Lysozyme Deposition Studies on Silicone Hydrogel Contact Lens Materials

by

Lakshman Nagapatnam Subbaraman

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Vision Science

Waterloo, Ontario, Canada, 2005

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

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

Abstract

Over 60 proteins have been detected in the tear film and among these lysozyme has attracted the greatest attention. Several techniques for elucidating the identity, quantity and conformation of lysozyme deposited on soft contact lenses have been developed. Lysozyme also deposits on the newly introduced silicone hydrogel (SH) lens materials, but in extremely low levels compared to conventional hydrogel lenses. Hence, a major analytical complication with the study of the SH contact lens materials relates to the minute quantity of deposited lysozyme.

The first project of this thesis involved the development of a method whereby lysozyme mass extracted from SH lens materials would be preserved over time and would be compatible with an optimized Western blotting procedure. This methodological development was incorporated into a clinical study (CLENS-100® and Silicone Hydrogels – CLASH study) wherein the difference in the degree of total protein, the difference in lysozyme deposition and activity recovered from lotrafilcon A SH lens material when subjects used surfactant containing rewetting drops (CLENS-100®) versus control saline was investigated. The remaining experiments were *in vitro* experiments wherein the lenses were doped in artificial lysozyme solution containing ^{125}I-labeled lysozyme. These experiments were performed to gain insight into the kinetics of lysozyme deposition on SH lens materials and also the efficacy of a reagent in extracting lysozyme from SH lens materials.

A protocol was developed whereby the percentage loss of lysozyme mass found on lotrafilcon A SH lenses was reduced from approximately 33% to <1% ($p<0.001$), following extraction and resuspension. The results from the CLASH study demonstrated that when subjects used a surfactant containing rewetting drop instead of a control saline drop total protein deposition (1.2±0.7 μg/lens versus 1.9±0.8 μg/lens, $p<0.001$), lysozyme deposition (0.7±0.5 μg/lens versus 1.1±0.7 μg/lens, $p<0.001$) and percentage lysozyme denaturation (76±10% versus 85±7%, $p=0.002$) were all reduced. The results from the kinetics study demonstrated that lysozyme accumulated rapidly on etafilcon A lenses (1 hr, 98±8 μg/lens), reached a maximum on the 7th day (1386±21 μg/lens) and then reached a plateau (p=NS). Lysozyme accumulation on FDA Group II and SH lenses continued to increase across all time periods, with no plateau being observed ($p<0.001$). The results from the extraction efficiency study showed that 0.2% trifluoroacetic acid/ acetonitrile was 98.3±1.1% and 91.4±1.4% efficient in extracting lysozyme deposited on etafilcon A and galyfilcon lenses, while the lysozyme extraction efficiency was 66.3±5.3 % and 56.7±3.8% for lotrafilcon A and balafilcon lens materials ($p<0.001$).

The results from these studies re-emphasize that novel SH lens materials are highly resistant to protein deposition and demonstrate high levels of biocompatibility.

Acknowledgements

At the outset I gratefully acknowledge the Almighty who has blessed me in completing my project successfully with the help, support and co-operation of the following:

- My supervisors Dr Lyndon Jones and Dr Michelle Senchyna who have been guiding me in my project right from the beginning till the successful completion. My thanks to them for having extended much needed support and guidance all these two years and for giving me strength of mind whenever there was any sagging in my enthusiasm.
- My committee members Dr Anthony P Cullen and Dr Tom Singer who have rendered invaluable assistance in my pursuit.
- Mary-Ann Glasier who has taught me the lab techniques untiringly in my research activities.
- All the members in the lab, who have been of immense help to me whenever I needed.
- Dr Heather Sheardown in McMaster University for helping me to radiolabel the proteins.
- My fellow scholars who helped me in my pleasant domestic living - who made a home away from home - but for which I could not have concentrated on my studies and research.
- All the members of staff and faculty of the School of Optometry and CCLR.
- All those who directly or indirectly extended necessary assistance in my academic and research activities.

Dedication

Dedicated to my parents and brothers who stay tens of thousands of miles away and constantly pray for my progress and success.

Table of Contents

List of Figures

List of Tables

List of Symbols and Abbreviations

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

ml	millilitre
mM	millimolar
MRB	modified reconstitution buffer
ng	nanogram
NVP	N-vinyl pyrrolidone
OD	optical density
PV	PureVision™
PVDF	polyvinylidene difluoride
R^2	correlation coefficient
RB	reconstitution buffer
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH	silicone hydrogel
TBS	Tris-buffered saline
TBS-Tw	Tris-buffered saline with 0.05% Tween® 20
Tris	Tris (hydroxymethyl) aminomethane
USAN	United States Adopted Names Council
UV	ultraviolet
v/v	volume/volume
WB	Western blotting
w/v	weight/volume

1 Introduction

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

Contact lenses suffer from the same problems of deposition that other biomaterials exhibit, being rapidly coated with a variety of proteins, lipids and mucins. [2-9] The first event observed at the interface between a contact lens and tear fluid is protein adsorption.[10, 11] Of late, the study of interaction of tear proteins with contact lenses has become an important field of research, following the widespread use of contact lenses in many physiological and pathological conditions. Tears have a rich and complex composition, allowing a wide range of interactions and competitive processes. Protein adsorption on contact lenses is the overall result of various types of interactions between the different components present, i.e. the chemical composition and the surface charge, the structure of the protein molecules, the nature of the medium (tears) and many other solutes present in tears.

Several techniques for elucidating the identity, quantity and conformation of deposits on soft contact lenses have been developed. Using these methods, many studies have

demonstrated that lysozyme is the principal soilant on the lens surface. Lysozyme also deposits on the newly introduced silicone hydrogel (SH) lens materials, but in extremely low levels compared to conventional hydrogel lenses. [12-14] Hence, a major analytical complication with the study of the SH contact lens materials relates to the minute quantity of deposited lysozyme.

This thesis is partially focused on optimization of existing analytical techniques, such that the lysozyme deposits extracted from SH contact lenses can be accurately and sensitively analyzed. This project will also involve the use of ^{125}I labeled-lysozyme to gain insight into the kinetics of lysozyme deposition on SH lens materials and also the efficacy of a reagent in extracting lysozyme from SH lens materials. Finally, this thesis will also investigate the impact on protein deposition by treating SH lenses with a novel rewetting agent, following collection of lenses from carefully controlled clinical studies.

With a greater understanding of protein deposition on contact lens materials, further enhancements in contact lens materials can be made. This will aid practitioners in prescribing the correct contact lens material for their patients.

2 Literature Review

2.1 Contact lenses

Contact lenses are one of the most widely used biomedical devices in the world. [9] They have been studied extensively with respect to their level of deposition, primarily due to their non-invasive use and easy recovery compared to other biomaterials in contact with biological fluids. [1] Contact lens materials can broadly be classified into two types, (a) water-containing soft (hydrogel) and (b) non-water containing rigid gas-permeable (RGP) materials.

2.1.1 Conventional contact lens materials

Hydrogels are water-absorbing, hydrophilic polymeric materials. The amount of water adsorbed by the hydrogel is described by the term "equilibrium water content" (EWC) and this factor strongly influences the polymer's surface, mechanical and transport properties. [15] The first successful material of this type (poly-2-hydroxyethyl methacrylate [polyHEMA]) was developed by Wichterle and Lim in the late 1960's as a general purpose surgical material. [15] Over 90% of contact lens wearers use hydrogel lenses, due to their increased initial comfort and reduced sensation of dryness. [16]

The corneal metabolic requirements of patients vary with each individual [17] and some patients with higher prescriptions, may exhibit corneal edema and develop chronic hypoxic complications if fitted with low water content materials, due to the increased thickness of the lenses. [18, 19] These patients require high water content hydrogels to

reduce such complications. Two principal strategies are available to increase the water content of hydrogels above that of polyHEMA. Small quantities of charged groups such as methacrylic acid or larger amounts of hydrophilic, neutral groups such as polyvinyl alcohol or N-vinyl pyrrolidone are added to polyHEMA or methyl methacrylate to raise their equilibrium water contents to 60% or greater.

Commercially available contact lens materials can be divided into various groups depending on their charge and water content. The FDA currently classifies contact lens materials into four groups, depending upon their charge and water content (Table 2.1).

Table 2.1 FDA classification of hydrogel contact lens materials

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

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

There are several different wearing modalities utilized for hydrogel lenses. They are as follows:

1. Daily wear (DW) lenses are cleaned and removed each night and are discarded after a period of time. This period varies from one day to one year, but usually after 30 days or less.
2. Extended wear (EW) contact lenses are worn for 7 days and 6 nights, with the lens disposal occurring after this time-frame.

3. Continuous wear (CW) modality is a relatively new modality, which requires little, if any, lens maintenance. Under this modality, contact lenses remain on the ocular surface for up to 30 days and nights without removal, after which they are discarded.

In order to prevent corneal infection, the corneal tissues require sufficient oxygen to function without compromising cellular processes. Clearly, this is more difficult under closed-eye conditions, when the lid severely limits oxygen transport to the cornea. In order to provide a healthy ocular surface under EW and CW conditions it is important that the lens material provides a substantial amount of oxygen to the ocular surface. This has been achieved through the recent development of novel silicone hydrogel contact lens materials.

2.1.2 Silicone hydrogel contact lens materials

Preliminary attempts (in the 1970's and early 1980's) to use silicone within hydrogel lenses in the silicone elastomers failed due to the exposure of hydrophobic silicone on the surface of the lens material. This resulted in increased lens binding to the cornea, enhanced lipid deposition and decreased in-eye wettability of the lens. [20] In order to cope with this problem a surface modification process was required, which would increase the hydrophilicity of the lens surface and make the surface more wettable and hence more biocompatible. [21] Additionally, the surface treatment should maintain a stable tear film layer, be non-irritating, provide low bacterial adherence and minimize deposition of substances from tears. [22]

Consequently, these silicone hydrogel (SH) lenses were developed and were first introduced into the market in 1999. [23] The addition of silicone to the lens increases the material's oxygen transmission, while the hydrogel component allows for fluid transport and lens movement. The combination of these two components allows for safe, extended wear of lenses when compared to conventional lens materials. [20, 24] These lens materials transmit 5-6 times more oxygen than the conventional polyHEMA-based lenses and hence could provide safe overnight wear for up to 30 continuous nights. [24]

Currently four silicone hydrogel lenses are available in the North American market. Table 2.2 summarizes the differences between the four SH materials.

Table 2.2 Characteristics of currently available silicone hydrogel lenses

Proprietary name	**Focus® Night & Day™**	**PureVision™**	**Acuvue® Advance™**	**O_2 Optix**
Manufacturer	CIBA Vision	Bausch & Lomb	Vistakon	CIBA Vision
Water content (%)	24	36	47	33
Oxygen Permeability (Dk)	140	99	60	110
Centre thickness (mm) -3.00D	0.08	0.09	0.07	0.08
Oxygen Transmissibility (Dk/t) at 35˚C	175	110	86	138
FDA group	I	III	I	I
USAN	Lotrafilcon A	Balafilcon A	Galyfilcon A	Lotrafilcon B
Stiffness (g/mm^2)	130	110	55	100
Base Curve (mm)	8.4, 8.6	8.6	8.3, 8.7	8.6
Total Diameter (mm)	13.8	14	14	14.2
Year of introduction	1999	1999	2003	2004

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

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

The surfaces of the lotrafilcon (A and B) lenses are permanently modified in a gas plasma reactive chamber to create an ultrathin (25 nm), high refractive index, continuous hydrophilic surface. [23, 25] Balafilcon lenses are surface treated in a gas plasma reactive chamber, which transforms the silicone components on the surface of the lenses into hydrophilic silicate compounds. [23, 26] This results in glassy, discontinuous silicate islands and the hydrophilicity of these areas bridges over the underlying hydrophobic balafilcon material. Galyfilcon lenses incorporate a long chain, high molecular weight molecule called Hydraclear™, which maintains flexibility and moisture. This wetting agent is present throughout the lens and hence no surface treatment is required for these lenses. [27]

With the introduction of these novel SH lens materials, hypoxia and oedema related problems have been solved; however, as will be discussed in the following sections, the problems such as tear related deposition and wettability still remain unsolved.

2.2 Contact lens deposits

One of the major problems with hydrophilic contact lenses is that they are susceptible to spoilage from constituents of the tear film, which include a wide variety of proteins, lipids and mucins. [2-9] At extreme levels of build-up, these deposits are associated with diminished visual acuity [28] and a feeling of dryness and discomfort. [29] Deposits can ultimately lead to more serious clinical conditions such as hypersensitivity reactions and giant papillary conjunctivitis. [30-33] Moreover, these deposits potentially increase the risk of bacterial attachment by providing a solid substrate and shelter. [34-36]

The adsorption of tear derived substances at the contact lens interface is highly complex and dependent upon a number of factors. Notable amongst these are the material's equilibrium water content (EWC), [3] surface charge, [37] protein size/ charge [38] and age of the lens material. [29] However, the relative importance of the different components of deposited films to these clinical effects remains largely unknown.

As already mentioned, the newly introduced SH contact lenses have significantly increased oxygen transmission due to the incorporation of siloxane groups. [14, 39, 40] The incorporation of silicone results in an increased degree of hydrophobicity, which results in increased lipid deposition compared with other non silicone-containing materials. [24] However, these lens materials do deposit extremely low levels of protein compared to conventional hydrogel lenses, with typical levels being in the < 20 μg/lens range. [12-14] Among the conventional lens materials, Group I lenses typically attract less than 10 μg of

protein, Groups II and III lenses approximately 30 µg, and Group IV lenses 1000 µg or more. [2, 3, 5, 7, 37, 41-49]

2.2.1 Protein deposits on contact lenses

Proteins primarily are deposited onto the contact lenses from the tear fluid. Table 2.3 lists the average concentration of some important tear proteins. Proteins deposit on the contact lenses as films and these protein films are invisible during the early stages of the spoilation process, but with the advancement of time, the protein denatures and they assume a thin, translucent, whitish appearance. [50] These protein deposits remain primarily on the surface, but in high water content lenses they may penetrate into the lens matrix. [51] All proteins in the tear film have the potential to form contact lens deposits, although, several factors ultimately influence the type, quantity and structure of such deposits.

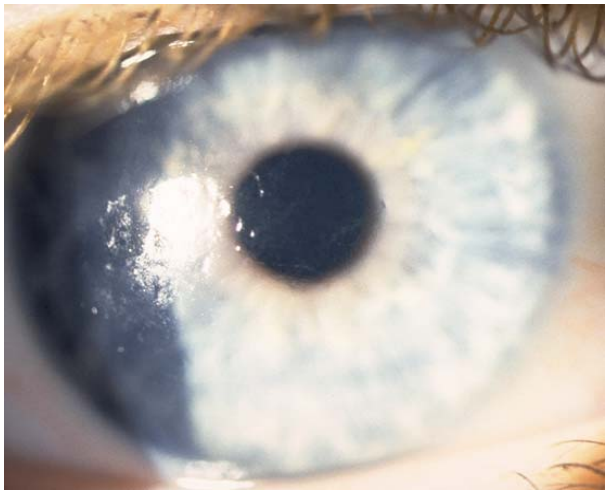


Figure 2.1 Protein deposits on contact lens.
(Picture courtesy of Dr. Lyndon Jones)

Table 2.3 Average concentration of some important tear proteins [52]

Component	Average concentration (mg/100ml)
Total Protein	751
Lysozyme	236
Lactoferrin	184
Tear specific pre-albumin	123
Albumin	130
Immunoglobulin A	30
IgG	12.6
IgM	0.086
IgE	0.01

Protein deposition onto hydrogel lens materials is a highly complex process, depending upon the charge and size of the protein, environmental pH, charge and water content of the substrate and competition between the various tear film constituents that are present. [2, 41, 53] Protein has been the major focus of both identification and quantitation studies of contact lens deposits. Estimates of the total amounts vary but fall within a reasonably well-defined range. Group I lenses typically attract less than 10 μg of protein, Groups II and III lenses approximately 30 μg, and Group IV lenses 1000 μg or more. [2, 3, 5, 7, 37, 41-49] Silicone hydrogel lens materials deposit extremely low levels of lysozyme compared to conventional hydrogel lenses, with typical levels being in the < 20 μg/lens range. [12-14]

Tear film proteins frequently detected on hydrogel contact lenses include lysozyme, lactoferrin and albumin, [45, 50, 53] and among these lysozyme has been the most widely studied. [2, 7, 38, 54, 55]

2.3 Lysozyme

Lysozyme (also called Muramidase) is a bacteriolytic enzyme that was discovered by Fleming in 1922. [56] It is found in mammalian urine, saliva, tears, milk, cervical mucus, leukocytes and kidneys. [52] Tear lysozyme is derived from the acinar and ductal epithelial cells of both main and accessory lacrimal glands. [57, 58]

2.3.1 Lysozyme structure

Lysozyme is a compact globular protein molecule with a molar mass of 14,500 D. [58] It has a slightly ellipsoidal shape, and its dimensions are 45 X 30 X 30 Å. It is a compact protein of 129 amino acids which folds into a compact globular structure. [59] The 129 amino acid sub-units are cross-linked by four disulphide bridges. [60] There is a close cluster of basic groups (Arginine 45 and 68 in one region, Arginine 61 and 73 in a second and Arginine 5, 125 and 128 in a third) which form the highly positively charged surface regions of lysozyme, which give it a very high isoelectric point of 11.1. The polypeptide chain forms five helical segments, a 3 stranded anti-parallel Beta sheet that comprises one wall of the binding cleft. A deep cleft contains the active site (described later in Section 2.3.1.3) which divides the molecule into two domains. These domains are linked by alpha-helix residues. One domain consists of residues that have Beta Sheet structure; the other domain has in its residues that are helical in nature. [59]

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

Lysozyme, in its native globular state contains two fragments which have immunologic activity and encompass two independent antigenic determinants. [61] One of these immunologically active fragments consists of two peptides, derived from the NH_2-terminus and the COOH-terminus of lysozyme linked together by a disulphide bond. The second immunologically active component isolated was a large fragment derived from the region located between residues Leucine 57 and Arginine 107 of the lysozyme sequence. [61] This peptide which contains two disulphide bridges, is also capable of binding to anti-lysozyme antibodies. Similar structure was obtained for lysozyme in further experiments conducted using epitope mapping. [62, 63] The location of these regions in the three-dimensional structure of lysozyme in shown in Figure 2.2.

