Evaluation of a Novel Glycoprotein On Commercial Contact Lenses

by

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

Waterloo, Ontario, Canada, 2015

Author's Declaration

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

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

Abstract

Purpose

The purpose of this thesis was to evaluate the interaction of a novel glycoprotein, known as proteoglycan 4 (PRG4), with various commercial contact lenses.

Methods

PRG4 was investigated for its effects on commercial contact lenses. Both bovine PRG4 and recombinant human PRG4 (rhPRG4) were examined on six silicone hydrogels (balafilcon A, senofilcon A, lotrafilcon B, comfilcon A, delefilcon A, narafilcon A) and one conventional hydrogel (etafilcon A). Lens parameters, such as material wettability, bacterial adhesion and viability, and location of sorbed PRG4, were investigated in the following:

- The effect of PRG4 on the wettability of both commercially available silicone and conventional hydrogel lens materials was investigated *in vitro*. Additionally, the substantivity of PRG4 onto the lens surface was also examined (Chapter 3)
- Using a novel labeling technique, rhPRG4 was fluorescently tagged and visualized, using confocal microscopy, to elucidate the sorption profile within various commercial lens materials (Chapter 4)
- The antibacterial effect of bovine PRG4- and rhPRG4-treated lenses was investigated. *Staphylococcus aureus* was radiolabeled with ^{3}H-uridine and the bacterial suspension was exposed to various lenses. The total bacterial adhesion was measured by using a Beta counter to detect the ^{3}H isotope, and viability was determined using an agar

plate counting method for each lens type (Chapter 5)

Results

The lens material largely influences how PRG4 interacts with the contact lens. The presence of a surface treatment and/or ionically charged monomers has a significant impact on the wettability and sorption of PRG4. Generally, the incorporation of PRG4 onto relatively hydrophobic surfaces appears to enhance the surface wettability, though PRG4 exhibited greater substantivity on relatively hydrophilic surfaces.

PRG4 did not demonstrate significant antibacterial properties against *S. aureus*. However, PRG4 did not significantly increase bacterial adhesion, even though proteins on lens surfaces are known to attract more bacteria. In addition, it is possible that PRG4 is denatured to a certain extent within the lens, which may ultimately serve as a source of nutrients for the bacteria to thrive on. Future work is required to investigate this supposition.

Conclusions

The results from this thesis have demonstrated that PRG4 can have a significant positive impact on lens material wettability and perhaps other metrics as well, which can possibly translate to enhanced lens wear comfort and lower drop-out rates. Since the lens composition is the major contributing factor in how PRG4 interacts with the material, lens manufacturers can potentially use this information to develop lenses to better incorporate PRG4 for various wear modalities. The unique combination of contact lens and PRG4 is a fairly novel area of research and has the potential for future additional studies.

Acknowledgements

First and foremost, I wish to sincerely thank Dr. Lyndon Jones for accepting me into the Centre for Contact Lens Research. I am truly fortunate and grateful to be given this unforgettable graduate experience. His helpful guidance and endless encouragements have allowed me to succeed in becoming a better person. His unyielding commitment and dedication to contact lens research has been a source of inspiration and a reminder to always strive for excellence.

I would also like to acknowledge Dr. Lakshman Subbaraman for all of his assistance during the course of my Master's. You have been a wonderful mentor and I deeply appreciate the trust and support that you have shown me.

Additionally, I would also like to thank the members of my committee, Drs. David McCanna, Vivian Choh and Kristine Dalton. Your suggestions and insights during our meetings have been invaluable and an instrumental component to the success of this thesis.

I wish to show my appreciation to Dr. Tannin Schmidt for supplying the PRG4 for my thesis. You have always ensured that the PRG4 shipment would arrive on time, which I am truly thankful for. It has been an honor in collaborating with your research group at the University of Calgary, and I hope to see PRG4s potential commercial application in the near future.

To Miriam Heynen, Jaya Dantam, and Negar Omali, thank you for sharing your expertise and the countless hours of training. The lab is truly fortunate to have you all.

Thank you to the Graduates in Vision Science (GIVS) and members of the School of Optometry. You are my second family away from home, and I am truly blessed to have met each and every one of you. You have all been a significant factor in making my graduate experience worthwhile, and I wish you all great success in your future endeavors.

This thesis was made possible through the generous funding by Natural Sciences and Engineering Research Council (NSERC) of Canada, Canadian Institutes of Health Research (CIHR), and the Canadian Optometric Education Trust Fund (COETF).

Dedication

I dedicate this thesis to my friends and family.

Thank you all for your support and believing in me.

I would also like to specially dedicate this thesis to Anita.

You have been with me every step of the way and providing me the strength in overcoming my obstacles.

Your support and endless love was the source to my motivation, and this would not have been made possible without you.

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List of Symbols and Abbreviations

ABA	4-azidobenzoic acid
ACA	advancing contact angle
AFM	atomic force microscopy
ANOVA	analysis of variance
CFU	colony forming units
CH	conventional hydrogel
CHO	Chinese hamster ovary
CLSM	confocal laser scanning microscopy
CPM	counts per minute
Dk	oxygen permeability
DMA	*N*,*N*-dimethylacrylamide
DMSO	dimethyl sulfoxide
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
FM0411M	2-ethyl [2-[(2-methylprop-2-enoyl)oxy]ethyl] carbamate
GAG	glycosaminoglycan
HA	hyaluronic acid
HEMA	hydroxyethyl methacrylate
HPLC	high performance liquid chromatography
HSD	Honestly Significantly Different
IBM	isobornyl methacrylate
ISO	International Organization for Standardization
kDa	kilodalton
M3U	α-[[3-(2-[[2-(methacryloyloxy)ethyl] carbamoyloxy]ethoxy)propyl] dimethylsilyl]-ω-[3-(2-[[2-(methacryloyloxy)ethyl] carbamoyloxy] ethoxy)propyl]poly([oxy[(methyl) [3-[ω-methylpoly(oxyethylene)

	oxy]propyl]silylene] /[oxy[(methyl)(3,3,3-trifluoropropyl)]silylene]/ oxy (dimethylsilylene)]
MA	methacrylic acid
mg	milligram
ml	millilitre
mM	millimolar
mPDMS	monofunctional polydimethylsiloxane
MWCO	molecular weight cut off
NVA	*N*-vinyl aminobutyric acid
NVP	*N*-vinyl-pyrrolidone
OCA	optical contact angle
OD	optical density
PBS	phosphate buffered saline
PBVC	poly[dimethysiloxy] di [silybutanol] bis[vinyl carbamate]
PEX	hemopexin
pHEMA	poly(hydroxyethyl methacrylate)
PRG4	proteoglycan 4
PVP	polyvinylpyrrolidone
rhPRG4	recombinant human proteoglycan 4
RPM	rounds per minute
SH	silicone hydrogel
SMB	somatomedin-B
TAIC	1,3,5-triprop-2-enyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione
TEGDMA	tetraethyleneglycol dimethacrylate
TPVC	tris-(trimethylsiloxysilyl) propylvinyl carbamate
TRIS	trimethylsiloxy silane
TSA	tryptic soy agar
TSB	tryptic soy broth
µg	microgram

µl	microliter
USAN	United States adopted name
UV	ultraviolet
VMA	*N*-Vinyl-*N*-methylacetamide
γ	interfacial tension
θ	theta

Chapter 1 – Introduction

1.1 Contact lenses

The prevalence of individuals with some form of refractive error, such as myopia, has increased substantially between the 1970's and 2000's, and these rates show no signs of diminishing into the new millennium.[1,2] To correct for the reduced visual acuities, spectacles (glasses), contact lenses, and refractive surgery are made available to patients. Despite spectacles being one of the mostly commonly used methods for vision correction, the use of contact lenses is growing in popularity, particularly amongst young adults.[3] This observation can be best explained by the convenience and cosmetic benefits that contact lenses have to offer.[4-6]

