In Vitro Competitive Binding of Major Tear Proteins to Contact Lenses

by

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AUTHOR'S 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.

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ABSTRACT

Purpose

To study the impact of incubation solution complexity on protein deposition to silicone hydrogel (SH) contact lenses and the effect of contact lens cleaning solutions on protein activity and removal.

Methods

The SH materials investigated in this thesis included senofilcon A (SA), lotrafilcon B (LB), and balafilcon A (BA). Contact lenses were incubated in both a simple saline solution containing only one protein and a more complex artificial tear solution (ATS) containing numerous proteins, lipids, and mucins. Protein deposition was determined at days 1, 7, 14, and 28.

- For the first *in vitro* experimental chapter (Chapter 3), ^{125}Iodine radiolabeled albumin, lactoferrin, and lysozyme were the proteins of interest. Protein deposition to the different SH contact lenses was quantified using a gamma counter.
- For the second *in vitro* experimental chapter (Chapter 4), ^{125}Iodine radiolabeled lysozyme was the protein of interest and the effect of a contact lens care regimen (OPTI-FREE RepleniSH) on lysozyme deposition to SH lenses was determined.

The effect of contact lens cleaning solutions on lysozyme activity and removal was also determined.

- For the third *in vitro* experimental chapter (Chapter 5), the effect of cleaning solutions on lysozyme activity was determined. OPTI-FREE RepleniSH (OFR), BioTrue (BT), and Clear Care (CC) were the cleaning solutions used in this study. Lysozyme activity was determined at hours 1, 12, 24, and 48 using the turbidity assay.

Results

The three proteins investigated exhibited unique deposition profiles which were impacted by the complexity of the incubation solution and the SH materials. In Chapter 3, BA lenses incubated in a complex analogue of the human tear film accumulated the most lysozyme and albumin. Lactoferrin deposited in greater amounts when LB lenses were incubated in a simple saline solution containing only lactoferrin. Protein deposited similarly on SA lenses, independent of incubation solution used.

In Chapter 4, the cleaning efficacy of OFR was determined for different SH materials. Lysozyme was more efficiently removed when both SA and LB lenses were incubated in a complex ATS. As seen in Chapter 3, SA lenses were not impacted by the complexity of the incubation solution. The protein removal efficacy of multi-purpose solution (MPS) for BA lenses was similar independent of incubation solution used. BA lenses incubated in ATS attracted the greatest amount of lysozyme; however, LB lenses incubated in the same solution allowed for the greatest protein removal by OFR.

In Chapter 5, MPS had varying effects on lysozyme activity. For OFR, there was an enhanced lysozyme activity throughout the 48-hour time period. For BT, the activity at hr 1 was relatively low, but an increase was seen by hr 12, which remained stable up to hr 48. For CC, there was a denaturing effect on lysozyme, and the activity was decreased significantly throughout the duration of the experiment.

Conclusions

Protein deposition profiles varied when lenses were incubated in either a simple saline solution or a complex artificial tear solution and deposition patterns varied between SH materials. The efficacy of a contact lens care regimen (OFR) to remove deposited lysozyme from SH lenses was dependent on both lens material and incubation solution. Lysozyme activity was impacted differently by the three care regimens, with OFR seemingly enhancing activity, BT stabilizing it, and CC exhibiting decreased activity. Future *in vitro* studies should use more appropriate analogues of the human tear film in order to better mimic the in vivo situation, as the complexity of the incubation solution has a clear impact on protein deposition profiles.

// ACKNOWLEDGEMENTS

DEDICATION

I lovingly dedicate this thesis to my parents and brothers, who have constantly provided me endless support. To my mother, I thank you for your fortitude and belief in me no matter what. To my father, for raising me with selflessness and helping me to journey through life with intellect, I thank you.

Thank you to Aysha, my best friend who has been like a sister to me, whose constant optimism allowed me to see the light at the end of the tunnel.

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TABLE OF CONTENTS

List of Figures ix

List of Tables xi

List of Symbols and Abbreviations xii

1. Introduction 1

  1.1 Contact lenses 1

    1.1.1 Deposition profiles 3

  1.2 Contact lens solutions 4

    1.2.1 Multi-purpose solutions 4

    1.2.2 Hydrogen peroxide-based solutions 4

  1.3 Human tear film 5

    1.3.1 Tear film deposits 5

    1.3.2 *In vitro* vs. *ex vivo* studies 5

      1.3.2.1 Incubation solution 6

  1.4 Protein deposition 7

    1.4.1 Lysozyme 7

    1.4.2. Lactoferrin 8

    1.4.3 Albumin 9

  1.5 Protein Quantification Techniques 10

    1.5.1 Amino acid analysis 10

    1.5.2 Bicinchoninic Acid (BSA) Assay 10

    1.5.3 Enzyme-linked immunosorbent assay (ELISA) 10

    1.5.4 High performance liquid chromatography (HPLC) 11

    1.5.5 Micrococcal assay 11

    1.5.6 Radiolabeling 12

    1.5.7 Western blotting (WB) 12

2. Thesis Rationale 14

3. Composition of incubation solution impacts *in vitro* protein uptake to silicone hydrogel contact lenses 16

3.1 Overview 17
3.2 Introduction 19
3.3 Methods 21
3.4 Results 25
3.5 Discussion 32
3.6 Acknowledgements 35
4. Effect of multi-purpose solution on competitive binding protein to silicone hydrogel contact lenses..36
4.1 Overview 37
4.2 Introduction 39
4.3 Methods 40
4.4 Results 43
4.5 Discussion 48
4.6 Conclusion 52
4.7 Acknowledgements 53
5. Impact of contact lens cleaning solutions and lens cases on lysozyme activity 54
5.1 Overview 55
5.2 Introduction 57
5.3 Methods 58
5.4 Results 62
5.5 Discussion 72
6. General Discussion and Conclusion 77
7. Future Studies 82
References 84
Chapter 1 References 84
Chapter 3 References 97
Chapter 4 References 103
Chapter 5 References 107
Chapter 7 References 111
Appendices 112

LIST OF FIGURES

Figure 3-1. Flowchart depicting layout of two-part study, where each time point contained three replicates 22

Figure 3-2. Lysozyme deposition to senofilcon A, lotrafilcon B and balafilcon A lenses using a single protein solution and a complex ATS solution. Incubation points: 1, 7, 14 and 28 days 27

Figure 3-3. Lactoferrin deposition to senofilcon A, lotrafilcon B and balafilcon A lenses using a single protein solution and a complex ATS solution. Incubation points: 1, 7, 14 and 28 days 29

Figure 3-4. Albumin deposition to senofilcon A, lotrafilcon B and balafilcon A lenses using a single protein solution and a complex ATS solution. Incubation points: 1, 7, 14 and 28 days 31

Figure 4-1. Histology cassettes holding four lenses....42

Figure 4-2. Effect of a multi-purpose cleaning solution (OPTI-FREE RepleniSH) on lysozyme deposition to senofilcon A lenses using a single protein solution (Solution A) and a complex ATS (Solution B). Incubation time points: 1, 7, 14 and 28 days....44

Figure 4-3. Effect of a multi-purpose cleaning solution (OPTI-FREE RepleniSH) on lysozyme deposition to lotrafilcon B lenses using a single protein solution (Solution A) and a complex ATS (Solution B). Incubation time points: 1, 7, 14 and 28 days....46

Figure 4-4. Effect of a multi-purpose cleaning solution (OPTI-FREE RepleniSH) on lysozyme deposition to balafilcon A lenses using a single protein solution (Solution A) and a complex ATS (Solution B). Incubation time points: 1, 7, 14 and 28 days....47

Figure 5-1. Contact lens cases and solutions used in experiment 60

Figure 5-2. Effect of multi-purpose solution (MPS) concentration on lysozyme activity using a fixed amount of lysozyme. (Solutions were placed in polypropylene tubes)...62

Figure 5-3: Effect of OPTI-FREE RepleniSH (OFR) and saline on lysozyme activity using a high (500µg) and low (10µg) protein concentration in an OFR lens case. Time points: 1, 6, 12, 24, and 48 hours (n=3) 64

Figure 5-4. Effect of BioTrue (BT) and saline on lysozyme activity using a high (500µg) and low (10µg) protein concentration in a BT lens case. Time points: 1, 6, 12, 24, and 48 hours (n=3)............................66

Figure 5-5. Effect of Clear Care (CC) and saline on lysozyme activity using a high (1500µg) and low (20µg) protein concentration in a CC neutralization cassette. Time points: 1, 6, 12, 24, and 48 hours (n=3).......68

Figure 5-6. Effect of saline on lysozyme activity when high concentrations of lysozyme are placed in MPS and polypropylene, control cases (500µg) and neutralization cassettes (1500µg). Time points: 1, 6, 12, 24, and 48 hours (n=3)..70

Figure 5-7. Effect of saline on lysozyme activity when low concentrations of lysozyme are placed in MPS and polypropylene, control cases (10µg) and neutralization cassettes (20µg). Time points: 1, 6, 12, 24, and 48 hours (n=3) 71

LIST OF TABLES

Table 1-1. Classification of contact lens materials through the FDA 2

Table 1-2. Properties of common silicone hydrogel contact lens materials 3

Table 3-1. Components of the Saline Solution 23

Table 3-2. Components of the Complex Artificial Tear Solution 24

Table 4-1. Properties of silicone hydrogel contact lenses 41

Table 4-2. Properties of the cleaning solution OPTI-FREE RepleniSH 42

Table 5-1. Properties of contact lens care regimens investigated 59

Table 5-2. Outline of experimental procedure 61

LIST OF SYMBOLS AND ABBREVIATIONS

ATS	artificial tear solution
BA	balafilcon A
BCA	bicinchoninic acid
BCL	bovine colostrum lactoferrin
BML	bovine milk lactoferrin
BSA	bovine serum albumin
BT	BioTrue
CC	Clear Care
CLPC	contact lens induced papillary conjunctivitis
Dk	oxygen permeability
Dk/t	oxygen transmissibility
EA	etafilcon A
ELISA	enzyme-linked immunosorbent assay
FDA	US Food and Drug Administration
GA	galyfilcon A
GPC	giant papillary conjunctivitis
HEL	hen egg lysozyme
HPLC	high performance liquid chromatography
hr	hour
ICL	iodine monochloride
ISO	International Organization for Standardization
kDA	kiloDalton
LB	lotrafilcon B
µg	microgram
µL	microliter
mg	milligram
min	minutes
mL	millitre

MPS	multi-purpose solution
ng	nanogram
OD	optical density
OFE	OPTI-FREE Express
OFR	OPTI-FREE RepleniSH
PBS	phosphate buffer saline
pHEMA	poly-2-hydroxyethyl methacrylate
PP	polypropylene
PVP	polyvinylpyrrolidone
SA	senofilcon A
SDS	sodium dodecyl sulfate
sec	seconds
SH	silicone hydrogel
TMB	3,3′,5,5′-tetramethylbenzidine
TFA/ACN	trifluoroacetic acid/acetonitrile
USAN	United States Adopted Names
WB	Western Blotting

1 INTRODUCTION

1.1 CONTACT LENSES

Contact lenses have become increasingly popular to correct refractive errors, mainly due to their cosmetic and optical advantages over spectacles [1-4]. Contact lenses are more convenient to wear in certain weather conditions (such as rain and humidity) or to prevent fogging in the winter. Athletes also benefit from the use of contact lenses in their activities and the incidence of injury to the face as a result of direct impact is greatly reduced. It may be more convenient to wear contacts in a sport that requires constant movement, running, jumping or tackling to ensure steady vision. Contact lenses also provide increased peripheral vision. Additionally, managing various medical conditions requires the use of contact lenses. Patients suffering from keratoconus may not have the visual ability to wear spectacles due to significant corneal distortion. Finally, a patient with a high refractive error requires thicker lenses and perhaps a limited choice of frames and may choose to wear contact lenses over spectacles. The use of contact lenses is aesthetically appealing for many patients and has the potential to increase self-esteem and confidence in social settings.

The prevalence of myopia has increased significantly over the past few decades, with over 40% of young adults in the Unites States [5] and 70% in East Asian countries [6] now being myopic. Interestingly, the prevalence of myopia has increased since 1971 by 66% [5]. Over 125 million people worldwide wear contact lenses [1], and the different materials available for soft lenses have been classified under four Food and Drug Administration (FDA) groups (Table 1-1). Two properties of contact lens materials include oxygen permeability (Dk) and oxygen transmissibility (Dk/t). Diffusion and solubility are two factors that determine the Dk of a material, which provides a guide to the ability of a material to deliver oxygen to the cornea [7]. The rate at which oxygen molecules can pass through a polymer is diffusion, whereas solubility is the number of oxygen molecules within the polymer [7]. Dk/t refers to the Dk of a specific material of a given thickness [7]. Conventional hydrogel contact lens materials were introduced in the 1970's [8,9] and consist of poly-2-hydroxyethyl methacrylate (pHEMA) and exhibit relatively low DK/t, which may lead to hypoxic complications [10,11]. Silicone hydrogel (SH) lenses were introduced in 1999 [12], nearly 30 years after conventional lenses' debut to the market. The high Dk/t of these materials

caused fewer hypoxic complications [12,13] and as a result, the number of pHEMA-based hydrogel lens wearers decreased rapidly, accounting for approximately 25% today [14].

Table 1-1: Classification of contact lens materials through the FDA

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

Low= <50%, high= >50%

An important contact lens property relates to its wettability, which determines the ability of the tear film to uniformly spread across the lens surface. As presented in Table 1-2, in order to improve the in-eye wettability of hydrophobic SH materials, various treatments or surface modifications have been used. As an example, senofilcon A (SA) and galyfilcon A (GA) lenses contain an internal wetting agent (polyvinyl pyrrolidone (PVP)), which is incorporated in the material [15], lotrafilcon B (LB) undergoes a plasma treatment [16], and balafilcon A (BA) undergoes a plasma oxidation process [17].

The combination of silicone and hydrogel components give SH lenses unique properties of high Dk/t and ion permeability. These key features allow for SH lenses to be worn for extended periods of time, with certain materials being approved for up to 30 days without removal [18]; however, deposition of tear film components, such as lipids [19,20] and proteins [21,22], may cause ocular complications and/or limit the length of time such lenses can be worn without removal occurring.

Table 1-2: Properties of common silicone hydrogel contact lens materials

USAN	Balafilcon A	Comfilcon A	Galyfilcon A	Lotrafilcon A	Lotrafilcon B	Senofilcon A
Trade Name	PureVision	Biofinity	Acuvue Advance	Air Optix Night & Day Aqua	Air Optix Aqua	Acuvue Oasys
Manufacturer	Bausch & Lomb	Cooper Vision	Johnson & Johnson	CIBA Vision	CIBA Vision	Johnson & Johnson
FDA Category	III	I	I	I	I	I
Centre thickness (mm) at -3.00D	0.09	0.08	0.07	0.08	0.08	0.07
Water content (%)	36	48	47	24	33	38
Oxygen permeability (Dk)	91	128	60	140	110	103
Oxygen transmissibility (Dk/t) at 35°C	101	160	86	175	138	147
Surface modifications	Plasma oxidation process	No surface treatment	No surface treatment. Internal wetting agent (PVP)	25nm plasma coating (high refractive index)	25nm plasma coating (high refractive index)	No surface treatment. Internal wetting agent (PVP)

PVP= polyvinyl pyrrolidone

1.1.1 Deposition Profiles

It is evident that pHEMA-based and SH contact lens materials result in different deposition profiles, due to variations in water content, surface charge and surface hydrophilicity [23,24]. The relatively hydrophilic nature of pHEMA-based lenses results in them accumulating relatively low amounts of lipids and relatively higher amounts of proteins than SH materials; this trend is reversed for the more hydrophobic SH materials [19,25-31]. When comparing *in vitro* study data, 1434-1800μg of lysozyme deposit onto pHEMA lenses [32-34], whereas SH lens materials accumulate significantly less protein (<20μg per lens) [27,35]. Studies have further shown that SH materials accumulate a greater percentage of denatured protein [34] compared to pHEMA lenses. The SH material, LB, accumulates at most 25% active lysozyme [27,34,35], whereas the conventional lens material, specifically etafilcon A (EA), accumulates over 75% active lysozyme [27,34,35]. With respect to lipids, the SH lens material, BA,

accumulates up to 600µg per lens compared to 20µg accumulated onto the conventional EA lens material [27], although more recent work has questioned this amount and demonstrated lower amounts [36,37]. In a recent study [29], specific lipids, such as cholesterol and phosphatidylcholine, accumulated in greater amounts onto SH lens materials compared to FDA group IV lenses.

1.2 CONTACT LENS SOLUTIONS

The main purpose of contact lens care regimens is to disinfect the lens [38,39] and to remove tear film deposits [37,40-42]. Contact lens care regimens have the ability to modify both the chemical and physical properties of the lens surface [37]. For example, the hydrophilicity of contact lenses is maximized through the addition of various wetting and lubricating ingredients to contact lens care solutions [43].

1.2.1 Multi-Purpose Solutions

Multi-purpose solutions (MPS) have the largest market share today [14] and a recent study has shown that practitioners recommend this type of care regimen to 77% of their contact lens patients [14]. MPS consist of anti-microbial agents [44], chelators, buffer systems, surfactants, and wetting agents [45,46]; however, their ability to remove proteins from contact lenses is typically less than 50% [40,47]. Biocompatibility of the MPS with the anterior eye is important [48], particularly because of the potential release of MPS from the lens onto the corneal surface [49].

1.2.2 Hydrogen Peroxide-Based Solutions

According to market research conducted US practitioners, hydrogen peroxide (H_2O_2) systems are recommended to approximately 22% of contact lens wearers [14]. At a concentration of 3%, H_2O_2 systems have proven effective against bacteria and fungi [50,51]. Neutralisation of the peroxide into water and oxygen is accomplished by platinum discs or tablets containing catalytic enzymes [52], providing a preservative-free environment [45]. The care regimens AOSept and Clear Care use platinum discs for the neutralization process, which reduce the initial 3% H_2O_2 to 0.9% within the first couple of minutes [53].

1.3 HUMAN TEAR FILM

Consisting of lipid, protein, and mucin components, the human tear film carries out a number of vital functions for the eye [54]. Acting as a lubricant for the conjunctiva, cornea, and eyelids [55], the tear film allows for a smooth optical surface [56]. The tear film also provides nutrients to the avascular cornea and washes exogenous components from the anterior eye [55]. The tear lipids both hinder evaporation and stabilize the tear film [54], while tear proteins participate in a number of roles, including protection against microorganisms and controlling immune responses [57]. The mucins in the tear film provide lubrication to the ocular surface, decreasing friction during blinking [58], and protect the cornea and conjunctiva by coating foreign bodies in order to shield the epithelial surfaces [54].

1.3.1 Tear Film Deposits

Human tear film components deposit on the contact lens once it is inserted onto the anterior surface of the eye. These deposits may result in reduced visual acuity [59], decreased wettability [60], and may cause a dryness sensation or discomfort [61]. Furthermore, some deposits may lead to increased bacterial adhesion [62] and inflammatory complications, such as giant papillary conjunctivitis (GPC) [63]. A number of symptoms are associated with GPC, such as itchiness and decreased lens tolerance due to contact lens awareness [64]. The mechanical strain caused by the contact lens in rubbing against the upper tarsal conjunctiva [63] and denatured proteins deposited on the lens surface can trigger this condition [65].

1.3.2 *In Vitro* vs. *Ex Vivo* Studies

In vitro studies are those that occur outside the living organism, whereas *ex vivo* studies are those experiments that are conducted in a laboratory setting outside of the living organism. Contact lens studies that conduct experiments on patient-worn lenses are commonly considered *ex vivo* studies. Both *in vitro* and *ex vivo* studies allow an experimenter to conduct trials that do not require an intact organism. Nonetheless, each study design has its advantages and disadvantages. *In vitro* studies are generally inexpensive, less time consuming, and provide the ability to control variables. However, one of

the major disadvantages of *in vitro* studies is that they do not closely mimic the in vivo situation and hence struggle to provide accurate, truly predictive results.

Naturally, the protein sorption profiles of *in vitro* contact lens studies differ from those of *ex vivo* studies [27,32-35,66-69]. A number of effects are lacking in the *in vitro* model, such as blinking, surface drying, and cleaning processes, and therefore it is not uncommon that *in vitro* and *ex vivo* models elicit different deposition patterns. Despite all of the differences between *in vitro* and *ex vivo* study designs, SH materials have not shown great differences between protein deposition profiles; for example, worn SA lens materials accumulate about 7µg [37,67,68] (25% of which is lysozyme [67,68]), whereas incubated SA lenses accumulate 6-13µg of lysozyme [33,34]. Over 7µg of total protein [69-71], of which less than 25% is lysozyme [67,68], accumulate on patient-worn LB contact lenses; however, *in vitro* models accumulate 6-10µg of lysozyme per lens [34,40]. Worn BA lenses accumulate 5-34µg of protein [37,67,68], where 32-50% [40,72] is lysozyme. *In vitro* studies have shown that about 10µg of lysozyme deposit onto BA lenses [33,72]. Conventional hydrogel materials, unlike SH materials, show differences between *in vitro* and *ex vivo* deposition patterns; for example, with regards to ionic pHEMA lenses, *in vitro* models accumulate 1434-1800µg of lysozyme [32-34], whereas *ex vivo* models accumulate 985-991µg of lysozyme [27,66].

1.3.2.1 *Incubation solution*

For *in vitro* studies, the composition of incubation solution plays a pivotal role in protein deposition onto contact lens materials. In a number of previous studies [33-35,40,73-75], the incubation solution consists of a simple saline that contains a single protein of interest. The competitive nature and complexity of the human tear film is not considered, hence deposition patterns are not an appropriate representation of the in vivo situation. Few studies [76-78] have incorporated the complexity of the tear film in order to understand the deposition behaviour and competitive nature of both lipids and proteins when depositing onto pHEMA-based contact lens materials. Specifically, Mirejovsky and colleagues [76] confirmed that lipid deposition patterns seen on hydrogel lenses incubated in an artificial tear solution (ATS) that contained proteins, glycoproteins, and lipids were similar to patient-worn study lenses. Similarly, Prager and Quintana [77] determined that protein deposition patterns were dependent on

incubation solution composition (both simple and complex solutions), lens material used, and lens exposure time to the ATS. It is, however, unclear whether similar competitive behaviour is present when various SH materials are incubated in a more complex ATS. A recent study conducted by Ng and colleagues [79], determined that both lactoferrin and lipids impact lysozyme deposition to both SH and conventional hydrogel lens materials, where deposition patterns were lens material-dependent. FDA group I and II lenses accumulated less lysozyme in the presence of lipids; however, the presence of lactoferrin decreased the amount of lysozyme accumulated onto group IV lenses [79]. Overall, the complexity of the tear film impacted the deposition pattern of protein, whereas previous studies that incubated lens materials in a simple solution did not show similar deposition patterns.

1.4 PROTEIN DEPOSITION

1.4.1 Lysozyme

Lysozyme was discovered in the early 20th century and is a bacteriolytic protein [80] with immune and anti-tumour properties [81]. Spanning a length of 129 amino acids [82], this small (14.5 kDA) positively charged protein is derived from the lacrimal glands [83], constituting 1.9 mg/mL of the human tear film [84]. Lysozyme is one of the most abundant proteins in the tear film and has also been detected in other bodily fluids, although in much lower concentrations [85]. Biological fluids in which lysozyme is found include blood serum, mucous, and saliva. The antimicrobial properties of lysozyme allows for the hydrolyzing of glycosidic bonds in the peptidoglycan (outer sugar coating) of gram-positive bacteria [81]. The hydrolysis of the β-1,4 linkage between N-acetylmuramic acid and N-acetyl-D-glucosamine residues causes the cell wall to rupture, leading to cell lysis [86]. Other studies have investigated protein-lipid interactions [87] and protein adsorption to interfaces [88] using lysozyme as a model protein [81].

Lysozyme concentration in the tear film remains relatively constant when tear flow is stimulated (reflex tears) [89,90] and during open or closed eye conditions [84]. Pietsch and Pearlman reported that lysozyme concentrations are highest between 11 and 20 years of age [91], and the concentration decreases at a rate of 1mg/dL per year after the age of 40 [92]. In the tear film of lens wearers, lysozyme has a slightly reduced biological activity compared to non-lens wearers, as determined by electrophoresis [93], which could lead to an increased risk of corneal inflammation and infection [94].

Kramann and colleagues further determined a decrease in lysozyme protein with contact lens-wear [95]. With regards to overnight wear, Choy and colleagues found that there was no change in lysozyme concentrations when orthokeratology (ortho-k) lenses were worn [96].

