Protein Deposition and Bacterial Adhesion to Conventional and Silicone Hydrogel Contact Lens Materials

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Introduction

Contact lenses suffer from the same problems of deposition that other biomaterials exhibit, being rapidly coated with a variety of proteins, lipids and mucins. The first event observed at the interface between a contact lens and tear fluid is protein adsorption. Protein deposits on contact lenses are associated with diminished visual acuity, dryness and discomfort and lid-related inflammatory changes.

The aim of this thesis was to determine the quantity and the conformational state of lysozyme deposited on contact lens materials over various time periods and also to determine the clinical relevance of protein deposits on contact lenses. The specific aims of each chapter of this thesis were as follows:

- Chapter 4: To determine the total lysozyme deposition on conventional and silicone hydrogel contact lens materials as a function of time by artificially doping lenses with ^{125}I-labeled lysozyme.
- Chapter 5: To determine the conformational state of lysozyme deposited on conventional and silicone hydrogel contact lens materials as a function of time using an *in vitro* model.
- Chapter 6: To quantify the total protein, total lysozyme and the conformational state of lysozyme deposited on a novel, lathe-cut silicone hydrogel contact lens material after three-months of wear.

- Chapter 7: To determine the relationship between protein deposition and clinical signs & symptoms after one-day wear of etafilcon lenses in a group of symptomatic and asymptomatic lens wearers.
- Chapter 8: To determine the influence of individual tear proteins (lysozyme, lactoferrin and albumin) on the adhesion of Gram positive and Gram negative bacteria to conventional and silicone hydrogel contact lens materials.

Methods

- Chapter 4: Conventional hydrogel FDA group I (polymacon), group II (alphafilcon A and omafilcon A), group IV (etafilcon A and vifilcon A), polymethyl methacrylate and silicone hydrogel lens materials (lotrafilcon A, lotrafilcon B, balafilcon A, galyfilcon A and senofilcon A) were incubated in a lysozyme solution containing ^{125}I-labeled lysozyme for time periods ranging from 1 hour to 28 days. After each time period, lysozyme deposited on contact lens materials was determined using a Gamma Counter.
- Chapter 5: Conventional hydrogel FDA groups I, II, IV and silicone hydrogel lens materials were incubated in lysozyme solution for time periods ranging from 1 hour to 28 days. After each time period, the lysozyme deposited on the lenses was extracted and the sample extracts were assessed for lysozyme activity and total lysozyme.
- Chapter 6: 24 subjects completed a prospective, bilateral, daily-wear, nine month clinical evaluation in which the subjects were fitted with a novel, custom-made, lathe-cut silicone hydrogel lens material (sifilcon A). After 3 months of wear, the lenses were collected and total protein, total lysozyme and active lysozyme deposition were assessed.

- Chapter 7: 30 adapted soft contact lens wearers (16 symptomatic and 14 asymptomatic) were fitted with etafilcon lenses. Objective measures and subjective symptoms were assessed at baseline and after hours 2, 4, 6 and 8. After 2, 4, 6 and 8 hour time points, lenses were collected and total protein, total lysozyme and active lysozyme deposition were assessed.
- Chapter 8: Three silicone hydrogel (balafilcon A, lotrafilcon B & senofilcon A) and one conventional hydrogel (etafilcon A) lens materials were coated with lysozyme, lactoferrin and albumin. Uncoated and protein-coated contact lens samples were incubated in a bacterial suspension of *Staphylococcus aureus* 31 and two strains of *Pseudomonas aeruginosa* (6294 & 6206). The total counts and the viable counts of the adhered bacteria were assayed.

Results

- Chapter 4: Lysozyme accumulated rapidly on conventional hydrogel FDA group IV lenses, reached a maximum on day 7 and then plateaued with no further increase. PMMA showed a deposition pattern similar to that seen on lotrafilcon A and lotrafilcon B silicone hydrogel lenses. After 28 days, conventional hydrogel FDA group IV lenses deposited the most lysozyme.
- Chapter 5: After 28 days, lysozyme deposited on group IV lenses exhibited the greatest activity. Lysozyme deposited on polymacon, lotrafilcon A and lotrafilcon B exhibited the lowest activity. Lysozyme deposited on omafilcon, galyfilcon, senofilcon, and balafilcon exhibited intermediate activity.

- Chapter 6: The total protein recovered from the custom-made lenses was 5.3±2.3 μg/lens and the total lysozyme was 2.4±1.2 μg/lens. The denatured lysozyme found on the lenses was 1.9±1.0 μg/lens and the percentage of lysozyme denatured was 80±10%.
- Chapter 7: Correlations between subjective symptoms and protein deposition showed poor correlations for total protein/ lysozyme and any subjective factor, and only weak correlations between dryness and active lysozyme. However, stronger correlations were found between active lysozyme and subjective comfort.
- Chapter 8: Different tear proteins had varying effects on the adhesion of bacteria to contact lens materials. Lysozyme deposits on contact lenses increased the adhesion of Gram positive *Staphyloccocus aureus* 31 strain, while albumin deposits increased the adhesion of both the Gram positive *Staphyloccocus aureus* and Gram negative *Pseudomonas aeruginosa* 6206 & 6294 strains. Lactoferrin deposits increased the total counts of both the Gram positive and Gram negative strains, while they reduce the viable counts of the Gram negative strains.

Conclusions

- Chapter 4: Lysozyme deposition is driven by both the bulk chemistry and also the surface properties of conventional and silicone hydrogel contact lens materials. The surface modification processes or surface-active monomers on silicone hydrogel lens materials also play a significant role in lysozyme deposition.
- Chapter 5: The reduction in the activity of lysozyme deposited on contact lens materials is time dependent and the rate of reduction varies between lens materials. This variation

in activity recovered from lenses could be due to the differences in surface/ bulk material properties or the location of lysozyme on these lenses.

- Chapter 6: Even after three-months of wear, the quantity of protein and the conformational state of lysozyme deposited on these novel lens materials was very similar to that found on similar surface-coated silicone hydrogel lenses after two to four weeks of wear. These results indicate that extended use of the sifilcon A material is not deleterious in terms of the quantity and quality of protein deposited on the lens.
- Chapter 7: In addition to investigating the total protein deposited on contact lenses, it is of significant clinical relevance to determine the conformational state of the deposited protein.
- Chapter 8: Uncoated silicone hydrogel lens materials bind more Gram positive and Gram negative bacteria than uncoated conventional hydrogel lens materials. Lysozyme deposited on contact lens materials does not possess antibacterial activity against all bacterial strains tested, while lactoferrin possess an antibacterial effect against certain Gram negative strains tested in this study.

This thesis has provided hitherto unavailable information on contact lens deposition and its influence on subjective symptoms and bacterial binding. These results suggest that protein deposition has a significant potential to cause problems. Therefore, it is important that practitioners advise their patients regarding the importance of lens disinfection and cleaning and appropriate lens replacement schedules. These results will also be useful for the contact lens industry and the general field of biomaterials research.

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Finally, I would like to thank my parents and my brothers for their love, support and encouragement.

Dedication

To my parents and my brothers.

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List of Symbols and Abbreviations

Å	Angstrom
AA	Acuvue® Advance™
ACN/TFA	Acetonitrile/ 0.2% Trifluoroacetic acid
AV	Acuvue® 2
BioStab	BioStab™ Biomolecule Storage Solution
CH	conventional hydrogel
cm	centimetre
CW	continuous wear
Dk/t	oxygen transmissibility
DPM	disintegrations per minute
DTT	Dithiothreitol
DW	daily wear
EDTA	Ethylenediamine tetra acetic acid
EW	extended wear
FDA	US Food and Drug Administration
FND	Focus® Night & Day™
GLB	gel loading buffer
HEMA	poly-2-hydroxyethylene methacrylate
KDa	KiloDalton
µg	microgram
µl	microlitre

mg	milligram
ml	millilitre
mM	millimolar
MRB	modified reconstitution buffer
ng	nanogram
NVP	N-vinyl pyrrolidone
OD	optical density
PV	PureVision™
PVDF	polyvinylidene difluoride
RB	reconstitution buffer
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH	silicone hydrogel
TBS	Tris-buffered saline
TBS-Tw	Tris-buffered saline with 0.05% Tween® 20
Tris	Tris (hydroxymethyl) aminomethane
USAN	United States Adopted Names
UV	ultraviolet
v/v	volume/volume
WB	Western blotting
w/v	weight/volume

1 Introduction

The use of artificial materials (or "biomaterials") within the body as replacement prostheses has seen a rapid rise over the last few years. [1] As a consequence, the study of interactions between implantation materials and body tissues has become increasingly important. Once inserted, the biomaterial undergoes various interactions with the host biological environment and the "biocompatibility" of the given material depends upon many factors, related to both the host and implanted material. To date, soft contact lens is one of the biomaterials that has received great clinical exposure.

Over the last 35 years, the number of contact lens wearers has increased from 10 million to 140 million, with the vast majority (over 90%) of the patients being fitted with soft, hydrophilic lenses. [2] Recent reports suggest that the contact lens industry is healthy and the worldwide soft contact lens market is estimated at $5.3 billion, while the U.S. market is estimated at $1.9 billion. [3] However, it has been observed that 35% of contact lens wearers discontinue from lens wear due to various issues including, discomfort and dryness, [4] possibly as a direct consequence of lens deposition. [5, 6]

Contact lenses suffer from the same problems of deposition that other biomaterials exhibit, being rapidly coated with a variety of proteins, lipids and mucins. [6-13] The first event observed at the interface between a contact lens and tear fluid is protein adsorption.[14, 15] Of late, the study of interaction of tear proteins with contact lenses has become an important field of research, following the widespread use of contact lenses in many physiological and pathological conditions. Tears have a rich and complex

composition, allowing a wide range of interactions and competitive processes. Protein adsorption on contact lenses is the overall result of various types of interactions between the different components present, i.e. the chemical composition and the surface charge, the structure of the protein molecules, the nature of the medium (tears) and many other solutes present in tears.

To improve the biocompatibility of contact lenses with the ocular surface, there needs to be a better understanding of the way in which contact lenses interact with the corneal surface and the tear film, and of the lens-related factors contributing to infection and inflammatory responses. The aim of this thesis is to gain a greater understanding of the interaction of tear proteins with conventional and novel silicone hydrogel lens materials and also to determine the influence of tear protein deposits on the subjective symptoms and bacterial adhesion to contact lens materials.

Chapter 2 of this thesis provides an overview of literature concerning various contact lens materials, the tear film, tear film proteins, deposition of tear proteins on contact lens materials, various methods used to determine the protein deposited on contact lenses and also on the major corneal infections related to contact lens wear. Chapter 3 lists the various objectives of this thesis. Chapter 4 determines the quantity of total lysozyme deposited on various conventional and silicone hydrogel contact lens materials over time. Chapter 5 discusses the conformational state of the lysozyme deposited on various conventional and silicone hydrogel contact lens materials as a function of time using an *in vitro* model. Chapter 6 determines the protein deposition on a

novel, lathe-cut silicone hydrogel contact lens material after three months of wear. Chapter 7 discusses the relationship between protein deposition and clinical signs and symptoms after one-day wear of etafilcon contact lenses in a group of symptomatic and asymptomatic contact lens wearers. Chapter 8 determines the effect of protein deposits on bacterial adhesion to conventional and silicone hydrogel contact lens materials. Chapter 9 provides a summary of all the results from this thesis and chapter 10 recommends a list of projects that can be conducted as a continuation of this research work, which would be valuable additions to the literature and would help in the development of safer and more comfortable contact lenses.

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2 Literature Review

2.1 Contact lenses

Contact lenses are one of the most widely used biomedical devices in the world. [1] They have been studied extensively with respect to their level of deposition, primarily due to their non-invasive use and easy recovery compared to other biomaterials in contact with biological fluids. [2] Contact lens materials can broadly be classified into two types based on their modulus and elasticity, (a) water-containing soft (hydrogel) and (b) non-water containing rigid gas-permeable (RGP) materials.

2.1.1 Conventional hydrogel contact lens materials

Hydrogels are water-absorbing, hydrophilic polymeric materials. The amount of water adsorbed by the hydrogel is described by the term "equilibrium water content" (EWC) and this factor strongly influences the polymer's surface, mechanical and transport properties. [3] The first successful material of this type (poly-2-hydroxyethyl methacrylate [polyHEMA]) was developed by Wichterle and Lim in the late 1960's as a general purpose surgical material. [3] The hydrophilicity of polyHEMA arises from the presence of hydrophilic side groups. [3, 4] As noted above, the amount of water absorbed by a hydrogel is expressed as the EWC, which represents a percentage ratio between weight of water in the gel and weight of the hydrated gel. EWC is a crucial factor for oxygen permeability of polyHEMA-based hydrogels, because oxygen permeation occurs primarily through the water filled channels of the swollen gel.

Oxygen delivery to the cornea depends upon both the oxygen permeability (Dk) of the material and the thickness (t) of the lens. [4] The Dk of conventional hydrogels is directly related to the amount of water that a polymer can hold, since the oxygen dissolves into the water-phase of the material and diffuses through the lens from the anterior to the posterior lens surface. [4] The Dk increases logarithmically with increasing water content of the material. [5] Unfortunately, this reliance on water to maximize Dk has been a severely limiting factor for the development of hydrogels, since water has a Dk value of only 80 barrer. [6]

Morgan and Efron estimated that polyHEMA has a Dk of only 9-10 barrers. [7] In order to increase the Dk of a conventional hydrogel contact lens material beyond that of polyHEMA, it is necessary to incorporate monomers that will bind more water into the polymer. Two principal strategies are available to increase the water content of hydrogels above that of polyHEMA. Small quantities of charged groups such as methacrylic acid or larger amounts of hydrophilic, neutral groups such as polyvinyl alcohol or N-vinyl pyrrolidone are added to polyHEMA or methyl methacrylate to raise their equilibrium water contents to 60% or greater. [4, 8] However the incorporation of these more hydrophilic monomers also results in contact lenses with decreased strength and elasticity. [8] On the other hand, copolymerization with hydrophobic monomers like methyl methacrylate (MMA) and tetraethyleneglycol dimethacrylae (TEGDMA) decreases water content, while increasing lens stiffness and reducing elasticity. [8]

Commercially available hydrogel contact lens materials are divided into various groups depending upon their charge and water content. The United States Food and Drug Administration (FDA) currently classifies contact lens materials into four groups, depending upon their charge and water content (Table 2.1).

Table 2-1: FDA classification of hydrogel contact lens materials

FDA Classification	Group I	Group II	Group III	Group IV
Water Content	Low	High	Low	High
Charge	Non-Ionic	Non-Ionic	Ionic	Ionic

Low = < 50% water; High = > 50% water; Ionic = Charged; Non-Ionic = No charge

From a clinical perspective, it must be remembered that oxygen delivery to the cornea depends upon both the Dk of the material and the lens thickness (t), with thinner lenses providing the cornea with more oxygen, since there is less of a barrier for the oxygen to diffuse through. The most widely cited figures for the minimum acceptable Dk/t are 24 x 10^{-9} units for daily wear and 87 x 10^{-9} units for overnight or extended wear. [9] Recently, a level of 125 x 10^{-9} units has been reported as a requirement to prevent stromal anoxia during closed-eye conditions. [10, 11]

Hydrogel contact lenses are used for different wearing modalities. They are as follows:

1. Daily wear (DW) lenses are cleaned and removed each night and are discarded after a period of time. This period varies from one day to one year, but usually after 30 days or less.

2. Extended wear (EW) contact lenses are worn for 7 days and 6 nights, with the lens disposal occurring after this time-frame. The advantages of extended wear are that this wearing modality offers the convenience of less handling and maintenance and therefore fewer lens solutions and potentially lower costs. Patients can awake in the morning seeing clearly. The disadvantages of extended wear with conventional soft materials are the associated hypoxic complications that occur.
3. Continuous wear (CW) refers to wearing contact lenses while sleeping overnight for periods of up to thirty days and nights consecutively. Under this modality, contact lenses remain on the ocular surface for up to 30 days and nights without removal, after which they are discarded.

To maintain normal corneal physiology and to prevent corneal infection, the corneal tissues require sufficient oxygen to function without compromising cellular processes. Clearly, this is more difficult under closed-eye conditions, when the lid severely limits oxygen transport to the cornea. In order to provide a healthy ocular surface under extended wear and continuous wear conditions, it is important that the lens material transmits a substantial amount of oxygen to the ocular surface. It is clear that the conventional hydrogel lens materials provide inadequate oxygen transmissibilities for safe, edema-free overnight wear. This awareness of the short-comings of conventional hydrogel materials resulted in the development of novel silicone hydrogel materials that would transmit increased amounts of oxygen to the corneal surface.

2.1.2 Silicone hydrogel contact lens materials

2.1.2.1 Development of silicone hydrogel contact lenses

Preliminary attempts (in the 1970's and early 1980's) to use silicone within hydrogel lenses in the silicone elastomers failed due to the exposure of hydrophobic silicone on the surface of the lens material. [12] This resulted in increased lens binding to the cornea, enhanced lipid deposition and decreased in-eye wettability of the lens. [13] In order to cope with this problem, lens materials had to undergo a surface modification process, which would increase the hydrophilicity of the lens surface and make the surface more wettable and hence more biocompatible. [14] Additionally, the surface treatment should maintain a stable tear film layer, be non-irritating, provide low bacterial adherence and minimize deposition of substances from tears. [15]

The most common silicone is polydimethylsiloxane (PDMS), which features a repeating $(CH_3)_2SiO$ unit (Figure 2.1). [12] Due to the presence of the Si-O backbone, PDMS possesses relatively higher oxygen permeability when compared to polyHEMA-based polymers. [16] However, its highly hydrophobic characteristic induces increased lipid affinity, poor wettability and corneal adhesion**.** [12, 16] To overcome these issues, PDMS had to be modified significantly before being considered as a contact lens material. The main modification strategy involved combining the high oxygen permeability property of PDMS with the hydrophilic nature and wettability of polyHEMA. [12] The major challenge in designing these copolymers is combining the hydrophobic silicone macromers with the hydrophilic monomers. [12] The early copolymerizations resulted in opaque and phase-separated materials. However successful materials have been generated, involving

strategies including the use of hydrophilic block siloxane copolymers, siloxane graft copolymers and modification of trimethylsiloxy silane (TRIS), which led to the development of commercial contact lenses based on silicone technology. [16]

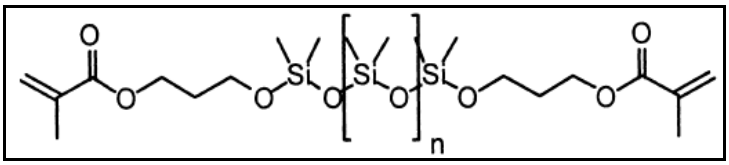

Figure 2-1: The structure of PDMS

Two key approaches significantly contributed to the development of silicone hydrogel contact lens materials. [12, 16] The first approach involved the incorporation of fluorine into silicone hydrogels, resulting in fluorosilicone hydrogels. The reactive site is shown in Figure 2.2. These types of fluorosiloxane-containing monomers enhance the relative compatibility with hydrophilic monomers, obtaining the desired transparent copolymers. [17] Furthermore, fluorosilicone hydrogels improved deposit resistance due to the presence of the fluorinated group. [16, 17]

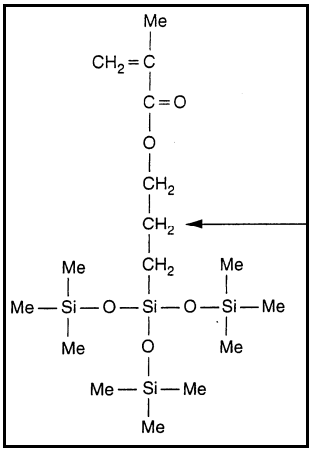

Figure 2-2: Modification site of TRIS by the introduction of hydrophilic groups.

The other approach involved the development of siloxane macromers. A typical siloxane macromer is copolymerized by combining siloxane monomers (such as TRIS), hydrophilic monomers (such as NVP or DMA) and fluorinated monomers. [16] These types of hydrophilic macromers are the major polymeric components used in silicone hydrogel formulations. [16] Figure 2.3 shows the structure of a siloxane macromer used in silicone hydrogel lenses.

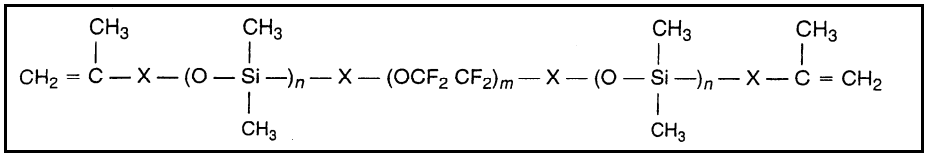

Figure 2-3: Structure of siloxy-based polyfluoroether macromer.

Consequently, silicone hydrogel lenses were developed and were first introduced into the market in 1999. [12] The addition of silicone to the lens increases the material's oxygen transmission, while the hydrogel component allows for fluid transport and lens movement. The combination of these two components allows for safe, extended wear of lenses when compared to conventional lens materials. [4, 13] These lens materials transmit 5-6 times more oxygen than the conventional polyHEMA-based lenses and hence could provide safe overnight wear. [4]

2.1.2.2 Properties of silicone hydrogel contact lenses

Currently six silicone hydrogel lenses are available in the North American market. Table 2.2 summarizes the differences between the six silicone hydrogel lenses that are currently available.

Table 2-2: Characteristics of currently available silicone hydrogel lenses

Proprietary name	Night & Day™	O_2 Optix™	PureVision™	Acuvue® Advance™	Acuvue® OASYS™	Biofinity®
USAN	Lotrafilcon A	Lotrafilcon B	Balafilcon A	Galyfilcon A	Senofilcon A	ComfilconA
Manufacturer	CIBA Vision	CIBA Vision	Bausch & Lomb	Johnson & Johnson	Johnson & Johnson	CooperVision
Water content (%)	24	33	36	47	38	48
Oxygen Permeability (Dk) †	140	110	91	60	103	128
Centre thickness (mm) -3.00D	0.08	0.08	0.09	0.07	0.07	0.08
Oxygen Transmissibility (Dk/t) †† at 35˚C	175	138	101	86	147	160
FDA group	I	I	III	I	I	I
Modulus (MPa)	1.5	1.0	1.1	0.4	0.7	0.75
Surface Treatment	25nm plasma coating with high refractive index	25nm plasma coating with high refractive index	Plasma oxidation process	No surface treatment. Internal wetting agent.	No surface treatment. Internal wetting agent.	No surface treatment
Principal monomers	DMA+TRIS+ Siloxane monomer	DMA+TRIS+Siloxane monomer	NVP+TPVC+NVA+ PBVC	mPDMS+ DMA+HEMA+silo xane macromer+ PVP+ EGDMA	mPDMS+ DMA+HEMA+silox ane macromer+ PVP+ TEGDMA	Unpublished

DMA (N,N-dimethylacrylamide); EGDMA (ethyleneglycol dimethacrylate); HEMA (poly-2-hydroxyethyl methacrylate); mPDMS (monofunctional polydimethylsiloxane) NVP (N-vinyl pyrrolidone); TEGDMA (tetraethyleneglycol dimethacrylate); TPVC (tris-(trimethylsiloxysilyl) propylvinyl carbamate); TRIS (trimethylsiloxy silane); NVA (N-vinyl aminobutyric acid); PBVC (poly[dimethysiloxy] di [silylbutanol] bis[vinyl carbamate]); PVP (polyvinyl pyrrolidone).

† The oxygen permeability of a material is referred to as the Dk. The units of 10^{-11} cm^2/s ml O_2/ml X mm Hg are often omitted for convenience. Dk value is a physical property of a contact lens material and describes its intrinsic ability to transport oxygen. "D" is the diffusion coefficient – a measure of how fast dissolved molecules of oxygen move within the material and "k" is a constant representing the solubility coefficient or the number of oxygen molecules dissolved in the material.

†† Oxygen transmissibility is referred to as Dk/t, with units of 10^{-9} cm/s ml O_2/ml X mm Hg. Here "t" is the thickness of the lens or sample of the material, and "D" and "k" are as defined above.

CIBA Vision's Night & Day™ material, lotrafilcon A, employs a co-continuous biphasic or two channel molecular structure, in which two phases persist from the front to the back surface of the lens. [18-21] The siloxy phase facilitates the solubility and transmission of oxygen and the hydrogel phase transmits water and oxygen, allowing good lens movement. The two phases work concurrently, to allow the co-continuous transmission of oxygen and aqueous salts. Lotrafilcon A is comprised of a fluoroether macromer co-polymerised with the monomer TRIS and N,N-dimethyl acrylamide (DMA), in the presence of a diluent. [12, 22] The resultant silicone hydrogel material has a water content of 24% and a Dk of 140 barrers. [23] CIBA Vision's O2Optix™ material, lotrafilcon B, is based upon the technology used in Night & Day™ and is surface treated in the same manner. It has a water content of 33% and a Dk of 110 barrers and is less stiff than the lotrafilcon A material. The surfaces of the lotrafilcon A and B lenses are permanently modified in a gas plasma reactive chamber to create an ultrathin (25 nm), high refractive index, continuous hydrophilic surface. [12, 18-21]

Balafilcon lenses are surface treated in a gas plasma reactive chamber, which transforms the silicone components on the surface of the lenses into hydrophilic silicate compounds. [12, 22] This results in glassy, discontinuous silicate islands and the hydrophilicity of these areas bridges over the underlying hydrophobic balafilcon material. Balafilcon A is a homogeneous combination of the silicone-containing monomer polydimethylsiloxane (a vinyl carbamate derivative of TRIS) co-polymerized with the hydrophilic hydrogel monomer N-vinyl pyrrolidone (NVP). [16, 22, 24, 25] This silicone hydrogel material has a water content of 36% and a Dk of 101 barrers.

Vistakon's Acuvue® Advance™ material, galyfilcon A, incorporates a long chain, high molecular weight molecule called Hydraclear™, which maintains flexibility and moisture. [26-28] This wetting agent is present throughout the lens material and hence no surface treatment is required for these lenses. [26] This lens material has a water content of 47%. It was the first of the so-called "second generation" silicone hydrogels [29] and has a UV blocker, with a reported Class 1 UV protection, blocking >90% of UVA and >99% of UVB rays. [26-28, 30] Vistakon's Acuvue® OASYS™ (senofilcon A) has a light blue tint, Class 1 UV blocking capabilities and a 1-2-3 inversion marker. [31]

CooperVision's Biofinity® lens is manufactured from comfilcon A and has a significantly higher oxygen permeability [32] than would be predicted from its water content, implying that the chemistry upon which it is based is different to that employed in the current silicone hydrogels, which are all based on the highly oxygen permeable

monomer TRIS. This silicone hydrogel material has a water content of 48% and a Dk of 128 barrers.

Once the contact lens is inserted onto the eye, it interacts to a great extent with the tear film. The next section provides an overview of the tear film, its structure, functions and various components that are present in the tear film.

2.2 The tear film

The tear film is by far the most dynamic unit in the lacrimal functional unit. The tear film consists of a variety of components, including electrolytes, salts, proteins, lipids, mucins and peptides. The normal pH value of the tear film is between 7.14 and 7.82, with a mean value of 7.4-7.5, which is similar to plasma pH. [33] The tear film has several specific functions and they are listed below:

1. It provides lubrication to the ocular surface and nurtures the anterior tissues of the eye.
2. It provides a regular optical surface for the eye's optical system, by filling the irregularities of the corneal epithelium.
3. The corneal epithelium and stroma receive oxygen that is dissolved in the tear film.
4. The tear film is an integral part of the ocular surface defense mechanism.
5. It removes cellular debris and metabolic waste from the cornea and conjunctiva with blink.

In 1946, Wolff proposed that the tear film was a three-layered structure, consisting of an anterior lipid layer, middle aqueous layer and deeper mucin layer (Figure 2.4). [34] Another model with six layers has been proposed by Tiffany, [35] which included the original three layers proposed by Wolff, along with air-lipid, lipid-aqueous and aqueous-mucus interfaces. Dilly suggested that there are dissolved mucins in the aqueous layer, which decrease in concentration towards the lipid layer. [36] Currently the most

accepted concept is that the tear film is a bilayered structure, consisting of an aqueous/mucinous phase, with an overlying superficial lipid phase. [37]

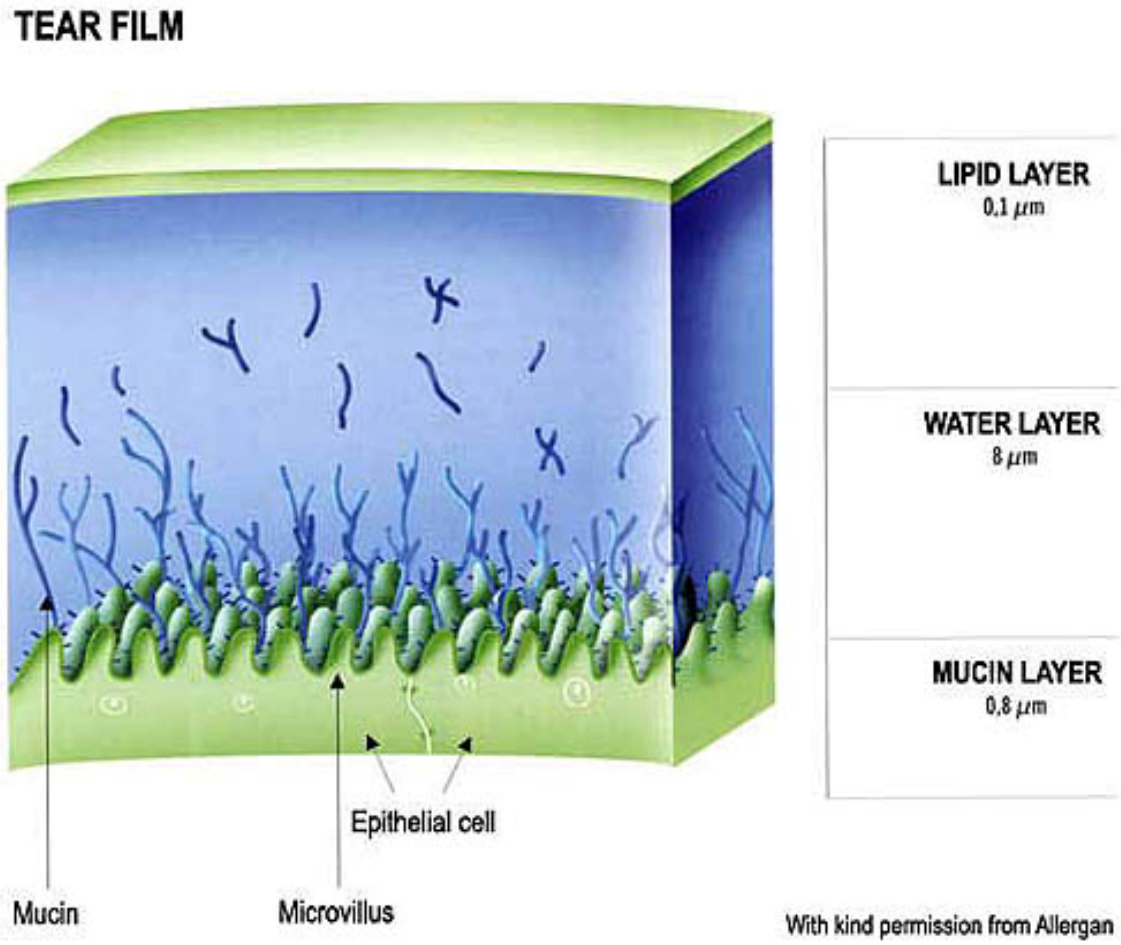

Figure 2-4: Layers of the tear film

Composition of the tear film, showing the three layers: an outer lipid layer, an intermediary aqueous layer and inner layer of mucins (Reproduced from http://www.lea-test.fi/en/eyes/images/pict7b.jpg)

2.3 Layers of the tear film

2.3.1 The lipid layer

Lipid forms the superficial/outermost layer of the tear film and is derived primarily from the meibomian glands in the eyelids. [38] It is believed that the lipid layer

has two distinct regions - a relatively thick outer layer, containing non-polar lipids such as wax esters, sterol esters, hydrocarbons, and triglycerides; and a thin polar inner layer, predominantly consisting of phospholipids. [39] The polar phase of the lipid layer owes its surfactant properties which facilitate mixing with both aqueous and non-polar lipids. It acts base for the more superficially located non-polar lipids. [40] The major classes of lipids are the wax monoesters and sterol esters, which comprise approximately three-quarters of the meibomian gland fluid. [40, 41] Studies using specular microscopy have reported lipid thickness values of 100 nm. [42] Using specular reflectometry, it was found that the lipid layer thickness was 13-70 nm. [43] The refractive index of meibomian lipid was found to be 1.4766 at 589 nm and 35°C. [44]

2.3.1.1 Functions of the lipid layer

1. To provide an effective barrier and prevent evaporation. [45]
2. To provide a surfactant layer that acts as an effective bridge between the non-polar lipid layer and the aqueous mucinous layer. [46]
3. To act as a lubricant to facilitate the movement of the eyelids during blinking. [47]
4. To maintain compression and expansion of the lipid film during blinks, to prevent tear overflow. [47]
5. To form a barrier for preventing contamination of the tear film. [44]
6. To provide a smooth surface for refraction of the incoming rays of light. [47, 48]

2.3.2 The aqueous layer

The aqueous layer of the tear film forms the bulk of the tear film thickness. The aqueous layer is approximately 6.5-7.5 μm thick. [49] This intermediary watery phase is mainly produced by the main lacrimal gland, and also by the accessory glands of Krause and the accessory glands of Wolfring. This layer is composed of electrolytes, protein enzymes and metabolites. The bulk of the aqueous component of the tears is not only composed of water, but contains numerous electrolytes, proteins, peptide growth factors, vitamins, anti-microbials, cytokines, immunoglobulins, and hormones. The composition of the tear film varies in response to environmental and bodily conditions. Electrolytes present in the tear film include sodium, potassium, magnesium, calcium, chloride, bicarbonate, and phosphate ions. These are largely responsible for modifying the osmolality of tears, [50] act as a buffer to maintain pH at a relatively constant level [51] and maintain epithelial integrity. [52]

To date, over 90 human tear proteins have been identified (Table 2.4), [53-55] of which the primary proteins are lysozyme, lactoferrin, and lipocalin. [56] Other important proteins of note include serum albumin, IgG, ceruloplasmin, transferrin, and monomeric IgA. [56] The tear proteins are explained in greater detail in an upcoming section (Section 2.4). Numerous peptide growth factors including EGF, HGF, TGF β are also found in the aqueous layer. [54, 55]

2.3.2.1 Functions of the aqueous layer

1. This layer is responsible for creating a conducive environment for the epithelial cells of the ocular surface, carrying essential nutrients and oxygen to the cornea, allowing cell movement over the ocular surface. [57]
2. The major tear proteins found in this layer (lysozyme, lipocalin, lactoferrin and IgA) are responsible for antimicrobial activity. [57-59]
3. Washing away debris, toxic substance and foreign bodies with blink. [59]
4. Many of the growth factors that are present in the aqueous phase play a significant role in corneal physiology. [59]
5. Quantitatively, this is the most important layer.

Table 2.3 lists some of the various components of the aqueous and lipid phase, their concentrations and other physical characteristics.

Table 2-3: Summary of tear film components of the aqueous and lipid phase

Component	Basal (or Open) Eye Tear Fluid	Closed Eye (sleep) Tear Fluid	Reflex Lacrimation/ during SCL wear
Water [60]	98 – 98.5%	-	-
Oxygen [61]	155mm Hg	55mm Hg	-
Fluid volume [62]	6.2 – 9.0 µl	-	-
pH [63]	Range 6.5 - 7.8	-	SCL: range 6.6 – 7.3
Osmolarity [64, 65]	302 – 325 mOsm/l	< 300mOsm/l	Reflex: 285 mOsm/l SCL: 307 mOsm/l
Flow rate[62, 66]	0.5 - 1.2 µl/min	Almost stagnant	Reflex: > 1.2 µl/min
Turnover rate [67]	14-15 %/min	-	-
Refractive index [68]	1.335 – 1.53	-	-

Surface tension [46]	40 – 46 mN/m	-	-
Evaporation rate [69, 70]	10.1x10-7 $gm.cm^{-2}.sec^{-1}$ 14.7 ± 6.0 $gm.cm^{-2}.sec^{-1}$	-	-
Viscosity [60, 71]	5 - 8 mPa.s 9 mPoises	-	-
Tonicity [60]	Equal to 1.0% NaCl	Equal to 0.9% NaCl	-
Ions Content [72]	Sodium 145 mEq/l Potassium 20 mEq/l Chloride 128 mEq/l Bicarbonate 26 mEq/l Calcium 2.11 mg/dl Mg++ and Zn++ Trace only	-	-
Glucose Content [73]	128-166 µM 7.5 mg%	- 5 mg%	-
Antioxidants [74]	Ascorbic acid 665 µM Tyrosine 45 µM Glutathione 107 µM Cysteine 48 µM Uric acid 328 µM	Ascorbic acid 116 µM Tyrosine 16 µM Glutathione 131 µM Cysteine 20 µM Uric acid 13 µM	-
Total Protein Concentration [66, 75, 76]	Range 4.0 – 9.4 mg/ml	18 - 43 mg/ml	Reflex: 6.0 mg/ml SCL: Range 8-14 mg/ml
Secretory IgA Concentration [75-77]	0.54 - 0.85 mg/ml	8.4 - 5.5 mg/ml	Reflex: 0.23 mg/ ml SCL: 0.84 mg/ml
Lactoferrin Concentration [75, 78, 79]	0.8 – 6.3 mg/ml	1.8 mg/ml Similar to basal tears	Reflex: 1.8 mg/ml Similar to basal tears
Lipocalin Concentration [75, 80, 81]	1.2 – 2.2 mg/ml	2 –3 mg/ml Similar to basal tears	Reflex: 1.2 – 1.7 mg/ml
Lysozyme Concentration [77, 79, 82]	1.2 - 4.6 mg/ml	1.8 mg/ml Similar to basal tears	Reflex: 1.6 mg/ml Similar to basal tears SCL: 1.4 – 1.7 mg/ml
Enzyme Content [83, 84]	Peroxidase 5.2 – 7.7 U/ml Secretory phospholipase A2 1.45 µg/ml Gelatinase 0.3 ng/ml β-lysin 0.3 ng/ml	-	-
Lipid layer Content [85]	0.13 – 1.8 mg/ml	-	-
Lipid components (percentage by weight) [85, 86]	Sterol esters 27 - 29.5%w Waxy esters 32 – 35%w Triacylglycerols 4.0%w Free sterols 1.8 – 2.0%w Free fatty acids 0.5 - 2.1%w Polar lipids 15.0 - 16.0%w Cholesterol (sterols) 15%w Hydrocarbon 7%w	-	-

2.3.3 The mucin layer

Mucins are primarily produced in the goblet cells of the conjunctiva and the crypts of Henle in the conjunctival fornices. [87] A secondary source of mucin is from the squamous epithelial cells of the ocular surface (cornea and conjunctiva), [36, 88, 89] with a small contribution from the lacrimal gland. Goblet cell mucin forms a gel in the deepest layer of the tear film, while soluble mucin is found in the aqueous layer. [90]

Mucins are high molecular weight glycoproteins, that have at least 50 to 80% of their mass as carbohydrate, O-linked to serine and threonine residues present within tandem repeats of amino acids in their protein backbone. [91-93] Mucins are hydrophilic in nature and their molecular mass range from 3×10^5 to over 4×10^7 kDa. [91-93] To date, at least 20 distinct human mucins have been cloned (MUC1– MUC20, including 3A, 3B, 5AC, and 5B). [94, 95] Mucins are classified as either transmembrane or secretory mucins. [91] Secreted mucins can further be sub-classified as gel-forming or soluble, based on their ability to form polymers. Of these, MUCs 1, 3A, 3B, 4, 12, 13, 15, 16, 17 and 20 have been characterized as membrane associated. MUC 2, 5AC, 5B, 6, 7 and MUC9 are classified as secretory mucins. [96] In this category, MUC 2, 5AC, 5B, 6 are categorized as gel-forming mucins and MUC7 & MUC9 are classified as soluble mucins. [96] Several reports have shown alterations of mucin products in the ocular surface in dry eye. [97-99]

2.3.3.1 Functions of the mucin layer

1. One of the primary functions of mucin is lubrication, facilitating the eyelid margins and palpebral conjunctiva to slide smoothly over one another during blinking and ocular rotational movements. [100]
2. Mucus plays an important role in overcoming this hydrophobicity of the corneal surface, when non-wetting occurs in some areas of the cornea. [100]
3. Mucus threads protect the cornea and conjunctiva from abrasion by covering foreign bodies with a slippery coating. [101]
4. Mucus also helps in wetting the ocular surface and in glycocalyx formation. [36]
5. The ocular surface glycocalyx acts as a barrier to pathogens. [101]

2.4 Tear Proteins

Using a proteomic technique, 97 proteins have been identified in the tear film. [55] The various tear proteins that were identified in the study [55] are mentioned in Table 2.4.