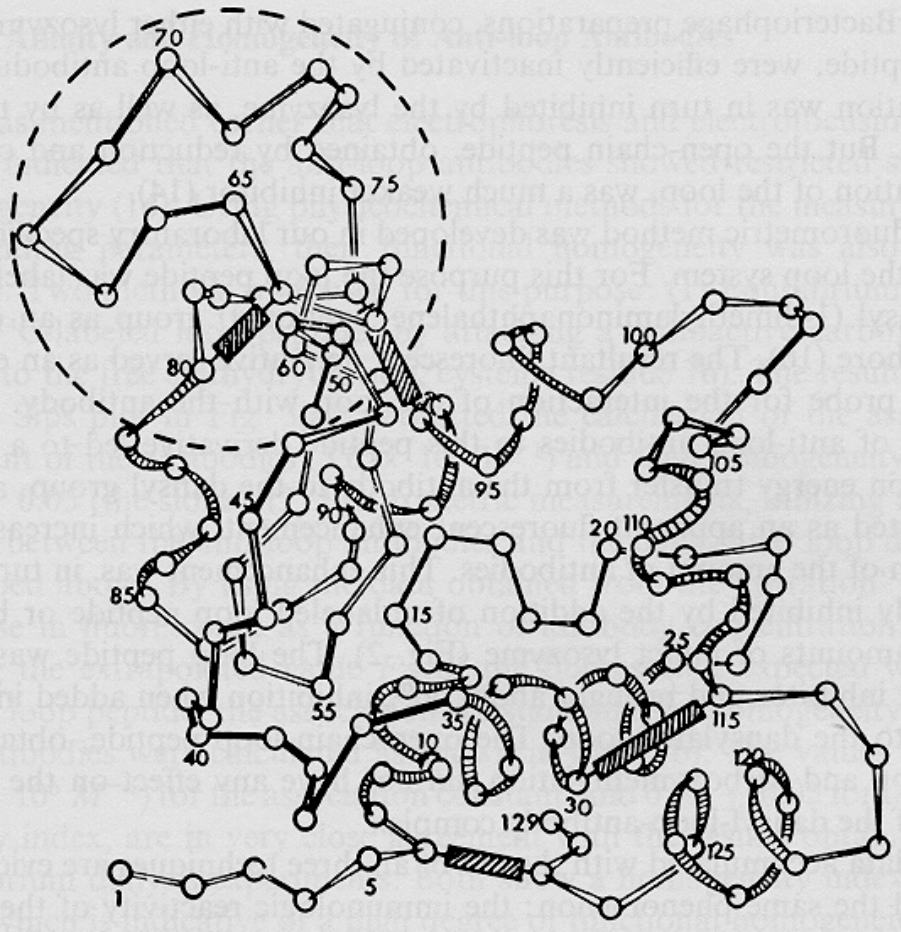


Figure 2.2 Schematic drawing of the main chain conformation of lysozyme. [59]

2.3.1.2 Structural comparison of human lysozyme versus hen egg lysozyme

X-Ray crystallographic studies [60, 64] and far-UV circular dichroism studies [65, 66] suggest that the two enzymes namely, human lysozyme and hen egg lysozyme have very similar secondary structures. Even though the sequences in these two lysozymes are different, [67] sizable numbers of substitutions are non-conservative; hence the structures and functions are highly similar. [59] Moreover, if the ionic strength was maintained constant within a certain range (pH 5 to 9), the activity of both these enzymes were found to be same. [59, 68]

2.3.1.3 Structure – Activity Relationship (SAR)

Lysozyme is instrumental in destroying certain species of bacteria which are gram-positive. These gram-positive bacteria possess an outer coat of a peptideglycan (sugar) polymer (or peptidoglycan), which, in gram-negative bacteria, is only transiently stained since those bacteria are covered by a second, outer lipid membrane. [69]

Lysozyme hydrolyzes or breaks up the glycan (sugar polymer) components of the peptidoglycan of gram-positive bacteria. Specifically, lysozyme breaks β-1,4 glycosidic bond of the oxygen bridge between the repeating glycan units of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG), which is responsible for its anti-bacterial properties.

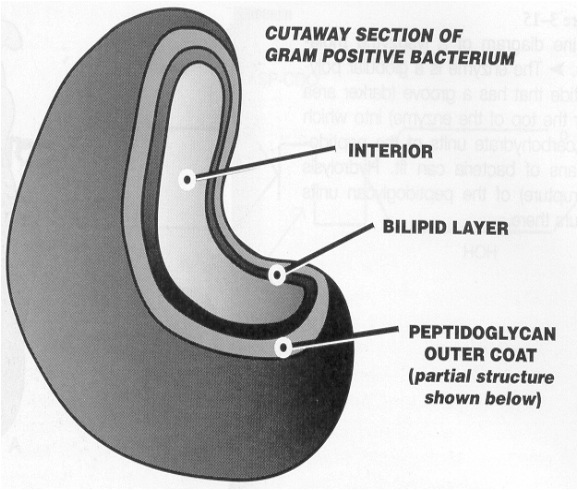


Figure 2.3 Cut-away diagram of a gram-positive bacterium. [69]

The gram-positive bacterium has two boundary layers: a bilipid layer (membrane) and a peptidoglycan layer (outer coat).

A portion of the bacterial peptidoglycan is able to fit in a groove on the outer surface of the enzyme that contains the active site (Figure 2.4). The active site contains two amino acid components (Glutamine and Aspartic Acid) whose carboxylate groups participate in the hydrolysis. [59] The molecular mechanism of lysozyme catalysis at the active site involves multiple steps, in which a proton is donated by an uncharged Glutamine residue at the active site by breaking the glycosidic bond. [69] At completion, the original forms of the enzymes are regenerated and the hydrolyzed chains of the peptidoglycan leave the active site of the enzyme.

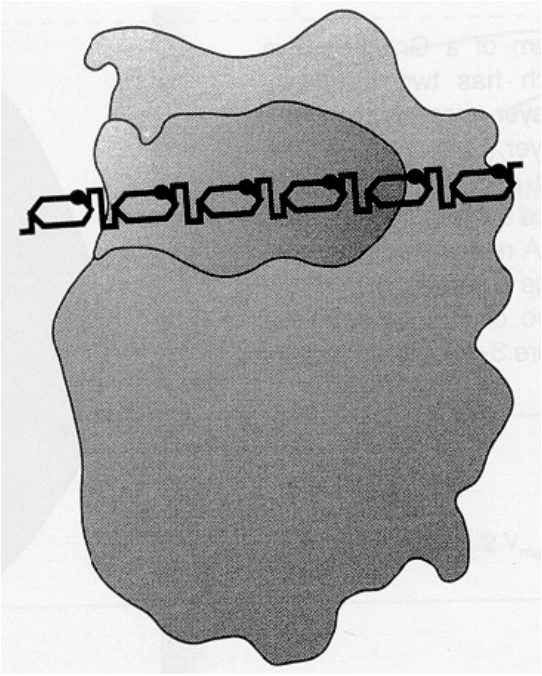


Figure 2.4 Outline diagram of a lysozyme molecule. [69]

The enzyme is a globular polypeptide that has a groove (darker area near the top of the enzyme) into which the carbohydrate units of the peptidoglycans of bacteria can fit. Hydrolysis of the peptidoglycan units occurs here.

2.3.2 Lysozyme and contact lenses

The major proteins that are deposited on contact lenses include lysozyme, lactoferrin and albumin, [45, 50, 53] and among these lysozyme has been the most widely studied. [2, 7, 38, 54, 55] Karageozian first reported that the principal component of deposits that presented problems with contact lens wear was lysozyme, which may be selectively adsorbed and denatured on the lens surface. [70] Many others have since confirmed the predominance of lysozyme in lens deposition. [7, 42, 44-46, 71, 72]

Lysozyme is a major component in tears and of contact lens deposits. It accounts for approximately 40% of total protein found in tears and is the major protein (approximately 36 to 95% depending on lens type) deposited on hydrogel contact lenses. [73, 74] In addition to its bactericidal properties (described earlier), lysozyme is also reported to have anti-inflammatory properties in the tear film, although the mechanism through which this action occurs is unknown. [75] Exploration of lysozyme deposition (quantity and conformation) on a number of different traditional and silicone hydrogel surfaces is of growing interest due to observations that patients using silicone hydrogel lenses are prone to develop papillary conjunctivitis, [76-78] possibly due to the denaturation of lysozyme on the lens materials.

Lysozyme is a positively charged molecule and this, coupled with its small size, results in its increased adsorption onto negatively charged substrates such as FDA group IV contact lens materials. [3, 7, 38, 55, 79] However, silicone hydrogel lens materials deposit extremely

low levels of lysozyme compared to conventional hydrogel lenses, with typical levels being in the < 20 μg/lens range. [12-14]

In order to measure the low levels of lysozyme deposited on SH lens materials an accurate method to quantify protein levels in nanogram quantities is required. The following section discusses the options available to quantify such low levels of protein.

2.4 Methods to quantify proteins deposited on contact lenses

Several microscopic, photometric and imaging techniques have been used to investigate protein deposits on contact lenses. [5, 41, 44, 80, 81] The major limitation of microscopic and imaging techniques is that they are generally not suitable for accurate quantitation purposes. [9] Various biochemical assays including Enzyme-Linked Immunosorbent Assay (ELISA), High Performance Liquid Chromatography (HPLC) and Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) are useful because of their increased sensitivity, accuracy and ability to target specific proteins.

2.4.1 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a widely used method for investigation of tear film proteins. ELISA utilizes antibodies bound to solid surfaces, such as plastic or polystyrene micro-titre plates to quantify protein of interest. [82] ELISA relies on antibody recognition of the protein of interest in solution. A colorimetric, fluorescent or chemiluminescent reaction is used to quantify the amount of protein bound to the well, followed by detection of the specific signal. [82]

The major advantage of ELISA is the ability to process high number of samples (up to 96) at the same time and this method also reduces the need for handling the sample. The disadvantage with this method is that it can cross-react with non-targeted proteins, or the interaction between antibody and target can be disrupted by other sample components. [83]

2.4.2 High Performance Liquid Chromatography (HPLC)

HPLC separates proteins based on molecular size in a column support through a tightly packed matrix (such as glass, plastic or silica beads) under high pressure. There are several types of HPLC and Size Exclusion HPLC is the widely used method for quantifying individual proteins in solution. The major advantage of HPLC is that several proteins can be quantified from a single experiment. However, Fullard (1988) found an unusually high absorbance for lysozyme, which may decrease the accuracy of the assay and could lead to erroneous conclusions. [84]

2.4.3 Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis followed by Immuno blotting

Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis (SDS-PAGE) is a specific type of gel electrophoresis that uses sodium dodecyl sulphate (SDS) as a detergent to confer a negative charge to a protein and polyacrylamide as a matrix to separate proteins according to size. Electrophoresis refers to the migration of charged molecules in solution in response to an electric field. Electrophoresis is simple, rapid and highly sensitive and can be used to study the properties of a single charged species, and as a separation technique. [85] Once physically separated, proteins are visualized using antibodies and chromogenic, fluorescent or chemiluminescence is used to quantify the binding of the antibodies to the protein. Resolution of proteins that migrate together in the gel is accomplished by the specificity of the antibody; a single antibody will recognize a small portion of the protein of interest, thereby reducing the potential of visualizing co-migrating tear film components. In addition immunological visualization allows

identification of variations in protein conformation, such as unexpected polymerization or interaction with other proteins. [86, 87]

2.4.3.1 PhastSystem™

The PhastSystem™ (Amersham Pharmacia Biotech, Baie d'Urfe, QC, Canada) is an automated mini-gel system that combines both electrophoresis and immunoblotting into a single apparatus. This system is different from that of traditional larger gel electrophoresis systems, in that it utilizes pre-cast, extremely small (4.5 cm X 5 cm) SDS gels for protein separation. These extremely small SDS gels confer increased sensitivity with a minute sample volume requirement, compared to other electrophoresis and immunoblotting systems. (Figure 2.5)

Figure 2.5 The Phast System™

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

directly into wells cut into a gel. These disposable well combs are then automatically applied to the mini-gel for electrophoretic separation. The temperatures, voltage, current and power are automatically regulated throughout the separation process. Western blotting is similarly automated and simplified by the PhastSystem™. The small size of the gels and the small sample volume applied to the gel make the PhastSystem™ unique among all other systems. The low sample volumes and small pre-cast gels also provide an added level of sensitivity and flexibility, especially for proteins in low concentrations. [88, 89] Several researchers have used the PhastSystem™ to detect extremely low concentrations of tear film proteins. [88-90]

Whilst the methods such as HPLC or ELISA can provide other advantages such as enhanced efficiency in quantifying several proteins at the same time (HPLC) or provide greater sample throughput (ELISA), electrophoretic separation followed by immunoblotting using the PhastSystem™, provides us with the best tool for quantifying individual proteins in extremely low concentrations, while at the same time allowing visualization of the protein to assess cross-reactivity between the antibody and protein of interest and possible aggregation or polymerization of the protein. Using this technique, a method has been optimized to quantify lysozyme deposition on SH lenses. [13]

The following section discusses the possible difficulties associated with the analysis of lysozyme deposited on SH lenses through this optimized procedure, which primarily arises due to its deposition in low quantities.

2.5 Difficulties associated with contact lens deposit analysis

2.5.1 Lysozyme and its stability in contact lens deposit analysis

Lysozyme is a relatively stable protein when compared to most other proteins. [7] The properties of lysozyme do not change at pHs between 1 and 8. [91] A study by Ikeda and colleagues (1967) showed that lysozyme denatures only when the pH is above 12. [92] The thermal stability of lysozyme in various solutions was studied by Hamaguchi and Sakai (1965) and they found that its structure is not affected by heat up to 55° C. [93] Moreover, by increasing the temperature only the internal fold of lysozyme is disrupted and no changes occur in the helical part at high concentrations of organic solvents.

Despite its stability, lysozyme might undergo changes in its conformation during the process of contact lens deposit analysis. In the optimized procedure developed in our lab, the various steps involved include extraction of deposits using an extraction buffer, lyophilization, storage and resuspension. [13] Any of these steps could potentially alter the native conformational state of lysozyme and could result in failure to be recognized by the antibody.

To-date, no study has been conducted on the effect of storage on lysozyme deposits that have been extracted from contact lens materials. In two recent studies undertaken on tear and salivary samples, it was demonstrated that a reduction in lysozyme quantity occurs as a function of storage time. [94, 95] Preliminary results in our laboratory have demonstrated that lysozyme deposits extracted from SH contact lens materials also demonstrate a loss in total mass after lyophilization and resuspension, as a function of storage time when

assessed by Western blotting. These data (unpublished) indicate that this loss in mass is particularly problematic with lysozyme deposition on lotrafilcon A lens materials. This loss represents a potential source of error when quantifying total lysozyme deposition. Moreover, the amount of lysozyme extracted from SH materials is very low, such that even a minimal loss would be significant in the interpretation of the total amount of lysozyme deposited. Hence there is a need to devise a method whereby lysozyme mass extracted from SH lens materials would be preserved over time and would be compatible with the optimized Western blotting procedure. [13]

2.5.2 Elution of proteins from contact lens materials

To-date, elution of proteins from the material remains the best option to quantify specific proteins deposited onto contact lenses. [6, 41, 42, 96] The accurate quantification of a protein deposited on a surface requires that all or at least a known percentage of the protein of interest be removed. Hence the ability of an extraction buffer to extract the proteins for analysis is a focus of major interest.

The most common method for protein extraction involves the use of a combination of detergents and reducing agents to break chemical bonds between the adsorbed proteins and the contact lens surface. Some of the agents that have been incorporated in elution mixtures include sodium dodecyl sulphate (SDS), dithiothreitol (DTT), urea, NaOH, ethylene diamine tetraacetic acid (EDTA) and Tris-HCL. [5, 7, 29, 41, 42, 45, 46, 53, 97-101]

2.5.2.1 Review of various extraction procedures in the literature

One of the significant earlier works on deposit removal was conducted by Wedler in 1977. [45] He considered the removal of deposits on contact lenses by various chemical reagents including urea, guanidine hydrochloride, potassium thiocyanate, potassium perchlorate, hydroxylamine, EDTA, SDS and DTT. However, he found that the deposits were most effectively removed from the lenses by the combination of heat, SDS and DTT.

Many other researchers have used these techniques since then, particularly SDS. [5, 29, 41, 42, 46, 53, 97-99] SDS, a stong detergent and DTT, which cleaves the disulphide bonds between and within the proteins, are required to break apart chemical bonds and solubilize proteins for quantification. Urea, a hydrogen bond cleaving agent, is used for the denaturation of proteins and as a mild solubilization agent for insoluble or denatured proteins. EDTA is a chelating agent that binds metal ions; however some studies demonstrate that EDTA is not effective in extracting protein deposits. [45] Tris-HCL is a buffering agent that maintains the pH of the extraction solution. In addition to chemical "mixtures" to extract proteins, heat and sonication have been utilized to increase the efficiency of protein extraction, theoretically by agitating the biomaterial in the extraction solution and helping to break apart the bonds. [102, 103] However, it became apparent from the study by Yan and co-workers (1993) that these common extraction procedures may fail to remove even 75% of the total material on lenses. [46]

Keith and co-workers (1997) developed a quick, simple and efficient extraction technique. [74] They used an extraction solvent consisting of 50:50 mix of 0.2% trifluoroacetic acid and acetonitrile. Extraction efficiency for lysozyme from laboratory deposited group IV lenses was found to be approximately 100%. A similar extraction procedure has been adopted by many other researchers since then. [12, 104] However, the efficacy of this technique in extracting lysozyme from SH lens materials has not been reported.

2.5.3 Radiotracer technique to determine extraction efficiency

For studies where proteins are eluted from lenses it is vital to ensure that the protein extraction method is effective. Some researchers have utilized the UV spectrophotometer to assess protein extraction efficiency. [13, 105] Using this technique, it is possible to determine the extraction efficiencies of lenses with no UV blocking properties such as etafilcon A. However, it is not possible to use this method on SH lenses as, unlike etafilcon A lenses (Group IV), SH lenses deposit extremely low levels of lysozyme, making UV spectrophotometry too insensitive.

Hence, a more sensitive method to estimate the efficiency of lysozyme removal from SH lenses has to be employed. One of the positive avenues is the use of radioactively labeled lysozyme. The isotope ^{125}I has been used to radioactively label lysozyme and other proteins in previous studies. [2, 48, 103, 106, 107] The isotope is incorporated into the protein and radioactivity can be used to assess the level of protein binding. Thus, I sought to quantify the percentage of lysozyme extracted by 0.2% trifluoroacetic acid/ acetonitrile from SH and Group IV contact lens materials by artificially doping lenses with ^{125}I-labeled lysozyme.

2.6 Kinetics of lysozyme deposition

Following insertion onto the eye, hydrogel contact lenses rapidly adsorb components from the tear film, particularly protein, [2, 3, 7, 37, 47, 48] lipid, [5, 29, 79, 96, 108] and mucin. [8] A number of studies have investigated the kinetics of protein or lipid deposition on contact lens materials *in vitro*, [8, 38, 48, 54, 109-113] and *in vivo* [41, 42, 44, 79, 98, 104, 114, 115] on conventional hydrogel lens materials. However, to-date no study has investigated the kinetics of protein or lipid deposition on SH lens materials.

