Fundamentals of Protein Adsorption at the Solid-Liquid Interface over Short Time Periods

by

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AUTHOR'S DECLARATION

This thesis consists of material of which I authored or co-authored: See Statement of Contributions included in the 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.

STATEMENT OF CONTRIBUTIONS

I would like to acknowledge the names of my co-authors who contributed to this thesis:

- Dr. Lyndon Jones, PhD
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ABSTRACT

Purpose: To directly measure the quantity and degree of denaturation of biomaterial-adsorbed proteins over short time periods.

Methods: Contact lenses were used as model biomaterials as they are widely used, readily available, and have a wide variety of material properties. The proteins lysozyme, lactoferrin, and albumin were investigated, as they are major protein components of bodily fluids, notably tears. Time points within the first few minutes of protein-material interactions were concentrated on.

- A novel technique to measure the activity of surface adsorbed lysozyme was developed (Chapter 3)
- Direct comparison of traditional extraction procedures and the novel surface technique for measuring the activity of adsorbed lysozyme (Chapter 4)
- The effect of competitive adsorption between lysozyme and lactoferrin and the effect on lysozyme activity (Chapter 5)
- Investigations of using I^{125} radiolabeling for protein adsorption experiments with contact lenses (Chapter 6)
- The effect of competitive adsorption of an artificial tear solution to the deposition of lysozyme, lactoferrin, and albumin (Chapter 7)

Results: Using the novel technique, the activity of surface adsorbed lysozyme and any biologically relevant lysozyme can be measured and distinguished within the first few minutes of lysozyme-material interaction. Using the novel technique in conjunction with protein extraction provides detailed activity information about deposited biologically relevant adsorbed lysozyme and lysozyme which is in underlying protein layers or in the bulk of the material. Lactoferrin co-adsorption with lysozyme did not affect the surface activity of lysozyme, but did decrease the activity of desorbed lysozyme.

Radiochemical experiments using I^{125} can provide sensitive measurements of protein adsorption to contact lens materials, but extra steps need to be taken to limit and measure the amount of unbound I^{125} in solution and to quantify the 'apparent mass' adsorbed unbound I^{125} represents.

Lotrafilcon B was the only lens material to show decreased protein adsorption due to competitive adsorption effects. This effect occurred when lysozyme and lactoferrin were competing and when lysozyme, lactoferrin, and albumin were competing with components from the artificial tear solution.

Conclusions: This thesis has developed and refined methods to measure biomaterial-adsorbed proteins over short time periods. These techniques can be utilized in the future to measure both the quantity and degree of denaturation of adsorbed proteins to contact lenses and other biomaterials.

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LIST OF SYMBOLS

Θ	angle
cm	centimeter
^{o}	degrees
^{o}C	degrees centigrade
$	dollar
>	greater than
<	less than
L	liter
µg	microgram
µL	microliter
mg	milligram
mL	milliliter
mm	millimeter
nM	millimolar
M	molar
MW	molecular weight
nm	nanometer
pH	negative logarithm hydronium ion concentration
%	percentage
rpm	revolutions per minute
s	seconds

LIST OF ABBREVIATIONS

HEMA	2-hydroxyethyl methacrylate
ATS	artificial tear solution
AFM	atomic force microscopy
ATR	attenuated total internal reflection
CD	circular dichroism
CSS	complex buffered saline solution
CLSM	confocal laser scanning microscopy
CA	contact angle
CH	conventional hydrogel
CPM	counts per minute
ELISA	enzyme linked immunosorbent assay
EWC	equilibrium water content
FITC	fluorescein isothiocyanate
FDA	Food and Drug Administration (United States)
FTIR	Fourier transform infrared spectroscopy
GPC	giant papillary conjunctivitis
HSD	honest significant difference
IgE	immunoglobulin E
IgG	immunoglobulin G
IEC	immunoglobulin-enzyme complex
IR	infrared spectroscopy
ICl	iodine monochloride
I^{125}	iodine-125
MS	mass spectrometry

MALDI-MS	matrix-assisted laser desorption ionization mass spectrometry
MMA	methyl methacrylate
NSERC	Natural Science and Engineering Council of Canada
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PBSI	phosphate buffered saline plus sodium iodide
pHEMA	poly(2-hydroxyethyl methacrylate)
PMMA	poly(methyl methacrylate)
PVP	polyvinyl pyrrolidone
P	probability
QCM	quartz crystal microbalance
RM-ANOVA	repeated measures analysis of variance
SIMS	secondary ion mass spectrometry
SH	silicone hydrogel
SPR	surface plasmon resonance
TIRF	total internal reflection fluorescents
TCA	trichloroacetic acid
UV	ultraviolet
XPS	X-ray photoelectron spectroscopy

CHAPTER 1 INTRODUCTION

1.1 *Protein Fundamentals*

1.1.1 *Protein Structure*

Proteins make up the largest organic component of biological cells. Within cells and living organisms proteins function as enzymes, transportation mechanisms, and have roles in immune defense, gene regulation, and cell signaling.[1,2] Proteins are polymers, consisting of about 20 different monomer units known as amino acids.[1,2] Amino acids (see Figure 1-1) have the general structure: $H_2NCH\mathbf{R}COOH$ and differ chemically by their R groups.[1,2] These R groups or side chains vary by structure, chemical polarity, isoelectric point, and molecular weight.[1,2] These properties are listed in Table 1-1.

Figure 1-1: Amino acid general structure

Figure 1-2: Polypeptide demonstrating peptide bonds (green)

Table 1-1: The Properties of Major L-Amino Acids Found in Proteins[1,3]

Name	Abbreviations		Molecular Weight	Side Chain	Polarity	Isoelectric Point
Alanine	Ala	A	89	$-CH_3$	NP	6.01
Arginine	Arg	R	174	$-(CH_2)_3-NH-C(=NH)-NH_2$	P	10.76
Asparagine	Asn	N	132	$-CH_2-C(=O)-NH_2$	P	5.41
Aspartic Acid	Asp	D	133	$-CH_2-COOH$	P	2.77
Cysteine	Cys	C	121	$-CH_2-SH$	P	5.07
Glutamic Acid	Glu	E	146	$-[CH_2]_2.COOH$	P	3.22
Glutamine	Gln	Q	147	$-(CH_2)_2-C(=O)-NH_2$	P	5.55
Glycine	Gly	G	75	-H	NP	5.97
Histidine	His	H	155	H_2C- (imidazole ring: N, NH)	P	7.59
Isoleucine	Ile	I	131	$-CH(CH_3)-C_2H_5$	NP	6.02
Leucine	Leu	L	131	$-CH_2-CH-(CH_3)_2$	NP	5.98
Lysine	Lys	K	146	$-[CH_2]_4-NH_2$	P	9.74
Methionine	Met	M	149	$-[CH_2]_2-S-CH_3$	NP	5.74
Phenylalanine	Phe	F	165	$-CH_2-C_6H_5$	NP	5.48
Proline	Pro	P	115	(complete structure) O, C–OH, HN (pyrrolidine ring)	NP	6.48
Serine	Ser	S	105	$-CH_2-OH$	P	5.68
Threonine	Thr	T	119	$-CH(OH)-CH_2$	P	5.87
Tryptophan	Trp	W	204	$-CH_2$ (indole ring, N H)	NP	5.89
Tyrosine	Tyr	Y	181	$-H_2C-C_6H_4-OH$	P	5.66
Valine	Val	V	117	$-CH-(CH_3)_2$	NP	5.97

NP – non-polar, P – polar. pI values are given for the whole amino acid.

Linking amino acids together into a polypeptide requires peptide bonds.[1,2] These form bonds between the amino and carboxyl groups, as shown in Figure 1-2. One end of the polypeptide, the N-terminus, will contain a free amino group while the other end of the polypeptide, the C-terminus, will contain a free carboxyl group.[1,2]

Hydrogen bonding between adjacent carbonyl and amide groups will arrange the polypeptide into secondary structures, such as α-helices and β-sheets.[1,2] The most common secondary structure in proteins is the α-helix, in which the polypeptide chain is a twisting cylindrical spiral.[1,2] To complete a 360^0 turn, 3.6 amino acid residues are needed. The α-helix structure is stabilized by hydrogen bonding between amide and carbonyl groups 4 amino acid residues apart. The structure of β-strands contains segments of the polypeptide chain laying side by side. When the adjacent segments progress towards the same terminus the β-sheet lies parallel. When the adjacent segments progress towards the opposite terminus the β-sheet lies antiparallel. Both parallel and antiparallel strands can occur in the same β-sheet, as shown in Figure 1-3. There is stabilizing hydrogen bonding between carboxyl and amine groups of the adjacent segments.

Figure 1-3: Parallel and antiparallel beta sheets (dotted lines represent hydrogen bonding)

Tertiary structure describes the three-dimensional conformation of the protein. The tertiary structure of hen egg white lysozyme,[4] bovine lactoferrin,[5] and bovine serum albumin,[6] from the protein data bank[7] are shown in Figure 1-4, Figure 1-5, and Figure 1-6 respectively. Tertiary structure is stabilized by a variety of non-covalent interactions, which will be discussed in greater detail in the next section.

Some proteins can be made up of more than one polypeptide chain and are identified as having quaternary structure. A classic example is hemoglobin, which contains two α-globulin and two β-globulin polypeptides associated together.[8]

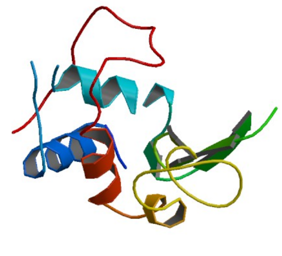

Figure 1-4: 3D structure of hen egg white lysozyme (ribbon model)

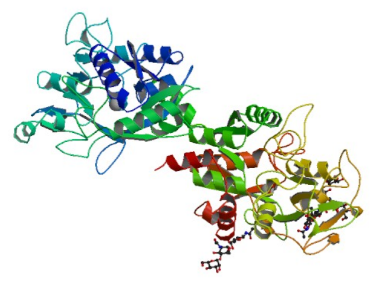

Figure 1-5: 3D structure of bovine lactoferrin (ribbon model)

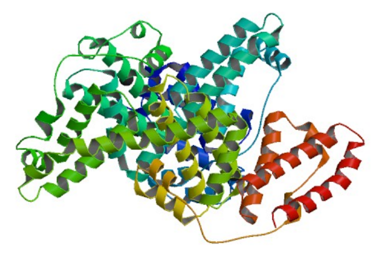

Figure 1-6: 3D structure of bovine serum albumin (ribbon model)

Images from the protein data bank[7]

1.1.2 Influences on Protein Structure

The structure of a protein will be stabilized at its isoelectric point.[9,10] At this point there is electrostatic attraction between the evenly distributed positive and negative charges that will promote a compact structure.[9,10] A shift away from the isoelectric point causes repulsion in the distributed charges, resulting in an expanded protein structure.[9,10] In addition to ionic groups, dipoles and induced dipoles play a role in stabilizing protein structure.[9,10] The van der Waals interactions between groups in the protein and surrounding media favour a compact protein structure.[9,10]

There are a variety of non-polar groups attached to protein backbones.[1,2] Water-polar interactions are more favourable than water interactions with non-polar groups, or non-polar groups interacting with each other.[9,10] In aqueous solution, hydrophobic R groups tend to reposition themselves from the protein exterior to the protein interior, which is referred to as hydrophobic effect.[9,10] The subsequent intramolecular hydrophobic interactions promote a compact protein structure.

Hydrogen bonding, dipole interactions, and hydrophobic interactions limit the rotational mobility and the bond lengths and angles of the protein chain and R groups.[9,10]

1.1.3 Factors Influencing Protein Adsorption

Proteins are relatively stable molecules but will often adsorb onto polymeric or metallic surfaces.[11–22] In simple terms, proteins will adsorb onto surfaces if the Gibbs free energy (G) is lowered:

Equation 1-1: Gibbs Energy

$$G=H-TS$$

where temperature is represented by T, enthalpy by H and entropy by S.[9] If adsorption can decrease H or increase S then adsorption will be energetically favourable. There are two key forces that drive protein adsorption: charge interactions, and the hydrophobic effect.

Protein and a sorbent surface in aqueous solution at a specific pH generally will not be at the isoelectric point of both the protein and the surface.[23] Thus, there will be an unequal charge distribution in the protein molecule, the sorbent surface or both. Opposite charges between the protein and the surface will cause a net attraction for protein adsorption. Lysozyme, a protein that has a net positive charge at physiological pH, demonstrates this property as it readily adsorbs onto surfaces with net negative charge.[24] Like charges will cause a net repulsion for protein adsorption, but does not automatically prevent adsorption. For instance, albumin has a net negative charge at physiological pH and is known to deposit on surfaces with the same or opposite charge.[25,26] Additionally, during adsorption the distributed charges on the protein overlap with the distributed

charges on the surface. The charge density per area of the adsorbed protein may not be equal, thus an electric potential will arise and counter-ions will move in to balance the charge differential and can become trapped between the protein and the surface.[27] These counter-ions reduce the attraction of the protein to the sorbent surface and maximal attraction will occur at the isoelectric point of the protein-surface complex, where there will be minimal counter-ions.

Proteins in aqueous solution tend to shield their hydrophobic groups from water. It is not always possible, due to the structure of the protein, to shield all hydrophobic groups from water. There will remain some portion to interact with hydrophobic surfaces.[28] Rearrangement of the protein structure to allow internally shielded hydrophobic groups to interact with a hydrophobic surface will be energetically favourable, as it increases entropy.[10] These rearrangements may cause denaturation of the protein[29] from significant secondary and tertiary structural rearrangements or loss if its biological function.

1.2 The Tear Film

The human tear film is about 3μm thick[30,31] and consists of three general layers: lipid, aqueous, and mucin. The outmost layer is the lipid layer, which at 50-100nm[32] acts as a barrier separating the ocular environment from the outside environment and functions to impede tear evaporation.[33] The middle aqueous layer is the thickest and consists of electrolytes and proteins.[34,35] The major functions of the aqueous layer are to allow the movement of nutrients and waste, as well as providing antimicrobial activity.[36,37] The total concentration of protein (basal) in tears ranges from 3.5-9.5mg/mL,[34,38,39] with lysozyme (0.7-3mg/mL),[39–41] lactoferrin (0.7-3.2mg/mL),[40–43] lipocalin (0.5-3.5mg/mL),[43,44] and albumin (0.013-0.38mg/mL)[42,45] being major components.[37] Laying

on the ocular surface are transmembrane mucins, which lubricate the ocular surface and provide a barrier against foreign bodies[46] and make up the 20-50nm thick mucin layer,[47] which acts to wet the surface cells of the cornea and stabilize the tear film above it.

1.3 Biomaterials

A biomaterial can be defined as any material that interacts with bodily fluids.[48] They may function as medical devices and can be used to correct vision, increase blood circulation, act as joint replacements, and form scaffolds for tissue replacement, among other uses,[49] and are made up of a variety of polymers and metals.

The desirable properties of a biomaterial will depend on its purpose. A scaffolding material will want to promote cell deposition and eventual degradation, while a heart valve will want to resist deposition of cells, especially thrombocytes, and remain intact for life.

1.3.1 Polymers

Biomaterials are typically made up of polymers. Similar to polypeptides, polymers are made up of long chains of repeating units known as monomers. Both amino acids and biomaterial monomers can have a wide range of unique properties due to the utilization of a carbon backbone, as carbon atoms can form stable bonds with many different atoms.[50] Carbon chemistry in biomaterials can be from natural compounds, synthetic, or a combination of both as silicone can also form stable bonds with many different atoms and is utilized in biomaterials to increase their oxygen permeability. As in proteins, the functional groups in the polymer backbone interact with each other and the environment and form the basis for the polymers' properties and modifying the chemistry of the

monomers can have a large impact on the properties of the polymer.[50] For example a polymer can become more or less flexible by modifying the chemistry of the monomers. The stability of polymers can also be greatly improved by changing the cross-link density, which involves covalently linking long polymer chains together by the addition of monomers with active carbon-carbon double bonds.[50] These added monomers are known as ‘cross-linking agents’. An example of polymers chains and cross-linked polymers chains is shown in Figure 1-7 and Figure 1-8 respectively.

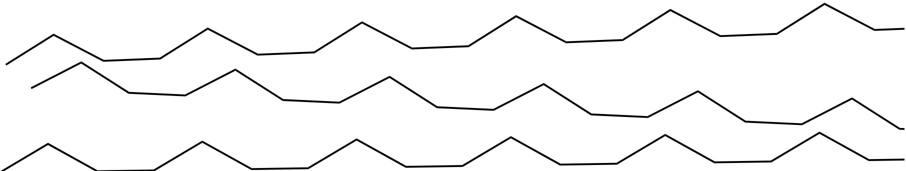

Figure 1-7: Example of polymers chains

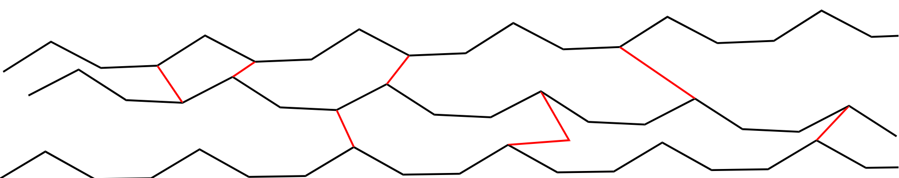

Figure 1-8: Example of cross-linking (red lines) in polymers chains

1.3.2 Surface Characterization

Surface characterization of any biomaterial is important in ascertaining how it may interact with biological systems. Surface characterization involves analyzing the chemical structure, hydrophobicity, topography, morphology and ionic groups of the surface.[51–53] The techniques to characterize surfaces generally comprise a variety of microscopic, spectroscopic, and thermodynamic methods.[51,54] Surface characterization will be briefly outlined below, however an extensive review regarding the surface characterization techniques for biomaterials has been written by Merrett et al.[55]

Chemical analysis will provide information regarding the chemical composition of the surface. This can be accomplished using a number of techniques, including X-ray photoelectron spectroscopy,[56,57] attenuated total reflectance Fourier transform infrared spectroscopy,[58,59] mass spectrometry,[60,61] auger electron spectroscopy,[62,63] and Raman spectroscopy.[64,65]

Thermodynamic analysis determines information about the surface energy. Common approaches involve contact angle measurement using Wilhelmy plate,[66,67] sessile drop,[68,69] and captive bubble methods.[70,71]

Topographical analysis produces high resolution, three-dimensional images of the biomaterial surface. Techniques to acquire these images include electron spectroscopy,[72,73] scanning tunneling microscopy,[74,75] and atomic force microscopy.[76,77]

In the case of protein adsorption, surface characterization can indicate if particular proteins will deposit by measuring the hydrophobicity and the surface charge at physiological pH, the potential location of deposition by measuring surface topography, and the general state of the adsorbed proteins by measuring the hydrophobicity.

1.3.3 Protein Adsorption

After surface characterization it is important to measure protein adsorption directly. Protein adsorption to biomaterials can be quantified in a variety of approaches. Quantifying proteins in solution, either in mass-depletion experiments or following protein extraction, can be undertaken using gel electrophoresis,[78–80] enzyme linked immunosorbent assays (ELISA),[81–84] colorimetric assays,[85–87] fluorescent assays,[88–90] and UV spectroscopy.[91–93] Quantifying proteins on biomaterials directly can be obtained

using in situ techniques such as ellipsometry,[13,94–96] quartz crystal microbalance,[97–99] attenuated total internal reflection infrared spectroscopy,[12,17,22] total internal reflection fluorescence,[11,100–103] surface plasmon resonance,[104–107] and atomic force microscopy.[19,108,109] Ex situ techniques to quantify protein adsorption include colorimetric assays,[15,110,111] ELISA,[81,112,113] confocal laser scanning microscopy,[57,114–118] X-ray photoelectron spectroscopy,[57,119–121] mass spectrometry,[122–127] and radiochemical experiments.[14,20,118,120,128–136]

In addition to quantifying adsorbed proteins it is important to understand the degree of protein denaturation on materials, as denatured protein can cause discomfort,[137] an immunological response,[138–141] bacterial adhesion[142,143] and thrombosis.[138] Denaturation can be determined by looking at changes in the secondary and tertiary structure, typically by nuclear magnetic resonance,[144–148] circular dichroism,[149–151] attenuated total internal reflection infrared spectroscopy,[12,73,152–154] and Raman spectroscopy[155–158] or by measuring the loss in biological function of the protein.[159–166]

1.3.4 Contact Lens Materials

Contact lenses are widely-used biomaterials, with an estimated world-wide market of $7.6 billion USD annually. The US market is approximately $2.5 billion USD, with 37 million wearers in the US.[167] Starting in the 1930s, contact lens materials have been continuously changing.[168] Poly(methyl methacrylate) (PMMA) was the first contact lens material utilized. It is formed by the polymerization of methyl methacrylate (MMA), as shown in Figure 1-9.[169] PMMA has excellent optical properties, is practically physiologically inactive, however the oxygen permeability is very poor.[170,171]

Figure 1-9: Polymerization of methyl methacrylate to poly(methyl methacrylate)

Methyl methacrylate has relatively good wettability, however it can be made more hydrophilic by adding a hydroxyl group to create 2-hydroxyethyl methacrylate (HEMA), as shown in Figure 1-10. HEMA (like MMA) can be polymerized to form poly(2-hydroxyethyl methacrylate) or pHEMA.

Figure 1-10: Methyl methacrylate modified to form 2-hydroxyethyl methacrylate

HEMA was first developed and applied to contact lens manufacture by Wichterle and Lim.[172,173] Unhydrated, HEMA is glassy, making it easy to machine cut, and when

hydrated can be used as a soft contact lens. The ability for HEMA and other hydrogels to attract water is described by their equilibrium water content (EWC)[50]:

Equation 1-2: Equilibrium water content

EWC = weight of water/weight of the hydrated material x 100%.

EWC is one of two factors that determine the classification of a contact lens material into one of four groups. Contact lenses are also classified based on their ionic content.[174] The Food and Drug Administration (FDA) classification system is shown in Table 1-2.

Table 1-2 Food and Drug Administration (FDA) Classification of Contact Lens Materials

FDA Group	EWC	Water Content Level	Ionic content	Ionic Classification
I	< 50%	Low	< 0.2%	Non-ionic
II	> 50%	High	< 0.2%	Non-ionic
III	< 50%	Low	> 0.2%	Ionic
IV	> 50%	High	> 0.2%	Ionic

The EWC of pure HEMA is 38% and its ionic content is below 0.2%, which would classify it as a FDA Group I material. A notable example of a commercially available contact lens material strictly containing HEMA is polymacon. The EWC of HEMA can be increased or decreased by adding different monomers. For instance, including MMA with HEMA will decrease the EWC compared to pure HEMA, while increasing the EWC can be accomplished by including N-vinyl pyrrolidone, such as in FDA group II material alphafilcon A, or methacrylic acid, such as in the FDA group IV material etafilcon A. The vast majority of commercially available materials incorporate HEMA either solely or with the addition of monomers. These are categorised under the broad category “conventional hydrogels” (CH).

Beginning in the late 1990's, commercial soft contact lenses incorporating silicone moieties became available.[174] Their popularity has steady increased and now encompass 66% of all new fits and re-fits of contact lenses in the US.[167] While incorporating silicone groups vastly improves the oxygen permeability of contact lenses, it is hydrophobic and poorly wettable.[168] The first generation of so called "silicone hydrogels" (SH) attempted to improve the wettability by modifying the surfaces of the materials and 'hiding' the underlying silicone. The surfaces of lotrafilcon A and lotrafilcon B are modified in a gas reactive plasma chamber, resulting in a continuous 5nm thick hydrophilic layer.[175,176] The silicone on the surface of balafilcon A is oxidized to hydrophilic silicate using gas reactive plasma.[177] This results in glassy 'islands' which bridge over the underlying silicone. The second generation of SH have no surface modifications, rather they incorporate an internal wetting agent to improve the surface wettability. These materials are senofilcon A and galyfilcon A, and have polyvinyl pyrrolidone (PVP) incorporated.[176] The third generation of SH also have no surface modification, but in contrast to galyfilcon A and senofilcon A, comfilcon A incorporates its wetting agent into the material backbone in an attempt to make the material inherently wettable.[178]

1.3.5 Contact Lens Properties

There are several properties that an ideal lens material should have, which will be explained in greater detail below:[179]

1) Meet or surpass the oxygen requirements of the cornea
2) Not provoke physiological response
3) Be highly wettable during wear

4) Have good physical stability and durability

5) Resist deposits from the tear film

6) Exhibit excellent optical transparency

7) Require minimal maintenance by patients

8) Be cost-effective to manufacture and purchase

1.3.5.1 Corneal Oxygen Requirements

The cornea is a unique tissue in the sense that it is avascular and thus obtains its oxygen requirements mainly from oxygen in the atmosphere.[179] Contact lenses lie on top of the cornea and act as a barrier between atmospheric oxygen and the cornea. Thus, the health of the cornea when wearing a contact lens is dependent upon the ability of the material to transport oxygen. The permeability (P) of a material is dependent upon the diffusion coefficient (D) and the solubility of oxygen in that material (k), summarized as:[168]

Equation 1-3: Oxygen permeability

$$P = Dk$$

The permeability is measured in Barrers and will depend also on the thickness of the material (t). Thus the permeability for a given thickness (or oxygen transmissibility) is:[168]

Equation 1-4: Oxygen transmissibility

$$P/t = Dk/t$$

measured in Barrer/mm. Holden and Mertz[180] describe the material transmissibility requirements as 24 Dk/t during the day and 87 Dk/t overnight during sleep, though later

work by Harvitt and Bonanno [181] using different criteria indicate that 125 Dk/t is the threshold for overnight wear.

1.3.5.2 Physiological Response

In addition to preventing oxygen from reaching the cornea, contact lenses may block adequate nutrient delivery and waste product removal to and from the cornea. Water and ions may also be prevented from reaching the posterior surface of the lens, which can cause it to stick to the cornea.[182] A more elastic material (as compared with a stiffer one) also tends to more readily adhere to the corneal surface. Thus, the lens 'elasticity' or modulus plays an important role in preventing a physiological response. Stiffer lenses (particularly if the fit is not optimized) may mechanically irritate tissues during blinking and are thought to be a cause of papillary conjunctivitis.[183,184] Contact lens materials themselves should also be inert and not provoke an immune response.[185]

1.3.5.3 Wettability

The term "wettability" describes how a fluid spreads across a solid surface.[186] For contact lenses, we are interested in how the tear film spreads across the lens surface as a continuous tear film is important for visual clarity and patient comfort.[49] Measuring wettability is typically done by measuring the contact angle (CA) by Wilhelmy plate,[66,67] sessile drop,[68,69] or captive bubble[70,71] techniques. An example of CA for two representative contact lenses using the sessile drop technique is shown in Figure 1-11. For wettability determinations, two types of CA are measured, the advancing angle after the material has been exposed to air and the receding angle after the material has been exposed to water.[49] The difference between the advancing and receding CA is known as hysteresis (see below):

Equation 1-5: Hysteresis

$$CA_{Hysteresis} = CA_{Advancing} - CA_{Receding}$$

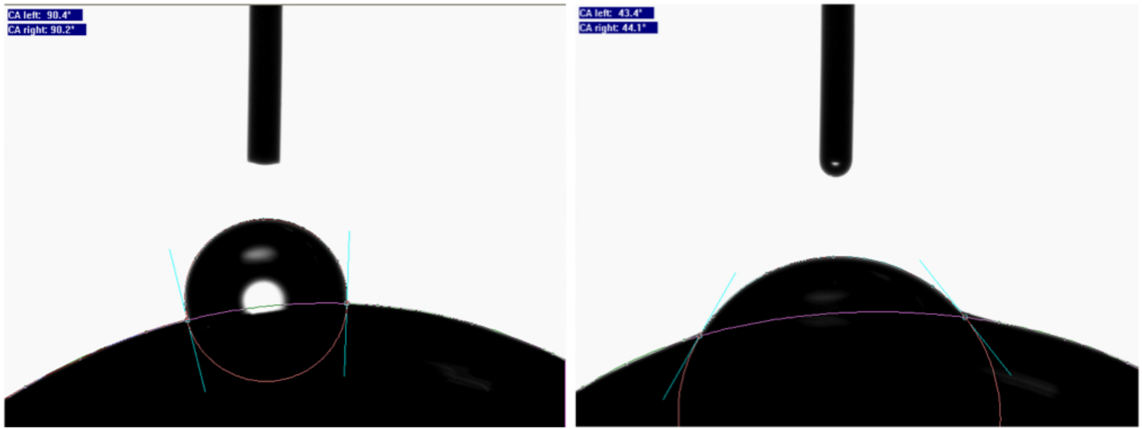

Figure 1-11: Advancing contact angles on relatively non-wettable (left) and wettable (right) contact lenses.

Contact lens CA will be influenced by the inherent material properties[135,143,186,187] and there is some evidence indicating that adsorption of tear film components can influence CA.[188,189] Despite the variety of CA, there is very little evidence that CA measurement alone will predict discomfort in patients.[190–194]

1.3.5.4 Durability and Stability

Contact lens materials should remain usable throughout the planned replacement schedule, without tearing or losing any of their ‘ideal’ properties. There is evidence to suggest that low water content lens materials last longer than high water content lens materials.[195,196] However, these lifespans are much longer than the replacement schedules used with modern soft lenses, which are typically 4 weeks or less.[197] The parameters of the contact lens materials should also remain stable after insertion onto the ocular surface, as there remains the potential for materials to change their EWC or lose oxygen transmissibility.[198]

1.3.5.5 Deposition

Upon interacting with the tear film contact lenses can rapidly adsorb proteins,[12,18,114,125,131,150,199,200] lipids,[92,201–204] and mucins.[205,206] These deposits, particularly protein, have a number of consequences for patients, including discomfort,[92,207] visual deficiencies,[208] bacterial adhesion,[142,143] and, more seriously, inflammatory reactions such as giant papillary conjunctivitis (GPC).[139–141] In contrast, there is evidence demonstrating that deposits may have benefits for patients such as increasing contact lens wettability[188,189] and removal of adhered bacteria.[209] It may be that an ideal contact lens should be selective in its adsorption rather than completely resistant to all deposits.

1.3.5.6 Optical Transparency, with Minimal Fuss and Cost

Contact lenses are tools for vision correction, and thus any technological improvements must not affect optical transparency.[210] While contact lenses are great tools for vision correction, patients remain notorious for poor compliance in proper and safe use of contact lenses.[211–216] There is a need for contact lens use to decrease in complexity, such as with daily disposable wear versus planned replacement, all while remaining cost effective for the patient and the manufacturer.

1.3.6 Protein Adsorption and Contact Lenses

It is known that proteins readily adsorb to contact lenses and can subsequently denature, causing problems for patients.[92,142,143,207,208] Silicone hydrogels show reduced protein adsorption compared to CH, but the deposited protein tends to be denatured.[133,164,166,202] Of particular consequence when considering denatured protein are severe complications such as GPC associated with SH wear.[217,218] GPC manifests as

inflammation in the papillae of the upper tarsal conjunctiva (see Figure 1-12), and causes irritation, burning, itching, redness, and contact lens intolerance.[140,217] It is characterized as both a type I hypersensitivity reaction (involving IgE), and as a type IV hypersensitivity reaction (mediated by cells).[219,220] One explanation for the immune system reacting to unfolded self-proteins is a popular yet controversial theory called the 'hygiene hypothesis'.[221,222] First noted by Strachan,[223] it argues that exposure to infectious agents may decrease the incidence of allergic responses. Consequently a lack of exposure to infectious agents could cause the immune system to be hyper-sensitive to the denatured protein on contact lenses. Regardless of the exact mechanism, it is clear that quantifying both the level of protein deposition and degree of denaturation onto contact lenses is important for patient comfort and ongoing success.

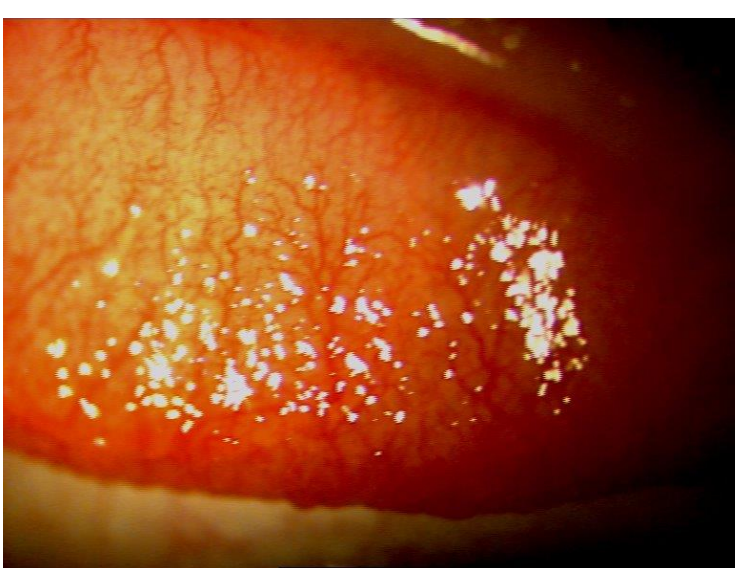

Figure 1-12: An example of giant papillary conjunctivitis. Image courtesy of the Centre for Contact Lens Research.

Studies investigating protein adsorption onto contact lens materials typically focus on adsorption at typical replacement intervals of 1 to 4 weeks.[80,131,133,166,224–227] While these

long time points provide insight into the levels of protein adsorption experienced by patients over the course of one wear cycle, there is a lack of understanding regarding protein adsorption to contact lens materials during the first few minutes of interaction with the tear film. Both the amount of protein adsorption and the degree of denaturation remain poorly understood during this early time interval. A further gap in our knowledge arises from the current techniques to measure the degree of protein denaturation at short time intervals. Secondary and tertiary structure of adsorbed proteins can be determined via nuclear magnetic resonance,[144–148] circular dichroism,[149–151] attenuated total internal reflection infrared spectroscopy,[12,73,152–154] and Raman spectroscopy.[155–158] However, since the biological function of proteins will only partly depend on its secondary and tertiary structure it may be better to measure biological function directly. Measuring biological function on contact lenses is primarily focused on lysozyme, and requires removal of lysozyme from the contact lenses. Extracting all of the lysozyme from contact lens materials and measuring the biological activity can provide a general overview of the degree of denaturation of lysozyme, however in terms of eliciting a biological response only loosely bound protein that can desorb into solution or protein in the outer surface layer of deposits can interact with biological systems. Additionally, there are inherent problems with any extraction procedure, as some protein could remain adsorbed to the material (as no methods provide 100% removal)[228–232] and the physical process of removal may affect protein denaturation.

Thus, there remains a need to both measure and understand protein adsorption to contact lens materials over the initial minutes and hours of the interaction of proteins with these biomaterials.

CHAPTER 2   EXPERIMENTAL TECHNIQUES

The materials and methods are detailed within each experimental chapter, however the following will be a brief overview of the major experimental techniques used in this thesis.

2.1 Micrococcal Activity Assays

Lysozyme antimicrobial activity against *Micrococcus lysodeikticus* was first described by Fleming in 1922.[1] Lysozyme catalyzes the hydrolysis of the glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in bacterial cell walls, causing the cells to lyse.[2] As the cells are lysed, a solution containing *M. lysodeikticus* will become clearer. This effect can occur within minutes, depending on the concentration of lysozyme and can be visible to the naked eye, as demonstrated in Figure 2-1. Intact cells and cellular components (after lysis) scatter light differently. Intact cells, which are close to 1μm in diameter, are much better at scattering light than smaller cellular components, thus a solution of cellular components appears clearer.

Figure 2-1: *Micrococcus lysodeikticus* before lysozyme exposure (left) and after 2 minutes of exposure (right)

Spectroscopic examinations of this phenomenon focus on specific wavelengths (450nm, 570nm, 700nm)[3–8] using a spectrometer. The classical Micrococcal Activity Assay involves *M. lysodeikticus* in cuvettes, to which small samples of lysozyme are added, and the change in absorption at a chosen wavelength is measured at short intervals for a few minutes. The rate of adsorption change is related to the mass of lysozyme added, therefore standard curves can be created with known amounts of lysozyme, as shown in Figure 2-2, to which the rates of absorption change from unknown samples of lysozyme are compared, to determine the corresponding masses of lysozyme.

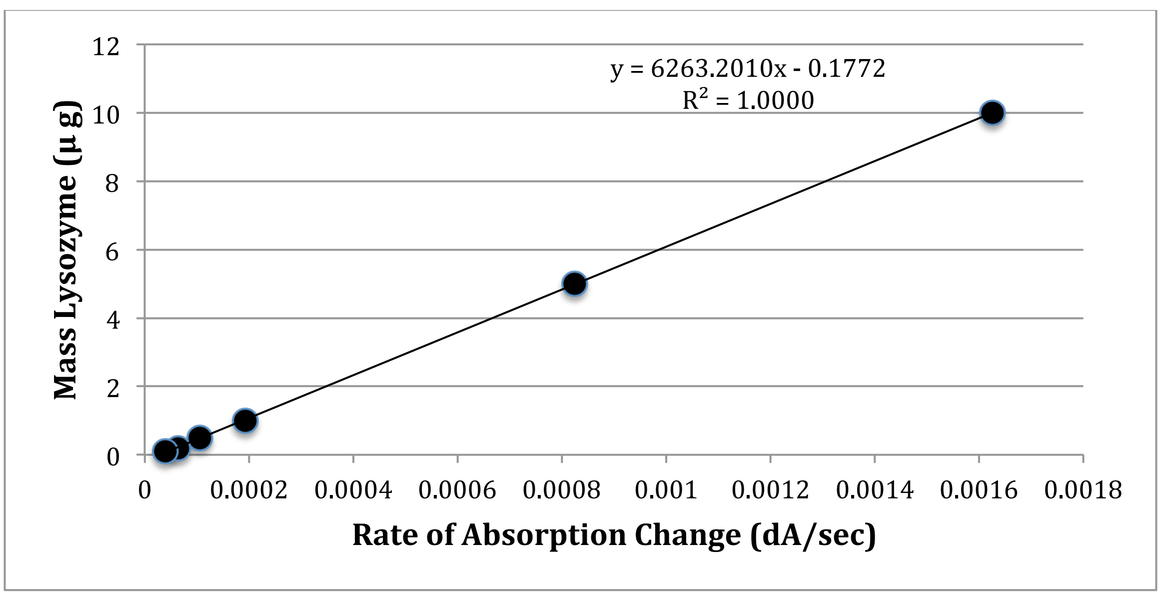

Figure 2-2: Standard curve of the rate of absorption change based on the mass of lysozyme in solution

2.2 I^{125} Radiochemical Experiments

Iodine-125 (I125) is often used as a tracer ion in protein adsorption experiments. Its half-life is ~two months and it emits gamma rays as it decays, which can be readily detected with a gamma counter, allowing for sensitive measurement of protein in the nanogram range. Proteins were radiolabeled by the iodine monochloride (ICl) method as previously described.[9,10] The ICl method typically labels tyrosine residues, and a lesser extent histidine, though the tyrosine bond is stronger.[11]

An intrinsic problem when using any label (fluorescent, radiochemical) to measure protein adsorption is the disassociation of the label from the protein. Since only the signal from the label is used to quantify protein adsorption, any label that adsorbs onto the test material can lead to anomalously high apparent protein adsorption. This has been shown for proteins labeled with I^{125} using a variety of labeling techniques including the ICl method.[12] Proteins labeled with I^{125} have been shown to disassociate with more than half of all I^{125} labels after just three days of incubation in saline solution.[12] This effect can be reduced if the labeled protein is incubated with other biomolecules (such as other proteins), but not prevented entirely.

Due to restrictions when using radioisotopes, protein adsorption kinetic experiments from Chapters 6-7 were done with 10^6 counts per minute (CPM) of radiolabeled protein per mL of incubation solution. This worked out to be ~1% of the total protein incubated with contact lenses. Increasing the amount of radiolabeled protein can increase the sensitivity of this technique, however there is a risk of reaching the detector limit for high protein adsorbing materials and, as mentioned above, more tracer iodide could disassociate into solution.

As only the signal from the I^{125} label is counted (in CPM) extra steps must be taken to convert CPM to masses of each protein. To accomplish this, aliquots of 100µL from incubation solution controls (without lenses) were counted to generate a conversion factor (in µg/CPM) for each incubation solution and time point. The CPM for each sample is then multiplied by the corresponding conversion factor to determine the mass of protein in the sample.

CHAPTER 3 MEASURING THE KINETICS AND ACTIVITY OF ADSORBED PROTEINS: IN VITRO LYSOZYME DEPOSITED ONTO HYDROGEL CONTACT LENSES OVER SHORT TIME PERIODS*

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Measuring the kinetics and activity of adsorbed proteins: *In vitro* lysozyme deposited onto hydrogel contact lenses over short time periods

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Abstract: A new process has been developed to determine the biological activity of an intact layer of lysozyme deposited onto a biomaterial surface. This process is applied to a number of common hydrogel contact lenses. The activity of the surface-adsorbed protein is measured using a standard micrococcal activity assay, with extra steps to distinguish between protein on the surface and protein in solution. This is in contrast to protein extraction work in which the activity of all adsorbed protein is measured. For ionic materials, which are known to deposit large amounts of protein, particularly positively charged proteins such as lysozyme, there is evidence for loosely bound protein re-entering the solution, thus making it impossible to truly separate out the surface-adsorbed protein. This optimized process provides the first quantification of the biological activity of an intact layer of surface-adsorbed protein at a hydrogel interface. © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2012.

Key Words: protein adsorption, contact lens, protein activity on biomaterials, protein surface activity, lysozyme kinetics

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INTRODUCTION

In any biomaterial application, protein is rapidly deposited onto the material of interest. There are a wide variety of very sensitive techniques that can be used to measure the amount of adsorbed protein, including ellipsometry,[1–3] quartz crystal microbalance (QCM),[4–6] and radiolabeling.[7–10] After deposition, the conformation of proteins may change from a native state to a non-native or denatured state.[11–15] For the particular case of contact lenses, it has been well established that tear film proteins rapidly deposit onto contact lenses[14,16–25] and that protein deposited on siloxane-based materials tends to be relatively low in quantity, but a higher percentage of the deposited protein is denatured.[13–16] Denatured protein on any biomaterial poses a risk for end users, because it has been implicated as a cause of various inflammatory reactions.[26] A specific example in the case of contact lenses is contact lens-induced giant papillary conjunctivitis,[27–29] which is characterized by mucous discharge, ocular itching, redness, and increased patient awareness of the lenses.[30–32] Thus, it is clear that in addition to characterizing the amount of protein deposited on a biomaterial it is even more important to characterize the (secondary or tertiary) structure and biological activity of adsorbed protein. Although some studies have used Fourier transform infrared spectroscopy or circular dichroism to determine changes in secondary structure on adsorption,[33–38] biological activity of an enzyme may depend only on a part of the total tertiary structure, and thus there is not yet a definitive way to correlate changes in secondary structure with changes in biological activity. Despite multilayer formation of proteins to some ionic biomaterials, the outermost molecules will have the strongest impact on a biological response from the host because it is these molecules that are in direct contact with the biological system. In particular, the question that should be addressed is the activity of the absorbed layer and not the activity of all protein molecules that constitute an absorbed layer. There is a significant difference between these two quantities, because it is likely only the protein at the interface with the surrounding solution that can truly contribute to the biological activity of the adsorbed layer.

Contact lenses are widely used biomaterials, being worn by 120 million people worldwide and comprising a market in excess of $(US)6 billion.[39] Contact lenses are ideal model biomaterials because these materials have been extensively characterized by atomic force microscopy,[40–43] X-ray

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photoelectron spectroscopy,[41,44] and contact angle measurements.[45–47] Contact lenses also represent an ideal system for testing general concepts in biomaterials science, because they are easily inserted and removed from the "host" organism, unlike many other biomaterials, which are typically implanted for extended periods of time.

Lysozyme, a relatively small, compact, and globular protein, has a molar mass of 14,500 Da, dimensions of 45 Å × 30 Å × 30 Å and a net positive charge at physiological pH.[48] Lysozyme, which has a concentration of 1.9 mg/mL in human tears and accounts for 30% of total protein in tears,[49] is often utilized as a model protein for contact lens protein conformational studies.[11–15] Because lysozyme is bacteriolytic,[50] turbidometric micrococcus activity assays are standard tools to determine its biological activity.[51–57] Many previous studies measuring lysozyme activity on contact lenses extracted the protein from the lenses using highly surface active solvents, which was followed by *ex situ* measurement of the protein activity.[13–16] There are a number of implicit assumptions used in this approach – notably that only surface-adsorbed protein is removed, and that the extraction process does not affect the state of the protein. However, the extraction process may remove lysozyme from both the immediate surface of the contact lenses and from within the bulk of the material. In addition, there is no way to independently determine the effect of the extraction solvent on the activity of extracted protein. Finally, because the host response will primarily depend on the outermost protein layer deposited on a biomaterial, the use of an extraction method to determine overall protein activity is not an ideal approach.

In a notable exception to the procedure mentioned above, Zhang et al.[58] have reported on the activity of proteins on contact lenses without protein extraction. Unfortunately, their technique does not make an attempt to distinguish between lysozyme bound to the surface and lysozyme that had become desorbed from the surface and was present in the surrounding solution. This results in an inability to make definitive conclusions about the activity of surface bound protein. A more complete assay would involve distinction of surface-adsorbed lysozyme from that which has desorbed from the lens, both of which can contribute to the biological activity.

Quantifying the amount of biologically active lysozyme in solution is commonly undertaken using a micrococcal assay.[12,13,15] The basic principle behind the technique is to expose *Micrococcus lysodeikticus* cells to a solution of lysozyme. Biologically active lysozyme will lyse the cell, resulting in a decrease in the concentration of cells, whereas increasing the amount of cell wall fragments. Because intact cells and cell wall fragments scatter light differently, the optical extinction of the solution at a particular wavelength of incident light can be correlated to changes in the intact cell concentration. In particular, decreases in the turbidity are observed because cells are destroyed. This process can be calibrated and used to quantitatively determine the amount of active lysozyme in the solution. To the best of our knowledge, there are no examples of techniques that are able to measure specifically the biological activity of an intact protein layer adsorbed onto the surface of a biomaterial.

In this study, we present a method that can be used to determine the amount of surface-adsorbed biologically active lysozyme on any material and apply it to a number of different contact lens materials. The premise of the experimental technique is simple: if there is active protein adsorbed onto the biomaterial surface, then that protein should lyse bacterial cells in a similar manner to those in solution. We first establish the technique on a simple PDMS-coated polystyrene cuvette, showing that we can distinguish active from inactive (thermally denatured) protein, and surface adsorbed versus protein in solution. Subsequently, we use the technique to perform a detailed study of lysozyme that has deposited onto commercially available contact lenses as a function of protein deposition time. The results show that it is relatively easy to measure the activity of an intact layer of surface-adsorbed lysozyme, that different commercial lenses show very different behavior in terms of the activity of surface-adsorbed lysozyme, and finally allow us to make conclusions about amounts of protein that are easily desorbed from lenses on rinsing. We also examine the effect of thermal treatment on adsorbed protein layers, and make comparisons with extraction activity studies. The measurements are done as a function of exposure time, with a focus on shorter times that are not normally the focus of biomaterial investigations. The reason for this is that we expect the surface activity to develop rapidly, and may even saturate after formation of the first monolayer.