Table 2-4: List of tear proteins identified using tear proteomic approach [55]

	Protein	Function - location
1	Lactoferrin	Transfer/carrier – secretory granule
2	Poly Ig receptor	Immune response – plasma membrane
3	Serum albumin	Transfer/carrier - extracellular
4	Basic proline-rich protein	Unclassified - extracellular
5	Ig Alpha 1 bur	Immune response – plasma membrane
6	IGHA1 Protein	Immune response - plasma membrane
7	Zn-alpha2-glycoprotein	Lipid and fatty acid metabolism, glycolysis - extracellular
8	Lipocalin 1	Transfer/carrier - extracellular
9	Proline Rich 4	Unclassified - extracellular
10	Ig alpha 1	Immune response – plasma membrane
11	Haptoglobin	Serine protease - extracellular
12	Lacritin	Unclassified - extracellular
13	Cystatin S	Protease inhibitor - cytoplasm
14	IgG Kappa	Immune response - plasma membrane
15	Keratin 9	Cell structure - cytoplasm
16	Immunoglobin J	Immune response – endoplasmic reticulum
17	Prolactin induced protein	Unclassified - extracellular
18	Keratin 14	Cell structure - cytoplasm
19	Keratin 10	Cell structure - cytoplasm
20	Lysozyme	Carbohydrate metabolism - extracellular
21	Cystatin SN	Protease inhibitor - cytoplasm
22	Secretoglobin, family 2A, member 1 (Lipohilin C)	Unclassified - extracellular
23	Secretoglobin, family 1D, member 1 (Lipophilin A)	Transfer/carrier - extracellular
24	Alpha1-antichymotrypsin	Protease inhibitor - extracellular
25	Ig Variable	Immune Response - plasma membrane
26	HRPE773	Unclassified - unknown

27	Protein Len, Bence Jones	Immune response - plasma membrane
28	Ig Mu	Immune response - extracellular
29	Ig alpha chain C	Immune response - plasma membrane
30	DMBT1 (gp 340)	Extracellular transport and import - extracellular
31	Annexin A1	Fatty acid metabolism, cell motility – plasma membrane
32	Lactate dehydrogenase A	Glycolysis - cytoplasm
33	Annexin A2	Transfer/carrier - nucleus
34	Heat shock 70 KDa 1B	Chaperone - cytoplasm
35	Triosephosphate isomerase	Glycolysis, metabolism - cytoplasm
36	Immunoglobin lambda	Immune response - plasma membrane
37	Aldehyde dehydrogenase 1	Dehydrogenase - unknown
38	Heat shock 27 KDa protein 1	Chaperone, Cell structure (muscle contraction vision) - cytoplasm
39	IGHM Protein	Immune response
40	Keratin 1	Cell Structure – plasma membrane
41	proapolipoprotein	Transporter lipid and fatty acid
42	Peroxiredoxin 1	Antioxidation and free radical removal - cytoplasm
43	Keratin 6B	Cell Structure - cytoplasm
44	Neutrophil gelatinase-associated Lipocalin	Small molecule transport - extracellular
45	Keratin 6A	Cell structure - cytoplasm
46	Transferrin	Transfer/carrier - extracellular
47	Selenium binding protein	Immune response - cytoplasm
48	Pyruvate kinase	Glycolysis - unknown
49	Prostatic binding protein	Transfer/carrier - cytoplasm
50	Hornerin	Unclassified – plasma membrane
51	Peroxiredoxin 2	Antioxidation and free radical removal - cytoplasm
52	Glutathione S-transferase	Detoxification - cytoplasm
53	Immunoglobin lambda light chain	Immune response – plasma membrane
54	S100 Calcium binding protein A11	Signaling, DNA replication - cytoplasm
55	Ezrin	Cell structure - cytoplasm
56	Peroxiredoxin 5	Antioxidation and free radical removal - cytoplasm

57	Cyclophilin A	Transport/immune response - cytoplasm
58	Eukaryotic translation elongation factor	Translational regulation - cytoplasm
59	S100 Calcium binding protein A9	Cell communication, immune response - cytoplasm
60	Actin, beta	Cell structure - cytoplasm
61	Serine Proteinase inhibitor	Proteolysis - extracellular
62	S100 Calcium binding protein A8	Cell Communication - cytoplasm
63	S100 Calcium binding protein A6	Signal transduction - cytoplasm
64	Polyubiquitin	Proteolysis - cytoplasm
65	S100 Calcium binding protein P	Cell Communication - cytoplasm
66	S100 Calcium binding protein A4	Macrophage mediated immunity - cytoplasm
67	Cystolic malate dehydrogenase	Dehydrogenase - cytoplasm
68	PGK1	Glycosysis - cytoplasm
69	Skeletal muscle alpha-actin	Cell structure - cytoplasm
70	Thioredoxin peroxidase	Antioxidation, free radical removal - cytoplasm
71	Enolase 1	Glycolysis - cytoplasm
72	Peptidylprolyl isomerase A (Cyclophilin A)	Protein folding, immune response - cytoplasm
73	Calmodulin	Protein phosphorylation, Ca mediated signaling, muscle contraction - cytoplasm
74	Isocitrate dehydrogenase 1	Tricarboxylic acid pathway - cytoplasm
75	Ig G1 H Nie	Immune response – plasma membrane
76	Aldehyde dehydrogenase Isozyme 3	Other carbon metabolism - cytoplasm
77	Aldehyde dehydrogenase 1A1	Other carbon metabolism - unknown
78	Alpha-1-antitrypsin	Proteolysis - extracellular
79	Heat shock 70KDa protein 6	Protein folding, stress response - Nucleus
80	Heat shock 70KDa protein 8	Protein folding, stress response - nucleolus
81	Hemopexin	Transfer/carrier protein - extracellular
82	Argininosuccinate synthetase	Amino acid synthesis - cytosol
83	Villin 2	Cell structure - cytoplasm
84	Gelosin isoform a	Cell structure - unknown

85	Protein Rei, Bence-Jones	Immune response – plasma membrane
86	Transcobalamin 1	Vitamin metabolism, Cation transporter - extracellular
87	NGAL (Lipocalin 2)	Small molecule transport - extracellular
88	Secretory leukocyte peptidase inhibitor	Protease inhibitor - extracellular
89	Beta-2 microglobin	Cell Structure - extracellular
90	Cystatin C	Protease inhibitor - extracelluar
91	Mesothelin/megakaryocyte potentiating factor	Ligand-mediated signaling, cell adhesion - unknown
92	Keratin 2a	Cell structure - cytoplasm
93	Pre-pro-megakaryocyte potentiating factor	Ligand-mediated signaling, cell adhesion - unknown
94	Clusterin (apolipoprotein J)	Apoptosis - extracellular
95	Complement Component 3	Immune response - extracellular
96	Secretoglobin, family 2A member 2 (Mammaglobin 2)	Unclassified - extracellular
97	Ig J	Immune response – plasma membrane

The following section provides a brief overview of some of the major tear proteins that have been widely studied and published in the literature.

2.4.1 Lysozyme

Lysozyme (also called Muramidase) is a bacteriolytic enzyme that was discovered by Fleming in 1922. [102] It is found in mammalian urine, saliva, tears, milk, cervical mucus, leukocytes and kidneys. [103] Tear lysozyme is derived from the acinar and ductal epithelial cells of both main and accessory lacrimal glands. [104, 105]

2.4.1.1 Lysozyme structure

Lysozyme is a compact globular protein molecule with a molar mass of 14,500 Da. [105] It has a slightly ellipsoidal shape, and its dimensions are 45 X 30 X 30 Å. [106] It is a compact protein of 129 amino acids which folds into a compact globular structure. [106] The 129 amino acid sub-units are cross-linked by four disulphide bridges. [107] There is a close cluster of basic groups (Arginine 45 and 68 in one region, Arginine 61 and 73 in a second and Arginine 5, 125 and 128 in a third) which form the highly positively charged surface regions of lysozyme, which give it a very high isoelectric point of 11.1. Lysozyme is a relatively stable protein when compared to most other proteins. [108] The properties of lysozyme do not change at pHs between 1 and 8. [109] A study by Ikeda and colleagues showed that lysozyme denatures only when the pH is above 12. [110] The thermal stability of lysozyme in various solutions was studied by Hamaguchi and Sakai and they demonstrated that its structure is not affected by heat up to 55˚ C. [111] Moreover, by increasing the temperature only the internal fold of lysozyme is disrupted and no changes occur in the helical part at high concentrations of organic solvents.

The polypeptide chain forms five helical segments, a 3 stranded anti-parallel Beta sheet that comprises one wall of the binding cleft. A deep cleft contains the active site (described later in Section 2.4.1.4) which divides the molecule into two domains. These domains are linked by alpha-helix residues. One domain consists of residues that have Beta Sheet structure; the other domain has in its residues that are helical in nature. [106]

2.4.1.2 Structural aspects of lysozyme – from the viewpoint of an antibody

Lysozyme, in its native globular state contains two fragments which have immunologic activity and encompass two independent antigenic determinants. [112] One of these immunologically active fragments consists of two peptides, derived from the NH_2-terminus and the COOH-terminus of lysozyme linked together by a disulphide bond. The second immunologically active component isolated was a large fragment derived from the region located between residues Leucine 57 and Arginine 107 of the lysozyme sequence. [112] This peptide which contains two disulphide bridges, is also capable of binding to anti-lysozyme antibodies. Similar structure was obtained for lysozyme in further experiments conducted using epitope mapping. [113]

2.4.1.3 Structural comparison of human lysozyme versus hen egg lysozyme

X-Ray crystallographic studies [107, 114] and far-UV circular dichroism studies [115, 116] suggest that the two enzymes namely, human lysozyme and hen egg lysozyme have very similar secondary structures. Despite the fact that the sequences in human lysozyme and hen egg lysozyme are different, [117] sizable numbers of substitutions are non-conservative;

however the structures and functions are highly similar. [106] Moreover, if the ionic strength was maintained constant within a certain range (pH 5 to 9), the activity of both these enzymes were found to be same. [106, 118]

2.4.1.4 Structure – Activity Relationship of lysozyme

Lysozyme is instrumental in destroying certain species of bacteria which are gram-positive. [103, 106] These gram-positive bacteria possess an outer coat of a peptideglycan (sugar) polymer (or peptidoglycan), which, in gram-negative bacteria, is only transiently stained since those bacteria are covered by a second, outer lipid membrane. [119] Lysozyme hydrolyzes or breaks up the glycan (sugar polymer) components of the peptidoglycan of gram-positive bacteria. [119] Specifically, lysozyme breaks β-1,4 glycosidic bond of the oxygen bridge between the repeating glycan units of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG), which is responsible for its anti-bacterial properties. [119]

A portion of the bacterial peptidoglycan is able to fit in a groove on the outer surface of the enzyme that contains the active site. [106] The active site contains two amino acid components (Glutamine and Aspartic Acid) whose carboxylate groups participate in the hydrolysis. [106] The molecular mechanism of lysozyme catalysis at the active site involves multiple steps, in which a proton is donated by an uncharged Glutamine residue at the active site by breaking the glycosidic bond. [119] At completion, the original forms of the enzymes are regenerated and the hydrolyzed chains of the peptidoglycan leave the active site of the enzyme.

2.4.2 Lactoferrin

Lactoferrin is a large basic glycoprotein (pI 7.5-8.5) which is member of the transferrin family. [59] Its primary role in the tear film is to chelate iron, which during a bacterial invasion, would halt the growth of most bacteria. [59] Lactoferrin is produced in the acinar epithelium of the main and accessory lacrimal glands. [120] The major form of lactoferrin has a molecular weight of 78 kDa, while the minor form corresponds to an 83 kDa in gel electrophoresis. Kijlstra and co-workers investigated the various forms of lactoferrin and showed that the two isoforms were not due to iron or carbohydrate changes within the structure. [121] Instead, it was found that an increase in iron ions in human tears produces a protein species around 52 kDa. [121] It is considered that the minor form, in being slightly larger than the major lactoferrin species, is actually a precursor of lactoferrin.

Fullard and Tucker showed that the concentration of lactoferrin remained relatively constant through a series of non-stimulated and stimulated tear samples. [81] Therefore, lactoferrin reductions are correlated with decreases in tear production from the lacrimal gland. [122] Lactoferrin concentrations, being not significantly reduced during the reflex action of tearing, are usually around 1.7 to 2.5 mg/ml for basal tears and 1.5 to 1.8 mg/ml during stimulation. [66, 123] No differences in lactoferrin concentration has been found between men and women, and similarly, no significant differences for an age range from 17-60 years using reflex tears. [78]

In another study, it was found that the concentration of lactoferrin was significantly decreased in the tears of patients with non-Sjogren syndrome, Sjogren

syndrome and Stevens-Johnson syndrome, when compared with control subjects. [124] Previous studies have also proposed that lactoferrin and other glycoproteins adsorbed on to contact lens materials can promote bacterial adhesion, since their carbohydrate moieties may act as receptors for bacterial lectins. [125, 126] Another study demonstrated that lactoferrin deposited on the lens surface promotes the adhesion of *Pseudomonas aeruginosa* strain Paer 1; nevertheless, once adherent, this protein reduces the proportion of viable bacteria on the lens surface. [127] Lactoferrin is considered to play an important role in the specific defense of the ocular surface against a variety of bacteria. [59, 128, 129] Lactoferrin forms complexes by binding to acidic macromolecules (including immunoglobulins and serum albumin). [77] Lactoferrin in the tears interacts with both human lysozyme and bacterial lipid A showing that individual proteins play many roles, both protecting and interacting with other tear components. [130-132] Lactoferrin can inhibit the formation of classical C3 convertase of the complement system preventing complement activation in the tear film and thus decreasing inflammation. [133]

2.4.3 Albumin

The presence of albumin in the tear fluid is due to serum leakage from the vessels of the conjunctiva and acts as an indicator of the integrity of the blood/tear barrier. [104, 134] Albumin has molecular weight (apparent) of 60 kDa and a neutral isoelectric point. During an eye infection [135, 136] and during the closed eye state/ sleep, [137] there is often an increase in vascular permeability and a concomitant increase in albumin concentration. [75] The concentration of tear albumin ranges from 10-73 μg/ml with no change with contact lens wear after 6 months. [79] In related studies looking into the role of albumin in fluids,

an increase in gastric mucus viscosity occurred with increasing albumin concentration. [138] This may be of significant importance as this may have an influence on friction and movement of contact lenses over the ocular surface. An albumin to lactoferrin ratio of 2:1 has been suggested to indicate Sjögren's syndrome. [139] To-date, there are very few reports on albumin deposition on either conventional hydrogel lens materials [140-143] or silicone hydrogel contact lens materials. [144] It has previously been shown that contact lens-induced papillary conjunctivitis is associated with increased albumin deposits on hydrogel contact lenses. [140] Previous studies have also shown that albumin adsorbed onto the contact lens surface could modulate the adherence ability of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* on hydrogel contact lens materials. [145-147]

2.4.4 Immunoglobulins

Immunoglobulins (Ig) are a large group of proteins whose molecular weights range between 100-500 kDa. [56] Immunofluorescent staining in both the main and accessory lacrimal glands shows IgA, IgG, IgM, IgD and IgE, with the secretory form of IgA (sIgA) being the predominant immunoglobulin. [66] IgA (145 kDa) may form dimers spontaneously through association with a cysteine-rich polypeptide known as the J (joining) chain. sIgA (500 kDa) is a combination of two IgA molecules (heavy chain 64 kDa; light chain 28 kDa) with a secretory piece (85 kDa; T-piece) joined by disulfide bonds and the J chain. [148] IgA and the J chain are produced by lymphocytes (plasma cells) transported into acinar cells and secreted into tears as s IgA. [149]

sIgA levels in tears vary from 0.5 – 1.0 mg/ml which is much higher than found in serum. [75, 76] In closed eye condition, sIgA is secreted from the lacrimal gland and its concentration can be as high as 8.6 mg/ml. [75] There is no significant difference in the concentrations of sIgA during daily contact lens wear and non-contact lens wear. [76] During extended contact lens wear, sIgA decreases in concentration as compared to daily lens wear (2.42 mg/ml to 0.72 mg/ml). [150] Previous studies have shown varied results for the level of sIgA in the tears of contact lens wearers, with both increases [151] and unchanged levels of sIgA being detected. [152] Macki and Seal did not demonstrate a change in the amount of sIgA in tears of patients with Keratoconjunctivitis Sicca. [153]

Secretory IgA is considered one of the most important immunoglobulins in the defence of the ocular surface. It interacts with other proteins and with bacterial contaminants. [75, 76] sIgA is thought to modulate the ability of microorganisms to colonize the ocular surface. [154] It has been shown that sIgA has specific antigenic activity against *Staphylococcus intermedius* and *Pseudomonas aeruginosa* enhancing the effectiveness of polymorphonuclear leukocytes phagocytic activity towards the bacteria. [155]

2.4.5 Lipocalin

The lipocalins are a family of lipid binding proteins with a molecular weight between 18 and 25 kDa. [156, 157] In tears, associated members of the lipocalin family include apolipoprotein D and tear specific prealbumin, now referred to simply as tear lipocalin. [157] The non-reduced form of lipocalin can dimerise and these have been previously labeled as tear protein G observed in protein gel electrophoresis. [158] Lipocalins

in tears are regulated proteins produced by acinar cells. [80] Lipocalins comprise 15 - 33% of tear protein ranging in concentration from 1.2 - 1.7 mg/ml. [159] Six isoforms with isoelectric points ranging through 4.6-5.4 are known, although recently another four isoforms have been identified through HPLC and two dimensional electrophoresis techniques. [159]

Tear lipocalin binds to and may have a direct functional role with both lactoferrin and lysozyme in the tear film. [160, 161] Tear lipocalins bind a broad array of lipids including fatty acids, cholesterol, phospholipids and glycolipids. [80] Lipids that contaminate the corneal surface after tear film drying could potentially de-stabilize the reforming tear film. Removal of such lipids by lipocalin may prevent the repeated drying cycle on the ocular surface. [58] Glasgow and co-workers showed that tear lipocalins could interact with both the air and aqueous phase of solutions such as the tear film. [58] The lipid binding ability of lipocalins suggests that it could potentially be an important secondary component used in tear film stabilization.

2.4.6 Phospholipase A2

Phospholipases consist of a family of cytosolic and secretory lipolytic enzymes. In tears, group II (secretory) phospholipase A2 (sPLA2; 14 kDa; pI > 10.5) is found in concentrations averaging 1.450 μg/ml. [84] This lipolytic enzyme catalyses the hydrolysis of the fatty acid acyl ester bond at the sn-2 position (CO-OCH linked to saturated or unsaturated aliphatic groups) of phosphocholine, ethanolamine, glycerol, inositol and other alcohol lipids. [162] Group II sPLA2 is released from specialized acinar cells

predominately in the centre of the lobules of the lacrimal gland (Aho et al. 1996, Gillette and Allansmith 1980). [120, 162] Gram-negative organisms covered with lipopolysaccharides external to their peptidoglycan layer are resistant to lysis by lysozyme. Group II PLA2 might act synergistically with lysozyme (and also complement) to lyse these bacteria. [162, 163] Group II PLA2 in the presence of calcium can kill staphylococci and other gram-positive bacteria. [164] It has been suggested that Secretory PLA2 could potentially remodel or remove peroxidised or senescent phospholipids from the tear film. [165]

2.4.7 Other tear proteins of significance

The complement system is one of the main effector mechanisms of the immune response on the ocular surface. All components of the complement system were found to be activated in tears with a great increase in their concentration during sleep. [123] Lactoferrin, decay-accelerating factor (DAF) and CD59, are known to control or regulate the complement system. These proteins are present in both the tears and on the plasma membranes of corneal and conjunctival epithelia. [123, 166] Additionally, all three regulators have been shown to bind to hydrogel lenses. [166, 167] This could assist in damping the activation of complement on the lens, and also minimize the inflammatory effect of other deposited proteins, or inversely the bound regulators could incite inflammation.

The corneal epithelium produces gelatinase B, an enzyme that participates in the wound healing process that follows mechanical, thermal or laser injury of the cornea. [168] Gelatinase B is a matrix metalloproteinase (apparent molecular weight of 92kDa, also known as MMP-9) that is found in ng/ml quantities in the tear fluid of healthy subjects.

An 84 kDa active form has a much greater activity in patients with ocular rosacea. [83] MMP-9 activity is correlated with a delayed tear clearance and elevated tear interleukin-1α concentrations. [83] Interleukin-1 increases the production and activity of enzymes of the matrix metalloproteinase family, including collagenases and gelatinases, that degrade extracellular matrix and may contribute to the development of the eyelid and ocular surface disease in rosacea. [169, 170] Collagenases have the potential to break down the corneal epithelial basement membrane and hence to dissolve collagen. These processes could lead to corneal epithelial erosion and possible ulcerations. [171]

Table 2-5: Summary of major tear proteins and their significance

Protein	MW (kDa)	Produced in	Role	Concentration
Lysozyme [66, 120, 162]	14.5	acinar & intralobular epithelial cells of main & accessory lacrimal glands (secreted from lysosome vesicle)	Bactericidal	0.5-4.6 mg/ml decreased in Sjogren's syndrome and HSV
Lactoferrin [59, 78, 121, 123, 162]	78	acinar & intralobular epithelial cells of main & accessory lacrimal gland (mainly acinar cells)	Bacteriostatic, anti-inflamatory	0.8-6.3 mg/ml decreased in Sjogren's syndrome
Albumin [53, 79, 81]	60	serum derived - enters tear film by capillary leakage	carrier protein	<0.1 mg/ml increases during ocular inflammation
IgA [76, 81]	145	Plasma cells of the lacrimal gland - migrating to acinar epithelia	immune defence	0.1 - 0.9 mg/ml decreased in Sjogren's syndrome
Lipocalin [58, 80, 157, 161]	18-25	Acinar cells	Binds lipids	1.2-1.7 mg/ml
Phospholipase [84, 162]	14	some acinar cells, and central lobular cells of the main lacrimal gland	Lipolytic enzyme	1.45 g/ml

2.5 Contact lens deposits

One of the major problems associated with hydrophilic contact lenses is that they are susceptible to spoilage from constituents of the tear film, which include a wide variety of proteins, [108, 172-176] lipids, [177-181] and mucins. [182] At extreme levels of build-up, these deposits are associated with diminished visual acuity, [183, 184] reduced wearing time [185] and a feeling of dryness and discomfort. [179, 186-188] Deposits can potentially lead to more serious clinical conditions such as hypersensitivity reactions and contact lens associated papillary conjunctivitis. [189-192] Moreover, these deposits are known to increase the risk of bacterial attachment, by providing a solid substrate and shelter. [146, 147, 193]

The adsorption of tear derived substances at the contact lens interface is highly complex and is dependent upon a number of factors. Notable amongst these are the material's water content, [141, 173, 174, 194-197] hydrophobicity, [141, 143, 173, 198, 199] charge, [175, 194, 196, 200, 201] pore size, [194, 202] surface roughness, [143] age of the lens material [179] and the size or charge of the protein. [203] However, the relative importance of the different components of deposited films to these clinical effects remains largely unknown.

As already mentioned, the newly introduced silicone hydrogel contact lenses have significantly increased oxygen transmission due to the incorporation of siloxane groups. [23, 204, 205] The incorporation of silicone results in an increased degree of hydrophobicity, which results in increased lipid deposition compared with other non silicone-containing materials. [4, 206, 207] However, these lens materials do deposit extremely low levels of

protein compared to conventional hydrogel lenses, with typical levels being in the < 20 μg/lens range. [204, 208, 209]

2.5.1 Protein deposits on contact lenses

Proteins primarily are deposited onto the contact lenses from the tear fluid. [210] Proteins deposit on the contact lenses as films and these protein films are invisible during the early stages of the spoilation process, but with the advancement of time, the protein denatures and they assume a thin, translucent, whitish appearance. [211] These protein deposits remain primarily on the surface, but in high water content lenses they may penetrate into the lens matrix. [212] All proteins in the tear film have the potential to form contact lens deposits, although, several factors ultimately influence the type, quantity and structure of such deposits. [1]

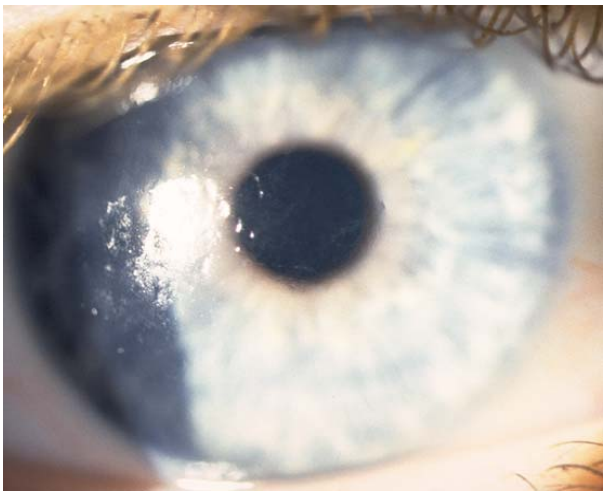

Figure 2-5 Protein deposits on contact lens.
(Picture courtesy of Dr. Lyndon Jones)

Protein deposition onto hydrogel lens materials is a highly complex process, depending upon the charge and size of the protein, environmental pH, charge and water content of the substrate and competition between the various tear film constituents that are present. [142, 167, 173] Protein has been the major focus of both identification and quantitation studies of contact lens deposits. Estimates of the total amounts vary but fall within a reasonably well-defined range. Group I lenses typically attract less than 10 μg of protein, Groups II and III lenses approximately 30 μg, and Group IV lenses 1000 μg or more. [108, 126, 167, 172-176, 178, 213-217] Silicone hydrogel contact lens materials deposit extremely low levels of lysozyme compared to conventional hydrogel lenses, with typical levels being in the < 20 μg/lens range. [204, 208, 209] Using mass spectrophotometry, recent studies have shown that over 60 proteins can be detected on worn silicone hydrogel contact lens materials. [218, 219] The various proteins that were detected on the contact lens materials are listed in the following table (Table 2.6).

Table 2-6: List of tear proteins detected on silicone hydrogel contact lenses identified using tear proteomic approach [219]

Major tear proteins
1. Lysozyme
2. Lipocalin 1
3. Lactoferrin
Immunoglobulin family
4. Heavy chain of IgA2
5. Light chain of IgA1
6. Immunoglobulin J chain
7. Large anti-HSV-glycoprotein D single chain antibody
8. Heavy chain of IgM
9. Ig kappa light chain
10. Ig rheumatoid factor H
11. Immunoglobulin heavy chain variable region
12. Poly-Ig receptor (secretory component)

Proline Rich Proteins

13. Proline rich 4 isoform 1 or 2 (lacrimal)
14. Nasopharyngeal carcinoma associated proline rich 4
15. Basic proline-rich protein (Lacrimal) (pHL E1F1)

Apolipoproteins

16. Apolipoprotein J/Clusterin
17. Apolipoprotein

Albumin

18. Serum albumin

Hemoglobins

19. Hemoglobin subunit alpha
20. Hemoglobin subunit beta

Complement proteins

21. C9 complement protein
22. Complement component C3

Keratins

23. Keratin 1 (stratum corneum/keratinocytes – keratin 10/9 pair)
24. Keratin 2 (stratum corneum/keratinocytes – keratin 10/9 pair)
25. Keratin 3 (cornea – keratin 12 pair)
26. Keratin 4 (stratified epithelium – keratin 13 pair)
27. Keratin 5 (stratified epithelium – keratin 14/15 pair)
28. Keratin 6
29. Keratin 9 (stratum corneum/keratinocytes – keratin ½ pair)
30. Keratin 10 (stratum corneum/keratinocytes – keratin ½ pair)
31. Keratin 12 (cornea – keratin 3 pair)
32. Keratin 13 (stratified epithelium – keratin 4 pair)
33. Keratin 14 (stratified epithelium – keratin 5 pair)
34. Keratin 15 (stratified epithelium – keratin 5 pair)
35. Keratin 18 (simple epithelia – keratin 8 pair)
36. Keratin, type I (Hair keratin)

Proteoglycans and mucins/mucin-like proteins

37. Heparan sulphate proteoglycan
38. DMBT-1, isoform b precursor variant

Other proteins

39. Prolactin-induced protein
40. Heat shock protein 27
41. Predicted protein, similar to Mammaglobin A precursor
42. Retinoic acid receptor responder 1, isoform 1 or 2
43. Peptide PB saliva

44. Peptide PA saliva
45. Transferrin
46. Aldehyde dehydrogenase
47. Alpha - tubulin
48. Chain A, Human cathepsin G
49. Dermcidin preproprotein
50. Fibrinogen betaB
51. Fibronectin
52. Ficolin 2 isoform b
53. Lacritin
54. Pre-pro-megakaryocyte potentiating factor
55. Tubulin 5 - beta
56. Vitronectin precursor
57. Mesothelin or CAK1 antigen precursor
58. Matrix Gla protein
59. Heterogenous nuclear ribonucleoproteins A2/B1 isoform A2
60. Heterogenous nuclear ribonucleoproteins A1
61. Heterogenous nuclear ribonucleoproteins A3
62. Plasminogen
63. E-cadherin
64. Cystatin SA-III
65. hCG22067
66. Alpha-S1-casein
67. Actin
68. Phospholipid transfer protein isoform a

Tear film proteins frequently detected on hydrogel contact lenses include lysozyme, lactoferrin and albumin, [142, 211, 215, 218-220] and among these lysozyme has been the most widely studied. [108, 173, 194, 203, 221] The following section provides a brief overview of lysozyme deposition on contact lenses.

2.5.2 Lysozyme and contact lenses

The major proteins that are deposited on contact lenses include lysozyme, lactoferrin and albumin, [142, 211, 215] and among these lysozyme has received the greatest attention. [108, 173, 194, 203, 221] Karageozian first reported that the principal component of deposits that presented problems with contact lens wear was lysozyme, which may be selectively adsorbed and denatured on the lens surface. [222] Many others have since confirmed the predominance of lysozyme in lens deposition. [108, 126, 213, 215, 216, 223, 224]

Lysozyme is a major component in tears and of contact lens deposits. [55, 219] It accounts for approximately 40% of total protein found in tears and is the major protein (approximately 36 to 95% depending on lens type) deposited on hydrogel contact lenses. [195, 225] In addition to its bactericidal properties (described earlier), lysozyme is also reported to have anti-inflammatory properties in the tear film, although the mechanism through which this action occurs is unknown. [82] Exploration of lysozyme deposition (quantity and conformation) on a number of different conventional and silicone hydrogel surfaces is of growing interest due to observations that patients using silicone hydrogel lenses are prone to develop papillary conjunctivitis, possibly due to the denaturation of lysozyme on the lens materials. [190, 192, 226-229]

Lysozyme is a positively charged molecule and this, coupled with its small size, results in its increased adsorption onto negatively charged substrates such as FDA group IV contact lens materials. [108, 195, 199, 203, 230-232] Studies to date, suggest that the novel silicone hydrogel lens materials deposit extremely low levels of lysozyme compared to

conventional hydrogel lenses, with typical levels being in the < 20 μg/lens range. [204, 208, 209] Once lysozyme firmly adsorbs onto contact lens materials, it tends to undergo conformational changes, [108, 208, 209, 232] which might potentially result in a variety of immunological responses, including contact lens associated papillary conjunctivitis. [190, 192, 226-229] Some studies have speculated that the conformational state of the deposited protein could have an influence on various subjective symptoms in contact lens wearers; [1, 233] however, to-date, no study has determined the relationship between subjective symptoms and the conformational state of the deposited protein. Therefore, in addition to investigating the total quantity of the deposited protein, it is of significant clinical relevance to study the conformational state of the deposited protein.

Several studies have investigated the kinetics of protein or lipid deposition on contact lens materials *in vitro*, [143, 172, 182, 197, 203, 221, 234-236] and *in vivo* [126, 167, 180, 213, 230, 237-240] on conventional hydrogel contact lens materials. Although, the quantity and/or conformational state of lysozyme deposited on silicone hydrogel lens materials have been reported, [204, 208, 209] to-date, no study has examined the deposition of lysozyme or the conformational state of lysozyme deposited on silicone hydrogel contact lens materials as a function of time. Knowing the rate of protein deposit accumulation and the duration over which lysozyme denatures is clinically relevant to patient symptoms and in determining the most appropriate replacement frequency. In addition, this information would be very useful for designing clinical investigations of hydrogel lenses and associated lens care products.

2.6 Methods used to determine protein aggregation

In this section, a brief overview of various physical methods that are used to determine protein aggregation and some of the advantages and disadvantages of each of these techniques are discussed.

2.6.1 Atomic Force Microscopy

Atomic Force Microscopy (AFM) is a direct, in-situ method for visualizing the surface morphology of protein aggregates that are being studied. [241] AFM works by a flexible force-sensing cantilever which is scanned over the surface of the sample in a pattern to reveal atomic level resolution. [241, 242] Some disadvantages of AFM include issues of the uniformity of the sample being measured, the possibility of damage to the sample from the force being applied by the AFM measurement. [243] Moreover, this is only a surface morphology technique, and AFM does not provide any information about the actual composition of the aggregate being measured. [243]

2.6.2 Electron Microscopy

Electron Microscopy (EM) works by a focused beam of electrons being scanned across the surface of the sample producing backscattered and secondary electrons as a way to image the sample. [241] The EM technique is extremely useful for determining the morphology of the sample, especially when used in conjunction with other, complimentary physical methods. [241] EM is a very sensitive method which has the capability to detect at atomic resolution and to see "a" species rather than "*the*" dominant

species more accurately. [241] Another well-known disadvantage of EM is the possibility of the electron beam damaging the sample by either causing nucleation or changing the morphology of the fibrils already present in the sample. [244]

2.6.3 Circular Dichroism

Circular Dichroism (CD) is a direct, usually in-situ measurement and involves the detection of circularly polarized light in the absence of a magnetic field. [245] CD is also capable of giving information about the secondary structure of the protein, [245, 246] so that a conformational change, for example, can be followed over time. Another useful advantage of this technique is the ability to differentiate between the formation of α-helices and β-sheets by their signature spectra. [245] One of the requirements for this method is that the sample should be optically active. [245]

2.6.4 Dyes

The use of dyes is a commonly employed technique for the detection of protein aggregation due to the sensitivity of the technique. [242] It is usually an ex-situ method as the concentrations of proteins used for these experiments must be increased in order to be detected by the dyes used. [243] This results in a fluorescence spectrum that shows an increase in the amount of fluorescence with the formation of more aggregates. [247] A disadvantage of this technique is that, as with some other techniques, different species deposited on the biomaterial cannot be differentiated from one another and all contribute to the fluorescence in a combined, averaged way. [248] Recently, tear proteins such as

lysozyme and albumin were tagged with fluorescent dyes. [144, 249, 250] Using confocal microscopy, the location of these fluorescently-tagged proteins have been determined on conventional and novel silicone hydrogel contact lens materials. [144, 249, 250] These studies have demonstrated that the location of the proteins depends upon the nature of the protein and also on the polymer make-up and/ or surface modification processes of the conventional and silicone hydrogel lens materials. [144, 249, 250]

2.6.5 Fluorescence Spectroscopy with Extrinsic and Intrinsic Fluorophores

Fluorescence spectroscopy involves measuring the fluorescence intensity of either fluorophores to gain information on conformation changes occurring in the protein. [247] This technique is advantageous in that it is broadly applicable, is sensitive enough and provides good signal to noise ratios and the kinetics of deposition can be readily measured. [247] There are several sub-categories of fluorescence spectroscopy which can be used to elucidate information on folding, membrane-protein interactions and fibril formation. [247] Although fluorescence spectroscopy is a powerful technique to follow structural changes, other complementary techniques should be used in conjunction with it to verify the fluorescence observed. [243]

2.6.6 Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) is a direct, usually ex-situ method that can be used to detect the β-structure of proteins as well as the orientation of adjacent β-sheets to determine whether they are in a parallel or anti-parallel

configuration. [251] Therefore, FTIR can provide quantitative information about the larger aggregates being formed and information about the morphology of these aggregates. [243]

2.6.7 Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance Spectroscopy (NMR) is a powerful, direct, method that has been used in monitoring protein aggregation in both the solid state and in solution. [252] Using solid state NMR, the amount of aggregates that solidify can be monitored and their conformational changes can be observed. [252] The use of solid-state NMR typically requires the use of labeled amino acid residues in order to obtain a reasonable signal. [252] One possible disadvantage is that at the higher concentrations required for solution NMR, proteins with a tendency to aggregate often become insoluble. [253] Due to the concentration and aggregation issues, typically, 2-D and polynuclear NMR techniques are required to monitor solution aggregation processes. [253]

2.6.8 Mass Spectrometry

Mass spectrometry, in particular matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry, is a direct, ex-situ method that has been used to monitor the formation of protein complexes. [251] Since MALDI-TOF has the ability to monitor species in the molecular weight range up to approximately 300,000 g/mol, both smaller and larger proteins can be detected. [254] One of the disadvantages of this method is that the use of MALDI-TOF typically requires the use of one of a number of buffer, salt, and co-solvent conditions. [255]

2.6.9 Quartz Crystal Oscillator Measurements

This technique employs an oscillating quartz crystal microbalance to detect the growing weight of protein fibrils on the surface of existing seeds. [256] The mass change is taken as a direct measurement of aggregation. [256] The main disadvantage of this sensitive technique is that quartz crystal oscillator measurements detect a mass change but do not provide information about the heterogeneity or homogeneity of the sample. Perhaps the use of these microbalance methods in conjunction with mass spectrometry could alleviate this problem.

2.6.10 Sedimentation Studies

Sedimentation studies are used to separate soluble from insoluble proteins using centrifugation. [242] In theory, the aggregated and thus heavier protein(s) should become separated from the monomeric protein. [242] However, there is evidence for the formation of soluble oligomeric intermediates that are in some cases hypothesized to be toxic, [257-259] and this technique would not be able differentiate between monomeric and soluble oligomeric protein or between insoluble intermediates and fibrils. [242] Sedimentation could, however, be used in conjunction with other techniques to distinguish between the species contained in a heterogeneous sample. [242]

2.6.11 Size-Exclusion Chromatography

Size exclusion chromatography is a method of separating aggregates of various sizes. [260] In combination with one or more of the other technique(s), size-separated

aggregates can be detected, although it is not clear if the separation process causes or just reports the separated sizes. [251] To date, size exclusion chromatography appears to be the most useful method for separating out smaller protein subsequently detected by NMR or absorbance measurements. [251]

In addition to the above-mentioned techniques, other physical techniques employed to determine protein aggregation are absorbance spectroscopy, [242] calorimetry, [251] flow birefringence, [261] light scattering, [262] turbidity [242] and X-ray diffraction. [242, 263] The next section specifically deals with various techniques that are employed to quantify protein deposition on contact lens materials.

2.7 Methods to quantify proteins deposited on contact lenses

Several microscopic, photometric and imaging techniques have been used to investigate protein deposits on contact lenses. [126, 167, 178, 264, 265] The major limitation of microscopic and imaging techniques is that they are generally not suitable for accurate quantitation purposes. [1] Various biochemical assays including Enzyme-Linked Immunosorbent Assay, High Performance Liquid Chromatography and Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis are useful because of their increased sensitivity, accuracy and ability to target specific proteins.

