Impact of *in vitro* Tear Film Composition on Lysozyme Deposition and Denaturation

by

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Authors Declaration

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.

I understand that my thesis may be made electronically available to the public.

Abstract

Purpose

To study the impact of lactoferrin and lipids on the kinetic deposition and denaturation of lysozyme on conventional and silicone hydrogel contact lens materials.

Methods

The contact lens materials investigated in this thesis included two silicone hydrogel lenses [AIR OPTIX AQUA; lotrafilcon B and ACUVUE OASYS; senofilcon A] and two conventional hydrogel lenses [ACUVUE 2; etafilcon A and PROCLEAR; omafilcon A]. All four lens materials were incubated at 37^{o}C in four different solutions: a complex artificial tear solution (ATS) that consisted of various proteins, lipids, mucins and electrolytes; an ATS without lactoferrin; an ATS without lipids; and an ATS without lactoferrin and lipids. Following each time point (4 and 12 hours, 1, 2, 3, 5, 7, 14, 21 and 28 days), the lens materials were removed from the incubation solutions for lysozyme analysis using the methods below:

- To quantify the kinetic uptake of lysozyme to different contact lens materials, I^{125}-radiolabelled lysozyme was added to each incubation solution. The total amount of lysozyme deposited on each lens type was quantified using a gamma counter. (Chapter 3)
- To study the activity of lysozyme deposited to contact lenses, a novel fluorescence-based lysozyme activity assay was compared to the classical micrococcus turbidity assay. Potential interactions with different lens materials and protein extraction solvents were evaluated and the detection range for active lysozyme was determined. (Chapter 4)

- To investigate the kinetic denaturation of lysozyme deposited to different contact lens materials, the fluorescence-based activity assay and the enzyme-linked immunosorbent assay were used. (Chapter 5)

Results

The presence of both lactoferrin and lipids significantly decreased lysozyme uptake to lotrafilcon B. Lysozyme deposition on senofilcon A was greater in the absence of lipids after day 21, however the opposite was seen with etafilcon A, where lysozyme uptake was lower without lipids in the ATS. Lactoferrin and/or lipids had no effect on lysozyme adsorption to omafilcon A.

The novel fluorescence-based lysozyme activity assay demonstrated a high sensitivity and a wide linear range of detection, which covers the amount of lysozyme typically extracted from contact lenses. Using this novel technique, kinetic lysozyme activity on both silicone hydrogel materials was shown to decrease in the presence of lipids in the ATS. In addition, lactoferrin had a protective effect on lysozyme activity for lysozyme sorbed to senofilcon A. Moreover, the presence of lactoferrin and/or lipids did not exhibit any effect on lysozyme denaturation with conventional hydrogel lens materials.

Conclusions

The presence of lactoferrin and lipids in an artificial tear solution impacted lysozyme deposition and denaturation of lysozyme on various contact lens materials. The use of a complex artificial tear solution to investigate the adsorption of lysozyme and its reduction in activity on contact lenses exhibited results that were comparable to previous *ex vivo* studies, particularly results seen with silicone hydrogel lens materials. It is important for future *in vitro* studies, when developing

tear film models, to consider the effects of various tear film components when investigating protein deposition and denaturation on contact lenses.

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Dedication

This thesis is dedicated to my family.

Thank you Mom and Dad for your unconditional love, support and trust in the decisions I make in life. I am truly honored to have you as my parents.

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Thank you to my Grandma and Grandpa for being my source of motivation in life.

I hope I've made you proud.

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List of Symbols and Abbreviations

ATS	artificial tear solution
BSA	bovine serum albumin
CLPC	contact lens induced papillary conjunctivitis
cm	centimeters
D	diopters
Dk	oxygen permeability
Dk/t	oxygen transmissibility
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESR	electron spin resonance
FDA	US Food and Drug Administration
FTIR-ATR	Fourier transform infrared-attenuated total reflectance
GPC	giant papillary conjunctivitis
HEL	hen egg lysozyme
HEMA	polyhydroxyethylmethacrylate
HPLC	high performance liquid chromotography
IEP	isoelectric point
kDA	kiloDalton
Lac	lactoferrin
Lcf	lactoferrin
Lip	lipids
µg	microgram
µL	microlitre

MA	methacrylic acid
mg	milligram
ml	millilitre
ng	nanogram
OD	optical density
PBS	phosphate buffer saline
PC	phosphorylchlorine
pHEMA	poly-2-hydroxyethyl methacrylate
PVP	poly(vinylpyrrolidone)
RGP	rigid gas permeable
RFU	relative fluorescence units
SD	standard deviation
sec	second
sIgA	secretory immunoglobulin A
TFA/ACN	trifluoroacetic acid/ acetonitrile
Tris	trimethylsiloxy silane
USAN	United States Adopted Names

1. Introduction

1.1 Contact lenses

Contact lenses are one of the most widely used biomedical devices in the world.[1] The idea of using contact lenses to correct vision was first introduced in 1508 and has evolved tremendously over the ensuing 500 years.[2] There are two main types of contact lens materials currently prescribed: soft hydrogel materials, which account for approximately 90% of contact lens fits, and rigid gas-permeable (RGP) materials which account for the remaining 10% of fits worldwide.[3] The major factors that account for the greater preference for soft lenses over RGP lenses include ease of fit for the clinician, faster initial lens adaptation, and improved comfort for many patients.[4]

1.1.1 Conventional hydrogel lenses

Poly-2-hydroxyethyl methacrylate (pHEMA) has been the pre-eminent material utilized in the production of soft hydrogel contact lenses since the 1970s. The incorporation of pHEMA in contact lenses allows superior water absorbance, [5] which increases lens wettability and initial comfort over rigid lenses. Soft lens materials with a higher water content provide greater oxygen permeability (Dk), as the water phase dissolves oxygen and permits diffusion to the cornea.[6] Achieving higher Dk levels for contact lens materials is essential to reduce hypoxic complications such as corneal edema and acidosis.[7-9] The maximum Dk of conventional hydrogels (which depend upon the water phase for oxygen transport) is dictated by the Dk of water, which has 80 Dk units. Therefore, although most hydrogel lenses contain high water

content, they do not provide the appropriate level of oxygen transmissibility (Dk/t) required for overnight or extended lens wear, in which oxygen levels are further reduced by eye closure.[10]

1.1.2 Silicone hydrogel lenses

In order to reduce the incidence of contact lens induced hypoxia, particularly during closed eye wear, scientists incorporated siloxane monomers into conventional hydrogel-based materials, to result in a new class of materials termed "silicone hydrogels". In these materials, the siloxane components primarily improve oxygen permeability and the hydrogel components offer the water absorbing abilities that permit these materials to be soft and flexible.[11, 12] Following their introduction in 1999, silicone hydrogel contact lenses became increasingly popular and are now the most commonly prescribed class of contact lens materials worldwide.[13] These lenses greatly reduce hypoxia-related ocular complications for both daily and extended lens wear,[14-16] as their oxygen transmissibility is multiple times greater than most conventional hydrogel lenses.[17, 18] The silicone component, however, creates a more hydrophobic lens surface, which consequently decreases lens wettability.[19] Thus, many silicone hydrogel lens materials undergo surface modifications or incorporation of internal wetting agents to overcome this issue. Examples of surface modifications include a plasma coating over the surface of lens materials such as lotrafilcon A and B, as well as various other plasma treatments.[11, 19-21] In a different approach, poly(vinyl pyrrolidone) has been integrated into silicone hydrogel lens materials, including senofilcon A and galyfilcon A, to increase the hydrophilicity of the contact lens. [22]

The differing compositions and properties of conventional and silicone hydrogel lens materials ultimately make each lens type unique. The United States Food and Drug

Administration (FDA) classifies and groups these lens materials based on their ionic charge and water content as follows:

Table 1-1. FDA classification of conventional and silicone hydrogel contact lenses

FDA Classification	**Group I**	**Group II**	**Group III**	**Group IV**
Water Content	Low < 50% water	High > 50% water	Low < 50% water	High > 50% water
Charge	Non ionic < 0.2% MA	Non Ionic < 0.2% MA	Ionic > 0.2% MA	Ionic > 0.2% MA

FDA (Food and Drug Administration); MA (Methacrylic acid)

Water content and ionic charge of a lens material impacts the interaction between the lens and the tear film, leading to very specific deposition profiles during lens wear. The following sections will discuss the three distinctive layers of the tear film and the major components in each layer.

1.2 Tear film

The anterior surface of the eye is covered by a thin fluid film (approximately 3-7μm thick) known as the tear film.[23-25] The tear film covers the underlying cornea and conjunctiva and serves to protect against foreign substances from the external environment, as well as maintaining the health of the eye.[26] Most textbook models broadly divide the tear film into three layers: the lipid layer, which is the most superficial layer, the middle aqueous layer and the inner-most mucin layer. Each layer contains various macromolecules that have specific tasks and assist in maintaining ocular health and function of the eye.[26] While some recent publications propose that these layers are not distinct and consist of various graded layers, the description below (for simplicity) will report on the composition of the tear film based on this trilaminar structure.

1.2.1 Lipid Layer

The meibomian glands are the primary source for the secretion of the lipid layer of the tear film.[27] These sebaceous glands are lined in parallel to each other in the upper and lower tarsal plates, perpendicular to the lid margins.[28] The lipid layer can be further divided into two phases, a polar and non-polar phase.[29-31] The polar phase, which is closest to the external environment, is mostly composed of phospholipids such as phosphatidylcholine and phosphatidylethanolamine,[32] and makes up less than 10% of the lipid layer. In comparison, the non-polar phase, which is situated closest to the aqueous layer and forms the majority of the lipid layer, is composed primarily of cholesterol and cholesterol esters, as well as hydrocarbons and triglycerides.[29, 30, 32, 33]

The lipid layer, which is approximately 0.1μm thick, serves as a protective barrier for both the eye and the tear film from foreign contaminants. [34] In addition, it provides stability for the tear film as it prevents the evaporation of tears when eyelids are opened, as well as maintaining a smooth tear film for the refraction of incoming light.[33, 35, 36] Lastly, the lipid layer acts as a lubricant which assists in eyelid movement during the process of blinking.[35]

Given that the lipid layer plays a big role in tear film stability, any disturbance to the lipid layer composition would have significant clinical complications. Meibomian gland dysfunction (MGD) is a chronic disease that is characterized by blockage of, or changes in, meibomian secretions.[37] As a consequence, the amount of lipids in the tear film is decreased, which promotes rapid evaporation of tears, causing dry eye.[37] Hence, the lipid layer is considered an important component in the tear film in maintaining the tear film intact over the anterior surface of the eye.

1.2.2 Aqueous Layer

The aqueous layer is mainly produced by the lacrimal gland, and also by the accessory glands of Krause and Wolfring. [38] The aqueous layer makes up the bulk of the tear film, with a thickness of about 6-7 μm and is composed of water and various water-soluble proteins, vitamins, cytokines, immunoglobulins, hormones, electrolytes and metabolites.[38, 39] The various electrolytes present in the aqueous layer include sodium, potassium, calcium, bicarbonates, chloride, and phosphate ions. These electrolytes regulate tear osmolality, as well as acting as a buffer to maintain the physiological pH of tears.[38] In previous studies, over 400 proteins have been identified in the human tear fluid.[40-42] These different proteins and antimicrobial agents in the aqueous layer are largely responsible for the prevention of viral and bacterial infections in the

eye.[26] Amongst the large class of proteins, lysozyme, lactoferrin, lipocalin and secretory immunoglobulin A are found to be the most abundant in tears,[43] and are all commonly involved in antibacterial activities. The following paragraphs provide a brief description of the major proteins and their function in the aqueous layer.

Lysozyme was discovered by Alexander Fleming in 1922 when he noticed that his nasal mucous, containing lysozyme, had wiped out an area of cultured bacterial cells.[44] This observation was later discovered to be caused by lysozyme, which catalyzes the hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in the peptidoglycan of Gram positive bacteria.[45] As a result, the cell wall ruptures, which leads to bacterial cell lysis. Lysozyme is primarily found in biological fluids such as mucous, saliva and tears and in only small concentrations in blood serum. The concentration of lysozyme in tears ranges from 1.2 - 4.6mg/mL in the open eye, which is higher than all other bodily fluids.[46] Lysozyme has a relatively low molecular weight (14 kDa) and is positively charged at pH 7.4.[47, 48] It is relatively stable at temperatures up to 55° C and in solutions with pH as low as 1.0.[48, 49]

Lactoferrin was first discovered as an iron chelator in human milk, but later in the 1960s, it was recognized to have bacteriostatic effects. [50, 51] Like lysozyme, lactoferrin is also found in many biological secretions such as saliva, milk and nasal secretions. In tears, the concentration of lactoferrin ranges from 1.7 - 2.5mg/mL.[26, 52, 53] Lactoferrin acts as an iron sequestering agent that allows it to chelate iron from bacteria, preventing bacterial growth.[54] Studies have also suggested that lactoferrin can enhance the activity of other proteins such as lysozyme and secretory IgA to support the ocular defence mechanism. [55-57]

Lipocalin has a molecular weight of 18-25 kDa and ranges from 1.2 – 1.7mg/mL in tears.[58, 59] Lipocalin has multiple binding sites that allow it to bind to a large diversity of macromolecules, including lipids and proteins.[60-62] Previous studies have suggested that lipocalin plays a large role in maintaining tear film stability by removing lipid contaminants from the ocular surface that prevent the proper formation of the tear film.[61, 62] This is beneficial for proteins such as lysozyme, which are inactivated by long chain fatty acids.[63] Furthermore, it has been suggested that negatively charged lipocalin in the tear film may bind to the positively charged proteins, lysozyme and lactoferrin, to perform synergistic antibacterial activities.[60]

Immunoglobulins are a large group of proteins found in lacrimal glands, with secretory immunoglobulin A (sIgA) being the most predominant.[52] The concentration of sIgA in tears ranges from 0.07 – 2.4mg/mL, which is much higher than in blood serum.[26] The molecular weight of sIgA is 450kDa and is formed by two IgA molecules in conjunction with a secretory component.[64] Secretory IgA has various roles in protecting the ocular surface from bacteria; it prevents microorganisms from binding to the ocular surface and promotes phagocytosis of bacteria by polymorphonculear leukocytes.[65, 66]

1.2.3 Mucin Layer

Conjunctival goblet cells are the primary source of mucin secretion, which form the mucin layer that maintains a wettable corneal surface. [67, 68] The mucin layer is 0.2 – 1.0μm thick and contains immunoglobulins, enzymes, urea, salts and glucose.[69] Mucins are large glycoproteins with molecular weights ranging from 3×10^5 to over 4×10^7 kDa and are classified as transmembrane or secretory mucins. [70] Transmembrane mucins form a protective barrier against pathogens at the epithelial cell-tear film interface, whereas secretory mucins move within

the tear film. [71] One of the primary functions of the mucin layer is to lubricate the palpebral conjunctiva, allowing smooth movements of the eyelid during blinking. [67]

The moment a contact lens is fitted on the ocular surface, the tear film immediately interacts with the contact lens material, leading to various lens depositions. The next section will discuss the major tear film components that accumulate on silicone and conventional hydrogel lenses.

1.3 Contact lens deposition

1.3.1 Major deposited proteins

Amongst all tear film components, proteins are the primary component that deposits on conventional hydrogel contact lenses during lens wear. Lysozyme, lactoferrin and albumin are examples of major proteins that can typically be detected on worn contact lenses.[38, 72-75]

Lysozyme is a small compact protein that exhibits a strong positive charge in the tear film environment (pH 7.4) and selectively accumulates on various contact lens materials, particularly FDA group IV lenses materials.[48, 73, 76, 77] Studies have reported that FDA group IV lens materials deposit approximately 1000µg of lysozyme, whereas the remaining FDA group I, II and III lens materials accumulate typically less than 60µg.[76, 78, 79] Like lysozyme, lactoferrin is also positively charged, however less than 20µg of lactoferrin adsorb onto any of the four FDA materials[74, 80] due to its greater molecular weight, fewer positive binding sites and overall lower charge.[81-83] In contrast, the protein albumin has an overall negative charge and is found in lower concentrations in tears (10 - 24µg/mL).[84] Albumin adsorbs in greater amounts on FDA group I lens materials compared to lysozyme and lactoferrin.[75]

Protein adsorption onto biomaterials such as contact lenses is a complex process and influenced by various factors. Protein charge, size and concentration play key roles in the deposition process, especially if other tear components are present to compete for binding sites.[81, 83 85] Lens materials with an ionic charge will allow strong electrostatic interactions with oppositely charged proteins, thus further enhancing the level of uptake.[86, 87] Protein deposition will also increase if the lens material has big enough pore sizes that allow the protein to diffusively penetrate into the matrix of the lens.[87]

The process that leads to the deposition of proteins onto contact lenses can be divided into 3 steps:[88]

1. Transport of protein towards the liquid-solid interface
2. Attachment of protein at the interface
3. Conformational changes of the adsorbed protein

The initial transport step is simply the diffusion of the protein across the tears towards the lens surface. Hence, higher concentrated proteins in the tears have a higher chance of interacting with the surface compared to proteins that are less abundant.[85] When the protein binds to the surface of the lens it may undergo structural rearrangements in order to achieve the most thermodynamically favourable state.[88] Proteins that have a strong structural stability undergo minimal structural changes, whereas proteins with lower internal coherence will adsorb to the surface even if structural rearrangements are unfavourable.[81] Consequently, the adsorbed protein partially loses its tertiary and secondary structure and becomes "denatured", which may result in a loss of its biological function.[89, 90] There are several variables that influence the degree of denaturation of adsorbed proteins, including the amount of time the protein is bound to the surface, the hydrophobicity of the surface and the location of the deposited lysozyme on or in the lens material. [79] Lysozyme denaturation increases over time, and the rate is higher for lens materials that are relatively hydrophobic.[79] It has further been suggested that lysozyme which penetrates into the lens matrix is less likely to denature than when tightly bound to the lens surface.[48, 79, 91]

1.3.2 Major deposited lipids

Silicone hydrogel lenses deposit higher amounts of lipids as compared to conventional hydrogel lenses.[73, 92-94] The deposition of lipids is primarily due to the adherence between the hydrophobic lipids with the hydrophobic sites on the lens material.[95] Hence, because silicone-based lens materials are relatively hydrophobic, tear film lipids are more likely to adhere during lens wear. Over 45 different types of lipids have been identified in the tear film,[29-31] but cholesterol, cholesteryl esters, triglycerides and waxy esters have been found to be major components in lipid deposits on contact lenses.[96-98] Cholesterol and phosphatidycholine has also been recently shown to accumulate in higher amounts on silicone hydrogel lenses compared to FDA group IV lenses. [92]

Like proteins that undergo structural changes following adsorption, lipids can undergo oxidative degradation, which occurs when lipids lose electrons under oxidative stress. [99] To date, there are a limited number of studies that have investigated the effects of contact lens wear on lipid oxidation. However, a previous study has associated increased amounts of lipid peroxidation products in tears with patients who suffer from dry eyes. [100]

1.4 Complications from contact lens deposition

Contact lenses are susceptible to spoilage from the various tear film constituents such as lipids,[101, 102] proteins[103, 104] and mucins.[75, 105] These deposits may potentially decrease visual acuity and may cause dryness and discomfort, which ultimately leads to intolerance and discontinuation of lens wear.[106-110] Certain tear film deposits have further shown to promote bacterial adhesion,[111-113] which may increase the risk of ocular infections such as bacterial keratitis.

Another clinical concern associated with contact lens deposits is the initiation of inflammatory responses. Denatured protein on contact lenses has been associated with a condition known as contact lens induced papillary conjunctivitis (CLPC).[114, 115] Symptoms of CLPC typically include ocular redness, increased mucus production due to ocular irritation, burning, itching, increased lens awareness and lens intolerance.[116] CLPC can develop during rigid and soft hydrogel lens wear, however it is more commonly seen in soft hydrogel lens wearers and symptoms are also typically noted sooner with hydrogel lens wear.[116-118] Although the exact cause of CLPC is unclear, many factors may promote its development. It was previously suggested that tear film deposits on the lens surface act as an antigenic stimulus, which leads to the production of various immunoglobulins and immune response initiators such as C3 anaphylatoxin (C3a). These immunoglobulins, along with C3a, interact with mast cells and basophils, leading to a release of vasoactive amines, which are responsible for the development of CLPC symptoms. In addition, the presence of a contact lens can also cause mechanical trauma to the conjunctiva, which leads to a production of inflammatory mediators and a subsequent recruitment of leukocytes to the conjunctiva. These leukocytes will interact with immunoglobulins and C3a, thus further increasing the discharge of vasoactive amines.[116]

Hence, the development of CLPC can be initiated by the presence of deposits on contact lenses, as well as the physical presence of the contact lens that induces trauma to the conjunctiva.[119]

Several *in vitro*[75-77] and *ex vivo*[104, 120] studies have investigated the deposition of proteins and lipids on conventional and silicone hydrogel contact lenses over the past few decades. These studies have used numerous methods to identify and quantify protein and lipids deposits, which will be discussed in the subsequent sections. For the purpose of this thesis, the sections will solely focus on protein quantification techniques.

1.5 Quantification of protein deposits on contact lenses

The quantification and identification of deposited proteins typically require the extraction of the tear film deposit from the lens material prior to analysis. In the past, different solvents have been used to remove the protein from the contact lens, including sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), urea and TrisHCl.[102, 121-123] The use of heat and sonication in combination with extraction solvents increases the efficiency of protein removal from biomaterials.[124, 125] Unfortunately, most extraction procedures are ineffective in removing 100% of the protein deposited on a lens.[126] More recently, Keith et al.[72] introduced a more efficient extraction solvent, consisting of trifluoroacetic acid and acetonitrile, which can remove up to ~100% of the total lysozyme from certain lens materials. Many studies have since utilized this technique.[79, 104, 120, 123, 127] Following the elution procedure, the extracted proteins can be quantified using a variety of methods, that will be described below.

1.5.1 Bicinchoninic acid (BCA) analysis

The principle behind the BCA analysis is based on two separate reactions: Proteins in an alkaline solution containing copper ions (Cu^{+2}) reduce the Cu^{+2} ions into cuprous ions (Cu^{+1}).[128] The bicinchoninic acid in the solution then reacts with the Cu^{+1} ions, resulting in the formation of a purple colored complex.[128] The color intensity of this reaction product increases in proportion to increasing protein concentrations in the sample and can be measured spectrophotometrically. This method is highly sensitive and shows low protein-to-protein variation compared to other protein assays.[128] However, in samples containing multiple proteins, the BCA assay will be unable to quantify individual proteins.

