fig. 3.6).

Figure 3.6: Dynamic cyclic Π-A profile of human meibomian lipid s seeded with and without 1 mol % β-carotene at 35°C. Micrographs show the appearance of the films at different Π. Scale bar represents 50µm.

3.5.5 **Effect of bovine lung surfactant on the surface activity of meibomian lipids.**

Lung SP (10µL; fraction 4) was applied between the barriers to the surface of the subphase in the trough at 20°C, isocycles were conducted until equilibrium was reached, then heated to 35°C, allowed to equilibrate, then cooled back to 20°C. Except for the first isocycle, just after the film had been spread, the films were very similar in their Π-A profiles. At 20°C, area at take-off was less in the equilibrium cycle compared with the first cycle, heating to 35°C slightly increased hysteresis and cooling back to 20°C returned the profile to that seen before heating. Π<sub>max</sub> of ~27mN/m was almost identical under all conditions (Fig. 3.7A).
It was interesting to note that the hysteresis on expansion was less pronounced with lung SPs (Fig 3.7A) than previously observed for lysozyme or lactoferrin (Tragoulias et al, 2005).

Pure meibomian lipid films demonstrated a gradual rise in $\Pi$ until $\Pi_{\text{max}}$ of ~12mN/m and no phase transitions were noted. The seeding of meibomian lipid films with small amounts of lung SP (2.5-5µL; 1-2µg) had a strong influence on the $\Pi$-$\mathcal{A}$ profiles of meibomian lipid films and showed hybrid characteristics. The $\Pi_{\text{max}}$ reached by these mixed films was much higher than that for meibomian lipids alone, which was typically about 24mN/m at equilibrium for 20µg of meibomian lipids applied to the subphase. However, despite the difference in $\Pi$, significant hysteresis was not observed in meibomian films seeded with lung surfactants. This feature has not been observed with surface activity tests using other proteins.

With a smaller ratio of lung SP, the profiles were dominated by meibomian lipid characteristics (Fig. 3.7B), except that there was little change in the $\Pi$-$\mathcal{A}$ profiles when first applied at 20°C and at its equilibrium cycle. It showed the characteristics of reduced $\Pi_{\text{max}}$ on heating and increased $\Pi$ on cooling. With higher ratios of lung SP, the hysteresis in the curves was markedly decreased and similar to low ratios of lung SP, it reached a stable $\Pi_{\text{max}}$ quickly. There was still a slight decrease in $\Pi_{\text{max}}$ on heating to 35°C, and a slight expansion on cooling.
L meibum at 35°C

Cooled to 20°C

Equilibrium isocycle at 20°C

1st isocycle at 20°C

Equilibrium isocycle at 35°C

A (cm²)

Π (mN/m)

0 10 20 30 40 50 60 70 80

0 10 20 30 40 50 60 70 80

B

20µL meibum at 35°C
Figure 3.7: Comparative isocycles at 20°C and 35°C of lung SP alone (A; 3.9μg) and mixtures of lung SP with meibomian lipids (B; 1μg lung SP + 17.5μg meibum; 20μg pure meibum) (C; 2μg lung SP + 15μg meibum). The shapes of the individual surface pressure-area cycles are exported as separate loops for visual comparison.
Microscopically the meibomian film seeded with lung SPs was more fluid at equivalent $\Pi$ than meibomian lipid films alone (compared with Fig 6 in ref 4) and overall seemed to be a more even film than with meibomian lipids alone. However, for the first few isocycles, there were distinct blobby dark patches at high $\Pi$ which were not apparent once a number of isocycles had been completed. This could be indicative of the lung SP and the meibomian lipids not having mixed completely, even though they were thoroughly mixed as a solution before applying them to the subphase. Upon heating to 35°C, the film was very fluid and its features could not be discerned, even at $\Pi_{\text{max}}$. When cooled back to 20°C, the film looked like a normal meibomian lipid film except that the equivalent appearances occurred at much higher $\Pi$ (Fig. 3.8).

![Figure 3.8: Micrographs showing the appearance of a mixture of lung SP (2μg) and meibomian lipids (20μg) seeded with a fluorescent marker. Isocycles were carried out at 20°C, then at 35°C then cooled back to 20°C. Note e.g. "26 iso1" means the micrograph was taken at a pressure of 26mN/m during isocycle 1. The blurriness seen in "7.6 iso1 20°C", and "24 35°C" indicate that the film was moving very fast (shutter speed 0.01s). The arrows in iso1 20°C indicate the same feature at different pressures. The arrows in "24 iso2" and "Cooled 33.8" highlight some subtle substructures in a relatively even film. Scale bar represents 50μm.](image-url)
3.5.6 **Surface activity of keratin at 35°C.**

The $\Pi_{\text{max}}$ at equilibrium achieved by keratin spread onto the air-buffer interface (5µL) demonstrated strong surface activity with a $\Pi_{\text{max}}$ of approximately 28mN/m (Fig. 3.9). The profile showed more hysteresis compared with meibomian lipid films and the film also appeared to be more elastic (less fluid) as evidenced by the constant increase in $\Pi$ after take off at 65cm$^2$. The solvent (control) showed no surface activity (Fig. 3.9).

![Graph](image.png)

**Figure 3.9:** Dynamic cyclic $\Pi$-$A$ profile of keratin (5µL, 9.2mg/mL) at 35 °C.
3.5.7 Penetration and effect of keratin on the surface activity of meibomian lipids at 35°C.

A typical protein penetration profile can be categorized into four phases (Fig. 3.10).

A) Initial Π drop phase: Once the predetermined target Π is achieved, the molecules in the surface layer relax.

B) Lag/Induction phase: The protein is normally injected into the subphase once the pressure stabilises. There is a lag before a change in Π is noted due to the time taken for diffusion of the protein through the subphase to the surface.

C) Penetration phase: Π increases constantly at a sharp rate as more molecules adsorb to the surface film. The penetration phase could have two stages, indicated by multiple gradients. The first (1) steeper gradient represents new molecules adsorbing to the surface film and the second (2) gradient usually indicates existing molecules unfolding at the surface (Graham and Phillips, 1979), as well as new molecules adsorbing to the surface.

D) Equilibrium phase: The Π profile reaches a plateau and there is little change in Π. The proteins in the film are in equilibrium with proteins in the subphase.

Figure 3.10: Representation of a typical penetration profile
For penetration of keratin into a meibomian lipid film, an initial drop in $\Pi$ up to 1.5mN/m was observed after the target pressure was reached due to relaxation of the meibomian lipid film. Injection of 1µL of keratin solution into the subphase resulted in a gradual increase in $\Pi$ until a $\Pi_{eq}$ of 10mN/m occurred at around 4000s. When 7µL of keratin solution was added, diffusion and penetration appeared faster and $\Pi_{eq}$ was greater (~16mN/m) compared with when 1µL was added (Fig. 3.11).

**Figure 3.11:** A series of penetration curves at 35°C showing the penetration of different concentrations of keratin (9.2mg/mL) and control solutions into a human meibomian film (20µL, 1mg/mL) compressed to a predetermined surface pressure of 5mN/m.
3.5.8 Dynamic-cyclic Π-A profile of fluorescently tagged human meibomian lipids before and after penetration of different concentrations of keratin at 35°C

Π-A profile’s of human meibomian lipid films before penetration, showed a constant increase in Π with little hysteresis at 35°C. Micrographs taken prior to penetration illustrate an amorphous mass and exhibited few small dark patches. Following penetration of 1μL keratin there was a notable increase in Π<sub>max</sub>, ~19mN/m with 1μL keratin and ~26mN/m with 7μL keratin. While the main influence of 1μL keratin was an increase in Π<sub>max</sub>, the addition of 7μL keratin revealed a steeper compression profile that more closely resembled the pure keratin profile, and larger hysteresis (Fig. 3.12) that was comparable to that of major proteins such as lysozyme in our previous work (Tragoulias et al, 2005). Micrographs taken after penetration illustrate a more stable and stiffer (slow moving) meibomian film. At low to medium Π (particularly 8-18mN/m), dark stringy patches of lipids are observed and dominate the film, but appear to be squeezed out under higher Π (Fig. 3.12). It is likely that the stringy appearance of these dark domains is formed by the unique influence of keratin, as such a microscopic appearance/structure of meibum has not been observed in our previous work with other proteins (Tragoulias et al, 2005).
Figure 3.12: Dynamic cyclic Π-A profile of human meibomian lipids (20µL, 1mg/mL) before and after penetration of different amounts of keratin (9.2mg/mL) at 35 °C. Micrographs show the appearance of the films at different Π. Scale bar represents 50µm.
3.6 Discussion

The results of the study strongly indicate that even small amounts of some of the newly identified components of the tear film, such as keratin and tear SPs, have a strong effect on the surface activity of meibum.

Keratin had a strong effect and this could be important in diseases such as MGD. In terms of the pathogenesis of obstructive MGD, hyperkeratinisation of the meibomian gland duct leads to obstruction of the ducts and the levels of keratin in meibum increase up to 10% (Ong et al, 1991). This is normally associated with its presumed role in obstructing secretion of meibum from the ducts due to hyperkeratinisation. However, if as indicated by the studies here, the keratin is mixed with meibomian lipids, this increase could also have important structural and physicochemical implications in meibomian films of patients with MGD. Not only does keratin have important implications for the surface pressure of meibomian films, but the increased hysteresis and steeper compression profile of meibomian films after penetration of keratin, also indicates that keratin influences the viscoelasticity of meibum. It is likely that it would make the films more rigid and more subject to fracture under the blinking cycle. The finding that changes in protein concentration (including keratin) could alter the viscoelasticity of meibum is also supported by Borchman et al (2010). Using principal component analysis to identify compositional differences between normal meibomian lipid samples and samples from MGD patients, it was identified that MGD lipid samples contain greater levels of protein compared to normal meibum samples, resulting in meibum that is more viscous and ordered (less fluid) in patients with MGD.

Differential expression of keratin in tear samples of subjects with keratoconus has also been reported in preliminary studies by Pannebaker et al (2010), and although the levels of keratin used in our penetration experiments are likely to be higher than identified in keratoconus...
subjects, it is evident from our work that even low concentrations of keratin in an aqueous environment could interact with and consequently affect the surface activity of meibomian lipids (9.2μg/1μL keratin diffused in 80mL subphase). A limitation of the current study was that the keratin used in these studies was a denatured commercial product, and its structural conformation would differ from native keratin. Further studies of meibomian lipid samples from MGD and keratoconus subjects need to be conducted to ascertain the physicochemical changes caused by proteins including keratin.