2.7.1 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a widely used method for investigation of tear film proteins. ELISA utilizes antibodies bound to solid surfaces, such as plastic or polystyrene micro-titre plates to quantify protein of interest. [266] ELISA relies on antibody recognition of the protein of interest in solution. A colorimetric, fluorescent or chemiluminescent reaction is used to quantify the amount of protein bound to the well, followed by detection of the specific signal. [266]

The major advantage of ELISA is the ability to process high number of samples (up to 96) at the same time and this method also reduces the need for handling the sample. The disadvantage with this method is that it can cross-react with non-targeted proteins, or the interaction between antibody and target can be disrupted by other sample components. [267]

2.7.2 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) separates proteins based on molecular size in a column support through a tightly packed matrix (such as glass, plastic or silica beads) under high pressure. There are several types of HPLC and Size Exclusion HPLC is the widely used method for quantifying individual proteins in solution. The major advantage of HPLC is that several proteins can be quantified from a single experiment. However, Fullard (1988) found an unusually high absorbance for lysozyme, which may decrease the accuracy of the assay and could lead to erroneous conclusions. [77]

2.7.3 SDS-PAGE followed by Immuno blotting

Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis (SDS-PAGE) is a specific type of gel electrophoresis that uses sodium dodecyl sulphate (SDS) as a detergent to confer a negative charge to a protein and polyacrylamide as a matrix to separate proteins according to size. Electrophoresis refers to the migration of charged molecules in solution in response to an electric field. Electrophoresis is a simple, rapid and highly sensitive technique and this can be used to study the properties of a single charged species, and as a separation technique. [268] Once physically separated, proteins are visualized using antibodies and chromogenic, fluorescent or chemiluminescent substrate is used to quantify the binding of the antibodies to the protein. Resolution of proteins that migrate together in the gel is accomplished by the specificity of the antibody; a single antibody will recognize a small portion of the protein of interest, thereby reducing the potential of visualizing co-migrating tear film components. In addition, immunological

visualization allows identification of variations in protein conformation, such as unexpected polymerization or interaction with other proteins. [269, 270]

2.7.3.1 PhastSystem™

The PhastSystem™ (Amersham Pharmacia Biotech, Baie d'Urfe, QC, Canada) is an automated mini-gel system that combines both electrophoresis and immunoblotting into a single apparatus (Figure 2.6). This system is different from that of traditional larger gel electrophoresis systems, in that it utilizes pre-cast, extremely small (4.5 cm X 5 cm) SDS gels for protein separation. These extremely small SDS gels confer increased sensitivity with a minute sample volume requirement, compared to other electrophoresis and immunoblotting systems.

Figure 2-6: The PhastSystem™

Unlike other gel electrophoresis systems, the protein-containing loading buffer, in volumes from 0.3 µl to 1 µl, is applied to well combs with up to 12 lanes rather than

directly into wells cut into a gel. These disposable well combs are then automatically applied to the mini-gel for electrophoretic separation. The temperatures, voltage, current and power are automatically regulated throughout the separation process. Western blotting is similarly automated and simplified by the PhastSystem™. The small size of the gels and the small sample volume applied to the gel make the PhastSystem™ unique among all other systems. The low sample volumes and small pre-cast gels also provide an added level of sensitivity and flexibility, especially for proteins in low concentrations. [148, 271] Several researchers have used the PhastSystem™ to detect extremely low concentrations of tear film proteins. [148, 208, 209, 232, 271-276]

Whilst the methods such as HPLC or ELISA can provide other advantages such as enhanced efficiency in quantifying several proteins at the same time (HPLC) or provide greater sample throughput (ELISA), electrophoretic separation followed by immunoblotting using the PhastSystem™, provides us with the best tool for quantifying individual proteins in extremely low concentrations. While at the same time it allows visualization of the protein to assess cross-reactivity between the antibody and protein of interest and possible aggregation or polymerization of the protein. Using this technique, a method has been optimized to quantify lysozyme deposition on SH lenses. [209]

2.7.4 Radiolabeling technique

A number of studies have found that artificial tear solutions can be an attractive option in determining the binding affinity of different components and the mechanisms involved in such binding. [197, 277-285] The major advantages of *in vitro* studies are that

several experimental variables can be eliminated, the quantity of the bound component can be determined by labeling the species fluorometrically or radiometrically, and several experimental hypotheses can be tested without engaging in a resource consuming clinical trial. A number of quantitative protein methods have been applied to the analysis of protein deposits on contact lenses. However, many of these techniques require complex extraction procedures which may not be 100% efficient. The radiochemical assay is:

(1) quantitative and reproducible with a low detection limit

(2) able to assess a large number of samples

(3) compatible with all contact lens materials

(4) not dependent on complex extraction techniques

(5) able to detect surface as well as bulk protein.

The isotope ^{125}I has been extensively used to radioactively label lysozyme and other proteins in previous studies. [172, 173, 286-288] The isotope is incorporated into the protein and radioactivity can be used to assess the level of protein binding.

2.8 Corneal complications associated with contact lens wear

Corneal complications associated with soft contact lens wear can be broadly classified as metabolic, infective, inflammatory, mechanical and allergic (Table 2.7).

Table 2-7: Corneal complications associated with soft contact lens wear

Metabolic	Changes to corneal physiology	Epithelium	Altered Metabolism, mitosis, increased thickness and decreased exfoliation
		Stroma	Decreased Keratocyte density
		Endothelium	Altered metabolism, polymegethism and pleomorphism
Infective	Microbial Keratitis (MK)	Most common bacterium is *Pseudomonas aeruginosa*	
Inflammatory	Contact Lens Acute Red eye (CLARE)	Often caused by microbial contamination of lenses	
	Contact Lens Peripheral Ulcer (CLPU)		
	Infiltrative Keratitis (IK)		
Mechanical	Superior Epithelial Arcuate Lesion (SEAL)	Lens design, lens material, lens modulus, foreign bodies, dehydration	
	Corneal staining & Erosions		
Allergic	Contact Lens Papillary Conjunctivitis (CLPC)	Lens deposits, solution toxicity	

The following section provides an overview of the incidence and risk factors, pathogenesis and microbiology associated with Microbial Keratitis (MK), Contact Lens Acute Red eye (CLARE) and Infiltrative Keratitis (IK).

2.8.1 Microbial Keratitis

2.8.1.1 Incidence and risk factors

Over the years, several studies were conducted to identify the risk factors and incidence rates that are associated with contact lens-related microbial keratitis (MK).

Table 2-8: Annualized incidence (per 10000 wearers) of contact lens related microbial keratitis

Study	Incidence per 10000 wearers (Confidence Intervals)	
	Daily wear soft contact lens	Overnight wear soft contact lens
Poggio et al (1989) [289]	4.1 (2.9-5.2)	20.9 (15.1-26.7)
MacRae et al (1991) [290]	5.2 (0-15.4)	18.2 (8.2-27.8)
Nilson & Montan (1994) [291]	2.2 (0.4-3.9)	13.3 (4.1-22.6)
Cheng et al (1999) [292]	3.5 (2.7-4.5)	20.0 (10.3-35.0)
Lam et al (2002) [293]	3.1 (2.1-4.0)	9.3 (4.9-13.7)
Morgan et al (2005) [294]	6.4 (4.1-9.9)	96.4 (37.5-245.2)

One of the overwhelming risk factors for the development of MK is overnight wear of soft contact lenses. Schein and co-workers reported that the relative risk of MK following extended wear of soft lens was four times greater than daily wear. [295] Dart and co-workers showed that when compared to rigid contact lenses, overnight wear of soft contact lens wear carry 21 times greater risk of MK, while daily wear increases the risk by approximately 4 times. [296] A study by Poggio and Abelson suggested an increased risk of MK with disposable soft contact lenses. [297] A retrospective study from Sweden reported an increased risk for conventional daily wear compared to disposable daily wear. [291] Other studies found an increased risk of MK with disposable lenses compared to daily wear or extended wear of conventional soft contact lenses. [298-300]

With the introduction of high Dk silicone hydrogel contact lenses, it was assumed that the risk of developing MK would be much less, as these lenses reduce many of the clinical signs of hypoxia. [301] Preliminary studies that evaluated high Dk lenses indicated that there was no incidence of MK with these lenses. [302, 303] However, MK has been reported following extended wear of these lenses as well. [304-306] Some of the other risk factors for MK include trauma, altered corneal sensation, altered tear volume and altered tear composition, smoking and non compliance with lens care techniques. [76, 193, 293, 295, 307-309]

2.8.1.2 Pathogenesis

Three main factors are required for MK to occur: exposure to infectious micro-organisms, binding of these micro-organisms to the cornea and, in the case of the majority of ocular pathogens, a compromised cornea with an epithelial break. [56, 310] MK is most often caused by *Pseudomonas aeruginosa*. [56, 310, 311] *Pseudomonas* keratitis requires corneal trauma, particularly an epithelial defect in order for it to develop. [312] Studies have shown that lens wear by itself increased the organisms' adhesion to the cornea [307] while extended wear of contact lenses further enhances its adhesion to the corneal epithelium. [313] Solomon and co-workers showed in an animal model, that hypoxia along with the presence of a contact lens was a major risk factor for the development of *Pseudomonas keratitis*, even more than the presence of the organism and epithelial defect. [314] It was hypothesized that corneal hypoxia led to a break in the epithelium which served as a portal of entry for the bacteria.

2.8.1.3 Microbiology

A number of microorganisms have been implicated in MK. Approximately one third of contact lens-related MK have been found to be associated with gram positive cocci such as *staphylococci* and *streptococci*, but two thirds is associated with gram negative rods, especially *Pseudomonas aeruginosa*. [315] Fungi are more commonly seen in therapeutic contact lens wear [316] and account for approximately 12% of the cases, whereas they account for 3% of cases associated with cosmetic or aphakic contact lens wear. [315] The following table summarizes the organisms that are frequently isolated from cases of patients with MK with silicone hydrogels and HEMA-based hydrogel lenses. [56]

Table 2-9: Bacteria isolated from cases of MK [56]

Bacteria	Silicone hydrogel	HEMA-based hydrogel
Gram negatives	75%	73%
Pseudomonas aeruginosa or spp	42%	66%
Serratia marcescens	8%	4%
Alcaligenes xylosidans	8%	-
Acinetobacter spp	8%	-
Haemophillus influenzae	-	1%
Morganella morganni	-	1%
Escherichia coli	-	1%
Unidentified	8%	-
Gram Positives	25%	25%
Streptococcus viridans	17%	-
Coagulase negative staphylococci	-	13%
Staphylococcus aureus	-	6%
Corynebacterium spp.	8%	3%
Propionibacterium spp.	-	3%
Bacillus cereus	-	1%
Unidentified	-	1%

2.8.2 Contact Lens Acute Red Eye (CLARE) and Infiltrative Keratitis (IK)

2.8.2.1 Incidence and risk factors

Contact Lens Acute Red Eye (CLARE) has been reported following overnight wear of silicone hydrogel lenses as well, with an annualized incidence rate of 0.7 and 0.8 per 10,000 for extended wear (7 days) and continuous wear (30 days) respectively. [303] Studies conducted at the Cornea and Contact Lens Research Unit (CCLRU) and L.V. Prasad Eye Institute give incidence rates ranging from 1.0 to 7.0% of eyes following overnight wear of conventional hydrogel lenses and 2.5 to 12.5% for silicone hydrogel lens wearing eyes. [317] Overnight wear of lenses is the major risk factor for CLARE. Earlier studies concluded that the condition developed as a result of debris being trapped under a tight immobile lens, [318, 319] however later studies found no correlation between lens tightness and CLARE. [320] Bacterial contamination of contact lenses is another risk factor for the development of CLARE. [321]

Infiltrative Keratitis (IK) is the general term used to describe all infiltrative events not categorized as Contact Lens-induced Peripheral Ulcer (CLPU), CLARE or Microbial Keratitis. IK is a relatively mild inflammatory event and has been reported following conventional and silicone hydrogel lens wear. [322] Studies conducted at the CCLRU and L.V. Prasad Eye Institute give incidence rates ranging from 2.0 to 4.0% of eyes for disposable hydrogel lenses and 5.6 to 17.8% for continuous wear of silicone hydrogel lenses. [317] Since the condition resembles CLARE, it has been suggested that bacterial contamination of lenses or lens cases are a major risk factor. In addition, mechanical

trauma has also been postulated to lead to IK, as a large number of cases have been isolated from silicone hydrogel wearing eyes. [317]

2.8.2.2 Pathogenesis

One of the key elements in the chain of events that lead to CLARE has been demonstrated to be due to the colonization of contact lenses with bacteria and also the inflammatory state of the lens wearing eye. [317] A state of sub-clinical inflammation exists during eye closure [137, 323] which promotes the growth of the normal ocular microbiota. [324] These changes contribute to a pro-inflammatory state in the closed eye, which in addition to the accumulation of bacterial toxins and other by-products predispose to the development of CLARE. In addition to the accumulation of bacteria on the lens, corneal trauma can also lead to corneal infiltration. Trauma to the cornea, potentially triggers an immune response which can then lead to infiltration by Polymorphonuclear leukocytes (PMNs) that is observed in IK. [325]

2.8.2.3 Microbiology

Several Gram positive and Gram negative bacteria are seen in the contact lenses of patients with CLARE, IK and CLPU and the following table (Table 2.10) lists the various bacteria that are associated with these adverse responses:

Table 2-10: Micro-organisms associated with CLARE, IK and CLPU

Micro-organism	Adverse responses
Gram negatives	
Abiotrophia defectiva	IK
Acinetobacter spp.	CLARE,IK
Aeromonas hydrophilia	CLARE
Alcaligenes xylosoxidans subsp. *Dentrificans*	IK
Branhamella catarrhalis	IK
Enterobacter cloacae	IK
Eshericia coli	CLARE,IK
Haemophillus influenzae	CLARE,IK
Haemophillus parainfluenzae	CLARE,IK
Klebsiella oxytoca	CLARE,IK
Klebsiella pneumoniae	CLARE
Neisseria spp	IK
Pseudomonas aeruginosa	CLARE,CLPU
Serratia liquefaciens	CLPU,IK
Serratia marcescens	CLARE,IK
Stentrophomonas maltophilia	CLARE
Gram Positives	
Non-haemolytic *Streptococcus* spp.	IK
Staphylococcus aureus	CLPU,IK
Streptococcus pnuemoniae	CLARE,CLPU,IK
Streptococcus viridans	CLARE,IK
Fungus	
Yeast	IK
Mould	IK

2.9 Bacterial adhesion to contact lenses

Several factors regulate the adhesion of bacteria to biomaterials including surface properties of the biomaterial, bacteria-biomaterial surface interactions, van der Waals forces, properties of the bacterial cell and the electrolyte concentration of the suspending liquid. [326-329] There are several steps involved in the adhesion of bacteria to contact lens materials. Initially, bacteria adhere to contact lenses non-specifically by removing water

molecules surrounding the lenses using their hydrophobic surface components. [326, 330] Following this reversible initial attachment, the bacteria adhere to the biomaterial strongly by an adhesion receptor mediated specific adhesion. [326, 330] After initial adhesion, bacteria adhered to the contact lens surface proliferates on the substratum within the polysaccharide-rich glycocalyx, forming micro-colonies. [328] As these micro-colonies grow, they coalesce with neighboring micro-colonies to form fully-developed biofilms. [328]

It has been demonstrated that *Pseudomonas aeruginosa* can use several cell surface structures to adhere to epithelial cells. [331-335] The lipopolysaccharide (LPS) which is involved in the adhesion of *Pseudomonas aeruginosa* to corneal epithelial cells [334, 335] is also believed to play a role in bacterial adhesion to contact lenses. It was demonstrated that altering the LPS of *Pseudomonas aeruginosa* increased its adhesion to conventional hydrogel FDA group IV etafilcon A lens materials. [336] It has also been suggested that the changes in the outer membrane proteins of *Pseudomonas aeruginosa* may have an effect on their adhesion to contact lenses. [336] Cowell and colleagues demonstrated that growth of *Pseudomonas aeruginosa* Paer1 under conditions of nitrogen or carbon limitation also altered the ability of this strain to adhere to etafilcon A lens materials. [337] A study by Stapleton and co-authors showed that the adhesion of *Pseudomonas aeruginosa* to low water content, non-ionic contact lenses was significantly greater than to ionic lenses and the maximal adhesion was observed after 45 minutes. [338] In addition, factors such as proteins and lipids deposited from tears on the contact lens surface are likely to alter the bacterial adhesion to a contact lens. [127, 145-147, 193, 307, 336, 339-342] Other factors that play a

role in the adhesion of bacteria to contact lenses include lens material, [336, 338, 343-348] duration of wear, [193, 336, 349] and surface roughness. [350, 351] To-date, few studies have investigated the adhesion of bacteria to novel silicone hydrogel lens materials; [344-347] however, none of these have determined the influence of tear components on the adhesion of bacteria to silicone hydrogel lens materials.

2.10 Summary

A review of the literature proves that most of the work conducted to date has focused on protein deposition on conventional hydrogel contact lens materials with little knowledge about the quantity and the conformational state of protein deposition on novel silicone hydrogel lens materials. Moreover, there is a clear need for further investigation into the quantity and the conformational state of protein deposited on conventional and silicone hydrogel contact lens materials as a function time. To date, there is no evidence concerning the relationship between the conformational state of deposited protein and subjective symptoms and also the effect of tear protein deposits on bacterial adhesion to novel silicone hydrogel contact lens materials. These are the areas that will be addressed in this thesis.

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3 Thesis Objectives

The aim of this thesis was to gain a greater understanding of the interaction of tear proteins with conventional and novel silicone hydrogel contact lens materials and also to determine the influence of tear protein deposits on the subjective symptoms and bacterial adhesion to contact lens materials.

The specific aims of this thesis are as follows:

1. To determine the total lysozyme deposition on conventional and silicone hydrogel contact lens materials as a function of time by artificially doping lenses with ^{125}I-labeled lysozyme.
2. To determine the conformational state of lysozyme deposited on conventional and silicone hydrogel contact lens materials as a function of time using an *in vitro* model.
3. To quantify the total protein, total lysozyme and the conformational state of lysozyme deposited on a novel, lathe-cut silicone hydrogel contact lens material after three-months of wear.
4. To determine the relationship between protein deposition and clinical signs & symptoms after one-day wear of etafilcon lenses in a group of symptomatic and asymptomatic lens wearers.
5. To determine the influence of individual tear proteins (lysozyme, lactoferrin and albumin) on the adhesion of Gram positive and Gram negative bacteria to conventional and silicone hydrogel contact lens materials.

4 Kinetics of *in vitro* lysozyme deposition on silicone hydrogel, PMMA and FDA groups I, II and IV contact lens materials

This chapter is published as follows:

Lakshman N Subbaraman, Mary-Ann Glasier, Michelle Senchyna, Heather Sheardown, Lyndon Jones. Kinetics of *in vitro* lysozyme deposition on silicone hydrogel, PMMA, FDA groups I, II and IV contact lens materials. *Curr Eye Res*. 2006; 31(10): 787-96.

4.1 Abstract

We sought to compare the kinetics of *in vitro* lysozyme deposition on silicone hydrogel (SH), PMMA, FDA groups I, II and IV contact lenses. Lenses were incubated in ^{125}I-labeled lysozyme for time periods ranging from 1 hour to 28 days and radioactive counts were determined. SH lenses and PMMA deposited less lysozyme than conventional hydrogel lenses ($p<0.05$). Lysozyme accumulation on group IV lenses reached a maximum on the 7th day and then plateaued. PMMA showed a deposition pattern similar to that seen on lotrafilcon A and lotrafilcon B SH lenses. These results reiterate that kinetics of lysozyme deposition is highly material dependent.

Key words: contact lens; deposition; kinetics; radiolabeled lysozyme; silicone hydrogel.

4.2 Introduction

Following insertion onto the eye, hydrogel contact lenses rapidly adsorb components from the tear film, particularly protein, [1-6] lipid, [7-11] and mucin. [12] These deposits create a number of problems for patients, including discomfort, reduced visual acuity, dryness and reduced lens life. [9, 13-15] Protein deposits may be more problematic as they can lead to adverse immunological responses, including Giant Papillary Conjunctivitis. [16-18] The newly introduced silicone hydrogel (SH) contact lenses offer excellent comfort and performance and superior physiological responses compared to traditional contact lens biomaterials. [19-21] Moreover, recent data suggest that these lenses deposit extremely low levels of protein when compared to conventional hydrogel (CH) lens materials. [22-26]

It is widely recognized that the adsorption of proteins at the contact lens surface is complex and depends upon a number of factors. Notable among these are material water content and surface charge. [3-7, 10, 27-31] A number of studies have investigated the kinetics of protein or lipid deposition on contact lens materials *in vitro*, [1, 12, 32-38] and *in vivo* [10, 27, 39-45] on CH lens materials. Although, the quantity and/or conformation of lysozyme deposited on SH lens materials have been reported, [22-24] to-date, no study has examined the deposition of lysozyme on SH lens materials as a function of time. Knowing the rate of protein deposit accumulation and the duration at which protein accumulation reaches either a maximum or a plateau level could be clinically relevant to patient symptoms and in determining the most appropriate replacement frequency. In addition, this information would be very useful for designing clinical investigations of hydrogel lenses and

associated lens care products. As lysozyme approximates 90% of the protein found on group IV contact lens, it is often used as the "model protein" for protein accumulation. [6, 43, 46]

A number of studies have found that using model artificial tear solutions in an *in vitro* model can be an attractive option for determining the binding affinity of different components and the mechanisms involved in such binding. [35, 47-55] The principal advantages of this method are that most of the experimental variables are eliminated, quantitation can be enhanced by labeling the species fluorometrically or radiometrically, and simple hypotheses can be tested without indulging in resource-consuming clinical studies. However, the real world provides a complex set of variables which cannot be excluded from having an influence in clinical lens spoilage. Although, *in vitro* results may not be directly transferable to the *in vivo* state, *in vitro* studies will provide valuable guidance for further *in vivo* studies.

A number of quantitative analytical methods have been applied to the analysis of protein deposits on contact lenses. [42, 44, 56-59] However, many of these techniques require complex extraction procedures which may not be 100% efficient. The radiochemical assay is quantitative and reproducible with a low detection limit, able to assess a large number of samples, compatible with all contact lens materials, not dependent on complex extraction techniques and able to detect surface as well as bulk protein deposition. The isotope ^{125}I has been extensively used to radioactively label lysozyme and other proteins in previous studies. [1, 2, 60-62] The isotope is incorporated into the protein and radioactivity

can be used to assess the level of protein binding. Hence in this study, we used an *in vitro* radiolabeling method using ^{125}I to gain insight into the kinetics of lysozyme deposition on all the currently available SH lenses and compared these data with the results from polymethyl methacrylate (PMMA), FDA Group I, Group II and Group IV CH lens materials.

4.3 Materials and Methods

4.3.1 Reagents and materials

Chicken egg white lysozyme was purchased from Sigma-Aldrich (St. Louis, MO, USA). The lenses that were used in the study included five types of SH lenses, five types of CH lenses and one PMMA lens. The SH lenses examined were Night & Day™ (CIBA Vision, Duluth, GA, USA), O_2Optix™ (CIBA Vision, Duluth, GA, USA), PureVision™ (Bausch & Lomb, Rochester, NY, USA), Acuvue® Advance™ (Vistakon, Johnson & Johnson, Jacksonville, FL, USA) and Acuvue® OASYS™ (Vistakon, Johnson & Johnson, Jacksonville, FL, USA). The properties of these lens materials are described in Table 4.1. The CH lens materials examined were Optima™ FW (Bausch & Lomb, Rochester, NY, USA), Proclear® (CooperVision, Norfolk, VA, USA), SofLens™ 66 (Bausch & Lomb, Rochester, NY, USA) Acuvue® 2 (Vistakon, Johnson & Johnson, Jacksonville, FL, USA) and Focus® Monthly (CIBA Vision, Duluth, GA, USA). The properties of these lens materials are described in Table 4.2. PMMA lenses were ordered from Cardinal Laboratories (Cambridge, ON, Canada). Sterile, 5ml non-pyrogenic, polypropylene round bottom tubes were purchased from Falcon (Franklin Lanes, NJ, USA). All other reagents purchased were analytical grade and obtained from Sigma (St. Louis, MO, USA).

Table 4-1: Properties of silicone hydrogel lens materials evaluated in the study

Proprietary name	Night & Day™	O_2 Optix™	PureVision ™	Acuvue® Advance™	Acuvue® OASYS™
USAN	Lotrafilcon A	Lotrafilcon B	Balafilcon A	Galyfilcon A	Senofilcon A
Manufacturer	CIBA Vision	CIBA Vision	Bausch & Lomb	Johnson & Johnson	Johnson & Johnson
Water content (%)	24	33	36	47	38
Oxygen Permeability (Dk)	140	110	91	60	103
Centre thickness (mm) -3.00D	0.08	0.08	0.09	0.07	0.07
Oxygen Transmissibility (Dk/t) at 35˚C	175	138	101	86	147
FDA group	I	I	III	I	I
Surface Treatment	25nm plasma coating with high refractive index	25nm plasma coating with high refractive index	Plasma oxidation process	No surface treatment. Internal wetting agent (PVP) that also coats the surface	No surface treatment. Internal wetting agent (PVP) that also coats the surface
Principal monomers	DMA+TRIS+ Siloxane monomer	DMA+TRIS+ Siloxane monomer	NVP+TPVC+ NVA+ PBVC	mPDMS+ DMA+HEMA +siloxane macromer+ PVP+ EGDMA	mPDMS+ DMA+HEMA +siloxane macromer+ PVP+ TEGDMA

DMA (N,N-dimethylacrylamide); EGDMA (ethyleneglycol dimethacrylate); HEMA (poly-2-hydroxyethyl methacrylate); mPDMS (monofunctional polydimethylsiloxane) NVP (N-vinyl pyrrolidone); TEGDMA (tetraethyleneglycol dimethacrylate); TPVC (tris-(trimethylsiloxysilyl) propylvinyl carbamate); TRIS (trimethylsiloxy silane); NVA (N-vinyl amino acid); PBVC (poly[dimethysiloxy] di [silylbutanol] bis[vinyl carbamate]); PVP (polyvinyl pyrrolidone).

Table 4-2: Properties of conventional hydrogel lens materials evaluated in the study

Proprietary name	Optima™ FW	SofLens™ 66	Proclear®	Focus® Monthly	Acuvue® 2
USAN	polymacon	Alphafilcon A	Omafilcon A	Vifilcon A	Etafilcon A
Manufacturer	Bausch & Lomb	Bausch & Lomb	Cooper Vision	CIBA Vision	Johnson & Johnson
Water content (%)	38	66	62	55	58
FDA group	I	II	II	IV	IV
Surface Treatment	None	None	None	None	None
Principal monomers	pHEMA	HEMA+ NVP	HEMA+PC	HEMA+ PVP+MA	HEMA+MA

HEMA, poly(2-hydroxyethyl methacrylate); MA, methacrylic acid; NVP, N-vinyl pyrrolidone; PC, phosphorylcholine; PVP, polyvinyl pyrrolidone.

4.3.2 Preparation of artificial lysozyme solution

Artificial lysozyme solution was prepared at a concentration of 1.9 mg/ml using Phosphate Buffered Saline (PBS), pH 7.4. Lysozyme was labeled with ^{125}I using the Iodine mono-chloride (ICl) method. [60, 63, 64] ^{125}I-labeled lysozyme was used as the isotopic tracer to quantify protein adsorption to the contact lenses. Radiolabeled lysozyme was dialyzed extensively against Phosphate Buffered Saline to remove free iodide and the percentage of free iodide was determined based on the precipitation of protein with trichloroacetic acid and the radioactivity of the supernatant. In all cases, the samples which had free iodide of less than 1% were used in the study. ^{125}I-labeled lysozyme was added to unlabeled solution such that the samples had a counting rate of 10^5 disintegrations per minute/ml (DPM/ml).

4.3.3 Incubating lenses in artificial lysozyme solution

Four lenses from each lens type were incubated for each time period, resulting in a total of 444 lenses being evaluated in the study. Lenses were removed from their packaging and thoroughly rinsed with PBS to ensure that no packaging solution remained on the lens surface and interfered with the deposition process. The lenses were then placed in 1ml of the labeled lysozyme solution and allowed to incubate at a temperature of 37°C with constant rotation for various time periods. Our previous work (unpublished) has shown that the deposition process differs with various conventional and SH lens materials, thus we elected to incubate the materials for slightly differing time periods, particularly during the early loading period. The time points chosen for the incubation process are described in Table 4.3.

Table 4-3: Table representing various time points for which the lenses were incubated in the artificial lysozyme solution

Silicone hydrogel lens materials	Conventional hydrogel lens materials and PMMA
½ day, 1 day, 2 days, 3 days, 5 days, 7 days, 14 days, 21 days and 28 days	1 hour, 6 hours, ½ day, 1 day, 2 days, 3 days, 5 days, 7 days, 14 days, 21 days and 28 days

The lysozyme-labeled incubation solution was changed every 7 days to simulate the availability of lysozyme within the tears during in-eye wear. Work in our laboratory (unpublished) has indicated that such a process adequately mimics that seen during wear. The lysozyme solution that was regularly changed had an identical volume and radioactive counting rate on each occasion.

Following the specified periods of incubation (Table 4.3), the lenses were aseptically removed from the labeled lysozyme solution using forceps and were rinsed briefly in saline to remove unbound protein from the lens surface. The lenses were then placed in sterile 5ml (12 X 75 mm), non-pyrogenic, polypropylene round bottom tubes and subsequently radioactive counts were determined using a Gamma Counter (Perkin Elmer Wallac Wizard 1470 Automatic Gamma Counter, Wellesley, MA, USA). The amount of protein adsorbed to the lenses was calculated by dividing the counts deposited on the lenses by the specific activity of the protein solution. [1]

4.3.4 Data Analysis

Statistical analysis was conducted using Statistica 7 software (StatSoft Inc, Tulsa, OK, USA). All data are reported as mean ± SD. Repeated Measures Analysis of Variance was used to determine statistically significant differences between deposition across various time points and differences between materials at any time point. Post-hoc multiple comparison testing was undertaken using the Tukey-HSD test. In all cases, a p value of <0.05 was considered significant.

4.4 Results

Figure 4.1 compares the kinetics of lysozyme deposition on alphafilcon A, omafilcon A, polymacon and PMMA. Figure 4.2 compares the kinetics of lysozyme deposition on the two group IV lens materials (etafilcon A and vifilcon A). Figure 4.3 displays the kinetics of lysozyme deposition on the five SH lens materials.

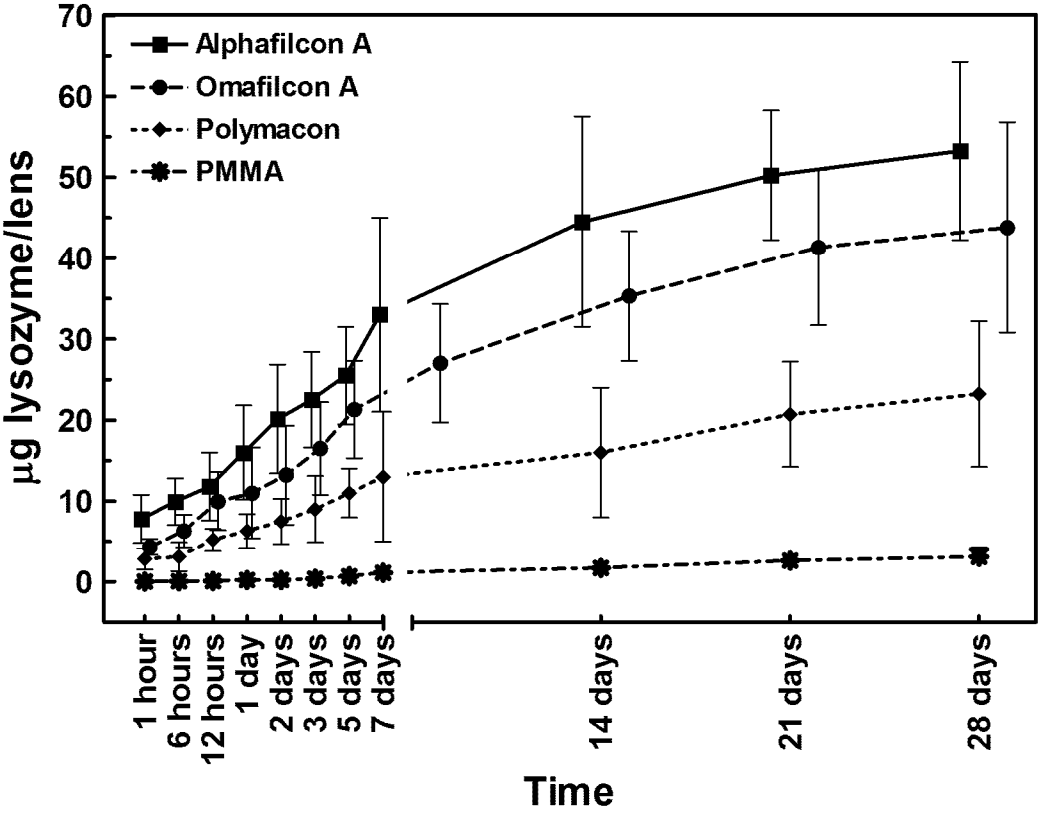

Figure 4-1: Comparison of the kinetics of lysozyme deposition on PMMA, alphafilcon A, omafilcon A and polymacon lens materials.

Error bars represent mean±SD (n=4).

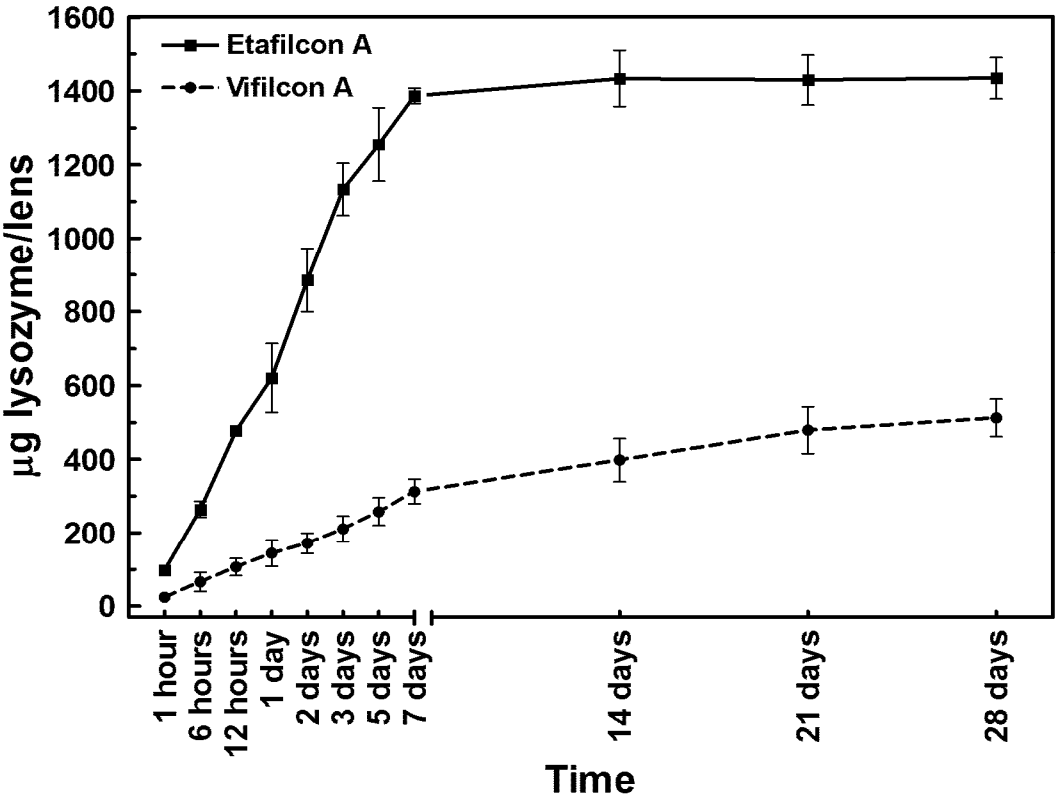

Figure 4-2: Comparison of the kinetics of lysozyme deposition on etafilcon A and vifilcon A lens materials.

Error bars represent mean±SD (n=4).

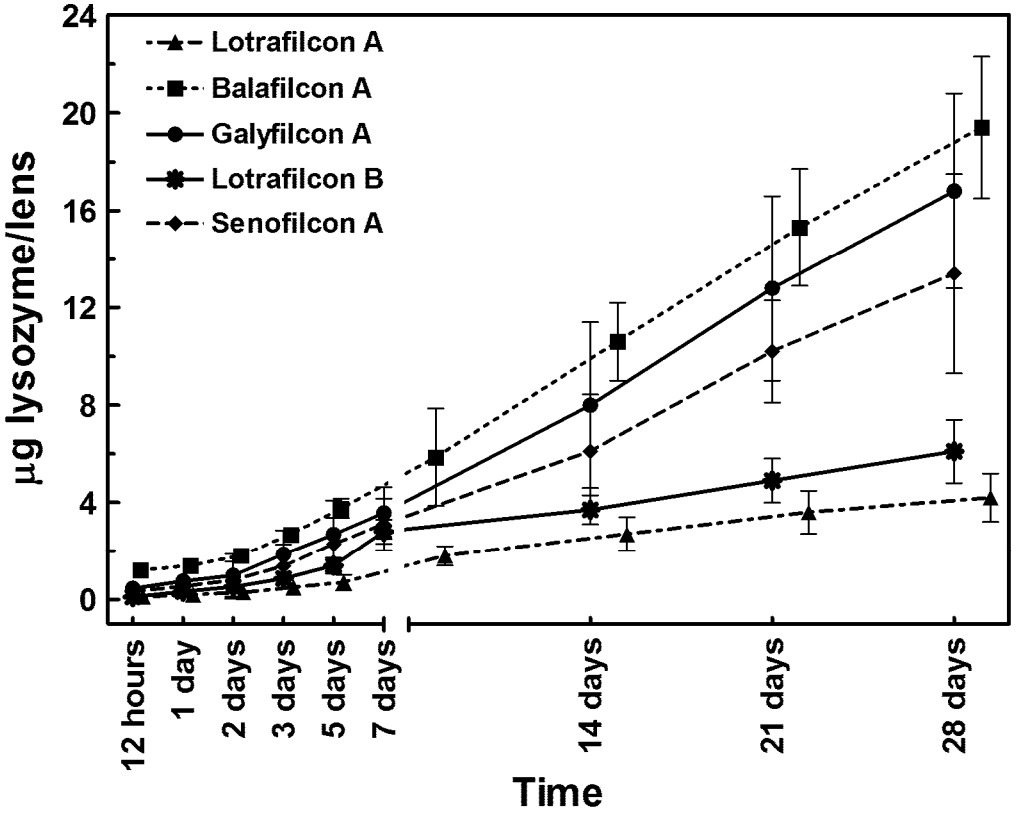

Figure 4-3: Comparison of the kinetics of lysozyme deposition silicone hydrogel contact lens materials.

Error bars represent mean±SD (n=4).

The etafilcon A material deposited significantly more lysozyme than all other lens materials ($p<0.001$) and the amount of lysozyme increased significantly between days 1 and 7 ($p<0.001$) and then reached a plateau, with no further increase occurring ($p>0.05$). FDA group IV vifilcon A material deposited significantly higher amounts of lysozyme than all other lens materials ($p<0.05$) but, significantly lower lysozyme than FDA group IV etafilcon A lens material at all points ($p<0.05$).

After 28 days of incubation, both the FDA group II lens materials (alphafilcon and omafilcon) deposited significantly more lysozyme than the group I material (polymacon) and PMMA ($p<0.05$). There was no significant difference between the two group II materials ($p>0.05$), while there was a significant difference between group I and PMMA ($p<0.05$). Among the SH lens materials, Tukey-HSD post-hoc analysis showed that there were no significant differences among the five SH lens materials until day 7 ($p>0.05$). At day 28, there were significant differences in lysozyme deposition between lotrafilcon A & lotrafilcon B versus all other SH lenses ($p<0.01$) and senofilcon A versus balafilcon A lens materials ($p<0.05$). There were no significant differences between PMMA versus lotrafilcon A & lotrafilcon B ($p>0.05$), lotrafilcon A versus lotrafilcon B and galyfilcon A versus senofilcon A lens materials ($p>0.05$).