1.4.2 Lactoferrin

Lactoferrin is a positively charged iron-binding protein, with a molecular weight of 80 kDA [97,98]. The length of the protein spans as a single polypeptide chain [99] made up of 692 amino acids [100,101]. Lactoferrin is mainly secreted from lacrimal gland acini [102,103] and has a concentration of 1.5-2.2 mg/mL in the human tear film [97]. The concentration of lactoferrin in the blood plasma is 0.2 µg/mL, which is significantly lower in comparison to human tears [104,105]. A major source of lactoferrin protein is breast milk [106], however varying amounts are found in mammals. Post-natal breast milk, known as colostrum, has concentrations of lactoferrin up to 7 mg/mL, whereas mature lactation milk contains 7-fold less [107-109].

Lactoferrin is a part of the non-specific immune system [108,110] and plays a role in the first line of defense against microbial infections, as it is conveniently situated at the mucosa [106]. The mucosa is a moist tissue lining organs and various areas in the body cavity, where pathogens may enter the body [106]. Lactoferrin is not only known to protect lysozyme from losing its activity [111], but it aids in lysozyme's accessibility to bacteria [112]. It does this by interfering with the lipopolysaccharide layer of gram-negative bacteria by increasing its permeability [113]. Lactoferrin is involved in inhibiting the growth of and killing gram-positive bacteria [114-116] and is also fungicidal, particularly towards Candida species [108,110]. Lactoferrin also acts to sequester iron from bacteria, which is required for growth [108,110]. It is further known to possess anti-inflammatory [117-119], anti-oxidant [120,121], and anti-tumour [122-124] properties. Lactoferrin performs anti-viral activity against a number of DNA and RNA viruses [106].

Lactoferrin has a number of important functions in the tear film; it stimulates iron adsorption [125] and supports the defense mechanism against bacteria [126-128] by targeting the microorganisms growing in the conjunctival sac [125,129]. The concentration of lactoferrin in the tear film remains quite constant

when the eye is placed under particular conditions; for example, reflex tears and closed eye conditions provide the same concentration of lactoferrin (1.8 mg/mL); however, there is greater lactoferrin when the eye is open (2.6 mg/mL) [84]. Interestingly, with a decrease in tear production, due to dry eye, there is a decrease in lactoferrin concentration [130,131]. Similar to lysozyme, Choy and colleagues determined there was no change in lactoferrin concentration when ortho-k lenses were worn over-night compared to over-night sleep without ortho-k lens-wear [96]. Carney and colleagues demonstrated that during extended contact lens-wear, there was no change in tear lactoferrin concentration after 1 night, 1 week, or 6 months [132]. A decrease in lactoferrin of approximately 50% has however been found in lens wearers that developed GPC, compared to lens wearers without this condition (0.876±0.42 vs 1.73±0.46 mg/mL) [102,133].

1.4.3 Albumin

Albumin is a negatively charged protein with a molecular weight of 66 kDA [134] and is built as a single polypeptide chain of 585 amino acids [135]. Albumin is synthesized in the liver [136] and is mixed into the human tear film by leaking out of conjunctival capillaries [137]. It is the most abundant protein found in blood plasma, constituting 50 mg/mL [138] and is found in the tear film at a concentration ranging from 0.02 to 0.5 mg/mL [139,140]. Fatty acids and hormones are both insoluble products of the human serum and hence require a carrier, such as albumin [141], which consists of four distinct binding sites [135]. Albumin is known as a major antioxidant in human plasma [142-144], scavenging reactive oxygen species [145] and has a half-life of approximately 19 days in the human body [146,147].

Researchers test the alteration in tear protein concentration in order to conclude if an increase or decrease in ocular concentration is seen when the eye is under certain conditions [96,148]. For example, during closed eye conditions or inflammation of the conjunctiva, limbal blood vessel dilate and allow for serum proteins to leak into the tear film, which increases the concentration of albumin in the tear film [148]. Non-lens wearers have a lower albumin concentration in their tear film compared to contact lens wearers (0.045 ± 0.032 vs. 0.059 ± 0.054 mg/mL), as determined by Baleriola-Lucas and colleagues [148]. The concentration of albumin increases significantly overnight, both with and without contact lens-wear [96]. Without lens-wear, albumin concentrations increased 9-fold in tears when comparing values

before sleep (0.02 [0.01-0.05] mg/mL) and after sleep (0.20 [0.15-0.58] mg/mL) [96]. Furthermore, participants wearing ortho-k lenses presented with albumin concentrations 13-fold greater in their tears than those not wearing lenses [96]. Choy and colleagues suggested a few reasons for the significant increase, such as intrinsic diurnal rhythm, a hypoxic environment, and cellular leakage [96].

1.5 PROTEIN QUANTIFICATION TECHNIQUES

1.5.1 Amino Acid Analysis

Automated amino acid analysis in the picomole range is well recognized, as stated by Bohlen in a 1982 publication [149]. High-sensitivity amino acid detection is determined by post-column derivatization of amino acids by use of fluorogenic reagents, such as fluorescamine [150] and OPA[2]/2-mercaptoethanol [151]. As the detection sensitivity of amino acid analysis increases, contamination issues become more important, as interference limits the degree of sensitivity attainable [149]. Contamination by ammonia in the buffer system causes buffer change and artifactual peaks [152-154]. Hydrochloric acid is a major source of contamination that interferes with buffer preparation and peptide hydrolysis [155,156].

1.5.2 Bicinchoninic Acid (BCA) Assay

Introduced in 1985 by Smith and colleagues [157], the BCA assay is considered one of the most popular colorimetric methods in the detection and quantification of protein [158,159]. It is a copper-based assay that combines two separate reactions - the reduction of copper ions (Cu^{2+}) to cuprous ions (Cu^{+}) using an alkaline medium and the colorimetric detection of the BCA- Cu^{+} complex [160]. BCA forms a purple-coloured complex between the peptide bonds of the protein and copper [157]. The intensity of the colour change is dependent on the concentration of protein present in the sample, whereby colour production increases proportionally with increasing protein concentration [157].

1.5.3 Enzyme-Linked Immunosorbent Assay (ELISA)

Theorized and developed by the principle investigator Peter Perlmann, along with a colleague, Eva Engvall, the ELISA technique incorporates the principle of an immunoassay coupled with a reporter

labeling-enzyme [161]. In 1971, alkaline phosphatase was used as the reporter label that quantitatively measured IgG in rabbit serum [162]. In the late 1960s, a group of researchers in Villejuif, France reported successful coupling of antigens or antibodies with enzymes (alkaline phosphatase and glucose oxidase are a couple of examples) [161]. A study conducted by Avrameas [163] coupled enzymes to proteins with glutaraldehyde.

A protein is detected by an antibody that is covalently bond to an enzyme [164]. A substrate (dye) that will cause a colour change in the presence of enzyme is added once protein is bound to the specific antibody [164]. The intensity of the colour change is proportional to the amount of protein in the sample [164]. The ELISA is advantageous in that it can process up to 96 samples at once and is not as time-consuming as other protein quantification techniques, such as Western Blotting.

1.5.4 High Performance Liquid Chromatography (HPLC)

Used to both identify and quantify protein, the HPLC technique separates proteins based on molecular size by mass transfer between stationary and mobile phases [165]. A solvent is added to the proteins in a sample mixture, followed by flowing through a chromatographic column in order to separate the proteins [165]. Retention time identifies each individual protein as it exits the HPLC [165].

Size exclusion-HPLC (SE-HPLC) is used to fractionate tear protein samples which is followed by another technique (sandwich ELISA, for example) to quantify the protein, as SE-HPLC alone cannot quantitatively identify protein [166]. Protein fractioning in aqueous solutions is advantageous in that it minimizes protein exposure to conformational change and denaturation and is less time-consuming [166].

1.5.5 Micrococcal Assay

A protein has the ability to undergo conformational changes- specifically, when lysozyme deposits onto contact lens materials, it is likely to denature and lose its antimicrobial properties [167]. Lysozyme activity has been determined using a turbidity assay, where *Micrococcus lysodeikticus* bacteria is presented as the target for lysozyme protein [168]. An occurrence known as the ‘clearing phenomenon’

takes place [169,170], where the yellow colour of bacteria is diminished into a near clear tone as the optical density (OD) of the buffer solution decreases. The N-acetylglucosamine linkages in the cell wall of this organism are hydrolyzed [169,170] and hence the activity of lysozyme is determined over a time-interval. When greater concentrations of protein are found, the sample solution is more likely to clear at a faster rate. Studies [34,35,171] have determined the kinetic denaturation of lysozyme extracted from both silicone and conventional hydrogel contact lenses.

1.5.6 Radiolabeling

In 1958, the iodine monochloride (ICL) method was introduced to conjugate isotopes with proteins [172], by binding to the aromatic ring of tyrosine [173]. There are 3 tyrosine residues in lysozyme [174], 22 in lactoferrin [175], and 18 in albumin [176], although, not all of them may be accessible for conjugation. The iodine atoms are in a reactive state as soon as the conjugation reaction occurs [172], which is quite preferable. Radiolabeling is advantageous in that protein does not need to be extracted from contact lens materials in order for protein to be quantified.

This method of protein quantification is quick and simple; however, there is a possibility of isotope-detachment from the protein of interest causing free isotopes to generate false positive results. False positives can be minimized by the use of a dialyzing process that decreases free isotopes in solution. Radiolabeling is strictly a quantification technique and does not provide the specific location of protein, that is, whether protein has been adsorbed to the lens surface or absorbed into the lens matrix. Therefore, proteins are conjugated with fluorescent probes in order to use confocal scanning microscopy to determine location-specific sorption profiles.

1.5.7 Western Blotting (WB)

In the WB technique, protein transfers from a sodium dodecyl sulfate (SDS) polyacrylamide gel to an adsorbent membrane [177], where the blotted proteins provide an exact replica of the gel [178]. Proteins are transferred to the adsorbent membrane once separated using SDS-polyacrylamide gel electrophoresis (PAGE) [179], followed by staining with antibodies in order to view the proteins. This

technique has the ability to detect proteins of low abundance [178]. Before the WB, molecular probes did not have easy access to the separated proteins in the gel matrix [178]. The introduction of WB allowed proteins to be equally accessible by ligands [178]. Although the WB has a number of advantages, it is outcompeted by the ELISA technique when comparing time efficiency, as the ELISA is quicker and processes up to 96 samples at once.

2 THESIS RATIONALE

Conventional hydrogel and silicone hydrogel (SH) contact lenses present with a number of differences, the most obvious being the higher oxygen transmissibility found in SH lenses. SH lenses are also more resistant to deformation due to their higher modulus, and are typically less wettable compared to conventional hydrogels. Contact lenses are categorized under the Food and Drug Administration (FDA) into four groups: Groups I and III are low (<50%) water content and groups II and IV are high (>50%) water content. Silicone is relatively hydrophobic, and as a result most SH lenses require surface modification in order to increase wettability and to improve on-eye performance.

The deposition profile of tear film components to contact lens materials is distinctly different between both material groups, showing that the more hydrophobic SH lenses attract greater quantities of lipid and only small amounts of protein, while poly-2-hydroxyethyl methacrylate (pHEMA)-based conventional lenses accumulate primarily proteins. Protein deposition became a major focus of numerous previous studies, particularly because of the ocular response that may result from it. Studies have associated lens deposits with an increase in discomfort, dryness, and certain inflammatory responses; however lens age might be an equally important factor. An inflammatory reaction that has earned attention is giant papillary conjunctivitis (GPC), which arises due to both mechanical reasons and has also been associated with denatured protein deposits on lenses, resulting in an immune response.

To investigate the deposition profile of major tear film components to different contact lens materials, numerous *ex vivo* and *in vitro* studies have been conducted in the past. *In vitro* experiments are performed in order to have complete control over the surrounding variables. They allow narrowing in on a specific question and can determine individual variables in a complex environment. *Ex vivo* studies might be closer to the truth, however, human data are typically more variable and less cost efficient.

The purpose of this thesis was to use a more advanced *in vitro* method and to compare these findings to *ex vivo* data. The contact lens deposition profile of three major tear film proteins was determined using solutions of different complexity for incubation. A common practice used in previous years was

determining protein deposition, particularly lysozyme, lactoferrin, and albumin, when each of these proteins was individually used in a phosphate buffered saline. This method did not accurately mimic the complexity of the human tear film and lacked competitiveness between tear components, which include hundreds of proteins, numerous lipids and mucins. In this thesis work a complex artificial tear solution (ATS) was used to better simulate the human tear film. The purpose of the first experimental chapter (Chapter 3) was to determine whether the complexity of an incubation solution impacts protein deposition to SH lenses. In this study, one solution contained saline and a single protein (Solution A), whereas the other solution contained saline, proteins, lipids, and mucins (Solution B). The use of three different contact lens materials permitted a comparison between lens materials. These findings were then compared with patient-worn lenses in Appendix A.

Contact lens care regimens disinfect the worn lens and remove protein deposits from the lens. The second experimental chapter investigated how efficiently cleaning solutions removed deposited proteins from contact lenses when the incubation solution for the contact lenses was either very complex or much more simplified. This study is similar to the one described in Chapter 3, with the addition of cleaning steps. In Chapter 4 the impact of other tear film components was investigated to determine whether they enhance or reduce the binding strength of certain proteins to different lens materials.

Contact lens care systems are essential for reusable contact lenses for disinfection and overall cleaning of the lenses between wearing cycles. A number of multi-purpose solutions (MPS) and hydrogen peroxide cleaning systems are currently available on the market. Cleaning systems allow for a biologically healthy state for the eye and keep proteins, which are deposited on the lens surface, "active" so that they may contribute to microbial killing and not induce inflammatory responses. Hence, the purpose of Chapter 5 was to investigate the effect of MPS and a hydrogen peroxide-based system on lysozyme activity. Denatured proteins present with ocular adverse inflammatory effects and proteins depositing onto the lens may cause bacterial adhesion and increase the risk of microbial keratitis.

This thesis work will provide valuable information for future study designs using *in vitro* models to look at contact lens deposition.

3 COMPOSITION OF INCUBATION SOLUTION IMPACTS *IN VITRO* PROTEIN UPTAKE TO SILICONE HYDROGEL CONTACT LENSES

This chapter is published as follows:

Jadi S, Heynen M, Luensmann D, Jones L. Composition of incubation solution impacts *in vitro* protein uptake to silicone hydrogel contact lenses. Mol Vis. 2012;18:337-47.

The format of Figures 3-2, 3-3, and 3-4 has been modified for this thesis.

	Concept/ Design	Acquisition of data	Analysis	Write-up/ Publication
Jadi	Y	Y	Y	Y
Heynen	-	Y	-	-
Luensmann	Y	-	Y	Y
Jones	Y	-	-	Y

3.1 OVERVIEW

Purpose: To determine the impact of incubation solution composition on protein deposition to silicone hydrogel (SH) contact lenses using a simplistic and a complex model of the tear film.

Methods: Three SH materials - senofilcon A (SA), lotrafilcon B (LB) and balafilcon A (BA) - were incubated in two different solutions; Solution A was a simplistic augmented buffered saline solution containing a single protein, whereas Solution B was a complex artificial tear solution (ATS), containing the augmented buffered saline solution in addition to proteins, lipids and mucins (pH= 7.4). The proteins of interest (lysozyme; lactoferrin; albumin) were radiolabeled with ^{125}Iodine (2% protein of interest) and the accumulation of the conjugated protein to the lens materials was determined after 1, 7, 14, and 28 days of incubation. Protein deposition was measured using a gamma counter and the raw data was translated into absolute amounts (µg/lens) via extrapolation from standards.

Results: After 28 days, lysozyme uptake was significantly lower on BA lenses when incubated in Solution A (33.7µg) compared to Solution B (56.2µg), $p<0.001$. SA lenses deposited similar amounts of lysozyme when incubated in either Solution A (2.6µg) or Solution B (4.1µg), $p>0.05$. LB lenses also deposited similar amounts of lysozyme for both solutions (Solution A: 5.0µg vs. Solution B: 4.7µg, $p>0.05$). After 28 days, BA lenses accumulated approximately twice the amount of lactoferrin than the other lens materials, with 30.3µg depositing when exposed to Solution A and 22.0µg with Solution B. The difference between the two solutions was statistically significant ($p<0.001$). LB materials deposited significantly greater amounts of lactoferrin when incubated in Solution A (16.6µg) compared to Solution B (10.3µg), $p<0.001$. Similar amounts of lactoferrin were accumulated onto SA lenses regardless of incubation solution composition (Solution A: 8.2µg, Solution B: 11.2µg, $p>0.05$). After 28 days, albumin deposition onto BA lenses was significantly greater when lenses were incubated in Solution B (1.7µg) compared to Solution A (0.9µg), $p<0.001$. Similar amounts of albumin were deposited on SA lenses when incubated in either solution (0.6µg vs. 0.7µg, $p>0.05$). LB lenses incubated in Solution A deposited more albumin compared to Solution B (0.9µg vs. 0.6µg), $p=0.003$.

Discussion: Protein deposition onto SH materials varied when contact lenses were incubated in either a complex ATS compared to a single protein solution. More lysozyme accumulated onto BA lenses incubated in a complex analogue of the human tear film, whereas more lactoferrin deposited onto SA lenses independent of incubation solution composition. In order to better mimic the *ex vivo* environment, future studies should use more appropriate analogues of the tear film.

Key Words: Contact lens deposition, lysozyme, lactoferrin, albumin, silicone hydrogel contact lenses, tear film, competitive protein uptake

3.2 INTRODUCTION

Silicone hydrogel (SH) lenses became increasingly popular over the last decade primarily due to their higher oxygen permeability, leading to reduced hypoxic complications compared to poly-2-hydroxyethyl methacrylate (pHEMA)-based lenses [1,2]. A recent survey indicated that 54% of all contact lens-wearers in the United States (US) were fitted with SH materials for daily wear, as compared with only 15% using hydrogel lenses [3]. This has changed greatly since 2005, where only 22% of the lens wearers in the US were fitted with silicone hydrogel lenses [4].

Contact lenses are prone to protein deposition, the amounts of which are dependent on the chemical composition of the lens materials [5,6]. A number of studies have shown that deposition onto contact lenses may cause discomfort [7] acute red eye [8], and inflammatory reactions [9]. Deposited proteins denature over time and hence may cause inflammatory responses to the palpebral conjunctiva, such as giant papillary conjunctivitis [10]. Contact lens wear can lead to microbial keratitis through infection of the cornea by pathogenic organisms, such as gram-negative *Pseudomonas aeruginosa*, which adhere to the protein-coated lens material [11]. Tear film deposits may further reduce visual acuity [12] and surface wettability [13].

A number of different tear film proteins have been detected in the proteomic profiles deposited on SH contact lenses, including albumin, lipocalin, lactoferrin, and lysozyme [14,15]. Many other proteins have been identified in the human tear film, some examples are complement C3 [16], IgE [17], IgG [18], and secretory phospholipase A2 [19]. Using antibody arrays, a number of chemokines, cytokines, and growth factors have been detected in the human tear film [20], as well as proteases and protease inhibitors detected through mass spectrometers [21]. There are more than 100 different proteins identified in the tear film [21,22], constituting a protein concentration of around 8mg/mL [21,23]. Lysozyme is primarily used as the “model protein” for *in vitro* studies investigating deposition on lenses. The main reasons for this are the high abundance of this positively charged protein in the tear film and the fact that it accounts for approximately 90% of the deposited protein on ionic (negatively charged) pHEMA-based lenses [16,24].

Most SH contact lenses available today are non-ionic and deposit substantially less protein than ionic conventional hydrogels [25]. Deposition profiles are often determined using simplified *in vitro* models, however, there are several differences between *in vitro* and *ex vivo* results when comparing protein accumulation on contact lenses [14,26-28]. The *in vitro* model typically lacks the effect of blinking, surface drying, the cleansing process of contact lenses between hours of wear, and the physiological events that are naturally occurring in the eye. As a result, the level of lysozyme deposition determined on ionic pHEMA lenses is typically slightly lower on worn lenses compared to data collected on *in vitro* deposited lenses (*ex vivo*= 985-991µg of lysozyme [26,29], *in vitro*= 1434-1800µg of lysozyme [27,30,31]). In comparison to pHEMA, SH lenses deposit much lower amounts of lysozyme, averaging <20 µg/lens [26,32]. SH materials generally accumulate similar amounts of protein, except for the ionic SH material balafilcon A (BA), which deposits much great amounts of protein per lens [5,30]. Subbaraman and colleagues illustrated in an *in vitro* study that senofilcon A (SA) and lotrafilcon B (LB) lenses deposited 3.7µg and 6.1µg of lysozyme, whereas BA deposited approximately three times that amount (19.4µg) after two weeks of incubation [30]. *Ex vivo* data from Subbaraman have further shown that after two weeks of lens-wear, SA and LB deposit similar amounts of total protein - 4.6µg and 6.6µg respectively- whereas BA deposits approximately 26.9µg [33], which is only marginally higher compared to the lysozyme *in vitro* results. Zhao and colleagues demonstrated a similar pattern, where BA lenses deposited the greatest amount of protein and SA the least; however, SA lenses deposited significantly less protein (0.1µg [5]) than findings by Subbaraman and colleagues (4.6µg).

In vitro deposition studies have limitations when single protein solutions are utilised, as they cannot accurately mimic the ocular tear film, due to their lack of other tear film components, including other proteins, lipids and mucins [30]. The use of more complex artificial tear solutions (ATS) on pHEMA-based contact lenses has shown to impact lipid and lysozyme uptake onto the lens material [34-36]. Whether proteins that are different in size and charge respond in a similar fashion when depositing to SH lenses is not clear, therefore the purpose of this *in vitro* study was to compare the amount of protein uptake on different SH lens materials using two different *in vitro* models. The first model uses an augmented buffered saline solution with a single protein added, whereas the second model uses a far more complex ATS, consisting of the augmented buffered saline solution as its base, for lens incubation.

3.3 METHODS

A single protein solution and a complex ATS were used to investigate potential differences in protein deposition to SH materials, using radiolabeled lysozyme, lactoferrin and albumin.

Three SH contact lens materials were investigated in this study, senofilcon A (SA, ACUVUE OASYS, Johnson & Johnson, Jacksonville, FL), lotrafilcon B (LB, Air Optix, CIBA VISION, Duluth, GA) and balafilcon A (BA, PureVision, Bausch & Lomb, Rochester, NY). These lenses have been categorized in different Food and Drug Administration (FDA) groups, with both SA and LB belonging to FDA group I (low water content <50%, non-ionic), whereas BA belongs to FDA group III (low water content <50%, ionic).

Two independent studies were performed in parallel to investigate the deposition of a single protein when added to a saline solution compared to a complex ATS. To identify the protein of interest in the solution and on the lens, proteins were conjugated with Iodine-125 (125I). The conjugated proteins included hen egg lysozyme (HEL) bovine colostrum and milk lactoferrin (BCL/BML), and bovine serum albumin (BSA). The iodine monochloride (ICL) method [37,38] was used to radiolabel the proteins of interest, by covalently binding 125I to the tyrosine ring [39,40]. The radiolabeled proteins were added to the incubation solutions at a concentration of 2% of the individual protein concentration. Control solutions not containing a contact lens were used in order to verify radioactivity in the solution and decay over time.

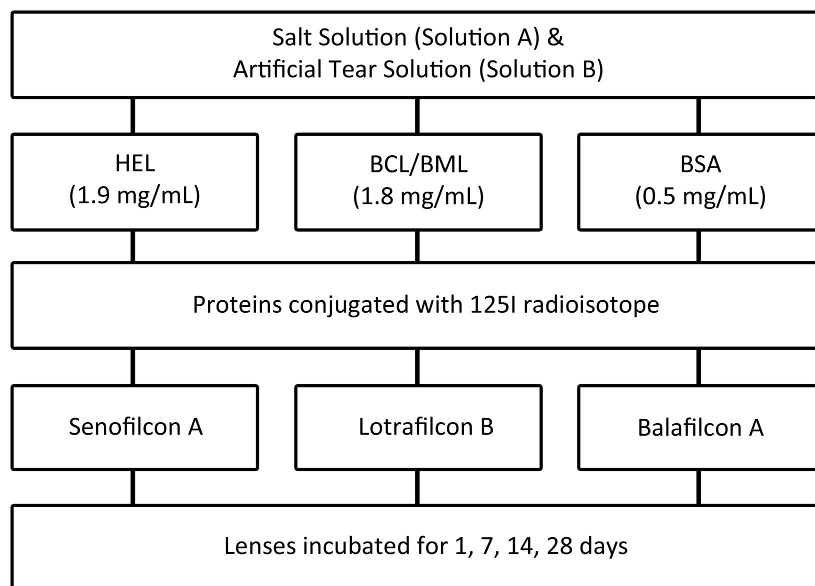

Figure 3-1: Flowchart depicting layout of two-part study, where each time point contained three replicates.