1.5.2 High performance liquid chromatography (HPLC)

HPLC is a chromatographic method used to separate proteins based on molecular size,[129] for the purposes of identifying and quantifying proteins in a complex solution, like the tear film. The separation procedure involves mass transfer between stationary and mobile phases. The proteins in the sample are added to a solvent, which is then forced to flow through a chromatographic column to separate the individual proteins in the mixture. As each protein component exits from the HPLC, a detector identifies and quantifies the individual proteins in the solution based on their retention time. [129] Unlike the BCA assay, the HPLC method is able to quantify individual proteins in a complex protein mixture. However, a previous study[130] had found that lysozyme exhibits unusual high absorbance in the HPLC, which consequently interferes with the HPLC measurements.

1.5.3 Western blotting and enzyme-linked immunosorbent assay (ELISA)

Western blotting is another technique used to quantify proteins extracted from contact lenses. This technique identifies and quantifies proteins in a sample using antibodies specific to the protein of interest.[131] The proteins in the sample are first separated by gel electrophoresis and subsequently transferred to a nitrocellulose or polyvinylidene fluoride (PVDF) membrane.[131] The membrane is then stained by antibodies to visualize the proteins.

The enzyme-linked immunosorbent assay (ELISA) is a similar technique that uses antibodies for protein detection. ELISA relies on the recognition of the protein under test by a detection antibody that is covalently bonded to an enzyme.[132] Once the antibody is bound to the protein of interest, a substrate (dye) that produces a visible color in the presence of the enzyme is added.[132] The intensity of the signal produced by the enzyme-substrate reaction is proportional to

the amount of a specific protein in the sample. The ELISA technique can process up to 96 samples at once, which is a major advantage over the Western blotting technique.

1.5.4 Radiolabelling of proteins

Radiolabelling is a highly sensitive *in vitro* technique, which provides a quick and simple way to quantify the protein of interest deposited onto contact lenses. This technique allows a simultaneous detection of proteins on the lens surface and in the bulk and does not necessitate the protein be extracted from the lens. Furthermore, this technique has been shown to be compatible with all contact lens materials.[75, 76, 78, 80, 133-136] A disadvantage of this technique is the possibility of the radioactive isotope detaching from the protein of interest and interacting with other components in a complex solution or with the contact lens material and therefore generating false results. Hence a dialyzing procedure is often utilized in order to reduce the amount of free radioactive isotopes in the solution.

1.5.5 Methods to determine protein denaturation

The conformational state of deposited proteins on contact lenses is of clinical relevance due to its association with ocular inflammatory conditions. Different techniques have been used to study the structural arrangement of deposited proteins, such as electron spin resonance (ESR) spectroscopy and Fourier transform infrared-attenuated total reflectance spectroscopy (FTIR-ATR).[137, 138] The micrococcal turbidity assay is specifically used for lysozyme and relies on the lytic activity of this enzyme against *Micrococcus lysodeikticus* bacteria.[73, 77, 79, 120, 123] These Gram positive bacteria form a yellow colored solution, when suspended in a buffer. When active lysozyme is then added, the decrease in optical density (OD) of the solution can be monitored over time. The greater the concentration of active lysozyme, the quicker the solution becomes

transparent. This method has been used previously to monitor the kinetic denaturation of lysozyme extracted from silicone and conventional hydrogel lens materials.[79]

Enzchek® is a novel fluorescence-based lysozyme activity assay that effectively detects active lysozyme in cell culture.[139, 140] Like the turbidity assay, the fluorescence-based assay relies on the lytic activity of lysozyme on the fluorescently tagged *Micrococcus lysodeikticus*. As the bacteria are lysed, the reaction products release a fluorescence signal that is used to quantify the amount of active lysozyme in the sample. Although this novel method has been shown to have high sensitivity and a wide range of detection,[141] to date it has not been used in contact lens research.

1.6 In vitro, in vivo and ex vivo studies

In vitro, (Latin: “in glass”), refers to experimental investigations that are conducted outside of a living organism. The purpose is to study biological properties in a controlled environment, rather than in a human or animal. In contrast, *in vivo*, (Latin: “in life”), refers to studies that are done in/on a living organism. Sometimes, the living tissue can be extracted from a human or animal and studied in another artificial environment outside of the organism. This type of experimentation is referred to as *ex vivo*, (Latin: “out of the living). *In vitro* and *ex vivo* studies are often preferred over *in vivo* studies as they allow the experimental testing on certain aspects of a living organism that would typically be impossible to conduct on an intact organism. However, the main disadvantage for conducting *in vitro* studies is that they often struggle to closely mimic *in vivo* conditions in order to produce results that correlate with real life outcomes. The following table summarizes the advantage and disadvantages of conducting *in vitro* and *in vivo* studies.

Table 1-2. Advantages and disadvantages of *in vitro* and *in vivo* studies

	In vitro	***In vivo***
Advantages	• Study biological properties that would normally be inconvenient if conducted *in vivo* • Relatively inexpensive and quicker than *in vivo studies* • Experimental variables are controlled by researcher • Key components can be isolated from the system and studied individually	• Results directly translate to real life outcomes • Includes all parameters that normally influence the result in real life
Disadvantages	• Difficult to mirror *in vivo* conditions • Results may not always correlate with *in vivo* results	• Cost and time demanding • Difficult to make cause and effect conclusions with multiple uncontrolled variables present

Over the past years, a large number of *in vitro* studies[76, 78, 81, 82, 136] have been conducted to investigate protein deposition on contact lens materials, however, most of these studies incubate their contact lenses in solution containing only one protein of interest.[75, 77, 80, 81, 135] These incubation solutions do not take into account the impact of other tear film components and as a result protein sorption profiles differ between *in vitro* and *ex vivo* studies.[73, 77, 104, 142]

2. Thesis Rationale

Tear film deposition on contact lens materials have been investigated extensively. Protein deposits are often associated with reductions in visual acuity and may lead to symptoms of dryness and discomfort during lens wear. In more severe cases, denaturation of proteins on the lens material may trigger immunological responses, leading to ocular complications such as papillary conjunctivitis. Thus, protein adsorption and changes in protein conformation during the deposition process have attracted much attention from both clinicians and scientists.

Lysozyme is a major protein constituent of the human tear film and has been shown to selectively deposit to various lens materials, particularly negatively charged hydrogel materials. For these reasons, lysozyme is often used as a model protein for contact lens deposition studies. A number of previous *in vitro* studies have investigated lysozyme accumulation and denaturation on both silicone and conventional hydrogel lenses. However, these studies often incubated their lenses in a simplistic solution composed of lysozyme dissolved in phosphate buffered saline. These incubation solutions are not accurate portrayals of the human tear film, which contains numerous proteins, lipids, mucins and electrolytes. To enable the interactions between tear film components and to allow competitive uptake during the deposition process, a more complex artificial tear solution was used to study the impact of major tear film components on lysozyme deposition and denaturation on contact lens materials.

In the first study of this thesis (Chapter 3), an artificial tear solution was used to investigate the impact of major tear film components on the deposition profile of lysozyme to silicone and conventional hydrogel lenses. The tear film protein lactoferrin and tear lipids were selected, as they represent primary components of the tear film. Lactoferrin is a positively

charged protein which has a similar abundance in the tear film as lysozyme and plays a major role in defence against ocular surface infection. Tear lipids are primarily found in the outermost layer of the tear film and prevent excessive evaporation of the tears, thus maintaining the stability of the tear film. Some of these lipids have primarily hydrophobic properties and deposit in relatively high amounts on silicone hydrogel lenses.

In Chapter 4 of this thesis, a novel fluorescence-based assay was compared with the classical micrococcus turbidity assay to determine the activity of lysozyme. The traditional micrococcus turbidity assay is time consuming and has difficulties detecting low levels of active lysozyme. Recently, a novel fluorescence-based lysozyme activity assay was developed for cell culture studies, which is more time efficient and can detect lower amounts of active lysozyme when compared to the turbidity assay. This novel method had not previously been used in contact lens studies and a validation study was undertaken to investigate its utility for investigating lysozyme activity with soft lens polymers.

The last study of this thesis (Chapter 5) investigated the effects of lactoferrin and lipids on lysozyme activity when deposited on silicone and conventional hydrogel lenses. Lactoferrin is known for its bacteriostatic function, and in combination with lysozyme it has a synergistic effect on the lytic activity of lysozyme against Gram positive bacteria. However, it was unclear whether lactoferrin may also have an effect on the activity of lysozyme during the adsorption process. Various contact lens materials were used to confirm the hypothesis that lysozyme denatures more quickly on relatively hydrophobic surfaces. In addition, the presence of lipid may change the hydrophilicity of the contact lens surface, which could impact the denaturation rate of a subsequent lysozyme layer.

The results from this thesis will determine the impact of major tear film components on the deposition profile of lysozyme to different contact lens materials. It will also confirm whether the use of an artificial tear film model that closely resembles the human tear film is able to more accurately mimic the amount and conformational state of deposited lysozyme that occurs *in vivo* on contact lens materials. These findings will help in designing future *in vitro* deposition studies.

3. Impact of Tear Film Components on Lysozyme Deposition to Contact Lenses

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Ng A, Heynen M,Luensmann D, Jones L. Impact of tear film components on lysozyme deposition to contact lenses. Optom Vis Sci 2012; 89:392-400.

3.1 Overview

Purpose: To investigate the impact of lactoferrin and lipids on the kinetic deposition of lysozyme on silicone and conventional hydrogel lenses, using a complex artificial tear solution.

Methods: Two silicone hydrogel lenses [AIR OPTIX AQUA; lotrafilcon B and ACUVUE OASYS; senofilcon A] and two conventional hydrogel lenses [ACUVUE 2; etafilcon A and PROCLEAR; omafilcon A] were investigated. Lenses were incubated in four different solutions: A complex artificial tear solution (ATS) consisting of various salts, lipids, proteins and mucins, an ATS without lactoferrin (ATS w/o Lac), an ATS without lipids (ATS w/o Lip) and an ATS without lactoferrin and lipids (ATS w/o Lac & Lip), each containing 2% radiolabeled (125I) lysozyme (1.9mg/ml). After each time point (4, 12hours and 1, 2, 3, 5, 7, 14, 21, 28 days), the amount of lysozyme per lens was quantified.

Results: After 28 days, lotrafilcon B lenses incubated in ATS deposited significantly less lysozyme (9.7±1.4µg) than when incubated in solutions not containing lactoferrin and lipids (>11.8 µg) ($p<0.001$). Lysozyme uptake to senofilcon A lenses was higher in ATS w/o Lip (5.3±0.1µg) compared to other solutions (<3.9µg) ($p<0.001$). Etafilcon A lenses deposited the most lysozyme in all solutions compared to the rest of the lens types ($p<0.001$). For etafilcon A lenses, less lysozyme was deposited when incubated in ATS w/o Lip (588.6±0.4µg) compared to the other solutions (>642.6µg) ($p<0.001$). Omafilcon A lenses in ATS w/o Lac accumulated significantly less lysozyme (12.8±1.0µg) compared to the other solutions (>14.2µg) ($p<0.001$).

Conclusions: An ATS containing lactoferrin and lipids impacts lysozyme deposition on both silicone and conventional hydrogel contact lenses. When using in vitro models to study protein

deposition on contact lenses, more complex models should be used to better mimic the human tear film.

3.2 Introduction

Contact lenses are biomedical devices used to correct vision and it is estimated that over 125 million people in the world are contact lens wearers[1] Despite their popularity, dryness and lens discomfort, particularly at the end of the day, continue to be common concerns and have become the primary reasons for discontinuation of contact lens wear.[2-4] The cause for dryness and discomfort has been linked to tear film deposition onto contact lenses in some studies.[5-7]

A number of *in vitro*[8-10] and *ex vivo*[11, 12] studies have investigated tear film protein deposition on silicone and conventional hydrogel lens materials. Simple *in vitro* models are often used to predict *ex vivo* results due to lower costs and time commitments compared to more complex *in vivo* studies. *In vitro* studies that have investigated protein deposition on contact lenses have typically relied on the use of very simplistic tear film models,[9, 13] which may not be a very accurate portrayal of the human tear film. Very few studies have evaluated the differences between *ex vivo* and *in vitro* models and it is relatively unclear which components of the human tear film primarily influence the accumulation of certain tear proteins to contact lenses.[14, 15]

The tear film is a complex aqueous solution consisting of various lipids, proteins, metabolites and electrolytes.[16, 17] Within this complex composition, lysozyme is found to be the most abundant protein.[18, 19] Lysozyme is a small bacteriolytic enzyme (14 kDa)[20, 21] that interferes with the specific bonds responsible for protecting and maintaining the shape of the exoskeleton in the bacterial cell wall.[22] Lysozyme has a large net positive charge thus allowing it to bind relatively easily to negatively charged substrates.[23-25] Lysozyme has drawn much attention in contact lens deposition studies in recent years due to its selective absorbance to ionic contact lens materials.[23] Protein deposits on contact lenses can result in discomfort,[3, 4] decreased

visual acuity[26] and inflammatory conditions involving papillary changes in the tarsal conjunctiva known as giant papillary conjunctivitis.[11, 27, 28]

Tear film deposition on contact lens materials has been previously shown to be mediated by the physical properties of the lens surface such as surface charge and hydrophobicity.[23, 29, 30] Lysozyme binds in high amounts to ionic polyhydroxyethylmethacrylate (pHEMA) hydrogels, while only a fraction of this level of deposition is typically seen on silicone hydrogel lenses.[5, 9, 10, 31-33] However silicone hydrogel materials appear to deposit lipids relatively quickly[34] and at higher amounts compared to pHEMA lenses.[32] In addition, another major protein found in the tear film, lactoferrin, has shown similar characteristics to lysozyme in selectively binding onto ionic pHEMA-based contact lenses.[25, 35]

As described above, it is accepted that the composition of contact lens materials determines subsequent tear film deposition. However, little is known about the importance of the tear components on protein uptake and whether it has the same impact for different materials. Therefore, the purpose of this study was to investigate the impact of individual tear film components on the kinetics of lysozyme deposition to silicone and conventional hydrogel lens materials using a complex artificial tear solution (ATS).

3.3 Materials and Methods

In this *in vitro* study, two silicone hydrogel and two conventional pHEMA-based lens materials were investigated. All lenses were unworn, with a power of -3.00 Diopters (D) and their individual properties are summarized in Table 3-1.

Table 3-1. Properties of contact lens materials investigated in this study

	Silicone Hydrogel		Conventional Hydrogel	
USAN	Lotrafilcon B	Senofilcon A	Etafilcon A	Omafilcon A
Proprietary Name	AIR OPTIX AQUA	ACUVUE OASYS	ACUVUE 2	PROCLEAR
Manufacturer	CIBA Vision	Johnson & Johnson	Johnson & Johnson	CooperVision
Water Content (%)	33%	38%	58%	62%
Surface Treatment	25nm plasma coating	No surface treatment. Internal wetting agent (PVP)	None	None
FDA group	I	I	IV	II
Ionicity	Non ionic	Non ionic	Ionic	Non ionic
Principal Monomers	DMA+TRIS+ Siloxane monomer	mPDMS+DMA+ HEMA + siloxane macromer +TEGDMA	HEMA+MA	HEMA+PC

DMA - N,N-dimethylacrylamide; FDA - Food and Drug Administration; HEMA - (poly-2-hydroxyethyl methacrylate); MA - methacrylic acid; mPDMS - monofunctional polydimethylsiloxane; PC - phosphorylcholine; PVP - polyvinyl pyrrolidone; TEGDMA - tetraethyleneglycol dimethacrylate; TRIS - trimethylsiloxy silane; USAN United States Adopted Names

3.3.1 *Artificial Tear Solution (ATS)*

Contact lenses were incubated in an ATS consisting of a mixture of proteins, lipids, mucins, salts, glucose (all Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and urea (EMD Science, VWR, Mississauga, Canada) and were adjusted to a physiological pH of 7.34. The detailed composition of the ATS is based on a previous study by Lorentz et al[36] (see Table 3-2). In order to examine the impact of lactoferrin and lipids on lysozyme deposition, the contact lenses were incubated in four different solutions (Table 3-3).

Table 3-2. Artificial tear solution components

Salt Components (mg/mL)		**Lipid Components** (mg/mL)	
NaCl	5.26	Oleic Acid	0.018
KCl	1.19	Oleic Acid Methyl Ester	0.012
Na_2CO_3	1.27	Triolein	0.016
$KHCO_3$	0.30	Cholesterol	0.0018
$CaCl_2$	0.07	Cholesteryl Oleate	0.024
$Na_3C_6H_5O_7$	0.44	Phosphotidylcholine	0.0005
Urea	0.072		
Glucose	0.036	**Protein Components** (mg/mL)	
Na_2HPO_4	3.41	Bovine Albumin	0.20
HCl	0.94	Hen Egg Lysozyme	1.90
		Bovine Mucin	0.15
ProClin 300	200µL per litre of solution	Bovine IgG	0.02
		Lactoferrin	1.80

Table 3-3. List of solution combinations used for incubation

Solution	
1	Complex ATS with all its tear film components (ATS)
2	ATS without lactoferrin (ATS w/o Lac)
3	ATS without lipids (ATS w/o Lip)
4	ATS without lactoferrin and lipids (ATS w/o Lac & Lip)

ATS (artificial tear solution); Lac (lactoferrin); Lip (lipids)

3.3.2 *Pre-treatment of vials and lenses*

Previous work in our laboratory demonstrated that the glass vials (6mL, Wheaton Science Products, Millville, NJ, USA) used during the lens incubation period bound proteins and lipids to their walls. Specifically, up to 80% of the lipids from the ATS bound to the glass walls of the vials within four days of incubation, resulting in a reduction in their concentration within the soaking solution. Therefore, to prevent loss of macromolecules to the glass surface during lens incubation, the vials were pre-treated for 7 days with 1.5mL of solution at 37^{o}C. The solution used for the pre-treatment phase matched the subsequent solution used for lens incubation, with the exception of lactoferrin, which was excluded from the pre-treating solution due to cost considerations. Each solution was replaced with fresh solution for incubating the lens materials.

Prior to the incubation, lenses were removed from their blister packages using sterile silicone tipped forceps, rinsed in a saline solution for 2 seconds and then immersed in 5mL of an augmented buffered saline solution containing the salt components listed in Table 3-2, at room temperature for 24 hours. This solution was adapted from a previous study conducted by

Lorentz and colleagues[36]. This pre-soaking step was performed to remove any blister pack solution and prevent potential carryover into the incubation solutions.

3.3.3 *Incubation of lenses*

Following pre-treatment, the pre-treating solution was removed from the vials and subsequently replaced by 1.5mL of one of the four incubation solutions. Lenses were placed in individual vials and incubated at 37^{o}C. In order to study the kinetics of the lysozyme deposition, lenses were incubated for ten different time periods (4 and 12 hours, 1, 2, 3, 5, 7, 14, 21, and 28 days). Three replicates for each condition were used, thus a total of 480 contact lenses was used in the study. Each lens type was incubated in 1.5mL of incubation solution, with the exception of the etafilcon A lenses, which were soaked in 6.0mL. The reason for this extra volume is that previous work (unpublished) demonstrated that the amount of lysozyme uptake to this lens material is more than that available in 1.5mL,[9, 10] which resulted in total depletion of the lysozyme from this volume of solution before the lens material was "saturated". For this reason, a higher volume of incubation solution for etafilcon A lenses were used to ensure a sufficient amount of lysozyme for maximum uptake.

3.3.4 *Quantifying lysozyme deposits on lenses*

To quantify the amount of lysozyme deposited on lenses, each solution contained 98% hen egg lysozyme (HEL), with the remaining 2% lysozyme being conjugated with a radioactive iodine isotope (I^{125}), which was used as a reporter molecule. Following each time point, lenses were removed from the solution using sterile forceps and rinsed in a complex salt solution for 2 seconds to remove unbound lysozyme from lenses. Afterwards, lenses were blotted dry on lens paper and inserted into culture tubes (VWR, Mississauga, ON, Canada). Lysozyme uptake on

each lens was quantified by counting radioactive lysozyme using a gamma counter (1470 Wallac Wizard PerkinElmer, Woodbridge, ON, Canada).

3.3.5 *Statistical analysis*

Data analysis was conducted using Statistica 8 software (StatSoft Inc, Tulsa, OK, USA). A repeated measures analysis of variance (ANOVA) was used to determine statistically significant differences between lysozyme deposition across various time points and between materials. A post-hoc Tukey-HSD (Honestly Significant Difference) test was performed. A p value of <0.05 was considered significant. Lysozyme deposition was considered to plateau when no significant increase was found between the respective time point and day 28 ($p>0.05$).

3.4 Results

A comparison of kinetic lysozyme uptake between the four materials revealed that etafilcon A lenses deposited significantly higher amounts of lysozyme with all four solutions at any time point, compared to the other lens materials ($p<0.001$). The amount of lysozyme deposited on the etafilcon A material after only 4 hours of incubation was significantly greater than the amount of lysozyme deposited on the other three materials after 28 days of incubation ($p<0.001$). In contrast, senofilcon A lenses deposited lower amounts of lysozyme in comparison to the other 3 lens types throughout the 28 day period ($p<0.001$).

Figure 3-1 displays the kinetic lysozyme uptake to lotrafilcon B using the four different solutions. Within the first 4 hours of incubation, lysozyme deposition when incubated in the ATS was significantly lower (6.1±0.4µg) compared to the ATS without lactoferrin (8.4±0.4µg), the ATS without lipids (12.3±0.5µg) and the ATS without lactoferrin and lipids (13.4±0.9µg) (all

$p<0.001$). There was a significant difference in average lysozyme uptake to lotrafilcon B lenses between all four incubating solutions following the 28 days ($p<0.001$). Furthermore, incubation in ATS without lactoferrin and lipid yielded significantly greater lysozyme uptake compared to the other solutions throughout the 28 days ($p<0.001$). Incubation in ATS without lipids displayed significantly greater lysozyme deposition compared to the amounts deposited from an ATS without lactoferrin ($p<0.001$). At the end of the 28 day incubation period, the total amount of lysozyme deposited on lotrafilcon B lenses when incubated in ATS was significantly less (9.7±1.5µg) than in ATS without lactoferrin and lipids (17.0±1.6µg) and ATS without lactoferrin (11.8±0.4µg) and ATS without lipids (15.8±0.8µg) ($p<0.001$). The amount of lysozyme uptake reached a plateau at 12 hours when exposed to ATS without lactoferrin and lipids, whereas incubation in ATS without lactoferrin and ATS without lipids displayed a plateau in deposition at the beginning of day 1 ($p>0.05$). Lastly, a plateau was reached on day 5 of incubation in the ATS ($p>0.05$).

