CONFOCAL MICROSCOPY TO STUDY LYSOZYME SORPTION

A NOVEL USE OF CONFOCAL MICROSCOPY TO STUDY LYSOZYME SORPTION TO SILICONE HYDROGEL AND CONVENTIONAL HYDROGEL

CONTACT LENS MATERIALS

By

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ABSTRACT

The purpose of this study was to observe penetration profiles of lysozyme on a variety of contact lens materials by confocal microscopy, to analyze influential factors that are involved in these penetration curves and to suggest possible mechanisms related to the in-eye clinical performance of these materials.

An FITC-lysozyme conjugate was synthesized in-house by amine reaction. Contact lenses were incubated in a lysozyme solution with a final concentration of 1.9 mg/mL for various periods before undergoing microscopic analysis. Optimal parameters for confocal scanning were successfully obtained to acquire desired fluorescence signals on various contact lenses. Measurement units were converted into absolute amounts of lysozyme using lysozyme data from ¹²⁵I gamma counting studies. A rhodamine labeled dextran solution was applied to distingush the surface of the contact lenses under examination. The data from these studies were then used to calculate the theoretical numbers of layers of adsorbed lysozyme on the lens surface.

The results show that there were distinct differences in lysozyme penetration in the twelve hydrogel materials examined.

A pure pHEMA lens, with a water content of 38%, deposited lysozyme primarily on the lens surface after 24 hours, with full penetration occurring after 4-weeks of incubation. Three types of non-ionic contact lens materials with water contents > 50%exibited rapid penetration within the lens bulk after 24-hours incubation, with increased deposition within the matrix after 4 weeks. Two ionic, high water content polymers (Acuvue 2 and Focus Monthly) exhibited markedly different penetration profiles, particularly after 24 hours, with very rapid and total penetration in Acuvue 2, as compared with partial penetration in Focus Monthly.

Modern silicone hydrogel contact lenses can be nominally divided into first generation, plasma-modified materials and second generation materials which incorporate an internal wetting agent such as polyvinyl pyrrolidone (PVP). These materials exhibited different lysozyme deposition profiles. Lysozyme fully penetrated PureVision after 24 hours, whereas no lysozyme penetration occurred on lenses manufactured from Focus Night & Day or O₂Optix , even after 4 weeks.

Lenses manufactured from Acuvue Advance and Acuvue OASYS, two second generation silicone hydrogel lenses, also displayed their own characteristic deposition profile. Acuvue Advance always exhibited a partial penetration of lysozyme within the matrix, even after 4 weeks of doping. Interestingly, Acuvue OASYS showed a similar profile to Focus Night & Day and O_2Optix , with predominantly surface deposition occurring.

To confirm possible surface adsorption of lysozyme on surface-coated Focus Night & Day and O_2Optix , a rigid polymethylmethacrylate (PMMA) contact lens was used as a model of surface adsorption. A mounting medium containing rhodamine labeled dextran was scanned to distinguish the lens surface, as it was assumed that no surface penetration of the very high molecular weight dextran would occur. Using this model, it was confirmed that surface adsorption of lysozyme occurred on these plasmacoated lens materials, which is similar to that seen with PMMA. In a further experiment, it was seen that lysozyme sorption on Acuvue OASYS exhibits a penetration profile which is different to that seen in Focus Night & Day and O₂Optix, with lysozyme just penetrating the lens surface. The results from the studies described above demonstrated that in 24 hours lysozyme sorption did not achieve a complete monolayer. However, after 4 weeks multi-layer adsorption occurred, with the more hydrophilic materials depositing the most lysozyme.

The quantitative measurement of lysozyme penetration on and into contact lens materials by confocal microscopy combined with ¹²⁵I labelling offers a valuable tool to discover the potential mechanisms of interactions between protein and polymer materials. This study reveals some important information that may be beneficial to contact lens development and will prove to be valuable in other more broad areas of biomedical research in which polymers and biological fluids come into contact.

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LIST OF ABBREVIATIONS

CLAPC	Contact lens associated papillary conjunctivitis
DABCO	1,4-diazabicyclooctane
DMA	N,N-dimethylacrylamide
DMSO	Dimethyl sulfoxide
EGDMA	Ethyleneglycol dimethacrylate
FITC	Fluorescein isothiocyanate
GPC	Giant papillary conjunctivitis
HEL	Hen egg lysozyme
HEMA	2-hydroxyethyl methacrylate
LSCMs	Laser Scanning Confocal Microscopes
MAA	Methacrylic acid
MMA	Methyl methacrylate
mPDMS	Monofunctional polydimethylsiloxane
NCVE	N-carboxyvinyl ester
NVP	N-vinyl pyrrolidone
PBVC	Poly[dimethylsiloxyl] di [silylbutanol] bis[vinyl carbamate]
PC	Phosphorylcholine
PDMS	Poly dimethylsiloxane
PEO	polyethylene oxide
РММА	Poly(methyl methacrylate)

PVP	Polyvinyl pyrrolidone
TEGDMA	Tetraethyleneglycol dimethacrylae
TPVC	Tris-(trimethylsiloxysilyl) propylvinyl carbamate
TRIS	Trimethyl siloxy silane
XPS	X-ray photoelectron spectroscopy

1.0 INTRODUCTION

Approximately 125 million people use contact lenses worldwide. Contact lens spoilation is a well-known clinical complication, due to the deposition of tear film components, such as proteins, lipids and mucin. These deposits, particularly denatured proteins at the lens surface, lead to discomfort, reduced vision and increased inflammatory responses. Ocular biocompatibility, involving the interaction between tear components and contact lens materials, is an area of significant research when developing new contact lens materials.

Current soft contact lens materials are divided into two major categories: carbonbased conventional hydrogels and silicone-based silicone hydrogels. The most outstanding difference between the two groups of materials is the significantly higher oxygen permeability offered by silicone-based polymers, which deliver sufficient oxygen to the cornea to allow safe overnight wear for up to 30 days. Another difference is that silicone hydrogels deposit much less tear proteins, particular lysozyme, than conventional hydrogel lenses based on pHEMA/MAA copolymers.

The investigation of protein deposition on contact lens materials requires an interdisciplinary approach, including input from clinicians, protein biochemists, physical chemists, polymer chemists and an understanding of polymer engineering and polymer processing, as well as the biological responses of tears to polymers.

Thus far, numerous studies have focused on measurement of the amounts of individual proteins deposited, competitive sorption between tear components, and the status of the protein (native or denatured) on contact lens materials. However, the location of tear components on the lens material has attracted little attention. This project studies the location of lysozyme, a very abundant tear protein deposited on contact lenses, in an attempt to develop a suitable technique to observe the location of a protein on a biomaterial.

2.0 LITERATURE REVIEW

2.1 History of Contact Lenses

The earliest concept of contact lenses was described by Leonardo de Vinci in 1508. The first contact lens made from glass was made in 1887 to correct optical defects. Poly(methyl methacrylate) (PMMA) replaced glass as a lens material in the 1940s. While it had similar properties to glass, including acceptable surface wettability and excellent durability, its poor oxygen permeability remained a critical problem. To avoid the corneal anoxia induced by oxygen deficiency, PMMA lenses were designed to have a small diameter and to float on the precorneal tear film to ensure that the cornea received oxygen during each blink and movement of the lens (Lloyd, 2001). Copolymerization with methacrylic acid (MAA) and trimethyl siloxy silane (TRIS) resulted in the rigid gas permeable contact lenses with higher oxygen permeability that became popular in the late 1970s.

In parallel with these developments, in the early 1960s, OttoWichterle developed a new material specifically intended for contact lens use (Nicolson, 2001). Poly 2-(hydroxyethyl methacrylate) (pHEMA), shown in Figure 2.1, was designed to be a highly water swollen gel. Soft hydrogel lenses fabricated from this material became commercially available in the early 1970s, and brought a great revolution in the field of contact lens practice for their outstanding wettability and instant comfort. However, since oxygen transport in these materials occurs primarily through the water swollen

pores of the gel, hydrogel materials based on pHEMA have relatively low oxygen permeability that make them poorly suited to extended wear applications, where patients would obtain maximum convenience from their lenses by sleeping in them overnight.

Because soft contact lenses cover the entire cornea, the oxygen from the atmosphere must diffuse through the lens in order to reach the cornea. Therefore, the oxygen transmissibility of hydrogel lenses is related to both the water content of hydrogel and the thickness of lens. Since higher water content lenses have the potential for higher oxygen permeability, approaches aimed at copolymerizing HEMA with highly hydrophilic monomers such as methacrylic acid (MAA) and N-vinyl pyrrolidone (NVP) have been made to improve oxygen permeability of the lens (Bohnert, 1988).

However, when attempting to achieve higher oxygen permeability, the most successful approach has been due to the incorporation of silicone into the hydrogel. These silicone-based hydrogels are highly permeable due to the very high oxygen permeability of silicone rubbers (Ho, 1988). In 1999, two silicone hydrogel contact lenses were launched, under the trade names of Focus Night & Day and PureVision. These were the first lenses approved for continuous wear (in-eye wear for periods of up to 30 days without removal) by the US Food and Drug Administration (FDA).

2.2 Chemistry of Lens Materials

2.2.1 Soft Contact Lenses

Polymers are very large molecules with long chains of repeat units. Any polymer used in the manufacture of contact lenses must meet a series of structure-based requirements (Sariri, 2004). It must be chemically stable, biologically inert and optically clear. The polymers used in contact lens manufacture or suggested as contact lens materials can be divided into two major groups, based on their modulus and elasticity: rigid or soft polymers. In the rigid group of contact lens materials, PMMA is the principal polymer, which is carbon-based. The monomer structure of methyl methacrylate (MMA) is shown in Figure 2.1. For the soft contact lenses, pHEMA is the principal hydrophilic monomer used. The hydrophilicity arises from the presence of hydrophilic side groups. pHEMA hydrogels do not dissolve but swell in water. The amount of water absorbed by a hydrogel is expressed as the equilibrium water content (EWC), which represents a percentage ratio between weight of water in the gel and weight of the hydrated gel. As noted above, EWC is a crucial factor for oxygen permeability of pHEMA-based hydrogels, because oxygen permeation occurs primarily through the water filled channels of the swollen gel. pHEMA alone has a maximum water content of approximately 38%. Therefore to increase the water content of the swollen hydrogel lens, copolymers of HEMA with other hydrophilic monomers, particularly ionic methacrylic acid (MAA) or non-ionic N-vinyl pyrrolidinone (NVP), can lead to water contents that are greater than 50% (w/w). However the incorporation of these more hydrophilic monomers also results in lenses with decreased strength and elasticity.



