INTERFACIAL VISCOELASTICITY OF HUMAN MEIBOMIAN LIPID FILMS

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Thesis submitted to fulfil the requirements for the degree of

Doctor of Philosophy

School of Natural Sciences
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Parramatta Campus, NSW, Australia
October, 2011
I dedicate this thesis to my beloved Grandparents, who had a dream to see their children and grandchildren achieve the success of education they were not offered.

Grandpa, I wish you were here to see me finish. I miss you.
Acknowledgments

Throughout my PhD candidature, I have met many individuals who have supported me. Foremost, I would like to thank my primary supervisor, Professor Thomas Millar who gave me this wonderful opportunity to embrace a new and exciting venture. I learnt extraordinary things, got to travel the globe, and was able to meet amazing individuals who have changed my life. I am now more confident in my own abilities and am not afraid to take the leap of faith. Thank you for believing in me.

Secondly, I would like to thank my co-supervisors Professor John Bartlett and Dr Howard Ketelson. Howard, I want to thank you and James for the initial training on the pendant drop rheometer. It was invaluable to me. I also enjoyed the experience of working at Alcon, and loved Texas! Thank you for looking after me while I was there.

I would like to also give a huge thank you to my alter ego at Stanford University, Danielle Leiske. It was you who initially gave me a crash course in rheology and started my love affair with this topic. Thank you for looking after me while I was visiting Stanford, and for providing me with plenty of information and resources throughout this entire journey. You patiently answered my deluge of dumb questions and gave me the confidence to solve all the problems I encountered. The best decision I ever made was to take a chance and share a room at my first ever conference with someone I had never met before, who lived on the other side of the world. You are a dear friend and I am so glad you were a part of this journey with me.
Special thanks also go out to Professors Gerry Fuller (Stanford University) and Clay Radke (UC Berkeley). Without their expertise in rheology and their useful discussions and critical analysis of my work, this thesis would not have been possible. I want to also thank my fellow PhD students Chendur Palaniappan (UWS) and Colin Cerratani (UC Berkeley); and office mates Mark, Alan, Arianne, Santosh and Poonam for their support throughout this candidature.

I would like to acknowledge the University of Western Sydney for providing me with financial assistance through the UWS Postgraduate Research Award. Special thanks goes to the School of Natural Sciences, for proving funding for the instrumentation used in this thesis, as well as funds for conference travel.

Lastly but very importantly, I would like to thank my family. Without your patience, understanding, support, and most of all love, the completion of this work would not 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.

Shiwani R. Raju
October, 2011
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Symbols and Abbreviations

Below is a list of symbols and abbreviations used throughout the text. The typical units for each physical quantity are provided in brackets. Some symbols and abbreviations have a short description of the term. Symbols may also have multiple meanings and are listed immediately after the first meaning.

\( A \)  
- surface area (cm\(^2\)), and mean molecular area (Å\(^2\)/molecule)

\( A_0 \)  
- initial surface area (cm\(^2\))

\( \Delta A \)  
- amplitude
  - The height of the sine wave curve in oscillatory rheology. It is also sometimes referred to as the strain in oscillatory rheology (dimensionless, usually expressed as %).

ADSA  
- axisymmetric drop shape analysis

Alb  
- albumin

ATB  
- artificial tear buffer

BAM  
- Brewster angle microscopy

\( Bo \)  
- Boussinesq number
  - Dimensionless number used to describe whether flow is experienced from the surface or from the bulk.

De  
- Deborah number
  - Dimensionless number used to characterise the fluidity of a material.

DPPC  
- 1,2-dipalmitoyl-sn-glycero-3-phosphocholine

\( E \)  
- dilatational modulus
  - Similar to \( G \) however, stress is applied in an extensional manner rather than in shear (mN/m).

\( E^* \)  
- complex dilatational modulus (mN/m)

\( E' \)  
- dilatational elastic modulus (mN/m)

\( E'' \)  
- dilatational viscous modulus (mN/m)
EOBO  (poly) ethylene oxide–(poly) butylene oxide
A non-ionic, di-block copolymer

\(G\)  
shear modulus
It is also known as the *rigidity* modulus. Defined as the linear relationship between \(\tau\) and \(\gamma\) (\(G = \tau / \gamma\)) (Pa or mN/m).

\(G^*\)  
complex shear modulus
Derived from \(G\) however, in oscillatory tests, it is mathematically split into its real and imaginary components where \(G^* = G' + iG''\) (mN/m).

\(G'\)  
shear elastic modulus, also known as the *storage* modulus (mN/m)

\(G''\)  
shear viscous modulus, also known as the *loss* modulus (mN/m)

ISR  
interfacial stress rheometer
A shear rheometer developed by Gerry Fuller and co-workers (Stanford University, CA)

Lf
 lactoferrin

LVR  
linear viscoelastic range
In oscillatory tests, the material must be tested within this range otherwise the calculations are rendered invalid.

Lys  
lysozyme

Muc  
mucin

Mw  
molecular weight

NBD-PC  
1-acyl-2-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino dodecanoyl]-sn-glycero-3-phosphocholine

\(\gamma\)  
interfacial tension (mN/m), and shear strain
Shear strain is defined as the ratio of the relative displacement of one plane with respect to a fixed plane; divided by the distance between the 2 planes (dimensionless, usually expressed as %).

\*  
Pa is used when the bulk modulus is calculated whereas mN/m is used when interfacial modulus is calculated. Pa.s is used for bulk viscosity whereas mN-s/m is used for interfacial viscosity values. Both units are indicated when this thesis has used these values in both contexts, otherwise, only the unit which is appropriate for this thesis has been indicated in brackets.
\( \dot{\gamma} \)  
Shear rate  
Change of shear strain per unit of time. It is also known as the velocity gradient (s\(^{-1}\)).

\( \gamma_0 \)  
Strain amplitude  
Used in oscillatory rheology (dimensionless, usually expressed as \%).

Initial interfacial tension (mN/m)

\( \delta \)  
Phase angle  
The lag or difference between stress and strain (°).

\( \eta \)  
Shear viscosity  
The linear relationship between \( \tau \) and \( \dot{\gamma} \) (\( \eta = \tau / \dot{\gamma} \)) (Pa.s or mN-s/m).

\( \eta^* \)  
Complex shear viscosity (mN-s/m)

\( \tau \)  
Shear stress  
Defined as the unit of force per area (Pa).

\( \tau_0 \)  
Stress amplitude  
Used in oscillatory rheology (dimensionless, usually expressed as \%).

\( \tau_y \)  
Yield point/stress  
This is the stress which corresponds to the material transitioning from an elastic deformation, to a permanent and irrecoverable deformation state (Pa).

\( \omega \)  
Frequency (rad/s or Hz)

\( \Pi \)  
Surface pressure (mN/m)

\( \Pi \text{-A profile} \)  
Surface pressure-area profiles (obtained from compression/expansion isocycles from the Langmuir trough)

\( \Pi_{\text{max}} \)  
Maximum surface pressure (mN/m)
Abstract

Purpose: The meibomian lipid layer is a thin, multilayered structure which forms the outermost layer of the tear film. Remarkably, this layer is able to adapt and maintain its structure, even during blink cycles when the lipid film is under stress. Stress arises from either the dragging of the eyelids across the surface, or the opening and closing motion of the eyelids which rapidly compresses and expands the film. As such, this layer must have properties of viscosity and elasticity, which would provide the film with both the necessary strength and flexibility to maintain its integrity under these enormous forces. Therefore, the purpose of this study was to determine the viscoelasticity of human meibomian lipid films at the air-liquid interface. Meibomian lipids from various animal species which have different lipid compositions were also measured and compared to human meibomian lipid films. The effect of whole tears, commercial proteins and a di-block copolymer, EOBO was also measured and compared to human meibomian lipid films.

Methods: Viscoelasticity of meibomian lipid films were measured under both oscillatory shear and dilatational methods. The shear properties (G) were measured with the ISR, which utilises a commercial Langmuir trough. Here, both human and animal meibomian lipids were spread on the surface of the ATB subphase. Films were compressed to a desired surface pressure and viscoelasticity was measured as a function of frequency. The dilatational properties (E) were measured using the pendant drop rheometer. Here, an aqueous drop was suspended from the tip of stainless steel needle in air giving a characteristic pendant shape. Human meibomian lipids were spread on the surface of the drop where viscoelasticity was measured as a function of amplitude, frequency, time and temperature. The subphase of the drop was also varied to using
whole tears, as well as commercial proteins and EOBO dissolved in ATB.

Complementary information on meibomian lipid films were obtained from Π-A profiles carried out on the Langmuir trough. The appearances of these films were monitored using BAM and fluorescence microscopy. Experiments were conducted at both 20°C and 37°C.

Results: Shear viscoelasticity of human meibomian lipid films increased overall with surface pressure, where the film transitioned from a Newtonian fluid-like film at low pressures (only G” present, with frequency dependant modulus) to a viscoelastic gel with increasing pressure (G’ and G” moduli present with G’ greater than G” and moduli independent of frequency). Animal meibomian lipid films showed similar trends to human lipid films with subtle differences. The exception was the kultarr lipid film which demonstrated Newtonian behaviour at all surface pressures. Π-A profiles and BAM of these lipid films demonstrated similar findings. The absence or presence of particular lipid species between meibomian lipid samples between humans and animals could not be correlated to a particular difference in viscoelasticity, surface pressure or film morphology.

Dilatational viscoelasticity also demonstrated that human meibomian lipid films were capable of forming a viscoelastic gel at the air-liquid interface however, moduli (E’ and E”) demonstrated slight frequency dependence, indicating rearrangement of the lipid molecules on the surface. Aging of the film demonstrated differences in the first 30 minutes, but remained stable afterwards. Lowering the temperature to 20°C made the film more rigid, however the overall characteristics observed at 37°C were retained. The adsorption of commercial proteins to the lipid interface enhanced the viscoelasticity of the meibomian lipid film, indicated by the overall increase of the dilatational moduli.
Surprisingly, the use of whole tears as the subphase did not mimic the values obtained from the use of commercial proteins however, similar trends were observed. This indicates that proteins from whole tears contribute towards the viscoelasticity of the meibomian-protein interface \textit{in vivo}. The presence of EOBO lowered the dilatational viscoelasticity of the meibomian lipid film, indicating that this molecule was interrupting important lipid-lipid and lipid-protein interactions that occur at the meibomian lipid interface. However, \Pi-A profiles and fluorescence microscopy demonstrated EOBO integrating into the meibomian lipid layer and enhancing surface pressure, suggesting that EOBO needs further analysis and characterisation.

\textbf{Conclusions}: The information reported in this thesis has provided a sound basis for understanding the mechanical properties of the meibomian lipid interface \textit{in vivo}. Shear and dilatational measurements provided complementary information where the formation of a viscoelastic gel at the surface layer of the tear film is most likely a mechanism where the lipid film can maintain its integrity. This is supported by clinical studies that show limited rearrangements of the surface of the tear film between blink cycles. For a better understanding of how different components, such as proteins and lipids effect the viscoelasticity of the meibomian lipid layer, a more systematic approach of analysing these molecules need to be considered for future experiments. Analysing these lipid and lipid-protein/polymer films at higher resolutions, such as neutron scattering experiments would also allow a better understanding of the overall biophysical properties of the meibomian lipid layer and would particularly provide complementary information about the viscoelastic properties of the meibomian lipid interface.
Chapter 1

Introduction
1.1 Overview

Biological interfaces have become of great interest because of their essential roles as cell membranes (Edidin, 2003) and lung surfactant (Pérez-Gil, 2008). The multidisciplinary approach of combining physical and chemical sciences to study these interfaces, as well as the use of advanced computing and imaging techniques, has led to a better understanding of their supramolecular interactions, structure, and consequently, functions. As a result, this approach has now formed a basis for studying other more complex interfaces. One such interface is the tear film: this dynamic entity plays a crucial role in the proper functioning of one of our most important sensory organs— the eye.

1.2 Structure of the tear film

The tear film forms a thin (3–8µm thick) (King-Smith et al, 2004), multilayered structure that covers the exposed surface of our eyes. It has three layers: a mucin layer against the ocular surface, a middle aqueous-protein layer which comprises the major component of tears, and an outer lipid layer interacting with air (Wolff, 1954) (Fig. 1.1). Although the tear film as a whole is an interface, its multilayered structure means that within the film there are a number of interfaces. A solid-liquid interface exists between the ocular surface and the anchored mucin layer. There is a liquid-liquid interface between the aqueous layer and the lipid layer. Lastly, an air-liquid interface exists between the external environment and the outer, non polar phase of the tear film lipid layer.
While knowledge of these interfaces has existed, for many years, most research on the tear film had focused on the individual layers and their components. This has led to many reviews dealing with the protective role of the tear film, including the specific roles of individual components (Rolando and Zierhut, 2001; Craig, 2002; Tiffany, 2003; Tiffany, 2008; Craig et al, 2010; Uchina and Tsubota, 2010). In the case of the mucin layer, it was originally only thought to be a physical barrier against debris and pathogens on the ocular surface (Gipson, 2004; Mantelli and Argüeso, 2008). The aqueous layer was thought to mostly play an immune role due to the large amount of anti-microbial and anti-inflammatory proteins present (lactoferrin, lysozyme, albumin, immunoglobulin A) (Selinger et al, 1979; Sack et al, 1992; Zierhut et al, 2002; Flanagan and Willcox, 2009). The lipid layer was studied as a
blanket preventing evaporation of the aqueous layer and therefore, protecting the ocular surface from dryness and subsequent corneal abrasions and scarring (Mishima and Maurice, 1961; Craig and Tomlinson, 1997; Mathers, 2004).

It was through a series of seminal papers from Holly and co-workers (Holly and Lemp, 1971; Holly, 1973; Holly, 1973; Holly, 1974; Holly and Lemp 1977) that started addressing how the tear film maintains its structure to carry out its most important function: to provide the cornea an optically smooth, refractive surface to ensure proper vision. Holly and his co-workers forayed into integrating physical and chemical sciences to study the tear film structure, which also facilitated an understanding of how this structure breaks down, especially during ocular diseased states. This formed the concept that in order for the tear film to maintain its dynamic structure, each of its defined layers must interact with each other. Consequently, interfaces were paramount to this idea.

### 1.3 Holly’s model of the tear film; the role of mucins

Holly and his co-workers brought to the forefront the importance of considering the physicochemical properties of the tear film. Properties such as surface tension, viscosity and elasticity, were recognised as not only being useful for spreading the tear film across the ocular surface, but these properties also enabled the maintenance of the tear film structure on the surface (without spillage), even during blink cycles when the tear film is under enormous stress. Clinically, it was observed that the ocular surface of patients suffering from various ocular diseases showed the presence of dry spots due to the break down of the tear film (Holly and Lemp, 1971; Holly and
Chapter 1 Introduction

Lemp, 1977). It is here that Holly and co-workers began to study the solid-liquid interface (cornea-mucin interface) and more specifically, what mechanisms allow the tear film to spread evenly over the hydrophobic cornea. Since mucins and proteins comprise a large component of tears and are in direct contact with the cornea, it was not surprising that he studied mucins and proteins as possible surfactants and wetting agents (Holly and Lemp, 1971; Holly, 1973; Holly, 1974; Holly and Lemp, 1977; Holly and Hong, 1982).

Holly and Lemp (1971) first established that a clean and mucin free corneal epithelial surface has a critical surface tension of 28mN/m (dyn/cm). In this same study, they established that in order for any liquid to spread and completely wet the corneal epithelial, its surface tension must be lower than 28mN/m. It was hypothesised that proteins in the aqueous layer may play a role in lowering surface tension of tears; however, a previous study by Miller (1969) reported that the surface tension of whole tears was 46mN/m, which was not low enough to wet the cornea. While they considered the use of surface active components such as detergents, which could easily achieve a surface tension lower than the mucin free corneal surface, they ruled this out because a surface tension this low can also cause emulsification of the tear film lipid layer (lipid layer of the tear film forming micelles in the aqueous phase), leading to further instabilities.

On the basis of these results and ideas, Holly and Lemp instead believed that some component or components of tears must be *increasing* the critical surface tension of the corneal epithelium, and the likely candidates were the ocular mucins based on the
presence of mucins found in other epithelial cells (e.g. digestive and respiratory tract). In a range of contact angle experiments, he observed that a mucin coated cornea was easily wettable by tears, as opposed to a cornea free of mucins. Holly and Lemp believed that the ocular mucins secreted by the goblet cells, adsorb to the corneal surface and render it hydrophilic. In this process, the critical surface tension is raised and therefore, the cornea can be completely wettable by tears, ensuring a spread tear film.

While Holly established the role of mucins as wetting agents at the solid-liquid interface, he did not disregard the fact that mucins, along with tear proteins, are surfactants. In his understanding of tear film formation and break-up (Holly, 1973a; Holly, 1973b; Holly and Lemp, 1977), he extrapolated that the tear film lipid layer must play a role in tear film stability by further lowering the surface tension of tears. However, after spreading meibomian lipids over saline, a surface tension of 60mN/m was obtained, which was not low enough to spread tears over the corneal surface (as discussed above) (Holly, 1973b). Working within the context of the lipid layer being reformed on each blink cycle, Holly suggested that the surface tension of this lipid interface must be lowered by the interaction of tear proteins and/or mucins. This lipid-protein/mucin interaction would then further lower the surface tension of tears, and aid in its stability. He supported his theory by conducting Langmuir trough experiments (Langmuir, 1917; Roberts, 1984; Murray and Nelson, 1996) measuring the surface pressure (inverse of surface tension) of proteins and mucins only (commercial samples), and proteins and mucins mixed with lipids (petroleum oil used as a model) (Holly, 1974).
Holly believed that mucins were the most surface active component in tears because when combined experimentally with lipids, a surface tension of 42–37mN/m (surface pressure of 30–35mN/m) was produced, which was lower than the other proteins tested. For this reason, plus the fact that the interactions of proteins would likely stiffen the lipid film rendering it brittle, Holly disregarded the role of proteins at the lipid interface. Based on the hydrophilic nature of mucins and their ability to form gels, he advocated mucins as the key surfactant and proposed a new model of the tear film (Fig. 1.2). Holly’s model of the tear film incorporated a gel-like (viscoelastic) meibomian lipid interface (through the interaction of mucins at the surface), as well as a mucin gradient in the aqueous layer, which also give it gel-like characteristics rather than a simple watery layer.

Figure 1.2: Holly’s model of the tear film. A more integrated structure than the previous three layered model of the tear film. This model incorporates a viscoelastic lipid interface through the interaction of mucins at the surface, and an aqueous layer with a mucin gradient giving it surfactant and gel-like properties. Adapted from Holly (1973a). Formation and rupture of the tear film. Exp. Eye Res. 15: 515–525, with permission from Elsevier.
Since Holly’s work, there has been more detailed information about the components of the tear film that has become available. The introduction of DNA technology has detected 19 different types of mucin genes which have been reviewed extensively in terms of their structure, function and regulation (Argüeso and Gipson, 2001; Dartt, 2004; Gipson, 2004; Mantelli and Argüeso, 2008). Membrane bound mucins such as MUC1, MUC3A, MUC3B MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20 and MUC21, form the corneal surface layer (glycocalyx) while secreted mucins such as MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC9 and MUC19, are distributed in the aqueous layer and exhibit gelling properties.

These latest findings have not critically impacted Holly’s model of the tear film, but rather added specific details to it about the distribution of mucins (Argüeso and Gipson, 2001), particularly within the aqueous and corneal surface layers. Conversely, the interaction of mucins at the lipid interface was still unknown due to the little investigation in this area. In addition, since Holly, the physicochemical and interfacial research on the tear film from a fundamental standpoint had been idle for almost two decades. Instead, this type of research shifted towards clinical situations such as dry eye (break-up time, interferometry, thickness, evaporation, and artificial tears), and in applied sciences (contact lens wettability and lens cleaning solutions).

1.4 The aqueous layer; the role of proteins

1.4.1 Function of the aqueous layer

Following Holly, progression of tear film research reverted to being done in the context of a model comprising three distinct layers. The lacrimal gland is responsible
for the production of the aqueous fluid of the tear film. This layer is essential for providing the avascular cornea with oxygen, nutrients and trace elements. The combination of metal ions, inorganic salts, glucose, urea, bicarbonate ions and proteins (Rolando and Zierhut, 2001; Tiffany, 2003), have resulted in an osmolarity of 310–317mOsmols/L in tears collected from the lacrimal lake (Tomlinson and Khanal, 2005). Furthermore, this layer is able to resist microbial infections, wash away any foreign debris or toxic chemicals, and aid in ocular wound repair (Rolando and Zierhut, 2001; Tiffany, 2003). In recent times, better collection techniques and proteomics (allow analysis and usage of much smaller sample sizes, which is especially important in the case of dry eye patients), have not only led to the qualitative discovery of the major proteins (Fullard and Synder, 1990), but quantitative discovery of more than 60 other proteins (Gachon et al., 1979; Li et al., 2005; Ohashi et al., 2006). Some of these have been assigned a mitogenic role e.g. lacritin (Sanghi et al., 2001), which plays a role in aiding in epithelial renewal. Others have contributed to the immune role of tears and the surfactant property of tears, e.g. complement proteins (Willcox et al., 1997) and lung surfactant proteins (Bräuer et al., 2007), respectively.