Single protein solution

The single protein solution (A) consisted of an augmented buffered saline solution containing different salts, glucose, and urea (Table 3-1). This was adapted from Van Haeringen [41] and further modified at the Centre for Contact Lens Research [42]. A single protein of interest was added to the solution.

Table 3-1: Components of the Saline Solution

Component	mM (mmol/mL)	MW (g/mol)
$C_6H_{12}O_6$*	0.2	180.2
$CaCl_2$*	0.5	147
H_2O	-	-
HCl (10 M)*	-	-
KCl#	16	74.55
KHCO*	3	100.12
Na_2CO_3+	12	105.99
Na_2HPO_4*	24	141.96
$Na_3C_6H_5O_7$^	1.5	294.1
NaCl*	90	58.44
$(NH_2)_2CO$!	1.2	60.06
ProClin 300*	-	-

$C_6H_{12}O_6$ (glucose), $CaCl_2$ (calcium chloride), H_2O (Milli-Q gradient), HCl (10 M, hydrochloric acid), KCl (potassium chloride), $KHCO_3$ (potassium bicarbonate), Na_2CO_3 (sodium carbonate), Na_2HPO_4 (sodium hydrogen phosphate), $Na_3C_6H_5O_7$ (trisodium citrate), NaCl (sodium chloride), $(NH_2)_2CO$ (urea)
* Sigma-Aldrich, Oakville, ON, # BDH Inc., Toronto, ON, + EMD Chemicals Inc., Gibbstown, NJ, ^ Caledon Laboratories LTD., Georgetown, ON, ! EM Science, Gibbstown, NJ

The saline solution was prepared with Milli-Q water in a glass beaker using a stir bar for constant mixing. The individual components were added to the solution. Finally, ProClin-300 (200μL/L solution, Sigma, Oakville, ON), an antimicrobial agent, was added to the solution in order to inhibit bacterial growth. If necessary, NaOH was used to adjust the solution to a physiological pH of 7.4 [43]. The pH was further determined at each study time point using pH paper (VWR, Mississauga, ON) to verify an unchanged environment of the solution at a pH of about 7 on a scale of 1-12.

The solution was split into three batches and one protein, either HEL (1.9mg/mL), BCL/BML (1.9mg/mL) or BSA (0.5mg/mL) was added to make Solution A.

Complex ATS

The complex ATS (B) consisted of the saline solution described above, plus proteins, lipids and mucins (Table 3-2). All lipids and proteins were purchased from Sigma-Aldrich, Oakville, ON.

Table 3-2: Components of the Complex Artificial Tear Solution

Lipids	C (mg/mL)	Proteins	C (mg/mL)
Cholesterol [34-36]	0.0018	Albumin [44]	0.5
Cholesteryl oleate [34]	0.024	IgG [45-47]	0.02
Oleic acid [35,36]	0.0018	Lactoferrin [34,36]	1.8
Oleic acid methyl ester [34]	0.012	Lysozyme [34,36]	1.9
Phosphatidyl choline [48,49]	0.0005	Mucin [34,36]	0.15
Triolein [34]	0.016	Saline solution	-

The concentration of cholesterol was adapted through a formulation from a couple of studies- Haberland and colleagues state in a study that the concentration of cholesterol that will dissolve is 0.0018 mg/mL [50], whereas a study undertaken by Saatci and colleagues states that the concentration of this lipid is found in higher amounts in the tear film [51]. The IgG concentration was adapted from a number of literature values [45-47]. Coyle and Sibony provide a range of IgG concentration found in the tear film that better relates to this study [47].

The saline solution was prepared as described above and all proteins were added (Table 3-2). Concentrated lipids were mixed in a separate flask with hexane-ether and sonicated for 5 minutes in order to break down the lipids into micelles. The lipid and protein saline solution were combined and nitrogen purged with nitrogen for 10 minutes to adjust the pH and evaporate hexane-ether.

Contact Lens Incubation

All lenses were individually soaked in 5mL of the prepared saline for 24 hours, to remove any packaging solution components from the lenses. The lenses were handled with silicone-tipped tweezers in a sterile environment. Screw-capped glass vials (6mL, VWR, Mississauga, ON) were autoclaved and pre-treated for 4-7 days with the same solution used for lens incubation, to coat the vials and minimize adsorption of elements to the walls of the vials during the lens incubation. During the pre-treatment phase, the concentration of lactoferrin (1.8 mg/mL) was halved to 0.9 mg/mL due to quantity and cost limitations. For similar reasons, both IgG and lactoferrin were omitted when pre-treating the complex ATS vials.

In order to fully submerge the lens, each lens was incubated in 1.5 mL of solution at 37°C and placed on a rotatory shaker at 60rpm (VWR, Mississauga, ON). Time periods of 1, 7, 14, and 28 days were investigated using three replicates per lens type and time point, resulting in a total of 216 contact lenses being examined in the study.

After each incubation period, lenses were removed from the incubation solution, rinsed in saline twice, placed in a 12x75mm culture tube (VWR, Mississauga, ON), air-dried for 12 hours to evaporate off unbound iodine. The Wallac Wizard 1470 Gamma Counter (Perkin Elmer, Woodbridge, ON) was used to quantify the amount of protein deposited on the lens.

3.4 RESULTS

This study consisted of two experiments, undertaken in parallel, to compare the deposition of lysozyme, lactoferrin and albumin to SH materials, when incubated in a single protein versus a complex ATS solution.

The pH of both solutions used for incubation (Solutions A and B) was checked at each time-point. The results were in good agreement with the human tear film, which has a pH of approximately 7.4 [43]. Control solutions, not containing a contact lens, confirmed the anticipated amount of radioactivity in each solution, permitted us to monitor the radioactive decay over time and protein quantification.

Data analysis was conducted using Statistica 9 (StatSoft Inc. Tulsa, OK). A repeated measures ANOVA (analysis of variance) was used to compare protein deposition to the different lens materials over time. Factors included in the ANOVA were: protein of interest, contact lens material, and time point. Tukey's HSD (Honestly Significant Difference) test was used for post-hoc comparisons; $p<0.05$ was considered significant.

LYSOZYME

Results are presented in Figure 3-2. All lens types showed an increase in lysozyme deposition between days 1 and 28, independent of solution used for incubation ($p<0.001$).

After 1 day of incubation, SA lenses accumulated similar amounts of lysozyme when incubated in either solution (Solution A: 0.28±0.03μg, Solution B: 0.31±0.03μg; $p=1.00$). Slightly more lysozyme was found after 28 days: SA lenses accumulated 4.06±0.19μg when incubated in Solution B and 2.57±1.33μg using Solution A, however, this difference was also not statistically significant ($p=0.20$). LB lenses deposited similar amounts of lysozyme with both solutions (Solution A: 0.74±0.08μg, Solution B: (0.58±0.09μg); $p=1.00$) after 1 day of incubation. This amount increased after 28 days to 4.99±0.01μg and 4.70±0.20μg using Solution A and B respectively ($p=1.00$). BA accumulated similar amounts of lysozyme after 1 day (Solution A: 4.69±0.19μg, Solution B: 4.96±0.19μg; $p=1.00$) independent of the solution used, but deposited significantly higher amounts after 14 and 28 days when incubated in Solution B (Day 28: Solution A = 33.68±1.81μg; Solution B = 56.22±1.59μg; $p<0.001$) (Figure 3-2).

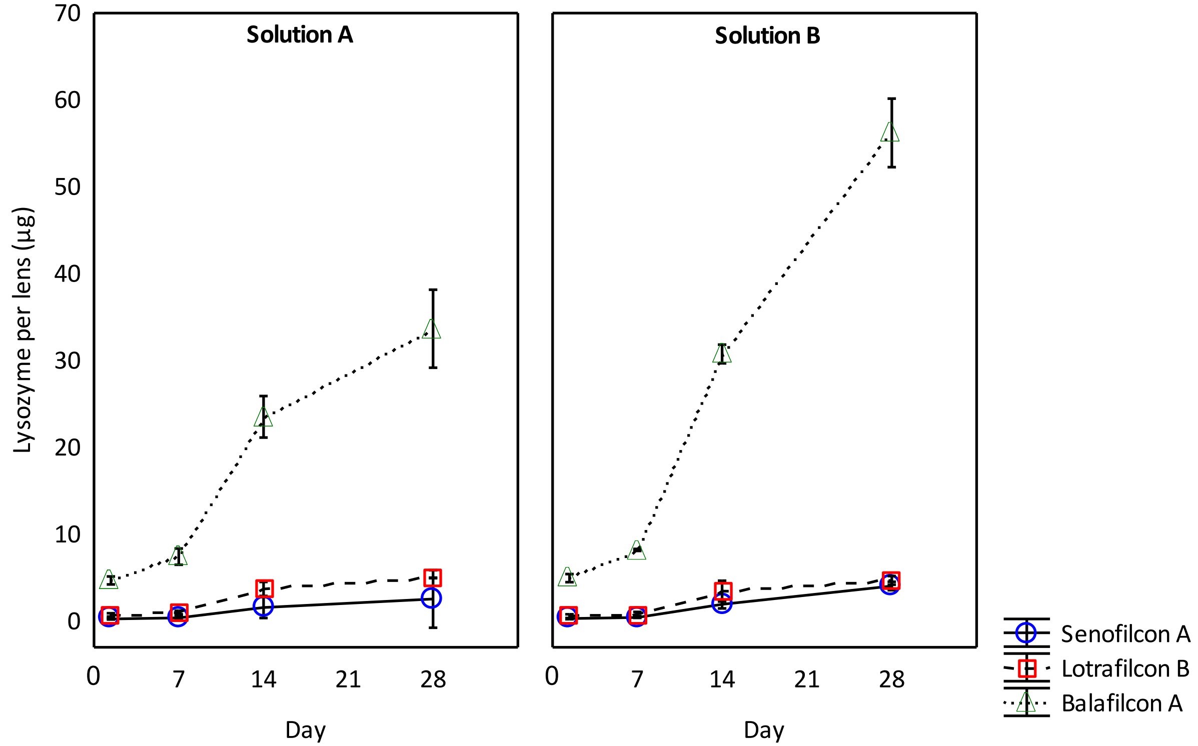

Figure 3-2: Lysozyme deposition to senofilcon A, lotrafilcon B and balafilcon A lenses using a single protein solution and a complex ATS solution. Incubation points: 1, 7, 14 and 28 days.

Lysozyme deposition increased between each time point for both solutions (A and B) over a period of 28 days ($p<0.001$), with Solution B depositing significantly more lysozyme than Solution A by day 28 ($p<0.001$). Independent of lens type, lysozyme deposition increased from day 1 to 28, depositing significantly greater amounts of protein between each time point, for both Solutions A and B. ($p<0.001$).

LACTOFERRIN

Results are presented in Figure 3-3. From day 1 to 28, the amount of lactoferrin deposition to SH lenses increased independent of solution used for incubation ($p<0.001$).

After 1 day of incubation, SA lenses accumulated similar amounts of lactoferrin using Solution A (0.81±0.09μg) and Solution B (0.81±0.04μg) ($p=1.00$). After 28 days, slightly less lactoferrin was seen when incubated in Solution A (8.17±0.70μg) in comparison to Solution B (11.21±0.28μg) ($p=0.17$). LB lenses incubated in Solution A (5.34±0.17μg) accumulated slightly more lactoferrin than Solution B (2.16±0.07μg) after 1 day, however this difference was not significant ($p=0.13$). After 28 days however, LB deposited significantly more lactoferrin when incubated in Solution A compared to Solution B (Solution A: 16.62±0.86μg, Solution B: 10.28±1.66μg; $p<0.001$). BA lenses also attracted slightly higher amounts of lactoferrin when incubated in Solution A (5.75±0.86μg) compared to Solution B (2.62±0.66μg) after 1 day ($p=0.14$) which became statistically significant after 28 days, where Solution A allowed for 30.25±2.10μg of deposits on the lenses compared to Solution B (22.04±0.51μg) ($p<0.001$) (Figure 3-3).

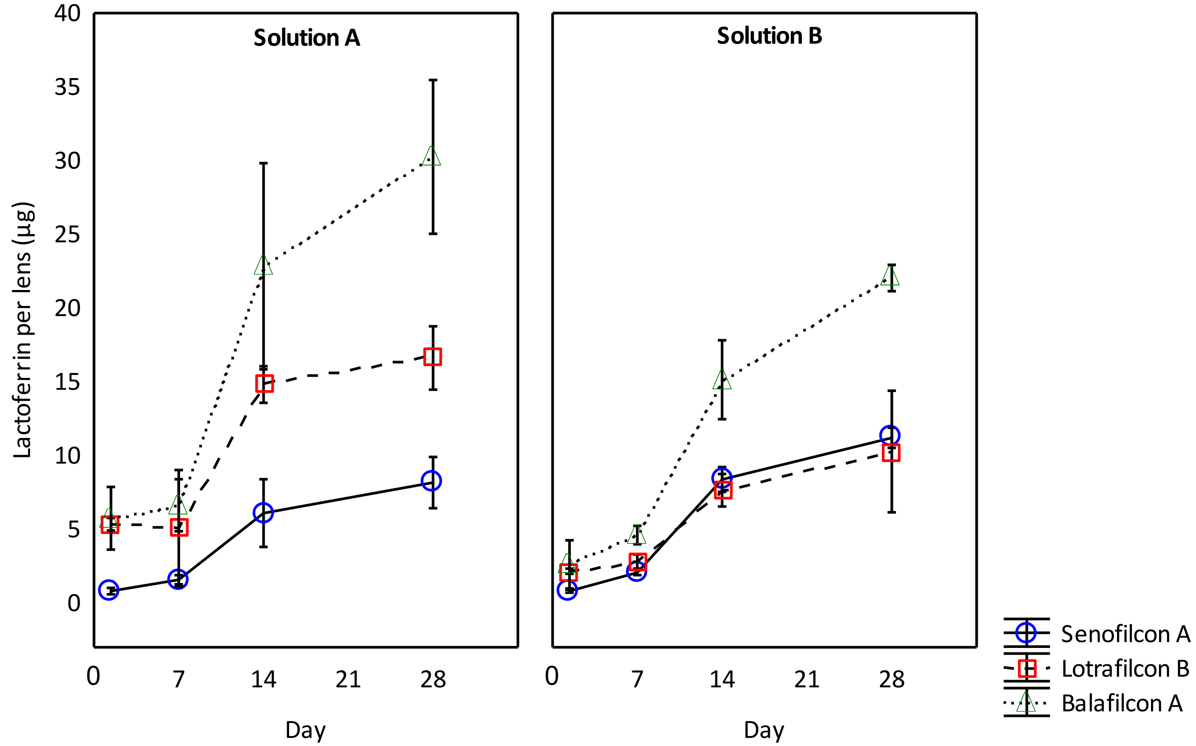

Figure 3-3: Lactoferrin deposition to senofilcon A, lotrafilcon B and balafilcon A lenses using a single protein solution and a complex ATS solution. Incubation points: 1, 7, 14 and 28 days.

There was an increase in lactoferrin deposition between days 1 and 28 ($p<0.001$), with Solution A depositing significantly more lactoferrin than Solution B ($p=0.017$). Independent of lens type, lactoferrin deposits similarly on lens materials incubated in Solution A at day 1 and 7 ($p=0.91$). However, there was an increase in the rate of accumulation between the other time points (day 7 and 14 ($p<0.001$); day 14 and 28 ($p=0.001$)). With regards to Solution B, there was not a significant difference between lactoferrin deposits at days 1 and 7 ($p=0.52$), 14 and 28 ($p=1.00$), but a significant difference between days 7 and 14 ($p<0.001$).

ALBUMIN

Results are presented in Figure 4. In general, there was an increase in albumin deposition to SH lenses over time, independent of solution used for incubation ($p<0.001$).

After 1 day of incubation, SA lenses accumulated similar amounts of albumin when incubated in either solution (Solution A: 0.32±0.02µg, Solution B: 0.45±0.02µg; $p=0.70$). A similar result was also seen after 28 days, showing 0.56±0.06µg when incubated in Solution A and 0.70±0.09µg using Solution B ($p=0.53$). LB deposited significantly more albumin in Solution A (0.66±0.05µg) compared to Solution B (0.40±0.06µg) after 1 day of incubation ($p=0.04$). Likewise more albumin was accumulated after 28 days when lenses were incubated in Solution A (0.92±0.03µg) compared to Solution B (0.58±0.08µg) ($p=0.003$). After 1 day of incubation, BA lenses deposited similar amounts of albumin with both solutions (Solution A (0.57±0.07µg); Solution B (0.73±0.03µg) ($p=0.38$)). With a longer incubation of 28 days, BA lenses accumulated significantly less ($p<0.001$) albumin in Solution A (0.85±0.00µg) in comparison to Solution B (1.68±0.04µg) (Figure 4).

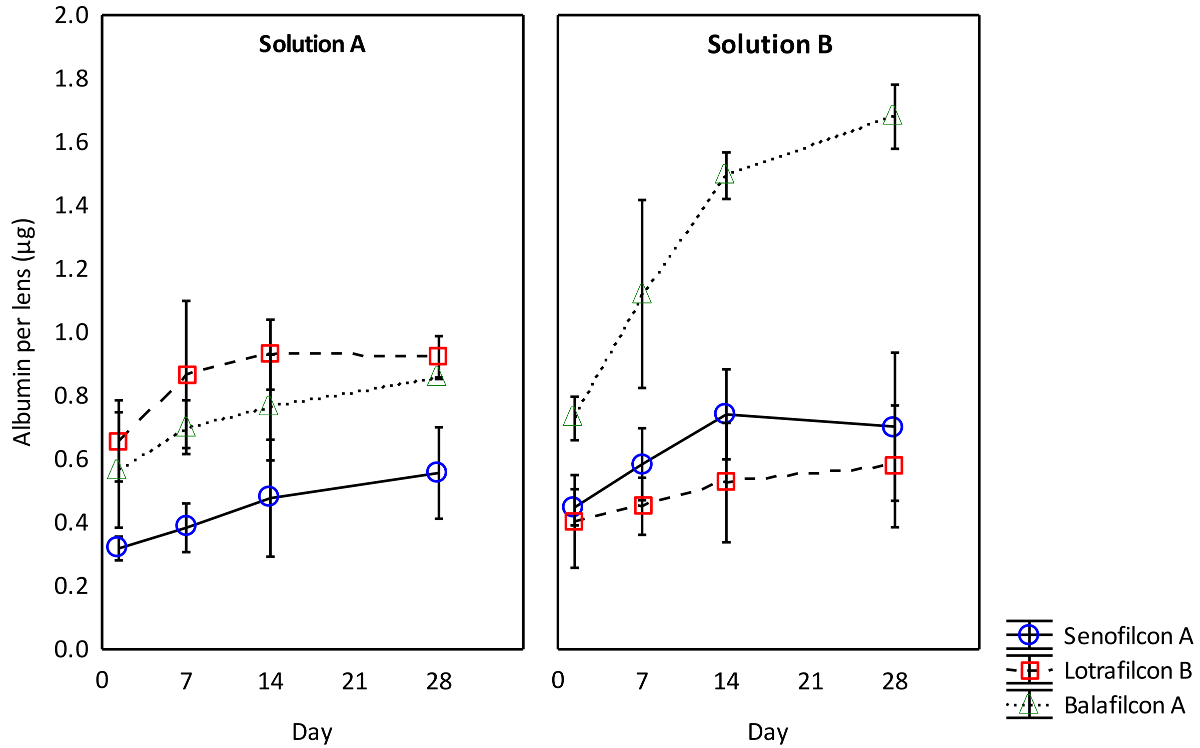

Figure 3-4: Albumin deposition to senofilcon A, lotrafilcon B and balafilcon A lenses using a single protein solution and a complex ATS solution. Incubation points: 1, 7, 14 and 28 days.

There was an increase in albumin deposition between day 1 and day 28 and also between each time-point ($p<0.001$), with Solution B depositing significantly more albumin than Solution A ($p=0.008$). Independent of lens type, the amount of albumin increased from day 1 to day 7 ($p=0.01$) using Solution A and a plateau was seen after this time point (day 7 and 14 ($p=0.18$), day 14 and 28 ($p=0.40$)). With regards to Solution B, there was a significant difference between each time point, as protein deposition increased significantly at each measurement; day 1 and 7 ($p=0.02$), day 7 and 14 ($p<0.001$), day 14 and 28 ($p<0.001$)).

3.5 DISCUSSION

The human tear film contains a variety of proteins, lipids, and mucins, each of which differs in size, charge, and concentration [29,52,53]. Positively charged [1] lysozyme (14.5 kDA [54]) and the iron-binding protein lactoferrin (80 kDA [55]) constitute major proportions of the tear film, measuring 1.9 mg/mL [56] and 1.5-2.2 mg/mL [55], respectively. Lysozyme contains three positive binding sites [57], whereas lactoferrin has one [58]. Albumin, a negatively charged protein, has a molecular weight of 66 kDA [59] and is found in the tear film at a concentration ranging from 0.02 to 0.5 mg/mL [60,61].

The contact lens materials investigated in this study were incubated in a non-competitive, single protein solution and a complex ATS, consisting of multiple proteins, mucins, and lipids. This complex ATS, according to the Vroman effect, will allow for sequential adsorption of proteins to the lens surface [52,62,63]. Blood plasma proteins undergo the Vroman effect when adsorbing onto artificial surfaces, particularly the displacement of fibrinogen by other plasma proteins [64]. Sariri and Sabbaghzadeh have demonstrated competitive protein binding onto soft contact lens surfaces and the ability of proteins to displace one another [65]. To-date, only a few studies have determined the impact of other tear components during the sorption process, and no data were available on proteins of different charge, size and abundance and their interaction with SH materials [25,30]. It was predicted that the negatively charged albumin would deposit to only a minor extent onto negatively charged materials, due to electrostatic repulsion [66].

The three SH lenses investigated in this study differed in material composition, water content and surface modification. The SA material contains a copolymerization of HEMA and N, N-dimethyl acrylamide with (3-methylacryloxy-2hydroxypropyloxy) propylbis (trimethylsiloxy) methylsilane [67]. In addition, an internal wetting agent (polyvinyl pyrrolidone (PVP)) is incorporated into SA lenses to improve wettability [68-70]. Lysozyme contributes 6-13µg of the total protein deposition per SA lens in *in vitro* studies [27,30], whereas *ex vivo* studies report up to 7µg of total protein per lens deposited [5,28,33], with lysozyme contributing about 25% [28,33], demonstrating that more lysozyme is deposited *in vitro* (6-13µg vs. 1.75µg). For SA lenses, there was no statistically significant difference in deposition of any of the proteins investigated whether Solution A or B was used for incubation (p=NS).

This suggests that this material is unaffected by incubation solution composition. Given the complex nature of the ATS, this result suggests that little competition for protein deposition occurs with this material, and that protein deposition is driven by non-competitive factors. After 28 days, SA also deposited the lowest amount of all three proteins, as compared with the other two materials. This low level of deposition has been seen in other *in vitro* and *ex vivo* studies [27,28,30,33], and may be attributed to the neutral surface charge and, specifically, the presence of PVP, which for both contact lenses and other biomaterial applications has also been shown to exhibit low levels of protein deposition [30,71].

The LB material has a co-continuous biphasic- siloxy and hydrogel phase, which aids the lens in maintaining oxygen and salt transmission [67]. This lens material is coated by hydrophilic plasma to improve hydrophilicity of the surface [67,72] and this plasma coating (25nm thick) limits access to the underlying polymer, hence decreasing protein deposition on this material and within the matrix [6,73]. *In vitro* studies on LB show that lysozyme contributes about 6-10µg of total protein deposited per lens [25,27]. *Ex vivo* studies illustrate that >7µg [74,75] of total protein per lens is deposited, with <25% as lysozyme [28,33]. After 28 days of incubation, no differences in the amounts of lysozyme deposited on LB lenses were measured between the two incubation solutions (p=NS). This may be due to the size of lysozyme, which is the smallest of the three proteins and may outcompete the other two proteins, appearing as if it is accumulating on the lens material without competition from other proteins. A significant difference in both lactoferrin and albumin accumulation occurred ($p<0.05$), with the simplistic incubation solution (Solution A) producing the greatest deposition. These data suggest that when exposed to Solution A, which has no lysozyme, the other two proteins of interest can deposit freely, without the competitive binding that lysozyme exhibits. After 28 days, in comparison with the other two materials, LB deposits more protein than SA, but less than BA.