HEMA

MMA



NVP

Figure 2.1: Chemical formulas of common monomers used in conventional hydrogel lenses (Lloyd, 2001), with permission.

MAA

On the other hand, copolymerization with hydrophobic monomers like methyl methacrylate (MMA) and tetraethyleneglycol dimethacrylae (TEGDMA) decreases water content, while increasing lens stiffness and reducing elasticity. As an example, Kunzler (Kunzler, 1999) describes a formulation of 94% (w/w) pHEMA copolymerized with 6% (w/w) methacrylic acid, resulting in a material with a water content of 70%.

However, there are several basic limitations of high-water-content hydrogels. Firstly, materials with high-water-content usually possess poor tear strength. Secondly,

such materials exhibit high deposition of tear-film proteins, particularly when negatively charged ionic hydrogels interact with "positively charged" lysozyme (Minarik, 1989). In addition, thin (<80 µm centre thickness) high-water-content lenses may induce epithelial dehydration, which can in turn lead to harmful effects to corneal epithelial cells (Orsborn, 1988).

2.2.2 Silicone Hydrogel Lenses

The need for higher oxygen permeability led to consideration of other potential polymer candidates. Silicone, a broad class of synthetic polymers, with its Si-O backbone has unique properties that include high oxygen permeability. The most common silicone is polydimethylsiloxane (PDMS), which features a repeating $(CH_3)_2SiO$ unit (Tighe, 2000), as shown in Figure 2.2.



Figure 2.2: The structure of PDMS (Lloyd, 2001), with permission.

As a result of this Si-O backbone, PDMS possesses excellent oxygen permeability compared with pHEMA-based polymers. However, its highly hydrophobic characteristic induces high protein and lipid affinity, poor wettability and corneal adhesion. Therefore, PDMS must be modified before being considered as a contact lens material. The main modification strategy involves combining the high oxygen permeability of PDMS with the hydrophilic nature and wettability of pHEMA. The major challenge in designing these copolymers is combining the hydrophobic silicone macromers with the hydrophilic monomers. The early copolymerizations resulted in opaque and phase-separated materials. However successful materials have been generated, involving strategies including the use of hydrophilic block siloxane copolymers, siloxane graft copolymers and modification of TRIS. This has led to the production and development of commercial contact lenses based on silicone technology (Kunzler, 1999).



Figure 2.3: Modification site of TRIS by the introduction of hydrophilic groups. (Tighe, 2000), with permission

Two key approaches significantly contributed to the birth of silicone hydrogel contact lenses (Tighe, 2000). The first approach involved the incorporation of fluorine into silicone hydrogels, resulting in fluorosilicone hydrogels. The reactive site is shown in Figure 2.3. This type of fluorosiloxane-containing monomers enhances the relative

compatibility with hydrophilic monomers, obtaining the desired transparent copolymers. Furthermore, fluorosilicone hydrogels improved deposit resistance due to the presence of the fluorinated group (Kunzler, 1996). The other approach was the development of siloxane macromers. A typical siloxane macromer is copolymerized by combining siloxane monomers (such as TRIS), hydrophilic monomers (such as NVP or DMA) and fluorinated monomers (Kunzler, 1999). Such hydrophilic macromers are the major polymeric components used in silicone hydrogel formulations. Figure 2.4 is the structure of a siloxane macromer used in silicone hydrogel lenses.

$$CH_{3} CH_{3} CH_{2} CH_{2} CH_{2} CH_{2} CH_{2} CH_{2} CH_{2} CH_{3} CH_{3}$$

Figure 2.4: An example of a siloxy-based polyfluoroether macromer (Tighe, 2000), with permission.

Since silicones tend to be highly surface active, surface modification to improve wettability proved necessary. Gas plasma techniques were used to modify the surface of the first commercially available silicone hydrogel lenses, PureVision and Focus Night & Day. Plasma polymeric films have universal adhesion capabilities that can deposit on almost any solid substrate. Reactions involved in plasma modification include ablation, etching or direct deposition. The improved wettability of silicone hydrogel lenses treated by plasma modification is typically due to the hydroxyl groups created in the oxygenation process, which increase the hydrophilicity of the surface, thus enhancing patient comfort.

Although both of the original silicone hydrogel lenses were surfaced-modified using gas plasma techniques, they exhibit very different surface properties (Tighe, 2000). The PureVision lens is treated using plasma oxidation, resulting in the formation of SiO₂ containing glassy islands on the surface. The "island" structures are isolated rather than connected and thus still allow a flexible lens. The surface of Focus Night & Day (and more recently O₂Optix) lenses is chemically uniform. The gas plasma changes the surface structure and coats it with a 25 nm thin hydrophilic layer. More recently released silicone hydrogel lenses, such as Acuvue Advance and Acuvue OASYS, use polyvinyl pyrrolidone (PVP) as an internal wetting agent and no surface treatment is required, as PVP acts as a surface modifying agent when the lens is hydrated (Steffen, 2004).

2.3 Protein Adsorption at Biomaterials Interfaces

Proteins are macromolecules that are made up of 20 different amino acids. Due to the presence of an N-terminal residue (-NH2), a terminal carboxyl group (-COOH) and a variety of functional groups, proteins can be positive, negative or neutral depending on the pH of surrounding environment. The complexity of protein structure ensures its characteristic three-dimensional structure; the amphiphilic nature of these macromolecules contributes to their surface activity. It has been widely shown that proteins have a strong tendency to nonspecifically adsorb at any solid surface need a

reference for this statement. Protein adsorption is a ubiquitous event that occurs immediately upon exposing a surface to a protein-containing fluid. As such, it has been widely described as the initial and fate determining step in defining the response to biomaterials. In blood contacting materials for example, the initial phase of protein adsorption is followed with host responses including platelet adhesion and activation, intrinsic coagulation, complement activation and leukocyte interactions (Albert, 1996; Courtney, 1994). The effect of adsorbed proteins on contact lens materials is less defined, although protein adsorption has been associated with end of day discomfort and other disease states (Castillo, 1986a; Meisler DM, 1995).

2.3.1 Interaction forces of protein adsorption

The interaction forces between proteins and polymer interfaces include hydrophobic, van der Waals and electrostatic interactions (Van Oss, 1994). The hydrophobic effect has been suggested as the major driving force of protein adsorption found at many experimental systems (Mille, 2000). Competitive protein adsorption is known as the Vroman effect that describes a dynamic adsorbed protein layer. The resulting exchange of different proteins based upon the affinity of proteins to the solid surface (Vroman, 1967), and demonstrates the importance of studying protein adsorption in a relevant context.

2.3.2 Methods for mediating protein repulsion

Grafting hydrophilic polymers to a broad range of polymers is a popular strategy to minimize protein adsorption (Chen, 2005; Kingshott, 2002; Lee, 1997). PEO, a linear

polymer with repeat unit of -CH₂CH₂O- has been particularly widely studied due to its non-cytotoxic and biologically inert nature (Roberts, 2002) as well as the excellent levels of protein repulsion that are observed with high density PEO layers. Current methods used to graft PEO to a substrate include physical adsorption (McPherson, 1997); heat, radiation, or plasma mediated crosslinking of adsorbed PEO (Lopez, 1992); covalent attachment of PEO chains (Chen, 2005; Gombotz, 1991; Unsworth, 2005b) and incorporation of PEO into polymeric network (Chen, 2004). PEO layer conformation undergoes a continuous change from a mushroom to a brush structure as more PEO molecules react with grafting sites (Szleifer, 1996). While there are a variety of mechanisms proposed for the resistance of PEO grafted surfaces to protein adsorption, three major interaction forces have been widely touted as having significant effects on the interactions of the PEO grafted surface with proteins. Steric repulsion resulting from compression of the PEO layer by the approaching protein molecules has been widely speculated as a contributing factor. Such compression decreases the PEO chain conformational entropy, thus leading to a thermodynamically unfavourable interfacial interaction (McPherson, 1995; Van Oss, 1994). PEO is also believed to change the structure of water at the interface. This water layer is responsible for the strong longrange hydrophobic attractions that occur between the surface and the protein, thus altering the van der Waals interactions (Israelachvili, 1996). Furthermore, PEO attenuates both attractive and repulsive electrostatic forces at the surface by the disruption of the electrical double layer (Burns, 1995).

PEO conformation, chain density, molecular weight and end-group chemistry have all been speculated to effect protein adsorption at the polymer interface. Szleifer (Szleifer, 1997) found that lysozyme levels decreased with increased PEO grafting density and chain length. However, arguments exist at the effect of chain length. Some experimental results have shown that a high surface coverage of PEO with short chains performed a significantly reduced protein adsorption (Lopez, 1992; Wu, 2000). Unsworth et al. (Unsworth, 2005a) demonstrated that chain length effects are much less important than graft density effects. However, because current experimental designs face the difficulty of separating the effects of chain density and length of PEO, further welldesigned experiments are required to better understand the role of these various factors.

Surprisingly, while PEO modification has been widely examined for minimizing the effects of protein adsorption on materials intended for use in blood contacting (Archambault, 2004; Li, 2004) as well as other biotechnology based (Jin, 2004; Stolnik, 2001) applications, there are no reports in the literature about the effects of PEO modification on contact lens materials. PEO has been reported to undergo oxidation *in vivo*; it is unknown whether these effects have led to the lack of PEO in ophthalmic applications. Surface and bulk modification with other hydrophilic polymer chains, presumably intended to increase the hydrophilicity of the surface and decrease protein adsorption have been used in contact lenses however. Polyvinyl pyrrolidone (PVP), which has been used in dialysis and other applications (Hatcher, 2003; Hoenich, 2000) has been incorporated in Acuvue Advance and Acuvue OASYS lenses as an internal

wetting agent. Presumably, the PVP chains will migrate to the surface acting as PEO (Chen, 2004), resulting in a protein repellent interfacial layer. Similarly, phosphorylcholine (PC), which has also been used for modification of materials in blood contacting applications with excellent results (Feng, 2006; Feng, 2005), has been incorporated in Proclear Compatibles lenses, presumably in part to decrease the deposition of proteins on the surfaces of these lenses.

2.4 Physiology of the Cornea and External Eye

The physiological structure of the eye is shown in Figure 2.5.



Figure 2.5: The essential structures of the eye (Lloyd, 2001), with permission.