Since deposition is a time-dependent process, empirically derived values will be highly dependent upon the age of lenses tested. Previous studies show that lenses recovered within the first few minutes of wear demonstrate coatings of some degree and the process of deposition continues over time. [41, 42, 116, 117] Sack and co-workers (1996) found that lysozyme gets deposited on Group IV lenses at a constant rate of 2.2 μg/ minute during the initial part of open-eye wear; this later leveled to a steady state. [98] Lin and co-workers (1991) showed that accumulation of components is not consistent, with some entities showing faster binding rates. [42] Keith and co-workers (2003) have accurately plotted the medium term build-up of lysozyme on Group IV lenses. [104] They found a mean concentration of 55 μg/ lens after 15 minutes of wear, which reached a maximum at around 1300 μg/ lens after six days of wear and also that the time to reach plateauing varied from day four to eleven between subjects. [104] Consistent with this study, Jones and co-workers (2000) determined that surface deposition on Group IV lenses plateaued on day one, with intramatrix deposition continuing to increase up to seven days. [118] Surface protein on Group II lenses also peaked within one day, but total protein accumulation

continued for up to 30 days. [118] The concept of plateauing is also supported by Richards (1992) and Tripathi (1992). [44, 119] In comparing the deposition on Group II lenses, Jones and co-workers (1996) found an increase in lipid by 80% and of protein by 152% at three months compared with one month of wear. [29] Gellatly and co-workers (1988) noted that only 3% of patients whose lenses had been worn for an estimated total of 2600 hours or less showed a significant visible deposition, whereas 80% of patients whose lenses were older than 2600 hours showed this degree of deposition. [28] Maissa and co-workers (1998) observed more surface proteins on ionic materials after three months compared with one month of wear, but no change over this time for non-ionic materials. [79] Jones and co-workers (2000) observed that lipid accumulation ceased by the end of day one on Group IV lenses but continued unabated for at least four weeks for Group II lenses. [118] Lin and co-workers (1991) observed that lysozyme accumulation as measured by SDS-PAGE, increased with wearing times up to one week on Acuvue® lenses (Group IV), but after 24 hours wear lysozyme accumulation did not increase on the SeeQuence lens (Group I). [42]

It is widely recognized that the adsorption of proteins at the contact lens surface is complex and depends upon a number of factors. Notable among these are material water content and surface charge. [3, 7, 37, 42, 46, 47, 79, 96, 120] Knowing the rate of protein deposit accumulation and at what point in lens wearing time the accumulation reaches either a maximum or a plateau level could be clinically relevant to patient symptoms and be helpful in designing clinical investigations of hydrogel lenses and associated lens care products. As the most abundant tear protein, lysozyme approximates 90% of the protein found on group IV contact lens and is therefore often used as the prototypical marker for

protein accumulation. [7, 74, 98] Although, the quantity and conformation of lysozyme deposited on SH lens materials have been reported, [12-14] no study has looked at the deposition of lysozyme as a function of time in SH lens materials.

2.6.1 Advantages of *in vitro* experiments and radiotracer technique

Soft lenses are not always obtained for analysis from clinical sources. A number of studies have found artificial tear solutions an attractive option in determining the binding affinity of different components and the mechanisms involved in such binding. [110, 121-129] The principal advantages are that many experimental variables are eliminated, quantitation can be enhanced by labeling the species fluorometrically or radiometrically, and simple hypotheses can be tested without having to engage in a resource consuming clinical trial.

A number of quantitative protein methods have been applied to the analysis of protein deposits on contact lenses. However, many of these techniques require complex extraction procedures which may not be 100% efficient. The radiochemical assay is:

1. Quantitative and reproducible with a low detection limit.
2. Able to assess a large number of samples.
3. Compatible with all contact lens materials.
4. Not dependent on complex extraction techniques.
5. Able to detect surface as well as bulk protein.

Hence in this thesis, I wanted to gain insight into the kinetics of lysozyme deposition on SH lenses and compare it with Group II and Group IV conventional hydrogel lens materials using the radiotracer technology.

2.7 Influence of rewetting drops on protein and lysozyme deposition

In addition to oxygen transmission, adequate contact lens wettability is critical to achieving long-term physiological compatibility and successful, problem-free contact lens wear. [130] With conventional lens materials, wettability reduces over the wearing period and replacing a lens frequently results in the maintenance of a more wettable, "cleaner" surface. [29, 131, 132] When it comes to in-eye wettability, SH lenses are inherently hydrophobic and hence, as already discussed, require a surface modification process to provide a hydrophilic, wettable surface. [23, 25] Of additional concern is the fact that SH materials deposit greater quantities of lipid than conventional materials, [12] which may act to negate the impact of the surface treatment and result in a hydrophobic surface. To-date, no work has been published that examines the wettability of SH lenses in eye.

One of the means of modifying the lens surface to enhance wettability is to treat the lens with a wetting agent such as a surfactant. Surfactant-containing contact lens care systems are extensively used within the contact lens care industry for cleaning purposes. However, because of the ability of surfactants to associate at interfaces, it can be expected that surfactants will also adsorb onto the surface of hydrogels and thus potentially influence the surface wetting characteristics. These effects may have significant implications in terms of perceived patient comfort on both initial insertion and at the end of the wearing period. Surface modification by the addition of surfactants, which has until now been neglected, may be more important to the patient than any cleaning action. To date, only one study has examined this effect, [133] and this provided confirmation that surfactants can positively influence initial lens comfort.

An appreciation of the physicochemical basis behind lens wettability may enable targeted development of solutions, whose sole function is to produce enhanced lens comfort through in-eye surface modification of materials. Hence it is important to investigate the impact of treating SH lenses during their wearing period with a novel rewetting agent that has been specifically developed to reduce in-eye deposition. Hence, this thesis will also investigate the effect of a surfactant containing rewetting drop on protein deposition on one type of SH lens material, following its collection from a carefully controlled clinical study.

3 Thesis Objectives

This thesis will involve the analysis of contact lenses following their collection from carefully controlled clinical trials and also by artificially soiling lenses to fulfill four objectives:

1. To devise a method whereby lysozyme mass would be preserved over time and would be compatible with the optimized Western blotting procedure.
2. To determine the efficiency of 0.2% trifluoroacetic acid/ acetonitrile in extracting lysozyme from SH and Group IV contact lens materials by artificially doping lenses with ^{125}I labeled lysozyme.
3. To determine lysozyme deposition as a function of time in Group IV, Group II and SH contact lens materials by artificially doping lenses with ^{125}I labeled lysozyme.
4. To determine the difference in the degree of total protein, the difference in lysozyme deposition and activity recovered from lotrafilcon A SH lens material when subjects used a surfactant containing rewetting drop (CLENS-100®) versus a saline control, following its removal from a carefully controlled clinical study.

4 Materials and Methods

4.1 Stabilization study

The sample variables examined in this study included: (1) the presence or absence of gel loading buffer (GLB); (2) temperature (-20°C and -70°C); (3) composition of two reconstitution buffers (RB and MRB) and (4) the presence or absence of a biomolecule stabilizing agent (BioStab™ Biomolecule Storage Solution). These six conditions with the two buffers (see Figure 4.1) were examined systematically as described below. Each trial was conducted in triplicate, resulting in a total of 60 samples being measured.

4.1.1 Reagents and materials

All PhastSystem™ pre-cast gels, buffer strips, well combs, filter paper and ECL-Plus™ kits were purchased from Amersham Pharmacia Biotech (Baie d'Urfe, QC, Canada). Immuno-Blot® PVDF (polyvinylidene difluoride) membrane was purchased from Bio-Rad Laboratories (Mississauga, ON, Canada). Polyclonal rabbit anti-human lysozyme was purchased from Cedarlane Laboratories (Hornby, ON, Canada) and goat anti-rabbit IgG-HRP was purchased from Sigma (St. Louis, MO, USA). Human lysozyme (neutrophil) was purchased from Calbiochem (La Jolla, CA, USA). A product developed specifically for stabilizing proteins and enzymes (BioStab™ Biomolecule Storage Solution – BioStab) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents purchased were analytical grade and obtained from Sigma (St. Louis, MO, USA).

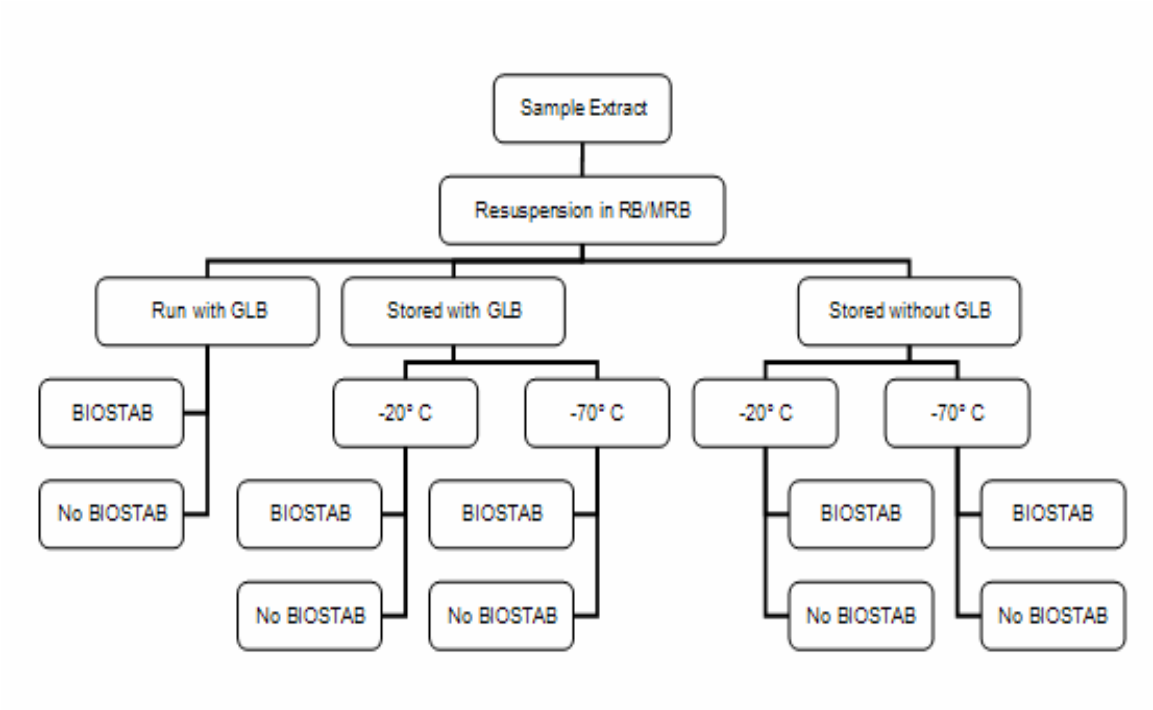
4.1.2 Protein deposit extraction from contact lenses

Twelve lotrafilcon A (Focus® Night & Day™ CIBA Vision, Atlanta, GA) SH contact lenses were collected following 4 weeks of daily wear use, during which subjects had disinfected the lenses with AOSept (CIBA Vision). Lenses were aseptically collected using non-powdered surgical gloves and placed in individual glass vials containing 1.5 ml extraction solution consisting of a 50:50 mix of 0.2% trifluoroacetic acid and acetonitrile (ACN/TFA). [74] The lenses were incubated in darkness at room temperature for 24 hours. Two 0.6 ml aliquots of ACN/TFA was transferred to sterile eppendorf tubes and lyophilized to dryness in a Savant Speed Vac (Halbrook, NY, USA). Dried protein pellets were stored at –70^{o}C prior to reconstitution.

4.1.2.1 Sample processing following extraction

Figure 4.1 describes the sample processing following resuspension of the lyophilized sample extracts. Four 600 µl aliquots (2 X 600 µl from the right eye lens and 2 X 600 µl from the left eye lens of the same subject) of lyophilized lens extracts were taken and three of them were resuspended in 20 µl of either a standard reconstitution buffer (RB - 10mM Tris-HCl; 1mM EDTA, pH 12) or a modified reconstitution buffer (MRB - 10mM Tris-HCl; 1mM EDTA, 0.9% saline, pH 12). Three of these 20 µl aliquots were pooled to total 60 µl and this volume was added to the fourth 600 µl aliquot of lyophilized Focus® Night & Day™ (FND) lens extract.

Figure 4.1 Schematic of lysozyme processing following its extraction from lotrafilcon A lens material, lyophilization and resuspension.



4.1.2.2 Addition of enzyme stabilizer

0.5 µl of the initial stock was taken and checked for neutrality using pH paper (Hydrion Papers, Micro Essential Laboratory, Brooklyn, NY, USA). Once neutrality was confirmed, 4 µl of the sample was added to 10 polypropylene sample tubes (Axygen MAXYMmum Recovery ™, Axygen Scientific, INC, CA, USA). 2.5 µl of BioStab was added to five samples and the same quantity of MilliQ water was added to the remaining five samples, which acted as the control group.

4.1.2.3 Addition of gel loading buffer

Six of the 10 samples were diluted with gel loading buffer (GLB - 5% SDS; 100 mM Tris, pH 7.4; 30% Glycerol; 1 mM EDTA; 0.02% bromophenol blue). The remaining four samples were stored under various conditions without gel loading buffer.

4.1.2.4 Storage

One set of samples were run without storage (fresh) and further samples were stored for 48 hours, under various conditions (Figure 4.1).

4.1.2.5 Lysozyme standard range

Human lysozyme standard curves were run on each Western blot so that four points falling within the linear range of detection were produced, to facilitate regression analysis of sample extracts. Standards were prepared fresh on the day of analysis from a 0.2

μg/μL working solution of purified human neutrophil lysozyme. Standards were diluted with dilution buffer and then mixed with gel loading buffer (5% SDS; 100 mM Tris, pH 7.4; 30% Glycerol; 1 mM EDTA; 0.02% bromophenol blue). The optimal set of concentrations for lysozyme quantification was determined to be 20, 10, 5 and 2.5 ng/μL.

4.1.2.6 Electrophoresis and immunoblotting

Prepared sample extracts and standards were boiled for three minutes after which 1 μL of each was loaded directly onto 8 X 1μl combs. All samples were subjected to SDS-PAGE on 10-15% gradient gels with a 13 mm stacking zone and 32 mm gradient zone on an automated minigel system (Amersham Pharmacia Biotech PhastSystem™) using the manufacturer's specified conditions.

Once separated, proteins were analyzed via Western blotting. PVDF membranes, that had been previously activated by soaking in 100% methanol for 60 seconds followed by transfer buffer (20 mM Tris, 150 mM glycine; 10% (v/v) methanol) for 25 minutes, were placed on the separation section of the gels for transfer in the PhastSystem™ using manufacturer's specified conditions. Once transfer was complete, PVDF membranes were blocked for 2 hours in 20% blocking buffer [Tris-buffered saline (TBS) (50 mM Tris; 100mM NaCl pH 7.4); 20% (w/v) skim milk powder; 0.05% (v/v) Tween®-20]. Following blocking, membranes were washed 3 times, each 10 minutes in duration in 50 mL of TBS with 0.05% Tween-20 (TBS-Tw). The membranes were then incubated at room temperature in 5% blocking buffer containing 1:1000 polyclonal rabbit anti-human

lysozyme for 1 hour and placed overnight at 4°C. Post washing, blots were incubated in 5% blocking buffer for 1 hour at room temperature with 1:10 000 goat anti-rabbit IgG-HRP conjugated secondary antibody. Following 2 washes/10 minutes each with TBS-Tw and one wash of 10 minutes with TBS, bound antibody was visualized by enhanced chemiluminescence (ECL Plus®). Optical density data was gathered by surrounding each of the eight bands with a fixed dimension box and was quantified on a Storm840® Imaging System (Molecular Dynamics, Sunnyvale, CA, USA) (Figure 4.2) set at 800V, high sensitivity and ImageQuant 5.1 software (Molecular Dynamics, Sunnyvale, CA, USA).

Figure 4.2 Molecular Dynamics Storm840® Imaging System

4.1.2.7 Quantification of densitometric data

Quantification of lysozyme concentration found in each lens extract was carried out by densitometric analysis. Optical density (OD) was measured from digitized images of the PVDF membranes using ImageQuant 5.1 software. The OD of the four points derived from the lysozyme standard curve was graphed versus the concentration loaded. Linear

regression was performed and the equation of the line-of-best-fit was used to calculate the lysozyme concentration in the sample extracts. The OD obtained from the digitized images of the sample extracts was substituted into the equation:

$$x = (y - b)/m$$

where y is the OD of the sample extract, m is the slope of the line calculated by linear regression, b is the y intercept as calculated by linear regression, and x is the concentration of purified lysozyme. A single digitized image contained 8 bands, 4 bands representing purified lysozyme and 4 bands representing sample extracts. Lysozyme bands migrated to a position representing a molecular weight of 14 kDa on the gels. All samples were analyzed on duplicate gels to account for loading errors.

Comparison of lysozyme band intensity in stored versus fresh samples enabled calculation of percentage mass loss of lysozyme.

4.1.2.8 Negative controls - Testing for BioStab cross-reactivity in Western blotting

In order to test if the enzyme stabilizer itself had any possible cross-reactivity during the Western blotting procedure, BioStab in conjunction with buffer alone was subjected to SDS-PAGE and Western blotting to PVDF membranes using the PhastSystem™, as described above.

4.2 Extraction Efficiency Study

4.2.1 Reagents and materials

The lenses that were used in the study are described in Table 4.1.

Table 4.1 Lens parameters used in extraction efficiency study

Proprietary name	**Focus® Night & Day™**	**PureVision™**	**Acuvue® Advance™**	**Acuvue ®**
Manufacturer	CIBA Vision	Bausch & Lomb	Vistakon	Vistakon
Water content (%)	24	36	47	58
Oxygen Permeability (Dk)	140	99	60	22
Centre thickness (mm) -3.00D	0.08	0.09	0.07	0.07
Oxygen Transmissibility (Dk/t) at 35˚C	175	110	86	31
FDA group	I	III	I	IV
USAN	Lotrafilcon A	Balafilcon A	Galyfilcon A	Etafilcon A
Stiffness (g/mm^2)	130	110	55	35
Base Curve (mm)	8.4, 8.6	8.6	8.3, 8.7	8.4, 8.7
Total Diameter (mm)	13.8	14	14	14

Chicken egg white lysozyme was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sterile, 5ml non-pyrogenic, polypropylene round bottom tubes were purchased from

Falcon (Franklin Lanes, NJ, USA). All other reagents purchased were analytical grade and obtained from Sigma (St. Louis, MO, USA).

4.2.2 Doping of lenses in artificial lysozyme solution

Figure 4.3 describes this experiment in a graphical format.