1.4.2 Integrative role of proteins; function of lipocalin

While the other major tear proteins predominantly play a role in the immune protection for the eye and are strictly confined within the aqueous layer, tear lipocalin (15–33% of total tear protein mass) has been shown to have a possible integrative role in the tear film. Previous research has shown that tear lipocalin has some antimicrobial activity (Selsted and Martinez, 1982; Fluckinger et al., 2004), but more recently, lipocalin has been established as the principal lipid binding protein
that scavenges harmful lipids from the ocular surface (Gasymov et al., 2005). Since lipocalin has a lipid binding domain (Glasgow et al., 1995), there has been some thought that it can also potentially interact with the lipid layer of the tear film (Mudgil and Millar, 2008; Millar et al., 2009). The fact that lipocalin can have a role in all three layers of the tear film is akin to the role of mucins suggested by Holly (1973b). As a result, there had been a renewed interest in the physical role proteins may play in the tear film (Nagyova and Tiffany, 1999; Gouviea and Tiffany, 2005; Tragoulias et al., 2005, Millar et al., 2006; Mudgil et al., 2006; Millar et al., 2009).

Nagyova and Tiffany (1999) speculated that lipocalin could play a significant role in lowering the surface tension of tears. Using a pooled sample of whole tears, they separated free lipids from whole tears and compared its surface tension against whole tears with lipids (46mN/m). The surface tension of whole tears minus the lipids was significantly higher (53mN/m), but this was restored to the original value (46mN/m) when the lipids were added back. In the same study, extending Holly’s work of surface pressure measurements of proteins (Holly, 1974; Holly and Hong, 1982), they used a capillary method to measure the surface tension of individual solutions of proteins found in tears, as well as in combination with other proteins and lipids (from the aqueous layer). They established that the surface tension closest to whole tears was attained from a combination of the four major proteins found in tears (lysozyme, lactoferrin, lipocalin and immunoglobulin A), and free lipids. Because free lipids were essential in obtaining this surface tension, they hypothesised that it must have been the interaction of tear lipocalin with these free lipids that was responsible for the surface tension of tears.
In a follow up study by Gouviea and Tiffany (2005), similar interactions between lipocalin (both apo and holo forms), free lipids and tear proteins were investigated in terms of their contribution to the viscosity of tears. The authors believed that although gel-forming mucins are present in the aqueous layer, there is not enough mucin to contribute towards the non-Newtonian viscosity that whole tears exhibit (Tiffany, 1991). Except for β-lactoglobulin (a commercially available lipocalin used to model tear apo-lipocalin), the other individual proteins showed Newtonian viscosity. However, when the proteins were in combination with each other, they showed non-Newtonian viscosity like whole tears. The viscosities of both apo and holo forms of lipocalin, and both apo and holo forms of β-lactoglobulin were also compared. Apo forms of both proteins showed non-Newtonian viscosities however, holo forms were Newtonian. It is here that the authors concluded that a combination of protein interactions, plus the interaction of proteins (lipocalin) with lipids contributed towards the viscosity of human tears. On this basis, it is possible that lipocalin, along with the other tear proteins, may interact with the meibomian lipid interface and alter the physicochemical properties in this lipid layer as well.

1.5 The meibomian lipid layer

1.5.1 Anatomy of the meibomian glands and function of the lipid layer

Research on the tear film lipid layer initially focused on the anatomy of the holocrine, lipid secreting glands, the meibomian glands (Wolff, 1954; Butovich et al, 2008). The secretions from these small openings found in the tarsal plates (30–40 glands in the upper lid, while the lower lid has 20–30 glands) (Wolff, 1954) are collectively known as the meibomian lipids (Fig. 1.3). These lipids form a thin
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(~100nm thick) (Bron et al., 2004), multilayered amorphous film at the air-tear interface, known as the meibomian lipid layer. There are a number of functions assigned to this interface: it is believed to provide an optically smooth surface for the refraction of light to the cornea; prevent evaporation of the aqueous layer of tears; allow lubrication of the eye lids during blinking; and lower the surface tension of tears, aiding in its stability (Holly, 1973b; Bron and Tiffany, 1998; McCulley and Shine, 2001; Bron et al., 2004).

Figure 1.3: Lower eye lid margin of the koala, Phascolarctos cinereu and brushtail possum, Trichosurus vulpecula. Arrows indicate: a) the meibomian glands; b) the orifices (gland openings); and c) the meibomian lipid secretions from hard squeezing of the glands. Glands and orifices are more difficult to see in the brushtail possum due to the pigmentation of the lid margins.

1.5.2 Composition and structure of the meibomian lipid layer

While it was observed that meibomian lipids form the outermost layer of the tear film, very little was known about how these lipids were structured and orientated at the two interfaces (the aqueous and air phases). Compositional studies of meibomian lipids in humans and animals using various techniques have shown a predominance
of wax and sterol esters (60–75%); and small amounts of triglycerides, diesters, free sterols and fatty acids, hydrocarbons and polar lipids (Nicolaides et al, 1981; McCulley and Shine, 1997; Bron and Tiffany, 1998; McCulley and Shine, 2003; Bron et al, 2004; Butovich et al, 2008; Butovich, 2009). McCulley and Shine (1997) were first to propose a model of the meibomian lipid layer. They highlighted a distinct arrangement comprising two phases: a non-polar phase consisting of hydrocarbons, and wax and sterol esters at the air-liquid interface; and a polar surfactant phase consisting of phospholipids, free fatty acids and alcohols, and triglycerides that integrated with both the aqueous and non-polar lipid layers.

While this model provides a good foundation for understanding the structure and orientation of the lipid molecules in the meibomian lipid layer, this model has never been tested. Furthermore, recent evidence has suggested that the nature of the polar lipids is contentious and may be species specific (Butovich et al, 2007). Phospholipids have been consistently reported in rabbit meibomian lipid samples, but not in other species (Tiffany, 1979; Greiner et al, 1996; McCulley and Shine, 2003; Butovich et al, 2007). Butovich et al, (2007) and Chen et al, (2010) demonstrate that little, if any phospholipids are present in human meibomian lipids. Saville et al, (2010) has shown that phospholipids are found in meibomian lipids, but in their study, a qualitative approach was used. This finding of little or no phospholipids has critical implications for the organisational lipid layer model, as proposed by Shine and McCulley (1997) because it incorporated phospholipids as the acting surfactant between the non polar lipids, and the aqueous layer of the tear film. These findings have indicated that other molecules must be performing the necessary surfactant role, and the most likely candidates are the tear proteins (Millar et al, 2006).
The importance of protein-protein and protein-lipid interactions to the physical properties of the tear film was first highlighted by Tiffany (Nagyova and Tiffany, 1999; Gouviea and Tiffany, 2005); however, how this process occurs with protein-lipid interactions have been speculative. Up until this point, proteins and mucins interacting with the meibomian lipid layer were thought to only occur through adsorption (Holly, 1974) rather than integration. By fluorescently tagging lipids and proteins, Millar and co-workers demonstrated that certain proteins such as lipocalin, lysozyme and mucins, were able to penetrate and integrate into the meibomian lipid layer (Millar et al, 2006; Mudgil et al, 2006; Mudgil and Millar, 2008; Millar et al, 2009). These findings were analogous to lung surfactant and cell membrane structures in vivo and therefore, Millar and co-workers proposed a new model of the tear film, incorporating proteins within the meibomian lipid layer (Fig. 1.4). Millar renewed fundamental interest in interfacial and physicochemical research on the tear film by extending the works of Holly and Tiffany. In particular, he recognised that the meibomian lipid interface was critical for the stability and proper functioning of the tear film as a whole.
Figure 1.4: Millar’s model of the tear film. An adaption of Holly’s model of the tear film however, with a more complex outer layer, which not only contains meibomian lipids, but also integrated ocular mucins and denatured proteins (Millar et al, 2006).

1.5.3 Secretion and formation of the meibomian lipid layer

Meibomian lipids are delivered into the eye lid marginal reservoirs and secreted through the lid orifices (Fig. 1.3) (Bron et al, 2004). The mechanical forces associated with blinking allow the steady delivery of the meibomian lipids to the lid margin (Berger and Corrsin, 1974; Chew et al, 1993; Korb et al, 1994). Blinking is also responsible for spreading these lipids onto the aqueous layer, forming a continuous lipid film over the surface (Berger and Corrsin, 1974; Bron et al, 2004). Upon the formation of a new blink, it is the subsequent blink cycles which continually thicken the meibomian lipid layer (Korb et al, 1994). Through interferometry, it has been observed that this structure is maintained over several blink cycles rather than being reformed with each blink (Yokoi et al, 1996; Goto et al, 2003a; Goto et al, 2003b; Goto, 2004; Yokoi and Komuro, 2004; King-Smith et
This indicates that new lipid molecules are gradually being transferred onto this surface.

Interferometry (Bron et al., 2004) and more recently, the assessment of the lipid layer using quantum dots (Khanal and Millar, 2010), has also shown the jetting of lipids onto this lipid layer, further highlighting the importance of blinking to the secretion and formation of the meibomian lipid layer. It is the inability to blink properly (proptosis of the eye) (Kaercher and Bron, 2008), and disorders of the meibomian glands† (abnormalities of the secretion, the obstruction of the gland orifices, and the inflammation and loss of glands) (Driver and Lemp, 1996; Millar et al., 2010) which have subsequently lead to the thinning and break-up of the meibomian lipid layer (Yokoi et al., 1996; Craig and Tomlinson, 1997; Goto et al., 2003; Olsen et al., 2003; Yokoi and Komuro, 2004; King-Smith et al., 2009). Thinning and break-up of the tear film lipid layer is the leading cause of increased tear evaporation and consequently, dry eye‡ and other related ocular problems.

1.5.4 Mechanical properties of the meibomian lipid layer

While blinking is important for the meibomian lipid layer formation (Berger and Corrsin, 1974), the mechanical action of blinking places an extraordinary amount of stress on this lipid interface. There is the mechanical stress from the drag action of the eye lids across the surface of the meibomian lipid layer (shear stress), and also the stress from the changing surface area of the eye as it opens and closes (dilatational stress). Furthermore, in each blink cycle (about 5–10 seconds) the lipid

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† The MGD report (2011) provides an extensive review of meibomian gland disorders
‡ The DEWS report (2007) provides an extensive review of dry eye
layer is subject to temperature and compositional changes. Remarkably, this complex structure (in normal subjects) is able to adapt and retain its structure for the functionality of the eye.

Holly (Holly, 1973\textsuperscript{a}; Holly, 1973\textsuperscript{b}; Holly, 1974), Millar (Tragoulias \textit{et al}, 2005; Millar \textit{et al}, 2006; Mudgil \textit{et al}, 2006; Mudgil and Millar, 2008; Millar \textit{et al}, 2009) and their co-workers have now well established the surfactant role played by meibomian lipids and the integrated proteins in maintaining the integrity of the tear film. As this lipid-protein interface is able to maintain its structure during blinking (Yokoi \textit{et al}, 1996; Goto \textit{et al}, 2003\textsuperscript{b}; Bron \textit{et al}, 2004; Goto, 2004; Yokoi and Komuro, 2004; King-Smith \textit{et al}, 2009), this interface must consequently have unique mechanical properties, such as viscosity and elasticity that allow these changes. These gel-like (viscoelastic) properties have also been observed in spreading and kinetic studies of the tear film lipid layer where natural tear particles (Owens and Phillips, 2001), and interference patterns (Bron \textit{et al}, 2004; Yokoi \textit{et al}, 2008) have been used to track the movement and spread time of the tear film lipid layer. In the opening motion of the eye lids, the meibomian lipid layer moves as a whole unit indicating some elasticity; yet there is also a lag effect, or slow dragging of the film even when the lid is stationary, indicating a viscous property (Bron \textit{et al}, 2004).

While it certainly appears that the meibomian lipid layer is viscoelastic, at the time of beginning my PhD, these properties had never been systematically tested. More recently, Yokoi \textit{et al}, (2008) conducted an \textit{in vivo} study assessing the spread kinetics
of the lipid layer in normal and aqueous deficient dry eyes. While they alluded that the meibomian lipid film was viscoelastic, their study did not directly measure this. Instead, viscoelasticity was inferred from applying the Voigt model to their data. This study nevertheless, brought to the forefront the clinical relevance for the potential use of viscoelasticity as a non-invasive diagnostic tool for dry eye because they were able to note differences in elasticity of normal patients versus dry eye patients. This study highlighted the need to conduct a systematic evaluation of the viscoelasticity of meibomian lipid films.

1.6 Aim

The aim of this thesis is to determine the interfacial viscoelastic properties of human meibomian lipid films in vitro, with the intent of providing a fundamental basis for clinical and in vivo studies. Before introducing the experimental sections, the next chapter provides a background into the concepts of Rheology, which is the study of flow (viscosity) and deformation (elasticity) behaviour. In addition, it also highlights the definitions and jargons used in rheology, and an explanation of the techniques and instrumentation used in this thesis is given.
Chapter 2

Understanding Rheology
2.1 Overview

This thesis will largely examine the viscoelasticity, or rheology of the meibomian lipid interface. By general consensus, rheology is a difficult topic, especially if the audience is not trained in engineering and mathematical sciences. Furthermore, the majority of the literature and text books on this subject assume a prior knowledge of engineering units and often, they are littered with complex mathematical equations and jargon, making it difficult to understand for those not directly involved in the field. To overcome this problem, background information is given here to enable a better understanding of the purpose and interpretation of methodologies and results that are presented in this thesis. Consistent with this theme is: specific reference to everyday materials as examples; the presentation of mathematics critical to this thesis; and the terminology and jargon clearly defined. For additional information, it may be useful to consult An Introduction to rheology (Barnes et al, 1989), and The rheology handbook (Mezger, 2006).

2.2 Defining rheology

Rheology is defined as the study of deformation and flow of matter, under an applied stress (coined by Eugene C. Bingham, 1920). More specifically, it is studying the deformation or elastic behaviour of a solid, and the flow or viscous behaviour of a liquid. A simple analogy would be that the elasticity of a solid such as a rubber band would only be determined once it is stretched (an applied stress). Similarly the viscosity of a liquid such as oil, would only be determined if you stir it.
2.2.1 **Applications of rheology**

Rheology is relevant to almost all industrial applications; this includes food sciences (Rao, 2007), pharmaceutical and cosmetic sciences (Barry, 1970; Gallegos and Franco, 1999), paint sciences (Lambourne and Strivens, 1999), and essentially in any other processes that involves the flow of a material. In part, the advancement of rheology has been necessary with the introduction of synthetic polymers, which often have been designed to modulate the physical and structural properties of materials. In food science and paint applications, rheology has been used extensively to study the stability of products and hence, improve its shelf life and storage (Bergh *et al.*, 1989; Dickinson, 1999). Rheology has also been crucial to the functionality of many products, e.g. applying paint on a wall, but ensuring that it does not drip as it dries.

In terms of improving quality and texture using rheology, the tempering of chocolate (Afoakwa *et al.*, 2007) is a prime example where being able to process the material at a specific temperature, is crucial to the viscosity of the final product. Another example is in the automobile industry where the viscosity of engine oil is critical for its lubricating properties– this plays a major role in the efficiency of fuel consumption (Taylor, 1997; Coy, 1998). In cosmetic applications, quick dry nail polish, waxes used for hair removal (hard versus soft wax) and make-up skin foundation (available in either a liquid or solid form), are all examples of how rheology can influence the functionality of a product. From these examples, it is clear that rheology is an important consideration for the processing of a material and consequently, it is measured and manipulated in almost all commercial and industrial applications.
2.2.2 Types of stress

Materials can be subjected to a number of different types of stresses (Fig. 2.1). The most common being a shear stress which is defined as a stress being applied in a parallel or tangential manner. In shear stress, the volume or surface area is constant yet, there is a change of shape to the material. Extensional or normal stress is relatively more complex and is defined as stress being applied in a perpendicular manner. In extensional rheology, the volume or surface area (dilatational stress) normally changes. Shear rheology is the more extensive rheology used due to the difficulties and complexities associated with producing and analysing extensional flows. As a result, shear stress has formed a basis for many rheological models.

![Figure 2.1: Types of stress applied to a material. Arrows indicate direction of force.](image)

- **(a)** Shear stress is the force applied in a parallel manner—volume or surface area does not change, but shape does.
- **(b)** Extensional stress is the force applied in a perpendicular manner—volume changes.
- **(c)** Dilatational stress is an extensional stress where volume and surface area changes, but shape is retained.

2.2.3 Stress and strain relationship

The behaviour of a material is associated with a stress and strain relationship. When a stress (τ) or force is applied to a solid, its deformation results in an instant strain (γ). In Hooke’s *True theory of elasticity* (1678), Hooke described the deformation of a spring and stated that the power of any spring is in the same proportion with the tension thereof. In other words, there is a linear relationship between the stress...
applied to the spring, and the resulting extension or strain from the stress. Therefore, if the stress was removed, the spring would return to its original state prior to the deformation. The linear relationship between τ and γ is known as the modulus of rigidity, or shear modulus \( G \). This is calculated as,

\[
G = \frac{\tau}{\gamma}
\]

Classic solids, like the spring obey Hooke’s law (\( \tau = G\gamma \)) and are known as Hookean solids (Fig. 2.2a).

**Figure 2.2:** Stress and strain relationship in classical models of rheology. (a) Application of shear stress to a Hookean solid. The shear stress \( \tau \), is the force (F) per unit of area (A) required to produce the motion, F/A. Shear strain \( \gamma \), is the change in angle as a result of displacement of one plane with respect to another \( \Delta x \), divided by the distance between the two planes (h), \( \Delta x/h \). The linear relationship between stress \( \tau \) and strain \( \gamma \) is known as the shear modulus \( G \), and is calculated as \( G= \tau/\gamma \). (b) Application of shear stress to a Newtonian fluid using the two-plate method. A shear stress required for motion is applied to the upper plate, while the lower plate remains stationary. The velocity (V) is obtained \( \Delta x/t \), followed by the velocity gradient or shear rate \( \dot{\gamma} \) between the two plates. This model represents a linear relationship between shear stress \( \tau \) and shear rate \( \dot{\gamma} \) and consequently, viscosity \( \eta \) is calculated as \( \tau/\dot{\gamma} \).

Similarly, a shear stress may be applied to a fluid however, the resultant deformation will cause the fluid to flow even when the stress is removed (this is observed in water when we see swirling even after stirring). A classical fluid such as water obeys Newton’s theory associated with shear flow (Principia, 1687). Newton stated that the
resistance which arises from the lack of slipperiness (now known as viscosity) of the parts of liquid, other things being equal, is proportional to the velocity with which parts of the liquid are separated from another. In other words, if two layers of liquid slide past each other, there is a linear relationship between the force required to separate the liquid layers (shear stress, $\tau$), and the velocity gradient (shear rate, $\dot{\gamma}$) between the two layers (Fig. 2.2b). Consequently the flow, or shear viscosity ($\eta$) can be calculated as,

$$\eta = \frac{\tau}{\dot{\gamma}}$$

Fluids which obey Newtonian’s law ($\tau = \eta \dot{\gamma}$) are known as Newtonian fluids.

### 2.2.4 Non-linearity in rheology

Hookean solids and Newtonian fluids represent ideal linear models and are classical extremes in rheology. In reality, most materials exhibit a combination of both solid-like and fluid-like behaviour (viscoelastic), depending on the stress and how it is applied. For instance, a viscoelastic material such as toothpaste will behave like a solid under small stresses (gravitational force), but when the stress is increased, e.g. from squeezing, the paste will flow and behave as a fluid. On the contrary, a viscoelastic material such as cornstarch in water, behaves like a fluid in low shear rates, e.g. as you stir it, yet it behaves as a solid in high shear rates or with large stress, e.g. punching or rolling the solution. Once the stress is removed, the solution will behave as a liquid again. A good example of non-linearity in rheology is known as shear thinning behaviour (shear viscosity is lowered with increasing shear rate), and this is seen with toothpaste (Fig. 2.3a). The behaviour of corn starch in water is an example of shear thickening (viscosity is increased with increasing shear rate). Another more complicated example of shear thinning behaviour can be seen in
plastics. Plastics behave as solids under low stress and return to their original shape once the stress is removed. However, once a plastic reaches a point of permanent deformation in high stress, known as the *yield point/stress* \( (\tau_y) \), it will behave as another shear thinning fluid (Fig. 2.3b). This type of fluid is referred to as a Bingham plastic.

![Figure 2.3: Shear thinning and thickening behaviour of materials](image)

(a) Shear thinning and thickening behaviour observed as a function of viscosity \( (\eta) \). The viscosity of a Newtonian fluid is independent of shear rates \( (\dot{\gamma}) \). In shear thinning behaviour, viscosity decreases with shear rates, whereas viscosity increases in shear rate with shear thickening fluids. (b) Shear thinning and thickening behaviour observed as a function of stress \( (\tau) \). Newtonian fluids have a linear relationship with stress and shear rate. In shear thickening behaviour, stress is increased with increasing shear rates, whereas stress is decreased with shear rate in shear thinning behaviour. Normal shear thinning behaviour is also observed once a yield stress \( (\tau_y) \) is reached (Bingham plastic), otherwise the material behaves as an ordinary solid.