The cornea is the outermost portion of the anterior ocular surface. Its major functions include light refraction to the crystalline lens and barrier protection from harmful invasion of ocular pathogens. Unlike most tissues in the body, the cornea receives nutrients from the tears and aqueous humour rather than from the blood vessels, as if it contained blood vessels it would not be transparent and would be unable to transmit light to the retina. The cornea is comprised of three cellular layers (Lloyd, 2001). The epithelial layer is responsible for blocking foreign materials and absorbing oxygen and other nutrients provided in tears. The stromal cell layer is located beneath the epithelial layer and comprises about 90 percent of the thickness of the cornea. This layer maintains the strength and elasticity of the cornea and consists of a highly hydrated collagen mesh, interspersed with small numbers of corneal fibroblast cells. The endothelial cell layer is the innermost single layer of cells and is responsible for pumping excess water out of the stromal layer, thus avoiding corneal edema and maintaining corneal transparency.

The lack of blood vessels within the cornea requires that oxygen needs are met directly via diffusion through the tear layer from the atmosphere. Therefore, a contact lens placed in the front of the cornea obstructs the supply of oxygen. Under conditions of inadequate oxygen supply, corneal edema may be induced, which consequently leads to a variety of pathologic responses including keratitis, epithelial microcysts and limbal hyperemia (Fonn, 2002).

2.5 **Biocompatibility of Contact Lens Materials**

While oxygen permeability is a crucial factor for success of contact lens materials, ocular compatibility also plays an important role in the performance of lenses. Biomaterials are defined as a group of materials designed for use in physiological

interfaces (Ratner, 2004). While there has been rapid progress in the development of new biomaterials, interactions with the biological environment can lead to a number of adverse effects. It has been noted that the initial and fate determining step of any biomaterial interaction with a physiological system is the adsorption of proteins (Brash, 2000). In the blood, this involves the adsorption of plasma proteins and the subsequent protein mediated thrombotic and immune responses. In ocular applications, lens spoliation and end of day discomfort induced by the deposition of tear components including proteins, lipids and mucin have become major problems since the introduction of soft hydrogel contact lenses (Bontempo, 2001; Brennan, 2000). Deposit formation and build up on hydrogel contact lenses are directly associated with discomfort and reduced visual acuity (Jones, 1996). These deposits are also responsible for a variety of clinical symptoms including inflammation and infection. Of particular interest is giant papillary conjunctivitis (GPC) (Allansmith, 1977; Stapleton, 2003). The common symptoms of GPC include itching, contact lens awareness and decreased lens tolerance (Porazinski, 1999). Contact lens induced GPC or contact lens associated PC (CLAPC) is found in all types of lens materials and is known to involve both immunologic and mechanical effects (Donshik, 2003). It has similar pathology, but is associated with contact lens wear. It is believed that lens deposits on the lens surface (e.g. denatured protein) act as an antigen stimulator resulting in type I hypersensitivity, inducing the elevated local concentration of IgE and histopathological changes in the epithelial layers at conjunctival biopsy (Ballow, 1989; Barishak, 1984). In addition, these surface attached tear deposits will increase the roughness of contact lenses (Baguet, 1995) and induce

physical trauma to the conjunctival epithelium, leading to releasing of neutrophil chemotactic factors (Elgebaly, 1991) that gather various inflammatory cells at injured conjunctiva. Furthermore, the occurrence of GPC has been shown to decrease as a result of lens frequent replacement (Donshik, 1999), compared with extended lens wear (Levy, 1997). The recent shift to increased use of extended wear silicone hydrogel lenses has resulted in an increased clinical incidence of contact lens associated papillary conjunctivitis (CLAPC), which has similar pathology condition with GPC, is particularly found in contact lens wear. Internal data indicate that higher denaturation of lysozyme with longer wearing time in Acuvue 2 lenses is associated with lower comfort scores based on subject's response (Subbaraman, 2006b). Furthermore, the relatively low amount of lens deposits on silicone hydrogel lenses has been shown to consist primarily of denatured protein (Suwala, 2006). Conversely, conventional hydrogel materials, while taking up significantly higher amounts of native protein, with relatively little denatured material. Combining these observations, it seems possible that the presence of denatured protein located at the lens – tear interface is a contributing factor to CLAPC and can be correlated to the clinical symptoms observed.

Because of the critical roles of proteins in determining biocompatibility, an understanding of the fundamental mechanisms involved in the interactions between tear proteins and lens materials is important for the design and development of new deposition resistant polymers. The eye has several advantages for studies of biocompatibility, including its accessibility and possibility of studying the initial stages of spoliation under in vivo conditions. A contact lens can easily be removed and analyzed after only a few minutes of wear. Therefore, contact lenses are one of the most suitable devices for *ex vivo* biocompatibility studies.

2.6 Tear Film and Tear Proteins

2.6.1 Structure and Functions of the Tear Film

The tears film refers to the fluid present in the precorneal film and in the conjunctival sac. The normal volume of tear fluid on the ocular surface is approximately 5-10 μ l. It is believed that structurally the tear film consists of three layers: lipid, aqueous and mucin (Holly, 1977; Tiffany, 1994). The superficial lipid layer is about 40-90 nm thick, depending on measurement methods (King-Smith, 2004). The function of the lipid layer is to reduce tear evaporation from the aqueous phase. The aqueous phase of the tears, the major component of the film comprising approximately 98% of the total thickness, contains a wide variety of organic and inorganic substances, including as many as 60 proteins (Zhou, 2006). The mucin layer is composed of a high molecular weight glycoprotein that is located at the corneal interface. Mucin plays a role in removing lipids and debris from the surface of the anterior eye. The major functions of tear film include maintenance of light refraction, lubrication, supply of nutrients to the cornea and conjunctiva, and defence of the ocular surface from infections (Ohashi, 2006).

2.6.2 Tear Proteins Important to Contact Lens Wear

Most tear proteins are produced by the main and accessory lacrimal glands (Van Haeringen, 1981). The protein concentrations vary greatly, depending on whether those being examined are unstimulated, emotional or irritated tears (Farris, 1985). The major tear proteins known to deposit on hydrogel contact lenses include lysozyme, albumin, lactoferrin and lipocalin. Table 2.1 summarizes some of the properties of these tear proteins.

	Average concentration	Molecular	Isoelectric point and
Components	in tear (mg/ml)	weight (kDa)	charge in tears
Lysozyme	2.36	14.4	11.0, +
Albumin	1 30	68	47_
mounn	1.50	00	٦./, "
Lactoferrin	1.84	82	9.0, +
Lipocalin	1.23	21	5.5, -

Table 2.1: Major tear proteins deposited on contact lenses (Tighe, 2001).

Lysozyme

Lysozyme is a bacteriolytic enzyme that works in a similar fashion to penicillin, by hydrolyzing the $\beta(1-4)$ glycosidic bond at the cell wall of microorganisms (McClellan, 1997). Lysozyme has been extensively studied as a model protein to examine mechanisms of protein stability, folding and denaturation (Chang, 2002; Jolles, 1984). The type of lysozyme most commonly investigated is hen egg-white lysozyme (HEL), which was the first enzyme submitted to amino acid sequence and X-ray crystallographic analysis (Jolles, 1984). The molecular weight of HEL is 14,400 Da and its isoelectric point is 11.0, which ensures that it has a positive charge in the physiological environment. Tear lysozyme makes up to approximately 40% of tear proteins (Selinger, 1979). In humans, the concentration of lysozyme is higher in tears than in any other body fluid (Jolles, 1967). People with reduced amounts of tears (such as those who suffer from 'dry eye" symptoms) exhibit normal lysozyme concentrations (Avisar, 1979). The activity of lysozyme can be measured by assessing the clearing rate of a suspension of *Micrococcus lysodeikticus*, which is often used to determine the activity of lysozyme (Senchyna, 2004).

Albumin

Tear albumin, especially in contact lens-wearing eyes, is derived from its leakage from serum, due to conjunctival stimulation (Barishak, 1984). Serum albumin weighs 60% (w/w) of plasma proteins and its main function is regulation of colloidal osmotic pressure of the blood.

Adsorbed serum albumin demonstrated prevention of platelet adhesion, which is involved in the initial stage of thrombus formation (Kim, 1987). In studies of contact lens deposition, albumin demonstrates distinctly different deposition profiles to lysozyme. Albumin deposited more on neutral pure pHEMA lenses than lysozyme, whereas lysozyme accumulated most on pHEMA/MAA lenses, due to electrostatic

attraction (Taylor, 1998). Albumin is believed to irreversibly adsorbing onto the surface of hydrogel lenses (Castillo, 1984; Garrett, 1999b).

Lactoferrin

Tear lactoferrin, an iron carrying protein, makes up 25% of the composition of human tears. Similar to lysozyme, lactoferrin is a tear protein involved in non-specific outer eye defences, by reducing the available essential metal ions for microbial metabolism (McClellan, 1997). Its serum counterpart is transferrin, which is also an iron transporter. Similar to lysozyme, lactoferrin concentration does not significantly change in either basal or reflex tears in contact lens wearers (Farris, 1981). However, in the case of giant papillary conjunctivitis, reduced lactoferrin levels were found that may contribute to increased attachment of bacteria and ocular inflammation (Ballow, 1987). As a major tear protein deposited on hydrogel contact lenses, lactoferrin has been paid much less attention than lysozyme or albumin.

Lipocalin

Tear lipocalin was originally called "tear prealbumin" because it was found to migrate more anodally than serum albumin (Redl, 2000). Tear lipocalin is the principal lipid binding protein in tears (Glasgow, 1999) and has been found deposited on soft contact lenses (Baguet, 1995; Bruinsma, 2002). Some researchers have used low-cost bovine β-lactoglobulin instead of tear lipocalin for *in vitro* experiments, because they

both belong to the lipocalin family and share common structural features (Kontopidis, 2004).