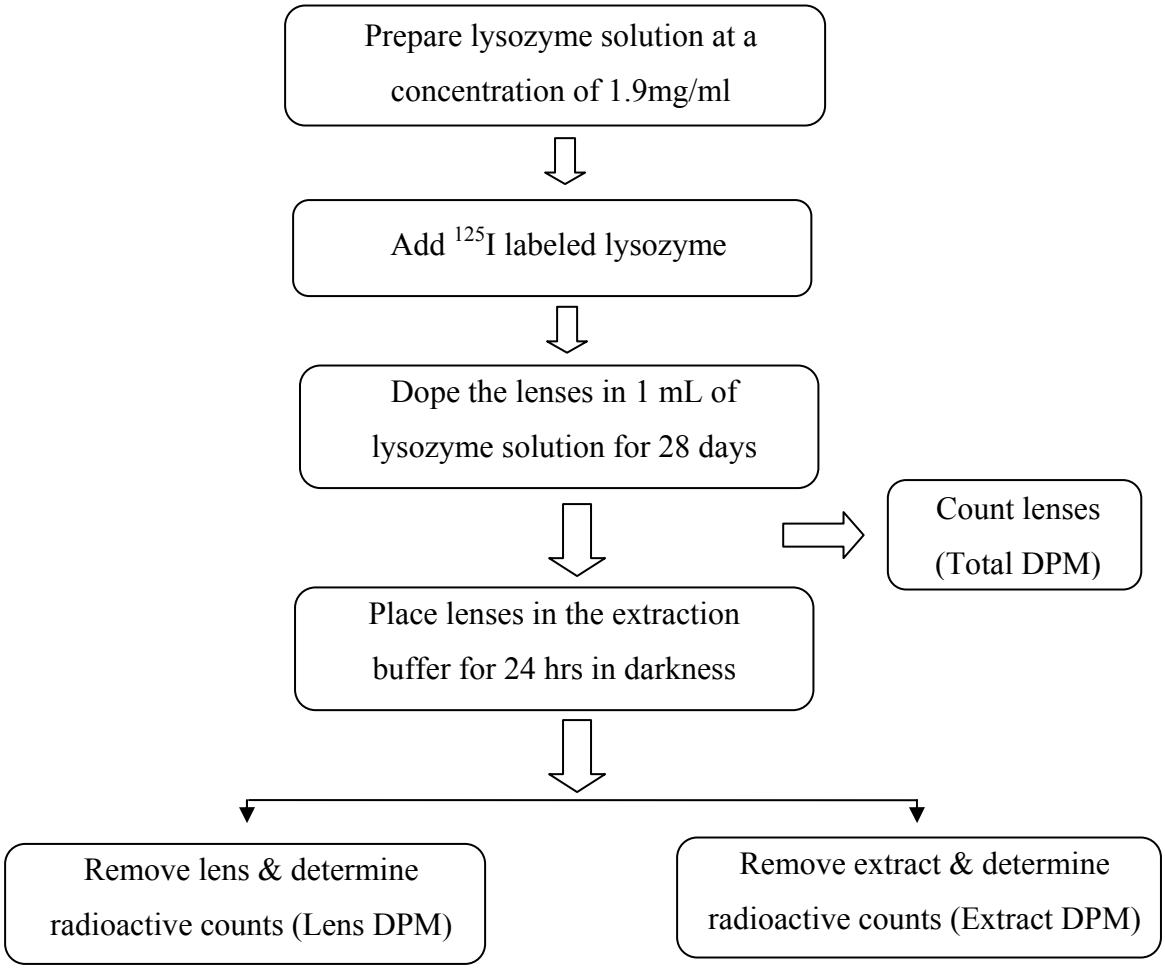


Figure 4.3 Schematic of protocol adopted to determine the extraction efficiency of 0.2% TFA/ACN in extracting lysozyme from Group IV and SH lenses.

Artificial lysozyme solution was prepared at a concentration of 1.9 mg/ml using Phosphate Buffered Saline (PBS), pH 7.4. Lysozyme labeled with ^{125}I was used as the isotopic tracer to quantify protein adsorption to new contact lenses. ^{125}I labeled lysozyme was added to unlabeled solution such that the samples had a counting rate of 10^5 disintegrations per minute/ml (DPM/ml).

As soon as the lenses were opened from the blister pack, they were initially rinsed with Phosphate Buffered Saline (PBS), pH 7.4 to ensure that the packaging solution did not enter the artificial lysozyme solution. The lenses were then placed in 1ml of the labeled lysozyme solution and were allowed to incubate at a temperature of 37° C with constant rotation for 28 days. The labeled lysozyme solution was replaced with identical quantity and counting rate every seven days, to simulate the process of replenishment of the tears.

Following 28 days of doping, the lenses were aseptically collected using forceps and were rinsed briefly in saline to remove unbound protein sticking on to the lenses. The lenses were then placed in sterile, 5ml (12 X 75 mm), non-pyrogenic, polypropylene round bottom tubes and were counted in the Beckman Gamma 5500 Counter (Figure 4.4). This count gave the total quantity of labeled protein that was deposited on the lens (Total DPM).

Figure 4.4 Beckman Gamma Counter

4.2.3 Extraction of proteins from contact lenses

The lenses were then placed in individual glass vials containing 1.5 ml of an extraction solution of 50:50 mixture of 0.2% trifluoroacetic acid and acetonitrile. [74] The lenses were incubated in darkness at room temperature for 24 hours. Following this the lenses were carefully removed from the vials and were placed in sterile, 5ml (12 X 75 mm), non-pyrogenic, polypropylene round bottom tubes and the lenses were counted in the Gamma Counter (Beckman Gamma 5500). This count gave the total quantity of labeled protein that was remaining on the lens following its extraction (Lens DPM). Apart from measuring the lens DPM, the extract was also counted which gave counts of the extracted protein (Extract DPM). The percentage of lysozyme extracted from the lens was calculated by the formula used by Prager and co-workers, [122]

$$\% \text{ extracted} = \text{Total DPM} - \text{Lens DPM} / \text{Total DPM} \times 100$$

4.3 Kinetics Study

4.3.1 Reagents and materials

Chicken egg white lysozyme was purchased from Sigma-Aldrich (St. Louis, MO, USA). The lenses that were used in the study included three types of SH lenses and two types of conventional hydrogel lenses. The SH lenses included Focus® Night & Day™ (Ciba Vision, Atlanta, GA, USA), PureVision™ (Bausch & Lomb, Rochester, NY, USA) and Acuvue® Advance™ (Vistakon, Johnson & Johnson, Jacksonville, FL, USA) (Table 2.2). The conventional hydrogel lens materials that were used in this study are described in Table 4.2. Sterile, 5ml non-pyrogenic, polypropylene round bottom tubes were purchased from Falcon (Franklin Lanes, NJ, USA). All other reagents purchased were analytical grade and obtained from Sigma (St. Louis, MO, USA).

Table 4.2 Conventional hydrogel lens materials evaluated in the kinetics study

	Acuvue ®	**Soflens 66**
Water Content	58 %	66 %
Monomers	HEMA/ MA	HEMA/ NVP
USAN	Etafilcon A	Alphafilcon A
Manufacturer	Vistakon	Bausch & Lomb
FDA category	Group IV	Group II
ISO category	Filcon 1b	Filcon 4a
Manufacture	Moulded	Moulded
Back Optic Zone Radius	8.7 mm	"Medium"
Total Diameter	14 mm	14.2 mm
Center thickness	0.07 mm	0.10 mm

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

Figure 4.5 describes this experiment in a graphical format.

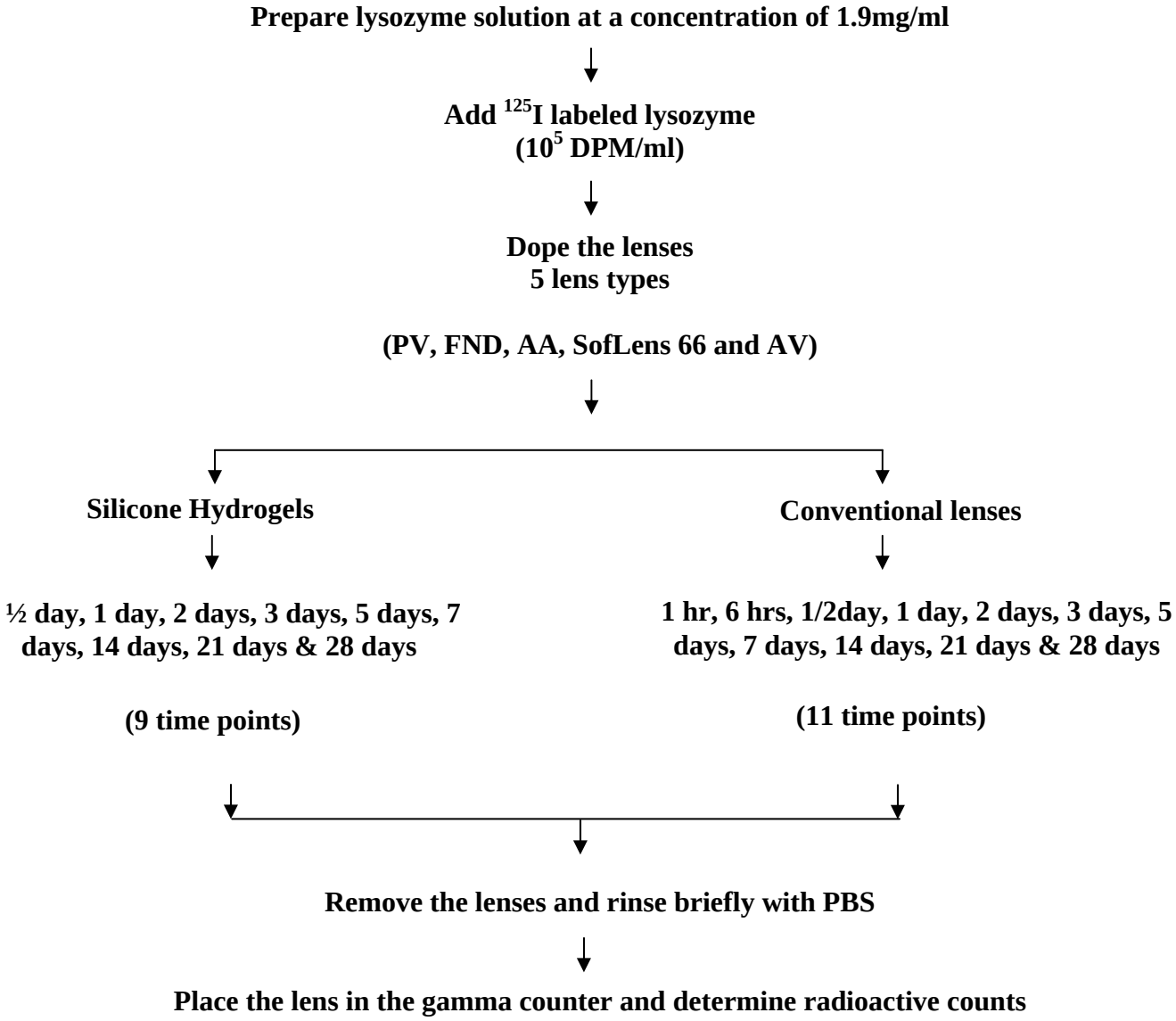


Figure 4.5 Schematic of protocol adopted to determine the kinetics of lysozyme deposition

4.3.2 Doping of lenses in artificial lysozyme solution

Artificial lysozyme solution was prepared at a concentration of 1.9 mg/ml using Phosphate Buffered Saline (PBS), pH 7.4. Lysozyme labeled with ^{125}I was used as the isotopic tracer to quantify protein adsorption to new contact lenses. ^{125}I labeled lysozyme was added to unlabeled solution such that the samples had a counting rate of 10^5 disintegrations per minute/ml (DPM/ml).

As soon as the lenses were opened from the blister pack, they were initially rinsed with Phosphate Buffered Saline (PBS), pH 7.4 to ensure that the packaging solution did not enter the artificial lysozyme solution. The lenses were then placed in 1ml of the labeled lysozyme solution and were allowed to incubate at a temperature of 37°C with constant rotation for different time periods (Figure 4.5). The labeled lysozyme solution was replaced with identical quantity and counting rate, once in seven days in order to simulate the process of replenishment of the tears.

Following the specified days of doping (Figure 4.5), the lenses were aseptically collected using forceps and were rinsed briefly in saline to remove unbound protein sticking on to the lenses. The lenses were then placed in sterile, 5ml (12 X 75 mm), non-pyrogenic, polypropylene round bottom tubes and were counted in the Gamma Counter (Beckman Gamma 5500). The amount of protein adsorbed to the lenses was calculated by dividing the counts on the lenses by the specific activity of the protein. [48]

4.3.3 Data Analysis

All data are reported as mean ± SD. Analysis of Variance was used to determine statistically significant differences between deposition across various time points and differences between materials at any time point. Post hoc multiple comparison testing was undertaken using Bonferroni test. In all cases, a p value of <0.05 was considered significant.

4.4 Influence of rewetting drops on protein and lysozyme deposition

4.4.1 Collection of human worn contact lenses

One type of contact lens [lotrafilcon A, (Focus® Night & Day™ (FND), CIBA Vision] was collected from a clinical study within the Center for Contact Lens Research at the University of Waterloo, following completion of specified wear and care regimens. The study was conducted as a 2-month prospective clinical trial, using a randomized cross-over design. Participants wore a pair of FND lenses on a daily wear basis. Each participant was issued one care system (OPTI-FREE® EXPRESS®) which was used in accordance with the manufacturer's specifications. All participants were assigned to use either CLENS-100® (Alcon) or an unpreserved saline drop MINIMS sodium chloride (Bausch and Lomb) four times a day. After the first wearing period of 28 days, the lenses were replaced with a new set and the second drop was used.

Upon completion of the 28-day wearing period, lenses were collected aseptically (using non-powdered surgical gloves) and placed in individual, sealed glass vials. The right eye lens was placed in 1.5ml of 50:50 mix of 0.2% trifluoroacetic acid and acetonitrile (ACN/ TFA). All lenses collected in ACN/TFA were maintained in the dark at room temperature for 24 hours. [74]

4.4.2 Reagents and materials

All PhastSystem™ pre-cast gels, buffer strips, well combs, filter paper and ECL-Plus™ kits were purchased from Amersham Pharmacia Biotech (Baie d'Urfe, QC, Canada).

Immuno-Blot® PVDF (polyvinylidene difluoride) membrane was purchased from Bio-Rad Laboratories (Mississauga, ON, Canada). Polyclonal rabbit anti-human lysozyme was purchased from Cedarlane Laboratories (Hornby, ON, Canada) and goat anti-rabbit IgG-HRP was purchased from Sigma (St. Louis, MO, USA). Human lysozyme (neutrophil) was purchased from Calbiochem (La Jolla, CA, USA). Chicken egg lysozyme and lyophilized *Micrococcus lysodeikticus* cells were purchased from Sigma (St. Louis, MO, USA). All other reagents purchased were analytical grade and obtained from Sigma (St. Louis, MO, USA).

4.4.3 Protein deposit extraction from contact lenses

Lenses collected in ACN/TFA were incubated in the dark at room temperature for 24 hours. Two 0.70 ml aliquots of ACN/TFA was transferred to sterile eppendorf tubes and lyophilized to dryness in a Savant Speed Vac (Halbrook, NY, USA). Dried protein pellets were stored at –70^{o}C prior to reconstitution.

4.4.4 Sample processing following extraction in ACN/TFA-based extraction buffer

Preliminary results in our laboratory demonstrated that lysozyme deposits extracted from silicone hydrogel contact lens materials demonstrated a loss in total mass as a function of storage time when assessed by Western blotting. This loss represents a potential source of error when quantifying total lysozyme deposition.

Hence, prior to electrophoresis, lyophilized protein pellets were reconstituted in modified reconstitution buffer (10 mM Tris-HCl; 1 mM EDTA, with 0.9% saline) pH 12.0 and BioStab™ Biomolecule Storage Solution (Sigma Aldrich). Reconstituted samples were used for Western blot, lysozyme activity analysis and total protein.

4.4.4.1 Lysozyme standard range

Human lysozyme standard curves were run on each Western blot so that four points falling within the linear range of detection were produced, to facilitate regression analysis of sample extracts. Standards were prepared fresh on the day of analysis from a 0.2 μg/μL working solution of purified human neutrophil lysozyme. Standards were diluted with dilution buffer and then mixed with gel loading buffer (5% SDS; 100 mM Tris, pH 7.4; 30% Glycerol; 1 mM EDTA; 0.02% bromophenol blue). The optimal set of concentrations for lysozyme quantification was determined to be 0.01, 0.005, 0.0025, 0.001 μg/μL.

4.4.5 Electrophoresis and immunoblotting

Once prepared, samples were subjected to SDS-PAGE followed by Western blotting to PVDF membranes using the PhastSystem™ (Amersham-Pharmacia Biotech) as described earlier (Section 4.1.2.6) and also in a previously published paper. [13]

Lysozyme was identified using a rabbit anti-human lysozyme polyclonal antibody (Calbiochem), followed by a peroxidase conjugated goat anti-rabbit secondary antibody

(Sigma-Aldrich). Individual standard curves of purified human neutrophil lysozyme (Calbiochem) were run on each gel to facilitate regression analysis. Immunoreactivity was visualized by incubating with ECL Plus chemiluminescent substrate (Amersham-Pharmacia Biotech). Optical densities of the resulting bands were quantified from digitized images created with a Molecular® Dynamics Storm™ 840 Imager using ImageQuant™ 5.1.

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

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

4.4.6 Measurement of lysozyme activity

The extracts were assayed for lysozyme activity using a fresh suspension of *Micrococcus lysodeikticus* for each sample. Two milligrams of desiccated *micrococcyl* cells were weighed out and suspended in a volume of 50 mM sodium phosphate buffer (pH 6.3) such that an initial optical density (OD) of 0.9-1.0 was achieved at 450 nm (Multiskan Spectrum ELISA Plate Reader, fitted with a micro-cuvette, ThermoLabsystems). 1 ml of buffered *micrococcyl* solution was used for each sample tested. Each 1 ml volume was placed in a 1.5 ml cuvette and incubated in the cuvette housing at 30^{o} C. All samples were compared to the results of 0.1 μg human neutrophil lysozyme standard added to a 1 ml volume from the *same batch* of buffered *micrococcyl* solution. The initial optical density at 450 nm (OD450), was taken for all samples (time=0) as well as at 30 second intervals

for a length of five minutes after the addition of the appropriate volume of sample (added in a volume of no more than 10 μl). The deviation in OD450 between each time point from time zero was calculated and plotted.

Linear regression analysis was then applied to the curve. Experiments with R^2 values higher than 0.98 were only used. The equation of the regression line was calculated to give the slope. Specific activity (SA) for the standard and each sample tested was then determined using the equation:

$$SA = slope \times 1000/mg\ of\ lysozyme\ used$$

As both the standard and sample specific activities were known (and were determined from the same *micrococcyl* stock solution), one could directly compare the effects of the same quantity of lysozyme on the *micrococcyl* solution. Finally, to determine the amount of lysozyme from the lens extract still in native form, the following formula was employed:

$$Native\ lysozyme = total\ lysozyme\ (sample\ SA / standard\ SA)$$

where total lysozyme was pre-determined via Western blot analysis as described above.

The denatured lysozyme component was derived by:

$$Denatured\ lysozyme = total\ lysozyme - native\ lysozyme$$

The final calculation was the percent of denatured lysozyme:

$$\%\ denatured\ total\ lysozyme = (denatured\ lysozyme / total\ lysozyme) \times 100$$

4.4.7 Measurement of total protein deposition

Amido Black Dot Blot Protein Assay was used to determine the total protein deposited on the lenses. In this method, Nitrocellulose membrane (Bio-Rad Laboratories, CA, USA) was pre-treated with 15% (v/v) phosphoric acid and 10% (v/v) methanol for one minute. This pre-treated membrane was blotted between two sheets of filter paper and air-dried at room temperature. This membrane was placed in water briefly and was positioned into the Bio-Dot ® Microfiltration Apparatus (Bio-Rad Laboratories). A fresh set of protein standards was prepared by diluting 2.0mg/ml BSA stock standard in Phosphate-buffered saline (PBS) at pH 7.2. Protein solutions (concentrations ranging between 0.5 and 16 μg/ml) were applied to membrane in 50 μl volumes. Following the application of stock and the sample solutions, all the wells were rinsed with 100μl of PBS. After rinsing, the membrane was air-dried and was immersed in 1.5% glutaraldehyde for 5 minutes. Then the membrane was washed twice with 1N NaOH to remove glutaraldehyde and then with 0.05% (v/v) HCl solution to neutralise NaOH. The membrane was then immersed in Amido Black Stain [0.1% (w/v) amido black, 5% (v/v) methanol, 10% (v/v) glacial acetic acid]. The membrane was then destained with large volumes of 40% (v/v) methanol, 10% (v/v) glacial acetic acid and was dried at room temperature. Stained protein dot-blots were imaged on the Syngene® with GeneSnap™ using white light and were quantified using Gene Tools™.