### 2.2.5 Importance of time in rheology

A time scale is important when a material transitions from an elastic state to a viscous one. For instance, the viscosity of ketchup is decreased the longer you shake the bottle (with a constant shear stress or rate). In this case, the transition from a solid state to viscous state is a matter of seconds. Another example of time dependant flow can be seen with *silly putty*. If you leave this silicone polymer sitting on a bench over a long time scales (hours), it will eventually flatten out and flow, behaving as a
Chapter 2 Understanding Rheology

liquid. However, the polymer can be moulded into any shape, and under a short time scale (minutes), it will retain its shape and behave as a solid. Similarly, if you pull apart the polymer very slowly, it will flow. If it is pulled apart very quickly, a third class of behaviour is observed where the material will fracture in a brittle and solid-like manner. In this case, silly putty has demonstrated behaviour in a more extreme case of time dependant flow, described as glassy behaviour.

Glass ordinarily behaves like a solid however, its structure is more like a supercooled liquid. It has been observed that antique glass found in cathedrals has a noticeably thinner appearance at the top of the panes than at the bottom. This has suggested that the glass has indeed flowed, although the flow process has taken many centuries! Time dependant flow of materials, particularly fluids, is often characterised by the Deborah number (De) (Reiner, 1964). De is a dimensionless number defined as the time of stress relaxation divided by the experimental time of observation. The smaller the De number, the more fluid the material is.

2.2.6 Temperature and pressure in rheology

Transitions of flow behaviour can also been seen with temperature changes. For instance, cooking oil is visibly thinner and less viscous with an increase in temperature. On the other hand, the addition of corn starch to foods such as gravy, thickens the gravy making it more viscous with temperature, even when the shear rate is constant. Likewise, pressure can also alter flow behaviour. Oils commonly become very viscous and even solidify (become crystalline in structure) under high pressures.
2.2.7 Re-defining rheology

It is clear that defining a solid or liquid is not as simple as just looking at the stress and strain relationship. Factors such as shear load (stress), time, temperature and pressure can all influence the properties of a material. The original coining and definition of rheology was very broad and could technically include any material possessing flow and deformation behaviour. Today, rheology has been re-defined to exclude classical extremes (Newtonian fluids and Hookean solids). It has instead centred more on the unusual behaviour of materials which fall in between these extremes, i.e., viscoelastic materials.

While glass and even other solids such as steel can exhibit liquid like properties, waiting centuries to see flow (e.g. in glass), or even months and days is not an appropriate time scale in rheology. Flow behaviour must be seen in real time laboratory settings and therefore, materials such as glass and steel are studied as solids, with material properties of tensile strength, ductility, and brittleness. On the other hand, semi-solid materials such as gels, adhesives and plastics, show relatively obvious signs of flow behaviour, and are regarded as viscoelastic and non-Newtonian fluids. Fluids which are not viscoelastic, yet do not follow Newton’s law are also considered non-Newtonian fluids§ and their properties are also studied. Semi-solids and both classes of non-Newtonian fluids are all regarded as complex fluids. Today, rheology is re-defined as the deformation and flow behaviour of these complex fluids (Larson, 1999).

§ All viscoelastic fluids are non-Newtonian, but not all non-Newtonian fluids are viscoelastic
2.3 Linear viscoelasticity

A viscoelastic material means that this material simultaneously possesses both viscous and elastic properties. While shear load, temperature and pressure may all influence the viscoelastic properties of a material, it is the time scale and the strength of deformation which ultimately determines whether one material property may dominate over the other. For instance, margarine is solid-like at room temperature, whereas heating it transforms it to a liquid-like state. Temperature has influenced the material properties of margarine in an extreme sense however, even at the solid state, margarine possesses fluid-like properties which can only be determined when its material properties are probed as a function of time. Indeed, the viscosity (and elasticity) of margarine at room temperature could be decreased with constant shearing motions in a manner of seconds e.g. as you apply it on bread. In comparison, if a tub of the same margarine is inverted over a piece of bread, one may have to wait hours or days until the bread is ready.

Although rheologists use the terms Newtonian or Hookean to describe classical liquids and solids respectively, these models can be combined to describe the behaviour of a viscoelastic material that shows Hookean solid-like behaviour or Newtonian fluid-like behaviour in a given situation. In a viscoelastic material the viscous behaviour can be related to Newton’s law, while the elastic behaviour can be related to Hooke’s law. These linear aspects of fluids and solids have been combined to develop time dependant linear viscoelastic models. These models are in turn used to determine the viscoelastic properties of a material (non-linear viscoelasticity also exists, but since this is not relevant in this thesis, they will not be described here).
2.3.1 Oscillatory rheology

Rheological tests can be conducted in either a static manner, where the strain or stress applied to the material is constant (creep and relaxation tests), or in a dynamic mode where stress, strain and even time, can be varied. It is also important to note that in some practical situations, it is more appropriate to apply a strain and measure stress, rather than in the classical situation discussed above where a stress is applied and the resultant strain is measured (Section 2.2.3). Rheological instruments are designed on both principles: either stress controlled or strain controlled. An oscillatory test is a dynamic test which has become very popular particularly because it allows the user to probe a material on different time scales (frequency sweeps, see below). The mathematics used to derive oscillatory shear deformation is relatively complex and will not be discussed here in detail. Briefly, the material is deformed in a sinusoidal manner and the time dependant functions are translated and represented as a function of frequency ($\omega$) (Fig. 2.4).

![Oscillatory Rheology Diagram](image)

**Figure 2.4: The sine wave function used in oscillatory rheology.** Geometrically, it is a circle plotted as a function of time. The frequency (angular) is represented as the amount of waves (one wave =360°) per unit of time, in radians per second. The amplitude is the height of the wave from centre position.
The sinusoidal strain applied to a material is represented as,
\[ \gamma(t) = \gamma_0 \sin(\omega t) \]
where \( \gamma_0 \) is the strain amplitude and \( \omega \) is the angular frequency. The resultant stress (linear) from the sinusoidal strain is represented as,
\[ \tau = \tau_0 \sin(\omega t + \delta) \]
where \( \tau_0 \) is the stress amplitude and \( \delta \) is the phase angle or lag. If the material probed is perfectly elastic (ideal Hookean solid), there would be phase angle of zero between the strain applied and the resultant stress (which is analogous to the stress/strain relationship in a spring) (Fig. 2.5a). However, if the material probed is perfectly viscous (Newtonian), a phase lag of 90° would exist (Fig. 2.5b). Materials which have phase angles between 0° and 90° are viscoelastic (Fig. 2.5c).

Figure 2.5: Stress and strain relationship in oscillatory rheology. An ideal elastic solid (Hookean) has a phase angle of 0° (a). A perfectly viscous liquid (Newtonian) has a phase angle of 90° (b). Materials which have phase angles between 0° and 90° are viscoelastic (c).

If the phase angle is zero, the relative amplitudes of stress and strain define the shear modulus \( (G) \) as described by Hooke’s law. However, in oscillatory shear, the phase angle is non-zero and the shear modulus is complex \( (G^*) \). Mathematically it can be
split into real and imaginary components,

\[ \tau = \gamma_0 [G' \sin(\omega t) + G'' \cos(\omega t)] \]

\[ G^* = G' + iG'' \]

In this case, the real component is the in-phase elastic contribution \((G')\), also known as the \textit{storage} modulus (energy is conserved or recoverable, e.g. a spring returns to its original state once deformation stress is removed). The imaginary component is the out-of-phase viscous contribution \((G'')\) which is also known as the \textit{loss} modulus (energy is lost or unrecoverable, e.g. a fluid never returns to its original state after deformation has ceased). It is important to note that the imaginary component in the shear complex modulus is not a measure of viscosity, but rather the ratio of the behaviour that is out of phase in regards to the elasticity. The viscosity measured in oscillatory experiments is also complex \((\eta^*)\) and is calculated as,

\[ \eta^* = \eta' + i\eta'' \]

where \(\eta'\) is the dynamic viscosity and \(i\eta''\) is the imaginary elasticity (the recoverable energy in regards to viscosity). This is derived from the complex shear modulus \(G^*\) divided by the angular frequency \(\omega\) \((\eta^* = G^*/i\omega)\).

\textbf{2.3.2 Parameters in oscillatory rheology}

In oscillatory tests, four control parameters are typically varied: amplitude, frequency, time and temperature.

\textbf{2.3.2.1 Varying amplitude}

An amplitude or strain sweep (Fig. 2.6a), which is varying the amplitude while keeping all other test parameters constant, is often the first experiment to be
conducted on a new material. This test is primarily conducted to determine the linear viscoelastic range (LVR), i.e., the range in which material properties follow the linear viscoelastic models and therefore, modulus values are not dependent on amplitude. For a material to be in the LVR, both $G'$ and $G''$ are independent of amplitude (strain) (Fig. 2.6b). Once the LVR is determined, a strain closest to, or at the limiting value ($\gamma_L$) is used for all other experiments. Amplitude sweeps are also used determine the yield point in viscoelastic solids (Fig. 2.6c). This is the point outside the LVR where irreversible deformation has occurred and as a result, $G''$ starts to dominate over $G'$. The cross over point is known as the flow point, which literally means that the material has started to flow.

![Diagram](image_url)

**Figure 2.6: Amplitude sweep in oscillatory tests.** (a) Amplitude (strain) ($\gamma$) is varied over time yet frequency, temperature and total experiment time is kept constant. (b) Sample demonstrating fluid-like behaviour since $G'' > G'$. The LVR is the range before both moduli show dependence of strain. The point at which the moduli start showing dependence of strain is known as the limiting value ($\gamma_L$). (c) Sample demonstrating semi-solid like behaviour since $G' > G''$. After $\gamma_L$ is reached, the point (flow point) at which $G''$ starts to dominate indicates flow behaviour.
2.3.2.2 Varying frequency

Once the amplitude or strain rate in the LVR has been determined, a frequency sweep is conducted on the test material, i.e., varying the frequency, yet keeping all other parameters constant (Fig. 2.7a). Frequency is a time dependant variable and therefore, it allows you to probe the material properties of a sample at short time scales (high frequencies), long time scales (low frequencies), as well as intermediate time scales. Since time is such an important variable in rheology, a frequency sweep conducted in oscillatory tests outlines the mechanical blueprint of a material. For instance, the rheology of a material as a function of frequency is particularly useful in paint applications e.g., non drip paint needs to behave like a shear thinning fluid at high frequencies (during the application process), but it also needs to behave like a solid at low frequencies as this ensures the paint does not drip once it is on the walls (under gravitational force).

Figure 2.7 represents classic frequency plots. Figure 2.7b shows that $G''$ is more dominant than $G'$ initially, demonstrating fluid-like properties however, as frequency is increased the material becomes more rigid and solid-like, $G' > G''$. The frequency at which $G'$ becomes greater than $G''$ is often referred to as the crossover point, and is inversely proportional to the average relaxation time of the material. In other words, if deformations are so fast (high frequency) that the material cannot relax, the material will store energy and the dominant modulus will be $G'$. If the deformation is much slower (low frequency), the material will have time to respond to the stress and flow like a fluid ($G'' > G'$).
In contrast, Figure 2.7c, shows the $G' > G''$ indicating that the material is more solid-like however, both moduli are independent of frequency. This is indicative of classical gel behaviour (Larson, 1999). Sometimes there is a slight dependence of frequency with the moduli ($G' > G$), but this is still considered a semi-solid gel, depending on the type of material. Often, a frequency dependency is observed when molecules are reorganising at the surface under deformation, or if surface molecules are exchanging with the bulk molecules (or vice versa). This type of behaviour is not uncommon with proteins and polymer melts. On the other hand, this behaviour could also be referred to as glassy, indicating a material which is much more rigid than a gel. This is normally observed when the the ratio between $G'$ and $G''$ is large, and have higher moduli (Fig. 2.7d). Distinguishing between a gel and glass is not a simple task, and often the material has to be very well characterised before any conclusions can be made.
Figure 2.7: Frequency sweep in oscillatory tests. (a) Frequency (ω) is varied over time yet amplitude, temperature and total experiment time is kept constant. (b) Sample demonstrating fluid-like behaviour at low frequencies (G" > G'), yet becomes more solid-like at higher frequencies (G' > G''). (c) Sample demonstrating classical gel behaviour since G' > G'', yet both moduli are independent of frequency. (d) Sample demonstrating more rigid behaviour since the ratio of G' over G'' is much larger and the moduli values are higher. This rigid gel-like material is also considered glassy.

2.3.2.3 Varying temperature and time

A temperature or time sweep is conducted if the amplitude and frequency are kept constant. The importance of time and temperature in rheology have already been discussed in this chapter, but combining these variables with an oscillatory test allows one to determine how both the relative elastic and viscous components change with time and temperature. This results in understanding more detail about the material properties. In particular, independence of G' as a function of time is
correlated with stability of emulsions (Fig. 2.8a). The longer the independence of $G'$ from time, the more stable the emulsion. On the other hand, temperature dependence of $G'$ and $G''$ is useful in hardening processes where a transition of dominance from $G''$ to $G'$’s is advantageous at a critical temperature (e.g. corn starch used to thicken gravy and other food products) (Fig. 2.8b).

Figure 2.8: Time and temperature sweep in oscillatory tests. (a) Time sweep used as a variable to measure the stability of an emulsion. The longer the independence of $G'$ with respect to time, the more stable the emulsion. (b) A sample demonstrating ‘hardening’ and increasing rigidity over time. The point where $G'$ crosses over $G''$ and starts dominating is known as the gel point.

Time, temperature, shear rates, and shear stresses can all influence the rheology of complex fluids. Although other rheological techniques, such as rotational tests (not discussed directly in this thesis however, examples that have been discussed, such as shear thinning use rotational test methods) can measure these properties, the advantage of a dynamic oscillatory test is that it allows the material to be analysed as its relative elastic ($G'$) and viscous ($G''$) contributions separately. As this technique provides a clear advantage, as well as being increasingly popular for rheology, this
thesis will use a number of oscillatory rheometers to measure the viscoelastic properties of the meibomian lipid interface.

2.4 Interfacial rheometry

Throughout this chapter, various rheological parameters (shear load, temperature, time) and test methods (oscillatory, creep, relaxation) have been highlighted. Today, there are a variety of rheometers used to measure the viscoelasticity of a material; some of these are versatile enough to do many of the tests mentioned above, as well as measure the various parameters. For a good review of rheometers and instrumentation used in rheology, refer to Macosko (1994).

While this thesis will use oscillatory rheometry to explore the viscoelasticity of the meibomian lipid interface, before an oscillatory rheometer can be chosen, it is essential to determine the type of rheological information required from the material. More specifically, is it the bulk properties or interfacial properties of a material which needs to be assessed? Traditional rheology has centred on the bulk properties, particularly due to the nature of the materials being tested and the need for them in industry (polymer melts and solutions, and colloidal dispersions) (Larson, 1999). It is only more recently that the need for studying interfaces has arisen.

Interfaces are important because any system where two or more immiscible phases exist (gases, water, oil), the unique properties of the interfaces determine how these systems mix and co-exist together. An example of this is foams and emulsion systems in the food science literature. There has been emerging evidence that the mechanical (rheological) properties of the interfacial layers of the dispersed droplets
are critical to its stability and hence, prevent them from fusing together and coalescing (Dickinson, 1997; Benjamins and Lucassen-Reynders, 1998; Wilde, 2000; Rodríguez Patino et al, 2001; Murray, 2007). In most cases, the stability of foams and emulsions has been improved by surfactants such as proteins and lipids (Dickinson, 1999; Dickinson, 2001; Bos and Vilet, 2001; Rodríguez Patino et al, 2003; Murray, 2007).

While great advancements in interfacial rheology have been made in the food science literature and in other industrial applications, similar interfacial systems also exist in biological systems. As a result, interfacial rheology has now been extended to these interfaces. For instance, lung physiology groups have applied rheology to study the stability of pulmonary surfactant (Miller et al, 1997; Wüstneck et al, 2002; Wüstneck et al, 2005). Pulmonary surfactant is a mixture of lipids (phospholipids) and proteins (surfactant proteins B and C) which line the surface of alveoli and prevent lung collapse during breathing (Pérez-Gil, 2008). The tear film’s outer lipid interface is strikingly similar to pulmonary surfactant in that it is also a mixture of lipids and proteins, and is subjected to a constant stress from blinking—akin to the stress from breathing that is applied to pulmonary surfactant. Therefore, interfacial rheology could be applied to study the tear film’s lipid interface, and is the purpose this thesis.

The need for applying rheology to interfaces has led to a surge in developing the theoretical models and consequently, the development of the necessary experimental
tools (Miller et al., 2010). Shear ($G$) or dilatational (extensional) ($E$) stress could be applied to an interface, similarly to how it is applied to the bulk (as discussed above). Since biological interfaces experience a combination of both forms of stress, it would be ideal that rheometers capable of applying both types of stresses be used. Indeed, this thesis reports the viscoelastic properties of the meibomian lipid interface obtained from both oscillatory shear and dilatational rheometry. The interfacial shear rheometer used was the interfacial stress rheometer that was developed by Fuller and co-workers (Brooks et al., 1999). These experiments were conducted in the Fuller laboratory at Stanford University. The interfacial dilatational rheometer used was the pendant drop rheometer (Benjamins et al., 1996). This thesis predominately features viscoelastic results obtained from the pendant drop technique.

**The theoretical background in this chapter has largely focused on shear rheology. However, extensional and dilatational models have been derived from shear rheology. This will be explained in Chapter 4.**
Chapter 3

Interfacial shear viscoelasticity of human and animal meibomian lipid films††

†† Excerpts from this chapter have been published in Leiske et al. (2010). The interfacial viscoelastic properties and structures of human and animal Meibomian lipids. Exp. Eye Res. 90: 598–604.
Chapter 3 Interfacial shear viscoelasticity of human and animal meibomian lipid films

3.1 Introduction

Meibomian lipids are a complex mixture of different lipid species (Section 1.5.2). While it has been discussed that the composition of meibomian lipids from animal species varies from humans (Section 1.5.2), there are also variances within human meibomian lipid samples, particularly of lipids from patients with ocular diseases such as dry eye, to normal patients free of disease (Shine and McCulley, 1991; Shine and McCulley, 1996; Shine and McCulley, 2000; Oshima et al, 2009; Borchman et al, 2012). Although the exact nature of these changes within humans, and its effect to the structure and functionality of the meibomian lipid interface and the tear film as a whole is still relatively unknown, a comparison of animal models with humans may provided a basis. In terms of the whole tear film, it has been generally observed that animals have longer interblink periods (time between two blink cycles) than humans (Blount, 1927; Stevens and Livermore, 1978; Kirsten and Kirsten, 1983; Hall, 1945; Korb et al, 1998). This suggests that their tear film structure is more stable.

Since the meibomian lipid interface plays a major role in promoting tear film stability, presumably there is something unique about the structure and composition of these animal lipids. Differences in the meibomian lipids between species may reflect functional adaptations to environmental conditions, such as to reduce evaporation in dry deserts, or to behavioural changes such as diurnal versus nocturnal. Nevertheless, the longer interblink cycles suggest that their properties assist in increasing the stability of the tear film and hence, this needs to be investigated.
While the overall aim of this chapter (and thesis) is to establish the viscoelasticity of the human meibomian lipid interface, a rheological comparison of animal meibomian lipid films with human lipid films will provide a basis of how these compositional changes influence the mechanical and structural properties of these complex, multilayered films. More specifically, can viscoelasticity be directly related to the presence or absence of a particular lipid component? By establishing which lipid species contribute to the desirable film properties, this information may ultimately be useful in clinical situations, such as designing dry eye treatments that emulate these properties.

The shear rheological properties of human and animal meibomian lipid films were obtained using the interfacial stress rheometer (ISR). In addition to probing surface rheology, these experiments were supported by Π-A measurements obtained from the Langmuir trough. Since lipid molecules organise differently as the level of film compression changes (changing surface pressure), rheological properties were examined at different surface pressures in order to correlate viscoelastic data with molecular organisation. Brewster angle microscopy (BAM) was used to monitor the microscopic appearance of these films.

### 3.2 Materials and Methods

#### 3.2.1 Meibomian lipid collection

Lipids from a range of animals from different climates and interblink periods were collected and used in this study. This included laboratory species such as the rabbit (Oryctolagus cuniculus) and rat (Rattus norvegicus). The rabbit’s interblink period
has been observed to be over 5 minutes (Korb et al., 1998), while the rat also has a period of more than 2 minutes (Stevens and Livermore Jnr., 1978). Bovine lipids were used since their interblink period and lipid composition is very similar to humans (about 3 seconds) (Blount, 1927; Ponder and Kennedy, 1927; Hall, 1945; Korb et al., 1998). Australian marsupial lipids were used in this study because these animals are usually exposed to harsh, arid environments. Some of these are also nocturnal desert species such as the kultarr (Antechinmys laniger), while others are tree dwelling nocturnals such as the phascogale (Phascogale tapoatafa). Other diurnal species have unusually long interblink periods for instance, the koala (Phascolarctos cinereus) has been observed for more than 10 minutes without blinking, while the wallaby (Macropus rufogriseus) has been observed for more than 2 minutes without blinking. Generally, these Australian marsupials have interblink periods of more than 1 minute, which is considerably larger than humans.