1.1.1 Conventional hydrogels

Originally, contact lenses were made from a hard plastic material known as Perspex, but the majority of lenses currently prescribed are soft (conventional hydrogel) lenses.[7] Hydrogels, in general, are three-dimensional networks of polymers that have the capacity to absorb large amounts of water.[8] Wichterle and Lim[9] pioneered the development of the first Food and Drug Administration (FDA) approved hydrogel lens in 1970. This lens was made from the polymer poly-2-hydroxyethyl methacrylate (pHEMA or polymacon).[10] The advantages of pHEMA are that it is optically transparent, flexible, and physically resistant to changes in temperature and pH, thus making it appropriate as a biomedical device on the eye.[9] Since then, pHEMA has evolved substantially and takes on several variations, as seen by the wide assortment of lenses currently on the market. Despite the material diversity

amongst lenses, they all addressed a common issue, which was a lack of oxygen being delivered to the cornea during lens wear from the original Perspex lenses that often resulted in corneal oedema and the formation of hypoxic signs such as microcysts.[11,12] Oxygen permeability (Dk) in conventional hydrogel lenses is primarily governed by the amount of oxygen that is dissolved in the water component of the lenses.[13,14] Since pHEMA has a maximum water content of approximately 38%, polymacon lenses have a Dk of approximately 10 units.[15] Therefore, most pHEMA based lenses contain additional polymers, such as methacrylic acid, that have higher water binding affinities to increase the lens overall water content and indirectly its oxygen permeability.[16] However, conventional hydrogel lenses still have relatively mediocre Dk values of < 45 units,[15] and work conducted by Holden and Mertz[17] suggested that a minimum value of 87 units was required by the cornea, especially during overnight lens wear.

1.1.2 Silicone hydrogels

The end of the 20th century (1998-99) saw the introduction of a new line of contact lens materials known as "silicone hydrogels". These materials were essentially conventional pHEMA-based hydrogels that incorporated elements of silicone rubber.[14,18,19] The end result was a material with significantly enhanced Dk because it did not use water but rather the siloxane moieties to transport oxygen through the lens, while maintaining the transparency and flexibility of a conventional hydrogel lens. Issues of contact lens induced hypoxia were reduced,[20-22] as eye care practitioners began prescribing silicone hydrogels over conventional hydrogel lenses for their superior oxygen permeability.[23] However, these siloxane components inherently increased the materials hydrophobicity and, as a result, the water

content was significantly less than that of conventional hydrogels.[24] As with conventional hydrogels, silicone hydrogel lenses have undergone several stages of enhancements, with the primary goal of enhancing the water content and surface wettability of the lens. These two characteristics may potentially have a role in modulating lens wear comfort and governing the material's biocompatibility on the ocular surface.

1.1.3 Lens grouping system

Given the vast diversity of commercially available lenses todays, the FDA has categorized lenses into groups based on their material ionicity containing a negative charge and water content.[25] Four FDA approved groups were initially developed in an attempt to categorize the numerous types of conventional hydrogel lenses, as shown in Table 1-1. However, this simplistic grouping system did not adequately reflect the level of complexity in silicone hydrogel technology. With the inclusion of hydrophobic siloxane polymers, it fundamentally changed how the material interacted with care solutions and tear film components compared to conventional lenses. Therefore, a fifth FDA group had been exclusively proposed for silicone hydrogels, which was further divided into four subgroups, as shown in Table 1-2.[26,27]

Table 1-1. Approved FDA grouping system for conventional hydrogel lenses.

FDA Group	**I**	**II**	**III**	**IV**
Ionic	No	No	Yes	Yes
Water Content	<50%	>50%	<50%	>50%
Example Lens	Polymacon (soflens 38)	Proclear (omafilcon A)	Durasoft 2 (phemfilcon A)	Acuvue 2 (etafilcon A)

Table 1-2. Proposed FDA grouping system for silicone hydrogel lenses.[25,26]

FDA Group	V-A	V-B	V-C	V-D
Ionic	No	No	No	Yes
Water Content	<50%	<50%	>50%	-
Surface Treated	Yes	No	-	-
Example Lens	Air Optix (lotrafilcon B)	Acuvue Oasys (senofilcon A)	Biofinity (comfilcon A)	PureVision (balafilcon A)

1.2 Biomaterial wettability

The phenomenon known as "wettability" is commonly reported as the interaction between a fluid/aqueous phase with a solid surface.[28] Another description of wettability is in terms of hydrophilicity and hydrophobicity, which describes the materials preference for water.[29] A material is said to be wettable (hydrophilic) if the fluid phase spreads over the surface with relative ease until it reaches equilibrium. Conversely, a material that is not wettable (hydrophobic) will resist the spread of water. Generally, water molecules will assemble in the form of a droplet in order to have the majority of molecules sequestered within the bulk to minimize surface area. The cohesive force that holds the droplet together is known as surface tension, as shown in Figure 1-1.[30] In the case of a pure liquid, molecules within the bulk have a net force of zero because each molecule experiences an equal attractive "pulling" force from neighboring molecules (Figure 1-1). However, molecules at the surface experience a net force that pulls it towards the center, resulting in the liquid to adopt a shape with the smallest surface area and surface free energy, hence a droplet.

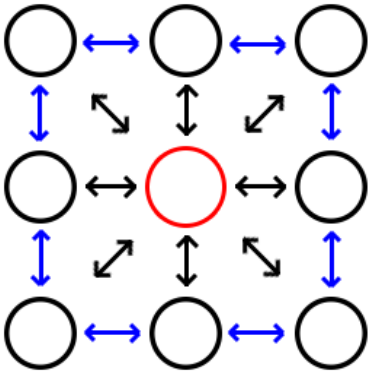

Figure 1-1. Attractive forces between water molecules. Molecules in the center (red circle) are equally attracted to neighboring molecules. Molecules at the surface are held together by a cohesive force known as surface tension (blue arrows).

When the liquid phase interacts with a solid surface, another force needs to be considered, known as interfacial tension. Attractive forces between molecules in these two phases become significant and can further influence the shape of the liquid droplet.[29] Interfacial tension is particularly relevant in the development of biomaterials and their compatibility, since they are in contact with living tissues and biological fluids.

Despite wettability being a widely accepted material property, it does not have its own set of units. Wettability is often a term that is loosely used in the sense that a material is or is not wettable. However, the use of contact angle measurements has provided an objective method of measuring wettability. This concept was initially proposed by Young[31] and later perfected by Dupré in 1869,[32] where the Young-Dupré equation was formulated (1):

$$\cos\theta = (\gamma GS - \gamma SL)/\gamma GL \qquad (1)$$

where θ is the angle formed at the interfacial tension (γ) between the solid (S), liquid (L), and gaseous (G) phase. Therefore, the smaller the contact angle, the "more" wettable the material surface is.

1.2.1 Significance of wettability

The application of wettability and contact angle measurements has proven useful in the evaluation of biomaterials, particularly in the design of contact lenses.[33] The success of a contact lens is primarily driven by the patient through their perceived comfort rating. There is a multitude of factors that influences lens wear comfort, but, there is a general acceptance that biocompatibility is a significant factor. It should be borne in mind that biocompatibility refers not only to the material characteristics but also its interaction with the surrounding environment.[34,35] A biocompatible contact lens can remain on the ocular surface without affecting the natural processes that take place, such as the formation and integrity of the overlying tear film. When lenses are worn, they lie within the aqueous layer of the tear film, thus disrupting its overall integrity and structure.[36] The pre-lens tear film that covers the anterior portion of the contact lens is thinner and more unstable than the tear film that covers the cornea in the absence of the lens. Consequently, there is a decrease in the tear break-up time,[37,38] which correlates to symptoms of dryness and discomfort during lens wear.[39-42] These symptoms are largely responsible for lens discontinuation, with dropout rates as high as 34%,[43,44] which is why manufacturers have acknowledged that wettability is an important lens material metric to enhance lens biocompatibility.