There has been increasing interest and speculation about the roles of SPs, in particular SP-B and SP-C, since their discovery in the tear film (Bräuer et al, 2007a; Bräuer et al, 2007b; Bräuer and Paulsen, 2008). Although the properties of lung surfactants have been well studied, these studies evaluating surface activity of lung surfactant and phospholipid mixtures do not accurately reflect the biophysical properties of the complex and predominantly non-polar meibomian lipid system (Longo et al, 1993; Taneva and Keough, 1994; Bi et al, 2002), thus making it impossible to compare with our data. Our findings indicate that even minor amounts of SPs considerably reduce the surface tension (increased surface pressure) of meibomian films and are likely to be a key surfactant for the lipid layer of the tear film. Supporting this idea was the concentrations used. At the time of this study, the concentration of tear SPs was unknown, but Bräuer’s group have quantified low microgram amounts of SP-B’s, which is comparable with the amounts used in our study (Bräuer, personal communication).

Unlike other tear proteins previously tested by our group (Millar et al, 2006; Millar et al, 2009), meibomian film-SP interactions did not result in a large hysteresis or reorganisation of meibomian lipids, and only altered the appearance of meibomian films in higher concentrations. It is likely that this unique behaviour has something to do with the extremely hydrophobic nature of these proteins.
Compared with lung surfactant films, the SPs interact very differently with meibomian lipids. In the lungs, it is believed that the SPs are desorbed and adsorbed to the lung surfactant as the alveoli collapse and expand (Zuo et al, 2008; Orgieg et al, 2010). The consistent increase in pressure during isocycles of mixed SP and meibomian lipids indicate that they are not being adsorbed and desorbed. If this were the case, a flattening of the curve at lower surface areas and a $\Pi_{\text{max}}$ similar to that of meibomian lipids alone would be expected as this would indicate desorption. Such flattening is observed during isotherms of pulmonary surfactant films (Zuo et al, 2008), and this is modelled as multilayer formation which allows layers of phospholipids to be pushed into the subphase stabilised by SPs. With meibomian lipids, the stability of the isocycles and a similarity in appearance to normal meibomian lipid films suggest that they are acting as true surfactants in the TFLL and remain at the aqueous lipid interface.

Although tear SPs are similar in amino acid sequence to lung SPs, there is considerable post-translational processing of lung SPs, including cleavage (Whitsett et al, 1986; Voorhout et al, 1992; Bräuer et al, 2007b). Such post-translational processing of tear SPs is yet to be determined, and therefore tear SPs may have slightly different characteristics from those shown here. Ideally, for future experiments, the effect of tear SPs on the TFLL should also be evaluated. A limitation for such an experiment is the establishment of a recombinant system for expression of tear SPs in sufficient quantities for functional studies. Although this is currently being pursued, it is proving to be very difficult and has not yet been successful. Success in this recombinant technology would also provide the opportunity to synthesize and purify SPs in sufficient quantity to fluorescently tag them. Using tagged SPs in meibomian lipid film experiments would help to determine whether they are an interfacial anchor at the aqueous lipid interface or whether they penetrate into meibomian films to form an integral part of the surface layer.
An unusual finding was that the physicochemical contribution of β-carotene within meibomian films was strongly influenced by temperature. At 20°C, β-carotene had a condensing effect on meibum at low pressures, but was squeezed out at high pressures and had a minimal effect on the final surface pressure. However, at 35°C, the condensing effect was not observed and β-carotene remained within the meibomian film, causing a slight increase in surface pressure. It is likely that the highly fluid state of meibum and the thermodynamics at this temperature contributed to this behaviour, but the exact mechanisms are still unclear. Nevertheless, it appears to have a minor physicochemical role, in addition to its established antioxidant activities (Snodderly, 1995; Chen et al, 2009). The physiological effects of β-carotene could be further understood by investigating the effects of eye drops containing β-carotene on the lipid layer of the tear film using high magnification interference microscopy as described by King-Smith et al (2011).
4 Effects of phospholipids and proteins on meibomian lipid films

4.1 Introduction

Interactions between the major aqueous proteins and meibomian lipids have been shown to be likely determinants of the physicochemical parameters influencing tear stability (Nagyova and Tiffany, 1999; Tragoulias et al, 2005; Mudgil et al, 2006; Millar et al, 2009). In particular, it has been demonstrated that major proteins of the tear film (lactoferrin, lysozyme and lipocalin) are surfactants (Tragoulias et al, 2005; Mudgil et al, 2006; Millar et al, 2009), and current scientific evidence indicates that they are an integral part of the TFLL; there is no evidence to say that they are not. Since meibomian lipid composition appears to be changed in certain disease states (Shine and McCulley, 1998; Shine and McCulley, 2000), these changes could in turn affect the physicochemical properties of the TFLL. Although this could happen simply due to the change in the lipid composition, the change in the lipid composition is also likely to affect how components from the aqueous adsorb to and unfold in the TFLL. Since there is currently no mechanism for collecting the TFLL from the ocular surface, a strategy to test this concept is to evaluate meibomian lipid films by seeding with particular lipids (oleic acid, cholesterol and cholesteryl oleate) and determine how this influences protein binding and penetration.

Other components, particularly phospholipids, are also strong surfactants and it has been proposed that phospholipids provide a stabilising interface between the aqueous phase and the non-polar lipid phase of the TFLL to promote TFLL stability, formation and spread over the aqueous phase (Greiner et al, 1996; Shine and McCulley, 2003). Since the McCulley and Shine model of the TFLL, it is now evident that human meibomian lipids contain very little or no phospholipids. (Butovich et al, 2007; Butovich et al, 2008; Chen et al, 2010). However, this does not exclude the TFLL having phospholipids acquired from the aqueous, which has
been found to contain relatively high levels of phospholipids compared with meibum
(Borchman et al, 2007; Butovich et al, 2008; Dean and Glasgow, 2012). In order to test
whether adsorption of phospholipids from the aqueous to the TFLL is likely to affect its
properties, the adsorption of phospholipids from the subphase into human meibomian lipid
films spread onto the surface of the aqueous subphase was investigated.

4.2 Methods

4.2.1 Penetration of lysozyme into meibomian lipid films seeded with 10 mol %
cholesterol or oleic acid
Meibomian lipids seeded with 10 mol % cholesterol or oleic acid (total number of molecules
was kept constant at 3.44 x 10^{16}) were spread over the buffered subphase in a Langmuir
trough so that the predetermined target pressure (5mN/m) could be achieved within a surface
area of 16 cm² - 30 cm². Although 10 mol % seeding is unphysiological, the strategy was that
if a high percentage of cholesterol or oleic acid had no effect then lower percentages would
also have no effect. Therefore, if an effect were seen with the high percentages, lower
percentage mixtures would be used.

After compression of the lipid films to the target pressure (5mN/m), 35μL of lysozyme
solution dissolved in 25mM sodium phosphate buffer (3.2mg/mL pH 7.4) (Sigma Aldrich
Australia; Batch# L-6876), was injected into the artificial tear subphase (80mL), half the
sample injected outside each barrier. The final concentration of lysozyme is about 2000 times
less than that reported in whole tears (Fullard and Snyder, 1990). The reason for using a much
lower concentration was both financial and scientific: if there was an effect at a much lower
concentration then there should also be a greater effect at higher concentrations.

4.2.2 Surface activity measurement of phospholipids
Surface activity of DPPC at the air-buffer interface was evaluated by dissolving the lipids in
chloroform and applying 6μL of the DPPC solution (DPPC, 0.5mg/mL, Sigma Aldrich,
Australia; Batch# L-079H5228) onto the air-buffer interface of the Langmuir trough.
Dynamic compression-expansion isocycles were subsequently conducted to evaluate surface activity.

Surface activity of phospholipids in the aqueous subphase was assessed by dissolving the phospholipids in 25mM sodium phosphate buffer (0.379 mg/mL, pH 7.4), and injecting 6 mL of the phospholipid solution (Sigma Aldrich, Australia; Batch# L-079H5228) into the artificial subphase (3 mL outside each barrier) subsequent to barrier compression to the predetermined surface area normally occupied by meibomian lipids at a surface pressure of 5mN/m. Adsorption was monitored over a 1-2 hour period and isocycles were conducted after absorption to evaluate the surface activity. The final concentration of dissolved phospholipid solution in the subphase was much lower than the physiological concentration reported by Kawashima et al (2003).

4.2.3 Penetration of phospholipids into meibomian lipid films

Human meibomian lipids (20µL, 1mg/mL dissolved in chloroform) were spread over the buffered subphase so that predetermined target pressure could be achieved within a surface area of 16cm² and 30cm². Upon compression of the meibomian lipids to the target pressure (5mN/m), 6mL of the phospholipid solution, was injected into the artificial tear subphase, half the sample injected outside each barrier.

4.2.4 Pre and post penetration isocycles

A minimum of three isocycles were conducted prior to compression of barriers to the target pressure. These expansion-compression cycles allowed us to evaluate the surface activity of meibomian lipids prior to the injection of lysozyme or phospholipids. The adsorption and penetration of these components into the meibomian lipid layer was monitored over a 3-4 hour period. Isocycles were also conducted after the absorption to evaluate the surface activity of meibomian lipids after phospholipid or lysozyme absorption/penetration. Experiments were conducted at 35 °C.
4.3 Results

4.3.1 The effect of cholesterol and oleic acid on the interaction of lysozyme with human meibomian lipids

4.3.1.1 Penetration profile of lysozyme into meibomian films with and without 10 mol % cholesterol or oleic acid

The results indicate that the penetration profile of lysozyme into films seeded with cholesterol or oleic acid is similar to that of lysozyme penetration into pure meibomian films, i.e. changes in meibomian lipids did not influence the interaction of proteins with meibomian lipids. This was further supported by the comparable Π-A profile data after lysozyme penetration of meibomian films seeded with and without cholesterol or oleic acid (Fig. 4.1).