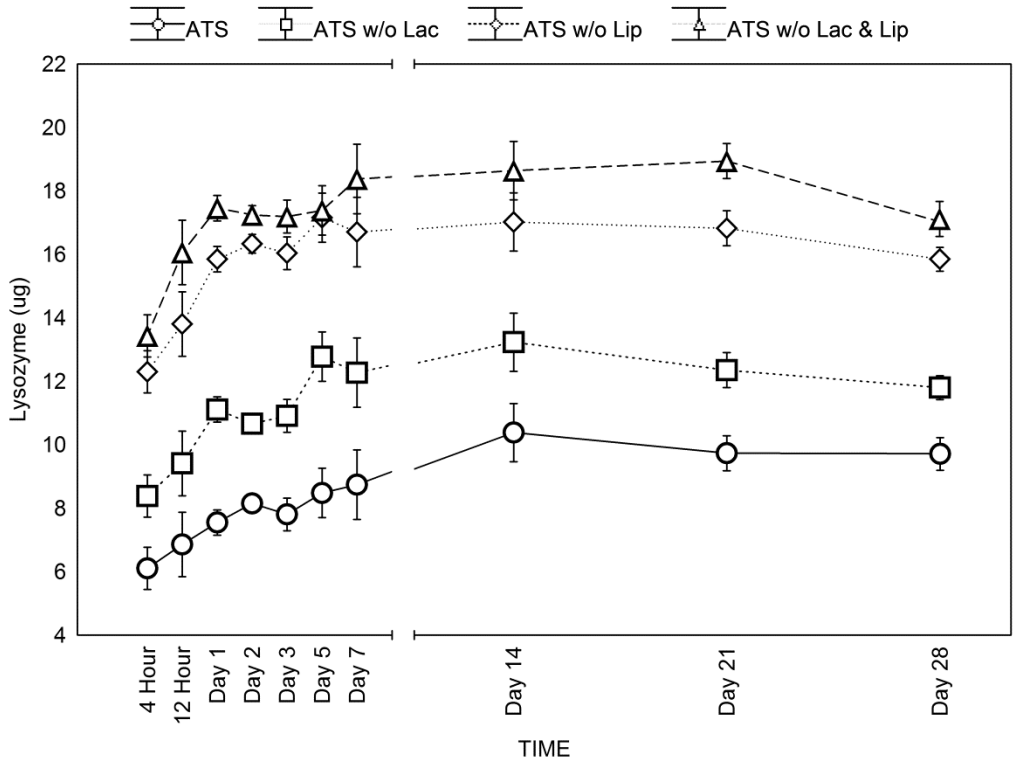

Figure 3-1. Kinetic lysozyme uptake to lotrafilcon B lenses over a period of 28 days. The solutions used for incubation were a complex artificial tear solution with multiple tear film components (ATS), an ATS without lactoferrin (ATS w/o Lac), an ATS without lipids (ATS w/o Lip) and an ATS without lactoferrin and lipids (ATS w/o Lac & Lip).

Figure 3-2 shows the kinetic lysozyme deposition on senofilcon A lenses after incubation in all four solutions. Following the first 4 hours of incubation, the amount of lysozyme uptake between the four incubation solutions was no higher than 1.5±0.1µg. There were no significant differences in lysozyme uptake between the four incubation solutions until after 12 hours of incubation, where lysozyme uptake was significantly greater in ATS without lipids compared to ATS ($p<0.001$). Higher amounts of lysozyme were also detected using ATS without lipids between study day 2 and day 28 compared to ATS without lactoferrin ($p<0.001$). In addition, lysozyme deposition with ATS without lipids was greater than with ATS without lactoferrin and lipids from day 21 onwards ($p<0.001$). At the end of the day 28, total lysozyme accumulated

from incubating in ATS without lipids was significantly higher (5.3±0.1µg) compared to the other solutions, where deposition was no more than 3.9µg ($p<0.001$). Lysozyme deposition reached a plateau on senofilcon A lenses at day 14 when incubated in ATS, ATS without lactoferrin and ATS without lactoferrin and lipids ($p>0.05$). There was no plateau when lenses were incubated in ATS without lipids by the end of the 28 day incubation period ($p<0.001$).

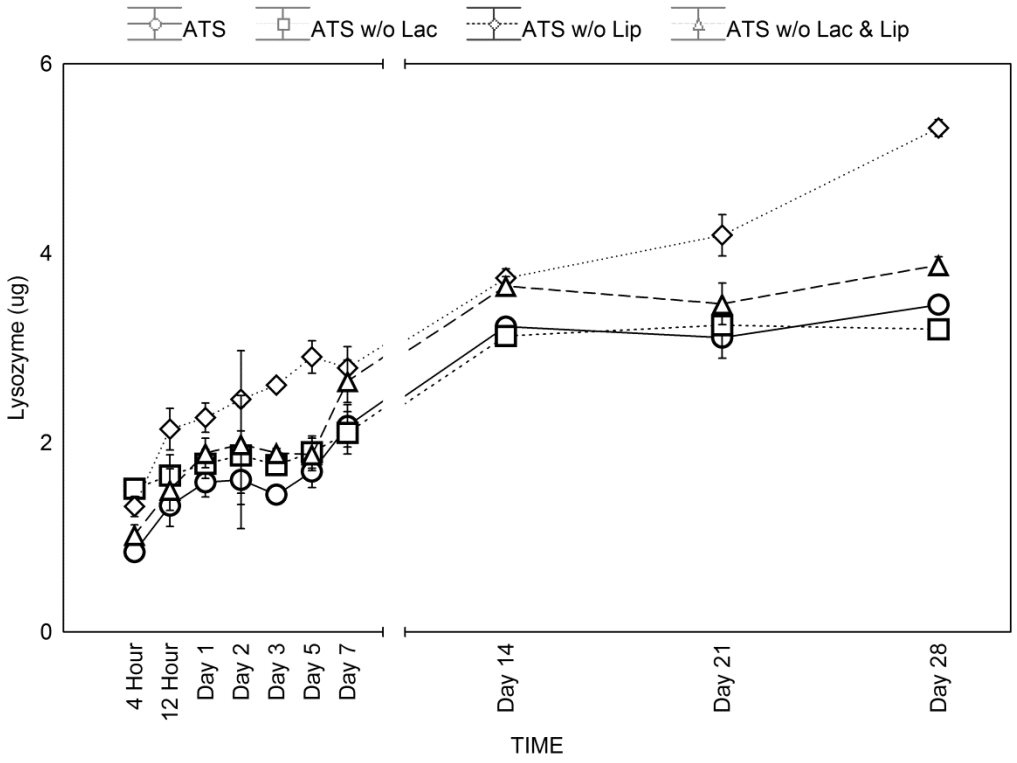

Figure 3-2. Kinetic lysozyme uptake to senofilcon A lenses over a period of 28 days. The solutions used for incubation were a complex artificial tear solution with multiple tear film components (ATS), an ATS without lactoferrin (ATS w/o Lac), an ATS without lipids (ATS w/o Lip) and an ATS without lactoferrin and lipids (ATS w/o Lac & Lip).

The kinetic lysozyme deposition on etafilcon A lenses after incubation with all four solutions is displayed in Figure 3-3. Within 4 hours of incubation, etafilcon A lenses accumulated greater than 225.8±0.2µg of lysozyme in each of the incubation solutions. After the

first 12 hours, kinetic lysozyme deposition on etafilcon A lenses was significantly less using the ATS without lipids compared to the ATS without lactoferrin and lipids ($p<0.001$). Lysozyme uptake from incubating in ATS without lipids was significantly less after day 3 compared to ATS without lactoferrin ($p<0.001$). Furthermore, after day 5, lysozyme deposition was found to be significantly less when incubated in ATS without lipids compared to ATS ($p<0.001$). Total lysozyme accumulated from incubating in ATS without lipids was significantly lower (588.6±0.4µg) compared to the other solutions where the total amount of lysozyme uptake was more than 642.6µg at the end of day 28 ($p<0.001$). A plateau in lysozyme deposition on etafilcon A lenses was reached in all incubating solutions by day 7 of incubation ($p>0.05$).

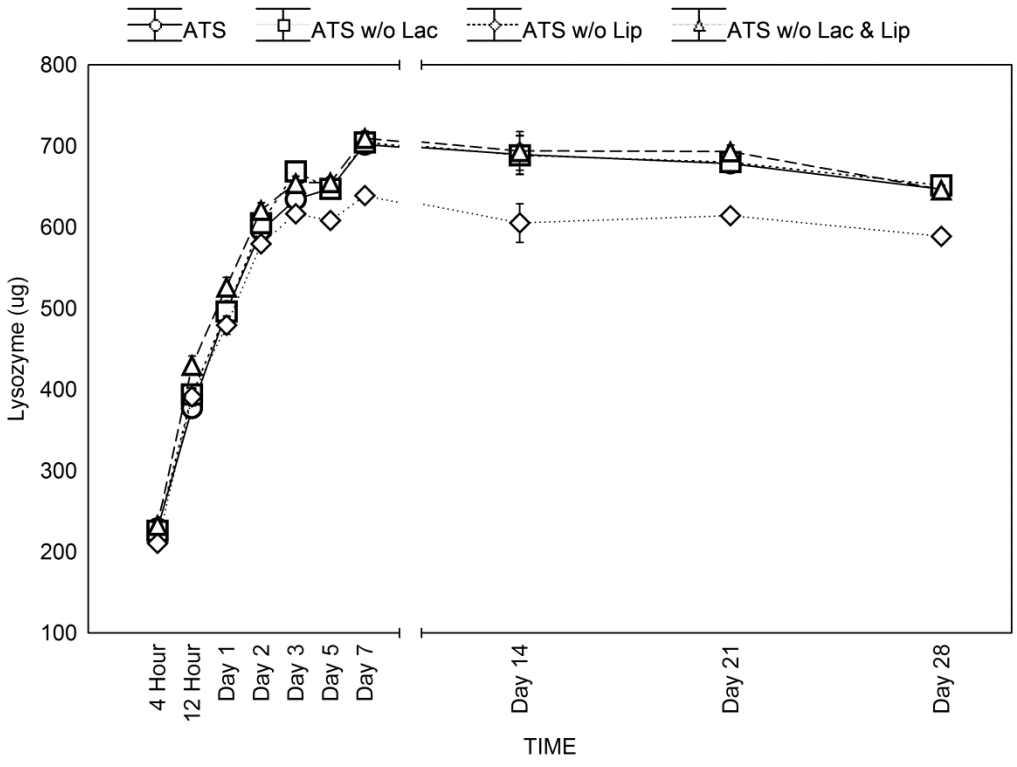

Figure 3-3. Kinetic lysozyme uptake to etafilcon A lenses over a period of 28 days. The solutions used for incubation were a complex artificial tear solution with multiple tear film components (ATS), an ATS without lactoferrin (ATS w/o Lac), an ATS without lipids (ATS w/o Lip) and an ATS without lactoferrin and lipids (ATS w/o Lac & Lip).

Figure 3-4 shows the kinetic lysozyme uptake to omafilcon A using the four solutions. Overall, lower amounts of lysozyme were detected on omafilcon A lenses when incubated in ATS compared to the ATS without lipids or ATS without lactoferrin and lipids ($p<0.001$). Lysozyme deposition was also shown to be significantly lower in ATS without lactoferrin compared to ATS without lipids and ATS without lactoferrin and lipids ($p<0.001$). After 4 hours, omafilcon A lenses deposited more than 6.3±0.3µg of lysozyme in each of the incubation solutions. Incubating in ATS displayed significantly lower lysozyme deposition from the beginning of day 1 to day 7, compared to ATS without lipids ($p<0.001$). However, the lysozyme deposited from these two solutions were comparable after day 7 ($p>0.05$). Lysozyme uptake using the ATS without lactoferrin and ATS without lactoferrin and lipids were comparable in the first week of incubation ($p>0.05$). However, lysozyme deposition became significantly greater in ATS without lactoferrin and lipids following 14 days of incubation ($p<0.001$). In addition, the exposure to ATS without lactoferrin displayed comparable amounts of lysozyme accumulation for the first 2 weeks compared to ATS and ATS without lipids ($p>0.05$) up to day 14, where lysozyme deposition became significantly less in ATS without lactoferrin ($p<0.001$). At the end of day 28, total lysozyme deposition from incubating in ATS without lactoferrin was significantly less (12.8±1.0µg) compared to the other solutions, where uptake was more than 14.2µg ($p<0.001$). Lysozyme uptake began to plateau at day 14 in ATS without lactoferrin and in ATS without lactoferrin and lipids, whereas a plateau began at day 21 in ATS and in ATS without lipids ($p>0.05$).

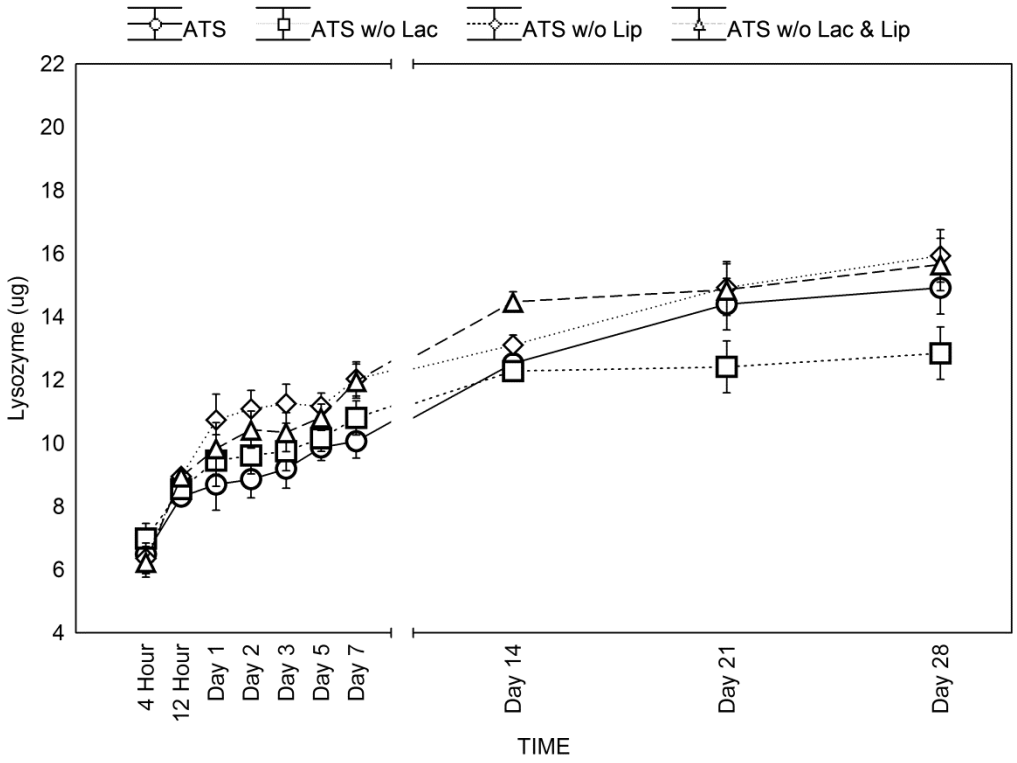

Figure 3-4. Kinetic lysozyme uptake to omafilcon A lenses over a period of 28 days. The solutions used for incubation were a complex artificial tear solution with multiple tear film components (ATS), an ATS without lactoferrin (ATS w/o Lac), an ATS without lipids (ATS w/o Lip) and an ATS without lactoferrin and lipids (ATS w/o Lac & Lip).

The following data points had one measurement removed from the triplicate measurements: Lysozyme sorption to senofilcon A in ATS w/o Lip and in ATS w/o Lac and Lip on Day 3 (Fig. 3-2), to etafilcon A in ATS w/o Lip on Day 1 and Day 21 (Fig. 3-3) and to omafilcon A in ATS w/o Lip on Day 14 (Fig. 3-4). These values were greater than 2 standard deviations from the mean of the other two measurements and were considered an outlier. In each case, no more than 1 data point was removed from each triplicate.

3.5 Discussion

This study outlines the effects of lactoferrin and lipids on lysozyme deposition to conventional and silicone hydrogel lens materials monitored over a period of 28 days using an *in vitro* model.

3.5.1 Silicone hydrogel lenses

Lipid deposition on silicone and conventional hydrogel materials have been previously investigated and results have shown lipid uptake to silicone hydrogels is greater than on conventional hydrogels.[32, 34, 37] The two silicone hydrogel lenses in this study, lotrafilcon B and senofilcon A, possess a lower water content of 33% and 38% respectively, compared to the pHEMA-based materials, etafilcon A and omafilcon A with 58% and 62%, which may influence the adherence of ATS components to the lens material. In addition, siloxane-based contact lens materials are more hydrophobic, allowing lipids to be more readily deposited because of the favorable hydrophobic to hydrophobic interaction at the lens surface.[38] This could explain the greater amount of lysozyme deposition on lotrafilcon B and senofilcon A lenses when incubated in ATS without lipids (Figure 3-1 and 3-2): Without the presence of lipids in the solution, there are more sites available for lysozyme to attach to the lens material. On the contrary, when lipids are introduced, the accumulation of lipids on FDA group I lenses may perhaps outcompete lysozyme uptake because of the thermodynamically favorable binding of lipids. Although it has been shown that proteins bind more readily to hydrophobic than hydrophilic surfaces,[39, 40] our results suggest that the hydrophobic characteristic of group I lenses still have a greater attraction for lipids than for lysozyme and lactoferrin, since the presence of lipids markedly reduces the

binding of lysozyme. This has significant importance when developing an *in vitro* incubating solution to investigate protein deposition.

Lotrafilcon B and senofilcon A lens materials attract only low amounts of protein compared to ionic conventional lens materials.[5, 9, 10, 32, 33, 41] Lysozyme and lactoferrin both have a net positive charge, however their charges are unevenly distributed around their protein structure, thus allowing these tear film proteins to bind to various materials.[42] Lysozyme deposition on lotrafilcon B lenses was greater with lactoferrin absent in the ATS than when incubated in the complex ATS, which possibly may be due to competition with lactoferrin for binding sites on the lens surface. A previous study by Santos et al[43] has examined the protein content deposited on lotrafilcon B lenses that were worn on a daily basis for one month and found higher amounts of lactoferrin deposited on these lenses than lysozyme. This may suggest lotrafilcon B lenses have a stronger affinity to lactoferrin than lysozyme, which would explain the impact of the presence of lactoferrin on lysozyme uptake to lotrafilcon B lenses. On the contrary, there were no differences in lysozyme uptake to senofilcon A lenses with or without lactoferrin present in the ATS. This difference in deposition behavior between the two silicone hydrogel lenses could be due to the differences in the surface treatment processes of each lens type. Lotrafilcon B lenses are treated in a gas plasma reactive chamber to create a permanent ultrathin (25nm) continuous hydrophilic surface. Senofilcon A lenses do not have any surface treatment but contain an internal wetting agent (polyvinyl pyrrolidone; PVP) to assist lens wettability.[44-46] Santos et al [43] previously compared the wettability of a similar silicone hydrogel lens containing PVP (galyfilcon A) with lotrafilcon B lenses. The authors reported that the surface treatment on lotrafilcon B lenses and the internal wetting agent (PVP) in galyfilcon A lenses exhibited comparable effects on lens wettability. Thus, the application of a surface

treatment or the incorporation of an internal wetting agent in lenses does not seem to account for the differences in lysozyme uptake, but rather the differences in chemical composition of the lens materials that alters the deposition of lysozyme.

3.5.2 Conventional hydrogel lenses

Lysozyme bound to the etafilcon A material in the presence of tear film components at very high levels (greater than 200µg after 4 hours of incubation) compared to the rest of the lenses investigated (less than 20µg after 28 days of incubation). These results agree with previous studies that have shown protein deposition on FDA group IV lenses are greater than on group I and II lenses.[29, 47-49] This is largely due to the ionic interactions between tear film proteins such as lysozyme and the lens material. FDA group IV lenses typically contain negatively charged methacrylic acid (MA) to increase the water content of the material, which results in a strong electrostatic attraction to net positively charged proteins such as lysozyme.[13, 47, 50] At a normal physiological pH, the ionized groups in MA repel one another, causing an increase in distance between chains, allowing pores to be formed.[51] These sequences of events allow proteins to adsorb and penetrate into the hydrogel matrix. In contrast, previous reports have shown the presence of phosphorylcholine in contact lenses hinders protein deposition,[52-55] which further supports the data from this study, where omafilcon A lenses deposited low amounts of lysozyme.

Lactoferrin and lysozyme are abundant antibacterial proteins from the tear film, both being positively charged. Although similar, lactoferrin is greater in size (74 kDa) and possesses a relatively weaker positive charge compared to lysozyme.[25] Sariri and colleagues[25] have previously shown that ionic etafilcon A lenses deposit both lactoferrin and lysozyme, however

greater amounts of lysozyme were found compared to lactoferrin and the differences in size and relative charge strength account for these uptake differences. In the current study, no differences were seen in lysozyme uptake to etafilcon A lenses incubated in an ATS compared to an ATS without lactoferrin and lipids (Figure 3-3). However, removing lipids from the ATS decreased the amount of lysozyme deposition. These results suggest that lipids may either promote the binding of lysozyme to the lens surface or prevent lactoferrin adsorption from the ATS, thus presenting more attachment sites for lysozyme to bind. The former is unlikely, as a previous study has shown that the addition of lipids to an incubating solution decreases the amount of lysozyme uptake on group IV lenses.[13] Bontempo and Rapp's proposed reason is that lysozyme attachment on group IV lenses promotes attachment of non polar lipids and as a result hinders further lysozyme binding. Thus, it remains unclear whether lipids inhibit lactoferrin adsorption on group IV lenses. Lactoferrin, a member of the transferrin family of proteins, has the ability to bind to various cells and anions, which implies it has various binding motifs and binding sites.[56] A study that investigated the interactions between tear proteins with the meibomian lipid film demonstrated that both lysozyme and lactoferrin are capable of binding to lipids.[57] It is possible that lipids have a greater attraction to lactoferrin than lysozyme and as a consequence, the adsorption of lactoferrin on etafilcon A lenses is reduced in the presence of lipids, allowing lysozyme to better compete for binding sites on the lens. However, future studies should focus on understanding the impact of lipids and other tear film components on the deposition of lactoferrin on both silicone and conventional hydrogel lens materials.

Previous studies showed that group II lens materials, such as omafilcon, are more prone to deposit lipids and less protein compared to group IV materials.[29, 58, 59] Although there were overall no significant differences ($p>0.05$) in lysozyme deposition between the two ATSs

without lipids (ATS without Lip and ATS without Lac and Lip), both deposited more on the omafilcon material compared to the other two solutions containing lipids. These findings further support the hypothesis that with the presence of lipids in an incubating solution, lysozyme deposition on omafilcon A lenses would be less, compared to an environment where lipids are absent.