The BA material has a biphasic character due to copolymerization of the TRIS derivative vinyl carbamate and N-vinyl pyrrolidone [67]. Hydrophilic glassy silicate 'islands' can be seen on the surface of BA lenses [6] due to the oxidation of TRIS [67]. BA is considered ionic (FDA Group III) due to its incorporation of N-vinyl aminobutyric acid and as a result, this material typically accumulates more tear proteins, particularly those that are positively charged, compared to other SH lenses [5,27,30]. Furthermore,

unlike other SH lenses, the surface of BA is more porous, allowing for protein to penetrate through the matrix [72,73]. Previous *in vitro* studies report that lysozyme deposits approximately 10µg of protein per lens [30,76]. Of the total amount of protein depositing on worn BA lenses (5-34µg) [5,33], lysozyme accounts for 32% [76] to 50% [25]. Lysozyme accumulated significantly more on BA lenses ($p<0.05$) when incubated in the complex ATS. This is an interesting phenomenon, as it would be predicted that there would be no difference between the two solutions because of lysozyme's ability to deposit on a negatively charged material in large amounts, independent of incubation solution. One potential explanation could be that when exposed to a complex ATS that there is an initial deposition of the positively charged lysozyme, which acts to partially neutralize the surface charge of the BA material, allowing some binding of the negatively charged albumin, which then results in a "layering" of proteins on top of this initial layer [77,78]. Lactoferrin, as expected, deposited significantly more on the BA material when incubated in the simplistic solution. This is due to the decrease in available binding sites on the negatively charged BA material, due to lysozyme's competitive behavior. In contrast, albumin deposited more when lenses were incubated in the complex ATS (Solution B). The low level when exposed to Solution A is expected, as both BA and albumin are negatively charged and exhibit mutual electrostatic repulsion. The higher level when exposed to the complex Solution B can be attributed to the partial neutralization of the BA material by the positively charged lysozyme and lactoferrin, allowing albumin for an increased opportunity to bind to the BA surface. Of the three materials examined, BA deposits the highest amount of all three proteins.

Patient-worn senofilcon A lenses deposit approximately 7µg [28,33] of total protein, whereas lenses incubated in Solution A and B deposited approximately two times more protein (11.30µg and 15.97µg, respectively (sum of 125I data from all three proteins). Lotrafilcon B lenses in Solution A deposited approximately three times more total protein (22.53µg) than what has been found in *ex vivo* studies (>7µg [14,74,75]), whereas Solution B lenses accumulated roughly two times more total protein (15.56µg). *Ex vivo* studies on balafilcon A have found 5-34µg [5,33] of total protein, whereas BA lenses incubated in Solution A accumulated two times more protein (64.78µg) versus approximately three times more total protein when using Solution B (79.94µg). A number of reasons may account for these differences. The naturally occurring physiological events of the eye, blinking, and surface drying are all lacking in this *in vitro* model. The lens surface in vivo dries between blinks as the lid wipes over the

material [79] and this drying is known to influence deposition onto lens materials from the tear film [80]. Most importantly, *ex vivo* studies typically contain the use of a care regimen each day, which would be predicted to decrease protein accumulation on the lens material over time.

In conclusion, this study confirms that there are differences in amounts of protein deposition onto SH materials incubated in either a single protein or complex ATS incubation solution. The results showed that protein accumulation was further dependent on incubation time, the nature of the protein (size, concentration, and charge) and type of SH material. BA was the greatest accumulator, as previously reported. With regards to lysozyme deposition, no impact of the type of solution was seen for SA and LB lenses, however, BA lenses incubated in Solution B deposited greater amounts of lysozyme. Greater amounts of lactoferrin also accumulated on LB and BA lenses when incubated in Solution A, whereas the opposite trend was seen for SA lenses, which deposited more lactoferrin with Solution B. Finally, BA lenses deposited greater amounts of albumin when incubated in Solution B, whereas LB lenses accumulated greater albumin when incubated in Solution A, while less solution impact was found using SA lenses.

The diversity of the results in this study highlights the importance of using appropriate *in vitro* models, as the outcome for protein accumulation to certain contact lens - protein interactions is strongly impacted by the competitive nature of the respective tear film components.

3.6 ACKNOWLEDGEMENTS

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4 EFFECT OF MULTI-PURPOSE SOLUTION ON COMPETITIVE BINDING PROTEIN TO SILICONE HYDROGEL CONTACT LENSES

4.1 OVERVIEW

Purpose: The purpose of this *in vitro* study was to determine the effect of a multi-purpose solution on removing protein deposited on silicone hydrogel (SH) lenses incubated in either a simple protein solution or complex artificial tear solution (ATS).

Methods: Three SH materials - senofilcon A (SA), lotrafilcon B (LB) and balafilcon A (BA) - were incubated in two different solutions (n=4 for each lens type); Solution A was a simple saline solution containing a single protein, whereas Solution B was a more complex ATS, containing proteins, lipids and mucins. Lysozyme protein was radiolabeled with ^{125}Iodine and protein deposition was determined after 1, 7, 14, and 28 days of incubation. Half of the lenses were cleaned with OPTI-FREE RepleniSH (OFR) five times a week and compared to the un-cleaned lenses.

Results: At the end of the 28 days, SA lenses incubated in Solution A accumulated significantly less lysozyme than the lenses incubated in Solution B (1.24µg vs. 3.81µg; $p<0.001$). A similar trend was also seen with LB lenses (Solution A: 2.57µg, Solution B: 13.66µg; $p<0.001$). BA lenses incubated in Solution A accumulated 22.37µg of lysozyme, whereas Solution B accumulated 28.18µg ($p<0.001$). When SA lenses were cleaned with OFR, 47% of protein was removed when lenses were incubated in Solution A and 87% was removed when lenses were incubated in Solution B. Both incubation solutions allowed for approximately 0.50µg of lysozyme to accumulate on the SA lenses after cleaning. For LB lenses, OFR removed 46% when lenses were incubated in Solution A and 75% when lenses were incubated in Solution B. There was a plateau at 0.60µg and 1.40µg for Solution A- and Solution B-incubated lenses, respectively. For BA lenses, about a 70% cleaning efficacy is found for lenses incubated in either Solution A or Solution B.

Discussion: The two different incubation solutions had an impact on the amount of lysozyme depositing on SA, LB, and BA lenses. Lenses incubated in the ATS typically accumulated greater amounts of lysozyme, which highlights the importance of choosing more appropriate models when conducting *in vitro* experiments. The care regimen, OFR, had a greater efficacy when removing protein from SA and

LB lenses incubated in a complex ATS; however, a similar cleaning efficacy was found for BA lenses, regardless of incubation solution.

Key Words: Contact lens deposition, lysozyme, multi-purpose solution, silicone hydrogel contact lenses, tear film

4.2 INTRODUCTION

Proteins enter the tear film through different pathways, including the lacrimal [1,2] and meibomian glands [3], ocular surface cells [4], and by leakage through the blood vessels [5]. It is therefore not surprising that de Souza was able to detect 491 different types of proteins in the human tear film [6]. The total protein concentration is approximately 8mg/mL [6,7] and the most abundant proteins are lysozyme and lactoferrin, which together account for approximately 50% of the total protein [2,8]. The exposure of biomaterials, such as contact lenses, typically results in deposition of tear proteins to the contact lens surface and penetration into the lens matrix [9,10]. The deposition profile is very material-specific and silicone hydrogel (SH) contact lens materials have shown to accumulate significantly less protein than conventional hydrogel lenses [11]; however, the protein that deposits onto the SH surface is mainly denatured [12,13]. Denatured proteins have been linked to giant papillary conjunctivitis [14], which is an inflammatory reaction to proteins, occurring at the palpebral conjunctiva [15].

Contact lens multi-purpose solutions (MPS) and hydrogen peroxide-based systems are used by contact lens-wearers in order to clean and disinfect the lenses between wearing cycles [16,17]. Cleaning solution components have the ability to change the chemical and/or physical properties of the lens surface, as well as stick onto the lens surface or penetrate through the lens matrix [18]. A study by Zhao and colleagues determined that the amount of protein removed from lenses was dependent on the MPS used as well as the lens type; OPTI-FREE Express (OFE) and OPTI-FREE RepleniSH (OFR) removed the most protein from lotrafilcon B lenses (1.7µg (OFR) and 3.6µg (OFE) compared to less than 1µg for other MPS tested) [18]. Proteins were most efficiently removed from balafilcon A lenses regardless of the lens care solution used (5.4 to 23.5µg) [18]. For senofilcon A lenses, AQuify showed a better efficacy of protein removal compared to the other products [18].

A more recent *in vitro* study, conducted by Luensmann and colleagues, reported that the efficiency of protein removal from contact lenses using various care regimens ranged from 2.9% to 62.4% and only a minor difference was seen between manual lens rubbing and soaking alone [11]. In this study, only a single protein solution was used for incubation and the results suggested that lotrafilcon B lenses accumulated small amounts of lysozyme, only, but this deposit was bound more tightly bound compared

to etafilcon A, which accumulated high amounts of lysozyme, but which released a higher percentage during the cleaning cycle [11]. This could be explained, as denatured proteins typically bind more tightly to contact lenses [19], and the percentage of denatured protein recovered from SH lenses is typically higher compared to the content determined on conventional hydrogels [12,13].

The purpose of this *in vitro* study was to determine the amount of protein deposition onto three SH lens types when regularly cleaned with a MPS care regimen. A single protein solution and a complex artificial tear solution (ATS) were used to determine lysozyme deposition rates. In a previous study [20] differences were found for the amount of protein depositing when other tear film components were present and the current study will determine whether these incubation solutions also impact MPS cleaning efficacy.

4.3 METHODS

In this *in vitro* study, a simple saline solution and a more complex ATS were used to determine the deposition of lysozyme onto different SH lenses, while undergoing frequent cleaning cycles with OFR.

Incubation solutions

A single protein solution (Solution A) and complex ATS (Solution B) were used for the contact lenses incubation. Solution A contained a number of salts, glucose, and urea, and the composition details are outlined in recent publication by Lorentz and colleagues [21]. Lysozyme (1.9mg/mL) was added as a single protein of interest to Solution A. The process of making both solutions has been described in detail in a recent publication by Jadi et al. [20]. In brief, Solution B consisted of the saline solution described above with an addition of proteins (albumin, lactoferrin, lysozyme, and IgG), mucins, and lipids (cholesterol, cholesteryl oleate, OAME, oleic acid, phosphatidylcholine, and triolein) [21]. The pH of both solutions was within human tear film limits at about 7.4 [22].

Lysozyme was conjugated with iodine-125 (^{125}I) through the iodine monochloride method [23]. The radiolabeled protein was added to the incubation solutions at a concentration of 2%. Control solutions,

not containing a contact lens, were used to verify radioactive decay over time.

Three SH contact lens materials (Table 4-1) were studied in this experiment - senofilcon A (SA, Acuvue Oasys, Johnson & Johnson, Jacksonville, FL), lotrafilcon B (LB, Air Optix; CIBA VISION, Duluth, GA), and balafilcon A (BA, PureVision; Bausch & Lomb, Rochester, NY).

Table 4-1: Properties of silicone hydrogel contact lenses

	Senofilcon A	Lotrafilcon B	Balafilcon A
FDA Group	I: low water (<50%), non-ionic	I: low water (<50%), non-ionic	III: low water (<50%), ionic
Water content (%)	38	33	36
Charge	Neutral	Neutral	Negative
Oxygen permeability (Dk)	103	110	91
Surface modifications	No surface treatment. Internal wetting agent (PVP)	25nm plasma coating (high refractive index)	Plasma oxidation process

PVP= polyvinyl pyrrolidone

Contact lens incubation

Each contact lens was pre-soaked in saline for 24 hours in order to remove the blister-pack solution. Glass jars (250mL; Qorpak, Bridgeville, PA), containing histology cassettes (VWR, Mississauga, ON), were pre-treated for 4–7 days with the same solution used for lens incubation. This was done to coat the jars and cassettes and to minimize adsorption of solution components to the walls of the glass and cassettes during the lens incubation. IgG was not used in the pre-treatment of ATS vials due to cost limitations.

Contact lenses were placed in histology cassettes that held four lenses securely, incubated at 37 °C, and placed on a rotatory shaker (VWR, Mississauga, ON) at 60 rpm for 1, 7, 14, and 28 days (Figure 4-1). Four replicates were used for each lens type and time point, resulting in 576 lenses total.

Figure 4-1: Histology cassettes holding four lenses

Following the incubation, 50% of the lenses (test group) underwent a cleaning procedure using OFR (Table 4-2). The other 50% (control group) were not cleaned with OFR during the study.

Table 4-2: Properties of the cleaning solution OPTI-FREE RepleniSH

Disinfectant	Components	Buffer
ALDOX (0.0005%) PolyQuad (0.001%)	TearGlyde- Tetronic 1304 and C9-ED3A	Sodium citrate

After each time period, the test lenses was removed from the incubation solution and placed in OFR overnight. The next morning, lenses were rinsed in saline twice and placed in a culture tube (12x75mm; VWR, Mississauga, ON). The tubes were left to dry for about 12 hours in order for unbound iodine to evaporate. The control lenses were treated in the same way, without undergoing the cleaning cycle at this time point. The radioactive protein content on each lens was then determined using the Wallac Wizard 1470 Gamma Counter (Perkin Elmer, Woodbridge, ON) and the total amount of lysozyme deposited on the contact lens was calculated.

The incubation solution was replenished once a week to minimize chances of the solution becoming dilute between cleaning cycles. Lenses in the test group were cleaned five times a week in the evenings

and removed from cleaning solution the following day. Lenses in the control group did not undergo any cleaning.

Statistica 9 (StatSoft Inc. Tulsa, OK), was used to conduct data analysis. A repeated measures analysis of variance (ANOVA) was used to compare the amount of lysozyme deposition onto the different SH materials over time (Main Factors: incubation solution, contact lens material, and time point). Tukey's honestly significant difference (HSD) test was used for post-hoc comparisons; $p<0.05$ was considered significant.

4.4 RESULTS

This study consisted of two experiments performed simultaneously. A single protein solution and a complex ATS were used to incubate SH lenses in order to compare the deposition of lysozyme onto the materials and to determine the protein removal following a cleaning cycle. Figures 4-2 to 4-4 outline the deposition profile of lysozyme with and without frequent cleaning cycles using OFR over a 28-day time period.

Figure 4-2 outlines the deposition of lysozyme protein on SA lenses. After 1 day, SA lenses incubated in either Solution A or Solution B accumulated similar amounts of lysozyme (Solution A: 1.73±0.37 µg, Solution B: 1.76±0.04 µg; $p=1.00$). However, after 28 days, SA lenses incubated in Solution A accumulated lower amounts in comparison to the results found on day 1. This pattern was not seen for SA lenses incubated in the complex Solution B. A statistically significant difference in deposition was seen between these two solutions after the 28 days; SA lenses accumulated significantly more when incubated in Solution B (3.81±0.65 µg) compared to Solution A (1.24±0.47 µg) ($p<0.001$).

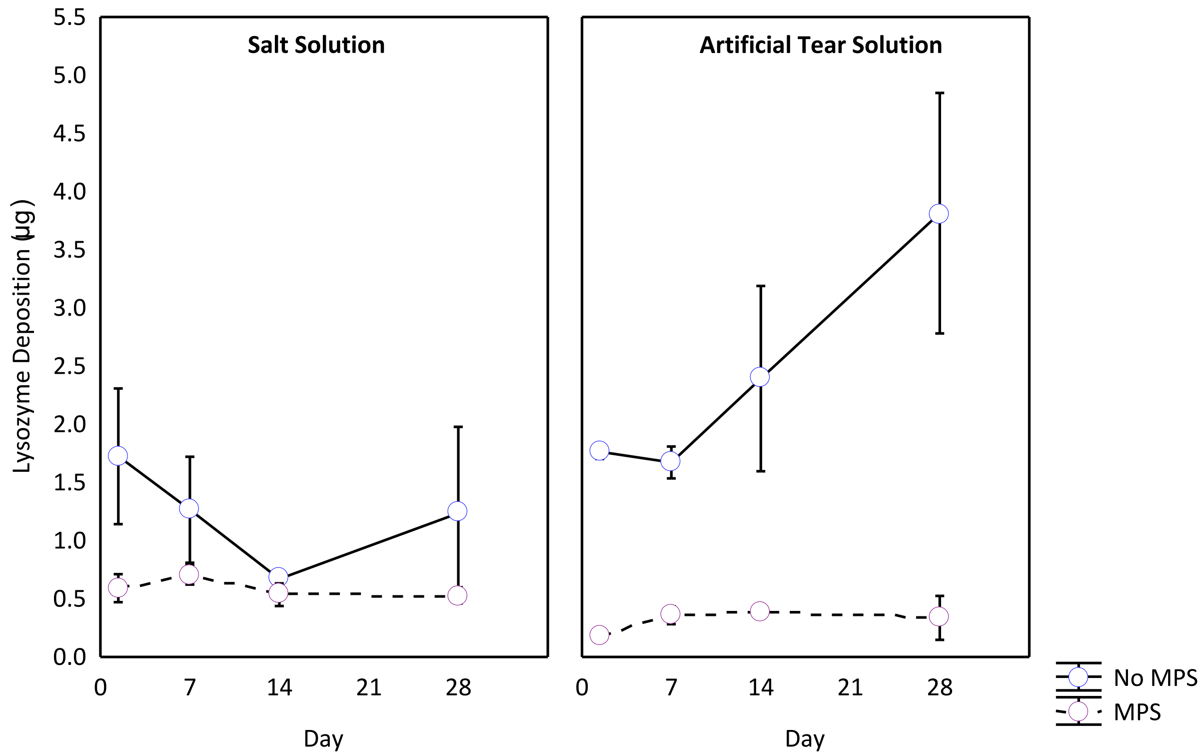

Figure 4-2: Effect of a multi-purpose cleaning solution (OPTI-FREE RepleniSH) on lysozyme deposition to senofilcon A lenses using a single protein solution (Solution A) and a complex ATS (Solution B). Incubation time points: 1, 7, 14 and 28 days.

With regards to the efficiency of protein removal when SA lenses were incubated in Solution A, OFR removed the highest percentage of lysozyme (66%) after 1 day of incubation (p=0.006). Following this, 44% was removed after a week (p=0.22) and the least removed (19%) after 14 days (p=1.00). At day 28, lenses in the test group had undergone 20 cycles of over-night soaking in MPS and, as a result, 57% less lysozyme was found on these SA lenses in comparison to the non-cleaned control lenses (p=0.08). On average, nearly 50% of the protein deposited onto the SA lenses was removed with OFR when lenses were incubated in a simple protein solution (Solution A). Similar amounts of lysozyme were detected on the cleaned SA lenses, which did not change/increase over time (p>0.05).

When the SA lenses were incubated in Solution B, OFR removed significant amounts of lysozyme, regardless of the time point. After 1 day of incubation, 90% of protein was removed ($p<0.001$); high removal efficiencies were also observed after 14 (84%) and 28 (91%) days. Overall, a better protein removal efficiency was seen when SA lenses were incubated in the more complex Solution B; however, the amount of lysozyme remaining on lenses are quite similar regardless of the incubation solution used, plateauing at about 0.50μg.

Figure 4-3 shows the lysozyme deposition for LB lenses over the 28-day time period. LB lenses incubated in a simple protein solution accumulated similar amounts of lysozyme as those lenses incubated in a complex ATS after 1 day; Solution A: 2.93±0.43 μg, Solution B: 2.83±0.60 μg, $p=1.00$. A significant increase in lysozyme deposition was seen with lenses incubated in Solution B between 7 and 28 days ($p<0.001$); however, this was not apparent for LB lenses incubated in Solution A, which showed a plateau immediately after day 1 ($p>0.05$). After 28 days, LB lenses accumulated 2.57±0.16 μg of lysozyme when incubated in Solution A, which was significantly lower compared to LB lenses incubated in Solution B (13.66±4.29 μg) ($p<0.001$).

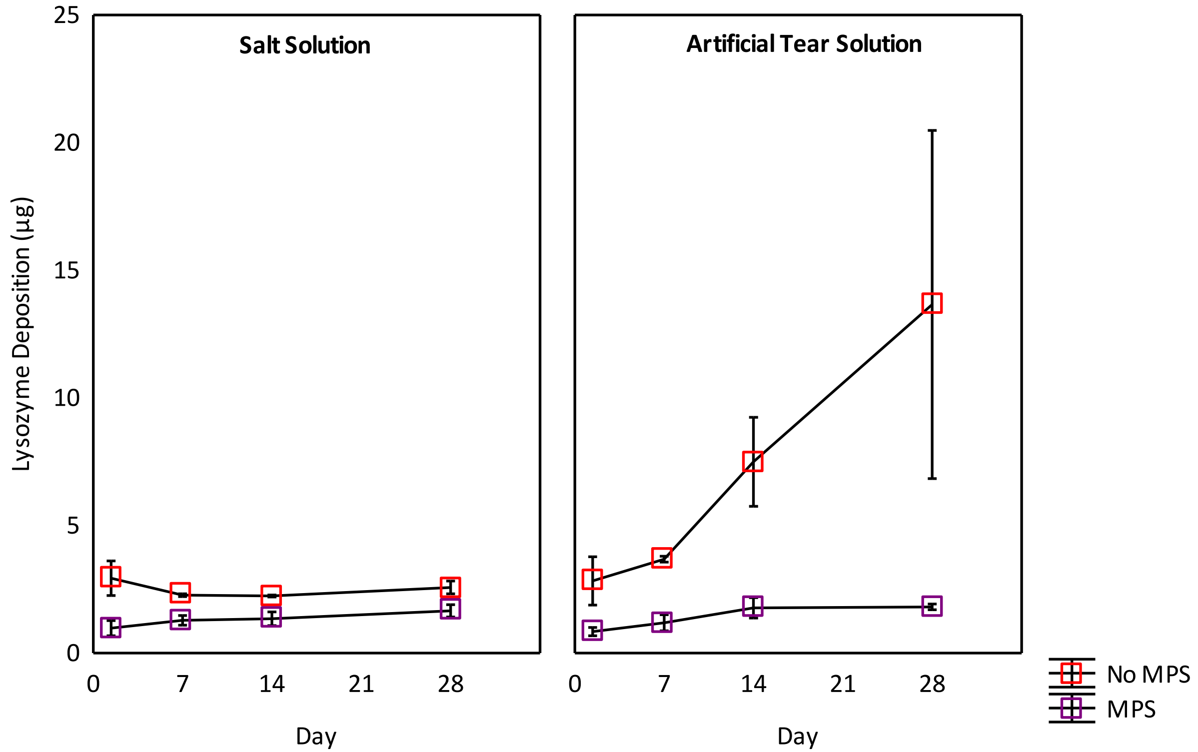

Figure 4-3: Effect of a multi-purpose cleaning solution (OPTI-FREE RepleniSH) on lysozyme deposition to lotrafilcon B lenses using a single protein solution (Solution A) and a complex ATS (Solution B). Incubation time points: 1, 7, 14 and 28 days.

Following the OFR cleaning cycle, 67% of the lysozyme was removed from the LB lenses after 1 day of incubation in Solution A. A slightly reduced efficiency was seen after 7, 14 and 28 days, showing 44%, 40%, and 35%, respectively. Significant differences were not seen between LB lenses that did or did not undergo a cleaning cycle for the 14 and 28-day time points ($p>0.05$). The amount of protein remaining on the cleaned LB contact lenses after each time point was similar throughout the experiment, plateauing at about 0.60µg.

When LB lenses were incubated in Solution B, the amount of protein removed was overall greater in comparison to lenses incubated in Solution A ($p<0.01$). At day 1, there was a 70% protein removal

efficiency (p=0.04), followed by 68% at day 7 (p=0.01), 76% by day 14 (p<0.001), and 87% at day 28 (p<0.001). Overall, 75% of the protein deposited throughout the Solution B-incubated LB lenses was removed. This allowed for a plateauing effect seen between days 1 and 28 at about 1.40µg, which was just over half the amount remaining when incubating LB lenses in Solution A.