MATERIALS AND METHODS

Micrococcal activity assays as previously described[12,13,15] are utilized to determine the activity of lysozyme. *M. lysodeikticus* (Sigma–Aldrich, Oakville, ON, Canada, ATCC # 4698) was diluted in phosphate buffered saline (PBS; pH 7.4) to approximately 2 mg/mL or 1 mg/mL the day before performing the assay, to allow the bacteria to swell at ∼4°C overnight. Prepared *M. lysodeikticus* was left to rotate on a platform shaker at room temperature for ∼30 min and then incubated at 37°C for ∼30 min. The *M. lysodeikticus* was diluted with PBS buffer to an optical density of ∼1.2 at 450 nm, then divided into 1 mL aliquots, to which samples and standards were added. Optical extinction measurements were acquired every 30 s at 30 ± 1°C for 5 min (Multiskan Spectrum ELISA Plate Reader, Thermo Labsystems, Finland). Standards (250 ng, 150 ng, 50 ng, and 12.5 ng) using hen egg lysozyme (Sigma–Aldrich, Oakville, ON, Canada) were measured concurrently with samples. Standard curves were plotted using linear regression of the change in absorbance over time versus mass of lysozyme, to which the change in absorbance of the samples were compared, to extrapolate an active mass of lysozyme in the samples. Note that the resulting surface activity measurements are determined in units of activity for an equivalent mass of protein in solution.

In this study, we considered a number of different contact lens materials and a simple PDMS polymer (General Electric RTV 108). The contact lenses used (shown in Table I)

TABLE I. Contact Lens Materials Evaluated in This Study

Name	USAN	Manufacturer	Principle Monomers	Diameter (mm)
Acuvue OASYS	Senofilcon A	Johnson & Johnson	mPDMS, DMA, HEMA, siloxane macromer, TEGDMA, and PVP	14
Air Optix	Lotrafilcon B	CIBA Vision	DMA, TRIS, and siloxane monomer	14.2
Acuvue 2	Etafilcon A	Johnson & Johnson	HEMA and MAA	14
PureVision	Balafilcon A	Bausch & Lomb	NVP, TPVC, NVA, and PBVC	14
Biofinity	Comfilcon A	CooperVision	M3U, FMM, TAIC, IBM, HOB, NMNVA, and NVP	14

mPDMS, monofunctional polydimethylsiloxane; DMA, *N,N*-dimethylacrylamide; HEMA, 2-hydroxyethyl methacrylate; TEGDMA, tetraethyleneglycol dimethacrylate; PVP, polyvinyl pyrrolidone; TRIS, trimethyl siloxy silane; MAA, methacrylic acid; NVP, N-vinyl pyrrolidone; TPVC, tris-(trimethylsiloxysilyl) propylvinyl carbamate; NVA, *N*-vinyl aminobutyric acid; PBVC, poly[dimethylsiloxyl] di [silylbutanol] bis[vinyl carbamate]; M3U, αω-bis(methacryloyloxyethyl iminocarboxy ethyloxypropyl)-poly(dimethylsiloxane)-poly(trifluoropropylmethylsiloxane)-poly(methoxy-poly-(ethyleneglycol)propylmethyl-siloxane); FMM, α-methacryloyloxyethyl iminicarboxyethyloxypropyl-poly(dimethylsiloxy)-butyldimethylsilane; TAIC, 1,3,5-triallyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione; IBM, isobornyl methacrylate; HOB, 2-hydroxybutyl methacrylate; NMNVA, *N*-methyl-*N*-vinyl acetamide.

include both conventional and silicone hydrogel materials, which accumulate varying amounts of protein.[12,15,59–61] Lenses were soaked in PBS for 24 h before protein incubation to remove any storage solution from the lenses. The lenses were then removed from PBS, blotted on lens paper, and placed in 6 mL screw cap vials (VWR, Mississauga, ON), which contained 1.5 mL of lysozyme dissolved in PBS at a concentration of 2 mg/mL or 0.2 mg/mL. Due to the short incubation periods (as short as 10 s), samples could not be incubated at temperatures above ambient, thus the experiment was undertaken at room temperature. A subset of our lenses (those used for thermal stability measurements as described below) were incubated in lysozyme solution for 24 h at 37°C. After incubation, contact lenses were removed from the screw cap vials and swirled in two containers of 100 mL PBS for 5 s each, blotted on lens paper and placed into 1.5 mL of PBS in 24-well plates in preparation for activity measurements.

To measure the activity of surface-adsorbed lysozyme, a repeater pipette was used to transfer 500 μL of 2 mg/mL *M. lysodeikticus* into each well of 24-well plates (VWR, Mississauga, ON), with each well containing previously incubated contact lenses. The plates were then put into a SpectraMax M5[e] plate reader (Molecular Devices, SunnyVale, CA). Each well in the plates was scanned every 30 s for 30 min while the plates were kept at 30 ± 1°C to allow for turbidity measurements, as in previously established micrococcal assays.[62,63] The optical extinction at a wavelength of 450 nm was measured in each well. The plates were shaken for 10 s in between each read interval and allowed to settle for 100 ms before the next reading. The software used was Softmax Pro (Molecular Devices, SunnyVale, CA). Lysozyme standards (10 μg, 5 μg, 1 μg, 0.5 μg, 0.2 μg, and 0.1 μg) using hen egg lysozyme were measured concurrently with samples, to calibrate the measurements to known protein amounts. Standard curves were plotted using linear regression of the change in absorbance over time versus mass of lysozyme, to which the change in absorbance of the samples were compared, to determine an active mass of lysozyme in the samples.

The procedure above is essentially the same as Zhang et al.[58] and measures all active lysozyme in the sample well. This single procedure cannot distinguish between surface-adsorbed protein and any protein that had become desorbed from the lens and was now in solution. Because the final step in the rinsing process includes placing the protein-coated lens into a buffer solution that is initially free of protein, it is possible for reversibly adsorbed protein to desorb from the lens. To make this important distinction, an additional step was introduced into the process. The 24-well plates containing contact lenses ready for activity measurements, were first incubated at 30 ± 1°C for 30 min. This allowed for similar amounts of protein to desorb from the lenses into solution as would occur with lenses in solution undergoing activity measurements. The contact lenses were then removed (as described above) from the wells and activity assays were performed on the remaining solution, to determine the amount of protein that was released from the lens during the time frame of the assay. We can subtract these activity values from our studies on lenses which had remained in the wells to obtain the contribution solely due to the protein still adsorbed onto the lens.

After deposition of protein to a particular contact lens, it is possible that the simple process of lens removal is a strong enough perturbation to dislodge very weakly bound protein (as we will detect this, both as part of the surface active protein and desorbed protein). To investigate this further, two different techniques for removing the lenses were investigated on balafilcon A and etafilcon A contact lenses. The first technique involved slow removal of lenses from the solution. A second, more vigorous, technique involved swirling the lenses clockwise five times, then removing/reimmersing the lenses five times, followed by complete and rapid removal of the lenses from the solution. Very loosely bound protein, however, may be removed by even the more gentle process, in which case it will not be possible to unambiguously quantify the activity of adsorbed protein.

In addition to the activity of adsorbed proteins, it is important to consider the resistance of adsorbed protein to

denaturing. A particularly tractable example is that of thermal stability. The thermal stability of the adsorbed lysozyme was determined by heating the samples to 80°C for 2 h. A 24-well plate containing prepared contact lenses, after 24 h incubation in 1.5 mL of 2 mg/mL lysozyme, and predetermined amounts of lysozyme soaking in 1.5 mL PBS was placed into an oven at 80°C and incubated for 2 h. After incubation, the plate was rapidly cooled in ice before assaying in the Molecular Devices SpectraMax M5[e] plate reader, as previously described. A similar test was also done for lysozyme adsorbed on a simple PDMS-coated substrate. A thin layer of PDMS was added to one side of the inside of 4.5 mL polystyrene cuvettes (VWR, Mississauga, ON) and left at 75°C to cure overnight. The cuvettes were then incubated at 37°C with 3 mL of 1 mg/mL lysozyme for ~22 h. The cuvettes were rinsed with water to remove any unbound or loosely bound lysozyme and then either incubated at 37°C or, for studying thermally denatured protein 80°C, for ~1 h before assaying. Micrococcal activity assays were performed, as described above, with the exception that 1 mL of ~1.2 OD *M. lysodeikticus* was added to the cuvettes containing lysozyme deposited onto PDMS.

Statistical analysis

Data analysis was undertaken using Statistica 8 (Statsoft, Tulsa, OK). Significance levels of $p \leq 0.05$ were considered to be significant. Data were analyzed using repeated measures one-way analysis of variance. Tukey HSD *post hoc* comparisons were performed, where applicable.

RESULTS

Activity of lysozyme adsorbed onto PDMS

As a test of our technique, a preliminary study (without considering kinetics) was undertaken using a PDMS substrate and lysozyme activity was determined before and after heating the sample to 80°C.

Significantly more active lysozyme was detected on PDMS when incubated at 37°C (~45 ng), compared with after heating the sample to 80°C (<1 ng; $p < 0.05$) as seen in Figure 1. These findings demonstrate that micrococcal assays can be used to detect and quantify the activity of lysozyme adsorbed to biomaterial surfaces and also that the denatured lysozyme (as suspected) shows no evidence for biological activity.

Time dependence of lysozyme activity on contact lens materials

The activities of lysozyme deposited to the different contact lens materials over time is shown in Table II. "Total active lysozyme" was determined for the contact lenses in the solution using the micrococcal assay. The activity of protein that was released from the lens into the solution was further measured and this amount was subtracted from the "total active lysozyme" to calculate the "surface active lysozyme". Note that for etafilcon A lenses, the concentration of the protein in the solution was 0.2 mg/mL and for balafilcon A lenses both 2 mg/mL and 0.2 mg/mL concentrations were studied. "BD" refers to values that were below the detection limit of the experiment.

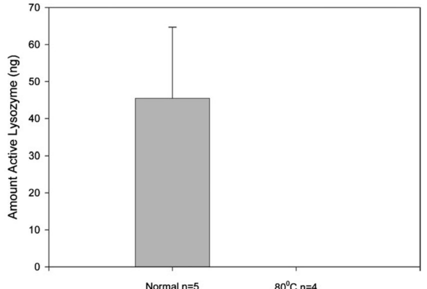

FIGURE 1. Comparison of the activity of lysozyme deposited onto PDMS under normal assay conditions versus thermal denaturation. Error bars represent mean ± SD.

The observation that lysozyme on etafilcon lenses from times greater than or equal to 15 min started to exhibit significant desorption led us to use the time points between 10 s and 7.5 min to calculate the mean of the surface active protein amounts for each lens type (Table III). This number quantifies the mass equivalent biological activity of the adsorbed lysozyme layer. We consider the contact lens to have a total surface area of two sides to be 3.34 cm^2. We further use calculate surface monolayer coverage of lysozyme on a surface to be 207 ng/cm^2 or 310 ng/cm^2 depending on the orientation of lysozyme.[65] Using these two references, we calculated the amount for a theoretical monolayer of lysozyme on a contact lens to be 692–1035 ng and thus determined the percentage of active lysozyme in a surface layer for each lens type (Table III). For these calculations, the mean value of the "surface active lysozyme" from Table III was used.

Figure 2(a) shows the data for lysozyme deposited to senofilcon A (a), lotrafilcon B (b), and comfilcon A (c) contact lenses. For both senofilcon and lotrafilcon, the total active lysozyme and surface active lysozyme showed little if any time dependence. Only about 30 ng of active protein was found on the lens surface, and this remained essentially constant over the range of the experiment. Two of the three replicates for solution activity at 10 s, and total activity at 1 h were omitted as they were below detection, resulting in $n = 1$ for those values. No significant differences were seen between the activity of a surface layer on senofilcon A and lotrafilcon B lenses($p < 0.05$), which is in agreement with findings by Subbaraman et al.[15] who extracted lysozyme after 1 day of incubation (Table IV). (We note that the amount of active lysozyme was below detection at the 1 h time point.)

It is interesting to note that although there was measurable surface lysozyme activity on both senofilcon A and lotrafilcon B at most times with less than an hour of exposure to protein solution, it was below detection at the 1 h

TABLE II. Total and Surface Active Lysozyme on Contact Lenses in μg/Lens (Mean ± SD)

Lens Type	Value	Time								
		10 s	1.5 min	3 min	4.5 min	7.5 min	15 min	30 min	1 h	2 h
Senofilcon A (2 mg/mL)	Total active lysozyme	0.113 ± 0.007	0.125 ± 0.0317	0.130 ± 0.003	0.144 ± 0.007	0.133 ± 0.003	0.139 ± 0.002	0.146 ± 0.008	0.117[a]	0.149 ± 0.010
	Surface active lysozyme	0.025 ± 0.007[a]	0.029 ± 0.032	0.029 ± 0.005	0.037 ± 0.007	0.028 ± 0.008	0.028 ± 0.007	0.031 ± 0.011	0.005 ± 0.010[a]	0.068 ± 0.125
Lotrafilcon B (2 mg/mL)	Total active lysozyme	0.143 ± 0.023	0.129 ± 0.003	0.143 ± 0.005	0.127 ± 0.012	0.147 ± 0.006	0.135 ± 0.015	0.135 ± 0.012	BD	0.140 ± 0.021
	Surface active lysozyme	0.053 ± 0.023	0.033 ± 0.009	0.037 ± 0.020	0.026 ± 0.013	0.035 ± 0.008	0.021 ± 0.016	0.032 ± 0.012	BD	0.058 ± 0.135
Comfilcon A (2 mg/mL)	Total active lysozyme	0.120 ± 0.013	0.119 ± 0.016	0.154 ± 0.012	0.205 ± 0.022	0.244 ± 0.008	0.260 ± 0.011	0.300 ± 0.014	0.395 ± 0.048	0.400 ± 0.033
	Surface active lysozyme	0.023 ± 0.014	0.002 ± 0.019	0.012 ± 0.013	0.027 ± 0.024	0.046 ± 0.009	0.020 ± 0.015	0.059 ± 0.117	0.033 ± 0.049	0.023 ± 0.071
Balafilcon A (2 mg/mL)	Total active lysozyme	0.356 ± 0.019	0.823 ± 0.070	1.07 ± 0.063	1.494 ± 0.197	1.704 ± 0.190	1.946 ± 0.201	2.248 ± 0.134	3.678 ± 0.045	2.906 ± 0.196
	Surface active lysozyme	0.124 ± 0.020	0.351 ± 0.087	0.281 ± 0.065	0.440 ± 0.250	0.342 ± 0.227	−0.164 ± 0.406	−0.262 ± 0.251	0.604 ± 0.293	−1.359 ± 0.505
Balafilcon A (0.2 mg/mL)	Total active lysozyme	BD	0.039 ± 0.032	0.127 ± 0.005	0.177 ± 0.017	0.216 ± 0.044	0.318 ± 0.017	0.340 ± 0.008	0.374 ± 0.025	0.360 ± 0.09
	Surface active lysozyme	BD	0.039 ± 0.032	0.116 ± 0.010	0.149 ± 0.018	0.162 ± 0.049	0.208 ± 0.030	0.172 ± 0.034	0.164 ± 0.063	0.114 ± 0.101
Etafilcon A (0.2 mg/mL)	Total active lysozyme	0.991 ± 0.068	3.554 ± 0.480	4.543 ± 0.242	5.144 ± 0.585	5.693 ± 0.757	6.994 ± 1.505	9.535 ± 0.095	9.854 ± 1.098	12.396 ± 0.749
	Surface active lysozyme	0.045 ± 0.314	1.127 ± 0.644	0.430 ± 0.596	1.087 ± 1.207	1.352 ± 0.868	−0.729 ± 1.628	−2.556 ± 1.468	−4.198 ± 2.716	−1.738 ± 0.802

BD, below detection.
[a]Denotes $n = 1$.

TABLE III. Surface Active Lysozyme on Contact Lenses (Mean ± SD)

Lens Type	Surface Layer Active Lysozyme (ng/Lens)	Surface Active Lysozyme % (Range min–max)
Senofilcon A	30 ± 10	3–4
Lotrafilcon B	37 ± 10	4–5
Comfilcon A	22 ± 10	2–3
Balafilcon A (2 mg/mL)	310 ± 80	30–45
Balafilcon A (0.2 mg/mL)	90 ± 20	9–13
Etafilcon A (0.2 mg/mL)	810 ± 500	78–117

TABLE IV. Comparison of Lysozyme Activity Deposited (μg) on Contact Lenses From Different *In Vitro* Studies (Mean ± SD)

Lens Type	Total Active Lysozyme (Hour 2) This Study	Subbaraman et al.[15] (Day 1; 1.9 mg/mL)
Senofilcon A	0.15 ± 0.01	0.4 ± 0.2
Lotrafilcon B	0.14 ± 0.02	0.2 ± 0.2
Comfilcon A	0.40 ± 0.03	–
Balafilcon A	2.91 ± 0.20	0.8 ± 0.1
Balafilcon A	0.36 ± 0.09 (0.2 mg/mL)	
Etafilcon A	12.40 ± 0.75 (0.2 mg/mL)	560 ± 36

FIGURE 2. Kinetics of active and total lysozyme deposited onto non-ionic: (a) senofilcon A (2 mg/mL), (b) lotrafilcon B (2 mg/mL), and (c) comfilcon A (2 mg/mL). Error bars represent mean ± SD, $n = 3$, * Denotes $n = 1$. In all cases: (●) total activity and (○) activity of the surface-adsorbed lysozyme after correction for desorbed lysozyme.

incubation time point. In addition, there was no evidence for surface activity on these materials after 24 h of incubation for both thermally treated and untreated lenses (not included in time dependence due to different incubation temperature). This may indicate long-term structural changes in the adsorbed protein which would be relevant on time frames for normal use. In this case, the small measured activity between 10 s and 1 h of exposure would be considered a transient event, with a long-term state of complete inactivity.

In contrast to senofilcon and lotrafilcon, comfilcon A contact lenses [Fig. 2(c)] show a clear increase in the amount of active lysozyme as the incubation time increases. In a technique that does not differentiate between desorbed protein and protein remaining adsorbed to the biomaterial, it would appear that the adsorbed surface protein maintains a significant biological activity (certainly much greater than senofilcon A or lotrafilcon B). However, after correcting for the protein which had desorbed into surrounding solution, the actual activity of the adsorbed layer was even less than that of senofilcon A or lotrafilcon B (Table III). This is a clear indication that on comfilcon A, there is some amount of protein that is reversibly adsorbed and desorbs from the surface and either retains or regains its biological activity. At the same time, the protein that remained on the surface had very little biological activity. It is not necessarily surprising that the protein desorbs in this way, because while the lens is incubated in lysozyme solution there is both adsorption and desorption going on. The actual measured uptake of protein is the final result of the competition between adsorption and desorption. Once the lens is removed from the incubation vial and transferred to the well plate, there is no more adsorption because the surrounding solution is originally free from protein, and desorption is the only transport mechanism.

Balafilcon A contact lenses incubated in a 2-mg/mL protein solution [Fig. 3(a)], at time points greater than or equal to 15 min, showed lysozyme activity which was surprisingly higher in solution compared with the total active lysozyme, which contained both surface active lysozyme and solution active lysozyme. Although these differences are of only slight statistical significance, the resulting calculation of surface active lysozyme led to values less than zero. Some of

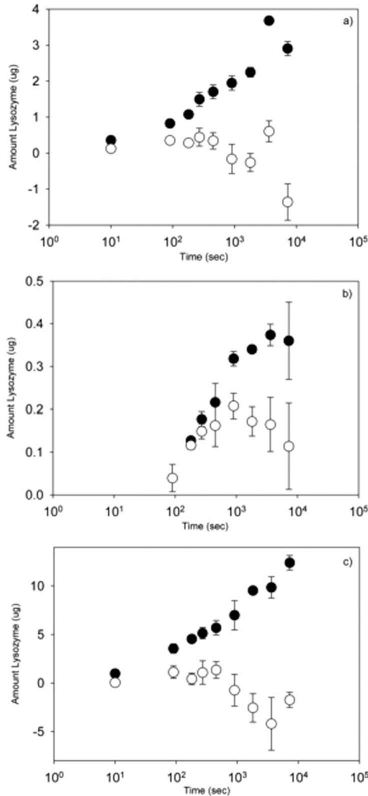

FIGURE 3. Kinetics of active and total lysozyme deposited onto ionic: (a) balafilcon A (2 mg/mL), (b) balafilcon A (0.2 mg/mL), and (c) etafilcon A (0.2 mg/mL). Error bars represent mean ± SD, $n = 3$, * Denotes $n = 1$. In all cases: (●) total activity and (○) activity of the surface-adsorbed lysozyme after correction for desorbed lysozyme.

the buried layers of lysozyme may have been removed from the lenses by physical forces during to the lens removal process, hence resulting in greater solution activity. This was further investigated by comparing the two different removal techniques using balafilcon A lenses. Although slow removal typically resulted in more solution active lysozyme than vigorous removal, up to 100 ng more, we were unable to find a lens removal process that removed this effect. From Figure 3(a), total active lysozyme increased slightly over time, whereas surface active lysozyme showed a linear trend for incubation times up to 7.5 min; and although the amount of biologically active lysozyme that was released from the lens in the solution increases, or more specifically the amount of active lysozyme in solution that has desorbed from the lenses (results not shown) increased when more lysozyme deposited on balafilcon A lenses, the surface active lysozyme remained relatively constant.

The incubation of balafilcon A contact lenses in a solution with a lower protein concentration (0.2 mg/mL) showed total active lysozyme increasing over time, whereas surface active lysozyme saturated after 15 min, which is similar to the results seen with balafilcon A using the higher lysozyme concentration (2 mg/mL). However, the amount of activate lysozyme on balafilcon A contact lenses incubated in a 0.2-mg/mL solution was approximately 2× lower compared with balafilcon A lenses incubated in a 2-mg/mL solution. One possible reason could be the different concentrations lead to different mechanisms for adsorption, which has been described previously.[65]

Etafilcon A contact lenses incubated in 0.2 mg/mL lysozyme solution showed, for times <15 min, total activity increasing with time, whereas surface active lysozyme remained essentially constant. At time points greater than or equal to 15 min, Etafilcon displayed the same behavior as balafilcon, resulting in negative amounts of calculated surface active lysozyme.

The data on lysozyme activity for senofilcon A, lotrafilcon B, balafilcon A, and etafilcon A contact lenses in this study are substantially lower compared with previous *in vitro* studies (Table IV). To date, there are no published data on lysozyme activity for comfilcon A lenses. Major differences are seen between the measured surface activity (left) and the measured activity using extraction techniques[15] (far right column). However, it needs to be considered that extraction techniques measure the biological activity of all lysozyme removed from the lens. The surface activity determined in this study is the biological activity of the outermost protein layer on the lens surface. In that sense, the quantity in the left column of data will be very similar to that of a protein monolayer.

The comparison between the data in Figures 2 and 3 shows that our technique is able to quantify the activity of the layer of strongly bound protein to a biomaterial, and also to measure the amount that becomes desorbed into solution. This works well for materials in which the protein layer is fairly strongly bound to the material. In particular, in cases where the seemingly gentle process of removing a protein-coated lens disrupts the protein layer, this technique can lead to anomalous negative values.

Thermal stability of lysozyme adsorbed to contact lenses

The structural stability of lysozyme was further investigated by heating the samples for 2 h at 80°C, followed by activity measurements. In standard solutions, this heating process removed 100% of the biological activity for lysozyme solution containing 1 μg or less protein, and removed 94% and 80% for solutions of 5 μg and 10 μg of lysozyme, respectively. Comfilcon A, balafilcon A, and etafilcon A were the only lens types that had detectable amounts of surface active lysozyme after 1 day of incubation. Heating lysozyme-

coated comfilcon A lenses resulted in activity levels below the detection threshold. Similarly, thermal treatment of lysozyme-coated etafilcon lenses resulted in a 90% decrease in the activity. This is similar to the solution controls and suggests that adsorption onto etafilcon does not enhance the thermal stability of lysozyme. Applying the same treatment to lysozyme (2 mg/mL)-coated balafilcon lenses only resulted in a ~41% reduction for biological activity. By comparison, heating lysozyme on PDMS resulted in 100% loss of its biological activity. Previous studies have shown that large amounts of lysozyme can deposit within the bulk material of balafilcon A and etafilcon A lenses,[7,61] which could have protected the protein stability for balafilcon A during the heating process, but had no protective effect for lysozyme accumulated to etafilcon A. Because this study was measuring the activity of the surface layer only, it could be speculated, that after the thermal treatment, protein which was previously protected inside the material matrix may have traveled in the solution phase and readsorbed to the surface, hence give rise to an apparent increase in activity. Whether this is impacted by the porosity of the lens or specific interaction between the protein and the material has not been determined.

DISCUSSION

All of the FDA group I materials tested had similar amounts of surface active lysozyme, despite differences in total activity. The overall percentage of active lysozyme was low (2–5% compared with solution standards). There are a number of possible reasons for this finding: (a) all protein molecules have reduced their activity to 2–5%, (b) 95–98% of the adsorbed protein is inactive, but 2–5% has remained active, and (c) the adsorption has caused a change in the shape of the protein which affects the number of molecules/monolayer. We note that these are not exclusive effects, and the actual reason for the low activity of the adsorbed layer can be any combination of these three (or even other) reasons. In any event, these are significant findings, because other work has shown that protein deposited on FDA group I materials retains a high amount of activity 2 days after incubation[15] using extraction methods before activity analysis. When comparisons are made to previous work (Table IV), our total activity results for senofilcon A and lotrafilcon B were similar. Despite this immediate loss in biological activity, irreversible denaturation of lysozyme may take days after depositing on FDA group I materials.

Of the four silicone hydrogel lenses tested, balafilcon A showed the greatest amount of total activity after 2 h. Previous work[15] has reported that lysozyme extracted from balafilcon A lenses retains its biological function (~90% after 1 day incubation) which is a much larger percentage than our results for surface activity.

Etafilcon A was the only conventional hydrogel investigated in this study. Etafilcon A is known to adsorb substantial amounts of lysozyme,[7,64] which largely remains active.[12,13,15] Previous studies have further determined that lysozyme on etafilcon A lenses is distributed evenly throughout the lens material.[7,61] In this study, for etafilcon A lenses at least 85% of the surface layer of deposited lysozyme retained its biological function. Other studies have shown that more than 90% of the lysozyme extracted from etafilcon A lenses after more than 2 weeks of incubation[12,15] still retained its biological function. Etafilcon A lenses were the only lens type investigated that showed an intact layer of deposited lysozyme with similar percentage of retained activity because total lysozyme extracted from lenses in previous studies.[12,15] These results suggest that lysozyme does not rapidly lose its biological function on depositing to etafilcon A contact lens.

Table IV compares total active lysozyme after 2 h from this study to previously published work using protein extraction after 1 day. This comparison utilizes total activity as a rather than surface activity as the extraction process follows that protocol more closely. We note reasonable agreement between the two, suggesting that either the total protein deposited is not much more than a monolayer, or that underlying layers are irreversibly denatured. One exception was balafilcon A incubated in a 2-mg/mL solution which had ~3.5× the activity compared with extraction studies. The problems associated with extraction studies become evident with this lens type. Previous work has shown the extraction efficiency with balafilcon A is only 53%.[66] In addition, the remaining differences may highlight the denaturing effect of the extraction process on lysozyme. Future work will make direct comparisons between the two techniques in identically prepared samples.

Limitations in this current technique were found when determining the amount of surface active lysozyme on the ionic lenses (balafilcon A and etafilcon A). These lenses had large amounts of solution active lysozyme, which produced negative results for the surface active lysozyme calculations. One possible explanation for this result is that the ionic nature of balafilcon A and etafilcon A lenses attract loosely bound, positively charged lysozyme to their surfaces which easily desorbs from the lenses, due to shear forces when the lenses are removed from solution. However, it is important to keep in mind that the calibration, as well as the measurements of activity of desorbed protein, is done with the protein in solution, whereas the surface activity involves measuring the activity of adsorbed protein. It is not necessarily true that the same amount of lysozyme will give the same activity in the two different cases, and this difference could contribute to the anomalous negative results.

This current technique measures different features of lysozyme activity compared with previous methods, which required extracting protein before analysis. We were able to determine the activity of a surface layer of lysozyme, which might be of great relevance for biocompatibility testing of biomaterials. Extraction techniques, despite their limitations, are effective at probing the total activity of a protein layer. Given the strengths of both techniques, they should be utilized together to achieve a complete lysozyme activity assay.

CONCLUSIONS

This study has established an effective technique to evaluate the activity of an intact lysozyme layer coating on a

biomaterial. The results showed that protein can rapidly deposit and can rapidly lose its biological function in as little as 10 s of exposure. The quantity and biological activity of adsorbed lysozyme was influenced by the material composition, reiterating previous findings that silicone hydrogels deposit less lysozyme than conventional hydrogels and the biological activity of lysozyme is reduced (to near zero) on silicone hydrogels versus conventional hydrogels. Comparisons between our technique and previous protein extraction show reasonable agreement once proper comparisons are made. The ability to measure the activity of the outermost surface layer of deposited lysozyme will provide a better understanding between interaction of biomaterials and host response.

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REFERENCES

1. Keuren JF, Wielders SJ, Willems GM, Morra M, Lindhout T. Fibrinogen adsorption, platelet adhesion and thrombin generation at heparinized surfaces exposed to flowing blood. Thromb Haemost 2002;87:742–747.
2. Spaeth K, Brecht A, Gauglitz G. Studies on the biotin–avidin multilayer adsorption by spectroscopic ellipsometry. J Colloid Interface Sci 1997;196:128–135.
3. Puleo DA, Bizios R, Mora MF, Wehmeyer JL, Synowicki R, Garcia CD. Investigating Protein Adsorption via Spectroscopic Ellipsometry. Biological Interactions on Materials Surfaces. New York: Springer; 2009. p 19–41.
4. Teichroeb JH, Forrest JA, Jones LW, Chan J, Dalton K. Quartz crystal microbalance study of protein adsorption kinetics on poly(2-hydroxyethyl methacrylate). J Colloid Interface Sci 2008; 325:157–164.
5. Welle A. Competitive plasma protein adsorption on modified polymer surfaces monitored by quartz crystal microbalance technique. J Biomater Sci Polym Ed 2004;15:357–370.
6. Hayakawa T, Yoshinari M, Nemoto K. Characterization and protein-adsorption behavior of deposited organic thin film onto titanium by plasma polymerization with hexamethyldisiloxane. Biomaterials 2004;25:119–127.
7. Luensmann D, Zhang F, Subbaraman L, Sheardown H, Jones L. Localization of lysozyme sorption to conventional and silicone hydrogel contact lenses using confocal microscopy. Curr Eye Res 2009;34:683–697.
8. Karlsson LM, Tengvall P, Lundstrom I, Arwin H. Penetration and loading of human serum albumin in porous silicon layers with different pore sizes and thicknesses. J Colloid Interface Sci 2003; 266:40–47.
9. Ihlenfeld JV, Cooper SL. Transient in vivo protein adsorption onto polymeric biomaterials. J Biomed Mater Res 1979;13:577–591.
10. Lindblad B, Johansson A. ^{125}I-fibrinogen uptake on peripheral venous cannulas: A comparison between different cannula materials and coatings. J Biomed Mater Res 1987;21:99–105.
11. Glasier MA, Keech A, Sheardown H, Subbaraman LN, Jones L. Conformational and quantitative characterization of lysozyme extracted from galyfilcon and senofilcon silicone hydrogel contact lenses. Curr Eye Res 2008;33:1–11.
12. Suwala M, Glasier MA, Subbaraman LN, Jones L. Quantity and conformation of lysozyme deposited on conventional and silicone hydrogel contact lens materials using an in vitro model. Eye Contact Lens 2007;33:138–143.
13. Senchyna M, Jones L, Louie D, May C, Forbes I, Glasier MA. Quantitative and conformational characterization of lysozyme deposited on balafilcon and etafilcon contact lens materials. Curr Eye Res 2004;28:25–36.
14. 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.
15. Subbaraman LN, Jones L. Kinetics of lysozyme activity recovered from conventional and silicone hydrogel contact lens materials. J Biomater Sci Polym Ed 2010;21:343–358.
16. Jones B, Sack R. Immunoglobulin deposition on soft contact lenses: Relationship to hydrogel structure and mode of use and giant papillary conjunctivitis. CLAO J 1990;16:43–48.
17. Lin ST, Mandell RB, Leahy CD, Newell JO. Protein accumulation on disposable extended wear lenses. CLAO J 1991;17:44–50.
18. 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.
19. Meadows DL, Paugh JR. Use of confocal microscopy to determine matrix and surface protein deposition profiles in hydrogel contact lenses. CLAO J 1994;20:237–241.
20. 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.
21. Hart DE. Influence of contact lens material surface characteristics and replacement frequency on protein and lipid deposition. Optom Vis Sci 1999;76:616–617.
22. Ichijima H, Kawai T, Yamamoto K, Cavanagh HD. Determination of protein deposits on RGP lenses by X-ray photoelectron spectroscopy. CLAO J 2000;26:18–20.
23. 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.
24. Bontempo AR, Rapp J. Protein and lipid deposition onto hydrophilic contact lenses in vivo. CLAO J 2001;27:75–80.
25. 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(Pt 1):5–12.
26. Janatova J. Activation and control of complement, inflammation, and infection associated with the use of biomedical polymers. ASAIO J 2000;46:S53–S62.
27. Spring TF. Reaction to hydrophilic lenses. Med J Aust 1974;1: 449–450.
28. Refojo MF, Holly FJ. Tear protein adsorption on hydrogels: A possible cause of contact lens allergy. Cont Intraoc Lens Med J 1977; 3:23–36.
29. Hart DE, Schkolnick JA, Bernstein S, Wallach D, Gross DF. Contact lens induced giant papillary conjunctivitis: A retrospective study. J Am Optom Assoc 1989;60:195–204.
30. Elhers WH, Donshik PC. Giant papillary conjunctivitis. Curr Opin Allergy Clin Immunol 2008;8:445–449.
31. Dumbleton K. Adverse events with silicone hydrogel continuous wear. Cont Lens Anterior Eye 2002;25:137–146.
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. Fu K, Griebenow K, Hsieh L, Klibanov AM, Langer R. FTIR characterization of the secondary structure of proteins encapsulated within PLGA microspheres. J Control Release 1999;58:357–366.
34. van Stokkum IH, Linsdell H, Hadden JM, Haris PI, Chapman D, Bloemendal M. Temperature-induced changes in protein structures studied by Fourier transform infrared spectroscopy and global analysis. Biochemistry 1995;34:10508–10518.
35. Heaton RJ, Haris PI, Russell JC, Chapman D. Application of SPR & FTIR spectroscopy to the study of protein–biomaterial interactions. Biochem Soc Trans 1995;23:502S.
36. Sasahara K, Demura M, Nitta K. Partially unfolded equilibrium state of hen lysozyme studied by circular dichroism spectroscopy. Biochemistry 2000;39:6475–6482.
37. Roux P, Delepierre M, Goldberg ME, Chaffotte AF. Kinetics of secondary structure recovery during the refolding of reduced hen egg white lysozyme. J Biol Chem 1997;272:24843–24849.
38. Laurents DV, Baldwin RL. Characterization of the unfolding pathway of hen egg white lysozyme. Biochemistry 1997;36: 1496–1504.

39. Nichols J. Contact lens 2010. Contact Lens Spectrum 2011;26:24–28.
40. Gonzalez-Meijome JM, Lopez-Alemany A, Almeida JB, Parafita MA. Surface AFM microscopy of unworn and worn samples of silicone hydrogel contact lenses. J Biomed Mater Res B Appl Biomater 2009;88:75–82.
41. Teichroeb JH, Forrest JA, Ngai V, Martin JW, Jones L, Medley J. Imaging protein deposits on contact lens materials. Optom Vis Sci 2008;85:1151–1164.
42. Guryca V, Hobzova R, Pradny M, Sirc J, Michalek J. Surface morphology of contact lenses probed with microscopy techniques. Cont Lens Anterior Eye 2007;30:215–222.
43. Gonzalez-Meijome JM, Lopez-Alemany A, Almeida JB, Parafita MA, Refojo MF. Microscopic observation of unworn siloxane-hydrogel soft contact lenses by atomic force microscopy. J Biomed Mater Res B Appl Biomater 2006;76:412–418.
44. Bruinsma GM, Rustema-Abbing M, de Vries J, Stegenga B, van der Mei HC, van der Linden ML, Hooymans JM, Busscher HJ. Influence of wear and overwear on surface properties of etafilcon A contact lenses and adhesion of *Pseudomonas aeruginosa*. Invest Ophthalmol Vis Sci 2002;43:3646–3653.
45. Cheng L, Muller SJ, Radke CJ. Wettability of silicone-hydrogel contact lenses in the presence of tear-film components. Curr Eye Res 2004;28:93–108.
46. Maldonado-Codina C, Morgan PB. In vitro water wettability of silicone hydrogel contact lenses determined using the sessile drop and captive bubble techniques. J Biomed Mater Res A 2007;83:496–502.
47. Lorentz H, Rogers R, Jones L. The impact of lipid on contact angle wettability. Optom Vis Sci 2007;84:946–953.
48. Imoto T, Johnson L, North A, Phillips D, Rupley J. Vertebrate lysozymes. In: Boyer Peditor. The Enzyme. New York: Academic Press; 1972.
49. Fullard R, Snyder C. Protein levels in nonstimulated and stimulated tears of normal human subjects. Invest Ophthalmol Vis Sci 1990;31:1119–1126.
50. Fleming A. On a remarkable bacteriolytic element found in tissues and secretions. Proc R Soc Lond Ser B 1922;93:306–317.
51. Vantrappen G, Ghoos AY, Peeters T. The inhibition of lysozyme by bile acids. Am J Dig Dis 1976;21:547–552.
52. Morsky P. Turbidimetric determination of lysozyme with *Micrococcus lysodeikticus* cells: Reexamination of reaction conditions. Anal Biochem 1983;128:77–85.
53. Ito Y, Yamada H, Nakamura S, Imoto T. Purification, amino acid sequence, and some properties of rabbit kidney lysozyme. J Biochem 1990;107:236–241.
54. Brecher AS, Rasmusson E, Riley C, VanDenBerghe S. The effect of chlorambucil upon lysozyme activity. Fundam Clin Pharmacol 1999;13:107–112.
55. Chhabra S, Sachdeva V, Singh S. Influence of end groups on in vitro release and biological activity of lysozyme from a phase-sensitive smart polymer-based in situ gel forming controlled release drug delivery system. Int J Pharm 2007;342:72–77.
56. Wu Y, Daeschel MA. Lytic antimicrobial activity of hen egg white lysozyme immobilized to polystyrene beads. J Food Sci 2007;72: M369–M374.
57. Shugar D. The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. Biochim Biophys Acta 1952;8: 302–309.
58. Zhang S, Borazjani RN, Salamone JC, Ahearn DG, Crow Jr SA, Pierce GE. In vitro deposition of lysozyme on etafilcon A and balafilcon A hydrogel contact lenses: Effects on adhesion and survival of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Cont Lens Anterior Eye 2005;28:113–119.
59. Luensmann D, Heynen M, Liu L, Sheardown H, Jones L. The efficiency of contact lens care regimens on protein removal from hydrogel and silicone hydrogel lenses. Mol Vis 2010;16:79–92.
60. Subbaraman LN, Glasier MA, Senchyna M, Sheardown H, Jones L. Kinetics of in vitro lysozyme deposition on silicone hydrogel, PMMA, and FDA groups I, II, and IV contact lens materials. Curr Eye Res 2006;31:787–796.
61. Zhang F, Glasier MA, Sheardown H, Jones L. The use of confocal microscopy to determine the location of lysozyme deposition in conventional hydrogel and silicone hydrogel contact lens materials. Invest Ophthalmol Vis Sci 2006;47:2405 (E-abstract).
62. Ito Y, Yoshikawa A, Hotani T, Fukuda S, Sugimura K, Imoto T. Amino acid sequences of lysozymes newly purified from invertebrates imply wide distribution of a novel class in the lysozyme family. Eur J Biochem 1999;259:456–461.
63. Dai Y, Wang C. A mutant truncated protein disulfide isomerase with no chaperone activity. J Biol Chem 1997;272:27572–27576.
64. Garrett Q, Garrett RW, Milthorpe BK. Lysozyme sorption in hydrogel contact lenses. Invest Ophthalmol Vis Sci 1999;40:897–903.
65. Morrissey BW, Fenstermaker CA. Conformation of adsorbed gamma-globulin and beta-lactoglobulin. Effect of surface concentration. Trans Am Soc Artif Intern Organs 1976;22:278–284.
66. Subbaraman LN, Glasier MA, Senchyna M, Sheardown H, Jones L. Extraction efficiency of an extraction buffer used to quantify lysozyme deposition on conventional and silicone hydrogel contact lens materials. Eye Contact Lens 2007;33:169–173.

CHAPTER 4 EXTRACTION VERSUS IN SITU TECHNIQUES FOR MEASURING SURFACE-ADSORBED LYSOZYME*

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Author	Concept/Design	Acquisition of Data	Analysis	Write Up/Publication
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ORIGINAL ARTICLE

Extraction versus *In Situ* Techniques for Measuring Surface-Adsorbed Lysozyme

Brad Hall*, Chau-Minh Phan†, Lakshman Subbaraman‡, Lyndon W. Jones§, and James Forrest||

ABSTRACT

Purpose. To compare two techniques for measuring the activity of lysozyme deposited onto hydrogel contact lens and to image the binding of *Micrococcus lysodeikticus* to contact lenses.

Methods. Using a previously described protein extraction technique and a recently developed *in situ* technique, we measured the time-dependent activity of adsorbed lysozyme on six different contact lens materials during the first minute and up to 1 week of interaction with the material surface. Total activity of extracted lysozyme, total *in situ* activity, and the activity of the outer surface layer of sorbed lysozyme were determined using the two different techniques. Micrococcal cellular interaction with surface-adsorbed lysozyme was imaged using confocal microscopy.

Results. The differences between total extracted activities, total *in situ* activities, and surface activities were both measurable and material specific. In most cases, total extracted activity is greater than total *in situ* activity, which, in turn, is greater than surface activity. After 1 week, etafilcon A had the highest extracted activity at 137 μg/lens, followed by omafilcon A, balafilcon A, comfilcon A, senofilcon A, and lotrafilcon B at 27.4, 2.85, 2.02, 0.46, and 0.27 μg/lens, respectively. Micrococcal cell adhesion was greatest on contact lenses with high contact angles, such as balafilcon A, omafilcon A, and senofilcon A and lowest on contact lenses with low contact angles, such as etafilcon A, comfilcon A, and lotrafilcon B. Subsequent removal/prevention of adhered micrococcal cells was greatest on balafilcon A, which had the highest surface activity, and lowest on lotrafilcon B, which had the lowest surface activity.

Conclusions. This study has measured and made direct comparisons between two established techniques for measuring the activity of adsorbed lysozyme. The extraction technique determines the activity of underlying layers of lysozyme or lysozyme within the matrix of the material. Conversely, the *in situ* technique allows conclusions to be drawn about only the biologically relevant lysozyme including the activity of just the outer surface of adsorbed lysozyme.

(Optom Vis Sci 2014;91:1062–1070)

Key Words: protein adsorption, contact lens, protein activity on biomaterials, protein surface activity, lysozyme kinetics, silicone hydrogel

The interaction between a biomaterial and protein in solution is one of the fundamental problems in biomaterials science and bioengineering. This problem can be further divided into measuring the quantity of adsorbed protein and the biological activity (related to structure) of that protein. Measuring the amount of adsorbed protein on a flat substrate is relatively straightforward and many techniques are used, including ellipsometry,[1,2] quartz crystal microbalance,[3,4] surface plasmon resonance,[5,6] and atomic force microscopy.[7] Similar measurements on commercial materials are not as straightforward, and techniques such as Western blotting, enzyme-linked immunosorbent assays, and radiolabeling using I125 or C14 have been used.[8–10] From an application standpoint, biological structure and resultant activity rather than the amount of adsorbed protein are often more important, as denatured proteins may lead to an immunological response.[11] Additionally, denatured protein can provide a substrate for bacterial adhesion,[12,13] whereas protein that remains active can remove bacterial cells.[14] In the case of contact lenses, for example, accumulation of denatured protein is associated with the development of giant papillary conjunctivitis[15–17] and discomfort.[18]

Measuring the tertiary or secondary structure of adsorbed protein deposits has been undertaken with limited success.[19,20] Given that

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biological function may relate to only part of the structure, the best approach is probably to measure the biological activity of the adsorbed protein directly. More specifically, it is desirable to measure the activity of the intact biomaterial-adsorbed protein layer. A technique that can measure *in situ* the biological activity of an intact deposit of lysozyme has recently been described.[21] More commonly, the protein deposit is removed or extracted from the material and then activity is measured *ex situ*.[22] This extraction technique has been used extensively to characterize the activity of protein adsorbed onto soft contact lenses.[9,23–25] There are a number of potential drawbacks to the extraction approach. For example, the extraction process cannot remove all the protein adsorbed onto contact lenses. This efficiency is material dependent and can be as low as 54% of the total adsorbed protein.[24–26] Additionally, the highly surface active solutions used to remove the protein may affect protein activity.[25] Finally, the extraction process does not distinguish between surface-adsorbed protein and protein deposited within the material matrix as the removal process removes protein from the surface and bulk areas of the lenses and transfers it to solution. If we consider an example of multilayer adsorption, where the protein in near or immediate contact with the material surface was denatured and inactive, but the outer-top surface maintained full biological activity, then an *in situ* measure would indicate "high" activity, whereas an extraction method (which measures activity of all molecules in the layer) would suggest a lower activity. For comparison with *in vivo* systems, this distinction can be very important.

Lysozyme is the typical model protein for biological activity measurements.[24,27–30] It is abundant in the tear film, accounting for 30% of the total protein with a concentration of 1.9 mg/mL,[31] and the biological activity can be measured using a standard micrococcal activity assay.[9,21,23,24,27–30]

In this article, we make direct comparisons between *in situ* and *ex situ* measures of the protein activity of lysozyme deposits on commercial contact lenses. By using the same experimental protocols for the lens preparation and protein deposition before activity measurements, we can make quantitative and direct measurements of *in vitro* and extracted lysozyme activity for a number of common soft lens materials.

Experimental Technique

Contact Lens Preparation

Preparation and subsequent deposition of lysozyme onto the lenses were completed in the same manner before activity measurements. Four silicone hydrogel and two conventional hydrogel contact lenses (all commercially available), known to deposit lysozyme,[9,21,23,32–34] were investigated (Table 1).

The contact lenses were removed from their packaging solutions, rinsed with phosphate-buffered saline (PBS; pH 7.4) to remove any residual packaging solution, and then placed into individual wells of a 12-well plate (VWR, Mississauga, ON) containing 5 mL of PBS. The plates were then sealed with parafilm and placed on a shaker for 24 hours at ambient temperature. The lenses were then removed, blotted on lens paper, and placed into 6-mL screw cap glass vials (VWR), which contained 1 mL of lysozyme dissolved in PBS at a concentration of 1.9 mg/mL (etafilcon A was incubated in 0.19 mg/mL lysozyme as the high lysozyme accumulation interferes with the accuracy of *in situ* activity measurements). The lenses were incubated at 37°C and removed after specific time intervals in minutes: 10, 100, 1000 (~1 day), and 10,000 (~1 week), with the exception of a subset of lenses that were incubated at ambient temperature and removed after 1 minute (as it was not feasible to reliably incubate at 37°C for this short time). Removed lenses were swirled in two containers of 100 mL PBS for 5 seconds each, blot dried on lens paper, and then placed either in 24-well plates for *in situ* activity measurements or into 6-mL polyethylene vials (VWR) for lysozyme extraction.