Animal and human ethics were approved for the collection of meibomian lipids. The animal eyelids were dissected post-mortem (the animals usually died of natural causes, or were killed as part of other studies). Bovine eyelids were obtained from a slaughterhouse. Meibomian lipids were extruded by hard squeezing of the eyelid margins. Human samples were collected by the same method from a volunteer with no clinical signs of dry eye disease. The extracted lipids were an opaque solid ranging in colour from white to pale yellow. These were gently scraped off the lid margin with a stainless steal spatula and dissolved in chloroform (HPLC grade, Labscan, Australia).
3.2.2 **Preparation of lipid solutions and buffers**

The lipids were dried (under vacuum centrifugation), weighed and then reconstituted in chloroform at a concentration of 1mg/mL. Human samples collected with this technique have been analysed by mass spectrometry for composition by Butovich and co-workers (Butovich *et al.*, 2007; Butovich *et al.*, 2008; Butovich, 2009a). These samples have been found to contain lipid species only and there has been no evidence of protein contamination. Detailed compositional analysis of lipids from the particular animal species mentioned above is currently still in progress by Butovich and Millar (2009). An artificial tear buffer (ATB) (6.626g/L NaCl, 1.716g/L KCl, 1.376g/L NaHCO$_3$, 0.147g/L CaCl$_2$, 0.100g/L NaH$_2$PO$_4$, 4.18g/L MOPS, pH 7.4), which has been derived from the artificial tear solution described by Mirejovsky *et al.*, (1991) was used as the subphase for all experiments. Ion exchanged purified water with a resistivity of 18.2MΩ.cm (Millipore, Milli Q) was used in all experiments and buffers.

3.2.3 **Surface pressure measurements of meibomian lipid films**

A double barrier Langmuir trough coupled with a Wilhelmy balance (Nima Technology, Ltd, UK) was used to carry out surface pressure (Π) measurements at 20°C. Details regarding optimization of the experimental procedure are described in Tragoulias *et al.* (2005). Briefly, meibomian lipids solutions were deposited by touching micro drops of solution to the surface of the ATB subphase between the barriers. 15 minutes was allowed for the chloroform to evaporate before compression–expansion cycles (isocycles) were started. Changes in film surface pressure were measured with respect to mean molecular area (A) (Π-A profiles). The
trough was compressed and expanded at a rate of 15cm²/min. The amount of material added to the trough was adjusted in order to maximize compression while maintaining zero pressure before compression. The assumed molecular weight (Mw) of 650g/mol for meibomian lipids was used for calculating molecular area (Butovich, 2009a). These experiments, as well as the rheological experiments discussed in this chapter and subsequent chapters, were conducted at least three times to ensure reproducibility.

3.2.4 Measuring the shear rheology of meibomian lipid films

The interfacial stress rheometer (ISR) was used to probe the interfacial shear rheology of meibomian lipid films. The validation and merits of this technique have been described elsewhere (Brooks et al, 1999; Reynaert et al, 2008) however briefly, the ISR (Fig. 3.1) utilises a commercial Langmuir trough (KSV Instruments, Ltd., Helsinki, Finland) which is surrounded by two Helmholtz coils. These coils apply an oscillating magnetic field to move a magnetized, teflon-coated rod along its major axis at sinusoidal strains. The rod floats at the interface in a rectangular, quartz channel. The long axis of the channel is placed perpendicular to the trough barriers so that compression of the lipid layer throughout the trough (inside and outside the channel) is uniform. The channel helps to maintain the lateral position of the rod during experiments, in addition to defining the velocity profile created by the rod motion. The resulting stress is calculated by using a calibration constant to convert the strength of the magnetic field to force (the rheometer was calibrated prior to deposition of every monolayer). Rod position was monitored using a CCD camera (Basler Electric Company, Highland, IL) and strain was ascertained from the images.
Lipids were deposited to surface pressures between 4–6mN/m to achieve sufficiently high compression, since the length of the channel physically did not allow enough area compression. Films were compressed to the minimum surface area and expanded a few times to enable an equilibrium molecular organisation of the film prior to experiments. An amplitude sweep found a strain of 2.4% to be in the LVR and was used for all experiments. Frequency sweeps were conducted at a range of 0.06–12rad/s (0.01–2Hz) at specific surface pressures. For less elastic fluids, data collection at low surface pressures was limited by the sensitivity of the instrument. Experiments were conducted at room temperature (20–25°C).

**Figure 3.1: Interfacial Stress Rheometer.** This rheometer measures the shear rheology of an interface. It utilises a commercial Langmuir trough surrounded by two Helmholtz coils. These coils apply a magnetic field to oscillate a magnetized, teflon-coated rod along its major axis at sinusoidal strains. Rod position is monitored using a CCD camera.

**3.2.5 Brewster angle microscopy of meibomian lipid films**

Morphology of meibomian lipid films was observed using a homebuilt Brewster angle microscope (BAM) (Hönig and Möbius, 1991; Hönig and Möbius, 1992).
Briefly, BAM utilises a commercial Langmuir trough and under the Brewster angle, p-polarized light is not reflected at the air-water interface. However, upon the formation of a lipid film, the Brewster angle is modified and hence light is reflected. The degree and areas of brightness correlates to molecular density and organisation of the lipid molecules, giving insight into the structure of these films. A HeNe laser (model 1125P, Uniphase, Manteca, CA) was used as the illumination source for the p-polarized light (power 10mW, diameter 632.8nm). A CCD camera and controller (model C2400, Hamamatsu, Bridgewater, NJ) was used as the detector. The BAM resolution was 10µm and the field of view was 1.1mm in the focal plane. Horizontal lines in the images are artefacts of the technique and do not represent material structure. Meibomian lipid solutions were deposited on the ATB subphase in the same manner as described above. Images were captured over several isocycles at room temperature.

3.3 Results

3.3.1 Dynamic $\Pi$-$A$ profiles of human and animal meibomian lipid films

The dynamic $\Pi$-$A$ profile of human meibomian lipid films demonstrated a film that exhibited a liquid-expanded characteristic until compression of the film, where a condensed and more solid-like phase transitioned (Fig. 3.2). The film reached a maximum surface pressure ($\Pi_{\text{max}}$) of 23mN/m with some hysteresis, suggesting there was lag and some stress relaxation occurring at the interface. $\Pi$-$A$ profiles of the animal meibomian lipid films were qualitatively similar to humans with subtle differences (Fig. 3.3). Bovine, wallaby, phascogale, and kultarr films (Fig. 3.3a–d)
all reached very similar $\Pi_{\text{max}}$ in the range of 20–25 mN/m. Koala and rabbit films (Fig. 3.3e–f) reached $\Pi_{\text{max}}$ of 30 mN/m, which is indicative of a more surface active film. Koala films also had larger hysteresis compared with the other films, indicating a potentially more viscous film. Likewise, koala lipid films took longer to reach equilibrium (20 isocycles, versus 3 for humans), indicating greater molecular reorganisation occurring at the interface than the other lipid films. In terms of the lift off positions of the lipid films, with the exception of wallaby (Fig. 3.3b), films took off at a range between 40–45 Å$^2$/molecule. Wallaby lipids took off at 70 Å$^2$/molecule, indicating a larger liquid-expanded phase. Collapse was not evident for any of the species tested.

**Figure 3.2: $\Pi$-A profile of human meibomian lipid film.** Film exhibited a liquid-expanded characteristic until compression, where a condensed and more solid-like phase transitioned. $\Pi_{\text{max}}$ of 23 mN/m was reached, with some hysteresis. Take off point was at 45 Å$^2$/molecule.
Figure 3.3: Π-A profiles of animal meibomian lipid films. Lipid profiles were generally very similar to human lipid films (Fig. 3.2) and to each other, with subtle differences in hysteresis, Π_{max} and lift off points. Koala profile includes the first isocycle (dashed black line), and the equilibrium isocycle (20th cycle) (solid red line).
3.3.2 **Interfacial shear rheology of meibomian lipid films as a function of pressure**

3.3.2.1 **Human meibomian lipid films**

The interfacial shear modulus and complex viscosity for human meibomian lipid films is shown in Figure 3.4. The shear modulus demonstrated that at low surface pressures (5–10mN/m), the meibomian film behaved more like a viscous fluid since the shear viscous modulus (G’’) increased linearly with frequency, while the shear elastic modulus (G’) remained zero (Fig. 3.4a). At the surface pressure of 15mN/m, both G’ and G’’ increased an order of magnitude and subsequently, the film became more solid-like (apparent from the dominance of G’). At the pressure of 20mN/m, both moduli were invariant with frequency, indicating the formation of a classical gel. Overall, the moduli increased two orders of magnitude as the surface pressure of the lipid film increased. This is an indication that the film became more strengthened with film pressure, correlating with Π-A profiles (Fig. 3.2) that showed phase transitions from liquid-expanded to liquid-condensed when film pressure was increased. When complex viscosity was plotted as a function of frequency (Fig. 3.4b), shear thinning behaviour was evident at pressures of 15–20mN/m. At the lower surface pressures, the film behaved like a Newtonian viscous fluid (viscosity not changing with shear rate).
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Figure 3.4: Interfacial shear viscoelasticity of human meibomian lipid films. (a) Shear modulus ($G$) as a function of frequency ($\omega$) demonstrated that at low surface pressures, (5–10mN/m) lipid films exhibited fluid-like, Newtonian characteristics. $G’$ started to grow in at 15mN/m where eventually the film transitioned into a classical gel. (b) Complex viscosity ($\eta^*$) as a function of frequency demonstrated shear thinning behaviour in lipid films where $G’$ started to appear– films at higher surface pressures

3.3.2.2 Animal meibomian lipid films

The shear viscoelasticity of the animal lipid films tested are shown in Figure 3.5. Bovine, wallaby, phascogale and rabbit lipids (Fig. 3.5a–d) generally followed similar trends to humans with some subtle differences. In humans, the film remained fluid at low surface pressures (5–10mN/m) however, with the exception of phascogale (Fig. 3.5c), the other species demonstrated elasticity ($G’$) from surface pressures of 5mN/m (bovine, wallaby) and 10mN/m (rabbit). $G’$ and $G’’$ of these animal lipid films increased with film pressure and formed gels akin to humans. The koala lipid films (Fig. 3.5e) were different from human lipid films because these films were not able to reach higher pressures, even though $\Pi$-A profiles demonstrated that they were able to (Fig. 3.3). Generally, the moduli of the koala lipid films were higher than human lipid films at the equivalent surface pressure by an order of magnitude, suggesting a stronger film is formed at the air-liquid interface.
The viscoelasticity of the kultarr lipid film (Fig 3.5f) was the most different to human lipid films and the other animal films tested. With the exception of a small elastic contribution at 22mN/m (data not shown), the film remained fluid-like at all surface pressures, demonstrating zero G’ and a linear increase in G” with frequency. Furthermore, G” was independent of surface pressure, unlike the other lipid films. The complex viscosity of the animal lipid films (Fig 3.6) demonstrated similar trends to human lipid films, with the exception of the kultarr lipid films (Fig. 3.6f). In these animal lipid films, shear thinning behaviour was observed at pressures where elasticity was evident. Newtonian behaviour was observed in fluid films, such as those films at low pressures with zero G’ and the kultarr lipid films. The complex viscosity of the kultarr lipid films also did not increase with surface pressure, correlating with shear viscoelastic results (Fig. 3.5f).
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Figure 3.5: Interfacial shear viscoelasticity of animal meibomian lipid films. Viscoelastic profiles of bovine, wallaby, phascogale and rabbit (a–d) were generally very similar to humans (Fig. 3.4a). These films formed gels at higher surface pressures (10–20 mN/m). Koala lipid films (e) were not able to reach high surface pressures however, they were still able to form gels at equivalent pressures to human lipid films, although the moduli overall were higher than human lipid films. Kultarr lipid films (f) were drastically different from human lipid films and the other animal species. The film remained fluid-like at all surface pressures with no contribution of $G'$. 
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Figure 3.6: Complex viscosity of animal meibomian lipid films. With the exception of the kultarr lipid films (f), bovine, wallaby, phascogale, rabbit and koala lipid films (a–e) all showed evidence of shear thinning behaviour at surface pressures where there was an elastic ($G'$) contribution. This behaviour was also observed with human meibomian lipid films (Fig 3.4b). Kultarr films demonstrated Newtonian behaviour at all surface pressures.

3.3.3 BAM images of meibomian lipid films

BAM showed that upon deposition, human meibomian lipid films appeared to be inhomogeneous with dark regions as large as several hundred micrometers (Fig. 3.7a). These dark regions (subphase) were surrounded by material (lipids) that was inhomogeneous on shorter length scales. Between surface pressures of
5–15mN/m (Fig. 3.7b–c) the dark regions decreased in size while the surrounding material became more uniform. At pressures of 15mN/m and above (the pressure at which the elasticity of the film increased, Fig. 3.4) the film was homogeneous on length scales as small as the resolution limit of BAM (Fig. 3.7c). Upon expansion, the appearance of the film was similar to just after deposition however, the material retained inhomogeneity on a smaller length scale (large dark regions in the film were no longer present) (Fig. 3.7d). Bovine and koala meibomian lipid films‡‡ showed similar transitions in morphology (data not shown). The kultarr meibomian lipid films were similar to human films upon deposition however, film morphology did not change during compression or expansion isocycles (Fig. 3.7e–h), correlating with the lack of change observed in rheology.

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**Figure 3.7: BAM images of human and kultarr meibomian lipid films.** The morphology of the human meibomian lipid film became more uniform and homogenous as surface pressure was increased (a–d). Kultarr lipid morphology (e–h) did not change with film pressure.

‡‡ Not all animal lipids were examined by BAM. Bovine lipids were tested because they have a similar composition to humans. Koala and kultarr lipids were tested because these lipid films had very obvious differences in their rheology compared with humans.
3.4 Discussion

3.4.1 Interfacial shear rheology of human meibomian lipid films

Through interference microscopy, past researchers have predicted that the meibomian lipid interface behaves as a gel *in vivo* (Yokoi *et al.*, 1996; Goto *et al.*, 2003; Bron *et al.*, 2004; Goto, 2004; Yokoi and Komuro, 2004). Likewise, previous studies using bulk rheology have shown that tear film lipids (free lipids in the bulk) contribute significantly to the non-Newtonian behaviour of tears (Gouveia and Tiffany, 2005). However, this is the first study to show that *meibomian lipids* are capable of forming a 2-dimensional gel at the air-water interface using direct rheological measurements. In this study we used the ISR, a rheometer that measures the *shear* properties of this lipid interface (Brooks *et al.*, 1999). The ISR utilises a Langmuir trough and therefore, it is advantageous in that it allows one to probe the surface at different surface pressures (phase changes can be correlated to viscoelasticity), and at a constant surface area. A constant surface area is important in rheological measurements because it prevents Marangoni stress (defined as stress that arises due to surface tension gradients) complicating surface flows by influencing the interfacial stress which is to strictly come from the forced strain.

This is the first time that the solid-like behaviour observed in the meibomian lipid film, has been reported in lipid-only systems by groups using constant surface area techniques at the air-water interface. On the other hand, lipid monolayers dominated by fluid-like behaviour are common; the behaviour of meibomian lipids at low surface pressures is similar to previously published results for 1,2-dipalmitoyl-sn-
glycero-3-phosphocholine (DPPC) and lung surfactant (Anseth et al, 2005; Nishimura et al, 2008). In both cases, the addition of other materials increased the elasticity of the otherwise fluid lipids. Cholesterol myristate enhanced elasticity of DPPC films but also left the films brittle and subject to breakage (Nishimura et al, 2008). Lung surfactant was shown to gel in the presence of air pollutant and epithelial cell supernatant, but this was attributed to film penetration by proteins. In contrast to these systems, meibomian lipids are a lipid only system capable of deforming without breakage under large strains. Presumably, the diverse combination of lipid species found in meibomian lipids behaves synergistically to enhance the elasticity and ductility of the film without the addition of proteins or other large molecules.

As a function of surface pressure, both elasticity and viscosity increased in meibomian lipid films. On the other hand, DPPC films as a function of pressure did not measure any elasticity, although viscosity increased (personal communication with Danielle Leiske). II-A isotherms of DPPC film is more dramatic than the meibomian lipid film in that it shows three distinct phase transitions (Ma and Allen, 2006), with the solid phase being very pronounced (sharp rise in pressure). This crystalline structure has also been observed microscopically and therefore, it is unusual that DPPC did not show any elasticity in these shear experiments, even at higher surface pressures. It is most likely the multilayered nature of the meibomian lipid interface that accounts for this elasticity. Furthermore, this highlights the need to use other rheological techniques to characterise the meibomian lipid interface. In the case of DPPC films, although shear rheology has indicated fluid-like behaviour,
extensional rheology has conversely indicated a very elastic response (Miano et al., 2006).

### 3.4.2 \( \Pi - A \) profiles and BAM of human meibomian lipid films

The increase in elasticity of the meibomian lipid interface with surface pressure coincided with changes in molecular organisation of the film, and in its morphology. In terms of molecular organisation, \( \Pi - A \) profiles indicated that the film indeed transitioned from a liquid-expanded phase to a more solid-like condensed phase with film pressure. The observed hysteresis in the \( \Pi - A \) profiles is indicative of lag occurring at the surface and hence, this explains the increased viscosity with increased surface pressure.

In terms of film morphology, at low surface pressures the large holes in the film are likely due to low densities in lipids, similar to those observed by Mudgil and Millar (2008) using fluorescence microscopy, and Kaercher, et al., (1993) by BAM. Maruyama et al., (1998) also observed self-assembled domains at zero pressures in docosanoic acid (C\(_{22}\)) monolayers. They postulated that these domains were a result of strong intermolecular attractions between the long hydrophobic chains which consequently, produced an elastic monolayer. A similar mechanism may be present in meibomian lipid films. Although the majority of the acyl chains in wax esters have sixteen to eighteen carbons in human meibomian lipids, the cholesterol ester species include very long chains (C\(_{18}\)–C\(_{34}\)) (Butovich, 2009\(^b\)) which prevent meibomian lipids from forming a 2-dimensional gas phase. Instead, the lipids condense into islands directly upon deposition, but these islands are not yet dense enough to reduce the surface tension of the interface. As the film is compressed, the transition to
classic gel behaviour was observed, along with a homogeneous appearance above a pressure of 15mN/m. This is likely due to increased local packing. Higher molecular packing accompanied with intrinsically strong interactions between the molecules leads to increased mechanical strength. When these films were expanded, the regions of low lipid density observed at deposition were no longer present, which indicates some reorganisation of the lipids as a result of compression.

### 3.4.3 Comparison of animal and human meibomian lipid films

It was not surprising that human and bovine lipids exhibited similar Π-A profiles, viscoelastic properties and film morphologies; the composition of meibomian lipids from both species are very similar (Butovich and Millar, 2009). However, meibomian lipids of several of the other species studied contained lipid species with ratios relatively higher than human or bovine lipids. For instance, wallaby lipids contain a high percentage of triglycerides (Butovich and Millar, 2009). This could have affected the packing of the other lipid species and therefore, may have been the reason why the take-off point of 70Å²/molecule was much earlier than humans (45Å²/molecule). Rabbit lipids contain a large amount of phospholipids (Tiffany, 1979; Greiner et al, 1996; McCulley and Shine, 2003; Butovich et al, 2007) and this could have been a possible reason why there was a higher Πₘₐₓ of rabbit lipid films versus human lipid films.

It is unusual that the koala lipid film exhibited a higher Πₘₐₓ than human films because their composition is strikingly very similar (personal communication with Igor Butovich). Koala lipid films also had larger hysteresis compared with other
films, indicating a potentially more viscous film. Likewise, koala lipid films took longer to reach equilibrium (20 isocycles, versus 3 for humans), indicating greater molecular reorganisation occurring at the surface than the other films. A compositional analysis of the lipids from the other species is still under progress but nevertheless, there still exists subtle and minor differences in composition compared with human lipid films (Butovich and Millar, 2009). Remarkably, the surface pressure dependence of these lipid films and the shear modulus were very similar to that of human and bovine lipid films at room temperature. This simply implies that a range of lipid types at different ratios, versus specific composition of lipids are able to collectively interact to form films with similar properties.

The large hysteresis in the isocycles of koala lipid film correlates with higher shear values at equivalent surface pressures compared with humans. This may also be evidence of irreversible formation of domains during compression cycles. However, the film morphology, as witnessed by BAM for multiple cycles were not drastically different from human and bovine lipid films. At this time it is not known precisely why the koala lipid film exhibited such strong history dependence. The irreversible changes that occurred in the film with compression were on length scales beyond the resolution of the BAM. However, it seems that whatever unique properties koala meibomian lipid films exhibit, result in extremely stable tear films in vivo.

In contrast to the high elasticity of the other animal lipid films, the kultarr lipid film consistently showed no elasticity, and no morphological changes upon compression. Although the composition of these lipids are not drastically different from human
and bovine lipids, kultarr lipid films do not seem to form strong intermolecular interactions and the reason for this is unknown at this point. Part of the problem associated with testing lipids from exotic species such as the koala and kultarr is that the eyelids and thus, the lipids, are hard to obtain. Therefore, the anomalies associated with the koala and kultarr lipid films could not be re-evaluated or further tested. In addition, there is also the possibility of lipid variations within species and this could account for the koala Π-A profile showing high surface pressures which were not able to be obtained during the rheological measurements (different samples were used).