Following the application of lysozyme into the subphase with unseeded meibum on the surface, there was an initial drop in Π from Π_{init} of 5mN/m as the film relaxed. This was followed by an increase in Π (around 500s), which was an indication of lysozyme adsorbing to the meibomian film. Π_{eq} (equilibrium pressure) was reached around 2000s. Oleic acid seeded meibomian film revealed a similar Π_{eq} and the penetration rate was also comparable, with a less than 300s difference to reach Π_{eq}. Meibomian films seeded with cholesterol reached a slightly higher Π_{eq} but the difference was less than 3mN/m and the penetration rate was comparable across all three films (Fig. 4.1).
Lysozyme (35µL, 3.2mg/mL) penetration into meibomian lipid films with and without 10 mol % cholesterol or oleic acid. Note, films were pre-set at 5mN/m and given time to relax before lysozyme was applied to the subphase.

4.3.1.2 Dynamic cyclic Π-A profile of meibomian lipid films before and after penetration with lysozyme

Π-A profiles of human meibomian lipids before penetration, showed a constant increase in Π with little hysteresis at 35°C. This addition of cholesterol or oleic acid did not have a major effect on the surface activity of meibomian lipid films (Fig. 4.2), and this supports the findings discussed in Chapter 2.

Figure 4.1: Lysozyme (35µL, 3.2mg/mL) penetration into meibomian lipid films with and without 10 mol % cholesterol or oleic acid. Note, films were pre-set at 5mN/m and given time to relax before lysozyme was applied to the subphase.

Figure 4.2: Dynamic isocycle Π-A profiles of meibomian lipid films with and without 10 mol % cholesterol or oleic acid before penetration of lysozyme (35µL, 3.2mg/mL) at 35°C.
The Π-A profile of a pure meibomian film after lysozyme penetration (Fig. 4.3) illustrates sigmoidal characteristics (characteristic of proteins) that were not seen prior to penetration. The Π_{max} after penetration is much higher indicating the presence of lysozyme molecules in the meibomian lipid films that were not present prior to penetration. This is supported by a larger surface area needed to reach the starting Π of 5mN/m, which indicates that the molecules are occupying more area.

The Π-A profile of meibomian films seeded with oleic acid or cholesterol after lysozyme penetration was similar to meibomian films alone. Hysteresis, take off, Π_{max}, and the area occupied at 5mN/m were comparable to that of pure meibomian films after lysozyme penetration. However, cholesterol seeded films did reveal slightly increased hysteresis compared with other films after lysozyme penetration (Fig. 4.3).

**Figure 4.3:** Dynamic cyclic Π-A profile of meibomian lipid films with and without 10 mol % cholesterol or oleic acid after penetration of lysozyme (35µL, 3.2mg/mL) at 35°C.
4.3.2 Surface activity of phospholipids

4.3.2.1 Surface activity of phospholipids spread onto the air-buffer interface

Π-A profile of DPPC spread onto the air-buffer interface (6µL, 0.5mg/mL) revealed a predominant fluid expanded phase up to 32mN/m (approximately 25cm²). Further compression of the film resulted in a transition to liquid condensed phase, until it reached Π\text{max} ≈41mN/m. The isocycles demonstrated nominal hysteresis, but in comparison to DPPC experiments at 20°C, the Π at which the phase transition occurred was increased in our experiments at physiological temperature. The increased temperature also lead to a notable reduction in area of the phase transition; a concept confirmed by Crane et al (1999) (Fig. 4.4A).

4.3.2.2 Adsorption profile of phospholipids injected into the subphase and cyclic Π-A profile of phospholipids following adsorption at 35°C

After injection of DPPC into the subphase, a slow induction phase was observed for 500s followed by a very slow increment in Π to 0.75m/Nm. A subsequent minor drop in Π was observed, due to evaporation of the subphase which causes a decrease in the baseline. The data indicates that nominal adsorption of DPPC to the air-buffer interface occurred (Fig. 4.4B).

In contrast to the adsorption profile of DPPC spread onto the air-buffer interface, isocycles conducted after adsorption measurement revealed large increase in Π (Π\text{max} of ~45mN/m) and strong hysteresis. The data from this profile illustrates evidence of strong surface activity that was not seen in surface pressure-time (Π-T) profiles and indicates a rearrangement of the molecules on the surface once external energies are applied (decreasing the surface area) (Fig. 4.4C).
Figure 4.4: Surface activity measurement of phospholipids at 35°C. A) Dynamic cyclic Π-A profile of phospholipids spread onto the air-buffer interface (6μL, 0.5mg/mL). B) Penetration profile of phospholipids injected into a buffered aqueous subphase (6mL, 0.5mg/mL) where the barriers had been compressed to a predetermined surface area normally occupied by meibomian lipids at 5mN/m (20µL, 1mg/mL). C) Dynamic cyclic Π-A profile of phospholipids at the air-buffer interface after adsorption from the aqueous subphase.

4.3.3 Interaction of phospholipids in an aqueous environment with human meibomian lipids

4.3.3.1 Penetration of human meibomian lipids by phospholipids

4.3.3.1.1 Surface area-time profile of phospholipids

The results from the injection of DPPC into the subphase (with human meibomian lipids on the surface), indicate that there was nominal penetration of DPPC into a meibomian lipid surface layer. The Π-T profile showed there was an initial fall in Π caused by the relaxing and rearrangement of molecules in the meibomian lipid film. Adsorption was observed after 750s and evidenced by stabilisation in Π. A very slow adsorption phase was observed until an equilibrium pressure of 7mN/m was reached around 9000s (Fig. 4.5).
Figure 4.5: Penetration of phospholipids (6mL, 0.5mg/mL) into a human meibomian film (20µL, 1mg/mL) compressed to a predetermined surface pressure of 5mN/m at 35°C.

4.3.3.1.2 Dynamic cyclic Π-A profile of human meibomian lipids before and after penetration of phospholipids

Π-A profiles of human meibomian lipids before penetration demonstrated a gradual increase in surface pressure and little hysteresis. Following penetration of DPPC, a notable rise in Π_{\text{max}} was observed (∼28mN/m) (Fig.4.6). Surprisingly, given the hysteresis observed in isocycles of DPPC after adsorption to an air aqueous interface, there was little hysteresis after DPPC phospholipids adsorbed to a meibomian lipid film.

Figure 4.6: Dynamic cyclic Π-A profile of meibomian lipid film (20µL, 1mg/mL) before and after penetration of phospholipids (6mL, 0.5mg/mL) at 35°C.
4.4 Discussion

An aim of this study was to evaluate the hypothesis that changes in the lipid composition of the TFLL would affect the binding of proteins to the TFLL and thereby, the surface pressure of meibomian films. However, based on penetration profiles and Π-A profiles, there is strong evidence that changes in components such as cholesterol or oleic acid do not affect the ability of proteins to bind to meibomian lipids or resulting protein-lipid interactions. The surprising outcome of these results could be explained by the possibility that proteins have a dominant influence on surface pressure and hence, mask/mitigate the influence of changes in lipid components on the surface activity/behaviour of films.

In our study, the difference in surface pressure and surface activity of films with varying lipid components was minimal, with the maximum surface pressure varying between 8-12mN/m. However, the addition of lysozyme, even at extremely diluted concentrations caused a large increase in surface pressure (Π_max difference ~20mN/m), increase in elasticity and significant rearrangement of molecules (hysteresis).

The idea that lipids could have a minor role compared to proteins in the surface pressure/tension of films is supported by previous work of Holly and Tiffany (Holly, 1973b; Nagyova and Tiffany, 1999). Although Tiffany has shown that it is the interaction of both lipids and proteins that are responsible for the surface tension of tears, he also noted that meibomian lipids alone were poor contributors to surface tension, while β-lactoglobulin (lipocalin), demonstrated a strong effect on surface tension (Nagyova and Tiffany, 1999). Holly also demonstrated that the surface tension (spreading pressure) of meibomian lipids alone was as little as 10 dynes/centimetre, but the film pressure increases dramatically in the presence of mucins (Holly, 1973b).
Whilst changes in lipid composition could have other biological consequences i.e. evaporation or viscoelasticity, our data indicate that changes in lipid composition do not have an obvious effect on protein binding or surface pressure.

Another aim of this study was to evaluate if phospholipids (DPPC) in an aqueous environment are capable of interacting and altering the surface activity of meibomian lipid films. It was interesting to observe that although there was absorption of DPPC into meibomian lipid film under static conditions (fixed area, penetration profile), DPPC demonstrated significantly increased surface activity (penetration), and lowered the surface tension of meibomian films (increased surface pressure) under dynamic conditions (pressure-area isocycles). An explanation could be that under static conditions some DPPC molecules are limited to the aqueous-lipid interface and occupy space at the interface under static conditions (therefore a strong increase in surface pressure is not observed). However, under dynamic conditions, holes occur in the meibomian lipid film allowing free surface for more DPPC molecules to bind. Once this had occurred, the phospholipids become trapped and hence contribute to the high surface pressure of the film.

McCulley and Shine established the idea that polar lipids, in particular phospholipids, act as an important interface between non polar lipids and the aqueous phase of the tear film (McCulley and Shine, 1997; Shine and McCulley, 2003). Whilst, recent studies analysing the composition of meibum have questioned the presence of phospholipids and therefore their contribution to TFLL structure and dynamics, the data suggest that free phospholipids in the aqueous phase of tears could contribute to the interfacial role described by McCulley and Shine. More recently, Glasgow’s group (Dean and Glasgow, 2012) have identified lysophosphatidylcholines as the most abundant phospholipids in tears and postulate that they exist in sufficient concentrations to retard evaporation. While it has been established that tear lipocalin binds phosphatidylcholines (Dean and Glasgow, 2012), and also that holo-tear lipocalin adsorbs to meibomian lipid films (Mudgil and Millar, 2008; Millar et al, 2009),
further studies are required to determine whether free/unboundlysophatidylcholines would have similar physicochemical effects to those identified in this study using DPPC.

It was also interesting to note that the surface activity of DPPC from the aqueous subphase was significantly different from the surface activity of DPPC spread onto the air-buffer interface. A significant difference in hysteresis and surface activity was noted, and could be due to micelle formation of DPPC that have adsorbed from the aqueous phase. Further work at the atomic scale using X-ray reflectivity or neutron scattering (using deuterated phospholipids) need to be conducted to understand the structural contribution of aqueous phospholipids to meibomian films under various physicochemical parameters.