Determination of Lysozyme Activity

The extraction process was performed as described previously.[9,23,25,26,35] In brief, all lenses were then placed into 6-mL polyethylene vials containing an extraction solvent consisting of acetonitrile (ACN) and 0.2% trifluoroacetic acid (TFA) (ACN and 0.02% TFA were used for senofilcon A as the higher concentration disintegrates this material) and then incubated in the dark at ambient temperature for 24 hours. After incubation,

TABLE 1.

Properties of contact lens materials evaluated in this study

USAN	Proprietary name	Manufacturer	Water content, %	FDA group	Principal monomers
Senofilcon A	ACUVUE OASYS	Johnson & Johnson	38	I	mPDMS, DMA, HEMA, siloxane macromer, TEGDMA, PVP
Lotrafilcon B	AIR OPTIX	CIBA Vision	33	I	DMA, TRIS, siloxane monomer
Comfilcon A	BIOFINITY	CooperVision	48	I	M3U, FMM, TAIC, IBM, HOB, NMNVA, NVP
Balafilcon A	PUREVISION	Bausch + Lomb	36	III	NVP, TPVC, NVA, PBVC
Omafilcon A	PROCLEAR	CooperVision	62	II	HEMA, PC
Etafilcon A	ACUVUE 2	Johnson & Johnson	58	IV	HEMA, MA

mPDMS, monofunctional polydimethylsiloxane; DMA, *N,N*-dimethylacrylamide; HEMA, 2-hydroxyethyl methacrylate; TEGDMA, tetraethylene-glycol dimethacrylate; PVP, polyvinyl pyrrolidone; TRIS, trimethyl siloxy silane; M3U, ax-bis(methacryloyloxyethyl iminocarboxy ethyloxypropyl)-poly(dimethylsiloxane)-poly(trifluoropropylmethylsiloxane)-poly(methoxy-poly-(ethyleneglycol)propylmethylsiloxane); FMM, α-methacryloyloxyethyl iminicarboxyethyloxypropyl-poly(dimethylsiloxy)-butyldimethylsilane; TAIC, 1,3,5-triallyl-1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione; IBM, isobornyl methacrylate; HOB, 2-hydroxybutyl methacrylate; NMNVA, *N*-methyl-*N*-vinyl acetamide; NVP, *N*-vinyl pyrrolidone; TPVC, tris-(tri-methylsiloxysilyl)propylvinyl carbamate; NVA, *N*-vinyl aminobutyric acid; PBVC, poly[dimethylsiloxyl] di [silylbutanol] bis[vinyl carbamate]; PC, phosphorylcholine; MA, methacrylic acid.

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1-mL aliquots of the resultant solution were transferred to sterile Eppendorf tubes (VWR) and dried using a Savant Speed Vac (Holbrook, NY). The dried lysozyme samples were stored at −80°C before reconstitution in 100 μL of tear dilution buffer (10 mM trimethyl siloxy silane, 1 mM ethylenediaminetetraacetic acid, NaCl [0.9% wt/vol], pH 8.0).

For lysozyme activity measurements, *Micrococcus lysodeikticus* (Sigma-Aldrich, Oakville, ON, Canada; American Type Culture Collection # 4698) was diluted in PBS, pH 7.4, to concentrations of 1 to 1.5 mg/mL and allowed to swell at 4°C for 24 hours. The prepared *M. lysodeikticus* was removed and placed on a shaker for 30 minutes and subsequently incubated at 37°C for 30 minutes. The *M. lysodeikticus* was added to individual wells in 24- or 96-well plates, containing contact lens or contact lens extracts in PBS, to a volume of 2 and 0.1 mL, respectively, and an optical density of 1.2 at 450 nm. Optical extinction measurements were obtained at 31°C every 30 seconds for 30 minutes (SpectraMax M5e plate reader, Molecular Devices, Sunnyvale, CA). Contact lens and contact lens extract absorbance versus time was compared with standard curves, which were plotted using linear regression of the change in absorbance over time versus mass of lysozyme, to extrapolate active masses of lysozyme in the samples. Note that standard curves were plotted using known amounts of lysozyme in solution, and the resulting masses are equivalent to masses of lysozyme in solution.

The activity of lysozyme in the lens extracts models the activities of all the adsorbed lysozyme, including lysozyme deposited within the matrix of the lens materials, whereas the activity of lysozyme for lenses directly in solution models the activities of the most biologically relevant lysozyme, which is lysozyme located in the outer surface layer of adsorbed protein and any protein desorbed into solution to which the host's biological systems can interact. To determine the activity of the surface-adsorbed layer of lysozyme, an extra step was implemented in which contact lens samples were incubated in 1.5 mL of PBS at 31°C for 30 minutes to allow for lysozyme desorption into solution. The contact lenses were removed from the well plate, and the activity of the remaining solution was measured as described above. The activity values of lysozyme desorbed into PBS are then subtracted from the activity of lysozyme-coated lenses (whose activity is influenced by both surface-adsorbed lysozyme and desorbed lysozyme in PBS) to calculate the activity of surface-adsorbed lysozyme solely.

Confocal Imaging of M. lysodeikticus Adhesion onto Contact Lenses

Although the *in vitro* activity measurements give a quantitative value of the activity of the adsorbed protein layer, they do so on the basis of calibration with known amounts of lysozyme in solution. Using this calibration involves an implicit assumption that the lysing efficiency of the adsorbed lysozyme is the same as that in solution. The activity measurements are essentially dependent on the speed at which lysozyme can catalyze the hydrolysis of the glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in bacterial cell walls, thus causing the cells to lyse.[36] It is likely then that if *M. lysodeikticus* cells are localized very near or on the protein-covered lens materials, then the cells would be able to interact with a larger number of lysozyme molecules simultaneously (or interact for longer times) than possible with random interactions in solution. Determination of the ability for *M. lysodeikticus* to localize near the contact lens surface was measured using a confocal imaging technique, as previously described.[37] Briefly, prepared contact lens samples were exposed to 2 mL of *M. lysodeikticus* solution with an optical density of 1.2 at 450 nm for 5 minutes. To stain the cells for imaging, lenses were then rinsed in 100 mL of PBS, placed in a scanning plate covered with 100 μL of a mixture of STYO 9 and propidium iodide (Molecular Probes, Burlington, ON, Canada), and incubated at 31°C for 15 minutes. Samples were then imaged using a confocal microscope. ImageJ (Bethesda, MD) software was used to determine the area on the contact lenses covered by *M. lysodeikticus* cells.

Statistical Analysis

Data were analyzed using repeated-measures analysis of variance. Tukey honestly significant difference *post hoc* comparisons were performed, where applicable; $p < 0.05$ was taken to be significant. The Student *t* test was used to assess pairwise differences where applicable.

RESULTS

The measured activities (compared with solution standards) are summarized in Table 2 for the 1-week time point.

Fig. 1 shows the results of lysozyme activity measurements after four silicone and two conventional hydrogel contact lens materials were exposed to lysozyme solutions for times ranging from 1 minute to 1 week. In this figure, we plot the activity of the extracted deposit as solid circles, the total *in situ* activity (as defined in the "Experimental Technique" section) as hollow circles, and the derived *in situ* surface activity as solid triangles. The extracted protein may include any protein that had been adsorbed (which

TABLE 2.

Activity of lysozyme on contact lenses (in micrograms per lens, mean ± SD, n = 4)

Lens type	Activity	10,000 min (~1 week)
Senofilcon A	Extracted	0.46 ± 0.13
	Direct method	0.18 ± 0.03
	Surface	0.03 ± 0.04
Lotrafilcon B	Extracted	0.27 ± 0.08
	Direct method	0.15 ± 0.01
	Surface	0.02 ± 0.01
Comfilcon A	Extracted	2.02 ± 0.18
	Direct method	0.79 ± 0.06
	Surface	0.41 ± 0.09
Balafilcon A	Extracted	2.85 ± 0.21
	Direct method	3.19 ± 0.26
	Surface	1.25 ± 0.29
Omafilcon A	Extracted	27.38 ± 4.68
	Direct method	3.91 ± 0.09
	Surface	0.36 ± 0.12
Etafilcon A	Extracted	137.31 ± 13.79
	Direct method	3.92 ± 0.09
	Surface	0.29 ± 0.10

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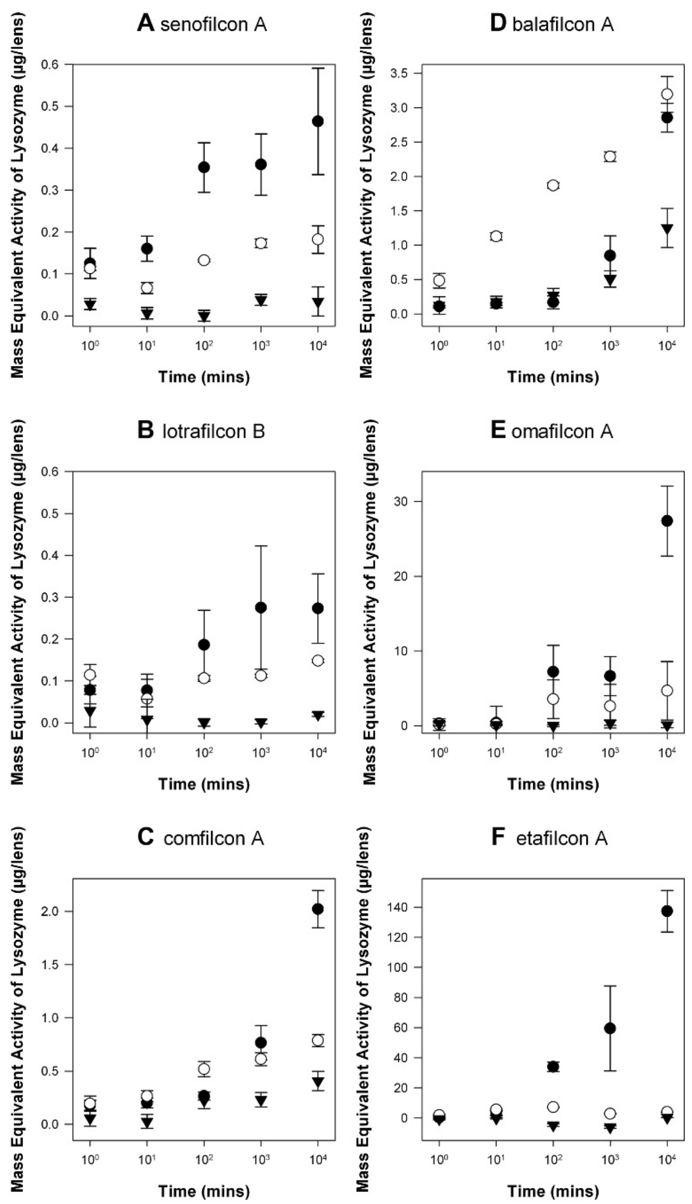

FIGURE 1.

Kinetics of active lysozyme deposited onto (A) senofilcon A, (B) lotrafilcon B, (C) comfilcon A, (D) balafilcon A, (E) omafilcon A, and (F) etafilcon A (0.2 mg/mL). Error bars represent mean ± SD, n = 4. In all cases: (●) total activity from extraction, (○) total activity from direct measurement, (▼) activity of the surface-adsorbed lysozyme after correction for desorbed lysozyme. Note that panels A and B are on the same scale, different from panels C to F.

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can be many times the amount of protein in the outermost layer of an adsorbed deposit), whereas the total direct method activity includes surface-adsorbed protein as well as any protein that was desorbed from the lens after exposure to the bacterial solution (as that solution initially contains no lysozyme). The surface activity involves *only* the protein that remains on the lens surface during the entire activity measurement. Generally, over time, the extracted activity increases, whereas total *in situ* and surface activity tend to plateau, although there are significant differences between all quantities, and these differences are material dependent.

Activity of Silicone Hydrogels

For the nonionic silicone hydrogels (Fig. 1A to C), the extracted protein has an activity that is greater than or equal to either of the *in situ* measurements. For senofilcon A, the extracted activity is higher than the *in situ* activity after 10 minutes or longer, whereas the extracted activity from comfilcon A was statistically higher only after 10,000 minutes ($p < 0.0005$). Lysozyme activity from *ex situ* and *in situ* measurements was not statistically different for lotrafilcon B. In all cases where distinction is possible, the surface activity was the lowest measured quantity. Note the relatively high variability for activity measurements using the extraction technique.

For balafilcon A (Fig. 1D), there is a surprising reversal in this trend. Whereas for the initial time points, the extraction activity results were not distinguishable from the "*in situ* surface activity," the total measurement in the *in situ* method was greater than that of the extraction measurement for 10 minutes $\leq t \leq$ 1000 minutes ($p < 0.0005$). This is puzzling, as the lenses were treated in exactly the same manner until either the *in situ* assay or the extraction process was reached. Because the total integrated adsorbed protein must be greater than the total direct measure, this observation means that the amount of protein that simply desorbs from the lens into the solution during the activity assay measurements is much greater than that which is removed by extraction or that the extraction has had a significant effect on the activity of the extracted protein. To confirm this surprising observation, we undertook a direct comparison between the extraction efficiencies of the traditional extraction mixture (as described above) and PBS. Prepared contact lenses were placed in 1.5 mL of either a mixture of ACN/TFA or PBS and left in the dark at ambient temperature for 24 hours. From the resultant solution, aliquots of 100 μL were immediately used for activity measurements, whereas a subset of ACN/TFA extracts were subjected to drying and −80°C storage. The results of this test are shown in Fig. 2. It is clear that for balafilcon A, a PBS solution extracts more active lysozyme than a mixture of ACN and TFA ($p < 0.005$), whereas storage at −80°C after extraction does not affect activity. Previous work has shown that the extraction process used in this study can only extract about 54% of lysozyme from balafilcon A lenses,[26] which may explain this difference in activity.

Activity of Conventional Hydrogels

Omafilcon A is a nonionic polyHEMA-based material. Compared with the other nonionic lenses in this study, there is an order of magnitude greater extracted activity and direct method activity

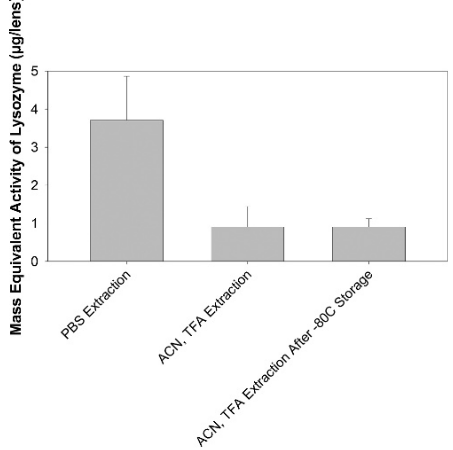

FIGURE 2.
Comparison of the activity of lysozyme extracted from balafilcon A contact lenses using a mixture of PBS, ACN and TFA, and ACN and TFA and then −80°C storage for 24 hours. Error bars represent mean ± SD, n = 3.

(Fig. 1E). The extracted activity and direct method activity are statistically different only at day 7 ($p < 0.0005$).

Etafilcon A (Fig. 1F) was incubated with 1/10 of the lysozyme concentration compared with all other lens types but still had the greatest extracted activity after 7 days ($p < 0.0005$). *Ex situ* activity measurements were statistically greater than *in situ* activity measurements at all time points after 100 minutes ($p < 0.05$).

Adhesion of *M. lysodeikticus* to Contact Lens Materials

Attraction and subsequent adhesion of *M. lysodeikticus* to contact lens materials may affect the efficiency of surface-adsorbed lysozyme compared with lysozyme in solution. This interaction should be understood to improve the direct comparison of extraction and *in situ* measurements. Representative images of *M. lysodeikticus* cells that adhered to contact lens materials are shown in Fig. 3.

Percent coverage of cells on lysozyme-treated and untreated contact lenses is shown in Fig. 4. Lysozyme-treated contact lenses had a significantly lower percent coverage of the contact lens surface by *M. lysodeikticus* than untreated lenses for senofilcon A, comfilcon A, etafilcon A, and omafilcon A ($p < 0.05$). There were no cells visible on comfilcon A and etafilcon A for lysozyme-treated lenses.

DISCUSSION

Micrococcal activity assays are used to determine the biological activity of lysozyme after or during adsorption onto biomaterials. In this study, we make direct comparisons between direct *in situ* measurements of activity and measurements after protein extraction. Generally, there is a good agreement between these two

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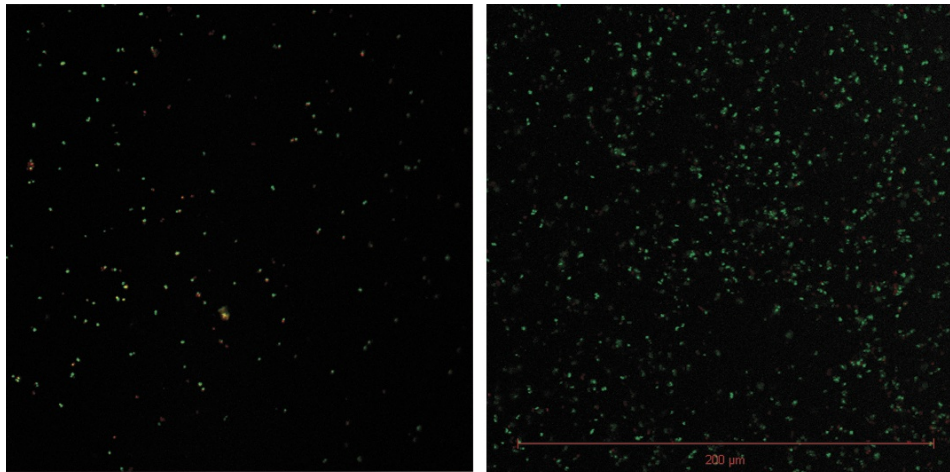

FIGURE 3.
Micrococcus lysodeikticus adhesion onto untreated senofilcon A (left) and balafilcon A (right) contact lenses. Stained with STYO 9 and propidium iodide.

measurements until about 10 minutes, where there is more extracted activity than *in situ*, with the notable exception of balafilcon A. This is not unexpected, as the initial buildup of lysozyme monolayers will require some time before there is more lysozyme buildup than is biologically relevant (lysozyme that can interact with the host's biological systems). The differences between extracted activities and *in situ* activities after 10 minutes will be caused by protein removed in underlying layers and within the matrix of the material. There are instances then where either measurement is more appropriate than the other. Determining the biologically relevant active lysozyme on materials provides insight into material biocompatibility, whereas understanding the denaturation extent of underlying lysozyme presents an opportunity to develop processes, such as cleaning solutions, capable of removing it.

Surface activity measurements are not directly measured but calculated from two separate measurements. As a result, materials that deposit relatively high amounts of lysozyme, such as the conventional hydrogel lenses omafilcon A and etafilcon A, are

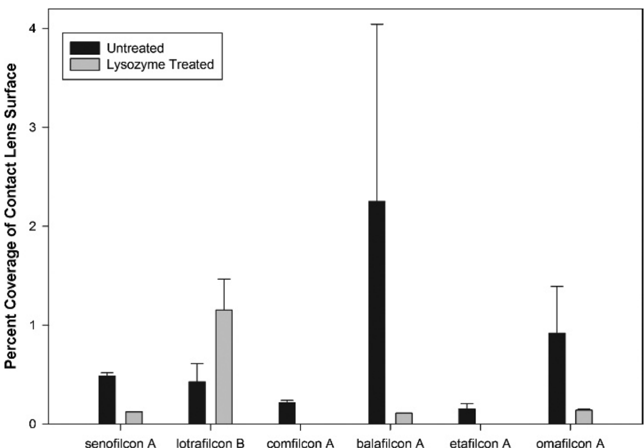

FIGURE 4.
Surface coverage of *M. lysodeikticus* on untreated (black) and lysozyme-treated (gray) contact lenses.

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prone to significant desorption of lysozyme from these materials, resulting in larger errors in surface activity determination. This process is not unexpected, as a lysozyme-coated material placed in a solution will approach equilibrium of adsorption and desorption; however, the desorption can lead to negative values when we calculate surface activity. As a result, this technique is not well suited to lead to strong conclusions about the surface activity of lysozyme on omafilcon A and etafilcon A materials. Despite this limitation for lenses that deposit high amounts of lysozyme, this surface technique presents a unique opportunity to probe the activity of intact deposits of adsorbed lysozyme.

The surface activities of lysozyme deposited on senofilcon A and lotrafilcon B are relatively constant through all time points and average about 24 and 16 ng, respectively. These values are not significantly different and indicate that senofilcon A and lotrafilcon B are qualitatively the same, as demonstrated previously.[9,21] Lotrafilcon B and senofilcon A are both classified as Food and Drug Administration (FDA) group I materials (nonionic, low water content) and represent the first and second generation of silicone hydrogels, respectively,[38] which may explain their similar effect on lysozyme.

Comfilcon A is a later "generation" of silicone hydrogels[39] but is also classified as an FDA group I material. The main difference between comfilcon A, senofilcon A, and lotrafilcon B is the increased water content and reported wettability of comfilcon A owing to incorporation of inherent wetting agents in the material backbone.[39] These properties may result in comfilcon A behaving more similarly to a conventional hydrogel,[40] which would help explain the relatively higher amount of activity in all measurements compared with senofilcon A and lotrafilcon B.

If we take the contact lens to be a spherical cap with a diameter of 14 mm and a radius of curvature of 13 mm, then the surface area will be 3.34 cm^2. Because adsorbed lysozyme will have a coverage of 207 ng/cm^2 or 310 ng/cm^2 depending on its orientation,[33] 692 to 1035 ng will be the theoretical amount of lysozyme for monolayer coverage of a contact lens. By comparing our *in situ* and surface activity measurements to the theoretical maximum, we can draw conclusions about the overall activity of the adsorbed lysozyme.

Balafilcon A is known to deposit large amounts of lysozyme compared with other silicone hydrogels because of its net negative charge and large pore size.[41,42] It is the only material investigated to show about 100% of the activity expected of the 1035-ng theoretical maximum amount of lysozyme that could be present in the outer surface layer,[33] which occurred after 1 week. This is in contrast to previous work,[9,24] which demonstrated by extraction that about 63% of the total lysozyme deposited is active. *This contrast is precisely why surface activity measurements are important in understanding biocompatibility.* Extraction measurements could lead to the conclusion that balafilcon A has low biocompatibility in terms of protein activity; however, surface activity measurements demonstrate a high biocompatibility in terms of biologically relevant lysozyme. To the best of our knowledge, this is the only study to directly compare extraction, *in situ*, and surface activity measurements.

Both omafilcon A and etafilcon A, despite a high amount of activity from extraction, showed a plateau of activity from the *in situ* activity measurements at about 4 to 5 μg. This may be the maximum amount of biologically relevant lysozyme on contact lenses. Using the theoretical maximum of lysozyme per monolayer, we can calculate the maximum amount of monolayers that are biologically relevant on contact lenses to be about four to seven. The fact that this is greater than a single monolayer could indicate that the surface-adsorbed protein is adsorbing not as a simple layer but as a structure with more surface area. Alternatively, and more likely, this is a result of the fact that activity on the lens surface has been compared with a calibration in solution.

There is a limitation when using the turbidimetric assay. It is not as sensitive as the fluorescence-based technique used by Ng et al.[35] with contact lens extracts. Ng et al.[35] determined the detection limit of the turbidimetric assay to be 20 ng and the detection limit of the fluorescence-based assay to be 2 ng. This discrepancy has also been discussed previously.[27] Despite the enhanced sensitivity of the fluorescence-based assay, Ng et al. found only 3 ng of deviation between the two methods,[35] which may not be a relevant difference for activity measurements after extraction. Using a fluorescence-based assay for *in situ* and subsequently surface activity measurements warrants further investigation.

In a previous study,[21] where we introduced the technique to measure activity of an intact layer, we did not distinguish between two different mechanisms for the surface activity. One possibility is that the bacterial cell interacts with lysozyme that is adsorbed onto the lens. A second possibility is that the bacterial cell interacts with lysozyme that was initially adsorbed to the lens but had desorbed into solution. There is a clear and important difference as if it is the first case, then the calibration (where we compare the activity of lysozyme in solution) is not necessarily valid because the actual lysing occurs on the biomaterial and not in solution. To test this, the following was implemented. Balafilcon A lenses were prepared for confocal imaging as previously described; however, both untreated and treated lenses were placed together and exposed to *M. lysodeikticus* cells simultaneously. If solution active lysozyme lyses the *M. lysodeikticus* cells, then there would be the same number of cells for both lenses as the protein-coated lens would act as a source of lysozyme in solution for *both* lenses. If surface-adsorbed lysozyme lyses the *M. lysodeikticus* cells *on the lens*, then there would be more cells on the untreated surfaces. We found more *M. lysodeikticus* cells on lysozyme-untreated versus lysozyme-treated lenses ($p < 0.005$), which indicates that surface-adsorbed lysozyme interacts directly with *M. lysodeikticus* cells at the surface. Adsorbed lysozyme lysing bacterial cells directly at the surface could potentially, owing to the bacterial cell proximity to the biomaterial surface, cause the cell contents to adsorb onto the material. These cellular contents may cause inflammatory reactions and discomfort. In the case of lipopolysaccharides, they are known to cause keratitis and conjunctivitis.[43] It is clear that any investigation of protein activity on biomaterials must characterize the surface-adsorbed protein.

Contact lens materials with surface active lysozyme may be able to remove adhered *M. lysodeikticus* cells and/or prevent their adherence to the lens material surface. This reduction was highest for lysozyme-coated omafilcon A and balafilcon A. This is expected, as omafilcon A and balafilcon A have relatively high surface activities. Lenses with the relatively lowest surface activities (senofilcon A and lotrafilcon B) showed moderate and no reduction, respectively. Lysozyme-coated etafilcon A and

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comfilcon A completely desorbed and/or prevented adhesion of *M. lysodeikticus* cells, although we would expect that there will be a relatively small amount of cells.

The adhesion of *M. lysodeikticus* cells to the contact lenses in this study was material dependent. Previous studies have suggested that bacterial adhesion to biomaterials will be dependent on the hydrophobicity of the material and will favor hydrophobic materials.[44–47] Senofilcon A, omafilcon A, and balafilcon A all had relatively large amounts of *M. lysodeikticus* cells on the surface in the absence of lysozyme compared with lotrafilcon B, comfilcon A, and etafilcon A, which had relatively low amounts of cells. Previous work[48–50] has determined that the contact angles are relatively higher for senofilcon A, omafilcon A, and balafilcon A than the contact angles of lotrafilcon B, comfilcon A, and etafilcon A. This suggests that the attraction and subsequent adhesion of *M. lysodeikticus* are dependent on the hydrophobicity of the material. To the best of our knowledge, this is the first study to use this confocal imaging technique to image surface-bound *M. lysodeikticus*.

CONCLUSIONS

We have measured the activities of lysozyme extracted from deposits and lysozyme still adsorbed onto contact lenses and imaged the adhesion of *M. lysodeikticus* on contact lenses. Using these methods, we have shown that lysozyme activity and *M. lysodeikticus* adhesion are material dependent. Individually, the extraction activities demonstrate the activity of underlying layers of lysozyme or lysozyme within the matrix of the material. *In situ* measurements allow conclusions to be drawn about only the biologically relevant lysozyme, which we have shown to encompass about four to seven monolayers, and surface activity measurements reveal the activity of just the outer surface layer of lysozyme. Together, this suite of techniques provides a powerful method to characterize biomaterial and protein interactions for future lysozyme activity studies.

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REFERENCES

1. Mora MF, Wehmeyer JA, Synowicki R, Garcia CD. Investigating protein adsorption via spectroscopic ellipsometry. In: Puleo DA, Bizeos R, eds. Biological Interactions on Materials Surfaces. New York, NY: Springer; 2009:19–41.
2. Spaeth K, Brecht A, Gauglitz G. Studies on the biotin-avidin multilayer adsorption by spectroscopic ellipsometry. J Colloid Interface Sci 1997;196:128–35.
3. Welle A. Competitive plasma protein adsorption on modified polymer surfaces monitored by quartz crystal microbalance technique. J Biomater Sci Polym Ed 2004;15:357–70.
4. Teichroeb JH, Forrest JA, Jones LW, Chan J, Dalton K. Quartz crystal microbalance study of protein adsorption kinetics on poly(2-hydroxyethyl methacrylate). J Colloid Interface Sci 2008; 325:157–64.
5. Green RJ, Davies J, Davies MC, Roberts CJ, Tendler SJ. Surface plasmon resonance for real time in situ analysis of protein adsorption to polymer surfaces. Biomaterials 1997;18:405–13.
6. Green RJ, Frazier RA, Shakesheff KM, Davies MC, Roberts CJ, Tendler SJ. Surface plasmon resonance analysis of dynamic biological interactions with biomaterials. Biomaterials 2000;21:1823–35.
7. Cullen DC, Lowe CR. AFM studies of protein adsorption: 1. Time-resolved protein adsorption to highly oriented pyrolytic graphite. J Colloid Interface Sci 1994;166:102–8.
8. Chow LM, Subbaraman LN, Sheardown H, Jones L. Kinetics of in vitro lactoferrin deposition on silicone hydrogel and FDA group II and group IV hydrogel contact lens materials. J Biomater Sci Polym Ed 2009;20:71–82.
9. Subbaraman LN, Jones L. Kinetics of lysozyme activity recovered from conventional and silicone hydrogel contact lens materials. J Biomater Sci Polym Ed 2010;21:343–58.
10. Prager MD, Quintana RP. Radiochemical studies on contact lens soilation. I. Lens uptake of 14C-lysozyme from simple and complex artificial tear solutions. J Biomed Mater Res 1997;36:119–24.
11. Janatova J. Activation and control of complement, inflammation, and infection associated with the use of biomedical polymers. ASAIO J 2000;46:S53–62.
12. Willcox MD, Harmis N, Cowell BA, Williams T, Holden BA. Bacterial interactions with contact lenses; effects of lens material, lens wear and microbial physiology. Biomaterials 2001;22:3235–47.
13. Bruinsma GM, van der Mei HC, Busscher HJ. Bacterial adhesion to surface hydrophilic and hydrophobic contact lenses. Biomaterials 2001;22:3217–24.
14. Williams TJ, Schneider RP, Willcox MD. The effect of protein-coated contact lenses on the adhesion and viability of gram negative bacteria. Curr Eye Res 2003;27:227–35.
15. Donshik PC, Ehlers WH, Ballow M. Giant papillary conjunctivitis. Immunol Allergy Clin North Am 2008;28:83–103, vi.
16. 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.
17. Hart DE, Schkolnick JA, Bernstein S, Wallach D, Gross DF. Contact lens induced giant papillary conjunctivitis: a retrospective study. J Am Optom Assoc 1989;60:195–204.
18. Subbaraman LN, Glasier MA, Varikooty J, Srinivasan S, Jones L. Protein deposition and clinical symptoms in daily wear of etafilcon lenses. Optom Vis Sci 2012;89:1450–9.
19. Mannucci LL, Moro F, Cosani A, Palumbo M. Conformational state of lacrimal proteins adsorbed on contact lenses. Curr Eye Res 1985;4:734–6.
20. Castillo EJ, Koenig JL, Anderson JM, Kliment CK, Lo J. Surface analysis of biomedical polymers by attenuated total reflectance-fourier transform infra-red. Biomaterials 1984;5:186–93.
21. Hall B, Jones L, Forrest JA. Measuring the kinetics and activity of adsorbed proteins: in vitro lysozyme deposited onto hydrogel contact lenses over short time periods. J Biomed Mater Res (A) 2013; 101:755–64.
22. Yagami T, Sato M, Nakamura A, Shono M. One of the rubber latex allergens is a lysozyme. J Allergy Clin Immunol 1995;96:677–86.
23. Suwala M, Glasier MA, Subbaraman LN, Jones L. Quantity and conformation of lysozyme deposited on conventional and silicone hydrogel contact lens materials using an in vitro model. Eye Contact Lens 2007;33:138–43.

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24. Senchyna M, Jones L, Louie D, May C, Forbes I, Glasier MA. Quantitative and conformational characterization of lysozyme deposited on balafilcon and etafilcon contact lens materials. Curr Eye Res 2004;28:25–36.
25. Glasier MA, Keech A, Sheardown H, Subbaraman LN, Jones L. Conformational and quantitative characterization of lysozyme extracted from galyfilcon and senofilcon silicone hydrogel contact lenses. Curr Eye Res 2008;33:1–11.
26. Subbaraman LN, Glasier MA, Senchyna M, Sheardown H, Jones L. Extraction efficiency of an extraction buffer used to quantify lysozyme deposition on conventional and silicone hydrogel contact lens materials. Eye Contact Lens 2007;33:169–73.
27. Helal R, Melzig MF. Determination of lysozyme activity by a fluorescence technique in comparison with the classical turbidity assay. Pharmazie 2008;63:415–9.
28. 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;28:842–9.
29. Morsky P. Turbidimetric determination of lysozyme with *Micrococcus lysodeikticus* cells: reexamination of reaction conditions. Anal Biochem 1983;128:77–85.
30. Wu Y, Daeschel MA. Lytic antimicrobial activity of hen egg white lysozyme immobilized to polystyrene beads. J Food Sci 2007;72: M369–74.
31. Fullard RJ, Snyder C. Protein levels in nonstimulated and stimulated tears of normal human subjects. Invest Ophthalmol Vis Sci 1990; 31:1119–26.
32. Ng A, Heynen M, Luensmann D, Jones L. Impact of tear film components on lysozyme deposition to contact lenses. Optom Vis Sci 2012;89:392–400.
33. Garrett Q, Garrett RW, Milthorpe BK. Lysozyme sorption in hydrogel contact lenses. Invest Ophthalmol Vis Sci 1999;40:897–903.
34. Luensmann D, Glasier MA, Zhang F, Bantseev V, Simpson T, Jones L. Confocal microscopy and albumin penetration into contact lenses. Optom Vis Sci 2007;84:839–47.
35. Ng A, Heynen M, Luensmann D, Subbaraman LN, Jones L. Optimization of a fluorescence-based lysozyme activity assay for contact lens studies. Curr Eye Res 2013;38:252–9.
36. Ibrahim HR, Matsuzaki T, Aoki T. Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. FEBS Lett 2001;506:27–32.
37. McCanna DJ, Jones LW. Membrane permeability of *Staphylococcus aureus* aggregates exposed to contact lens care solutions. Invest Ophthalmol Vis Sci 2012;53:E-Abstract 6089.
38. Guillon M, Maissa C. Use of silicone hydrogel material for daily wear. Cont Lens Anterior Eye 2007;30:5–10.
39. Szczotka-Flynn L. Looking at silicone hydrogels across generations. Optom Manag 2008;43(5):68–71.
40. Teichroeb JH, Forrest JA, Ngai V, Martin JW, Jones L, Medley J. Imaging protein deposits on contact lens materials. Optom Vis Sci 2008;85:1151–64.
41. González-Méijome JM, López-Alemany A, Almeida JB, Parafita MA, Refojo MF. Microscopic observation of unworn siloxane-hydrogel soft contact lenses by atomic force microscopy. J Biomed Mater Res B Appl Biomater 2006;76:412–8.
42. 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;63:319–25.
43. Liang H, Baudouin C, Labbe A, Pauly A, Martin C, Warnet JM, Brignole-Baudouin F. In vivo confocal microscopy and ex vivo flow cytometry: new tools for assessing ocular inflammation applied to rabbit lipopolysaccharide-induced conjunctivitis. Mol Vis 2006; 12:1392–402.
44. Kodjikian L, Casoli-Bergeron E, Malet F, Janin-Manificat H, Freney J, Burillon C, Colin J, Steghens JP. Bacterial adhesion to conventional hydrogel and new silicone-hydrogel contact lens materials. Graefes Arch Clin Exp Ophthalmol 2008;246:267–73.
45. Kodjikian L, Burillon C, Roques C, Pellon G, Renaud FN, Hartmann D, Freney J. Intraocular lenses, bacterial adhesion and endophthalmitis prevention: a review. Biomed Mater Eng 2004;14:395–409.
46. Pascual A, Fleer A, Westerdaal NA, Verhoef J. Modulation of adherence of coagulase-negative staphylococci to Teflon catheters in vitro. Eur J Clin Microbiol 1986;5:518–22.
47. Magnusson KE. Hydrophobic interaction—a mechanism of bacterial binding. Scand J Infect Dis Suppl 1982;33:32–6.
48. Menzies KL, Jones L. The impact of contact angle on the biocompatibility of biomaterials. Optom Vis Sci 2010;87:387–99.
49. Lorentz H, Rogers R, Jones L. The impact of lipid on contact angle wettability. Optom Vis Sci 2007;84:946–53.
50. Maldonado-Codina C, Morgan PB. In vitro water wettability of silicone hydrogel contact lenses determined using the sessile drop and captive bubble techniques. J Biomed Mater Res (A) 2007; 83:496–502.

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CHAPTER 5 KINETICS OF COMPETITIVE ADSORPTION BETWEEN LYSOZYME AND LACTOFERRIN ON SILICONE HYDROGEL CONTACT LENSES AND THE EFFECT ON LYSOZYME ACTIVITY*

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Kinetics of Competitive Adsorption between Lysozyme and Lactoferrin on Silicone Hydrogel Contact Lenses and the Effect on Lysozyme Activity

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ABSTRACT

Purpose: To determine the effect of competitive adsorption between lysozyme and lactoferrin on silicone hydrogel contact lenses and the effect on lysozyme activity.

Methods: Three commercially available silicone hydrogel contact lens materials (senofilcon A, lotrafilcon B and balafilcon A) were examined, for time points ranging from 10 s to 2 h. Total protein deposition was determined by I^{125} radiolabeling of lysozyme and lactoferrin, while the activity of lysozyme was determined by a micrococcal activity assay.

Results: Senofilcon A and balafilcon A did not show any relevant competitive adsorption between lysozyme and lactoferrin. Lotrafilcon B showed reduced protein deposition due to competitive adsorption for lactoferrin at all time points and lysozyme after 7.5 min. Co-adsorption of lactoferrin and lysozyme decreased the activity of lysozyme in solution for senofilcon A and lotrafilcon B, but co-adsorption had no effect on the surface activity of lysozyme for all lens types investigated.

Conclusions: Competition between lysozyme and lactoferrin is material specific. Co-adsorption of lysozyme and lactoferrin does not affect the activity of surface-bound lysozyme but can reduce the activity of subsequently desorbed lysozyme.

Keywords: Competitive protein adsorption, contact lens, lysozyme activity, protein deposition, silicone hydrogel

INTRODUCTION

In any biomaterial application, contact with bodily fluids leads to protein deposition within the first few minutes of interaction. These adsorbed proteins may subsequently change their conformation from a native state to a denatured state, which can lead to problems for the host, including thrombosis and inflammation.[1]

The contact lens is an example of a widely used biomaterial, with an estimated 38 million wearers in the United States alone.[2] During wear, contact lenses invariably sorb proteins from the surrounding tear film.[3–7] These deposits are known to affect wearers by decreasing their visual performance,[8] decreasing comfort,[5,9] and upon denaturing can lead to inflammatory responses including giant papillary conjunctivitis (GPC).[10–12]

Measuring the quantity of protein on a lens can be achieved using a variety of techniques, including quartz crystal microbalance,[13,14] surface plasmon resonance,[15,16] ellipsometry,[17,18] atomic force microscopy,[19] and radiolabeling using I125[20,21] or C14,[22] while protein conformational analysis involves investigating the changes of secondary or tertiary protein structure[4,23] or the changes in protein biological activity by *ex-situ*[6,21,24] or *in*-situ[25] measurements.

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Despite the variety of techniques, they are typically focused on measuring single protein species. Given that biological fluids contain a variety of molecules – including many different protein species – it is imperative to understand how co-adsorption or competitive adsorption affects protein deposition, and more importantly, how it affects protein state.

Lysozyme is one of the key proteins of interest in protein-biomaterial conformational studies,[6,21,24,25] and is a well-known biofouler of contact lenses.[3,6,24,26–33] One of its major biological functions is to induce bacterial lysis, which can be measured using Micrococcal activity assays.[21,25,34–36] In addition to lysozyme, lactoferrin is also found in tears and is known to deposit on contact lenses.[20,29,37] In mixed solutions, lactoferrin can have a synergistic effect on the bacteriolytic function of lysozyme.[38,39] In particular, lactoferrin has been observed to increase the activity of lysozyme with bacterial cells.[40] To date, however, there is very little understanding of how co-adsorption and competitive adsorption of lysozyme and lactoferrin onto biomaterials affects the biological functions of these proteins, particularly surface-adsorbed protein.

The fact that lactoferrin can increase the activity of lysozyme in bulk solution does not necessarily mean the same applies on adsorbed deposits. The activities of such deposits are very relevant for real biomaterial use. Using a recently reported technique to determine the activity of surface-adsorbed lyosyme,[25] we aim to determine the activity of co-adsorbed lysozyme and lactoferrin deposits, as compared to simple lysozyme deposition.

EXPERIMENTAL TECHNIQUE

Figure 1 provides a brief overview of the experimental technique detailed below. All contact lens materials utilized were prepared in the same manner prior to activity and total protein quantification.

The contact lens materials (as shown in Table 1) were removed from their packaging solution, rinsed in 100 mL of phosphate buffered saline (PBS), pH 7.4, to remove any residual packaging solution and then placed into individual wells of a 24-well plate (VWR, Mississauga, ON) containing 1.5 mL of PBS. The plates were sealed with parafilm and placed on a shaker for 24 h at ambient temperature. The lenses were removed, blotted on lens paper and placed into 6 mL screw cap glass vials (VWR), which contained 1.5 mL of protein dissolved in PBS. Protein uptake measurements were done on lenses, which had incubated in a mixture of 2 mg/mL lysozyme (from hen egg, Sigma-Aldrich, Oakville, Canada) and 2 mg/mL lactoferrin (from bovine, Sigma-Aldrich). Both lysozyme and lactoferrin were radiolabeled with an iodine tracer (^{125}I) using the

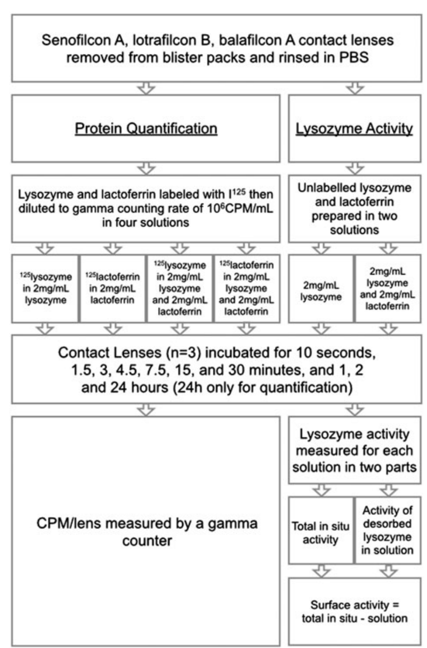

FIGURE 1 Flowchart depicting the layout of this study.

iodine monochloride method, as previously described.[41,42] In an effort to reduce anomalously high apparent protein deposition due to free iodine in solution, the protein solutions were placed in dialysis bags (Pierce, Rockford, IL) and were then immersed in 500 mL of PBS at ambient temperature for 24 h. The radiolabeled proteins were subsequently diluted in unlabeled protein solution to a gamma counting rate of 10^6 counts per minute (CPM)/mL. Total protein measurements included four parts: each protein quantified individually at a concentration of 2 mg/mL, and each protein quantified while competing with the other with both proteins at a concentration of 2 mg/mL. All lenses were incubated at ambient temperature and removed after 10 s, 1.5, 3, 4.5, 7.5, 15 and 30 min, and 1, 2 and 24 h. Removed lenses were swirled in two containers of 100 mL PBS for five seconds each, dried on lens paper and placed into either radioactivity counting vials to dry overnight or into 1.5 mL of PBS in 24-well plates for activity measurements.

Radioactivity counting vials were counted using a gamma counter (Wallac Wizard 1470 Gamma Counter Perkin Elmer, Woodbridge, ON) providing results in CPM. In order to be able to convert from CPM to μg lysozyme or lactoferrin, 100 μL of the doping solution

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TABLE 1 Properties of contact lenses evaluated in this study.

USAN	Proprietary name	Manufacturer	Water content (%)	FDA group	Principle monomers
Senofilcon A	ACUVUE OASYS	Johnson & Johnson	38	I	mPDMS, DMA, HEMA, siloxane macromer, TEGDMA, PVP
Lotrafilcon B	AIR OPTIX	Alcon	33	I	DMA, TRIS, siloxane monomer
Balafilcon A	PUREVISION	Bausch & Lomb	36	III	NVP, TPVC, NVA, PBVC

mPDMS, monofunctional polydimethylsiloxane; DMA, N,N-dimethylacrylamide; HEMA, 2-hydroxyethyl methacrylate; TEGDMA, tetraethylene- glycol dimethacrylate; PVP, polyvinyl pyrrolidone; TRIS, trimethyl siloxy silane; NVP, N-vinyl pyrrolidone; TPVC, tris-(tri- methylsiloxysilyl) propylvinyl carbamate; NVA, N-vinyl aminobutyric acid; and PBVC, poly[dimethylsiloxyl] di [silylbutanol] bis[vinyl carbamate].

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controls (without lenses) were pipetted into radioactivity counting vials and left to dry over night.

The substrate used for lysozyme activity measurements, *Micrococcus lysodeikticus* (Sigma-Aldrich, ATCC # 4698), was diluted in PBS, pH 7.4, to concentrations of 1.5 mg/mL and allowed to swell at 4 °C for 24 h. The prepared *M. lysodeikticus* was removed and placed on a shaker for 30 min and subsequently incubated at 37 °C for 30 min. The *M. lysodeikticus* was added to individual wells in 24-well plates, containing contact lenses in PBS, to a volume of 2 mL and an optical density of 1.2 at 450 nm. Optical extinction measurements were obtained at 31 °C every 30 s for 30 min (SpectraMax M5e plate reader, Molecular Devices, SunnyVale, CA). Optical absorbance versus time data were compared to standard curves, which were plotted using linear regression of the change in absorbance over time versus mass of lysozyme, to extrapolate active masses of lysozyme in the samples. Note that standard curves are plotted using lysozyme in solution, and all the resulting masses are equivalent to masses of lysozyme in solution.

The most biologically relevant model for the activity of lysozyme on contact lenses is a direct measure of the activity of a contact lens, which has been exposed to lysozyme, and then allowed to reach an equilibrium with buffer solution. In this case, the active protein can either be on the lens itself, or in the solution having been desorbed from the lens. To determine the activity of only the adsorbed layer of lysozyme (independent from protein which has desorbed into solution), an extra step was required. In this case, contact lens samples previously exposed to lysozyme solution for specific times were incubated in 1.5 mL of PBS at 31 °C for 30 min to allow for protein desorption into solution. The contact lenses were then removed and the activity of the remaining solution was measured as described above. These activity values represent the activity of only the desorbed protein and can be subtracted from measurements of lenses in solution to obtain the contribution solely from lysozyme adsorbed onto the lens. We note that the same calibration curve is used to quantify both the in solution and adsorbed lysozyme. This means that activity is always measured in units of ''solution equivalent mass of lysozyme''.

To test the affect of lactoferrin in solution to lysozyme activity in solution, 100 ng of lysozyme and either 0, 10, 25, 50 or 100 ng of lactoferrin were diluted in PBS to a volume of 1.5 mL within individual wells of a 24-well plate. Subsequently, 0.5 mL of prepared *M. lysodeikticus* was added to the wells, and the lysozyme activity was measured.