Figure 4-4 shows the data collected with BA lenses. At day 1, BA lenses accumulated 10.21±0.28 µg when incubated in Solution A and accumulated slightly less when incubated in Solution B (9.48±0.64 µg) (p=0.095). BA lenses incubated in both solutions accumulated more lysozyme over time and accumulated over twice the amount from day 1 to 28 for Solution A-incubated lenses (22.37±0.68 µg) and three times the amount for lenses incubated in Solution B (28.18±0.26 µg); p<0.001.

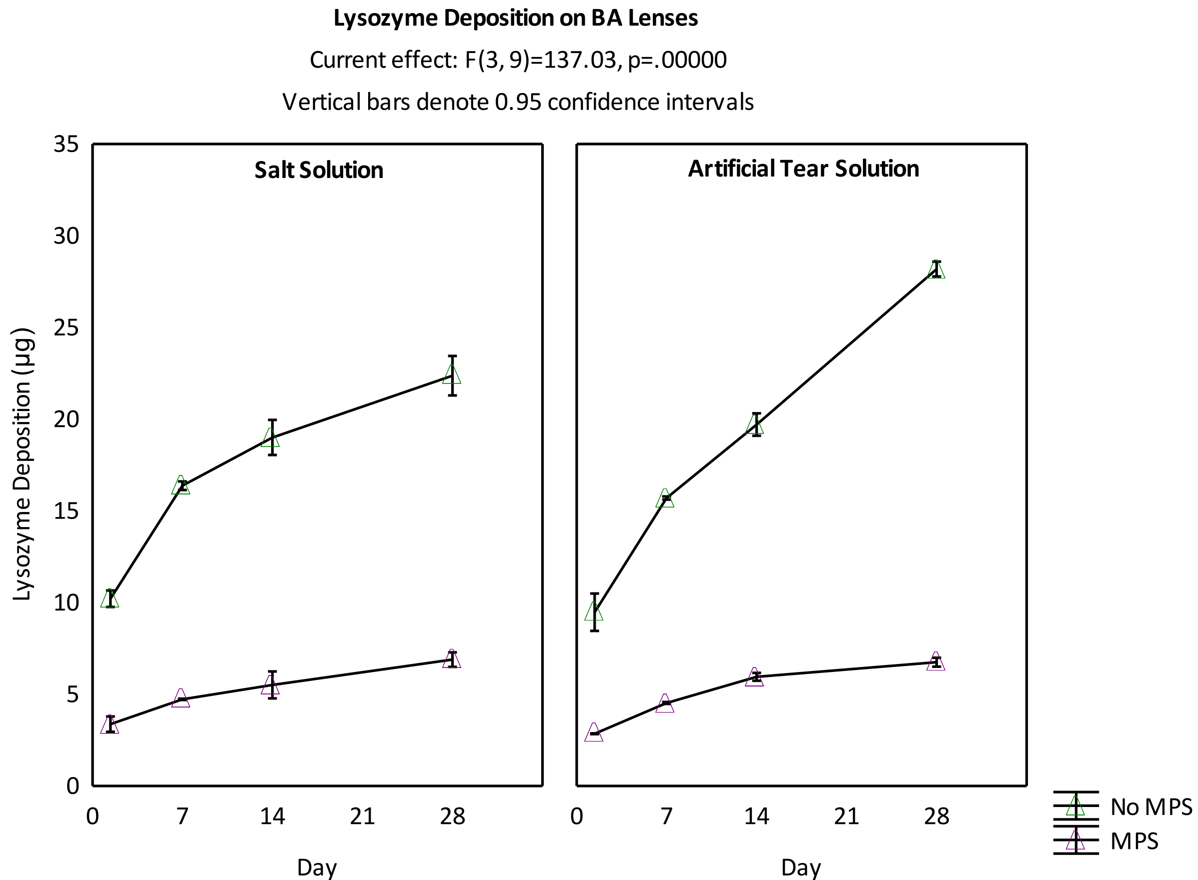

Figure 4-4: Effect of a multi-purpose cleaning solution (OPTI-FREE RepleniSH) on lysozyme deposition to balafilcon A lenses using a single protein solution (Solution A) and a complex ATS (Solution B). Incubation time points: 1, 7, 14 and 28 days.

BA lenses incubated in either Solution A or Solution B have shown similar trends in protein efficiency when cleaned with OFR. BA lenses incubated in Solution A experience a similar lysozyme removal efficiency independent of the length of incubation time. The removal efficiency was 67% on day 1, 71% on days 7 and 14 and 69% on day 28. The removal efficiency was significant at all time points ($p<0.001$). On average, 70% of Solution A's deposited protein is removed throughout the 28-day time period. The remaining protein on the lenses, following the cleaning cycles, increased slightly with each time point, which was different for SA and LB lenses.

When BA lenses were incubated in Solution B, OFR removed approximately 70% of the accumulated protein throughout days 1 to 14 and 76% on day 28 ($p<0.001$). The total amount of lysozyme remaining on the lens increased throughout each time point, which was similar to the pattern seen with Solution A; between days 1 and 7 ($p<0.001$) and days 7 and 14 ($p=0.001$). A plateau began to surface near day 28, as there was not a significant difference between the amount of lysozyme accumulated at days 21 and 28 ($p=0.063$).

4.5 DISCUSSION

The purpose of contact lens cleaning systems is to remove macromolecules, such as lipids, proteins and other surface debris, and to eliminate microorganism growth. A number of cleaning systems have been introduced that claim to further maintain wettability and comfort for the lens-wearer [24]. A number of studies have been conducted to test the efficacy of contact lens cleaning solutions against microbial growth and tear film deposits [13,24-26]. For multi-purpose care regimens, manufacturers often recommend manual lens rubbing to maximize the cleaning efficiency, since soaking alone may not be able to remove deposits sufficiently, such as cosmetics and proteins [27,28]. The visual presence of proteins is found on contact lenses and this may result in a hazy-look to the lens – this is decreased significantly when lenses are rubbed prior to placing in a cleaning solution [27].

Lysozyme deposition onto SH lens materials and the efficiency of an MPS to remove this protein was the focus of this study. Two incubation solutions were used in order to determine the impact of tear film

components after 28 days of incubation and daily cleaning procedures. Solution A was a single protein solution that contained a saline base along with the protein lysozyme in a physiological concentration of 1.9mg/mL [2]. Solution B was a complex ATS with the same saline base as Solution A; however, other proteins, lipids, and mucins were added to allow for competitive binding. Studies have demonstrated that proteins have the ability to displace one another when competing for binding sites onto artificial surfaces, including contact lenses [29,30].

A number of studies have shown that the ionic BA material typically accumulates more tear film proteins compared to other SH lenses, including SA and LB lenses [12,31], which is in agreement with our current results. We have recently demonstrated that the composition of the incubation solution can impact protein deposition profiles to contact lenses [20]. This study further confirmed that only SA lenses accumulated similar amounts of protein- albumin, lactoferrin, lysozyme- regardless of the incubation solution used. BA and LB lenses showed varying deposition profiles, depending on the protein and solution composition [20]. In a recent study undertaken by Ng and colleagues [32], SA lenses accumulated greater lysozyme (5.3µg) when incubated in an artificial tear solution that did not contain lipids than other solutions of varying complexity (3.9µg). It was demonstrated that LB lenses accumulate significantly less lysozyme (9.7µg) when incubated in a complex solution containing lactoferrin protein and a variety of lipids compared to a simple solution (11.8µg).

This study consisted of two experiments run in parallel investigating three different SH contact lens materials, which differed in their composition, surface treatment, and water content (Table 4-1). The SA lens material contains a copolymerization of HEMA and N, N-dimethyl acrylamide with (3-methylacryloxy-2hydroxypropyloxy) propylbis (trimethylsiloxy) methylsilane [33]. Additionally, SA lenses contain an internal wetting agent, polyvinyl pyrrolidone (PVP), increasing surface hydrophilicity [34]. In previous *ex vivo* studies it was determined that lysozyme contributes nearly 25% [35,36] of the total protein (7µg) deposited onto SA lenses [18,35,36], which is similar to the total amount of lysozyme (6µg) deposited onto these lenses over a 2-week time period in *in vitro* studies [12,31]. After immediate incubation, lysozyme deposition onto SA lenses was driven by non-competitive factors (p=NS) as Solution A and Solution B deposited similar quantities; however, this trend was not witnessed as further time points were reached. By day 28, SA lenses accumulated significantly greater amounts of lysozyme

when incubated in the more complex ATS ($p<0.001$). This shows that lysozyme is affected in non-competitive ways when depositing onto the lens material, as with greater complexity of the incubation fluid, more lysozyme deposits. It is possible that lysozyme deposits initially, followed by other negatively charged proteins, such as albumin, and further packing of lysozyme on top, which will be attracted, electronically, to the albumin [37,38]. OFR was used as the MPS of interest in determining its efficacy in removing protein from the lenses. Whether lenses were incubated in a simple or complex solution, the final mass of lysozyme on the contact lenses was similar and did not change/increase over time, averaging at 0.50µg. This means that a higher percentage of protein was removed from SA lenses incubated in Solution B, as they accumulated the most after the 28-day time period.

The LB lens material is contains both a siloxy and hydrogel phase, allowing the maintenance of oxygen and salt diffusion [33]. The hydrophilicity of the lens surface is optimized using a 25nm surface plasma coating [39,40]. This coating provides a boundary, controlling access to the underlying polymer, which reduces protein penetration into the lens matrix [11,40]. *Ex vivo* studies have shown that lysozyme deposits approximately 25% of the total protein accumulating on LB lenses [35,36], which equals 2µg of lysozyme [41,42]. *In vitro* studies have shown that up to 12µg of lysozyme deposit on LB lenses [11,32]; however, this study demonstrates that LB lenses may accumulate even greater, showing variability at day 28 when incubated in Solution B (13.66µg). Like SA lenses, our current study suggests that primarily non-competitive factors drive the accumulation of lysozyme to LB lenses during the start of the incubation period; there was no significant difference in the amount of lysozyme deposited from Solution A and Solution B (p=NS). For the following three time points, there was a significant difference in the amount of lysozyme that deposited to LB lenses. LB lenses that were incubated in Solution A accumulated significantly less lysozyme than those incubated in Solution B ($p<0.001$). The deposition pattern of LB lenses incubated in Solution B is different than that seen for Solution A-incubated lenses in that a plateau was not reached. Greater variability is seen for LB lenses incubated in a complex ATS and this may be due to the increased interactions between proteins, lipids, and mucins and the overall complexity of the incubation solution used. The accumulation of lysozyme to LB lenses may be caused by non-competitive factors as more lysozyme deposited when other tear film components were available in the solution. A similar "sandwich-phenomenon" may be responsible as described above [37,38]. When lenses incubated in Solution A were placed in OFR for cleaning, approximately 50% of the

total protein was removed by the end of the 28-day time period; however, for LB lenses incubated in Solution B, the care regimen was able to remove 75% of total lysozyme. There was an evident plateau at about 0.60μg throughout the cleaning process between days 1 and 28 for Solution A-incubated LB lenses, whereas lenses incubated in Solution B reached a plateau at near 1.40μg following the cleaning cycle.

The BA lens material incorporates a co-polymerization of TRIS derivative vinyl carbamate and N-vinyl pyrrolidone, providing it with biphasic characteristics [33]. On the surface of the BA material are hydrophilic glassy silicate 'islands' [40], which result from the oxidation of TRIS [33]. In addition to this, the surface of BA lenses is porous, allowing molecules, such as proteins, to penetrate through the matrix [43]. N-vinyl aminobutyric acid is incorporated into this lens material, which provides its ionic character, hence attracting positively charged proteins to the material [18,31]. *Ex vivo* studies reported that numerous proteins deposit on BA lenses; however, lysozyme contributes for up to 50% [11,44]. *In vitro* studies done on lysozyme deposition have concluded that up to 50μg of lysozyme may accumulate on the lens [11,12]. After a day of incubation, this current study found significantly more lysozyme on BA lenses incubated in simple lysozyme solution compared to lenses incubated in a complex ATS ($p<0.001$), suggesting that competitive factors may drive the binding of lysozyme to the lenses. Since lysozyme is overall positively charged and BA is an ionic lens, it is expected of lysozyme to deposit at greater amounts than the other proteins due to electrostatic attraction, its small size, and overall abundance. By the end of the 28-day time point, significantly more lysozyme accumulated on the BA lenses incubated in the complex ATS ($p<0.001$). When the lenses were subject to over-night cleaning with OFR, the cleaning efficiency was similar independent of the incubation solution. Nearly 70% of the accumulated protein was removed from both sets of lenses. Despite the frequent cleaning cycles, a small increase in lysozyme accumulation was seen over the 28-day period with both incubation solutions. A plateau was reached by day 28 for Solution B-incubated lenses.

Studies have determined the efficacy of certain cleaning solutions in removing proteins from contact lens materials. In a study undertaken by Luensmann and colleagues [11], it was determined that the removal efficiency of lysozyme from LB lenses was less than 10% when using either a hydrogen peroxide system (Clear Care) or a MPS (COMPLETE MPS Easy Rub). With regards to BA lenses, on average,

approximately 60% of accumulated lysozyme was removed when lenses were subject to the different care regimens [11]. Jung and Rapp found that up to 50% of protein was removed from hydrophilic contact lenses incubated in a more complex tear solution that contained proteins and glycoprotein when cleaned with a regimen, such as OPTI-FREE [25]. In a study conducted by Zhao and colleagues [18], it was determined that higher amounts of protein could be removed from LB lenses when OPTI-FREE cleaning solutions were used. They further recovered over 20µg of deposited protein from worn-BA lenses regardless of lens care solution used [18].

Limitations of this study include placing contact lenses in histology cassettes in order to increase efficiency in time during incubation and MPS-soaking. The cassettes provided lenses with a boundary and may not allow lysozyme to effectively bind to the lens materials and hence will maintain its conformation; loose binding will allow appreciable removal of protein from the lens and hence can be a plausible explanation for the large percentages of lysozyme removed from the lens materials.

4.6 CONCLUSION

In this *in vitro* study, the amount of lysozyme depositing to SH lenses was impacted by the complexity of the incubation solution, which subsequently impacted the protein removal efficiency when using a MPS. The largest difference in lysozyme deposition was found for LB lenses, which accumulated approximately four times more lysozyme when incubated in a complex ATS compared to a simple protein solution. Both SA and LB lenses showed a plateauing pattern in protein deposition when cleaned regularly with the MPS independent of the complexity of the incubation solution. However, cleaned BA lenses show a steady increase in deposition over the 28-day time period.

Based on the range of deposition amounts found in this study, it is important that *in vitro* models take into consideration the complexity - or lack thereof - of an incubation solution. The interaction between individual proteins and other tear film components, such as lipids, significantly impacts protein deposition pattern to SH contact lenses.

4.7 ACKNOWLEDGEMENTS

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5 IMPACT OF CONTACT LENS CLEANING SOLUTIONS AND LENS CASES ON LYSOZYME ACTIVITY

5.1 OVERVIEW

Purpose: The purpose of this *in vitro* study was to determine both the effect of different contact lens cleaning solutions and lens cases on lysozyme activity.

Methods: A high (500µg) and a low (10µg) concentration of lysozyme were used to represent the amount deposited on patient-worn hydrogel and silicone hydrogel (SH) contact lenses. Three different contact lens cleaning solutions (OPTI-FREE RepleniSH (OFR), BioTrue (BT), and Clear Care (CC)) were placed in their respective lens cases and a control solution, phosphate-buffered saline (PBS), was placed in all test solution cases as well as polypropylene (PP) plastic vials. Lysozyme concentrations were increased (high: 1500µg, low: 20µg) when using neutralization cassettes, to replicate the fact that lens cases hold 2 lenses. The activity of lysozyme at 1, 12, 24, and 48 hrs was determined.

Results: For OFR, the high lysozyme concentration showed an enhanced activity of 222% at the 1-hr time point and dropped significantly to 180% at 12 hrs ($p<0.001$). Lysozyme activity reduced further to 132% by hr 24 ($p<0.001$). No significant change was seen between the 24-hr (132%) and the 48-hr (126%) time points ($p>0.05$). The low lysozyme concentration started at 195% (1 hr), dropped to 138% (12 hrs; $p<0.001$) and remained unchanged for the subsequent time points ($p>0.05$). For BT, the high lysozyme concentration started at 65% activity (1 hr) and significantly increased to 99% by 12 hrs ($p<0.001$), followed by a lack of change for successive time points ($p>0.05$). The low lysozyme concentration exhibited an activity of 45% at 1 hr, which increased significantly to 86% (12 hrs; $p<0.001$); no further change was seen after this time point ($p>0.05$). For CC, the high lysozyme concentration exhibited a low activity of 32% (1 hr), followed by an increase to 48% at the 12-hr time point ($p<0.001$). No further change was seen at 24 hrs ($p>0.05$) and a slight increase to 54% was noted at 48 hrs ($p<0.001$). The low lysozyme concentration dissolved in CC exhibited 32% activity at 1 hr, which significantly increased to 44% by 12 hrs ($p<0.001$). Between 12 and 24 hrs there was a plateau ($p>0.05$) followed by a final peak (95%) at 48 hrs ($p<0.001$). When high concentration lysozyme was dissolved in PBS and placed in different lens cases, greatest initial activity (hr 1; 128%) was seen when OFR lens cases were used ($p<0.001$); however, when the final time point (hr 48) was reached, lysozyme was more stable when placed in BT lens cases (BT case = 42%; OFR case = 34%). For low concentration lysozyme

dissolved in PBS, initial lysozyme activity (hr 1; 61%) was also highest when placed in OFR lens cases ($p<0.001$); however, by hr 48, greater lysozyme activity was found in CC neutralization cassettes (CC cassettes = 48%; OFR case = 18%; $p<0.001$).

Conclusion: The three contact lens cleaning solutions showed varying impact on the activity of lysozyme when exposing a high or a low lysozyme concentration to the solution. When lysozyme was dissolved in OFR, the activity of protein was enhanced over 100%. Lysozyme placed in BT solution, exhibited an initial increase, after which the activity remained constant throughout the 48-hr time period. For CC, lysozyme activity was lower compared to the other two care regimens. Lysozyme activity was further impacted by the lens case, resulting in an initial increase when lysozyme dissolved in PBS was placed in OFR cases.

Key Words: contact lens care regimens, hydrogen peroxide solution, lysozyme activity, *Micrococcus lysodeikticus*, multi-purpose solution

5.2 INTRODUCTION

Discovered in the early 20th century, lysozyme was considered an enzymatic protein with antimicrobial properties [1]. It is found in a high concentration in the human tear film, constituting 1.9 mg/mL [2]. This positively charged [3] protein (14.5 kDa [4]) plays an important role in the human tear film as it hydrolyzes glycosidic bonds in the peptidoglycan of bacteria and prevents microbial colonization [5]. Once lysozyme deposits onto contact lens materials, it is likely to denature [6] and may lose its antimicrobial activity. An *ex vivo* study has shown that up to 90% of lysozyme that deposited onto worn silicone hydrogel (SH) contact lens surfaces was denatured [7]. A number of negative consequences may result from inactive protein deposits, including an immunological stimulus [8] causing papillary conjunctivitis [9], reduced visual acuity [10], and an overall discomfort with the lenses [11].

Introduced in the 1970's [12,13], conventional, poly-2-hydroxyethyl methacrylate (pHEMA)-based contact lenses exhibit a relatively low oxygen transmissibility, which can result in hypoxic complications in some wearers [14,15]. SH lenses, which were introduced in 1999, have a higher oxygen permeability [13,16], and exhibit a different deposition profile of tear film components compared with pHEMA-based materials. PHEMA-based lenses typically accumulate greater amounts of protein and lower amounts of lipid, in comparison to SH lenses, which attract more lipids and only very little protein [17-19]. A study conducted by Jones et al., determined that ionic pHEMA lenses accumulated approximately 100x more protein than SH lenses; however, SH lenses accumulated 30x more lipid than ionic pHEMA-based lenses [20]. Specifically, the ionic pHEMA lens, etafilcon A, accumulated 1000µg of lysozyme per lens, whereas the SH lens, balafilcon A, accumulated approximately 10µg [20].

Contact lens care systems, such as multi-purpose solutions (MPS) and hydrogen peroxide systems, are used to disinfect lenses [21,22] and to remove tear film deposits between wear-times [23-26]. Cleaning solution components have the ability to change the chemical and/or physical properties of the lens surface [24]. The components may also stick onto the lens surface or penetrate through the lens matrix [24]. Contact lens cases are specific to their cleaning solution and are considered class II devices under the Food & Drug Administration (FDA). The FDA and the International Organization for Standardization (ISO) tests contact lens case performance and prepares standards for testing lens care solutions [27].

A recent study conducted by Barniak and colleagues [28] investigated the effect of MPS on lysozyme activity and its ability to prevent denaturation of lysozyme in solution after adding sodium dodecyl sulfate (SDS). Better maintenance of lysozyme activity was seen with BioTrue and ReNu, compared to OPTI-FREE (EXPRESS and RepleniSH), AQuify, and COMPLETE MPS Easy Rub Formula [28]. Findings from this study are of interest; however, it remains unclear how the activity of lysozyme changes over time when exposed to these care regimens, without adding SDS. Our current study reports lysozyme activity changes when lysozyme is dissolved in different contact lens care regimens and lens cases for up to 48 hrs.

5.3 METHODS

A recent in-house study determined the effect of varying MPS concentrations on lysozyme activity when a fixed amount of lysozyme was used. BioTrue (BT), Clear Care (CC), and OPTI-FREE RepleniSH (OFR) were diluted with phosphate-buffered saline (PBS) to four different concentrations (2.67%, 5.33%, 8.33%, and 16.67%) in order to determine if MPS concentrations have a denaturing, renaturing, or no effect on lysozyme activity. The diluted care regimens and the control solution (1x PBS) were placed in individual Eppendorf tubes using a volume of 50µL. Lysozyme (83ng) was dissolved in 25µL of PBS and added to the solution, followed by a final addition of 300µL of bacteria. Lysozyme activity was determined immediately as described below.

For the main experiment, two different concentrations of lysozyme were used, which was representative of the amount deposited onto *ex vivo* lenses- a high concentration (500µg) was used to mimic lysozyme deposition on ionic conventional hydrogel lens lysozyme deposits [8,29], and a low concentration (10µg) was used to simulate deposition rates on SH lenses [7,8]. Since the CC neutralization cassettes hold two lenses, the amount of lysozyme was increased to 1500µg and 20µg for conventional and SH lenses, respectively.

The activity of lysozyme was determined for up to 48 hrs when added to three different contact lens cleaning solutions, BT, CC, and OFR, which are described in Table 5-1.

Table 5-1: Properties of contact lens care regimens investigated

Property	BioTrue (BT) Bausch & Lomb	Clear Care (CC) CIBA Vision	OPTI-FREE RepleniSH (OFR) Alcon
Disinfectant	Polyaminopropyl biguanide (0.00013%), polyquaternium (0.0001%)	Hydrogen peroxide (3%)	ALDOX (0.0005%) PolyQuad (0.001%)
Components	Hyaluronan (lubricant), sulfobetaine, poloxamine	Sodium chloride (0.79%), Phosphonic acid, Pluronic 17R4	TearGlyde- Tetronic 1304, C9-ED3A
Buffer	Boric acid, sodium borate, edetate disodium, sodium chloride	Phosphate	Sodium citrate

Lysozyme was dissolved in PBS at a concentration of 2mg/mL. Low and high concentration lysozyme stocks were prepared separately for each test solution, including the PBS control solution. Once both the high and low concentration solutions were prepared, this was considered hr 0. The lens cases specific to the different care regimens were used in the study, in addition to polypropylene (PP) plastic control vials (Figure 5-1).

Figure 5-1: Contact lens cases and solutions used in experiment

For OFR and BT lens cases, the right lens compartments were filled with 3mL of low concentration lysozyme solution (10µg total protein) and the left lens compartments were filled with 3mL of high concentration lysozyme solution (500µg total protein). For CC, three cases were filled with 9mL high concentration lysozyme CC solution (1500µg total protein) and three cases were filled with low concentration lysozyme CC solution (20µg total protein). This resulted in three replicates for each solution and lens case.

In addition, the PBS control solutions containing the respective amounts of lysozyme were filled in the original lens cases of the different care regimens using the solution quantities mentioned above (n=3 for each). PBS was further investigated using PP vials (3mL; n=3).