Many of the available lens materials (with the exception of PMMA) are replaced every 14 or 28 days. Table 4.4 provides the amount of lysozyme deposited on each lens material after these two time points. To place the results in the context of general biomaterials research investigating protein deposition on hydrogel materials, the data in $\mu g/cm^2$ are also provided.

Table 4-4: Lysozyme deposited on various lens types represented in both µg/lens and µg/cm^2 after 14 and 28 days of incubation in ^{125}I labeled lysozyme solution

Lens Type	FDA Group	Lysozyme deposited (µg/lens)		Lysozyme deposited (µg/cm^2)	
		14 days	28 days	14 days	28 days
PMMA	-	1.7 ± 0.6	3.1 ± 0.9	0.9 ± 0.3	1.6 ± 0.5
Polymacon	I	16 ± 8	23.2 ± 9	5.2 ± 2.6	7.5 ± 2.9
Alphafilcon A	II	44.5 ± 13	53.3 ± 11	14.3 ± 4.2	17.1 ± 3.5
Omafilcon A	II	35.3 ± 8	43.8 ± 13	11.3 ± 2.6	14 ± 4.2
Vifilcon A	IV	356 ± 48	512.3 ± 51	115.7 ± 15.6	166.5 ± 16.6
Etafilcon A	IV	1433.5 ± 76	1434.5 ± 56	465.8 ± 24.7	466.2 ± 18.2
Lotrafilcon A	I	2.7 ± 0.7	4.2 ± 0.9	0.9 ± 0.2	1.4 ± 0.3
Lotrafilcon B	I	3.7 ± 0.6	6.1 ± 1.3	1.2 ± 0.2	2 ± 0.4
Balafilcon A	III	10.6 ± 1.6	19.4 ± 2.9	3.4 ± 0.5	6.3 ± 0.9
Galyfilcon A	I	8 ± 3.4	16.8 ± 4	2.6 ± 1.1	5.5 ± 1.3
Senofilcon A	I	6.1 ± 3.2	13.4 ± 4.1	2 ± 0.9	4.4 ± 1.3

4.5 Discussion

Radiochemical analysis offers great sensitivity for quantification of small amounts of material and has previously been used to measure deposition of tear components on contact lenses. [1, 2, 48] The principal advantages of radiolabeling through the ICl method is that the number of potential oxidation steps in which ^{125}I is transferred to the protein is limited. During the time of addition to the reaction mixture, iodine atoms are already in a reactive state and the number of substitution or oxidation reactions that can occur is equal to the number of active iodine atoms. [65] Previous studies have shown that the ICl-labeled proteins do not exhibit preferential adsorption relative to the unlabeled protein. [66] From a health hazard aspect, the ICl method can be carried out with minimal exposure to radiation. [65] Lysozyme was selected for these studies because it is recognized as a prominent lens soilant and a significantly greater quantity of lysozyme binds to ionic, high-water-content (group IV) lenses than to other types of lenses and is therefore often used as the prototypical marker for protein accumulation. [6, 43, 46] This is the first study to look at the kinetics of lysozyme deposition on SH lens materials and compare it with PMMA, FDA group I, group II and group IV lens materials.

Figure 4.2 shows that etafilcon A lenses attracted substantial quantities of protein, which was significantly greater than that measured on the FDA group II, PMMA and SH lenses ($p < 0.001$). This finding is in accordance with other previous studies examining protein and lysozyme deposition on different lens groups [2, 3, 5, 6, 8, 10, 67] and occurs because methacrylic acid imparts a negative charge to the material and thus thermodynamically favors the deposition of lysozyme, which is a positively charged

species at physiological pH. The deposition of lysozyme on etafilcon A may also be due to increased affinity of the material for protein uptake. Previously published *in vitro* studies investigating the kinetics of protein deposition on conventional hydrogel contact lens materials, using artificial tear solutions containing lysozyme [1, 2, 32, 33, 37, 38, 48] or albumin [1, 34, 35], indicate that the proteins deposit rapidly on hydrogel materials, occurring within minutes of exposure. [1, 32, 34, 35, 37, 48]

Lysozyme accumulated rapidly on etafilcon A, reached a maximum on the 7th day and then maintained a plateau, with no further increase occurring (Figure 4.2). The kinetics of lysozyme deposition on etafilcon A lenses broadly follows a three-phase process, with initial adsorption, followed by a rapid increase in which saturation occurs rapidly, and finally a plateau. [32, 33, 35, 36, 38, 39, 41] Its adsorption neutralizes the charge on the lens surface to such an extent, that the charge on the lens surface is reversed. [68] Hence the deposition plateaus after seven days of incubation. All these factors indicate that the ionicity, water content, and chemical composition of the underlying polymer has a significant impact on the extent of protein deposited. [1, 32, 35, 38] Our data supports this view.

Although vifilcon A is a FDA group IV material, these lenses deposited significantly lower amounts of lysozyme than etafilcon A lenses. These results are in agreement with a previously published *in vitro* paper, where an approximately four-fold increase in lysozyme deposition on etafilcon A than vifilcon A lenses after 24 hours of incubation was reported. [32] Etafilcon A (HEMA+MA) has an increased negative charge

when compared to vifilcon A (HEMA+PVP+MA), due to an increased concentration of MA. An increase in charge density will result in an increase in the effective pore size and this may promote the diffusive penetration of lysozyme. [69]

Both the FDA group II lens materials deposited significantly lower amounts of lysozyme than the FDA group IV lens materials. Both alphafilcon A (HEMA+ NVP) and omafilcon A (HEMA+PC) are non-ionic, high water content lens materials. Both these lenses have HEMA in common, which has a neutral charge; alphafilcon A has NVP, a hydrophilic monomer which has a neutral charge and omafilcon A has PC which is zwitterionic. The ability of PC-containing lens materials to be resistant to protein-fouling is well documented in the literature. [70, 71]

In the group I, II and SH materials, lysozyme deposition was significantly less than that seen with the group IV materials. Figure 4.1 indicates that the group II material deposited approximately 30 μg in the first week and then the weekly amount reduced from 15 μg in the second week to <5 μg in the final week, suggesting that a plateau effect is occurring. Figure 4.3 shows that balafilcon A lenses deposit lysozyme at a rate of approximately 5 μg/lens per week, galyfilcon A lenses deposit at a rate of approximately 4 μg/lens per week, senofilcon A lenses deposit at a rate of 3 μg/lens per week and lotrafilcon A & B deposit at a rate of 1-2 μg/lens per week.

Results from this study clearly demonstrate that SH lens materials deposit significantly less lysozyme compared to traditional hydrogel group IV lens materials. SH

contact lens materials represent a new family of biomaterials that have properties unlike any other previously developed for contact lens use. In addition to differences in surface modification, there are fundamental differences in the bulk chemistry of the polymers. The difference in the quantity of lysozyme deposition measured between the five SH materials could be related to differences in either the bulk material or surface treatment. The lotrafilcon A, lotrafilcon B and balafilcon A lens materials are surface treated in an attempt to improve the wettability of the materials and to reduce the degree of deposition. [72, 73] Galyfilcon A and senofilcon A lenses incorporate a long chain, high molecular weight molecule called Hydraclear™, and the manufacturers claim that this maintains flexibility and moisture. [73] This wetting agent is present throughout the lenses and hence no surface treatment is required for these lenses. [73]

Although three of the currently available SH lens materials undergo surface treatment, some of the studies assessing the *in vitro* wetting angle of the two SH materials indicates that they remain relatively hydrophobic compared with conventional hydrogels, [70, 74, 75] with the balafilcon A material exhibiting a higher wetting angle than lotrafilcon A. The surface treatment of the balafilcon A material exposes more hydrophobic sites than the 25-nm homogenous surface modification that is used on the lotrafilcon A material. [76] In addition to differences in surface modification, there are fundamental differences in the bulk chemistry of the polymers, as described in Table 4.1. Some of the earlier studies have shown that proteins have a greater affinity for hydrophobic than for hydrophilic surfaces. [77-79] Proteins such as lysozyme which have high internal stability do not normally adsorb onto hydrophilic surfaces unless there is an electrostatic attraction. [80]

Balafilcon A is relatively more hydrophobic than other SH lens materials, as the surface treatment of the balafilcon A exposes more hydrophobic sites; [76] this might be the reason for these lenses depositing more lysozyme than other SH materials. In addition, balafilcon A is relatively more porous when compared to other SH lens materials. [81] Hence, lysozyme can easily penetrate into the matrix of these lens materials, resulting in increased amounts of deposition than other SH lens materials. The plasma coating on some of the SH lens materials can create an imperturbable surface state, [82, 83] which could result in lower adsorption of lysozyme in these materials than when compared to CH lens materials. Thus, because of their unique surface and bulk properties, these newly developed SH lens materials are highly resistant to protein deposition.

Our data are derived using an *in vitro* model and it is noteworthy to compare our results with those obtained from *in vivo* studies investigating lysozyme deposition on contact lens materials. Previous *in vivo* studies have shown that lenses recovered within the first few minutes of wear demonstrate protein deposition of some degree and the process of deposition continues over time. [27, 39, 41, 42, 84, 85] Sack and co-workers showed that lysozyme is rapidly deposited on FDA Group IV lenses. Further temporal studies showed that during open eye conditions, deposition is linearly related to the duration of wear and the deposition eventually leveled off such that similar levels of protein were recovered from the lenses after 12 to 14 hours of open eye or several days of extended wear. [43] Keith and co-workers determined lysozyme deposition as a function of time on etafilcon lenses. [41] They found a mean concentration of 55 μg/lens after 15 minutes of wear, which reached a maximum at around 1300 μg/lens after six days of wear, with a

plateau occurring between days four to eleven for various subjects. [41] Consistent with this study, Jones and co-workers determined that surface deposition reached a plateau on day one, while the intra-matrix deposition continued to increase up to seven days on etafilcon lenses. [39] Surface protein on alphafilcon also peaked within one day, but total protein accumulation continued for up to 30 days. [39] The concept of plateauing on etafilcon lenses is also supported by Richards and Tripathi. [44, 56] Lin and co-workers also showed that lysozyme accumulation, increased with wearing times up to one week on etafilcon lenses. [27] Previous data from lotrafilcon A and balafilcon A studies are also consistent with the data reported in this study, in which the material deposited <20 μg of lysozyme after a month of wear. [22, 23] Thus, our *in vitro* data reported in this study is consistent with that reported for FDA group II, IV and SH lenses collected from *in vivo* studies and leads us to believe that our kinetic results are highly predictive of that which would be seen in lenses worn by clinical subjects.

Examination of Figures 4.1 and 4.3 indicate that replacement schedules can play an important role in controlling protein deposition. Currently, many patients replace their lens materials after 14 days rather than 28. Our data indicate that FDA group II materials would exhibit approximately 25% less lysozyme deposition if replaced in such a manner and that balafilcon and galyfilcon as much as 50% less lysozyme. These data are of interest to patients and clinicians alike, particularly given that protein denaturation may also decrease if materials are replaced more frequently.

One of the limitations of this study was that the lenses were incubated in a solution containing only one type of protein, namely lysozyme. An important factor to consider in the process of deposition relates to whether other substances from the tear film (for example, mucins, lipids or other proteins) concurrently deposit onto the material surface at different rates. Hence, it is necessary in future studies, to investigate the kinetics of deposition using a complex artificial tear solution and to look at the protein-protein, protein-lipid and protein-mucin interactions. It is clear that SH lenses only deposit small amounts of lysozyme and previously published studies suggest that much of this lysozyme is denatured. [22, 23] Therefore, it would be interesting to investigate the activity of lysozyme recovered from these lenses at various time points. Hence, in addition to investigating the kinetics of deposition, the kinetics of denaturation and how they relate to wearing period, the influence of various care regimens, and the degree to which other tear proteins denature also require investigation.

4.6 Conclusions

In conclusion, the results from this study indicate that lysozyme deposition is driven by both the bulk chemistry and also the surface properties of CH and SH lens materials. The surface modification processes or the inclusion of high molecular entities or surface-active monomers on SH lens materials also play a significant role in lysozyme deposition on SH lens materials. The results from this study reiterate that SH lens materials deposit very low amounts of lysozyme. SH lenses that are surface modified with a 25nm plasma coating (lotrafilcon A & lotrafilcon B) showed a deposition pattern similar to PMMA lens materials. SH lenses that use a surface modification system based

on PVP (galyfilcon and senofilcon) had similar deposition patterns, while balafilcon lenses (which undergo a plasma oxidation process) showed the highest level of lysozyme deposition.

The next chapter of this thesis will discuss the conformational state of lysozyme recovered from various conventional and silicone hydrogel contact lens materials as a function of time using an *in vitro* model.

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5 Kinetics of lysozyme activity recovered from conventional and silicone hydrogel contact lens materials

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5.1 Abstract

Purpose: To determine the activity of lysozyme recovered from various conventional and silicone hydrogel (SH) contact lens materials as a function of time, using an *in vitro* model.

Methods: Polymacon, omafilcon, etafilcon, vifilcon, lotrafilcon A, lotrafilcon B, balafilcon A, galyfilcon A and senofilcon A contact lenses (n=5) were incubated in lysozyme solution for time periods ranging from 1 hour to 28 days. Following the specified incubation period, the lysozyme deposited on the lenses was extracted and the sample extracts were assessed for lysozyme activity and total lysozyme.

Results: There was no significant difference (NSD) between omafilcon and polymacon lens materials for the initial three days ($p>0.05$), however there was a significant difference between the two lenses from five to 28 days ($p<0.05$). There was NSD ($p>0.05$) between etafilcon and vifilcon lens materials at all time points and significant differences were seen among various SH lens materials at different time points. After 28 days, lysozyme deposited on etafilcon (90±3%) and vifilcon (91.4±3%) exhibited the greatest activity. Lysozyme deposited on polymacon (17.8±4%), lotrafilcon A (23.4±4%) and lotrafilcon B (24±5%) exhibited the lowest activity. Lysozyme deposited on omafilcon, galyfilcon, senofilcon, and balafilcon exhibited 38±3%, 62.3±8%, 47±6% and 61±7% of activity respectively.

Conclusions: The reduction in activity of lysozyme deposited on contact lens materials is time dependant and the rate of reduction varies among lens materials. This variation in activity recovered from lenses could be due to the differences in surface/ bulk material properties or the location of lysozyme on these lenses.

Key words: contact lens; lysozyme; protein activity; silicone hydrogel; tear protein.

5.2 Introduction

Following insertion onto the eye, hydrogel contact lenses rapidly sorb components from the tear film, particularly proteins, lipids and mucins. [1-4] At extreme levels of build-up, these deposits are associated with diminished visual acuity, [5] dryness and discomfort, [6, 7] lid-related inflammatory changes and contact lens associated papillary conjunctivitis. [8-11] Moreover, these deposits could potentially increase the risk of bacterial attachment, by providing a solid substrate and shelter. [12-14]

It was originally believed that the protein layer which is bound to the lens material is highly homogeneous, consisting almost entirely of denatured lysozyme. [15] Sack and co-workers were the first to demonstrate that the "Lens Bound Protein Layer" retained a certain amount of biological activity and that the conformational state of the deposited protein is highly dependent on the hydrogel structure. [4] It is also of interest to note that some of the earlier studies have suggested that the allergenicity of adsorbed proteins on hydrogel lenses is related to their degree of denaturation. [10, 11, 16]

Using mass-spectrometry, it was recently shown that lysozyme, lipocalin, lactoferrin, lacritin, proline-rich 4 and Ig alpha are the tear proteins that are frequently detected in the proteomic profile of contact lens deposition on siloxane-based "silicone hydrogel" (SH) lenses. [17, 18] Among these unique tear proteins, lysozyme is a bacteriolytic enzyme with a relatively small molecular weight (14 kDa) and a positive charge at neutral pH. Lysozyme is one of the major proteins that is found on contact lens materials and, as lysozyme approximates 90% of the protein found on group IV contact lens, it is

often used as the "model protein" for protein accumulation. [4, 19-24] Once lysozyme firmly adsorbs onto contact lens materials, it tends to undergo conformational changes, [4, 24-26] which might potentially result in a variety of immunological responses, including contact lens associated papillary conjunctivitis. [8, 10, 11, 27] Recently, our group has also shown that the subjective comfort of symptomatic contact lens wearers is closely associated with the activity of lysozyme recovered from polyHEMA-based hydrogel lenses worn for as little as one day. [28] Therefore, in addition to investigating the total quantity of the deposited protein, it is of significant clinical relevance to study the conformational state of the deposited protein.

Although, the conformational state of lysozyme deposited on hydrogel lens materials after a specified period has been reported, [24, 26, 29, 30] to-date, no study has examined the activity of lysozyme deposited on polyHEMA-based conventional hydrogel (CH) and SH lens materials as a function of time. Knowing the rate of protein deposit accumulation and the duration over which lysozyme denatures is clinically relevant to patient symptoms and in determining the most appropriate replacement frequency. In addition, this information would be very useful for designing clinical investigations of hydrogel lenses and associated lens care products. Commercially available contact lens materials are classified into four different groups based on their water content and charge by the United States Food and Drug Administration (FDA). Therefore, it is of interest to determine whether the water content and charge of the lens material affect the biological activity of the deposited lysozyme. Hence, the purpose of this study was to compare the activity of lysozyme deposited on FDA group I, FDA group II & FDA group IV CH lens

materials and the first & second generation SH lens materials, as a function of time using an *in vitro* method.

5.3 Materials and Methods

5.3.1 Reagents and materials

The four types of CH lenses examined were FDA group I Optima™ FW (Baush & Lomb), FDA Group II Proclear® (CooperVision), FDA group IV Focus® Monthly (CIBA Vision) and FDA Group IV Acuvue® 2 (Vistakon, Johnson & Johnson). Five types of SH lens materials were examined, including Night and Day™ (CIBA Vision), O_2Optix™ (CIBA Vision), PureVision™ (Bausch & Lomb), Acuvue® Advance™ (Vistakon, Johnson & Johnson) and Acuvue® OASYS™ (Vistakon, Johnson & Johnson). The properties of each material are described in Tables 5.1 and 5.2. PhastSystem™ components were described previously. [26] Immuno-Blot® PVDF (polyvinylidene difluoride) membranes were purchased from Bio-Rad Laboratories (Mississauga, ON, Canada). Polyclonal rabbit anti-human lysozyme was purchased from Cedarlane Laboratories (Hornby, ON, Canada) and goat anti-rabbit IgG-HRP was purchased from Sigma (St. Louis, MO, USA). Lysozyme and lyophilized *Micrococcus lysodeikticus* cells were also purchased from Sigma (St. Louis, MO, USA). All other reagents purchased were of analytical grade.

Table 5-1: Properties of silicone hydrogel lens materials evaluated in the study

Proprietary name	Night & Day™	O_2 Optix™	PureVision ™	Acuvue® Advance™	Acuvue® OASYS™
USAN	Lotrafilcon A	Lotrafilcon B	Balafilcon A	Galyfilcon A	Senofilcon A
Manufacturer	CIBA Vision	CIBA Vision	Bausch & Lomb	Johnson & Johnson	Johnson & Johnson
Water content (%)	24	33	36	47	38
Oxygen Permeability (Dk)	140	110	91	60	103
Centre thickness (mm) -3.00D	0.08	0.08	0.09	0.07	0.07
Oxygen Transmissibility (Dk/t) at 35˚C	175	138	101	86	147
FDA group	I	I	III	I	I
Surface Treatment	25nm plasma coating with high refractive index	25nm plasma coating with high refractive index	Plasma oxidation process	No surface treatment. Internal wetting agent that also coats the surface	No surface treatment. Internal wetting agent that also coats the surface
Principal monomers	DMA+TRIS+ Siloxane monomer	DMA+TRIS+ Siloxane monomer	NVP+TPVC+ NVA+ PBVC	mPDMS+ DMA+HEMA +siloxane macromer+ PVP+ EGDMA	mPDMS+ DMA+HEMA +siloxane macromer+ PVP+ TEGDMA

DMA (N,N-dimethylacrylamide); EGDMA (ethyleneglycol dimethacrylate); HEMA (poly-2-hydroxyethyl methacrylate); mPDMS (monofunctional polydimethylsiloxane) NVP (N-vinyl pyrrolidone); TEGDMA (tetraethyleneglycol dimethacrylate); TPVC (tris-(trimethylsiloxysilyl) propylvinyl carbamate); TRIS (trimethylsiloxy silane); NVA (N-vinyl aminobutyric acid); PBVC (poly[dimethysiloxy] di [silylbutanol] bis[vinyl carbamate]); PVP (polyvinyl pyrrolidone).

Table 5-2: Properties of conventional hydrogel lens materials evaluated in the study

Proprietary name	Optima™ FW	Proclear®	Focus® Monthly	Acuvue® 2
United States Adopted Name (USAN)	polymacon	Omafilcon A	Vifilcon A	Etafilcon A
Manufacturer	Bausch & Lomb	Cooper Vision	CIBA Vision	Johnson & Johnson
Water content (%)	38	62	55	58
FDA group	I	II	IV	IV
Surface Treatment	None	None	None	None
Principal monomers	polyHEMA	polyHEMA+ PC	polyHEMA+ PVP+MA	polyHEMA+ MA

polyHEMA, (poly-2-hydroxyethyl methacrylate); MA, methacrylic acid; NVP, N-vinyl pyrrolidone; PC, phosphorylcholine; PVP, polyvinyl pyrrolidone.

5.3.2 Incubation of lenses in lysozyme solution

Lysozyme solution was prepared at a concentration of 1.9 mg/ml using Phosphate Buffered Saline (PBS), at pH 7.4. Five lenses from each lens type were incubated for each time period, resulting in a total of 425 lenses being evaluated in the study. Lenses were removed from their packaging and thoroughly rinsed with PBS to ensure that no packaging solution remained on the lens surface and interfered with the deposition process. In order to achieve this, lenses were removed from the packaging solution using forceps and were placed in vials containing PBS with the lenses' concave side up for one hour. The vials were then placed on a plate shaker for two minutes and this process was performed a total of three times with fresh PBS each time. Lenses were then placed in

1ml of the lysozyme solution and were allowed to incubate at a temperature of 37°C with constant rotation for various time periods. Our previous work (unpublished) has shown that the deposition process differs with various conventional and SH lens materials, thus we elected to incubate the materials for slightly differing time periods, particularly during the early loading period. The CH lens materials were incubated for one hour, twelve hours, one, two, three, five, seven, 14, 21 and 28 days; whereas the SH lens materials were incubated for twelve hours, one, two, three, five, seven, 14, 21 and 28 days.

5.3.3 Protein deposit extraction and sample processing following extraction

After the specified period of incubation, the lenses were rinsed with PBS in order to remove any unbound protein on the lens surface. The lenses were then placed into an extraction solvent consisting of acetonitrile/ trifluroacetic acid (ACN/TFA) and were incubated in the dark at room temperature for 24 hours. [26, 31, 32] Two 0.70 ml aliquots of ACN/TFA was transferred to sterile eppendorf tubes and lyophilized to dryness in a Savant Speed Vac (Halbrook, NY, USA). Dried protein pellets were stored at –80ºC prior to reconstitution. Prior to electrophoresis/Western blotting and lysozyme activity analysis, lyophilized protein pellets were reconstituted in modified reconstitution buffer – MRB (10 mM Tris-HCl; 1 mM EDTA, with 0.9% saline) pH 12.0 and BioStab® Biomolecule Storage Solution (Sigma Aldrich). [32]

5.3.4 Electrophoresis and immunoblotting

The total lysozyme deposited on the lens materials was determined using electrophoresis and immunoblotting techniques. [26, 32] Lysozyme standards were prepared fresh on the day of analysis from a 1.0 μg/μL frozen stock of purified neutrophil lysozyme with MRB, pH 8.0 and subjected to SDS-PAGE followed by Western blotting to PVDF membranes. [26, 32]

5.3.5 Negative control - extraction and Western blot analysis of lenses

All lens materials (n=3 per material) were extracted in ACN/TFA solution and were subjected to SDS-PAGE and Western blotting, as described above.

5.3.6 Measurement of lysozyme activity

The extracts were assayed for lysozyme activity using a fresh suspension of *Micrococcus lysodeikticus* for each sample. [26, 29] *Micrococcal* cells were suspended in 50 mM sodium phosphate buffer (pH 6.3) to an initial optical density of 1.0 at 450 nm (Multiskan Spectrum ELISA Plate Reader, fitted with a micro-cuvette, ThermoLabsystems). Standards and samples were applied to 1 ml cells in 10 μl or less. Kinetic measurements were taken at 30 sec intervals (450 nm) for 5 minutes at 30^{o} C, with stirring for 10 seconds after each measurement except time zero. Lysozyme standard (5, 10, 50, 100 ng) was run concurrently with samples. The change in absorbance was plotted against time, from 30 seconds to between 3 and 5 minutes, to determine the slope or initial velocity. A native lysozyme standard curve was constructed of mass lysozyme

standard against initial velocity by least-squares, as described previously. [26] R^2 values were always at least 0.98 and the equation of the regression line was calculated to give the slope. Finally, to determine the amount of lysozyme from the lens extract still in native form, the following formula was employed: Active lysozyme = total lysozyme (sample specific activity/ standard specific activity), where total lysozyme was pre-determined via Western blot analysis as described above. The denatured lysozyme component was derived by: Denatured lysozyme = total lysozyme - active lysozyme. The final calculation was the percent of active lysozyme: % active lysozyme = (active lysozyme / total lysozyme) X 100.

5.3.7 Statistical Analysis

Statistical analysis was conducted using Statistica 8 software (StatSoft Inc, OK, USA). All data are reported as mean ± SD. Two repeated measures ANOVAs (one for CH and the other for SH) were performed, with the material and time course as the factors**.** Post-hoc multiple comparison testing was undertaken using the Tukey-HSD test. In all cases, a p value of <0.05 was considered significant.

5.4 Results

Figure 5.1 compares the kinetics of lysozyme activity recovered from polymacon (FDA group I) and omafilcon A (FDA group II). Figure 5.2 compares the kinetics of lysozyme activity recovered from the two FDA group IV lens materials (etafilcon A and

vifilcon A). Figure 5.3 displays the kinetics of lysozyme activity recovered from the five SH lens materials. Repeated Measures ANOVA for the four CH lens materials showed a significant interaction between lenses and time (F-statistic = 62.969, $p<0.001$); similarly, a significant interaction was seen between the lenses and time for the five SH lens materials (F-statistic = 22.919, $p<0.0001$).

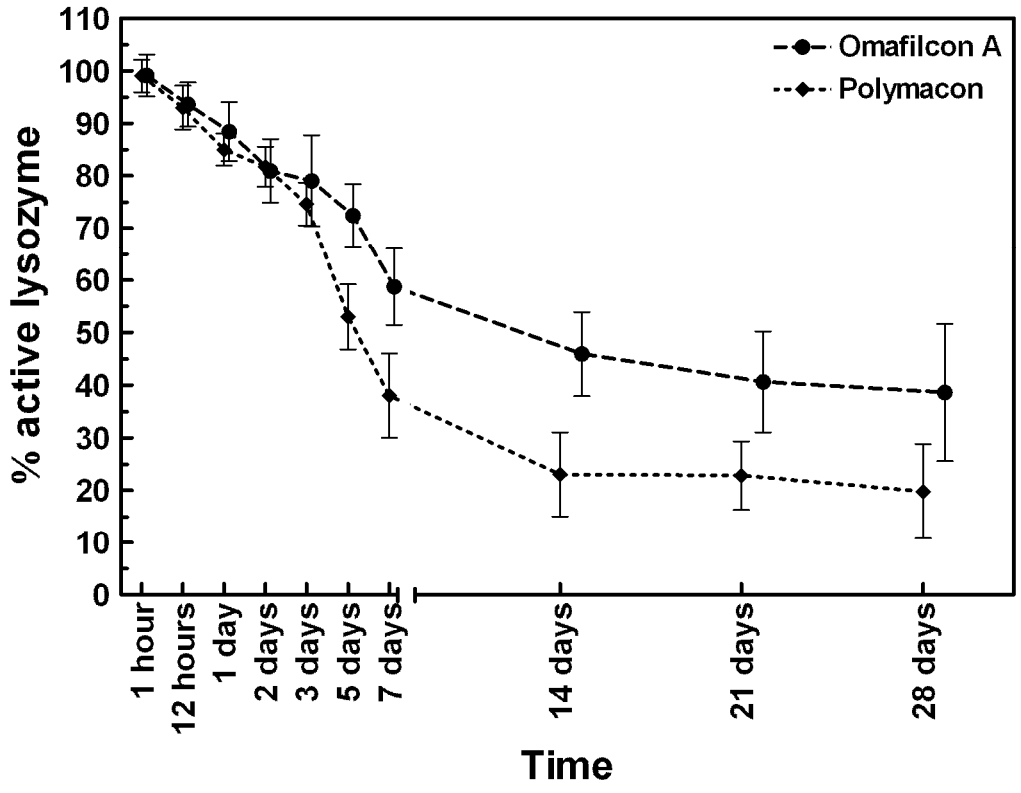

Figure 5-1: Comparison of the kinetics of percentage active lysozyme recovered from polymacon and omafilcon A lens materials.

Error bars represent Mean ± Standard Deviation, (n=5).

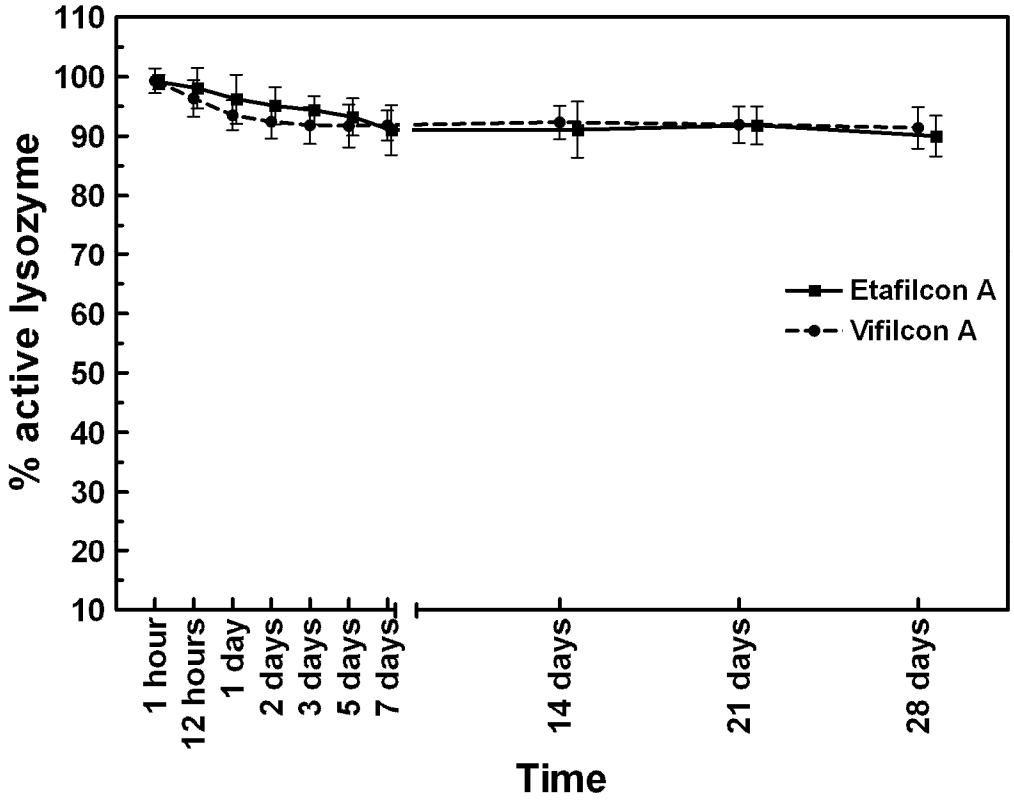

Figure 5-2: Comparison of the kinetics of percentage active lysozyme recovered from etafilcon A and vifilcon A lens materials.

Error bars represent Mean ± Standard Deviation, (n=5).

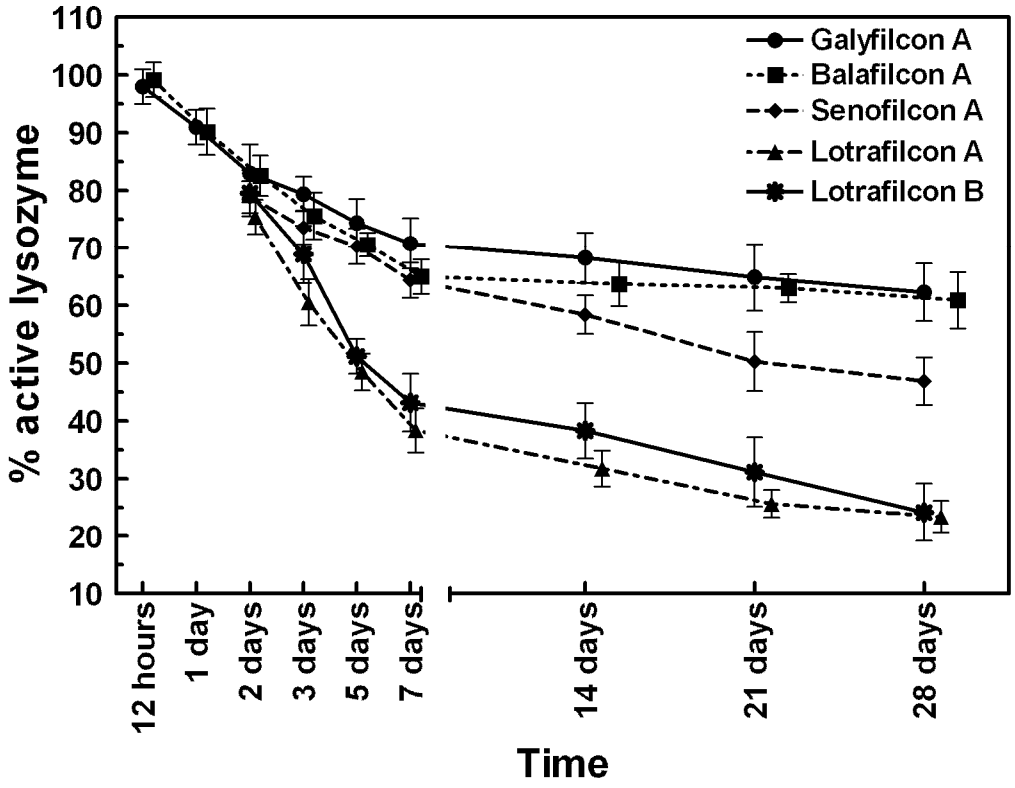

Figure 5-3: Comparison of the kinetics of percentage active lysozyme recovered from silicone hydrogel lens materials.

Error bars represent Mean ± Standard Deviation, (n=5).

Figure 5.1 shows that there was a gradual reduction in the activity of lysozyme deposited on omafilcon A (FDA group II) and polymacon (FDA group I) lens materials from one hour to 14 days (all $p<0.01$), with the exception of between the first and second days (both $p>0.05$) and second and third days (both $p>0.05$) of incubation. However, there was no statistically significant reduction in the activity of lysozyme deposited on both lens materials from days 14 to 28 (all $p>0.05$). There was no significant difference between these two materials until day three ($p>0.05$ for all time points), however there

was a significant difference between the two lens materials from day 5 through to day 28 ($p<0.05$ for all time points).

Figure 5.2 shows that there was no significant difference (all $p>0.05$) between the two FDA group IV lens materials (etafilcon A and vifilcon A) across any time point evaluated in this study. There was a significant difference between the one hour time point and 21, 28 day time points for both the FDA group IV lens materials ($p<0.05$).

Figure 5.3 shows that there was a gradual reduction in the activity of lysozyme recovered from all the five lens materials until day seven (all $p<0.05$), and there was no statistically significant further reduction in activity for the galyfilcon A and balafilcon A materials (all $p>0.05$) after day seven. For senofilcon A, lotrafilcon A and lotrafilcon B lens materials, the 21 and 28 day time points were significantly lower than all other time points ($p<0.05$), except the 14 day time point ($p>0.05$). Among the five SH lens materials, there was no significant difference among them until day three ($p>0.05$). At the end of 28 days, there was no significant difference between galyfilcon A and balafilcon A lens materials ($p=0.64$) and no significant difference between lotrafilcon A and lotrafilcon B lens materials ($p=0.72$). Lysozyme recovered from senofilcon A exhibited significantly higher activity than those recovered from lotrafilcon A & B (both $p<0.05$), but significantly lower activity than that recovered from galyfilcon A and balafilcon A lens materials (both $p<0.05$).

After 28 days, lysozyme deposited on etafilcon A and vifilcon A exhibited the greatest activity (90±3% and 91.4±3% respectively), which was statistically greater than all other lens types ($p<0.001$). There was no significant difference between etafilcon A and vifilcon A lens materials at the end of 28 days ($p=0.96$). Lysozyme deposited on polymacon (17.8±4%), lotrafilcon A (23.4±4%) and lotrafilcon B (24±5%) exhibited the lowest activity. Lysozyme deposited on omafilcon, galyfilcon, senofilcon, and balafilcon exhibited 38±3%, 62.3±8%, 47±6% and 61±7% of activity respectively.

In a clinical situation, SH lens materials that were evaluated in this study are typically replaced every 14 or 28 days. Table 5.3 provides the amount of denatured lysozyme in microgram per lens that was deposited on each lens material after the one, seven, 14 and 28 days of incubation. No signal was seen on Western blots run on control lens materials after being subject to the same procedures, confirming that there was no background interference from the lens polymer. The percentage reduction in the activity of lysozyme when subjected to the extraction process was found to be 2.1 ± 0.7%.

Table 5-3: Denatured lysozyme deposited on conventional hydrogel and silicone hydrogel lens materials in µg/lens (Mean ± standard deviation).

Lens	Day 1 (µg DL/lens)	Day 7 (µg DL/lens)	Day 14 (µg DL/lens)	Day 28 (µg DL/lens)
Polymacon	0.8 ± 0.5	6.8 ± 3	10.8 ± 4	16.5 ± 6
Omafilcon A	1 ± 1	10 ± 3	18 ± 5	25 ± 6
Etafilcon A	23 ± 7	120 ± 14	121 ± 12	135 ± 13
Vifilcon A	8 ± 3	23 ± 7	28 ± 5	43 ± 9
Lotrafilcon A	BD *****	0.4 ± 0.3	1.2 ± 0.8	2.4 ± 1
Lotrafilcon B	BD *****	0.2 ± 0.2	1.1 ± 0.7	2.2 ± 1.3
Balafilcon A	0.1 ± 0.1	1.9 ± 0.9	3.9 ± 1	7.8 ± 3
Galyfilcon A	0.1 ± 0.1	1 ± 0.5	2.2 ± 1	3.5 ± 1.6
Senofilcon A	BD *****	0.3 ± 0.2	0.9 ± 0.6	3.1 ± 1.7

* BD = Below Detection

5.5 Discussion

Protein deposition on any polymeric surface is a complex process. Previous studies have proposed that there are distinct steps that contribute to the kinetics of protein sorption onto polymeric surfaces. [33, 34] Initially, there is a rapid and reversible uptake of proteins in the first few minutes until a pseudo equilibrium is reached, resulting in up to 50 to 60% of the surface being covered with a random arrangement of adsorbed molecules, which would facilitate further protein uptake. [34] After adsorption, protein molecules on the polymer surface will undergo structural transition as a function of time, which will lead to an optimal interaction between the polymer surface and the protein. [33, 34] With an increase in the surface-protein interaction, there is an increase in the entropy of the protein and the protein is less likely to desorb from the surface. Moreover, some of the proteins which are bound to the polymer will penetrate into the matrix of the material through a process of simple diffusion and complex electrostatic attraction [35] which might result in increased conformational changes to the protein, depending on the polymer make-up.

Protein denaturation is commonly defined as any non-covalent change in the structure of a protein and this change may alter the secondary, tertiary or quarternary structures of the molecule. Some analytical methods are sensitive to detect very slight changes in structure, while others require rather large alterations in the protein structure before these changes can be observed. For enzymatic proteins such as lysozyme, denaturation can be defined as the loss of structural configuration which will render the enzyme inactive. Determination of loss of enzymatic activity can be a very sensitive

measure of denaturation, since procedures including the *micrococcal* assay are capable of detecting very low levels of activity. This study compared the kinetics of the activity of lysozyme deposited on FDA group I, II & IV CH lens materials and the first & second generation SH lens materials, using a modified *micrococcal* assay.