Statistical Analysis

Data analysis was conducted using STATISTICA 12 (StatSoft Inc., Tulsa, OK). The difference in protein quantity and lysozyme activity for all time periods was analyzed using repeated measures analysis of variance. A *post-hoc* analysis using Tukey's HSD was used where applicable, $p < 0.05$ was taken to be significant.

RESULTS

In order to determine the effect lactoferrin has on the biological activity of lysozyme deposits on contact lenses, we must first measure the actual amount of each protein on the lens. As part of this, we measure both independent and co-adsorbed protein on the three lenses considered in this study. These data are shown in Figure 2. This is done by radiolabeling the specific protein of interest and then treating lenses with either single or mixed protein solutions. Figure 2 demonstrates that differences between single protein adsorption and co-adsorption of the two proteins can be significant and material specific. For the cases of senofilcon A and balafilcon A (Figure 2a and c), there is no clear difference in the deposition for each protein when adsorbing isolated or co-adsorbing for all time points measured ($10\,s \leq t \leq 1\,d$), $p > 0.05$. This signifies that during the time scale of these studies, there is no evidence for competition for binding sites between proteins and very little interaction between the proteins. In contrast, for lotrafilcon B, there is a significant

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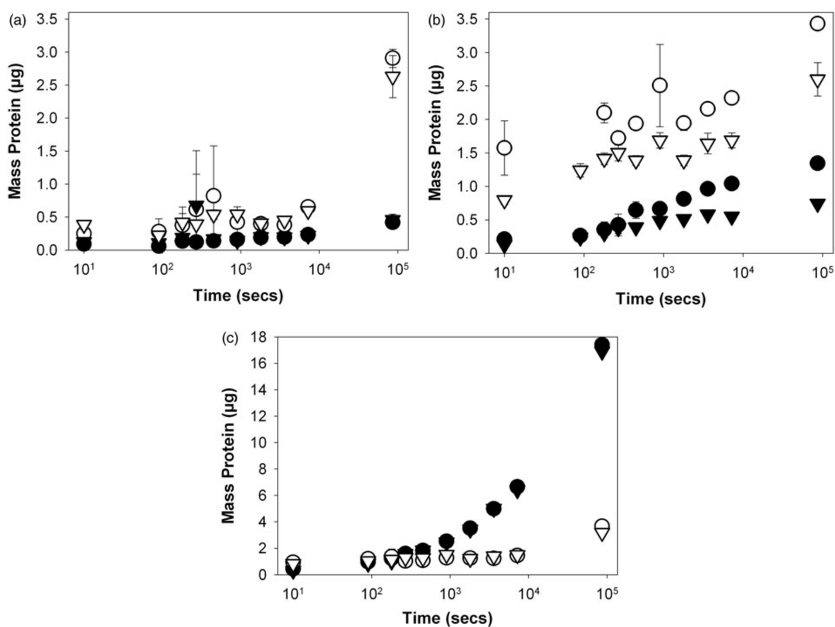

FIGURE 2 Lysozyme and lactoferrin deposition onto: (a) senofilcon A, (b) lotrafilcon B and (c) balafilcon A. Error bars represent mean ± SD, $n = 3$, In all cases: (●) lysozyme, (▼) lysozyme competing with lactoferrin, (○) lactoferrin and (▽) lactoferrin competing with lysozyme. Note the difference in scale between (c) and (a and b).

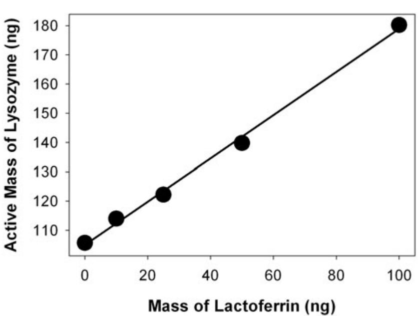

FIGURE 3 Equivalent active masses of lysozyme in solution when 100 ng of lysozyme interacts in solution with different masses of lactoferrin.

difference between the simply adsorbed and co-adsorbed protein. Lysozyme and lactoferrin individual adsorption is significantly higher for both proteins compared to when they are co-adsorbing, $p = 0.019$ for both proteins. For lotrafilcon B, it is clear that co-adsorption decreases the sorbed mass of both proteins. It may be that lysozyme and lactoferrin are competing for binding sites, or that interaction between lysozyme and lactoferrin decreases their affinity for the lotrafilcon B material.

After determination of how much of each protein deposits onto the study materials, the next step is to measure the biological activity of lysozyme. Prior to activity measurements on adsorbed lysozyme, we measure the effect lactoferrin has on lysozyme activity when both proteins are in solution (Figure 3). This figure clearly demonstrates that lactoferrin can increase the activity of lysozyme in solution. Since both proteins can co-adsorb onto contact lens materials, we investigated whether lactoferrin can similarly affect the activity of lysozyme when lactoferrin co-adsorbs with lysozyme on contact lens materials.

Figure 4 shows the activity of lysozyme when presented alone (●) and with lactoferrin co-adsorption (○) for both total *in situ* activity and surface activity. It is clear from Figure 4, that the activities are material dependent. The *y*-axis in either case is a measure of the activity in units of mass of equivalent activity of

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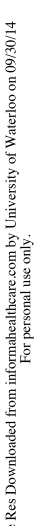
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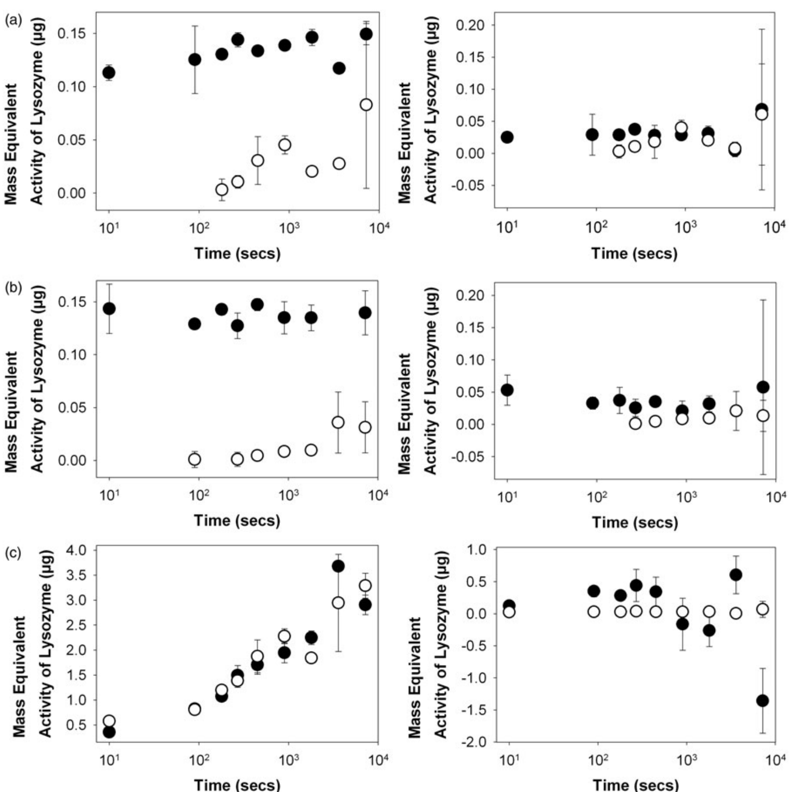

FIGURE 4 Lysozyme total activity (left) deposited onto: (a) senofilcon A, (b) lotrafilcon B, (c) balafilcon A and lysozyme surface activity (right): (a) senofilcon A, (b) lotrafilcon B and (c) balafilcon A. Error bars represent mean ± SD, $n = 3$. In all cases: (•) lysozyme, (○) lysozyme + lactoferrin. Note the difference in scale between (c) and (a and b).

lysozyme in solution. For this reason, the mass values should not be literally interpreted as a mass. Even with this caveat, it is remarkable that the activity values correspond to mass values, which are 5–20% of the total amount of protein for senofilcon and lotrafilcon, and about 30% of the total amount for balafilcon. We also note that lysozyme adsorbed onto balafilcon has 10 times the activity of either lotrafilcon or senofilcon.

For the non-ionic hydrogels (Figure 4a and b), the total *in situ* lysozyme activity for simply adsorbed lysozyme deposits is significantly greater ($p = 0.0015$ and $p = 0.00018$, respectively) than those made made by co-adsorbing lysozyme with lactoferrin. This is a puzzling observation. One possible explanation for senofilcon A and lotrafilcon B is that co-adsorption with lactoferrin may decrease desorption of lysozyme. Since the desorbed lysozyme is the largest contributor to the total *in situ* activity, this would certainly result in activity similar to that reported in Figure 3(a and b). The desorption of lysozyme was tested directly and it was determined that the total amount of desorbed lysozyme does not depend on whether the lysozyme is formed by adsorption from a pure or mixed protein solution. This result combined with Figure 4 suggest that while the total amount of desorbed lysozyme is the same from lysozyme that has been simply adsorbed versus co-adsorbed from solution onto either senofilcon or lotrafilcon, *the actual amount of active lysozyme is different*. This would seem to indicate that the presence of lactoferrin co-adsorbed onto the lens may result in a lower activity of the desorbed lysozyme. This has a significant impact on the ability to make strong ''real-world'' statements about protein

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activity from adsorbed layers using only simple one-protein studies.

Figure 4 clearly suggests that lactoferrin co-adsorption has no effect on lysozyme surface activity. However, we must remember that the protein content in the outer surface layer (that able to interact with the assay bacteria) will not be the same at all time points. In order to present a more accurate comparison, we must compare the amount of active lysozyme for isolated adsorption and co-adsorption with lactoferrin to the masses of the proteins that deposit.

Table 2 lists the total adsorption per lens. This is not necessarily the best metric in order to think about the interaction of that protein when adsorbed onto the lens and worn by a user. In such a system, it is really only the outer surface layer of the adsorbed layer that can interact with the environment. Clearly, the outer surface layer must depend on the structure of the deposits. As a first approximation, we assume the protein deposits uniformly over the entire surface of the lens. This is equivalent to saying that a monolayer is deposited over the entire lens before any part of the lens has more than one layer.

In order to estimate the protein content of the outer surface layer, the following procedure was implemented. If we take the surface area of a contact lens to be[25] 3.34 cm^2, and use the maximum density that surface-adsorbed lysozyme and lactoferrin can obtain based on their dimensions and orientation, 310 ng/cm^2 and 244 ng/cm^2, respectively,[43,44] we can approximate the amount of each protein in the outer surface layer using the following equations:

$$A_{\text{Surface}} = A_{\text{lysozyme}} + A_{\text{lactoferrin}}$$

$$3.34\,\text{cm}^2 = \left[(\text{mass lysozyme})/310\,\text{ng/cm}^2 + (\text{mass lactoferrin})/244\,\text{ng/cm}^2\right] / \#\ \text{monolayers}$$

This approximation treats every layer of protein deposition the same, which we note they are not, but still provides a reasonable approximation of the outer surface layer. Similarly, the same approximation was used for isolated lysozyme. Since adsorbed isolated lactoferrin does not have any detectable activity using the micrococcal assay, the mass of isolated lactoferrin in the outer surface layer is omitted from Table 3. Approximated values of isolated lysozyme each protein co-adsorbed on the hydrogels investigated are listed in Table 3.

TABLE 2 Mass of protein deposited onto contact lenses (in μg, $n = 3$).

Lens type	Time (s)	Mass of lysozyme	Mass of lysozyme when competing with lactoferrin	Mass of lactoferrin	Mass of Lactoferrin when competing with lysozyme
Senofilcon A	10	0.089 ± 0.026	0.121 ± 0.059	0.245 ± 0.072	0.381 ± 0.072
	90	0.058 ± 0.018	0.106 ± 0.035	0.278 ± 0.196	0.222 ± 0.061
	180	0.133 ± 0.056	0.186 ± 0.085	0.372 ± 0.182	0.416 ± 0.237
	270	0.119 ± 0.027	0.675 ± 0.831	0.612 ± 0.535	0.391 ± 0.046
	450	0.137 ± 0.018	0.167 ± 0.068	0.819 ± 0.759	0.536 ± 0.073
	900	0.157 ± 0.055	0.142 ± 0.037	0.419 ± 0.236	0.538 ± 0.052
	1800 (30 min)	0.186 ± 0.043	0.197 ± 0.018	0.395 ± 0.046	0.404 ± 0.084
	3600 (1 h)	0.193 ± 0.035	0.194 ± 0.063	0.377 ± 0.024	0.447 ± 0.030
	7200 (2 h)	0.231 ± 0.023	0.219 ± 0.024	0.652 ± 0.019	0.593 ± 0.016
	86,400 (24 h)	0.416 ± 0.055	0.456 ± 0.087	2.904 ± 0.138	2.628 ± 0.320
Lotrafilcon B	10	0.209 ± 0.075	0.126 ± 0.019	1.572 ± 0.406	0.794 ± 0.068
	90	0.262 ± 0.041	0.236 ± 0.028	–	1.239 ± 0.099
	180	0.352 ± 0.117	0.300 ± 0.051	2.098 ± 0.148	1.412 ± 0.086
	270	0.424 ± 0.163	0.378 ± 0.086	1.720 ± 0.103	1.500 ± 0.121
	450	0.644 ± 0.124	0.386 ± 0.024	1.935 ± 0.058	1.387 ± 0.075
	900	0.664 ± 0.022	0.483 ± 0.053	2.506 ± 0.614	1.685 ± 0.116
	1800 (30 min)	0.811 ± 0.029	0.511 ± 0.049	1.942 ± 0.108	1.388 ± 0.079
	3600 (1 h)	0.960 ± 0.072	0.579 ± 0.015	2.155 ± 0.086	1.641 ± 0.155
	7200 (2 h)	1.040 ± 0.080	0.547 ± 0.038	2.318 ± 0.095	1.687 ± 0.112
	86,400 (24 h)	1.343 ± 0.073	0.741 ± 0.035	3.428 ± 0.077	2.599 ± 0.250
Balafilcon A	10	0.425 ± 0.058	0.312 ± 0.074	0.940 ± 0.181	0.820 ± 0.053
	90	0.971 ± 0.228	0.842 ± 0.074	1.196 ± 0.185	1.035 ± 0.104
	180	1.219 ± 0.071	1.009 ± 0.245	1.359 ± 0.578	1.147 ± 0.068
	270	1.587 ± 0.199	1.569 ± 0.162	1.025 ± 0.152	1.278 ± 0.118
	450	1.831 ± 0.118	1.832 ± 0.059	1.071 ± 0.101	1.262 ± 0.041
	900	2.516 ± 0.119	2.441 ± 0.062	1.266 ± 0.148	1.518 ± 0.179
	1800 (30 min)	3.512 ± 0.332	3.423 ± 0.097	1.245 ± 0.087	1.210 ± 0.107
	3600 (1 h)	4.986 ± 0.240	4.997 ± 0.218	1.220 ± 0.070	1.391 ± 0.071
	7200 (2 h)	6.648 ± 0.276	6.406 ± 0.162	1.448 ± 0.063	1.490 ± 0.085
	86,400 (24 h)	17.396 ± 0.673	16.893 ± 0.205	3.661 ± 0.375	3.175 ± 0.212

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TABLE 3 Protein outer surface layer coverage (in ng, $n = 3$).

	Lens type								
	Senofilcon A			Lotrafilcon B			Balafilcon A		
Time	Iso Lys	Co Lys	Co Lac	Iso Lys	Co Lys	Co Lac	Iso Lys	Co Lys	Co Lac
10 s	89	121	381	211	126	–	425	89	745
1.5 min	58	106	222	265	196	661	971	1035	–
3.0 min	133	186	416	356	166	684	1035	509	414
4.5 min	120	597	345	429	180	673	1035	563	372
7.5 min	137	167	536	652	175	678	1035	577	361
15 min	158	142	538	673	223	640	1035	622	326
30 min	187	197	404	823	200	658	1035	705	260
1.0 h	194	194	447	976	256	614	1035	747	227
2.0 h	233	219	593	1035	215	646	1035	835	158

After calibrating our activity measurements to only the lysozyme contained in the outer surface layer (Figure 5), we find there is very little difference of lysozyme activity alone and lysozyme activity when co-adsorbed with lactoferrin for all lens types. This is not surprising as lactoferrin is thought to increase the activity of lysozyme by facilitating the interaction between lysozyme and bacterial cells.[40] If the bacterial cells are already attracted to the material surface, as demonstrated previously (unpublished), then lactoferrin will not be effective at facilitating the interaction between lysozyme and the bacterial cells, and thus will not be able to increase the surface activity of lysozyme when both proteins are co-adsorbed onto materials. In this case, the enhancement of activity demonstrated for protein solution in Figure 3 appears to be irrelevant for lysozyme deposits on silicone hydrogel biomaterials.

The percentage of active lysozyme adsorbed solely or co-adsorbed with lactoferrin is less than 100% for all materials, though there are some material differences. Lysozyme activity on balafilcon A is generally greater than 40%, while lysozyme activity on senofilcon A and lotrafilcon B are generally lower than 40%. Lysozyme activity on senofilcon A is generally above 20%, while on lotrafilcon B it is lower than 20%. It may be that (1) our calibration is off and there is not a complete monolayer formed, and thus less lysozyme present in the outer surface layer or (2) that a large percentage of the deposited lysozyme is denatured in the outer surface layer, which is unaffected by co-adsorption with lactoferrin. These two possibilities are not mutually exclusive.

Based on our approximation from Table 3, senofilcon A was the only hydrogel that did not deposit enough protein to form a complete monolayer for either isolated lysozyme or co-adsorption of lysozyme and lactoferrin. Since senofilcon also showed no difference between total lysozyme deposited for isolated lysozyme and co-adsorption of lysozyme and lactoferrin, it is no surprise that for all time points, the mass of adsorbed lysozyme is the same for both cases.

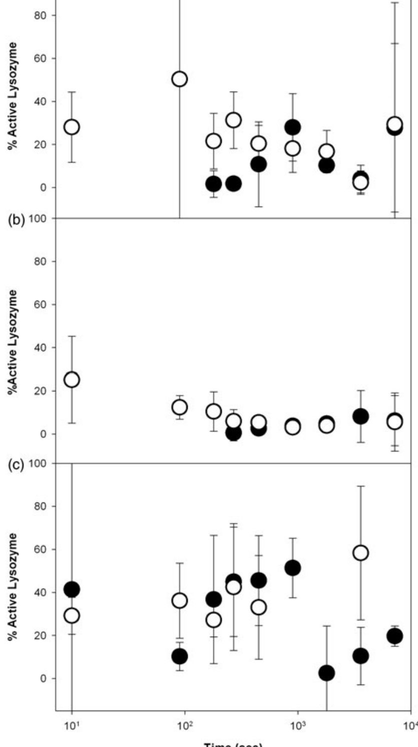

FIGURE 5 Percentage of active lysozyme deposited onto: (a) senofilcon A, (b) lotrafilcon B and (c) balafilcon A. Error bars represent mean ± SD, $n = 3$. In all cases: (○) lysozyme and (●) lysozyme + lactoferrin.

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Lotrafilcon B did not have enough deposited protein to reach full monolayer coverage for isolated lysozyme deposition, but did after 1.5 min when lysozyme was co-adsorbing with lactoferrin, with the outer surface layers showing about a 3:1 ratio for lactoferrin to lysozyme coverage for all time points. Balafilcon A reached a monolayer of deposition for both isolated and co-adsorbed protein in as little as 3 min.

DISCUSSION

Lysozyme and lactoferrin are proteins found within the tear film.[45] When a contact lens is worn, these proteins can compete for binding sites and/or interact with each other and additional tear film components. Previous studies have examined the impact of protein–protein interaction and/or competition on the adsorption of tear film proteins to contact lenses.[14,46–49] To the best of our knowledge, this is the only study to investigate protein competitive adsorption on silicone hydrogels over short time periods, and the subsequent effect on lysozyme activity.

Determining the activity of lysozyme not only provides insight into the general state of proteins on a contact lens, which if unfolded can cause inflammation such as GPC,[10–12] but clinically denatured lysozyme has been correlated with patient discomfort.[50] We demonstrate in this manuscript that adsorbed lysozyme can still be active against Gram-positive bacteria, but it should be noted that lens-adsorbed lactoferrin can kill adhered Gram-negative bacteria.[51,52] Thus, understanding the quantity and quality of contact lens adsorbed lysozyme and lactoferrin is important for understanding patient responses.

Senofilcon A and lotrafilcon B are non-ionic silicone hydrogels. Lactoferrin deposition on these materials is higher than lysozyme deposition, reaching 2.9 µg and 3.4 µg, respectively, after one day, compared to 0.4 µg and 1.3 µg for lysozyme. Lactoferrin depositing more than lysozyme on low water content, non-ionic (FDA group I) materials has been demonstrated previously,[46] and may be due to inherent material properties.

Lactoferrin deposition on balafilcon A lenses was higher than all other silicone hydrogels investigated after one day, at 3.6 µg. Previous work has also demonstrated lactoferrin deposition on balafilcon A lenses is higher than other silicone hydrogels.[20] Balafilcon A has a net negative charge,[53] therefore it is not unexpected that positively charged lactoferrin would have greater deposition on balafilcon A than the non-ionic lenses in this study. Lysozyme deposition was also highest on balafilcon A lenses compared to senofilcon A and lotrafilcon B after one day.

For balafilcon A, lysozyme deposition was much higher than lactoferrin deposition, which differs from the other silicone hydrogels investigated. One possible explanation for this is that lactoferrin has a weaker net positive charge than lysozyme,[54] thus the electrostatic attraction for lysozyme will be greater than for lactoferrin.

Lotrafilcon B was the only lens type to show competitive binding between lysozyme and lactoferrin. Competition for binding sites reduced lactoferrin deposition compared to isolated adsorption in as little as 10 s, whereas lysozyme deposition was reduced after 7.5 min compared to isolated lysozyme adsorption. The presence of lactoferrin has been shown to decrease the deposition of lysozyme for up to 28 d of incubation on lotrafilcon B lenses.[48] The surface of lotrafilcon B is modified in a gas plasma reactive chamber to create a thin (25 nm) hydrophilic layer, which has been shown to resist protein penetration into the underlying material matrix.[55] This resistive layer may cause lysozyme and lactoferrin to compete for surface binding sites, reducing the amount either can deposit compared to isolated adsorption.[55,56] Senofilcon A and balafilcon A lenses did not show competition for binding sites between lysozyme and lactoferrin.

The total lysozyme activity on balafilcon A was the same in the case of lysozyme adsorbed solely or lysozyme co-adsorbed with lactoferrin. This is not unexpected as the total mass of lysozyme deposited on balafilcon A was the same for lysozyme adsorbed solely or co-adsorbed with lactoferrin. Senofilcon A and lotrafilcon B showed the opposite trend for total lysozyme activity. Total lysozyme activity was higher for lysozyme adsorbed solely versus lysozyme co-adsorbed with lactoferrin for senofilcon A and lotrafilcon B. This effect cannot be explained merely by deposition differences between isolated lysozyme and lysozyme lactoferrin co-adsorption, as only lotrafilcon B had reduced lysozyme deposition when competing with lactoferrin. We determined experimentally (unpublished) that there are no differences in protein desorption for these materials between lysozyme adsorbed solely and co-adsorbed with lactoferrin. Thus we can conclude that the lysozyme desorbing from these non-ionic lenses when co-adsorbing with lactoferrin is more denatured than when lysozyme adsorbs solely. Lactoferrin can increase the activity of lysozyme in solution, but co-adsorption and subsequent desorption appears to denature lysozyme. If lysozyme and lactoferrin were desorbing into solution individually, we would expect the total activity to be higher, since the activity of lysozyme in solution would increase, as demonstrated in Figure 2. These proteins may be desorbing as aggregates; perhaps single protein aggregates and/or multi-protein aggregates. Aggregates could be formed as the proteins unfold to have hydrophobic

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interactions with the lens surface, and while doing so they become entangled. From Figure 4, we see activity of lysozyme on the material surface is unaffected by co-adsorption of lactoferrin for senofilcon A, lotrafilcon B and balafilcon A.

Though there are no observed differences in Figure 4 for surface activity, it is important to calibrate the activity in terms of how much lysozyme is in the outer surface layer for each material (Figure 5). We can see from Figure 5 that the percentage of active lysozyme is unaffected by co-adsorption with lactoferrin.

In conclusion, co-adsorption and/or competition of lysozyme and lactoferrin decrease their subsequent deposition on lotrafilcon B, but does not affect their deposition on senofilcon A or balafilcon A. Lactoferrin co-adsorption with lysozyme has no effect on the surface activity of lysozyme, but can decrease the activity of lysozyme, which desorbs from non-ionic silicone hydrogels.

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DECLARATION OF INTEREST

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REFERENCES

1. Janatova J. Activation and control of complement, inflammation, and infection associated with the use of biomedical polymers. ASAIO J 2000;46:S53–S62.
2. Nichols JJ. Contact lenses 2012. Contact Lens Spectr 2013; 28:24–29.
3. Garrett Q, Garrett RW, Milthorpe BK. Lysozyme sorption in hydrogel contact lenses. Invest Ophthalmol Vis Sci 1999; 40:897–903.
4. Mannucci LL, Moro F, Cosani A, Palumbo M. Conformational state of lacrimal proteins adsorbed on contact lenses. Curr Eye Res 1985;4:734–736.
5. 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.
6. Suwala M, Glasier MA, Subbaraman LN, Jones L. Quantity and conformation of lysozyme deposited on conventional and silicone hydrogel contact lens materials using an in vitro model. Eye Contact Lens 2007;33:138–143.
7. Refojo MF, Holly FJ. Tear protein adsorption on hydrogels: a possible cause of contact lens allergy. Contact Intraocular Lens Med J 1977;3:23–36.
8. Gellatly KW, Brennan NA, Efron N. Visual decrement with deposit accumulation of Hema contact lenses. Am J Optom Physiol Opt 1988;65:937–941.
9. Pritchard N, Fonn D, Weed K. Ocular and subjective responses to frequent replacement of daily wear soft contact lenses. CLAO J 1996;22:53–59.
10. Elhers WH, Donshik PC. Giant papillary conjunctivitis. Curr Opin Allergy Clin Immunol 2008;8:445–449.
11. Hart DE, Schkolnick JA, Bernstein S, Wallach D, Gross DF. Contact lens induced giant papillary conjunctivitis: a retrospective study. J Am Optom Assoc 1989;60:195–204.
12. Allansmith MR, Korb DR, Greiner JV, Henriquez AS, Simon MA, Finnemore VM. Giant papillary conjunctivitis in contact lens wearers. Am Acad Ophthalmol 1977;83: 697–708.
13. Welle A. Competitive plasma protein adsorption on modified polymer surfaces monitored by quartz crystal microbalance technique. J Biomater Sci Polym Ed 2004;15: 357–370.
14. Teichroeb JH, Forrest JA, Jones LW, Chan J, Dalton K. Quartz crystal microbalance study of protein adsorption kinetics on poly(2-hydroxyethyl methacrylate). J Colloid Interface Sci 2008;325:157–164.
15. Green RJ, Frazier RA, Shakesheff KM, Davies MC, Roberts CJ, Tendler SJ. Surface plasmon resonance analysis of dynamic biological interactions with biomaterials. Biomaterials 2000;21:1823–1835.
16. Green RJ, Davies J, Davies MC, Roberts CJ, Tendler SJ. Surface plasmon resonance for real time in situ analysis of protein adsorption to polymer surfaces. Biomaterials 1997; 18:405–413.
17. Mora MF, Wehmeyer JL, Synowicki R, Garcia, CD. Investigating Protein Adsorption via Spectroscopic Ellipsometry. In: Puleo DA, Bizios R, editors. Biological Interactions on Materials Surfaces. Springer New York; 2009. pp. 19–41.
18. Spaeth K, Brecht A, Gauglitz G. Studies on the biotin-avidin multilayer adsorption by spectroscopic ellipsometry. J Colloid Interface Sci 1997;196:128–135.
19. Cullen DC, Lowe CR. AFM studies of protein adsorption: 1. Time-resolved protein adsorption to highly oriented pyrolytic graphite. J Colloid Interface Sci 1994;166:102–108.
20. Chow LM, Subbaraman LN, Sheardown H, Jones L. Kinetics of in vitro lactoferrin deposition on silicone hydrogel and FDA group II and group IV hydrogel contact lens materials. J Biomater Sci Polym Ed 2009;20:71–82.
21. Subbaraman LN, Jones L. Kinetics of lysozyme activity recovered from conventional and silicone hydrogel contact lens materials. J Biomater Sci Polym Ed 2010;21: 343–358.
22. Prager MD, Quintana RP. Radiochemical studies on contact lens soilation. I. Lens uptake of 14C-lysozyme from simple and complex artificial tear solutions. J Biomed Mater Res 1997;36:119–124.
23. Castillo EJ, Koenig JL, Anderson JM, Kliment CK, Lo J. Surface analysis of biomedical polymers by attenuated total reflectance-Fourier transform infra-red. Biomaterials 1984;5:186–193.
24. Glasier MA, Subbaraman LN, Senchyna M, Jones L. A solid-phase assay for the quantitation of total protein eluted from balafilcon, lotrafilcon, and etafilcon contact lenses. Curr Eye Res 2008;33:631–640.
25. Hall B, Jones L, Forrest JA. Measuring the kinetics and activity of adsorbed proteins: In vitro lysozyme deposited

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onto hydrogel contact lenses over short time periods. J Biomed Mater Res Part A 2013;101A:755–764.
26. Subbaraman LN, Glasier MA, Senchyna M, Sheardown H, Jones L. Extraction efficiency of an extraction buffer used to quantify lysozyme deposition on conventional and silicone hydrogel contact lens materials. Eye Contact Lens 2007;33:169–173.
27. Zhao Z, Wei X, Aliwarga Y, Carnt NA, Garrett Q, Willcox MDP. Proteomic analysis of protein deposits on worn daily wear silicone hydrogel contact lenses. Mol Vis 2008;14:2016–2024.
28. Teichroeb JH, Forrest JA, Ngai V, Martin JW, Jones L, Medley J. Imaging protein deposits on contact lens materials. Optom Vis Sci 2008;85:1151–1164.
29. Lin ST, Mandell RB, Leahy CD, Newell JO. Protein accumulation on disposable extended wear lenses. CLAO J 1991;17:44–50.
30. Jones L, Senchyna M, Glasier M-A, Schickler J, Forbes I, Louie D, et al. Lysozyme and lipid deposition on silicone hydrogel contact lens materials. Eye Contact Lens 2003;29: S75–S79; discussion S83–S84, S192–S194.
31. Zhang S, Borazjani RN, Salamone JC, Ahearn DG, Crow Jr SA, Pierce GE. In vitro deposition of lysozyme on etafilcon A and balafilcon A hydrogel contact lenses: Effects on adhesion and survival of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Contact Lens Anterior Eye 2005;28: 113–119.
32. Deng XM, Castillo EJ, Anderson JM. Surface modification of soft contact lenses: Silanization, wettability and lysozyme adsorption studies. Biomaterials 1986;7:247–251.
33. Subbaraman LN, Glasier MA, Senchyna M, Sheardown H, Jones L. Kinetics of in vitro lysozyme deposition on silicone hydrogel, PMMA, and FDA groups I, II, and IV contact lens materials. Curr Eye Res 2006;31:787–796.
34. Morsky P. Turbidimetric determination of lysozyme with *Micrococcus lysodeikticus* cells: reexamination of reaction conditions. Anal Biochem 1983;128:77–85.
35. Vantrappen G, Ghoos AY, Peeters T. The inhibition of lysozyme by bile acids. Am J Dig Dis 1976;21:547–552.
36. Ito Y, Yamada H, Nakamura S, Imoto T. Purification, amino acid sequence, and some properties of rabbit kidney lysozyme. J Biochem 1990;107:236–241.
37. Green-Church KB, Nichols JJ. Mass spectrometry-based proteomic analyses of contact lens deposition. Mol Vis 2008;14:291–297.
38. Leitch EC, Willcox MD. Synergic antistaphylococcal properties of lactoferrin and lysozyme. J Med Microbiol 1998;47:837–842.
39. Leitch EC, Willcox MD. Lactoferrin increases the susceptibility of S. epidermidis biofilms to lysozyme and vancomycin. Curr Eye Res 1999;19:12–19.
40. Ellison RT, Giehl TJ. Killing of gram-negative bacteria by lactoferrin and lysozyme. J Clin Invest 1991;88:1080–1091.
41. Ceska M, Sjodin AV, Grossmuller F. Some quantitative aspects of the labelling of proteins with 125I by the iodine monochloride method. Biochem J 1971;121:139–143.
42. 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.
43. Messina GML, Satriano C, Marletta G. A multitechnique study of preferential protein adsorption on hydrophobic and hydrophilic plasma-modified polymer surfaces. Colloids Surf B Biointerfaces 2009;70:76–83.
44. Morrissey BW, Fenstermaker CA. Conformation of adsorbed gamma-globulin and beta-lactoglobulin. Effect of surface concentration. Trans Am Soc Artif Intern Organs 1976;22:278–284.
45. Fullard RJ, Snyder C. Protein levels in nonstimulated and stimulated tears of normal human subjects. Invest Ophthalmol Vis Sci 1990;31:1119–1126.
46. Carney FP, Morris CA, Milthorpe B, Flanagan JL, Willcox MDP. In vitro adsorption of tear proteins to hydroxyethyl methacrylate-based contact lens materials. Eye Contact Lens 2009;35:320–328.
47. Bohnert JL, Horbett TA, Ratner BD, Royce FH. Adsorption of proteins from artificial tear solutions to contact lens materials. Invest Ophthalmol Vis Sci 1988;29:362–373.
48. Ng A, Heynen M, Luensmann D, Jones L. Impact of tear film components on lysozyme deposition to contact lenses. Optom Vis Sci 2012;89:392–400.
49. Jadi S, Heynen M, Luensmann D, Jones L. Composition of incubation solution impacts in vitro protein uptake to silicone hydrogel contact lenses. Mol Vis 2012;18: 337–347.
50. Subbaraman LN, Glasier M-A, Varikooty J, Srinivasan S, Jones L. Protein deposition and clinical symptoms in daily wear of etafilcon lenses. Optom Vis Sci 2012;89: 1450–1459.
51. Williams TJ, Schneider RP, Willcox MDP. The effect of protein-coated contact lenses on the adhesion and viability of gram negative bacteria. Curr Eye Res 2003;27:227–235.
52. Subbaraman L, Borazjani R, Zhu H, Zhao Z, Jones L, Willcox MDP. Influence of protein deposition on bacterial adhesion to contact lenses. Optom Vis Sci 2011;88:959–966.
53. Santos L, Rodrigues D, Lira M, Oliveira ME, Oliveira R, Vilar EY, et al. The influence of surface treatment on hydrophobicity, protein adsorption and microbial colonisation of silicone hydrogel contact lenses. Contact Lens Anterior Eye 2007;30:183–188.
54. Sariri R, Tighe B. Effect of surface chemistry on protein interaction with hydrogel contact lenses. Iran Polym J 1996; 5:259–266.
55. Luensmann D, Zhang F, Subbaraman L, Sheardown H, Jones L. Localization of lysozyme sorption to conventional and silicone hydrogel contact lenses using confocal microscopy. Curr Eye Res 2009;34:683–697.
56. Luensmann D, Glasier M-AA, Zhang F, Bantseev V, Simpson T, Jones L. Confocal microscopy and albumin penetration into contact lenses. Optom Vis Sci 2007;84: 839–847.

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CHAPTER 6 ANALYSIS OF USING I^{125} RADIOLABELING FOR QUANTIFYING PROTEIN ON CONTACT LENSES*

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Hall	Y	Y	Y	Y
Heynen	Y	Y	Y	Y
Jones	Y			Y
Forrest	Y			Y

Abstract

Purpose. To investigate the accuracy of I^{125} radiolabeling to quantitatively determine the deposition of protein onto various commercially available contact lens (CL) materials.

Methods. Commercially available silicone hydrogel and conventional hydrogel CL materials were examined for times ranging from 10 seconds to one week. Adsorption of free I^{125} was measured directly for the CL. The use of dialyzing labeled proteins and/or using NaI to compete with free I^{125} uptake was investigated as ways to minimize effects due to free I^{125}.

Results. At all time points and with all lens materials there was 0.3 µg/lens or greater of apparent mass attributable to free I^{125} uptake. Dialyzing labeled proteins significantly reduced free I^{125} uptake for all materials investigated. The benefit of using dialyzed protein was most prominent at shorter times, as free I^{125} is continuously generated over time. Using NaI can reduce free I^{125} uptake for some lens materials, but this is shown to directly affect protein deposition on some materials.

Conclusions. Periodic replenishment of incubation solutions with freshly dialyzed labeled protein to limit free I^{125} generation is recommended, but the incorporation of NaI onto the buffer solution is not. Irrespective of the exact procedure to limit free I^{125} uptake, extra steps must be performed to quantify the amount of I^{125} adsorbed onto contact lens materials, to determine thresholds of confidence with respect to the actual protein deposition that occurs.

Keywords: Silicone hydrogel, contact lens, protein deposition, I^{125} radiolabeling, free I^{125}

Introduction

Any material in contact with bodily fluids is at risk for protein deposition. This is one of the fundamental problems in biomaterials research. In the ocular environment, contact lenses are in constant contact with tear fluid and are notoriously prone to protein deposits from the tear film.[1–5] These protein deposits can lead to severe complications for some patients, including bacterial colonization of lenses[6,7] and giant papillary conjunctivitis.[8,9] It is therefore imperative to determine accurately the deposition of protein from the tear film onto contact lenses.

Silicone hydrogels (SH) are increasing in popularity with patients and currently account for over 60% of all new fits and re-fits of contact lenses in the USA.[10] Protein sorption by SH materials is relatively low compared to conventional poly[2-hydroxyethyl methacrylate] (pHEMA) based contact lenses,[2,3,11,12] though the protein sorbed on SH materials is often reported to be denatured.[11,13–15] Due to the low amounts of protein deposited on SH materials, the methodologies that can be utilized to measure protein deposition are more limited than those for conventional lenses. In particular, any technique must be sensitive enough to measure the small amount of protein deposited, even over the short time periods (<24 hours) associated with normal daily wear. The use of chemical extraction of the deposited protein from the lens material prior to analysis is undesirable, as some protein may not be extracted, regardless of the extraction methodology employed.[16]

One common technique to measure protein adsorption that is generally well suited to SH lenses is the use of I^{125} radiolabeling of protein.[12,17–19] Radiolabeling using I^{125} is sensitive enough to measure the low amounts of protein that deposits onto SH materials, can accomplish this without the need for scintillation fluid and does not require chemical extraction of the adsorbed protein. However, the use of radiolabelled protein is not without its complications. The biggest concern is that the I^{125} tracer can dissociate from the protein of interest and the free tracer ion can bind to the material under test. This has been observed and quantified in metallic biomaterial applications.[20,21] Since only the radioactivity of the material is measured, the binding of the free tracer ion leads to anomalously high apparent protein deposition. This effect reduces the accuracy of the technique, and always leads to greater estimates of adsorbed protein. The radioactivity of the tag also limits its use to solely in vitro experiments and cannot be utilized for clinical investigations.

This manuscript quantifies the use of I^{125} radiolabeling to determine the in vitro deposition of protein onto various commercially available contact lens materials for time periods ranging from seconds to a week. Disadvantages of the technique will be discussed and potential solutions proposed and tested.

Materials and Methods

Contact Lens Pre-Treatment

The contact lenses (and their properties) used for this study are provided in Table 1.

Contact lenses were removed from their packaging solution, swirled in 100mL of either phosphate buffered saline (PBS) pH 7.4 or PBS plus 0.01M NaI (PBSI) pH 7.4 for 5s to

remove any residual packaging solution. The lenses were then placed into individual wells of 24-well plates (VWR, Mississauga, ON, Canada) containing 2mL of PBS or PBSI. The plates were sealed with parafilm and placed onto a shaker for 24 hours at ambient temperature.

Effect of Labeled Lysozyme Concentration and Dialysis on Protein Deposition

Lysozyme (from hen egg, Sigma-Aldrich, Oakville, ON, Canada) was radiolabeled with an iodine tracer (I^{125}) using the iodine monochloride (ICl) method, as previously described.[22,23] Unbound I^{125} was removed by passing the labeled samples through two 3mL syringe packed with AG 1-X4 (100-200 dry mesh in chloride form) (Bio-Rad, Hercules, CA, USA).

To test the effectiveness of dialysis to reduce the amount of unbound I^{125}, half of the labeled lysozyme was placed into dialysis cassettes (7000 MWCO) (Pierce, Rockford, IL, USA) and immersed into 500mL of PBS at ambient temperature for 24 hours, while half was not. After dialysis was complete, dialyzed labeled lysozyme and non-dialyzed labeled lysozyme was added to solutions of 2mg/mL of unlabeled lysozyme. The amount of radioactive lysozyme added was varied to give rise to gamma counting rates of 10^6, $5x10^5$, $2x10^5$CPM (counts per minute)/mL. Then 1.5mL of the lysozyme solutions containing dialyzed or unanalyzed labeled lysozyme were pipetted into 6mL screw cap glass vials (VWR, Mississauga, ON). Prepared senofilcon A, lotrafilcon B and balafilcon A contact lenses were added. The vials were sealed with parafilm and incubated at 37^0C for 24 hours. After incubation, the contact lenses were removed, swirled for 5s each in two containers of 100mL PBS and placed into radioactivity counting vials. The count rate

from each of the lenses was measured using a gamma counter (Wallac Wizard 1470 Gamma Counter Perkin Elmer, Woodbridge, ON, Canada) providing results in CPM. To convert CPM to mass of lysozyme, 100µL samples of the doping solution controls (without lenses) were counted.

Free I^{125} Generated Over Time

Even after dialysis, labeled proteins may continuously release some of the iodine tracer. To measure this effect, lysozyme, lactoferrin, and albumin were radiolabeled with an iodine tracer (I^{125}) using the ICl method,[22,23] dialyzed for 24 hours, and then diluted in 1.9mg/mL lysozyme, 1.8mg/mL lactoferrin (from bovine, Sigma-Aldrich, Oakville, ON, Canada), 0.2mg/mL albumin (from bovine, Sigma-Aldrich, Oakville, ON, Canada) respectively, to a gamma counting rate of 10^6 CPM/mL. The protein solutions contained either buffered saline solution (BSS) as described in Table 2, or buffered saline solution containing artificial tear components (ATS), as previously described,[12,17,24] outlined in Table 3. Of each of the six resultant solutions, 1.5mL was added to 6mL - vials, which were capped, sealed with parafilm, and incubated at 37^0C for 1 or 7 days. In order to measure only I^{125} in solution, we first denature and aggregate all (labeled and unlabeled) protein by reacting 1mL from the vials with 0.5mL of 20% trichloroacetic acid (TCA) for 10 minutes. The 1.5mL resultant solution was centrifuged at 3000rpm for 2 minutes. After this process there should be no protein in the supernatant liquid, and thus measuring the count rate of the supernatant is a measure of the amount of free tracer ion. In order to measure this, 100µL aliquots were pipetted into counting tubes from the supernatant and the original incubation vial.

Since radioactivity in the supernatant will be solely from free I^{125}, while the original incubation vial will contain both free I^{125} and labeled protein, the percent of the total CPM from just free I^{125} can be calculated as follows (accounting for the dilution by TCA):

Free I^{125} % = $CPM_{supernatant}/CPM_{incubation\ solution} * 100\%$.

Kinetics of Lysozyme and Lactoferrin Deposition with and without Dialysis

It is important to understand the effect of free I^{125} on any measurements of protein uptake as a function of time. To test this, lysozyme and lactoferrin were radiolabeled with an iodine tracer (I^{125}) using the ICl method,[22,23] some were dialyzed while others were not, then diluted in 2mg/mL lysozyme and 2mg/mL lactoferrin in PBS to a gamma counting rate of 10^6 CPM/mL. Aliquots of 1.5mL of each solution were added to 6mL vials to which prepared senofilcon A, lotrafilcon B and balafilcon A contact lenses were added. The vials were capped, sealed with parafilm, and incubated at 37^0C for 30, 60, and 120 minutes, and at ambient temperature only for 0.17, 1.5, 3, 4.5, 7.5, and 15 minutes due to the multiple short time points. After incubation the lenses were removed, swirled for 5s each in two containers of 100mL PBS and placed into radioactivity counting vials.

Effect of PBSI on Contact Lens Uptake of Lysozyme and Free I^{125}

It could be argued that if the system is in chemical equilibrium, the amount of free I ions should be fixed at some value. The unbinding of I from the protein is the result of the drive of the overall system to attain chemical equilibrium. If this is the case, then adding I into solution directly could lessen the rate at which the tracer I becomes released into solution. It is also possible that the NaI could inhibit the adsorption of free I^{125} to the

contact lens materials. To measure this, senofilcon A, lotrafilcon B, comfilcon A, balafilcon A, and etafilcon A contact lenses were prepared in either PBS or PBSI as described above. Contact lenses were placed into 6mL vials containing 1.5mL of 1.9mg/mL of lysozyme containing 10^4 CPM/mL of free I^{125} diluted in either PBS or PBSI and incubated at ambient temperature for 1 minute, 2 hours, and 24 hours. Contact lenses were removed, swirled for 5s twice in two containers of 100mL of either PBS or PBSI and placed into radioactivity counting vials. If PBSI can limit the uptake of free I^{125}, then this may have an effect on the apparent lysozyme adsorption kinetics by contact lenses. To investigate this, senofilcon A, comfilcon A, and balafilcon A contact lenses were prepared in either PBS or PBSI as described above. Contact lenses were placed into 6mL vials containing 1.5mL of 1.9mg/mL of lysozyme containing 10^6 CPM/mL of I^{125} tagged lysozyme, diluted in either PBS or PBSI and incubated at 37^0C for 1 minute, 2 hours, and 1 and 7 days. Contact lenses were removed, swirled for 5s twice in two containers of 100mL of either PBS or PBSI and placed into radioactivity counting vials. In order to distinguish between tightly bound protein and transient protein, contact lenses were immersed in 1.5mL of PBS within the counting vials for 24 hours at ambient temperature. The PBS and any desorbed protein was then removed and placed into separate radioactivity counting vials. Individual lenses and desorbed protein in PBS were counted using a gamma counter.

Statistical Analysis

Data analysis was conducted using STATISTICA 10 (StatSoft Inc, Tulsa, OK, United States). The difference in protein (and apparent protein) quantity and for all time periods was analyzed using repeated measures analysis of variance (RM-ANOVA). A post-hoc

analysis using Tukey's HSD was used where applicable, $P<0.05$ was taken to be significant.

Results

Effect of Labeled Lysozyme Concentration and Dialysis on Protein Deposition

The effect of dialysis and the concentration of radioactivity (CPM/mL) on contact lens uptake of lysozyme are shown in Figure 1. It is evident from this figure that the use of labeled protein that has not been dialyzed results in much higher apparent adsorbed mass of lysozyme. The contact lenses from each concentration of radioactivity are compared to standards of known mass. Since the actual mass of lysozyme is independent of the fraction of radiolabelled lysozyme, the masses in Figure 1 should be the same at each concentration of radioactivity. The most probable reason for the apparent differences as a function of total radioactivity is that there are differences in the amount of free I^{125} in each solution and that this free I^{125} eventually deposits onto the contact lens materials and is therefore interpreted as adsorbed protein. Using dialyzed labeled lysozyme significantly reduces the free I^{125} in solution, but there are still some concentration differences. This implies that there is still a measurable amount of free I^{125} in the incubation solution even after dialysis. Quantification of this effect is complicated by the fact that the free I^{125} seems to affect each contact lens material differently.