The basis for using rheology to compare a range of animal meibomian lipids to human lipids was to observe whether compositional differences affect the mechanical and structural properties of the lipid interface, and if these differences can be attributed to the presence of a specific lipid species. Besides the kultarr lipids, this study highlighted that compositional differences in the meibomian lipids between species had very subtle effects in rheology, as well as in surface pressure measurements and film morphology. Furthermore, it was inconclusive whether these minor effects were due to a certain class of lipids. For more insight into the possible compositional effects of lipids to the mechanical properties of the meibomian lipid interface, it would be ideal to conduct a more systematic study in the future by seeding these lipids with molar ratios of lipid species already found in meibomian lipids (cholesterol and its esters, free fatty acids and alcohols). This information will also be clinically significant because the lipids of patients who suffer from dry eye have variances in their composition compared with normals (Shine and McCulley,
1991; Shine and McCulley, 1996; Shine and McCulley, 2000; Oshima et al, 2009; Borchman et al, 2012). Not only can rheology be used as a diagnostic tool for assessing dry eye, but understanding these differences may lead to the development of dry eye treatments which better emulate the desirable properties of normal meibomian lipid films.

The secondary reason for comparing animal and human meibomian lipid films was to observe whether structural properties of these lipid films can be correlated to the functionality of the lipid interface, i.e., promoting film stability (prevention of localised thinning and evaporation). It is very likely that the animals which have very long interblink periods, such as the koala and rabbit have a unique lipid interface and tear film structure as a whole. This study did not show any evidence to support this theory however, only the meibomian lipid interface was tested. The stability of the tear film is due to a complex interaction of all three layers and therefore, it is likely that the aqueous layer also plays a critical role in achieving such long, stable tear films. The ideal situation would be to measure the rheology of the meibomian lipid interface with whole tears from the animals (as the subphase). The ISR would not allow this measurement because it requires the subphase volume to be at least 100mL. On the other hand, the pendant drop apparatus (Chapter 4) utilisles small volumes and could easily emulate the animal tear film models. This will certainly be a consideration in future experiments.

Finally, it is important to note that these experiments were part of a pilot study to understand the rheology of meibomian lipid films. Therefore, these experiments were
initially conducted at room temperature. While conducting physiochemical experiments at room temperature is the norm for other lipid systems, meibomian lipids have a melting range between 30–34°C (Butovich *et al.*, 2007), which is much lower than pure lipids. This means that physiologically, their structure is markedly different to room temperature. Indeed, the biophysical properties of the meibomian lipid interface studied by Mudgil and Millar (2011) have demonstrated a more relaxed film (less solid) at 37°C. Essentially, for a better understanding of the shear rheological properties of the meibomian lipid interface, future experiments at 37°C need to be conducted.
Chapter 4

Interfacial dilatational viscoelasticity of human meibomian lipid films; interactions with proteins
4.1 Introduction

Biological interfaces are commonly subjected to a mixture of mechanical stresses. Indeed, the meibomian lipid layer in vivo not only undergoes a shear stress, which arises from the drag action of the eyelids across the surface during a blink cycle, but the surface area of this interface also changes in a blink cycle, undergoing a dilatational deformation. So far, we have observed that meibomian lipids are capable of forming a 2-dimensional gel at the air-liquid interface under shear deformation (Chapter 3). However, it is also likely that this lipid interface will behave differently under a different type of deformation. Therefore, one of the main purposes of this chapter is to characterise the meibomian lipid interface using dilatational rheology in the expectation of providing complementary data to support the shear rheology findings.

The dilatational rheometer that was used in this study is the pendant drop apparatus (Benjamins et al, 1996). The particular advantage of this technique is that it allows small sample sizes to be analysed (as small as 1µL), as opposed to other interfacial techniques, such as the ISR which requires at least 100mL of subphase volume. This means that for the first time, it is possible to use whole tears as the subphase in these experiments, rather than to mimic the tear film with the use of commercial proteins, which is the case when using other rheometers. Using whole tears is particularly important because it consists of a complex array of tear proteins, ions, and also uncharacterised components which may interact with the meibomian lipid layer and affect its rheology. Therefore, the use of whole tears would predict a more realistic representation of how the meibomian lipid interface behaves rheologically in vivo.
While the pendant drop apparatus provides a great opportunity to utilise whole tears, it also enables the modelling of the tear film by using commercial proteins in the subphase that are at the same concentrations as those found in whole tears. Proteins have generally thought to play a major role in the biophysical properties of the meibomian lipid interface (Section 1.4). Consequently, here we were able to elucidate the role of major tear protein (s) or protein combinations to the rheology of the meibomian lipid interface by systematically comparing its rheology with the meibomian lipid-tear interface. Therefore, the aim of this chapter is to not only investigate the dilatational rheology of the human meibomian lipid interface, but to also investigate its interaction with tears and commercial proteins.

4.2 Materials and Methods

4.2.1 Sample preparation
The collection and preparation of human meibomian lipids were conducted as described in Sections 3.2.1 & 3.2.2 however, the concentration of meibomian lipids used in these current experiments was 0.5mg/mL, and hexane (HPLC grade, Labscan, Australia) was used as the spreading solvent (the high density of chloroform was not ideal as a spreading agent on the pendant drop because it fell to the bottom of the drop). The model proteins tested were lysozyme (Lys), lactoferrin (Lf n), human albumin (Alb) and bovine submaxillary mucin (referred to as mucin (Muc) for the remainder of the chapter). All proteins were obtained from Sigma Chemical Company (Sydney, Australia). Protein solutions were made at concentrations based on those reported in stimulated and non-stimulated whole tears (Fullard and Snyder, 1990). Lys (3.2mg/mL), Lfn (1.8mg/mL), Alb (0.1mg/mL) and
a combination of Lys (3.2mg/mL) and Lfn (1.8mg/mL), were dissolved in ATB. The concentration of Muc in tears has been controversial where some authors have used 1–2mg/mL (Holly, 1973), while others have used between 0.1–0.2mg/mL (Tiffany et al, 1998; Pandit et al, 1999; Bron et al, 2004). Here, we have used concentrations of 0.2mg/mL, 1mg/mL, and 2mg/mL dissolved in ATB to compare whether concentration had any rheological effect.

In order to collect enough tears so that a series of experiments could be done on the same sample, reflex tears were collected from a group of volunteers by stimulation using onions. The tears were pooled, centrifuged to remove particulate matter, and then stored in small aliquots (100µL) at -80°C until used. The total protein concentration of these tears using the BCA method (Stich, 1990) was estimated to be approximately 7mg/mL. Ion exchange purified water with a resistance of 18.2 MΩ (Millipore, Milli Q) was used in all solutions.

4.2.2 **Pendant drop rheometry**

The pendant drop technique has been extensively described elsewhere (Benjamins et al, 1996; Monteux et al, 2004; Miano et al, 2006) but briefly, liquid drops ranging in size from 1–25 µL are formed at the tip of a stainless steel needle attached to a syringe that is controlled by an electronic dosing unit. The drop is suspended in air, giving it the pendant shape (Fig. 4.1). An auxiliary syringe (also controlled by an electronic dosing unit) may be used to add another liquid into the subphase of the drop or onto the surface of the drop to form a coating. Drops are imaged using a CCD camera, and software based on the axisymmetric drop shape analysis (ADSA) method described by Neumann and co-workers (Rotenberg et al, 1982; Cheng et al,
1990; Cheng and Neumann, 1992) is used to calculate the dynamic interfacial tension \( (\gamma) \) and surface area \( (A) \) of the drop. A piezoelectric device is used to oscillate (pulse) the volume of the drop via a sine wave function, controlling frequency \( (\omega) \) and amplitude \( (\Delta A) \) (Fig. 2.4).

Changes in drop volume result in changes in \( A \), and are calculated as,

\[
A = A_0 + \Delta A \sin(\omega t)
\]

where \( A_0 \) is the surface area prior to oscillations. Consequently, changes in \( A \) result in changes in \( \gamma \) and are calculated as,

\[
\gamma = \gamma_0 + \Delta \gamma \sin(\omega t + \delta)
\]

where \( \gamma_0 \) is the interfacial tension prior to oscillations, and \( \delta \) is the phase angle which may arise.

The complex dilatational modulus \( (E^*) \) is calculated by correlating these changes to a stress and strain relationship (as described in Section 2.2.3), where changes in \( A \) correspond to strain, while changes in \( \gamma \), correspond to the resultant stress. Therefore, \( E^* \) can be defined as,

\[
E^* = \frac{d\gamma}{dA/A} = \frac{d\gamma}{d\ln A}
\]

---

§§The dilatational modulus, \( E \) (\( E^* \) for complex modulus) is derived similarly to the shear modulus, \( G \) as described in Chapter 2 (Sections 2.2.2 & 2.2.3) however, the major difference between these is that \( E \) uses Young’s modulus in its theory and calculations, whereas \( G \) uses Hooke’s modulus.
Furthermore, $E^*$ can be separated into its real ($E'$) and imaginary ($E''$) components,

$$E^* = E' + iE''$$

where $E'$ represents the in-phase elastic contribution and $E''$ represents the out-of-phase viscous contribution.

**Figure 4.1: The pendant drop.** Typical drops of the same volume hanging from the tip of a needle giving a pendant shape. Interfacial tension and drop surface area are calculated based on the shape of the drop (broken line)-- ADSA profiling. Drops with higher interfacial tensions appear rounder; the addition of surfactants lowers tension, making the drop shape more elongated.

### 4.2.3 Measuring the dilatational viscoelasticity of meibomian lipid films

Viscoelastic measurements were obtained using a commercial contact angle tensiometer (OCA-20, Dataphysics, Germany) via the pendant drop mode (Fig. 4.2). Typically, a water drop with a volume of ~15µL was formed at the tip of a blunt, stainless steel needle (15 gauge, Hamilton Company, Nevada, USA), giving a characteristic surface tension of ~72mN/m. An auxiliary syringe with a fine, sharp pointed needle (32 gauge) was used to apply 0.5µL of the meibomian lipid solution to the surface of the drop. The solvent was given 5 minutes to evaporate before the
drop was lowered into a temperature controlled chamber (First Ten Angstroms, Portsmouth, Virginia) set at 37°C. A further 25 minutes was allowed for the drop to stabilise prior to oscillations. Due to evaporation, the drop volume was readjusted every few minutes via the electronic dosing unit attached to the main syringe.

Analysis of meibomian lipid films at 20°C was also attempted but the meibomian lipids failed to spread properly over the drop surface at this temperature. This was indicated by inconsistent viscoelastic data (data not shown), and the data obtained at the two different temperatures not varying as expected. Therefore, drops coated with meibomian lipids were first heated at 37°C to ensure a spread film (lowered into chamber for 5 minutes), and then temperature of the chamber was switched to 20°C. This cooling process took approximately 30–45 minutes, almost equivalent to the 30 minutes allowed for equilibration of the drop at 37°C. To rule out any aging effects of the interface to rheology, viscoelasticity was measured as a function of time (see below).

**Figure 4.2: Commercial set up of the pendant drop apparatus.** A pendant drop is suspended in a thermostatic chamber. An auxiliary syringe is used to coat the drop with surfactants. Drops are monitored and imaged using a CCD camera, where the ADSA profiling tool is incorporated in the commercial software. The piezoelectric unit is used to oscillate drops via a sine wave function.
4.2.3.1 **Amplitude sweep of meibomian lipid films**

An initial amplitude sweep of the meibomian lipid film was carried out to ascertain the LVR (Section 2.3.2.1). The drop was oscillated at strain amplitudes varying from 1–10%, while the frequency was kept at 1.257 rad/s. The typical amount of iteration steps between amplitudes was 15, and this was divided on a linear scale. The number of cycles per step was set at 3, and 300 images per iteration step were taken for drop shape analysis. Therefore, total experiment time was approximately 4 minutes.

Amplitude sweeps of meibomian lipid films were carried out at both 20°C and 37°C however, only 37°C data are presented here since it is physiologically relevant and trends across both temperatures were similar. A strain amplitude of 2% (or relative area change \( \frac{dA}{A} \) of 0.02) was in the LVR and determined to be the most appropriate strain as non-linear effects were seen at larger strains (Fig. 4.3). This strain was also consistent with other lipid films using the pendant drop technique (Miano *et al*, 2006), as well as our previous work on the ISR which determined a

![Figure 4.3: Amplitude sweep of human meibomian lipid film at 37°C. A strain amplitude of 2%, or relative area change (\( \frac{dA}{A} \)) of 0.02 was in the LVR (see arrows). Non-linear effects were seen above this amplitude.](image-url)
strain of 2.4% to be most appropriate for meibomian lipids at constant area. Therefore, all experiments without proteins were conducted at this strain.

**4.2.3.2 Frequency sweep of meibomian lipid films**

A frequency sweep of meibomian lipid films ranging over 2 orders of magnitude (0.125–12.56 rad/s) was conducted. At 20°C, the typical amount of iteration steps between frequencies was 15–20, and this was divided on a log scale. The amount of cycles per step was set at 5, and 300 images per iteration step were taken for drop shape analysis. Therefore, total experiment time was approximately 15–20 minutes. At 20°C, evaporation of the drop was very minimal but was counteracted with the thermostatic chamber being half filled with water. Evaporation of the drop was significant at 37°C and therefore, experimental oscillations were shortened by reducing the number of cycles per step to 3. Furthermore, the sweep was segmented into 4 consecutive stages (10 iterations between 12.56–1.25 rad/s, 4 iterations between 1.25–0.37 rad/s, 1 iteration at 0.25 rad/s, and 1 iteration at 0.125Hz) so the drop could be readjusted to the starting volume after each stage. Duration of each step was typically from 45–120 seconds, with total experimental time being 5–6 minutes.

**4.2.3.3 Time sweep of meibomian lipid films**

To some extent, meibomian lipid films were aged in experiments prior to oscillations (during heating/cooling stages), as well as being aged in protein experiments (see below). Therefore, the effect of aging to the viscoelasticity of the meibomian lipid film was tested to ensure that aging of the film had no significant effect on the data. Viscoelasticity was measured at the following time intervals after the application of
lips on the surface: 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes. The oscillation frequency was kept at 3.14 rad/s and at each time interval, 4 measurements were taken. The amount of cycles per step was set at 3, and 300 images per measurement step were taken for drop shape analysis. Measurements typically took ~2 seconds at each time interval. Data were collected at 37°C since this temperature was physiologically relevant. Furthermore, it was assumed that trends would be similar at 20°C. Drop volume was maintained at 15µL throughout the experiment.

4.2.3.4 Temperature sweep of meibomian lipid films

Besides conducting experiments at 20°C and 37°C, the effect of temperature on the viscoelasticity of meibomian lipid films was systematically tested. Viscoelasticity was measured at a temperature range of 43–18°C at one degree intervals. The oscillation frequency was kept at 3.14 rad/s and at each temperature interval, 4 measurements were taken. The amount of cycles per step was set at 3 and 300 images per measurement step were taken for drop shape analysis. Measurements at each time interval typically took ~2 seconds while overall, the experiment took ~1 hour since it took some time for the temperature to change and stabilise. Drop volume was maintained at 15µL throughout the experiment as previously described.

4.2.4 Measuring the dilatational viscoelasticity of meibomian lipid films with the interaction of proteins

4.2.4.1 Protein only experiments

Before the viscoelasticity of meibomian lipid films with adsorbed proteins were measured, adsorbed films of just proteins were examined in order to elucidate individual contributions compared with whole tears. A drop of ~5µL of water was
ejected from the main syringe. Using the auxiliary syringe, ~15µL of protein solution was injected into the small drop giving a total volume of ~20µL. The drop was then lowered into the thermostatic chamber and aged for 1 hour prior to oscillation experiments to ensure an equilibrium surface tension was reached. Furthermore, this allowed sufficient time for viscoelasticity not to change, as evidenced by previous studies (Benjamins and Lucassen-Reynders, 1998; Freer et al, 2004). The aging time was also sufficient for the drop volume to be reduced to 15µL, ensuring that the original protein concentration was restored. The drop volume of 15µL was maintained throughout the experiment with the addition of water supplied from the main syringe via the electronic dosing unit. This ensured that the osmolarity of drop was also kept constant.

Frequency sweeps as described above were conducted at both 20°C and 37°C, except the strain amplitude of each protein solution was unique in order to be in LVR. A strain amplitude of 3% was used for Lys, Lfn and Lys + Lfn mixture. A strain amplitude of 5% was used for Alb and Muc solutions. For whole tears, 15µL was added similarly to the protein solutions described above. The drop was then lowered into the thermostatic chamber and aged for 1 hour at either 20°C (no temperature switching was required here) or 37°C (aging time included the time it took to add 15µL of tears). A similar frequency sweep described above was conducted however, a strain amplitude of 3% was used.

4.2.4.2 Meibomian lipid films with adsorbed proteins

_in vitro_ models of the tear film were created by the formation of drops of whole tears/protein solutions at the tip of the needle (as described above) followed by
meibomian lipid solution (0.5µL) being spread on the surface of the drop using the auxiliary syringe. The drop was lowered into the thermostatic chamber set at 37°C and then aged for 1 hour prior to oscillation experiments. For 20°C experiments, the temperature was switched to 20°C after 5 minutes to ensure that the meibomian lipids were properly spread on the surface of the pendant drop. During the aging process, osmolarity and protein concentration of the drop was maintained by maintaining the drop volume (15µL) with the addition of water, as described previously. A frequency sweep was conducted at both 20°C and 37°C, except the strain amplitude of tears and protein solutions coated with meibomian lipids was varied from the strain amplitudes of pure protein solutions and whole tears. With meibomian lipids on the surface, a strain amplitude of 2% was found to be in the LVR and hence, this was used for these experiments.

4.3 Results

4.3.1 Dilatational viscoelasticity of meibomian lipid films

4.3.1.1 Viscoelasticity as a function of time: effect of aging

The aging effect on the meibomian lipid film at 37°C is shown in Figure 4.5. The most notable effect was observed in the first 15 minutes when E’ increased from 15mN/m to 20mN/m. This is most likely due to the film equilibrating and reorientating into a stable formation. From 30–45 minutes, there was a slight change in both moduli, where E’ increased from 23mN/m to 25mN/m and E” changed from 5.5mN/m to 6mN/m. From 60 minutes onwards, there were minimal changes in viscoelasticity where a value of 25mN/m for E’ was maintained, while a value of 6mN/m for E” was maintained. Oscillation experiments with pure meibomian lipid
films at 37°C were started 30 minutes after lipids were spread on the surface of the pendant drop. Although some minimal changes occurred at this time, the total experimental time was very short (6 minutes) and therefore, any aging effect would have been minimised. Experiments at 20°C were started after 45 minutes, so aging here would have had a minimal effect.

![Graph](image)

**Figure 4.4: Time sweep of human meibomian lipid film at 37°C.** Most change in viscoelasticity was observed in the first 15 minutes. Minimal changes occurred from 45 minutes onwards.

### 4.3.1.2 Viscoelasticity as a function of frequency

The interfacial dilatational moduli for human meibomian lipids demonstrated that $E'$ was more dominant than $E''$ at both 37°C and 20°C, indicating that films were more solid-like, regardless of temperature (Fig. 4.5). At 20°C, $E'$ and $E''$ were both higher than the moduli at 37°C ($E'$ of 51–108 mN/m versus 28–45 mN/m and $E''$ of 23–51 mN/m versus 5–12 mN/m), indicating the formation of a relatively stronger and rigid film at cooler temperatures. Films also demonstrated that the moduli were dependant on frequency, where both $E'$ and $E''$ increased with frequency. This is an
indication that the films are gel-like (Section 2.3.2.2) however, these films are not considered to be perfect gels as there was still some dependence of the moduli with frequency.

![Graph showing frequency sweep of human meibomian lipid films.](image)

**Figure 4.5: Frequency sweep of human meibomian lipid films.** The film exhibited a soft, gel-like characteristic at 37°C where there was a slight dependence on frequency by both E’ and E”. A more pronounced effect was seen at 20°C where the viscoelasticity was more than 2-fold higher than at 37°C, indicating the formation of a stronger and more rigid film. Both films demonstrated gel-like characteristics however, these are not considered perfect gels as there was some dependence of the moduli with frequency.

### 4.3.1.3 Viscoelasticity as a function of temperature

The dilatational viscoelasticity of the meibomian lipid interface decreased with increasing temperature (Fig. 4.6). E’ dominated over E” at all temperatures and essentially, the film changed from a rigid solid to a softer solid-like film at temperatures over 32°C. On the other hand, shear rheology (G) demonstrated more obvious transitions from solid-like to fluid-like behaviour (Fig. 4.7). G’ tended to dominate at temperatures below 27°C. At 35°C, G’ was only apparent at high surface pressures, whereas at 37°C, the film was purely viscous at all surface pressures.
Chapter 4 Interfacial dilatational viscoelasticity of human meibomian lipid films; interactions with proteins

Figure 4.6: Temperature sweep of human meibomian lipid film under dilatational deformations. Viscoelasticity decreased with increasing temperature; however, $E'$ still dominated over $E''$ and hence, the film remained a viscoelastic solid.