Table 5-2: Outline of experimental procedure

	OFR, BT, and PBS	CC
Volume of stock solution (mL)	0.833	5
Volume of test solution (mL)	9.167	25
Volume in lens case (mL)	3	9
Mass in lens case (μg)	L= 10 H= 500	L= 20 H= 1500
Initial concentration in lens case (mg/mL)	0.167	0.333
Volume removed from lens case (μL)	25	25
Dilution of sample (mL)	1.224	1.216
Final concentration in assay (mg/mL)	0.00333	0.00672
Final mass in assay (ng)	83	168

L=low lysozyme concentration, H=high lysozyme concentration

The cases were tightly closed and kept at room temperature in a Styrofoam container for the duration of the experiment. At each time point of 1, 12, 24, and 48 hrs, three samples of 25μL of each low concentration case compartment were taken to determine lysozyme activity and hence nine measurements were obtained for each solution-lens case combination and time point. For high lysozyme concentrations, each 25μL sample underwent a 50-fold dilution with the respective test solution prior to analysis.

A micrococcal turbidity assay [7,8,30] was performed on all samples, which consisted of 25μL aliquots in 300μL of bacteria. *Micrococcus lysodeikticus* (*M. lysodeikticus*) bacteria were prepared at 1mg/mL of 50 mM phosphate buffer (pH 6.3) and diluted to an optical density of 1.1 at 450nm. Readings were taken at 32°C with the SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) and lysozyme activity was then determined every 30 seconds over a period of 15 minutes. Standards with known mass were prepared at hr 0 and were used to convert the rates of lysozyme activity computed by the SoftMax Pro 5.4.1 software (Molecular Devices, Sunnyvale, CA) into mass of lysozyme present in each well. Negative control samples containing only the contact lens cleaning solutions without the addition of lysozyme were included to correct for background noise.

Statistica 9 (StatSoft Inc. Tulsa, OK) was used to analyze the data and repeated measures ANOVA (analysis of variance) was performed in order to compare the activity of lysozyme when placed in the

different solutions. Factors included in the ANOVA were: solution of interest, lysozyme concentration, and time point. Tukey's HSD (Honestly Significant Difference) test was used for post-hoc comparisons, where $p<0.05$ was considered significant.

5.4 RESULTS

The impact of different contact lens care solutions on lysozyme activity was investigated over a 48-hr time period. PBS was included as a control solution as it contained neither surfactants nor disinfectants.

In a recent in-house study, lysozyme activity was determined after being added to MPS, which was diluted with different amounts of PBS. A fixed amount of lysozyme was added to all dilutions. The results are shown in Figure 5-2.

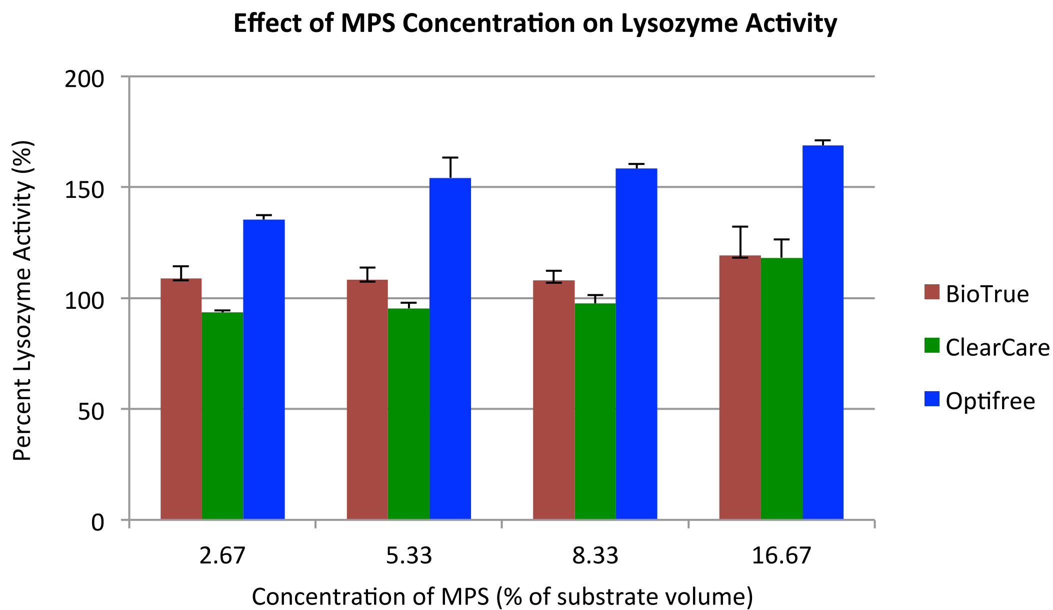

Figure 5-2: Effect of multi-purpose solution (MPS) concentration on lysozyme activity using a fixed amount of lysozyme. (Solutions were placed in polypropylene tubes).

It was confirmed that varying concentrations of OFR enhanced the activity of lysozyme to up to 170%; however, when a low concentration (2.67%) of MPS was used, the apparent increase of lysozyme activity was slightly less (135%). Regardless of the concentration of BT and CC, the solutions' effect on lysozyme was relatively similar throughout the experiment, with slightly increased activities when high concentrations of MPS were used. The concentration of care regimen present in the assay of the main experiment was 16.67%.

OPTI-FREE REPLENISH

The results of the two different lysozyme solutions placed in OFR lens cases are presented in Figure 5-3. All conditions (high and low concentration lysozyme dissolved in OFR and in PBS) showed an overall decrease in lysozyme activity from hr 1 to 48, where the difference in activity was significant for each of the conditions ($p<0.001$).

For OFR solution, there was a significant difference in lysozyme activity for high and low concentrations ($p<0.001$). After 1 hr of exposure to OFR solution, the activity of the high lysozyme concentration increased to 222±28%. After 12 hrs, lysozyme activity significantly decreased to 180±15% ($p<0.001$) and continued to decrease to 132±13% after 24 hrs ($p<0.001$), after which it remained relatively unchanged (126±10% at 48 hrs; $p=1.00$). When the low concentration of lysozyme was used, the activity of the protein was enhanced to 195±11% after 1 hr of exposure to OFR and reduced to 138±5% after 12 hrs ($p<0.001$). The following time points, 24 and 48 hrs, exhibited no significant change in activity ($p>0.05$).

For PBS, there was a significant difference in lysozyme activity for the high and low protein concentrations ($p<0.001$). High concentration of lysozyme in PBS was enhanced less strongly compared to OFR ($p<0.001$) and exhibited an activity of 128±18% after 1 hr. At hr 12, the activity dropped significantly to 62±9% ($p<0.001$). Lysozyme activity was further reduced after 24 hrs ($p<0.001$) and plateaued between the last two time points (34±8% at 48 hrs; $p=1.00$). When the low concentration of lysozyme in PBS was placed in OFR cases, the activity decreased to 61±7% after 1 hr and continued to reduce to 41±6% by 12 hrs ($p=0.004$). At 24 hrs, the activity halved to 19±3% ($p=0.003$), and then remained unchanged at 48 hrs ($p=1.00$).

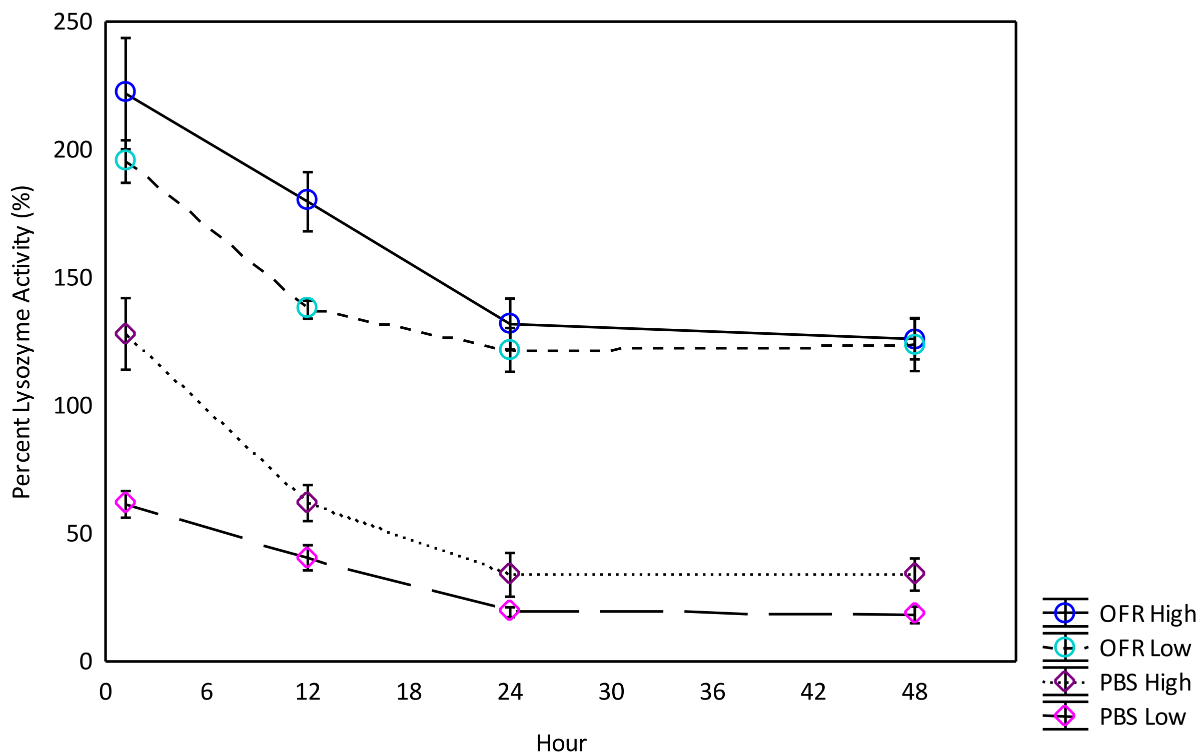

Figure 5-3: Effect of OPTI-FREE RepleniSH (OFR) and phosphate-buffered saline (PBS) on lysozyme activity using a high (500µg) and low (10µg) protein concentration in an OFR lens case. Time points: 1, 12, 24, and 48 hrs (n=3).

BIOTRUE

The effect of BT lens cases on lysozyme activity is presented in Figure 5-4. The high and low lysozyme concentrations in BT showed an overall significant increase in activity from hr 1 to 48 ($p<0.001$); however, the high and low concentrations of lysozyme in PBS did not indicate a significant change in activity between the first and final time point ($p=1.00$). Lysozyme activity was overall significantly greater when high concentrations of lysozyme were placed in BT lens cases, independent of solution used ($p<0.05$).

There was a significant difference in lysozyme activity for the high and low concentrations with the use of BT solution ($p=0.001$). At the 1-hr time point, the high lysozyme concentration showed an activity of 65±15% followed by a significant increase to 99±8% at 12 hrs ($p<0.001$). No further change was seen for the successive time points ($p=1.00$). For the low concentration of lysozyme, the initial activity was at 45±3% for the first time point, which was significantly less than the activity of the high concentration ($p=0.006$). As seen with high concentration lysozyme, the activity increased by 12 hrs to 86±8% ($p<0.001$). No further change was noted after 12 hrs ($p>0.05$).

After 1 hr, nearly 20% less activity was found in high concentrations of lysozyme placed in BT cases when immersed in PBS (46±14%) in comparison to BT solution (65±15%); $p=0.01$. No changes were seen between 12 and 24 hrs ($p>0.05$), followed by a significant decrease in activity at 48 hrs (42±4%; $p=0.001$). When the low concentration of lysozyme in PBS was placed in BT cases, the activity of lysozyme initially decreased to 36±4%, followed by an increase to 50±7% at hr 12 ($p<0.001$). At hr 24, there was an additional increase in lysozyme activity (59±5%; $p<0.001$). A significant reduction was seen between the last two time points, resulting in 35±4% activity after 48 hrs ($p<0.001$).

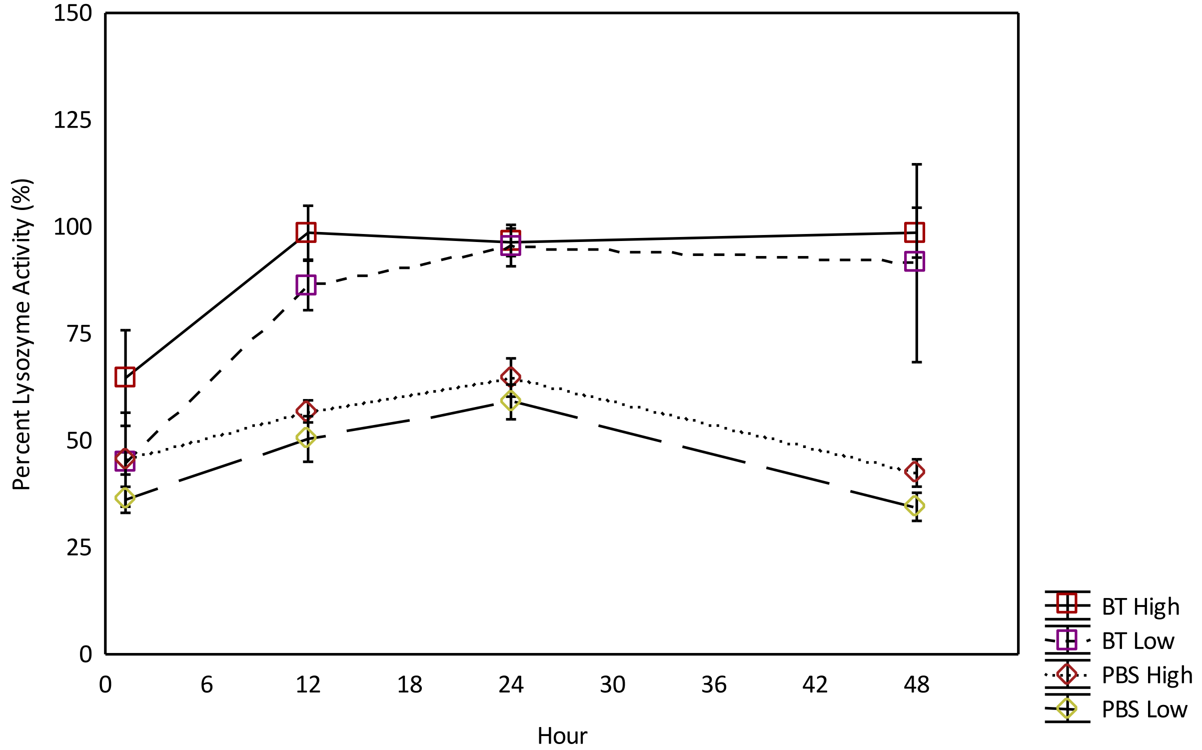

Figure 5-4: Effect of BioTrue (BT) and phosphate-buffered saline (PBS) on lysozyme activity using a high (500µg) and low (10µg) protein concentration in a BT lens case. Time points: 1, 12, 24, and 48 hrs (n=3).

CLEAR CARE

The effect of the CC neutralization cassettes on lysozyme activity is presented in Figure 5-5. All conditions (high and low concentration lysozyme) showed an overall increase in lysozyme activity from hr 1 to 48, where the overall difference in activity was significant for each of the conditions ($p<0.001$).

For the CC solution, there was a significant difference in lysozyme activity for the high and low concentrations ($p<0.001$). After 1 hr, the high concentration of lysozyme exhibited an activity of 32±2%, followed by a significant increase to 48±4% by hr 12 ($p<0.001$). The 24-hr time point did not show a

significant change in activity ($p=0.89$); however, there was a slight increase in activity by hr 48 (54±3%; $p<0.001$). For low concentration lysozyme, the initial 1-hr showed a low activity of 32±1%, followed by a slight increase to 44±2% by 12 hrs ($p<0.001$). The activity subtly plateaued between hrs 12 and 24 ($p=1.00$). After 48 hrs, the activity of low concentration lysozyme in CC increased drastically to 95±4% ($p<0.001$).

For protein exposed to PBS, there was an overall significant difference in lysozyme activity between the high or low concentrations ($p<0.001$). Similar to CC in neutralization cassettes, the high lysozyme concentration in PBS had a low activity of 24±4% at the 1-hr time point. The activity increased to 36±1% at 12 hrs ($p<0.001$), plateaued at hr 24 ($p=1.00$), and finally decreased significantly to 32±4% ($p<0.001$). When the low concentration of lysozyme in PBS was placed in CC neutralization cassettes, the activity of lysozyme decreased to 25±1%, increased to 33±2% by 12 hrs ($p<0.001$), and remained unchanged for hr 24 ($p=0.03$). A final increase to 48±2% was seen at the 48-hr time point ($p<0.001$).

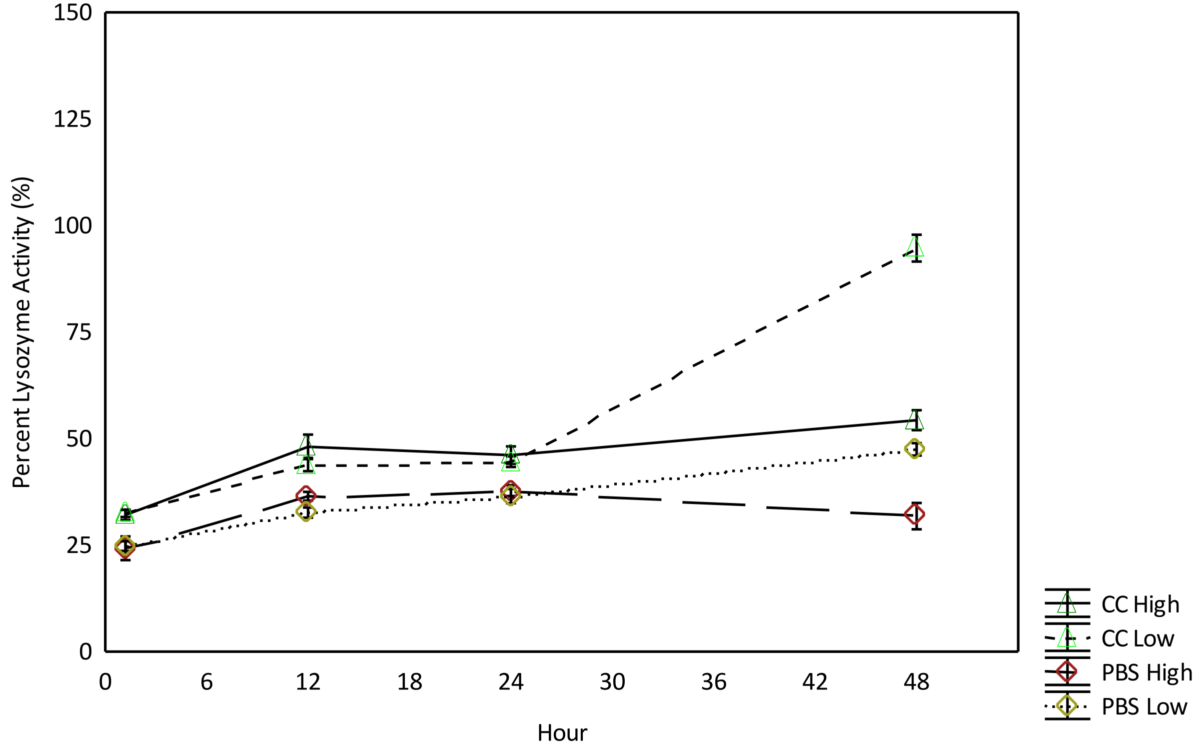

Figure 5-5: Effect of Clear Care (CC) and phosphate-buffered saline (PBS) on lysozyme activity using a high (1500µg) and low (20µg) protein concentration in a CC neutralization cassette. Time points: 1, 12, 24, and 48 hrs (n=3).

An interesting trend occurred for both lysozyme concentrations when both CC and PBS were used to determine lysozyme activity over time- a peak was seen at hr 48 ($p<0.001$). Commonly, there was a plateauing effect between the 12- and 24-hr time points ($p>0.05$).

PHOSPHATE-BUFFERED SALINE

Figure 5-6 represents the effect of lens cases on lysozyme activity when a high concentration of lysozyme was dissolved in PBS and placed in different cases. Similar to both BT cases and CC neutralization cassettes, lysozyme dissolved in PBS and placed in PP plastic vials had a low 1-hr lysozyme activity reading (40±3%). The activity increased to 53±10% at 12 hrs ($p=0.008$) and continued to increase to 66±4% by hr 24 ($p=0.03$). At hr 48, lysozyme activity dropped significantly to 20±2% ($p<0.001$). Comparing 1-hr time point activities, it was seen that lysozyme was most stable (128±18%) when placed in OFR lens cases ($p<0.001$). By the end of the 48-hr time period, lysozyme activity was similar when placed in OFR, BT, or CC cases ($p>0.05$).

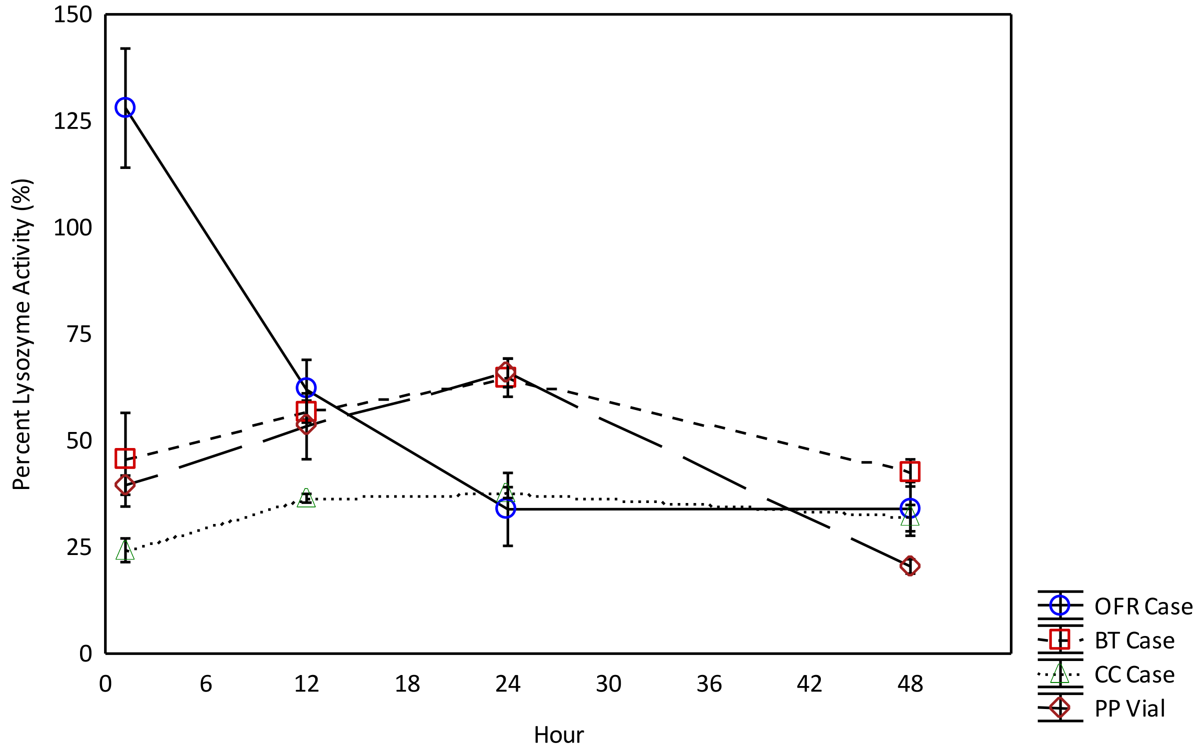

Figure 5-6: Effect of phosphate-buffered saline on lysozyme activity when high concentrations of lysozyme are placed in multi-purpose and polypropylene, control cases (500µg) and neutralization cassettes (1500µg). Time points: 1, 12, 24, and 48 hrs (n=3).

Overall, when a high concentration of lysozyme was placed in OFR cases, there was a significant decrease in activity between 1 and 48 hrs ($p<0.001$). For both BT cases and CC neutralization cassettes, hrs 1 and 48 showed no significant change in activity ($p>0.05$). Finally, when PBS was filled in PP vials, there was an ultimate decrease in lysozyme activity after 48 hrs ($p<0.001$).

Figure 5-7 depicts the effect of lens cases on lysozyme activity when a low concentration of lysozyme was dissolved in PBS and placed in different cases. Similar to BT cases, lysozyme dissolved in PBS that was placed in PP plastic vials had a lower 1-hr lysozyme activity reading compared to OFR (PP vial= 36±3%; OFR case= 61±7%). The activity increased to 54±5% at 12 hrs ($p<0.001$) and then significantly

decreased to 39±4% at hr 24 and 13±3% by hr 48 (p<0.001). At the 1-hr time point, lysozyme activity was highest when placed in OFR cases (61±7%; p<0.001); however, activity was lowest by hr 48 when placed in either OFR cases or PP vials compared to the other cases.