1.2.2 Measurement techniques

There is a variety of ways to measure lens material wettability, both *in vivo* and *in vitro*. A few examples of *in vivo* measurements involve the use of a slit lamp biomicroscope for visual inspection,[42,45,46] measuring the non-invasive tear break-up time over the lens

surface using placido rings from a corneal topographer (Figure 1-2),[47,48] or interference fringes [49,50] to analyze tear film thickness and stability. However, *in vitro* wettability measurements are often assessed first to produce baseline measurements, which are accomplished using three well-established techniques: sessile drop, captive bubble, and Wilhelmy plate.[28,30,51,52]

Among the three techniques listed, the sessile drop is the most commonly used to measure contact lens wettability.[53-56] Although the sessile drop is predominately used for *in vitro* studies, Haddad et al.[57] were successful in performing this technique *in vivo.* For *in vitro* measurements, the contact lens material is placed onto a curved mount after blot drying away excess solution from the lens surface. The lens-mount is placed directly underneath a syringe that suspends approximately 5 µl of high performance liquid chromatography (HPLC) grade water. As it was alluded to earlier, purified liquid is used because molecules within the bulk will experience the same cohesive forces from neighboring molecules. The lens-mount is raised until it contacts the droplet. The attractive forces between liquid and

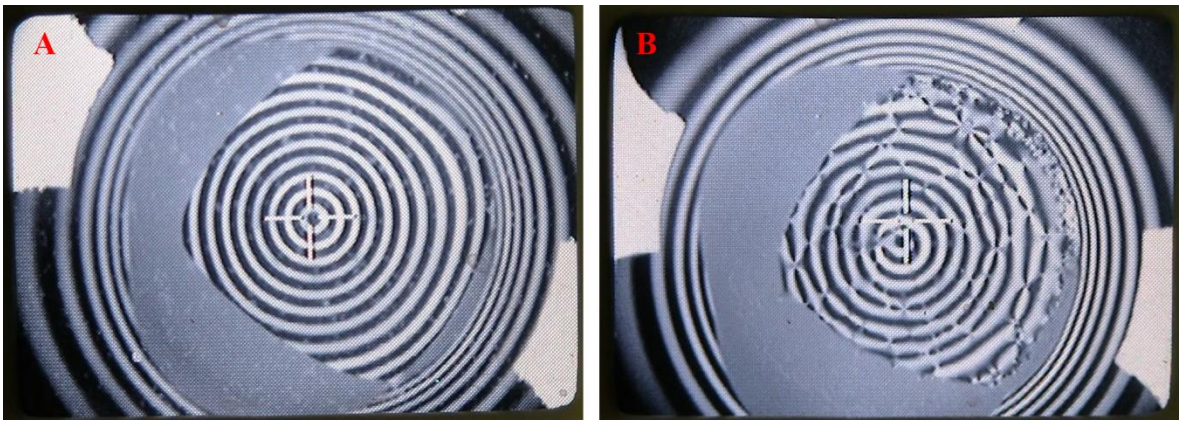

Figure 1-2. Placido rings measured from a corneal topographer on a A) wettable lens surface and B) poorly wettable lens surface. Images courtesy of Hendrik Walther.

solid molecules through interfacial tension will dictate the degree of spreading, where the liquid will eventually stabilize. A goniometer, which is an instrument that measures angles, is used in conjunction with a camera to image the lens and water droplet and, through data analysis, a contact angle is derived by using the Young-Dupré equation, as shown in equation (1). Specifically, the type of contact angle that is calculated from the sessile drop technique is known as the advancing contact angle because the droplet is spreading across the lens surface.[58,59] Software analysis examines the lens curvature, as well as the shape of the droplet, and calculates the advancing contact angle at the solid-liquid interface, as shown in Figure 1-3.

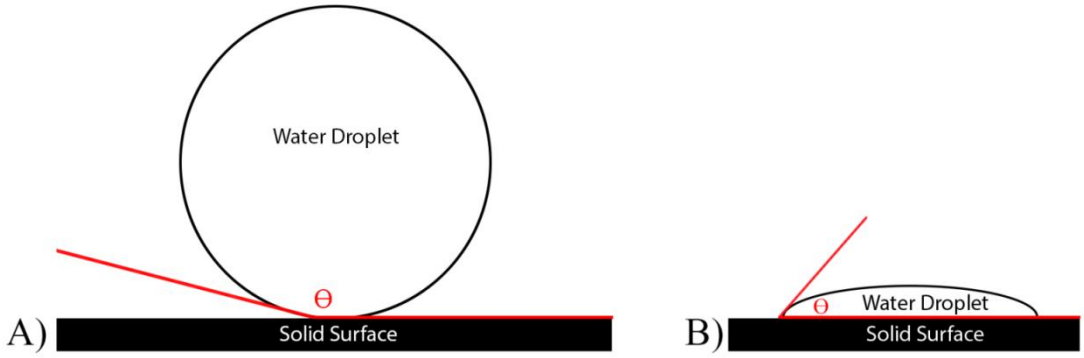

Figure 1-3. Schematic diagram of the sessile drop technique on a A) hydrophobic surface measuring a high advancing contact angle (poor wettability) and B) hydrophilic surface measuring a low advancing contact angle (good wettability).

The relative degree of difficulty in performing the sessile drop method is low compared to the other techniques mentioned, which is why it is most commonly used for wettability analysis. However, the sessile drop has several disadvantages that must be considered. Its major criticism is that the measurement is conducted in air, which puts the

lens at risk of dehydrating and reporting an unreliable value.[60] Consequently, polymers can undergo chain-rotation to achieve a more favourable energy state; in pHEMA contact lenses this phenomenon causes the hydrophobic groups that are present within the lens to orientate towards the surface and become exposed to air.[61] Additionally, the water droplet placed onto the surface can dehydrate since this technique is performed in air.[28] The overall ramification of dehydration is that the droplet could potentially withdraw/evaporate and is no longer an accurate measure of the advancing contact angle but, instead, the receding contact angle. Fortunately, the sessile drop method is a relatively quick technique, making these issues generally non-significant, although they should still be considered.

The captive bubble technique was developed to address the issues of wettability measurements in air. The lens is submerged in a liquid environment with the anterior surface facing down. A syringe is placed directly underneath the contact lens and an air bubble is dispensed; as the air bubble contacts the lens and expands, it will continually push out the surrounding water.[62] The angle between the lens surface and the air bubble is calculated, which is known as the receding contact angle, as shown in Figure 1-4.[28]

Although the captive bubble technique addresses the issues of dehydration of the substrate, it is a fairly difficult technique to perform and requires a higher level of expertise relative to the sessile drop method. Also, the captive bubble technique typically takes longer to conduct due to the setup of the apparatus.[28] This method has also been criticized for not producing representative results because wettability is measured in a liquid environment. This will often generate a lower contact angle and, additionally, it is difficult to differentiate between lenses as they are all in a similar state of hydration.[63] Lastly, there are potential

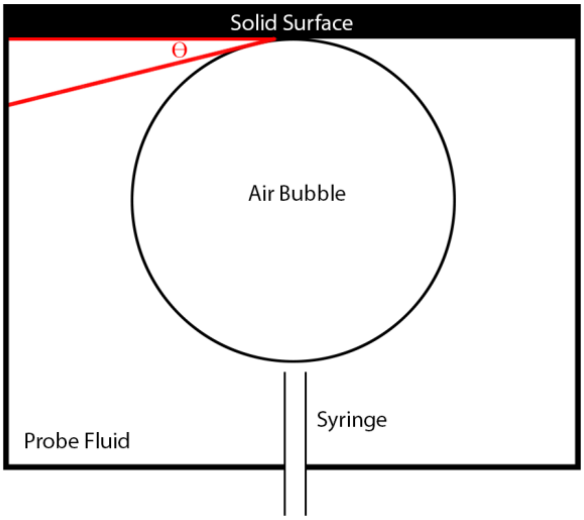

Figure 1-4. Schematic diagram of the captive bubble technique dispensing an air bubble via syringe to measure the receding contact angle.