Protein denaturation on any polymeric surface or a contact lens material is influenced by several factors, including contact time of the protein with the substrate, chemical composition of the substrate, surrounding pH, type of protein and temperature of the surrounding medium. [33, 36-39] Several studies also suggest that proteins that are exposed to hydrophobic surfaces are more likely to denature than those that are exposed to hydrophilic surfaces. [21, 33, 36, 39-42] It has also been suggested that the degree of denaturation of a protein is dependent on the location of the protein in a polymer; proteins that are located in the matrix of the lens material are less likely to denature than those that are adsorbed on the lens surface. [4, 39, 43] The clinical relevance of protein denaturation on hydrogel lenses relates to the impact of this protein on inflammation and lens comfort. Previous studies have suggested that protein denaturation is closely linked to inflammatory conditions such as giant papillary conjunctivitis. [8-11] In addition, recent work has suggested that even over a short period of wear that protein denaturation can impact on lens comfort, with little correlation being shown with total amounts of either lysozyme or total protein. [28]

Figure 5.1 shows that there was no significant difference in the percentage active lysozyme recovered from FDA group I polymacon and FDA group II omafilcon A lens

materials for up to three days of incubation ($p>0.05$) and the percentage active lysozyme recovered from polymacon was significantly lower for the remaining time periods tested. Polymacon is the USAN for hydrogel materials fabricated from poly-2-hydroxyethyl methacrylate (polyHEMA), which is a non-ionic and low water content material (Table 5.2) with an average pore radius of 4-8 Å at its equilibrium condition of hydration. [44] Lysozyme is a compact globular protein molecule of molar mass of 14,500 Da, with a slightly ellipsoidal shape, and its dimensions are 45 X 30 X 30 Å. [45] Due to the small pore size of polyHEMA hydrogels, penetration of even the smallest of proteins such as lysozyme will be prevented. [44] The deposition of lysozyme on polyHEMA hydrogels should entirely be a surface adsorption phenomenon, due to the scarcity of cationic and anionic binding sites on the lens material. Lysozyme that is bound to the lens material will presumably be held by hydrophobic and non-ionic hydrophilic interactions. These forces in turn, would favour the exposure of non-ionic amino acids that are normally buried within the protein interior, resulting in increased denaturation of the protein. However, the presence of a hydrophobic cross-linking agent in the material and other trace impurities including methacrylic acid (MA) present during polyHEMA processing may increase the pore size of the contact lens network. [46] This would force the lysozyme to penetrate into the matrix through the small pores of polyHEMA, which would result in further changes in the conformational state of the protein, potentially resulting in further loss of activity.

Omafilcon A is a copolymer of polyHEMA and phosphorylcholine (PC), which is non-ionic and has high water content, and is hence classified as a FDA group II material.

Using confocal microscopy and fluorescently-tagged lysozyme, it has been shown that lysozyme rapidly penetrates into the bulk of the omafilcon material after as little as 24 hours exposure. [47, 48] The ability of PC-containing hydrogels to resist protein-deposition in both the eye and following contact with blood is well documented in the literature. [23, 49-53] Moreover, it has been shown that PC-based omafilcon has a higher resistance to dehydration than other CH lenses, presumably due to the high water affinity of the incorporated PC. [54] However, these lens materials have a relatively high water contact angle, when assessed using a sessile drop technique, suggesting that these materials are relatively more hydrophobic than ionic FDA group IV lens materials. [55]

Figure 5.2 shows that the percentage active lysozyme recovered from both the FDA group IV lens materials, etafilcon A and vifilcon A, was significantly greater than that measured on all the other lens materials examined, at the end of 28 days ($p < 0.001$). This finding is in accordance with other previous *ex vivo* and *in vitro* studies which have examined the degree of denatured lysozyme recovered from FDA group IV [4, 24-26] and SH lens materials. [24-26] Previous studies have also shown that both etafilcon A [3, 23-26, 56-60] and vifilcon A [21, 23] deposit significantly higher amounts of lysozyme than other CH and SH lens materials. Both these lens materials have a negative charge, due to the presence of the ionic monomer methacrylic acid (MA). Among these two materials, etafilcon A (a copolymer of polyHEMA and MA only) has an increased concentration of MA, so etafilcon A has a substantially higher negative charge than vifilcon A (a copolymer of polyHEMA, PVP and MA). An increase in charge density will result in an increase in the effective pore size, provoked by the repulsion of the charged groups, and this may

promote the diffusive penetration of lysozyme into MA-containing hydrogels. [61] Therefore, the dynamics of lysozyme deposition will be radically different on these lens materials when compared to that seen in a polyHEMA hydrogel. Using confocal microscopy, a previous *in vitro* study from our group has determined that lysozyme is primarily located within the bulk of these FDA group IV hydrogels, with relatively little surface-located lysozyme being determined. [47, 48] Using a sessile drop technique, it was shown that the CH group IV lens materials are more wettable than other lens materials, indicating that these lenses are more hydrophilic. [55] Therefore, we speculate that the activity of lysozyme recovered from CH group IV lens materials is significantly higher than that recovered from other lens materials because lysozyme is exposed to a more hydrophilic surface and the sorbed lysozyme is located primarily in the bulk of the hydrogel rather than on the surface, resulting in reduced denaturation. Despite, the fact that lysozyme also penetrates into the omafilcon material, there is a large difference in the activity of lysozyme recovered from group IV and group II lens materials. This begs the question of whether the high amounts of lysozyme deposited on etafilcon and vifilcon lens materials actually helps to prevent subsequent denaturation, and this area requires further experimentation.

Currently available SH lens materials are made up of highly complex monomers and contain multiple monomer components (Table 5.1). However, regardless of the polymer make-up, the hydrophobic and surface active nature of the silicone component necessitates additional modification or surface treatment. These SH lens materials can be broadly classified into three groups, based upon their surface treatment. [62-70] The two

CIBA Vision materials (lotrafilcon A and lotrafilcon B) have a plasma-coated surface consisting of a high refractive index polymer. [65-67] Bausch & Lomb's balafilcon A has a plasma-treatment that converts the hydrophobic silicon at the surface into a highly wettable "glassy" surface that consists of silicate. [63, 64] The two Johnson and Johnson materials (senofilcon A and galyfilcon A) do not undergo a surface modification process, but rather incorporate polyvinyl pyrrolidone (PVP) during the polymerization process, which then acts to assist with surface wetting and hydrophilicity. [68-70]

Among the SH materials, balafilcon A and galyfilcon A materials had the highest percentage of lysozyme activity at the end of 28 days. This could be attributed to the location of lysozyme on these lens materials. Using confocal microscopy, we have shown that there was a significant amount of lysozyme penetration into the bulk of the matrix of balafilcon material, with a gradual build-up of lysozyme as a function of time for up to 28 days. [47, 48] Lysozyme can easily penetrate into the interior matrix of the balafilcon material due to its macroporous nature, as shown by scanning electron microscopy and atomic force microscopy. [62, 63] Under conditions of dehydration and hydration, it was found that the diameter of these macropores could be as high as 0.5 μm, which is significantly larger than the pore size of CH lenses. It is therefore reasonable to assume that these macropores are sufficiently large enough to permit the diffusion of lysozyme, with relatively low structural transitions. In addition, the incorporation of N-vinyl aminobutyric acid imparts an overall negative charge to the balafilcon material (it is the only FDA group III SH material commercially available) and this will also increase the amount of lysozyme that sorbs onto the material.

In the galyfilcon A lens material, it was found that a significant amount of lysozyme remained on the surface, but a significant amount of lysozyme also penetrated into the bulk of the material, when assessed by confocal microscopy. [47, 48] Surprisingly, senofilcon A, which undergoes a similar process of incorporating PVP into the lens to aid wettability as that used in galyfilcon A, showed very little penetration of lysozyme, with most lysozyme remaining on the surface. [47, 48] This difference in the location of lysozyme on lens materials may help to explain the differences in the active lysozyme recovered from these two lens materials, despite their similar compositions.

SH lens materials that were surface modified with a 25nm plasma coating (lotrafilcon A & B) showed a deposition pattern similar to polyHEMA lens materials. These materials are surface treated by coating them with a 25nm thick cross-linked hydrocarbon-containing plasma polymer. [62, 65, 66] It would appear that the "sealed-in" nature of the surface of lotrafilcon A and lotrafilcon B, coupled with their very low water content (Table 5.1) and lack of surface charge, produces lens materials that are highly resistant to protein deposition. Therefore, the deposition of lysozyme on lotrafilcon A and B lens materials will entirely be a surface adsorption phenomenon. The results from confocal microscopy also support this view. [47, 48] This would result in lysozyme remaining entirely on the lens surface, with no penetration occurring, thereby resulting in further denaturation of the deposited protein.

One of the limitations of this study was that the lenses were incubated in a solution containing only one type of protein, namely lysozyme. An important factor to

consider in the process of deposition relates to whether other substances from the tear film (for example, mucins, lipids or other proteins) concurrently deposit onto the material surface at different rates. Moreover, certain tear proteins such as lactoferrin are known to protect lysozyme from losing their activity. [71-73] Hence, it is necessary in future studies to investigate the activity of lysozyme using a complex tear solution and to look at the protein-protein, protein-lipid and protein-mucin interactions. It would also be of interest to study the activity of other major tear proteins such as lactoferrin, once they deposit onto conventional and silicone hydrogel contact lens materials. In addition to investigating the kinetics of denaturation and how they relate to the wearing period, the influence of various care regimens on denaturation also require investigation.

5.6 Conclusions

In conclusion, the results from this study suggest that the reduction in the activity of lysozyme deposited on contact lens materials is time dependent and that the rate of reduction in the activity of lysozyme varies between lens materials. This reduction in the loss of biological activity could be due to the differences in surface or bulk properties of the lens materials and also depend on the location of lysozyme on these lens materials. These results reiterate that the levels of lysozyme activity are highly variable between lens materials and also that SH lens materials deposit very low amounts of lysozyme. After 28 days, lysozyme recovered from CH FDA group IV lens materials (etafilcon A and vifilcon A) retained the highest amount of percentage activity, while lysozyme recovered from SH lenses that are surface modified with a 25nm plasma coating (lotrafilcon A & B) retained an activity similar to that of a CH FDA group I lens material.

Among SH lenses, lenses that use a surface modification system based on PVP (galyfilcon A) and lenses that undergo a plasma oxidation process (balafilcon A) retained similar levels of percentage active lysozyme.

To our knowledge, this is the first study to investigate the kinetics of lysozyme activity recovered from SH and CH lens materials. It provides hitherto unavailable information on the rate of reduction of lysozyme activity deposited on contact lens materials, which will be useful for contact lens practitioners, the contact lens industry and the general field of biomaterials research.

The next chapter of this thesis will determine the total protein, total lysozyme and active lysozyme deposition on a novel, lathe-cut silicone hydrogel contact lens material (sifilcon A) after three months of wear.

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6 Protein deposition on a novel, lathe-cut silicone hydrogel contact lens material (sifilcon A)

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6.1 Abstract

Purpose: To determine the quantity of total protein, total lysozyme and the conformational state of lysozyme deposited on a novel, lathe-cut silicone hydrogel (SiHy) contact lens material (sifilcon A) after three months of wear.

Methods: 24 subjects completed a prospective, bilateral, daily-wear, nine month clinical evaluation in which the subjects were fitted with a novel, custom-made, lathe-cut SiHy lens material. The lenses were worn for three consecutive three-month periods, with lenses being replaced after each period of wear. After 3 months of wear, the lenses from the left eye were collected and assessed for protein analysis. The total protein deposited on the lenses was determined by a modified Bradford assay, total lysozyme using Western blotting and the lysozyme activity was determined using a modified *micrococcal* assay.

Results: The total protein recovered from the custom-made lenses was 5.3±2.3 µg/lens and the total lysozyme was 2.4±1.2 µg/lens. The denatured lysozyme found on the lenses was 1.9±1.0 µg/lens and the percentage of lysozyme denatured was 80±10%.

Conclusions: Even after three-months of wear, the quantity of protein and the conformational state of lysozyme deposited on these novel lens materials was very similar to that found on similar surface-coated SiHy lenses after two to four weeks of wear. These results indicate that extended use of the sifilcon A material is not deleterious in terms of the quantity and quality of protein deposited on the lens.

Key words: contact lens, deposition, lysozyme activity, lathe cut silicone hydrogel, tear protein

6.2 Introduction

Protein deposition on contact lens materials is a complex process and is dependent upon several factors, including chemical composition of the lens material, material water content and surface charge. [1-9] In addition to the aforementioned factors, studies have shown that the fabrication process and the surface regularity of the lens material can also influence the deposition of tear components on to lens materials. [10-14] Previous *in vitro* and *ex vivo* studies have shown that proteins increasingly accumulate on lens materials over time, with longer incubation times or in-eye wearing periods resulting in increased deposition. [5, 6, 10, 12, 15-26]

A recent study that investigated the proteomic profile of contact lens deposition on silicone hydrogel (SiHy) lens materials using mass-spectrometry identified lysozyme, lipocalin, lactoferrin, lacritin, proline-rich 4 and Ig alpha as frequently recognized tear proteins. [27] Among the unique tear proteins that are found on hydrogel lens materials, lysozyme is a bacteriolytic enzyme with a relatively small molecular weight (14 kDa) and a positive charge at neutral pH. Once lysozyme firmly adsorbs onto contact lens materials, it tends to denature fairly rapidly, [3, 28-30] which might potentially result in a variety of immunological responses, including contact lens associated papillary conjunctivitis (CLAPC). [31-34]

Currently available SiHy lens materials are cast-moulded and are available only in a limited range of parameters, which limit their usage. A novel SiHy lens material has been developed by CIBA Vision (sifilcon A) which is lathe cut, allowing for an increased

parameter range to be fitted, and for practitioners to "custom-design" the lens in terms of diameter, base-curve and back vertex power. [35, 36] This lens is also unique in that it is currently the only SiHy lens to be replaced on a three monthly basis, which is considerably longer than other currently available SiHy lens materials, which are replaced every two or four weeks. The use of such a lens has been welcomed by contact lens practitioners, as it opens up the opportunity to fit SiHy lenses to patients with parameters that are typically outside those currently available. [37, 38]

Several *ex vivo* studies which have investigated protein deposition on commercially available cast-moulded SiHy lens materials after two or four-weeks of wear have shown that SiHy lenses deposit significantly lower quantities of protein than conventional FDA group II or IV lens materials. [28, 29, 39-43] Similar results were obtained through *in vitro* studies which investigated the protein deposition on contact lenses by artificially "soiling" various lens materials with tear proteins for time periods ranging from one-hour to one-month. [16, 17, 30, 44-46] A previous *ex vivo* study that examined the protein deposition on SiHy lens materials after two weeks of wear has shown that lotrafilcon-based, galyfilcon and senofilcon lens materials deposit 5 to 7 μg of total protein per lens, while balafilcon lens material deposits 27 μg of total protein per lens. [41] However, to-date no study has investigated the protein deposition on a lathe-cut SiHy lens material, particularly one used for three months of wear. Moreover, no study has investigated the conformational state of the deposited protein on any contact lens material after three months of lens wear. Thus, the purpose of this study was to determine the

quantity of total protein, total lysozyme and the conformational state of lysozyme deposited on a novel, lathe-cut SiHy lens material after three months of in-eye wear.

6.3 Materials and Methods

6.3.1 Study design and collection of worn contact lenses

Ethics clearance was obtained from the Office of Research Ethics at the University of Waterloo before commencement of the study. The study was carried out in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants prior to enrolment in the study. This study was conducted as a prospective, bilateral, daily wear, nine-month clinical evaluation at the Centre for Contact Lens Research, School of Optometry, University of Waterloo.

All the participants were adapted daily-wear hydrogel soft lens wearers. A total of 26 participants (18 females and 8 males; mean age, 31.6 ± 10.75 years; Range, 18-54 years) were enrolled, of which two participants discontinued from the study. Of these, one discontinued due to poor visual quality and the other discontinued due to discomfort and dryness with the lenses. The sifilcon A lens materials were ordered for the participants based on the screening examination and trial lens fitting, and were fitted according to the manufacturer's guidelines. [35] Lenses were worn for three consecutive three-month periods, with lenses being replaced after each period of wear. The commercially available multi-purpose solution CLEAR CARE® (CIBA Vision®, Duluth, GA) was used during this study. Upon removal at the end of each daily wear

period, participants were instructed to rub the lenses for five seconds with the CLEAR CARE® solution, prior to soaking in fresh solution overnight. Participants were advised to exercise special caution when using the peroxide-based care system with the rubbing step, to avoid getting any peroxide in their eye. They were advised to remove both lenses and place them in the open "baskets" of the lens case before they began the rub/rinse step with either lens, to avoid removing the second lens with a peroxide-laden finger. No enzyme removal systems or stand-alone surfactant cleaners were allowed. Participants habitually using rewetting drops were permitted to continue to use these drops as required.

6.3.2 Collection of worn contact lenses

Upon completion of three-months of daily wear, lenses from the left eye were collected (using non-powdered surgical gloves) and placed in individual, sealed glass vials containing 1.5 ml of a 50:50 mix of 0.2% trifluoroacetic acid and acetonitrile (ACN/ TFA). [29, 40, 47, 48] The vials were incubated in the dark at room temperature for 24 hours following which the aliquots of lens extracts were transferred to sterile Axygen microcentrifuge tubes and evaporated to dryness in a Savant Speed Vac (Halbrook, NY, USA). Dried protein pellets were stored at –80ºC for up to two weeks prior to reconstitution.

6.3.3 Reagents and materials

Sifilcon A lenses (O_2OPTIX Custom™) were provided by CIBA Vision® (Duluth, GA). The known properties of this material are given in Table 6.1. The PhastSystem™ components were described in detail previously. [29] Immuno-Blot® PVDF (polyvinylidene difluoride) membranes and protein assay reagents were purchased from Bio-Rad Laboratories (Mississauga, ON, Canada). Polyclonal rabbit anti-human lysozyme was purchased from Cedarlane Laboratories (Hornby, ON, Canada) and goat anti-rabbit IgG-HRP was purchased from Sigma (St. Louis, MO, USA). Human lysozyme (neutrophil) and BioStab Biomolecule Storage Solution (Sigma # 92889) were purchased from VWR (Toronto, ON, Canada). *Micrococcus lysodeikticus* and all other reagents purchased were of analytical grade and obtained from Sigma Aldrich.

Table 6-1: Properties of the lens materials investigated in the current study

	O_2OPTIXCustom	O_2OPTIX
United States Adopted Name	sifilcon A	lotrafilcon B
Water content	32%	33%
Modulus	1.1 MPa	1.0 MPa
Handling tint	Light green	Light blue
Wearing schedule	Daily wear	Daily wear and extended wear up to six nights
Replacement schedule	Quarterly	Two-week
Dk	82	110
Centre thickness (@ -3.00D)	0.07mm	0.08mm
Dk/t (@-3.00D)	117	138
Sphere powers	+20.00 to -20.00 DS in 0.25 steps	+6.00 to -10.00 DS
Surface treatment	25nm plasma coating	25nm plasma coating
Manufacturing process	Lathe cut	Cast mould
Monomers	undisclosed	DMA+TRIS+Siloxane monomer

DMA (N,N-dimethylacrylamide); TRIS (trimethylsiloxy silane)

6.3.4 Measurement of total lysozyme deposition - Electrophoresis and immunoblotting

Lenses collected in ACN/TFA were incubated in the dark at room temperature for 24 hours and the samples were processed. [40, 48] Lysozyme standards were prepared fresh on the day of analysis from a 1.0 μg/μL frozen stock of purified human neutrophil lysozyme with modified reconstitution buffer, pH 8.0 and subjected to SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) followed by Western blotting to PVDF membranes. [29, 40, 48]

6.3.4.1 Negative control - extraction and western blot analysis of unworn lenses

Three new, unworn sifilcon A lenses were extracted in ACN/TFA solution and were subjected to SDS-PAGE and Western blotting, as described above.

6.3.5 Measurement of lysozyme activity

The contact lens extracts were assayed for lysozyme activity using a fresh suspension of *Micrococcus lysodeikticus* for each sample. [29, 30, 40] *Micrococcal* cells were suspended in 50 mM sodium phosphate buffer (pH 6.3) to an initial optical density of 1.0 at 450 nm (Multiskan Spectrum ELISA Plate Reader, fitted with a micro-cuvette, ThermoLabsystems). Human neutrophil lysozyme standard (2.5, 5, 12.5, 50, 150, 250 ng) was run concurrently with the samples. The mass of active lysozyme in contact lens extracts was extrapolated from the native lysozyme standard curve. [29, 30, 40]

6.3.6 Measurement of total protein deposition

A Bradford assay [49, 50] was used to determine the total protein deposited on the sifilcon A lens material; however, modifications were made to quantify the contact lens extracts. Bovine Serum Albumin (0, 0.5, 1, 2, 4, 6, 8, 10 µg) was used as the protein standard. All tubes were neutralized with 25µl 0.5M phosphate pH 7.2 and 145µl water, and neutrality was confirmed with pH paper. The total volume for this initial sample preparation was 190µl (20µl + 25µl + 145µl water). Calcium chloride (10µl, 500mM) was added and all tubes were mixed well by vortexing. The samples were allowed to precipitate for 5 minutes and 1 ml of 99.9% ethanol was added. The tube was vortexed

and centrifuged (15000g for 1 minute) and the supernatant was aspirated, avoiding the pellet. 1 ml of 90% (v/v) ethanol was then added which was followed by vortexing, centrifuging and aspiration. The samples were then dried in the SpeedVac for 15 minutes. 50 µl of the Bradford reagent was added to the samples and the samples were placed statically for 10 minutes at room temperature. The tubes were then placed in boiling water for 30 seconds to dissolve the precipitates, followed by vortexing. Once the tubes returned to room temperature, they were centrifuged briefly, such that the Bradford reagent sedimented at the bottom of the tube. 200 µl of cold NaCl was added to the samples and were vortexed twice. 200 µl of the sample was then transferred to a 96-well microtitre plate and the absorption ratio was read within 10 minutes. Samples were read on three occasions and the average of these was recorded. A standard curve was prepared and the line of best-fit was determined. The sample A595 values were compared with the curve to calculate the amount of total protein in the samples.

6.4 Results

Table 6.2 shows the total protein, total lysozyme, denatured lysozyme in microgram per lens and the percentage denatured lysozyme after three months of daily wear of the sifilcon A lens material. For comparison purposes, Table 6.2 also shows the protein deposition data from another *ex vivo* study from our group [41] which reports on the total protein, total lysozyme, denatured lysozyme in microgram per lens and the percentage denatured lysozyme after two weeks of wear of lotrafilcon A, lotrafilcon B, balafilcon A, galyfilcon A and senofilcon A lens materials.

No signal was seen on Western blots run on unworn sifilcon A lenses after being subject to the same procedures, confirming that there was no background interference from the lens polymer.

Table 6-2: Protein deposition on sifilcon A lens material after three months of lens wear.

This table also shows the protein deposition on five other silicone hydrogel lens materials after two-weeks of lens wear from a previously published study. [41] In both studies CLEAR CARE® was used as the care regimen

	Sifilcon A	Lotrafilcon A *	Lotrafilcon B *	Balafilcon A *	Galyfilcon A *	Senofilcon A *
Total protein deposition (in µg/lens)	5.3 ± 2.3	5.2 ± 2.2	6.6 ± 3.4	26.9 ± 9.3	6.3 ± 3.4	4.6 ± 2.5
Total lysozyme deposition (in µg/lens)	2.4 ± 1.2	1.1 ± 0.8	1.4 ± 1.1	13.3 ± 9.0	1.9 ± 1.4	0.9 ± 0.6
Denatured lysozyme (in µg/lens)	1.9 ± 1.0	0.8 ± 0.7	1.1 ± 0.9	7.3 ± 5.8	1.1 ± 1.0	0.6 ± 0.3
Percentage denatured lysozyme	80 ± 10	74 ± 17	78 ± 14	53 ± 17	53 ± 19	66 ± 19

* Data from a previously published ex vivo study. [41]

6.5 Discussion

This is the first study to report on protein deposition levels on a novel, custom-made, lathe-cut SiHy lens material after three months of daily wear. Traditionally, lathe cut lens materials undergo a "polishing" step to eliminate any surface irregularities. However, the sifilcon A lens materials do not undergo this polishing step because these lenses undergo a state-of-the-art custom lathing process through the InnoLathe™ manufacturing technology (CIBA Vision, personal communication), obviating the necessity for this final polishing step. The finished lenses then undergo a similar plasma surface treatment to that seen with CIBA Vision's other SiHy materials, which are permanently modified in a gas plasma reactive chamber using a mixture of trimethylsilane oxygen and methane to create a permanent, ultrathin (25nm), high refractive index, continuous hydrophilic surface. [51-55]

Figures 6.1 and 6.2 show the AFM images of lotrafilcon B and sifilcon A lens materials, respectively, at 20X20μm scan size. The AFM image of the lotrafilcon B lens material (Figure 6.1) shows that the surface of these lenses exhibit characteristic multiple linear marks, that are similar to that previously reported for lotrafilcon A [56, 57] and lotrafilcon B lenses. [58] Surprisingly, these characteristic linear "lines" are not seen on the surface of the lathe-cut sifilcon A material (Figure 6.2), as linear marks are commonly seen on lenses that are fabricated through a lathe-cutting process. [59, 60] Interestingly, the surface of the sifilcon A lens material demonstrates a porous, "sponge-like" surface topography similar to that of galyfilcon A and senofilcon A lens materials. [58] This is of interest, as it appears that lathing a "rubbery", siloxane-based material such as sifilcon A

produces a different topography to that typically seen in lathed HEMA materials. It is clear from Figures 6.1 and 6.2 that the surface topography of the lathe-cut sifilcon A material is "less smooth" than that of the lotrafilcon B lens material. It has previously been shown with HEMA-based materials that lathe-cut lenses have surfaces that are generally "rougher" than those fabricated by cast-moulding. [59, 61]

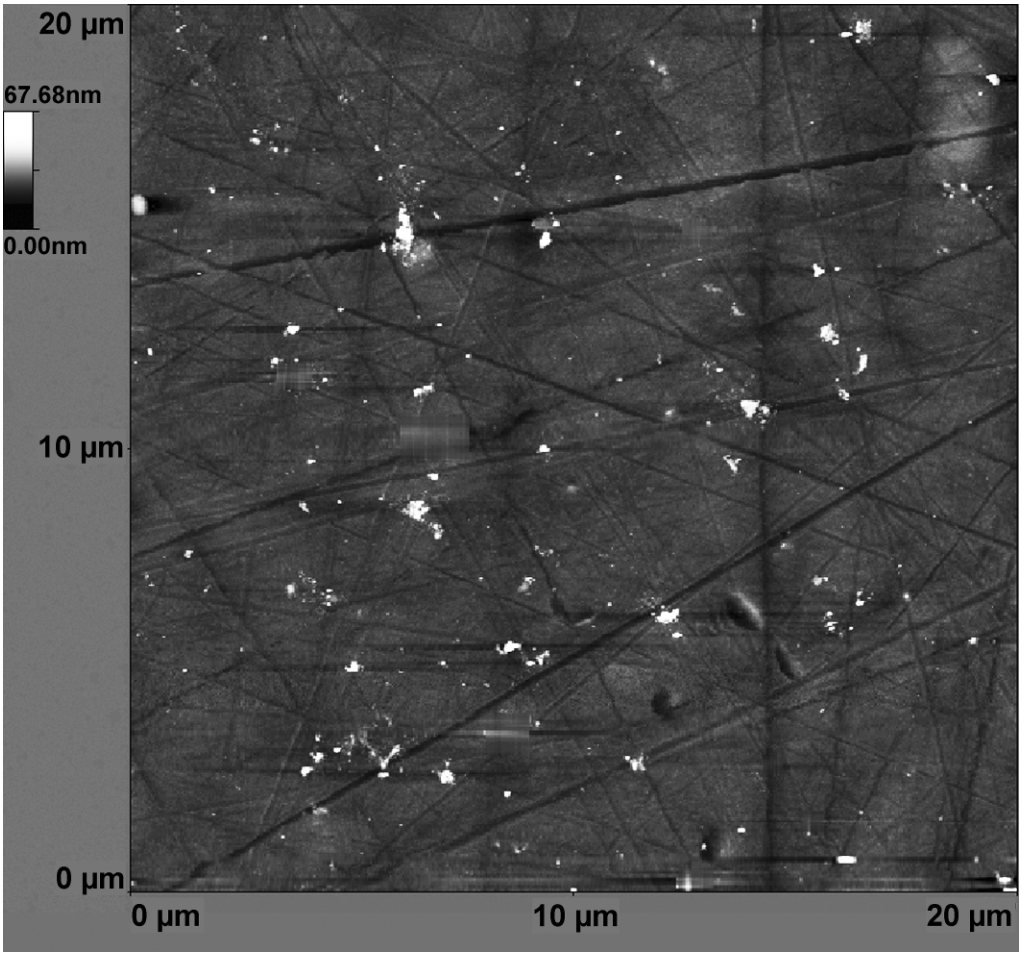

Figure 6-1: AFM image an unworn lotrafilcon B lens material.

Atomic Force Microscopy (AFM) image (20x20µm) of a rinsed, unworn, lotrafilcon B lens (-10.00D) fabricated via a moulding process.

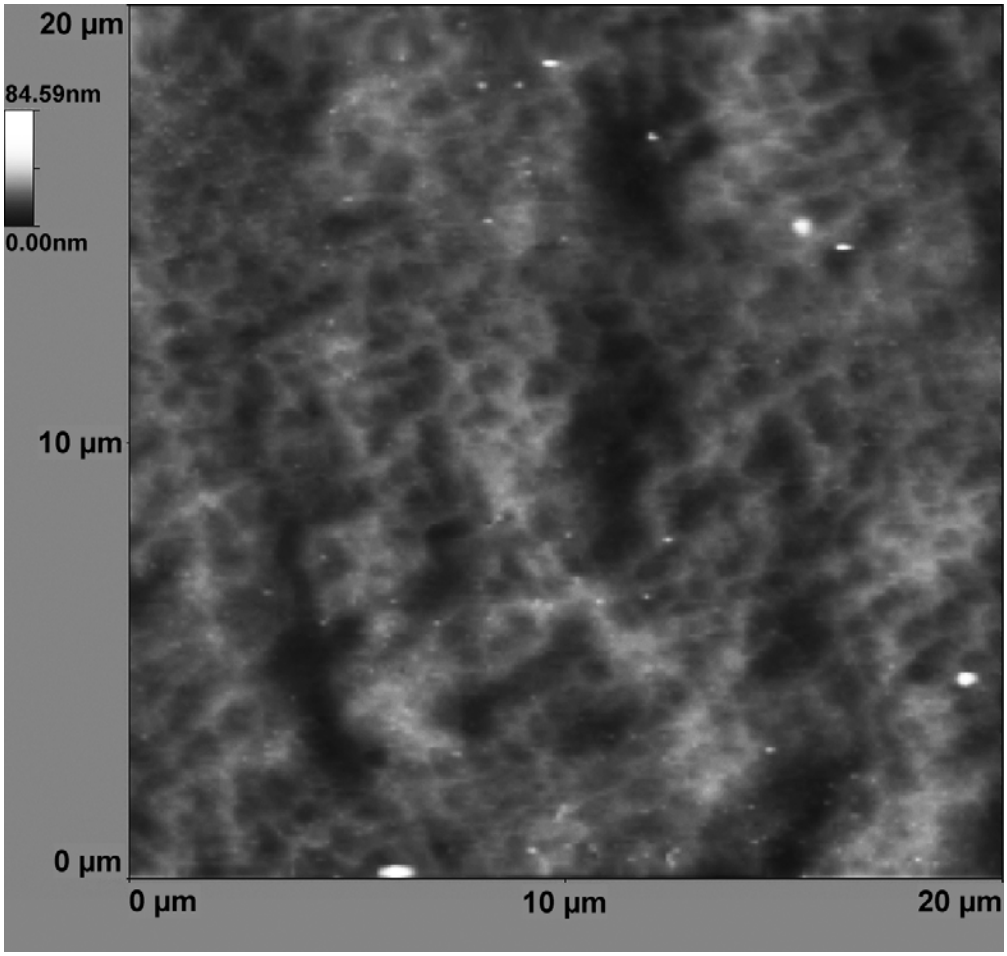

Figure 6-2: AFM image of an unworn sifilcon A lens material.

Atomic Force Microscopy (AFM) image (20x20µm) of a rinsed, unworn, sifilcon A lens (-10.00D) fabricated via a lathing process.

It is noteworthy to compare the total protein deposited on the sifilcon A lens material with that deposited on a conventional hydrogel contact lens after three months of wear. In this study, sifilcon A deposited 5.3±2.3 µg/lens of total protein (Table 6.2) after three months of wear, as compared with a conventional hydrogel FDA group II material (vasurfilcon A), which deposited 106±16 µg/lens of total protein [62] after the same period of wear. In the other study, [62] Bausch & Lomb's ReNu Multi-Purpose was used as the

care regimen, which was used with a "Rub-and-Rinse" format. The Vasurfilcon A lens material has methyl methacrylate and N-Vinyl pyrrolidone as its principal monomers, thus it is non-ionic, high water content (74%) material, which is classified as a FDA group II material. It has also been shown in an earlier *in vitro* study using a radiolabeling technique that FDA group II lens materials deposit significantly lower amounts of lysozyme than a high water content, ionic FDA group IV lens material, but deposit higher amounts of lysozyme than most SiHy lens materials. [16] Despite the fact that the care regimen and the methods used to examine the total protein were different in these two studies, this information puts into perspective the fact that SiHy lens materials still deposit substantially less protein than conventional lens materials, even after periods of wear longer than that typically seen with most SiHy lenses.

It is also interesting to note that the total protein and total lysozyme deposited on the sifilcon A lens material is similar to that found on similar surface-coated SiHy lens materials (such as lotrafilcon A and lotrafilcon B) after two-weeks of lens wear. [41] The results from the other *ex vivo* study [41] showed that lotrafilcon A, lotrafilcon B, galyfilcon A and senofilcon A deposited 5.2±2.2, 6.6±3.4, 6.3±3.4 and 4.6±2.5 μg of total protein per lens respectively, while balafilcon A deposited 26.9±9.3 μg of total protein per lens after two-weeks of wear (Table 6.2). It is also of interest to note that the total protein and total lysozyme deposition on the sifilcon A lens material is similar to that seen on galyfilcon A and senofilcon A lens materials. As mentioned previously, the AFM images (Figure 6.2) shows that sifilcon A lens has a similar surface topography to that of galyfilcon A and senofilcon A lens materials. [58] Both this study and the other *ex vivo*

study [41] used the peroxide-based CLEAR CARE® solution as the care regimen. However, in this study the participants used the solution in a "Rub-and-Rinse" format, whereas in the other study [41] the solution was used only in the "Rinse" format. Despite this difference, the levels of deposition were remarkably similar. More work looking at the role of "Rubbing-and-Rinsing" and its role in keeping the sifilcon A lenses "clean" of deposits is warranted, as this study only investigated protein deposition and not other tear-derived deposits, particularly lipid, which are known to be an issue with SiHy lenses. [28, 63-65]

Previous studies which have compared the protein uptake by contact lens materials that are fabricated by means of spin-casting, cast-moulding and lathe-cut processes have shown that lathe-cut lens materials typically attract more deposits than the other fabricating methods. [10-12] This enhanced deposition could be due to the presence of an increased number of binding sites on the lens material, which are caused by the fabrication defects on the lathe-cut lenses. A study by Fowler and Gaertner, [14] using a scanning electron microscope, also demonstrated that there was "heaping-up" of deposits in the lathe-cut areas of worn contact lenses. In contrast, a study by Kaplan and Gundel [13] showed that there was no significant difference in deposition between polished and unpolished lathe-cut lens materials. The results from this study would suggest that factors such as material water content, surface charge or treatment and material composition play a far bigger role in determining the deposition of tear proteins on hydrogel lenses than the manufacturing method.

This is the first study to determine the conformational state of lysozyme deposited on a SiHy lens material after three-months of wear. The results from this study show that after three months of lens wear, the percentage denatured lysozyme recovered from these materials was 80 ± 10% (Table 6.2). The percentage denatured lysozyme recovered from these lenses is comparable to that from the lotrafilcon A lens material worn on an extended wear basis for 30 days [28] and comparable to lotrafilcon A and lotrafilcon B lens materials after two-weeks of daily lens wear. [41] It is highly unlikely that lysozyme which is irreversibly bound to the lens polymer will have retained any biological activity after three-months of wear. The 20% "active" lysozyme which is recovered from the sifilcon A lens material is more likely to be adsorbed "firmly" but not "irreversibly" on to the lens material. In addition, it is speculated that the lysozyme which has retained its biological activity might have adsorbed either "reversibly" or "irreversibly" on the lenses on the day when the lens was collected for laboratory analysis.

6.6 Conclusions

In conclusion, the results from this study indicate that the extended use of the sifilcon A lens material used in the O_2OPTIX Custom™ SiHy lens is not deleterious in terms of the amount and the quality of protein that is deposited on the lens. These results indicate that even after three months of wear, the quantity of protein and the conformational state of lysozyme deposited on these novel lenses (when worn using a care regime employing a rub-and-rinse format) is very similar to that found on similar surface-coated SiHy lenses (lotrafilcon A and lotrafilcon B) after two to four weeks of wear. These results reiterate that SiHy lens materials, although fabricated via a lathe-

cutting process, deposit low quantities of total protein and total lysozyme even after extended period of daily wear. Further work is required to determine the location of lysozyme on these lathe-cut lens materials. Lysozyme forms only a portion of the total protein deposited on the sifilcon A lens material; hence the quantity of other protein types deposited should also be investigated. It would also be of interest to study lipid deposition on these lens materials and also to determine the impact of care regimen on protein/ lipid deposition.

The next chapter of this thesis will discuss the relationship between protein deposition and clinical signs & symptoms after one-day wear of etafilcon A contact lenses in a group of symptomatic and asymptomatic contact lens wearers.

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7 Influence of protein deposition on subjective symptoms in one day wear of etafilcon lenses in symptomatic and asymptomatic contact lens wearers

7.1 Abstract

Purpose: To determine the relationship between clinical signs & symptoms and protein deposition over eight hours wear of etafilcon lenses in symptomatic and asymptomatic contact lens wearers.

Methods: 30 adapted soft contact lens wearers (16 symptomatic and 14 asymptomatic) were fitted with etafilcon lenses. *In vivo* wettability, non-invasive tear break-up time (NITBUT) and subjective symptoms (vision, comfort and dryness) were assessed at baseline and after hours 2, 4, 6 and 8. After 2, 4, 6 and 8 hour time points, lenses were collected and total protein, total lysozyme and active lysozyme deposition were assessed.

Results: There was a significant reduction ($p<0.05$) in the NITBUT at 8 hours in both groups. In the symptomatic group, there was a significant reduction in subjective comfort and dryness ratings at 6 and 8 hour measurement with respect to baseline ($p<0.05$). There was a significant increase in total lysozyme and total protein deposition ($p<0.05$) across all time points in both groups; most of the lysozyme remained active (>94% at 8h). Pearson's correlations between subjective symptoms and protein deposition showed poor correlations for total protein/ lysozyme and any subjective factor ($r<0.3$; $p>0.05$), and only weak correlations between dryness and % active lysozyme ($r=0.3–0.4$ for all time points; $p<0.05$). However, stronger correlations were found between active lysozyme and subjective comfort ($r=0.7$-0.8; $p<0.05$).

Conclusions: In addition to investigating the total protein deposited on contact lenses, it is of significant clinical relevance to determine the conformational state of the deposited protein.

Key words: comfort, contact lens, deposition, lysozyme activity, tear protein.