Our findings that aqueous tear lipids could have an important role in determining the structural and dynamic characteristics of the meibomian lipid films is supported by Borchman et al (2007). Their work using spectroscopic and fluorescent probe techniques indicates that structural differences between meibomian and aqueous lipids could affect the evaporation rate of the tear film, as well the structural packing of lipids within the TFLL depending on the nature of interaction between lipids at the aqueous-lipid interface (Borchman et al, 2007).
5 Viscoelasticity of cholesterol, its esters and β-Carotene

5.1 Introduction

Surface tension is just one determinant of tear stability. Mechanical properties such as the viscoelasticity of tears are also important in its ability to withstand the range of forces experienced during blinking. Tiffany (1991) has shown that tear fluid can withstand the high shear rates during blinks, as well as low shear rate during open eye, due to its non-Newtonian properties i.e. viscosity changes due to shear rate.

In terms of the TFLL, elasticity is a solid property and ensures that the film has structure which can be maintained over several blink cycles. Conversely, viscosity is a fluid property and in the TFLL, it ensures flow and spread of the film. Both properties are important; a film that comprises only elasticity would be brittle and break up, especially during the high forces experienced by blinking, and a purely viscous film would have little structural integrity, and therefore the functionality of the TFLL would be lost. Ideally, a combination of viscosity with elasticity is needed for the TFLL to allow flexibility and still ensure that the structure is maintained.

Interference patterns of the TFLL have indicated a viscoelastic gel-like structure that is stable for multiple blinks (Goto and Tseng, 2003; Yokoi et al, 2008). This contrasts with the aqueous layer that appears to be swept away during a blink (Khanal and Tomlinson, 2005; Khanal and Millar, 2010). These observations have been confirmed in a study by Leiske et al (2010), where the viscoelastic properties of meibomian lipid films were evaluated in vitro and it was concluded that meibomian lipid films behave like a two dimensional gel at the air-liquid interface. The study also revealed that the ability of meibomian lipids to stretch and deform under large stresses without breaking, and is a characteristic that could help its spread during blinks (Leiske et al, 2010).
Given that viscosity and elasticity are essential properties of the TFLL to enable its proper function, it is of interest to determine how particular TFLL components influence these properties. As alluded to in earlier chapters, since the exact nature of meibomian lipids is unknown, seeding meibomian lipids with particular components is a mechanism for gleaning knowledge about the possible roles of particular components in terms of viscosity and elasticity.

An oscillating pendant drop method was chosen to investigate the viscoelasticity of meibomian lipid films and the effect of increasing cholesterol and its esters in these films. This technique was chosen in preference to other techniques such as the Langmuir trough, shear rheometer and other forms of surface rheometers (Miano et al, 2006; Leiske et al, 2010) because sample volumes of both the aqueous subphase and the lipid film are small. This offers the potential, based on the studies carried out here, to use whole tears as the subphase.\(^7\)

### 5.2 Materials and solutions

Cholesterol (Sigma Aldrich, Australia; Batch #045K5311), cholesteryl oleate (Sigma Aldrich, Australia; Batch #045K5311098K5016), oleic acid (Nu-Chek Prep, U.S.A; Batch #U-46A-110-R) or \(\beta\)-carotene (Sigma Aldrich, Australia; Batch #068K2561) were mixed with human meibomian lipids (assumed molecular weight 350) in the mole fraction range of 0 to 1 (0 to 100 mol %). The total number of molecules was kept constant at \(1.72 \times 10^{17}\). Chloroform was used as the solvent and was of HPLC grade (Labscan).

### 5.3 Pendant drop method

The pendant drop technique measures the dilatational rheology of interfaces and this method has been described in detail elsewhere (Logio et al, 2001; Miano et al, 2006; Ravera et al, \(7\) Studies in this chapter were conducted with the technical assistance and expertise of my colleague Shiwani Raju.)
2010). Briefly, aqueous drops ranging in size from 10-25µL are formed at the end of a stainless steel needle attached to a syringe.

The hanging drop gives the ‘pendant’ shape (Fig. 5.1). The dilatational viscoelastic modulus (E*) is obtained by correlating changes in surface tension over changes in drop surface area, according to Gibbs (Benjamins et al, 1996; Monteux et al, 2004; Miano et al, 2006). This represents a stress and strain relationship which forms a basis for many rheological models. To give changes in drop surface tension and area, drop volume is varied by oscillating (pulsing) the drop at a specific amplitude and frequency range using a sine wave function. Drop surface area and tension is calculated by software based on the drop shape analysis tool developed by Neumann and co-workers (Rotenberg et al, 1982; Cheng et al, 1990; Cheng and Neumann, 1992). The dilatational viscoelastic modulus (E*) can be split further into its real (E’) and imaginary (E”) components, where E’ represents the elastic contribution of the material, and E” represent the viscous contribution. Viscoelastic measurements were obtained using a commercial contact angle tensiometer (OCA-20, Dataphysics, Germany) via the pendant drop mode (Fig. 5.2).

In these experiments, a strain amplitude of 2% (dA/A) was determined to be in the linear viscoelastic regime and so this amplitude was chosen for all experiments. The surface tension across all samples and experiments was maintained between 42-60mN/m (surface pressure 12-30mN/m). Besides the magnitude of the strain put on the sample, the speed of application is also important as this can alter the viscoelastic properties. Therefore, measurements of these samples were conducted using a frequency range over 3 orders of magnitude, 0.02-3 Hz. Amplitude and all other experimental parameters were kept constant, while the frequency of the oscillations was varied (frequency sweep). The drop sub-phase was artificial tear buffer (ATB) or ATB with lysozyme (3.2mg/mL; Sigma Aldrich, Australia; Batch #L-6876), and was a total volume of 15µL. The drop was coated with 0.5µL of lipids (0.5mg/mL) using an auxiliary syringe and then lowered and equilibrated into a thermostatic chamber set at 35 °C.
If the sub-phase had no protein, the drops were equilibrated for 15 minutes. With a protein sub-phase, drops were aged for 1 hour so an equilibrium surface tension was reached. Before the oscillations, the drop volume was monitored and topped up to the starting volume to replace evaporative loss. Experimental time was typically 5-6 minutes. However, due to evaporation of the drop at 35°C, the frequency sweep was segmented into 4 consecutive stages (3-0.2Hz, 0.2-0.06Hz, 0.04Hz, and 0.02Hz). This meant that the drop could be topped up to the starting volume after each stage (duration for one stage was typically from 45sec-120sec).

Figure 5.1: A typical drop hanging from the tip of a needle giving the pendant shape.
5.4 Results

5.4.1 Viscoelasticity of meibomian films and lysozyme
Films of meibomian lipids and lysozyme individually demonstrated gel like behaviour, which is indicated when the modulus is independent of frequency (Larson, 1999). The complex modulus values for meibomian films were ~20mN/m higher at higher frequencies than lysozyme films, indicating that meibum was more elastic and solid-like than protein films. The corollary is that protein films are more “flexible”. Films of lysozyme coated with meibum, demonstrated a 2 fold increase in the complex modulus (E*) to ~100mN/m (Fig. 5.3). This indicates that the adsorption and interaction of lysozyme with meibum, increases the viscoelasticity and stability of the film.
### 5.4.2 Viscoelasticity of meibomian films with and without 1 mol % cholesterol or 1 mol % β-carotene

The addition of 1 mol% β-carotene had a minimal influence on the viscoelastic properties of meibomian lipid films, with the modulus values of meibomian films with and without β-carotene being comparable across the frequency range. Addition of 1 mol % of cholesterol gave a higher $E^*$ across all frequency ranges, albeit a small increase (Fig. 5.4).
The effect of cholesterol and β-carotene on meibomian films was also investigated with lysozyme in the subphase. The addition of lysozyme to the subphase and its interaction with meibomian films increased viscoelasticity and made the meibomian film more resistant to changes in frequency. Lysozyme also further mitigated any minor influence cholesterol or β-carotene might have had on the viscoelasticity of human meibomian films (Fig. 5.5).

![Figure 5.5: Complex viscoelasticity as a function of frequency of lysozyme (LZ) coated human meibomian films seeded with 1 mol % cholesterol or 1 mol % β-carotene.](image)

5.4.3 Viscoelasticity of meibomian films seeded with and without oleic acid or cholesteryl oleate

Seeding of meibomian lipid films with 5 mol % oleic acid also had very minimal effect on the viscoelasticity of meibomian lipid films. A minimal difference in E* (~10mN/m) was observed at low frequencies, but the E* was comparable at higher frequencies (less than 5mN/m difference in E*) (Fig. 5.6).
Figure 5.6: Complex viscoelasticity as a function of frequency of meibomian films with and without 5 mol % oleic acid.

However, the addition of 10 mol % cholesteryl olate caused a 40mN/m decrease in E* of meibum at high frequency ranges indicating that the addition of cholesteryl olate made the film relatively “weaker” (Fig. 5.7).

Figure 5.7: Complex viscoelasticity as a function of frequency of meibomian films with and without 10 mol % cholesteryl olate.
5.4.4 Viscoelasticity of meibomian films seeded with and without varying molar ratios of cholesterol

The addition of molar ratios of cholesterol, higher than expected in disease states (see Chapter 1) had a strong effect on the viscoelasticity of meibomian films, particularly at higher frequencies. Seeding the meibomian film with 5 mol % cholesterol caused $E^*$ to increase by at least 20mN/m (~30mN/m) at higher frequencies. Meibomian films seeded with 10 mol % cholesterol caused a doubling of $E^*$. Such films are likely to be extremely elastic i.e. “stiff” (Fig. 5.8).

Note: During initial experiments with cholesterol, keeping the total number of molecules at $1.72 \times 10^{17}$ caused a distortion in the pendant drop shape. This made the calculation of surface tension impossible because the software requires the drop shape to be axis symmetrical. Therefore the total number of molecules was reduced to $8.6 \times 10^{16}$ (the molar ratio of cholesterol and meibum was still maintained in these experiments). Nevertheless cholesterol at the higher ratios significantly increased the viscoelasticity of meibomian films.