3.5.3 Comparison to in vitro and ex vivo studies

From the current study, the presence of lactoferrin and lipids created variations in lysozyme deposition on both silicone and conventional hydrogel lens materials, which varied between lens materials. The lysozyme uptake patterns are in reasonable agreement with a previous *in vitro* study by Subbaraman et al,[9] and both studies showed that etafilcon A lenses demonstrate a large increase in lysozyme deposition during the first week of incubation and then shows a plateau for the remaining 3 weeks, while omafilcon A lenses illustrate a steady increase in lysozyme deposition throughout the 4 weeks. However, lysozyme uptake was drastically lower for etafilcon A and omafilcon A lenses in this current study when incubated in the complex ATS (647.0±1.57µg and 14.9±0.6µg respectively) compared to the results from Subbaraman and co-workers[9] (1434.5±56µg and 43.8±13µg respectively), which used a single protein solution for incubation. We believe this is due to the presence of lipids within the ATS competing for binding sites in the lens. Moreover, differences in the lysozyme deposition curve were noted for the senofilcon A material between the two studies. In this study, lysozyme only slowly increases over the course of the 28 days and shows a plateau effect after day 14. In the previous study,[9] lysozyme uptake continues to increase over time and does not plateau by the end of 28 days. Subaraman et al[9] also showed differences in the amount of lysozyme quantified on lotrafilcon B and senofilcon A lenses (6.1±1.3µg and 13.4±4.1µg respectively) at the end of

28 days compared to when incubated in ATS in the current study (9.7±0.5µg and 3.45±0.3µg respectively). Our data would also suggest that there are other factors apart from lactoferrin and lipid that influence deposition, as variations in lysozyme uptake between this current study and the Subbaraman *in vitro* study[9] were evident when comparing the data from the ATS without lactoferrin and lipids. Thus, this comparison between the use of a single protein solution and an incubating solution that contains multiple tear film components demonstrates the existence of competitive interactions of tear film components on and throughout contact lens materials.

To evaluate the accuracy of the ATS used in this study as a model of the tear film, *ex vivo* studies were reviewed to compare lysozyme deposition on worn lenses to the lenses incubated in the ATS. Boone et al[11] quantified lysozyme deposition on bi-weekly worn lotrafilcon B and senofilcon A lenses using western blot. The amount of lysozyme deposited on lotrafilcon B lenses (0.3±0.2µg) and senofilcon A lenses (1.6±1.6µg) were lower compared to the quantified amounts in the current study (10.4±0.3µg and 3.2±0.1µg respectively). The differences may be reflected by the use of lens care solutions during these *ex vivo* studies, which remove a certain amount of lysozyme from the lenses. On the contrary, *ex vivo* studies typically found higher amounts of lysozyme deposition (~1000µg) on worn etafilcon A lenses compared to the results from this study.[31, 33, 60] The lower amount of lysozyme deposition obtained from *in vitro* incubated etafilcon A lenses compared to the worn lenses could perhaps be explained by the rapid decrease in lysozyme concentration in the ATS as lysozyme deposits on the lenses. This is different *in vivo* when fresh tears are secreted and spread over the surface of the cornea with each blink, whereas the lenses in this study were kept in the same ATS throughout the entire incubation process until they were retrieved for lysozyme quantification. Future *in vitro* studies should aim to replenish the ATS in the vials during the incubation period. In addition, the

current in vitro model is limited in a way that it does not expose lenses in an air-water interface, which would have been a better representation for lens wear. Future studies would require the development of an artificial eye model that incorporates tear replacement with blinking movements to better mimic in vivo conditions during contact lens wear. Furthermore, because the efficiency of protein removal by lens care solutions are dependent on lens material and protein type, [61, 62] the incorporation of utilizing lens care solutions on incubated lenses could further investigate and explain the differences in protein removal on different lenses by various lens care products as shown in previous *ex vivo* studies.

3.6 Conclusion

In summary, both lactoferrin and lipids exhibit an impact on the kinetic uptake of lysozyme to silicone and conventional hydrogel materials. The amount of lysozyme deposited on each lens type, as well as the relative effect of lactoferrin and/or lipids on lysozyme deposition, was lens material dependent. Overall, the presence of lipids exhibited a greater effect than lactoferrin in reducing lysozyme deposition on both FDA group I and II lenses. In comparison, the presence of lactoferrin decreased the amount of lysozyme deposition on group IV lenses. Interestingly, by adding lipids to the ATS, the effect of decreasing lysozyme uptake on etafilcon A lenses by lactoferrin was diminished. The deposition patterns of lysozyme from the current study that utilized a newly developed artificial tear solution varied from previous *in vitro* studies that used simplified tear film models. Interactions between tear film components and their competitive binding on these lens materials might be responsible for this difference. In conclusion, when using *in vitro* models to study protein deposition on contact lenses, more complex models should be used to better mimic the composition of the human tear film.

4. Optimization of a Novel Fluorescence-Based Lysozyme Activity Assay for Contact Lens Studies

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4.1 Overview

Purpose: To optimize a novel fluorescence-based lysozyme activity assay to investigate the conformational state of lysozyme in solution and to determine the impact of extraction and evaporation procedures on lysozyme activity.

Methods: The fluorescence-based lysozyme activity assay, Enzchek (Molecular Probes Inc, Eugene, OR) which utilizes fluorescently quenched *Micrococcus lysodeikticus*, was compared to the classical lysozyme turbidity assay using four differently concentrated lysozyme samples (20, 10, 5.0 and 2.0ng/µL). Furthermore, six differently concentrated lysozyme samples (2.0, 1.0, 0.5, 0.25, 0.125 and 0.01µg/µL) were quantified using the fluorescence-based assay in the presence of extraction solvents consisting of 0.2% and 0.02% trifluroacetic acid/ acetonitrile (TFA/ACN) and following evaporation procedures.

Results: A standard curve was generated by the fluorescence-based assay ranging from 2ng – 150ng. The total active lysozyme quantified in the four lysozyme samples were not significantly different between the two assays ($p>0.05$) and the concordance correlation coefficient was determined to be 0.995. However an average discrepancy between the two assays was found to be 0.474ng, with the turbidity assay typically reporting higher active lysozyme measurements. The sensitivity of the fluorescence-based assay was higher than the classical turbidity assay when quantifying 20ng or less active lysozyme. Following extraction and evaporation procedures and the addition of lens extracts, the total active lysozyme recovered was 95% or greater.

Conclusions: In comparison to the classical turbidity assay, the fluorescence-based assay is a very sensitive method, making it a favourable technique, particularly when studying contact lens materials that deposit relatively low levels of lysozyme.

4.2 Introduction

Lysozyme, also known as muramidase or N-acetylmuramide glycanhydrolase, is a ubiquitous bacteriolytic enzyme found in most biological secretions, including saliva, mucous and tears.[1, 2] This abundant protein has been explored extensively in many areas of research including physicochemical,[3, 4] crystallographic,[5] enzymatic,[6, 7] and immunological studies.[8] Lysozyme was first discovered in 1922 by Alexander Fleming [9] and since then has been well known for its antibacterial property. Fleming discovered that his nasal drippings, containing natural lysozyme, triggered the death of bacterial cells in his Petri dish.[9] This phenomenon is now known to be caused by the lysozyme-catalyzed hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan of the bacterial cell wall.[10] Consequently, the damaged bacterial cell wall loses its structural integrity and ruptures, which results in subsequent cell death.

The concentration of lysozyme found in human tears is the highest of all body fluids (1.7 to 1.9 mg/ml in the open eye),[11] accounting for 40% of the total protein content of tears.[12, 13] This enzyme provides antibacterial protection against microbes that may cause ocular infections such as bacterial keratitis.[14] Lysozyme concentration decreases significantly in the tears of patients with Sjögren's syndrome and in patients exhibiting ocular toxicity due to long term practolol therapy (a beta adrenergic blocking agent for treatment of cardiovascular disorders).[15] Patients diagnosed with herpes simplex virus infection and children with malnutrition also demonstrate reduced lysozyme levels in their tears.[15] Thus, the quantification of lysozyme in patients' tears provides a valuable diagnostic aid to detect symptoms of various health complications.

Lysozyme has shown to selectively deposit onto both conventional and silicone hydrogel contact lens materials,[16-20] with conventional hydrogel lenses accumulating higher levels of lysozyme than silicone hydrogel materials.[16, 20, 21] However, although silicone hydrogel lenses deposit significantly lower quantities of protein, the degree and the rate of lysozyme denaturation is relatively higher on these materials.[22] The conformational state of these deposited proteins is of significant interest because higher levels of denatured proteins have been associated with an ocular inflammatory response known as contact lens associated papillary conjunctivitis (CLPC).[23-26] Higher amounts of denatured proteins have further been linked to reduced comfort during contact lens wear.[27]

In the past, *in vitro*[17, 22] and *ex vivo*[28] studies have investigated the conformational state of lysozyme on contact lenses, by measuring the activity of extracted lysozyme from lens materials using a turbidimetric lysozyme activity assay.[17, 22, 28-30] This turbidity assay utilizes *Micrococcus lysodeikticus* as a substrate and relies on the lytic activity of lysozyme. This method determines the kinetic changes in the conformational state of lysozyme; however it is often time consuming when processing a large number of samples. Furthermore, the turbidity assay is also relatively low in sensitivity and has a narrow linear range of detection,[31] which presents some difficulties when quantifying active lysozyme from contact lens materials that deposit low amounts of lysozyme.[22] Most recently, a novel fluorescence-based lysozyme activity assay kit became commercially available, which has been shown to have high sensitivity in cell culture studies.[32, 33] However, to our knowledge, this assay has not previously been investigated in studies looking at the activity of lysozyme extracted from contact lens materials. Thus, the aim of this study was to optimize this novel fluorescence-based assay to investigate the conformational state of

lysozyme in solution and to determine the impact of extraction and evaporation procedures on lysozyme activity.

4.3 Materials and Methods

To compare a novel fluorescence-based assay with the classical turbidity assay, four differently concentrated solutions of hen egg lysozyme (HEL, Sigma-Aldrich, St. Louis, MO, USA) (at 20, 10, 5.0, and 2.0ng/µL) were used in this study. Three replicates were prepared to assess the validity and repeatability of the fluorescence-based assay.

4.3.1 Classical turbidimetric assay

The turbidity assay was adapted from previous contact lens studies.[17, 22, 28] Fresh *Micrococcus lysodeikticus* was re-suspended in sodium phosphate buffer (pH 6.3) and adjusted to an initial optical density (OD) of 1.1 at 450nm (Multiskan Spectrum ELISA Plate Reader, ThermoLab systems). Samples and standards of no more than 10µL were added in cuvettes containing 1.0mL bacteria. The assay was set to run for 5 minutes at 31^{o}C and the change in OD at 450nm was recorded at 30 second intervals, with stirring for 10 seconds after each measurement. The rate in OD change was calculated and subsequently compared to a standard curve to determine the mass of active lysozyme in each sample. The standard curve was generated by adding different volumes (10, 8, 6, 4, and 2µL) of a 12µg/µL lysozyme standard into a cuvette. The lower limit of detection for the turbidity assay was established by the lower quantity of lysozyme that produces a detectable change in OD.

4.3.2 Fluorescence-based lysozyme activity assay

The EnzChek® lysozyme activity assay (Molecular Probes Inc, Eugene, OR, USA) measures total active lysozyme using fluorescently-quenched *Micrococcus lysodeikticus*. As the bacteria are lysed in the presence of lysozyme, an increase in fluorescent signal in proportion to the amount of active lysozyme in the sample is seen.[31] In this study, the substrate was re-suspended in a 1X reaction buffer (0.1M sodium phosphate, 0.1M NaCl, pH 7.5, containing 2mM sodium azide as preservative). Standards and samples (50µL) were added to 50µL of substrate using 96 well black microplates (Costar, Fisher Scientific, VWR, Mississauga, Canada). The assay was set to run for 30 minutes using excitation/emission of 485/530nm at 37^{o}C (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). Given that up to 96 samples can be processed at once for each 30 minute assay, the time required to process each sample is significantly less compared to the turbidity assay. To follow the kinetics of the reaction, fluorescence was measured every 2 minutes, with 5 seconds of plate shaking before each reading. The change in fluorescence over time for each sample was compared to a standard curve to determine the total amount of active lysozyme. The standard curve was generated by performing a 2X serial dilution using a 12µg/µL lysozyme standard. The limit of detection for the fluorescence-based assay was established by the lowest quantity of lysozyme that produces a detectable change in fluorescence.

4.3.3 Using the fluorescence-based assay for contact lens extracts

The steps involved in the quantification of contact lens deposits include (1) the extraction of the proteins from contact lenses and (2) the drying of the extracted proteins by evaporation followed by (3) the quantification method.[17, 22, 28] To investigate the effects of (1) and (2) on

lysozyme conformation, 50µL of six differently concentrated HEL samples (2.0, 1.0, 0.5, 0.25, 0.125, and 0.01µg/µL) were added to 500µL of two differently concentrated protein extraction solvents: 0.2% and 0.02% trifluroacetic acid/ acetonitrile (TFA/ACN). The use of TFA/ACN is a common technique for protein extraction and has been previously used to remove deposited protein from patient-worn contact lenses.[12, 17, 22, 28, 34] Following the incubation in extraction buffer, all samples were dried down using a Savant Speed Vac (Halbrook, NY, USA). The negative controls were the six HEL samples measured without the addition of extraction solvents and evaporation. All samples were diluted to fall within the detection range and then measured using the fluorescence-based assay.

The effect of the contact lens extracts on the novel fluorescent-based assay was also investigated. To determine the impact of lens extracts on the outcome of the fluorescence-based assay, two silicone hydrogel lenses: lotrafilcon B (Air Optix Aqua, CIBA Vision) and senofilcon A (Acuvue OASYS, Johnson & Johnson Vision Care) and two conventional hydrogel lenses: etafilcon A (Acuvue 2, Johnson & Johnson Vision Care) and omafilcon A (Proclear, CooperVision) were investigated. Following a 24 hour pre-soaking step in saline solution to remove any blister pack solution, lenses were individually immersed for 24 hours in either 0.2% or 0.02% TFA/ACN solvents. The lens materials were not exposed to proteins prior to extraction. In previous studies,[28, 34, 35] the 0.2% TFA/ACN solvent was used to extract proteins from lotrafilcon B, etafilcon A and omafilcon A lenses, whereas 0.02% TFA/ACN was used for senofilcon A. Six differently concentrated lysozyme samples (as described above) were then added to 500uL of the lens extracts for each lens type. The negative controls were lysozyme samples in the extraction solvents (without lens extracts). Subsequently, all samples were dried

down by evaporation, diluted to fall within the range of detection and immediately processed by the fluorescence-based assay.

4.3.4 Lysozyme quantification

In addition to determining active lysozyme, the total amount of lysozyme present in all samples was quantified by an enzyme-linked immunosorbent assay (ELISA).[36] Initially, three replicates of 50µL of each sample were added into flat bottom 96-well plates (Maxisorp certified, Thermo Scientific, VWR, Mississauga, Canada) and kept at room temperature for 2 hours. Subsequently, the wells were washed 3x using 300µL of the wash buffer (Phosphate buffered saline, PBS) and 200µL of 3% bovine serum albumin in PBS was added to block any additional binding sites. After 2 hours, the wells were washed again 3x, followed by adding 50µL of 1/1300 rabbit anti-chicken lysozyme antibody (Meridian Life Science, MJS BioLynx, Memphis, TN, USA) to the sample. After 1.5 hours of incubation, wells were once again washed 3x and 50µL of the 1/5000 donkey anti-rabbit IgG-horseradish peroxidase linked antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added. Following 1 hour of incubation, wells were washed again 3x and 50µL of 3,3'5,5',-tetramethylbenzidine reagent (Sigma-Aldrich Canada, Oakville, ON, Canada) was added. The plate was incubated for 5 minutes in the dark and finally 50µL of 1M H_2SO_4 was added to stop the reaction. The OD of each well was measured at 450nm using the SpectraMax M5 Multi-Mode Microplate Reader). The OD measurements were compared to a standard curve to determine the total amount of lysozyme in each sample.

4.3.5 Percentage of active lysozyme

The percentage of active lysozyme quantified in each sample was calculated as follows:

% Active Lysozyme = (Active Lysozyme / Total Amount of Lysozyme) X 100%

4.3.6 Statistical analysis

Data analysis was conducted using Statistica 8 software (StatSoft Inc, Tulsa, OK, USA). The correlation of the active lysozyme measurements quantified by the two activity assays was assessed by calculating the concordance correlation coefficient.[37] A comparison between the two assays was further done using a Bland-Altman plot.[38] In addition, a repeated measures analysis of variance (RM-ANOVA) was used to determine statistically significant differences in lysozyme activity after extraction and evaporation and between the four lens extracts. A post-hoc Tukey-HSD (Honestly Significant Difference) test was performed and a p-value of <0.05 was considered significant.

4.4 Results

The standard curve produced by the classical turbidity assay is seen in Figure 4-1. The range of detection for the turbidity assay was found to be between 20-120ng. The linear regression equation and the correlation coefficient for the standard curve were $y = 0.0003x - 0.0013$ and $R^2 = 0.995$. Figure 4-2 displays the standard curve of the fluorescence-based activity assay, which ranged from 2ng to 150ng. The linear regression equation and the correlation coefficient of the standard curve were $y = 0.0308x + 0.3653$ and $R^2 = 0.997$.

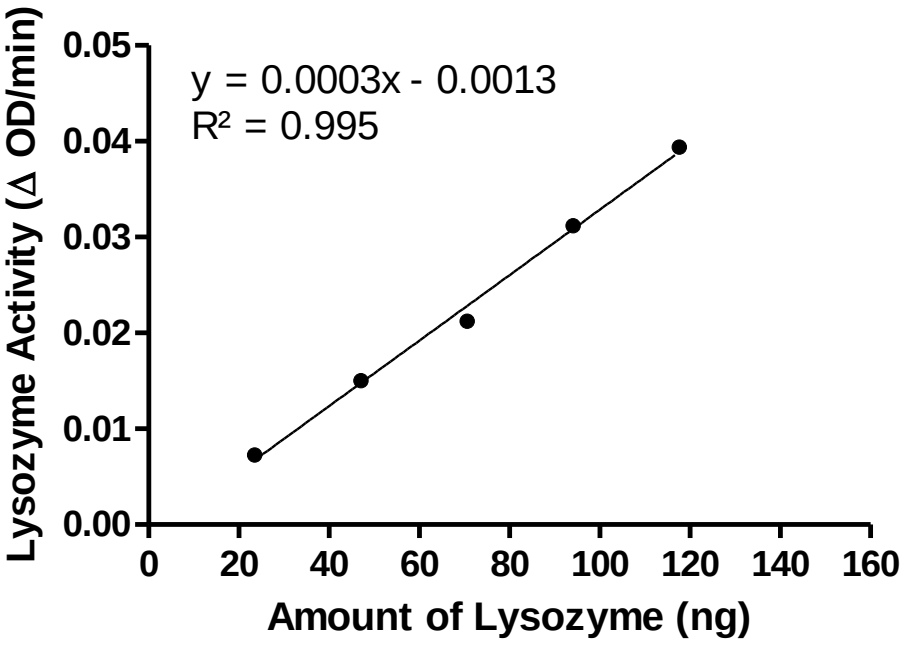

Figure 4-1. The standard curve of lysozyme was determined with the turbidity assay, by measuring the change in optical density (OD) of the *Micrococcus lysodeikticus* solution over time (minutes).

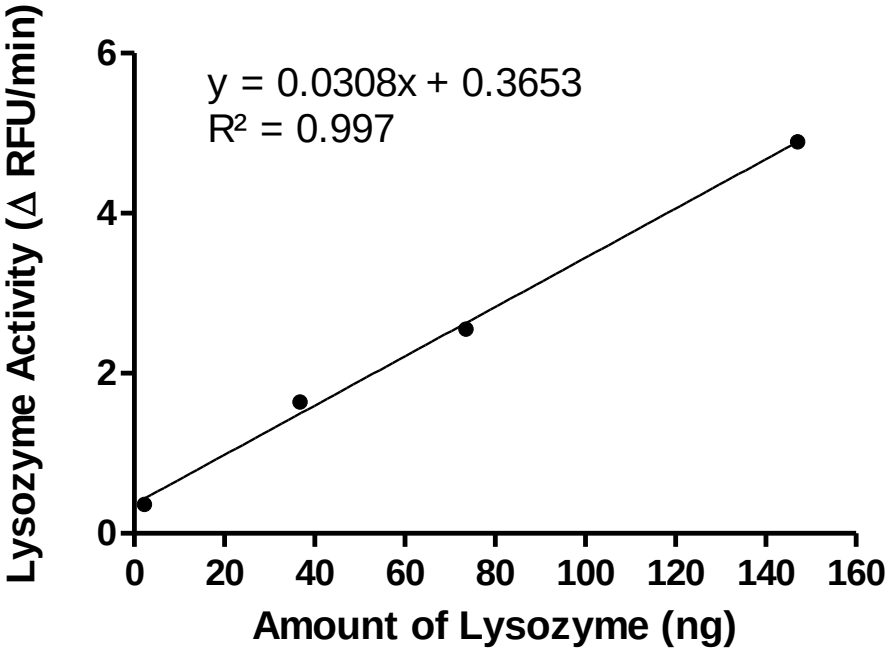

Figure 4-2. The standard curve of lysozyme was determined with the fluorescence-based assay, by measuring the change in fluorescence (RFU) of the fluorescently-tagged *Micrococcus lysodeikticus* in the solution over time (minutes).

Measurements of active lysozyme generated by the turbidity assay and the fluorescence-based assay are shown in Figure 4-3. The concordance correlation coefficient between the results from the two assays was $p_c = 0.995$ and the upper and lower 95% confidence interval were calculated to be 0.998 and 0.983 respectively.