optical problems when measuring the contact angle accurately. The light from the goniometer is originally in air and enters the liquid medium with a higher refractive index, thus making it difficult to precisely locate the air bubble on the lens surface in a vertical plane to the light path.[28]

The development of the Wilhelmy plate method served to combine aspects from both the sessile drop and captive bubble. Essentially, the contact lens being studied is cut into a strip which suspends vertically from a microbalance. The advancing contact angle is determined as the strip is lowered into a fluid-filled container. Once submerged, the lens strip is withdrawn and the receding contact angle is obtained, as shown in Figure 1-5.[30] Despite having the dual-function convenience, the obvious disadvantage of this technique is the sample preparation of cutting the contact lens into a strip. Furthermore, the data analysis is also relatively complex.[63] Another variable that may produce differences in measurements is

the speed at which the substrate is submerged and withdrawn from the liquid medium. It was demonstrated by Cain et al.[64] that increasing the rate of submersion will increase the contact angle.

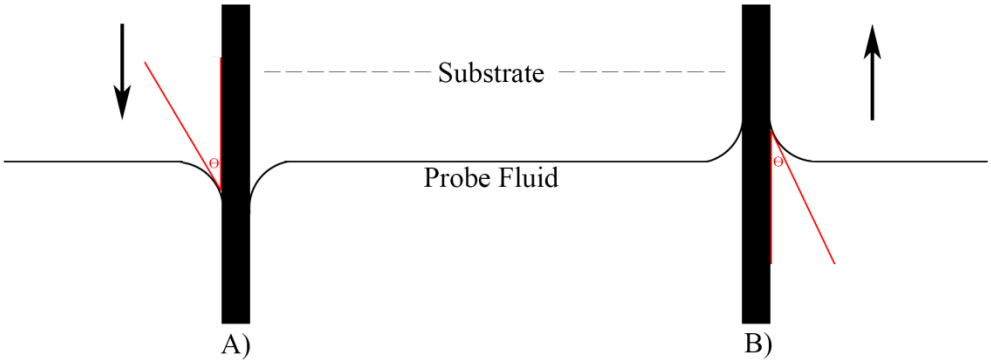

Figure 1-5. Schematic diagram of the Wilhelmy plate method. A) The advancing contact angle is measured as the substrate is immersed into the probe fluid; B) The receding contact angle is measured as the substrate is withdrawn out of the probe fluid.

What should be borne in mind is that no technique is "superior" over another since each method has its advantages and disadvantages. Despite the captive bubble selected as the ISO international standard for rigid gas-permeable lens analysis, there is currently no selected ISO standard analysis for soft hydrogel lenses.[63] Nonetheless, certain methods may be deemed more appropriate to use, which can depend on the objectives of the study or the resources at hand, for example. In this thesis, the sessile drop was selected as the primary tool for wettability analysis for its relative ease and convenience; additionally, the data can be compared directly with the numerous published studies that have also used this technique.

As alluded to earlier, the material wettability can have a potentially significant impact on the perceived comfort during lens wear. However, wettability can also have a significant

effect at the microscopic level, where its immediate effect is not so readily realized. It is believed that a surface with poor wettability is prone to accumulating hydrophobic and denatured molecules onto the lens surface, which can potentially have immense consequences over an extended period of time.[65] An example would be deposition of molecules from the tear film onto the contact lens material. Therefore, the following section will review the types of deposition that can occur on various commercial contact lenses.

1.3 Contact lens deposition

Contact lenses are considered biomaterials because they are in direct contact with the ocular surface. The process of molecules adhering to the lens surface is known as adsorption, whereas absorption describes molecules depositing within the lens material. Molecules will often undergo both adsorption and absorption, and this characteristic is generally described as "sorption". For instance, molecules from the tear film have the tendency to sorb onto contact lenses. However, the sorption profile of tear film constituents is not indiscriminate and largely depends on the materials chemical properties.[66] For instance, conventional hydrogel lenses are known to sorb large amounts of proteins,[67,68] whereas lipids accumulate significantly more on silicone hydrogel lenses.[69-71] These affinities are believed to be due to the ionicly charged polymers within the lens matrix, such as the methacrylic acid[72,73] and hydrophobic siloxane components present in conventional and silicone hydrogels respectively. Since hydrogels are networks of polymers with "empty cavities" to occupy water molecules, these pocket of space can serve as reservoirs for deposits to accumulate in. Generally, lenses with larger pores will deposit more tear film constituents.[73] The overall size

of proteins and lipids will also influence the sorption kinetics onto the lens, such that smaller molecules will deposit more readily.

1.3.1 Complications

Deposition of tear film constituents onto contact lenses has been generally viewed as being undesirable. The presence of these deposits can adversely affect visual acuity,[74] and can cause symptoms of dryness and discomfort due to the deposits disrupting the smooth lens surface over which the eyelid sweeps.[75,76] These symptoms have plagued the contact lens industry, with dropout rates as high as 34% amongst contact lens wearers.[43,44]

Further investigation of these deposits has revealed that proteins often become denatured once they interact with lens materials, and protein denaturation is much more pronounced on silicone hydrogels than it is for conventional lenses.[67,77,78] The denatured protein can elicit an immunological response on the ocular surface, resulting in a condition known as papillary conjunctivitis, which can increase the symptoms of discomfort felt by wearers.[79]

Additionally, a study conducted by Hart claimed there are “dry spots” on the lens surface that may attract lipid deposits and contribute to symptoms of dryness and discomfort.[80] However, new research suggests that lipid deposition may in fact be beneficial to lens wearers because it may actually improve material wettability.[55,81] It has been speculated from these studies that select lipids, such as phosphatidylcholine, are responsible for this effect and might possibly be included into various contact lenses and ophthalmic solutions.

Furthermore, contact lenses are susceptible to bacterial contamination by the natural flora of the ocular and skin surfaces. The lens itself is an ideal medium for bacteria because the ocular surface is at an optimal temperature and tear proteins can serve as nutrients.[82] The potential for irreversible vision loss can occur due to the cornea becoming infected, which is a condition known as microbial keratitis.[83] Additional complications that may arise are acute red eye and peripheral ulcers, both of which can cause pain and discomfort to the wearer.[84] The most prevalent bacteria that have been isolated from cases of contact lens-induced microbial keratitis are the Gram-positive *Staphylococcus* and Gram-negative *Pseudomonas*.[85-87]

Given the severity of microbial keratitis, several manufacturers have developed cleaning solutions with anti-microbial properties. The active ingredients in most cleaning solutions today are polyhexamethylene biguanide and 3% hydrogen peroxide,[23,88] which have biocidal properties to neutralize a wide spectrum of bacteria. Surface coatings on lenses and storage cases have also been proposed as a method to prevent the attachment and proliferation of bacteria.[89,90]

Numerous contact lens bacterial binding studies have been published. The majority of these studies examined the adhesion of various bacterial strains on the lens surface.[84,89,91-97] The general findings were that adhesion is affected by the surface properties of the microbes under consideration, the surface to which these microbes attaches to, as well as the surrounding medium. A variety of conventional and silicone hydrogel lenses were examined, and it was shown that bacteria adsorbs significantly more onto silicone hydrogel materials.[91,92,98] It is believed that the hydrophobic domains present in silicone hydrogels are

responsible for the increase in bacterial attachment. However, these results are inconsistent with another published study reporting that microbial keratitis occurs approximately five times more with conventional hydrogel lenses than with silicone hydrogels.[99] One theory is that bacteria may not actually adhere onto the lens surface but to tear deposits, such as lysozyme, which binds in large quantities on conventional hydrogel lenses.