2.7 Interaction between Lysozyme and Lens Materials

Protein deposition on contact lenses has been extensively studied both ex vivo and in vitro (Bohnert, 1988; Jones, 1997; Myers, 1991; Soltys-Robitaille, 2001). Most of the research has focused on identification and quantification of the components of the adsorbed protein layer. Among tear proteins, lysozyme has been mostly studied because lysozyme plays a dominant role in lens spoliation (Castillo, 1986b). Lysozyme is both reversibly and irreversibly adsorbed on copolymers of pHEMA/MAA (Castillo, 1985). Some studies have clearly demonstrated that the charge rather than the size of lysozyme is primarily responsible for its high levels of uptake, particularly on pHEMA/MAA materials (Garrett, 2000; Soltys-Robitaille, 2001). Myers and coworkers found a nearly equal distribution of visible protein deposits on the surface of pHEMA/MAA and pure pHEMA polymers (Myers, 1991). However, there was a significantly greater total protein accumulation on the pHEMA/MAA materials, which implies penetration of the protein into the lens matrix. After confocal microscopy was successfully applied to detect matrix tear proteins into contact lens materials in ex-vivo samples (Meadows, 1994), Garrett and colleagues demonstrated that lysozyme, with relatively low molecular weight and a net positive charge, does penetrate the matrix of pHEMA/MAA hydrogels, using fluorescence penetration curves (Garrett, 1999). The same group also reported that an increased amount of MAA in the pHEMA hydrogels increased
lysozyme sorption and penetration (Garrett, 2000), due to electrostatic attraction. Lysozyme deposition on silicone hydrogels has been found to be significantly less than on conventional hydrogel lenses (Jones, 2003); Senchyna, 2004). The relatively low amount of lysozyme present on these materials is predominantly denatured (Senchyna, 2004).

To date, there have been relatively few studies investigating the location of protein deposits on conventional lens materials (Garrett, 1999a; Meadows, 1994) and there has been no reports on the protein deposition profile of silicone hydrogel lenses. The deposition profile would be expected to differ between these two classes of materials, based upon their differences in surface and bulk composition.

2.8 Fluorescence Techniques for Detection of Biological Species

2.8.1 Fluorescence Principles

Fluorophores are defined as any functional component of a molecule that produces fluorescence by absorbing extra energy. These molecules are in a relatively low-energy and stable configuration in their ground state, but can be excited by the absorption of light energy from an external source to achieve a higher energy state. The energy of the excited state rapidly decays and results in the emission of light energy by returning the molecule to its ground state in a process called fluorescence. The schematic process of fluorescence is shown in Figure 2.6.

Theoretically, a fluorophore can repeatedly undergo this fluorescence process. This property makes fluorescence a highly sensitive technique for sample visualization. In reality, however, high-intensity and repeated illumination can cause degradation to the structure of a fluorophore, limiting its potential for fluorescence in a process known as photobleaching. A fluorophore is excited most efficiently by light of a particular wavelength, known as the excitation maximum. Similarly, the fluorescence output of a fluorophore is easiest to detect at its emission maximal wavelength. The emission maximum for the fluorophore always has a longer wavelength than the excitation maximum, due to energy loss during the transient excited lifetime. This difference between the excitation and emission maxima is called the Stokes Shift (Guilbault, 1990). The excitation and emission spectra of a fluorophore offers information about applied wavelengths to induce and detect fluorescence effectively. Furthermore, when using more than one fluorophore simultaneously, each fluorophore should generate its own distinct emission range, allowing for the simultaneous examination of multiple species labeled with different fluorophores.



Figure 2.6: The fluorescence principle.

Fluorescent dyes offer outstanding sensitivity and specificity for the detection and imaging of macromolecules, including proteins, nucleic acids, lipids and physiological ions, in cells. Fluorescence is therefore the predominant optical method used in confocal microscopy.

2.9 Confocal Microscopy

Laser Scanning Confocal Microscopes (LSCMs) became available in the mid 1980s and were immediately applied to biological and materials research. Advances in optics and electronics for microscope design have led to significant improvements in the current generation of confocal microscope relative to early models.

Confocal microscopy has been used in a wide variety of biomaterials applications, including for example investigation of the mechanisms of

microencapsulation in the controlled drug delivery area (Yeo, 2004), protein uptake studies in ion exchange chromatography for the optimization of chromatographic conditions (Dziennik, 2005), and observation of the morphology and cell assembly in scaffolds for tissue engineering (Zong, 2005).

Confocal microscopes can offer several benefits compared with conventional light and electron microscopes. The most outstanding advantage of confocal microscopy is its ability to noninvasively optically section a specimen. This eliminates any physical sectioning artifacts that are observed with conventional light and electron microscopes. Light that is outside of the optical plane is eliminated using a pinhole design (spatial filters), resulting in improved image contrast, clarity and detection sensitivity. Figure 2.7 graphically explains how a pinhole works. The resolution of a confocal microscope depends on a number of factors including the wavelength of the excitation/emission laser beam, the numerical aperture of the objective lens, the pinhole size, the refractive index of components in the light path and the alignment of the instrument (Cheng, 1994).

As shown in Figure 2.7, the mechanism by which a confocal microscope generates a three dimension reconstruction of an image can be summarized as follows. A scanning laser beam is directed at the focal plane in a specimen by a reflective dichroic mirror. Emitted fluorescent and reflected light is scattered in all directions, but the longer wavelength emitted fluorescent light passes through the dichroic mirror, while the reflected light is deviated by this mirror.A pinhole (confocal aperture) is placed in front of the photodetector, such that only the fluorescent light coming from the focal point can

be detected by the photodetector and out-of-focus information is largely obstructed. This is especially important when dealing with thick specimens if considering light scattering.



Figure 2.7: Schematic of a confocal microscope, (Cheng 1994) with permission.

The plane of focus is controlled by a fine-stepping motor, which moves the microscope stage up and down. Finally, a 3-D reconstruction of the specimen can be generated by stacking 2-D optical sections collected in series. This technique provides researchers with a clear picture of the architecture of materials and sub-cellular structures.

3.0 OBJECTIVES

The ultimate objective of this project was to develop a novel technique to characterise the depth of penetration of protein into hydrogel contact lens polymers and to determine the amount of protein deposited on the lens interface. The in vitro model used in this preliminary study only included a single protein, to avoid the potential confounding effects of protein-protein competition.

Newly introduced silicone hydrogel lenses have their own distinct properties and polymer constituents and have not been as widely studied as conventional materials. They have previously been shown to deposit only low levels of protein, most of which is denatured (Jones, 2003). One hypothesis tested in this thesis was that lysozyme only adsorbs on the surface of silicone hydrogel lenses, particularly on those with a plasma surface modification.

4.0 MATERIALS AND METHODS

4.1 Fluorescence Conjugation

Fluorescent dyes are commonly used in the detection of biological entities including proteins. Conjugation reactions involve the reactive side chains present in peptides and proteins. One of the major intrinsic reactive groups of proteins is the aliphatic ε -amine of the amino acid lysine. The other reactive amines in proteins are the α -amino groups of N-terminal amino acids. The presence of these amine functional groups and their relatively high reactivity with a variety of other functional groups make these aliphatic amine groups the most widely used groups for protein functionalization with fluorescent tags. There are four major classes of fluorescent reagents used to label amines include reactive esters, isothiocyanates, aldehydes and sulfonyl halides (Brinkley, 1992).

Fluorescein isothiocyanate (FITC) is a representative of the most commonly used fluorescent derivatization reagents for proteins. Isothiocyanates have intermediate reactivity and form thiourea bonds with proteins and peptides, as shown in Figure 4.1, at an optimal pH range of 9.0-9.5 in aqueous solution. The fluorescent properties of FITC include an absorbance maximum at 495 nm and an emission peak of 520 nm. FITC usually maintains good detectability in fluorescence assay systems, despite fluorescent quenching that occurs when it is conjugated to proteins.



Figure 4.1: Conjugate reaction of FITC and amine-containing proteins (Hermanson, 1996), with permission.

4.2 Photobleaching and Antifade Reagents

Theoretically, all fluorescent dyes will undergo some degree of photobleaching when exposed to the excitation light. The intensity and duration of the illumination directly impact the extent of photobleaching. Antifade reagents are used to sustain dye fluorescence by inhibiting the generation and diffusion of reactive oxygen species, thereby reducing photobleaching quantum efficiency. In this work, We have investigated the effect of "SlowFade" (Molecular Probes, Eugene, US), which is 1,4diazabicyclooctane (DABCO), which suppresses free radicals and then extends the constant fluorescence emission. SlowFade provides much better protection against photobleaching at a specific pH, so pre-equilibration of the specimen in the commercial buffer for at least 5 minutes is required.

4.3 Principles of Gel Filtration and Dialysis

Gel filtration chromatography is based on the principle of size exclusion. Separation occurs when small molecules diffuse into the gel pores and their flow is retarded, while larger molecules are eluted through the column faster due to their inability to enter the pores. There are two distinct applications for gel filtration chromatography: group separation and fractionation. Group separation separates the components of a sample into two groups based upon their size difference. High molecular weight substances such as lysozyme are excluded from the media of Sephadex and thus elute first, whereas free FITC with a lower molecular weight enters the pores of the Sephadex and elute later.

Sephadex is a bead-formed gel prepared by cross-linking dextran with epichlorohydrin. The gel swells in aqueous solutions. Different types of Sephadex have various degrees of cross-linking and different swelling ratios. The Sephadex G-25 medium used in the current work has a diameter ratio of 1.61 between hydrated and dried conditions and has the ability to remove low molecular weight contaminants from molecules larger than about 5000 Da. Sephadex G-25 has a working pH range of 2-13 without affecting its performance. In group separation applications, the column length is important for resolution and column diameter determines loading capacity. Usually short (5-30 cm), wide columns give satisfactory separation at high sample loading since the productivity decreases with increasing column length.

Dialysis is a separation process based on the selective diffusion of different sized substances through a semipermeable membrane. A dialysate, typically 200-300 times larger than the volume of the sample, results in the creation of a concentration gradient across the membrane. The molecules will then diffuse based on these concentration gradients, resulting in separation of low molecular weight species (which can pass through the membrane) from high molecular weight species, which cannot. In the current work, membranes used were composed of low-binding regenerated cellulose.

4.4 Materials and Suppliers

The key properties of all the soft contact lenses used in this thesis are summarized in Tables 4.1 and 4.2, including their breakdown into two distinct groups of conventional hydrogel and silicone hydrogel materials. USAN is a United States Adopted Name system that identifies components of monomers in contact lenses, according to FDA classification (Table 5.1). All contact lenses examined were unworn and a power of -3.00D was used in all cases. PMMA rigid contact lenses were ordered through a contact lens laboratory in Waterloo, ON, Canada. Other materials and suppliers are summarized in Table 4.3.