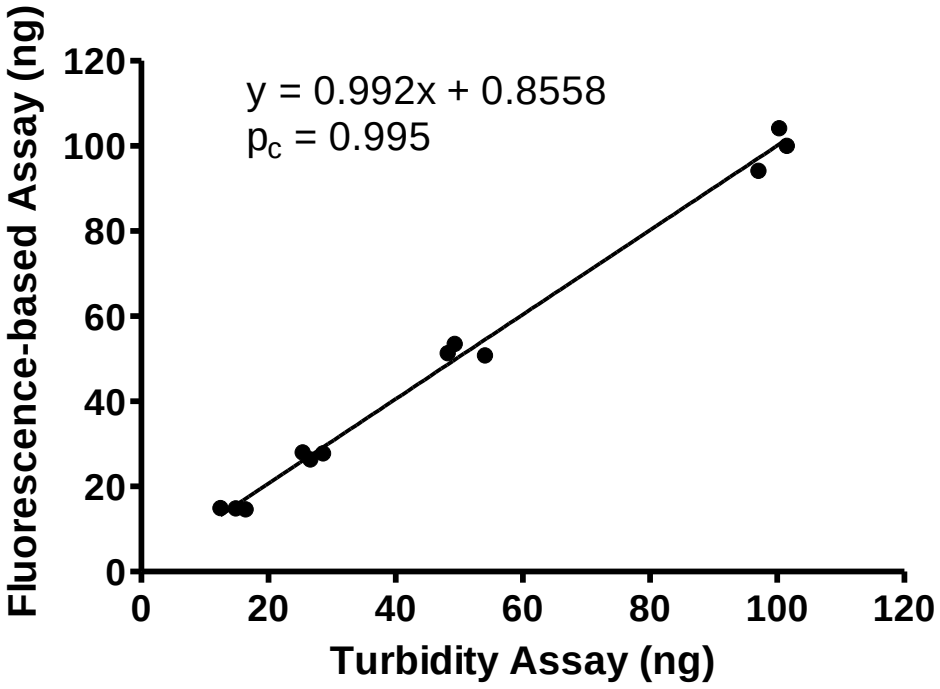

Figure 4-3. Correlation between active lysozyme measurements of four differently concentrated lysozyme samples (20, 10, 5.0, 2.0ng/µL) quantified by the fluorescence-based assay and the turbidity assay.

A comparison between the turbidity assay and the fluorescence-based assay was further made using a Bland-Altman plot (Figure 4-4). The bias of the distribution was calculated to be 0.474, indicating that the turbidity assay determined on average a slightly higher content of active lysozyme compared to the fluorescence-based assay. All data points were found to lie within the 95% limits of agreement, which ranged from -4.72 to 5.67.

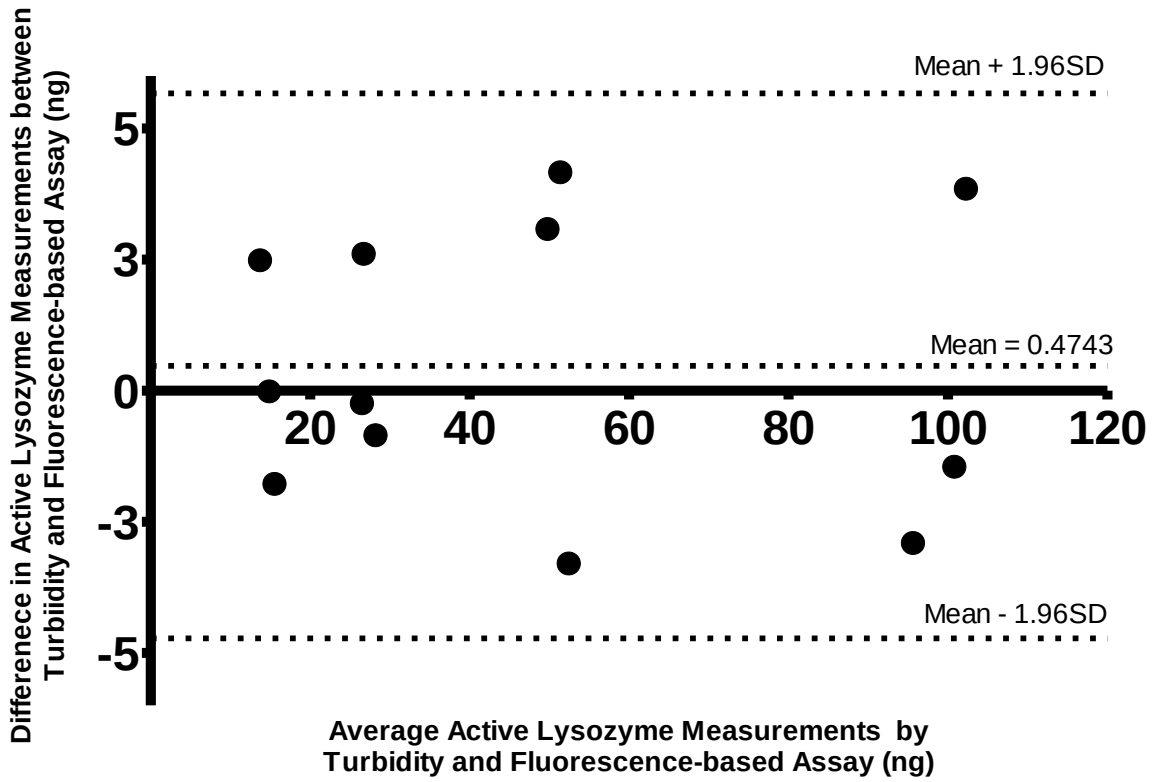

Figure 4-4. Bland-Altman plot of the amounts of active lysozyme quantified by the turbidity assay and the fluorescence-based assay. The Y-axis measures the difference in active lysozyme measurements between the two assays (turbidity – fluorescence-based assay) and the X-axis measures the average of the measurements from the two assays. The dashed lines represent the mean of the bias and bias ± 1.96 standard deviations (SD).

The effects of the extraction solution and evaporation process on lysozyme activity measured by the fluorescence-based activity assay are shown in Figure 4-5. There were no statistically significant differences found between the percentage of active lysozyme after adding the extraction solvents (0.2% and 0.02% TFA/ACN solvents) and evaporation compared to the control ($p > 0.05$). Samples that were evaporated without the addition of extraction buffers also showed no significant differences ($p > 0.05$). In addition, the percentages of active lysozyme recovered in each of the samples were all greater than 95%.

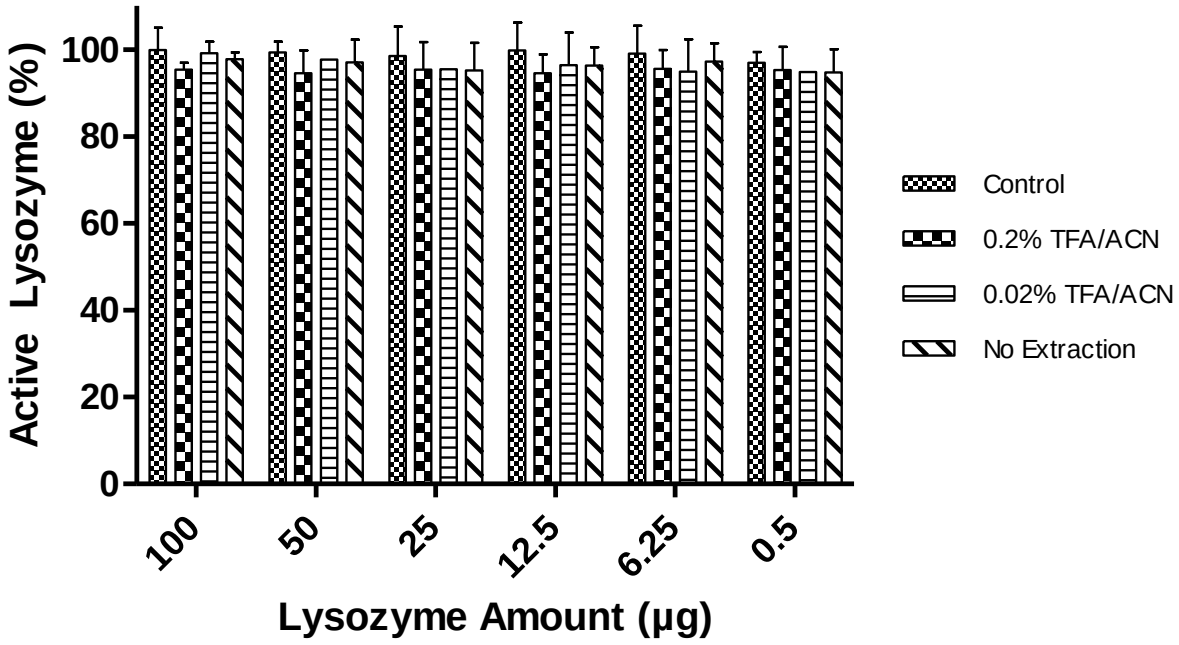

Figure 4-5. Percentage of active lysozyme recovered following the addition of extraction solvents (0.2% and 0.02% TFA/ACN) and evaporation. The percentage of active lysozyme was determined for different lysozyme concentrations using the fluorescence-based assay. There were no significant differences between samples following the addition of extraction solvents and evaporation ($p>0.05$).

The effect of contact lens extracts from lotrafilcon B, senofilcon A, etafilcon A and omafilcon A on lysozyme activity measured by the fluorescence-based assay is seen in Figure 4-6. The presence of lens extracts from different lens materials did not show any significant effect on the percentage of active lysozyme in the samples ($p > 0.05$). Similar to Figure 4-5, the percentages of active lysozyme recovered from all samples following the addition of lens extracts were no less than 95% of the total lysozyme added.

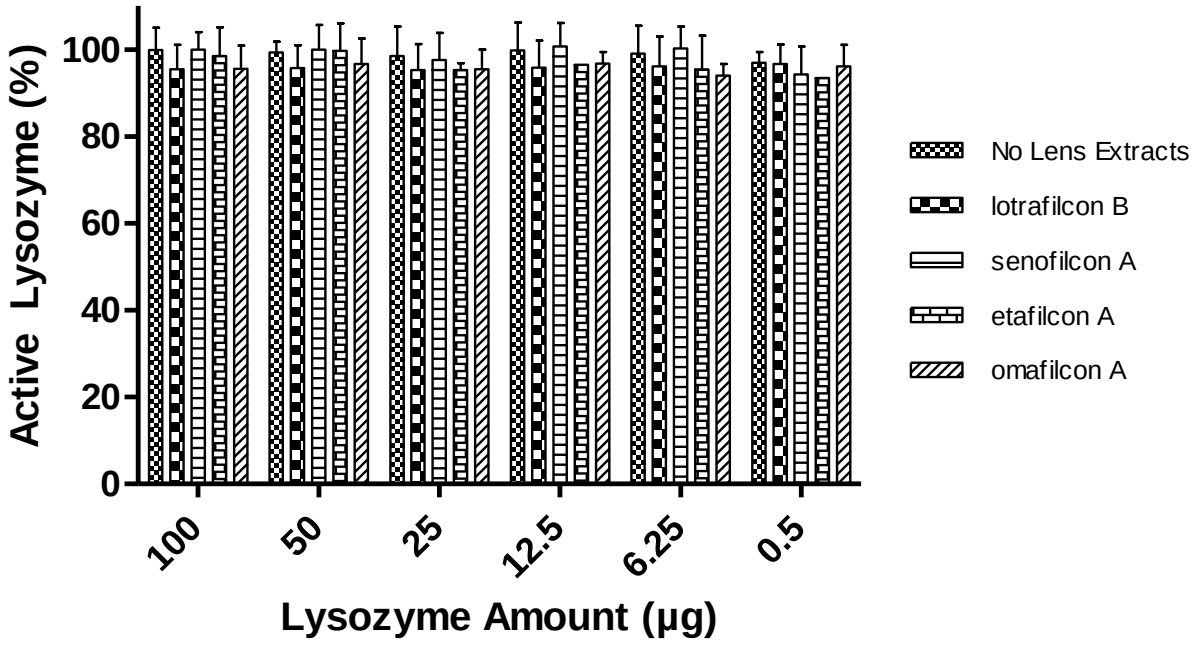

Figure 4-6. Percentage of active lysozyme recovered following the addition of contact lens extracts from lotrafilcon B, senofilcon A, etafilcon A and omafilcon A. Active lysozyme was quantified using the fluorescence-based assay. No significant differences were seen following the addition of lens extracts ($p>0.05$).

4.5 Discussion

This study investigated the accuracy and sensitivity of a novel fluorescence-based lysozyme activity assay in comparison to the classical turbidity assay. Furthermore, the compatibility of this novel technique for contact lens studies was examined.

4.5.1 Comparisons between assays

Similar to the turbidimetric assay, the novel fluorescence-based assay utilizes the gram positive bacteria, *Micrococcus lysodeikticus,* and relies on the bacteriolytic function of lysozyme to quantify the amount of active lysozyme in solution. Both assays have the ability to detect a

wide range of active lysozyme (Figures 4-1 and 4-2), however the current study determined that the fluorescence-based activity assay has a detection limit as low as 2ng, which provides a higher sensitivity compared to the turbidity assay (detection limit of 20ng). Helal and colleagues[31] have also reported similar findings in their recent work comparing the two assays. They demonstrated that the limit of detection for the turbidity assay was 2.3units/mL whereas the fluorescence-based assay had a detection limit of 0.47units/mL.[31] In comparison to the turbidimetric assay, the novel fluorescence-based assay generated comparable results when quantifying active lysozyme amounts that fell within the range of detection of both assays (Figure 4-3). The small discrepancy or bias between the two methods was 0.474ng, indicating the measurements from the turbidity assay were slightly higher than the fluorescence-based assay, although all data points fell within the mean ± 1.96SD (-4.77 and +5.67ng, Figure 4-4). While the two assays were comparable in quantifying active lysozyme, the bias between the two assays must be further evaluated in order to determine whether the discrepancy is large enough to be clinically relevant.

The concentration of lysozyme in the human tear film varies during the day and under different health conditions, with a high variability typically seen between individuals[39] Stolwijk et al.[40] reported that lysozyme levels in tears from patients diagnosed with pre-proliferative diabetic retinopathy have shown to have significantly higher tear lysozyme concentrations (2.77 ± 0.9mg/mL) compared to healthy controls (2.18 ± 0.6mg/mL). Another study by Gupta and co-workers [41] found that the tear lysozyme concentrations in patients diagnosed with severe and moderate acute adenovirus conjunctivitis had significantly lower tear lysozyme levels (0.9mg/mL and 1.13mg/mL respectively) compared to healthy subjects (1.45mg/mL). Thus,

despite the slight variations between the two assays in the current study, the deviations were less than 3ng which may not be large enough to be considered clinically significant.

4.5.2 Compatibility for contact lens studies

Extraction of protein deposits from contact lenses using a mixture of trifluoroacetic acid and acetonitrile was first used by Keith and colleagues[12] and has since been adapted in more recent contact lens deposition studies.[17, 22, 28] The proposed mechanism for the removal of proteins from the lens material is due to exchange of ions between the protein, solution and contact lens material, allowing the protein to readily solubilise in the extraction solvent.[12] The current study demonstrated that the active lysozyme recovered following incubation in 0.2% and 0.02% TFA/ACN solvent and/or evaporation was greater than 95% of the total lysozyme (Figure 4-5). This is in agreement with a recent study by Subbaraman et al,[22] who reported a reduction in lysozyme activity of 2.1 ± 0.7% using the turbidity assay. Previous studies that used TFA/ACN had demonstrated to efficiently remove over 90% of proteins from soft contact lenses, which allows for a representative analysis of the total content deposited.[12, 42] Future studies may consider simply analyzing active lysozyme deposited on contact lenses using the fluorescence-based assay by directly submerging the lens material into the substrate solution without the use of extraction procedures. However, this will limit to only one aspect of analysis of the many deposits found on contact lenses and more importantly, it is unsure whether the presence of a contact lens may or may not impede with the fluorescent readings and will require further investigation.

The Gram positive bacteria, *Micrococcus lysodeikticus*, used for the fluorescence-based assay, were conjugated with fluorescent molecules in a way that the fluorescence is quenched.

Fluorescence quenching refers to the process in which the fluorescent signal of a substrate is suppressed.[43] The intramolecular quenching is relieved when the bacterial cell walls are broken down by lysozyme. Thus, in theory, a fluorescent signal would only be generated in the presence of lysozyme or other molecules that can lyse bacterial cell walls. Previous work in our laboratory confirmed that samples containing lens extracts (without lysozyme) generated no lytic activity when quantified with the turbidity assay (unpublished). This was also found in the current study, where the presence of extracts from the two silicone hydrogel lenses, lotrafilcon B and senofilcon A and the two conventional hydrogel lenses, etafilcon A and omafilcon A, indicated no differences in lysozyme activity compared to lysozyme samples containing no lens extracts (Figure 4-6). The reduction in lysozyme activity of <5% was primarily caused by the extraction solvents and evaporation procedure, as mentioned previously, and not by the presence of contact lens extracts. Therefore, the results suggest that the novel fluorescence-based assay displays high specificity to lysozyme activity, even in the presence of other impurities in the solution.

Based on previous studies, the amount of lysozyme recovered from worn lenses ranges from over 1000μg per lens[19, 28] to as low as 0.3-0.9μg per lens.[29, 34] The detection range of the fluorescence-based assay was between 2-150ng, which falls well below the amount of lysozyme that deposits on contact lenses and could therefore be used in future studies.

4.6 Conclusion

The fluorescence-based lysozyme activity assay has shown to be comparable to the classical turbidity assay, with the advantage of a higher sensitivity, which allows the analysis of low lysozyme amounts of as little as 2ng. This is beneficial for studying contact lens materials that deposit relatively low levels of protein, since less sample volume will be required for the lysozyme assay, leaving ample volume for other analyses of the contact lens deposits. The activity of lysozyme was only marginally impacted by the 0.2% and 0.02% TFA/ACN solvent and/or evaporation process, leaving over 95% of the total lysozyme active. Furthermore, lens extracts from lotrafilcon B, senofilcon A, etafilcon A and omafilcon A caused no interferences with the fluorescent readings from the novel assay. In conclusion, the fluorescence-based lysozyme activity assay is a simple, sensitive and specific assay that is compatible for future studies that investigate the conformation state of lysozyme deposited on to contact lenses.

5. The Impact of Tear Film Components on the Conformational State of Lysozyme Deposited on Contact Lenses

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5.1 Overview

Purpose: To investigate the impact of lactoferrin and the presence of lipids on the kinetic denaturation of lysozyme deposited on silicone and conventional hydrogel lenses, using a complex artificial tear solution (ATS).

Methods: Two silicone hydrogel lenses [AIR OPTIX AQUA; lotrafilcon B and ACUVUE OASYS; senofilcon A] and two conventional hydrogel lenses [ACUVUE 2; etafilcon A and PROCLEAR; omafilcon A] were incubated in four different solutions: an ATS consisting of various salts, lipids, proteins and mucins, an ATS without lactoferrin, an ATS without lipids and an ATS without lactoferrin and lipids. At various time points over a 28 day period the percentage of active lysozyme per lens was determined using a fluorescence-based activity assay and an enzyme-linked immunosorbent assay (ELISA).

Results: After 28 days, the percentage of active lysozyme recovered from etafilcon A lenses in all solutions was significantly higher than all other lens materials ($p<0.001$). For lotrafilcon B, senofilcon A and omafilcon A lenses, lysozyme denaturation rates were greatest during the first week of incubation and reached a plateau after this time point ($p>0.05$). The inclusion of lipids in the ATS significantly increased the lysozyme denaturation on both silicone hydrogel materials ($p<0.001$), while the presence of lactoferrin had an impact on lysozyme recovered from senofilcon A lenses ($p<0.001$). Lysozyme activity on the conventional lenses was not significantly affected by either lactoferrin or lipids ($p>0.05$).

Conclusions: Lactoferrin and lipids have an impact on the conformational state of lysozyme deposited onto silicone hydrogel contact lenses, while conventional hydrogel lenses seem to be

unaffected. It is important for future *in vitro* studies to consider the impact of tear film components when investigating protein deposition and denaturation on contact lenses.

5.2 Introduction

Despite the development of disposable contact lenses, spoilage of contact lenses with various tear film components continues to be a problem for lens wearers. [1, 2] Immediately upon insertion of a contact lens it accumulates various tear film components, including lipids [3, 4] proteins [5, 6] and mucins [7, 8] and the accumulation of these tear film-derived deposits may be associated with lens discomfort, decreased visual acuity and increased symptoms of dryness.[1, 9-11]

The tear film spreads in a thin layer of approximately 3-7μm [12, 13] over the anterior surface of the eye and plays a key role in providing ocular defense against microbial agents.[14, 15] Various components contribute to the antibacterial defense system, including the abundant bacteriolytic enzyme lysozyme.[14] Lysozyme catalyzes the hydrolysis of β1,4 glycosidic bonds in the cell walls of gram positive bacteria,[16] enhances the phagocytosis of microorganisms by leukocytes and interacts with lactoferrin to provide ocular defense against bacteria.[17]

Numerous studies have demonstrated that lysozyme is one of the major proteins depositing on and within soft lens materials.[18-23] This low molecular weight (14kDa),[24, 25] and positively charged enzyme deposits on both conventional (polyHEMA-based) and silicone hydrogel lens materials, particularly ionic materials exhibiting a negative charge.[22, 23, 26-29] Previous studies have found that once lysozyme deposits onto contact lenses, it undergoes conformational changes, [27, 30, 31] which may cause lens discomfort[32] or ocular inflammatory reactions such as contact lens induced papillary conjunctivitis (CLPC).[33, 34] Thus, in addition to quantifying lysozyme deposition on contact lenses, determining the conformational state of the deposited lysozyme is also of great clinical significance.

Previous *in vitro* studies that examined lysozyme activity on contact lens materials often utilized simple tear film models in which to dissolve the lysozyme.[27, 30] However, these simple models do not accurately represent the complex composition of the tear film, and do not consider the potential impact of other tear components on the sorption of lysozyme and the ensuing conformational state of the lysozyme deposited onto the lens material. In recent studies, we have utilized more complex tear film models and found that the presence of lipids and other tear proteins, such as lactoferrin and albumin, have an impact on the amount of lysozyme deposited onto various lens materials. [29, 35] Thus, the aim of this study was to investigate the impact of specific tear film components on the kinetic conformational changes of lysozyme deposited on both conventional and silicone hydrogel lens materials using a complex artificial tear solution (ATS).

5.3 Materials and Methods

For this *in vitro* study, two silicone hydrogel lenses, lotrafilcon B (AIR OPTIX AQUA, CIBA Vision, Duluth, GA) and senofilcon A (ACUVUE OASYS, Johnson & Johnson, Jacksonville, FL) and two conventional hydrogel lenses, etafilcon A (ACUVUE 2, Johnson & Johnson, Jacksonville, FL) and omafilcon A (Proclear, CooperVision, Pleasanton, CA) were investigated. The dioptric power of the new, unworn lenses was -3.00D. The individual properties of each lens type are summarized in Table 5-1.