4.4.8 Data Analysis

All data are reported as mean ± SD. All data were tested for normality using Kolmogorov-Smirnov test. If the data proved to be parametric, then student t-test was used to determine statistically significant differences between the two care regimens. If the data proved to be non-parametric in nature, then a non-parametric Wilcoxon signed rank-sum test was used. In all cases, significance level was taken as $p < 0.05$.

5 Results

5.1 Stabilization study

Table 5.1 shows the percentage of lysozyme loss when the lyophilized sample extracts were resuspended in the "standard" reconstitution buffer, stored with and without the addition of BioStab, gel loading buffer and under the two storage temperatures (-20º C and -70º C). The addition of BioStab significantly reduced the amount of lysozyme mass loss.

Table 5.1 Percentage loss of lysozyme after 48 hours of storage when the samples were resusupended in the Standard Reconstitution Buffer.

	-20°C			-70°C		
	No Biostab	**With Biostab**	**p**	**No Biostab**	**With Biostab**	**p**
With GLB	32.9% ± 0.6	14.3% ± 2.5	< 0.001	31.3% ± 1.1	13.1% ± 1.9	< 0.001
Without GLB	33.5% ± 0.7	15.1% ± 1.5	< 0.001	31.7% ± 1.3	13.1% ± 1.3	< 0.001

Table 5.2 shows the percentage of lysozyme loss when the lyophilized sample extracts were resuspended in the "modified" reconstitution buffer, stored with and without the addition of BioStab, gel loading buffer and under the two storage conditions (-20º C and -70º C).

Table 5.2 Percentage loss of lysozyme after 48 hours of storage when the samples were resusupended in the Modified Reconstitution Buffer.

	-20°C			-70°C		
	No Biostab	**With Biostab**	**p**	**No Biostab**	**With Biostab**	**p**
With GLB	19.2% ± 4.5	0.9% ± 0.8	< 0.001	17.1% ± 2.8	0.6% ± 0.8	< 0.001
Without GLB	19.5% ± 4.3	1.4% ± 1.3	< 0.001	18.2% ± 1.9	1.1% ± 1.4	< 0.001

A four way analysis of variance was performed for all the data. The results indicated that buffer composition ($p<0.001$), storage temperature ($p=0.04$) and addition of BioStab ($p<0.001$) were all important in controlling loss of mass of lysozyme over time. However, no significant difference was found when the samples were stored with and without the addition of gel loading buffer ($p=0.373$).

No signal was seen on negative control Western blots run with BioStab, indicating that the enzyme stabilizer itself is not cross reactive with the Western blotting procedure used in this experiment.

5.2 Extraction efficiency study

The extraction efficiency for different lens types using the 0.2% TFA/ACN extraction buffer is detailed in Figure 5.1.

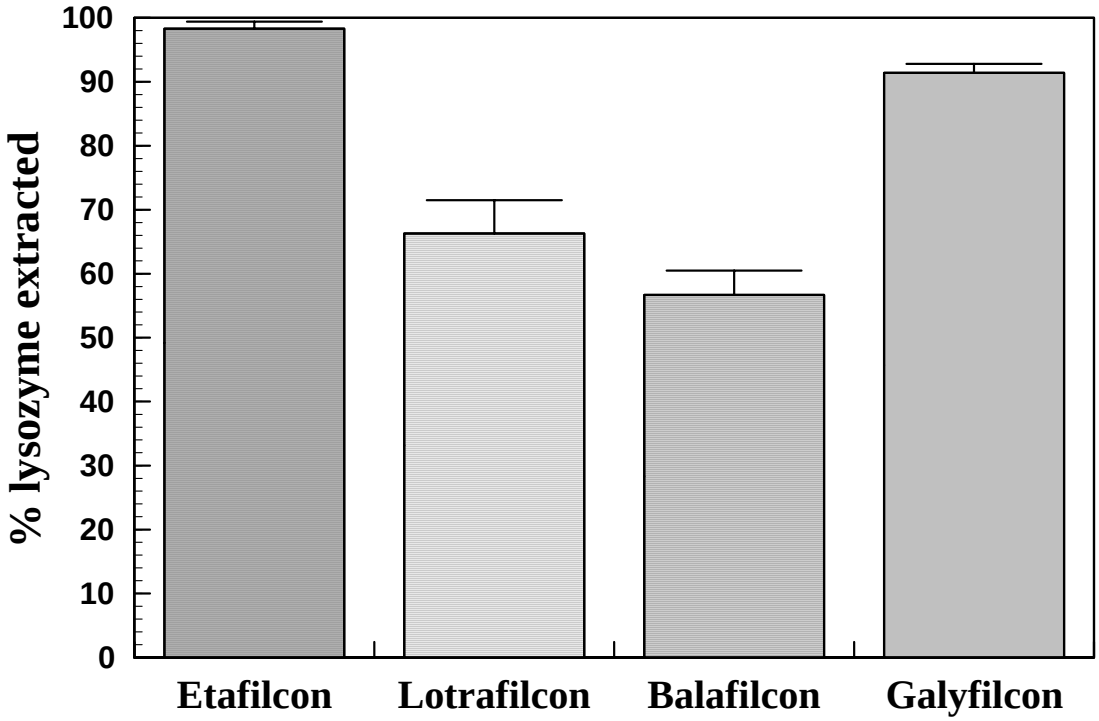


Figure 5.1 Percentage of lysozyme extracted by 0.2% TFA/ACN from different lens types

Analysis of Variance was performed for all the data. The results indicated that there were significant differences between etafilcon and lotrafilcon A ($p<0.001$) and etafilcon and balafilcon lens materials ($p<0.001$). Post hoc analysis by Tukey test showed that there was no significant difference between etafilcon and galyfilcon lens materials (p=NS).

5.3 Kinetics Study

Figures 5.2 and 5.3 show the kinetics of lysozyme deposition on alphafilcon and etaficon lens materials, while Figure 5.4 shows the kinetics of lysozyme deposition on the three types of SH lens materials.

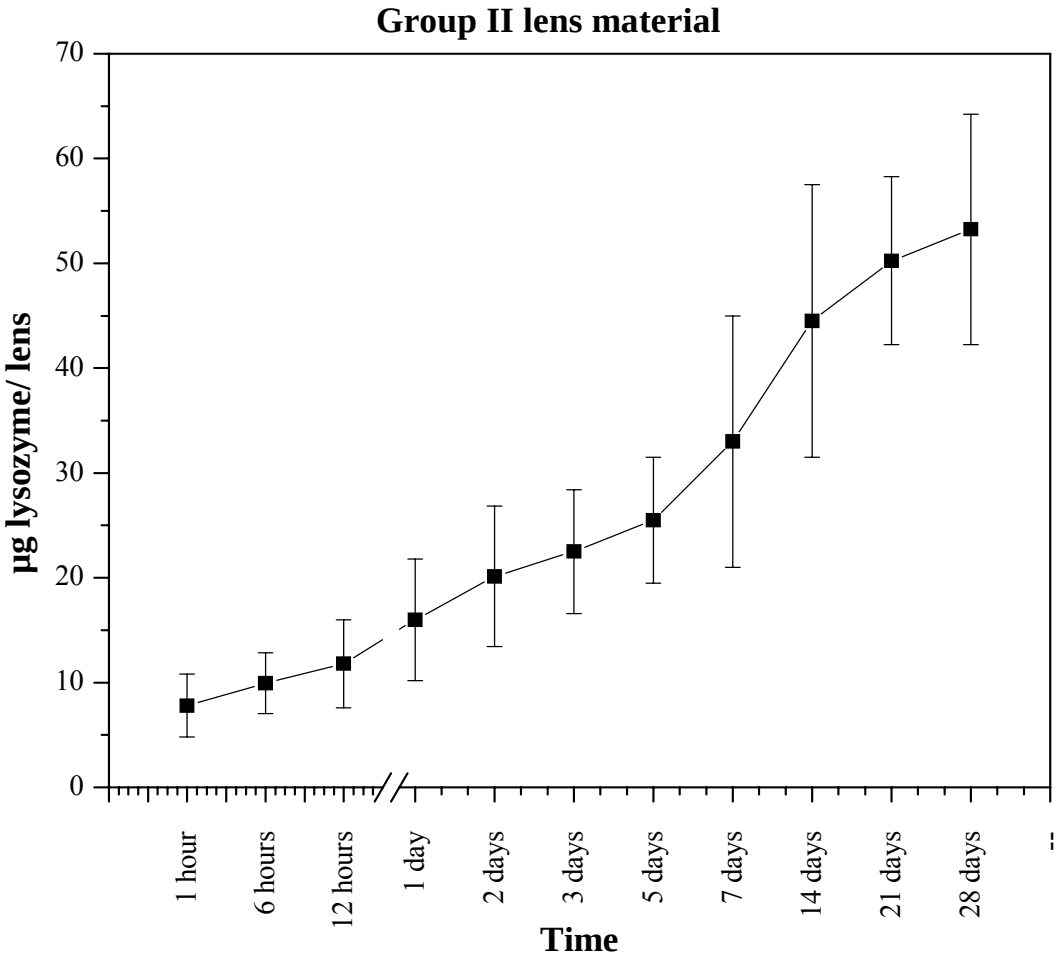


Figure 5.2 Lysozyme deposition curve for Alphafilcon A (Group II) lens material

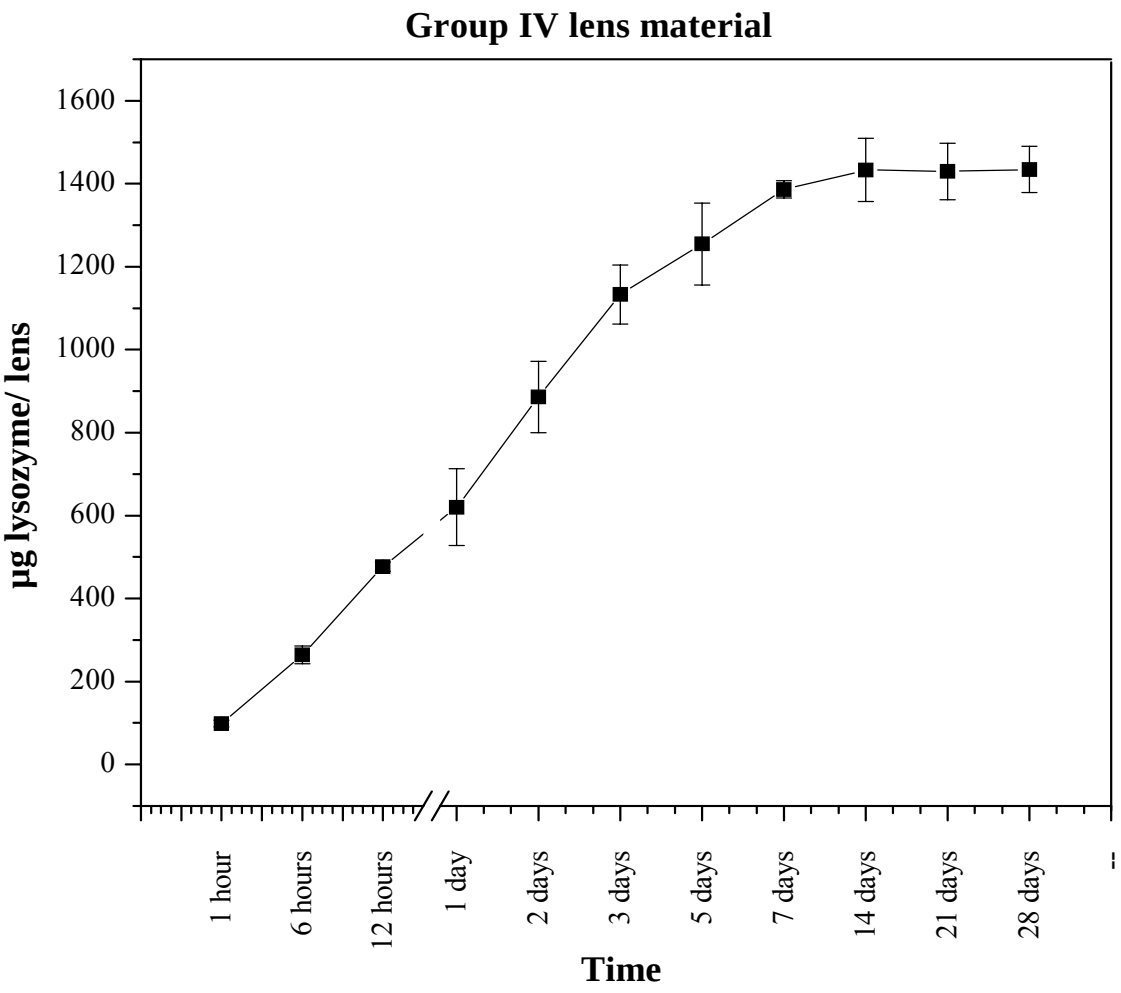


Figure 5.3 Lysozyme deposition curve for Etafilcon A (Group IV) lens material

Etafilcon lens material deposited significantly more lysozyme than all other lens materials ($p<0.001$). In the Group IV lens material, the amount of lysozyme increased significantly between days 1 and 7 ($p<0.001$) and then reached a plateau, with no further increase occurring ($p>0.05$).

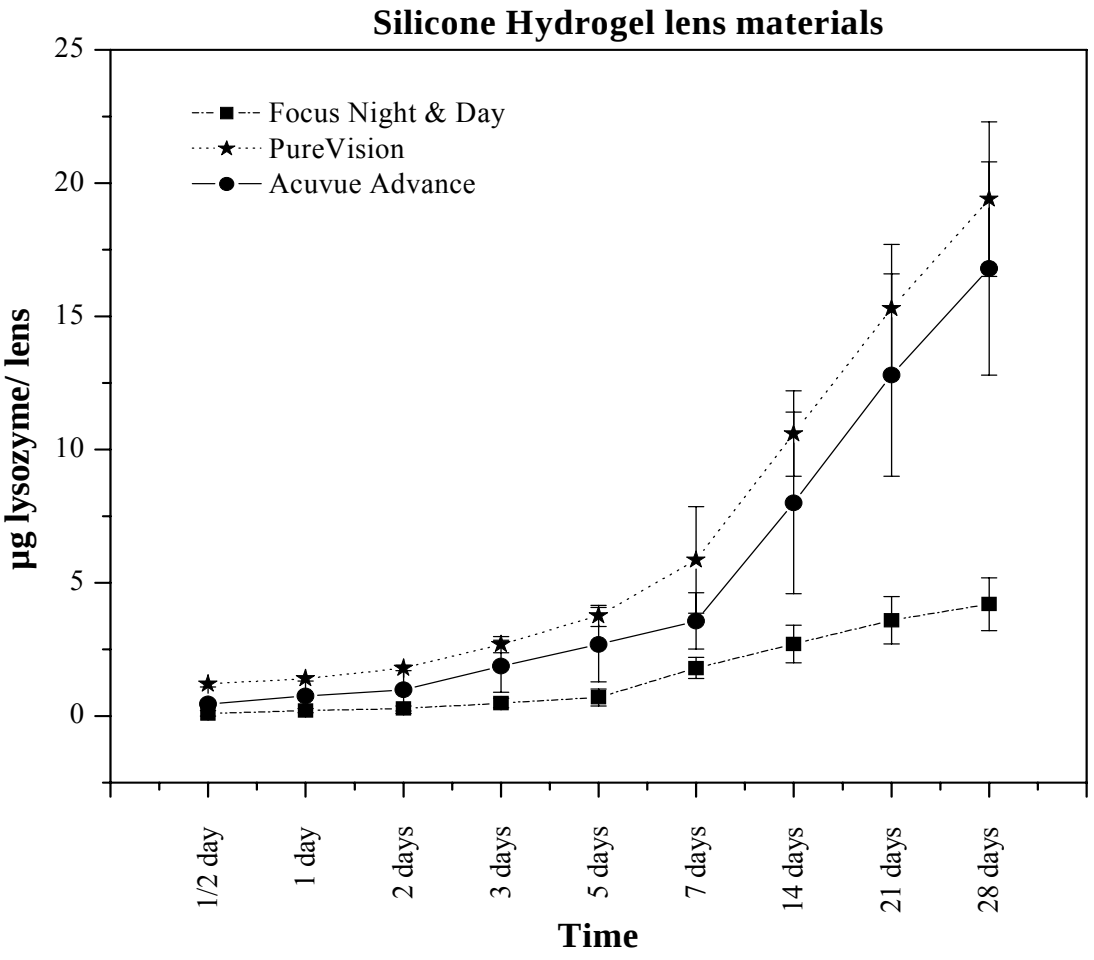


Figure 5.4 Lysozyme deposit curve on FND, PV and AA (SH) lens materials

With the Group II and SH lens materials there was a significant increase in lysozyme deposition across all time points ($p<0.001$). At 7 days, lotrafilcon A lenses deposited 1.8±0.4 µg of lysozyme per lens while balafilcon and galyfilcon lenses deposited 5.9±2 and 3.6±1 µg of lysozyme respectively. After 28 days of doping, lotrafilcon A lenses deposited 4.2±1 µg of lysozyme per lens while balafilcon and galyfilcon lenses deposited 19.4±3 and 16.8±4 µg of lysozyme respectively.

There was a significant difference in lysozyme deposition between the conventional lens materials and all the three SH lens materials across all time periods ($p<0.001$). Between SH lens materials, Bonferroni post-hoc analysis showed that there were no significant differences between lotrafilcon A, balafilcon and galyfilcon lens materials until 3 days ($p>0.05$). There were significant differences in lysozyme deposition between balafilcon versus galyfilcon ($p<0.05$), lotrafilcon A versus balafilcon lenses ($p<0.001$) and lotrafilcon A versus galyfilcon lenses ($p<0.001$) across all time periods after 5 days.

5.4 Influence of rewetting drops on protein and lysozyme deposition

5.4.1 Western blot quantification of lysozyme deposition

Lysozyme standard curves gave a range of R^2 values between 0.97 and 0.99, as demonstrated in Figure 5.5. Table 5.3 details the lysozyme deposition on FND lenses when subjects used an unpreserved saline drop versus a rewetting drop (CLENS-100®). The results demonstrate that lysozyme deposition was greater when subjects used unpreserved saline instead of CLENS-100® ($p<0.001$).