7.2 Introduction

One of the primary reasons for contact lens intolerance and discontinuation is dryness and discomfort that is associated with lens wear. [1-3] Contact lens-related dry eye has been associated with other correlates including alterations in functional visual acuity, [4, 5] reduced wearing time, [6] increased risk of bacterial adhesion, ocular surface desiccation and infection. [7, 8] Several studies have attempted to determine the potential mechanisms for contact lens-related dry eye and it has been observed that increased evaporation of the tear film, [9] rapid pre-lens tear film thinning, [10] limbal injection, [10] inflammation, [11-13] reduced lacrimation with concurrent increased osmolality [14, 15] and an increase in tear film osmolality [10] as significant factors that are typically associated with contact lens-related dry eye. In addition, higher water content contact lenses, [10, 16] reduced wettability of the lens surface [17-19] or any of the above-mentioned factors could also be associated with contact lens-related dry eye, confirming that this condition is multifactorial. Some studies have demonstrated that dryness and discomfort ratings become worse independently of the amount of dehydration or water content of the hydrogel lenses. [2, 20, 21]

The tear film is by far the most dynamic unit in the lacrimal functional unit, which consists of a variety of components, including proteins, lipids, mucins, peptides, electrolytes and salts. Using a proteomic technique, 97 proteins have been identified in the tear film [22] and many of these proteins are known to sorb onto contact lens materials. [23, 24] Protein deposition on contact lens materials is highly material dependent, with water content and surface charge having significant impacts on the amount of protein deposited.

[25-36] One of the major tear proteins that is recovered from FDA group IV contact lens materials is lysozyme. [28, 34, 36-40] Lysozyme is a bacteriolytic enzyme with a relatively small molecular weight (14 kDa) and a positive charge at neutral pH. Once lysozyme firmly adsorbs onto contact lens materials, it tends to undergo conformational changes, [28, 40-42] which might potentially result in a variety of immunological responses, including contact lens associated papillary conjunctivitis. [43-46]

In the past, several studies have determined the changes in tear film protein/ lipid levels in contact lens wearers, with some of these studies classifying contact lens wearers as being either "tolerant" or "intolerant". [11, 13, 47-61] However, these studies did not quantify the protein deposited on the lens materials *per se*, therefore it was not possible to determine the relationship between various clinical parameters and the amount of protein deposited on the contact lenses. Another study which quantified the protein deposited on the lens material, determined the effect of overnight eye closure on the rate and composition of protein deposition on the probable change in the rate of reflex-type tear secretion associated with eye closure. [38] Some studies have determined the link between protein deposition on contact lenses and subjective symptoms reported by lens wearers. [62-66] However, all these studies determined the deposition on lenses by employing relatively insensitive techniques such as visible deposition or video image analysis. [64-66]

Although some studies have speculated that the conformational state of the deposited protein could have an influence on various subjective symptoms in contact lens wearers, [62, 67] to-date, no study has determined the relationship between subjective

symptoms and the conformational state of the deposited protein, or indeed the differences in these factors in symptomatic and asymptomatic contact lens wearers. Thus, the purpose of this study was to investigate the impact of wearing time on clinical signs, subjective symptoms and the quantity of total protein & lysozyme deposition and the conformational state of the lysozyme deposited over an eight-hour wear period of a high water content ionic lens material (etafilcon A; Acuvue®; Johnson & Johnson) in a group of symptomatic and asymptomatic contact lens wearers. The other purpose of this study was to determine if there is any association between the clinical signs & symptoms and the protein deposition measured on the lenses.

7.3 Materials and Methods

Ethics clearance was obtained from the Office of Research Ethics at the University of Waterloo before commencement of the study. The study was carried out in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants prior to enrolment. The study was conducted at the Centre for Contact Lens Research, School of Optometry, University of Waterloo. This was a masked, non-dispensing and randomized study involving 30 previously adapted soft contact lens wearers. Participants were classified as being "symptomatic" or "asymptomatic" based on their responses to a pre-screening questionnaire. Patients who were classified as being symptomatic with their soft lenses were those subjects who reported reduced comfortable lens wear after a minimum of 6 hours of wear and who needed to resort to ocular lubricants in order to sustain their lens wear. All participants

were fitted with etafilcon A contact lenses (FDA group IV lens material; Acuvue®; Johnson & Johnson, Jacksonville, Florida) and the participants were not permitted to use any rewetting drops during the course of the study.

Each participant attended on two consecutive days, with a baseline and two study visits on each day. All the study visits were randomly determined based on a randomization table. During the baseline visit on day one, contact lenses were inserted into both eyes and, after the lenses had settled, objective and subjective measurements were determined. During the first study visit (which was randomly determined) on day one, objective and subjective measurements were determined and at the end of this visit, one lens was randomly removed from one of the participant's eyes for protein analysis. A new lens was re-inserted into that eye to ensure the subject was binocularly corrected. During the second study visit later that day (which was again randomly determined), objective and subjective measurements were taken and at the end of this visit, the lens from the other eye was collected for protein analysis. On the following day, the same procedures were repeated, for the remaining two time points, which were determined using a randomization table. Thus, each participant had lenses collected for analysis after 4 time periods (2 per day), with the periods of time being after two, four, six or eight hours of wear.

7.3.1 Clinical measurements

7.3.1.1 Objective measurements

The objective measurements were performed at baseline and after, two, four, six and eight hours of wear. Tear film stability was assessed by determining the non invasive tear breakup time (NITBUT) using the ALCON Eyemap® model EH-290 topography system (ALCON Inc., Forth Worth, Texas, USA). Participants were asked to blink three times before each measurement was taken. NITBUT was determined by measuring the time taken for distortions or discontinuities to appear in the reflected image of the concentric ring pattern. The time (in seconds) for the tear-film to rupture (and thus distort the rings) was measured to the nearest 0.1 of a second. Three measurements were taken on each eye and the average of these was used for analysis purposes.

Overall wettability of the contact lenses was assessed *in vivo* using the grid viewed on the ALCON Eyemap® (ALCON Inc., Forth Worth, Texas). The image of the placido disc was viewed on the monitor of the Eyemap and the *in vivo* wettability of the contact lenses was graded on a five-point scale (0 to 4), where "0" related to a lens exhibiting "severely reduced" wettability and "4" a lens with "perfect" wettability.

7.3.1.2 Assessment of subjective symptoms

Participants completed visual analogue scales at baseline, two, four, six and eight hour study visits. Participants rated the subjective symptoms of vision, comfort and dryness on a scale of 0-100 (0 = worst rating, 100 = best rating).

After two, four, six and eight hours of lens wear, lenses were collected by a gloved examiner and the lenses were briefly rinsed with saline to remove any residual, loosely adhered tear film and placed in individual, sealed glass vials containing a 50:50 mix of 0.2% trifluoroacetic acid and acetonitrile (ACN/ TFA). [37, 42] The vials were incubated in the dark at room temperature for 24 hours, then the aliquots of lens extracts were transferred to sterile Axygen microcentrifuge tubes and evaporated to dryness in a Savant Speed Vac (Halbrook, NY, USA). Dried protein pellets were stored at –80ºC for up to two weeks prior to reconstitution.

7.3.2 Analytical measurements

7.3.2.1 Reagents and materials

Immuno-Blot® PVDF (polyvinylidene difluoride) membranes were purchased from Bio-Rad Laboratories (Mississauga, ON, Canada). Polyclonal rabbit anti-human lysozyme was purchased from Cedarlane Laboratories (Hornby, ON, Canada) and goat anti-rabbit IgG-HRP was purchased from Sigma (St. Louis, MO, USA). Human lysozyme (neutrophil) and lyophilized *Micrococcus lysodeikticus* cells were also

purchased from Sigma (St. Louis, MO, USA). BSA standard was obtained from Pierce Biotechnology Inc (Rockford, IL, USA). All other reagents purchased were of analytical grade.

7.3.2.2 Measurement of total lysozyme deposition - Electrophoresis and immunoblotting

Lysozyme standards were prepared fresh on the day of analysis from a 1.0 μg/μL frozen stock of purified human neutrophil lysozyme with modified reconstitution buffer, pH 8.0 and subjected to SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) followed by Western blotting to PVDF membranes. [42, 68, 69]

7.3.2.3 Negative control - extraction and western blot analysis of unworn lenses

Three new, unworn etafilcon A lenses were extracted in ACN/TFA solution and were subjected to SDS-PAGE and Western blotting, as described above.

7.3.3 Measurement of lysozyme activity

The contact lens extracts were assayed for lysozyme activity using a fresh suspension of *Micrococcus lysodeikticus* for each sample. [40, 42, 69] *Micrococcal* cells were suspended in 50 mM sodium phosphate buffer (pH 6.3) to an initial optical density of 1.0 at 450 nm (Multiskan Spectrum ELISA Plate Reader, fitted with a micro-cuvette, ThermoLabsystems). Human neutrophil lysozyme standard (2.5, 5, 12.5, 50, 150, 250 ng) was run concurrently with the samples. The mass of active lysozyme in contact lens

extracts was extrapolated from the native lysozyme standard curve, as described previously. [40, 42, 69] The final calculation was the percent of active lysozyme: % active lysozyme = (active lysozyme / total lysozyme) X 100.

7.3.4 Measurement of total protein deposition

The total protein extracted from the lenses was determined using the Micro-BCA assay. Manufacturer's instructions were followed for the Micro BCA™ Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA). Phosphate buffered saline was used as the buffer. Each data point was the average of 3 determinations.

7.3.5 Statistical Analysis

Statistical analysis was conducted using Statistica 7 software (StatSoft Inc, OK, USA). All data are reported as mean ± standard deviation and range, unless otherwise indicated. A repeated measures analysis of variance (RM-ANOVA) was performed to determine significant differences at various time points and post-hoc multiple comparison testing was undertaken using the Tukey-HSD test. Pearson's correlations were performed to determine the relationship between various clinical signs and symptoms versus the analytical measures. In all cases, a p value of <0.05 was considered significant.

7.4 Results

Based on the participants' responses to the pre-screening questionnaire, 16 participants (mean age 24.73 ± 5.31) were classified as symptomatic and 14 participants (mean age 25.31 ± 4.78) were classified as asymptomatic.

7.4.1 Objective measurements

Figure 7.1 shows the NITBUT values over time for the symptomatic and asymptomatic groups. There was no significant difference in the NITBUT values between the two groups at any time point ($p>0.05$), but the eight hour time point was significantly lower than the baseline measurement in both the symptomatic and asymptomatic groups ($p<0.05$). Figure 7.2 shows the *in vivo* wettability over time for the symptomatic and asymptomatic participants. While *in vivo* wettability is seen to reduce over the course of the day for both groups, this reduction was not statistically significant for either group ($p>0.05$). There was also no significant difference between the two groups at any time point ($p>0.05$).

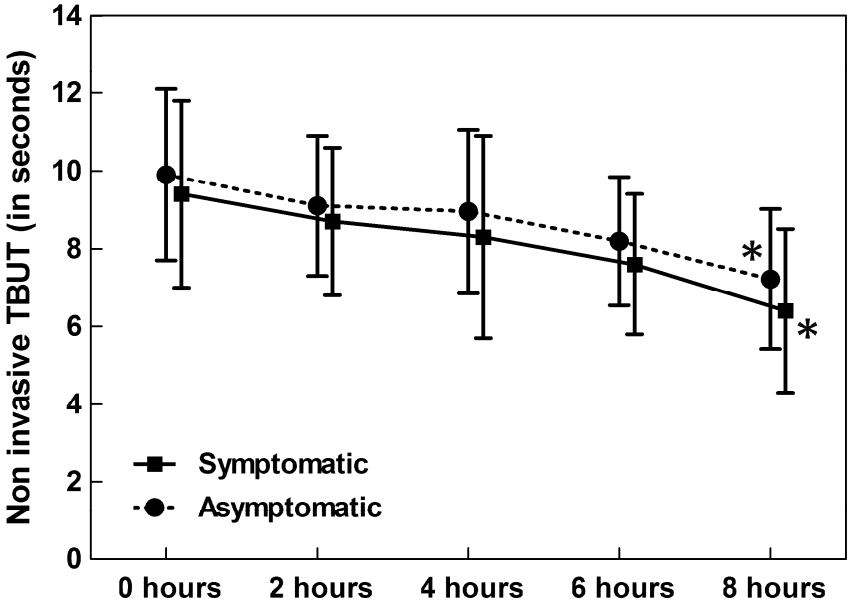

Figure 7-1: Non-invasive tear break up over time in symptomatic and asymptomatic contact lens wearers.

Error bars represent Mean ± SD. * represents $p<0.05$ within the same group from 0 hours (baseline visit).

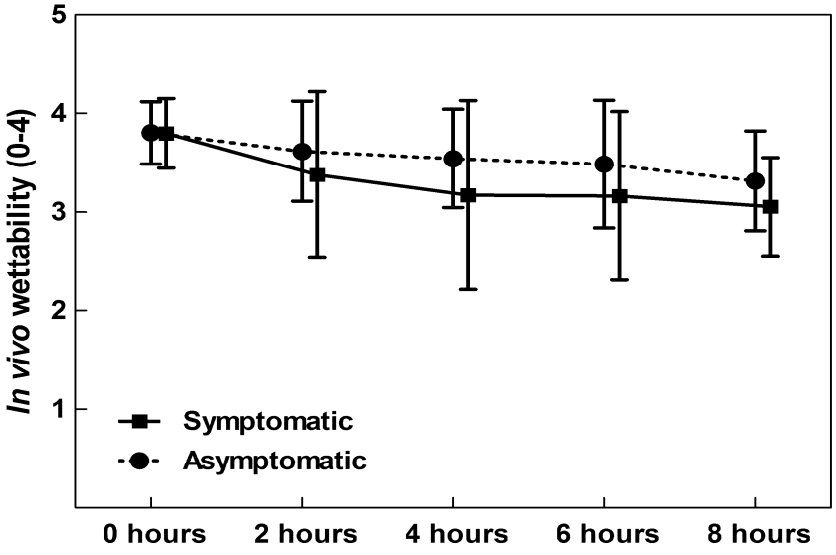

Figure 7-2: *In vivo* wettability over time in symptomatic and asymptomatic contact lens wearers.

Error bars represent Mean ± SD.

7.4.2 Subjective symptom ratings

Figure 7.3 shows that there was no significant difference for subjective vision ratings for both the groups over time ($p>0.05$) and also between the two groups at any time ($p>0.05$), although the symptomatic group showed lower ratings at all time points.

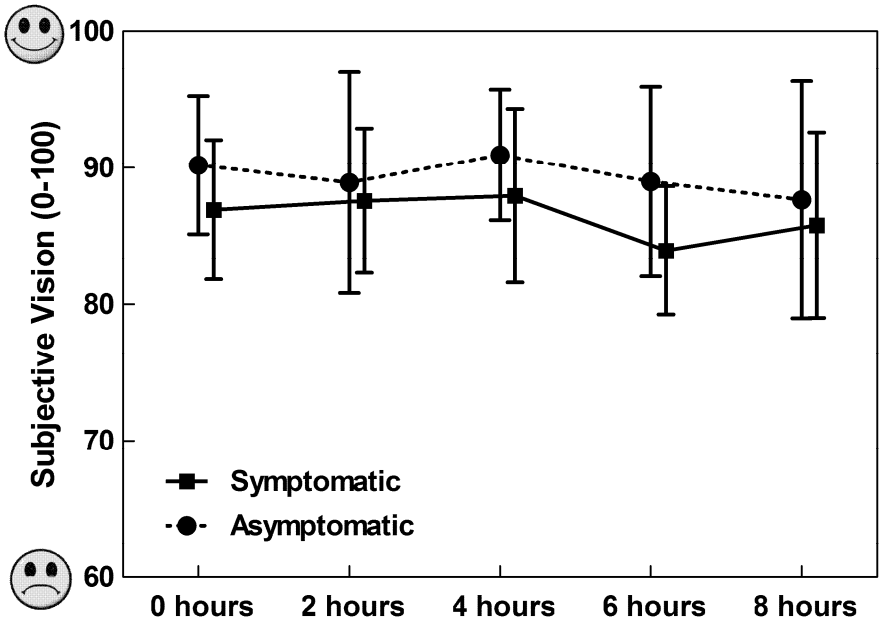

Figure 7-3: Subjective vision ratings over time in symptomatic and asymptomatic contact lens wearers.

Error bars represent Mean ± SD.

Figure 7.4 shows that there was no significant decrease in comfort over time in the asymptomatic group ($p>0.05$); however in the symptomatic group, the six and eight hour ratings were significantly lower than the baseline measurement ($p<0.05$). The symptomatic group had significantly lower comfort ratings than the asymptomatic group at the six and eight hour time points ($p<0.05$). However, there was no significant

difference between the two groups at other time points (all p>0.05), although the symptomatic group had lower comfort ratings at these times.

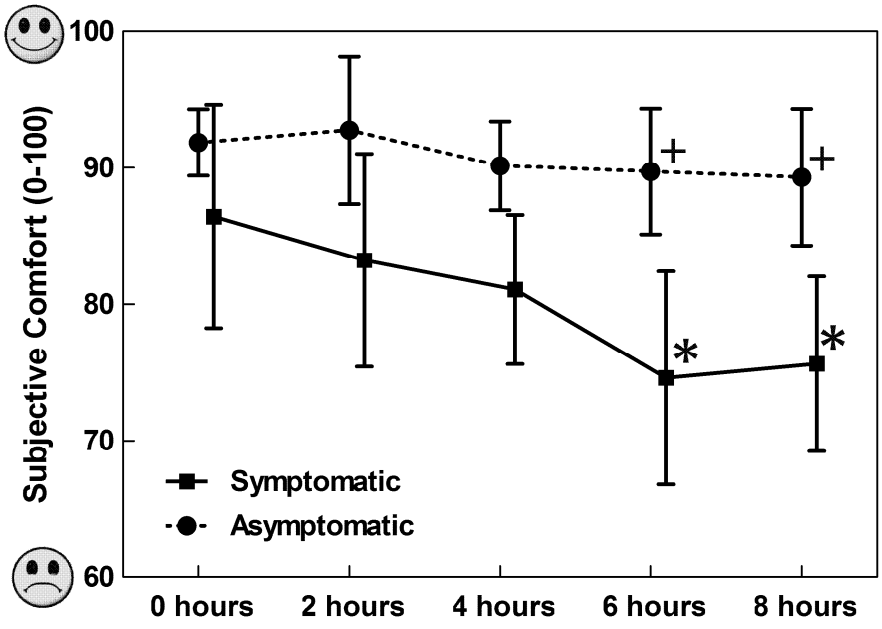

Figure 7-4: Subjective comfort ratings over time in symptomatic and asymptomatic contact lens wearers.

Error bars represent Mean ± SD. * represents p<0.05 within the same group from 0 hours; + represents p<0.05 between groups.

Figure 7.5 shows that there was no significant reduction in dryness ratings over time in the asymptomatic group (p>0.05); however in the symptomatic group, the six and eight hour ratings were significantly lower than the baseline measurement (p<0.05). The symptomatic group reported significantly more dryness than the asymptomatic group at the six and eight hour time points (p<0.05). However, there was no significant difference between the two groups at other time points (all p>0.05), although the symptomatic group had lower dryness ratings at these times.

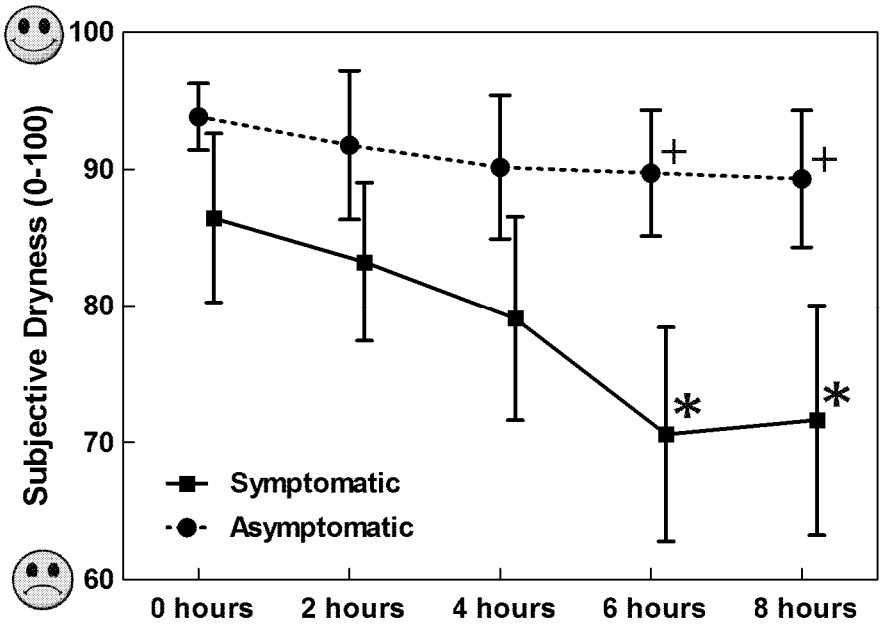

Figure 7-5: Subjective dryness ratings over time in symptomatic and asymptomatic contact lens wearers.

Error bars represent Mean ± SD. * represents $p<0.05$ within the same group from 0 hours; + represents $p<0.05$ between groups.

7.4.3 Analytical measurements

Table 7.1 shows the total protein, total lysozyme deposition and percentage active lysozyme recovered from the lenses at various time points in the asymptomatic and symptomatic participants. There was a gradual increase in total protein deposition and total lysozyme deposition on the lenses across the four time points ($p<0.05$) both in the asymptomatic and symptomatic group of participants. However there was no significant difference between the two groups at any time point ($p>0.05$). There was a gradual reduction in the activity of lysozyme deposited across the four time points, albeit it was not statistically significant ($p>0.05$). The percentage active lysozyme recovered from the

symptomatic wearers was lower at all time points, although this was not statistically significant (all $p>0.05$).

Table 7-1: Protein deposition on etafilcon lenses

Total protein, total lysozyme and percentage active lysozyme (mean±standard deviation) recovered from etafilcon contact lenses worn by asymptomatic and symptomatic subjects at four time points.

	Total protein (µg/lens)		Total lysozyme (µg/lens)		Percentage active lysozyme	
	Asymptomatic	**Symptomatic**	**Asymptomatic**	**Symptomatic**	**Asymptomatic**	**Symptomatic**
2 hours	147.87±43.41	183.89±55.04	123.23±36.18	141.45±42.34	99.15±1.06	97.95±1.96
4 hours	307.45±83.56	347.26±78.24	227.74±61.90	253.94±64.66	98.79±2.12	96.87±2.42
6 hours	371.78±79.57	417.66±86.93	288.21±61.68	321.28±66.87	98.19±3.06	95.98±2.62
8 hours	465.48±79.32	478.48±90.53	349.99±59.64	382.78±72.43	97.69±4.20	94.16±4.32

7.4.4 Correlations

Pearson's correlations between clinical signs and any of the protein deposition measures showed poor, insignificant correlations ($r<0.2$; $p>0.05$). Pearson's correlations between subjective symptoms and protein deposition showed poor correlations for total protein/ total lysozyme and any subjective factor ($r<0.3$; $p>0.05$) as shown in Tables 7.2 and 7.3, and only weak correlations between dryness and active lysozyme ($r=0.3$ to 0.4 for all time points; $p<0.05$) as shown in Table 7.4. However, stronger correlations were found between the active lysozyme and subjective comfort ($r=0.7$ to 0.8; $p<0.05$) as shown in Table 7.4.

Table 7-2: Correlation between total protein recovered from etafilcon contact lenses and various subjective symptoms at different time points.

	Comfort	Dryness	Subj. Vision
Total protein (2 hrs)	R = -0.16	R = -0.16	R = -0.25
Total protein (4 hrs)	R = -0.09	R = -0.29	R = -0.06
Total protein (6 hrs)	R = -0.09	R = -0.39	R = 0.11
Total protein (8 hrs)	R = 0.06	R = -0.32	R = 0.05

Table 7-3: Correlation between total lysozyme recovered from etafilcon contact lenses and various subjective symptoms at different time points.

	Comfort	Dryness	Subj. Vision
Total lysozyme (2 hrs)	R = -0.11	R = -0.13	R = 0.04
Total lysozyme (4 hrs)	R = -0.05	R = -0.35	R = 0.13
Total lysozyme (6 hrs)	R = -0.03	R = -0.12	R = 0.11
Total lysozyme (8 hrs)	R = 0.06	R = -0.23	R = 0.08

Table 7-4: Correlation between active lysozyme recovered from etafilcon contact lenses and various subjective symptoms at different time points.

* represents $p<0.05$.

	Comfort	Dryness	Subj. Vision
Active lysozyme (2 hrs)	R = 0.77 *	R = 0.37 *	R = 0.08
Active lysozyme (4 hrs)	R = 0.80 *	R = 0.36 *	R = 0.05
Active lysozyme (6 hrs)	R = 0.71 *	R = 0.47 *	R = 0.13
Active lysozyme (8 hrs)	R = 0.72 *	R = 0.31	R = 0.35

7.5 Discussion

To date, this is the only study that reports on the relationship between clinical signs, subjective symptoms and the conformational state of the lysozyme deposited on contact lenses. These results clearly suggest that there is a good correlation between lysozyme activity recovered from contact lenses and subjective comfort, even over short periods of lens wear. Previous studies have shown that tolerant contact lens wearers have fewer symptoms of discomfort and a more stable tear film (as measured by a higher maximum forced interval between blinks, tear meniscus height and volume and non-invasive tear film break-up time). [70] In the past, studies have also shown that the tear film of tolerant lens wearers showed lower levels and activity of secretory phospholipase A2, lower concentration of lipocalin and lower levels of peroxidised lipids. [47] It has also been shown that in the absence of lens wear, there were no differences between tolerant and intolerant lens wearers in conjunctival or limbal redness, lipid layer appearance, tear flow rate, tear film osmolality, and total protein, lactoferrin, lysozyme or secretory Immunoglobulin A concentrations in the tear film. [70]

Figure 7.1 shows that there was a significant reduction in the NITBUT after eight hours of lens wear in both symptomatic and asymptomatic contact lens wearers, but there was no significant difference between the two groups at any time point. When a contact lens is inserted into the eye, the tear film is disturbed and tear film break-up time reduces significantly. [71, 72] Previous studies have shown that the NITBUT of soft contact lens wearers to be in the range of 3 – 10 seconds, [59, 73, 74] which is similar to that found in the current study. A study by Guillon and colleagues showed that there was no difference in the tear film stability between asymptomatic and symptomatic contact lens wearers, although they found a significant difference between asymptomatic and symptomatic non-contact lens wearers. [72] However, another study by Glasson and colleagues showed that contact lens wear affected the stability of the tear film in tolerant contact lens wearers more than in intolerant contact lens wearers. [75] In their study, NITBUT decreased more dramatically in the tolerant contact lens wear group and it was also shown that the NITBUT of intolerant subjects was significantly lower initially before lens wear and remained low over 6 hours of lens wear. Another study by Fonn and colleagues also demonstrated a statistically significant decrease in pre-lens TBUT in symptomatic wearers during a 5 hour period, regardless of soft lens type, compared to no significant change in asymptomatic subjects. [20]

Figure 7.2 demonstrates that there was no significant reduction in the *in vivo* wettability of etafilcon contact lens materials over eight hours of wear in both symptomatic and asymptomatic contact lens wearers. Although high levels of total protein and total lysozyme deposited on etafilcon lens materials within a few hours of

wear (Table 7.1), it is quite clear that these deposits do not modify the measured *in vivo* wettability of these lenses to any appreciable extent. In the past, studies which determined the wettability of etafilcon lens materials over short periods of wear using indirect methods such as PLNITBUT also showed similar findings. [76] Previous *in vitro* and *ex vivo* studies which determined the influence of tear proteins on wettability of etafilcon lens materials also found that these deposits do not reduce the wettability of etafilcon lens materials over short periods of wear or over short periods of *in vitro* incubation. [77-79]

As expected, symptomatic contact lens wearers in this study showed a significant reduction in subjective comfort and dryness ratings over eight hours of wear, while the ratings of asymptomatic wearers remained relatively constant (Figures 7.4 and 7.5). These results are consistent with those from previous studies where symptomatic wearers showed a decrease in comfort and dryness ratings using visual analog scales over time. [2, 20, 70]

It is clear from Table 7.1 that etafilcon lenses attracted substantial quantities of total protein and total lysozyme even after 8 hours of lens wear in both the symptomatic and asymptomatic participants. This finding is in accordance with other previous *in vitro* and *ex vivo* studies that evaluated protein and lysozyme deposition on etafilcon contact lenses. [30, 36, 39-42, 80-84 31, 85] The increased affinity of lysozyme to the etafilcon material occurs because methacrylic acid imparts a negative charge to the material and thus

thermodynamically favors the deposition of lysozyme, which is a positively charged species at physiological pH.

After eight hours of wear, the percentage active lysozyme recovered from etafilcon lenses worn by symptomatic and asymptomatic lens wearers was over 94% (Table 7.1), which is significantly higher than those seen from novel silicone hydrogel lens materials. [41, 42, 69, 86-88] Previous *ex vivo* [41, 42] and *in vitro* [86, 87] studies have also demonstrated that the percentage active lysozyme recovered from conventional hydrogel FDA group IV etafilcon lens materials is significantly higher than those seen in novel silicone hydrogel and other groups of conventional hydrogel lens materials. Denaturation of a protein on any polymeric surface is dependent on several factors, including contact time of the protein with the substrate, chemical composition of the substrate, surrounding pH, type of protein, temperature of the surrounding medium and also the location of the protein in a polymer. [28, 67, 80, 89-92] Using confocal microscopy, it has recently been shown that lysozyme is primarily located within the bulk of etafilcon lens materials, with relatively little surface-located lysozyme [93, 94] resulting in significantly increased levels of lysozyme remaining active.

Although this study did not find a significant correlation between clinical signs & symptoms versus total protein and total lysozyme deposited on the lens materials (Tables 7.2 and 7.3), we were able to demonstrate a significant correlation between the activity of the lysozyme recovered from the lens materials and subjective comfort and dryness (Table 7.4). A previous study by Lever and colleagues which investigated the relationship

between total protein deposition and patient-rated lens comfort, found that there was no statistical correlation between these two factors. [62] This was the only study which attempted to determine the relationship between protein deposition on contact lenses and subjective comfort by quantifying the total protein deposited on lenses using biochemical techniques; [62] other studies estimated the relationship by evaluating the visible deposition/video image analysis of deposits on the lenses. [4, 63-66] Most studies that employed visible deposition/video image analysis showed that there was an association between visible deposition and comfort, [4, 64-66] however, one study that surveyed fifty comfortable and uncomfortable contact lens wearers did not show a difference in the amount of visible deposition on the lenses between the two groups. [63] Despite the fact there is no significant association between total protein deposited on contact lenses and subjective symptoms, it is extremely important to recognize that these findings do not eliminate total protein deposits as an important factor in contact lens wear. Indeed, protein deposition has a significant potential to cause problems as these deposits do play a significant role in modulating microbial adherence to lens materials. [95-98] Therefore, it is important that practitioners advise their patients regarding the importance of lens disinfection and cleaning and appropriate lens replacement schedules.

This study is the first to demonstrate that a significant correlation exists between subjective comfort and active lysozyme recovered from etafilcon lens materials, even over very short periods of wear. However, these results should be interpreted with caution as it would be erroneous to conclude that denatured lysozyme on contact lenses are solely responsible for the symptoms experienced by symptomatic contact lens

wearers. Rather they should be interpreted as lysozyme deposited on the contact lenses of symptomatic lens wearers tend to denature more than that seen in asymptomatic lens wearers. This is likely to happen because of the biochemical changes that occur in the tear film of symptomatic lens wearers, resulting in altered properties of the lens material, potentially leading to a change in the conformational state of the deposited lysozyme. Therefore, in addition to the other factors mentioned earlier, the conformational state of the deposited protein, inflammatory and sub-inflammatory mediators and the secretomotor response of the lacrimal system could also be significant factors in contact lens-induced dry eye, reiterating that this condition is multifactorial.

7.6 Conclusions

In conclusion, the results from this study suggests that even over a short period of wear, a significant correlation exists between subjective symptoms of comfort and dryness and the activity of lysozyme recovered from etafilcon contact lenses, with little correlation being shown with total amounts of either total protein or total lysozyme. Therefore, in addition to investigating the total quantity of the deposited protein, it is of significant clinical relevance to study the conformational state of the deposited protein. These results have tremendous implications, in that the novel contact lens materials that are being developed should possess properties that can retain the activity of the deposited protein, in addition to being deposit-resistant. Care regimens and multipurpose solutions should be capable of removing denatured proteins that are deposited on the lens materials.

Further work is required to determine if this important clinical finding is transferable to those patients who use silicone hydrogel lens materials. It would also be of interest to determine if there is a difference in the activity of lysozyme recovered from the tears of symptomatic and asymptomatic contact lens wearers. In addition to determining the activity of lysozyme, it would also be of interest to determine the activity of lactoferrin which is the other major tear protein found on contact lenses.

The next chapter of this thesis will determine the influence of individual tear proteins (lysozyme, lactoferrin and albumin) on the adhesion of Gram positive and Gram negative bacteria to conventional and silicone hydrogel contact lens materials.

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8 Influence of protein deposition on bacterial adhesion to contact lens materials

The following manuscript is currently under preparation:

Lakshman N Subbaraman**,** Lyndon Jones, Roya Borazjani, Hua Zhu, Zhenjun Zhao, Mark DP Willcox. Influence of protein deposition on bacterial adhesion to conventional and silicone hydrogel contact lens materials.

8.1 Abstract

Purpose: To determine the adhesion of Gram positive and Gram negative bacteria onto conventional hydrogel (CH) and silicone hydrogel (SH) contact lens materials with and without lysozyme, lactoferrin and albumin coating.

Methods: Four lens types (three SH – balafilcon A, lotrafilcon B & senofilcon A; one CH - etafilcon A) were coated with lysozyme, lactoferrin and albumin; uncoated lenses acted as controls. Uncoated and protein-coated contact lens samples were incubated in 1ml of bacterial suspension of *Staphylococcus aureus* (Saur 31) and two strains of *Pseudomonas aeruginosa* (Paer 6294 & 6206) for 24 hours at 37° C with PBS as the medium. The total counts of the adhered bacteria were determined using the ^{3}H-Thymidine method and the viable counts of the adhered bacteria were assayed by counting the number of colony-forming units on agar media.

Results: All three tested strains adhered at significantly lower levels to uncoated etafilcon A lenses when compared to uncoated SH lens materials ($p<0.05$). There was no significant difference between the total and viable counts ($p>0.05$) of all the three strains that were bound to uncoated lenses.

Effect of lysozyme coating: All four lens types with lysozyme-coating showed significantly increased binding (total and viable counts) of Saur 31 when compared to uncoated lenses ($p<0.05$). However, lysozyme-coating on all four lens materials did not have a significant influence on the adhesion (total and viable counts) of Paer 6206 and 6294 strains ($p>0.05$). There was no significant difference between the total and viable

counts ($p>0.05$) for all the three strains of bacteria which were bound to coated and uncoated lens materials.

Effect of lactoferrin coating: All four lens types with lactoferrin-coating showed significantly increased binding (total and viable counts) of Saur 31 when compared to uncoated lenses ($p<0.05$). There was no significant difference between the total and viable counts ($p>0.05$) of Saur 31 bound to uncoated and coated lenses. When compared to uncoated lenses, all four lactoferrin-coated lens types showed significantly higher total counts ($p<0.05$) of adhered Paer strains, while the lactoferrin-coated lenses showed significantly lower viable counts ($p<0.05$) of adhered Paer strains. There was a significant difference between the total and viable counts ($p<0.05$) that were bound to lactoferrin-coated lenses.

Effect of albumin coating: All four lens types with albumin-coating showed significantly increased binding (total and viable counts) of Saur 31, Paer 6206 and 6294 strains when compared to uncoated lenses ($p<0.05$). There was no significant difference between the total and viable counts ($p>0.05$) of all the three strains bound to uncoated and albumin-coated lenses.

Conclusions: These results demonstrate that uncoated SH lens materials bind more Gram positive and Gram negative bacteria than uncoated CH lens materials. These results also suggest that lysozyme deposited on contact lens materials does not possess antibacterial activity against certain bacterial strains, while lactoferrin possess an antibacterial effect against certain Gram negative strains tested in this study.

Key words: contact lens; deposition; *Pseudomonas*; silicone hydrogel; *Staphylococcus*.

8.2 Introduction

Contact lens-induced corneal adverse responses have been conveniently classified into:

a) serious sight threatening responses - microbial keratitis (MK)
b) significant adverse responses - contact lens acute red eye (CLARE), contact lens peripheral ulcers (CLPU) and infiltrative keratitis (IK)
c) non-significant adverse responses - asymptomatic infiltrative keratitis (AIK) and asymptomatic infiltrates (AI) [1]

Of these adverse responses, bacterial colonization of contact lenses is one of the initiating factors in MK, [2] CLARE, [3, 4] CLPU [5] and certain IK & AIK events. [6] Bacterial adhesion to the contact lens material is the first step in a series of events that leads to contact lens-related infections or inflammation. [7, 8] The colonization of bacteria on the contact lens allows the bacteria to multiply, which increases the contact time of the pathogen with the ocular surface, facilitating transfer of bacteria on to the cornea, thereby increasing the risk for infection. [8, 9]

The tear film is composed of several proteins including lysozyme, lactoferrin, & immunoglobulin A and polymorphonuclear leukocytes, which have an antibacterial and/ or bacteriostatic role. [10-12] In addition, mechanical defenses such as blinking and tear flow assist the eye in eliminating bacteria from the ocular surface. [10] The presence of a contact lens disrupts most, if not all, of these functions of the tear film, [13] and the contact lens provides a surface to which bacteria attach, further assisting bacteria in evading the

defense mechanisms of the eye. During contact lens wear, a protein-rich coating, or conditioning film, forms on the contact lens surface. [14-17] It has recently been shown that over 60 proteins are deposited onto contact lens materials and some of the major sorbed tear film components include lysozyme, lactoferrin and albumin. [18, 19] The exact composition of the conditioning film on contact lens materials is affected by several factors, including water content, [20-25] hydrophobicity, [22, 25-28] charge, [20, 23, 29, 30] pore size [20, 31] and surface roughness [27] of the lens material.

To date, several studies have investigated the ability of various bacteria to adhere to contact lens materials and these studies have shown that adhesion is dependent upon a variety of factors including lens material, [9, 32-38] tear film deposits, [9, 39-48] duration of wear, [9, 46, 49] and surface roughness. [50, 51] Most previous studies have determined the influence of deposits on bacterial adhesion only on conventional hydrogel contact lens materials. [9, 39-48] Albumin coated onto the surface of etafilcon A or polymacon contact lenses increased the adhesion of *Pseudomonas aeruginosa.* [44] Similarly, some strains of *Serratia marcescens* adhered more to etafilcon A lenses coated in an artificial tear fluid. [52] Another study showed that lysozyme adsorbed to etafilcon A contact lenses increases the adhesion of *Staphylococcus aureus* to lenses. [42] It has also been demonstrated that lactoferrin deposited on the lens surface promotes the adhesion of *Pseudomonas aeruginosa* strain Paer 1; nevertheless, once adherent, this protein reduces the proportion of viable bacteria on the lens surface. [43] To-date, few studies have investigated the adhesion of bacteria to novel silicone hydrogel lens materials; [33, 34, 36, 37] and none of these

studies have determined the influence of tear components on the adhesion of bacteria to silicone hydrogel lens materials.

Thus, the purpose of this study was to examine the effect of the contact lens conditioning film on bacterial adhesion by comparing the adhesion of three different bacterial strains to uncoated contact lenses and to contact lenses that are coated with individual major tear proteins (lysozyme, lactoferrin and albumin). The other purpose of the study was to determine whether the presence of these individual tear proteins had any effect on the viability of the attached bacteria.

8.3 Materials and Methods

8.3.1 Contact lenses

The silicone hydrogel lens materials examined in this study were lotrafilcon B (O_2Optix; CIBA Vision, Duluth, GA, USA), balafilcon A (PureVision™; Bausch & Lomb, Rochester, NY, USA) and senofilcon A (Acuvue® OASYS™; Vistakon, Johnson & Johnson, Jacksonville, FL, USA). The conventional hydrogel lens material examined was etafilcon A (Acuvue 2; Vistakon, Johnson & Johnson, Jacksonville, FL, USA). The properties of these lens materials are described in Table 8.1.

8.3.1.1 Treatment of uncoated lens materials

Three test and control lenses from each lens type were used for adhesion assays. Contact lenses were removed from their packaging and thoroughly rinsed with PBS to ensure that no packaging solution remained on the lens surface. In order to achieve this, lenses were removed from the packaging solution using forceps and were placed in vials containing PBS with the lenses' concave side up for one hour. The vials were then placed on a plate shaker for two minutes and this process was performed a total of three times with fresh PBS each time. These lenses were the "uncoated lenses" which acted as controls.