Table 5-1. Properties of contact lens materials investigated in this study

	Silicone Hydrogel		Conventional Hydrogel	
USAN	Lotrafilcon B	Senofilcon A	Etafilcon A	Omafilcon A
Proprietary Name	AIR OPTIX AQUA	ACUVUE OASYS	ACUVUE 2	PROCLEAR
Manufacturer	CIBA Vision	Johnson & Johnson	Johnson & Johnson	CooperVision
Water Content (%)	33%	38%	58%	62%
Surface Treatment	25nm plasma coating with high refractive index	No surface treatment, but incorporates an internal wetting agent (PVP)	None	None
FDA group	I	I	IV	II
Ionicity	Non ionic	Non ionic	Ionic	Non ionic
Principal Monomers	DMA+TRIS+ Siloxane monomer	mPDMS+DMA+ HEMA + siloxane macromer + PVP +TEGDMA	HEMA+MA	HEMA+PC

DMA - N,N-dimethylacrylamide; FDA - Food and Drug Administration; HEMA - (poly-2-hydroxyethyl methacrylate); MA - methacrylic acid; mPDMS - monofunctional polydimethylsiloxane; PC - phosphorylcholine; PVP - polyvinyl pyrrolidone; TEGDMA - tetraethyleneglycol dimethacrylate; TRIS - trimethylsiloxy silane; USAN United States Adopted Names

5.3.1 Artificial Tear Solution (ATS)

The ATS used in this study was a complex mixture of proteins, lipids, mucins, salts, glucose (all Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and urea (EMD Science, VWR, Mississauga, Canada) (Table 5-2). The composition of the ATS was adapted from a previous study by Lorentz et al[36] and has been used recently in our laboratory to investigate the effects of various tear film components on contact lens deposition.[29, 35, 37] To study the impact of lactoferrin

and lipids on the conformation of lysozyme after depositing on contact lens materials, four variations of the ATS were used for incubation (Table 5-3).

Table 5-2. Composition of the artificial tear solution used in the study

Salt Components (mg/mL)		**Lipids** (mg/mL)	
NaCl	5.26	Oleic Acid	0.018
KCl	1.19	Oleic Acid Methyl Ester	0.012
Na_2CO_3	1.27	Triolein	0.016
$KHCO_3$	0.30	Cholesterol	0.0018
$CaCl_2$	0.07	Cholesteryl Oleate	0.024
$Na_3C_6H_5O_7$	0.44	Phosphotidylcholine	0.0005
Urea	0.072		
Glucose	0.036	**Proteins** (mg/mL)	
Na_2HPO_4	3.41	Bovine Albumin	0.20
HCl	0.94	Hen Egg Lysozyme	1.90
		Bovine Mucin	0.15
Proclin 300	200µl per litre of solution	Bovine IgG	0.02
		Lactoferrin	1.80

Table 5-3. List of solution combination used for incubation

Solution	
1	Complex ATS with all its tear film components (ATS)
2	ATS without lactoferrin (ATS w/o Lcf)
3	ATS without lipids (ATS w/o Lip)
4	ATS without lactoferrin and lipids (ATS w/o Lcf & Lip)

ATS (artificial tear solution); Lcf (lactoferrin); Lip (lipids)

5.3.2 Pre-treatment of vials and lenses

Glass vials (6mL, Wheaton Science Products, Millville, NJ, USA) were pre-treated for 7 days at 37^{o}C with the same solution used during lens incubation, with the exception of lactoferrin, which was excluded due to cost considerations. The purpose of this pre-treatment step was to coat the walls of the vials with pre-treatment solution, which reduces the adsorption of ATS components during the lens incubation and impacts on subsequent deposition levels (in house data, not reported).

Twenty-four hours prior to the incubation, lenses were removed from their blister packages using sterile silicone tipped forceps, rinsed in a saline solution for 2 seconds and then pre-soaked, at room temperature, in 5mL of an augmented buffered saline solution containing the salt components of the ATS (Table 5-2). This procedure removed excess blister pack solution from the lenses.

5.3.3 Incubation of lenses

Following pre-treatment of the glass vials, the pre-treating solution was replaced with one of the four incubation solutions (Table 5-3). Lenses were removed from the saline solution and immediately placed in the vials. All lenses were incubated in 1.5mL of incubation solution except for etafilcon A, which was incubated in 6.0mL. The amount of lysozyme in 1.5mL would have been insufficient for etafilcon A, as this ionic lens type has been shown in previous studies[26, 27, 29] to accumulate high amounts of lysozyme. Thus, a higher volume was used for this lens type to allow for maximum uptake (in house data, not reported). All vials were capped and placed inside a 37^{o}C rotating incubator for 10 different time points (4 and 12 hours, 1, 2, 3, 5, 7,

14, 21, and 28 days). Each condition had 3 replicates, thus a total of 480 contact lenses were used in this study.

5.3.4 Protein extraction and evaporation

After each time point, lenses were removed from the vials and rinsed in saline solution for 2 seconds to remove unbound lysozyme from the lens surface. Subsequently, the lenses were incubated in a protein extraction solvent composed of trifluoroacetic acid and acetonitrile (TFA and ACN) for 24 hours in the dark. Lotrafilcon B, etafilcon A and omafilcon A were placed in a 0.2% TFA/ACN solvent, [21, 22, 38] whereas senofilcon A was placed in a 0.02% TFA/ACN solvent.[39] Previous studies from our group have shown that these extraction procedures results in lysozyme extraction efficiencies of >95%.[30, 40] Lenses were inserted into 1.5mL of extraction solvent, except for etafilcon A, which has been previously shown to require 4.0mL of solvent.[38] Following extraction, two 500µL of the extraction solution was aliquoted into eppendorf tubes and dried down to pellets in a Savant Speed Vac (Halbrook, NY, USA). Once dried, the samples were immediately re-suspended in a tear dilution buffer (TDB: 10mM Tris-HCl, 0.9% NaCl, 1mM EDTA, pH 8.0) for analysis.

5.3.5 Determining lysozyme activity

The conformational state of lysozyme extracted from the lens materials was assessed using the Enzchek® lysozyme activity assay (Molecular Probes Inc, Eugene, OR, USA). This assay has been recently optimized in our laboratory for contact lens studies (paper in submission).[40] Using 96 well black microplates (Costar, Fisher Scientific, VWR, Mississauga, Canada), 50µL of samples were added to 50µL of fluorescently tagged *Micrococcus lysodeikticus* in the wells. The fluorescent signal was measured kinetically every 2 minutes for

30 minutes using excitation/emission of 485/530nm at 37^{o}C (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). The change in fluorescence over time for each sample was compared to a standard curve to determine the amount of active lysozyme in each sample.

5.3.6 Quantification of total lysozyme

Total lysozyme deposited on the contact lenses was quantified using an enzyme-linked immunosorbent assay (ELISA).[41] Using flat bottom 96-well plates (Maxisorp certified, Thermo Scientific, VWR, Mississauga, Canada), samples of 50µL were added to the wells and incubated at room temperature for 2 hours. Subsequently, the wells were washed 3x using 300µL of the wash buffer (Phosphate buffered saline - PBS). Then, 200µL of blocking solution (3% bovine serum albumin in PBS) was added to each well and incubated for 2 hours at room temperature. The wells were washed again 3x, followed by the addition of 50µL of 1/1300 rabbit anti-chicken lysozyme antibody (Meridian Life Science, MJS BioLynx, Memphis, TN, USA) to the wells. After 1.5 hours of incubation, wells were once again washed 3x and 50µL of the 1/5000 donkey anti-rabbit IgG-horseradish peroxidase linked antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added. The incubation of the secondary antibody in the wells lasted one hour, before being washed 3x. Subsequently, 50µL of 3,3'5,5',-tetramethylbenzidine reagent (Sigma-Aldrich Canada, Oakville, ON, Canada) was added to each well. The plate was incubated for 5 minutes in the dark before 50µL of 1M H_2SO_4 was added to stop the reaction. The optical density (OD) of each well was measured at 450nm using the SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The absorbance values were compared to a standard curve to determine the total amount of lysozyme in each sample.

The percentage of active lysozyme quantified in each sample was calculated as follows:

$$\% \text{ Active Lysozyme} = \text{Active Lysozyme} / \text{Total Amount of Lysozyme} \times 100\%$$

5.3.7 Statistical analysis

Data analysis was conducted using Statistica 8 software (StatSoft Inc, Tulsa, OK, USA). A repeated measures analysis of variance (ANOVA) was used to determine statistically significant differences between the percentage active lysozyme across all time points and between materials and incubation solution. Further analysis was conducted using a post-hoc Tukey-HSD (Honestly Significant Difference) test. A p value of <0.05 was considered significant. The denaturation of lysozyme was considered to plateau when no significant difference was found between the respective time point and day 28 ($p>0.05$).

5.4 Results

The percentage of active lysozyme deposited onto the different lens materials after incubation in the complex tear solution (ATS) is shown in Figure 5-1. After 28 days, lysozyme deposited on etafilcon A had the greatest activity (84.4 ± 3.6%), which was significantly higher than the other lens materials ($p<0.001$). Lotrafilcon B and omafilcon A exhibited a similar reduction in activity, leaving a remaining 22.7% and 28.5% of active lysozyme respectively after 1 week ($p>0.05$ between lens types). The reduction in percentage active lysozyme on senofilcon A was not as high during the first week (60.7% active lysozyme) as compared to lotrafilcon B and omafilcon A, however, by the end of day 28, the amount of active lysozyme on senofilcon A was similar compared to lotrafilcon B and omafilcon A lenses (senofilcon A: 24.6 ± 1.0%; lotrafilcon B: 19.83 ± 1.1%, omafilcon A: 22.7 ± 1.5% ($p>0.05$).

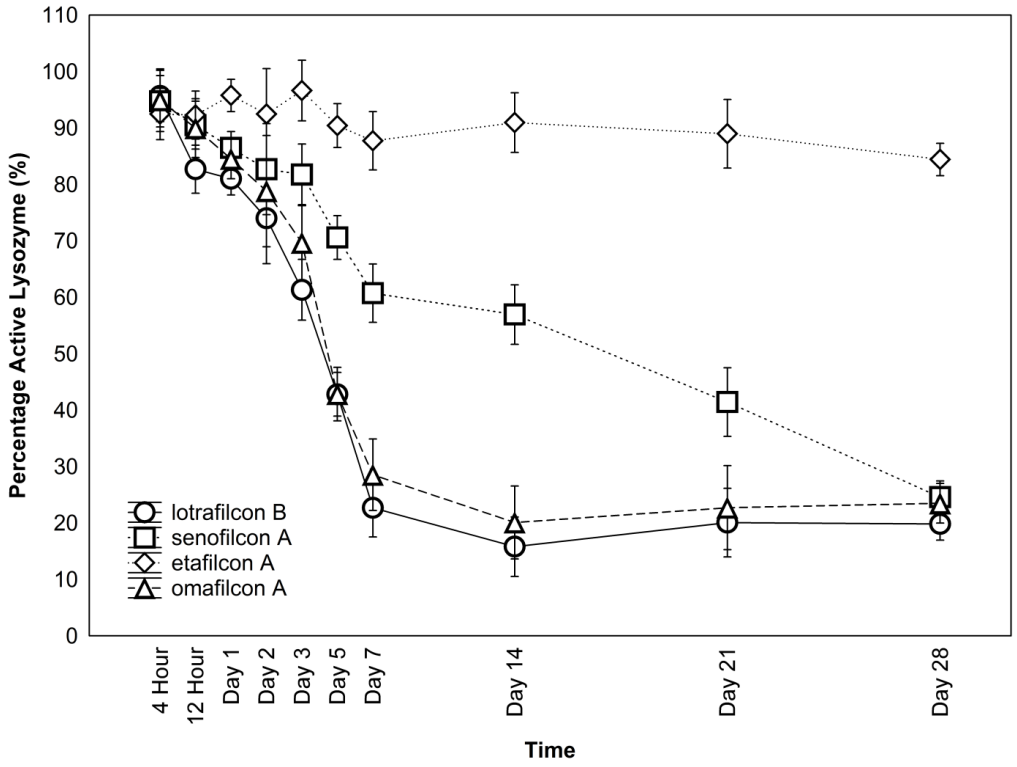

Figure 5-1. Kinetics of percentage active lysozyme recovered from contact lenses over a 28 day incubation period in the artificial tear solution (ATS). Contact lens materials included lotrafilcon B, senofilcon A, etafilcon A and omafilcon A.

The percentage active lysozyme recovered from all lens materials incubated in the ATS without lactoferrin, the ATS without lipids and the ATS without lactoferrin and lipids is illustrated in Figures 5-2 to 5-4 respectively. Lysozyme deposited on etafilcon A had significantly higher activity compared to the other lens materials on day 5 onward in the ATS without lactoferrin and in the ATS without lipids (Figures 5-2 and 5-3, $p<0.001$). Similarly, lysozyme activity on etafilcon A in the ATS without lactoferrin and lipids was significantly higher in comparison to the other lenses following day 2 of incubation (Figure 5-4). After 28 days of incubation in the ATS without lactoferrin, the percentage active lysozyme was 77.8 ± 4.6% for etafilcon A and ranged between 16.1 ± 4.0% to 32.2 ± 0.5% for lotrafilcon B,

senofilcon A and omafilcon A (Figure 5-2). When incubated in the ATS without lipids, the percentage active lysozyme on etafilcon A was 94.3 ± 0.2% and was between 34.8 ± 0.1% to 40.6 ± 3.6% for the other lens materials following the 4 week incubation period (Figure 5-3). Furthermore, in the ATS without lactoferrin and lipids, the percentage active lysozyme was 87.5 ± 5.3% for etafilcon A and ranged between 31.6 ± 8.9% to 40.8 ± 0.4% for the other lens materials (Figure 5-4). Overall, the rate of lysozyme denaturation on lotrafilcon B, senofilcon A and omafilcon A was similar throughout the 28 day incubation period in the ATS without lactoferrin, and the ATS without lipids and lactoferrin (Figures 5-2 and 5-4, $p>0.05$).

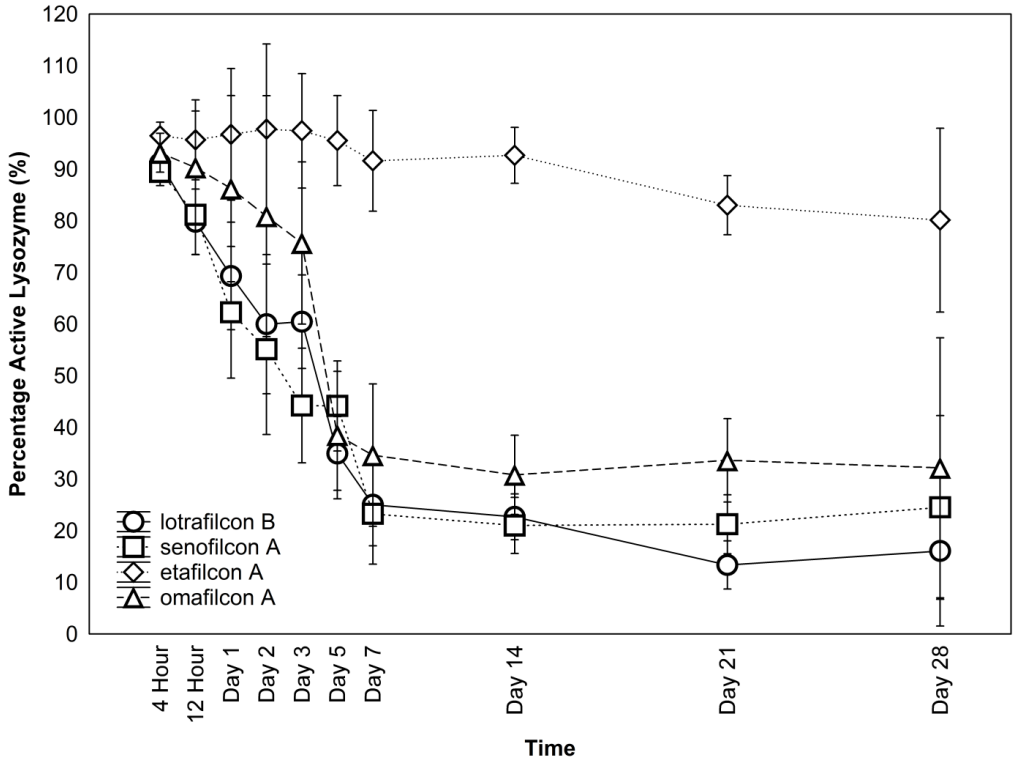

Figure 5-2. Kinetics of percentage active lysozyme recovered from contact lenses over a 28 day incubation period in the artificial tear solution without lactoferrin (ATS w/o Lcf). Contact lens materials included lotrafilcon B, senofilcon A, etafilcon A and omafilcon A.

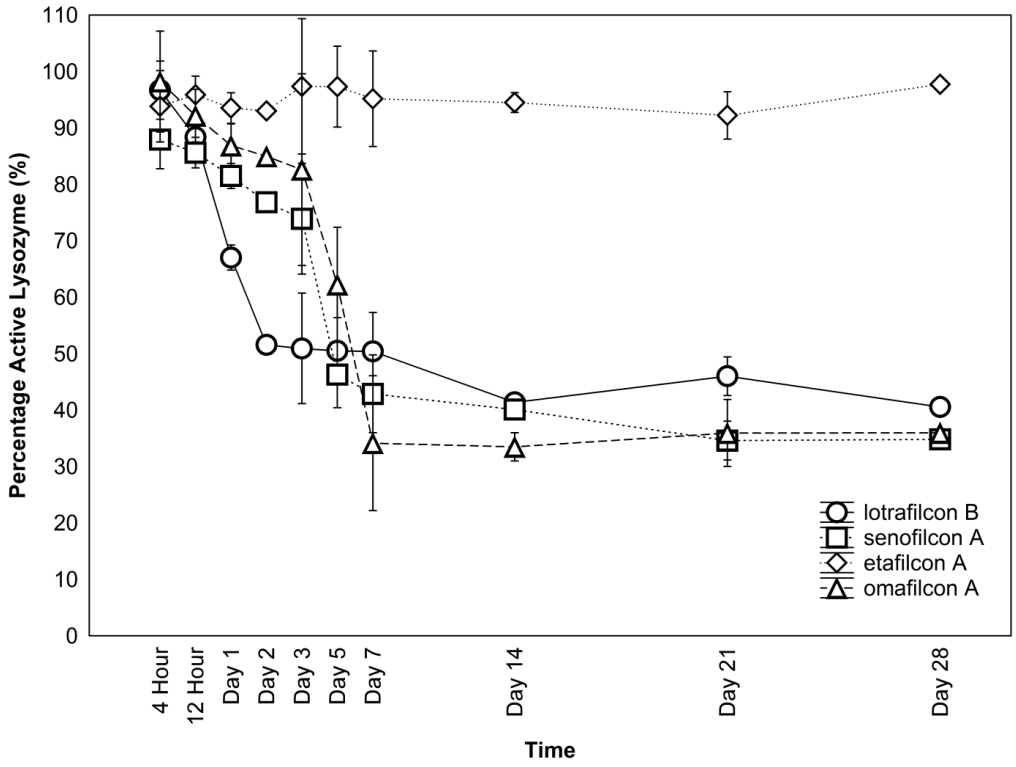

Figure 5-3. Kinetics of percentage active lysozyme recovered from contact lenses over a 28 day incubation period in the artificial tear solution without lipids (ATS w/o Lip). Contact lens materials included lotrafilcon B, senofilcon A, etafilcon A and omafilcon A.

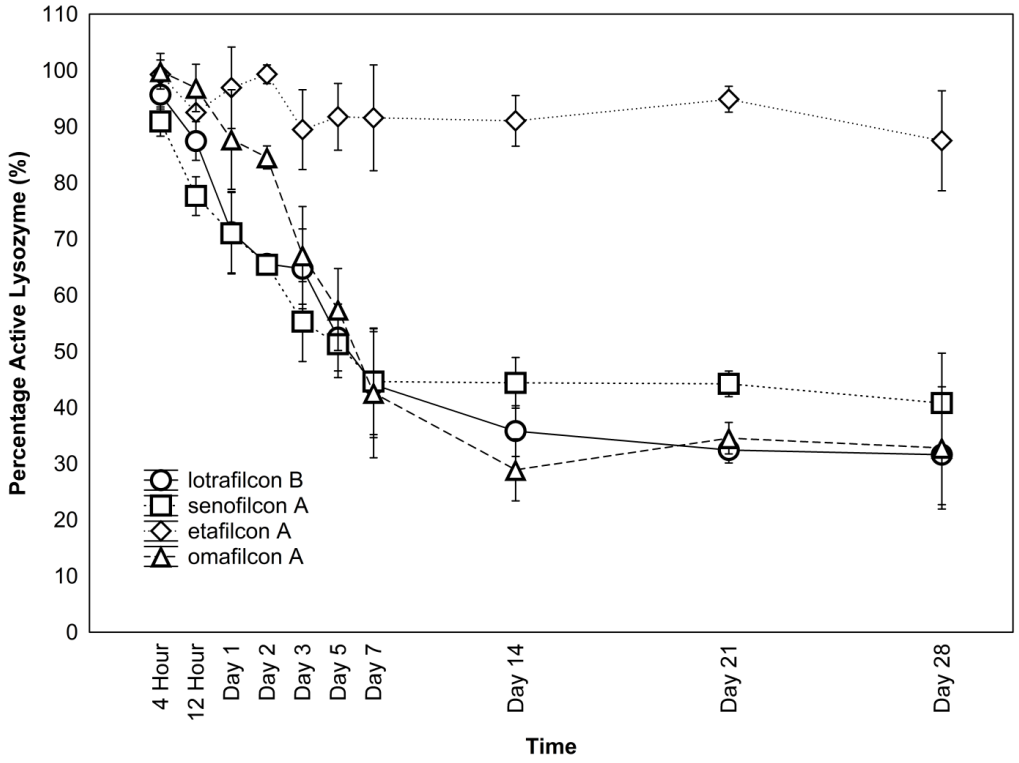

Figure 5-4. Kinetics of percentage active lysozyme recovered from contact lenses over a 28 day incubation period in the artificial tear solution without lactoferrin and lipids (ATS w/o Lcf & Lip). Contact lens materials include lotrafilcon B, senofilcon A, etafilcon A and omafilcon A.