Free I^{125} Generated Over Time

Compared to lysozyme in simple PBS, this release of bound I^{125} into solution containing free I^{125} may be different for other proteins, or solutions containing various proteins and lipids. Figure 2 demonstrates the free I^{125} generation over time in two different solutions

containing labeled albumin, lactoferrin, or lysozyme: a buffered saline solution or an artificial tear solution. For each protein type both solutions contain a significant amount of free I^{125} after one day of incubation, with lysozyme containing the least. Free I^{125} for all proteins increases after one week of incubation, with lysozyme still being the lowest. Generally, the free I^{125} is highest for ATS after one day and BSS after one week.

Kinetics of Lysozyme and Lactoferrin Deposition with and without Dialysis

It is almost certain that free I^{125} is continuously released from the labeled protein. Not surprisingly, the rate at which this release (and subsequent contact lens uptake) happens may be different for different proteins. We have investigated these effects on contact lens protein deposition kinetics and the results are shown in Figure 3. It is immediately clear from this figure that free I^{125} is taken up by each of the contact lens materials in significant quantities. At almost every time point for each protein and each contact lens material the non-dialyzed labeled protein results in erroneously high protein mass calculations. After just 2 hours for all lens types and each protein these differences are significant ($P<0.00006$). Dialyzing the labeled protein removes a significant amount of free I^{125} (~70-80%), but despite this, there is usually around 1-2.5% of the total CPM/mL of just free I^{125} after 24 hours. To accurately determine the protein deposition on contact lenses, it is vital to understand the mass equivalent radioactivity (CPM) attributed to free I^{125} uptake alone.

Effect of PBSI on Contact Lens Uptake of Lysozyme and Free I^{125}

Figure 4 quantifies the amount of free I^{125} bound to different contact lens materials, converting the CPM/lens to equivalent masses of protein. At all time points and lenses

there is 0.3 μg/lens or greater of erroneous mass due to free I^{125} in solution. Research with other biomaterials try to limit free I^{125} uptake by adding 0.01M of non-radioactive NaI in incubation solutions.[20,21,25–28] Pre-treating contact lenses with this 'cold' iodide may hinder the rate of later I^{125} adsorption. Including NaI in the incubation solutions may alter the rate of free I^{125} generation (due to the excess iodide) while the non-radioactive I^{127} may also compete with the I^{125} for adsorption onto contact lenses. Adding NaI to incubation solutions indeed reduces the amount of free I^{125} uptake for senofilcon A and balafilcon A after 24 hours but was only statistically significant for senofilcon A ($P<0.003$). There was no difference for lotrafilcon B, comfilcon A, or etafilcon A ($P>0.05$). Without NaI, the free I^{125} uptake on senofilcon A and balafilcon A increases over time.

If adding NaI to incubation solutions decreases the amount of free I^{125} uptake for some contact lens materials, we would expect an incubation solution with NaI and labeled lysozyme to have less apparent mass of lysozyme than without NaI. Indeed, this is the case with senofilcon A and balafilcon A, as shown in Figure 5. This difference was statistically significant for balafilcon A after 2 hours or longer ($P<0.02$). Also shown in Figure 5 are the values from Figure 4 of free I^{125} uptake by contact lenses alone (dashed lines). For senofilcon A, the kinetics of lysozyme deposition are below the apparent mass contribution from just free I^{125}, and therefore we have no ability to measure actual protein uptake for this lens material. For comfilcon A, we would expect there to be no difference between lysozyme deposition with and without NaI, since NaI did not impact I^{125} uptake. However, this is not the case and lysozyme deposition is increased in the presence of NaI. This same pattern is seen for balafilcon A, even though NaI did decrease free I^{125} uptake.

For both comfilcon A and balafilcon A lysozyme deposition is greater than the contribution from just free I^{125} and we can be confident in these values after 2 hours of incubation. This implies that NaI has a direct impact on the adsorption of lysozyme.

From Figure 5 we can also see differences in tightly bound protein (dotted lines), which was protein not removed from soaking in PBS or PBSI for 24h, compared to the total amount deposited (solid lines), which is the sum of the tightly bound protein and protein which was removed after soaking for 24h in PBS or PBSI. After 2 hours of incubation for comfilcon A, there is ~1 μg/lens of lysozyme that is reversibly bound and desorbs from the lens surface. For balafilcon A about half of the lysozyme that deposits is reversibly bound and desorbs into solution. Though we are not completely confident we can measure lysozyme depositing on senofilcon A, it is worth noting that all the 'deposits' are irreversibly bound.

Discussion

Quantifying protein deposition is a primary concern in any biomaterial application. Ideally, a technique will have the sensitivity to detect low levels of protein, will not be dependent upon protein extraction prior to quantification, and must solely interact with the protein of interest. This manuscript has investigated the merit and potential difficulties of using I^{125} radiolabeling to measure protein deposition on contact lens materials.

The main problem with using I^{125} labeling to measure protein adsorption is the uptake of the free iodide in solution. We are unable to distinguish between I^{125} bound to protein that adsorbs onto a lens and that which detaches from protein and then adsorbs onto the lens

as a free ion. This effect results in anomalously high apparent masses of protein. We see from Figure 4 that the free I^{125} deposits onto all the contact lens materials investigated and at amounts that correspond to significant apparent masses of protein. For some materials, such as senofilcon A, the net result of this effect is a complete inability to make a quantitative estimate of the actual protein adsorbed. Senofilcon A incorporates polyvinylpyrrolidone (PVP) as an internal wetting agent.[29] PVP has a high affinity for iodine[30] and this may explain the large uptake of free I^{125} compared to the other lens materials. Senofilcon A was also the only lens material to have protein deposition below the threshold of confidence.

Dialysis of the labeled protein prior to use can significantly reduce free I^{125}, and minimize the difficulties discussed above. In some instances there is an order of magnitude higher apparent protein deposition for protein solutions that have not undergone dialysis compared to those which have. The use of dialyses only offers temporary relief from this complication, as free I^{125} is continuously released from labeled protein. After just one week of labeled protein incubating there is up to 4 times more free I^{125} than after one day of incubation. This indicates that for studies with incubation times longer than one week, the incubation solution should be replenished with freshly dialyzed labeled protein.

From Figure 4, we see that adding NaI into the buffered solutions decreases the amount of free I^{125} for senofilcon A and balafilcon A, but does not have an effect for lotrafilcon B, comfilcon A, or etafilcon A. This result on its own suggests that NaI could be used in all I^{125} radiolabeled protein studies to reduce free I^{125} uptake. Indeed, there have been studies using metallic[20,21] and polymeric[25–28] biomaterials which have used NaI for this purpose. However, in the case of contact lens applications the effect of NaI on lysozyme

deposition is not completely understood. In particular the I may not be a simple bystander ion and can directly affect the adsorption of proteins. For balafilcon A, free I^{125} is reduced with NaI (Figure 4), however lysozyme deposition is increased (Figure 5). For comfilcon A there is no effect to free I^{125} uptake with NaI (Figure 4), but lysozyme uptake is greater (Figure 5). The deposition of lysozyme onto balafilcon A is influenced by the electrostatic attraction between positively charged lysozyme and negative charged balafilcon A, at pH 7.4. It is possible that the addition of NaI increases the negativity of balafilcon A and thus increases the deposition of lysozyme. The same mechanism may explain the similar result for comfilcon A, which would indicate that the interaction between lysozyme and comfilcon A is largely influenced by electrostatic interactions.

Our findings largely agree with that of Bohnert et al.[31] While our investigations focused on SH, they observed free I^{125} uptake by polymers and copolymers of HEMA and methyl methacrylate (MMA) when radiolabeling albumin, lysozyme and IgG. To reduce these complications they dialyzed the labeled protein for at least 24 hours before use and added unlabeled NaI to their incubation solutions. Dialysis was effective in reducing the free I^{125} uptake, however as in this study, the addition of NaI was not sufficient to prevent free I^{125} uptake entirely. When using I^{125} labeled proteins it is important for researchers to recognize that 'apparent masses of protein' can be introduced by two main factors: the amount of free I^{125} in solution and the adsorption of I^{125} to the contact lens materials. Caution must be exercised when interpreting past and future studies that do not recognize these limitations, attempt to limit their influence, and quantify the material specific free I^{125} uptake.

Conclusions

We have quantitatively investigated I^{125} radiolabeling to quantify protein deposition onto contact lens materials. The main conclusion from this work is that accurate results are only obtained by minimizing the amount of free I^{125}. This can be accomplished by dialyzing labeled proteins prior to use and periodic replenishment of incubation solutions with freshly dialyzed labeled protein. Irrespective of the exact procedure to limit free I^{125}, extra steps should be performed to quantify the amount of unbound I^{125} adsorbed onto contact lens materials to determine thresholds of confidence.

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References

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

2. Teichroeb JH, Forrest JA, Ngai V, Martin JW, Jones L, Medley J. Imaging protein deposits on contact lens materials. Optom Vis Sci 2008;85:1151–64.

3. Glasier MA, Subbaraman LN, Senchyna M, Jones L. A solid-phase assay for the quantitation of total protein eluted from balafilcon, lotrafilcon, and etafilcon contact lenses. Curr Eye Res 2008;33:631–40.

4. Myers RI, Larsen DW, Tsao M, Castellano C, Becherer LD, Fontana F, et al. Quantity of protein deposited on hydrogel contact lenses and its relation to visible protein deposits. Optom Vis Sci 1991;68:776–82.

5. Soltys-Robitaille CE, Ammon DM, Valint PL, Grobe GL. The relationship between contact lens surface charge and in-vitro protein deposition levels. Biomaterials 2001;22:3257–60.

6. Willcox MD, Harmis N, Cowell BA, Williams T, Holden BA. Bacterial interactions with contact lenses; effects of lens material, lens wear and microbial physiology. Biomaterials 2001;22:3235–47.

7. Bruinsma GM, van der Mei HC, Busscher HJ. Bacterial adhesion to surface hydrophilic and hydrophobic contact lenses. Biomaterials 2001;22:3217–24.

8. Allansmith M, Korb D, Greiner J, Henriquez A, Simon M, Finnemore V. Giant papillary conjunctivitis in contact lens wearers. Am Acad Ophthalmol 1977;83:697–708.

9. Hart DE, Schkolnick JA, Bernstein S, Wallach D, Gross DF. Contact lens induced giant papillary conjunctivitis: a retrospective study. J Am Optom Assoc 1989;60:195–204.

10. Nichols JJ. Contact Lenses 2013. Contact Lens Spectrum 2014;29:22–8.

11. Suwala M, Glasier MA, Subbaraman LN, Jones L. Quantity and conformation of lysozyme deposited on conventional and silicone hydrogel contact lens materials using an in vitro model. Eye Contact Lens 2007;33:138–43.

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

13. Ng A, Heynen M, Luensmann D, Subbaraman LN, Jones L. Optimization of a fluorescence-based lysozyme activity assay for contact lens studies. Curr Eye Res 2013;38:252–9.

14. Hall B, Jones L, Forrest JA. Measuring the kinetics and activity of adsorbed proteins: In vitro lysozyme deposited onto hydrogel contact lenses over short time periods. J Biomed Mater Res Part A 2013;101A:755–64.

15. Subbaraman LN, Jones L. Kinetics of lysozyme activity recovered from conventional and silicone hydrogel contact lens materials. J Biomater Sci Polym Ed 2010;21:343–58.

16. Subbaraman LN, Glasier MA, Senchyna M, Sheardown H, Jones L. Extraction efficiency of an extraction buffer used to quantify lysozyme deposition on conventional and silicone hydrogel contact lens materials. Eye Contact Lens 2007;33:169–73.

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

18. Subbaraman LN, Glasier MA, Senchyna M, Sheardown H, Jones L. Kinetics of in vitro lysozyme deposition on silicone hydrogel, PMMA, and FDA groups I, II, and IV contact lens materials. Curr Eye Res 2006;31:787–96.

19. Chow LM, Subbaraman LN, Sheardown H, Jones L. Kinetics of in vitro lactoferrin deposition on silicone hydrogel and FDA group II and group IV hydrogel contact lens materials. J Biomater Sci Polym Ed 2009;20:71–82.

20. Sheardown H, Cornelius R, Brash J. Measurement of protein adsorption to metals using radioiodination methods: a caveat. Colloid Surface B 1997;10:29–33.

21. Du YJ, Cornelius RM, Brash JL. Measurement of protein adsorption to gold surface by radioiodination methods: suppression of free iodide sorption. Colloid Surface B 2000;17:59–67.

22. Ceska M, Sjodin A, Grossmuller F. Some quantitative aspects of the labelling of proteins with 125I by the iodine monochloride method. Biochem J 1971;121:139–43.

23. 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;22:242–52.

24. Lorentz H, Heynen M, Kay LM, Dominici CY, Khan W, Ng WWS, et al. Contact lens physical properties and lipid deposition in a novel characterized artificial tear solution. Mol Vis 2011;17:3392–405.

25. Lopez G, Ratner B, Rapoza R, Horbett T. Plasma deposition of ultrathin films of poly(2-hydroxyethyl methacrylate): surface analysis and protein adsorption measurements. Macromolecules 1993;26:3247–53.

26. Wu Y, Simonovsky FI, Ratner BD, Horbett TA. The role of adsorbed fibrinogen in platelet adhesion to polyurethane surfaces: a comparison of surface hydrophobicity, protein adsorption, monoclonal antibody binding, and platelet adhesion. J Biomed Mater Res Part A 2005;74:722–38.

27. Rodrigues SN, Gonçalves IC, Martins MCL, Barbosa M a, Ratner BD. Fibrinogen adsorption, platelet adhesion and activation on mixed hydroxyl-/methyl-terminated self-assembled monolayers. Biomaterials 2006;27:5357–67.

28. Amaral IF, Sousa S, Neiva I, Marcos-Silva L, Kirkpatrick CJ, Barbosa M a, et al. Kinetics and isotherm of fibronectin adsorption to three-dimensional porous chitosan scaffolds explored by (125)I-radiolabelling. Biomatter 2013;3:1–11.

29. Steffen R, Schnider C. A next generation silicone hydrogel lens for daily wear. Part 1 - Material properties. Optician 2004;227:23–5.

30. Oster G, Immergut E. Ultraviolet and infrared spectral studies of polyvinylpyrrolidone. J Am Chem Soc 1954;76:1393–6.

31. Bohnert JL, Horbett TA, Ratner BD, Royce FH. Adsorption of proteins from artificial tear solutions to contact lens materials. Invest Ophthalmol Vis Sci 1988;29:362–73.

Tables

Table 1.
Properties of Contact Lens Materials Evaluated in this Study

USAN	Proprietary Name	Manufacturer	Water Content (%)	FDA Group	Principal Monomers
Senofilcon A	ACUVUE OASYS	Johnson & Johnson	38	I	mPDMS, DMA, HEMA, siloxane macromer, TEGDMA, PVP
Lotrafilcon B	AIR OPTIX	CIBA Vision	33	I	DMA, TRIS, siloxane monomer
Comfilcon A	BIOFINITY	CooperVision	48	I	M3U, FMM, TAIC, IBM, HOB, NMNVA, NVP
Balafilcon A	PUREVISION	Bausch + Lomb	36	III	NVP, TPVC, NVA, PBVC
Etafilcon A	ACUVUE 2	Johnson & Johnson	58	IV	HEMA, MA

mPDMS, monofunctional polydimethylsiloxane; DMA, N,N-dimethylacrylamide; HEMA, 2-hydroxyethyl methacrylate; TEGDMA, tetraethylene- glycol dimethacrylate; PVP, polyvinyl pyrrolidone; TRIS, trimethyl siloxy silane; M3U, ax-bis(methacryloyloxyethyl iminocarboxy ethyloxypropyl)-poly(dimethylsiloxane)-poly(trifluoropropylmethylsiloxane)-poly(methoxy-poly- (ethyleneglycol)propylmethyl-siloxane); FMM, a-methacryloyloxyethyl iminicarboxyethyloxypropyl-poly(dimethylsiloxy)-butyldimethylsilane; TAIC, 1,3,5-triallyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione; IBM, isobornyl methacrylate; HOB, 2-hydroxybutyl methacrylate; NMNVA, N-methyl-N- vinyl acetamide;NVP, N-vinyl pyrrolidone; TPVC, tris-(tri- methylsiloxysilyl) propylvinyl carbamate; NVA, N-vinyl aminobutyric acid; PBVC, poly[dimethylsiloxyl] di [silylbutanol] bis[vinyl carbamate]; MA, methacrylic acid.

Table 2.
Buffered Saline Solution Components

Name	Chemical Formula	mM (mmol/mL)	MW (g/mol)
Sodium chloride	$NaCl$	90	58.44
Sodium phosphate dibasic	Na_2HPO_4	24	294.1
Potassium chloride	KCl	16	74.55
Sodium carbonate	Na_2CO_3	12	105.99
Potassium bicarbonate	$KHCO_3$	3	100.12
Trisodium citrate	$Na_3C_6H_5O_7$	1.5	294.1
Urea	$(NH_2)_2CO$	1.2	60.06
Calcium chloride	$CaCl_2$	0.5	147
Glucose	$C_6H_{12}O_6$	0.2	180.2
Milli-Q water	H_2O	-	-
Hydrochloric acid	HCl	26	-
ProClin 300	-	0.2 mL/L	-

Table 3.
Artificial Tear Solution (ATS) Components

Name	Concentration (mg/mL)
Lipids	
Cholesteryl oleate	0.024
Triolein	0.016
Oleic acid methyl ester	0.012
Cholesterol	0.0018
Oleic Acid	0.0018
Phosphatidyl choline	0.0005
Proteins	
Lysozyme	1.9
Lactoferrin	1.8
Albumin	0.2
Mucin	0.15
IgG	0.02

Figures

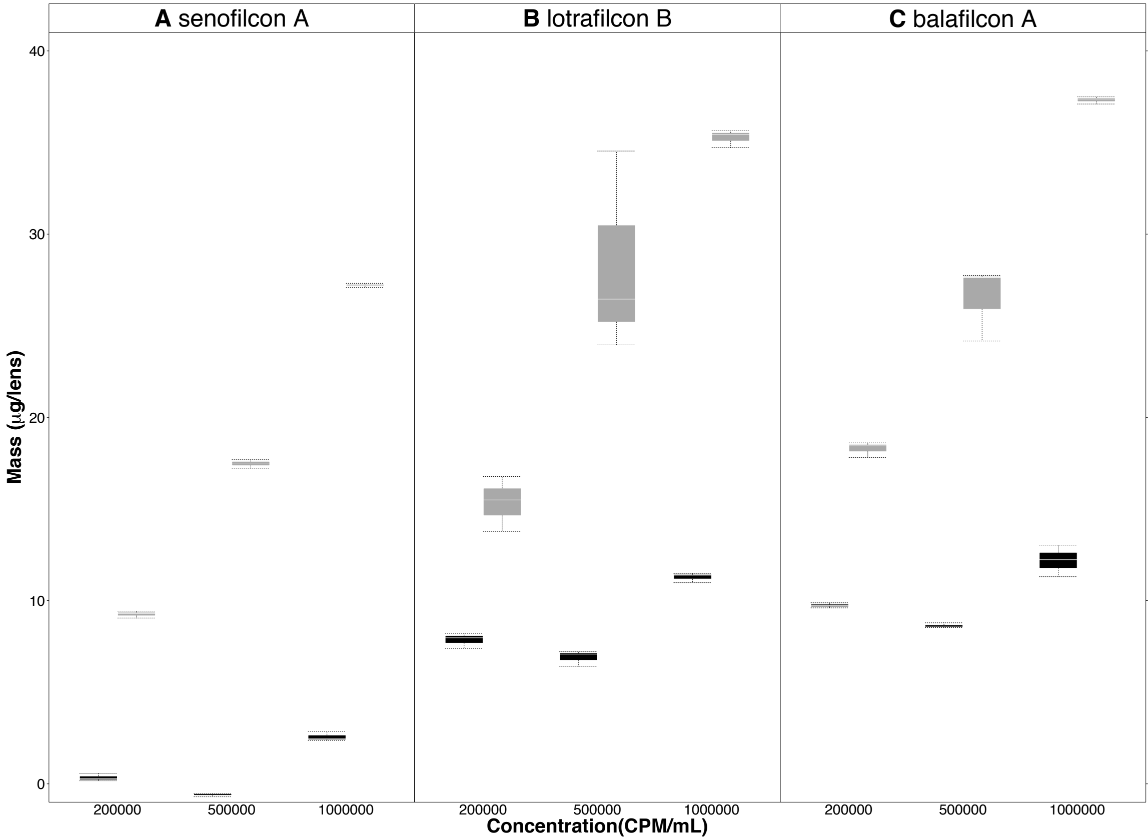

Figure 1. Box-and-whiskers plot of lysozyme deposited onto balafilcon A, lotrafilcon B, and senofilcon A contact lenses under different CPM/mL conditions, using incubation solutions containing labeled lysozyme that had either been through (■)dialysis or remained (■)undialyzed. The upper and lower extremities of the box represent the 75th and 25th percentiles, the bar within the box represents the median, and the whiskers represent the full extent of the data ranges, n=3.

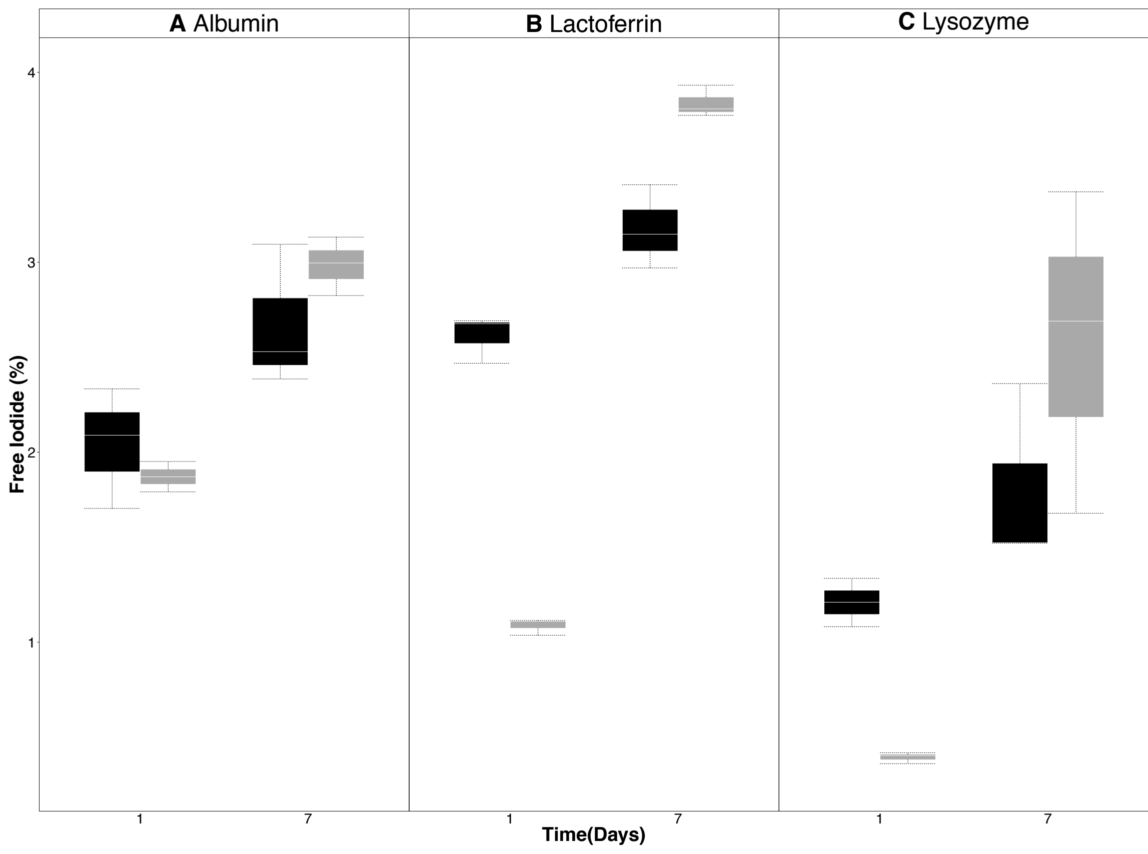

Figure 2. Box-and-whiskers plot of free iodide percentage over time for albumin, lactoferrin, and lysozyme in a (■)buffered saline solution and an (■)artificial tear solution. The upper and lower extremities of the box represent the 75th and 25th percentiles, the bar within the box represents the median, and the whiskers represent the full extent of the data ranges, n=3.

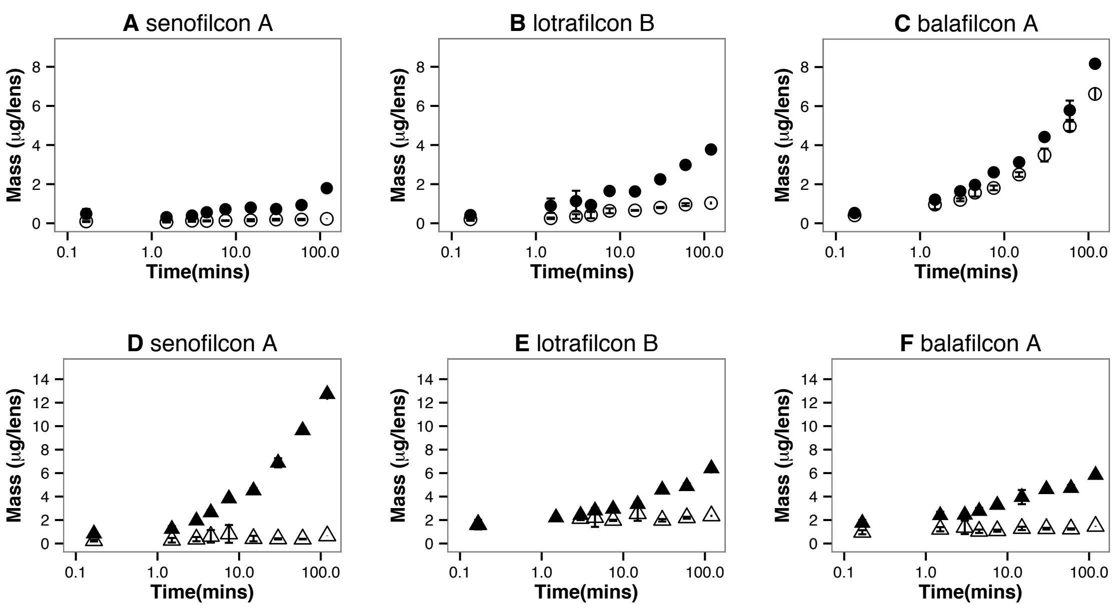

Figure 3. Kinetics of protein deposition when using labeled (●)lysozyme, (○)dialyzed lysozyme, (▲)lactoferrin, and (△)dialyzed lactoferrin on senofilcon A, lotrafilcon B, and balafilcon A. Error bars represent mean ± SD, n=3. Note the difference in scale for A, B and C compared to D, E and F.

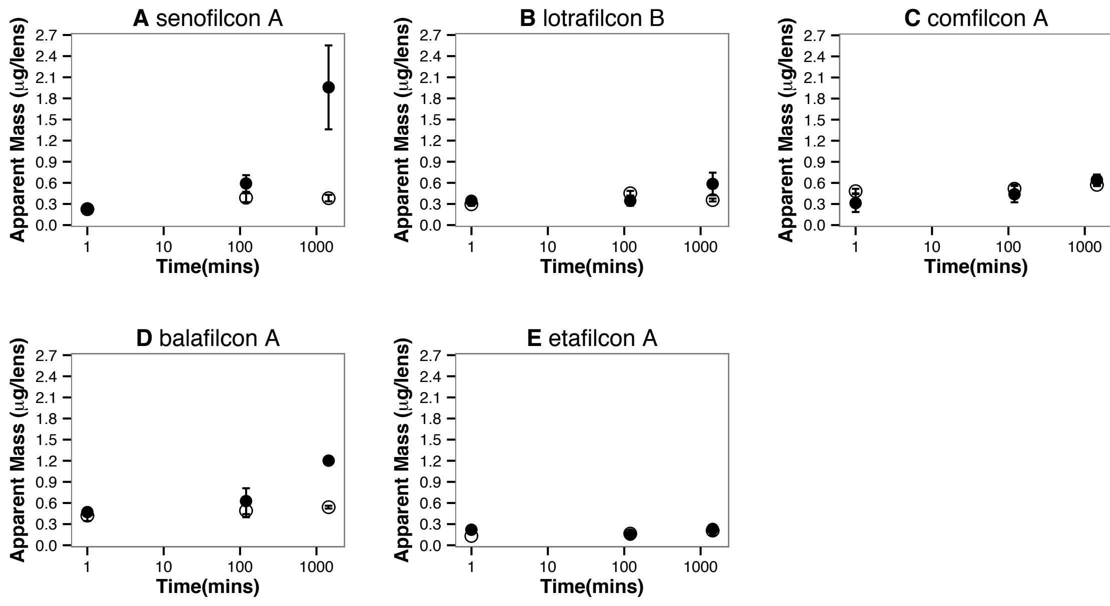

Figure 4. Kinetics of Free I^{125} depositing on senofilcon A, lotrafilcon B, comfilcon A, balafilcon A, and etafilcon A in (●)PBS and (○)PBSI incubation solutions. Error bars represent mean ± SD, n=3.

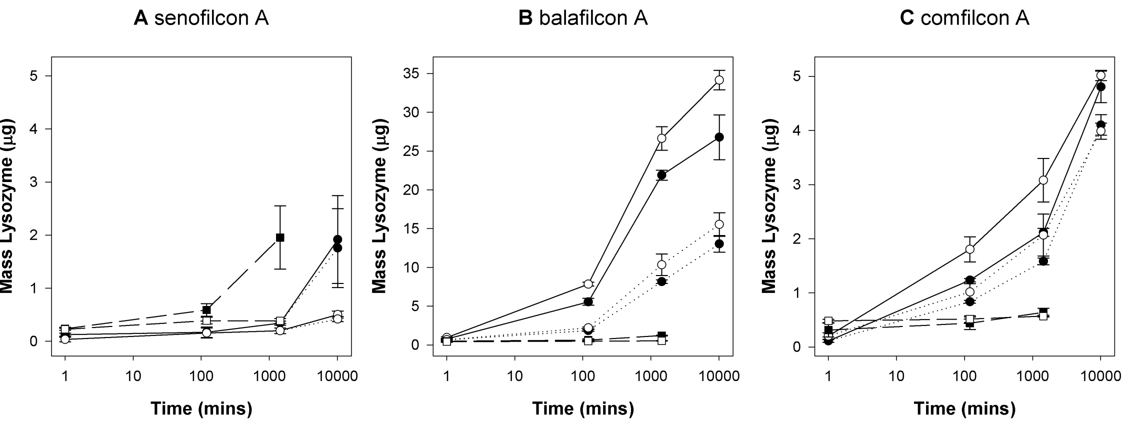

Figure 5. Kinetics of (---)free I^{125}, (—) total lysozyme, and (⋯)tightly bound lysozyme deposited on senofilcon A, comfilcon A, and balafilcon A in (●)PBS and (○)PBSI incubation solutions. Error bars represent mean ± SD, n=3.

CHAPTER 7 COMPETITIVE EFFECTS FROM AN ARTIFICIAL TEAR SOLUTION TO PROTEIN ADSORPTION*

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Hall	Y	Y	Y	Y
Jones	Y			Y
Forrest	Y			Y

Abstract

Purpose: To determine the competitive or co-adsorption effects of tear film components to the deposition of lysozyme, lactoferrin, and albumin (lys, lac, and alb) onto hydrogel contact lenses, and whether these proteins are reversibly bound.

Methods: Using a previously described artificial tear solution (ATS), we measured the time dependent adsorption of lys, lac, and alb onto 1 hydrogel and 4 silicone hydrogel contact lens materials between the first minute and up to one week of protein interaction with the material surface. Proteins were quantified using I^{125} radiolabeling of each protein individually in ATS and buffered saline. Extra steps were taken to limit the amount of unbound I^{125} and to quantify the amount of reversibly bound protein.

Results: Comfilcon A, balafilcon A, and etafilcon A did not show any relevant competitive adsorption between the ATS components and lys, lac, or alb until after 1 week. Competitive adsorption affects for lys, lac, and alb, were observed in as little as 1 minute on lotrafilcon B. Lotrafilcon B had no reversibly bound protein at any time points. The ionic materials balafilcon A and etafilcon A deposited significant amounts of reversibly bound lysozyme and lactoferrin in just 10 minutes. Senofilcon A apparent deposition was below our thresholds of confidence for this protein quantification method.

Conclusions: Both the competition between lys, lac, and alb and ATS components, and the reversibility of these bound proteins is material specific. Co-adsorption of lys, lac, and alb with ATS components can increase the reversibility of their adsorption.

Keywords: Silicone hydrogel, contact lens, protein deposition, competitive protein adsorption, artificial tear solution

Introduction

Protein interaction with biomaterials is a fundamental problem underpinning biomaterial use. Not only does this interaction occur rapidly,[1] but after adsorbing onto biomaterials, proteins may lose their structure or function and become denatured, possibly leading to thrombosis and inflammation.[2] In the case of contact lenses, denatured proteins can cause discomfort,[3,4] reduction in vision,[5] and, more seriously, inflammatory reactions such as giant papillary conjunctivitis (GPC).[6–8]

There are numerous studies which have focused on protein adsorption onto soft contact lenses for typical replacement schedules of 1 to 4 weeks, [9–16] but it is also fundamentally important to understand protein adsorption to biomaterials during the first few minutes of interaction. While a recent technique has been developed to measure the activity of lysozyme after these short time periods,[1] there is a lack of detailed knowledge regarding the amount of protein adsorbed. We have recently quantified lysozyme and lactoferrin adsorption in buffered saline individually and while co-adsorbing at these short time periods,[17] however the tear film is much more complicated. It is still not well understood how other proteins, lipids, and mucins from the tear film influence protein adsorption at short time points.

As proteins adsorb onto contact lenses they may rearrange their structure (denature) to lower the energy of the protein-substrate system.[18] Therefore, denatured protein should become more difficult to remove than if it had retained its native state, as it will have to additionally overcome the free energy of rearrangement.[18] Thus, if we can distinguish between loosely and tightly bound protein, we can gain insight into the protein state.

This study investigated the effects of competitive adsorption and co-adsorption of various tear film components on lysozyme, lactoferrin, and albumin adsorption onto contact lenses, using a recently developed artificial tear solution (ATS).[19] Additionally, extra steps were taken to distinguish loosely and tightly bound protein, the latter of which may be fully or partially denatured.

Materials and Methods

The contact lens materials utilized in this study and their properties are shown in Table 1. All contact lenses were prepared in the same manner prior to protein quantification.

Contact lenses were removed from their packaging solution, rinsed in 100mL of phosphate buffered saline (PBS) at pH 7.4 to remove any residual packaging solution and then placed into individual wells of a 24-well plate (VWR, Mississauga, ON) containing 2mL of PBS. The plates were sealed with parafilm and placed on a shaker for 24 hours at ambient temperature. Lenses were removed and placed into 6mL screw cap glass vials (VWR, Mississauga, ON) containing 1.5mL of either Solution 1(a, b, c) or Solution 2. Solutions 1a, 1b and 1c contained a solution containing a single protein in isolation. Solution 1a included lysozyme (hen egg), 1b included lactoferrin (bovine), and 1c included albumin (bovine serum) (all proteins were purchased from Sigma-Aldrich, Oakville, ON, Canada) dissolved into a buffered saline solution (pH 7.4) containing salts, urea, and glucose, as shown in Table 2. Solution 2 was an ATS as described previously,[12,13,19] and utilized the same buffered saline solution as Solution 1(a, b, c), but also contained lipids and a variety of proteins, as described in the bottom of Table 2.

To quantify the protein adsorption onto the contact lens materials, albumin, lysozyme, and lactoferrin were radiolabeled one at a time with an iodine tracer (I^{125}) using the iodine monochloride (ICl) method, as previously described.[20,21] There are many ways to label and quantity adsorbed protein and each method has its own advantages and disadvantages. Radiolabeling using I^{125} is sensitive enough to measure the low amounts of protein that deposit onto SH materials and does not require chemical extraction of the adsorbed protein. However, a known problem with any labeling technique is that the tracer can dissociate from the protein and the free tracer ion can bind to the material under test. For I^{125} radiolabeling, only the radioactivity of the material is measured, therefore the binding of the free tracer ion leads to anomalously high "apparent" protein adsorption, as previously reported in studies investigating metallic biomaterial applications.[22,23] In this current study, unbound I^{125} was reduced substantially by placing the labeled proteins into dialysis bags (Pierce, Rockford, IL, USA) and then immersing the bags into 500mL of PBS at ambient temperature for 24 hours, as previously described.[17]

Radiolabeled proteins were then removed from dialysis bags and diluted in either prepared Solution 1(a, b, c) or Solution 2, to a gamma counting rate of 10^6 CPM (counts per minute)/mL. In the ATS solution (Solution 2), only one protein at a time was labeled with the tracer. Screw cap vials containing contact lenses and incubation solutions were sealed with parafilm and incubated at 37^0C for 100, 1000, and 10000 minutes, and 1 and 10 minutes at ambient temperature with no parafilm needed, due to the short time periods. Contact lenses were removed from the various solutions, rinsed in 2 containers of 100mL PBS for five seconds each and placed into radioactivity counting vials containing 1.5mL of PBS. The contact lenses were immersed in PBS for 24 hours at ambient temperature,

to remove any loosely bound protein, before the PBS was removed and placed into separate radioactivity counting vials. Individual lenses and desorbed protein in PBS were counted using a gamma counter, (Wallac Wizard 1470 Gamma Counter Perkin Elmer, Woodbridge, ON, Canada) providing results in CPM. To convert CPM to mass of each protein, 100µL samples of each incubation solution (1a, 1b, 1c, and 2) were counted.

To determine the uptake of the free iodine tracer, additional steps were carried out. All lens types were prepared in PBS as described above. The contact lenses were placed into 6mL vials containing 1.5mL of 1.9mg/mL of lysozyme and 10^4 CPM/mL of free I^{125}. The contact lenses were then incubated at ambient temperature for 1 minute, 2 hours, and 24 hours, removed, rinsed for five seconds each in two containers of 100mL of PBS and placed into radioactivity counting vials. Note that due to regulatory handling constraints with unlabeled I^{125}, the measurements for the free I^{125} tracer were up to 24 hours versus ~1 week for the competitive adsorption measurements. Due to the same constraints, the uptake of the I^{125} tracer was measured in unlabeled lysozyme only, and the same threshold was used for all three proteins.

Data analysis was conducted using STATISTICA 12 (StatSoft Inc, Tulsa, OK, United States). The difference in protein quantity for all time periods was analyzed using repeated measures analysis of variance (RM-ANOVA). A post-hoc analysis using Tukey's HSD was used where applicable, $P<0.05$ was taken to be significant.

Results

The adsorption results are shown for lysozyme, lactoferrin, and albumin in Figures 1, 2, and 3 respectively. In each figure we show both the lens protein, which is adsorbed protein not removed after soaking in PBS, and the total protein, which is the sum of lens

protein and any protein removed during PBS soaking. For each contact lens material in each figure, there is a long dashed line, which represents the uptake of the I^{125} tracer on its own (i.e. with no protein in the solution). Any adsorption amount that lies below this threshold cannot be accurately measured. In such cases, we can only conclude that the adsorption amount is below the threshold. For example, Figure 1A shows this behavior for the case of senofilcon A. The uptake of just the I^{125} tracer is much higher than lysozyme, lactoferrin, or albumin adsorption (Figures 1-3) and thus we cannot determine accurately the amount of protein adsorption onto senofilcon A at these short time periods using this method.

From Figure 1, we can note that comfilcon A, balafilcon A, and etafilcon A do not show any significant differences in adsorption between isolated lysozyme (Solution 1a) and the analogous case of lysozyme with the components of the ATS (Solution 2), until after 1 week of incubation. After 1 week, we can see that the adsorption is higher for isolated lysozyme compared to lysozyme in the ATS ($P<0.0002$). For lotrafilcon B the adsorption was significantly higher for isolated lysozyme (Solution 1a) versus the ATS (Solution 2) after about 1 day ($P<0.0002$). Comparison of lens versus total protein shows that all of the lysozyme adsorbed to lotrafilcon B and comfilcon A was tightly bound and not removed during soaking for both isolated lysozyme (Solution 1a) and lysozyme in ATS (Solution 2). In contrast, significant amounts of loosely bound lysozyme were removed from etafilcon A and balafilcon A lenses incubated for 1 week ($P<0.02$) and 1 day ($P<0.0003$) respectively. After 1 week of incubation in ATS (Solution 2), 20 and 40 percent of the lysozyme on etafilcon A and balafilcon A respectively can be removed by soaking in PBS.

For lactoferrin (Figure 2), comfilcon A, balafilcon A, and etafilcon A do not show any significant differences in adsorption with isolated lactoferrin (Solution 1b) compared to lactoferrin in ATS (Solution 2). Similarly to lysozyme, isolated lactoferrin (Solution 1b) adsorption was significantly higher after just 1 minute of incubation ($P<0.0006$) compared to incubation in ATS (Solution 2), though this difference occurred sooner with lactoferrin. No significant amounts of lactoferrin could be removed by soaking in PBS for lotrafilcon B, etafilcon A, balafilcon A, and comfilcon A. Note that lactoferrin adsorption onto comfilcon A was below threshold until after 1 day.

As was the case for senofilcon A, we cannot accurately measure albumin adsorption to comfilcon A or balafilcon A as they are below the thresholds of I^{125} uptake alone (Figure 3). For etafilcon A, we can only accurately measure isolated albumin (Solution 1c) adsorption after 1 day, and there was no significant removal of albumin. We can accurately measure isolated albumin (Solution 1c) adsorption onto lotrafilcon B after 10 minutes and, similar to etafilcon A, there is no significant removal of albumin, but isolated albumin adsorption is much higher than when in ATS (Solution 2).

Discussion

We have determined the effect of competitive adsorption of lysozyme, lactoferrin, and albumin onto hydrogel contact lenses. It is clear from the results that there are key differences between isolated protein adsorption for all 3 proteins (Solutions 1[a, b, c]) and protein adsorption in an ATS (Solution 2). As a general rule, protein adsorption is decreased when competitively adsorbing with other tear film components.

We also note that for senofilcon A we cannot accurately measure any of the adsorption results using this radiolabel technique, as they are all below the threshold of confidence due to the amount of unlabelled I^{125} uptake, though we can say the adsorption of lysozyme, lactoferrin, and albumin is less than 2µg after one day. Senofilcon A incorporates polyvinyl pyrrolidone (PVP) as an internal wetting agent, which is known to have a high affinity for iodine.[24] The most likely explanation is that the PVP is binding free I^{125} in large quantities, causing our threshold of confidence for senofilcon A to be relatively high.

Lotrafilcon B was the only lens type in this study that demonstrated competitive adsorption effects in an ATS (Solution 2). Lysozyme, lactoferrin, and albumin adsorption are reduced when in an ATS (Solution 2) almost immediately, compared to isolated adsorption of each protein (Solutions 1[a, b, c]). The surface of lotrafilcon B undergoes plasma oxidation to create a 25nm thick hydrophilic layer, which prevents protein penetration.[25,26] The 'sealed-in' nature of this material may facilitate competitive adsorption between proteins and other tear film components. Previous work has shown, for lotrafilcon B, that components in an ATS can reduce the amount of adsorbed lysozyme,[13] and that lysozyme reduces the amount of adsorbed lactoferrin.[17] The observable differences in adsorption between isolated proteins (Solutions 1[a, b, c]) and protein in ATS (Solution 2) was seen in just 1 minute for lactoferrin, the quickest of all three proteins. Lactoferrin is much larger than lysozyme, at ~80 kDa versus ~14,[27,28] which may influence the increased competitive pressures for lactoferrin. The reversibility of the bound lactoferrin occurs only after longer incubation, if at all, compared to lysozyme. Similarly, when comparing loosely bound lysozyme and lactoferrin isolated

adsorption (Solutions 1[a and b]) versus that which occurs in ATS (Solution 2), the reversibility of these proteins is observed much sooner, if at all, in ATS. It may be worthwhile in future studies to determine if lysozyme incubation in ATS retains more activity than when it is adsorbed in isolation. It is important to note that lotrafilcon B was the only material where we could accurately measure albumin adsorption across our time scales. All other materials, with the exception of etafilcon A after 1 day incubation, had albumin adsorption levels too low to be measured accurately using the I^{125} labeling technique. We know from our thresholds that the upper limit of potential adsorption is quite low for albumin, generally below 1μg.

Comfilcon A had lysozyme and lactoferrin adsorption levels similar to lotrafilcon B and all of the protein adsorbed was tightly bound, although no competitive adsorption effects were observed until after 1 week for lysozyme. Both comfilcon A and lotrafilcon B are silicone hydrogel materials and as such are classified into the new FDA group V material group. The similarities between these non-ionic silicone hydrogel materials and relatively low water content may explain their similar results.[29] However, comfilcon A is a later "generation" of silicone hydrogels and incorporates internal wetting agents in the material backbone rather than using a surface-treatment to enhance wettability.[30]

For the ionic silicone hydrogel material balafilcon A, lysozyme adsorption was the highest compared to all other silicone hydrogels. This result is expected due to the net negative charge and large pore size of balafilcon A,[31,32] which attracts and allows the penetration of the small and net positively charged lysozyme.[33] The adsorption of lactoferrin to balafilcon A is also amongst the highest of all lenses investigated.

Etafilcon A had two orders of magnitude greater lysozyme adsorption than most other materials, and one order greater than balafilcon A. Etafilcon A was the only conventional hydrogel investigated and has a net negative charge and high water content. It is known to adsorb high amounts of lysozyme,[34–36] though interestingly low amounts of net positively charged lactoferrin,[11] which is similar to our results. Lactoferrin is larger than lysozyme and has a weaker net positive charge, which explains these differences.[33]

In addition to understanding how an ATS can affect protein adsorption, it is important to distinguish loosely bound protein from that which is tightly bound. Some fraction of the more tightly bound protein may be inactive (denatured) and could pose complications for contact lens wearers.[6–8] Previous work[1,37] has shown that the amount of biologically relevant active lysozyme on these lenses in ascending order is: lotrafilcon B < comfilcon A < balafilcon A < etafilcon A, which is the same ordering of amount of loosely bound protein in this work, which investigated the protein adsorption over much shorter time periods than the previous studies. Although lotrafilcon B had only tightly bound protein, there is still measurable biologically relevant lysozyme activity (0.14 ± 0.02 μg).[1] Despite a large amount of tightly bound lysozyme on contact lenses being inactive/denatured, some of the tightly bound lysozyme is still active. For a material such as etafilcon A, studies have shown that extracted lysozyme is nearly 100% active,[10,38] so it is likely that almost all the tightly bound lysozyme on this lens material is still active and less likely to lead to complications for contact lens wearers.

Conclusions

We have measured the competitive and co-adsorption effects of ATS components on lysozyme, lactoferrin, and albumin adsorption. All protein adsorption onto lotrafilcon B was decreased when incubated in ATS, while the other lens materials showed reduction only after 1 week of incubation. No significant amounts of protein adsorption onto lotrafilcon B lenses were reversibly bound, however reversibly bound lysozyme and lactoferrin were found more often and earlier on balafilcon A and etafilcon A.