Table 8-1: Properties of conventional and silicone hydrogel lens materials evaluated in the study

Proprietary name	Silicone Hydrogel			Conventional hydrogel
	O_2 Optix™	**PureVision™**	**Acuvue® OASYS™**	**Acuvue® 2**
United States Adopted Name (USAN)	Lotrafilcon B	Balafilcon A	Senofilcon A	Etafilcon A
Manufacturer	CIBA Vision	Bausch & Lomb	Johnson & Johnson	Johnson & Johnson
Water content (%)	33	36	38	58
Oxygen Transmissibility (Dk/t) at 35˚C	138	101	147	20
FDA group	I	III	I	IV
Surface Treatment	25nm plasma coating with high refractive index	Plasma oxidation process	None. Internal wetting agent	None
Principal monomers	DMA+TRIS+ Siloxane monomer	NVP+TPVC+NVA+ PBVC	mPDMS+ DMA+ polyHEMA+siloxane macromer+ PVP+ TEGDMA	polyHEMA+MA

DMA (N,N-dimethylacrylamide); MA, methacrylic acid; mPDMS (monofunctional polydimethylsiloxane); NVA (N-vinyl amino acid); NVP (N-vinyl pyrrolidone); polyHEMA (poly-2-hydroxyethyl methacrylate); PBVC (poly[dimethysiloxy] di [silylbutanol] bis[vinyl carbamate]); PVP (polyvinyl pyrrolidone); TEGDMA (tetraethyleneglycol dimethacrylate); TPVC (tris-(trimethylsiloxysilyl) propylvinyl carbamate); TRIS (trimethylsiloxy silane)

8.3.1.2 Protein coating on contact lenses

Three lenses from each lens type were coated with three different tear proteins: lysozyme, lactoferrin and albumin (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia). The tear proteins were coated onto the lenses such that their final concentration was similar to that found on worn contact lenses. In order to achieve this, lysozyme, lactoferrin and albumin solutions were prepared at a concentration of 1.9mg/ml, 1.9mg/ml and 0.5mg/ml respectively. The conventional hydrogel lens material was incubated in the lysozyme solution for 5 days, while the silicone hydrogel lens materials were incubated for 7 days. All the lenses were incubated in lactoferrin and albumin solutions for 7 days. After the specified incubation periods, the lenses were removed from the vials and washed in a plate shaker with PBS to remove loosely-bound protein. These lenses were the "protein-coated" lenses.

8.3.2 Bacterial preparation

Three bacterial strains were investigated in this study, of which one was a Gram positive strain (*Staphyloccus aureus* 31; Saur 31) and two were Gram negative strains (*Pseudomonas aeruginosa* 6206 and 6294; Paer 6206 and 6294). The Gram positive Saur 31 was isolated from a patient with contact lens induced peripheral ulcer at the Institute for Eye Research, Sydney, Australia. Both strains of Paer used in this study (6294 and 6206) were isolated from human MK specimens and were obtained from the School of Optometry, University of California, Berkeley, USA. Strain 6206 has been shown to be a cytotoxic strain, while strain 6294 is an invasive strain. [53]

Stock cultures of Saur 31, Paer 6206 and Paer 6294 were stored in 30% glycerol at -80º C. Bacteria were grown overnight in 10 ml of Tryptone Soy Broth (TSB; Oxoid, Sydney, Australia) at 37º C for 18 hours. Bacterial cells were harvested by centrifugation (Eppendorf 5810, Hamburg, Germany) for 10 minutes (3000 rpm at 18^0C) and washed in sterile phosphate buffered saline, pH 7.4 (PBS). The cells were then resuspended in sterile PBS and the concentration was adjusted using a spectrophotometer (Heliosβ, Unicam Instruments, Cambridge, UK) to give an optical density at 660nm of 0.1, which gave $1.0X10^8$ colony forming units (cfu)/ml. The suspension was serially diluted (1:10) to obtain the required concentration.

8.3.3 Determination of viable counts

The uncoated and protein-coated lenses were then placed in a 24 well plate containing 1 ml of bacterial suspension in each well and incubated at 37ºC for 24 hours. After 24 hours of incubation the lenses were removed from the incubator and shaken for 30 seconds on a plate shaker at 175 rpm to remove loosely adherent bacteria. The lenses were then washed three times in 1 ml of sterile PBS for 30 seconds, transferred to a sterile 5 ml plastic container (Greiner Bio-One Inc, Longwood, FL, USA) and vortexed vigorously for one minute in 2 ml of sterile PBS, using sterile magnetic stirring bars. For quantitation of viable bacteria per contact lens, the homogenate was serially diluted in PBS 1:10 by taking 100µl and adding it to 900µl of deneutralizing broth (Difco Laboratories Inc., MI, USA) in a micro-centrifuge tube. 50 µl of the serially diluted samples were plated in triplicate on nutrient agar plates (Oxoid, Sydney, Australia) and incubated for 18 hours at 37ºC. Dilutions with growth between 10-100 colonies were

counted on a colony counter. The number of colonies per dilution were recorded and used to calculate the number of colony forming units (cfu) per contact lens. The assay was repeated twice and the average values were computed to enumerate the viable counts of the bacteria adhered to contact lenses.

8.3.4 Determination of total counts

The total counts were determined using the ^{3}H-Thymidine method. Stock cultures of Saur 31, Paer 6206 and 6294 were grown in of 10mL Tryptone Soy Broth (TSB; Oxoid, Sydney, Australia) and ^{3}H-Thymidine (80Cui) for 18 hours. Bacterial cells were harvested by centrifugation (Eppendorf 5810, Hamburg, Germany) for 15 minutes (3000 rpm at 18^{0}C) and washed in sterile phosphate buffered saline, pH 7.4 (PBS). This was repeated a total of three times. The cells were then resuspended in sterile PBS and 1 Cui/ml of ^{3}H-Thymidine and the concentration was adjusted using a spectrophotometer (Heliosβ, Unicam Instruments, Cambridge, UK) to give an optical density at 660nm of 0.1. The protein-coated and uncoated lenses were then placed in a 24 well plate containing 1 ml of radiolabeled-bacterial suspension in each well and incubated at 37ºC for 24 hours. After 24 hours of incubation the lenses were removed from the incubator and shaken for 30 seconds on a plate shaker at 175 rpm to remove loosely adherent bacteria. The lenses were then washed three times in 1 ml of sterile PBS for 30 seconds. The vials were then placed with 500µL 0.2M NaOH at 80°C for 1 hour, following which they were allowed to cool down by placing them on ice. Scintillation fluid was then added to the vials and the counts per minute were determined using a Beta counter. A

standard curve was plotted to determine the (cfu)/lens values of total counts of adhered bacteria.

8.3.5 Statistical Analysis

Statistical analysis was conducted using Statistica 7 software (StatSoft Inc, OK, USA). All data are reported as mean ± SD. Repeated Measures Analysis of Variance was used to determine statistically significant differences. Post-hoc multiple comparison testing was undertaken using the Tukey-HSD test. In all cases, a p value of <0.05 was considered significant.

8.4 Results

8.4.1 Bacterial adhesion to uncoated lens materials

Figures 8.1, 8.2 and 8.3 show the adhesion of Gram positive Saur 31, Gram negative Paer 6206 and Paer 6294 strains respectively. All three tested strains adhered at a significantly lower level to uncoated etafilcon A lenses when compared to uncoated SH lens materials ($p<0.05$). Among the silicone hydrogels, the adhesion was highest on balafilcon A and lotrafilcon B lens materials, when compared to the senofilcon A material ($p<0.05$). Figures 8.1 to 8.3 show that there was no significant difference between the total and viable counts ($p>0.05$) of all the three strains that were bound to uncoated lenses.

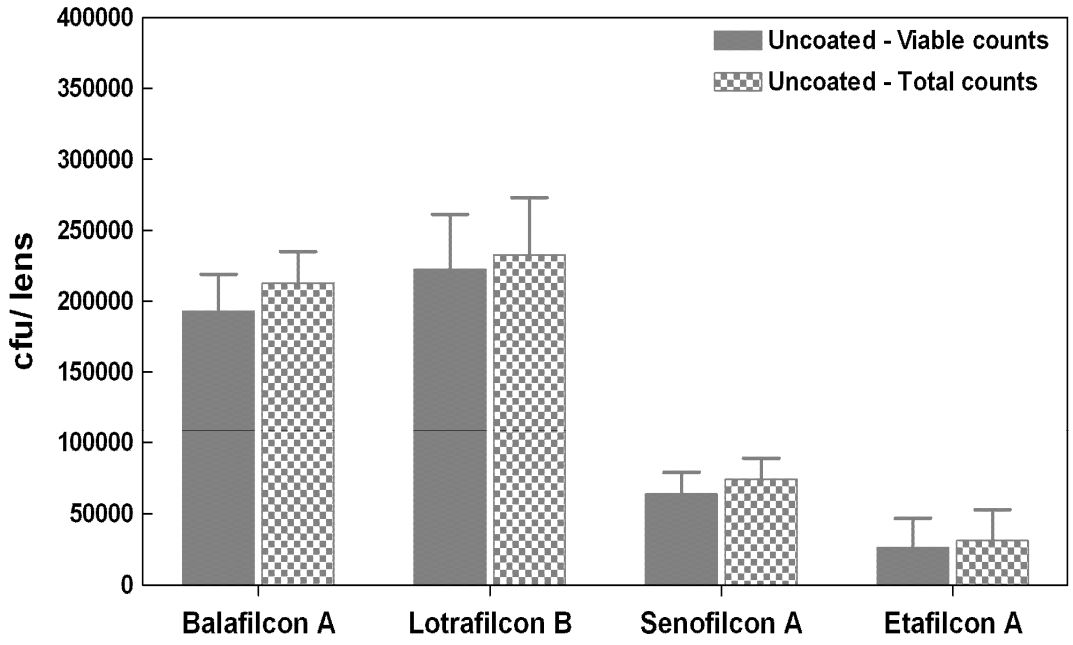

Figure 8-1: Adhesion of Gram positive Saur 31 to uncoated contact lens materials

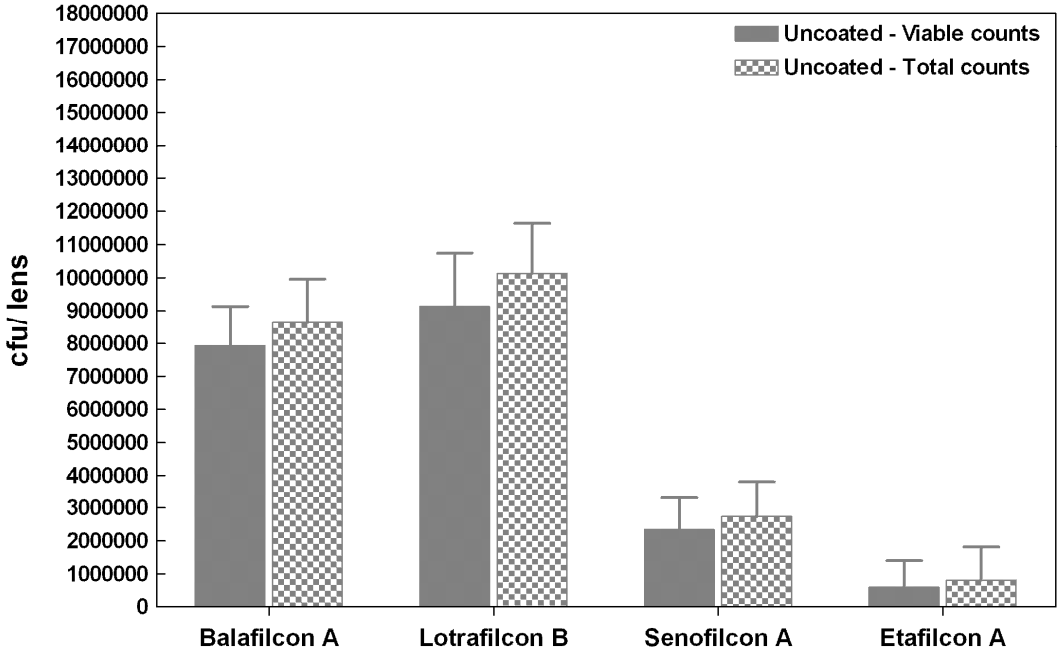

Figure 8-2: Adhesion of Gram negative Paer 6206 to uncoated contact lens materials

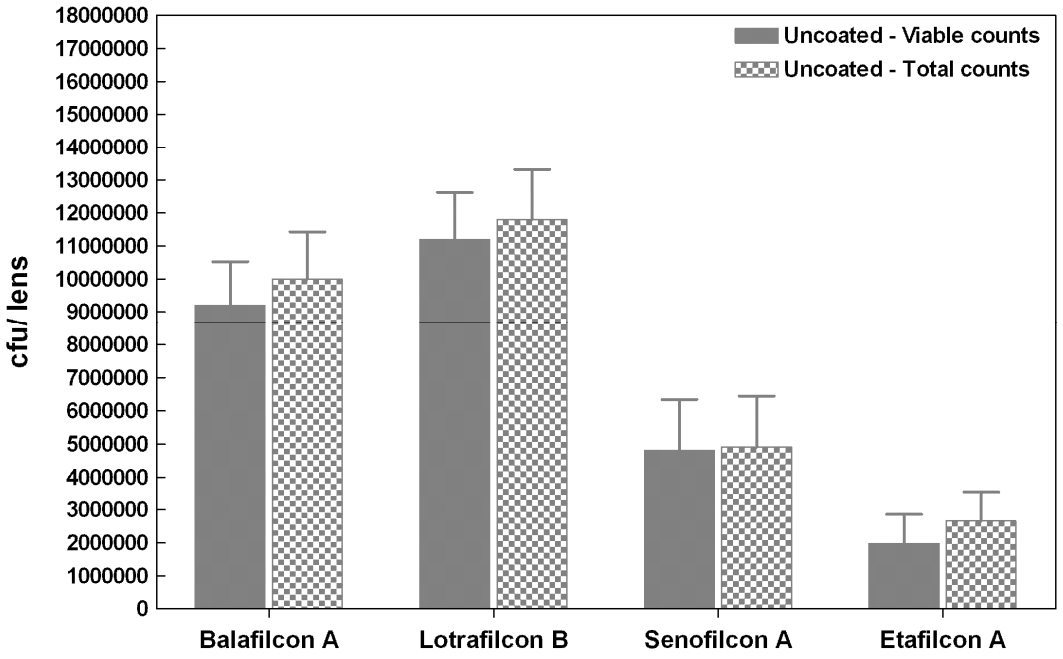

Figure 8-3: Adhesion of Gram negative Paer 6294 to uncoated contact lens materials

8.4.2 Effect of lysozyme-coating on bacterial adhesion

Figure 8.4 shows the adhesion of Gram positive Saur 31 to uncoated and lysozyme-coated contact lens materials. There was no significant difference between the viable and total counts for uncoated lenses ($p>0.05$ for all lens types) and no significant difference between the viable and total counts for lysozyme-coated lenses ($p>0.05$ for all lens types). When compared to uncoated lenses, there was a significant increase in the viable and total counts of Saur 31 adhered to the lysozyme-coated lenses for all lens types ($p<0.05$ for all lens types). Between the lysozyme-coated lens materials, there was no significant difference in Saur 31 adhesion between balafilcon A and lotrafilcon B ($p>0.05$) and no significant difference in Saur 31 adhesion between senofilcon A and

etafilcon A lens materials ($p>0.05$). Lysozyme-coated senofilcon A and etafilcon A lens materials showed significantly lower Saur 31 binding than lysozyme-coated balafilcon A and lotrafilcon B lens materials ($p<0.05$).

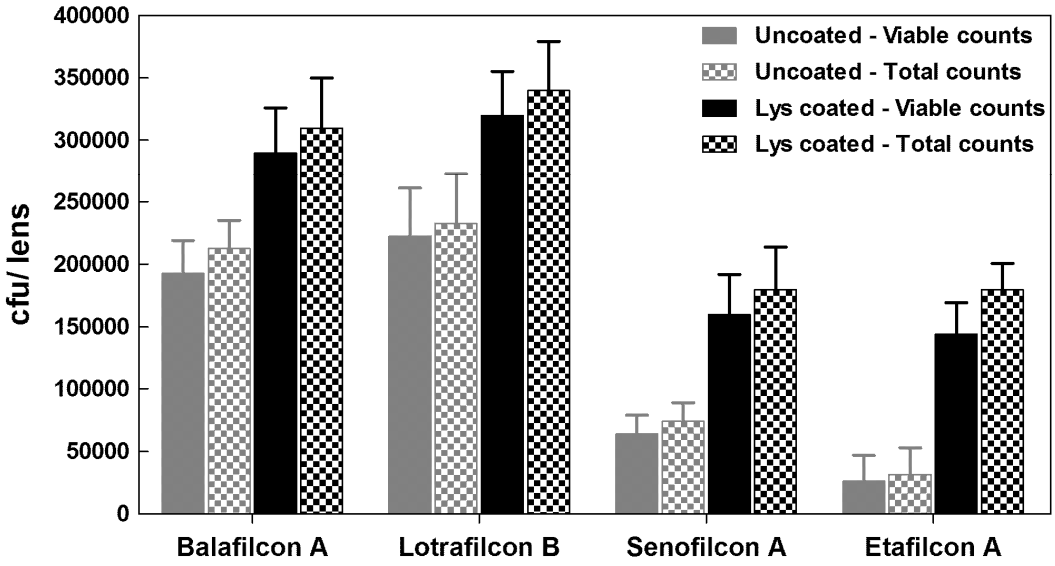

Figure 8-4: Adhesion of Gram positive Saur 31 to uncoated and lysozyme-coated contact lens materials

Figures 8.5 and 8.6 show the adhesion of Gram negative Paer 6206 and 6294 strains to uncoated and lysozyme-coated contact lens materials. There was no significant difference between the viable and total counts for uncoated lenses ($p>0.05$ for all lens types) and no significant difference between the viable and total counts for lysozyme-coated lenses ($p>0.05$ for all lens types). When compared to uncoated lenses, there was no significant increase in the viable and total counts of Paer 6206 and 6294 strains adhered to lysozyme-coated lenses for all lens types ($p<0.05$ for all lens types). Between

the lysozyme-coated lens materials, there was no significant difference in Paer 6206 and 6294 adhesion between balafilcon A and lotrafilcon B ($p>0.05$) and etafilcon A lens materials showed the lowest adhesion of Paer 6206 and 6294 strains. Lysozyme-coated senofilcon A showed significantly lower binding of Paer strains than lysozyme-coated balafilcon A and lotrafilcon B lens materials ($p<0.05$), but higher binding than etafilcon A lens material ($p<0.05$).

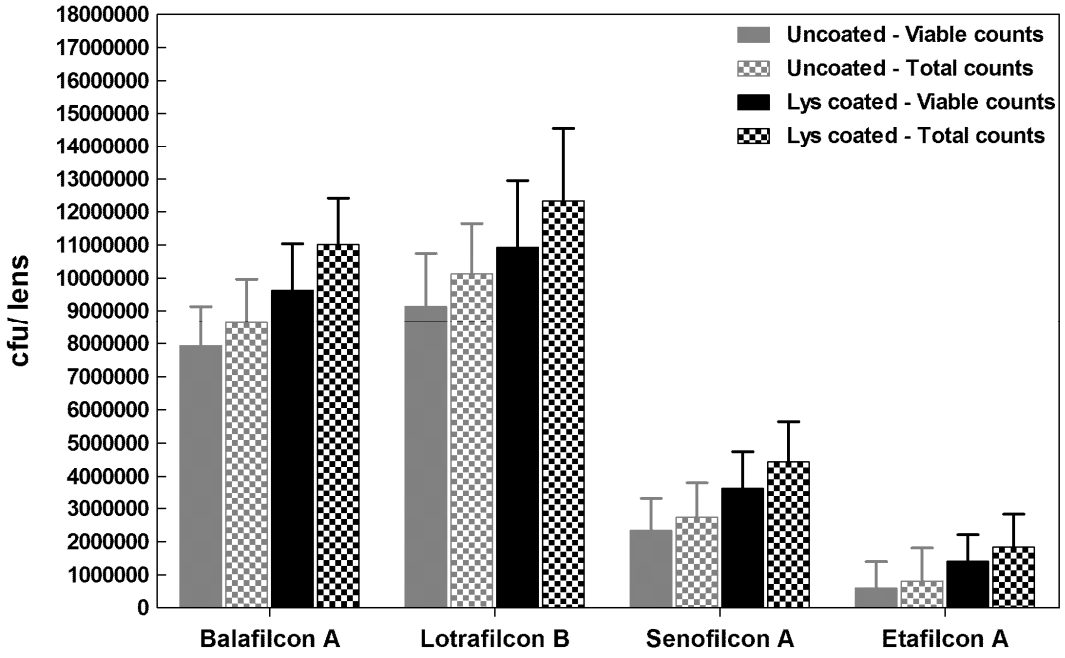

Figure 8-5: Adhesion of Gram negative Paer 6206 to uncoated and lysozyme-coated contact lens materials

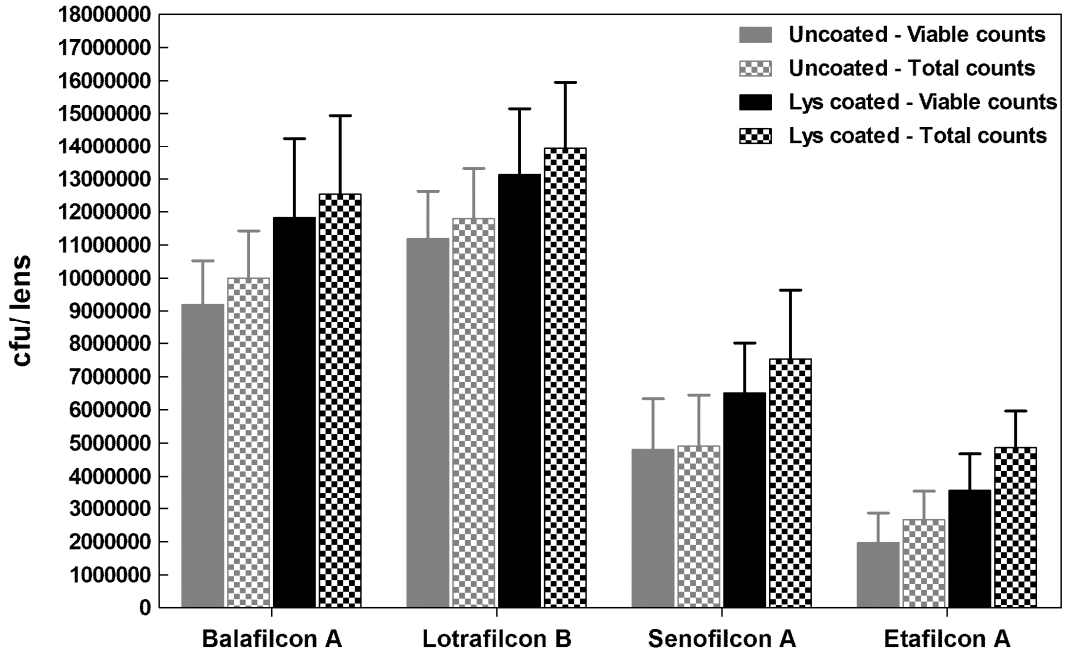

Figure 8-6: Adhesion of Gram negative Paer 6294 to uncoated and lysozyme-coated contact lens materials

8.4.3 Effect of lactoferrin-coating on bacterial adhesion

Figure 8.7 shows the adhesion of Gram positive Saur 31 to uncoated and lactoferrin-coated contact lens materials. There was no significant difference between the viable and total counts for uncoated lenses ($p>0.05$ for all lens types) and no significant difference between the viable and total counts for lactoferrin-coated lenses ($p>0.05$ for all lens types). When compared to uncoated lenses, there was a significant increase in the viable and total counts of Saur 31 adhered to the lactoferrin-coated lenses for all lens types ($p<0.05$ for all lens types). Between the lactoferrin-coated lens materials, there was no significant difference in Saur 31 adhesion between balafilcon A and lotrafilcon B ($p>0.05$) and lactoferrin-coated etafilcon A lens material showed the least adhesion of Saur 31. Lactoferrin-coated senofilcon A showed significantly lower binding of Saur 31

than lactoferrin-coated balafilcon A and lotrafilcon B lens materials ($p<0.05$), but higher binding than etafilcon A lens material ($p<0.05$).

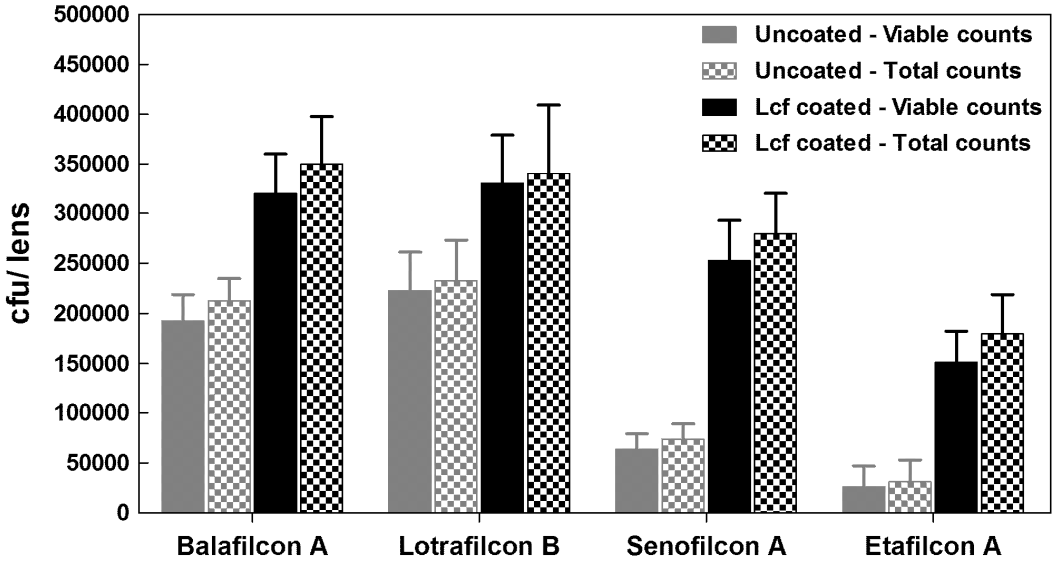

Figure 8-7: Adhesion of Gram positive Saur 31 to uncoated and lactoferrin-coated contact lens materials

Figures 8.8 and 8.9 show the adhesion of Gram negative Paer 6206 and 6294 strains to uncoated and lactoferrin-coated contact lens materials. There was no significant difference between the total and viable counts ($p>0.05$) of Paer strains that were bound to uncoated lenses, whereas there was a significant difference between the total and viable counts ($p<0.05$) that were bound to lactoferrin-coated lenses. When compared to uncoated lenses, all four lactoferrin-coated lens types showed significantly higher total counts ($p<0.05$) of adhered Paer strains, while the lactoferrin-coated lenses showed significantly lower viable counts ($p<0.05$) of adhered Paer strains.

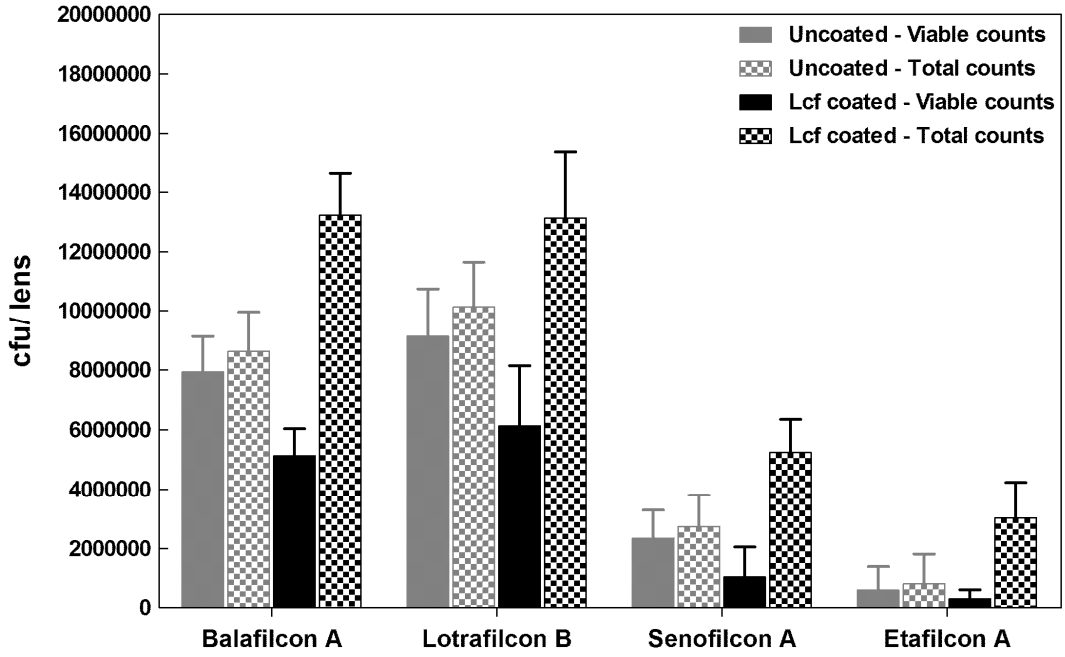

Figure 8-8: Adhesion of Gram negative Paer 6206 to uncoated and lactoferrin-coated contact lens materials

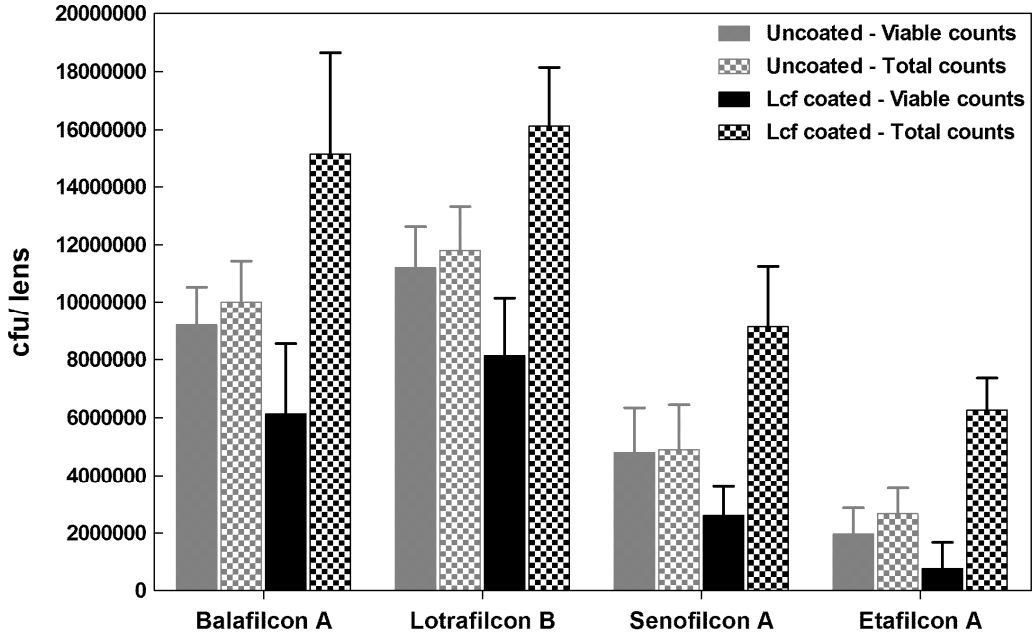

Figure 8-9: Adhesion of Gram negative Paer 6294 to uncoated and lactoferrin-coated contact lens materials

8.4.4 Effect of albumin-coating on bacterial adhesion

Figure 8.10 shows the adhesion of Gram positive Saur 31 to uncoated and albumin-coated contact lens materials. There was no significant difference between the viable and total counts for uncoated lenses ($p>0.05$ for all lens types) and no significant difference between the viable and total counts for albumin-coated lenses ($p>0.05$ for all lens types). When compared to uncoated lenses, there was a significant increase in the viable and total counts of Saur 31 adhered to the albumin-coated lenses for all lens types ($p<0.05$ for all lens types). Between the albumin-coated lens materials, there was no significant difference in Saur 31 adhesion between balafilcon A and lotrafilcon B ($p>0.05$) and no significant difference in Saur 31 adhesion between senofilcon A and etafilcon A lens materials ($p>0.05$). Albumin-coated senofilcon A and etafilcon A lens materials showed significantly lower Saur 31 binding than albumin-coated balafilcon A and lotrafilcon B lens materials ($p<0.05$).

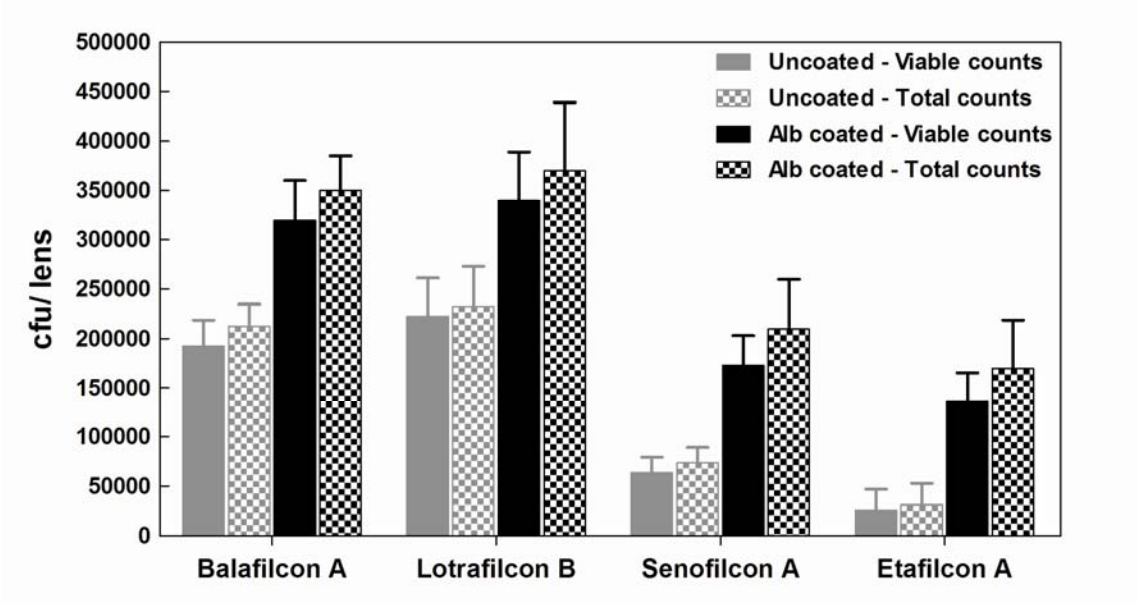

Figure 8-10: Adhesion of Gram positive Saur 31 to uncoated and albumin-coated contact lens materials

Figures 8.11 and 8.12 show the adhesion of Gram negative Paer 6206 and 6294 strains to uncoated and albumin-coated contact lens materials. There was no significant difference between the viable and total counts for uncoated lenses ($p>0.05$ for all lens types) and no significant difference between the viable and total counts for albumin-coated lenses ($p>0.05$ for all lens types). When compared to uncoated lenses, there was a significant increase in the viable and total counts of Paer 6206 and 6294 strains adhered to albumin-coated lenses for all lens types ($p<0.05$ for all lens types). Between the albumin-coated lens materials, there was no significant difference in Paer 6206 and 6294 adhesion between balafilcon A and lotrafilcon B ($p>0.05$) and no significant difference in the adhesion of Paer 6206 and 6294 strains between the albumin-coated senofilcon A and etafilcon A lens materials ($p>0.05$). Albumin-coated senofilcon A and etafilcon A lens materials showed significantly lower binding of Paer 6206 and 6294 strains than albumin-coated balafilcon A and lotrafilcon B lens materials ($p<0.05$).

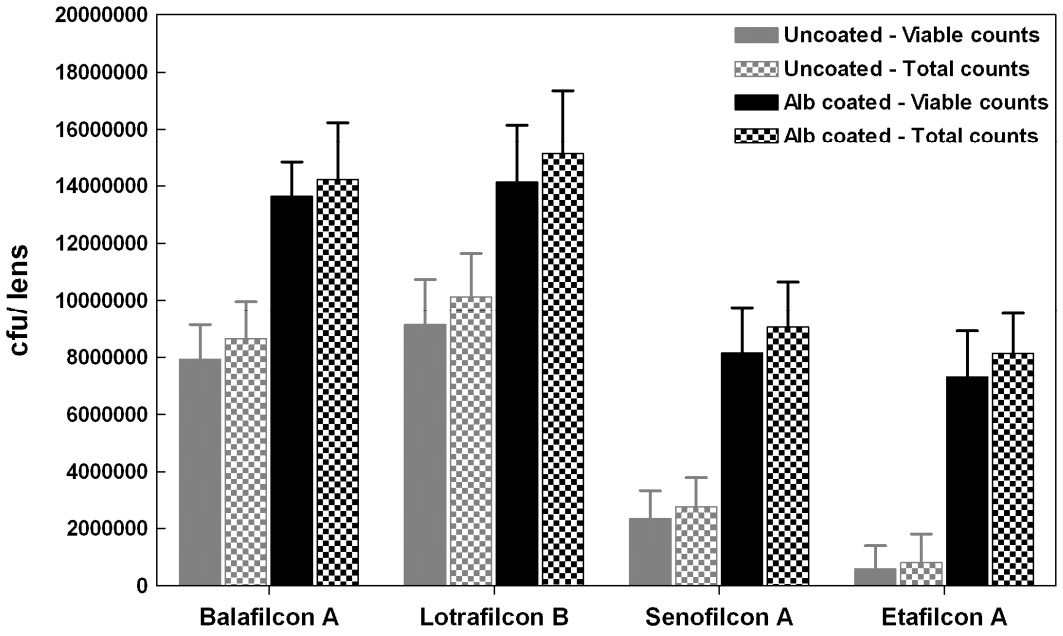

Figure 8-11: Adhesion of Gram negative Paer 6206 to uncoated and albumin-coated contact lens materials

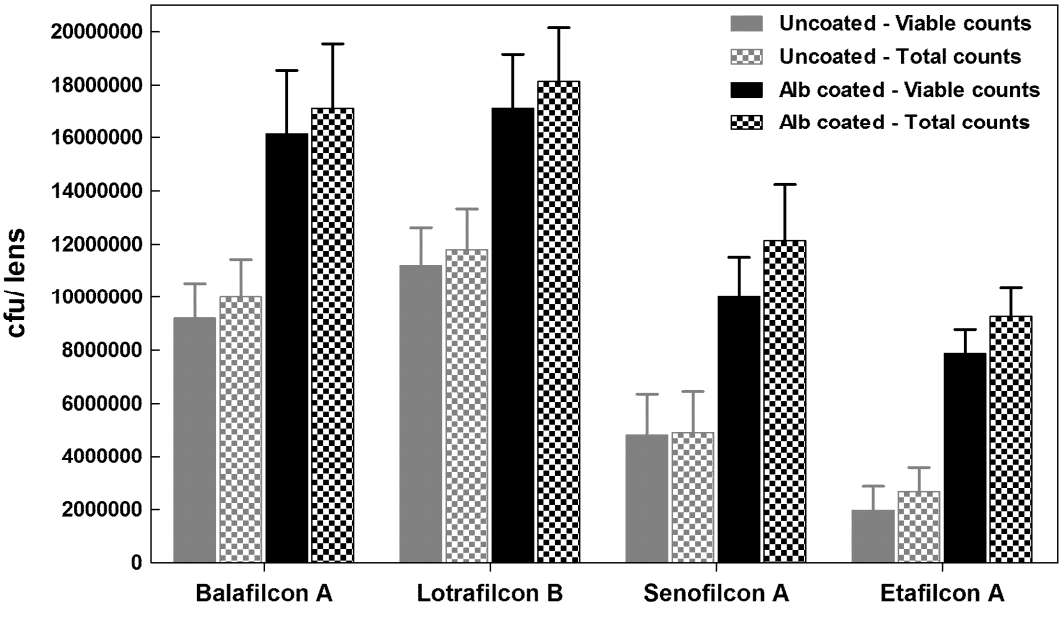

Figure 8-12: Adhesion of Gram negative Paer 6294 to uncoated and albumin-coated contact lens materials

8.5 Discussion

This is the first study to determine the effect of individual tear proteins on bacterial adhesion to silicone hydrogel lens materials. These results demonstrate that different tear proteins deposited on contact lenses have varying effects on the adhesion of bacteria to contact lenses. Historically, there has been disagreement concerning the effects of protein conditioning on the adhesion of bacteria to hydrogel contact lenses. Initial studies showed that bacteria readily adhered to unworn contact lenses, [38] but adhesion was shown to increase in patient-worn lenses [39, 41, 46, 54] Conversely, other studies showed that there was no increase, [45, 55] or inhibition of bacterial adhesion to patient-worn lenses [48] when compared to unworn lenses. However, all these studies had determined the influence of bacterial adhesion to worn lenses and did not determine the effect of the individual tear proteins. Later studies determined the influence of individual tear proteins on bacterial adhesion to contact lenses and these studies demonstrated that various proteins have the ability to modulate bacterial binding to conventional hydrogel contact lens materials.