Differences between incubation solutions for each material are shown in Figures 5-5 to 5-8. Figure 5-5 displays the kinetics of active lysozyme recovered from lotrafilcon B following incubation in all four solutions. Lysozyme activity dropped during the first 7 days before reaching a plateau in all solutions ($p>0.05$), with the exception of incubation in the ATS without lipids, where a plateau was reached at 52% active lysozyme on day 2. The percentage of active lysozyme, in all solutions, was not significantly different for the first week ($p<0.05$). After 7 days of incubation, there was significantly more active lysozyme in the two ATSs without lipids (ATS w/o Lip and ATS w/o Lcf & Lip) compared to the two ATSs with lipids (ATS and ATS w/o Lcf) ($p<0.001$). Throughout the 28 day incubation period, there was no significant

differences in active lysozyme between the two ATSs with lipids ($p>0.05$), and no significant differences between the two ATSs without lipids ($p>0.05$). At the end of the 28th day, the amount of active lysozyme was significantly higher in the ATS without lipids and the ATS without lactoferrin and lipids (45.6 ± 3.6% and 31.6 ± 8.9% respectively) compared to the ATS and ATS without lactoferrin (19.8 ± 1.1% and 16.1 ± 3.9% respectively) ($p<0.05$).

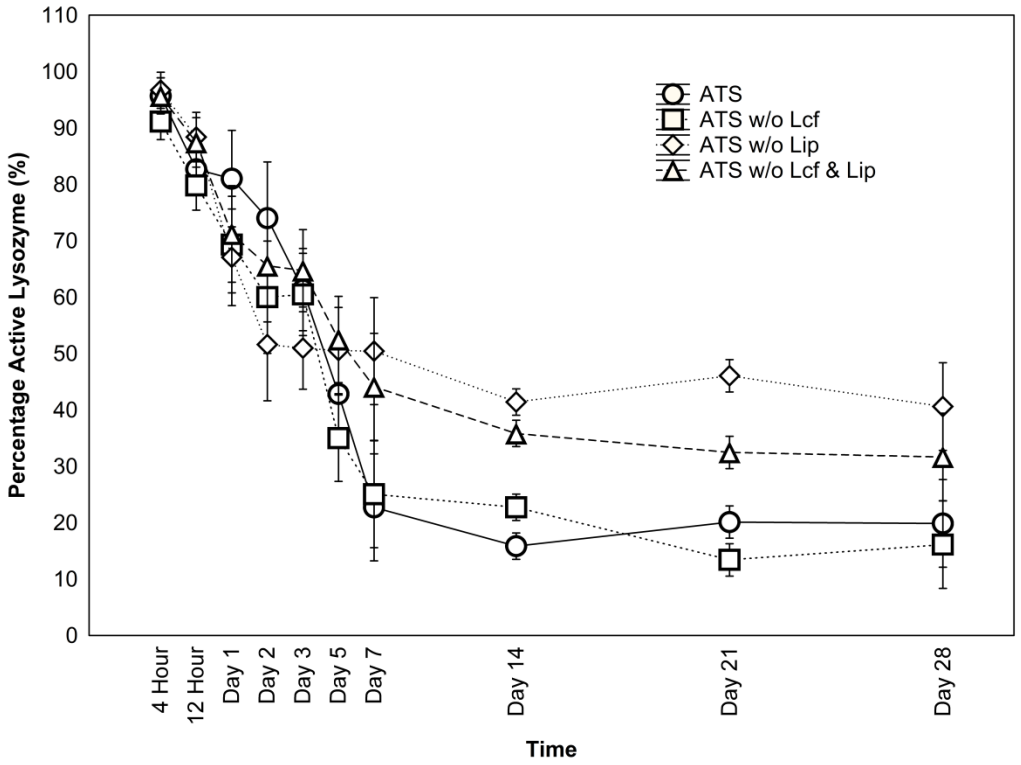

Figure 5-5. Kinetics of percentage active lysozyme recovered from lotrafilcon B lenses over a 28 day time period. The incubation solutions included a complex artificial tear solution with multiple tear film components (ATS), an ATS without lactoferrin (ATS w/o Lcf), an ATS without lipids (ATS w/o Lip) and an ATS without lactoferrin and lipids (ATS w/o Lcf & Lip).

Figure 5-6 shows the reduction in active lysozyme recovered from senofilcon A after incubation in all four solutions. During the first 4 hours of incubation, there were no significant differences in lysozyme activity in all solutions ($p>0.05$), until the 12th hour, where the

percentage of active lysozyme was significantly higher in the two ATSs with lactoferrin (ATS and ATS w/o lip) compared to the other 2 solutions ($p<0.001$). A reduction in lysozyme activity reached a plateau on day 7 in the ATS without lactoferrin ($p>0.05$) and was significantly lower compared to the other solutions from day 7 onward ($p<0.001$). Similarly, lysozyme denaturation reached a plateau on day 7 in the ATS without lipids and ATS without lactoferrin and lipids ($p>0.05$). There were no significant differences in the amounts of active lysozyme between the two ATSs without lipids from day 5 onward ($p>0.05$). Furthermore, incubation in the ATS displayed a gradual decrease in percentage active lysozyme during the 28 day incubation, without reaching a plateau ($p>0.001$). At the end of the 28-day incubation period, the ATS without lactoferrin and lipids ($40.7 \pm 0.4\%$) had significantly higher amounts of active lysozyme compared to the two ATSs that contained lipids (ATS; $24.6 \pm 1.0\%$ and ATS w/o Lcf; $19.4 \pm 9.8\%$) ($p<0.001$).

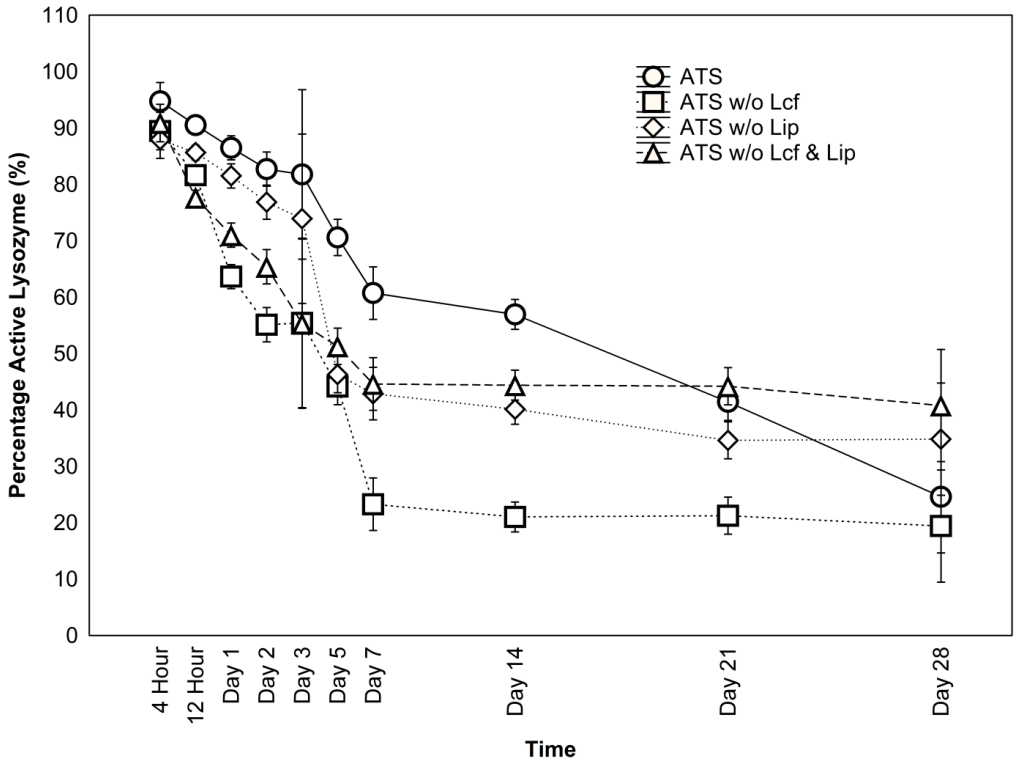

Figure 5-6. Kinetics of percentage active lysozyme recovered from senofilcon A lenses over a 28 day time period. The incubation solutions included a complex artificial tear solution with multiple tear film components (ATS), an ATS without lactoferrin (ATS w/o Lcf), an ATS without lipids (ATS w/o Lip) and an ATS without lactoferrin and lipids (ATS w/o Lcf & Lip).

The kinetics of active lysozyme recovered from etafilcon A following the incubation in all four solutions is displayed in Figure 5-7. There were no significant differences found in the amounts of active lysozyme, in all incubation solutions, throughout the 28 day time period ($p>0.05$). The percentage of active lysozyme in all incubation solutions remained over 77.8 ± 4.6% during the 28 day period.

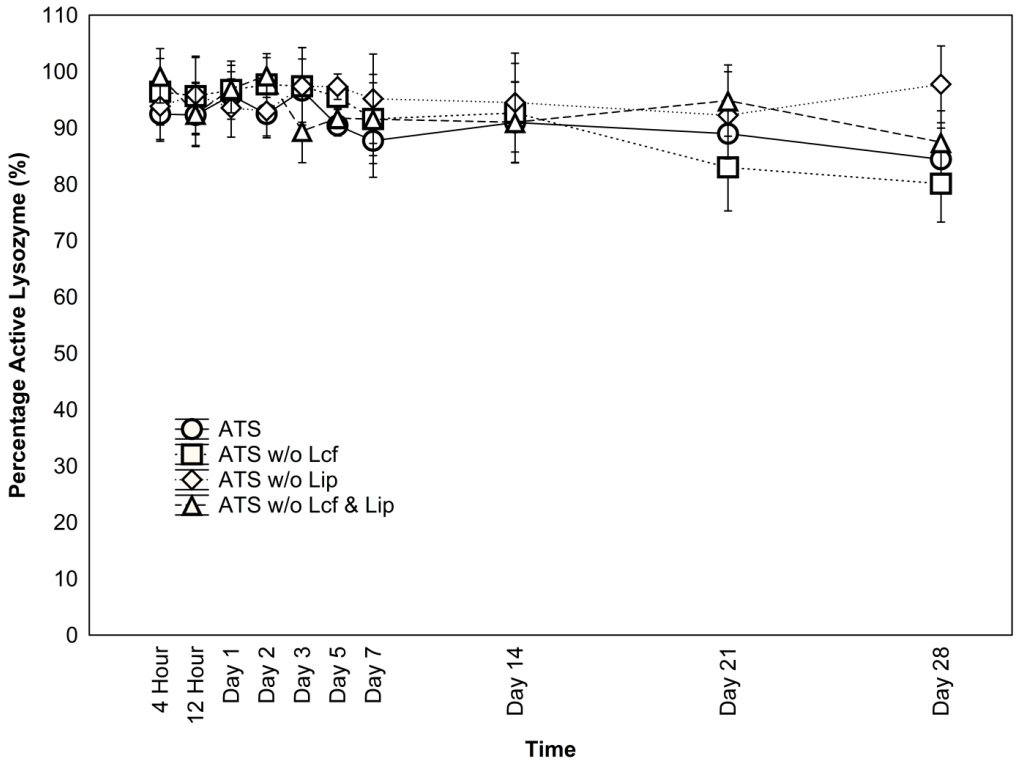

Figure 5-7. Kinetics of percentage active lysozyme recovered from etafilcon A lenses over a 28 day time period. The incubation solutions included a complex artificial tear solution with multiple tear film components (ATS), an ATS without lactoferrin (ATS w/o Lcf), an ATS without lipids (ATS w/o Lip) and an ATS without lactoferrin and lipids (ATS w/o Lcf & Lip).

Figure 5-8 shows the decrease in the amount of active lysozyme recovered from omafilcon A after incubation in all four solutions. There was a gradual decrease in percentage active lysozyme for all solutions until a plateau was reached on day 7 ($p>0.05$). There were no significant differences in lysozyme activity, between all incubation solutions, throughout the 28 day incubation period ($p>0.05$).

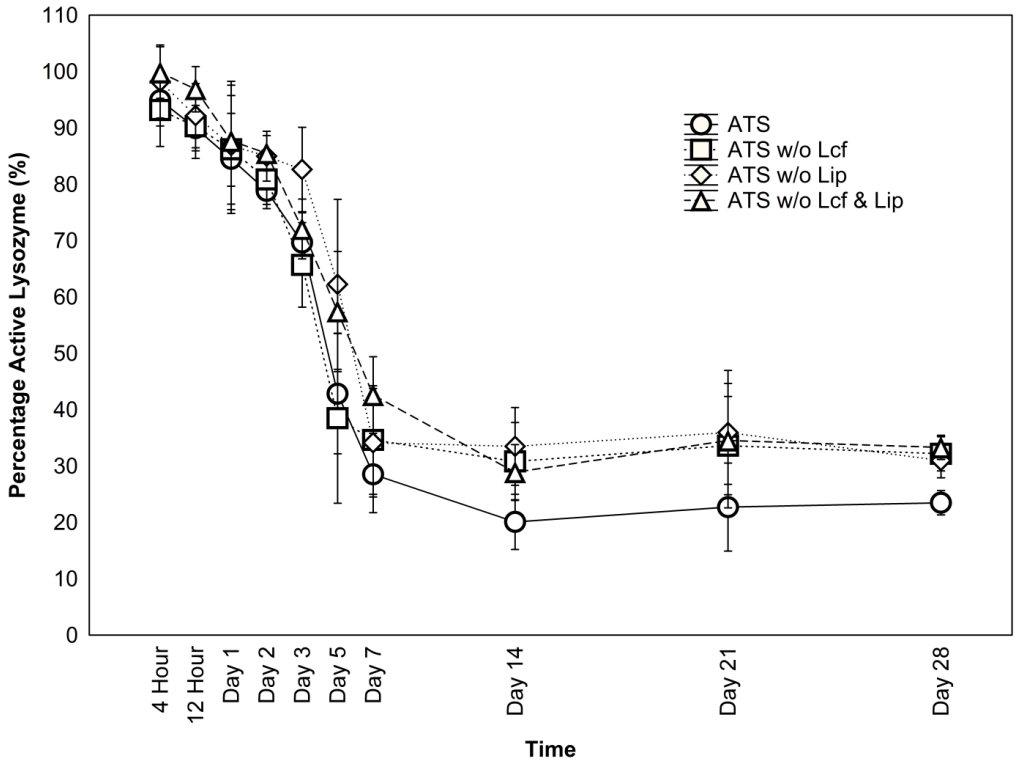

Figure 5-8. Kinetics of percentage active lysozyme recovered from omafilcon A lenses over a 28 day time period. The incubation solutions included a complex artificial tear solution with multiple tear film components (ATS), an ATS without lactoferrin (ATS w/o Lcf), an ATS without lipids (ATS w/o Lip) and an ATS without lactoferrin and lipids (ATS w/o Lcf & Lip).

Table 5-4 presents the amounts of total lysozyme deposited, expressed in micrograms per lens, and Table 5-5 reports the amount of denatured lysozyme, in micrograms per lens, following 1, 7, 14 and 28 days of incubation. The amount of denatured lysozyme (in micrograms) recovered from etafilcon A, in all solutions, was significantly higher than all other lens materials (Table 5).

Table 5-4. Total lysozyme deposited on silicone and conventional hydrogel contact lenses in all four incubation solutions, expressed in µg/lens (mean ± SD)

Lens	Solution	Day 1	Day 7	Day 14	Day 28
lotrafilcon B	ATS	0.8 ± 0.1	0.9 ± 0.8	1.2 ± 1	1.2 ± 1
	ATS w/o Lcf	1.2 ± 0.2	1.4 ± 0.8	1.5 ± 1.1	1.5 ± 0.1
	ATS w/o Lip	1.8 ± 1	1.9 ± 1.3	1.9 ± 0.2	2.0 ± 0.5
	ATS w/o Lcf & Lip	1.9 ± 0.1	2.0 ± 2.0	2.0 ± 0.4	2.1 ± 0.4
senofilcon A	ATS	1.6 ± 0.1	2.2 ± 0.2	3.2 ± 0.1	3.5 ± 0.1
	ATS w/o Lcf	1.8 ± 0.1	2.1 ± 0.6	3.1 ± 0.7	3.2 ± 0.2
	ATS w/o Lip	2.3 ± 0.3	2.8 ± 0.1	3.7 ± 0.5	5.3 ± 0.2
	ATS w/o Lcf & Lip	1.9 ± 0.2	2.7 ± 0.3	3.7 ± 0.5	3.9 ± 0.2
etafilcon A	ATS	493.2 ± 46	1303.2 ± 34	1389.5 ± 54	1394.0 ± 67
	ATS w/o Lcf	492.4 ± 26	1309.9 ± 37	1380.8 ± 47	1393.7 ± 38
	ATS w/o Lip	477.9 ± 51	1370.8 ± 52	1413.3 ± 36	1490.2 ± 50
	ATS w/o Lcf & Lip	526.6 ± 29	1319.7 ± 33	1374.5 ± 43	1392.8 ± 47
omafilcon A	ATS	8.7 ± 3	10.1 ± 5	15.5 ± 9	19.9 ± 5
	ATS w/o Lcf	9.4 ± 4	10.8 ± 2	15.3 ± 6	18.9 ± 9
	ATS w/o Lip	10.2 ± 4	11.9 ± 6	16.1 ± 1	20.9 ± 9
	ATS w/o Lcf & Lip	10.0 ± 7	12.0 ± 5	17.5 ± 3	20.6 ± 2

Table 5-5. Denatured lysozyme deposited on silicone and conventional hydrogel contact lenses in all four incubation solutions, expressed in µg/lens (mean ± SD)

Lens	Solution	Day 1	Day 7	Day 14	Day 28
lotrafilcon B	ATS	0.2 ± 0.1	0.7 ± 0.2	1.0 ± 0.1	1.0 ± 0.1
	ATS w/o Lcf	0.4 ± 0.4	1.0 ± 0.6	1.2 ± 0.3	1.3 ± 0.2
	ATS w/o Lip	0.6 ± 0.2	0.9 ± 0.5	1.1 ± 0.1	1.2 ± 0.6
	ATS w/o Lcf & Lip	0.5 ± 0.1	1.1 ± 0.6	1.3 ± 0.4	1.4 ± 0.3
senofilcon A	ATS	0.2 ± 0.1	0.9 ± 0.1	1.4 ± 0.2	2.6 ± 0.5
	ATS w/o Lcf	0.7 ± 0.1	1.6 ± 0.5	2.4 ± 1	2.6 ± 1
	ATS w/o Lip	0.4 ± 0.2	1.6 ± 0.1	2.2 ± 0.8	3.5 ± 0.6
	ATS w/o Lcf & Lip	0.6 ± 0.1	1.5 ± 0.2	2.1 ± 0.6	2.3 ± 1
etafilcon A	ATS	20.9 ± 9	160.1 ± 15	125.7 ± 20	217.3 ± 27
	ATS w/o Lcf	16.0 ± 8	113.1 ± 17	137.8 ± 22	309.3 ± 19
	ATS w/o Lip	28.3 ± 5	77.2 ± 16	77.7 ± 21	84.5 ± 12
	ATS w/o Lcf & Lip	16.3 ± 8	111.6 ± 11	123.5 ± 17	174.5 ± 18
omafilcon A	ATS	1.4 ± 0.8	7.2 ± 2	12.4 ± 6	15.4 ± 2
	ATS w/o Lcf	1.4 ± 0.3	7.1 ± 3	10.5 ± 3	12.8 ± 5
	ATS w/o Lip	1.6 ± 0.6	8.0 ± 1.5	10.3 ± 5	13.4 ± 7
	ATS w/o Lcf & Lip	1.2 ± 0.3	7.1 ± 3	12.4 ± 1	13.8 ± 6

5.5 Discussion

This study monitored the effects of lactoferrin and lipids on the activity of lysozyme recovered from silicone and conventional hydrogel lens materials over a 28 day incubation period.

5.5.1 Principles of protein adsorption and denaturation

Protein deposition and denaturation on contact lenses is a complex process that begins immediately upon exposure of the material to the protein of interest.[20, 26, 29, 42, 43] The sorption of proteins to biomaterials depends on a number of factors, including the hydrophobicity and ionic charge of the lens material,[20, 44] as well as the size[20] and abundance of the protein.[43, 45] Proteins typically have a higher affinity for hydrophobic surfaces than hydrophilic surfaces, however, the presence of ionic interactions can greatly influence protein deposition on any surface.[20, 44] For some lens materials, proteins accumulate on the contact lens surface and diffusely penetrate into the lens matrix,[46, 47] which increases the total amount of protein that sorbs to a lens.

Following the initial adsorption step, deposited proteins tend to rearrange their structure, allowing thermodynamically favourable interactions with the biomaterial surface.[44] Consequently, the protein partially loses its native structure or becomes "denatured", and may be unable to perform its biological function.[48] For instance, denatured lysozyme has reduced bacteriolytic activity compared to lysozyme in its native form.[49] Several factors influence the degree and rate of denaturation of proteins on contact lenses, including the duration of exposure[30] and the chemical makeup and hydrophobicity of the lens material.[30, 44, 50] Proteins in their native state are folded in such a way that hydrophilic amino acids are exposed to the exterior side of the molecule, whereas the hydrophobic amino acids are hidden in the interior.

When proteins adsorb to hydrophobic surfaces, secondary and tertiary conformation may change, allowing thermodynamically favorable adsorption.[44] Studies have suggested that lysozyme activity is less likely to reduce when proteins are able to accumulate in the lens matrix compared to lysozyme that is primarily bound to the contact lens surface.[18, 30, 50] Ultimately, these factors may influence the biocompatibility of the contact lenses, as denatured protein on contact lenses has been associated with lens discomfort and papillary conjunctivitis.[32-34]

5.5.2 Effects of lens material on lysozyme activity

Silicone hydrogel contact lenses have exceptional oxygen transmissibility.[51, 52] However, these siloxane-based lens materials are more hydrophobic than poly2-hydroxyethyl methacrylate (pHEMA)-based materials and require surface modifications or integration of wetting agents to improve the wettability of the lens material.[53-55] Lotrafilcon B is a silicone hydrogel material that is modified with a 25nm plasma coating on the lens surface. Previous studies[5, 22, 26, 29] have found low amounts of lysozyme deposited on lotrafilcon B, which may be largely influenced by its neutral surface charge, as well as the surface treatment that helps minimize the penetration of lysozyme into the lens matrix.[47] The fact that lysozyme deposits mainly on the lens surface[56] may explain the higher degree of lysozyme denaturation as determined in our current study (Figure 5-1 to 5-4).

Senofilcon A lenses contain an internal wetting agent (polyvinyl pyrrolidone - PVP) to enhance the surface wettability of the lens.[55] Lysozyme can be detected throughout the lens material and in slightly higher concentrations at the lens surface over time.[56] The similar lysozyme deposition profile (distribution and total sorption) for senofilcon A and lotrafilcon B lenses may explain the similar reduction in lysozyme activity in both lens types over time

(Figures 5-1 to 5-4). The only difference between these lens types was seen when lenses were incubated in the full artificial tear solution: senofilcon A lenses exhibited a slightly slower reduction in lysozyme activity over time compared to lotrafilcon B lenses (Figure 5-1). This may be due to the presence of lactoferrin in the solution and will be discussed later in this discussion.