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References

1. Hall B, Jones L, Forrest JA. Measuring the kinetics and activity of adsorbed proteins: In vitro lysozyme deposited onto hydrogel contact lenses over short time periods. J Biomed Mater Res Part A 2013;101A:755–764.

2. Janatova J. Activation and control of complement, inflammation, and infection associated with the use of biomedical polymers. ASAIO J 2000;46:S53–S62.

3. Pritchard N, Fonn D, Weed K. Ocular and subjective responses to frequent replacement of daily wear soft contact lenses. CLAO J 1996;22:53–59.

4. Jones L, Franklin V, Evans K, et al. Spoilation and clinical performance of monthly vs. three monthly Group II disposable contact lenses. Optom Vis Sci 1996;73:16–21.

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

6. Elhers WH, Donshik PC. Giant papillary conjunctivitis. Curr Opin Allergy Clin Immunol 2008;8:445–9.

7. Hart DE, Schkolnick JA, Bernstein S, et al. Contact lens induced giant papillary conjunctivitis: a retrospective study. J Am Optom Assoc 1989;60:195–204.

8. Allansmith M, Korb D, Greiner J, et al. Giant papillary conjunctivitis in contact lens wearers. Am Acad Ophthalmol 1977;83:697–708.

9. Lin ST, Mandell RB, Leahy CD, et al. Protein accumulation on disposable extended wear lenses. CLAO J 1991;17:44–50.

10. Subbaraman LN, Jones L. Kinetics of lysozyme activity recovered from conventional and silicone hydrogel contact lens materials. J Biomater Sci Polym Ed 2010;21:343–58.

11. Chow LM, Subbaraman LN, Sheardown H, et al. Kinetics of in vitro lactoferrin deposition on silicone hydrogel and FDA group II and group IV hydrogel contact lens materials. J Biomater Sci Polym Ed 2009;20:71–82.

12. Jadi S, Heynen M, Luensmann D, et al. Composition of incubation solution impacts in vitro protein uptake to silicone hydrogel contact lenses. Mol Vis 2012;18:337–47.

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

14. Keith DJ, Christensen MT, Barry JR, et al. Determination of the lysozyme deposit curve in soft contact lenses. Eye Contact Lens 2003;29:79–82.

15. Green-Church KB, Nichols JJ. Mass spectrometry-based proteomic analyses of contact lens deposition. Mol Vis 2008;14:291–297.

16. Zhao Z, Wei X, Aliwarga Y, et al. Proteomic analysis of protein deposits on worn daily wear silicone hydrogel contact lenses. Mol Vis 2008;14:2016–2024.

17. Hall B, Jones L, Forrest J. Kinetics of competitive adsorption between lysozyme and lactoferrin on silicone hydrogel contact lenses and the effect on lysozyme activity. Curr Eye Res 2014:In Press.

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

19. Lorentz H, Heynen M, Kay LM, et al. Contact lens physical properties and lipid deposition in a novel characterized artificial tear solution. Mol Vis 2011;17:3392–405.

20. Ceska M, Sjodin A, Grossmuller F. Some quantitative aspects of the labelling of proteins with 125I by the iodine monochloride method. Biochem J 1971;121:139–143.

21. 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.

22. Sheardown H, Cornelius R, Brash J. Measurement of protein adsorption to metals using radioiodination methods: a caveat. Colloid Surface B 1997;10:29–33.

23. Du YJ, Cornelius RM, Brash JL. Measurement of protein adsorption to gold surface by radioiodination methods: suppression of free iodide sorption. Colloid Surface B 2000;17:59–67.

24. Oster G, Immergut E. Ultraviolet and infrared spectral studies of polyvinylpyrrolidone. J Am Chem Soc 1954;76:1393–6.

25. Luensmann D, Zhang F, Subbaraman L, et al. Localization of lysozyme sorption to conventional and silicone hydrogel contact lenses using confocal microscopy. Curr Eye Res 2009;34:683–97.

26. Luensmann D, Glasier M-A, Zhang F, et al. Confocal microscopy and albumin penetration into contact lenses. Optom Vis Sci 2007;84:839–47.

27. Appelmelk BJ, An YQ, Geerts M, et al. Lactoferrin is a lipid A-binding protein. Infect Immun 1994;62:2628–32.

28. Emch AJ, Nichols JJ. Proteins identified from care solution extractions of silicone hydrogels. Optom Vis Sci 2009;86:E123–31.

29. Hutter JC, Green JA, Eydelman MB. Proposed silicone hydrogel contact lens grouping system for lens care product compatibility testing. Eye Contact Lens 2012;38:358–62.

30. Szczotka-Flynn L. Looking at silicone hydrogels across generations. Optom Manag 2008; 43:68–71.

31. González-Méijome JM, López-Alemany A, Almeida JB, et al. Microscopic observation of unworn siloxane-hydrogel soft contact lenses by atomic force microscopy. J Biomed Mater Res B Appl Biomater 2006;76:412–8.

32. 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;63:319–25.

33. Sariri R, Tighe B. Effect of surface chemistry on protein interaction with hydrogel contact lenses. Iran Polym J 1996;5:259–66.

34. Senchyna M, Jones L, Louie D, et al. Quantitative and conformational characterization of lysozyme deposited on balafilcon and etafilcon contact lens materials. Curr Eye Res 2004;28:25–36.

35. Subbaraman LN, Glasier M-A, Varikooty J, et al. Protein deposition and clinical symptoms in daily wear of etafilcon lenses. Optom Vis Sci 2012;89:1450–9.

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

37. Hall B, Phan C-M, Subbaraman L, et al. Extraction versus in situ techniques for measuring surface-adsorbed lysozyme. Optom Vis Sci 2014;91:1062–1070.

38. Suwala M, Glasier MA, Subbaraman LN, et al. Quantity and conformation of lysozyme deposited on conventional and silicone hydrogel contact lens materials using an in vitro model. Eye Contact Lens 2007;33:138–43.

Table 1.
Properties of Contact Lens Materials Evaluated in this Study

USAN	Proprietary Name	Manufacturer	Water Content (%)	FDA Group	Principal Monomers
Balafilcon A	PUREVISION	Bausch + Lomb	36	V	NVP, TPVC, NVA, PBVC
Comfilcon A	BIOFINITY	CooperVision	48	V	M3U, FMM, TAIC, IBM, HOB, NMNVA, NVP
Etafilcon A	ACUVUE 2	Johnson & Johnson	58	IV	HEMA, MA
Lotrafilcon B	AIR OPTIX	Alcon	33	V	DMA, TRIS, siloxane monomer
Senofilcon A	ACUVUE OASYS	Johnson & Johnson	38	V	mPDMS, DMA, HEMA, siloxane macromer, TEGDMA, PVP

NVP, N-vinyl pyrrolidone; TPVC, tris-(tri- methylsiloxysilyl) propylvinyl carbamate; NVA, N-vinyl aminobutyric acid; PBVC, poly[dimethylsiloxyl] di [silylbutanol] bis[vinyl carbamate]; M3U, ax-bis(methacryloyloxyethyl iminocarboxy ethyloxypropyl)-poly(dimethylsiloxane)-poly(trifluoropropylmethylsiloxane)-poly(methoxy-poly- (ethyleneglycol)propylmethyl-siloxane); FMM, a-methacryloyloxyethyl iminicarboxyethyloxypropyl-poly(dimethylsiloxy)-butyldimethylsilane; TAIC, 1,3,5-triallyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione; IBM, isobornyl methacrylate; HOB, 2-hydroxybutyl methacrylate; NMNVA, N-methyl-N- vinyl acetamide; HEMA, 2-hydroxyethyl methacrylate; MA, methacrylic acid; DMA, N,N-dimethylacrylamide; TRIS, trimethyl siloxy silane; mPDMS, monofunctional polydimethylsiloxane; TEGDMA, tetraethylene- glycol dimethacrylate; PVP, polyvinyl pyrrolidone.

Table 2.
Buffered Saline Solution and Artificial Tear Solution (ATS) Components

Name	Chemical Formula	Concentration	MW (g/mol)
Buffered Saline		**mM (mmol/mL)**	
Sodium chloride	$NaCl$	90	58.44
Sodium phosphate dibasic	Na_2HPO_4	24	294.1
Potassium chloride	KCl	16	74.55
Sodium carbonate	Na_2CO_3	12	105.99
Potassium bicarbonate	$KHCO_3$	3	100.12
Trisodium citrate	$Na_3C_6H_5O_7$	1.5	294.1
Urea	$(NH_2)_2CO$	1.2	60.06
Calcium chloride	$CaCl_2$	0.5	147
Glucose	$C_6H_{12}O_6$	0.2	180.2
Milli-Q water	H_2O	-	-
Hydrochloric acid	HCl	26	-
ProClin 300	-	0.2 mL/L	-
ATS Lipids		**(mg/mL)**	
Cholesteryl oleate		0.024	
Triolein		0.016	
Oleic acid methyl ester		0.012	
Cholesterol		0.0018	
Oleic Acid		0.0018	
Phosphatidyl choline		0.0005	
ATS Proteins		**(mg/mL)**	
Lysozyme		1.9	
Lactoferrin		1.8	
Albumin		0.2	
Mucin		0.15	
IgG		0.02	

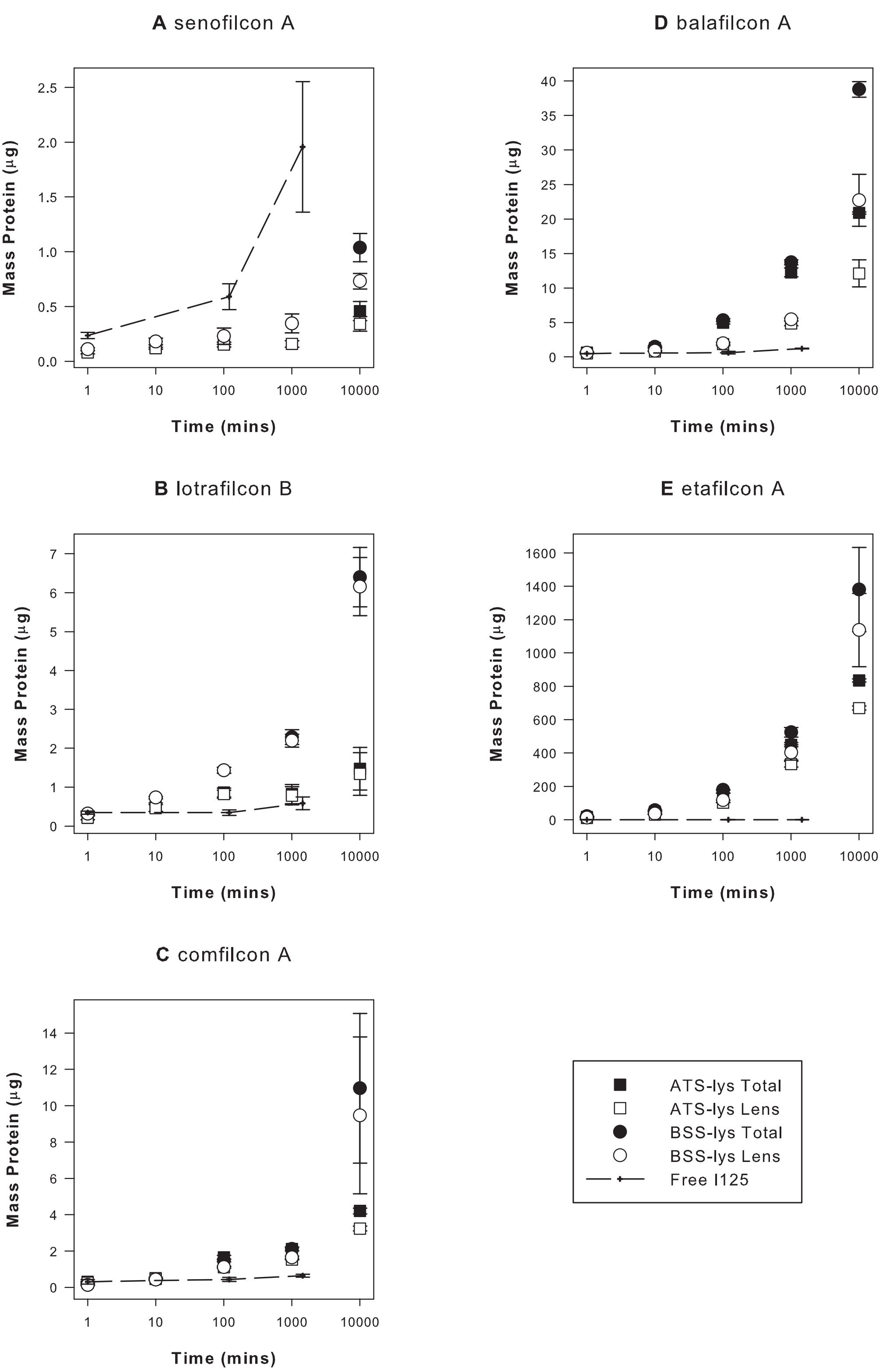

Figure 1: Kinetics of lysozyme adsorption in (●) buffered saline solution (BSS, Solution 1a), (■) artificial tear solution (ATS, Solution 2), and (---) unbound I^{125} onto contact lens materials. Filled symbols represent total lysozyme and unfilled symbols represent tightly bound lysozyme. Error bars represent mean ± SD, n=3. Note the increasing y-axis scales, ascending from A-E.

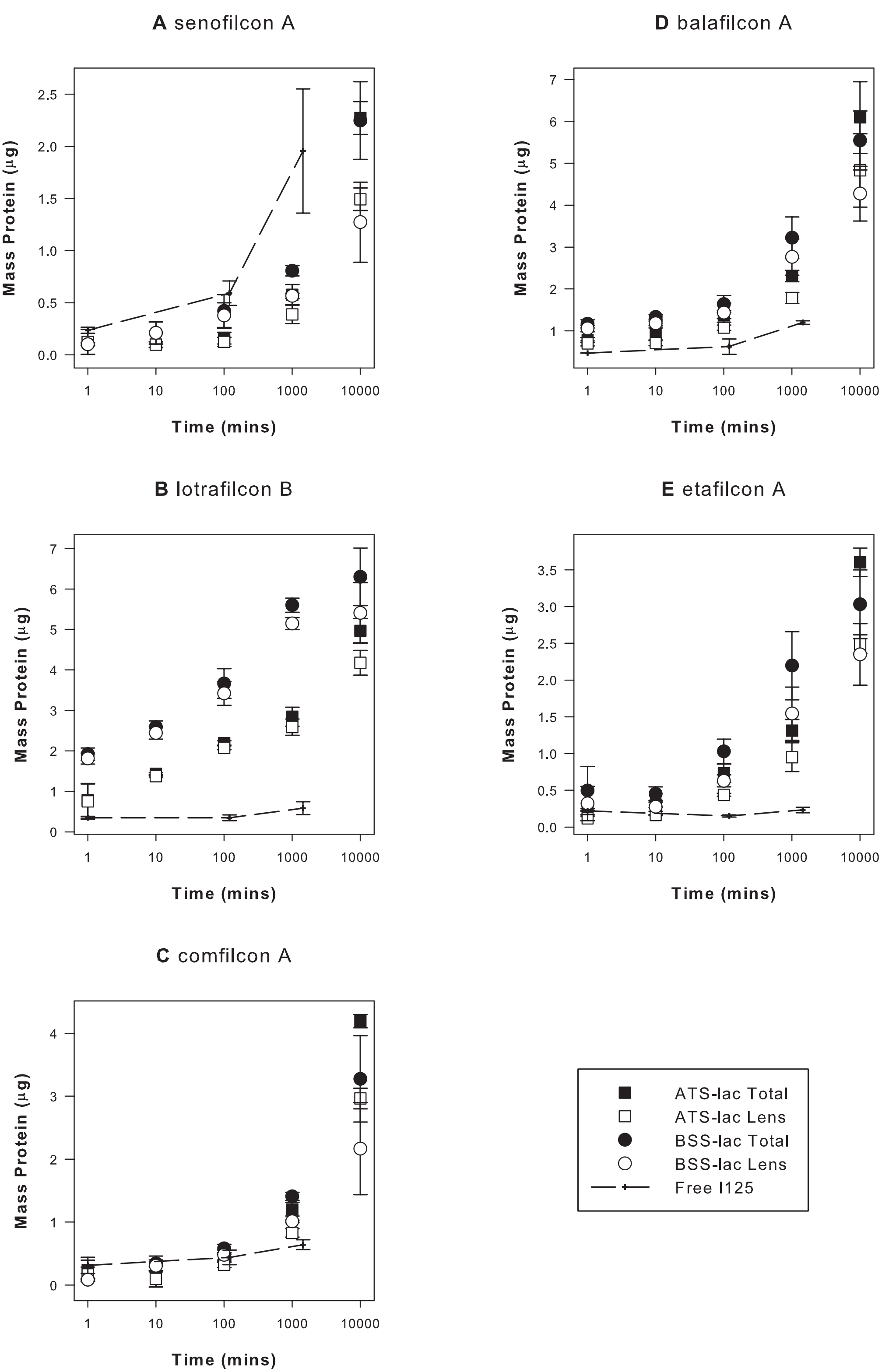

Figure 2: Kinetics of lactoferrin adsorption in (●) buffered saline solution (BSS, Solution 1b), (■) artificial tear solution (ATS, Solution 2), and (---) unbound I^{125} onto contact lens materials. Filled symbols represent total lactoferrin and unfilled symbols represent tightly bound lactoferrin. Error bars represent mean ± SD, n=3.

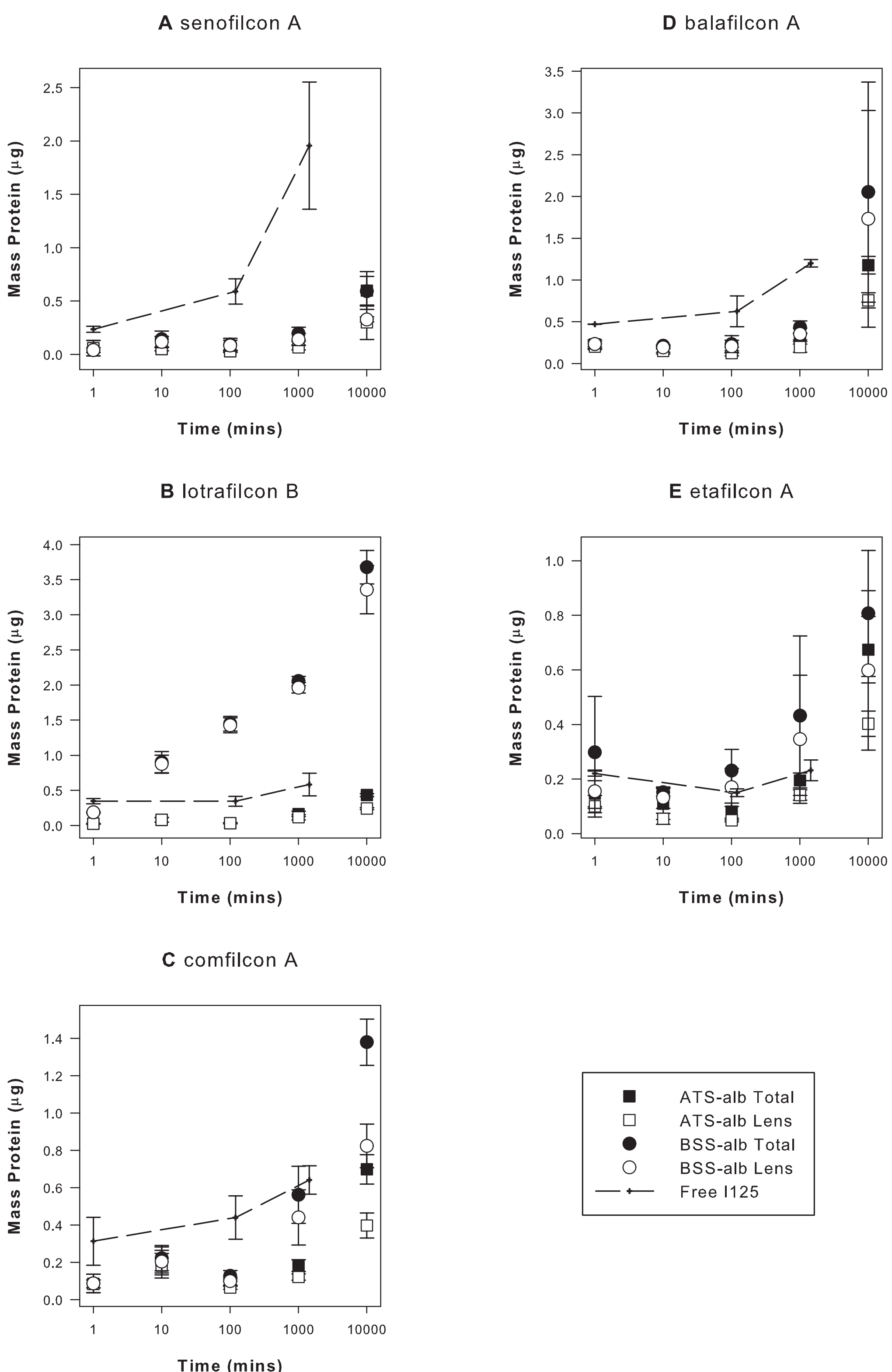

Figure 3: Kinetics of albumin adsorption in (●) buffered saline solution (BSS, Solution 1c), (■) artificial tear solution (ATS, Solution 2), and (---) unbound I^{125} onto contact lens materials. Filled symbols represent total albumin and unfilled symbols represent tightly bound albumin. Error bars represent mean ± SD, n=3.

CHAPTER 8 GENERAL DISCUSSION

Fundamental to understanding the biocompatibility of biomaterials is determining how they interact with bodily fluids. It is known that proteins from these fluids can deposit onto biomaterials within the first few minutes of interaction[1,2] and can lead to serious complications for users, with inflammation,[3] thrombosis[3] and bacterial adhesion[4,5] being commonly reported. While these biocompatibility issues are associated with denatured protein, it is difficult to draw conclusions about material biocompatibility based solely on the quantity of active or inactive protein. Distinction must be made between materials based upon the relative amounts of active protein compared to the total deposited. A material that deposits relatively large amounts of protein and keeps 50% of it active could have much greater denatured protein than a low depositing material with 20% active protein. Thus, it is imperative to have the ability to accurately measure protein quantity and quality on biomaterials.

This thesis has focused upon improving existing techniques to determine the quantity and quality of protein on biomaterials, with an emphasis on overcoming their limitations and improving their accuracy. This work has generated a broad understanding of the techniques currently available, their limitations, and where the novel methods outlined in this thesis fit within the literature. The focus was on contact lens materials, as they are widely used[6] and readily available. However, these findings have broader implications to the field of biomaterial research. In this general discussion, an overview of the current literature in this area will be presented.

8.1 Quantifying Protein

When examining biomaterial-protein interactions, it is first important to understand how much protein has deposited. Any technique to do so must be able to measure both the protein in the material matrix and on the surface, as there can be substantial amounts of protein in both.[7–9] There are many techniques that have been developed to measure protein in solution, including gel electrophoresis,[10–12] enzyme linked immunosorbent assays (ELISA),[13–16] colorimetric assays,[17–19] fluorescent assays,[20–22] and UV spectroscopy.[23–25] To utilize these techniques with biomaterials, typically adsorbed proteins are removed and re-suspended in solution.[15,25–28] This extraction process may not remove all of the protein of interest,[29] may remove substances from the material that interfere with the subsequent assay, and may detract from later protein activity measurements.[30] Protein extraction may not be required if mass depletion measurements are used. Mass depletion involves measuring the supernatant of the protein solution before and after exposure to materials. This can be undertaken using gel electrophoresis,[10–12] enzyme linked immunosorbent assays (ELISA),[13–16] colorimetric assays,[17–19] fluorescent assays,[20–22] and UV spectroscopy[23–25] and provides in situ protein adsorption information. These measurements will be concentration dependent and thus any material that adsorbs a significant amount of water from the incubation solution could affect the supernatant concentration. Most importantly, particularly in situations such as those assessed in this thesis where the quantity of protein deposited is often very low, mass depletion may not be sensitive enough to detect the low amounts of protein that can deposit onto some materials. There is therefore a need for a technique sensitive enough to accurately measure small amounts of protein adsorbed onto biomaterials.

Many different techniques have been developed as an attempt to fill this need, but as we will see, they all have their own advantages and disadvantages.

8.1.1 In situ

An accurate measurement of protein adsorption should limit its perturbation of the adsorption process and this thesis has previously provided reasons why extracting protein is not ideal. The best option would be to allow the adsorption process to remain undisturbed during measurement. It has been argued, somewhat controversially, that an interphase exists between adsorbed protein and that in solution that should be included in protein adsorption studies.[31] Removal of the material may alter or destroy this interphase, limiting our ability to measure it. Also, removal exposes adsorbed proteins to air, which may change the protein-material interactions of the adsorbed proteins.

It is worth first discussing the techniques that do not require removal of biomaterials from protein incubation solutions and their drawbacks.

8.1.1.1 Ellipsometry

Ellipsometry is commonly used to measure the thickness of protein layers on surfaces.[32–35] First described by Drude, it uses polarized light to examine interfaces.[36] Basically, a polarized beam of light is sent to and reflected from the surface of the biomaterial to a detector. A change in the polarization of the light is correlated to thickness changes at the solid-liquid interface and thickness changes in the nanometer range can be detected.[37] A simple setup is shown in Figure 8-1.

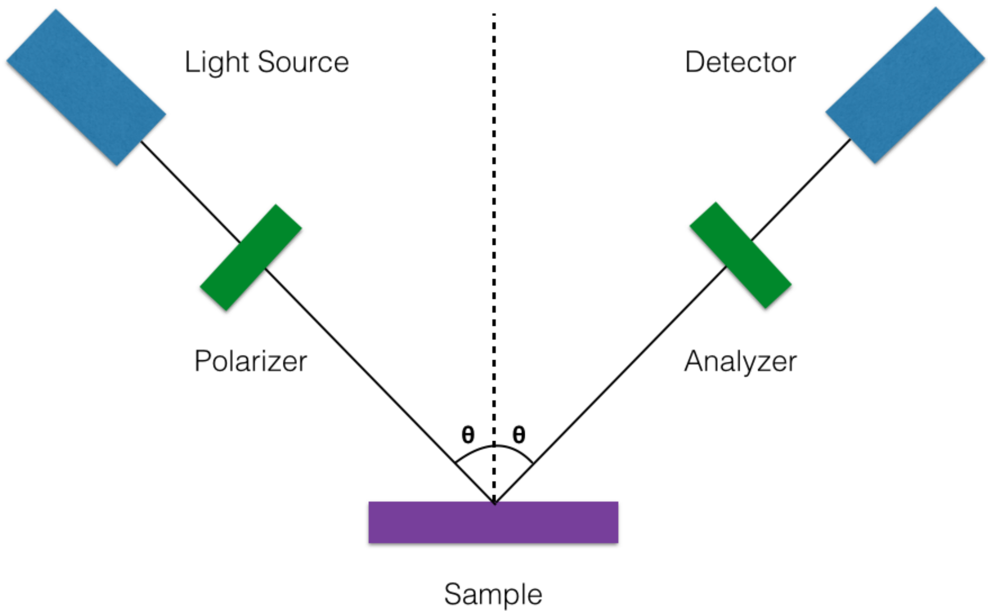

Figure 8-1: A simplified ellipsometer setup

Despite the sensitivity of ellipsometry there are several drawbacks. Ellipsometry measures thickness changes and thus models based on the dimensions of the protein of interest have to be used to determine how many protein layers there are in the measured thickness. This also precludes measurements of protein adsorption with more than just one biomolecule of interest (such as more proteins or lipids) as there is no way of discerning the contribution of each component to the thickness. Curved surfaces, such as contact lenses, can difficult for accurate determinations of thicknesses[38] since they can converge or diverge the incident light.

8.1.1.2 Quartz Crystal Microbalance

Quartz crystal microbalance (QCM) can be utilized to measure protein uptake on biomaterials.[9,39,40] Sauerbrey in 1959 demonstrated that mass additions to a piezoelectric quartz crystal would linearly decrease the crystal's resonance frequency.[41] Thus, with careful measurement of the crystal's resonance frequency, adsorbed mass to the crystal

can be quantified. QCM measurements were later adapted for use in liquid media, paving the way for protein adsorption studies.[42] The basic setup involves attaching two electrodes to the upper and lower crystal surface (typically gold or platinum) and applying an alternating electric field, which causes the crystal to oscillate at a characteristic frequency.[43,44] Thin films of the material of interest are added to the QCM crystal and subsequently exposed to protein solutions. Careful measurement of the resonance frequency of the crystal during adsorption can provide sensitive measurements of mass in the ng/cm^2 range.[9,45,46]

Similarly to ellipsometry, obtaining mass values based on the frequency changes requires modeling, which can be difficult for biomaterials that uptake significant amounts of water.[9] As different proteins can displace different amounts of water as they adsorb, comparisons between proteins must be interpreted with care.[9] Additionally, mass contributions from different co-adsorbing biomolecules (such as those in real or artificially created bodily fluids) are qualitative and not quantitative.[47] Biomaterials are also not examined directly, but analogues are merely created on the QCM crystal as thin films.

8.1.1.3 Attenuated Total Internal Reflection

Attenuated total internal reflection (ATR) is another technique to measure protein adsorption onto biomaterials in situ.[34,48,49] It utilizes the principle of total internal reflection and evanescent waves to probe the quantity and quality of adsorbed proteins. Light entering a trapezoidal crystal at certain angle will completely reflect from the internal surface 1+N times, with the angle being dependent on the refractive index of the crystal and sample media and the number of internal reflections (N) is dependent on the

incident angle and crystal thickness and length.[50] A typical setup using this approach is shown in Figure 8-2.

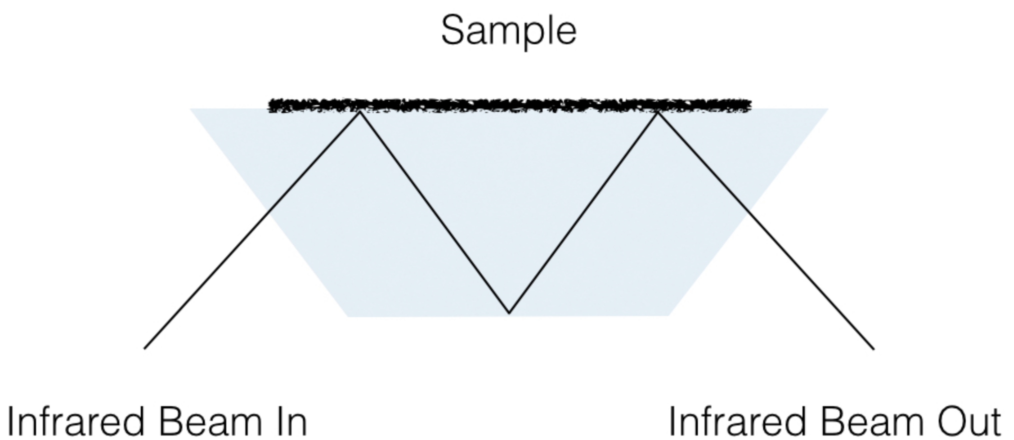

Figure 8-2: A simplified ATR setup

At each reflection there will be an evanescent wave created which penetrates into the sample.[50,51] Analyzing the interaction between the evanescent wave, the biomaterial, and any adsorbed protein allows information to be gathered about the quantity and conformation of deposited protein. Proteins absorb infrared light strongly at distinctive wavenumber bands at approximately 3300cm^{-1}, 1640cm^{-1}, and 1550cm^{-1} corresponding to N-H stretching, and amide regions I and II.[52,53] It is common for ATR techniques to be paired with Fourier transform infrared spectroscopy (FTIR) because of proteins strong infrared (IR) absorption.[54–56] Comparing the IR absorption of the untreated biomaterial to the material with deposited protein at these bands can give some information about the protein adsorption kinetics. Unfortunately, the information is more qualitative than quantitative as protein deposition is often reported as arbitrary “absorption units”, requiring other techniques to quantify the results.[54,56]

A major concern when undertaking ATR-FTIR measurements, especially in situ, is that water absorbs infrared wavelengths at almost exactly the amide I region that characterizes protein adsorption, but at orders of magnitude stronger.[50,57] The validity of subsequent subtraction of the absorption from water is questionable. As with ellipsometry and QCM, adsorption kinetics of different biomolecules is not possible using ATR-FTIR.

Total internal reflection fluorescence (TIRF) can be utilized to overcome the overwhelming absorption from water. By tuning the incident light to the excitation wavelength of a fluorophore attached to proteins, such as fluorescein isothiocyanate (FITC) 495nm[58–60] or the intrinsic fluorescence of proteins 290nm,[61,62] protein adsorption data can be obtained by measuring the intensities of the subsequently emitted light. The use of a fluorophore would allow for examination of competitive adsorption between multiple proteins, though the fluorophores may affect protein/protein and protein/surface interactions.[63,64] Regardless of the fluorescent technique used, careful calibration is required to obtain masses of absorbed protein. Biomaterials cannot be measured directly, but require 'analogues' to be created on the surface of the crystal.

Surface plasmon resonance (SPR) also utilizes ATR to measure the kinetics of protein adsorption.[65–68] When a thin metallic film (such as gold or silver) is added between the surface of the crystal and the biomaterial of interest, surface plasmons, oscillations of free electron density in the metal, can be created from the evanescent wave.[69] At a certain angle these surface plasmons can be resonantly excited and will absorb energy from the incident beam.[68] Biomaterial-adsorbed proteins will change the angle at which resonance occurs, and thus monitoring of this critical angle allows for determination of the protein adsorption kinetics.[70] Determination of adsorbed mass from changes in the critical angle

requires careful interpretation and mathematical analysis.[70] As with the other ATR techniques, protein adsorption to biomaterials is not measured directly, but thin films of analogues are added to the metallic surface.

8.1.1.4 Atomic Force Microscopy

Atomic force microscopy (AFM) can be used to obtain qualitative measurements of proteins adsorbed to biomaterials. A small tip, attached to a cantilever, traces the material directly in contact, not in contact but close to the surface, or constantly oscillating toward and away from the surface.[51] Laser light is focused onto the cantilever and reflected to a detector which is sensitive enough to convert the information to a three dimensional topographical map with less than 1nm resolution.[71] Using AFM, protein deposits can be imaged directly on biomaterials and the deposit height determined in situ.[72–74] Information can be gathered as to the deposition pattern of the protein, such as clumps or monolayer, in addition to deposit thicknesses. Figure 8-3 shows an AFM image of bovine serum albumin adsorbed to a lotrafilcon B contact lens material.

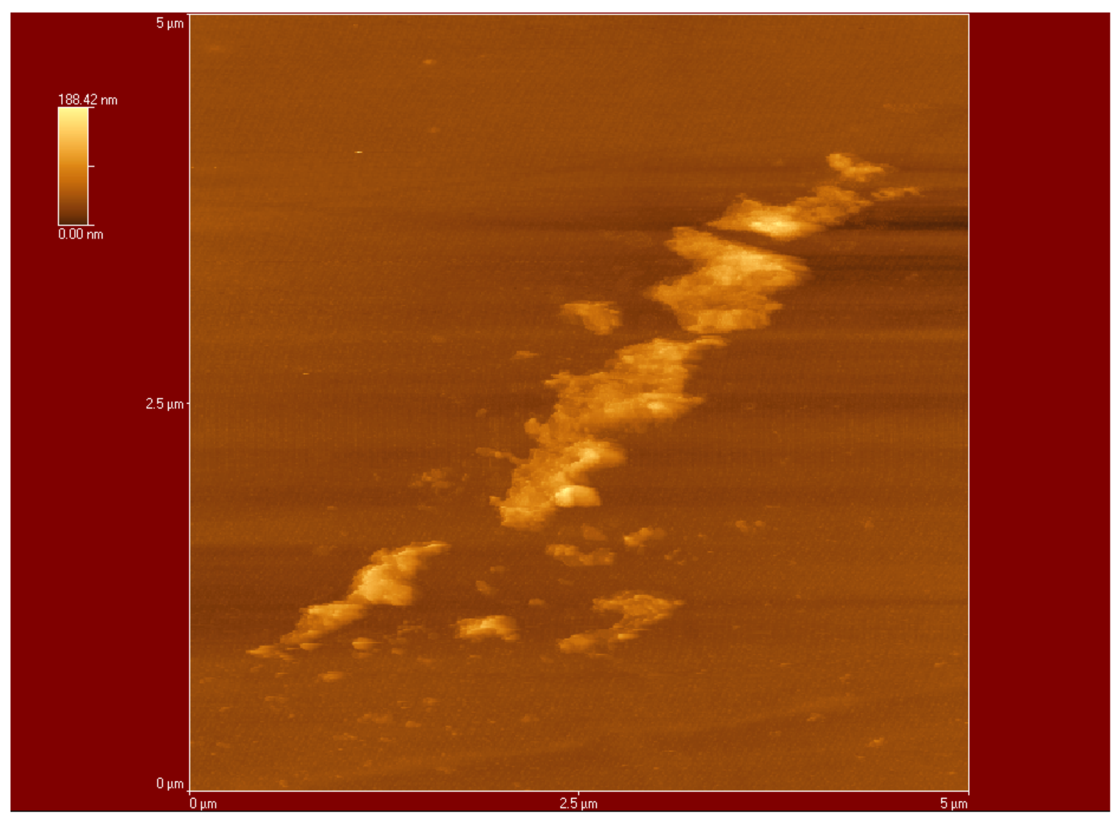

Figure 8-3: Bovine serum albumin adsorbed onto a lotrafilcon B contact lens

Since the height profile will be relative to the lowest height measured, smoother and flatter surfaced biomaterials may be easier to accurately determine the protein deposit thickness. Protein thickness determinations require unfouled material nearby as a reference, and may make interpretations difficult for monolayer or multilayer coverage.

8.1.1.5 Summary

In general, the above-mentioned techniques are sensitive enough to measure the small amounts of protein that can deposit onto biomaterials during the first few minutes of exposure to protein containing fluids. Additionally, they can be used to monitor the adsorption process in situ and in real time. A major problem with these techniques is that some do not measure actual biomaterials directly, but thin films of analogues. Careful mathematical modeling is needed for accuracy in the interpreted results, which may be

difficult as adsorption, desorption, thickness changes, denaturation, expulsion of water, etc. will be occurring simultaneously in real time.

8.1.2 Ex situ

Despite the advantages of in situ measurements, there is still a need for a technique sensitive enough to measure small amounts of protein on biomaterials directly. Most techniques involve removal of the biomaterial from the protein solution prior to analysis.

8.1.2.1 Colorimetric Assay

A popular technique to measure protein in solution is to use a colorimetric assay, such as the Lowry,[17] Bradford,[18] or bicinchoninic acid (BCA).[19] The presence of protein in samples causes a colour change in the reagents used and the total protein is eluded from adsorption spectra. Colorimetric assays are typically done with extracted protein,[15,20,75] though some research has been done using the BCA assay directly on biomaterials[76] including contact lens materials.[77,78] This thesis work attempted to utilize the BCA assay to measure protein adsorbed to contact lens materials, as described by Zhang et al.[78] but was not successful. There was significant interference from the contact lens materials themselves (1-3.5µg of apparent protein adsorption), which dwarfed any protein deposition for relatively low depositing silicone hydrogels. There was also significant interference from the blister pack solutions (2-28µg of apparent protein adsorption). These results are unpublished, but highlight a key problem with colorimetric assays. The results can be influenced by non-protein components (material monomers, surfactants, lipids) that lead to anomalous results. Colorimetric assays also cannot distinguish between different proteins.

Using an enzyme linked immunosorbent assay (ELISA), first established in the 1970s,[14,16,79] is another popular method of determining protein concentration in solution. This technique typically involves immunoglobulin-enzyme complexes (IECs) that bind to the protein. IECs that do not bind the proteins are removed, the enzyme's substrate is added, and a colour change occurs that can be read using a spectrometer. It is a sensitive technique and ELISAs have been used to quantify extracted protein[15,80] and protein adsorbed to materials.[13,81,82] The main concern when using this technique with biomaterials is that the region of the protein that the IEC binds to must be available. If the protein changes its conformation, denatures, binds to the material or other protein using this region it will not be measured. If the IEC binds to the material then anomalously high protein measurements will occur.

8.1.2.2 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) can be used to localize where proteins have adsorbed to biomaterials.[7,8,83–86] Proteins are conjugated to fluorescent labels before adsorption to biomaterials, which are subsequently excited with the incident laser light after adsorption. Only the emitted light from the focal plane can pass through a pinhole aperture and reach the detector. Thus thin "slices" of the biomaterial can be imaged for protein deposits and pieced together for a three dimensional image. A simplified schematic is shown in Figure 8-4.

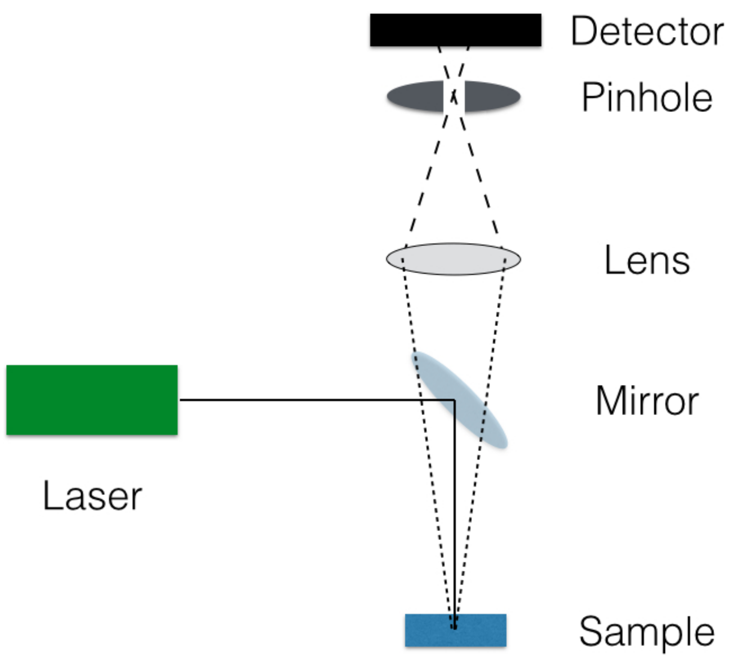

Figure 8-4: A simplified confocal laser scanning microscopy setup

With different fluorescent labels, different protein types could be imaged simultaneously. However, since CLSM requires the use of a fluorescent label, there is the possibility of label-surface, label-label, or protein-label interactions that could affect protein adsorption.[63,64] Another drawback is that the images acquired are not quantitative, but qualitative.

8.1.2.3 X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) can detect the adsorption of proteins by chemical analysis.[86–89] When a sample is irradiated with X-rays, the atoms in the sample will absorb them and emit electrons.[87,90] These emitted electrons have binding energies unique to each element and their intensities are proportional to the elements concentration.[87,90] Nitrogen is usually the element used to determine the concentration of adsorbed protein, but other trace elements present in the protein structure can be used (Fe, P, S) if present in high enough quantities.[87,90]

Relying on nitrogen or trace element concentration to determine the quantity of adsorbed protein means that distinguishing between different protein deposits on the same material is not possible with XPS solely. XPS also utilizes freeze-drying of the adsorbed proteins and biomaterial, which is not ideal.

8.1.2.4 Mass Spectrometry

Mass spectrometry (MS) is a powerful technique to reveal information about adsorbed proteins. It involves bombarding samples with electrons to eject ions from the sample.[90–92] These ions are subsequently separated by their mass/charge ratios and the results are output as a spectrum of ion abundance versus mass/charge ratio.

There are two commonly used methods for biomaterial bombardment: secondary ion mass spectrometry (SIMS) and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). The SIMS technique, pioneered by Benninghoven,[93] involves sputtering electrons onto the outermost 1-1.5nm of protein deposits to eject neutral and ionic molecules.[94,95] Laborious analysis of the spectrum is needed to construct the proteins from the component molecules measured.[96–98] MALDI-MS was developed by Hillenkamp[99] and aims to determine the molecular weights of deposits by limiting fragmentation.[90,91] This allows discrimination between different adsorbed biomolecules.[100–102] MALDI-MS can only detect proteins that are removed from the biomaterial by the matrix,[91] and thus tightly bound proteins or proteins in the bulk of the biomaterial may be missed.

8.1.2.5 Radiochemical Experiments

Instead of fluorescent labels, which can modify the properties of the labeled protein,[103] radiochemical experiments attach radioactive atoms to proteins and are thought not to affect protein properties or binding. Commonly C^{14} and I^{125} isotopes are used. The beta emissions from C^{14} require scintillation fluid in order to be counted, and thus experiments with C^{14} labeled proteins and biomaterials typically extract the labeled protein first.[104,105] A direct measurement of C^{14} labeled lysozyme adsorbed to contact lens materials was undertaken during this thesis work and is shown in Figure 8-5. From this figure we can see that it is possible to measure adsorbed protein in situ without extraction, even when the levels of lysozyme are very low. Despite this success, there are concerns about the validity and accuracy of the results. When comparing these results to previously established techniques of measuring adsorbed protein on the same silicone hydrogel materials,[106,107] the results in Figure 8-5 show much greater lysozyme deposition, which could be an indication of inaccuracy. In order to extrapolate adsorbed masses of lysozyme a standard curve must be generated of known amounts of lysozyme in scintillation fluid, which may react with the scintillation fluid very differently than adsorbed lysozyme. It is likely that the calibrations will be material specific.

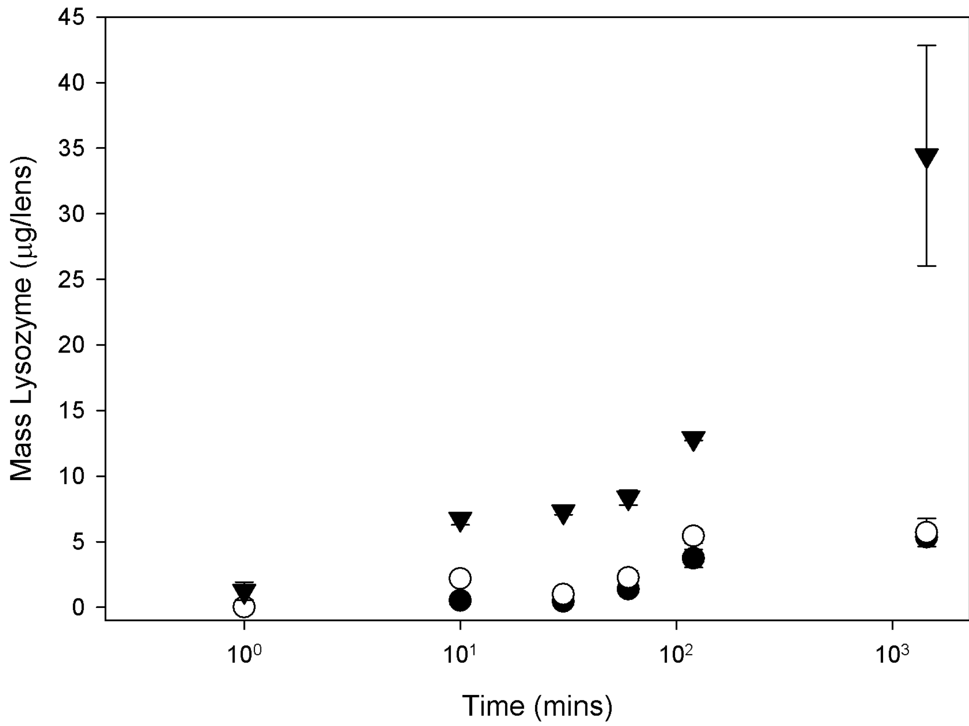

Figure 8-5: Lysozyme adsorption onto (●)senofilcon A, (○)lotrafilcon B, (▼)balafilcon A as measured by C^{14} methylated lysozyme. Error bars represent mean ± SD, n=3.