1.4 Contact lens modification

Due to the inherent hydrophobicity associated with silicone hydrogels, most of these early generation lenses underwent some form of lens modification to increase the surface hydrophilicity and wettability. It was noted that siloxane was fairly mobile within the hydrogel matrix and demonstrated the tendency to orient towards the surface of the lens because it was energetically favorable to be at the air interface.[100] As such, these lenses were rendered inadequate to support a stable tear film, particularly when used under the 30-night continuous wear modality. One method to increase wettability involved the use of plasma technologies to form hydrophilic polymers on the lens surface.[101] Plasma is essentially reactive gases that consist of high energy electrons and ions. Depending on the composition of gases in plasma, different results can be achieved. This technique was commonly applied to the first generation silicone hydrogel lenses, balafilcon A (Bausch + Lomb) and lotrafilcon A&B (CIBA Vision). With balafilcon A, these lenses are subjected to a reactive gas chamber undergoing plasma oxidation.[102] The organic silicone present in balafilcon A, trimethylsiloxy silane (TRIS), oxidizes into inorganic silicate, which is hydrophilic. Atomic force microscopy (AFM) topography images of these lenses reveal discontinuous silicate islands,

which are likely formed as a result of plasma oxidation.[103-105] In comparison, lotrafilcon lenses undergo a plasma polymerization process. These lenses are treated in a reactive gas chamber and a mixture of trimethylsilane oxygen and methane "coats" the lens surface with a 25-nm thick continuous coating containing hydrophilic groups.[14,106] Unlike plasma oxidation, this process produces a smooth surface[103,105,107] that completely occludes the underlying hydrophobic silicone, which accounts for the differences in wettability between these two lens materials.

Despite the advantages of surface treated lenses, the major drawback is that surface treatment is a highly expensive process. Johnson & Johnson developed senofilcon A and galyfilcon A as second generation silicone hydrogel lenses. These lenses do not undergo a surface treatment process, but rather incorporate an internal wetting agent, polyvinylpyrrolidone (PVP), into the bulk of the lens material to effectively "hide" the silicone.[108,109] PVP is a high molecular weight polymer that increases the lens wettability, owing to its excellent water retaining properties. However, studies on these lenses revealed increased amounts of lipid deposition, which has been linked to PVP because it is a polymer of *N*-vinyl pyrrolidone that is known to be lipophilic.[110-113]

The third generation of lenses does not utilize the techniques from previous generations to shield the silicone. Instead, these lenses are composed of two siloxy macromers that offer substantially higher oxygen permeability when combined together.[114] Issues of hydrophobicity are minimized because the unique blend of these polymers inherently produces hydrophilic domains. Examples of these lenses are comfilcon A and enfilcon A, which are manufactured by CooperVision.

1.5 Biomolecules

The development of contact lenses since the debut of polymacon has undergone monumental changes in terms of lens chemistry, with unique and innovative techniques being proposed. However, the use of biological molecules (biomolecules), or molecules naturally produced by living organisms, is an area that appears to be overlooked and not entirely appreciated by lens manufacturers. Biomolecules are molecules that are produced by living organisms, such as proteins, lipids, polysaccharides, and nucleic acids, which are often extracted or synthesized for various applications.[115] These biomolecules are advantageous because they are generally non-toxic, with an array of desirable functions. Such biomolecules have found their niche within the ophthalmic industry in therapeutic eye drops. A common active ingredient found in artificial tears is the biomolecule hyaluronic acid (HA). The innovative incorporation of HA has been shown to be an integral component for enhanced comfort and treatment of dry eye syndrome due to the molecules superior water binding properties.[116,117] Application of these eye drops onto the ocular surface can produce a corneal coating that can enhance tear film stability and reduce corneal staining.[116] Research conducted by Weeks et al.[118-120] has examined the effects of HA within a contact lens material and reported enhanced wettability and reduced lens deposition.

1.5.1 Glycoproteins and proteoglycans

Among the naturally occurring biomolecules, there is a sub-class of molecules known as glycoproteins. These are molecules with a protein core that have carbohydrate chains (glycans) covalently attached by glycosidic bonds through the process known as

glycosylation.[121] There are several reasons why proteins undergo glycosylation, and the degree of glycosylation can also affect the physical and chemical properties of the molecule.[122,123] For instance, the addition of carbohydrate chains can help stabilize the protein and/or assist in the folding process[124,125] and prevent degradation from proteolytic enzymes.[126] Carbohydrate chains are necessary for cellular communication and recognition.[127]

Proteoglycans are a specific class of glycoproteins that are extensively glycosylated, and as a result, the nomenclature "proteoglycan" reflects the shift from protein to carbohydrate.[128] The carbohydrate chains that are attached are known as glycosaminoglycans (GAGs), which are large, linear chains of polysaccharides that are unbranched with repeating disaccharide units (Figure 1-6).[129,130] The major biological function of proteoglycans is generally dictated by the properties of the bound GAGs, such as initiating and dictating inflammation.[131-133] A large majority of proteoglycans is found in the extracellular matrix,[115,134] specifically associated with loose connective tissue, and the associated GAGs have excellent water retention due to their high negatively charged density at physiological pH.[133] This feature allows for increased hydration and the ability to oppose mechanical forces, particularly around the joint.

Proteoglycan 4 (PRG4), also known as lubricin, is a proteoglycan coded by the *Prg4* gene[135] that is present in the synovial fluid and is an essential component for the lubrication of joints.[136,137] It was first discovered and isolated in bovine synovial fluid by Swann et al.[138] and was given the name lubricin in 1985.[139] PRG4 has a molecular weight of approximately

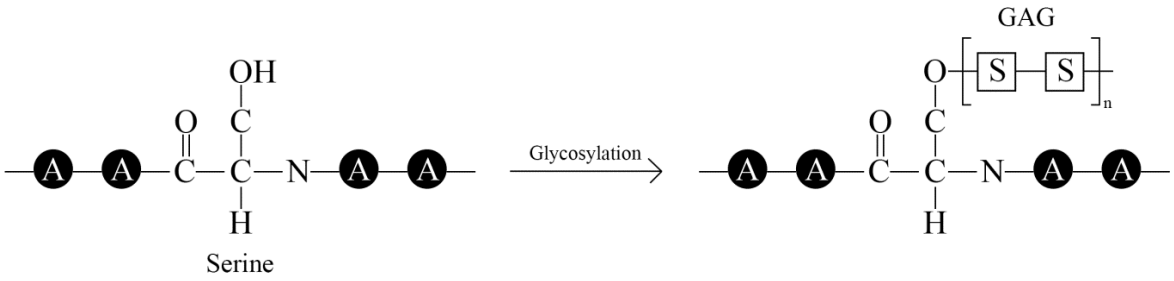

Figure 1-6. Illustration of a protein undergoing *O*-linked glycosylation. A glycosidic bond is formed between the serine oxygen and repeated disaccharide units, also known as GAGs. Legend: A = amino acid, C = carbon, GAG = glycosaminoglycan, H = hydrogen, N = nitrogen, O = oxygen, S = sugar.

227 kDa and undergoes extensive *O*-linked glycosylation at its central mucin-like protein core.[138,140] In this process, the glycans form glycosidic bonds with the hydroxyl oxygen, hence *O*-linked, of serine and threonine, which constitutes approximately 5 and 20 percent of PRG4's total amino acid residues respectively. Additionally, there are specialized hydrophobic domains called somatomedin B (SMB) and a hemopexin (PEX) that flank the protein backbone at the NH_2- and COOH-terminals, respectively (Figure 1-7).