![Graph of Complex Viscoelasticity vs Frequency](image)

**Figure 5.8:** Complex viscoelasticity as a function of frequency of meibomian films with and without 5 or 10 mol % cholesterol.
5.5 Discussion

The general consensus in ocular research is that changes in the concentration of particular components are likely to lead to altered physicochemical properties (e.g. viscoelasticity) and therefore decreased stability of the TFLL (Krenzer et al, 2000; Shine and McCulley, 2000; Sullivan et al, 2000; Joffre et al, 2008; Joffre et al, 2009). This understanding has also been the premise of evaporative dry eye classification (Report of the International Dry Eye Workshop, 2007). In our investigation, meibomian films seeded with extraordinarily high ratios of oleic acid (5 mol %) or β-carotene (1 mol %) that were equal to disease states, or even higher, did not demonstrate a considerable difference in viscoelasticity compared with normal meibomian films. Similarly, cholesterol at 1 mol % gave only a marginal increase in viscoelasticity. These results support the surface pressure data using the Langmuir trough, which indicated that meibomian films might have considerable tolerance to changes in composition. Similar to our findings, Leiske et al (2010) found very minor viscoelastic differences in human meibomian lipid films compared with those from various animal species. These experiments by Leiske et al were done on the premise that compositional differences in the meibomian lipids between species should be reflected in changes to the viscoelastic properties.

The addition of 5 and 10 mol % cholesterol to meibomian films resulted in greater increases in the viscoelastic modulus of meibomian films, and this change was evident by the film becoming very stiff and unstable. These concentrations of cholesterol were well beyond what is reasonably expected in any disease state and hence, serves more as a control to demonstrate that the viscoelastic modulus of meibomian films can be altered in this experimental setup. Interestingly, measurements of surface pressure of meibomian lipid films using a Langmuir trough showed little effect of seeding with cholesterol at 5 and 10 mol %. One possible explanation for this difference is that that the viscoelasticity of meibomian lipid films can be
altered independently of surface pressure changes. It is likely that there is more cholesterol in
the film (predominant over other lipid species) which contributes to the increased elasticity of
meibomian film causing the film to become brittle and hence unstable. This concept of
cholesterol displacement at low surface area is supported by investigations of cholesterol-
monoglyceride bilayer interactions (Crilly and Earnshaw, 1983). The authors proposed that an
increase in membrane interfacial viscoelasticity can be attributed to a reduction in the space
available for glycerol mono-oleate molecules as the concentration of cholesterol is increased,
resulting in minimal absorption of glycerol mono-oleate at the surface (Crilly and Earnshaw,
1983). In this case, it may be an oversimplification, because meibomian lipids are much more
complex in their structure and a large proportion comprises various cholesterol esters.
Cholesterol esters are classic structures in bulk, and form liquid crystals (Ginsburg et al,
1985; Alonso et al, 2001; Fisch, 2004). Particularly, saturated cholesteryl esters interdigitate
to form semicrystalline monolayers or multilayers at an aqueous interface and these resemble
their bulk characteristics (Ginsburg et al, 1985; Alonso et al, 2001).

Speculating that the cholesterol esters in meibomian lipid films have a tendency to form
liquid crystals, it is possible that cholesterol might disrupt these. A reason why this is evident
in the viscoelasticity experiments and not in the surface pressure measurements could be
because the cholesterol esters are not surfactant molecules and hence would be located in the
outer layer of the lipid layer and therefore, have a minimal contribution to the surface
pressure. In this position they will still influence the flow and resistance to the flow of the
film (viscoelasticity).

This idea is somewhat supported in that the addition of cholesteryl oleate caused instability
and a decrease in the viscoelasticity of meibomian films. This is a monounsaturated
cholesterol ester and would be expected to disrupt the semi-crystalline structures formed by
the meibomian cholesterol esters. Again, although the effect of cholesterol oleate was seen
with viscoelastic experiments, it was not noticeable in surface pressure measurements
(Langmuir trough, Chapter 2), or by other ocular researchers (Butovich et al, 2010). Other possibilities such as micelle formation also need to be considered in order to account for the sharp decrease in viscoelasticity of the meibomian lipid film.

Studies evaluating the surface activity of cholesteryl esters (oleate, linoleate and arachidonate) have reported low/varying pressures and unstable films when spread onto the subphase of a Langmuir trough, and it was proposed that the stability and behaviour of these compounds were determined by factors such as oxidation and ion concentration of the subphase (Kwong et al, 1971; Smaby and Brockman 1978). In our experiments, oxidation was minimised by storing the solution in chloroform, and the ion concentration of the subphase was also kept constant (ionic concentration equal to tears). To determine if indeed the oxidation state of the cholesteryl ester is important, experiments would need to be carried out using esters that had been deliberately oxidised. The results of some in-house experiments on oxidation of unsaturated fatty acids and their esters by a colleague, Burkhardt Schuett, indicated that oxidation is very slow without a potent oxidant. A recent study has shown that oxidation is unlikely to be a problem with meibomian lipids, because total molar levels of hydroperoxides, alkenyls and aldehydes were 0.012 in normals and 0.0009 in donors with MGD (Borchman et al, 2012). Hence, the relatively short timeframe used for the experiments here indicate that little oxidation would have occurred and thus, changes in viscoelasticity are likely to be properties of mixing with the meibomian lipids as discussed above.

It is clear that major proteins such as lysozyme have an important role in the viscoelasticity of meibomian films. The 2-fold increase in viscoelasticity across the frequency range indicates that it is likely to have an important role in physicochemical properties of tears, in addition to its antimicrobial properties. These findings are consistent with previous experiments which showed that lysozyme interacted with meibomian lipids to alter the surface pressure (Chapter 4); there is an expectation that if proteins are interacting with meibomian lipids to change their surface pressure, then it should also alter their viscoelasticity. Similar findings have been
reported by Nishimura et al (2008) in studies evaluating the effect of lysozyme on the viscoelastic properties of DPPC and cholesteryl myristate films.

One of the limitations of this study was that it used an artificial buffer as the subphase. However, these experiments have demonstrated that the technique itself is useful for evaluating the viscoelasticity of meibomian lipids. The small volumes used for the subphase mean that in future experiments, tears could be used as a subphase which could include samples from dry eye patients. This would allow for determination of the effects of complex compositional changes on the physicochemical properties of the tear film without unnecessary detailed analysis of the samples. This is particularly important where minor quantitative changes in a particular lipid or protein could have major influences on the biophysical function of the tear film.

When dealing with pathological tears, these experiments have a different dimension in that the pathological tears could contain enzymes such as lipases that affect the lipid layer. In addition, McCulley and Shine who have previously proposed that changes in the concentration of individual components can affect the function and formation of other components also reported that, changes in oxysterols can subsequently alter both the content of cholesterol and its esters, while minor increases in cholesterol can increase the lipase activity against triglycerides (Shine and McCulley, 1991). This would require quite complex experiments consisting of measuring the viscoelasticity of the pendant drop, allowing a change in composition through enzyme activity to occur, followed by analysis to determine if changes have occurred. Although it may not be possible to do this with whole tears in the first instance, the proof of principle could be tested with artificial enzyme solutions.

Yokoi et al (2008) have recently utilised a novel interferometry technique to analyse the rheological behaviour of the TFLL *in vivo*, in particular outlining information about the lipid layer thickness and spread after a blink. Although the researchers were able to make some
correlations on the velocity of TFLL spread between normals and dry eye patients, the technique was limited by the resolution of the interferometer, as well as the analytical software. Improvements in interferometry analytical techniques such as those now reported by King-Smith et al (2011) will allow scientists to compare the physicochemical behaviour of the TFLL between normals and dry eyes and thereby, more effectively evaluate the consequences of complex compositional changes that occur in the TFLL in vivo. Resolution is still a limitation of interferometry techniques because they rely on the interference of light and hence, can only detect layers on the surface that are greater than about 15 layers of lipids (20nm). To resolve this, it would be advantageous to support these studies at the atomic scale using X-ray or neutron reflectivity. It is likely that these will lead to a better understanding of the structural influence of major components such as cholesterol and wax esters on meibomian films.
6 X-Ray Reflectivity of meibomian lipid films

6.1 Introduction

The composition of the meibomian lipid secretion has been analysed by several groups (Nicolaides et al, 1981; Mathers and Lane, 1998; Butovich et al, 2007; Butovich, 2009; Chen et al, 2010) and although there is disagreement on some of the details, it is presumed that this complex mixture of lipids self assemble to give the lipid layer of the tear film its unique biophysical properties. The corollary is that in evaporative dry eye disease, the structure and biophysical properties of the lipid layer shift away from normal (Mathers, 2004). Therefore, it is critical to understand the structural features and molecular arrangements that enable meibomian lipids to spread over the aqueous layer of tear film, impart stability, retard evaporation, and facilitate visual acuity. Currently, the structure and arrangement of the meibomian lipid layer is unknown.

The physical chemistry literature contains many studies examining the properties and arrangements of molecules at air liquid interfaces (Bosio et al, 1987; Mendelsohn et al, 2009; Arseneault et al, 2010). Commonly used techniques are surface pressure measurements using the Langmuir trough or examining the film microscopically using either Brewster angle microscopy or fluorescence microscopy. Typically, these techniques have been applied to films of pure molecules or to binary mixtures of pure molecules. In biology, they have been used extensively for modelling phospholipid membranes and studying lung surfactant films (Lipp et al, 1997; Fullagar et al, 2003; Gross et al, 2006; Pavinatto et al, 2007; Fullagaret al, 2008). In all of these cases, the components in the films are well known and the data can be used to make plausible models of molecular behaviour at the interface.

Our group and others have been using these techniques to study meibomian lipid films in order to understand its structure and biophysical properties despite its complex and only
partially understood composition (Holly, 1974; Kaercher et al, 1992; Kaercher et al, 1995; Mudgil and Millar, 2010; Leiske et al, 2012). Earlier studies in this project using the Langmuir trough revealed that the behaviour of meibomian lipids is unique: it does not collapse under high pressure and it is also believed that when meibomian lipids are spread on a Langmuir trough and compressed, they form multiple layers as they do in vivo (Bron et al, 2004; Petrov et al, 2007; Mudgil and Millar, 2010). However, additional techniques are required to determine this. Techniques such as the Langmuir trough or Brewster angle microscopy do not allow the determination of the molecular structure of spread meibomian lipid films with atomic resolution. Molecular interactions are important and although microscopy has been valuable for obtaining some information about the behaviour of meibomian films, nanoscale resolution is needed to begin to resolve some of the molecular arrangements.

Such resolution at the atomic scale can be achieved using X-ray reflectometry which can resolve the electron density distribution within various macromolecules. In particular it can be used to analyse lipid films spread at the air liquid interface to obtain information on the packing and phase state under varying conditions. Here, we have used X-ray reflectometry to analyse meibomian lipid films and compared them with films seeded with other molecules: cholesterol, cholesterol esters, and β-carotene. It was expected that seeding the films with these lipids would differentially disrupt the films and give additional information about the likely arrangements of molecules in the films.