Radiochemical experiments using I^{125} do not need scintillation fluid as the emitted gamma rays can be measured directly. Adsorbed protein which has been tagged with I^{125} can be measured whether it is in the bulk of the material or on the surface of a variety of biomaterials without protein extraction,[85,88,94,108–115] and is commonly used instead of C^{14}. Since only the protein with the tracer ion will be quantified, it is possible to measure single protein species in a multi-component fluid.

Despite the assumed non-effect to protein properties or binding, I^{125} labeling suffers from one of the major problems associated with any label, that the I^{125} tracer can disassociate from the protein and adsorb to the biomaterial itself.[116,117] This was shown in Chapter 6. The effect is material specific and materials that deposit relatively high levels of protein are relatively unaffected, however low protein adsorbing materials can uptake substantial amounts of the iodine tracer compared to ‘real’ protein adsorption. An even

greater risk for inaccuracies are materials that incorporate monomers exhibiting strong interactions with iodine, such as polyvinyl pyrrolidine (PVP),[118] which can adsorb more tracer iodine than labeled protein, as seen in Chapter 6.

When using I^{125} radiochemical experiments, the generation of free I^{125} should be limited. Labeled protein should be used as soon as possible after labeling. Any generated free I^{125} can be reduced by dialysis of the labeled protein, however disassociation of the tracer iodine from protein is an ongoing process. From Chapter 6 we saw that the amount of free I^{125} can double or quadruple after a week of incubation with biomaterials. As a consequence, incubation solutions should be refreshed within one week with fresh, dialyzed, I^{125} labeled protein. Despite these precautions there will remain a small amount of free I^{125} in solution that should be measured. Biomaterials can then be exposed to the same concentration of unbound tracer iodine to determine the representative apparent deposition from just free I^{125}.

8.2 Protein Conformation

Arguably, more important to the biocompatibility than measuring the quantity of protein adsorbed to biomaterials is measuring the state of the protein. Adsorbed proteins may be in their native state or there could be some degree of denaturation, either structural changes and/or a loss of biological activity. Denatured protein is undesirable on biomaterials as it may lead to discomfort,[119] an immunological response,[3,120–122] bacterial adhesion[4,5] and loss of function to remove any adhered bacteria,[123] and potentially thrombosis.[3] Measuring the degree of denaturation of adsorbed proteins can be broken down into two broad categories: measuring the secondary and tertiary structural changes

and measuring changes in biological activity. In any approach to measure protein denaturation there is a need to avoid affecting the protein state by the chosen technique.

8.2.1 Measuring Secondary and Tertiary Structure

Proteins interacting with biomaterials may rearrange their structure in order to better associate with the material. For instance, a protein could rearrange its structure to allow normally internal hydrophobic groups to interact with a hydrophobic surface. These secondary and tertiary structural changes can be measured using sensitive techniques to gain an overall understanding of protein conformation.

8.2.1.1 Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) is a technique used to measure the quantum magnetic properties of atomic nuclei, specifically nuclei with a spin such as C13 and H1.[124] NMR can be used to determine the secondary and tertiary structure of proteins, including the structure of their component amino acids, adsorbed to biomaterials.[125–129] Due to its low sensitivity, NMR requires relatively long processing time[124,127] and protein adsorption is typically done using spheres.[125,128,129]

8.2.1.2 Circular Dichroism

Circular dichroism (CD) uses polarized light to determine the secondary and tertiary structure of adsorbed proteins.[130] CD measures the difference in absorption for left-handed and right-handed circularly polarized light passing through asymmetric samples.[130] Proteins are asymmetric and absorb UV light, thus absorption spectra using CD can be done in the UV range for proteins in solution[131–135] and adsorbed to biomaterials.[136–138] A simplified setup is shown in Figure 8-6.

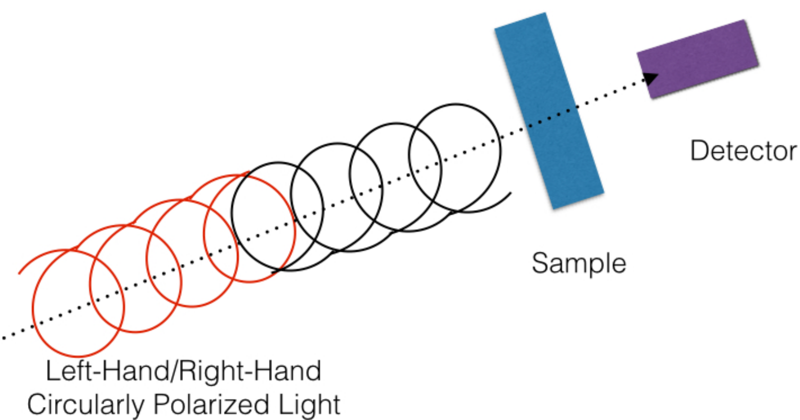

Figure 8-6: A simplified setup of circular dichroism spectroscopy

Using references of 100% α-helix, 100% β-sheet, etc. the fractional component of each can be interpreted for solution suspended and adsorbed proteins from the absorption spectra.[136,138] Due to the calibration needed from isolated secondary structural elements, the accuracy of this technique is lacking.[139]

8.2.1.3 Attenuated Total Internal Reflection Infrared Spectroscopy

As described in section 8.1.1.3, ATR-FTIR is a sensitive technique used to measure protein quantity, but is also routinely used to determine biomaterial-adsorbed protein secondary and tertiary structure.[52,55,140–142] Proteins absorb infrared light strongly at distinctive wavenumber bands, approximately 3300cm^{-1}, 1640cm^{-1}, and 1550cm^{-1}, corresponding to N-H stretching, and amide regions I and II.[52,53] The amide I region is the focus for structural information,[143–146] specifically the absorption at 1650-1658cm^{-1} is correlated to α-helix content[50,147] and the absorption at 1620-1640cm^{-1} is correlated to β-sheet content.[143,147] Comparing spectra of native to protein to adsorbed protein can provide insight into whether the protein is denatured. Denaturation effects will be protein specific, but some examples include increasing β-sheet content during aggregation and decreasing α-helix content with a corresponding increase in random coil content.[147]

In addition to the water absorption issues in the amide I region described in section 8.1.1.3,[50,57] the absorption by α-helices and β-sheets lie fairly close to one another and can make analysis difficult.[50]

8.2.1.4 Raman Spectroscopy

Raman spectroscopy is used to characterize the secondary and tertiary structure of proteins and is similar to infrared spectroscopy (IR).[148] Like IR, Raman measures intensities at specific wavenumbers, that correspond to molecular vibrations and rotations,[148] and relate them too protein structure.[148–151] An important region, as it is in IR, is the amide I at ~1640cm^{-1} which contains contributions from both the α-helices and β-sheets within the protein structure.[152] Raman relies on collecting inelastically scattered light from samples, rather than the absorption of infrared light,[148] which is a significant advantage over IR since water gives only a weak Raman signal in the amide I region. A simplified Raman setup is shown in Figure 8-7.

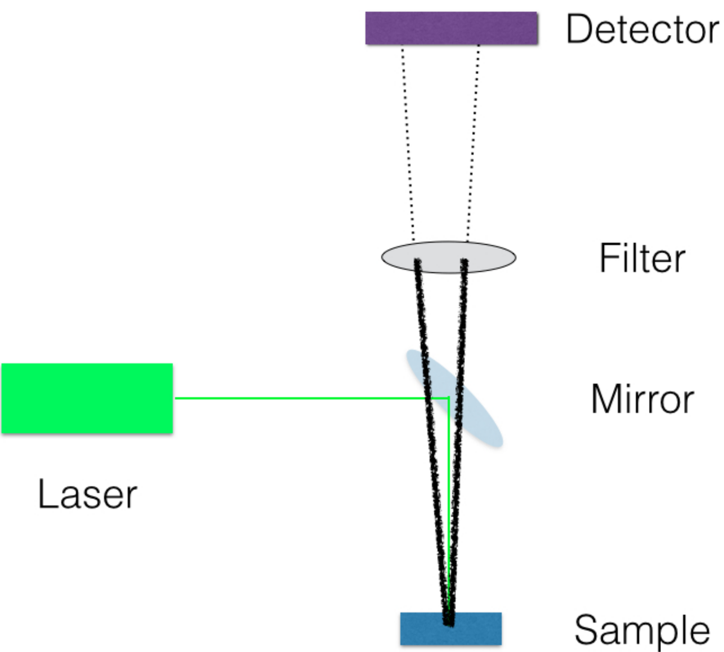

Figure 8-7: A simplified Raman spectroscopy setup

Raman spectroscopy has the potential to be used for investigating secondary and tertiary structural changes of adsorbed proteins, but it is difficult for materials without a high surface to volume ratio such as nanoparticles.[153] It is also difficult to distinguish between different biomolecules, since the spectrum will be the sum of all contributions in the sample.

8.2.2 Biological Function

It is clear from section 8.2.1 that measuring the secondary and tertiary structure of adsorbed proteins has had only limited success. Each technique, though powerful, can have accuracy issues from calibration, long processing times, and interference from the solvent, or lack of ability to distinguish between protein species. Even with a flawless technique to measure protein structure, the biological function of a protein will only relate to part of the structure. A decrease in α-helix content does not necessarily mean that the protein has lost its biological function since the active site of an enzyme could be unaffected. It may be that the best measure of surface adsorbed protein denaturation is to determine the loss of biological function.

8.2.2.1 Lysozyme Activity

Lysozyme is enzymatic, and thus is frequently used for biological activity measurements.[154–158] It catalyzes the hydrolysis of components in the bacterial cell wall, causing damage and eventual cell lysis. Based on this property, standard micrococcal activity assays have been developed to measure the biological activity of lysozyme in solution.[1,106,154–159] In Chapter 3 the micrococcal activity assay was modified to directly measure the biological activity of adsorbed lysozyme,[1] and used again in Chapters 4-5.[30,160] This technique directly measures the activity of biologically relevant lysozyme

rather than an overall measure of denaturation of all adsorbed lysozyme, as can be measured following lysozyme extraction. Biologically relevant biomaterial-adsorbed biomolecules are only those that can interact with biological systems. Biologically relevant lysozyme is therefore that which is in the outer surface layer of deposits and any lysozyme that desorbs back into solution. Extraction of the protein from the surface would be required to measure the activity of protein in the material bulk or underlying protein layers.[140]

Biological activity can also be determined when co-adsorbed with other biomolecules[141] since this is a specific measure of lysozyme biological function. By measuring the biological function of lysozyme on contact lenses, we have shown in Chapter 4 that lenses with more surface active lysozyme have reduced bacterial adhesion than those with less surface-adsorbed lysozyme or reduced surface activity of lysozyme.[140] Figure 8-8 demonstrates that bacterial adhesion is reduced when the contact lenses are coated in lysozyme, with the exception of lotrafilcon B that has adsorbed lysozyme which has lost its biological function, allowing for more adhesion.

Despite the clear advantages of measuring the biological function of lysozyme, it is important to remember that the amount of active surface-adsorbed lysozyme is determined based on calibrations from lysozyme in solution. It is possible that a material with 50% active lysozyme could have all "active" lysozyme but at a reduced efficiency. Conversely 25% of the lysozyme could be active, but functioning at an increased efficiency. It is likely that each calibration will be material specific and warrants further investigation.

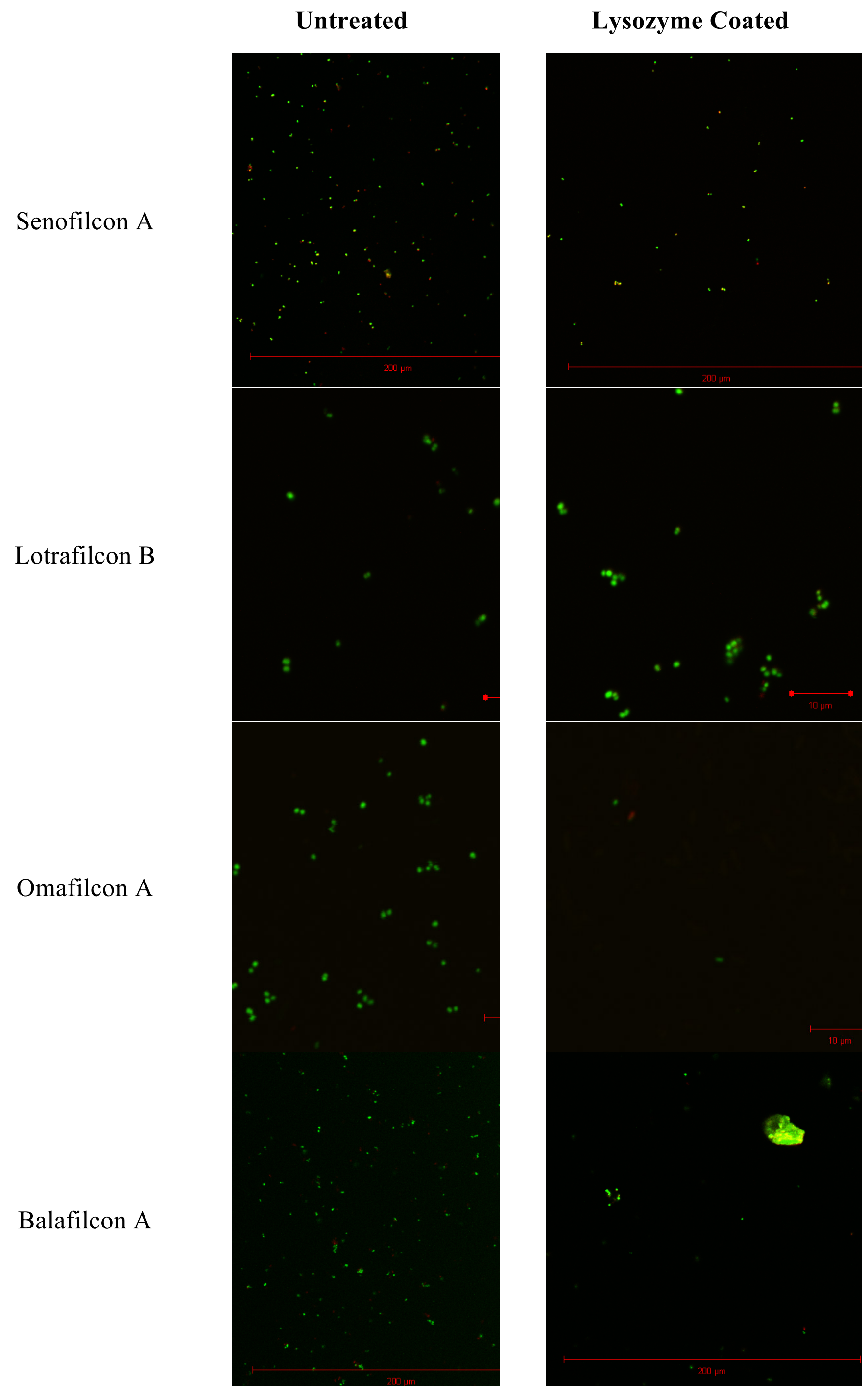

Figure 8-8: Micrococcus Lysodeikticus adhesion to untreated and lysozyme coated contact lenses. Stained with STYO 9 and propidium iodide

CHAPTER 9 CONCLUSIONS

There are a wide variety of techniques to measure both the amount of biomaterial-adsorbed protein and its degree of denaturation, and each has its advantages and disadvantages. To measure protein deposition over short time periods, on biomaterials, not just thin films, the method of choice should be I^{125} radiolabeling. As long as steps are taken to limit and measure any interaction of the iodine tracer with the material, it is sensitive enough to measure small amounts of deposited protein. I^{125} radiolabeling can also measure adsorbed protein from the bulk of the material and on the surface, without protein removal, and does not require lengthy processing times. To measure the denaturation of protein over short time periods, on biomaterials, the method of choice should be to measure the biological function of the adsorbed protein. The biological function will only partly depend on the secondary and tertiary forms, therefore it is better to measure the biological function directly. Any measure of the biological function of adsorbed proteins should include both the biologically relevant protein, protein in the outer surface layer and any protein that has desorbed into solution, and the non-biologically relevant protein, within the bulk of the material and underlying adsorption layers. The biologically relevant protein activity can be measured directly in solution. However, to measure the activity of non-biologically relevant protein, extraction is required.

Thus, in conclusion, investigations of protein adsorption onto biomaterials, over short time periods, should measure the protein adsorption with I^{125} radiolabeling and the adsorbed protein biological function directly and after extraction.

CHAPTER 10 PAPERS NOT INCLUDED IN THESIS

1. Hall, B, McCanna, D, Jones, L. Identification of coagulase negative staphylococci in daily disposable contact lens wearers. Letters in Applied Microbiology 2014;59(3):313-319.

This study aimed to identify and quantify the number of contaminating organisms on daily disposable (DD) soft contact lenses, which may be responsible for mild cases of keratitis that occur with this lens wear modality. Ten participants wore DD lenses, and 10 participants wore planned replacement (PR) lenses. Lenses were collected aseptically and analyzed for microbial contamination. Colony-forming units (CFU) were recorded, and representative colonies were used for identification using the API identification system. The DD lenses evaluated in this study were contaminated with coagulase-negative staphylococcus (CNS), ranging from 1 to 653 CFU. PR lenses showed more diversity in the types of contaminating micro-organisms and consisted of CNS, Gram-negative bacteria (Pseudomonas), a yeast (Candida) and a mold (Aspergillus), ranging from 1 to 230 CFU. CNS was the only type of micro-organism found on DD contact lenses and therefore may be the cause of any form of keratitis observed in DD lens wearers.

2. Hall, B, Jones, L, Dixon, B. Silicone allergies and the eye: fact or fiction? Eye Contact Lens 2014;40(1):51-57.

Objective: The purpose of this manuscript was to review the evidence concerning the role of an allergic reaction to silicone as the basis for the reported increase in contact lens-associated infiltrates in wearers of silicone hydrogel contact lenses.

Methods: A literature review was undertaken to investigate the antigenic properties of silicone and the causes of contact lens-associated inflammatory reactions.

Results: Immune cells cannot interact with silicone directly but can interact with antigens on these lenses. These antigens could be due to tear film deposits, microbial contamination, or components of care systems used with these lenses.

Conclusions: Inflammatory reactions associated with silicone hydrogel contact lens wear are not caused by an allergic reaction to silicone alone.

3. Hall, BJ, Jones, L. Contact lens cases: the missing link in contact lens safety? Eye Contact Lens 2010;36(2):101-105.

Purpose: To summarize a variety of issues associated with contact lens case contamination and discuss appropriate methods that can limit this.

Methods: A literature review was undertaken investigating the major factors associated with case contamination, with specific reference to the major pathogens associated with contamination, the role of bacterial biofilms, and methods that can limit contamination.

Results: The use of antimicrobial cases, regular case cleaning and case replacement, avoidance of topping up solutions, and not using tap water to rinse cases all appear to be important in avoidance of significant case contamination.

Conclusions: Contact lens case contamination is a significant public health concern and may contribute significantly to the development of microbial keratitis in contact lens wearers. Patients should be reminded that they must clean and disinfect their lens cases daily, should avoid the use of tap water for rinsing them, must not top up their solutions, must take into careful consideration where and how the cases are stored

4. Hall, B and Jones, L. Countering noncompliance with lens care and case technology. Contact Lens Spectrum 2010;25(12):50-51.

Objective: To summarize relevant contact lens care and case technology which may reduce hygienic non-compliance seen with contact lens wearers.

Methods: A literature review was undertaken investigating novel contact lens care system and case technology.

Results: Lens care and case technological improvements aim to increase lens and case replacement compliance, decrease microbial lens case contamination by antimicrobials and UV purification, and enhance the removal of deposits.

Conclusions: The development of novel contact lens cases will continue and is encouraged to aid in the compliance of contact lens wearers, though their effectiveness at reducing serious complications remains unproven.

CHAPTER 11 REFERENCES

11.1 Chapter 1 References

1. Karp G. Cell and Molecular Biology: Concepts and Experiments, Fourth. Hoboken, NJ: John Wiley & Sons, Inc.; 2005.

2. Voet D, Voet J, Pratt. C. Fundamentals of Biochemistry; Life at the Molecular Level, Second. Hoboken, NJ: John Wiley & Sons, Inc.; 2006.

3. Buxbaum E. Fundamentals of Protein Structure and Function, Vol. 31. New York, USA: Springer; 2007.

4. Diamond R. Real-space refinement of the structure of hen egg-white lysozyme. J Mol Biol. 1974; 82: 371–391.

5. Moore SA, Anderson BF, Groom CR, et al. Three-dimensional structure of diferric bovine lactoferrin at 2.8 A resolution. J Mol Biol. 1997; 274: 222–236.

6. Majorek KA, Porebski PJ, Dayal A, et al. Structural and immunologic characterization of bovine, horse, and rabbit serum albumins. Mol Immunol. 2012; 52: 174–182.

7. H.M. Berman, Westbrook J, Feng Z, et al. The protein data bank. Nucleic Acids Res. 2000; 28: 235–242.

8. Lukin J, Kontaxis G, Simplaceanu V, et al. Quaternary structure of hemoglobin in solution. Proc Natl Acad Sci. 2003; 100(2): 517–20.

9. Haynes CA, Norde W. Globular proteins at solid/liquid interfaces. Colloid Surface B. 1994; 2(6): 517–566.

10. Norde W. Adsorption of proteins from solution at the solid-liquid interface. Adv Colloid Interface Sci. 1986; 25(4): 267–340.

11. Lok BK, Cheng Y-L, Robertson CR. Protein adsorption on crosslinked polydimethylsiloxane using total internal reflection fluorescence. J Colloid Interface Sci. 1983; 91(1): 104–116.

12. Castillo EJ, Koenig JL, Anderson JM. Characterization of protein adsorption on soft contact lenses. 1984; 5(6): 319–325.

13. McClellan SJ, Franses EI. Adsorption of bovine serum albumin at solid/aqueous interfaces. Colloids Surfaces A Physicochem Eng Asp. 2005; 260(1): 265–275.

14. Carney FP, Morris CA, Milthorpe B, et al. In vitro adsorption of tear proteins to hydroxyethyl methacrylate-based contact lens materials. Eye Contact Lens. 2009; 35(6): 320–8.

15. Rabinow B, Ding Y, Qin C, et al. Biomaterials with permanent hydrophilic surfaces and low protein adsorption properties. J Biomater Sci Polym Ed. 1994; 6(1): 91–109.

16. Kothari S, Hatton P V., Douglas CWI. Protein adsorption to titania surfaces. J Mater Sci Mater Med. 1995; 6(12): 695–698.

17. Pitt WG, Cooper SL. FTIR-ATR studies of the effect of shear rate upon albumin adsorption onto polyurethaneurea. Biomaterials. 1986; 7(5): 340–7.

18. Castillo E, Koenig J, Anderson J, et al. Protein adsorption on hydrogels: II. Reversible and irreversible interactions between lysozyme and soft contact lens surfaces. Biomaterials. 1985; 6: 338–345.

19. Cullen DC, Lowe CR. AFM studies of protein adsorption: 1. Time-resolved protein adsorption to highly oriented pyrolytic graphite. J Colloid Interface Sci. 1994; 166(1): 102–8.

20. Rodrigues SN, Gonçalves IC, Martins MCL, et al. Fibrinogen adsorption, platelet adhesion and activation on mixed hydroxyl-/methyl-terminated self-assembled monolayers. Biomaterials. 2006; 27(31): 5357–67.

21. Xu LC, Siedlecki CA. Effects of surface wettability and contact time on protein adhesion to biomaterial surfaces. Biomaterials. 2007; 28(22): 3273–3283.

22. Fink D, Hutson T, Chittur K, et al. Quantitative surface studies of protein adsorption by infrared spectroscopy. Anal Biochem. 1987; 165: 147–154.

23. Matsumoto H, Koyama Y, Tanioka A. Interaction of proteins with weak amphoteric charged membrane surfaces: effect of pH. J Colloid Interface Sci. 2003; 264(1): 82–88.

24. Arai T, Norde W. The behavior of some model proteins at solid-liquid interfaces 1. Adsorption from single protein solutions. Colloids and Surfaces. 1990; 51: 1–15.

25. Elgersma A, Zsom RL., Norde W, et al. The adsorption of bovine serum albumin on positively and negatively charged polystyrene latices. J Colloid Interface Sci. 1990; 138(1): 145–156.

26. Norde W, Lyklema J. The adsorption of human plasma albumin and bovine pancreas ribonuclease at negatively charged polystyrene surfaces: I. Adsorption Isotherms. Effects of charge, ionic strength, and temperature. J Colloid Interface Sci. 1978; 66(2): 257–265.

27. Norde W. Ion participation in protein adsorption at solid surfaces. Colloids and Surfaces. 1984; 10: 21–31.

28. Latour R. Biomaterials: protein-surface interactions. Encycl Biomater Biomed Eng. 2005; 28: 1–15.

29. Wertz C, Santore M. Fibrinogen adsorption on hydrophilic and hydrophobic surfaces: geometrical and energetic aspects of interfacial relaxations. Langmuir. 2002; 18(3): 706–715.

30. Wang J, Fonn D, Simpson T, et al. Precorneal and pre- and postlens tear film thickness measured indirectly with optical coherence tomography. Invest Ophthalmol Vis Sci. 2003; 44(6): 2524–2528.

31. King-Smith PE, Fink BA, Fogt N, et al. The thickness of the human precorneal tear film: evidence from reflection spectra. Invest Ophthalmol Vis Sci. 2000; 41(11): 3348–59.

32. Olsen T. Reflectometry of the precorneal film. Acta Ophthalmol. 1985; 63(4): 432–8.

33. Craig J, Tomlinson A. Importance of the lipid layer in human tear film stability and evaporation. Optom Vis Sci. 1997; 74(1): 8–13.

34. Fullard RJ, Snyder C. Protein levels in nonstimulated and stimulated tears of normal human subjects. Invest Ophthalmol Vis Sci. 1990; 31(6): 1119–26.

35. Lemp M, Foulks G. The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop. Ocul Surf. 2007; 5(2): 75–92.

36. Smolin G. The defence mechanism of the outer eye. Trans Ophthalmol Soc UK. 1985; 104(pt 4): 363–366.

37. Craig JP, Willcox MDP, Argüeso P, et al. The TFOS International Workshop on Contact Lens Discomfort: report of the contact lens interactions with the tear film subcommittee. Invest Ophthalmol Vis Sci. 2013; 54(11): TFOS123–56.

38. Yamada M, Mochizuki H, Kawai M, et al. Decreased tear lipocalin concentration in patients with meibomian gland dysfunction. Br J Ophthalmol. 2005; 89(7): 803–5.

39. Glasson MJ. Differences in clinical parameters and tear film of tolerant and intolerant contact lens wearers. Invest Ophthalmol Vis Sci. 2003; 44(12): 5116–5124.

40. Rapacz P, Tedesco J. Tear lysozyme and lactoferrin levels in giant papillary conjunctivitis and vernal conjunctivitis. Eye Contact Lens. 1988; 14(4): 207–209.

41. Farris RL. Tear analysis in contact lens wearers. Trans Am Ophthalmol Soc. 1985; 83: 501–45.

42. Carney F, Morris C, Willcox M. Effect of hydrogel lens wear on the major tear proteins during extended wear. Aust N Z J Ophthalmol. 1997; 25(suppl 1): S36–S38.

43. Choy CK, Cho P, Benzie IF, et al. Effect of one overnight wear of orthokeratology lenses on tear composition. Optom Vis Sci. 2004; 81(6): 414–420.

44. Glasson M, Stapleton F, Willcox MDP. Lipid, lipase and lipocalin differences between tolerant and intolerant contact lens wearers. Curr Eye Res. 2002; 25(4): 227–235.

45. Sarac O, Gurdal C, Bostancı-Ceran B, et al. Comparison of tear osmolarity and ocular comfort between daily disposable contact lenses: hilafilcon B hydrogel versus narafilcon A silicone hydrogel. Int Ophthalmol. 2012; 32(3): 229–33.

46. Tiffany JM. Composition and biophysical properties of the tear film: knowledge and uncertainty. Adv Exp Med Biol. 1994; 350: 231–238.

47. Holly FJ, Lemp MA. Tear physiology and dry eyes. Surv Ophthalmol. 1977; 22(2): 69–87.

48. Williams D. Definitions in biomaterials. In: Proceedings of A Consensus Conference of the European Society for Biomaterials. Elsevier; 1987.

49. Menzies KL, Jones L. The impact of contact angle on the biocompatibility of biomaterials. Optom Vis Sci. 2010; 87(6): 387–99.

50. Tighe B. Contact Lens Materials. In: Phillips A, Speedwell L (eds). Contact Lens Practice. London: Butterworth-Heinemann; 2007. pp. 59–78.

51. Klee D, Höcker H. Polymers for Biomedical Applications: Improvement of the Interface Compatibility. In: Eastmond GC, Höcker H, Klee D (eds). Biomedical Applications Polymer Blends.Vol 149. Advances in Polymer Science. Springer Berlin Heidelberg; 1999. pp. 1–57.

52. Andrade JD, Hlady V. Protein adsorption and materials biocompatibility: A tutorial review and suggested hypotheses. Adv Polym Sci. 1986; 79: 1–63.

53. Andrade J. Surface and Interfacial Aspects of Biomedical Polymers. New York: Plenum Press; 1985.

54. Ratner BD, Tyler BJ, Chilkoti A. Analysis of biomedical polymer surfaces: polyurethanes and plasma-deposited thin films. Clin Mater. 1993; 13(1-4): 71–84.

55. Merrett K, Cornelius RM, Mcclung WG, et al. Surface analysis methods for characterizing polymeric biomaterials. J Biomater Sci Polym Ed. 2002; 13(6): 593–621.

56. Paynter RW, Martz H, Guidoin RG. An X-ray photoelectron spectroscopy study of the external surface of explanted microporous polyurethane vascular prostheses. Biomaterials. 1987; 8(2): 94–99.

57. Garrett Q, Chatelier RC, Griesser HJ, et al. Effect of charged groups on the adsorption and penetration of proteins onto and into carboxymethylated poly(HEMA) hydrogels. Biomaterials. 1998; 19(23): 2175–2186.

58. Castillo EJ, Koenig JL, Anderson JM, et al. Surface analysis of biomedical polymers by attenuated total reflectance-Fourier transform infra-red. Biomaterials. 1984; 5(4): 186–93.

59. Holmes-Farley SR, Whitesides GM. Reactivity of carboxylic acid and ester groups in the functionalized interfacial region of "polyethylene carboxylic acid" (PE-C02H) and its derivatives: differentiation of the functional groups into shallow and deep subsets based on a comparison of conta. Langmuir. 1987; 3(1): 62–76.

60. Benninghoven A. Surface investigation of solids by the statistical method of secondary ion mass spectroscopy (SIMS). Surf Sci. 1973; 35: 427–457.

61. Benninghoven A. Chemical analysis of inorganic and organic surfaces and thin films by static time-of-flight secondary ion mass spectrometry (TOF-SIMS). Angew Chem Int Ed Engl. 1994; 33: 1023–1043.

62. Palmberg PW, Rhodin TN. Auger electron spectroscopy of fcc metal surfaces. J Appl Phys. 1968; 39(5): 2425–2432.

63. Seah MP, Dench WA. Quantitative electron spectroscopy of surfaces: a standard data base for electron inelastic mean free paths in solids. Surf Interface Anal. 1979; 1(1): 2–11.

64. Kneipp K, Kneipp H, Itzkan I, et al. Ultrasensitive chemical analysis by Raman spectroscopy. Chem Rev. 1999; 99(10): 2957–76.

65. Yang D, Velamakanni A, Bozoklu G, et al. Chemical analysis of graphene oxide films after heat and chemical treatments by X-ray photoelectron and Micro-Raman spectroscopy. Carbon N Y. 2009; 47(1): 145–152.

66. Richardson RR, Miller JA, Reichert WM. Polyimides as biomaterials: preliminary biocompatibility testing. Biomaterials. 1993; 14(8): 627–35.

67. Han DK, Park KD, Ahn K-D, et al. Preparation and surface characterization of PEO-grafted and heparin-immobilized polyurethanes. J Biomed Mater Res Part A. 1989; 23(S13): 87–104.

68. Lander LM, Siewierski LM, Brittain WJ, et al. A systematic comparison of contact angle methods. Langmuir. 1993; 9(8): 2237–2239.

69. Harnett EM, Alderman J, Wood T. The surface energy of various biomaterials coated with adhesion molecules used in cell culture. Colloid Surface B. 2007; 55(1): 90–97.

70. Lelah MD, Grasel TG, Pierce JA, et al. The measurement of contact angles on circular tubing surfaces using the captive bubble technique. J Biomed Mater Res Part A. 1985; 19(9): 1011–1015.

71. Chen H, Zhang Z, Chen Y, et al. Protein repellant silicone surfaces by covalent immobilization of poly(ethylene oxide). Biomaterials. 2005; 26(15): 2391–9.

72. Ratner BD. Surface characterization of biomaterials by electron spectroscopy for chemical analysis. Ann Biomed Eng. 1983; 11(3-4): 313–36.

73. Oldani M, Schock G. Characterization of ultrafiltration membranes by infrared spectroscopy, ESCA, and contact angle measurements. J Memb Sci. 1989; 43: 243–258.

74. Hansma PK, Tersoff J. Scanning tunneling microscopy. J Appl Phys. 1987; 61(2): R1–R23.

75. Biegelsen DK, Bringans RD, Northrup JE, et al. Surface reconstructions of GaAs(100) observed by scanning tunneling microscopy. Phys Rev B. 1990; 41(9): 5701.

76. Siedlecki CA, Marchant RE. Atomic force microscopy for characterization of the biomaterial interface. Biomaterials. 1998; 19(4): 441–454.

77. Alaerts J, De Cupere VM, Moser S, et al. Surface characterization of poly(methyl methacrylate) microgrooved for contact guidance of mammalian cells. Biomaterials. 2001; 22(12): 1635–42.

78. Ramagli L, Rodriguez L. Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. Electrophoresis. 1985; 6(11): 559–563.

79. O'Farrell P. High resolution two-dimensional electrophoresis of proteins. J Biol Chem. 1975; 250(10): 4007–4021.

80. Lin ST, Mandell RB, Leahy CD, et al. Protein accumulation on disposable extended wear lenses. CLAO J. 1991; 17(1): 44–50.

81. Whittle JD, Bullett N, Short RD, et al. Adsorption of vitronectin, collagen and immunoglobulin-G to plasma polymer surfaces by enzyme linked immunosorbent assay (ELISA). J Mater Chem. 2002; 12(9): 2726–2732.

82. Butler J, McGivern P, Swanson P. Amplification of the enzyme-linked immunosorbent assay (ELISA) in the detection of class-specific antibodies. J Immunol Methods. 1978; 20: 265–383.

83. Stapleton F, Willcox MD, Morris C, et al. Tear changes in contact lens wearers following overnight eye closure. Curr Eye Res. 1998; 17(2): 183–8.

84. Yorde D, Sasse E, Wang T, et al. Competitive enzyme-liked immunoassay with use of soluble enzyme/antibody immune complexes for labeling. I. Measurement of human choriogonadotropin. Clin Chem. 1976; 22(8): 1372–1377.

85. Lowry O, Rosebrough N, Farr A, et al. Protein measurement with the folin phenol reagent. J Biol Chem. 1951; 193(1): 265–275.

86. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72: 248–54.

87. Smith P, Krohn R, Hermanson G, et al. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985; 150(1): 76–85.

88. Glasier MA, Subbaraman LN, Senchyna M, et al. A solid-phase assay for the quantitation of total protein eluted from balafilcon, lotrafilcon, and etafilcon contact lenses. Curr Eye Res. 2008; 33(8): 631–40.

89. Jones L, Haugland R, Singer V. Development and characterization of the NanoOrange protein quantitation assay: A fluorescence-based assay of proteins in solution. Biotechniques. 2003; 34(4): 850–861.

90. Noble JE, Knight AE, Reason AJ, et al. A comparison of protein quantitation assays for biopharmaceutical applications. Mol Biotechnol. 2007; 37(2): 99–111.

91. Stoscheck C. Quantitation of protein. Methods Enzymol. 1990; 182: 50–68.

92. Jones L, Franklin V, Evans K, et al. Spoilation and clinical performance of monthly vs. three monthly Group II disposable contact lenses. Optom Vis Sci. 1996; 73(1): 16–21.

93. Jones L, Evans K, Sariri R, et al. Lipid and protein deposition of N-vinyl pyrrolidone-containing group II and group IV frequent replacement contact lenses. CLAO J. 1997; 23(2): 122–126.

94. Mora MF, Wehmeyer JL, Synowicki R, et al. Investigating protein adsorption via spectroscopic ellipsometry. In: Puleo DA, Bizios R (eds). Biological Interactions on Materials Surfaces. Springer New York; 2009. pp. 19–41.

95. Spaeth K, Brecht A, Gauglitz G. Studies on the biotin-avidin multilayer adsorption by spectroscopic ellipsometry. J Colloid Interface Sci. 1997; 196(2): 128–35.

96. Keuren JF, Wielders SJ, Willems GM, et al. Fibrinogen adsorption, platelet adhesion and thrombin generation at heparinized surfaces exposed to flowing blood. Thromb Haemost. 2002; 87(4): 742–747.

97. Naderi A, Claesson PM. Adsorption properties of polyelectrolyte-surfactant complexes on hydrophobic surfaces studied by QCM-D. 2006; 22(18): 7639–7645.

98. Giamblanco N, Yaseen M, Zhavnerko G, et al. Fibronectin conformation switch induced by coadsorption with human serum albumin. Langmuir. 2011; 27(1): 312–9.

99. Teichroeb JH, Forrest JA, Jones LW, et al. Quartz crystal microbalance study of protein adsorption kinetics on poly(2-hydroxyethyl methacrylate). J Colloid Interface Sci. 2008; 325(1): 157–64.

100. Daly S, Przybycien T, Tilton R. Coverage-dependent orientation of lysozyme adsorbed on silica. Langmuir. 2003; 19: 3848–3857.

101. Lok BK, Cheng Y-L, Robertson CR. Total internal reflection fluorescence: a technique for examining interactions of macromolecules with solid surfaces. J Colloid Interface Sci. 1983; 91(1): 87–103.

102. Hlady V, Andrade J. Fluorescence emission from adsorbed bovine serum albumin and albumin-bound 1-anilinonaphthalene-8-sulfonate studied by TIRF. Colloids and surfaces. 1988; 32: 359–369.

103. Sanders A, Jennissen H. Monitoring fibrinogen adsorption kinetics by interfacial TIRF rheometry. J Mol Recognit. 1996; 9: 503–508.

104. Green RJ, Davies J, Davies MC, et al. Surface plasmon resonance for real time in situ analysis of protein adsorption to polymer surfaces. Biomaterials. 1997; 18(5): 405–13.

105. Heaton RJ, Haris PI, Russell JC, et al. Application of SPR & FTIR spectroscopy to the study of protein-biomaterial interactions. Biochem Soc Trans. 1995; 23(4): 502S.

106. Barker J, Jones M. The potential spread of infection caused by aerosol contamination of surfaces after flushing a domestic toilet. J Appl Microbiol. 2005; 99(2): 339–347.

107. Kooyman R, de Bruijn H, Eenink R, et al. Surface plasmon resonance as a bioanalytical tool. J Mol Struct. 1990; 218: 345–350.

108. Holland NB, Marchant RE. Individual plasma proteins detected on rough biomaterials by phase imaging AFM. J Biomed Mater Res Part A. 2000; 51(3): 307–15.

109. Teichroeb JH, Forrest JA, Ngai V, et al. Imaging protein deposits on contact lens materials. Optom Vis Sci. 2008; 85(12): 1151–64.

110. Rebeix V, Sommer F, Marchin B, et al. Artificial tear adsorption on soft contact lenses: methods to test surfactant efficacy. Biomaterials. 2000; 21(12): 1197–1205.

111. Zhang S, Borazjani RN, Salamone JC, et al. In vitro deposition of lysozyme on etafilcon A and balafilcon A hydrogel contact lenses: Effects on adhesion and survival of Pseudomonas aeruginosa and Staphylococcus aureus. Contact Lens Anterior Eye. 2005; 28(3): 113–19.

112. Bullett N, Whittle JD, Short RD, et al. Adsorption of immunoglobulin G to plasma-co-polymer surfaces of acrylic acid and 1,7-octadiene. J Mater Chem. 2003; 13(7): 1546–1553.

113. Cantarero L, Butler JE, Osborne JW. The adsorptive characteristics of proteins for polystyrene and their significance in solid-phase imunoassays. Anal Biochem. 1980; 105(2): 375–82.

114. Luensmann D, Glasier M-A, Zhang F, et al. Confocal microscopy and albumin penetration into contact lenses. Optom Vis Sci. 2007; 84(9): 839–47.

115. Luensmann D, Zhang F, Subbaraman L, et al. Localization of lysozyme sorption to conventional and silicone hydrogel contact lenses using confocal microscopy. Curr Eye Res. 2009; 34(8): 683–97.

116. Meadows DL, Paugh JR. Use of confocal microscopy to determine matrix and surface protein deposition profiles in hydrogel contact lenses. CLAO J. 1994; 20(4): 237–241.

117. Reichert U, Linden T, Belfort G, et al. Visualising protein adsorption to ion-exchange membranes by confocal microscopy. J Memb Sci. 2002; 199: 161–166.

118. Luensmann D, Heynen M, Liu L, et al. Determination of albumin sorption to intraocular lenses by radiolabeling and confocal laser scanning microscopy. J Cataract Refract Surg. 2009; 35(11): 2000–7.

119. McArthur S. Applications of XPS in bioengineering. Surf interface Anal. 2006; 38: 1380–1385.

120. Wagner MS, McArthur SL, Shen M, et al. Limits of detection for time of flight secondary ion mass spectrometry (ToF-SIMS) and X-ray photoelectron spectroscopy (XPS): detection of low amounts of adsorbed protein. J Biomater Sci Polym Ed. 2002; 13(4): 407–28.

121. McArthur S, McLean K, Kingshott P, et al. Effect of polysaccharide structure on protein adsorption. Colloid Surface B. 2000; 17: 37–48.

122. Canavan HE, Graham DJ, Cheng X, et al. Comparison of native extracellular matrix with adsorbed protein films using secondary ion mass spectrometry. Langmuir. 2007; 23(1): 50–6.

123. Eynde X, Bertrand P. ToF-SIMS quantification of polystyrene spectra based on principal component analysis (PCA)†. Surf Interface Anal. 1997; 25(11): 878–888.

124. Wagner MS, Graham DJ, Ratner BD, et al. Maximizing information obtained from secondary ion mass spectra of organic thin films using multivariate analysis. Surf Sci. 2004; 570(1-2): 78–97.

125. McArthur SL, McLean KM, St John HA, et al. XPS and surface-MALDI-MS characterisation of worn HEMA-based contact lenses. Biomaterials. 2001; 22(24): 3295–304.

126. Oleschuk RD, Mccomb ME, Chow A, et al. Characterization of plasma proteins adsorbed onto biomaterials by MALDI-TOFMS. Biomaterials. 2000; 21(16): 1701–10.

127. Kingshott P, St John HA, Griesser HJ. Direct detection of proteins adsorbed on synthetic materials by matrix-assisted laser desorption ionization-mass spectrometry. Anal Biochem. 1999; 273(2): 156–62.

128. Prager MD, Quintana RP. Radiochemical studies on contact lens soilation. I. Lens uptake of 14C-lysozyme from simple and complex artificial tear solutions. J Biomed Mater Res. 1997; 36(1): 119–24.

129. Kim D, Blanch H, Radke C. Direct imaging of lysozyme adsorption onto mica by atomic force microscopy. Langmuir. 2002; 18(15): 5841–5850.

130. Ceska M, Sjodin A, Grossmuller F. Some quantitative aspects of the labelling of proteins with 125I by the iodine monochloride method. Biochem J. 1971; 121(1): 139–143.

131. Jadi S, Heynen M, Luensmann D, et al. Composition of incubation solution impacts in vitro protein uptake to silicone hydrogel contact lenses. Mol Vis. 2012; 18: 337–47.

132. Bohnert JL, Horbett TA, Ratner BD, et al. Adsorption of proteins from artificial tear solutions to contact lens materials. Invest Ophthalmol Vis Sci. 1988; 29(3): 362–73.

133. Chow LM, Subbaraman LN, Sheardown H, et al. Kinetics of in vitro lactoferrin deposition on silicone hydrogel and FDA group II and group IV hydrogel contact lens materials. J Biomater Sci Polym Ed. 2009; 20(1): 71–82.

134. Wu Y, Simonovsky FI, Ratner BD, et al. The role of adsorbed fibrinogen in platelet adhesion to polyurethane surfaces: a comparison of surface hydrophobicity, protein adsorption, monoclonal antibody binding, and platelet adhesion. J Biomed Mater Res Part A. 2005; 74(4): 722–38.

135. Van Beek M, Jones L, Sheardown H, et al. Hyaluronic acid containing hydrogels for the reduction of protein adsorption. Biomaterials. 2008; 29(7): 780–789.

136. Tidwell CD, Castner DG, Golledge SL, et al. Static time-of-flight secondary ion mass spectrometry and x-ray photoelectron spectroscopy characterization of adsorbed albumin and fibronectin films. Surf Interface Anal. 2001; 31(8): 724–733.

137. Subbaraman LN, Glasier M-A, Varikooty J, et al. Protein deposition and clinical symptoms in daily wear of etafilcon lenses. Optom Vis Sci. 2012; 89(10): 1450–9.

138. Janatova J. Activation and control of complement, inflammation, and infection associated with the use of biomedical polymers. ASAIO J. 2000; 46(6): S53–S62.

139. Elhers WH, Donshik PC. Giant papillary conjunctivitis. Curr Opin Allergy Clin Immunol. 2008; 8(5): 445–9.

140. Allansmith M, Korb D, Greiner J, et al. Giant papillary conjunctivitis in contact lens wearers. Am Acad Ophthalmol. 1977; 83(5): 697–708.

141. Hart DE, Schkolnick JA, Bernstein S, et al. Contact lens induced giant papillary conjunctivitis: a retrospective study. J Am Optom Assoc. 1989; 60(3): 195–204.

142. Willcox MD, Harmis N, Cowell BA, et al. Bacterial interactions with contact lenses; effects of lens material, lens wear and microbial physiology. Biomaterials. 2001; 22(24): 3235–47.

143. Bruinsma GM, van der Mei HC, Busscher HJ. Bacterial adhesion to surface hydrophilic and hydrophobic contact lenses. Biomaterials. 2001; 22(24): 3217–24.

144. Fabris L, Antonello S, Armelao L, et al. Gold nanoclusters protected by conformationally constrained peptides. J Am Chem Soc. 2006; 128(1): 326–36.

145. Slocik JM, Naik RR. Probing peptide-nanomaterial interactions. Chem Soc Rev. 2010; 39(9): 3454–63.

146. Lundqvist M, Sethson I, Jonsson B-H. High-resolution 2D 1H-15N NMR characterization of persistent structural alterations of proteins induced by interactions with silica nanoparticles. Langmuir. 2005; 21(13): 5974–9.