PRG4 is considered as the body's main lubricating molecule, therefore, its function is not limited to the synovial fluid. PRG4 has been discovered in various parts of the body,[141-144] including recently on the ocular surface[145,146] where it may potentially have a role in preventing dry eye disease. Specifically, conjunctival epithelial cells were shown to transcribe and translate PRG4. Schmidt et al.[145] also demonstrated that lacrimal and meibomian glands contain PRG4 mRNA. Their work suggested that PRG4 may not only act as a boundary lubricant but could have additional properties. They examined PRG4 knockout

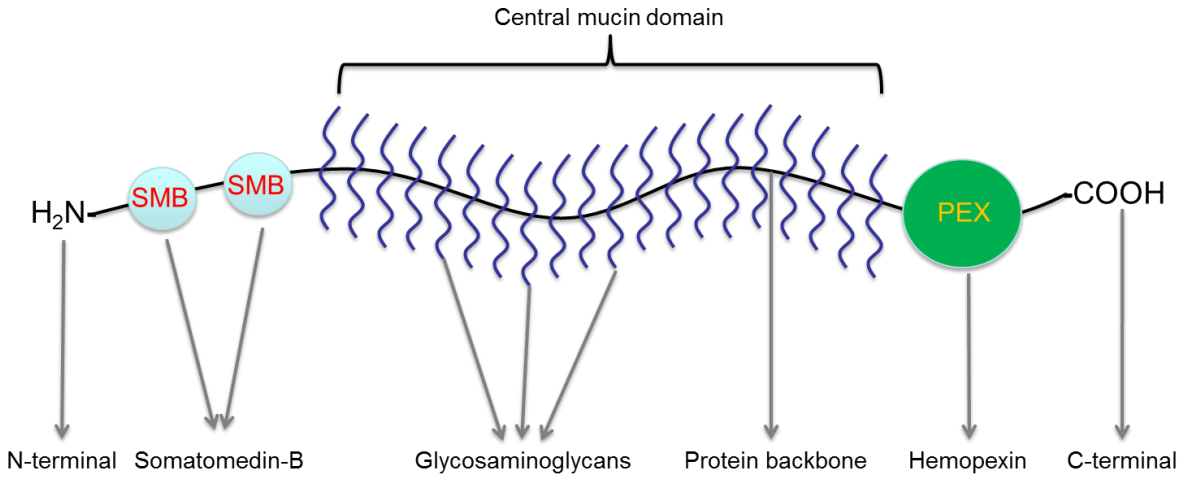

Figure 1-7. Schematic representation of proteoglycan 4.

mice and noticed an increase in fluorescein staining, which indicates that PRG4 may provide a protective effect on the cornea.[145] PRG4 has also been described to have anti-adhesive properties[147,148] for molecules such as proteins, lipids, and even bacteria.[149] Furthermore, the extensive *O*-linked glycosylations seen in PRG4 has been speculated to have wetting enhancing properties.

Most of the previous *in vitro* studies used the native form of PRG4 extracted from bovine stifle joints.[150,151] However, the extraction and purification process is costly and time-consuming and often resulted in low yields. Recombinant human PRG4 was later expressed, primarily for therapeutic evaluation of osteoarthritis.[152,153] Unfortunately, the recombinant version was not representative of the native PRG4 because its central core was truncated, which may inhibit some of the intrinsic properties that PRG4 possesses. Recent advances in molecular technology allowed for the expression of full-length recombinant human PRG4 (rhPRG4) in Chinese hamster ovary (CHO) cells. RhPRG4 was characterized by Samsom et

al.,[154] and they concluded that it exhibited similar levels of *O*-linked glycosylations and lubricating ability to that of bovine PRG4 and that it may be useful for clinical evaluations.

PRG4 has been extensively studied as a possible therapeutic agent for joint diseases, such as rheumatoid arthritis and osteoarthritis.[155] The novel application of PRG4 in the form of a therapeutic eye drop to alleviate symptoms of dry eyes and as an active component within commercial contact lens cleaning solutions due to its synergistic effect with HA has been considered.[156] Furthermore, the incorporation of PRG4 within model contact lenses, which are non-commercialized lens materials developed in lab, has also been found to significantly enhance the materials wettability.[157] Given the importance of biomaterial wettability and susceptibility to bacterial contamination, and the importance of biocompatibility in contact lens wearer comfort, the incorporation of PRG4 into commercial contact lenses has clinical implications because it has the potential to significantly improve the lens wearing experience and reduce drop-out rates. Therefore, the objective of this thesis is to evaluate the interaction of PRG4 with various commercial lenses and to determine if its potential application with contact lenses is viable.

Chapter 2 – Thesis Rationale

Contact lenses have undergone monumental amounts of research. The initial contact lens material was made out of glass in 1887 and has since evolved into the extremely complex lenses that are produced today.[158] The two primary forces driving the evolution of contact lenses are wearer comfort and ocular health during their wear. However, despite the many advancements seen with contact lenses over the past century, they remain far from ideal and approximately one in three patients will discontinue lens wear, primarily due to end of day discomfort.[43,44]

Various techniques have been proposed to improve the wettability and comfort of contact lenses, but none have considered the inclusion of naturally occurring biological molecules as a possible option. Biomolecules often have attractive properties that allow a particular part of the body to function with high efficiency, but are often difficult to reproduce synthetically. An example is in the knee joint, where the cartilage is subjected to immense frictional and compressive forces without significant damages to its structural integrity. The primary biomolecule responsible for this protective effect is a glycoprotein known as proteoglycan 4 (PRG4), which functions by reducing the friction at the joint, thus preserving the integrity of the cartilage. The PRG4 gene in humans has been isolated and PRG4 can be synthetically produced as a recombinant protein. Advantageously, the unique properties of PRG4 is not only limited to the joint but can be applied to other surfaces, such as contact lenses. The novel incorporation of PRG4 within model contact lens materials has been previously reported,[157,159,160] yet none have investigated its effect on commercial contact lenses.

In the first study of this thesis (Chapter 3), various commercially available contact lenses were treated with PRG4. The primary objective of this study was to determine if PRG4 can alter the surface wettability of these lenses. Previous work involving model, non-commercialized contact lenses examined the effect of PRG4 on the lens material and has shown that PRG4 can act as a wetting agent;[157] however, the lens chemistry of model materials is simplistic and not representative of the lenses on the market, particularly silicone hydrogels that have undergone significant wettability enhancements. Therefore, it was necessary to determine whether PRG4 can elicit the same wetting potential on a variety of commercial lenses, as it did with model lenses.

The second experiment of this thesis (Chapter 4) investigated the sorption profile of PRG4 within commercial lenses. Contact lenses are hydrogel products that are three-dimensional networks of polymers that have the ability to absorb water. However, this phenomenon is not only limited to water, but can apply to all molecules, such as proteins, lipids and cells. The sorption capability of contact lenses has been well documented and is largely material dependent; however, it is unknown how PRG4 interacts with the lens material. Since wettability is a surface characteristic, the results obtained can only partially elucidate the adsorptive profile of PRG4 on the surface, but gives no insight as to its distribution within the bulk of the lens. By fluorescently labeling PRG4, one can observe its location and relative concentration within a lens using confocal microscopy. With the numerous types of wetting enhancers in modern lenses, it is possible that these additional agents may or may not influence the penetration of PRG4 into the lens matrix. By knowing

where PRG4 is within the lens and how it interacts with the material, lens manufacturers could then tailor their lenses to better incorporate PRG4.