6.2 Materials and methods

Materials
DPPC (Sigma Aldrich, Australia), β-carotene (Sigma Aldrich, Australia), cholesterol (Sigma Aldrich, Australia), cholesteryl oleate (Nu-Chek Prep, U.S.A) and stearyl oleate (Nu-Chek Prep, U.S.A) were minimum 99% pure and therefore not purified further. Chloroform (Labscan) was used as the solvent and was HPLC grade.


**Collection of human meibomian lipids**

Meibomian lipids were collected as described previously in Chapter 2.

**Surface pressure measurements**

Human meibomian lipids, DPPC, wax ester, cholesteryl ester or a 1:1 mixture of the wax and cholesteryl ester were spread drop wise from a microsyringe onto the surface (air-buffer interface) of an artificial tear buffer (composition of buffer described in Chapter 2) in a single barrier minitrough (Nima Technology Ltd, UK; Working surface area 30cm$^2$ – 95cm$^2$). Π was monitored using a Wilhelmy paper plate. 50µL human meibomian lipids (1mg/mL; total 8.60 x 10$^{16}$ molecules; 100 mol %), human meibomian lipids containing 1 mol % cholesterol (total 8.60 x 10$^{16}$ molecules), human meibomian lipids containing 1 mol % β-carotene (total 8.60 x 10$^{16}$ molecules), 20µL DPPC (0.5mg/mL), and 40µL of 1:1 mixture of cholesteryl oleate and stearyl oleate (0.5mg/mL) were applied, and 10 minutes was allowed for evaporation of chloroform before isotherm cycles were performed.

By using the software supplied by NIMA Technology, dynamic Π-A profiles of the lipid films at the air-buffer interface of the Langmuir trough were collected. Compression and expansion cycles (isotherm cycles) were conducted over a surface area of 35-95 cm$^2$ and at a barrier rate of 15cm$^2$/min. The artificial tear buffer was kept at 20ºC by recycling water from a thermoregulated bath through a water jacket. Experiments could not be conducted at 35ºC due to the resulting increase in subphase evaporation and scatter of the X-Ray beam.

**X-ray Reflectometry**

The structures of films spread at the air-water interface were characterized using X-ray reflectometry at the Australian Nuclear Scientific and Technology Organization (ANSTO) in Sydney. A NIMA Technology Ltd Minitrough was mounted at the sample position of a Panalytical Ltd X’Pert Pro Reflectometer inside a Perspex box with kapton film windows to

---

8 A single barrier trough was used to allow unhindered access of the X-ray source to the surface of the trough.
minimize subphase evaporation during measurements. Films were characterized at a range of \( \Pi \) using Cu K\( \alpha \) X-ray radiation (\( \lambda = 1.54056 \, \text{Å} \)). The parallel incident X-ray beam was delivered to the sample surface via a Göbel mirror and collimated with 0.2 mm pre- and post-sample slits. Reflectivity data were collected over the angular range \( 0.05^\circ \leq \theta \leq 4.00^\circ \), with a step size of 0.010° and counting times of 10 s per step.

Structural parameters for these films were refined using the MOTOFIT reflectivity analysis software (Nelson, 2006) with reflectivity data as a function of the momentum transfer vector Q (= \( 4\pi(\sin\theta)/\lambda \)). In the fitting routines, the Levenberg-Marquardt method was used to minimize \( \chi^2 \) values by varying the structural parameters such as layer thickness, scattering length density (SLD) and interfacial roughness.

### 6.3 Results

#### 6.3.1 Spread Films of human meibomian lipids at 20°C

Dynamic equilibrium \( \Pi\)-A isocycles of human meibomian lipid films spread at the air-buffer interface (1mg/mL, 50μL) demonstrated strong hysteresis on expansion with a \( \Pi_{\text{max}} \) of ~10.5mN/m. Transition/phase states (\( \Pi_t \)) were observed at ~6mN/m and 9mN/m, indicating a transition towards a liquid condensed phase (Fig. 6.1).
Figure 6.1: Dynamic cyclic Π-A profile of human meibomian lipid film (50μL, 1mg/mL).

X-ray reflectivity data of the spread human meibomian lipid film (50μL, 1mg/mL) was collected at a range of surface pressures (0.6, 3.7 and 9.0mN/m). The points indicate observed experimental data while the solid lines are fits to these data based upon structural models (Fig. 6.2). In each case, a three-layer model was required to satisfactorily fit these data. The inset to Figure 6.2 shows the SLD profile as a function of depth from air through the human meibomian lipid film and in to the aqueous subphase. Refined structural parameters determined using these reflectivity data are listed in Table 6.1. In each instance, the refined structure shows layers of lower electron density adjacent to the water and air interfaces, with a denser layer in the centre of the film.

As the Π is increased there is a clear change in the internal molecular composition of meibomian lipid film, and an increase in film thickness (from 54Å to 60Å) as the molecules in the film become more closely packed. Compared to the uncompressed film (0.6mN/m), the compressed films show a decrease in SLD in the layer adjacent to the aqueous subphase (from 8.3(±1SD)×10^{-6}Å^{-2} to 7.1(±1SD)×10^{-6}Å^{-2}), indicating that water is squeezed out of this layer.
when the film is at higher Π. A thickening of this base layer is also evident with increasing Π (Table 6.1). The central and upper-most layers of the film also show significant changes in structure upon compression. With increasing Π from 0.6mN/m to 3.7mN/m the central portion of the film remains essentially unchanged; however, the surface region increases in SLD from 5.2×10^{-6} Å^{-2} to 6.9(±1SD)×10^{-6} Å^{-2}. X-ray reflectivity data collected near Π_{max}, 9mN/m, suggests a decrease in SLD for both the central and uppermost regions; although this decrease in electron density was offset in the upper layer by an increase in thickness.

**Figure 6.2:** X-ray reflectivity profiles of human meibomian lipid films (50μL,1mg/mL) at the air-buffer interface (a) in the uncompressed state (0.6mN/m, blue data); (b) at 3.7mN/m surface pressure (red data); and (c) at 9.0mN/m surface pressure (green data). Inset shows the equivalent SLD profiles.
**Table 6.1:** Refined structure of spread meibomian lipid films (50μL, 1mg/ml,) at the air-buffer interface as function of surface pressure (Scattering length density of the aqueous subphase: $9.45 \times 10^{-6} \text{Å}^{-2}$).

<table>
<thead>
<tr>
<th>Refined Parameter</th>
<th>Surface Pressure (±1SD) (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Upper layer</strong></td>
<td>(adjacent to air)</td>
</tr>
<tr>
<td>Thickness (Å)</td>
<td>17.9(3)</td>
</tr>
<tr>
<td>SLD ($\times 10^{-6} \text{Å}^{-2}$)</td>
<td>5.2(1)</td>
</tr>
<tr>
<td>Roughness (Å)</td>
<td>5.1(3)</td>
</tr>
<tr>
<td><strong>Middle layer</strong></td>
<td></td>
</tr>
<tr>
<td>Thickness (Å)</td>
<td>18.4(3)</td>
</tr>
<tr>
<td>SLD ($\times 10^{-6} \text{Å}^{-2}$)</td>
<td>12.7(1)</td>
</tr>
<tr>
<td>Roughness (Å)</td>
<td>2.8(2)</td>
</tr>
<tr>
<td><strong>Lower layer</strong></td>
<td>(adjacent to subphase)</td>
</tr>
<tr>
<td>Thickness (Å)</td>
<td>18.1(5)</td>
</tr>
<tr>
<td>SLD ($\times 10^{-6} \text{Å}^{-2}$)</td>
<td>8.3(1)</td>
</tr>
<tr>
<td>Roughness (Å)</td>
<td>3.3(2)</td>
</tr>
<tr>
<td>Total film thickness</td>
<td>54.4</td>
</tr>
</tbody>
</table>

**6.3.2 Spread Films of human meibomian lipids seeded with cholesterol at 20°C**

Dynamic equilibrium Π-A isocycles of human meibomian lipid films seeded with 1 mol % cholesterol and spread at the air-buffer interface demonstrated slightly higher Π$_{\text{max}}$ of ~13mN/m compared with meibomian films without cholesterol.
Similar to human meibum, strong hysteresis on expansion was observed. Transition states were observed at ~3mN/m, 7mN/m and 10mN/m (Fig. 6.3). The transition at lower $\Pi$ (3mN/m) was not observed with meibomian films without cholesterol (Fig. 6.3).

![Graph](image)

**Figure 6.3:** Dynamic cyclic $\Pi$-$A$ profile of human meibomian lipid film seeded with 1 mol % cholesterol.

X-ray reflectivity data of human meibomian lipid film seeded with 1 mol % cholesterol was collected at a range of $\Pi$ (1.9, 4.9 and 8.8mN/m). The refined structures associated with these data are given in Table 2. Comparison with Figure 6.2 indicates essentially the same behaviour, with little change in the structures compared with the equivalent meibomian lipid films without cholesterol. In a fashion similar to the unseeded meibomian lipid film, the thickness of meibomian lipid film with cholesterol also increased in thickness (from 53.3Å to 57.0Å) with increased compression.
Figure 6.4: X-ray reflectivity profiles of human meibomian lipid films seeded with 1 mol % cholesterol at the air-buffer interface (a) in the uncompressed state (1.9mN/m, blue data); (b) at 4.9mN/m surface pressure (red data); and (c) at 8.8mN/m surface pressure (green data). Inset shows the equivalent SLD profiles.
Table 6.2: Refined structure of spread meibomian lipid films seeded with 1 mol % cholesterol at the air-buffer interface as function of surface pressure (Scattering length density of the aqueous subphase: $9.45 \times 10^{-6}$ Å$^{-2}$).