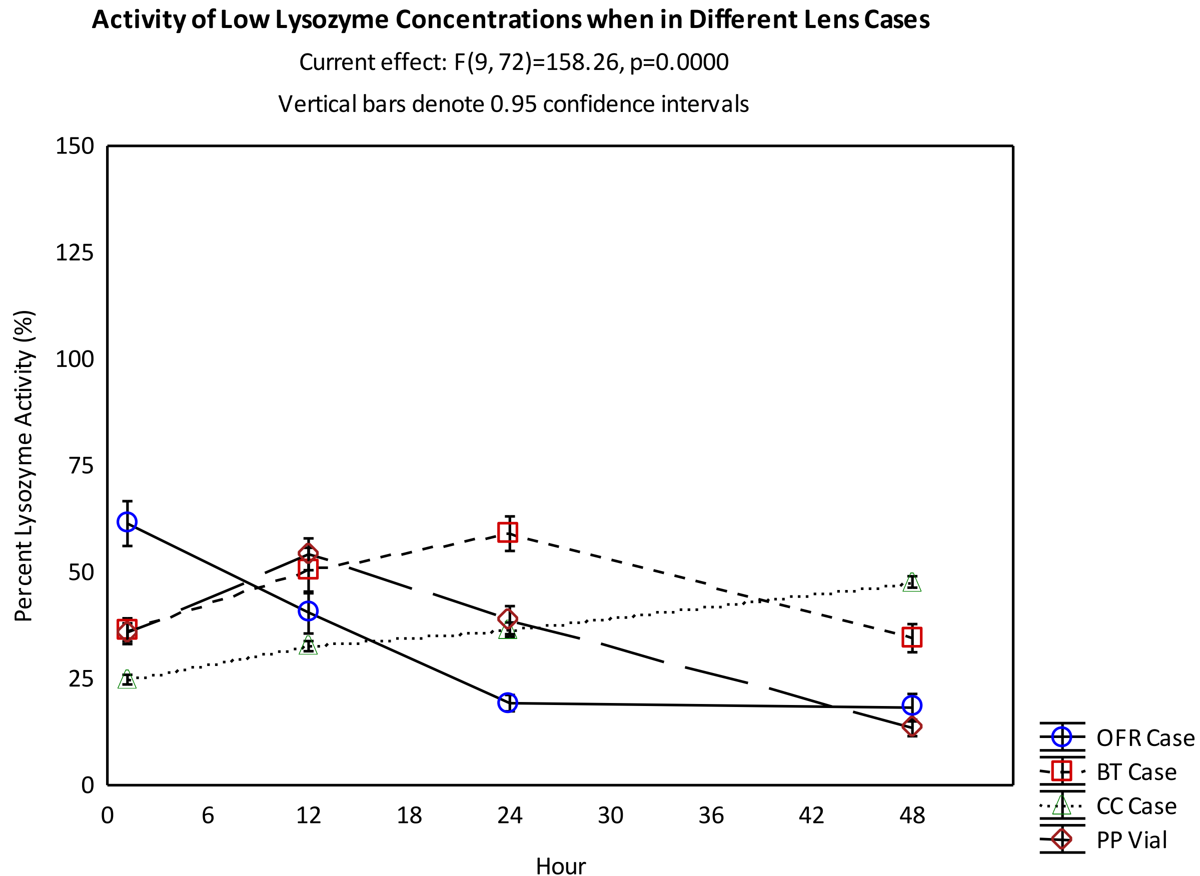

Figure 5-7: Effect of phosphate-buffered saline on lysozyme activity when low concentrations of lysozyme are placed in multi-purpose and polypropylene, control cases (10µg) and neutralization cassettes (20µg). Time points: 1, 12, 24, and 48 hrs (n=3).

A similar trend compared to high concentration lysozyme was found for low concentration lysozyme, where there was a significant decrease in activity between 1 and 48 hrs when PBS was placed in OFR lens cases (p<0.001). No significant change in lysozyme activity was noted between the first and final time points when PBS was placed in BT cases (p=1.00). When PBS was added to CC cassettes, there was

a significant increase in lysozyme activity over time (p<0.001). Lysozyme in PBS, when placed in PP vials, showed a significant decrease in activity between hrs 1 and 48 (p<0.001).

In summary, the activity of protein when either a high or low concentration of lysozyme was placed in PBS, specifically at the 12-hr time point, was similar between lens cases. For the high lysozyme concentration, during the first time point (1 hr), PBS in OFR lens cases exhibited a higher activity; however, a strong decrease in activity was seen as the final time point was reached. In contrast, the drop in low concentration lysozyme activity with OFR was steadier, reaching a similar activity level compared to the PP control vials at 48 hrs. Lysozyme placed in CC neutralization cassettes had the greatest activity by the end of the 48-hr time frame; however, OFR and BT exhibited a similar activity when the high lysozyme concentration was used.

5.5 DISCUSSION

Contact lens cleaning solutions have a number of functions, the major ones being to disinfect the lens after wear and to remove any tear film deposition components. One of the many proteins that may deposit onto the contact lens is lysozyme, a protein that carries antimicrobial properties [1]. Studies have demonstrated the efficacy of disinfection solutions against an array of microorganisms [31,32]. Interestingly, one of the biocides or disinfecting agents, PHMB, found in some cleaning solutions, including BT and CC, has been known to partly stick to glass [33] and to the manufacturers' bottles [31], which may cause differences in MPS' efficacy against microorganisms. The efficacy of a lens care solution is dependent on a number of factors, including pH, temperature, concentration of disinfectant, and the amount of organic matter present [34]. The antimicrobial efficacy is further dependent on the buffer system and surfactants [32]. The polyquad-based system in OFR has proven more effective in disinfecting than the polyhexanide-based MPS [35,36].

In this study, lysozyme activity was consistently over 100% throughout the 48 hrs of the experiment when exposed to OFR in the original lens cases, regardless of the lysozyme concentration. For high concentration lysozyme, the activity decreased from 222% to 180% and plateaued after 24 hrs. Certain

components in MPS seem to enhance the stabilization of lysozyme protein through synergistic actions [37] and hence, during this experiment, it appeared that there was an enhanced activity of lysozyme, where *M. lysodeikticus* bacteria was lysed at a faster than normal rate. For OFR lens cases, the rate of lysozyme activity was typically greater when a high concentration of lysozyme was used compared to a low lysozyme concentration. However, by the end of the 48-hr time period, the activity of lysozyme was similar for both concentrations in OFR solution. This would indicate that the ratio of active lysozyme to total lysozyme, as time progressed, was independent of the amount of lysozyme present in the OFR lens case. This would further confirm that the buffer system and the surfactants in the MPS play a critical role in maintaining antimicrobial activity of lysozyme [32]. We expect the activity of lysozyme to remain constant despite changing the concentration of lysozyme. Nevertheless, the results indicate that a lower concentration of lysozyme presents with lower activity levels. An explanation for this lies in the fact that lysozyme will adsorb onto the walls of the container, regardless of the amount present. When present in a higher concentration, the amount of enzyme that binds to the container does not significantly alter the activity. However, at a lower concentration, the amount present is sufficiently low that when it binds to the wall of the container, the amount of lysozyme present decreases enough for it to present as an apparent decrease in the activity level. Therefore, the decrease in activity is exaggerated due to a decrease in the amount present for the reaction and not entirely as a result of a decline in activity.

Lysozyme activity is enhanced not only in the presence of an MPS, but also in the presence of certain tear film components. In a study undertaken by Ellison and Giehl [38], it was shown that both lactoferrin and lysozyme protein exert an additive anti-microbial effect on bacteria [38], thereby potentially eliciting an enhanced activity of lysozyme when using a turbidity assay. If this current study had used an artificial tear solution (ATS) instead of PBS to dissolve the lysozyme, the resulting lysozyme activity would have been the summation of both the MPS and the lactoferrin-lysozyme synergy. Since this current study did not use lactoferrin in the turbidity assay, MPS was the only variable that could potentially enhance lysozyme activity. Similarly, a study conducted by Leitch and Willcox [39] confirmed that there is a synergistic relationship between lactoferrin and lysozyme against *Staphylococcus epidermidis* strains of bacteria [39]. Lysozyme has a direct antimicrobial effect on gram-negative bacteria through its adherence to the cell wall and hydrolysis of the N-acetylglucosamine linkages [40,41].

Lactoferrin makes the cell wall more susceptible to hydrolysis of bond linkages by lysozyme [38] due to its permeability and interference with the lipopolysaccharide layer [42].

When BT solution was placed in the respective BT lens case, the activity of lysozyme plateaued almost immediately; however, when PBS was placed in BT lens cases, the lysozyme activity decreased slightly over the 48 hours. After 1 hr, the activity of high concentration lysozyme and low concentration lysozyme in BT solution was 65% and 45%, respectively; both concentrations increased to a peak activity (99% and 86%, respectively) and did not change significantly after. This increase in activity began right after hr 1, which could be due to the stabilization of lysozyme as the time progressed. This could cause a renaturation of protein, allowing lysozyme to more rapidly lyse the bacteria during the turbidity assay.

A recent study undertaken by Barniak and colleagues determined the ability of MPS to prevent the chemical denaturation (using SDS) of lysozyme [43]. Two of the test solutions, BT and ReNu Fresh, presented with the ability to stabilize lysozyme after exposure to SDS. SDS is known to bind to lysozyme and change its conformational protein structure from a β-structure to an α-helix formation, as well as change its side chain residues [43,44]. OFR, OPTI-FREE *EXPRESS*, AQuify, COMPLETE MPS Easy Rub Formula, and PBS were among the test solutions that did not have a significant impact on the activity of lysozyme [43]. The component of BT, which preserves the active state of lysozyme, could be hyaluronic acid (HA), a glycosaminoglycan lubricant [45], which decreases denaturation of protein at the solid-liquid interface [46]. Certain surfactant ingredients in BT may play a pivotal role to keep protein in its active state including poloxamine surfactant, which reduces denaturation of hen egg lysozyme [47] and sulfobetaine 10, which aid in the refolding of lysozyme back into its active conformation [37] by binding to the protein without affecting its lysing function [48].

For CC solution, there was a slight increase in lysozyme activity throughout the 48-hr time frame; however, it exhibited a lower lysozyme activity compared to the other two MPS. An increase in activity was seen particularly for CC low lysozyme concentration at the 48-hr time point. Previous in-house work determined that a similar increase was found; however, this was at the 24-hr time point. A possible explanation for this increase may be that the protein is regaining activity in the neutral environment; however, more work is required to speculate what potentially can cause such a drastic increase. It

remained unclear from this experiment whether lysozyme deposition to the lens case, particularly CC neutralization cassettes, may have impacted the final outcome. For cases that attract more lysozyme, the total concentration in the solution may have been lower than expected, which would subsequently impact the activity readings. Future studies should therefore quantify the amount of lysozyme in the solution for each time point at which activity is determined. In addition to this, the amount of added lysozyme in the high concentration situation was not double the amount placed, it was three times greater thus, because there was an increase in the amount added, the activities may potentially be exaggerated for high lysozyme concentration readings and are not exactly comparable to OFR and BT findings.

CC is a hydrogen peroxide-based system, which denatures lysozyme and hence decreases the efficiency of active proteins, such as lysozyme [49]. Immediately after insertion of the contact lens basket into the 3% hydrogen peroxide solution, both an aggressive disinfection and a neutralization process occur simultaneously over a 6-hr time frame [50]. A platinum catalyst disc, attached to the lens basket, neutralizes the hydrogen peroxide solution [51]. This study demonstrated that of the three cleaning solutions used, CC was less effective in maintaining the activity of lysozyme compared to the other two MPS. Although hydrogen peroxide-based systems denature lysozyme, this may not present a critical problem to lens-wearers, as 3% hydrogen peroxide-based care solutions, such as AOSEPT, provide a high disinfection efficacy as shown by Hildebrandt [52] and Lowe [53]. The protein removal efficiency of CC is quite high [24] and thus the amount of protein remaining on the lens is quite low. Therefore, the increased denaturation of this very low amount of protein may be clinically irrelevant.

Proteins that deposit onto contact lenses typically undergo conformational changes and denature, which may cause inflammatory reactions, such as giant papillary conjunctivitis (GPC) [54,55]. A recent study by Wright et al. [37] investigated a new MPS solution- Bausch & Lomb's BioTrue without its two disinfectants, as they would interfere with the antimicrobial assessment of proteins. The solution, however, contained the buffer system and unique components found in BioTrue, such as hyaluronic acid [37]. It was demonstrated that contact lenses that are repeatedly exposed to this MPS allowed the proteins to stay in their active state [37]. Protein stabilizers, such as those found in MPS, may prevent

ocular responses caused by lens deposits by either preventing protein denaturation or reversing the process [37].

Future studies should determine the impact of MPS products on lysozyme activity when an ATS is used. Studies have previously shown that the organic load has a negative impact on contact lens disinfecting efficiency [56-58]. Interestingly, in the recent study by Hildebrandt and colleagues [52], the ATS utilized increased the efficacy of contact lens solutions against *Staphylococcus aureus,* particularly for OFR [52]. *In vitro* study designs should advance in order for a true mimic of *ex vivo* situations.

In summary, contact lens cleaning solutions varied in their effect on lysozyme activity, as OFR typically enhanced, BT stabilized, and CC decreased activity. This *in vitro* study confirmed not only that MPS had an effect on lysozyme activity, but also that the lens case in which solution was stored impacted activity over time.

6 GENERAL DISCUSSION AND CONCLUSION

The experiments conducted in this thesis investigated the effect of incubation solution complexity on protein deposition to silicone hydrogel (SH) contact lens materials and the subsequent removal efficiency using a multi-purpose solution (MPS). In addition to this, the activity of lysozyme was examined when placed in different contact lens cleaning solutions and lens cases. Furthermore, active and total lysozyme and total protein was determined for patient-worn SH lenses. This thesis provides a chapter-by-chapter series of studies performed and results are summarized in this section.

The third chapter of this thesis determined the effect of incubation solution on the deposition of the proteins albumin, lactoferrin, and lysozyme to three different SH contact lenses using a radioactive tracer. This study confirmed that the complexity of the incubation solution has a significant effect on the deposition profile of proteins to SH contact lenses. Greatest deposition of protein was seen on balafilcon A (BA) lenses, which accumulated significantly greater lysozyme when lenses were incubated in a complex artificial tear solution (ATS) compared to a simple salt solution containing only a single protein. The deposition of lysozyme to senofilcon A (SA) and lotrafilcon B (LB) lenses was less affected by the complexity of the incubation solution. LB and BA lenses that were incubated in the simple protein solution accumulated greater amounts of lactoferrin; however, SA lenses that were incubated in the ATS accumulated more lactoferrin. This may be because lactoferrin is larger in size than lysozyme and hence is outcompeted in the ATS by the much smaller lysozyme, which will accumulate in greater quantities when it is the only protein available in solution. SA lenses demonstrated that primarily non-competitive factors were affecting the deposition of albumin to these lenses. LB lenses accumulated greater albumin when lenses were incubated in the simple protein solution; however, BA lenses accumulated greatest albumin when incubated in the complex ATS. Again, albumin is not being out-competed by other proteins to bind onto LB lenses and, with regards to BA lenses, electronic repulsion may have caused less albumin to bind when no other proteins are available in solution, which may have resulted in a "sandwich phenomenon".

Chapter 4 investigated the effect of MPS on lysozyme removal after its deposition to various SH lenses, using either a simple protein solution or a complex ATS. When SA lenses were cleaned with OPTI-FREE RepleniSH (OFR), only small amounts of lysozyme remained on the cleaned lenses and no increase in deposition was seen throughout the 28-day time period, independent of the complexity of the incubation solution. Nearly 50% of the deposited lysozyme was removed when lenses were incubated in a simple solution and there was about a 90% removal efficiency when lenses were incubated in a complex ATS. When LB lenses incubated in a simple solution were cleaned, nearly half of the deposited protein was removed and resulted in a plateauing effect over time. However, the removal efficacy of OFR was increased by over 25% when cleaning LB lenses incubated in a complex ATS, concluding with a plateaued deposition pattern that was over two times greater than that of the simple protein solution. When BA lenses underwent 20 days of cleaning with OFR, the lenses incubated in either of the incubation solutions had the same amount of protein removed (approximately 70%). The plateau pattern that was witnessed for SA and LB lenses was not as evident for BA lenses, as there was a slight incline in the amount of lysozyme accumulated on the lenses as each time point was approached.

There was a significant change in deposition patterns seen for lysozyme deposition to SA, LB, and BA lenses when comparing Chapter 3 and Chapter 4. In Chapter 3, the deposition pattern of lysozyme for both SA and LB lenses was independent of incubation solution used; however, for Chapter 4, there was a significant increase in lysozyme deposition when both SA and LB lenses were incubated in a complex ATS. Although BA lenses accumulated significantly greater lysozyme when incubated in a complex ATS for both Chapters 3 and 4, there is double the amount of lysozyme deposited for Chapter 3. All of the variations seen between the two chapters could be attributed to a number of things. In the following discussion, I have grouped these into lens material, radiolabeling, and procedural factors.

Lens material-dependent variations seen in data would be due to a change in lot numbers, a change in the lens material chemical make-up, and a change in the blister-pack solution that houses the lens material. There was a short timeframe between conducting the two experiments and since then, there has not been any change in chemical make-up or blister-pack solution to either of the lens materials investigated. So, the lot number is essentially the only factor that could potentially affect deposition profiles. Even then, this would only permit a marginal change in the amount of protein accumulated

onto a lens material when different lot numbers are used. Therefore, this factor is unlikely to be a major contributor to the variation in results seen.

With regards to the radiolabeling technique, the iodine monochloride (ICL) method is repeatable and it is not expected that the procedure itself would cause significant variations in deposition profiles for lens materials. However, there may be differences in the amount of free iodine and the specific activity of the radiolabeled protein between the batches of radiolabeling protein used in the two chapters. There is a target percentage (<2%) of free iodine when performing the ICL method and this target value was never surpassed for either of the two experiments; however, the free iodine was not checked on a 28-day time course, so there may be alterations in the amount of free iodine available as time progressed for either of the Chapters. These differences could potentially alter deposition profiles for lenses; however, theoretically, there should be a uniform change to all materials. Free iodine may potentially cause false positive readings, but those readings would be expected for all lens types and so it is unclear as to why Chapter 4 had increased deposition values for SA and LB lenses, and decreased values for BA lenses. Further investigation is needed to understand whether or not the free iodine is the cause for these changes and hence future studies should measure free iodine in parallel with protein deposition measurements and determine whether there is a consistent pattern in the amount of free iodine present in solution. Dialysis was not used for either experiment and so future studies should incorporate dialysis into the methodology in order to limit the amount of free iodine in solution. Sufficient controls were used for both Chapters and the differences in specific activities were accounted for during the calculations to determine the amount of hot radiolabeled protein needed for each experiment.

Variations in experimental procedures could also account for the differences in deposition profiles for both Chapters 3 and 4. For both experiments, the container in which lenses were incubated was always pre-treated and exposed to the various macromolecules that may potentially bind to the container wall. For Chapter 3, 6 mL glass vials were used and pre-treated; however, due to the increase in lens number and steps involved for Chapter 4, larger containers (Qorpak bottles) were used. These bottles were also pre-treated, and in addition the histology cassettes in which lenses were placed were also pre-treated to minimize adsorption to the container wall. The pre-treatment procedure was consistent and hence it is not expected for this to be a major factor in the lack of repeatability between the two experiments.

Also, there was a variation between Chapter 3 and 4's method of rinsing lenses to remove loosely bound protein. For Chapter 4, the lenses were never removed from the histology cassettes when undergoing rinsing cycles, and so the cassettes themselves were being rinsed with the lenses inside. This was carried throughout the experiment for each of the lens types and so if this change in rinsing mechanism had an effect on deposition profiles, it would be expected that all lenses acted uniformly. It is clear that Chapter 3 and Chapter 4 had variations in lens material lot numbers, radiolabeling batches used, and experimental procedures; however, these variations were negated as best as possible to limit any sort of significant changes in deposition profiles.

The final chapter of this thesis (Chapter 5) explored the effect of contact lens cleaning solutions and respective lens cases on lysozyme activity by using OFR, BioTrue (BT), Clear Care (CC), and phosphate-buffered saline (PBS) as test solutions. To mimic typical amounts of lysozyme depositing to hydrogel and SH lenses, high and low lysozyme concentrations were dissolved in the solutions. For OFR and BT, 500µg/lens and 10µg/lens were added for high and low concentrations respectively, whereas for CC 1500µg and 20µg were used to represent two lenses stored in the same container. For OFR, there was greater lysozyme activity when the high protein concentration was used. Both concentrations of lysozyme demonstrated over 100% activity throughout the 48-hour time frame, which decreased slightly over time; however, after 24 hrs, there was no further change in activity for either lysozyme concentration. Similar to OFR, there was greater protein activity for high versus low concentrations of lysozyme that was dissolved in BT. After an initial increase in activity by 6 hrs, the activity remained relatively stagnant when lysozyme was dissolved as two separate concentrations. For the test solution, CC, there was an increase in lysozyme activity for low lysozyme concentration after both 6 and 48 hours; however, this was not seen with high concentrations as a plateauing effect was noted between the 12- and 24-hr time points. It was evident that OFR enhanced lysozyme activity, whereas CC reduced the activity and BT had no major effect throughout the experiment. When PBS was placed in different contact lens cases, the activity of the high concentration lysozyme was more consistent amongst time points than low concentration lysozyme activity. For both lysozyme concentrations, there was greater activity when lysozyme in PBS was placed in OFR lens cases for 1 hr compared to all other lens cases; however, by the end of the 48-hr time period there was slightly greater activity seen in BT lens cases when using high concentration lysozyme and CC neutralization cassettes when using low concentration

lysozyme. The OFR and BT lens case both contain a polypropylene base; however, the coating on the case may be specific to each manufacturer. No details are available on specific material make-up and so it is possible to speculate that the coatings on each lens case may potentially impact lysozyme activity. For the CC neutralization cassettes, there is no empirical evidence on whether or not the platinum disc may or may not have an effect on lysozyme activity. Overall, this study not only proved that contact lens solutions differ in their impact on lysozyme activity, but also confirmed that lens cases have an impact on protein activity.

To conclude, the complexity of incubation solutions has a direct impact on the deposition of tear film proteins to SH contact lens materials. The importance of using an artificial model that better mimics the human tear film is emphasized. Furthermore, differences were found in the cleaning efficiency with certain MPS, which likewise was related to the complexity of the incubation solution. Future studies should consider the complexity of the human tear film when designing *in vitro* studies in order to better replicate the ocular environment.

7 FUTURE STUDIES

A number of future studies could be performed in continuation of the results developed from this thesis. This thesis conducted a number of *in vitro* studies to investigate the effect of incubation solution complexity on protein deposition to silicone hydrogel (SH) contact lens materials, as well as determined the effect of a multi-purpose solution (MPS) on protein removal from lenses incubated in different solutions. The ability of different MPS to stabilize lysozyme protein was also investigated and further it determined total active lysozyme as well as total lysozyme and protein on worn lens materials. The overall purpose of future studies would be to enhance current understanding of the tear film interaction with contact lens materials, such as protein and lipid deposition, and to limit such by developing more advanced contact lens materials.

One future study may include the true mimic of the human tear film when investigating the deposition of proteins onto the contact lens materials. A substantial number of studies have evaluated the deposition of proteins to lenses; however, there have been several factors left out that could significantly affect the deposition pattern of proteins, such as the effect of blinking, surface drying, cleansing cycles, and physiological processes. *In vitro* experiments complement *ex vivo* data as individual components of the tear film can be investigated alone, in addition to a controlled setting with other tear film components. In vivo conditions are very complex and current *in vitro* studies typically lack exposure of lenses to an air-water interface, in order to mimic the effect of blinking, where the contact lens is exposed to air, resulting in an intermediate, hydrophobic state for the lens surface. Recently, “model blink cells” have been engineered to imitate the effect of blinking and to determine whether there is a significant effect on the deposition of macromolecules, such as proteins and lipids. During each “blink”, the contact lens surface is rewetted and there is a constant replenishing of artificial tear solution (ATS), which is typically lacking from *in vitro* studies. Replenishment of the incubation solution is often maintained on a daily or weekly basis (Chapter 4). As seen in Chapter 4 of this thesis, the use of cleaning solutions was maintained on a frequent basis. Future studies should attempt to clean lenses like a patient would, every day. Overall, the development of a model that could mimic all factors affecting the deposition of macromolecules to lens materials would be valuable.