147. Bower P, Louie EA, Long JR, et al. Solid-state NMR structural studies of peptides immobilized on gold nanoparticles. Langmuir. 2005; 21(7): 3002–7.

148. Shaw W, Long J, Campbell A, et al. A solid-state NMR study of dynamics in a hydrated salivary peptide adsorbed to hydroxyapatite. J Am Chem Soc. 2000; 15(12): 7118–7119.

149. Ranjbar B, Gill P. Circular dichroism techniques: biomolecular and nanostructural analyses- a review. Chem Biol Drug Des. 2009; 74(2): 101–20.

150. Mannucci LL, Moro F, Cosani A, et al. Conformational state of lacrimal proteins adsorbed on contact lenses. Curr Eye Res. 1985; 4(6): 734–6.

151. Tanaka M, Motomura T, Kawada M, et al. Blood compatible aspects of poly(2-methoxyethylacrylate) (PMEA)--relationship between protein adsorption and platelet adhesion on PMEA surface. Biomaterials. 2000; 21(14): 1471–81.

152. Maruyama T, Katoh S, Nakajima M, et al. FT-IR analysis of BSA fouled on ultrafiltration and microfiltration membranes. J Memb Sci. 2001; 192: 201–207.

153. Fu K, Griebenow K, Hsieh L, et al. FTIR characterization of the secondary structure of proteins encapsulated within PLGA microspheres. J Control Release. 1999; 58(3): 357–366.

154. Smith JR, Cicerone MT, Meuse CW. Tertiary structure changes in albumin upon surface adsorption observed via Fourier transform infrared spectroscopy. Langmuir. 2009; 25(8): 4571–4578.

155. Hédoux A, Guinet Y, Paccou L, et al. Analysis of the mechanism of lysozyme pressure denaturation from Raman spectroscopy investigations, and comparison with thermal denaturation. J Phys Chem B. 2011; 115(20): 6740–6748.

156. Hédoux A, Ionov R, Willart J-F, et al. Evidence of a two-stage thermal denaturation process in lysozyme: A Raman scattering and differential scanning calorimetry investigation. J Chem Phys. 2006; 124(1): 014703.

157. Filik J, Stone N. Analysis of human tear fluid by Raman spectroscopy. Anal Chim Acta. 2008; 616(2): 177–184.

158. Lord R, Yu N. Laser-excited Raman spectroscopy of biomolecules I. Native lysozyme and its constituent amino acids. J Mol Biol. 1970; 50: 509–524.

159. Helal R, Melzig MF. Determination of lysozyme activity by a fluorescence technique in comparison with the classical turbidity assay. Pharmazie. 2008; 63: 415–419.

160. Senchyna M, Jones L, Louie D, et al. Quantitative and conformational characterization of lysozyme deposited on balafilcon and etafilcon contact lens materials. Curr Eye Res. 2004; 28(1): 25–36.

161. Sack RA, Jones B, Antignani A, et al. 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(5): 842–849.

162. Morsky P. Turbidimetric determination of lysozyme with Micrococcus lysodeikticus cells: reexamination of reaction conditions. Anal Biochem. 1983; 128(1): 77–85.

163. Wu Y, Daeschel MA. Lytic antimicrobial activity of hen egg white lysozyme immobilized to polystyrene beads. J Food Sci. 2007; 72(9): M369–74.

164. Suwala M, Glasier MA, Subbaraman LN, et al. Quantity and conformation of lysozyme deposited on conventional and silicone hydrogel contact lens materials using an in vitro model. Eye Contact Lens. 2007; 33(3): 138–43.

165. Hall B, Jones L, Forrest JA. Measuring the kinetics and activity of adsorbed proteins: In vitro lysozyme deposited onto hydrogel contact lenses over short time periods. J Biomed Mater Res Part A. 2013; 101A(3): 755–764.

166. Subbaraman LN, Jones L. Kinetics of lysozyme activity recovered from conventional and silicone hydrogel contact lens materials. J Biomater Sci Polym Ed. 2010; 21(3): 343–58.

167. Nichols JJ. Contact Lenses 2013. Contact Lens Spectrum. 2014; 29(1): 22–8.

168. Nicolson PC, Vogt J. Soft contact lens polymers: an evolution. Biomaterials. 2001; 22(24): 3273–83.

169. Chiefari J, Chong YK, Ercole F, et al. Living free-radical polymerization by reversible addition−fragmentation chain transfer: the RAFT process. Macromolecules. 1998; 31(16): 5559–5562.

170. Ng CO, Tighe BJ. Polymers in contact lens applications VI. The “dissolved” oxygen permeability of hydrogels and the design of materials for use in continuous-wear lenses. Br Polym J. 1976; 8(4): 118–123.

171. Yang W-H. Oxygen permeability coefficients of polymers for hard and soft contact lens applications. J Memb Sci. 1981; 9(1-2): 53 – 67.

172. Wichterle O, Lim D. Hydrophilic gels for biological use. Nature. 1960; 185: 117–8.

173. Wichterle O, Lim D. Method for producing shaped articles from three-dimensional hydrophilic polymers. 1961: US patent 2976576.

174. Hutter JC, Green JA, Eydelman MB. Proposed silicone hydrogel contact lens grouping system for lens care product compatibility testing. Eye Contact Lens. 2012; 38(6): 358–62.

175. Weikart C, Miyama M, Yasuda H. Surface modification of conventional polymers by depositing plasma polymers of trimethylsilane and of trimethylsilane + O2. J Colloid Interface Sci. 1999; 211(1): 28–38.

176. Steffen R, Schnider C. A next generation silicone hydrogel lens for daily wear. Part 1 - Material properties. Optician. 2004; 227: 23–5.

177. González-Méijome JM, López-Alemany A, Almeida JB, et al. Microscopic observation of unworn siloxane-hydrogel soft contact lenses by atomic force microscopy. J Biomed Mater Res B Appl Biomater. 2006; 76(2): 412–8.

178. Szczotka-Flynn L. Looking at silicone hydrogels across generations. Optom Manag. 2008; 43: 68–71.

179. Jones L. Modern contact lens materials: A clinical performance update. Contact Lens Spectrum. 2002; 17(9): 24–35.

180. Holden BA, Mertz GW. Critical oxygen levels to avoid corneal edema for daily and extended wear contact lenses. Invest Ophthalmol Vis Sci. 1984; 25(10): 1161–7.

181. Harvitt DM, Bonanno JA. Re-evaluation of the oxygen diffusion model for predicting minimum contact lens Dk/t values needed to avoid corneal anoxia. Optom Vis Sci. 1999; 76(10): 712–9.

182. Tighe B. Silicone hydrogel materials: how do they work? In: Sweeny D (ed). Silicone Hydrogels: The Rebirth of Continuous Wear Contact Lenses. Oxford, England: Butterworth-Heinemann; 2000. pp. 1–21.

183. Skotnitsky C, Sankaridurg PR, Sweeney DF, et al. General and local contact lens induced papillary conjunctivitis (CLPC). Clin Exp Optom. 2002; 85(3): 193–197.

184. Skotnitsky CC, Naduvilath TJ, Sweeney DF, et al. Two presentations of contact lens-induced papillary conjunctivitis (CLPC) in hydrogel lens wear: local and general. Optom Vis Sci. 2006; 83(1): 27–36.

185. Hall BJ, Jones LW, Dixon B. Silicone allergies and the eye: fact or fiction? Eye Contact Lens. 2014; 40(1): 51–7.

186. Maldonado-Codina C, Efron N. Dynamic wettability of pHEMA-based hydrogel contact lenses. Ophthalmic Physiol Opt. 2006; 26(4): 408–18.

187. Santos L, Rodrigues D, Lira M, et al. The influence of surface treatment on hydrophobicity, protein adsorption and microbial colonisation of silicone hydrogel contact lenses. Contact Lens Anterior Eye. 2007; 30(3): 183–188.

188. Cheng L, Muller SJ, Radke CJ. Wettability of silicone-hydrogel contact lenses in the presence of tear-film components. Curr Eye Res. 2004; 28(2): 93–108.

189. Lorentz H, Rogers R, Jones L. The impact of lipid on contact angle wettability. Optom Vis Sci. 2007; 84(10): 946–953.

190. Morgan PB, Efron N. Comparative clinical performance of two silicone hydrogel contact lenses for continuous wear. Clin Exp Optom. 2002; 85(3): 183–92.

191. Keir N, Boone A, Woods CA, et al. In vivo and ex vivo wettability and the association with contact lens comfort. Cont Lens Anterior Eye. 2008; 31(6): 292.

192. Keir N, Jones L. Wettability and silicone hydrogel lenses: a review. Eye Contact Lens. 2013; 39(1): 100–8.

193. Cheung S-W, Cho P, Chan B, et al. A comparative study of biweekly disposable contact lenses: silicone hydrogel versus hydrogel. Clin Exp Optom. 2007; 90(2): 124–31.

194. Dumbleton KA, Woods CA, Jones LW, et al. Comfort and adaptation to silicone hydrogel lenses for daily wear. Eye Contact Lens. 2008; 34(4): 215–223.

195. Haig-Brown G. A clinical study of high water content contact lenses in the daily wear regime. J Br Contact Lens Assoc. 1985; 8: 12–16.

196. Jones L, Woods C, Efron N. Life expectancy of rigid gas permeable high water content contact lenses. Eye Contact Lens. 1996; 22(4): 258–261.

197. Morgan PB, Woods CA, Tranoudis IG, et al. International contact lens prescribing in 2013. Contact Lens Spectrum. 2014; 29(1): 30–35.

198. Morgan PB, Efron N. In vivo dehydration of silicone hydrogel contact lenses. Eye Contact Lens. 2003; 29(3): 173–6.

199. Myers RI, Larsen DW, Tsao M, et al. Quantity of protein deposited on hydrogel contact lenses and its relation to visible protein deposits. Optom Vis Sci. 1991; 68(10): 776–782.

200. Garrett Q, Griesser HJ, Milthorpe BK, et al. Irreversible adsorption of human serum albumin to hydrogel contact lenses: a study using electron spin resonance spectroscopy. Biomaterials. 1999; 20(14): 1345–56.

201. Bontempo A, Rapp J. Protein and lipid deposition onto hydrophilic contact lenses in vivo. Eye Contact Lens. 2001; 27(2): 75–80.

202. Jones L, Senchyna M, Glasier M-A, et al. Lysozyme and lipid deposition on silicone hydrogel contact lens materials. Eye Contact Lens. 2003; 29(1): S75–9; discussion S83–4, S192–4.

203. Lorentz H, Heynen M, Kay LM, et al. Contact lens physical properties and lipid deposition in a novel characterized artificial tear solution. Mol Vis. 2011; 17: 3392–405.

204. Jones L, Mann A, Evans K, et al. 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(10): 503–510.

205. Klein AE. Detection of mucin deposits on hydrogel contact lenses: evaluation of staining procedures and clinical significance. Optom Vis Sci. 1989; 66(1): 56–60.

206. Wedler FC, Illman BL, Horensky DS, et al. Analysis of protein and mucin components deposited on hydrophilic contact lenses. Clin Exp Optom. 1987; 70(2): 59–68.

207. Pritchard N, Fonn D, Weed K. Ocular and subjective responses to frequent replacement of daily wear soft contact lenses. CLAO J. 1996; 22(1): 53–59.

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

209. Williams TJ, Schneider RP, Willcox MDP. The effect of protein-coated contact lenses on the adhesion and viability of gram negative bacteria. Curr Eye Res. 2003; 27(4): 227–35.

210. Hui A, Boone A, Jones L. Uptake and release of ciprofloxacin-HCl from conventional and silicone hydrogel contact lens materials. Eye Contact Lens. 2008; 34(5): 266–71.

211. O'Donnell C, Efron N. Non-compliance with lens care and maintenance in diabetic contact lens wearers. Ophthalmic Physiol Opt. 2004; 24(6): 504–510.

212. Yung AMS, Boost M, Cho P, et al. The effect of a compliance enhancement strategy (self-review) on the level of lens care compliance and contamination of contact lenses and lens care accessories. Clin Exp Optom. 2007; 90(3): 190–202.

213. Claydon BE, Efron N. Non-compliance in contact lens wear. Ophthalmic Physiol Opt. 1994; 14(4): 356–364.

214. Collins MJ, Carney LG. Patient compliance and its influence on contact lens wearing problems. Am J Optom Physiol Opt. 1986; 63(12): 952–956.

215. Dumbleton K, Woods C, Jones L, et al. Patient and practitioner compliance with silicone hydrogel and daily disposable lens replacement in the United States. Eye Contact Lens. 2009; 35(4): 164–171.

216. Dumbleton K, Richter D, Woods C, et al. Compliance with contact lens replacement in Canada and the United States. Optom Vis Sci. 2010; 87(2): 131–139.

217. Donshik PC, Ehlers WH, Ballow M. Giant papillary conjunctivitis. Immunol Allergy Clin North Am. 2008; 28(1): 83–103.

218. Skotnitsky C, Jalbert I, O'Hare N, et al. Case reports of three atypical infiltrative keratitis events with high DK soft contact lens wear. Cornea. 2002; 21(3): 318–24.

219. Stapleton F, Stretton S, Sankaridurg PR, et al. Hypersensitivity responses and contact lens wear. Cont Lens Anterior Eye. 2003; 26(2): 57–69.

220. Butrus S, Portela R. Ocular allergy: diagnosis and treatment. Ophthalmol Clin North Am. 2005; 18(4): 485–92.

221. Okada H, Kuhn C, Feillet H, et al. The "hygiene hypothesis" for autoimmune and allergic diseases: an update. Clin Exp Immunol. 2010; 160(1): 1–9.

222. Fishbein AB, Fuleihan RL. The hygiene hypothesis revisited: does exposure to infectious agents protect us from allergy? Curr Opin Pediatr. 2012; 24(1): 98–102.

223. Strachan D. Hay fever, hygiene, and household size. BMJ. 1989; 299(6710): 1259–60.

224. Ng A, Heynen M, Luensmann D, et al. Impact of tear film components on lysozyme deposition to contact lenses. Optom Vis Sci. 2012; 89(4): 392–400.

225. Keith DJ, Christensen MT, Barry JR, et al. Determination of the lysozyme deposit curve in soft contact lenses. Eye Contact Lens. 2003; 29(2): 79–82.

226. Green-Church KB, Nichols JJ. Mass spectrometry-based proteomic analyses of contact lens deposition. Mol Vis. 2008; 14: 291–297.

227. Zhao Z, Wei X, Aliwarga Y, et al. Proteomic analysis of protein deposits on worn daily wear silicone hydrogel contact lenses. Mol Vis. 2008; 14: 2016–2024.

228. Subbaraman LN, Glasier MA, Senchyna M, et al. Extraction efficiency of an extraction buffer used to quantify lysozyme deposition on conventional and silicone hydrogel contact lens materials. Eye Contact Lens. 2007; 33(4): 169–73.

229. 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(5): 503–10.

230. Wedler FC. Analysis of biomaterials deposited on soft contact lenses. J Biomed Mater Res. 1977; 11(4): 525–35.

231. Yan G, Nyquist G, Caldwell KD, et al. Quantitation of total protein deposits on contact lenses by means of amino acid analysis. Invest Ophthalmol Vis Sci. 1993; 34(5): 1804–13.

232. Weathersby P, Horbett T, Hoffman A. A new method for analysis of the adsorbed plasma protein layer on biomaterial surfaces. ASAIO J. 1976; 22(1): 242–251.

11.2 Chapter 2 References

1. Fleming A. On a remarkable bacteriolytic element found in tissues and secretions. Proceedings of the Royal Society of London Series B, Containing Papers of a Biological Character. 1922; 93(653): 306–317.

2. Ibrahim HR, Matsuzaki T, Aoki T. Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. FEBS Lett. 2001; 506(1): 27–32.

3. Morsky P. Turbidimetric determination of lysozyme with Micrococcus lysodeikticus cells: reexamination of reaction conditions. Anal Biochem. 1983; 128(1): 77–85.

4. Ng A, Heynen M, Luensmann D, et al. Optimization of a fluorescence-based lysozyme activity assay for contact lens studies. Curr Eye Res. 2013; 38(2): 252–9.

5. Zhang S, Borazjani RN, Salamone JC, et al. In vitro deposition of lysozyme on etafilcon A and balafilcon A hydrogel contact lenses: Effects on adhesion and survival of Pseudomonas aeruginosa and Staphylococcus aureus. Contact Lens Anterior Eye. 2005; 28(3): 113–19.

6. Helal R, Melzig MF. Determination of lysozyme activity by a fluorescence technique in comparison with the classical turbidity assay. Pharmazie. 2008; 63: 415–419.

7. Subbaraman LN, Jones L. Kinetics of lysozyme activity recovered from conventional and silicone hydrogel contact lens materials. J Biomater Sci Polym Ed. 2010; 21(3): 343–58.

8. Sack RA, Jones B, Antignani A, et al. 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(5): 842–849.

9. Ceska M, Sjodin A, Grossmuller F. Some quantitative aspects of the labelling of proteins with 125I by the iodine monochloride method. Biochem J. 1971; 121(1): 139–143.

10. 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.

11. Krohn K, Knight L, Harwig J, et al. Differences in the sites of iodination of proteins following four methods of radioiodination. Biochim Biophys Acta. 1977; 490: 497–505.

12. Krohn K, Sherman L, Welch M. Studies of radioiodinated fibrinogen I. Physicochemical properties of the ICl, chloramine-T, and electrolytic reaction products. Biochim Biophys Acta. 1972; 285(1972): 404–413.

11.3 Chapter 8 References

1. Hall B, Jones L, Forrest JA. Measuring the kinetics and activity of adsorbed proteins: In vitro lysozyme deposited onto hydrogel contact lenses over short time periods. J Biomed Mater Res Part A. 2013; 101A(3): 755–764.

2. Norde W. Adsorption of proteins from solution at the solid-liquid interface. Adv Colloid Interface Sci. 1986; 25(4): 267–340.

3. Janatova J. Activation and control of complement, inflammation, and infection associated with the use of biomedical polymers. ASAIO J. 2000; 46(6): S53–S62.

4. Willcox MD, Harmis N, Cowell BA, et al. Bacterial interactions with contact lenses; effects of lens material, lens wear and microbial physiology. Biomaterials. 2001; 22(24): 3235–47.

5. Bruinsma GM, van der Mei HC, Busscher HJ. Bacterial adhesion to surface hydrophilic and hydrophobic contact lenses. Biomaterials. 2001; 22(24): 3217–24.

6. Nichols JJ. Contact Lenses 2013. Contact Lens Spectrum. 2014; 29(1): 22–8.

7. Meadows DL, Paugh JR. Use of confocal microscopy to determine matrix and surface protein deposition profiles in hydrogel contact lenses. CLAO J. 1994; 20(4): 237–241.

8. Luensmann D, Glasier M-A, Zhang F, et al. Confocal microscopy and albumin penetration into contact lenses. Optom Vis Sci. 2007; 84(9): 839–47.

9. Teichroeb JH, Forrest JA, Jones LW, et al. Quartz crystal microbalance study of protein adsorption kinetics on poly(2-hydroxyethyl methacrylate). J Colloid Interface Sci. 2008; 325(1): 157–64.

10. Ramagli L, Rodriguez L. Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. Electrophoresis. 1985; 6(11): 559–563.

11. O'Farrell P. High resolution two-dimensional electrophoresis of proteins. J Biol Chem. 1975; 250(10): 4007–4021.

12. Lin ST, Mandell RB, Leahy CD, et al. Protein accumulation on disposable extended wear lenses. CLAO J. 1991; 17(1): 44–50.

13. Whittle JD, Bullett N, Short RD, et al. Adsorption of vitronectin, collagen and immunoglobulin-G to plasma polymer surfaces by enzyme linked immunosorbent assay (ELISA). J Mater Chem. 2002; 12(9): 2726–2732.

14. Butler J, McGivern P, Swanson P. Amplification of the enzyme-linked immunosorbent assay (ELISA) in the detection of class-specific antibodies. J Immunol Methods. 1978; 20: 265–383.

15. Stapleton F, Willcox MD, Morris C, et al. Tear changes in contact lens wearers following overnight eye closure. Curr Eye Res. 1998; 17(2): 183–8.

16. Yorde D, Sasse E, Wang T, et al. Competitive enzyme-liked immunoassay with use of soluble enzyme/antibody immune complexes for labeling. I. Measurement of human choriogonadotropin. Clin Chem. 1976; 22(8): 1372–1377.

17. Lowry O, Rosebrough N, Farr A, et al. Protein measurement with the folin phenol reagent. J Biol Chem. 1951; 193(1): 265–275.

18. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72: 248–54.

19. Smith P, Krohn R, Hermanson G, et al. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985; 150(1): 76–85.

20. Glasier MA, Subbaraman LN, Senchyna M, et al. A solid-phase assay for the quantitation of total protein eluted from balafilcon, lotrafilcon, and etafilcon contact lenses. Curr Eye Res. 2008; 33(8): 631–40.

21. Jones L, Haugland R, Singer V. Development and characterization of the NanoOrange protein quantitation assay: A fluorescence-based assay of proteins in solution. Biotechniques. 2003; 34(4): 850–861.

22. Noble JE, Knight AE, Reason AJ, et al. A comparison of protein quantitation assays for biopharmaceutical applications. Mol Biotechnol. 2007; 37(2): 99–111.

23. Stoscheck C. Quantitation of protein. Methods Enzymol. 1990; 182: 50–68.

24. Jones L, Franklin V, Evans K, et al. Spoilation and clinical performance of monthly vs. three monthly Group II disposable contact lenses. Optom Vis Sci. 1996; 73(1): 16–21.

25. Jones L, Evans K, Sariri R, et al. Lipid and protein deposition of N-vinyl pyrrolidone-containing group II and group IV frequent replacement contact lenses. CLAO J. 1997; 23(2): 122–126.

26. Bontempo AR, Rapp J. Protein and lipid deposition onto hydrophilic contact lenses in vivo. CLAO J. 2001; 27(2): 75–80.

27. Glasier MA, Keech A, Sheardown H, et al. Conformational and quantitative characterization of lysozyme extracted from galyfilcon and senofilcon silicone hydrogel contact lenses. Curr Eye Res. 2008; 33(1): 1–11.

28. Yagami T, Sato M, Nakamura A, et al. One of the rubber latex allergens is a lysozyme. J Allergy Clin Immunol. 1995; 96(5 Pt 1): 677–86.

29. Subbaraman LN, Glasier MA, Senchyna M, et al. Extraction efficiency of an extraction buffer used to quantify lysozyme deposition on conventional and silicone hydrogel contact lens materials. Eye Contact Lens. 2007; 33(4): 169–73.

30. Hall B, Phan C-M, Subbaraman L, et al. Extraction versus in situ techniques for measuring surface-adsorbed lysozyme. Optom Vis Sci. 2014; 91(9): 1062–1070.

31. Vogler E. Protein adsorption in three dimensions. Biomaterials. 2012; 33(5): 1201–1237.

32. Mora MF, Wehmeyer JL, Synowicki R, et al. Investigating protein adsorption via spectroscopic ellipsometry. In: Puleo DA, Bizios R (eds). Biological Interactions on Materials Surfaces. Springer New York; 2009. pp. 19–41.

33. Spaeth K, Brecht A, Gauglitz G. Studies on the biotin-avidin multilayer adsorption by spectroscopic ellipsometry. J Colloid Interface Sci. 1997; 196(2): 128–35.

34. McClellan SJ, Franses EI. Adsorption of bovine serum albumin at solid/aqueous interfaces. Colloids Surfaces A Physicochem Eng Asp. 2005; 260(1): 265–275.

35. Keuren JF, Wielders SJ, Willems GM, et al. Fibrinogen adsorption, platelet adhesion and thrombin generation at heparinized surfaces exposed to flowing blood. Thromb Haemost. 2002; 87(4): 742–747.

36. Drude P. Ueber Oberflächenschichten. II Teil. Ann Phys Chem. 1889; 36: 865–897.

37. Rabe J, Novotny V, Swalen J, et al. Structure and dynamics in a Langmuir-Blodgett film at elevated temperatures. Thin Solid Films. 1988; 159: 359–367.

38. Merrett K, Cornelius RM, Mcclung WG, et al. Surface analysis methods for characterizing polymeric biomaterials. J Biomater Sci Polym Ed. 2002; 13(6): 593–621.

39. Naderi A, Claesson PM. Adsorption properties of polyelectrolyte-surfactant complexes on hydrophobic surfaces studied by QCM-D. 2006; 22(18): 7639–7645.

40. Giamblanco N, Yaseen M, Zhavnerko G, et al. Fibronectin conformation switch induced by coadsorption with human serum albumin. Langmuir. 2011; 27(1): 312–9.

41. Sauerbrey G. Verwendung von Schwingquarzen zur Wägung dünner Schichten und zur Mikrowägung. Zeitschrift für Phys. 1959; 155: 206–222.

42. Nomura T, Hattori O. Determination of micromolar concentrations of cyanide in solution with a piezoelectric detector. Anal Chim Acta. 1980; 115: 323–326.

43. Marx KA. Quartz crystal microbalance: a useful tool for studying thin polymer films and complex biomolecular systems at the solution-surface interface. Biomacromolecules. 2003; 4(5): 1099–120.

44. Höök F, Rodahl M, Kasemo B, et al. Structural changes in hemoglobin during adsorption to solid surfaces: effects of pH, ionic strength, and ligand binding. Proc Natl Acad Sci U S A. 1998; 95(21): 12271–6.

45. Messina GML, Satriano C, Marletta G. A multitechnique study of preferential protein adsorption on hydrophobic and hydrophilic plasma-modified polymer surfaces. Colloid Surface B. 2009; 70(1): 76–83.

46. Matsusaki M, Omichi M, Maruyama I, et al. Physical adsorption of human thrombomodulin (ART-123) onto polymeric biomaterials for developing an antithrombogenic blood-contacting material. J Biomed Mater Res Part A. 2008; 84A(1): 1–9.

47. Lord MS, Stenzel MH, Simmons A, et al. The effect of charged groups on protein interactions with poly(HEMA) hydrogels. Biomaterials. 2006; 27(4): 567–75.

48. Deng XM, Castillo EJ, Anderson JM. Surface modification of soft contact lenses: Silanization, wettability and lysozyme adsorption studies. Biomaterials. 1986; 7(4): pp. 247–251.

49. Popelka S, Machová L, Rypácek F. Adsorption of poly(ethylene oxide)-block-polylactide copolymers on polylactide as studied by ATR-FTIR spectroscopy. J Colloid Interface Sci. 2007; 308(2): 291–299.

50. Chittur K. FTIR/ATR for protein adsorption to biomaterial surfaces. Biomaterials. 1998; 19: 357–369.

51. Chan R, Chen V. Characterization of protein fouling on membranes: opportunities and challenges. J Memb Sci. 2004; 242(1): 169–188.

52. Oldani M, Schock G. Characterization of ultrafiltration membranes by infrared spectroscopy, ESCA, and contact angle measurements. J Memb Sci. 1989; 43: 243–258.

53. Miyazawa T. The characteristic band of secondary amides at 3100 cm^{-1}. J Mol Spectrosc. 1960; 4: 168–172.

54. Fink D, Hutson T, Chittur K, et al. Quantitative surface studies of protein adsorption by infrared spectroscopy. Anal Biochem. 1987; 165: 147–154.

55. Castillo EJ, Koenig JL, Anderson JM. Characterization of protein adsorption on soft contact lenses. 1984; 5(6): 319–325.

56. Pitt WG, Cooper SL. FTIR-ATR studies of the effect of shear rate upon albumin adsorption onto polyurethaneurea. Biomaterials. 1986; 7(5): 340–7.

57. Dong A, Huang P, Caughey WS. Protein secondary structures in water from second-derivative amide I infrared spectra. Biochemistry. 1990; 29(13): 3303–8.

58. Daly S, Przybycien T, Tilton R. Coverage-dependent orientation of lysozyme adsorbed on silica. Langmuir. 2003; 19: 3848–3857.

59. Lok BK, Cheng Y-L, Robertson CR. Protein adsorption on crosslinked polydimethylsiloxane using total internal reflection fluorescence. J Colloid Interface Sci. 1983; 91(1): 104–116.

60. Lok BK, Cheng Y-L, Robertson CR. Total internal reflection fluorescence: a technique for examining interactions of macromolecules with solid surfaces. J Colloid Interface Sci. 1983; 91(1): 87–103.

61. Hlady V, Andrade J. Fluorescence emission from adsorbed bovine serum albumin and albumin-bound 1-anilinonaphthalene-8-sulfonate studied by TIRF. Colloids and surfaces. 1988; 32: 359–369.

62. Sanders A, Jennissen H. Monitoring fibrinogen adsorption kinetics by interfacial TIRF rheometry. J Mol Recognit. 1996; 9: 503–508.

63. Luensmann D, Jones L. Impact of fluorescent probes on albumin sorption profiles to ophthalmic biomaterials. J Biomed Mater Res Part B Appl Biomater. 2010; 94(2): 327–36.

64. Guan A, Li Z, Phillips KS. The effect of fluorescent labels on protein sorption in polymer hydrogels. J Fluoresc. 2014: Epub ahead of print.

65. Green RJ, Davies J, Davies MC, et al. Surface plasmon resonance for real time in situ analysis of protein adsorption to polymer surfaces. Biomaterials. 1997; 18(5): 405–13.

66. Heaton RJ, Haris PI, Russell JC, et al. Application of SPR & FTIR spectroscopy to the study of protein-biomaterial interactions. Biochem Soc Trans. 1995; 23(4): 502S.

67. Barker J, Jones M. The potential spread of infection caused by aerosol contamination of surfaces after flushing a domestic toilet. J Appl Microbiol. 2005; 99(2): 339–347.

68. Kooyman R, de Bruijn H, Eenink R, et al. Surface plasmon resonance as a bioanalytical tool. J Mol Struct. 1990; 218: 345–350.

69. Green RJ, Frazier RA, Shakesheff KM, et al. Surface plasmon resonance analysis of dynamic biological interactions with biomaterials. Biomaterials. 2000; 21(18): 1823–35.

70. Silin V, Plant A. Biotechnological applications of surface plasmon resonance. Trends Biotechnol. 1997; 15: 353–359.

71. Jandt KD. Atomic force microscopy of biomaterials surfaces and interfaces. Surf Sci. 2001; 491(3): 303–332.

72. Cullen DC, Lowe CR. AFM studies of protein adsorption: 1. Time-resolved protein adsorption to highly oriented pyrolytic graphite. J Colloid Interface Sci. 1994; 166(1): 102–8.

73. Holland NB, Marchant RE. Individual plasma proteins detected on rough biomaterials by phase imaging AFM. J Biomed Mater Res Part A. 2000; 51(3): 307–15.

74. Teichroeb JH, Forrest JA, Ngai V, et al. Imaging protein deposits on contact lens materials. Optom Vis Sci. 2008; 85(12): 1151–64.

75. Soltys-Robitaille CE, Ammon DM, Valint PL, et al. The relationship between contact lens surface charge and in-vitro protein deposition levels. Biomaterials. 2001; 22(24): 3257–60.

76. Rabinow B, Ding Y, Qin C, et al. Biomaterials with permanent hydrophilic surfaces and low protein adsorption properties. J Biomater Sci Polym Ed. 1994; 6(1): 91–109.

77. Rebeix V, Sommer F, Marchin B, et al. Artificial tear adsorption on soft contact lenses: methods to test surfactant efficacy. Biomaterials. 2000; 21(12): 1197–1205.

78. Zhang S, Borazjani RN, Salamone JC, et al. In vitro deposition of lysozyme on etafilcon A and balafilcon A hydrogel contact lenses: Effects on adhesion and survival of Pseudomonas aeruginosa and Staphylococcus aureus. Contact Lens Anterior Eye. 2005; 28(3): 113–19.

79. Engvall E, Perlman P. Enzyme-linked immunosorbent assay, ELISA III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J Immunol. 1972; 109(1): 129–135.

80. Kothari S, Hatton P V., Douglas CWI. Protein adsorption to titania surfaces. J Mater Sci Mater Med. 1995; 6(12): 695–698.

81. Bullett N, Whittle JD, Short RD, et al. Adsorption of immunoglobulin G to plasma-co-polymer surfaces of acrylic acid and 1,7-octadiene. J Mater Chem. 2003; 13(7): 1546–1553.

82. Cantarero L, Butler JE, Osborne JW. The adsorptive characteristics of proteins for polystyrene and their significance in solid-phase imunoassays. Anal Biochem. 1980; 105(2): 375–82.

83. Luensmann D, Zhang F, Subbaraman L, et al. Localization of lysozyme sorption to conventional and silicone hydrogel contact lenses using confocal microscopy. Curr Eye Res. 2009; 34(8): 683–97.

84. Reichert U, Linden T, Belfort G, et al. Visualising protein adsorption to ion-exchange membranes by confocal microscopy. J Memb Sci. 2002; 199: 161–166.

85. Luensmann D, Heynen M, Liu L, et al. Determination of albumin sorption to intraocular lenses by radiolabeling and confocal laser scanning microscopy. J Cataract Refract Surg. 2009; 35(11): 2000–7.

86. Garrett Q, Chatelier RC, Griesser HJ, et al. Effect of charged groups on the adsorption and penetration of proteins onto and into carboxymethylated poly(HEMA) hydrogels. Biomaterials. 1998; 19(23): 2175–2186.

87. McArthur S. Applications of XPS in bioengineering. Surf interface Anal. 2006; 38: 1380–1385.

88. Wagner MS, McArthur SL, Shen M, et al. Limits of detection for time of flight secondary ion mass spectrometry (ToF-SIMS) and X-ray photoelectron spectroscopy (XPS): detection of low amounts of adsorbed protein. J Biomater Sci Polym Ed. 2002; 13(4): 407–28.

89. McArthur S, McLean K, Kingshott P, et al. Effect of polysaccharide structure on protein adsorption. Colloid Surface B. 2000; 17: 37–48.

90. Tsapikouni TS, Missirlis YF. Protein–material interactions: From micro-to-nano scale. Mater Sci Eng B. 2008; 152(1-3): 2–7.

91. Griesser HJ, Kingshott P, McArthur ally L, et al. Surface-MALDI mass spectrometry in biomaterials research. Biomaterials. 2004; 25(20): 4861–75.

92. Belu AM, Graham DJ, Castner DG. Time-of-flight secondary ion mass spectrometry: techniques and applications for the characterization of biomaterial surfaces. Biomaterials. 2003; 24(21): 3635–3653.

93. Benninghoven A. Surface investigation of solids by the statistical method of secondary ion mass spectroscopy (SIMS). Surf Sci. 1973; 35: 427–457.

94. Tidwell CD, Castner DG, Golledge SL, et al. Static time-of-flight secondary ion mass spectrometry and x-ray photoelectron spectroscopy characterization of adsorbed albumin and fibronectin films. Surf Interface Anal. 2001; 31(8): 724–733.

95. Wagner MS, Castner DG. Analysis of adsorbed proteins by static time-of-flight secondary ion mass spectrometry. Appl Surf Sci. 2004; 231-232: 366–376.

96. Canavan HE, Graham DJ, Cheng X, et al. Comparison of native extracellular matrix with adsorbed protein films using secondary ion mass spectrometry. Langmuir. 2007; 23(1): 50–6.

97. Eynde X, Bertrand P. ToF‐SIMS quantification of polystyrene spectra based on principal component analysis (PCA)†. Surf Interface Anal. 1997; 25(11): 878–888.

98. Wagner MS, Graham DJ, Ratner BD, et al. Maximizing information obtained from secondary ion mass spectra of organic thin films using multivariate analysis. Surf Sci. 2004; 570(1-2): 78–97.

99. Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem. 1988; 2301(29): 2299–2301.

100. McArthur SL, McLean KM, St John HA, et al. XPS and surface-MALDI-MS characterisation of worn HEMA-based contact lenses. Biomaterials. 2001; 22(24): 3295–304.

101. Oleschuk RD, Mccomb ME, Chow A, et al. Characterization of plasma proteins adsorbed onto biomaterials by MALDI-TOFMS. Biomaterials. 2000; 21(16): 1701–10.

102. Kingshott P, St John HA, Griesser HJ. Direct detection of proteins adsorbed on synthetic materials by matrix-assisted laser desorption ionization-mass spectrometry. Anal Biochem. 1999; 273(2): 156–62.

103. Bingaman S, Huxley VH, Rumbaut RE. Fluorescent dyes modify properties of proteins used in microvascular research. Microcirculation. 2003; 10(2): 221–31.

104. Prager MD, Quintana RP. Radiochemical studies on contact lens soilation. I. Lens uptake of 14C-lysozyme from simple and complex artificial tear solutions. J Biomed Mater Res. 1997; 36(1): 119–24.

105. Kim D, Blanch H, Radke C. Direct imaging of lysozyme adsorption onto mica by atomic force microscopy. Langmuir. 2002; 18(15): 5841–5850.

106. Subbaraman LN, Jones L. Kinetics of lysozyme activity recovered from conventional and silicone hydrogel contact lens materials. J Biomater Sci Polym Ed. 2010; 21(3): 343–58.

107. Subbaraman LN, Glasier MA, Senchyna M, et al. Kinetics of in vitro lysozyme deposition on silicone hydrogel, PMMA, and FDA groups I, II, and IV contact lens materials. Curr Eye Res. 2006; 31(10): 787–96.

108. Ceska M, Sjodin A, Grossmuller F. Some quantitative aspects of the labelling of proteins with 125I by the iodine monochloride method. Biochem J. 1971; 121(1): 139–143.

109. Jadi S, Heynen M, Luensmann D, et al. Composition of incubation solution impacts in vitro protein uptake to silicone hydrogel contact lenses. Mol Vis. 2012; 18: 337–47.

110. Bohnert JL, Horbett TA, Ratner BD, et al. Adsorption of proteins from artificial tear solutions to contact lens materials. Invest Ophthalmol Vis Sci. 1988; 29(3): 362–73.

111. Carney FP, Morris CA, Milthorpe B, et al. In vitro adsorption of tear proteins to hydroxyethyl methacrylate-based contact lens materials. Eye Contact Lens. 2009; 35(6): 320–8.

112. Chow LM, Subbaraman LN, Sheardown H, et al. Kinetics of in vitro lactoferrin deposition on silicone hydrogel and FDA group II and group IV hydrogel contact lens materials. J Biomater Sci Polym Ed. 2009; 20(1): 71–82.

113. Rodrigues SN, Gonçalves IC, Martins MCL, et al. Fibrinogen adsorption, platelet adhesion and activation on mixed hydroxyl-/methyl-terminated self-assembled monolayers. Biomaterials. 2006; 27(31): 5357–67.

114. Wu Y, Simonovsky FI, Ratner BD, et al. The role of adsorbed fibrinogen in platelet adhesion to polyurethane surfaces: a comparison of surface hydrophobicity, protein

adsorption, monoclonal antibody binding, and platelet adhesion. J Biomed Mater Res Part A. 2005; 74(4): 722–38.

115. Van Beek M, Jones L, Sheardown H, et al. Hyaluronic acid containing hydrogels for the reduction of protein adsorption. Biomaterials. 2008; 29(7): 780–789.

116. Sheardown H, Cornelius R, Brash J. Measurement of protein adsorption to metals using radioiodination methods: a caveat. Colloid Surface B. 1997; 10(1): 29–33.

117. Du YJ, Cornelius RM, Brash JL. Measurement of protein adsorption to gold surface by radioiodination methods: suppression of free iodide sorption. Colloid Surface B. 2000; 17(1): 59–67.

118. Oster G, Immergut E. Ultraviolet and infrared spectral studies of polyvinylpyrrolidone. J Am Chem Soc. 1954; 76(5): 1393–6.

119. Subbaraman LN, Glasier M-A, Varikooty J, et al. Protein deposition and clinical symptoms in daily wear of etafilcon lenses. Optom Vis Sci. 2012; 89(10): 1450–9.

120. Elhers WH, Donshik PC. Giant papillary conjunctivitis. Curr Opin Allergy Clin Immunol. 2008; 8(5): 445–9.

121. Allansmith M, Korb D, Greiner J, et al. Giant papillary conjunctivitis in contact lens wearers. Am Acad Ophthalmol. 1977; 83(5): 697–708.

122. Hart DE, Schkolnick JA, Bernstein S, et al. Contact lens induced giant papillary conjunctivitis: a retrospective study. J Am Optom Assoc. 1989; 60(3): 195–204.

123. Williams TJ, Schneider RP, Willcox MDP. The effect of protein-coated contact lenses on the adhesion and viability of gram negative bacteria. Curr Eye Res. 2003; 27(4): 227–35.

124. Silverstein R, Bassler G, Morrill T. Spectrometric identification of organic compounds, 5th ed. New York: Wiley; 1991.

125. Fabris L, Antonello S, Armelao L, et al. Gold nanoclusters protected by conformationally constrained peptides. J Am Chem Soc. 2006; 128(1): 326–36.

126. Slocik JM, Naik RR. Probing peptide-nanomaterial interactions. Chem Soc Rev. 2010; 39(9): 3454–63.

127. Lundqvist M, Sethson I, Jonsson B-H. High-resolution 2D 1H-15N NMR characterization of persistent structural alterations of proteins induced by interactions with silica nanoparticles. Langmuir. 2005; 21(13): 5974–9.

128. Bower P, Louie EA, Long JR, et al. Solid-state NMR structural studies of peptides immobilized on gold nanoparticles. Langmuir. 2005; 21(7): 3002–7.

129. Shaw W, Long J, Campbell A, et al. A solid-state NMR study of dynamics in a hydrated salivary peptide adsorbed to hydroxyapatite. J Am Chem Soc. 2000; 15(12): 7118–7119.

130. Hammes G. Spectroscopy for the Biological Sciences. New York, USA: John Wiley & Sons, Inc.; 2005.

131. Shanmugam G, Polavarapu PL. Vibrational circular dichroism spectra of protein films: thermal denaturation of bovine serum albumin. Biophys Chem. 2004; 111(1): 73–77.

132. Sasahara K, Demura M, Nitta K. Partially unfolded equilibrium state of hen lysozyme studied by circular dichroism spectroscopy. Biochemistry. 2000; 39(21): 6475–6482.

133. Laurents D, Baldwin RL. Characterization of the unfolding pathway of hen egg white lysozyme. Biochemistry. 1997; 36(6): 1496–1504.

134. Carrotta R, Manno M, Giordano FM, et al. Protein stability modulated by a conformational effector: effects of trifluoroethanol on bovine serum albumin. Phys Chem Chem Phys. 2009; 11(20): 4007–18.

135. Roux P, Delepierre M, Goldberg ME, et al. Kinetics of secondary structure recovery during the refolding of reduced hen egg white lysozyme. J Biol Chem. 1997; 272(40): 24843–24849.

136. Ranjbar B, Gill P. Circular dichroism techniques: biomolecular and nanostructural analyses- a review. Chem Biol Drug Des. 2009; 74(2): 101–20.

137. Mannucci LL, Moro F, Cosani A, et al. Conformational state of lacrimal proteins adsorbed on contact lenses. Curr Eye Res. 1985; 4(6): 734–6.

138. Tanaka M, Motomura T, Kawada M, et al. Blood compatible aspects of poly(2-methoxyethylacrylate) (PMEA)--relationship between protein adsorption and platelet adhesion on PMEA surface. Biomaterials. 2000; 21(14): 1471–81.

139. Khrapunov S. Circular dichroism spectroscopy has intrinsic limitations for protein secondary structure analysis. Anal Biochem. 2009; 389(2): 174–6.

140. Maruyama T, Katoh S, Nakajima M, et al. FT-IR analysis of BSA fouled on ultrafiltration and microfiltration membranes. J Memb Sci. 2001; 192: 201–207.

141. Fu K, Griebenow K, Hsieh L, et al. FTIR characterization of the secondary structure of proteins encapsulated within PLGA microspheres. J Control Release. 1999; 58(3): 357–366.

142. Smith JR, Cicerone MT, Meuse CW. Tertiary structure changes in albumin upon surface adsorption observed via Fourier transform infrared spectroscopy. Langmuir. 2009; 25(8): 4571–4578.

143. Jakobsen RJ, Wasacz FM, Brasch JW, et al. The relationship of bound water to the IR amide I bandwidth of albumin. Biopolymers. 1986; 25(4): 639–54.

144. Kato K, Matsui T, Tanaka S. Quantitative estimation of a-helix coil content in bovine serum albumin by Fourier transform-infrared spectroscopy. Appl Spectrosc. 1987; 41(5): 861–5.

145. Byler DM, Susi H. Examination of the secondary structure of proteins by deconvolved FTIR spectra. Biopolymers. 1986; 25(3): 469–87.

146. Yang W, Griffiths P, Byler D, et al. Protein conformation by infrared spectroscopy: resolution enhancement by Fourier self-deconvolution. Appl Spectrosc. 1985; 39(2): 282–287.

147. Jackson M, Mantsch HH. The use and misuse of FTIR spectroscopy in the determination of protein structure. Crit Rev Biochem Mol Biol. 1995; 30(2): 95–120.

148. Lord R, Yu N. Laser-excited Raman spectroscopy of biomolecules I. Native lysozyme and its constituent amino acids. J Mol Biol. 1970; 50: 509–524.

149. Hédoux A, Guinet Y, Paccou L, et al. Analysis of the mechanism of lysozyme pressure denaturation from Raman spectroscopy investigations, and comparison with thermal denaturation. J Phys Chem B. 2011; 115(20): 6740–6748.

150. Hédoux A, Ionov R, Willart J-F, et al. Evidence of a two-stage thermal denaturation process in lysozyme: A Raman scattering and differential scanning calorimetry investigation. J Chem Phys. 2006; 124(1): 014703.

151. Filik J, Stone N. Analysis of human tear fluid by Raman spectroscopy. Anal Chim Acta. 2008; 616(2): 177–184.

152. Williams RW, Dunker AK. Determination of the secondary structure of proteins from the amide I band of the laser Raman spectrum. J Mol Biol. 1981; 152(4): 783–813.

153. Strehle MA, Rosch P, Petry R, et al. A Raman spectroscopic study of the adsorption of fibronectin and fibrinogen on titanium dioxide nanoparticles. Phys Chem Chem Phys. 2004; 6: 5232–5236.

154. Helal R, Melzig MF. Determination of lysozyme activity by a fluorescence technique in comparison with the classical turbidity assay. Pharmazie. 2008; 63: 415–419.

155. Senchyna M, Jones L, Louie D, et al. Quantitative and conformational characterization of lysozyme deposited on balafilcon and etafilcon contact lens materials. Curr Eye Res. 2004; 28(1): 25–36.

156. Sack RA, Jones B, Antignani A, et al. 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(5): 842–849.

157. Morsky P. Turbidimetric determination of lysozyme with Micrococcus lysodeikticus cells: reexamination of reaction conditions. Anal Biochem. 1983; 128(1): 77–85.

158. Wu Y, Daeschel MA. Lytic antimicrobial activity of hen egg white lysozyme immobilized to polystyrene beads. J Food Sci. 2007; 72(9): M369–74.

159. Suwala M, Glasier MA, Subbaraman LN, et al. Quantity and conformation of lysozyme deposited on conventional and silicone hydrogel contact lens materials using an in vitro model. Eye Contact Lens. 2007; 33(3): 138–43.

160. Hall B, Jones L, Forrest J. Kinetics of competitive adsorption between lysozyme and lactoferrin on silicone hydrogel contact lenses and the effect on lysozyme activity. Curr Eye Res. 2014: In Press.