<table>
<thead>
<tr>
<th>Refined Parameter</th>
<th>Surface Pressure (±1SD) (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.9</td>
</tr>
<tr>
<td><strong>Upper layer</strong></td>
<td></td>
</tr>
<tr>
<td><em>(adjacent to air)</em></td>
<td></td>
</tr>
<tr>
<td>Thickness (Å)</td>
<td>17.7(2)</td>
</tr>
<tr>
<td>SLD ($\times 10^{-6}$ Å$^{-2}$)</td>
<td>6.0(1)</td>
</tr>
<tr>
<td>Roughness (Å)</td>
<td>4.0(2)</td>
</tr>
<tr>
<td><strong>Middle layer</strong></td>
<td></td>
</tr>
<tr>
<td>Thickness (Å)</td>
<td>18.1(2)</td>
</tr>
<tr>
<td>SLD ($\times 10^{-6}$ Å$^{-2}$)</td>
<td>12.6(1)</td>
</tr>
<tr>
<td>Roughness (Å)</td>
<td>3.8(2)</td>
</tr>
<tr>
<td><strong>Lower layer</strong></td>
<td></td>
</tr>
<tr>
<td><em>(adjacent to subphase)</em></td>
<td></td>
</tr>
<tr>
<td>Thickness (Å)</td>
<td>17.5(4)</td>
</tr>
<tr>
<td>SLD ($\times 10^{-6}$ Å$^{-2}$)</td>
<td>7.4(1)</td>
</tr>
<tr>
<td>Roughness (Å)</td>
<td>3.2(2)</td>
</tr>
<tr>
<td>Total film thickness</td>
<td>53.3</td>
</tr>
</tbody>
</table>
6.3.3 Spread Films of human meibomian lipids seeded with β-carotene at 20°C

Dynamic equilibrium Π-A isocycles of human meibomian lipid films seeded with 1 mol % β-carotene and spread at the air-buffer interface demonstrated slightly higher Π_{\text{max}} of ~13mN/m compared to meibomian films without β-carotene. Similar to pure meibum and meibomian films with cholesterol, strong hysteresis on expansion was observed. Transition states, similar to meibomian films seeded with cholesterol were observed at ~3mN/m, 8mN/m and 11mN/m (Fig. 6.3).

![Figure 6.5: Dynamic cyclic Π-A profile of human meibomian lipid film seeded with 1 mol % β-carotene.](image)

X-ray reflectivity data of human meibomian lipid film seeded with 1 mol % β-carotene were collected at a range of Π (1.9, 4.9 and 8.8mN/m). The refined structures associated with these data are given in Table 6.3. In comparison with the unseeded and cholesterol seeded meibomian lipid films, there are significant differences in the molecular structure of meibomian films in the presence of β-carotene.
Figure 6.6: X-ray reflectivity profiles of human meibomian lipid films seeded with 1 mol % β-carotene at the air-buffer interface (a) in the uncompressed state (1.5mN/m, blue data); (b) at 4.9mN/m surface pressure (red data); and (c) at 8.8mN/m surface pressure (green data). Inset shows the equivalent SLD profiles.

For both low (2.5mN/m) and medium (6.7mN/m) Π, β-carotene appears to be substantially more hydrophobic than the meibomian lipid and accumulates at the air interface of the film. As the Π is raised to 6.7mN/m, it can again be seen that water is squeezed out of the layer adjacent to the sub-phase in a fashion consistent with the pure and cholesterol-doped meibomian lipid films. At high Π (9.4mN/m), the β-carotene doped film adopts a structure that is similar to the meibomian systems described above, with water squeezed out of the layer adjacent to the subphase, a region of high electron density in the middle of the film and a layer of low electron density adjacent to the surface. Although X-ray reflectometry clearly indicates the molecular envelope of this composite film, there is insufficient scattering contrast between the β-carotene and the meibomian lipid components of the film to uniquely assign a location of the β-carotene at high Π. In order to achieve such an outcome, neutron reflectometry in concert with selective deuteration of the dopant would be required.
In contrast with the previous pure and cholesterol-seeded systems, the overall thickness of the β-carotene seeded film increases at medium $\Pi$, before decreasing at high $\Pi$. This fact, along with the substantial molecular re-ordering is highly suggestive of a phase transition for this system.

**Table 6.3**: Refined structure of spread meibomian lipid films seeded with 1 mol % β-carotene at the air-buffer interface as function of surface pressure (Scattering length density of the aqueous subphase: $9.45 \times 10^{-6}$ Å$^{-2}$).

<table>
<thead>
<tr>
<th>Refined Parameter</th>
<th>Surface Pressure (±1SD ) (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td><strong>Upper layer</strong></td>
<td></td>
</tr>
<tr>
<td><em>(adjacent to air)</em></td>
<td></td>
</tr>
<tr>
<td>Thickness (Å)</td>
<td>17.7(4)</td>
</tr>
<tr>
<td>SLD ($\times 10^6$ Å$^{-2}$)</td>
<td>10.7(1)</td>
</tr>
<tr>
<td>Roughness (Å)</td>
<td>3.6(2)</td>
</tr>
<tr>
<td><strong>Middle layer</strong></td>
<td></td>
</tr>
<tr>
<td>Thickness (Å)</td>
<td>19.3(5)</td>
</tr>
<tr>
<td>SLD ($\times 10^6$ Å$^{-2}$)</td>
<td>9.9(1)</td>
</tr>
<tr>
<td>Roughness (Å)</td>
<td>5.0(2)</td>
</tr>
<tr>
<td><strong>Lower layer</strong></td>
<td></td>
</tr>
<tr>
<td><em>(adjacent to subphase)</em></td>
<td></td>
</tr>
<tr>
<td>Thickness (Å)</td>
<td>18.7(5)</td>
</tr>
<tr>
<td>SLD ($\times 10^6$ Å$^{-2}$)</td>
<td>8.8(1)</td>
</tr>
<tr>
<td>Roughness (Å)</td>
<td>3.3(2)</td>
</tr>
<tr>
<td>Total film thickness</td>
<td>55.7</td>
</tr>
</tbody>
</table>
6.3.4 Spread films of 1:1 mixture of cholesteryl oleate and stearyl oleate at 20°C

Dynamic equilibrium Π-A isocycles of a 1:1 mixture of cholesteryl oleate and stearyl oleate spread at the air-buffer interface (40μL; 0.5mg/mL) demonstrated very little surface activity, with a Π_{max} of ~4mN/m, despite a baseline of 2.5mN/m. In contrast to meibomian films, very little hysteresis was observed and there were no transition states (Fig. 6.7).

![Dynamic cyclic Π-A profile of 1:1 mixture of cholesterol oleate and stearyl oleate (40μL, 0.5mg/mL).](image)

**Figure 6.7:** Dynamic cyclic Π-A profile of 1:1 mixture of cholesterol oleate and stearyl oleate (40μL, 0.5mg/mL).

X-ray reflectivity data of spread 1:1 mixture of the cholesterol and wax ester was collected at Π of 4mN/m. The best possible refined structural information associated with the data is given in Table 6.4. In contrast to previous films, reflectivity of the compressed 1:1 mixture showed very little structure and contrast compared to the aqueous subphase. Based on the available contrast, a two phase model was used to fit the data and the thickness of the film was ~44Å (Fig. 6.8, Table 6.4). An in depth understanding of wax and cholesteryl ester films will require the molecules to be deuterated and analysed using neutron scattering.
Figure 6.8: X-ray reflectivity profile of a 1:1 cholesteryl oleate: stearyl oleate film (40μL, 0.5mg/mL) at the air-buffer interface (Surface pressure 4mN/m). Inset shows SLD profile.

Table 6.4: Refined structure of spread 1:1 mixture of cholesteryl and stearyl oleate (40μL, 0.5mg/mL) at the air-buffer interface as function of surface pressure (Scattering length density of the aqueous subphase: 9.45×10^{-6} Å^{-2}).

<table>
<thead>
<tr>
<th>Refined Parameter</th>
<th>Surface Pressure (±1SD) 4 (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Top Layer</strong></td>
<td></td>
</tr>
<tr>
<td>Thickness (Å)</td>
<td>28.1(7)</td>
</tr>
<tr>
<td>SLD (×10^{-6} Å^{-2})</td>
<td>10.7(1)</td>
</tr>
<tr>
<td>Roughness (Å)</td>
<td>3.8(3)</td>
</tr>
<tr>
<td><strong>Base Layer</strong></td>
<td></td>
</tr>
<tr>
<td>Thickness (Å)</td>
<td>15.7(8)</td>
</tr>
<tr>
<td>SLD (×10^{-6} Å^{-2})</td>
<td>10.0(1)</td>
</tr>
<tr>
<td>Roughness (Å)</td>
<td>4.0(4)</td>
</tr>
</tbody>
</table>
6.3.5 Spread films of DPPC at 20°C

X-ray reflectivity data of spread DPPC film was collected at Π of 18mN/m (we were unable to record/retrieve the dynamic cyclic Π-A profile). The refined structures associated with this data are given in Table 6.5. In contrast to meibomian films, a compressed DPPC monolayer gave the expected two phase model, representing polar head groups adjacent to the air (~9Å) and hydrocarbon tail groups adjacent to the subphase (~15Å) (Figure 6.9, Table 6.5) with a total thickness of 24Å. The X-ray scattering length density was higher for the hydrophilic head groups than the tail groups. These values are in agreement with other X-ray studies of DPPC films (Wu et al, 2006).

![Graph of X-ray reflectivity profile](image)

**Figure 6.9:** X-ray reflectivity profile of spread DPPC film (0.5mg/mL; 20μL) at the air-buffer interface (Surface pressure 18mN/m). Inset shows SLD profile.
Table 6.5: Refined structure of spread DPPC film (0.5mg/mL; 20μL) at the air-buffer interface as function of surface pressure (Scattering length density of the aqueous subphase: 9.45×10^{-6} Å^{-2}).