The last study of this thesis (Chapter 5) explored the anti-adhesive potential of PRG4 on contaminated contact lenses with *Staphylococcus aureus*. Contact lenses are susceptible to contamination, particularly by the normal flora that inhabits skin and ocular surface, which are frequently contaminated with *S. aureus*. A contaminated lens, when worn, can increase the risk of microbial keratitis, which can ultimately lead to permanent vision loss if left untreated. Previous studies have reported PRG4 inhibiting the adhesion of *S. aureus* on a polystyrene surface. Since polystyrenes and hydrogels are two very different substrates, it is necessary to determine if these "anti-bacterial" properties could also be conferred on contact lenses treated with PRG4.

The results from this thesis will elucidate the potential of incorporating PRG4 on various commercial contact lenses as both a wetting and anti-bacterial agent. Additionally, the results will shed light regarding the material interaction to determine which lenses are best suited with the incorporation of PRG4. It is possible that these findings may one day lead to the inclusion of PRG4 in the material formulation or the blister package solution surrounding one or more commercially available contact lenses.

Chapter 3 – *In vitro* wettability analysis of proteoglycan 4 on hydrogel contact lenses

3.1 Overview

Purpose: The purpose of this study was to investigate the effect of bovine proteoglycan 4 (PRG4) on the wettability of various commercial contact lenses, and to assess its substantivity on the surface of each lens material.

Methods: Five contact lens materials – balafilcon A, senofilcon A, lotrafilcon B, comfilcon A, etafilcon A – were investigated under three conditions: (1) directly out of the blister pack; (2) phosphate buffered saline (PBS); (3) PRG4 solution at 300 μg/ml for 1 hour at 37˚C on a rotary shaker. Following incubation, the advancing contact angle (ACA) of these lenses was measured using an Optical Contact Angle analyzer to determine their wettability. Lenses then underwent eight cycles of rinsing in a preservative free saline solution, and the ACAs were re-measured after each rinse to assess substantivity.

Results: All lens materials, except balafilcon A, that were measured immediately after removal from its blister package demonstrated a significantly lower ACA compared to lenses incubated in PBS ($P<0.05$). Senofilcon A and balafilcon A lenses incubated in PRG4 displayed a significant reduction in ACA compared to their PBS control ($P<0.05$). However, the remaining lens materials displayed a significant increase in ACA after being coated with PRG4 ($P<0.05$). The substantivity results for PRG4 on senofilcon A lenses revealed that after

one cycle of rinsing the ACA increased and was not significantly different compared to control values ($P>0.05$). In comparison, balafilcon A lenses showed a gradual removal of PRG4 off its surface. Lotrafilcon B, comfilcon A, and etafilcon A lenses retained the glycoprotein with no significant signs of removal ($P>0.05$).

Conclusions: The amphiphilic structure of PRG4 significantly influences its wettability and substantivity to bound surfaces. Hydrophobic lenses showed improved wettability after being coated in PRG4, though PRG4 was weakly adsorbed onto these surfaces. Conversely, strong adhesion was observed on relatively hydrophilic lens surfaces, yet a decrease in surface wettability was seen.

3.2 Introduction

The novelty of silicone hydrogel (SH) contact lenses comes from their ability to be used in a continuous wear format, in which they are worn for up to 30 days without removal. This modality was achieved by incorporating siloxane components into the lens material, which allowed for greater oxygen transmissibility.[14,18,19] Prior to the advent of SH lenses, the majority of lenses worn were conventional hydrogel (CH) soft lenses.[23] However, conditions such as limbal hyperemia[161,162] and neovascularization[163,164] in the cornea can develop due to corneal hypoxia from wearing such lenses. Despite the fact that SH lenses transmit more oxygen to the cornea than CH materials, silicone is extremely hydrophobic and, consequently, the lens material is not relatively wettable.[165] The phenomenon known as "wettability" describes the ease or tendency of a fluid phase to spread over a solid surface.[28] Reasonably, a lens that is wettable will provide better comfort over a lens that is not due to increased biocompatibility on the ocular surface,[166] and because of this, lens manufacturers have developed various methods of improving their products wettability.[167] A few notable examples include plasma coatings onto the surface,[102,106] incorporation of internal wetting agents,[108] and unique polymer compositions that produce hydrophilic domains.[114] Although these techniques claim to improve wettability, patients still report symptoms of dryness and discomfort, particularly at the end of day,[76,168,169] and may discontinue their contact lens usage.

Lens wettability can be assessed *in vitro* using a variety of techniques, as described by French.[51] One of the most commonly used methods to measure wettability is the sessile drop technique,[53-56,170] where an inert water droplet is placed onto the surface of the test

material.[52] As the liquid phase interacts with the solid surface, the advancing contact angle (ACA) is measured using a goniometer. Generally, a large ACA (typically of >70º) equates to a poor wetting material, due to the surface resisting the spread of the liquid at the solid-liquid interface. Conversely, a wettable surface will cause the water droplet to completely spread, thus registering a lower ACA value.

Proteoglycan 4 (PRG4), also known as lubricin, is a glycoprotein that acts as a boundary lubricant in the lubrication of joints.[150,155,171] It was first discovered and isolated by Swann et al.[138] from bovine synovial fluid, and has since been found in various parts of the body. Recently, it has been found on the ocular surface, where it is believed to reduce friction between the cornea and conjunctiva,[146] as well as conferring a protective effect on the cornea.[145] Previous studies have shown that incorporation of bovine PRG4 into model contact lens materials will significantly enhance the material wettability,[157] but no studies to-date have investigated its role as a wetting agent with commercial lenses. It is hypothesized that a bovine PRG4 lens surface coating will improve the wettability of various commercial lenses due to the negatively charged hydrated sugars within PRG4,[172] which will promote an even and stable distribution of the tear film, thus providing enhanced ocular lubrication that can potentially result in increased wearer comfort.

The purpose of this study was to coat the surface of various commercial SH lenses and one CH lens with bovine PRG4 and to determine the material wettability by measuring the ACA using the sessile drop technique. In addition, the substantivity, which is a term that describes adherent qualities, was determined between PRG4 and the contact lens surfaces.

3.3 Materials and methods

3.3.1 Materials

Four commercial SH lenses [balafilcon A (Bausch + Lomb, Rochester, NY), senofilcon A (Johnson & Johnson, Jacksonville, FL), lotrafilcon B (Alcon Vision Care, Fort Worth, TX), comfilcon A (CooperVision, Pleasanton, CA)] and one CH [etafilcon A (Johnson & Johnson, Jacksonville, FL)] were investigated. The physical and chemical properties of these materials are found in Table 3-1. PRG4 was obtained from the University of Calgary, where it had been extracted from bovine synovial fluid and purified, as described.[150]

3.3.2 Pre-soaking of lenses

Using blunt metal forceps, each lens was removed from its blister package and dabbed onto lens paper (VWR Scientific Products, West Chester, PA) to remove excess blister pack solution. Lenses were placed in a 12-well plate, with each well containing 5 mL of phosphate-buffered saline (PBS). Lenses remained immersed overnight with gentle shaking at room temperature in order to remove components from the blister solution from the contact lens.

Table 3-1. Physical and chemical properties of silicone hydrogel lens materials.