This thesis confirmed that the complexity of the tear film should be maintained in future *in vitro* studies. *Ex vivo* studies could further be evaluated for the concentration of proteins and lipids that are currently not in ATS incubation solutions. Typically, only common proteins such as albumin, lactoferrin, and lysozyme, are added in the incubation solutions, in addition to a few other components such as IgG and mucins. However, the human tear film consists of more than 100 proteins [1] and over 40 lipids [2-4], of which most are typically lacking when using *in vitro* tear film models. In order to improve in these models, a more comprehensive number of proteins and lipids need to be included to truly mimic the effect of competitive binding of tear film components. Future *in vitro* models should consider findings of previous studies evaluating the concentration of specific proteins, lipids, and mucins found in the human tear film.

This thesis investigated the protein removal efficiency of MPSs (Chapter 4) and the effect of MPSs on lysozyme activity (Chapter 5). Chapter 4 determined the efficiency of protein removal by OPTI-FREE RepleniSH from SH contact lenses. Lysozyme is typically found in one of two forms, either active or denatured, as once the protein adsorbs onto the hydrogel contact lens material, it may undergo conformational changes [5,6]. Previous studies have shown that denatured protein binds more strongly to surfaces and is therefore less likely to be removed from hydrogel biomaterials [7,8]. The current experiment did not classify if primarily active or denatured lysozyme was removed during the cleaning cycle and should therefore be the focus for future studies. Chapter 5 determined that lens cases have varying effects on lysozyme activity when a control solution (phosphate-buffered saline (PBS)) is used. This chapter used a PBS control with each lens case and also investigated each MPS when used with its respective lens case. Future studies should use a single lens case as a variable, which would then be cycled through different MPSs and determine whether a certain MPS-lens case combination optimizes or decreases lysozyme activity.

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CHAPTER 1 REFERENCES

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APPENDICES

Appendix A

Ex Vivo Deposition of Proteins to Silicone Hydrogel Contact Lenses

OVERVIEW

Purpose: The purpose of this *ex vivo* study was to determine total protein and lysozyme deposition onto silicone hydrogel contact lenses.

Methods: Two silicone hydrogel (SH) materials - senofilcon A (SA) and lotrafilcon B (LB) - were investigated in this study. SA lenses were worn for two weeks and cleaned with either Clear Care or OPTI-FREE RepleniSH and LB lenses were worn for one month cleaned with Clear Care (n=20 per lens type). Total protein, total lysozyme, and total active lysozyme were determined using a Bradford assay, enzyme-linked immunosorbent assay (ELISA), and micrococcal assay, respectively. The effect of freezing on lysozyme activity was further investigated.

Results: Statistically significantly more protein deposited on LB lenses (9.23±1.60µg) than SA lenses (8.23±1.23µg); p=0.03. There was no significant difference between the amounts of total protein deposited on SA lenses cleaned with Clear Care or OPTI-FREE RepleniSH (p>0.05). The amount of lysozyme depositing on SA lenses was however higher compared to LB lenses (0.025±0.013 µg vs. 0.012±0.009 µg); p<0.001, while similar amounts of active lysozyme were recovered from both lens types. A decrease in active lysozyme of 27% (SA) and 54% (LB) was found when extracted protein samples were kept frozen for 3 months instead of 1 month.

Conclusion: This study confirmed that there is a difference in deposition patterns between silicone hydrogel contact lenses, even those within the same FDA group. However, these changes are very small and their likelihood of being clinically significant are very low.

Key Words: activity, lysozyme, protein deposition, silicone hydrogel contact lenses

INTRODUCTION

Over the years, the use of contact lenses for vision correction has become a popular choice, as they are convenient and more cosmetically appealing. A study conducted in 2009 reported that silicone hydrogel (SH) lenses were fit to 60% of all contact lens-wearers compared to 29% of lens fits with conventional poly(2-hydroxyethyl methacrylate) (pHEMA)-based lenses [1]. In a more recent survey conducted in 2010, the percentage of SH wearers increased to 66% and the number of conventional lens-wearers decreased to 25% [1]. This is in agreement with the market in Canada and the US, which reported that SH lenses currently account for about 70% of daily-wear lens fits [2]. With regards to replacement schedules, contact lenses that are replaced monthly are the most popular, accounted for 46% of contact lens fits and refits in 2011 [1]. Bi-weekly replaced lenses accounted for 33% [1].

SH lenses have a higher oxygen permeability compared to pHEMA-based lenses, due to the incorporation of siloxane groups [3,4]. However, the hydrophobic nature of silicone requires modifications of the contact lens surface to improve wettability of the lens material [5] and hence a number of different surface treatments have been developed [6,7]. For example, lotrafilcon A (Focus Night & Day) has a plasma coating to increase wettability, whereas balafilcon A (PureVision) undergoes a plasma oxidation process [8].

During contact lens wear, various tear film components such as proteins and lipids deposit on the lenses, which may result in reduced visual acuity [9], poor wettability [10], and reduced comfort [11]. These deposits may act as antigens [12] and hence cause ocular complications such as acute red eye [13] and inflammatory reactions [14], which may lead to giant papillary conjunctivitis [15]. The lens rubbing against the upper tarsal conjunctiva may further cause mechanical trauma with a subsequent increase of inflammatory markers in the tear film [12,16]. And finally, a protein coating on certain lens types can impact subsequent binding of bacterial organisms such as gram-negative *Pseudomonas aeruginosa*, which may increase the risk of developing microbial keratitis [17].

Contact lenses require a care regimen in order to disinfect the worn lens and to remove exogenous and tear film deposits [18-20]. A study conducted in 2011 reported that practitioners primarily recommend

multi-purpose solutions (MPS) (77%) to their patients, with hydrogen peroxide systems accounting for the remainder of the market (23%) [1]. The composition of MPSs is typically a complex combination of anti-microbial agents [21], surfactants, and buffer systems and although an important task is to remove tear film components from contact lenses, the removal efficiency is typically less than 50% [22,23].

The purpose of this *ex vivo* study was to determine the total amount of protein and percentage of lysozyme activity on worn lenses. Senofilcon A and lotrafilcon B lenses were investigated, which were regularly cleaned using a MPS or hydrogen peroxide-based system.

METHODS

Two SH contact lens materials were investigated in this study, senofilcon A (SA, ACUVUE OASYS, Johnson & Johnson) and lotrafilcon B (LB, Air Optix Aqua, CIBA VISION). Both lenses have been categorized in Group 1 under the Food and Drug Administration (FDA) (<50% water content, non-ionic).

In this study, SA lenses were cleaned with either OPTI-FREE RepleniSH or Clear Care, whereas LB lenses were only cleaned with Clear Care. Table 6-1 outlines the components of the two lens-care products.

Table 1: Composition of lens-care products used in the study

	Clear Care	OPTI-FREE RepleniSH
Disinfectant	Hydrogen peroxide (3%)	ALDOX (0.0005%) PolyQuad (0.001%)
Components	Sodium chloride (0.79%) Phosphonic acid Pluronic 17R4	TearGlyde- Tetronic 1304 and C9-ED3A
Buffer	Phosphate	Sodium citrate

SA lenses (n=20) and LB lenses (n=20, including 13 spherical and 7 multifocal designs) were collected from study participants after being worn for two or four weeks respectively. The lenses were collected ad hoc from participants involved in one of two ongoing studies at the Centre for Contact Lens Research (CCLR) and from volunteers at the School of Optometry and Vision Science. Details of the subjects are

described in Table 6-2. Lenses were removed by the participant at the end of the wearing period and placed in empty plastic vials (6mL, VWR, Mississauga, ON).

Table 2: Study participant details

	Age	Rx		Cleaning Solution
Senofilcon A	19	OD: -4.25	OS: -4.25	Clear Care
lens-wearers	19	OD: +4.75	OS: -1.50	Clear Care
	20	OD: -1.00	OS: -1.50	OPTI-FREE RepleniSH
	20	OD: -4.75	OS: -4.50	Clear Care
	21	OD: -4.50	OS: -3.75	Clear Care
	22	OD: -3.25	OS: -3.00	Clear Care
	22	OD: -3.25	OS: -3.25	OPTI-FREE RepleniSH
	23	OD: -3.50	OS: -4.25	Clear Care
	23	OD: -6.50	OS: -7.50	OPTI-FREE RepleniSH
	25	OD: -2.25	OS: -3.00	OPTI-FREE RepleniSH
	25	OD: -2.50	OS: -2.50	OPTI-FREE RepleniSH
	25	OD: -4.75	OS: -4.25	Clear Care
	27	OD: -1.25	OS: -1.50	Clear Care
	27	OD: -4.25	OS: -5.75	Clear Care
	28	OD: -5.50	OS: -5.00	Clear Care
	32	OD: -2.25	OS: -2.00	Clear Care
	36	OD: -3.00	OS: -2.75	OPTI-FREE RepleniSH
	40	OD: -2.50	OS: 2.75	OPTI-FREE RepleniSH
	49	OD: -3.50	OS: -3.50	OPTI-FREE RepleniSH
	50	OD: -3.75	OS: -3.75	OPTI-FREE RepleniSH
Lotrafilcon B	8	OD: -1.25	OS: -1.50	Clear Care
lens-wearers	10	OD: -1.50	OS: -1.75	Clear Care
	10	OD: -1.75M	OS: -1.50M	Clear Care
	10	OD: -2.25M	OS: -3.75M	Clear Care
	10	OD: -3.00M	OS: -3.00M	Clear Care
	10	OD: -3.25	OS: -3.25	Clear Care
	10	OD: -3.25M	OS: -3.25M	Clear Care
	14	OD: -1.50M	OS: -2.00M	Clear Care
	15	OD: -0.75M	OS: -0.75M	Clear Care
	15	OD: -1.25	OS: -1.75	Clear Care
	15	OD: -2.00	OS: -3.00	Clear Care
	16	OD: -2.25	OS: -1.75	Clear Care
	16	OD: -3.50	OS: -3.50	Clear Care
	16	OD: -5.50	OS: -4.25	Clear Care
	18	OD: -1.75	OS: -1.50	Clear Care
	22	OD: -2.50	OS: -2.25	Clear Care
	23	OD: -3.00	OS: -3.00	Clear Care
	24	OD: -3.50	OS: -3.50	Clear Care
	24	OD: -6.50	OS: -7.25	Clear Care
	27	OD: -2.25	OS: -2.25	Clear Care
	27	OD: -2.50	OS: -2.25	Clear Care
	27	OD: -5.00	OS: -4.00	Clear Care
	49	OD: -5.25M	OS: -3.50M	Clear Care

M= multifocal design

Two different solutions were used to extract the proteins from the lenses: Solution A containing acetonitrile/0.02% trifluoroacetic acid was used for SA lenses, and Solution B, containing acetonitrile/0.2% trifluoroacetic acid was used for LB lenses. The lower concentration is used for SA lenses as these lenses disintegrate when 0.2% solution is used (in-house data). This does not occur for LB lenses. Previous work has shown that both extraction methods are suitable to ensure adequate extraction of the deposited protein [24,25]. Details are seen in Table 6-3.

Table 3: Composition of the extraction solutions

Components	Solution A	Solution B
CH_3CN^*	250 mL	250 mL
$CF_3CO_2H^+$	50 µL	500 µL
H_2O	250 mL	249.5 mL

CH_3CN (acetonitrile), CF_3CO_2H (trifluoroacetic acid), H_2O (Milli-Q gradient)
[*] EMD Chemicals Inc., Gibbstown, NJ, [+] Caledon Laboratories LTD., Georgetown, ON

Both solutions were prepared prior to use. Lenses were placed individually in 1.5mL of the respective extraction solution and kept in the dark at room temperature for 24±1 hours. Aliquots of 650µL, 275µL, and 375µL were taken to measure total protein, total lysozyme, and total active lysozyme, respectively. All aliquots were dried down using the Savant SpeedVac apparatus (Halbrook, NY) and the protein pellets were stored at -80°C.

Measurement of Total Protein

A Bradford assay was performed to determine the amount of total protein deposited on each lens (µg), as previously described [26,27].

Measurement of Total Lysozyme

An enzyme-linked immunosorbent assay (ELISA) was used to determine the total amount of lysozyme deposited on each lens. The protocol outlined on the ELISA kit (CALBIOTECH, San Diego, FL) was followed, as described below.

Standards, controls, and samples were pipetted (25µL) into a 96-well plate and 100µL of anti-lysozyme enzyme conjugate solution was added to all wells. The plate was incubated and placed on a rotatory shaker (VWR, Mississauga, ON) at 10 rpm for 60 min. After the incubation period, the wells were emptied and washed 3x with 300µL of 1x wash buffer. The plate was blotted on absorbent paper and 100µL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added to all wells. The plate was once again incubated for 15 min at room temperature. Finally, 50µL of stop solution was added to all wells and the plate was gently mixed for 15-20 sec. Samples were read immediately after in the SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) at 450 nm.

Measurement of Total Active Lysozyme

Lysozyme activity of the lens extracts was determined using a micrococcal assay, as previously described [28-30]. *Micrococcus lysodeikticus* bacteria were prepared at a concentration of 1 mg per 1 mL of 50 mM phosphate buffer (pH 6.3) and then further diluted with this buffer to an optical density of about 1.1 at 450 nm using the Multiskan Spectrum ELISA Plate Reader (Thermo Labsystems). 10 µL of tear dilution buffer (pH 8.0, containing Tris, 0.9% NaCl, and EDTA) was added to the lens extracts and the activity of lysozyme was determined over 4 min reads at 30°C.

The percentage of active lysozyme deposited on the lens was calculated (total active lysozyme ÷ total lysozyme x 100%).

RESULTS

Total Protein Deposition

All LB lenses were cleaned with Clear Care. There was no significance difference between the amounts of protein deposited on single vision LB spherical compared to multifocal LB lenses ($p>0.05$). For this reason data from both LB lens types were combined. Likewise, similar amounts of protein were detected on SA lenses, independent of whether they were cleaned with OPTI-FREE RepleniSH or Clear Care

(p>0.05), which also permitted combining both groups. Statistically, significantly more protein deposited on LB lenses compared to SA lenses (9.23±1.60 vs. 8.23±1.23µg/lens; p=0.03), as seen in Figure 6-1.

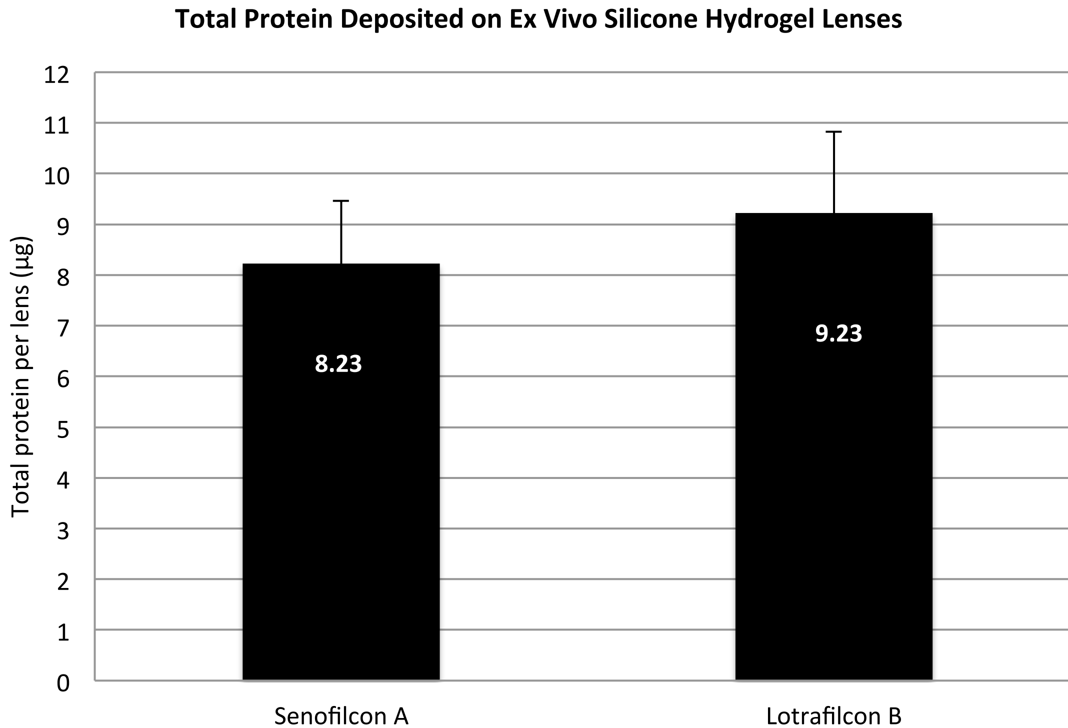

Figure 1: Total amount of protein (µg) deposited on senofilcon A and lotrafilcon B lenses

Total Lysozyme Deposition

Greater amounts of lysozyme accumulated on SA lenses compared to LB lenses (0.025 ± 0.013 vs. 0.012 ± 0.009 µg/lens). When investigating the impact of the different care regimens used by SA lens-wearers, there was no significant difference between the amounts of total lysozyme deposited on the lenses.

Total Active Lysozyme Deposition

The amount of active lysozyme deposited on SA and LB was similar for lenses cleaned with Clear Care solution (0.035 ± 0.025 vs. 0.055 ± 0.042 µg/lens). Interestingly, there was significantly more active

lysozyme on SA lenses that were cleaned with OPTI-FREE RepleniSH than those cleaned with Clear Care (0.075 µg vs. 0.035 µg/lens).

Freezing Effect

The different aliquots taken from the lens extraction were stored at -80°C for analysis at a later date, with an exception for total protein, which was analysed immediately. The effect of freezing on total lysozyme and total active lysozyme was determined at 1 and 3 months, as total lysozyme and total active lysozyme were frozen between 2 and 3 months.

Freezing had a significant impact on lysozyme activity. For SA lenses, the amount of active lysozyme determined after one month was 4.68µg, which decreased by 27% to 3.40µg after three months. The decrease in lysozyme activity was even more apparent for LB lenses (54%), with 1.55µg and 0.72µg being detected after one month and three months respectively.

DISCUSSION

One major drawback of this *ex vivo* study is that the patient populations for the two lens materials were different, as these lenses were collected opportunistically from two ongoing studies in the CCLR. The results must be considered in this context.

Previous studies have determined that the amount of total protein depositing on worn LB lenses is between 6.6 µg and 12.1 µg [31-33], which is similar to our current findings of 9.23 µg. The amount of total protein detected on worn SA lenses is typically slightly lower compared to LB lenses, estimating between 6.6 µg and 8.3 µg per lens [28,34,35]. This is again in agreement with our data of 8.23 µg. In order to improve the wettability of these materials, SA lenses contain the wetting agent polyvinyl pyrrolidone (PVP), whereas LB lenses undergo a surface modification to create a hydrophilic plasma coating. This may account for some of the differences in deposition between the materials. While the

results were statistically significant, the clinical significance of such a small difference is very dubious and it is unlikely that such a small difference is truly that relevant.

Lysozyme is one of the most prevalent tear film proteins, accounting for approximately 1.9 mg/mL [36] and numerous studies have shown that lysozyme deposition can be found on all types of contact lenses. Worn senofilcon A lenses accumulate between 0.9 µg [31] and 1.6 µg [28,34] of lysozyme, which is similar to the amount determined for lotrafilcon B lenses, which report between 0.3 µg [28] and 1.4 µg [31] per lens. The current study data suggest a lower lysozyme content of 0.025 µg and 0.012 for SA and LB lenses respectively; however, these data are incomparable to previous studies. Likewise, the amount of active lysozyme was found to be exceptionally low; 0.035 µg and 0.075 µg for SA lenses cleaned with Clear Care and OPTI-FREE RepleniSH, respectively, and 0.055 µg for LB lenses cleaned with Clear Care. Because the contact lens samples were frozen after collection, protein underwent conformational changes, thereby decreasing the mass of detected lysozyme when using an ELISA method. The detected mass of total lysozyme and active lysozyme for both SA and LB lenses was significantly less than that of previous studies, as samples were exposed to a thermally stressful environment. Even though direct comparison to previous studies cannot be made due to a change in environment for the protein, the exposure of lysozyme to thermal stress provides researchers with an understanding of what effects temperature have on protein.

Karlsson and colleagues established that the Western Blotting (WB) technique is more sensitive than the ELISA in quantifying total lysozyme [37]. This study determined total lysozyme deposited on lenses using the ELISA method. WB involves a fundamental step- electrophoresis. This process denatures and linearizes proteins thereby allowing inactive lysozyme to be detected. The detection range for the WB is within nanograms; however, the ELISA is capable of ascertaining levels among the picogram range. Albeit lacking sensitivity, ELISA is less time consuming and significantly more efficient considering it can read 96 samples at one time. The sensitivity of the lysozyme ELISA is 0.021 ng/mL; however, denatured protein is not detected because protein is not linearized during the procedure.

The impact of contact lens cleaning solutions on protein deposition and lysozyme activity was further investigated in this study. A minor effect of the care regimen was seen on lysozyme confirmation

extracted from SA materials, indicating slightly higher amounts of active lysozyme on lenses that were cleaned with Opti-FREE RepleniSH compared to Clear Care [28]. In a previous study, Zhao and colleagues found that after 30 days of LB lens wear, lenses accumulated less protein when cleaned with Clear Care (0.5µg) compared to Opti-FREE RepleniSH (1.7µg) [18]. The authors further report a similar pattern for SA lenses that were worn for two weeks, suggesting that Clear Care is more efficient in removing protein from the lenses than Opti-FREE RepleniSH [18]. Furthermore, an *in vitro* study undertaken by Luensmann and colleagues, found that 7.2% of lysozyme deposited on LB lenses could be removed by Clear Care, compared to <4% when using a MPS system [22].

The decrease in active lysozyme has been confirmed in an in-house study determining the effect of freezing on total lysozyme deposited on SA and balafilcon A (BA) lenses. It was shown that the activity of lysozyme decreased within a week of storage in a freezer. Tothova and colleagues, in a recent study, determined that there is an effect of freezing on serum amyloid A (SAA) samples [38]. SAA are a part of the apolipoprotein family and are associated with the high-density lipoproteins of the blood plasma. The researchers presented a trend showing a decrease in SAA concentrations over-time, with a significant decrease just after 2 days of storage; the initial concentration of SAA is 30.30 µg/mL and by the end of 3 weeks, there was just 13.94 µg/mL, reflecting a >50% drop in concentration [38]. There is a clear effect on proteins when they are stored in the freezer for long periods of time, which may be due to protein degradation [38]. Lysozyme, on the contrary, is an enzyme and does not present with a similar structure as SAA; however, a trend in denaturation is proven from this experiment. A way to protect the lysozyme from denaturation is by adding stabilizing agents, such as BioStab™, before the lysozyme aliquots are dried down and further frozen for storage. Work to further examine this would be worthwhile.

In conclusion, this study confirms that LB lenses accumulated greater amounts of protein compared than SA lenses, but these differences were likely clinically irrelevant. SA lenses accumulated similar amounts of total protein independent of whether lenses were cleaned with OPTI-FREE RepleniSH or Clear Care. Freezing the extracted protein samples up to 3 months had a significant effect on the amount of active lysozyme detected, with significantly lower amounts found with longer freezing periods. This highlights the importance of immediate data collection or the potential use of stabilizing reagents, to protect proteins against thermal stress, which causes denaturation.

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Appendix B

Permissions

Dear Salsabeel,

Congratulations on nearing the completion of your degree requirement. Permission is granted for republication as outlined in your message below. Please cite the paper with complete bibliographic information.

Best regards,

Jeff Boatright

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Jeffrey H. Boatright, PhD, FARVO
Associate Professor of Ophthalmology
Emory University School of Medicine

-------- Original Message --------

Subject: Permission to include MolVis Publication in MSc Thesis
Date: Wed, 4 Jul 2012 23:26:20 +0000
From: Salsabeel Jadi <sjadi@UWATERLOO.CA>
Reply-To: Salsabeel Jadi <sjadi@UWATERLOO.CA>
To: <MV-OFFICE@LISTSERV.CC.EMORY.EDU>

Good evening,

My name is Salsabeel Jadi and I am an MSc student at the University of Waterloo's School of Optometry.

I submitted a manuscript to MolVis, which was published in February '12. The title of the publication is: Composition of incubation solution impacts in vitro protein uptake to silicone hydrogel contact lenses.

This project serves as an integral part of my Master's thesis, and I would like to ask your permission to include it as one of the thesis chapters.

I sincerely thank you for your time and consideration.

Best wishes,

Salsabeel Jadi