<table>
<thead>
<tr>
<th>Refined Parameter</th>
<th>Surface Pressure (±1SD)</th>
<th>Surface Pressure (±1SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18 (mN/m)</td>
<td>18 (mN/m)</td>
</tr>
<tr>
<td>Hydrocarbon tails</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(adjacent to air)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thickness (Å)</td>
<td>14.7(3)</td>
<td></td>
</tr>
<tr>
<td>SLD (×10^{-6} Å^{-2})</td>
<td>10.3(1)</td>
<td></td>
</tr>
<tr>
<td>Roughness (Å)</td>
<td>3.9(3)</td>
<td></td>
</tr>
<tr>
<td>Polar head groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(adjacent to subphase)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thickness (Å)</td>
<td>9.4(4)</td>
<td></td>
</tr>
<tr>
<td>SLD (×10^{-6} Å^{-2})</td>
<td>12.8(1)</td>
<td></td>
</tr>
<tr>
<td>Roughness (Å)</td>
<td>2.3(2)</td>
<td></td>
</tr>
</tbody>
</table>
6.4 Discussion

Our understanding thus far from Langmuir trough work and fluorescence microscopy is that human meibum is a complex composite of lipids that endow meibomian films with unique biophysical properties, including the ability to defy collapse, self assemble to form different phases and stay homogeneous (despite changes in lipid composition) due to its unique fluid nature. Further investigations at the atomic scale to verify our previous findings strongly indicate that meibomian lipids form multiple phases/layers, even at the lowest surface pressures. Subjecting meibomian lipids to higher surface pressures, as one might expect in vivo during blinking, results in further reorganisation and increased thickness of the meibomian film with water being squeezed out of the layer adjacent to the subphase, but the overall structure of the film remains the same. These results are generally consistent with X-ray reflectivity evaluations of meibomian lipids reported by Fuller’s group (Leiske et al., 2012). However due to the low compression ratio and surface pressures achieved (Π_{max} of 10.5mN/m), sufficient multilayers were not formed to observe the Bragg peaks identified by their group. The findings support the concept that meibomian lipids reorganise over each other during the blinking cycle, thereby enabling the TFLL to maintain its structural integrity at high surface pressures (or low surface tension) of the eye, and this reorganisation prevents collapse.

A conclusion from Chapter 2 was that the unique multilayer formation and fluid nature of meibomian films could enable it to tolerate changes in its composition (levels comparable to pathological), and this has been further supported by the X-ray reflectivity profile. Refined structures of meibomian films seeded with cholesterol were similar to unseeded meibomian films, indicating that the increased concentration of cholesterol did not affect the self assembly or thickness of meibomian lipids. In contrast, β-carotene, caused significant molecular reorganisation of meibomian lipids films. As observed in previous Langmuir
trough investigations at 20°C (fluorescence microscopy and surface pressure area, Chapter 3), β-carotene at low surface pressures dominates at the air-water interface, but as the film is compressed at high surface pressures, carotene is squeezed out of the air water interface and the characteristic meibomian film structure is observed. Further work with deuterated lipids and neutron scattering is required to localise the position of carotene, cholesterol and other lipids within meibomian films at high pressures.

Establishing a correlation between the composition of meibum and its resulting biophysical characteristics proved more difficult. Wax and cholesteryl esters combined constitute nearly 70% of meibomian lipid composition and it was expected that a mixture of these components could emulate the key structural characteristics of meibum. However, X-ray reflectivity and Langmuir trough profiles of a 1:1 spread mixture of cholesteryl oleate and stearyl oleate demonstrated very low surface activity, as evidenced by minimum surface pressure and absence of peaks in the reflectivity data. The electron density of the spread cholesteryl-wax ester solution was essentially the same as the subphase and this suggests that the mixture was extremely unstable and had very little organised structure. Although the lack of organised structure was very surprising, the instability of non polar wax and cholesteryl mixtures has been alluded to by researchers such as McCulley and Shine. The authors proposed that non polar lipids by themselves are likely to be thermodynamically unstable and when spread could form unstable lipid droplets (McCulley and Shine, 1997; Shine and McCulley, 2003). Our findings indicate that polar components maybe key surfactants that enable spread of the TPLL and provide structural stability to the major non-polar constituents, a concept in line with hypotheses of previous researchers (McCulley and Shine, 1997; Shine and McCulley, 2003). Future modelling work should include a more complex system including key polar lipids such as O-acyl-omega-hydroxy-fatty acids (Butovich, 2009), and a mixture of wax esters of varying chain lengths to better model the structure and behaviour of a meibomian lipid film.
Previously, phospholipids have been used as experimental models to evaluate the biophysical properties and functionality of the meibomian lipid layer of tear film (Mudgil et al, 2006; Miano et al, 2007; Nishimura et al, 2008). However, the monolayer X-ray profile of DPPC (data comparable with Wu et al, 2006) was markedly different to the multilayered profile of meibomian films, and our results conclusively demonstrate that single component phospholipid systems or models cannot be representative of the physicochemical and structural properties of the primarily non polar meibomian lipid system. Our conclusion is supported by recent rheological studies (Leiske et al, 2010).
7 Conclusion

This Thesis has demonstrated that under controlled conditions in vitro, the physicochemical properties of the meibomian lipid films are resistant to large changes in lipid composition across a range of lipids. An analysis of its structure at the atomic scale reveals that this tolerance is most likely due to its ability to self assemble into complex multilayers at even the lowest surface concentration of molecules, and that this structure is able to reorganise in a dynamic manner according to the concentration of the molecules. This was indicated in the experiments where very high compressions of the meibomian lipid films (high number of molecules/surface area) increased the thickness and reorganisation of the films, but did not cause collapse. Therefore, the findings cast doubt on the general hypothesis that minor changes in the lipid composition could alter the physicochemical properties (Arcinega et al, 2010; Uchiyama et al, 2007; Uchiyama et al, 2010), and thereby destabilise the TFLL. Indeed, changes well beyond any physiologically realistic level of known lipid components such as cholesterol, cholesterol esters and wax esters, had little effect.

In contrast, minor changes in concentration of proteins such as SPs and keratin caused notable changes in the surface pressure of meibomian films, further supporting previous theory and model based investigations of the importance of the roles of proteins to this layer. There is also compelling evidence from this investigation that proteins from the aqueous strongly interact with the meibomian lipid film and alter both the surface tension and viscoelastic properties of meibomian films. These studies further support a previous concept that proteins have a considerably stronger effect on the variable responses of the surface tension of meibomian films compared with lipids (Nagyova and Tiffany, 1999).

The reliability of the findings is supported not only by reproducibility of results within the study, but baseline meibomian film data were comparable with studies previously published
by our group. Langmuir trough experiments were conducted on a minimum of two different occasions, and profiles (hysteresis, compression and expansion profiles) were reproducible with variability in maximum surface pressure of less than 2 mN/m even when using different batches of samples. The meibomian film profiles on the double barrier trough (baseline data) at both 20°C and 35°C were comparable to previously published studies (Millar et al, 2009; Mudgil and Millar, 2010; Millar, 2013). Similarly, X-ray reflectivity data of DPPC and pure meibomian films were consistent with findings by other groups (Wu et al, 2006; Leiske et al, 2010). Some variation in moduli was observed in repeats of viscosity experiments. However, we are not confident that the slight difference in E* between pure meibum and 1 mol % cholesterol or β-carotene is real. These findings have been discussed with other rheologists and they have indicated that these variances are quite acceptable. With respect to sensitivity of the method, our group has recently published data showing consistent variance in moduli with changes in temperature, and when using different sample types (lipids vs detergents vs polymers) (Raju et al, 2013).

A limitation of this study was that there could still be minor component lipids that were not tested, and these may have a major effect on the performance of the lipid film. If this were the case, then it is most likely that these would be surfactants because not many molecules are required compared with the non-surfactant molecules to influence the surface pressure. One such possibility is the recently identified (O-acyl)-omega-hydroxy-fatty acids (Butovich, 2009), that would have to be purified and evaluated using various techniques, similar to those in this investigation.

Future studies should also evaluate other parameters of TFLL and tear film stability, including evaporation. Changes in lipid composition are thought to correlate with evaporation rates of the tear film (Mathers, 2004; Joffre et al, 2008). As such, evaluating the evaporation rate of meibomian films seeded with different lipids and/or proteins at different surface pressures could provide important information in understanding the mechanisms of
evaporative dry eye. However, such an investigation should not only take into account the contribution of proteins, mucins and newly discovered SPs, but the methodology should also account for the physical characteristics of the TFLL, including thickness, re-organisation at different surface pressures and viscoelasticity, which could all influence evaporation rates.

Although these studies focused on the effect changes in ratios of lipids and proteins have on meibomian films, future research should evaluate the effect decreased lipids and proteins have on the physicochemistry of meibomian films – perhaps by removing moieties chromatographically. A limitation to such studies would be the ability to selectively extract lipid/protein components from limited sample material, as well as determining that only the targeted compound was extracted given that the exact composition of meibomian lipids is still unclear. Although changes in composition are unlikely to considerably alter physicochemical properties such as viscoelasticity or surface tension, changes in tear film components are still likely to influence the physiological function of the TFLL and/or tear film. Further investigations, similar to those conducted by Joffre et al (2009), evaluating the physiological effects of various lipid or protein components on conjunctival or other cell lines could also prove to be invaluable in understanding the relationship between changes in composition of the TFLL and pathophysiology of various ocular diseases.
References


Boesze-Battaglia K, Schimmel RJ (1997) Cell membrane lipid composition and
distribution: implications for cell function and lessons learned from photoreceptors and

Borchman D, Delamere NA, McCauley LA, Paterson CA (1989) Studies on the
distribution of cholesterol, phospholipid, and protein in the human and bovine lens.

meibum lipid composition with meibomian gland dysfunction using NMR and principal


Bosio L, Benattar JJ, Rieutord F (1987) X-ray reflectivity of a Langmuir monolayer on

Detection of surfactant proteins A and D in human tear fluid and the human lacrimal

Bräuer L, Johl M, Börgermann J, Pleyer U, Tsokos M, Paulsen FP (2007b) Detection and
localization of the hydrophobic surfactant proteins B and C in human tear fluid and the


Breustedt DA, Korndorfer IP, Redl B, Skerra A (2005) The 1.8-Å crystal structure of
human tear lipocalin reveals an extended branched cavity with capacity for multiple


Yeagle PL, Hutton WC, Huang C, Martin RB (1977) Phospholipid head-group
conformations; intermolecular interactions and cholesterol effects. *Biochemistry.* 16:
4344-4349.

Res.* 78: 399-407.

Yokoi N, Takehisa Y, Kinoshita S (1996) Correlation of tear lipid layer interference

Rheology of tear film lipid layer spread in normal and aqueous tear deficient dry eyes.


force microscopy and near field scanning optical microscopy studies. *J. Microsc.* 205:
136-146.

Zasadzinski JA, Stenger PC, Shieh I, Dhar P (2010) Overcoming rapid inactivation of
lung surfactant: Analogies between competitive adsorption and colloid stability.

Zuo YY, Veldhuizen RAW, Neumann AW, Peterson NO, Possmayer F (2008) Review:
Current perspectives in pulmonary surfactant – Inhibition, enhancement and evaluation.