Omafilcon A is a conventional hydrogel material composed of polyHEMA incorporated with phosphorylcholine (PC), a substance associated with improved biocompatibility for various biomaterials[57, 58] and, as contact lens materials, exhibit reduced protein adsorption.[59-62] However, despite its relative resistance to protein deposition, the amount of lysozyme that accumulates on omafilcon A typically ranges from 30 to 60μg,[26, 27, 29] which is higher than the amounts deposited on the silicone hydrogel materials lotrafilcon B and senofilcon A.[5, 26, 27, 29] Lysozyme has been previously shown to diffusely penetrate into the matrix of omafilcon A,[47] which may explain the relatively higher quantities of lysozyme that sorbs to this lens type. In addition, the surface wettability of omafilcon A, as determined by the sessile drop technique, was reported to be comparatively lower than other lens materials (ie exhibited a higher contact angle),[55] which may increase the kinetic denaturation of lysozyme, as previously shown.[30] This is in agreement with the current study, where the percentage of active lysozyme on omafilcon A dropped to between 20-41% in all four solutions during the first week of incubation, and remained at <36% of active lysozyme at the end of the 28 day period (Figures 5-1 to 5-4). These values were comparable to the amounts active lysozyme recovered from lotrafilcon B and senofilcon A, which also displayed high degrees of lysozyme denaturation during the 4 week incubation period.

In many previous studies, etafilcon A deposited significantly more lysozyme than other conventional and silicone hydrogel lens materials.[21, 22, 26, 29, 31] This is due to the ionic monomer methacrylic acid (MA) employed within the lens material attracting the strongly positively charged lysozyme.[63-65] In addition, etafilcon A has a relatively high water content and pore size,[66] permitting significant amounts of lysozyme to diffuse into the lens matrix, as shown by confocal microscopy imaging. [47] Thus, unlike the silicone hydrogel lenses lotrafilcon B and senofilcon A, the ionic pores of etafilcon A allows lysozyme to readily diffuse into the lens material, which may influence the conformational state of lysozyme. In addition, the contact angle of FDA group IV contact lenses is lower than certain silicone hydrogel lens materials, implying that these lenses have a more hydrophilic surface.[55] Etafilcon A-based materials have previously been shown to maintain a high percentage of lysozyme activity, [18, 27, 30, 31] which was further confirmed in the current study. During the 28 day period, >78% of the lysozyme deposited to etafilcon A remained active (Figure 5-7) and was significantly higher than other lens materials (Figure 5-1to 5-4). However, it is important to consider that despite the high percentage of active lysozyme recovered from etafilcon A, the actual amount of denatured lysozyme (by mass) per lens on etafilcon A (which was as high as 309 ± 19μg) was significantly higher than all other lens materials (which ranged from 0.2 to 15μg) (Table 5-5). For the etafilcon A material, the rapid thermodynamic interaction between the positively charged lysozyme and ionic surface will result in the majority of surface binding sites becoming occupied within hours of protein exposure, thus rapidly forming an initial monolayer of lysozyme, as described by Norde.[44] Some lysozyme will penetrate into the lens matrix and will less likely denature in comparison to the surface bound lysozyme. The high amounts of denatured lysozyme on etafilcon A could possibly be the surface bound lysozyme that initially formed the

surface monolayer. This is in agreement with a recent study by Hall et al.[67] where they discovered that the biological function of lysozyme deposited on etafilcon A lens materials rapidly reduces within the first 2 hours of exposure (up to 15% of surface bound lysozyme denatured). Subsequently, lysozyme in the solution begins to accumulate over the top of the existing monolayer[44] and will less likely denature, as they do not directly interact with the lens surface. As a result, the subsequent layers of lysozyme and the lysozyme deposited within the lens matrix make up the total active lysozyme detected on etafilcon A.

5.5.3 Effects of lactoferrin and lipids on lysozyme activity

Silicone hydrogel lenses accumulate relatively higher amounts of lipids than polyHEMA-based materials,[22, 37, 68] and it is suggested that these siloxane-based materials favourably adsorb lipids due to the hydrophobic to hydrophobic interactions between lipids and the lens surface.[4, 63, 69] In addition, the presence of lipids in an artificial tear solution has been previously shown to compete with the adsorption of lysozyme on lotrafilcon B lenses.[29] This may imply that lipid deposits render the surface more hydrophobic, and as a result increase the denaturation of lysozyme. This current study also supports this concept, given that lysozyme activity on lotrafilcon B was significantly lower in the ATS solutions containing lipids compared to the ATS solutions without lipids after the first week of incubation (Figure 5-5). Similar results were obtained with senofilcon A, where the percentage of active lysozyme was slightly higher in the ATS without lactoferrin and lipids and the ATS without lipids (although not significant) compared to the ATS and the ATS without lactoferrin by the end of the 28 day period (Figure 5-6). Moreover, the amount of active lysozyme in the two ATS solutions without lipids was significantly higher than when incubated in the ATS with lipids (without lactoferrin) throughout the 4 week incubation. Interestingly, the percentage of active lysozyme on

senofilcon A in the ATS (consisting of lactoferrin and lipids) did not drop below the ATS solutions without lipids until after day 21. Previous studies have shown that senofilcon A gradually deposits cholesterol (a major non-polar lipid in tears)[70, 71] over a 20 day period[68] and the addition of lipids in the incubation solution only impacted lysozyme deposition on senofilcon A after the 21st day of incubation.[29] This suggests that lysozyme activity on senofilcon A may not be affected by lipids until it reaches 3 weeks of lens incubation, which is of clinical relevance given that senofilcon A is a two-weekly replacement material. The presence of lactoferrin in the ATS may also influence the degree of lysozyme denaturation on senofilcon A, when compared to the other solutions. Chow et al[72] demonstrated that senofilcon A lenses accumulate similar amounts of lactoferrin to lysozyme, during the first 2 weeks of incubation.[26, 29] Lactoferrin is an antimicrobial protein that plays an important role in the ocular defense system against microbes, due to its inhibitory effect on bacterial growth. [73] Lactoferrin also maintains the antibacterial activity of lysozyme.[74-77] This may explain why lysozyme activity was significantly lower after the first week on senofilcon A lenses incubated in the ATS without lactoferrin, compared to the other solutions.

In comparison to the silicone hydrogel lenses, the presence of lipids and/or lactoferrin did not significantly affect the conformational state of lysozyme on the two pHEMA-based materials throughout the study period (Figures 5-7 and 5-8). Group IV lenses, such as etafilcon A, show a much lower affinity for lipids than proteins.[22, 63] Chow and colleagues[72] determined the kinetic deposition of lactoferrin on etafilcon A and found that the amount of lactoferrin deposited at the end of a 28 day period (11.3 ± 1.9μg) was only a fraction of the amount of lysozyme that deposits within the first 24 hours of exposure (>400μg).[26, 29] Although the concentrations of lysozyme and lactoferrin were similar in the simple ATS used in these previous experiments

(1.9mg/mL vs 1.8mg/mL), lactoferrin is much larger than lysozyme (74kDa vs 14kDa), and the isoelectric point is lower for lactoferrin than for lysozyme (IEP pH=8.7 vs pH=11)[78, 79] which explains the differences in the adsorbed quantities on etafilcon A. This suggests that the presence of lactoferrin and lipids would have minimal effects on lysozyme deposition quantity and activity for etafilcon A lenses due to the selective adsorption of lysozyme over both lactoferrin and lipids during incubation, which was confirmed in this study.

Similar to etafilcon A, the activity of lysozyme on omafilcon A was not significantly affected by lactoferrin or lipids (Figure 5-8). Previous studies have shown that omafilcon A accumulates relatively high amounts of lysozyme (compared with silicone hydrogel materials),[26, 29] and moderate amounts of lactoferrin[72] and lipids.[36] In addition, previous work from our group demonstrated that the quantity of lysozyme deposition was minimally impacted by the presence of lactoferrin and lipids.[29]

5.5.4 Comparison to in vitro studies

A number of previous studies have investigated protein denaturation on contact lenses, [22, 27, 30, 31] and these results were comparable with the values reported in this study for the hydrogel lenses. However, variations were noticed when comparing the outcomes obtained from previous studies for the silicone hydrogel lens materials examined. For example, the degree of lysozyme denaturation on lotrafilcon B and senofilcon A in this study (when incubated in the full ATS) was greater than that reported in previous in vitro work using a basic solution of PBS and lysozyme.[30] These differences appear to be largely due to the presence of lipids in the incubation solution, which impacts the adsorption and denaturation of lysozyme on the silicone hydrogel lenses.

Compared with the in vivo wearing situation, limitations of this study were that the lenses were constantly submerged in the ATS during incubation and the solutions were not replenished throughout the experiment. This does not accurately portray contact lens wear, since lenses are frequently exposed to an air-water interface and the tear film is replenished with various components in an in-eye situation. Therefore, future in vitro studies should consider incorporating an artificial eye model that exposes contact lenses to the air, while consistently coating and wetting the lens material with a thin layer of an artificial tear solution that is replenished on a regular basis.

5.6 Conclusion

The degree of lysozyme denaturation that occurs varies between lens types and is dependent on the hydrophobicity of the lens surface and the location of lysozyme in the lens material. The presence of lactoferrin and lipids in an incubation solution did not impact lysozyme activity on the conventional polyHEMA-based hydrogel lenses etafilcon A and omafilcon A. In contrast, the presence of lipids resulted in lower lysozyme activity for the silicone hydrogel lenses lotrafilcon B and senofilcon A. Additionally, the presence of lactoferrin preserved the activity of lysozyme on senofilcon A. Our results show that for in vitro studies examining deposition on silicone hydrogel materials that it is important to use complex models that closely mimic the human tear film. Future in vitro models should incorporate incubation solutions that are as close as possible to the composition of the human tear film and should consider methods to replenish the tear film components and build in the intermittent drying that occurs during the inter-blink period.

6. General Discussion and Conclusion

This thesis has, for the first time, investigated the impact of lactoferrin and lipids on the kinetic deposition and denaturation of lysozyme on contact lenses using a complex artificial tear solution. In addition, a novel fluorescence-based lysozyme activity assay was evaluated and utilized for the quantification of active lysozyme. The following section provides a summary of the results obtained in each study.

The study in Chapter 3 examined the effects of lactoferrin and lipids on lysozyme deposition to both conventional and silicone hydrogel contact lenses. The results have demonstrated that in the presence of lipids in the solution, the amount of lysozyme sorbed to lotrafilcon B and senofilcon A lens materials was significantly reduced. This may be largely influenced by the higher amount of lipids that deposit on silicone hydrogel lens materials compared to lysozyme.[1-3] In addition, the presence of lactoferrin in the solution significantly decreased the amount of lysozyme accumulated on lotrafilcon B, which was not observed with senofilcon A. This could be explained by the competition for binding sites between lysozyme and lactoferrin on the lens surface. Furthermore, the difference in deposition behavior between the two silicone hydrogel lenses may be influenced by the respective surface modification and overall material composition. The amount of lysozyme detected on etafilcon A lenses was significantly higher compared to all other lens materials, due to the strong ionic interactions between lysozyme and the negatively charged lens material.[4-6] Lysozyme deposition on etafilcon A lenses was significantly reduced when incubated in a solution without lipids. It was hypothesized that lipids impede the adsorption of lactoferrin to this lens type, which may explain the increased binding of lysozyme.[7] However, in the absence of lipids, lactoferrin can compete

for binding sites against lysozyme as they are both net positively charged proteins, which resulted in lower amounts of lysozyme deposits. Furthermore, omafilcon A lenses accumulated similar amounts of lysozyme, independent of whether lactoferrin and/or lipids were present or absent in the incubation solution.

The study in Chapter 4 evaluated a novel fluorescence-based lysozyme activity assay for contact lens studies. This method was compared with the lysozyme activity turbidity assay commonly used to measure lysozyme activity, and the results revealed that the two assays were comparable, with no significant differences between measurements. The turbidity assay typically reported slightly higher active lysozyme measurements than the fluorescence-based assay, nevertheless these discrepancies were not considered clinically significant. Both methods have a wide range of detection, however, the novel fluorescence-based assay exhibited a lower detection limit, which is more favourable for studying the conformational state of lysozyme on contact lenses that deposit relatively low levels of lysozyme. To determine the compatibility of the fluorescence-based assay for contact lens studies, typical extraction and evaporation procedures used in previous studies were investigated for interference with this assay. The results showed that neither the use of extraction solvents containing trifluoroacetic acid/acetonitrile and/or evaporation significantly affected lysozyme activity. Furthermore, contact lens extracts from the two silicone hydrogel lenses (lotrafilcon B and senofilcon A) and the two conventional hydrogel lenses (etafilcon A and omafilcon A) displayed no interference with the quantification of active lysozyme using the fluorescence-based activity assay.

The study in Chapter 5 investigated the effects of lactoferrin and lipids on lysozyme denaturation on both conventional and silicone hydrogel contact lenses, using the novel fluorescence-based assay evaluated in Chapter 4. Results from this study indicated that the

presence of lipids in an artificial tear solution increased the denaturation rate of lysozyme on both lotrafilcon B and senofilcon A lens materials. This could be associated with the relatively high levels of lipids that deposit on silicone hydrogel lens materials,[1-3] which may increase the surface hydrophobicity of these materials and hence increase lysozyme denaturation upon adsorption. In addition, lactoferrin helped maintain the activity of lysozyme on senofilcon A, which is in agreement with previous studies that suggested that lactoferrin has a synergistic effect on lysozyme activity against Gram positive bacteria.[8-11] In contrast, the presence of lactoferrin and/or lipids had no significant impact on the activity of lysozyme deposited on both hydrogel materials. This could be due to selective adsorption of lysozyme on these lens materials over both lactoferrin and lipids in the artificial tear solution.[1, 4, 12, 13] Lastly, etafilcon A retained the highest amount of active lysozyme (over 78% remained active) following the 28 day incubation period, whereas the other lens materials, lotrafilcon B, senofilcon A and omafilcon A, exhibited a high degree of lysozyme denaturation during the first week (as little as 23% active lysozyme) before reaching a plateau for the remaining 3 weeks of incubation. The hydrophobicity of the lens material and location of lysozyme deposition in/on the lens were suggested to impact conformational changes of lysozyme following adsorption.[14-17]

The current thesis project revealed that tear film components such as lactoferrin and lipids can alter the deposition profile of lysozyme through competitive interaction with various contact lens materials. These results also suggest that tear film deposits on contact lenses interact with each other and can potentially influence the conformational state of deposited proteins such as lysozyme. Silicone hydrogel lenses deposit relatively low amounts of lysozyme, possibly due to the competitive binding of lipids in the artificial tear solution. Interestingly, despite depositing low amounts of lysozyme, the reduction in lysozyme activity on these lens

materials is significantly greater compared to the conventional hydrogel lenses that deposited higher amounts of lysozyme. This begs the question of whether large amounts of lysozyme deposited on contact lens materials have a greater potential to maintain their conformational state than low amounts of deposited lysozyme. Furthermore, patients fitted with silicone hydrogel lenses are prone to develop contact lens induced papillary conjunctivitis (CLPC).[18, 19] The results from this thesis demonstrated that the degree of lysozyme denaturation on silicone hydrogel lenses is relatively high, particularly during the first week of incubation. However, the amount of denatured lysozyme, by mass, is relatively low. In contrast, etafilcon A maintained a relatively high percentage of active lysozyme throughout a 4 week period; however the amount of denatured lysozyme, by mass, is significantly higher compared to the amounts on silicone hydrogel lenses. These findings continue the debate on whether CLPC is mainly developed through mechanical trauma (due to the physical presence of a lens) or do denatured proteins on contact lenses play a significant role in triggering this immunological response.

In comparison to previous *in vitro* studies, the results from the current thesis work displayed some variations in the amounts of lysozyme deposited. In previous studies,[13, 20-22] the amount of lysozyme accumulated on senofilcon A, etafilcon A and omafilcon A was higher than that reported in Chapter 3, while lotrafilcon B displayed comparatively lower lysozyme deposition (Table 6-1).

Table 6-1. Total lysozyme deposition (µg/lens) to contact lenses after a 2 week exposure period

Lens type	**Current thesis work** (Chapter 3)*	**Current thesis work** (Chapter 5)**	**Previous *in vitro* studies** [13, 21] (using radiolabeling)	**Previous *ex vivo* studies** [1, 23] (using SDS-PAGE or Western blot)
Lotrafilcon B	10.4 ± 0.3	1.2 ± 1	Ranging between 3.7 ± 0.6 to 9.7 ± 1.5	1.4 ± 1.1
Senofilcon A	2.9 ± 0.1	3.2 ± 0.1	6.1 ± 3.2	1.6 ± 1.6
Etafilcon A	694.8 ± 5.8	1389.5 ± 54	1433.5 ± 76	985 ± 241
Omafilcon A	12.5 ± 0.2	15.5 ± 9	35.3 ± 8	N/A

Total lysozyme deposition to contact lenses quantified using radiolabeling* or ELISA**

This may have been largely influenced by the complex composition of the artificial tear solution used in this thesis as compared to the simple tear film models utilized previously. Furthermore, various components in the artificial tear solution could potentially have a greater preferential interaction with either the radiolabeled or non-radiolabeled lysozyme, which ultimately interferes with the accuracy of the radiolabeling technique. This would not normally occur in previous *in vitro* studies[13, 20-22] where other proteins, lipids and mucins were not present in the incubation solution. In addition, free iodine atoms could potentially interact with other components in the artificial tear solution that deposit on the lens material, consequently leading to false positive/negative results. Future work should investigate the differences in interactions between tear film components and radiolabeled or non-radiolabeled lysozyme, as well as incorporating a dialysis procedure to reduce the amount of free iodine in the radiolabeled samples. When compared with *ex vivo* results,[1, 23, 24] lysozyme uptake was similar to the current thesis work, particularly when using the ELISA for total lysozyme quantification (Table 6-1). In addition, the reduction in lysozyme activity on all lens materials was almost identical to previous *ex vivo* results. It is often difficult for *in vitro* studies to produce results that closely resemble

outcomes obtained in real life conditions. Factors such as variations in the human tear film concentration during the day and under different health conditions, blinking and the constant replenishment of fresh tears over the anterior surface of the eye all influence contact lens deposition but are extremely hard to mimic in an artificial environment. In comparison to previous *in vitro* models, the use of a complex artificial tear solution was relatively effective in closely mimicking the deposition and denaturation of lysozyme on contact lenses, especially the silicone hydrogel lenses, which are significantly impacted in the presence of lipids.

In conclusion, the kinetic deposition and denaturation of lysozyme on contact lenses is affected by lactoferrin and/or lipids. These effects were highly dependent on the chemical composition and properties of each lens material. The results from these studies further support the importance of using complex artificial tear solutions that strongly portray the tear film when conducting in vitro studies investigating the interaction between tear film constituents and soft contact lens polymers.

7. Future Studies

This final chapter will focus on potential future projects that could follow-on from the results accumulated in this thesis. The goal is to further enhance our understanding of the interactions between the tear film and contact lenses, which may ultimately facilitate in the future development of better and more biocompatible contact lens materials.

Developing an artificial eye model to mimic contact lens wear

The *in vitro* model used in this thesis had a number of limitations which may impact the lysozyme sorption profile and therefore deviate from *ex vivo* findings. Firstly, the lenses were not exposed to an air-water interface to simulate the blinking reflex, and this intermittent drying could potentially increase both lipid deposition and protein denaturation due to the increased hydrophobicity that occurs during the inter-blink period. Secondly, the incubation solutions were not replenished consistently as would occur during contact lens wear. Thirdly, the movement of the eyelid over contact lens during blink could impact tear film deposition on contact lenses. Finally, most lens wear schedules include an overnight cleaning cycle, which may further impact subsequent deposition profiles. Future studies should consider developing an artificial model that can expose contact lenses to the air while consistently spreading and replacing artificial tear fluid over and under the lens material and could consider including some option of lens cleaning at various phases during the incubation period.

Investigating the impact of lipocalin on other tear film deposits

Another major protein identified in the tear film is lipocalin.[1] This negatively charged protein interacts with the two positively charged proteins lysozyme and lactoferrin.[2]

Interestingly, tear lipocalin has been suggested to enhance the bacteriolytic function of lysozyme,[3] and also acts as a lipid scavenger, which facilitates the removal of lipid contaminants in tears through the lacrimal duct.[4] Lipocalin has a strong affinity for fatty acids, which is beneficial for lysozyme, as long chain fatty acids denature lysozyme in tears.[5-7] Hence, it would be interesting to investigate whether the presence of lipocalin in an artificial tear solution can impact the subsequent deposition and denaturation rate of lysozyme. In addition, given that lipocalin has a strong binding affinity for certain lipids, it would be interesting to determine if lipocalin can reduce the amount of lipids that deposit on silicone hydrogel contact lenses.

Studying the oxidative degradation of lipids on contact lens materials

Lipids can undergo oxidative degradation resulting from loss of electrons under oxidative stress. [8] The degradation of tear lipids has been previously suggested to occur in conjunction with contact lens wear due to lens-induced hypoxia.[9] Hence, it would be of interest to investigate the factors that influence lipid degradation on contact lenses and the effects of oxidized lipids on the denaturation of proteins deposited on contact lenses. Furthermore, it would also be interesting to study the potential impact of various tear film components on the stability of lipids deposited on contact lens materials.

Evaluating patient worn lenses and compare with tear film concentrations

Tear concentrations often vary between individuals and are largely influenced by the individual's age and health.[10-13] As a result, the variations in their tear film may impact the degree to which individual tear film components deposit on contact lenses. A future project could investigate how these differences in tear concentration influence contact lens deposition. For example, do patients with a relatively low tear concentration of lipids deposit less or more

lysozyme on their silicone hydrogel lenses? The *ex vivo* results from these studies would potentially coincide with the current thesis work in determining the impact of tear film components on lysozyme deposition to various contact lens materials.

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Appendix

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Best wishes,

Kurt

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From: Alan Ng[mailto:akl2ng@gmail.com]
Sent: Tuesday, June 26, 2012 10:24 AM
To: Zadnik, Kurt
Subject: Permission to include an OVS paper for my MSc thesis

Good morning Kurt,

My name is Alan Ng, I am a MSc student at the School of Optometry in the University of Waterloo. Earlier this year, I submitted a manuscript to OVS that was recently published in the April issue. The title of the OVS article was: Impact of Tear Film Components on Lysozyme Deposition to Contact Lenses.

Because this project was a major component in my MSc thesis, I would like to ask you for permission to include this article as one of my thesis chapters.

Thank you for your time and consideration. Sincerely, Alan Ng