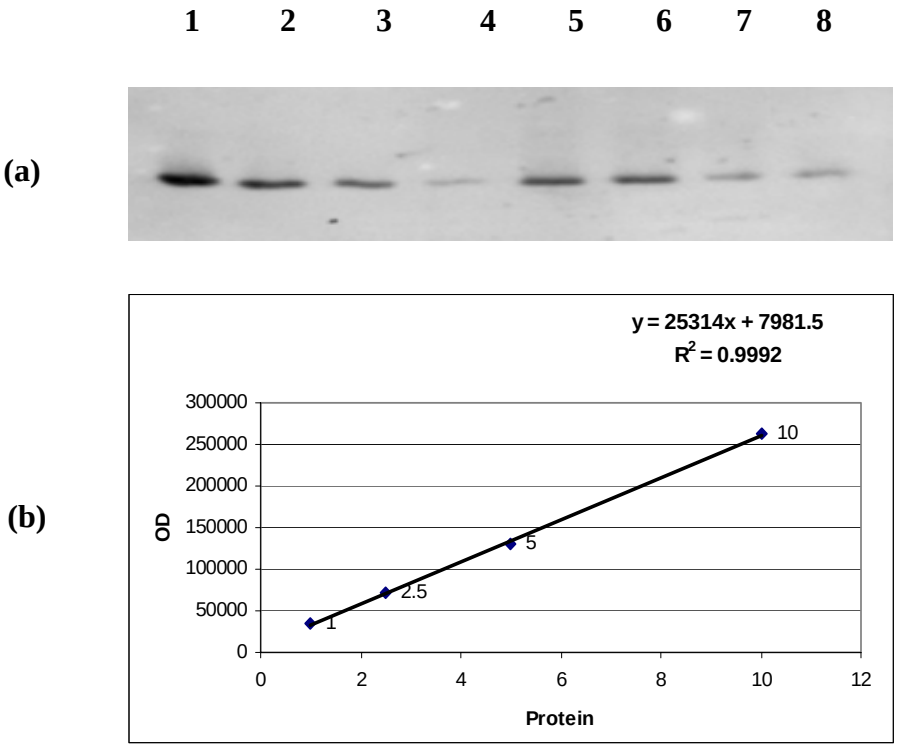


Figure 5.5 Western blot and regression analysis for lysozyme quantification

(a) An example of a western blot on FND lens extracts. Lanes 1-4: Lysozyme standard curve (Lane 1: 0.01; Lane 2: 0.005; Lane 3: 0.0025; Lane 4: 0.001 μg/μl). Lanes 5-6: Protein extracts from FND lenses when subjects used MINIMS sodium chloride. Lanes 7-8: Protein extracts from FND lenses when subjects used CLENS 100. (b) A typical regression curve created by graphing applied concentration of lysozyme standard (lanes 1- 4 from western blot) against the optical density of the resulting band immunoreactivity. Lysozyme concentration of lens extracts was quantified by extrapolation from this curve.

Table 5.3 Summary of lysozyme deposition data (N = 25).

MINIMS NaCl		CLENS-100®		p value (between care regimens)
Lysozyme (μg/lens)	Range (μg/lens)	Lysozyme (μg/lens)	Range (μg/lens)	
1.14 ± 0.7	0.53 to 3.13	0.73 ± 0.5	0.34 to 2.14	< 0.001

5.4.2 Assessment of lysozyme activity

A summary of the lysozyme activity results is presented in Table 5.4. The percentage of denatured lysozyme was reduced when subjects used CLENS-100® drops when compared with the control drops (p=0.002).

Table 5.4 Summary of lysozyme activity data (N = 25)

MINIMS NaCl		CLENS-100®		p value (between care regimens)
% Denaturation per lens	Range (%)	% Denaturation per lens	Range (%)	
85 ± 7	71 to 98	76 ± 10	64 to 97	0.002

5.4.3 Total protein

Table 5.5 details the total protein deposition found on FND lenses. Total protein deposited on FND lenses was significantly greater when MINIMS Sodium Chloride was used than when CLENS-100® was used ($p<0.001$).

Table 5.5 Summary of total protein deposition data (N = 25)

MINIMS NaCl		CLENS-100®		p value (between care regimens)
Total protein (μg/lens)	Range (μg/lens)	Total protein (μg/lens)	Range (μg/lens)	
1.86 ± 0.8	0.7 to 3.6	1.17 ± 0.7	0.5 to 2.8	< 0.001

6 Discussion and conclusions

There are over 60 proteins that have been detected in the tear film [134] and among these lysozyme has attracted the greatest attention. [2, 7, 38, 54, 55] Several studies have reported that lysozyme is the predominant tear film protein deposited on hydrogel lenses [7, 42, 44-46, 71, 72] and also that it plays an important role in ocular immunology. [30-33, 135, 136] Hence I chose to focus on the analysis of lysozyme deposited on SH lenses and also on its extraction and deposition as a function of time on SH lenses and compare it with what is seen on conventional hydrogel lens materials.

A method has been optimized in our laboratories to quantify the minute quantities of lysozyme deposited on SH lens materials. [13] One of the objectives of this thesis was to enhance this existing technique, such that the deposits extracted from SH contact lenses could be accurately and sensitively analyzed. Thus, the first project of this thesis involved the development of a method whereby lysozyme mass extracted from SH lens materials would be preserved over time and would be compatible with the optimized Western blotting procedure. This methodological development was incorporated into a clinical study wherein the difference in the degree of total protein, the difference in lysozyme deposition and activity recovered from lotrafilcon A SH lens material when subjects used surfactant containing rewetting drop (CLENS-100®) versus control saline was investigated.

The remaining studies were *in vitro* experiments wherein the lenses were doped in artificial lysozyme solution containing ^{125}I-labeled lysozyme. These experiments were performed to gain insight into:

1. the efficiency of extraction of the 50:50 mix of 0.2% trifluoroacetic acid and acetonitrile (ACN/TFA) (as this extraction reagent has been fully validated for traditional hydrogel lenses, but not for SH lenses)
2. the kinetics of lysozyme deposition on SH lenses and compare it with Group IV and Group II lens materials.

6.1 Stabilization Study

Preliminary work in our laboratories has shown that there is a substantial loss in lysozyme mass following extraction from SH lenses and subsequent processing (lyophilization, resuspension and storage). This is particularly true for lotrafilcon-based materials. Such loss in lysozyme mass has been previously reported by other groups looking at tears [94] and saliva, [95] but in these cases the concentration of lysozyme was significantly higher than that typically found on lotrafilcon-based hydrogel lenses, which typically deposit <5μg of lysozyme per lens. [12-14] An alternative reason for this loss in lysozyme mass is that lysozyme may undergo dimerization [99] or aggregation, [137] resulting in failure to be recognized by the antibody used in our Western blotting assay. However, preliminary work in our lab suggests that dimerized lysozyme would be detected with the polyclonal antibody used in this assay. Thus, my goal was to devise a protocol to reduce the degree of lysozyme loss, as this would serve as a significant tool for many research areas in which the examination of small amounts of lysozyme, in either solution or on the surface of biomaterials, is important.

Lysozyme is a globular protein which is relatively stable when compared to most other proteins found in tears. However, in the quantitation of lysozyme deposited on a contact lens, (which, depending on the quantification method, may require a significant degree of initial processing such as extraction, lyophilization, resuspension and storage), it is possible that the conformational state of any protein, including lysozyme, could be significantly altered. Altered conformation has significant implications if the quantitative technique being employed uses an antibody to recognize the protein of interest (for

example Western blotting or ELISA) and may be more critical than if the protein is being quantified by a method that does not involve antibody recognition (for example, High Performance Liquid Chromatography).

The stability of proteins in solution has been a major concern for biotechnologists and the pharmaceutical industry. Several studies have been conducted and it has been recognized that long term stability of proteins can be improved by adding substances such as sugars (eg Dextran, [138-140] Trehalose, [141-143] Sucrose [143, 144]), salts [145-149] and polyols such as Sorbitol. [150, 151] The current understanding of protein stabilization has been achieved by thermodynamic measurements of interactions and micro-environmental changes occurring upon addition of a stabilizing compound and also through Nuclear Magnetic Resonance Spectroscopy (NMR), Differential Scanning Calorimetry (DSC) and Circular Dichroism (CD). It is believed that the stabilizing phenomenon is a complex one and no single mechanism is responsible for stabilization.

This project set out to develop a protocol that would reduce the loss in lysozyme mass over time from an elute from a SH contact lens. The two potential protein stabilizers that were used in this study were 0.9% saline and a proprietary product developed for protein stabilization (BioStab™ Biomolecule Storage Solution). The presence of buffer or salt solution is believed to maintain the native conformational state of lysozyme. [148] The stabilizing effects of salts have been attributed mainly to their ability to mask the protein of interest from the surrounding solvents. The exclusion of harsh solvents from the protein surface leads to 'preferential hydration' of the protein or 'preferential exclusion'

of the additive from the protein surface, limiting their denaturing effect. BioStab™ Biomolecule Storage Solution is a solution which is free of DNAses, RNAses and proteases. This product is an aqueous solution which contains a non-ionic detergent and is non toxic. The producers and distributors of the product claim that this product increases storability of biomolecules such as enzymes, antibodies and DNA. Despite repeated attempts to obtain the exact chemical composition of the product, I was unable to obtain any further information and thus am unable to ascertain what components were exactly responsible for imparting such a protective effect during our analysis. However, examination of Tables 5.1 and 5.2 clearly demonstrate that this product has a marked influence in controlling the loss of lysozyme mass over storage time, with no apparent impact on its ability to be recognized by a suitable antibody.

The samples were tested by storing them with and without the addition of gel loading buffer in order to determine whether any of the components in the gel loading buffer was responsible for altering the structure of lysozyme. One of the major components in the gel loading buffer is glycerol (at a concentration of 30%). Glycerol itself has a potential stabilizing effect on protein molecules, [151, 152] however, we did not find any significant difference when the samples were stored with and without the addition of gel loading buffer.

6.1.1 Conclusion

A procedure has been optimized using a modified reconstitution buffer, BioStab™ Biomolecule Storage Solution and storage at -70°C in which the percentage loss of

lysozyme following extraction from lotrafilcon A contact lenses has been reduced from approximately 33% to less than 1%. This revised protocol will be of significant value for researchers interested in studying the deposition of proteins onto substrates in both ocular and non-ocular research areas.

This study was conducted on deposited lysozyme recovered from only one type of SH contact lens material. Further work must be undertaken to examine the impact of this protocol on other proteins and on proteins recovered from other types of SH lens materials.

6.2 Extraction Efficiency Study

This study specifically investigated the efficacy of the removal of lysozyme from etafilcon and three types of SH lens materials (lotrafilcon A, balafilcon and galyfilcon) using an *in vitro* deposition model. This technique was sensitive enough to investigate the extraction efficiency from SH lens materials, which deposit very low quantities of lysozyme. Extraction of proteins from hydrogel lenses using 0.2% TFA/ ACN is a commonly reported technique [12, 74, 104, 122, 153] and this buffer has been used previously for extraction of deposits from SH materials. [12, 13, 27] However, only one study has discussed the efficacy of removing lysozyme by the acid-based extraction technique and has compared that with SDS-based methods for SH lens materials. [13]

The results from this study demonstrate that the method developed by Keith and colleagues based upon TFA and ACN is 100% efficient in removing lysozyme from artificially spoiled etafilcon lens materials, which is consistent with the data from Keith and colleagues. [74] The mechanism for protein removal by this solution is thought to be due in part to an ion exchange interaction between the solution, protein and lens, along with strong solvation properties of the acetonitrile-trifluoroacetic acid mixture. [74] As the solution is acidic, it readily provides protons to interact with negative sites in the lenses. This can occur as an ionic interaction at an open negative site or as an exchange at an existing ionic bond between protein and lens. [154] In addition, the solution has strong solvation properties for proteins, in that it has ionic, aqueous and organic properties. Thus, the combination of these two properties provides a means of removing protein from lenses and readily solubilizing and maintaining it in solution. [154] However, the efficacy of

this method in extracting lysozyme from SH lens materials is lower. Although the current method could extract 91.4 ± 1.4 % of the lysozyme deposited on artificially spoiled galyfilcon lenses, it could extract only 66.3 ± 5.2 % and 56.7 ± 3.8 % from lotrafilcon A and balafilcon lenses respectively.

The possible reason for this reduced extraction efficiency from these two lens types could be due to the surface modification process that is involved with these lens materials. The surfaces of the lotrafilcon lenses are permanently modified in a gas plasma reactive chamber [23, 25] while the balafilcon lenses are surface treated in a gas plasma reactive chamber, which transforms the silicone components on the surface of the lenses into hydrophilic silicate compounds. [23, 26] No surface treatment is required for etafilcon and galyfilcon lenses. Hence this surface modification process in lotrafilcon A and balafilcon lens types could interfere with the buffer's ability to break the bonds between deposited lysozyme and the lens surface resulting in decreased extraction from these two lens materials.

It is a well established fact that lysozyme deposition is affected by the water content and the ionicity of lens materials. [2, 3, 5, 7, 37, 47, 79, 120, 155] However, it is not known if these factors affect lysozyme removal or if the extraction buffers are strong enough to overcome these effects. The ability of the components of an extraction buffer to break chemical bonds and encapsulate proteins for removal from the contact lens surface is determined by the extraction efficiency. Each type of protein deposited on a material may

have a different set of bonds and steric constraints, allowing it to remain on the surface. The extraction buffer should have the ability to break these interactions.

It is clear that SH lenses will still deposit significantly less lysozyme compared to traditional hydrogels. To overcome this issue of inefficiency, a new buffer has to be developed which has the capability to remove 100% of lysozyme from SH lenses, which will have minimal effect on lysozyme conformation and will be compatible with current and future methodologies used to quantify lysozyme concentration and conformation. Hence, the development of an efficient method to extract lysozyme from SH lens materials will be a valuable step in the investigation of the interaction of these contact lens materials with the human tear film.

6.3 Kinetics Study

Although radiochemical analysis offers great sensitivity for quantitation of small amounts of material, it has been used infrequently to measure deposition of tear components on contact lenses. Lysozyme was selected for these studies because it is a recognized prominent lens soilant and a significantly greater quantity of lysozyme binds to ionic, high-water-content (Group IV) lenses than to other types of lenses and is therefore often used as the prototypical marker for protein accumulation. [7, 74, 98] It is widely recognized that the adsorption of proteins at the contact lens surface is complex and depends upon a number of factors. Notable among these are material water content and surface charge. [3, 7, 37, 42, 46, 47, 79, 96, 120] This is the first study to look at the kinetics of lysozyme deposition on SH lens materials and compare it with Group IV and Group II lens materials.

Examination of Graph 5.3 shows that etafilcon A lenses (Group IV) attracted substantial quantities of protein, which was significantly greater than that measured on the alphafilcon A (Group II) and SH lenses ($p < 0.001$). This finding is in accordance with all other previous studies examining protein and lysozyme deposition on different lens groups. [2, 3, 5, 7, 47, 79, 156] This is because methacrylic acid imparts a negative charge to the material and thus thermodynamically favors the deposition of lysozyme which is a positively charged species. Lysozyme accumulated rapidly on Group IV lenses (1 hr, 98±8 μg/lens), reached a maximum on the 7th day (1386±21 μg/lens) and then reached a plateau, with no further increase occurring (p=NS).

Previously published *in vitro* studies investigating the kinetics of protein deposition on conventional hydrogel contact lens materials, most of which use lysozyme [38, 54, 104, 112, 113, 122] or albumin-containing [48, 109, 110] artificial tear solutions, indicate that the initial accumulation of protein on hydrogel materials is rapid, occurring within minutes of exposure to a protein-containing solution. [38, 48, 109, 110, 112, 122] The kinetics of lysozyme adsorption on Group IV lenses broadly follows a three-phase process, with initial adsorption, followed by a rapid increase in which saturation occurs rapidly, and finally a plateau. [38, 54, 110, 111, 113] They also indicate that the ionic binding capacity, water content, and chemical composition of the underlying polymer has a significant impact on the type and extent of protein deposited. [38, 48, 110, 113]

In the group II and SH lens materials, lysozyme deposited was significantly less than that seen with the group IV lenses. The amount of lysozyme deposition increased gradually across all time points and no plateau was achieved ($p<0.05$) (Figures 5.2 and 5.4). Hence, the kinetics of lysozyme deposition pattern in SH lenses were similar to that of Group II lenses in that no plateauing was seen even after 28 days of doping. At 7 days, FND lenses deposited 1.8±0.4 µg of lysozyme per lens while PV and AA lenses deposited 5.9±2 and 3.6±1 µg of lysozyme respectively. After 28 days of doping, FND lenses deposited 4.2±1 µg of lysozyme per lens while PV and AA lenses deposited 19.4±3 and 16.8±4 µg of lysozyme respectively. Results from this study also clearly demonstrates that SH contact lens materials deposit significantly less lysozyme compared to traditional hydrogel materials. Obvious potential factors relate to the differences in surface treatment and bulk composition between the currently available SH lenses.

SH contact lens materials represent a new family of biomaterials that have properties unlike any other previously developed for contact lens use. In addition to differences in surface modification, there are fundamental differences in the bulk chemistry of the polymers. The surfaces of the three (of the four commercially available SH lenses) are surface treated in an attempt to improve the wettability of the materials and to reduce the degree of deposition. [25] Galyfilcon lenses incorporate a long chain, high molecular weight molecule called Hydraclear™, which maintains flexibility and moisture. This wetting agent is present throughout the lenses and hence no surface treatment is required for these lenses. [27] Because of their unique surface and bulk properties, these newly developed SH lens materials are highly resistant to protein deposition. It is difficult to predict and explain the process of deposition in these newly developed SH lens materials.

6.3.1 Conclusions

To-date this is the first study to look at the kinetics of lysozyme deposition on SH lens materials. Radiochemical analysis is a sensitive and effective technique to determine the small quantities of lysozyme deposited on SH lenses. The kinetics of contact lens deposition depends on the chemical structure of lens material under consideration. Lysozyme deposition occurs rapidly with Gp IV materials before reaching a maximum, while SH and Gp II materials progressively accumulate lysozyme, with no plateau occurring.

The kinetics of material build-up on lenses has ramifications for both lens replacement frequency and the regularity of care and maintenance. Elucidating the apparent

differences between studies that do and do not show plateauing may be an important feature in relating deposition to symptomatology. Examination of methodology, differences in lens materials, denaturation of protein, and changes of the inflammatory state of the eye over longer wearing periods may explain these findings. All results analyzing the role of deposition in successful contact lens wear should be viewed against the background of lens age.

6.3.2 Future directions

One of the biggest drawbacks of this study was that the lenses were doped in a solution containing only one type of protein, namely lysozyme. An important factor to consider in the process of deposition relates to whether other substances from the tear film (for example mucins or other proteins) concurrently deposit onto the material surface at different rates. Hence it is necessary to look at the kinetics of deposition using a complex artificial tear solution and to look at the protein-protein, protein-lipid and protein-mucin interactions. It is clear that SH lenses only deposit small amounts of lysozyme and previously published studies suggest that much of this lysozyme is denatured. [12, 13] Hence it would be interesting to look at the activity of lysozyme recovered at various time points. In addition to looking at kinetics of deposition, the kinetics of denaturation and how they relate to wearing period, the influence of various care regimens, and the degree to which other tear proteins denature also require investigation.

6.3.3 Comparison of *in vitro* experiments to *in vivo* studies

Soft lenses are not always readily obtained for analysis from clinical sources. A number of studies have found artificial tear solutions an attractive option in determining the binding affinity of different components and the mechanisms involved in such binding. [110, 121-129] The principal advantages are that many experimental variables are eliminated, quantitation can be enhanced by labeling the species fluorometrically or radiometrically, and simple hypotheses can be tested without having to engage in a resource consuming clinical trial.