Figure 4.7: Temperature sweep of human meibomian lipid films as a function of surface pressure under shear deformations. Meibomian lipid films spread on the ISR (Section 3.2.4) were compressed to obtain a desired surface pressure at a set temperature. Viscoelastic measurements were taken at a constant frequency of 6.28 rad/s at each surface pressure. The film was purely viscous at all surface pressures at 37°C. $G'$ was measurable at 35°C, but only appearing at higher pressures. The film eventually transitioned to a solid at 27°C and below– when $G'$ became dominant over $G''$ at all pressures.
4.3.2  Effect of protein adsorption on the dilatational viscoelasticity of meibomian lipid films

4.3.2.1  Effect of whole tears on the viscoelasticity of meibomian lipid films

With whole tears only, and with meibomian lipids on its surface, generally $E'$ was dominant over $E''$ (Fig. 4.8) suggesting that the surface was behaving more like a viscoelastic solid rather than a viscoelastic liquid. Whole tears also demonstrated classical gel-like characteristics at 20°C where there was minimal increase in $E'$ and $E''$ with frequency. On the other hand, whole tears only at 37°C formed a viscoelastic film dependant on frequency. When the rheology of pure meibomian lipid films was compared with whole tears alone, at 37°C, $E'$ of meibomian lipids was greater than $E'$ of the tear only film, whereas $E''$ of both films were almost the same. At 20°C, both moduli of the pure meibomian lipid film were greater than the moduli of the whole tear film. This is an indication that meibomian lipids on water form a stronger and more structured film than tear proteins at the air-liquid interface.

The viscoelasticity of the meibomian lipid film was completely altered with the presence of whole tears in the subphase. At 37°C (Fig. 4.8a), there was a sharper increase in moduli with frequency rather than the relatively more subtle change observed in the lipid and tear only films. Furthermore, $E'$ and $E''$ values of the lipid-tear film appeared to be deviating away from each other with increasing frequency, as opposed to the moduli increasing at the same rate with frequency observed with the other films. Although $E''$ had generally increased overall with the interaction of tears, $E'$ was greater than the meibomian lipid film at higher
frequencies, but lower at slower frequencies, which may be an indication of mixed film behaviour. At 20°C (Fig. 4.8b), $E'$ for the most part was lower than $E'$ of the lipid only film and did not change with frequency, indicating classical gel behaviour. Although it appears that $E''$ was lost and unmeasurable at higher frequencies (>5rad/s), this was most likely due to the sensitivity of the technique.

Figure 4.8: Effect of tears on the viscoelasticity of human meibomian lipid films. The viscoelasticity of the meibomian lipid film was significantly altered with the presence of tear proteins and ions. At 37°C (a), pure tear and meibomian lipid films exhibited gel-like characteristics with less frequency dependence, whereas the lipid-tear film demonstrated a sharper increase in moduli with frequency and, $E'$ and $E''$ deviating away from one another with increasing frequency. At 20°C (b), the lipid-tear film demonstrated classical gel behaviour, as opposed to the frequency dependant behaviour of the lipid only film.

4.3.2.2 Effect of mucin concentration on the viscoelasticity of meibomian lipid films

Figure 4.9a illustrates the effect of different Muc concentrations (0.2mg/mL, 1mg/mL and 2mg/mL) on the viscoelasticity of meibomian lipid films. Only data collected at 37°C were demonstrated here as these were physiologically relevant. Furthermore, results at 20°C demonstrated very similar trends, but with higher dilatational values. Generally, the concentration of Muc in the subphase had minimal
effect on the meibomian lipid rheology, despite demonstrating a clear difference in $E'$ values for all three subphase concentrations (0.2mg/mL being the highest, followed by 2mg/mL and 1mg/mL, respectively). It is worthwhile noting that this variance was minor and within the realms of day to day variances associated with the experimental set up of the pendant drop. $E''$ values of the 0.2mg/mL subphase were slightly higher than the 1mg/mL and 2mg/mL subphases (these values just about overlapped each other).

Figure 4.9: Effect of mucin concentration on human meibomian lipid films at 37°C. (a) Muc subphase concentration demonstrated some variance on the viscoelasticity of the meibomian lipid film however, minor variances are considered insignificant as there are normal variances associated with the experimental set up of the pendant drop. (b) Adsorbed Muc only films demonstrated similar trends where there were minimal differences in viscoelasticity with the varying subphase concentrations. This indicates that Muc concentration generally has no significant effect on interfacial rheology.

A similar trend was also observed with adsorbed Muc only films (without the lipids on the surface) with varying subphase concentrations (Fig 4.9b). Here, the middle concentration of 1mg/mL had the highest viscoelasticity, followed by 0.2mg/mL and 2mg/mL, respectively. Since these results have indicated that subphase concentration of Muc had very minimal effect on rheology, we have used a concentration of
2mg/mL for further experiments. We found that this subphase concentration had the most reproducible data in terms of viscoelasticity and surface tension (data not shown).

4.3.2.3  **Effect of model tear proteins on the viscoelasticity of meibomian lipid films**

Generally, adsorbed protein only films exhibited gel-like characteristics at both 37°C and 20°C: $E'$ was greater than $E''$, and both moduli demonstrated either a very minimal or no dependence on frequency at both temperatures (Fig. 4.10). Besides Muc (Fig. 4.10 c–d), all other films of proteins, such as Lys (Fig. 4.10 a–b), Lfn (Fig. 4.10 e–f), Alb (Fig. 4.10 g–h) and Lys/Lfn mixture (Fig. 4.10 i–j), showed temperature dependence where the moduli increased with decreasing temperature. The reason for the Muc film demonstrating no change in moduli with temperature is unclear, but it could be associated with the large polysaccharide component of Muc.

Generally, the adsorption of proteins to the meibomian lipid interface increased $E'$ of lipid films irrespective of temperature. This indicates that these lipid-protein films have become strengthened and enhanced with the interactions of proteins and lipids at the surface. $E''$ of the meibomian lipid interface with the interaction of proteins was also increased at 37°C, but stayed similar or lowered compared with pure lipid films at 20°C, depending on the protein. The films at 20°C became more rigid as the relative ratio between $E'$ and $E''$ as a function of frequency increased; however, this was not unusual behaviour as there was an expectation that protein and lipid films will become more rigid at lower temperatures.
Chapter 4 Interfacial dilatational viscoelasticity of human meibomian lipid films; interactions with proteins

Figure 4.10: Effect of model proteins on the dilatational viscoelasticity of human meibomian lipid films. Model proteins such as Lys (a–b), Muc (c–d), Lfn (e–f), Alb (g–h), and a Lys/Lfn mixture (i–j) generally increased the viscoelasticity of the meibomian lipid film indicating a strengthened and enhanced film.
In terms of the specific behaviour of proteins, Lys (Fig. 4.10a–b), and Lfn (Fig. 4.10d–e) adsorption demonstrated frequency dependence of both moduli irrespective of temperature. There appears to be strong lipid dominance in these mixed films at 20°C because pure lipid films at this temperature demonstrate frequency dependence of moduli, whereas the pure protein films do not. At 37°C, both lipid and protein features could be dominating in these lipid-protein films because both pure lipid and pure protein films behave similarly.

Lys/Lfn adsorption to the lipid interface (Fig. 4.10i) at 37°C demonstrated a similar trend to the lipid-Lys and lipid-Lfn films discussed above. Surprisingly, the lipid-Lys/Lfn at 20°C was different to the lipid-Lys and lipid-Lfn films at 20°C, as the lipid-Lys/Lfn film demonstrated protein dominated characteristics (frequency independent moduli) rather than the lipid characteristics observed in the lipid-Lys and lipid-Lfn films.

The interaction of Muc with the meibomian lipid interface was more complicated (Fig. 4.10c–d). At both temperatures, only E’ was frequency dependant whereas E” did not change. This may be an indication of mixed film behaviour because frequency independant behaviour for E” is a protein feature that is particularly noticable at 20°C, whereas frequency dependant behaviour of E” is more characteristic of lipid films. The adsorption of Alb to the lipid interface at 37°C

*** Although a similar interaction between Muc and meibomian lipids has already been discussed in Section 4.3.2.2, the results here demonstrate a comparison of Muc interaction with lipids, with pure Muc and lipid only films, and with the other proteins tested.
(Fig. 4.10g) demonstrated similar behaviour to the lipid-Muc film, indicating mixed film behaviour. However, at 20°C (Fig. 4.10h), the lipid-Alb film demonstrated classical gel behaviour (as seen with the lipid-Lys/Lfn film), indicating a film dominated by protein characteristics.

4.3.2.4 Comparison of whole tears with model tear proteins

At both 37°C and 20°C, the viscoelasticity of films of whole tears alone demonstrated the complex modulus ($E^*$) was significantly lower compared with adsorbed films of model tear proteins alone (Fig. 4.11a–b). A similar trend, i.e. lower $E^*$, was also observed when meibomian lipids coated these protein drops (Fig. 4.11c–d). Despite the lower $E^*$ values of tears, the general viscoelastic profile (or shape of the data) reflected the profiles of the model tear proteins, except for Muc, which was more independent of frequency (Fig. 4.11a–b). In the presence of the lipid layer, some differences were seen in the profiles depending upon the temperature. The lipid-tear film demonstrated profile similarities to all lipid-protein films at 37°C, whereas at 20°C, it showed profile similarities with lipid-Alb, lipid-Muc and lipid-Lys/Lfn films (Fig. 4.11c–d).
4.4 Discussion

4.4.1 Dilatational rheology of meibomian lipid films

While we have previously ascertained that the meibomian lipid interface is able to form a 2-dimensional gel at the air-liquid interface under shear deformations (Chapter 3), this has been the first opportunity to study this interface under a different type of deformation. Here we used a novel technique, the pendant drop rheometer, to study the meibomian lipid interface under dilatational deformations. The dilatational viscoelasticity of meibomian lipids demonstrated gel-like behaviour at physiological temperatures however, unlike shear rheology where meibomian lipids formed a classical gel (G’ > G''), moduli were frequency independent, dilatational results indicated that there was still some dependence of the moduli with
frequency. While this frequency dependence is most likely due to the molecular rearrangements of the lipid molecules occurring at the surface, these results still indicate the formation of a gel-like structure, since the dependence of frequency was not strong.

Lowering the temperature to 20°C caused viscoelasticity of the lipid film to increase, making the film more rigid. The increase in viscoelasticity with lowering temperature was expected as the melting range of meibomian lipids is between 30–34°C (Butovich et al., 2007) although overall, these lipids start to soften even at 19°C (Tiffany and Marsden, 1986). Furthermore, Π-A isocycles conducted on the Langmuir trough have revealed that meibomian lipid films at 20°C reach higher surface pressures and are less expanded compared with a 37°C film, thereby indicating a more solid-like characteristic (Mudgil and Millar, 2011). Indeed, at an equivalent mean molecular area of meibomian lipids spread on the pendant drop, surface pressures of 10–13mN/m (tension of 60–57mN/m) was reached at 37°C, whereas pressures of 13–18mN/m (tension of 57–52mN/m) was reached at 20°C.

4.4.2 The effect of proteins on the dilatational rheology of meibomian lipid films

4.4.2.1 Effect of whole tears

The viscoelasticity of the meibomian lipid film was completely altered with the presence of tear proteins and ions compared with the lipid only and tear only films. It was observed that the rheology of the lipid-tear film demonstrated a higher dependence on frequency than the pure films. Also, the overall trend of the lipid-tear
film was altered where lipid features were exhibited at higher frequencies (higher elastic modulus, more solid-like), and protein characteristics were demonstrated at lower frequencies (lower moduli with little frequency dependence). This differs markedly from the model of lung surfactant where the viscoelasticity of DPPC films with mixtures of surfactant proteins B and C (lung surfactant) have shown gel-like behaviour (little frequency dependence), and an overall decrease in elasticity compared with pure DPPC films (Wüstneck et al., 2002; Wüstneck et al., 2005).

In the pulmonary system, proteins had a dominating effect on the lipid film at all frequencies, which is unlike the effect of tears on the meibomian lipid film. These differences are most likely associated with their distinct in vivo functional needs. Furthermore, the range of stresses applied to the tear film is entirely different from those applied to lung surfactant, which is almost always under a dilatational stress (Wüstneck et al., 2005). There needs to be a considerable amount of additional work to resolve which component(s), or combinations of components in the meibomian lipid-tear interface that leads to specific aspects of their rheological profiles.

Although the models used here of the meibomian lipid-tear film mixtures do not fully emulate the in vivo situation of the tear film, the findings are consistent with how the tear film might respond to the stresses that occur during a blink. The force applied to the meibomian lipid layer during the fast opening of motion of the eye in a blink cycle is akin to the high frequencies or forces applied to the in vitro films in dilatation. The lipid-tear film’s enhanced elastic properties under high frequencies resists collapse of the film which is consistent with in vivo findings of the lipid layer.
resisting collapse or break-up during a blink cycle. The additional viscosity and flexibility from the proteins ensure that the film is not brittle and subject to breakage when the eye has finished opening. Interference patterns between consecutive blinks have demonstrated that as the eye opens, the lipid-tear film relaxes, during which there is a slow upward drift of the lipid layer (Yokoi et al., 1996; Goto et al., 2003\textsuperscript{a}; Goto et al., 2003\textsuperscript{b}; Goto, 2004; Yokoi and Komuro, 2004; King-Smith et al., 2009). It is here that the high elasticity is not needed. This could be equated to the lower elasticity of the lipid-tear film at lower frequencies seen \textit{in vitro}.

The fact that the rheology of the meibomian lipid film with the interaction of tear proteins was entirely different at both physiological temperatures and 20°C to their individual elements (meibomian lipid film only and whole tear film only), indicates that there was a definite interaction of tear proteins with the meibomian lipid interface. This complements previous investigations by Millar and co-workers who have demonstrated that tear and model proteins interact with the meibomian lipid layer by increasing its surface pressure (Tragoulias \textit{et al.}, 2005; Millar \textit{et al.}, 2006; Mudgil \textit{et al.}, 2006; Mudgil and Millar, 2008; Millar \textit{et al.}, 2009). These findings add credence to the current models of the lipid layer of the tear film, which show the presence of proteins integrated into the layer (Millar \textit{et al.}, 2006; Butovich \textit{et al.}, 2008), rather than the tear film model interpreted by Holly and co-workers (Holly, 1973\textsuperscript{a}; Holly and Lemp, 1977\textsuperscript{b}). Moreover, the rheology of the lipid layer is affected by the presence of proteins in a complex and synergistic way i.e., not a simple summation of the viscoelastic forces between the separate entities.
4.4.2.2 **Effect of model proteins**

While it is known that tear proteins rheologically alter the bulk properties of whole tears (Gouviea and Tiffany, 2005) (and presumably the aqueous layer of the tear film *in vivo*), here we have demonstrated that they also play a role in altering the rheology of the meibomian lipid interface. The general observation was that the adsorption of model proteins to the meibomian lipid interface increased the viscoelasticity of the lipid interface, indicating an enhanced and strengthened film.

The viscoelastic profiles of the lipid-Lys film and lipid-Lfn film at both temperatures, and the lipid-Lys/Lfn film at 37°C, demonstrated frequency dependant moduli and looked strikingly similar to pure meibomian lipid only films, but with higher moduli. Unfortunately at 37°C, it was hard to determine whether these films were more protein or lipid dominated since pure protein films also behaved similarly to the pure lipid film at this temperature. This may be resolved with future experiments by systematically varying the concentration of the protein subphases. On the other hand, at 20°C, the lipid-Lys and lipid-Lfn films clearly demonstrated lipid dominated behaviour since pure Lys and Lfn films demonstrated frequency independent moduli at 20°C.

This was a surprising finding because Lys (14kDa) and Lfn (80kDa) are globular proteins (Graham and Phillips, 1979a; Kanyshkova *et al.*, 2001) which irreversibly denature at the surface and do not readily change their conformation upon adsorption (Graham and Phillips, 1979a; Graham and Phillips, 1979b; Graham and Phillips, 1980; Benjamins and Lucassen-Reynders, 1998). As such, they would form stable
and rigid structures and demonstrate gel behaviour (protein characteristics). Based on this knowledge, there was an expectation that the irreversible adsorption of protein molecules would dominate over the lipids. This was not the case for the lipid-Lys or lipid-Lfn films, but a mixed lipid-Lys/Lfn film demonstrated frequency independent moduli and therefore, protein characteristics were more dominant. It was unexpected that the lipid-Lys/Lfn film did not follow a similar trend to the individual lipid-Lys and lipid-Lfn films. Most likely, Lys and Lfn form a multimeric complex in the lipid-Lys/Lfn film, which dominates at the surface and does not readily allow the lipid molecules to unfold and interact.

The interaction of Muc and Alb with the meibomian lipid interface was more complicated. For lipid-Muc film, it appears that mixed film behaviour was observed at both temperatures since $E'$ demonstrated frequency dependence while $E''$ demonstrated frequency independence (protein-like feature). Lipid-Alb film also demonstrated mixed film behaviour at 37°C however, at 20°C, it demonstrated classical gel behaviour ($E' > E''$, moduli independent of frequency), which is a protein characteristic. It was surprising that the lipid-Muc film did not demonstrate classical gel behaviour because Muc in vivo do form gels (Pearson et al., 2000). Clearly, the presence of meibomian lipids on the surface altered the protein characteristics of the film.

Conversely, Alb is a globular protein (67kDa) (Graham and Phillips, 1979a) akin to Lys and Lfn. This explains the formation of a gel (lipid-Alb film) at 20°C because gels are stable structures which resist changes in molecular organisation. At 37°C,
most likely the lipids have had some dominance in the lipid-Alb film, which is why there was some dependence of the moduli with frequency. The fact that the lipid-Alb film at 20°C behaved different from the lipid-Lys and lipid-Lfn films at 20°C, despite being a similar class of proteins, could have something to do with the hydrophobic nature of Alb and the extra disulfide bridges present (Graham and Phillips, 1979a), which essentially allowed more rigidity to the film at 20°C. The heating of the film at 37°C most likely enabled more flexibility in the 3-dimensional structure of Alb and therefore, allowed the lipids to integrate and show some dominance (as evidenced by the frequency dependant E’ at 37°C).

4.4.2.3 Interaction of protein mixtures and other tear components

While the use of model proteins have brought to the forefront the potential rheological role of major proteins in tears in vivo, a conundrum still exists because of the unexpected response of the model proteins used in these studies. Put simply, the effects of whole tears as the subphase could not be mimicked by using solutions of the major proteins as the subphase. The possible reasons for this could be that there are non-major proteins in the tear film e.g. surfactant proteins B and C (Bräuer et al, 2007) that interact strongly with the lipid layer and was not tested here.

It could also be that particular combinations of proteins affect the rheology e.g. proteins, such as lipocalin forming multimeric complexes with Lys and Lfn. For this reason there was some expectation that perhaps the protein mixture of Lys and Lfn with meibomian lipids would behave more like the tear-lipid interface. Surprisingly, at physiological temperature, the lipid- Lys/Lfn showed no real difference in
rheology compared to the individual proteins with the lipid interface (lipid-Lys and lipid-Lfn films), so it was difficult to tell which protein was interacting more dominantly, whether the interaction of the proteins were equal, or if the two proteins were bound together and were interacting as a single entity.

Although dilataional values of tears (both alone and coated with lipids) was significantly lower than the model proteins (both alone and coated with lipids), the viscoelastic profile (shape of the data plots) of tears (both alone and coated with lipids) were very similar to the viscoelastic profile of the model proteins (both alone and coated with lipids). This indicates that there is some representation of the model proteins (both in combination and as single entities) in whole tears, although it is difficult to ascertain their specific rheological contribution at this stage. Furthermore, despite not seeing any difference with Lys/Lfn film compared with the single Lys and Lfn films (at 37°C), it should not be ruled out that other protein combinations may in fact reflect the rheology of whole tears. Of particular interest is tear lipocalin which has attracted some attention due to its affinity of binding to lipids (Gasymov et al, 2005). Furthermore, Tiffany and co-workers have demonstrated that holo lipocalin plays a rheological role (Gouviea and Tiffany, 2005) in the bulk properties of whole tears. Therefore, future experiments with both apo and holo forms of lipocalin with meibomian lipids, as well as its combinations with other proteins should be carried out.

Finally, in the studies here, the situation that occurs during dry eye was not addressed. It has been found that the osmolarity of the tears is likely to be much
higher in dry eye (Gilbard, 1985; Tomlinson et al., 2006; Liu et al., 2009). This could affect not only the meibomian lipid interface, but also the performance of proteins and how they interact with meibomian lipids. In some initial studies (Mudgil and Millar, 2011), it was found that increasing the levels of divalent cations had no effect on the surface pressure of meibomian lipid films. Certainly a future consideration will be to test different osmolarities on the rheology of the meibomian lipid layer.

4.4.3 Comparison of shear and dilatational rheology

It first has to be established that the data in these experiments were not simply an artefact of the technique used. This is important because very different values have been obtained in similar experiments involving shear rheometry (Chapter 3). The most obvious difference between techniques was observed with the modulus values. It is commonly observed that dilatational measurements provide modulus values much larger than shear values. Indeed, the dilatational moduli of meibomian lipid films demonstrated values approximately an order of magnitude higher than the equivalent films in shear experiments. This has also been observed with pure lipid films such as DPPC where shear rheology has resulted in values of 0.01–0.3mN/m (Nishimura et al., 2008), whereas dilatational rheology has shown values of 50–400mN/m (Wüstneck et al., 2002; Wüstneck et al., 2005; Miano et al., 2006).

Besides the difference in moduli, the two techniques are theoretically and in this instance, instrumentally different. While we have tried to keep the parameters of the two instruments consistent (such as frequency range, amplitude, temperature, thickness of the meibomian lipid films), the dilatational rheometer used, i.e., the
pendant drop apparatus, produced a film coating a drop†††. On the other hand, the shear rheometer (ISR) utilises a Langmuir trough and therefore, a Langmuir film was produced. Fundamentally, these are two very different types of films.