4.5 Lysozyme Labeling with FITC

Fluorescein isothiocyanate (FTIC) was stored at -20°C in a dessicator and protected from light until use. The FITC-lysozyme conjugate was synthesized by amine reaction at pH 9.0 in 0.1 M sodium bicarbonate. A mass ratio of 20:1 lysozyme:FTIC

was used (Garrett, 1999a). Briefly, 10 mg/mL of lysozyme in 0.1 M sodium bicarbonate (pH 9.0) was prepared. Since FITC is not stable in aqueous solutions, 10 mg FITC was disolved in dimethyl sulfoxide (DMSO) before immediately adding it to the lysozyme solution with gentle stirring. The conjugation reaction proceeded for 1 hour in the dark. As precipatition of the conjugated protein may occur during the reaction (as FITC-lysozyme is usually less soluble than native protein), the mixture was centrifuged at 12,000g for 1 minute, prior to removal of the free FITC by gel filtration.

Trade Name	USAN	Manufacturer	Centre thickness (µm)	Water content	Principal monomers
Optima FW	Polymacon	olymacon Bausch & Lomb		38%	HEMA
Proclear Compatibles	Omafilcon A	Cooper Vision	110	62%	HEMA, PC
SofLens 66	Alphafilcon A	Bausch & Lomb	100	66%	HEMA, NVP
OmniFlex	Lidofilcon A	Cooper Vision	120	70%	MMA, NVP
Acuvue 2	Etafilcon A	Vistakon	80	58%	HEMA, MAA
Focus Monthly	Vifilcon A	CIBA Vision	100	55%	HEMA, PVP, MAA

Table 4.1: Conventional hydrogel materials

HEMA 2-hydroxyethyl methacrylate; MAA methacrylic acid; MMA methyl methacrylate; NVP N-vinyl pyrrolidone; PC phosphorylcholine; PVP polyvinyl pyrrolidone.

Trade Name	USAN	Manufacturer	Centre thickness (µm)	Water content	Principal monomers
Focus Night & Day	Lotrafilcon A	CIBA Vision	80	24%	DMA, TRIS, siloxane macromer
O ₂ Optix	Lotrafilcon B	CIBA Vision	80	33%	DMA, TRIS, siloxane macromer
PureVision	Balafilcon A	Bausch & Lomb	90	36%	NVP, TPVC, NCVE, PBVC
Acuvue Advance	Galyfilcon A	Vistakon	70	47%	mPDMS, DMA, HEMA, siloxane macromer, EGDMA, PVP
Acuvue OASYS	Senofilcon A	Vistakon	70	38%	mPDMS, DMA, HEMA, siloxane macromer, TEGDMA, PVP

Table 4.2: Silicone hydrogel lens materials

DMA *N*,*N*-dimethylacrylamide; EGDMA ethyleneglycol dimethacrylate; HEMA 2hydroxyethyl methacrylate; mPDMS monofunctional polydimethylsiloxane; NCVE *N*carboxyvinyl ester; NVP N-vinyl pyrrolidone; PBVC poly[dimethylsiloxyl] di [silylbutanol] bis[vinyl carbamate]; PVP polyvinyl pyrrolidone; TEGDMA tetraethyleneglycol dimethacrylae; TPVC tris-(trimethylsiloxysilyl) propylvinyl carbamate; TRIS trimethyl siloxy silane.

Free FITC was removed using a Sephadex G25 column. Elution was performed using PBS (pH 7.4) to separate and exchange the buffer simultaneously. A portable UV light was used to monitor the separation process by observing fluorescence in the column. Following this, dialysis against PBS using a 7 kDa molecular weight cutoff dialysis cassette was performed until no free FITC was detected using a fluorescence spectrophotometer with an excitation wavelength of 488 nm. Generally three changes of

dialysate were used over a period of approximately 18 hours.

Table 4.3: Otl	ner materials	and suppliers
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Items	Suppliers
Hen egg lysozyme (HEL)	Sigma-Aldrich, St. Louis, MO
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, MO
Fluorescein isothiocyanate (FITC)	Molecular Probes, Eugene, OR
Rhodamine-labeled dextran	Molecular Probes, Eugene, OR
SlowFade antifade reagent	Molecular Probes, Eugene, OR
Sephadex G25 media	Amersham Biosciences, Baie d'Urfe
Dialysis cassett kit	Pierce, Rockford, IL
0.2 um syringe filters	Pall Corporation, Ann Arbor, MI
Bottle filters	Nalge Nunc Internal, Rochester, NY
Fluorescence spectrophotometer F4500	Hitachi, Tokyo, Japan
Multiskan Spectrum ELISA Plate Reader	Thermo Electron, Waltham, MA
Confocal microscope, Zeiss LSM 510	Carl Zeiss, Jena, Germany
META	

4.5.1 Determining the Degree of Labeling

The efficiency of fluorescent dyes binding to proteins is usually determined by spectroscopic techniques. FITC/lysozyme (dye/protein) ratio was determined by measuring FITC-lysozyme concentration using UV spectroscopy at 280 nm and the maximum absorbance of FITC (λ_{max} of FITC = 494 nm). Because most dyes have some absorption at 280 nm, the absorption of the conjugate at 280 nm must be corrected for the influence of the FITC, to obtain the correct lysozyme concentration. The molar absorption coefficient (Pace, 1995) for hen egg lysozyme is 34300 M⁻¹cm⁻¹ and can be correlated for absorption and concentration using the Beer-Lambert law:

$$A = \varepsilon lC \tag{4.1}$$

where ε is the molar absorption coefficient (M⁻¹cm⁻¹); *l* is the absorption pathlength (cm); and C is the protein molar concentration (M).

Therefore, from the absorbance measurements at 280nm and 494nm, the lysozyme concentration was determined using the followed equations:

$$A_{lysozyme} = A_{280nm} - A_{494nm} \times CF$$
 (4.2)

CF is the correct factor, which for FITC has a value of 0.30 as shown below.

$$CF = \frac{A_{280 \ freedye}}{A_{494 \ freedye}} \tag{4.3}$$

The lysozyme concentration follows from equation 4.4 below, where DF is dilution factor.

$$FITC - lysozyme(M) = \frac{\left[A_{280nm} - (A_{494nm} \times 0.30)\right] \times DF}{\varepsilon_{lysozyme}}$$
(4.4)

Degree of labeling is defined as

$$DOL = \frac{A_{494nm} \times DF}{\varepsilon_{FITC} \times M_{lysozyme}}$$
(4.5)

where ε for FITC is 68,000 at the data sheet of product. An example calculation for determining the efficiency of the labeling process is shown in Appendix D.

4.6 In vitro Lysozyme Sorption

The incubation solution was sterilized with 0.2 μ m syringe filters before doping with contact lenses. Unlabeled lysozyme was added to the FITC-lysozyme solutions to raise the concentration to the physiologic tear concentration of 1.9 mg/mL. The same concentration of unlabelded lysozyme was also prepared, to dilute FITC-lysozyme at different ratios, depending on the lysozyme amounts deposited on various lens materials. We found that less FITC-lysozyme in the incubation solution partially prevented the photobleaching of FITC, which seriously impacted the examination of lysozyme penetration curves in our preliminary experiments, especially when investigating Acuvue 2 lenses, which accumulated large amounts of lysozyme in the lens matrix. For these experiments we varied the % of FITC-lysozyme used. For example, FITC-lysozyme was 2% (v/v) in the incubation solution for Acuvue 2 lenses whereas undiluted FITC-lysozyme was used for doping the lens that accumulated the least amount of lysozyme (Focus Night & Day).

The hydrogel lenses summarized in Tables 4.1 and Table 4.2 were examined by confocal microscopy for protein uptake. In addition, a polymethyl methacrylate (PMMA)-based rigid contact lens was also investigated as a model of surface adsorption for lysozyme. Before incubation, the contact lenses were left in polypropylene tubes with 15 mL of sterile PBS for at least 30 minutes, to ensure that any influence of the lens packaging solution components was removed. The contact lenses were subsequently transferred into brown Eppendorf vials using sterile metal forceps and placed in a face

up position. Contact lenses were incubated in 1 mL of the protein solutions for various simulated wear periods, at a temperature of 37°C under constant rotation of 70 RPM.

4.7 Preparation of Microscopic Specimens

After specified periods of incubation, the contact lenses were removed from the doping vials for confocal microscopy. Approximately 40 μ L of mounting media (PBS, 0.5mg/mL dextran or antifade reagent) were placed onto the microscope slide prior to mounting the lens. Lenses were rinsed briefly in PBS and the center 4 mm of the lens was punched out. This piece of the lens was wicked dry using a Kimwipe to remove extra buffer and mounted onto the microscope slide. A coverslip was then carefully applied and sealed with nail polish to prevent evaporation of the mounting media and to stabilize the coverslip for use with immersion objectives.

Rhodamine-dextran was dissolved in PBS and vortexed to increase their solubility. The dextran solution was then centrifuged at 12,000 g for 1 min to remove any insoluble particles. In certain experiments, 0.5 mg/mL of dextran solution was applied as a mounting media to the lenses to aid in distinguishing the location of the lens surface.

4.8 Confocal Laser Scanning Microscopy

Lenses were examined by confocal microscopy using the scanning configurations shown in Table 4.4, with a Zeiss LSM 510 META confocal microscope. The processes

parameters, including pinhole diameter, detector gain, amplifer offset, amplifer gain, and scanning speed & area were optimized. Generally, each lens was scanned at 5 locations, at the centre, and at 3, 6, 9 and 12 o'clock, as previously described (Meadows, 1994). Each specimen was duplicated thus 10 scanned images were achieved through following microscopic analyses. The fluorescence signal of FITC was excited at 488 nm via an argon laser and the emission range was set between 505-530 nm. Excitation of rhodamine for imaging dextran was at 543 nm by a helium-neon laser and the fluorescence was detected between 560-615 nm. Each section of z stacks was set at 1 µm intervals. Lenses were scanned with a $40\times$ water immersion objective. Using the software provided with the microscope, the means of the fluorescence intensity were plotted as a function of the scanning depth. All control specimens were from various lenes incubated with 1.9 mg/mL unlabeled lysozyme that showed zero intensity at all parallel scans. 0.5 mg/mL rhodamine-dextran in PBS was used to distinguish the surface of Focus Night & Day, O₂Optix, Acuvue OASYS and PMMA lenses. The antifade reagent used in the mounting media prevented photobleaching during the scans.