However, the real world provides a complex array of variables that cannot be excluded from having an influence in clinical lens spoilage. Such effects include the cycle of evaporative drying and wetting created by the blinking action, the mechanical aspect of shear forces during blinking, differences in tear film protein and lipid composition, structure of the tear film, replenishment rate and volume of tears, wearing times, wearing conditions (for example outdoor or air-conditioned environments and proximity to chemical agents), ultraviolet light exposure, wearing mode (extended wear versus daily wear), interactions between the items under test and other tear film components, and the influence of external contaminants. Moreover, some evidence suggests that deposit formation is caused in part by the thinning and drying of the tear film, resulting in the lipid layer directly collapsing onto the lens surface and partitioning into the dry lens surface. [157, 158] Hence, *in vitro* results may not be directly transferable to the *in vivo* state. However, these *in vitro* studies will provide valuable guidance for further *in vivo* studies.

6.4 Influence of rewetting drops on protein and lysozyme deposition

A number of studies have investigated the effect of surfactants on hydrogel lens materials. [36, 105, 128, 159-166] To-date, this study is the first of its type which has examined the potential use of surfactant-containing rewetting drops with silicone hydrogel lenses. It is a well established fact that the major problem associated with silicone hydrogel lens wear is dryness and associated discomfort that develops over the course of the day. This study investigated the impact of treating SH lenses during their wearing period with a novel rewetting agent that has been specifically developed to reduce in-eye deposition on polyHEMA-based materials.

The results from this study demonstrate that total protein deposition (Table 5.5), lysozyme deposition (Table 5.3) and lysozyme denaturation (Table 5.4) were all significantly reduced when subjects used CLENS-100® compared with the control saline drop. The components of CLENS-100® include a surfactant designed to remove protein, lipids and debris, named Polyethylene glycol -11 lauryl ether carboxylic acid (also called RLM-100, a patented product); and the other important component is a substance that guards lenses against future protein build-up, named poloxamine. One other component is Tetronic® 1304, which helps the lenses to retain moisture and also helps to shield the lens from future protein build-up. Hence the total protein deposition and lysozyme deposition recovered from the lenses were significantly lower ($p<0.001$) when the subjects used CLENS-100® instead of MINIMS sodium chloride.

Generally, surfactants are used by the manufacturers, and include in them high molecular weight substances such as poloxamine (or poloxamer) or a lower molecular weight substance named tyloxapol. [167] Theoretically, it has been suggested that surfactants would alter the interfacial chemistry at the molecular layer between the contaminant and lens. Following this, polar micelles would form and they would entrap the debris and enable them to be rinsed away. Clinically, solutions containing a surfactant have been reported to promote mechanical cleaning as well as provide ongoing cleaning during wear. [166, 168, 169]

Table 5.4 indicates that the amount of denatured lysozyme recovered from lotrafilcon A lenses was 76 ± 10% when the subjects used CLENS-100® which was significantly lower than when subjects used MINIMS sodium chloride (85 ± 7%). The degree to which protein denaturation occurs is mediated by a number of factors, including contact time with the substrate, chemical composition of the substrate, protein type, surrounding pH, and temperature. Proteins are most likely to denature when exposed to strongly hydrophobic surfaces. [38, 170-174] The use of a surfactant containing rewetting drop could have probably increased the hydrophilicity of the contact lens surface. This would lead to lysozyme deposited on lenses getting exposed to a lesser hydrophobic surface resulting in lower denaturation.

Given the known link between protein deposition and immunological changes induced during lens wear, it is possible that reduced protein deposition and a reduction in denatured lysozyme could result in improved vision, reduced mucous discharge and palpebral lid changes. The results from this study were remarkably consistent and

strongly suggest that practitioners should consider prescribing surfactant-containing rewetting drops to patients who use lenses on a continuous wear basis. Silicone hydrogel chemistry is unique - new conditioning/ rewetting products will likely be needed as the popularity of this material grows. The results obtained would provide valuable information about the potential for surface modification of these polymers *in-situ*.

6.5 Summary

1. A procedure has been optimized using a modified reconstitution buffer, BioStab™ Biomolecule Storage Solution and storage at -70°C in which the percentage loss of lysozyme following extraction from lotrafilcon A contact lenses has been reduced from approximately 33% to less than 1%. This revised protocol will be of significant value for researchers interested in studying the deposition of proteins onto substrates in both ocular and non-ocular research areas.
2. The results from the extraction efficiency study showed that 0.2% trifluoroacetic acid/ acetonitrile was not 100% efficient in extracting lysozyme deposits from lotrafilcon A and balafilcon lens materials. However, it was efficient in extracting lysozyme deposited on etafilcon and galyfilcon lens materials.
3. The kinetics of lysozyme deposition on SH lenses was determined using an *in vitro* model. These results demonstrate that the kinetics of lysozyme deposition is material dependent and reiterates that SH lenses deposit very low amounts of lysozyme compared to conventional lenses.
4. The impact of treating SH lenses during their wearing period with a novel rewetting agent that has been specifically developed to reduce in-eye deposition was also investigated. The results from this study demonstrate that total protein deposition, lysozyme deposition and lysozyme denaturation were all reduced when subjects used a surfactant containing rewetting drop instead of a control saline drop.

References

1. Ratner B. New ideas in biomaterials science - a path to engineered biomaterials. *J Biomed Mater Res* 1993; 27: 837-850.

2. Bohnert J, Horbett T, Ratner B, Royce F. Adsorption of proteins from artificial tear solutions to contact lens materials. *Invest Ophthalmol Vis Sci* 1988; 29: 362-373.

3. Minarik L, Rapp J. Protein deposits on individual hydrophilic contact lenses: effects of water and ionicity. *CLAO J* 1989; 15: 185-188.

4. Hart DE, Tidsale RR, Sack RA. Origin and composition of lipid deposits on soft contact lenses. *Ophthalmology* 1986; 93: 495-503.

5. Jones L, Evans K, Sariri R, Franklin V, Tighe B. Lipid and protein deposition of N-vinyl pyrrolidone-containing group II and group IV frequent replacement contact lenses. *CLAO J* 1997; 23: 122-126.

6. Bontempo AR, Rapp J. Protein and lipid deposition onto hydrophilic contact lenses in vivo. *CLAO J* 2001; 27: 75-80.

7. Sack RA, Jones B, Antignani A, Libow R, Harvey H. Specificity and biological activity of the protein deposited on the hydrogel surface. Relationship of polymer structure to biofilm formation. *Invest Ophthalmol Vis Sci* 1987; 28: 842-849.

8. Castillo EJ, Koenig JL, Anderson JM, Jentoft N. Protein adsorption on soft contact lenses. III. Mucin. *Biomaterials* 1986; 7: 9-16.

9. Brennan NA, Coles MLC. Deposits and symptomatology with soft contact lens wear. *ICLC* 2000; 27: 75-100.

10. Wahlgren M, Arnebrant T. Protein adsorption to solid surfaces. *Trends Biotechnol* 1991; 9: 201-208.

11. Sariri R. Protein interaction with hydrogel contact lenses. *Journal of Applied Biomaterials & Biomechanics* 2004; 2: 1-19.

12. Jones L, Senchyna M, Glasier MA, Schickler J, Forbes I, Louie D, May C. Lysozyme and lipid deposition on silicone hydrogel contact lens materials. *Eye Contact Lens* 2003; 29: S75-79; discussion S83-74, S192-194.

13. Senchyna M, Jones L, Louie D, May C, Forbes I, Glasier M. Quantitative and conformational characterization of lysozyme deposited on balafilcon and etafilcon contact lens materials. *Curr Eye Res* 2004; 28: 25-36.

14. McNally J, McKenney C. A clinical look at a silicone hydrogel extended wear lens. *Contact Lens Spectrum* 2002: 38-41.

15. Wichterle O, Lim D. Hydrophilic gels for biological use. *Nature* 1960; 185: 117 - 118.

16. Barr J. Contact lenses and vision - the annual report. *Spectrum* 1997: 21-26.

17. Holden B, Sweeney D, Sanderson G. The minimum pre-corneal oxygen tension to avoid corneal edema. *Invest Ophthalmol Vis Sci* 1984; 25: 476-480.

18. La Hood D. Daytime edema levels with plus powered low and high water content hydrogel contact lenses. *Optom Vis Sci* 1991; 68: 877-880.

19. Sweeney D. Corneal exhaustion syndrome with long-term wear of contact lenses. *Optom Vis Sci* 1992; 69: 601-608.

20. Jones L, Dumbleton K. Silicone hydrogel contact lenses: Part 1 Evolution and current status. *Optometry Today* 2002; 42: 26-31.

21. Grobe GL, 3rd. Surface engineering aspects of silicone hydrogel lenses. *Contact Lens Spectrum* 1999; 14: 14-17.

22. Edwards K, Atkins N. Silicone hydrogel contact lenses: Part 2 Therapeutic applications. *Optometry Today* 2002; 42: 26-29.

23. Tighe B. Silicone hydrogel materials - how do they work ? In: Sweeney D. *Silicone Hydrogels: The Rebirth of Continuous Wear Contact Lenses.* Oxford: Butterworth-Heinemann, 2000; 1-21.

24. Jones L. Modern contact lens materials: A clinical performance update. *Contact Lens Spectrum* 2002; 17: 24-35.

25. Nicolson PC, Vogt J. Soft contact lens polymers: an evolution. *Biomaterials* 2001; 22: 3273-3283.

26. Lopez-Alemany A, Compan V, Refojo MF. Porous structure of Purevision versus Focus Night&Day and conventional hydrogel contact lenses. *J Biomed Mater Res* 2002; 63: 319-325.

27. Steffen R, Schnider CM. A next generation silicone hydrogel lens for daily wear. Part 1 - Material properties. *Optician* 2004; 227: 23-25.

28. Gellatly KW, Brennan NA, Efron N. Visual decrement with deposit accumulation of HEMA contact lenses. *Am J Optom Physiol Opt* 1988; 65: 937-941.

29. Jones L, Franklin V, Evans K, Sariri R, Tighe B. Spoilation and clinical performance of monthly vs three monthly group II disposable contact lenses. *Optom Vis Sci* 1996; 73: 16-21.

30. Allansmith MR. Immunologic effects of extended-wear contact lenses. *Ann Ophthalmol* 1989; 21: 465-467, 474.

31. Porazinski AD, Donshik PC. Giant papillary conjunctivitis in frequent replacement contact lens wearers: a retrospective study. *CLAO J* 1999; 25: 142-147.

32. Allansmith MR, Korb DR, Greiner JV, Henriquez AS, Simon MA, Finnemore VM. Giant papillary conjunctivitis in contact lens wearers. *Am J Ophthalmol* 1977; 83: 697-708.

33. Donshik PC. Contact lens chemistry and giant papillary conjunctivitis. *Eye Contact Lens* 2003; 29: S37-39; discussion S57-39, S192-194.

34. Aswad MI, John T, Barza M, Kenyon K, Baum J. Bacterial adherence to extended wear soft contact lenses. *Ophthalmology* 1990; 97: 296-302.

35. Butrus SI, Klotz SA, Misra RP. The adherence of Pseudomonas aeruginosa to soft contact lenses. *Ophthalmology* 1987; 94: 1310-1314.

36. Butrus SI, Klotz SA. Contact lens surface deposits increase the adhesion of Pseudomonas aeruginosa. *Curr Eye Res* 1990; 9: 717-724.

37. Minno GE, Eckel L, Groemminger S, Minno B, Wrzosek T. Quantitative analysis of protein deposits on hydrophilic soft contact lenses:1. Comparison to visual methods of analysis. 2. Deposit variation among FDA lens material groups. *Optom Vis Sci* 1991; 68: 865-872.

38. Garrett Q, Garrett RW, Milthorpe BK. Lysozyme sorption in hydrogel contact lenses. *Invest Ophthalmol Vis Sci* 1999; 40: 897-903.

39. Alvord L, Court J, Davis T, Morgan CF, Schindhelm K, Vogt J, Winterton L. Oxygen permeability of a new type of high Dk soft contact lens material. *Optom Vis Sci* 1998; 75: 30-36.

40. Morgan CF, Brennan NA, Alvord L. Comparison of the coulometric and polarographic measurement of a high-Dk hydrogel. *Optom Vis Sci* 2001; 78: 19-29.

41. Leahy CD, Mandell RB, Lin ST. Initial in vivo tear protein deposition on individual hydrogel contact lenses. *Optom Vis Sci* 1990; 67: 504-511.

42. Lin ST, Mandell RB, Leahy CD, Newell JO. Protein accumulation on disposable extended wear lenses. *CLAO J* 1991; 17: 44-50.

43. Tomlinson A, Simmons P. Surface deposits on low water content hydrogel contact lenses: Comparison of SEM and protein assay techniques. *ICLC* 1991; 18: 88-94.

44. Tripathi PC, Tripathi RC. Analysis of glycoprotein deposits on disposable soft contact lenses. *Invest Ophthalmol Vis Sci* 1992; 33: 121-125.

45. Wedler FC. Analysis of biomaterials deposited on soft contact lenses. *J Biomed Mater Res* 1977; 11: 525-535.

46. Yan GY, Nyquist G, Caldwell KD, Payor R, Mccraw EC. Quantitation of total protein deposits on contact lenses by means of amino acid analysis. *Invest Ophthalmol Vis Sci* 1993; 34: 1804-1813.

47. Myers RI, Larsen DW, Tsao M, Castellano C, Becherer LD, Fontana F, Ghormley NR, Meier G. Quantity of protein deposited on hydrogel contact lenses and its relation to visible protein deposits. *Optom Vis Sci* 1991; 68: 776-782.

48. Baines MG, Cai F, Backman HA. Adsorption and removal of protein bound to hydrogel contact lenses. *Optom Vis Sci* 1990; 67: 807-810.

49. Hu J, Tartaglia L, Kohler J, Lett J, Shih K. Gentle Touch, a lens material resistant to protein deposition. *CLAO J* 1995; 21: 93-95.

50. Franklin V, Bright A, Pearce E, Tighe B. Hydrogel lens spoilation Part 5: Tear proteins and proteinaceous films. *Optician* 1992; 204: 16-26.

51. Lowther G. Clinical aspects of lens spoilation. In: Ruben M and Guillon M. *Contact Lens Practice.* London: Chapman & Hall, 1994; 1119-1134.

52. Berman ER. *Biochemistry of the eye*, ed. Blakemore C. New York: Plenum Press, 1991.

53. Wedler FC, Illman BL, Horensky DS, Mowrey-McKee M. Analysis of protein and mucin components deposited on hydrophilic contact lenses. *Clin Exp Optom* 1987; 70: 59-68.

54. Castillo EJ, Koenig JL, Anderson JM, Lo J. Protein adsorption on hydrogels. II. Reversible and irreversible interactions between lysozyme and soft contact lens surfaces. *Biomaterials* 1985; 6: 338-345.

55. Garrett Q, Chatelier RC, Griesser HJ, Milthorpe BK. Effect of charged groups on the adsorption and penetration of proteins onto and into carboxymethylated poly(HEMA) hydrogels. *Biomaterials* 1998; 19: 2175-2186.

56. Fleming A. On a remarkable bacteriolytic element found in tissues and secretions. *Proc R Soc London (Biol)* 1922; 93: 306-309.

57. Janssen P, van Bijsterveld O. Origin and biosynthesis of human tear fluid proteins. *Invest Ophthalmol Vis Sci* 1983; 24: 623-630.

58. Imoto T, Johnson L, North A, Phillips D, Rupley J. Vertebrate Lysozymes. In: Boyer P. *The Enzyme.* New York: Academic Press, 1972; 665-868.

59. Osserman EF, Canfield RE, Beychok S. *Lysozyme*. New York: Academic Press, 1974.

60. Blake CC, Koenig DF, Mair GA, North AC, Phillips DC, Sarma VR. Structure of hen egg-white lysozyme. A three-dimensional Fourier synthesis at 2 Angstrom resolution. *Nature* 1965; 206: 757-761.

61. Arnon R, Sela M. Antibodies to a unique region in lysozyme provoked by a synthetic antigen conjugate. *Proc Natl Acad Sci U S A* 1969; 62: 163-170.

62. Craig L, Sanschagrin PC, Rozek A, Lackie S, Kuhn LA, Scott JK. The role of structure in antibody cross-reactivity between peptides and folded proteins. *J Mol Biol* 1998; 281: 183-201.

63. Davies DR, Cohen GH. Interactions of protein antigens with antibodies. *Proc Natl Acad Sci U S A* 1996; 93: 7-12.

64. Blake CC, Swan ID. X-ray analysis of structure of human lysozyme at 6 A resolution. *Nat New Biol* 1971; 232: 12-15.

65. Halper JP, Latovitzki N, Bernstein H, Beychok S. Optical activity of human lysozyme. *Proc Natl Acad Sci U S A* 1971; 68: 517-522.

66. Ikeda K, Hamaguchi K, Miwa S, Nishina T. Circular dichroism of human lysozyme. *J Biochem (Tokyo)* 1972; 71: 371-378.

67. Canfield RE, Kammerman S, Sobel JH, Morgan FJ. Primary structure of lysozymes from man and goose. *Nat New Biol* 1971; 232: 16-17.

68. Saint-Blancard J, Chuzel P, Mathieu Y, Perrot J, Jolles P. Influence of pH and ionic strength of the lysis of Micrococcus lysodeikticus cells by six human and four avian lysozymes. *Biochim Biophys Acta* 1970; 220: 300-306.

69. Whikehart DR. *Biochemistry of the eye*. 2nd ed. Philadelphia, Pennsylvania: Butterworth-Heinemann, 2003.

70. Karageozian HL. Use of the amino acid analyzer to illustrate the efficiency of an enzyme preparation for cleaning hydrophilic lenses. *Contacto* 1976; January: 5-10.

71. Hosaka S, Ozawa H, Tanzawa H, Ishida H, Yoshimura K, Momose T, Magatani H, Nakajima A. Analysis of deposits on high water content contact lenses. *J Biomed Mater Res* 1983; 17: 261-274.

72. Bilbaut T, Gachon AM, Dastugue B. Deposits on soft contact lenses. Electrophoresis and scanning electron microscopic examinations. *Exp Eye Res* 1986; 43: 153-165.

73. Castillo EJ, Koenig JL, Anderson JM. Characterization of protein adsorption on soft contact lenses. IV. Comparison of in vivo spoilage with the in vitro adsorption of tear proteins. *Biomaterials* 1986; 7: 89-96.

74. Keith D, Hong B, Christensen M. A novel procedure for the extraction of protein deposits from soft hydrophilic contact lenses for analysis. *Curr Eye Res* 1997; 16: 503-510.

75. Avisar R, Savir H, Shaked P, Menache R, Levinsky H. Lysozyme levels in human tears. *Annals of Ophthalmol* 1999; 31: 231-232.

76. Stern J, Skotnitsky C, O'Hare N, Tan T, Wong R, Sweeney D, Holden B. Comparison of the incidence of contact lens papillary conjunctivitis (CLPC) between six and thirty night high Dk soft extended wear schedules. *Invest Ophthalmol Vis Sci* 2001; 42: s597.

77. Skotnitsky C, Sankaridurg PR, Sweeney DF, Holden BA. General and local contact lens induced papillary conjunctivitis (CLPC). *Clin Exp Optom* 2002; 85: 193-197.