With shear experiments, the meibomian lipid interface was a 2-dimensional gel at room temperature. Increasing the temperature to physiological temperatures lowered the viscoelasticity overall, and transitioned the film into a viscous dominated film by 33°C (purely viscous at 37°C). It is most likely due to the melting of meibomian lipids that we did not observe any elasticity in the ISR measurements. Therefore, these lipids were able to flow around the rod, whereas below 33°C, they were solid islands stuck together.

Conversely, dilatational rheology showed subtle changes with temperature rather than the obvious transitions observed with shear rheology. The overall viscoelasticity decreased with increasing temperature but the film still remained a gel-like solid, even with higher temperatures. In the pendant drop technique, the measurements are derived from pulling molecules apart and pushing them back together. Therefore, even after the lipids melt, they would still occupy the same amount of space, which explains why the dilatational results did not change much. The dilatational moduli decreased because the surface tension of the meibomian lipid film was higher at

†††The other type of dilatational rheometer that has been used previously is the Langmuir trough with the oscillating barriers (Erni et al, 2005). While this rheometer would have made better comparisons with the ISR, this rheometer is less popular because it is not able to reach high frequencies and it does not mimic the mechanical action of the alveoli, and foams and emulsion systems as well as the pendant drop technique.
higher temperatures (surface tension of 60–57 mN/m was reached at 37°C, whereas 57–52 mN/m was reached at 20°C).

While shear experiments revealed the meibomian lipid interface to be purely viscous at physiological temperatures, dilatational experiments demonstrated the lipid film to be solid-like. The differences in the nature of the film could be attributed to the different types of deformations carried out on these films, rather than the experimental differences between set up of the films. Furthermore, the complexities associated with shear and dilatational rheology also lies in interpreting the results (not just with the theory and instrumentation) and therefore, it was difficult to directly compare the two approaches. To the best of our knowledge, there is only one study that has compared shear and dilatational rheology (Freer et al., 2004). Here, the authors have used proteins at the oil-water interface and have found that trends were different across the two techniques. Other authors have generally discussed that shear and dilatational provide useful complementary data. Certainly, here we have demonstrated that the meibomian lipid interface is able to form a gel-like structure under both shear and dilatational stresses and having this mechanical property suggests that this interface is playing some role in aiding tear film stability and formation.

4.4.4 Evaluation of the pendant drop rheometer

The major advantage of the pendant drop method is that it allows the use of small volumes. For the first time, here we have successfully been able to use whole tears as the subphase, while coating the drop with human meibomian lipids—creating an in vitro model of the tear film. This was a major advantage compared to other
rheological techniques because we have observed that creating a tear film model using commercial protein shows differences in rheology compared with whole tears.

The compositional complexities of tears make this fluid hard to mimic, and the difference in results between using whole tears and trying to emulate them with commercial proteins, highlight the need to use whole tears in understanding the rheology of the tear film in vivo. Moreover, there has also been interest in understanding the tear film structure and dynamics with patients suffering from dry eye and other ocular diseases. Not only can the pendant drop method be used to study the rheology of whole tears and meibomian lipids from these dry eye patients in the future, but it could also be used in both dynamic and static surface pressure/tension studies extending the works of Holly (Holly, 1973\textsuperscript{a}; Holly, 1973\textsuperscript{b}; Holly, 1974), Tiffany (Tiffany, 1991; Nagyova and Tiffany, 1999; Gouviea and Tiffany, 2005), and Millar (Tragoulias et al, 2005; Miano et al, 2005; Millar et al, 2006; Mudgil et al, 2006, Mudgil and Millar, 2008; Millar et al, 2009), and their co-workers. The versatility of the pendant drop apparatus certainly provides a useful tool for studying the biophysical properties of the tear film.

While the pendant drop apparatus has proven to have many advantages, like all instruments and techniques, it also has some limitations which may have had an impact on this study. Miller and co-workers (Leser et al, 2005) have observed that oscillation frequencies of more than 1 Hz (6.28 rad/s) for pendant drops at the air-water interface, deviate away from the Laplacian shape and therefore, measurements obtained past this critical frequency are artificial. Furthermore, the
more viscous a material gets, the lower the critical frequency. For lung surfactants, the frequency limit is 0.2 Hz or 1.256 rad/s (Wüstneck et al., 2005), which fortunately falls within the breathing frequency.

In this study, we have carried out frequency sweeps in the range of 0.02–2 Hz or 0.1256–12.56 rad/s. While this is past the critical frequency limit according to Miller (Leser et al., 2005), we did not see any change in the Laplacian shape for our drops past 1 Hz. Furthermore, frequency limit is dependant on the material and so far, meibomian lipids are not well characterised rheologically. This certainly needs to be considered for future experiments. The frequency ranges in these experiments were chosen for comparing data across both dilatational and shear measurements (Chapter 3). While it can not be ruled out that data obtained here may be artificial at the higher frequencies, the general trends were still similar, even if we disregarded the data past 1 Hz. Moreover, the values of the dilatational moduli in these studies are not critical per se (see below); it is the relative change in rheology between the meibomian lipid interface with and without proteins/tears that is important.

The need for applying rheology to interfaces has led to a surge in developing the theoretical models and consequently, the development of the necessary experimental tools (Miller et al., 2010). However, the main assumption made in these theoretical models is that the interfaces are monolayers. In pulmonary systems, phospholipids such as DPPC do form stable monolayers (Wüstneck et al., 2005; Pérez-Gil, 2008). Likewise, polymers often used in industrial applications also form monolayers (Fauré et al., 1998; Kiss et al., 2004). While instruments have their limitations, limitations
can also arise from the unusual nature of the sample material such as, meibomian lipids. The meibomian lipid interface *in vivo* forms a multilayered structure ~50–100 layers thick. At these thicknesses, we expect the lipid layer in the tear film to closely resemble a bulk phase, meaning the monolayer assumption is no longer valid.

This is quite a unique situation since there are no other multilayered systems being analysed using these physicochemical techniques (to our knowledge) and therefore, the theoretical models in rheology, as well as in physical chemistry have not taken into account this phenomenon. This presents a problem as the theoretical models in interfacial dilatational rheology use surface tension/interfacial tension in calculating the viscoelastic modulus. Interfacial tension is defined as the tension difference between the two immiscible phases. In terms of the tear film lipid interface (Fig. 1.1) there are two interfaces which could have two different interfacial tensions. There is the liquid-liquid interface between the aqueous subphase and the bulk lipids, and the air-lipid interface between the bulk lipids and the air. The dilemma exists in identifying which interfacial tension value to use in these calculations. Furthermore, without knowing the tension of the bulk lipids, it is impossible to calculate the interfacial tensions of the two interfaces.

To overcome this problem, we have not created lipid films as thick as *in vivo*. Instead, lipid films coating the pendant drop were approximately 10nm thick, or 3–5 layers of lipid molecules thick, depending on how these layers pack. Therefore, we assume that it is unlikely that the dominant tension was arising from the bulk of the lipids, but rather it was arising from the tension difference between water and air
(akin to how the surface tension is calculated in a monolayer). Nevertheless, the numerical values which were obtained from the dilatational viscoelastic measurements are more appropriate when used to compare the interface with relative changes within the same meibomian lipid system (changes in composition, the effect of proteins, temperature and time), rather than be compared with other monolayer systems such as pulmonary surfactant.

In terms of interfacial shear rheology and the use of the ISR, theoretical models assume that the flow properties arise predominantly from the surface viscosity rather than the bulk, which is defined as the Boussinesq (Bo) number (Brooks \textit{et al}, 1999; Erni \textit{et al}, 2005; Krägel and Derkatch, 2010). If $Bo \gg 1$, then the drag is experienced from the surface, whereas if $Bo \ll 1$, the drag has arisen from the bulk subphase (Edwards \textit{et al}, 1991). In the case of the meibomian lipid interface, the lipid films created on the ISR were much thinner than \textit{in vivo}, and equivalent to the thicknesses created on the pendant drop. Furthermore, this amount of lipids was still much smaller than the diameter of the oscillation needle, which meant that there were no significant velocity gradients within the lipid layer itself and $Bo \gg 1$. While the bulk forces of the meibomian lipid interface have not been an issue in this thesis, examining the bulk properties of meibomian lipids using more recent methods involving microrheology (Mason and Weitz, 1995; Crocker \textit{et al}, 2000) would be particularly useful for future experiments.
Chapter 5

Effect of a di-block copolymer, EOBO on the dilatational viscoelasticity of human meibomian lipid films
5.1 Introduction
Advances in polymer synthesis techniques and purification have enabled polymers with very specific structures and characteristics to be developed (Moad et al., 2008). Of particular interest are polymers which are designed to be of an amphiphilic nature, usually as copolymers in block formation. Their hydrophobic and hydrophilic regions make them an advantage in many biological applications because they are able to self-assemble at surfaces and interfaces (Alexandridis, 1997; Alexandridis and Lindman, 2000; Hamley, 2005). As such, they are particularly useful as stabilizing agents in emulsion systems (Müller et al., 1997; Riess and Labbe, 2004), as drug delivery tools through their ability to form micellular structures (Gaucher et al., 2005; Jain et al., 2011), and as a preventative against protein and plasma adhesion on medical implants (Amiji and Park, 1992; Gingell and Owens, 1994; Schorën et al., 1995; Kenausis et al., 2000). In ophthalmic applications, these copolymers are being used in similar ways, such as in dry eye formulations and as antifouling agents for contact lens wear.

Here, we have obtained a non-ionic, di-block copolymer that has been specifically designed for contact lens applications (Davies et al., 2011). The hydrophilic block consists of repeating monomers of ethylene oxide units (EO); this is covalently joined with the hydrophobic block of butylene oxide units (BO) (EOBO). While similar ethylene based polymers are already being successfully employed in ophthalmology, such as the tri-block copolymer, Pluronics™ (consists of a hydrophobic propylene oxide block, flanked by two hydrophilic ethylene oxide chains) (Jiao, 2008; Lin et al., 2009; Zhao et al., 2009; Thissen et al., 2010), the
advantage of a di-block copolymer like EOBO is that its molecular weight, block lengths and essentially the hydrophobe/hydrophilic ratio, can be more easily controlled (Alexandridis, 1997). As a result, the architecture of EOBO can be easily manipulated for specific properties and applications and therefore, it may be able to serve as a better alternative to Pluronics™.

While EOBO has been designed for various contact lens applications, its amphiphilic nature indicates that it would also interact with meibomian lipids and tear proteins. This provides a great opportunity to evaluate its interaction with the meibomian lipid interface and proteins on a fundamental basis and therefore, is the purpose of this chapter. Moreover, the interaction of this designer molecule with the lipid interface can be compared rheologically with proteins (Chapter 4).

As a starting point, the interaction of EOBO with the meibomian lipid interface and proteins (Lys) was characterised using dilatational rheology via the pendant drop apparatus. Complementary data focusing on the interaction of EOBO with meibomian lipids was obtained by surface pressure measurements using the Langmuir trough, as well the use of fluorescence microscopy to examine the appearance of these films. These results not only provide information about the basic interaction of tear film components with a controlled synthetic material, but also provide insight into whether EOBO can be used in other ophthalmic applications, such as in formulations for dry eye.
5.2 Materials and Methods

5.2.1 Materials

Human meibomian lipids were collected as described previously (Section 3.2.1) and dissolved in either chloroform (Section 3.2.2) or hexane (Section 4.2.1). These solutions were used for Langmuir trough and pendant drop experiments, respectively. EOBO (solid crystals), was supplied from Alcon™ Laboratories. The EO block consists of 45 repeating units while the BO block consists of 10 units; as such, EOBO has a Mw of 3050g/mol. Fluorescently tagged lipids, NBD-PC (1-acyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino dodecanoyl]-sn-glycero-3-phosphocholine) (Avanti Polar Lipids Inc., OR, USA) was used to seed films for fluorescence microscopy. Ion exchange purified water with a resistance of 18.2 MΩ (Millipore, Milli Q) was used in solutions.

5.2.2 Measuring the viscoelasticity of EOBO, and its interaction with meibomian lipids and lysozyme films

The dilatational viscoelasticity of EOBO (adsorbed films), plus its interactions with meibomian lipids and Lys films were obtained with the use of the pendant drop rheometer, described previously in Chapter 4 (Section 4.2.2). EOBO was dissolved in ATB at a concentration of 0.025mg/mL; although concentrations between 0.4–2mg/mL have been used typically in industry (personal communication with Howard Ketelson, Alcon™ Laboratories). Industrial concentrations were too high for use with the pendant drop apparatus because they caused a very low surface tension (~33mN/m) that made the pendant drop fall off once meibomian lipids were added to the surface of the drop. To measure the viscoelasticity of EOBO, the aqueous
polymer solution was applied as the subphase of the drop in the exact manner and aged for the same time (1 hour) as the protein solutions described previously (Section 4.2.4.1); EOBO molecules were essentially adsorbed to the air-liquid interface in this process. To investigate the effect of EOBO on the meibomian lipid interface, meibomian lipids were spread on the polymer drop surface prior to aging (Section 4.2.4.2). To investigate the effect of EOBO on Lys, a solution of EOBO (0.025mg/mL) with Lys (3.2mg/mL) was made in ATB, and its viscoelasticity was tested both with and without meibomian lipids on the surface.

Amplitude sweeps demonstrated that a strain amplitude of 2% was in the LVR for all drops (EOBO alone, EOBO with meibomian lipids, EOBO with Lys, EOBO with Lys and meibomian lipids) and determined to be the most appropriate strain as non-linear effects were seen at larger strains. A frequency sweep was then conducted on these drops, as described previously (Section 4.2.3.2). Experiments were carried out at both 20°C and 37°C. The results were compared with those from pure meibomian lipid films and lipid-Lys films.

5.2.3 Surface pressure measurements of films of EOBO, and its interaction with meibomian lipids

A set of preliminary surface pressure measurements were carried out on a Langmuir trough previously described in Section 3.2.3. Π-A isocycles of spread films of EOBO, meibomian lipids, and both EOBO and meibomian lipids together (molar ratios) were obtained at the air-interface. EOBO is of an amphiphilic nature so while it has previously been dissolved in ATB for pendant drop experiments, here we have dissolved EOBO in chloroform at a concentration of 0.25mg/mL. By dissolving
EOBO in chloroform, it allowed us to make 1% molar ratios of EOBO with 99% meibomian lipids (molar ratios can not be made with an aqueous and organic solution). The advantage of using molar ratios was that it enabled us to evaluate the interaction of both these molecules together at the surface, whereas an unknown amount of EOBO adsorbed to the meibomian lipid interface with pendant drop measurements. As this was a preliminary study, a 1% ratio was chosen as we were limited to the surface area of the trough. A higher percentage of polymer molecules would have meant a baseline over 0mN/m and therefore, an incomplete profile.

Solutions of pure EOBO (10µL), meibomian lipids (10µL), and meibomian lipids seeded with 1% EOBO (10µL), were deposited to the surface of the ATB subphase before isocycles were started. Experiments were conducted at both 20°C and 37°C.

5.2.4 **Fluorescence microscopy of meibomian lipid films, and its interaction with EOBO**

Spread films of pure meibomian lipids and meibomian lipids seeded with 1% EOBO were imaged using fluorescence microscopy. Pure EOBO films were also examined however, these micrographs did not us give enough information about the structure or phase behaviour of EOBO and hence, this data is not shown here. Furthermore, it was more essential to compare pure meibomian lipid films with lipid films seeded with EOBO molecules since the seeded film was still 99% meibomian lipids.

To examine these films, 1% (molar ratio) of the fluorescently tagged lipids, NBD-PC was mixed with the solutions of pure meibomian lipids and meibomian lipids with 1% EOBO. These mixtures were then spread on the ATB subphase of the Langmuir trough as previously described. The trough was placed under a Leica epifluorescence
microscope equipped with an excitation band pass filter of 450–490nm, a dichroic mirror with a reflection short pass of 510nm and a barrier filter with a line pass of 515nm for labelled lipids (fluorescing green). The film was then observed using a 40× objective giving a total magnification of 400×. Digital images were recorded using an Andor Ixon back illuminated DV887ECS-BV camera at a shutter speed of 0.01 seconds. Images were taken upon compression-expansion isocycles at various surface pressures. Fluorescein did not alter any film characteristics as there were no significant differences found in the Π-A profiles of films with the fluorescently tagged lipid compared with the original lipid film without the fluorescently tagged lipids. Experiments were conducted at both 20°C and 37°C however, images from 20°C only are presented here because at 37°C, films were moving too fast for images to be captured without blurring.

5.3 Results

5.3.1 Dilatational viscoelasticity of EOBO, and its interaction with meibomian lipid and lysozyme films

5.3.1.1 Frequency sweep of pure EOBO films

The dilatational moduli for EOBO films showed that E’ dominated over E”, with both moduli showing little frequency dependence and thereby, indicating gel-like characteristics (Fig. 5.1). Unlike meibomian lipid films and protein films (Fig. 4.5, Fig. 4.10), the moduli showed no temperature dependence; instead, values of 15–25mN/m for E’ and 2–4mN/m for E” obtained were similar at both 20°C and 37°C. Furthermore, the surface tensions of these films were also very similar with values of ~44mN/m at 20°C, and ~42mN/m at 37°C. Compared with meibomian
lipid films (Fig. 4.5), the moduli for EOBO films were much lower, indicating the formation of a relatively weaker gel than the meibomian lipid film.

**Figure 5.1: Frequency sweep of EOBO film at the air-water interface.** The EOBO film displayed a dominance of $E'$ over $E''$, with a slight dependence of $E'$ with frequency. This indicates the formation of a gel-like structure. The rheology of EOBO films showed no dependence on temperature as the moduli were almost the same at both 20°C and 37°C.

### 5.3.1.2 The effect of EOBO on meibomian lipid and lysozyme films

The interaction of EOBO (adsorption from the aqueous subphase) with the meibomian lipid interface demonstrated that at 37°C, there was no significant change in the viscoelasticity of the meibomian lipid interface (Fig. 5.2a), with surface tension lowering upon adsorption from ~53mN/m to ~39mN/m. This is an indication that lipid-lipid interactions that occur within the meibomian lipid film are more dominant than any interaction of EOBO with this film. A similar trend was also observed with the Lys film, where at 37°C, there was no significant change in viscoelasticity with the presence of EOBO (Fig. 5.2b). Again, surface tension of the
Lys-EOBO film was significantly lower at ~39mN/m compared with surface tension of the pure Lys film which was ~45mN/m.

Conversely at 20°C, the viscoelasticity of the meibomian lipid film and the Lys film was significantly lower in the presence of EOBO (Fig. 5.2c–d). The meibomian lipid interface demonstrated that both moduli decreased ~2-fold with the presence of EOBO (Fig. 5.2c), whereas the Lys interface demonstrated a ~3-fold decrease in $E'$ with the presence of EOBO (Fig. 5.2d). The change in surface tension was also similar to that which occurred at 37°C: the adsorption of EOBO to the meibomian lipid interface lowered surface tension from ~57mN/m to ~41mN/m, whereas the Lys-EOBO film had a tension of ~43mN/m compared with the tension of ~50mN/m with the pure Lys film. It is most likely due to the lower levels of lipid-lipid and protein-protein interactions that occur at the lipid-Lys interface at 20°C compared with physiological temperatures (optimal for lipid and protein activity), that has allowed some dominance of EOBO in these films. Furthermore, it appears that differences in surface tension observed with the presence of EOBO in these films were not making a difference to the rheology of the meibomian lipid and Lys films.
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Figure 5.2: Effect of EOBO on viscoelasticity of meibomian lipid and lysozyme films. At 37°C, EOBO had a minimal effect on both meibomian lipid (a) and Lys films (b), as the moduli did not change. Conversely at 20°C, both E’ and E” of the meibomian lipid interface was ~2-fold lower with the presence of EOBO (c), whereas E’ of the Lys film was ~3-fold lower with the presence of EOBO (d).

5.3.1.3 The effect of EOBO on meibomian lipid-lysozyme films

The interaction of EOBO with the meibomian lipid-Lys film is demonstrated in Figure 5.3. At both 20°C and 37°C, EOBO lowered the viscoelasticity of the lipid-Lys film, and rendered the film a classical gel (E’ > E”, with both moduli lacking frequency dependence). The film appeared to show similar characteristics to the pure EOBO film, except with higher moduli. This was in contrast to the lipid-Lys film without EOBO, which demonstrated a clear dependence of frequency at both 20°C and 37°C. The data suggest that EOBO shows some dominance in these lipid-Lys films over the lipid-protein interactions which occur. Furthermore, unlike the interaction of EOBO with the individual Lys and meibomian lipid films at 37°C (Fig. 5.2a and c, respectively), EOBO had clearly lowered the viscoelasticity of the
meibomian lipid-Lys film. This suggests that EOBO has disrupted the interaction of meibomian lipids with Lys at the air-interface.

Figure 5.3: Effect of EOBO on the viscoelasticity of meibomian lipid-lysozyme films. EOBO lowered the viscoelasticity of the lipid-Lys film at both 37°C (a) and 20°C (b). The overall profile of the lipid-Lys film in the presence of EOBO appeared more like the pure EOBO film, demonstrating classical gel behaviour. This was unlike the lipid-Lys film without EOBO, which demonstrated dilatational values clearly dependant on frequency.