4.9 Unit Conversion

By analysis of images obtained from the confocal microscope, fluorescence intensities can be directly plotted as a function of scanning depth in $1\mu m z$ step, which represented lysozyme penetration curves on the various lens materials. Fluorescence intensity was then transformed into microgram amounts based on the radioactive lysozyme uptake results measured on the various lens materials using equation 4.6. This

conversion assumes no thickness variation over the bulk of the lens and a spatially homogenous distribution of protein within the lens. An equation of the calculation is as follows:

$$Lysozyme(\mu g/\mu m) = \frac{\mu g/lens}{Q/lens} \times Q/\mu m$$
(4.6)

where μg /lens is the amount of lysozyme on each contact lens in micrograms measured using ¹²⁵I lysozyme, Q/lens is fluorescence quantum yield represented by emission intensity; and Q/ μ m is emission intensity of each micrometer section. In this way, spatial absolute amounts of lysozyme associated with the different regions of the lens could be determined.

Values used	Functions
40 × water immersion	This objective is for the aqueous mounting media.
1 airy unit = 69.6 μm	A smaller pinhole diameter increases the depth of focus and improves the display of the specimen sructure, while reducing the light intensity received by the detector.
300-900	Setting of image brigthness and contrast
Fixed -0.1	Setting of image background
Fixed 1	Amplication factor between 1 and 3
9 or 13	The signal-to-noise ratio is improved by reducing the scanning speed.
$\frac{230 \times 230 \ \mu \text{m}^2}{38 \times 38 \ \mu \text{m}^2}$	A smaller scanning area improves the maximal scanning speed.
Between 1-10%	The utilizable laser intensity
	Values used $40 \times \text{water}$ immersion 1 airy unit = $69.6 \ \mu\text{m}$ 300-900 Fixed -0.1 Fixed 1 9 or 13 $230 \times 230 \ \mu\text{m}^2$ $38 \times 38 \ \mu\text{m}^2$ Between 1-10%

Table 4.4: Parameters for confocal scanning

5.0 RESULTS AND DISCUSSION

5.1 Conventional Hydrogels

Protein sorption at liquid-solid interface includes adsorption of protein at the surface and the diffusion of protein molecules into the polymer matrix through water filled pores. The sorption behaviour of tear proteins is critical for understanding the mechanisms of contact lens spoliation. In the current study, various lens materials were examined in order to assess the effects of material on the uptake of protein. Based on the water contents of the materials and their charge, commercial contact lenses are classified into four groups by the Food and Drug Administration (FDA) (Table 5.1). These categories are useful for dividing the contact lenses tested into groups and for further discussion.

FDA categorization	Water content	Charge	Contact lenses tested	
Group I	=< 50%	Non-Ionic	Optima FW, Focus Night & Day, O ₂ Optix, Acuvue Advance, Acuvue OASYS	
Group II	=> 50%	Non-Ionic	Proclear Compatibles, Soflens 66, OmniFlex	
Group III	= < 50%	Ionic	PureVision	
Group IV	=> 50%	Ionic	Acuvue 2, Focus Monthly	

 Table 5.1: FDA classification of hydrogel contact lenses.

In addition to the FDA classifications, hydrogel lens materials are currently used in three major wearing modalities: daily wear, extended wear and continuous wear. Daily wear lenses are worn during the day only, removed for cleaning each night and typically replaced every 2 or 4 weeks. Daily disposable is a newer modality of daily wear that avoids tear deposits build up and offers much convenience to patients. However, a recent clinical trial suggests that patients prefer disposable extended-wear over daily disposable (Nichols, 2000). Extended wear lenses are worn overnight for up to 6 nights at a time and are then removed and replaced, with no solution or care regimen required for their maintenance. Continuous wear lenses are inserted and worn for up to 30 days and nights without removal, after which they are replaced. This latter modality is a significant challenge in terms of lens biocompatibility and wearing lenses in this way can induce a number of inflammatory and infective clinical complications.

In this study, all contact lenses tested were incubated for 24 hours, simulating overnight wear and up to four weeks, to investigate the maximum stage of lens spoilation that would be seen in typical lens wear. Furthermore, to investigate the kinetics of protein uptake into the lens material, four lens types, Acuvue 2, Focus Monthly, PureVision and Acuvue Advance were further examined and incubated for various time periods. The presented penetration curve of lysozyme was from a representative scanning image since there was no significant variation between 5 locations for a given specimen. Furthermore, such represented curve truly reflects the shape of the curve.

Lysozyme is a compact globular protein with a molecular weight of 14,400 Da and dimensions $45 \times 30 \times 30$ Å. Its high concentration in tears and relative importance for

contact lenses make it a natural choice for examination of sorption effects. In the current work, penetration data are presented such that the average fluorescence intensity for each 1 μ m image section was transformed into μ g of lysozyme based on radiolabeling results (Table 5.2 and Table 5.3) and the mass associated with each section plotted as a function of penetration depth.

	Optima FW	Proclear Compatibles	SofLens 66	OmniFlex	Acuvue 2	Focus Monthly
1 hour	2.8	4.3	7.8	6.8	98.3	24.6
24 hours	6.3	11.0	16.0	10.8	620	144.3
7 days	13.0	27.0	33.0	29.2	1386	312.8
14 days	16.0	35.3	44.5	37.4	1433	398
21 days	20.7	41.3	50.3	40.9	1429	479
28 days	23.2	43.8	53.3	48.6	1434	512

Table 5.2: Average amounts of lysozyme on conventional hydrogels (µg), (Subbaraman, 2006a).

	Focus Night& Day	O ₂ Optix	PureVision	Acuvue Advance	Acuvue OASYS
1 hour	N/A	N/A	0.8	0.2	N/A
24 hours	0.2	0.33	1.4	0.75	0.53
7 days	1.8	2.8	5.9	3.6	3.1
14 days	2.7	3.7	10.6	8.0	6.1
21 days	3.6	4.9	15.3	12.8	10.2
28 days	4.2	6.1	19.4	16.8	13.4

Table 5.3: Average amounts of lysozyme on silicone hydrogels (µg), (Subbaraman, 2006a).

Optima FW, a relatively pure pHEMA based contact lens, has a hydration of approximately 38% water. It is designed to have a thinner centre thickness (60µm) in order to minimize the barrier to oxygen permeability. Figure 5.1 summarizes the lysozyme sorption for this lens material after periods of 24 hours and 4 weeks of incubation in a 1.9 mg/mL lysozyme solution. It can be seen from the 24 hour penetration curve that over this period the lysozyme mainly remained on the outside of the lens, with low but measurable bulk amounts. After 4 weeks of incubation, lysozyme concentrations in the lens appeared to have achieved a nearly flat profile across the thickness of the lens material. Pure pHEMA has been reported to have an average pore radius of 4-8 Å at 41-42% hydration (Refojo, 1979). This material presumably would not be permeable to lysozyme given the dimensions of the protein. However, in the lens material, the presence of a hydrophobic cross-linking agent and other trace impurities including methacrylic acid present during pHEMA processing may increase the pore size

of lens network (Pinchuk, 1984), contributing to the higher accumulation of lysozyme in this "pure" pHEMA lens.



Figure 5.1: Lysozyme penetration curves of Optima FW in 24 hours and 28 days. Each data point means μg of lysozyme at 1 μm scanned section. The curves were from representative fluorescence images scanned by a confocal microscope.

Figure 5.2, 5.3 and 5.4, which summarize the lysozyme sorption onto three FDA group II lenses, show that these lens materials exhibit similar lysozyme penetration despite lens specific differences. Specifically, lysozyme rapidly penetrated the entire lens even after a period of only 24 hours. Of note, lysozyme sorption curves for both Proclear and SofLens 66 showed small peaks on or near the lens surfaces. Proclear is a pHEMA based lens which incorporates phosphorylcholine (PC), a synthetic phospholipid analog. Phospholipids are found in the outer lipid layers of cell membranes including red blood

cells and are thought to be responsible for cell membrane biocompatibility (Hayward, 1984). PC modification of biomaterials has been widely shown in a number of studies to significantly reduce protein adsorption to the material surface (Feng, 2006; Feng, 2005). Proclear with PC has shown resistance to deposition of both tear proteins and lipids (Young, 1997) as well as a higher resistance to dehydration than other conventional hydrogel lenses, presumably due to the high water affinity of the incorporated PC (Hall, 1999). Based on these facts, it is reasonable to assume that the small peak of lysozyme penetration at both sides of Proclear lenses is related to a PC-rich interfacial region that is generated in aqueous environments due to internalization of the PC components, resulting in highly porous region for PC's hydrophilicity (Court, 2001).



Figure 5.2: Lysozyme penetration into Proclear Compatibles in 24 hours and 28 days.



Figure 5.3: Lysozyme penetration into SofLens 66 in 24 hours and 28 days.



Figure 5.4: Lysozyme penetration into OmniFlex in 24 hours and 28 days.

While incorporation of NVP or the use of PVP chains has been used in some studies for reducing protein adsorption (Robinson, 2002), its effects tend to be much lower than those of other hydrophilic polymers such as polyethylene oxide (PEO) (Chen, 2004; Chen, 2005). However, PVP-containing contact lenses have been found to have high lipid accumulation, presumably due to the lipid solubility of pyrrolidone derivatives (Jones, 1997; Jones, 2000). The peak in protein sorption at the lens surface is presumably due to surface enrichment of the lens material with PVP altering the porosity and more important pore size of the surface layer. OmniFlex, another high water content and nonionic contact lens comprised mainly of MMA/PVP, does not show such characteristics.

Group IV lenses including Acuvue 2 (pHEMA/MAA) and Focus Monthly (pHEMA/MAA/PVP) have a significant negative charge due to the addition of the ionic methacrylic acid (MAA) monomer. These materials contain more than 50% water which results in higher oxygen permeability but, as a result of their higher porosity, pore size and charge, presumably these lens materials would be expected to show higher sorption of the globular and net positive lysozyme relative to other lens materials (Jones, 2003; Senchyna, 2004). Figure 5.5 and 5.6 shows the kinetics curves for these two group IV lenses. From the kinetics curves for the Acuvue 2 lenses, it can be observed that there is significantly more protein associated with these materials than those previously described and lysozyme sorption reaches an equilibrium after a period of \sim 7 days. *Exvivo* and *in-vitro* studies show similar results with lysozyme sorption that reaches a

plateau level after approximately 7 days of incubation or lens wear (Jones, 2000; Keith, 2003; Subbaraman, 2004).



Figure 5.5: The kinetics of lysozyme penetration into Acuvue 2 lenses.