78. Fonn D, MacDonald KE, Richter D, Pritchard N. The ocular response to extended wear of a high Dk silicone hydrogel contact lens. *Clin Exp Optom* 2002; 85: 176-182.

79. Maissa C, Franklin V, Guillon M, Tighe B. Influence of contact lens material surface characteristics and replacement frequency on protein and lipid deposition. *Optom Vis Sci* 1998; 75: 697-705.

80. Gudmundsson OG, Woodward DF, Fowler SA, Allansmith MR. Identification of proteins in contact lens surface deposits by immunofluorescence microscopy. *Arch Ophthalmol* 1985; 103: 196-197.

81. McArthur SL, McLean KM, St John HA, Griesser HJ. XPS and surface-MALDI-MS characterisation of worn HEMA-based contact lenses. *Biomaterials* 2001; 22: 3295-3304.

82. Wilson K, Walker J. *Principles and techniques of practical biochemistry*. 4th ed. Cambridge: Cambridge University Press, 1994.

83. Seal DV. The effect of ageing and disease on tear constituents. *Trans Ophthalmol Soc U K* 1985; 104 (Pt 4): 355-362.

84. Fullard R. Identification of proteins in small tear volumes with and without size exclusion HPLC fractionation. *Curr Eye Res* 1988; 7: 163-179.

85. Hames D. One-dimensional polyacrylamide gel electrophoresis. In: Hames D and Rickwood D. *Gel electrophoresis of proteins: a practical approach.* Oxford: IRL Press, 1990.

86. Coyle PK, Sibony PA, Johnson C. Electrophoresis combined with immunologic identification of human tear proteins. *Invest Ophthalmol Vis Sci* 1989; 30: 1872-1878.

87. Mii S, Nakamura K, Takeo K, Kurimoto S. Analysis of human tear proteins by two-dimensional electrophoresis. *Electrophoresis* 1992; 13: 379-382.

88. Kuizenga A, Vanhaeringen NJ, Kijlstra A. SDS-minigel electrophoresis of human tears - effect of sample treatment on protein patterns. *Invest Ophthalmol Vis Sci* 1991; 32: 381-386.

89. Kuizenga A, van Haeringen N, Kijlstra A. Identification of lectin binding proteins in human tears. *Invest Ophthalmol Vis Sci* 1991; 32: 3277 - 3284.

90. Kuizenga A, van Haeringen NJ, Meijer F, Kijlstra A. Analysis of human tear fluid components, inhibiting protein adhesion to plastic surfaces. *Exp Eye Res* 1996; 63: 319-328.

91. Ogasahara K, Hamaguchi K. Structure of lysozyme. XII. Effect of pH on the stability of lysozyme. *J Biochem (Tokyo)* 1967; 61: 199-210.

92. Ikeda K, Hamaguchi K, Imanishi M, Amano T. Effect of pH on the ultraviolet optical rotatory dispersion and circular dichroism of lysozyme. *J Biochem (Tokyo)* 1967; 62: 315-320.

93. Hamaguchi K, Sakai H. Structure of lysozyme. IX. The effect of temperature on the conformation of lysozyme. *J Biochem (Tokyo)* 1965; 57: 721-732.

94. Sitaramamma T, Shivaji S, Rao GN. Effect of storage on protein concentration of tear samples. *Curr Eye Res* 1998; 17: 1027-1035.

95. Ng V, Koh D, Fu Q, Chia SE. Effects of storage time on stability of salivary immunoglobulin A and lysozyme. *Clin Chim Acta* 2003; 338: 131-134.

96. Bontempo AR, Rapp J. Protein-lipid interaction on the surface of a hydrophilic contact lens in vitro. *Curr Eye Res* 1997; 16: 776-781.

97. Carney FP, Morris CA, Willcox MD. Effect of hydrogel lens wear on the major tear proteins during extended wear. *Aust N Z J Ophthalmol* 1997; 25 Suppl 1: S36-38.

98. Sack RA, Sathe S, Hackworth LA, Willcox MD, Holden BA, Morris CA. The effect of eye closure on protein and complement deposition on Group IV hydrogel contact lenses: relationship to tear flow dynamics. *Curr Eye Res* 1996; 15: 1092-1100.

99. Scott G, Mowrey-McKee M. Dimerization of tear lysozyme on hydrophilic contact lens polymers. *Curr Eye Res* 1996; 15: 461-466.

100. Francoise Gachon AM, Mallet J, Tridon A, Deteix P. Analysis of proteins eluted from hemodialysis membranes. *J Biomater Sci Polym Ed* 1991; 2: 263-276.

101. Stapleton F, Willcox MD, Morris CA, Sweeney DF. Tear changes in contact lens wearers following overnight eye closure. *Curr Eye Res* 1998; 17: 183-188.

102. Baguet J, Sommer F, Claudon-Eyl V, Duc TM. Characterization of lacrymal component accumulation on worn soft contact lens surfaces by atomic force microscopy. *Biomaterials* 1995; 16: 3-9.

103. Weathersby PK, Horbett TA, Hoffman AS. A new method for analysis of the adsorbed plasma protein layer on biomaterial surfaces. *Trans Am Soc Artif Intern Organs* 1976; 22: 242-252.

104. Keith DJ, Christensen MT, Barry JR, Stein JM. Determination of the lysozyme deposit curve in soft contact lenses. *Eye Contact Lens* 2003; 29: 79-82.

105. Franklin V. Cleaning efficacy of single-purpose surfactant cleaners and multi-purpose solutions. *Contact Lens & Anterior Eye* 1997; 20: 63-68.

106. Horbett TA, Weathersby PK. Adsorption of proteins from plasma to a series of hydrophilic-hydrophobic copolymers. I. Analysis with the *in situ* radioiodination technique. *J Biomed Mater Res* 1981; 15: 403-423.

107. Chinn JA, Posso SE, Horbett TA, Ratner BD. Post-adsorptive transitions in fibrinogen adsorbed to polyurethanes: changes in antibody binding and sodium dodecyl sulfate elutability. *J Biomed Mater Res* 1992; 26: 757-778.

108. Rapp J, Broich J. Lipid deposits on worn soft contact lenses. *CLAO J* 1984; 10: 235-239.

109. Castillo EJ, Koenig JL, Anderson JM, Lo J. Characterization of protein adsorption on soft contact lenses. I. Conformational changes of adsorbed human serum albumin. *Biomaterials* 1984; 5: 319-325.

110. Garrett Q, Milthorpe BK. Human serum albumin adsorption on hydrogel contact lenses *in vitro*. *Invest Ophthalmol Vis Sci* 1996; 37: 2594-2602.

111. Gachon AM, Bilbaut T, Dastugue B. Adsorption of tear proteins on soft contact lenses. *Exp Eye Res* 1985; 40: 105-116.

112. Kidane A, Szabocsik JM, Park K. Accelerated study on lysozyme deposition on poly(HEMA) contact lenses. *Biomaterials* 1998; 19: 2051-2055.

113. Monfils J, Tasker H, Townley L, Payor R, Dunkirk S. Laboratory simulation of protein deposition of vifilcon and etafilcon soft contact lens materials. *Invest Ophthalmol Vis Sci* 1992; 33(suppl.): 1292.

114. Alongi S, Rolando M, Marci A, Colonna A, Balestra G, Rizzetto R, Calabria G. Bacterial load and protein deposits on 15 day versus 1 day disposable hydrophilic contact lenses. *Cornea* 1998; 17: 146-151.

115. Keith D, Christensen M, Tudor M. The effect of multi-purpose solutions on the lysozyme deposit curve with soft contact lenses. *Invest Ophthalmol Vis Sci* 2001; 42: s594.

116. Hart DE, DePaolis M, Ratner BD, Mateo NB. Surface analysis of hydrogel contact lenses by ESCA. *CLAO J* 1993; 19: 169-173.

117. Hart D, Lane B, Josephson J, Tisdale R, Gzik M, Leahy R, Dennis R. Spoilage of hydrogel contact lenses by lipid deposits - tear film potassium depression, fat, protein, and alcohol consumption. *Ophthalmol* 1987; 94: 1315-1321.

118. Jones L, Mann A, Evans K, Franklin V, Tighe B. An in vivo comparison of the kinetics of protein and lipid deposition on group II and group IV frequent-replacement contact lenses. *Optom Vis Sci* 2000; 77: 503-510.

119. Richard NR, Anderson JA, Tasevska ZG, Binder PS. Evaluation of tear protein deposits on contact lenses from patients with and without giant papillary conjunctivitis. *CLAO J* 1992; 18: 143-147.

120. Fowler SA, Korb DR, Allansmith MR. Deposits on soft contact lenses of various water contents. *CLAO J* 1985; 11: 124-127.

121. Mirejovsky D, Patel AS, Rodriguez DD, Hunt TJ. Lipid adsorption onto hydrogel contact lens materials. Advantages of Nile red over oil red O in visualization of lipids. *Optom Vis Sci* 1991; 68: 858-864.

122. Prager MD, Quintana RP. Radiochemical studies on contact lens soilation. I. Lens uptake of ^{14}C-lysozyme from simple and complex artificial tear solutions. *J Biomed Mater Res* 1997; 36: 119-124.

123. Prager MD, Quintana RP. Radiochemical studies on contact lens soilation. II. Lens uptake of cholesteryl oleate and dioleoyl phosphatidylcholine. *J Biomed Mater Res* 1997; 37: 207-211.

124. Jung J, Rapp J. The efficacy of hydrophilic contact lens cleaning systems in removing protein deposits. *CLAO J* 1993; 19: 47-49.

125. Boot N, Kok J, Kijlstra A. The role of tears in preventing protein deposition on contact lenses. *Curr Eye Res* 1989; 8: 185-188.

126. Hong B, Bilbaut T, Chowhan M, Keith D, Quintana R, Turner F, Van Duzee B. Cleaning capability of citrate-containing versus non-citrate contact lens cleaning solutions: an in vitro comparative study. *ICLC* 1994; 21: 237-240.

127. Ho C, Hlady V. Fluorescence assay for measuring lipid deposits on contact lens surfaces. *Biomaterials* 1995; 16: 479-482.

128. Rebeix V, Sommer F, Marchin B, Baude D, Duc TM. Artificial tear adsorption on soft contact lenses: methods to test surfactant efficacy. *Biomaterials* 2000; 21: 1197-1205.

129. Guerrieri A, Sabbatini L, Zambonin PG, Ricci F, Pocobelli A, Cerulli L. Effect of anti-denaturant drugs on lysozyme deposit formation on soft contact lenses by liquid chromatography-electrochemical detection. *Biomaterials* 1995; 16: 1025-1030.

130. Holly F. Basic aspects of contact lens biocompatibility. *Colloids and Surfaces* 1984; 10: 343-350.

131. Bleshoy H, Guillon M, Shah D. Influence of contact lens material surface characteristics on replacement frequency. *ICLC* 1994; 21: 82-94.

132. Guillon M, Allary J, Guillon J, Orsborn G. Clinical management of regular replacement: Part I. Selection of replacement frequency. *ICLC* 1992; 19: 104-120.

133. Jones L, Tonge S, Goodall S, Tighe B. The influence of tear and non-tear derived surface active components on the wettability of group IV hydrogel lenses. *Optom Vis Sci* 1998; 75: 274.

134. Gachon A, Verrelle P, Betail G, Dastugue B. Immunological and electrophoretic studies of human tear proteins. *Exp Eye Res* 1979; 29: 539-553.

135. Chandler JW. Immunology of the ocular surface. *Int Ophthalmol Clin* 1985; 25: 13-23.

136. Chandler JW, Gillette TE. Immunologic defense mechanisms of the ocular surface. *Ophthalmology* 1983; 90: 585-591.

137. Gu Z, Zhu X, Ni S, Su Z, Zhou HM. Conformational changes of lysozyme refolding intermediates and implications for aggregation and renaturation. *Int J Biochem Cell Biol* 2004; 36: 795-805.

138. Sasahara K, McPhie P, Minton AP. Effect of dextran on protein stability and conformation attributed to macromolecular crowding. *J Mol Biol* 2003; 326: 1227-1237.

139. Allison SD, Chang B, Randolph TW, Carpenter JF. Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. *Arch Biochem Biophys* 1999; 365: 289-298.

140. Liao YH, Brown MB, Quader A, Martin GP. Protective mechanism of stabilizing excipients against dehydration in the freeze-drying of proteins. *Pharm Res* 2002; 19: 1854-1861.

141. Kaushik JK, Bhat R. Why is trehalose an exceptional protein stabilizer? An analysis of the thermal stability of proteins in the presence of the compatible osmolyte trehalose. *J Biol Chem* 2003; 278: 26458-26465.

142. Sola-Penna M, Meyer-Fernandes JR. Stabilization against thermal inactivation promoted by sugars on enzyme structure and function: Why is trehalose more effective than other sugars? *Arch Biochem Biophys* 1998; 360: 10-14.

143. Ueda T, Nagata M, Imoto T. Aggregation and chemical reaction in hen lysozyme caused by heating at pH 6 are depressed by osmolytes, sucrose and trehalose. *J Biochem (Tokyo)* 2001; 130: 491-496.

144. Remmele RL, Jr., Stushnoff C, Carpenter JF. Real-time in situ monitoring of lysozyme during lyophilization using infrared spectroscopy: dehydration stress in the presence of sucrose. *Pharm Res* 1997; 14: 1548-1555.

145. Takano K, Tsuchimori K, Yamagata Y, Yutani K. Contribution of salt bridges near the surface of a protein to the conformational stability. *Biochemistry* 2000; 39: 12375-12381.

146. Arakawa T, Bhat R, Timasheff SN. Preferential interactions determine protein solubility in three-component solutions: the $MgCl_2$ system. *Biochemistry* 1990; 29: 1914-1923.

147. Arakawa T, Bhat R, Timasheff SN. Why preferential hydration does not always stabilize the native structure of globular proteins. *Biochemistry* 1990; 29: 1924-1931.

148. Malzert A, Boury F, Renard D, Robert P, Proust JE, Benoit JP. Influence of some formulation parameters on lysozyme adsorption and on its stability in solution. *Int J Pharm* 2002; 242: 405-409.

149. Perez C, Griebenow K. Effect of salts on lysozyme stability at the water-oil interface and upon encapsulation in poly(lactic-co-glycolic) acid microspheres. *Biotechnol Bioeng* 2003; 82: 825-832.

150. Wimmer H, Olsson M, Petersen MT, Hatti-Kaul R, Peterson SB, Muller N. Towards a molecular level understanding of protein stabilization: the interaction between lysozyme and sorbitol. *J Biotechnol* 1997; 55: 85-100.

151. Grandori R, Matecko I, Mayr P, Muller N. Probing protein stabilization by glycerol using electrospray mass spectrometry. *J Mass Spectrom* 2001; 36: 918-922.

152. Knubovets T, Osterhout JJ, Connolly PJ, Klibanov AM. Structure, thermostability, and conformational flexibility of hen egg-white lysozyme dissolved in glycerol. *Proc Natl Acad Sci U S A* 1999; 96: 1262-1267.

153. Keith EO, Boltz M, Gadh R, Ghorsriz R, Mangatt D, Janoff LE. Adhesion of tear proteins to contact lenses and vials. *Biotechnol Appl Biochem* 2001; 34: 5-12.

154. Creighton TE. *Protein function - A practical approach*. Oxford: IRL Press, 1989.

155. Tomlinson A, Caroline P. Comparative evaluation of surface deposits on high water content hydrogel contact lens polymers. *CLAO J* 1990; 16: 121-127.

156. Tighe BJ, Jones L, Evans K, Franklin V. Patient-dependent and material-dependent factors in contact lens deposition processes. *Adv Exp Med Biol* 1998; 438: 745-751.

157. Franklin V, Pearce E, Tighe B. Hydrogel lens spoilation - deposit formation and the role of lipids. *Optician* 1991; 202: 19-26.

158. Tighe B. Blood, sweat and tears or some problems in the design of biomaterials. *J BCLA* 1990; 13: 13-20.

159. Bergmanson J, Barbeito R. Clinical assessment of ocular response to a multipurpose contact lens care solution. *ICLC* 1996; 23: 79.

160. Fowler SA, Allansmith MR. Removal of soft contact lens deposits with surfactant-polymeric bead cleaner. *CLAO J* 1984; 10: 229-231.

161. Miller R, Fainerman VB, Makievski AV, Kragel J, Grigoriev DO, Kazakov VN, Sinyachenko OV. Dynamics of protein and mixed protein/surfactant adsorption layers at the water/fluid interface. *Adv Colloid Interface Sci* 2000; 86: 39-82.

162. Reindel W, Ploscowe V, Minno G, Burke J, Castellano C, Hood D, Piccolo M, Snyder C, Steel S, Welcker S. Comparison study of hydrophilic contact lens surfactant cleaners: A clinical evaluation of residual deposits. *ICLC* 1989; 16: 232-236.

163. Simmons PA, Sun CM, Yamamoto BA, Edrington TB. Comparison of surfactant cleaning times on protein deposit removal from hydrogel lenses. *ICLC* 1995; 22: 16-22.

164. Simmons PA, Edrington TB, Pfondevida CJ, Lee M, Wechsler S. Comparison between evening and morning surfactant cleaning of hydrogel lenses. *ICLC* 1996; 23: 172-176.

165. Stein J, Keith D, Stone R, Turner F, Luna S. The relative effectiveness of five popular lens care regimens in minimizing protein deposition on soft contact lenses. *Optom Vis Sci* 1998; 75 S: 269.

166. Hood D, Rigel L. A clinical comparison of multi-purpose solutions. *Contact Lens Spectrum* 1995; 10: 31-36.

167. Phillips A, Stone J. *Contact lenses*. 3rd ed. Kent: Butterworth & Co (Publishers) Ltd, 1989.

168. House H, Leach N, Edrington T, Gunning J, Parsick S, Cyert L, Marsden H, Dvorak D, Toscano F, Walter D. Contact lens daily cleaner efficacy: multi-purpose versus single-purpose products. *ICLC* 1991; 18: 238-245.

169. Simmons PA, Ridder III WH, Edrington TB, Ho S, Lau KC. Passive protein removal by two multipurpose lens solutions: comparison of effects on in vitro deposited and patient worn hydrogel contact lenses. *ICLC* 1999; 26: 33-38.

170. Lee J, Li T, Park K. Solvation interactions for protein adsorption to biomaterial surfaces. In: Morra M. *Water in Biomaterials Surface Science.* Chichester: J Wiley & Sons Ltd, 2001; 127-146.

171. Garrett Q, Griesser HJ, Milthorpe BK, Garrett RW. Irreversible adsorption of human serum albumin to hydrogel contact lenses: A study using electron spin resonance spectroscopy. *Biomaterials* 1999; 20: 1345-1356.

172. Ratner B, Horbett T, Mateo N. Contact lens spoilation - Part 1: Biochemical aspect of lens spoilation. In: Ruben M and Guillon M. *Contact Lens Practice.* London: Chapman & Hall, 1994; 1083-1098.

173. Norde W. Adsorption of proteins from solution at the solid-liquid interface. *Adv Colloid Interface Sci* 1986; 25: 267-340.

174. Norde W, Anusiem A. Adsorption, desorption and re-adsorption of proteins on solid surfaces. *Colloids & Surfaces* 1992; 66: 73-80.