Miller and Ahearn reported that mucin, Immunoglobulin A, bovine serum albumin, lysozyme and lactoferrin coated onto unworn lenses enhanced the adhesion of *Pseudomonas aeruginosa* to hydrogel contact lens materials. [56] Williams and colleagues showed that lactoferrin coated onto the lens surface promotes the adhesion of *Pseudomonas aeruginosa* strain Paer 1; nevertheless, once adherent, this protein reduces the proportion of viable bacteria on the lens surface. [43] Albumin coated onto the surface of etafilcon A or polymacon contact lenses increased the adhesion of *Pseudomonas*

aeruginosa. [44] Similarly, some strains of *Serratia marcescens* adhered better to etafilcon A lenses coated in an artificial tear fluid. [52] Thakur and colleagues demonstrated that lysozyme adsorbed to a contact lens increases the adhesion of *Staphylococcus aureus* to etafilcon A contact lenses. [42] Some studies demonstrated that *Pseudomonas aeruginosa* can bind to ocular mucins. [11, 57] Willcox and colleagues showed that the bacteria isolated from adverse responses had the ability to adhere differently to various contact lens materials and for most material/ strain combinations there were increases in adhesion to worn lenses or no differences between adhesion to worn or unworn lenses. [9] It was suggested that the increase in adhesion seen for certain strains to worn lenses may indicate that the tear components, most likely proteins that are bound to the contact lenses, were conducive to bacterial adhesion. [9]

8.5.1 Bacterial binding to uncoated lenses

The results from this current study suggest that there was an increased binding of all three strains of bacteria to the tested uncoated silicone hydrogel lens materials, when compared with one type of uncoated conventional hydrogel lens material. Among the silicone hydrogels, the adhesion was highest on balafilcon A and lotrafilcon B lens materials, when compared to the senofilcon A material. Bacterial adhesion to a biomaterial is known to depend on the hydrophobicity or hydrophilicity of the biomaterial, and therefore on the nature of the polymer of the contact lens. [9, 33, 34, 37, 58, 59] Furthermore, the water content of the lens material is also known to affect the adhesion of bacteria, decreasing with increasing water content of the lens material. [34, 35, 60] The results from this study agree with these findings, in that etafilcon A, the material with the highest

water content (58%) of the four contact lenses demonstrated the lowest bacterial binding, while the opposite was true for the lotrafilcon B material. These results are in accordance with other published studies that have compared the bacterial adhesion to conventional and silicone hydrogel contact lens materials. [33, 34, 37]

8.5.2 Effect of lysozyme-coating on bacterial adhesion

It is clear from Figure 8.4 that lysozyme coating on all four contact lens types significantly increased the adhesion of Saur 31, when assayed by both total and viable counts. These results are in accordance with a previous study, where it was demonstrated that lysozyme deposits on contact lenses increased the adhesion of several strains of *Staphylococcus* to etafilcon A lenses. [42] However, the study by Thakur and colleagues did not examine whether the adherent bacteria were alive or dead. [42] Figures 8.5 and 8.6 demonstrate that lysozyme coatings on contact lenses had no effect on the adhesion of Paer 6206 and 6294 strains, when assayed by either viable or total counts. Moreover, these results demonstrate that there was no difference between the total counts and viable counts of all three adherent strains, suggesting that lysozyme deposited on contact lenses do not have an antibacterial effect against the tested bacterial strains.

The bactericidal effects of lysozyme are most potent against Gram positive bacteria which, unlike Gram negative bacteria, lack an extra outer membrane around the peptidoglycan layer targeted by lysozyme. [61, 62] Surprisingly, the results from this study show that lysozyme deposited on the lens materials do not possess an antibacterial effect against both the Gram positive and Gram negative strains tested in this study. It is known

that once lysozyme deposits on conventional and silicone hydrogel contact lens materials, it tends to undergo conformational changes, [16, 63-66] which could potentially result in the loss of antibacterial activity against these strains of bacteria.

8.5.3 Effect of lactoferrin-coating on bacterial adhesion

Figure 8.7 shows that lactoferrin coating on all four types of contact lenses significantly increased the adhesion of Saur 31, when assayed by both total and viable counts. However, there was no significant difference between the total and viable counts of bacteria adhered to lactoferrin-coated lenses, suggesting that lactoferrin deposited on contact lenses do not possess an antibacterial effect against Gram positive Saur 31 strain. Figures 8.8 and 8.9 also indicate that lactoferrin bound to the lens surface does indeed promote adhesion of Paer 6206 and 6294 strains. At the same time, this protein reduced the proportion of viable bacteria on the lens surface. Both lactoferrin and lysozyme are known to possess antibacterial properties and they occur in high concentrations in the tear fluid. [10, 67] These two proteins are known to deposit on all types of conventional and silicone hydrogel contact lens materials. [16, 18, 19, 64, 68, 69]

The striking contrast in the results of the viable and total counts for lactoferrin-coated lens samples can be explained based on the antibacterial mechanism of lactoferrin. Lactoferrin has traditionally been regarded as a bacteriostatic protein, since it sequesters the iron that bacteria require for growth. [70] Moreover, lactoferrin has also been demonstrated to possess direct bactericidal effects. [71, 72] Lactoferrin has also been shown to have a destabilizing effect on the lipopolysaccharide (LPS) of Gram negative bacteria,

by targeting either the metal cations that stabilize the negative charge of the LPS, [73, 74] or the LPS directly. [71, 75] Therefore, lactoferrin reduces the viability of the adhered Paer strains; however, there is an increase in the total counts of the adhered Paer strains. It has been proposed that lactoferrin and other glycoproteins adsorbed to the lens may promote bacterial adhesion since their carbohydrate moieties may act as receptors for bacterial lectins, resulting in increased total counts of the adhered Paer strains. [76, 77]

8.5.4 Effect of albumin-coating on bacterial adhesion

Figures 8.10 to 8.12 demonstrate that albumin-coating on all the contact lens types increased the adhesion of all three strains of bacteria, when assayed by both total and viable counts. These results are in agreement with a previous study which determined the effect of albumin coating on etafilcon and polymacon lens materials. [44] The results from the previous study demonstrated that *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* adhered significantly higher with increasing concentration of albumin on contact lenses. [44] However, the study by Taylor and colleagues did not evaluate whether the adhered bacteria were alive or dead, [44] while the results from this present study evaluated both the total and viable counts. These results suggest that albumin-coating on contact lenses increase the adhesion of all three strains of bacteria and also that the albumin deposits do not possess an antibacterial effect against the three bacterial strains tested in this study.

It has been demonstrated that bacteria prefer to attach to low-energy, hydrophobic surfaces rather than to high-energy, hydrophilic surfaces. [78] Some studies have concluded

that the adsorbed macromolecules on the surfaces will increase or reduce the surface wettability of the biomaterial. [79, 80] Such macromolecules could convert a hydrophilic surface into a hydrophobic one, which is favourable for bacterial binding, thereby increasing the bacterial attachment. It is possible that albumin deposits on the contact lenses increase the hydrophobicity of the surface, resulting in increased binding of all the strains of bacteria.

8.6 Conclusions

In conclusion, these results suggest that different tear proteins have varying effects on the adhesion of bacteria to contact lens materials. Lysozyme deposits on contact lenses increase the adhesion of Gram positive *Staphyloccocus aureus* 31 strain, while albumin deposits increase the adhesion of both the Gram positive *Staphyloccocus aureus* and Gram negative *Pseudomonas aeruginosa* 6206 & 6294 strains. Lactoferrin deposits increase the total counts of both the Gram positive and Gram negative strains, while they reduce the viable counts of the Gram negative strains. These results indicate that lysozyme and albumin do not possess antibacterial activity against all the three tested strains, while lactoferrin has an antibacterial effect against the Gram negative strains tested in this study.

Further investigation is required to determine the adhesion of more bacterial strains that are involved in the pathogenesis of contact lens-related infections. Furthermore, it will be of significant clinical relevance to evaluate the impact of various care regimens and the impact of “rub and rinse” steps on bacterial adhesion to contact

lenses. This study has demonstrated that lactoferrin-coating on contact lenses has the ability to reduce the viability of certain Gram negative strains of bacteria and that it may be worthwhile to develop antibacterial contact lenses with some form of lactoferrin coating, which might potentially reduce the risk of corneal infection.

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9 Conclusions

This thesis has for the first time determined the kinetics of lysozyme deposition and also the conformational state of lysozyme deposited on conventional and silicone hydrogel lens materials as a function of time. This thesis has also determined the protein deposition levels on a novel, lathe-cut silicone hydrogel lens material after three months of wear. Further, this thesis has also evaluated the relationship between protein deposition and clinical signs & symptoms and also the effect of individual tear protein deposition on bacterial adhesion to various contact lens materials. The following section summarizes the results from all these chapters.

Chapter 4 determined the kinetics of lysozyme deposition on conventional and silicone hydrogel lens materials and demonstrated that lysozyme deposition is driven by both the bulk chemistry and also the surface properties of conventional and silicone hydrogel contact lens materials. The surface modification processes or the inclusion of high molecular entities or surface-active monomers on silicone hydrogel lens materials also play a significant role in lysozyme deposition on these lens materials. The results from this study reiterate that silicone hydrogel lens materials deposit very low amounts of lysozyme. Silicone hydrogel lenses that are surface modified with a 25nm plasma coating (lotrafilcon A & B) showed a deposition pattern similar to PMMA lens materials. Silicone hydrogel lenses that use a surface modification system based on PVP (galyfilcon and senofilcon) had similar deposition patterns, while balafilcon lenses (which undergo a plasma oxidation process) showed the highest level of lysozyme deposition. However, this was still lower than that seen with most conventional materials.

Chapter 5 determined the lysozyme activity recovered from conventional and silicone hydrogel contact lens materials as a function of time and these results suggest that the reduction in the activity of lysozyme deposited on contact lens materials is time dependent and that the rate of reduction in the activity of lysozyme varies between lens materials. This reduction in the loss of biological activity could be due to the differences in surface or bulk properties of the lens materials and also depend on the location of lysozyme on these lens materials. These results reiterate that the levels of lysozyme activity are highly variable between lens materials and also that SH lens materials deposit very low amounts of lysozyme. After 28 days, lysozyme recovered from conventional hydrogel FDA group IV lens materials (etafilcon A and vifilcon A) retained the highest amount of percentage activity, while lysozyme recovered from SH lenses that are surface modified with a 25nm plasma coating (lotrafilcon A & B) retained an activity similar to that of a conventional hydrogel FDA group I lens material. Among silicone hydrogel lenses, lenses that use a surface modification system based on PVP (galyfilcon A) and lenses that undergo a plasma oxidation process (balafilcon A) retained similar levels of percentage active lysozyme.

Chapter 6 determined the protein deposition on a novel, lathe-cut silicone hydrogel lens material after three months of lens wear and these results indicate that the extended use of the sifilcon A lens material used in the O_2OPTIX Custom™ silicone hydrogel lens is not deleterious in terms of the amount and the quality of protein that is deposited on the lens as long as the lens is rubbed and rinsed. These results indicate that even after three months of wear, the quantity of protein and the conformational state of

lysozyme deposited on these novel lenses (when worn using a care regime employing a rub-and-rinse format) is very similar to that found on similar surface-coated silicone hydrogel lenses (lotrafilcon A and lotrafilcon B) after two to four weeks of wear. These results reiterate that silicone hydrogel lens materials, although fabricated via a lathe-cutting process, deposit low quantities of total protein and total lysozyme even after extended period of daily wear.

Chapter 7 determined the relationship between protein deposition and clinical signs & symptoms. These results suggest that even over a short period of wear, a significant correlation exists between subjective symptoms of comfort and dryness and the activity of lysozyme recovered from etafilcon contact lenses, with little correlation being shown with total amounts of either total protein or total lysozyme. Therefore, in addition to investigating the total quantity of the deposited protein, it is of significant clinical relevance to study the conformational state of the deposited protein. These results have tremendous implications, in that the novel contact lens materials that are being developed should possess properties that can retain the activity of the deposited protein, in addition to being deposit-resistant. Care regimens and multipurpose solutions should be capable of removing denatured proteins that are deposited on the lens materials.

Chapter 8 determined the influence of protein deposits on contact lenses on the adhesion of Gram positive and Gram negative bacteria. These results suggest that different tear proteins have varying effects on the adhesion of bacteria to contact lens materials. Lysozyme deposits on contact lenses increase the adhesion of Gram positive

Staphyloccocus aureus 31 strain, while albumin deposits increase the adhesion of both the Gram positive *Staphyloccocus aureus* and Gram negative *Pseudomonas aeruginosa* 6206 & 6294 strains. Lactoferrin deposits increase the total counts of both the Gram positive and Gram negative strains, while they reduce the viable counts of the Gram negative strains. These results indicate that lysozyme and albumin do not possess antibacterial activity against all the three tested strains, while lactoferrin has an antibacterial effect against the Gram negative strains tested in this study.

This thesis has provided hitherto unavailable information on contact lens deposition and its influence on subjective symptoms and bacterial binding. These results suggest that protein deposition has a significant potential to cause problems. Therefore, it is important that practitioners advise their patients regarding the importance of lens disinfection and cleaning and appropriate lens replacement schedules. These results will also be useful for the contact lens industry and the general field of biomaterials research.

10 Future work

This chapter recommends a list of projects that can be conducted as a continuation of this research work, which would be a valuable addition to the literature and would help in the development of safer and more comfortable contact lenses. Some of these include:

Quantifying other major proteins deposited on contact lenses:

Using a proteomic technique, 97 proteins have been identified in the tear film. [1] Many of these proteins are known to sorb onto contact lens materials, [2, 3] and lysozyme, lipocalin, lactoferrin, lacritin, proline-rich 4 and Ig alpha have been identified as the frequently recognized tear proteins on contact lenses. This thesis has studied lysozyme deposition on conventional and silicone hydrogel contact lens materials. Another recent study from our group has determined the kinetics of lactoferrin deposition on contact lens materials. [4] It will be of interest to quantify the other frequently recognized tear proteins found on novel silicone hydrogel contact lens materials.

Competitive interaction of proteins and other tear components:

An important factor to consider in the process of deposition relates to whether other substances from the tear film (for example, mucins, lipids or other proteins) concurrently deposit onto the material surface at different rates. It would be of interest to investigate the kinetics of deposition using a complex artificial tear solution consisting of a variety of proteins, mucins & lipids and evaluate the protein-protein, protein-lipid and protein-mucin interactions.

Relationship between lysozyme activity and symptoms in silicone hydrogel lens wearers:

Chapter 7 of this thesis has demonstrated that even over a short period of wear, a significant correlation exists between subjective symptoms of comfort & dryness and the activity of lysozyme recovered from etafilcon contact lenses, with little correlation being shown with total amounts of either total protein or total lysozyme. Further work is required to determine if this important clinical finding is transferable to those patients who use silicone hydrogel contact lens materials.

Determination of active lysozyme recovered from tears:

This thesis determined the activity of lysozyme deposited on various conventional and silicone hydrogel contact lenses. It will be of interest to determine the activity of lysozyme that is present in tears and investigate if there are any differences in the activity of lysozyme in tears of contact lens wearers and non-contact lens wearers. Further, it would be worthwhile to determine if these differences exist after overnight wear of contact lenses. It would also be of interest to determine if there is a difference in the activity of lysozyme recovered from the tears of symptomatic versus asymptomatic contact lens wearers and dry eyed versus non-dry eyed individuals.

Determination of the activity of lactoferrin deposited on contact lenses:

Our group has recently reported on the kinetics of lactoferrin deposition on conventional and silicone hydrogel contact lenses using an *in vitro* model. [4] It would be worthwhile to determine the activity of lactoferrin deposited on conventional and silicone hydrogel contact lens materials. In addition to investigating the activity of lactoferrin

deposited on contact lenses, it would be of clinical relevance to determine the kinetics of lactoferrin denaturation, how they relate to the wearing period, and also the influence of various care regimens on denaturation.

Effect of individual tear proteins on the wettability of contact lenses:

Chapter 8 of this thesis has speculated that certain tear proteins like lysozyme, lactoferrin and albumin once deposited on the contact lenses, will alter the wettability of the lenses. Our group recently reported on the effect of *in vitro* lipid doping on contact lens wettability using a sessile drop contact angle method. [5] It would be of interest to determine the impact of the major tear proteins on the wettability of conventional and silicone hydrogel contact lens materials.

Impact of care regimen on bacterial adhesion to contact lenses:

Chapter 8 of this thesis investigated the adhesion of three bacterial strains. It will be interest to investigate the adhesion of more bacterial strains that are involved in the pathogenesis of contact lens-related infections. Furthermore, it will be of significant clinical relevance to evaluate the impact of various care regimens and the impact of "rub and rinse" steps on bacterial adhesion to contact lenses.

Development of antibacterial lenses with lactoferrin coating:

Chapter 8 of this thesis has demonstrated that lactoferrin-coating on contact lenses has the ability to reduce the viability of certain Gram negative strains of bacteria and that

it may be worthwhile to develop antibacterial contact lenses with some form of lactoferrin coating, which might potentially reduce the risk of corneal infection.

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Appendices

Appendix A

Efficacy of an extraction solvent used to quantify lysozyme and albumin deposition on conventional and silicone hydrogel contact lens materials

This appendix has been published as two separate papers:

(1) Lakshman N Subbaraman, Mary-Ann Glasier, Michelle Senchyna, Heather Sheardown and Lyndon Jones. Extraction efficiency of an extraction buffer used to quantify lysozyme deposition on conventional and silicone hydrogel contact lens materials. *Eye Contact Lens*. 2007; 33(4):169-73.

(2) Lakshman N Subbaraman, Mary-Ann Glasier, Heather Sheardown and Lyndon Jones. Efficacy of an extraction solvent used to quantify albumin deposition on hydrogel contact lens materials. *Eye Contact Lens.* 2009; 35(2):76-80.

Abstract

Purpose: Extracting proteins from conventional hydrogel (CH) and silicone hydrogel (SH) lens materials using a mixture of trifluoroacetic acid/ acetonitrile (TFA/ACN) is a well established procedure for quantifying individual and total protein deposited on contact lenses. The purpose of this study was to determine the efficiency of TFA/ACN in extracting lysozyme and albumin from SH and a CH group IV lens material using an *in vitro* model.

Methods: One CH (etafilcon A) and four SH (lotrafilcon A, lotrafilcon B, balafilcon A and galyfilcon A) lens materials were incubated in both simple lysozyme solution and a complex Artificial Tear Solution (ATS) consisting of multiple tear components containing ^{125}I-labeled lysozyme. All the SH lenses were incubated in the lysozyme solution for 28 days, while etafilcon lenses were incubated for 7 days at 37^{o} C with constant rotation. The CH group IV lens material and four different SH lens materials were incubated in both simple albumin solution and an artificial tear protein solution containing ^{125}I-labeled albumin. All the lens materials were incubated in the albumin solution for 14 days. Following the incubation period, radioactive counts on the lenses were determined and the lenses were placed in an appropriate volume of the buffer for 24 hours in darkness. The lenses were removed from the buffer and radioactive counts were determined again to calculate the amount of lysozyme and albumin remaining on the lenses post-extraction.

Results: Extraction efficiencies for lysozyme from the ATS solution were 97.2%±1.2 for etafilcon A, 64.3%±6.2 for lotrafilcon A, 62.5%±5.6 for lotrafilcon B, 53.5%±5.8 for balafilcon A, and 89.2%±3.4 for galyfilcon A. Results were similar for the lysozyme extracted after incubating in the simple lysozyme solution.

Extraction efficiencies for albumin from the ATS solution were 97.2%±2 for etafilcon A, 77.3%±6.2 for lotrafilcon A, 73.5%±5.6 for lotrafilcon B, 81.5%±5.8 for balafilcon, and 91.2%±3.4 for galyfilcon A. Results were similar for the albumin extracted after incubating in the simple albumin solution.

Conclusion: TFA/ACN is extremely efficient at extracting lysozyme and albumin deposited on etafilcon lenses. However, it does not extract all the lysozyme/ albumin that is deposited on SH lenses and alternative extraction procedures should be sought.

Key words: contact lens; extraction; lysozyme; ^{125}I-labeled lysozyme; ^{125}I-labeled albumin; tear protein.

Introduction

Contact lenses suffer from the same problems of deposition that other biomaterials exhibit when in contact with a biological fluid, being rapidly coated with a variety of proteins, lipids and mucins from the tear film, in addition to several other substances. [1-8] Of all these deposit types, protein deposits have attracted the greatest degree of attention. Several microscopic, photometric and imaging techniques have been used to investigate deposits on contact lenses. [4, 9-16] The major limitation of microscopic and imaging techniques is that they are generally not suitable for accurate quantification purposes. [8] Therefore, various biochemical assays [6, 9, 17-19] have been developed and are typically employed because of their increased sensitivity, accuracy and ability to target specific proteins. In order to accurately perform these assays, it is critical that all the deposited proteins are extracted from the lens materials, because accurate quantification of the protein deposited on a surface requires that all - or at least a known percentage - of the protein of interest be removed. To-date, elution of proteins from the contact lens surface remains the best option to quantify specific proteins deposited on the surface of contact lenses. [5, 9, 18, 20, 21] Hence, the ability of an extraction buffer to maximally extract proteins for analysis is a point of major interest.

The most common method for protein extraction involves the use of a combination of detergents and reducing agents to break chemical bonds between the sorbed proteins and the contact lens surface. Some of the agents that have been incorporated in elution mixtures include sodium dodecyl sulphate (SDS), dithiothreitol (DTT), urea, NaOH, ethylene diamine tetraacetic acid (EDTA) and Tris-HCL. [4, 6, 9, 18, 21-

[29] One of the significant earlier works on deposit removal was conducted by Wedler [28] using various chemical reagents including urea, guanidine hydrochloride, potassium thiocyanate, potassium perchlorate, hydroxylamine, EDTA, SDS and DTT. It was found that the deposits were most effectively removed from the lenses by using a combination of heat, SDS detergent and DTT. Many other researchers have subsequently used these techniques, in particular those that are based upon SDS. [4, 9, 18, 22-27] In addition to chemical "mixtures" to extract proteins, heat and sonication have been utilized to increase the efficiency of protein extraction, theoretically by agitating the biomaterial in the extraction solution and helping to break apart the bonds. [16, 30] However, Yan and colleagues suggested that these common extraction procedures may fail to remove even 75% of the total material on the lenses. [26]

Keith and co-workers developed a quick, simple and efficient extraction technique [31] using an extraction solvent consisting of a 50:50 mix of trifluoroacetic acid and acetonitrile (TFA/ ACN). Using this method, the extraction efficiency for lysozyme from laboratory deposited FDA group IV lenses was found to be close to 100%. Similar extraction procedures have been adopted by many other researchers since then. [32-37] However, the efficacy of this technique in extracting lysozyme and albumin from silicone hydrogel (SH) lens materials has not been reported. Thus, the purpose of this study was to quantify the percentage of lysozyme and albumin extracted by TFA/ ACN from SH and etafilcon lens materials by artificially incubating lenses in ^{125}I-labeled lysozyme and ^{125}I-labeled albumin.

Materials and Methods

Reagents and materials

Lysozyme and albumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The lenses that were used in the study included four types of SH lenses and one type of conventional hydrogel (CH) lens. The SH lenses examined were Night & Day™ (CIBA Vision, Duluth, GA, USA), O_2Optix™ (CIBA Vision, Duluth, GA, USA), PureVision™ (Bausch & Lomb, Rochester, NY, USA), Acuvue® Advance™ (Vistakon, Johnson & Johnson, Jacksonville, FL, USA) and Acuvue® 2 (Vistakon, Johnson & Johnson, Jacksonville, FL, USA). The properties of these lens materials are described in Table A.1. Sterile, 5ml non-pyrogenic, polypropylene round bottom tubes were purchased from Falcon (Franklin Lanes, NJ, USA). All other reagents purchased were of analytical grade and were obtained from Sigma (St. Louis, MO, USA).

Table A-1: Silicone hydrogel lens materials and the conventional hydrogel lens material evaluated in the study.

Proprietary name	Night & Day™	O_2 Optix™	PureVision ™	Acuvue® Advance™	Acuvue® 2
USAN	Lotrafilcon A	Lotrafilcon B	Balafilcon A	Galyfilcon A	Etafilcon A
Manufacturer	CIBA Vision	CIBA Vision	Bausch & Lomb	Johnson & Johnson	Johnson & Johnson
Water content (%)	24	33	36	47	58
Centre thickness (mm) -3.00D	0.08	0.08	0.09	0.07	0.07
Oxygen Transmissibility (Dk/t) at 35˚C	175	138	101	86	21
FDA group	I	I	III	I	IV
Surface Treatment	25nm plasma coating with high refractive index	25nm plasma coating with high refractive index	Plasma oxidation process	No surface treatment. Internal wetting agent (PVP) that also coats the surface	None
Principal monomers	DMA+TRIS+ Siloxane monomer	DMA+TRIS+ Siloxane monomer	NVP+TPVC+ NCVE+ PBVC	mPDMS+ DMA+HEMA +siloxane macromer+ PVP+ EGDMA	HEMA+MA

DMA (N,N-dimethylacrylamide); EGDMA (ethyleneglycol dimethacrylate); HEMA (poly-2-hydroxyethyl methacrylate); mPDMS (monofunctional polydimethylsiloxane); NVP (N-vinyl pyrrolidone); TPVC (tris-(trimethylsiloxysilyl) propylvinyl carbamate); TRIS (trimethylsiloxy silane); NVA (N-vinyl amino acid); PBVC (poly[dimethysiloxy] di [silylbutanol] bis[vinyl carbamate]); PVP (polyvinyl pyrrolidone); MA (methacrylic acid).

Preparation of the doping solution

(a) Lysozyme solution

The concentrations of various tear components in the artificial tear solution (ATS) are described in Table A.2, along with a description of the concentration of various ATS components from previous *in vitro* doping experiments, for comparative purposes. [38, 39] The simple lysozyme doping solution was prepared at a concentration of 1.9 mg/ml, using Phosphate Buffered Saline (PBS) at a pH 7.4. Lysozyme was labeled with ^{125}I using the Iodine mono-chloride (ICl) method. [40-42] ^{125}I-labeled lysozyme was used as the isotopic tracer to quantify protein adsorption to the contact lenses. Radiolabeled lysozyme was dialyzed extensively against PBS to remove free iodide and the percentage of free iodide was determined based on the precipitation of protein with trichloroacetic acid and the radioactivity of the supernatant. In all cases, the samples which had free iodide of less than 1% were used in the study. ^{125}I-labeled lysozyme was added to unlabeled solution such that the samples had a counting rate of 10^5 disintegrations per minute/ml (DPM/ml).

(b) Albumin solution

The simple albumin doping solution was prepared at a concentration of 0.2 mg/ml, using Phosphate Buffered Saline (PBS) at a pH 7.4. A complex tear protein solution was prepared as given in Table A.2. Albumin was labeled with ^{125}I using the Iodine mono-chloride (ICl) method. [40-44] Free iodide was removed from the radiolabeled albumin by extensive dialysis against PBS and the percentage of free iodide was determined based on the precipitation of protein with trichloroacetic acid and the

radioactivity of the supernatant. [43, 44] In all cases, the samples which had free iodide of less than 1% were used in the study.

Table A-2: Table comparing the concentration of various tear components in artificial tear solutions for previous *in vitro* experiments and in the current study.

Component	Mirejovsky, *et al* (mg/ml)	Prager, *et al* (mg/ml)	Current study (mg/ml)
Lysozyme	1.9	1.9	1.9
Lactoferrin	1.8	1.8	1.8
α Acid Glycoprotein	0.5	0.5	-
Albumin	0.2	0.2	0.2
Mucin	0.15	0.15	0.15
γ Globulins	0.1	0.1	-
Cholesteryl linoleate	0.024	0.0262	0.075
Linalyl acetate	0.02	-	-
Triolein	0.016	0.0035	0.016
Oleic acid propyl ester	0.012	0.0311	-
Oleic acid methyl ester	-	-	0.024
Dicaproin	0.0032	-	-
Undecylenic acid, sodium salt	0.003	-	-
Dioleoyl	-	0.0142	-
Oleic acid	-	0.0018	0.008
Cholesterol	0.0016	0.0016	0.056
Sodium Chloride	6.626	-	6.63
Potassium Chloride	1.716	-	1.72
Sodium Bicarbonate	1.376	-	1.68
Lactic acid	0.27	-	-
Calcium chloride, dihydrate	0.147	-	0.147
Sodium dihydrogen phosphate, monohydrate	0.100	-	-
Buffers	3-(N-Morpholino)propanesulfonic acid @ 4.18 mg/ml	Hank's Balanced Salt Solution pH 7.4	HEPES MW238.3 (25mM) @ 5.9575 mg/ml pH 7.4

Incubating lenses in the tear solutions

Lenses were removed from their packaging and thoroughly rinsed with PBS to ensure that no packaging solution remained on the lens surface and interfered with the deposition process. Five lenses from each lens type were then placed in 1ml of the labeled ATS and labeled simple lysozyme/albumin solutions and were allowed to incubate at a temperature of 37°C with constant rotation. SH lenses were incubated in the lysozyme solution for 28 days, while etafilcon lenses were incubated for 7 days. All the lens materials were incubated in the albumin solution for 14 days. Previous data from our laboratory showed that etafilcon lenses accumulate enough quantity of lysozyme in a shorter period of time than the SH lenses, [45] so etafilcon lenses were incubated in the lysozyme solutions for a shorter span. The labeled ATS and lysozyme/albumin solutions were replaced once every seven days with a protein-doping solution at an identical volume and radioactivity. The labeled ATS and lysozyme/albumin solutions were replaced once after seven days, with a protein-doping solution at an identical volume and radioactivity.

Following the specified days of incubation, the lenses were aseptically removed from the labeled solutions using forceps and were rinsed briefly in saline to remove unbound protein from the lens surface. The lenses were then placed in sterile 5ml (12 X 75 mm), non-pyrogenic, polypropylene round bottom tubes and subsequently radioactive counts were determined in a Gamma Counter (Perkin Elmer Wallac Wizard 1470 Automatic Gamma Counter, Wellesley, MA, USA). This count gave the total quantity of labeled lysozyme/albumin that was deposited on the lens (Total DPM).

Extraction of lysozyme/ albumin from contact lenses

The lenses were then placed in individual glass vials containing the extraction solvent under test (TFA/ACN). The lenses were incubated in the extraction solvent in darkness at room temperature for 24 hours. Following this, the lenses were carefully removed from the vials and were placed in the sterile, 5ml (12 X 75 mm), non-pyrogenic, polypropylene round bottom tubes and radioactive counts were determined again on the lenses using a Gamma Counter. This count gave the total quantity of labeled lysozyme/ albumin that remained on the lenses following its extraction (Lens DPM). In addition to measuring the Lens DPM, radioactive counts were determined from the extraction buffer (Extract DPM), to ensure consistency of the results obtained.

The percentage of lysozyme/albumin extracted from the lens was calculated by the formula,

% lysozyme/ albumin extracted = (Total DPM – Lens DPM)/ Total DPM X 100

Data Analysis

Statistical analysis was conducted using Statistica 7 software (StatSoft Inc, OK, USA). All data are reported as mean ± SD. An Analysis of Variance was performed on all the data and post-hoc testing was undertaken using the Tukey-HSD test to determine statistically significant differences. In all cases, a p value of <0.05 was considered significant.

Results

Extraction efficiency for lysozyme

The extraction efficiencies for different lens types incubated in ATS and simple lysozyme solution using the TFA/ACN extraction buffer are reported in Table A.3.

Table A-3: Percentage of lysozyme extracted by TFA/ACN.

Table representing the percentage of lysozyme extracted from different lens materials using the 0.2% trifluoroacetic acid and acetonitrile extraction solvent when the lenses were incubated in the two tear solutions.

Lens Type	Percentage extracted in complex artificial tear solution	Percentage extracted in simple lysozyme solution
Etafilcon A	97.2 ± 1.2	99.3 ± 1.0
Lotrafilcon A	64.3 ± 6.2	66.3 ± 5.2
Lotrafilcon B	62.5 ± 5.6	64.4 ± 6.7
Balafilcon A	53.5 ± 5.8	56.7 ± 3.8
Galyfilcon A	89.2 ± 3.4	91.4 ± 1.4

The statistically significant differences between various lens materials for extraction efficiency using the extraction buffer are shown in Table A.4. There were no significant differences between the extraction efficiencies when the lenses were incubated in ATS and simple lysozyme solution ($p=0.74$).

Table A-4: Significant differences seen between different lens materials for lysozyme extraction efficiency.

Table shows the significant differences seen between different lens materials for extraction efficiency using 0.2% trifluoroacetic acid and acetonitrile extraction solvent.

Lens Types	p
Etafilcon versus all SH lens materials	<0.01
Galyfilcon versus other SH lens materials	<0.01
Balafilcon versus Lotrafilcon A	<0.05
Balafilcon versus Lotrafilcon B	<0.05
Lotrafilcon A versus Lotrafilcon B	0.63

Extraction efficiency for albumin

The extraction efficiencies for different lens types incubated in ATS and simple albumin solution using the TFA/ACN extraction solvent are reported in Table A.5.

Table A-5: Percentage of albumin extracted by TFA/ACN.

Table representing the percentage of albumin extracted from different lens materials using the 0.2% trifluoroacetic acid and acetonitrile extraction solvent when the lenses were incubated in two artificial tear solutions.

Lens Type	% extracted in complex artificial tear protein solution	% extracted in simple albumin solution
Etafilcon A	97.2 ± 2.0	97.3 ± 2.0
Lotrafilcon A	77.3 ± 6.2	80.3 ± 7.2
Lotrafilcon B	73.5 ± 5.6	72.4 ± 6.7
Balafilcon A	81.5 ± 5.8	80.7 ± 3.8
Galyfilcon A	91.2 ± 3.4	92.2 ± 3.4

The statistically significant differences between various lens materials for extraction efficiency using the extraction solvent are shown in Table A.6. There were no significant differences between the extraction efficiencies when the lenses were incubated in ATS and simple albumin solution (p=0.67).

Table A-6: Significant differences seen between different lens materials for albumin extraction efficiency.

Table shows the significant differences seen between different lens materials for extraction efficiency using 0.2% trifluoroacetic acid and acetonitrile extraction solvent.

Lens Types	p
Etafilcon A versus all SH lens materials	<0.05
Galyfilcon A versus other SH lens materials	<0.05
Lotrafilcon A versus lotrafilcon B	0.32
Lotrafilcon A versus balafilcon A	0.68
Lotrafilcon B versus balafilcon A	0.29

Discussion

Extraction of proteins from FDA group IV hydrogel lenses using 0.2% TFA/ACN is a commonly reported technique [31-33, 38, 46] and this buffer has previously been used for extraction of protein deposits from SH materials. [32, 34-37] Only one previous study has discussed the efficacy of removing lysozyme using this acid-based extraction technique on SH materials, [36] and this study compared the acid-based method to one based on SDS, and showed it to be a superior method.

When looking at the efficacy of an extraction solvent, some form of technique to accurately determine protein deposition on the biomaterial of interest is required. Previous researchers have utilized the UV spectrophotometer to assess extraction efficiency. [36, 47] However, many modern materials incorporate a UV blocking agent, negating the use of such a methodology. In addition, UV spectrophotometry is a relatively insensitive technique, and such a method would be too insensitive for analyzing SH materials, due to the extremely low levels of lysozyme or albumin deposited on these materials. [32, 34-36, 45] One method which is very sensitive at detecting very low quantities of lysozyme or albumin deposited on hydrogel materials is that based upon protein radiolabeling. [1, 19, 30, 40, 45, 48] The isotope ^{125}I has been extensively used to radioactively label lysozyme and other proteins in previous studies. [1, 19, 30, 40, 48] The isotope is incorporated into the protein and radioactivity can be used to assess the level of protein binding.

This unique study specifically investigated the efficacy of the TFA/ACN technique for removing protein from a FDA group IV material (etafilcon A) and four types of novel SH lens materials (lotrafilcon A, lotrafilcon B, balafilcon A and galyfilcon A) using an *in vitro* deposition model based on radiolabeled lysozyme or albumin. This radiolabel technique is sensitive enough to thoroughly investigate the extraction efficiency from SH lens materials, which deposit very low quantities of lysozyme/ albumin and could not easily be examined using any other technique.

Tables A3, A4, A5 and A6 clearly show that the method developed by Keith and colleagues based upon TFA/ACN is almost 100% efficient at removing lysozyme or albumin from artificially spoiled etafilcon lenses, which is consistent with previous work. [31] The possible mechanisms for protein removal by this solution could be partly attributed to an ion exchange interaction between the solution, protein and lens. [31] As the solution is acidic, protons from the solution interact with the negative sites on the lenses. This can occur as an ionic interaction at an open negative site or as an exchange at an existing ionic bond between protein and lens. [49] In addition, this solution has strong solvation properties for proteins, in that it has ionic, aqueous and organic properties. [31] Thus, these two combined properties of this solution provide a means of effectively extracting the protein from the lenses and readily solubilizes and maintains it in the solution. [31, 49]

However, examination of Tables A3, A4, A5 and A6 clearly demonstrate that the efficacy of this method in extracting lysozyme or albumin from SH lens materials is lower. Although the current method could extract approximately 90% of the lysozyme or

albumin deposited on artificially spoiled galyfilcon lenses, it was not as efficient at extracting lysozyme/albumin from the other three SH lens materials under test. The possible reason for this reduced extraction efficiency from these three lens types could be due to the surface modification process that is involved with these lens materials. The surfaces of the lotrafilcon A and lotrafilcon B lenses are permanently modified in a gas plasma reactive chamber [50, 51] while the balafilcon lenses are surface treated in a gas plasma reactive chamber, which transforms the silicone components on the surface of the lenses into hydrophilic silicate compounds. [50, 52] No surface treatment is required for etafilcon and galyfilcon lenses. It is possible that these surface modification processes in lotrafilcon A, lotrafilcon B and balafilcon lens types could interfere with the buffer's ability to break the bonds between deposited lysozyme/albumin and the lens surface resulting in decreased extraction from these two lens materials. A further explanation could be that once proteins deposit onto these relatively hydrophobic substrates that the protein deposited is more denatured than that found on more hydrophilic surfaces and thus more difficult to remove easily.

It is a well established fact that protein deposition is affected by the water content, hydrophobicity, charge, pore size and surface roughness of the lens material. [1, 2, 4, 6, 13, 31, 53-64] However, it is not known if these factors affect lysozyme or albumin removal or if the extraction buffers are strong enough to overcome these effects. The ability of the components of an extraction buffer to break chemical bonds and encapsulate proteins for removal from the contact lens surface is determined by the extraction efficiency. Each

type of protein deposited on a material may have a different set of bonds and steric constraints, allowing it to remain on the surface.

It is clear that SH lenses deposit significantly lower amounts of lysozyme or albumin compared to traditional hydrogel lens materials. To overcome this issue of inefficiency, a new buffer has to be developed which has the capability to remove a greater percentage of the deposited protein. Ideally, such a buffer should have a minimal effect on protein conformation and be compatible with current and future methodologies used to quantify lysozyme or albumin concentration and conformation.

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