At 1 hour of incubation, lysozyme had only partially penetrated into the Acuvue 2 lens, with the penetration curve showing high concentrations at the lens edge but lower concentrations in the bulk of the lens, However penetration of the protein into this lens material was relatively rapid, with a relatively flat concentration profile observed after 24 hours of incubation. The essential driving forces for lysozyme sorption in Acuvue 2

are presumably not only typical diffusion processes, which are likely higher with these lenses than the pHEMA materials due to higher pore size of these materials, but also electrostatic attractions. Therefore measurement of pore size of the contact lenses may help to differentiate lens porosity and explain the role of electrostatic forces for its high lysozyme accumulation (Gachon, 1986) The functions of MAA on bulk absorption of lysozyme have been studied. Garrett and coworkers (Garrett, 2000) reported that lysozyme amounts increased as a function of increasing percentage of MAA in hydrogels. Furthermore, the degree of lysozyme penetration into pHEMA/MAA polymers was found to increase not only due to charge effects as increases in both water content and pore size were observe with increasing MAA content.

In comparison, Focus Monthly lenses contain polyvinyl pyrrolidone (PVP) in addition to the MAA for increasing water content. As shown in Figure 5.6, the 24-hour penetration curve which shows lower sorption in the middle of the lens suggests that material specific differences between these lenses and the Acuvue 2 lens materials are responsible to slower protein penetration, even with similar water content (58% of Acuvue 2 *vs* 55% of Focus Monthly). Incubation of the lenses for additional time resulted in increased levels of protein in the lens interior. However, even after 3 weeks of incubation, significant surface adsorption remained with slightly lower levels of protein observed in the interior of the lens. Since the degree of penetration is proportional to the pore size (water contents) and/or charge density of contact lenses, lens specific differences are likely the cause of these differences.



Figure 5.6: The kinetics of lysozyme penetration into Focus Monthly lenses.

5.2 Silicone Hydrogel Lenses

Silicone hydrogel lenses have more complex monomer compositions. Commercially available materials contain multiple polymer components. However, regardless of the polymer makeup, the hydrophobic and surface active nature of the silicone component necessitates additional modification or surface treatment. Currently available silicone hydrogel lenses can therefore be divided into two categories depending upon their surface characteristics used to overcome their hydrophobic nature. PureVision, Focus Night and Day and O₂Optix are modified using a plasma treatment. PureVision has a silicate island surface created by plasma oxidation; Focus N&D and O₂Optix are modified by coating with a thin hydrophilic plasma film. Newer technology involves the incorporation of an internal wetting agent. Acuvue Advance and Acuvue OASYS represent a newer technology through the incorporation of a possible non-crosslinked PVP after lens copolymerization.



Figure 5.7: The kinetics of lysozyme penetration into PureVision lenses.

Figure 5.7 shows the kinetics results for the PureVison lenses. It can be seen that there was significant lysozyme penetration into the bulk of the lens matrix, with a gradual buildup of lysozyme as a function of incubations times of up to 28 days. The penetration of lysozyme into the interior of these matrices is likely due to the macroporous nature of this lens as shown by scanning electron microscopy and atomic force microscopy (Gonzalez-Meijome, 2006; Lopez-Alemany, 2002). Under conditions of dehydration and hydration, it was found that the diameter of macropores in the PureVision lens could be as high as $0.5 \,\mu m$, significantly larger than the pore size of conventional hydrogel materials. It is therefore reasonable to assume based on these protein sorption results that these macropores are sufficiently large and connected to permit the diffusion of lysozyme from the bulk solution without significant pore fouling. Furthermore, the size of these pores is likely sufficient to permit the rapid outward diffusion of the lysozyme into the mounting media prior to confocal microscopy analysis, resulting in the slightly lower surface protein concentration observed. Clearly the protein at the interface is not tightly bound as would be expected with a silicone rich surface and is able to release if this is the case.

In contrast, Focus N&D and O_2Optix , shown in Figures 5.8 and 5.9 respectively exhibited quite different protein uptake properties. The profiles show two sharp peaks of lysozyme deposition at or near the lens surface, implying only adsorption of lysozyme with little or no penetration into the lens material. Acuvue OASYS, which has no surface treatment, showed a similar penetration profile as Focus N&D and O₂Optix (Figure

5.10). This is presumably due to the lens having a relatively tight structure with low pore sizes. Surprisingly, Acuvue Advance, which is manufactured using a similar procedure to OASYS, showed quite different kinetics, with both lysozyme absorption and adsorption (Figure 5.11). As well, an uneven distribution of protein was noted consistently on these lens materials. The kinetics otherwise appeared consistent with a moving boundary.



Figure 5.8: Lysozyme adsorption onto Focus Night & Day in 24 hours and 28 days.



Figure 5.9: Lysozyme deposition to O₂Optix in 24 hours and 28 days.



Figure 5.10: Lysozyme sorption on Acuvue OASYS in 24 hours and 28 days.



Figure 5.11: The kinetics of lysozyme penetration into Acuvue Advance lenses.

Further investigation of these materials to confirm surface adsorption only were performed using a rhodamine conjugated dextran, with a MW of 2,000,000 Da to distinguish the "surface" of the lens as the large dextran molecule would not be expected to penetrate into the lens matrix. All contact lenses for scanning with the dextran were incubated for 4 weeks. As well, poly (methylmethacrylate) (PMMA) lenses were examined as controls as these rigid contact lens materials are believed only to permit water and ion permeation through their compact polymer network. Figure 5.12 shows that lysozyme and dextran show similar permeation characteristics on a PMMA lens, with peak and zero intensity occurring at the same location suggesting that dextran should be a reasonable model assuming that it does not penetrate the experimental lens materials.



Figure 5.12: Fluorescence curves of dextran and lysozyme on a PMMA lens. The curves were from the single surface of the lens.

It can be seen from Figures 5.13 and 5.14 for Focus N&D and O₂Optix, the fluorescence intensities for both dextran and lysozyme simultaneously showed a dramatic change at the lens "surface". Therefore, it seems that the silicone hydrogel materials with the plasma coating adsorbed lysozyme, with minimal penetration of the protein into the lens structure. It has been shown that the surface topography of the Focus N&D lenses is similar to that observed on a rigid lens (Gonzalez-Meijome, 2006; Merindano, 1998).



Figure 5.13: Fluorescence curves of dextran and lysozyme on a Focus Night & Day lens. The curves were from a single surface of the lens.



Figure 5.14: Fluorescence curves of dextran and lysozyme on an O₂Optix lenses lens. The curves were from a single surface of the lens.


Figure 5.15: Fluorescence curves of dextran and lysozyme on an Acuvue OASYS lens. The curves were from a single surface of the lens.

Further scanning of the OASYS lenses demonstrated considerable differences between the curves for lysozyme and dextran, as shown in Figure 5.15. When the fluorescence intensity for the dextran dropped to nearly zero, lysozyme intensity was at a maximum. This indicates that the lysozyme is partially adsorbed but that a small amount penetrates the lens surface. This would not be inconsistent with the OASYS lenses containing a wettable hydrophilic and porous PVP layer due to the incorporation of the wetting agent. While it is unknown how the PVP migrates to the lens surface, migration is presumably mediated by a hydrophilic driving force as shown previously with PEO modified silicone rubber (Chen, 2005). It is known that PVP is added later in the manufacturing process and is therefore not covalently linked to the lens structure. These non-crosslinked molecules may lead to a low fouling, low-friction surface, by extending tens of nanometers out of the lens surface. This interfacial PVP layer likely has a high water content, and high pore size region which allows for the penetration of lysozyme.

By using high MW dextran as a marker molecule and scanning the lenses with multiple laser channels, it is clearly possible to delineate differences between surface adsorption and absorption of protein. Silicone hydrogel lenses with a thin plasma layer as a surface coating adsorb protein while both adsorption and absorption were observed on OASYS lenses with an internal wetting agent even though these lenses appeared to show quite similar sorption features when lysozyme was applied in the absence of dextran.

As a general rule, protein sorption to a more hydrophobic adsorbent surface results in greater protein denaturation and a higher amount of irreversible adsorption (Arai, 1990). Furthermore, while most surfaces adsorb in monolayer amounts, lysozyme has the unusual characteristics of forming adsorption multilayers at a hydrophilic (Graham, 1979; Tilton, 1993) and hydrophobic (Schmidt, 1990)surface. It is known that the theoretical surface monolayer coverage of lysozyme is between 207-310 ng/cm² in a range of "side-on" or "end-on" arrangement (Garrett, 1999a). Depending on the penetration results of lysozyme and dextran, the lysozyme amounts at one surface of lenses was therefore calculated. Table 5.2 summarizes surface adsorption of lysozyme to three types of contact lenses after 24 hours and 28 days of incubation (See Appendix C for calculations). None of the lenses was covered by a complete monolayer after

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contacting lysozyme solution for 24 hours. Four week data showed significantly more lysozyme deposition. Acuvue OASYS showed the highest levels of lysozyme. Data of advanced contact angles by an *in-vitro* model revealed Acuvue OASYS lenses show higher values angles than Focus N&D and O_2Optix under unworn conditions (Jones, 2006), implying a more hydrophobic surface under the measurement conditions. It should be noted that the calculation of multilayer adsorption was based on the non-unfolding theory (Fersht, 1992) since lysozyme is a "hard" protein that is not easy to undergo unfolding.

Incubation times	Contact lenses	Single surface amounts (ng)	Single surface area (cm ²)	ng/cm ²	Theoretical layers	Lysozyme amounts (ng)
	Focus N&D	77	1.89	41	< 1	200
24 hours	O ₂ Optix	132	1.99	66	< 1	330
	Acuvue OASYS	188	2.00	94	< 1	530
	Focus N&D	1840	1.89	974	3.1-4.7	4,200
1 weeks	O ₂ Optix	2297	1.99	1154	3.7-5.6	6,100
4 weeks <u>Acuvue</u> 3665	3665	2.00	1832	5.9-8.8	13,400	

Table 5.4: Calculation of lysozyme adsorption

A range for a theoretical monolayer is 207-310 ng/cm² (Garrett, 1999a).

While it would be interesting to assess the levels of surface adsorption to the traditional hydrogel lenses, the double labelling experiments with the dextran were not performed with these materials as there was clearly matrix penetration. Acuvue 2 lenses

containing pHEMA and MAA for example showed some penetration of the large dextran molecule into the bulk of the lens, making delineation of the surface difficult (Appendix B: Figure B.1).