5.3.2 Π-A profiles of pure EOBO films, meibomian lipid films, and mixed films of EOBO with meibomian lipids

The dynamic Π-A profile of human meibomian lipids at 20°C have been discussed previously (Section 3.3.1) however, in these experiments we had added less lipids on the surface so the molar ratios were consistent with the seeded films. At 20°C, the meibomian lipid film still demonstrated a liquid-expanded characteristic, until higher compression where a condensed and more solid-like phase transitioned (surface pressure above 10mN/m) (Fig 5.4). Conversely, the film at 37°C was more relaxed (earlier lift off point) and remained in the liquid-expanded phase. The significant difference between the two temperatures was the Π_max, where it was 15mN/m at 20°C and 9mN/m at 37°C.
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The Π-A profile of pure EOBO films demonstrated a Π_{max} of 21mN/m and a lift off point of ~1900 Å^2/molecule (Fig. 5.4). The large lift off point was not surprising since EOBO has a relatively larger Mw than meibomian lipids. It appears that temperature did not significantly alter any film characteristics, but rather made the EOBO film slightly more relaxed at 37°C. Overall, the profile shape of the pure the EOBO film looked very similar to the profile of the meibomian lipid film, where the film appeared to be predominantly in the liquid-expanded to liquid-condensed phase. EOBO films demonstrated very little hysteresis (molecular rearrangement or molecular entanglements) compared with meibomian lipid films which demonstrated relatively larger hysteresis (not clear due to the scaling of the graph). This may be the reason why the viscoelasticity of meibomian lipid films were higher than EOBO films (more stress relaxation occurring at the surface).

In terms of the different phase behaviours observed in EOBO films, the unique structures of polymers means that they do not follow the traditional phases associated with Langmuir films per se, but have similar meanings (Fauře et al, 1998; Muñoz et al, 2000). It can be inferred from the EOBO profile that there was a semi-dilute phase from surface pressures of 0–3mN/m. This means that the polymer molecules are in a 2-dimensional disordered state– usually in parallel arrangement. This is also known as the pancake regime (dilute phase would be equivalent to a gas phase). Surface pressures of 3–10mN/m represents biphasic behaviour which means that the molecules are more condensed and as such, some molecules may have started to protrude into the water, orientating the hydrophobic head at the air-interface and hydrophilic tail submerged in water. This is also known as the mushroom regime.
From surface pressures of 10–20mN/m, the molecules have assembled into a 3-dimensional phase known as the brush formation (Muñoz et al., 2000). This is equivalent to a solid phase in traditional Langmuir films where the molecules are condensed and have self assembled with head and tail groups aligning upright at the air-interface.

The Π-A profile of EOBO mixed with meibomian lipids (1% EOBO) demonstrated mixed film behaviour (Fig. 5.4). While it seems that the overall shape was more like meibomian lipids, the higher $\Pi_{\text{max}}$ and lift off occurring earlier at $105 \text{ Å}^2$/molecule, suggests that EOBO has definitely had some impact on the film characteristics. Furthermore, temperature did not have any significant effect on the profile, highlighting the fact that EOBO has shown dominance in these films.

Figure 5.4: Π-A profiles of films of pure EOBO, meibomian lipids and 1% EOBO with meibomian lipids at the air-liquid interface. Pure meibomian lipid films at 20°C demonstrated a liquid-expanded to a condensed and more solid-like phase upon compression. The lift off point was $\sim45 \text{ Å}^2$/molecule and $\Pi_{\text{max}}$ was 15mN/m. The film at 37°C was more relaxed with a $\Pi_{\text{max}}$ of 9mN/m. Pure EOBO films demonstrated a $\Pi_{\text{max}}$ of 21mN/m and a lift off point of $\sim1900 \text{ Å}^2$/molecule. Temperature had very minimal effect. 1% EOBO films demonstrated mixed film behaviour where the overall profile shape looked more like meibomian lipids however; the higher $\Pi_{\text{max}}$ of 20mN/m, lift off occurring earlier at $105 \text{ Å}^2$/molecule, and temperature having a minimal effect, suggests that EOBO has had some impact on this film.
5.3.3 Microscopic appearance of meibomian lipid films, and its interaction with EOBO

At low surface pressure (2mN/m), the meibomian lipid film appeared to be amorphous, indicated by the NBD-PC evenly distributed (Fig. 5.5). As pressure of the film was increased, dark non-fluorescent patches (NBD-PC poor) started to appear (5mN/m) and merged into larger islands (8mN/m). These islands then appeared to condense into smaller regions (15–20mN/m), probably indicating areas of condensation of some of the meibomian lipids which excluded the NBD-PC.

The seeding of 1% EOBO into the meibomian lipid film markedly changed the appearance of the lipid film. At 0mN/m, the film had positive (fluorescent) and negative (non-fluorescent) 2D bubbles indicating that the meibomian lipids and the EOBO were both on the surface at the same time with a good deal of non mixing. This observation also indicated that the NBD-PC was preferentially partitioning the molecules, but it was not possible to determine if this was with the meibomian lipids or the EOBO. As surface pressure was increased, the diffuse grey areas indicated that the meibomian lipids and the EOBO were mixing (5–15mN/m) however, there were still patchy areas present, even at the highest surface pressure (20mN/m), indicating that there was still some non mixing occurring.
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Figure 5.5: Micrographs of a pure meibomian lipid film, and meibomian lipid film seeded with 1% EOBO (20°C). Pure meibomian lipid film appeared to be initially amorphous. As film pressure was increased, darker regions and islands started to appear, indicating the formation of lipid domains. The presence of 1% EOBO in the lipid film markedly changed the appearance of the film. At low surface pressures, both EOBO and meibomian lipids were occupying space on the surface however, these molecules were not mixing. As pressure was increased, diffuse grey patches formed indicating that these molecules started to integrate at the surface.

5.4 Discussion

5.4.1 Effect of EOBO on the dilatational rheology of meibomian lipids and lysozyme films

Up until now, this thesis has only observed the effects of other tear film constituents, namely proteins, on the viscoelasticity of the meibomian lipid interface (Chapter 4). Here, we have been able to investigate the effects of a controlled, synthetic designer molecule, an amphiphilic di-block copolymer (EOBO) on this interface. Remarkably, this is one of the very few studies which has examined the rheological effects of a block copolymer on lipid films. An initial frequency sweep conducted on adsorbed films of pure EOBO demonstrated E’ values of ~15–25mN/m, with the modulus demonstrating little frequency dependence. While, there are no reported dilatational values of this particular copolymer in literature, similar non-ionic polymers such as
Pluronics™ and polypropylene glycols have also demonstrated $E'$ values of ~20mN/m (Muñoz et al., 2003; Noskov et al., 2006; Noskov, 2010). The frequency dependence of $E'$ on these polymers had been attributed to the stress relaxation occurring inside the surface layer (due to polymer entanglements in the layer) (Noskov et al., 2006). This was most likely also occurring in the EOBO films. Surprisingly, shear elastic values of pure EOBO films were not attainable from the ISR due to the sensitivities of the instrument (personal communication with Danielle Leiske, Stanford University). This really substantiates the usefulness of the pendant drop rheometer and in general, the dilatational technique as it is a relatively novel method used to characterise polymer structures (only been used in the last decade) (Noskov, 2010).

Unlike proteins, the viscoelasticity of the meibomian lipid interface was not enhanced, but instead lowered (20°C) or unchanged (37°C) with the adsorption of EOBO. This was somewhat unexpected as the surface tension of the lipid interface at both temperatures lowered markedly with EOBO adsorption, indicating some interaction. This interaction was further supported by the fluorescence micrographs. Nevertheless, we have also previously observed that surface tension changes do not necessarily translate to a change in dilatational rheology (tears, Chapter 4). We speculate that the lack of change in rheology at 37°C was most likely due to the strong lipid-lipid interactions which occur within the meibomian lipid film and has prevented EOBO molecules cohesively integrating into this interface. Another possibility is that these lipid-lipid interactions were more dominant than any interaction of EOBO with this interface.
At 20°C, it appears that mixed film behaviour was displayed with the lipid-EOBO film because the dilatational values lay between the values of the pure meibomian lipid film and pure EOBO film. Lipid-lipid interactions at 20°C are weaker and this may have allowed some dominance of EOBO into this film. Ferri et al, (2005) also observed that the dilatational rheology of a DPPC film lowered in the presence of a Pluronics™ (22°C), but along with their surface pressure data, they suggested it was the repulsive behaviour between the polymer and lipid molecules that contributed towards this result. In our case, we also saw some evidence of non mixing of EOBO with meibomian lipids in the fluorescence micrographs (2d bubbles). Fuller and co-workers have also demonstrated the minimal interaction of EOBO with DPPC monolayers in shear rheology, X-ray reflectivity and surface pressure experiments (personal communication with Danielle Leiske, Stanford University). This was unlike proteins, which also had a lower (or similar) viscoelasticity to meibomian lipids, but when in combination with lipids, the viscoelasticity was markedly increased and enhanced.

The interaction of EOBO with Lys demonstrated a similar trend to the lipids where there was either no change in viscoelasticity (37°C), or the viscoelasticity was lowered (20°C). In this case, because both Lys and EOBO molecules were being adsorbed to the interface (instead of spread films, akin to the lipids), it is quite possible that at 37°C, Lys adsorbed to the air interface quicker and therefore, its presence dominated the film instead of EOBO. Furthermore, protein-protein interactions and the degree of protein unfolding at the interface at 37°C could have prevented the interaction of EOBO with Lys.
At 20°C, viscoelasticity of the EOBO-Lys film was almost the same as pure EOBO and this was most likely because EOBO molecules had adsorbed to the air interface first and therefore, prevented any interaction of Lys with EOBO. A similar finding was also observed with Leiske et al., (2009) where the shear rheology of Lys during adsorption to the air interface lowered drastically in the presence of EOBO. They also speculated that EOBO molecules adsorbed quicker than Lys to the air-interface. While this idea seems plausible, it should not be ruled out that Lys could have indeed adsorbed faster than EOBO, but once at the interface, EOBO simply displaced some or all of the Lys molecules off the surface. Even if some Lys molecules were still present at the interface, the relatively small amount of protein unfolding and protein-protein interactions at 20°C may not have been enough to prevent the dominance of EOBO to the viscoelasticity of this film.

While EOBO had demonstrated very little influence to the individual Lys and the meibomian lipid interface at physiological temperatures, its interaction with the integrated meibomian lipid-Lys film significantly lowered viscoelasticity at both 20°C and 37°C. It appears that EOBO has disrupted the important interactions between the meibomian lipids and the protein molecules which are vital for creating a proper tear film interface overall. At this stage, the mechanism of how EOBO interacts with proteins and lipids at interfaces remains speculative. One scenario that fits both temperatures is that EOBO adsorbs and penetrates the meibomian lipid interface first and creates a brush formation. Small angle neutron scattering experiments have shown that EOBO molecules (different block lengths to this study) were able to form a brush on solid surfaces (Griffiths et al, 1998) and therefore, also
likely to do the same at the lipid interface. This brush formation could then have created a steric hindrance to the Lys interaction with the lipids and thereby, lowered viscoelasticity of the lipid-Lys film. This may be a strong possibility because polymer brush formations have shown to create a steric hindrance to protein and plasma adhesion in other studies (Amiji and Park, 1992; Gingell and Owens, 1994; Schorën et al, 1995; Kenausis et al, 2000). Nevertheless, it is clear that the mechanism of how EOBO interacts with the meibomian lipid interface needs to be better understood and for the future, it should be examined at the neutron level (deuteration of the EOBO molecules).

5.4.2 Surface pressure measurements and fluorescence microscopy of the interaction of EOBO with meibomian lipid films

In terms of surface pressure and fluorescence microscopy experiments, results were difficult to correlate to the rheological data because the experimental set up was quite different (spread films of EOBO, versus adsorbed films), and as mentioned above, surface tension/pressure changes do not necessarily translate to rheological changes. In fact, where EOBO did not enhance the viscoelasticity of meibomian lipids, seeding meibomian lipid films with 1% EOBO did enhance surface pressure of the meibomian lipid film at both physiological and room temperatures. Whether or not surface pressure enhancement was due to a coupled interaction between the EOBO and meibomian lipid molecules, or simply because EOBO is a surface active molecule that has occupied space at the surface, was not conclusive. It certainly appears that the film had mixed film behaviour because of the higher $\Pi_{max}$ and earlier lift off point. Furthermore, considering that the film was 99% meibomian lipids,
there was not much difference in film characteristics with varying the temperature, further highlighting the fact that EOBO had shown some dominance in these films. It would also be interesting to see whether EOBO would affect Lys adsorption to the meibomian lipid film, akin to pendant drop experiments. This will certain be considered for future experiments.

Unfortunately, the overall profile shape of both pure meibomian lipid and EOBO films were also very similar and therefore, it was difficult to ascertain the role of EOBO in the seeded film. Fluorescence micrographs indicated that at all surface pressures, EOBO molecules were interacting with the meibomian lipid film. As film pressure was increased, EOBO was possibly being better integrated into the film as images looked similar to meibomian lipid films after lipocalin adsorption and penetration (Millar et al, 2009). Conversely, Leiske et al, (2009) observed through Π-A profiles that EOBO molecules were being squeezed out of DPPC films at high surface pressures. This was not the case here because the micrographs definitely indicated the presence of EOBO at the surface. Instead, a possibility could be that the two classes of molecules were simply not mixing at the interface and interaction was forced at the surface as barriers were being closed. Nevertheless, it would be interesting to see whether the rheology of the lipid-EOBO film could be correlated to surface pressure changes and is something that needs to be considered for future experiments. Indeed, changes in surface pressure observed in Π-A profiles of DPPC mixed with EOBO were correlated in shear experiments (Leiske et al, 2009).
5.4.3 **Application of EOBO in ophthalmology**

Although our results have not shown how EOBO is interacting with the meibomian lipid interface and proteins, we have demonstrated that EOBO is able to inhibit some lipid-protein interactions occurring at the meibomian lipid-Lys interface. This polymer has been developed specifically for contact lens applications and clearly, its use would be a success. Firstly, it could be used as a coating, where it would adopt a brush formation and prevent lipid and protein depositions. Secondly, it would be useful as a cleaning agent for worn lenses, especially if this polymer is able to displace lipids and proteins off surfaces. Furthermore, since the use of Pluronics™ are already very successful in ophthalmology, the use of EOBO would be an advantage since its architecture could be easily manipulated (Alexandridis, 1997), and the hydrophobic moiety of butylene oxide is more hydrophobic than the equivalent propylene oxide units found in Pluronics™ (Nace, 1996). As such, this may be an advantage for preventing fouling against the more recently developed silicone hydrogel contact lenses, which are preferred for their oxygen permeability and long term wear option, but have a relatively higher surface hydrophobicity which makes them more susceptible to lipid fouling (Jones *et al.*, 2003; Maziarz *et al.*, 2006; Stapleton *et al.*, 2006; Pucker *et al.*, 2010).

In terms of using EOBO in other ophthalmic applications, while some of our experiments have suggested that EOBO does not mix well with lipids and proteins, surface tension/pressure measurements suggest that EOBO is able to enhance surface pressure of the meibomian lipid film. Certainly, patients with dry eye whose lipid film is compromised may benefit with EOBO treatments as this would increase film
pressure. However, while film pressure may be improved, viscoelasticity of the film may decrease and cause further instabilities. At this stage, it is more important to elucidate which particular feature of the EOBO copolymer is responsible for a specific property, so EOBO can be used to target an exact problem. This would be tested by using EOBO samples with different block lengths, and should be carried out in the future.
Chapter 6

General summary, conclusions and future directions
The work presented in thesis primarily used *rheology* to characterise the tear film’s outer most layer– the meibomian lipid interface. Two distinct rheological techniques were used here; *shear* and *dilatational* rheology both established that meibomian lipids form a *viscoelastic* film at the air-liquid interface. This is presumably the case *in vivo* as well.

Under *shear* deformations, the meibomian lipid film demonstrated classical gel behaviour (Chapter 3). Meibomian lipids from a number of animal species, mainly Australian marsupials which have long interblink rates, were also tested and showed similarities with human lipids (kultarr excepted), despite differences in their lipid compositions. This either suggests that 1) a specific composition of lipid mixtures is not necessary to create this highly viscoelastic interface, rather the different ratios of lipids are able to synergistically combine to form similar viscoelastic films; or 2) meibomian lipids are resilient to minor compositional changes and therefore, require analysis of films at higher resolutions (X-ray reflectivity and neutron scattering experiments).

Under *dilatational* deformations, the meibomian lipid film formed a gel-like structure (Chapter 4), but not the classical gel observed in shear experiments. Here, the viscoelastic moduli demonstrated frequency dependency, which indicates that lipid molecules exhibit some reorganisation on the surface under stress. The differences between the shear and dilatational techniques have been already been discussed however, these experiments have essentially provided complementary information about the rheology of the meibomian lipid interface *in vitro.*
This chapter also highlighted the successful use of the pendant drop rheometer. This technique allows the use of small volumes and consequently, we were able to emulate an *in vivo* model of the tear film by using whole tears as the subphase, and then coating this drop with meibomian lipids. Not surprisingly, the rheology of the meibomian lipid film changed significantly with the adsorption of proteins and ions from tears. The lipid-tear film demonstrated mixed film behaviour where lipid features were dominant at higher frequencies, whereas protein characteristics were dominating at lower frequencies.

Commercial proteins (Lys, Lfn, Muc, Alb and Lys/Lfn mixture) were also tested here in the exact concentrations found in whole tears. Generally, the viscoelasticity of the meibomian lipid interface was enhanced by the interaction of proteins. However, these model proteins and combinations did not really replicate the viscoelastic values of the lipid-tear interface. Instead, there were similarities in the overall trend of the data (profile) between proteins and tears, indicating that these model proteins are playing some role in tears, and in the *in vivo* system.

While the contribution of proteins to the rheology of the meibomian lipid interface was highlighted in Chapter 4, Chapter 5 evaluated the effect of a synthetic molecule on this interface. The amphiphilic di-block copolymer, EOBO did not enhance the viscoelasticity of the meibomian lipid film like proteins did; instead EOBO lowered or did not change its viscoelasticity. Furthermore, EOBO lowered the viscoelasticity of the lipid-protein film, indicating that it is able to disrupt the important lipid-protein interactions that occur within this interface that is responsible for
Chapter 6 General summary, conclusions and future directions

maintaining the integrity of the tear film as whole. This data suggests that EOBO would be useful in contact lens applications e.g. as an anti-fouling agent to prevent lipid and protein depositions on lens surfaces.

In terms of future directions for this thesis, a systematic evaluation on the contribution of proteins to the meibomian lipid interface still needs to be conducted. While our results have shown some similarities between the model proteins used versus whole tears, there were also some differences observed, namely in the dilatational values obtained. Future experiments involving different protein combinations, as well as using proteins such as lipocalin, need to be conducted. Likewise, shear measurements have indicated minor difference observed with animal meibomian lipid films versus human lipid films, despite their compositions being different. While this could be due to the resilient nature of meibomian lipids, a systematic evaluation of meibomian lipids seeded with different classes of lipids already found in meibomian lipids, will provide better information.

Along with rheology, this thesis has also used other interfacial tools to examine the meibomian lipid interface. While surface pressure measurements and imagery have provided complementary data, it has also shown results opposite to viscoelasticity e.g. the EOBO study in Chapter 5. As mentioned above, examining the meibomian lipid interface at a higher resolution may provide an advantage, especially when coupled with rheology. As such, our preliminary data using X-ray reflectivity has shown that the meibomian lipid interface comprises three phases i.e., three different classes of lipids that form this interface (Palaniappan et al, 2010).
Neutron scattering experiments on this interface will provide an even better resolution as this technique allows the deuteration of molecules. Therefore, this technique would be able to ascertain exactly how the lipid molecules (deuterated) are orientated in the lipid interface. This would be especially useful in understanding the interaction of proteins at the meibomian lipid interface, as well as the interaction of polymers such as EOBO. Part of the difficulty associated with understanding the viscoelasticity results from the EOBO interaction with meibomian lipids and proteins was that it was unclear as to how this polymer was interacting with the film. Neutron scattering would certainly provide a better understanding of the structure and properties of the meibomian lipid interface and is something that will be considered for the future.

Finally, the information reported this thesis could be translated to clinical settings. Indeed, Yokoi et al. (2008) has modelled the velocity profiles of tear films of normal patients and aqueous-deficient dry eye patients into rheological data. As a starting point, we can certainly assess the viscoelasticity of the meibomian lipid interface from dry eye patients and compare them to normal samples. Likewise, tear samples can also be collected and analysed.

The information reported in this thesis has provided a sound basis for understanding the mechanical properties of the meibomian lipid interface in vivo. The formation of a gel at the surface layer of the tear film is most likely a stability mechanism, and is supported by clinical studies that show limited rearrangements of the surface of the tear film between blink cycles (Yokoi et al., 1996; Goto et al., 2003; Goto et al.,
2003\textsuperscript{b}; Goto, 2004; Yokoi and Komuro, 2004; King-Smith \textit{et al}, 2009). These findings demonstrate that in addition to lowering the overall surface tension of the tear film, meibomian lipids play a significant role in maintaining the integrity and structure of the tear film.
References


Monteux C, Fuller GG, Bergeron V (2004). Shear and dilational surface rheology of oppositely charged polyelectrolyte/surfactant microgels adsorbed at the air-