Lysozyme on Focus N&D and O_2Optix produced a typical multilayer of surface adsorption. It is possible that contributions of surface lysozyme to clinical symptoms of discomfort after extended wear can be explained by this study. High concentrations of denatured lysozyme at interface of the tears and contact lenses may lead to aggregation which in turn affects the tear film-derived biofilm initially deposited on the lens surface This may reduce wettability and enhance friction. Since even the (Jones, 2002). relatively low amounts lysozyme present on these lenses is on the surface, it will directly interact with the ocular environment. On the OASYS lenses, the thickness of the lysozyme layer at the interface is between 18 and 40 nm with some of it localized in the layer adjacent to the surface. Depending on the orientation, this represents 6-9 layers of lysozyme on the lens surface, which seems to build up as a function of time. This build up of lysozyme on the lenses may eliminate the low friction domains of extended noncrosslinked PVP wetting agent (Kim, 2002), increasing mechanical friction between eyelids, cornea, and contact lenses, and thus contributing to the clinical symptoms of decreased comfort with extended periods of wear and more serious complications such as contact lens associated papillary conjunctivitis (CLAPC) that is mainly induced by mechanical disturbances (Dumbleton, 2003). Also, Baguet and colleagues reported the increased surface roughness with more tear component deposits on hydrogel lenses

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(Baguet, 1995) which may also be a factor in decreasing patient comfort with lens wear. However, Acuvue OASYS is intended to 1-2 weeks extended wear. The thickness of built-up lysozyme from such period will be more useful.

However, the performance of contact lenses is multifactorial. Further well-designed investigations and various technique applications need to be pursued before confirming these hypotheses. In summary, based on all results we present, the quantitative confocal microscopy performed in the current work allows for the characterization of protein location (i.e. adsorption and absorption) on/in contact lens polymers. Application of this technique to better understanding the interactions of proteins with other biomaterials may provide greater insight into absorption effects, which are generally ignored.

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

In this study, confocal scanning microscopy was used to qualitatively examine the location of FITC labelled lysozyme in contact lenses. By combining this technique with radiolabeling techniques, the amount of lysozyme in 1 µm sections throughout the lens structure could be quantified. A total of 12 different commercially available contact lenses were examined including conventional hydrogel lenses, silicone hydrogel lenses and a rigid contact lens. As expected, different sorption profiles were found with a variety of factors influencing the lysozyme uptake including porosity of polymer network, and charge of polymer components.

Lysozyme partly penetrated Optima FW, a relatively pure pHEMA lens, in 24 hours and achieved an equilibrium penetration during the 4-week incubation. Three high water content (>50%) and non-ionic contact lenses, Proclear Compatibles, SofLens 66 and OmniFlex, exhibited quick penetration to the lens bulk, with high levels of accumulation in these lenses. Two high water content and ionic contact lenses showed significantly different profiles after 24 hours of protein exposure. Acuvue 2, a copolymer of pHEMA/MAA, showed a flat lysozyme penetration curve similar to the three high water content and non-ionic lenses, whereas Focus Monthly exhibited a curve which showed slower sorption and lower bulk lysozyme amounts compared with the surface. The presence of PVP in the latter lens is presumed to be a significant contributing factor to these differences. Methacrylic acid only is likely the most important factor in the

accumulation of lysozyme in Acuvue 2 lenses because of its negative charge at carboxyl groups in physiological environment. However, quick penetration of lysozyme in three non-ionic lenses (Proclear Compatibles, SofLens 66 and OmniFlex) suggests that pore size of contact lenses is also a dominant factor for penetrative speed. Therefore, the differences between Focus Monthly, a copolymer of pHEMA/MAA/PVP and Acuvue 2, which is comprised of pHEMA and MAA only are likely due to a combination of factors.

Silicone hydrogel lenses showed more distinct penetration profiles depending on their bulk and surface characteristics. By using a kinetics study, it was found that lysozyme penetrated the bulk of PureVision after 24 hours. With further incubation, the lysozyme continued to gradually build up in the lens matrix. By localising the lens surface using labelled dextran it was found that Focus N&D, O₂Optix and a PMMA rigid lens only showed surface adsorption of lysozyme even up to 4 weeks. Acuvue Advance containing the internal wetting agent PVP however showed quite different penetration kinetics at all six time points in a kinetics study. There was greater accumulation of lysozyme in the lens during the incubation periods, although the curves showed peaks in lysozyme sorption near the lens surface. Penetration of the lysozyme into the bulk of the lens material was also significant. It is believed that these sorption curves are the result of a porosity gradient in Acuvue Advance, probably due to the local concentration of PVP which increases the porosity of these materials. Lysozyme sorption may result in such clinical complaints as dryness after an extended period of wear. Acuvue OASYS

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was found to deposit less lysozyme than the Acuvue Advance lens, with a deposition curve similar to that obtained for Focus N&D and O₂Optix despite only subtle changes to the processing procedure. However, using dextran it was found that this lysozyme was deposited just below the lens surface. Higher water content in the silicone hydrogel lenses seemed to correlate with higher levels of lysozyme adsorption although there are clearly a number of other factors which affected this result. It is hypothesized that the thickness of lysozyme layers on the Acuvue OASYS lens after 4-weeks of incubation may negatively affect the lubricant effect of the extended non-crosslinked PVP, thus partially explaining the discomfort cited by contact lens wearers after extended periods of wear.

Recommendations:

In this work, it was found that localizing the lens surface is critical for accurate determination of the penetration characteristics of proteins in contact lenses. As well, additional scanning should be optimized in future work in order to obtain more accurate profiles of protein penetration. The confocal microscope suffers from some technical weaknesses which limit its application in these measurements because of photobleaching of fluorescent dyes during serial scanning. An alternative would be a two-photon microscope that avoids this technical shortfall. Therefore in the future it may be useful to examine the protein deposition profiles using this instrument.

Based on current results and methodology, additional studies examining the interaction between tear components and lens materials, including bulk and surface, are warranted. It would be of interest, for example, to:

1. Investigate the penetration profile of lysozyme in a combination of tear-film proteins to observe the competitive effects of other tear components.

2. Investigate the effect of protein size and charge in various lens materials by using simultaneous scanning of two or three proteins labeled with various fluorescent dyes.

3. Investigate lipid penetration profiles into contact lenses.

4. Investigate the desorption of proteins or lipids after exposing the lens materials to various care solutions

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8.0 APPENDIX A: Representative Confocal Images



Figure A 1: Confocal images (X-Z planes) of an O_2Optix lens. The graph is composed of three sections. The left red signals were dextran with red fluorescence. The middle green fluorescence represented lysozyme labeled with FITC. The right side was a combination image of dextran and lysozyme. The dark section between the top and bottom fluorescence signals were the matrix of O_2Optix , which indicates adsorption of dextran and lysozyme on the lens surface. This image was from a lens following incubation for 4 weeks.



Figure A 2: Confocal images (XY-Z planes) of Acuvue Advance (A) and Acuvue OASYS (B). The magnification was $400 \times$ and the area of each square section was $38 \times 38 \mu$ m. The change of fluorescence showed the partial penetration within theses lenses. The images were from 4-week incubation.



Figure A 3: Confocal images (XY-Z planes) of Optima FW (A), Acuvue 2 (B), SofLens 66 (C) and PureVision (D). The magnification was 400 × and scanned area of each square section was 38 × 38 μ m. The fluorescence throughout the bulk of lenses exhibited penetration of lysozyme. The images were from 4-week incubation.

9.0 APPENDIX B: Supplemental Curves of Confocal Microscopy.



Figure B 1: Fluorescence curves of dextran and lysozyme on an Acuvue 2 lens. The fluorescence intensities of dextran in the lens matrix and overlapped part of two curves indicate the penetration of dextran within the lens.



Figure B 2: Confocal scanning with/without antifade. There was no significant photobleaching to the curve with antifade reagent. The curves were from Acuvue 2 lenses with 4-week incubation.

10.0 APPENDIX C: Surface Area of Contact Lenses

One assumption is a contact lens is the part of a sphere. Figure 9.4 shows the relationship between a contact lens and a sphere. The surface area of contact lenses was calculated by the equation:

Surface =
$$2 \times \pi \times R \times h$$

Where R is the radius of the sphere, h is the height of a contact lens that is measured by a magnitude projector. Because the radius of lens base is 7 mm. R is calculated by the following equation.



Figure C 1: Schematic of a contact lens cut from a sphere. The top cap with a smaller circular base is assumed to be the contact lens.

	Tabl	e C	1:	Calcu	lation	of	lens	surf	ace	area
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Contact lenses	Average h (mm)	R (mm)	Surface area (mm ²)
Focus N&D	3.34	9.00	189
O ₂ Optix	3.79	8.36	199
Acuvue OASYS	3.83	8.31	200

11.0 APPENDIX D: An Example Calculation of Fluorescence

Labeling

1. The calculation of FITC-lysozyme concentration

Average of $A_{280} = 0.526$, Average of $A_{494} = 0.532$, the conjugate solution was diluted 10 times in PBS.

By the equation, lysozyme concentration (M) = $\{A_{280}-(A_{494} \times 0.30)\} \times DF /\epsilon = \{0.526-$

 (0.532×0.30) \times 10 \times /34,300 = 1.07 \times 10⁻⁴ M

So the mass concentration of lysozyme = $M \times MW$ of lysozyme = $1.07 \times 10^{-4} \times 14,400 =$ 1.54 mg/mL

2. The degree of labeling

 $A_{494} \times DF / 68,000 \times M = 0.532 \times 10/68,000 \times 1.07 \times 10^{-4} = 0.73$, which means an average of approximately 0.7 FITC molecules are conjugated to each lysozyme molecule.

12.0 APPENDIX E: Sephadex G-25 Column Packing

Calculation of the column volume was obtained using the following equation: $V = \pi R^2 \times h = 3.14 \times (1.25)^2 \times 15 = 74$ mL. Because the ratio between dried and wet Sephadex G-25 is 1g: 5 mL and the solvent is required to 1.5 times of the wet Sephadex volume, 15g of Sephadex G-25 media were weight out and dissolved in 115mL of PBS. This column media was degassed for 30 minutes with stirring to remove bubbles. After straightening the column by a leveler, the swollen G-25 slurry was poured into the column. A pump was used to pack the gel bed at a flow rate of 4mL/min until the height of the bed did not change. Once elution of the samples was complete, the Sephadex G-25 column was cleaned with 2 column volume of 0.2 M NaOH to remove precipitated protein and dyes, followed by rinsing of 5 column volume with PBS. 0.02% sodium azide in PBS was used as a storage solution to prevent microbial growth.

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