USAN	balafilcon A	senofilcon A	lotrafilcon B	comfilcon A	etafilcon A
Proprietary name	PureVision	Acuvue OASYS	Air Optix Aqua	Biofinity	Acuvue 2
Manufacturer	Bausch + Lomb	Johnson & Johnson	Alcon	CooperVision	Johnson & Johnson
Water content	36%	38%	33%	48%	58%
Surface treatment	Plasma oxidation	Internal wetting agent (PVP)	Plasma polymerization	None	None
Oxygen permeability	99 Dk	103 Dk	110 Dk	160 Dk	22 Dk
Monomers	NVP, TPVC, NVA, PBVC	mPDMS, DMA, HEMA, siloxane macromer, TEGDMA, PVP	DMA, TRIS, siloxane macromer	M3U, FM0411M, HOB, IBM, NVP, TAIC, VMA	HEMA, MA
FDA group	V	V	V	V	IV

DMA (N,N-dimethylacrylamide); **FM0411M** (2-ethyl [2-[(2-methylprop-2-enoyl)oxy]ethyl] carbamate); **HEMA** (poly-2-hydroxyethyl methacrylate); **HOB** ((2RS)-2-hydroxybutyl 2-methylprop-2-enoate); **IBM** (isobornyl methacrylate); **mPDMS** (monofunctional polydimethylsiloxane); **M3U** (α-[[3-(2-[[2-(methacryloyloxy)ethyl] carbamoyloxy]ethoxy)propyl] dimethylsilyl]-ω-[3-(2-[[2-(methacryloyloxy)ethyl] carbamoyloxy] ethoxy)propyl]poly([oxy[(methyl) [3-[ω-methylpoly(oxyethylene) oxy]propyl]silylene] /[oxy[(methyl)(3,3,3-trifluoropropyl)]silylene]/ oxy (dimethylsilylene)]); **NVA** (*N*-vinyl aminobutyric acid); **NVP** (N-vinyl-pyrrolidone); **PBVC** (poly[dimethysiloxy] di [silybutanol] bis[vinyl carbamate]); **PVP** (poly(vinylpryyolidone)); **TAIC** (1,3,5-triprop-2-enyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione); **TEGDMA** (tetraethyleneglycol dimethacrylate); **TPVC** (tris-(trimethylsiloxysilyl) propylvinyl carbamate); **TRIS** (trimethylsiloxy silane); **USAN** (United States adopted name); **VMA** (N-Vinyl-N-methylacetamide).

3.3.3 PRG4 solution and incubation

Aliquots from a PRG4 stock solution (2 mg/ml) were diluted with PBS to 300 µg/ml and 2 ml of solution was stored in 6 ml glass incubation vials (Wheaton, VWR, Mississauga, ON, Canada), sealed with Parafilm, and stored at 4 ºC. Prior to usage, incubation solutions were allowed to warm up to room temperature for one hour. Subsequently, pre-soaked lenses in PBS were removed from their well-plate and placed into their respective incubation vial with the anterior surface facing downwards. Lenses were incubated in the PRG4-solution at 37 ºC for one hour with gentle shaking and sealed with Parafilm.

3.3.4 Sessile drop technique

Immediately after incubation, the ACA of each lens material (n = 3) was measured. Lenses were removed from their incubation vials with blunt metal forceps and gently shaken to remove excess incubation solution. Lenses are normally blot dried on lens paper prior to measuring; however, blot drying may remove loosely bound PRG4 on the surface and potentially affect substantivity results. Instead, lenses were mounted on a custom curved convex mantle for one minute to air dry and placed directly beneath the syringe of an Optical Contact Angle analyzer (OCA, Dataphysics Instruments GmbH, Filderstadt, Germany), as shown in Figure 3-1. The syringe dispenses a 5 µl drop of high performance liquid chromatography (HPLC) grade water (EMD Chemicals, Gibbstown, NJ) at a constant rate of 2 µl/sec. A picture was taken once the droplet had settled after 2 seconds on the lens surface, as determined with a stopwatch (Figure 3-2), and the ACA was measured using the SCA 20 software, as described by Menzies et al.[173] This procedure was conducted two more times for

each lens type for the following negative control experimental conditions: (1) lenses straight out of their blister package, (2) lenses rinsed in PBS with no PRG4.

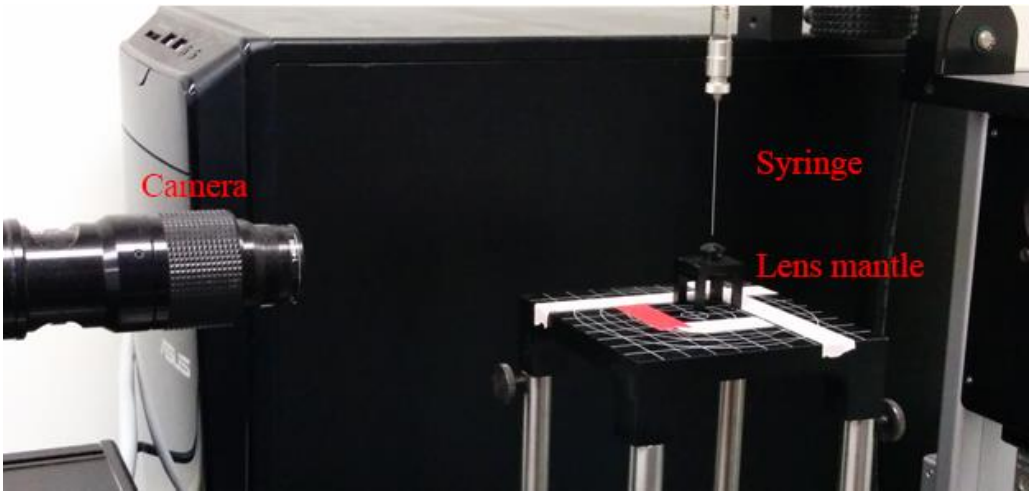

Figure 3-1. Optical Contact Angle analyzer apparatus consisting of a camera, syringe, and lens mantle to measure lens wettability.

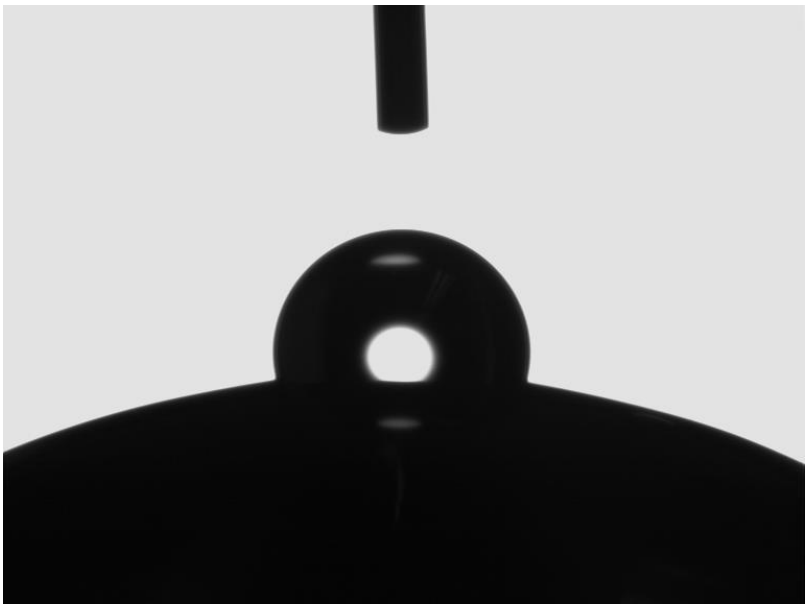

Figure 3-2. Image recorded by the Optical Contact Angle analyzer camera of the water droplet interacting with the lens surface.

3.3.5 PRG4 substantivity

Once the initial ACA measurement of each lens had been measured, denoted as t_0, the lens was removed off its mantle and placed in a cup containing 5 ml of unpreserved saline solution (Unisol, Alcon, Fort Worth, TX) for 5 minutes. Afterwards, lenses were placed back onto the mantle and its ACA was re-measured, up to a total of 8 cycles to determine the substantivity of PRG4 on the lens surface. This procedure was repeated for each control lens type, where lenses were rinsed in PBS with no PRG4 incubation.

3.3.6 Data analysis

Statistica 12 (StatSoft Inc. Tulsa, OK) was used to conduct the statistical analysis. ACAs from the first objective of this study were reported as means ± standard deviations where the data were analyzed using a factorial analysis of variance (ANOVA), with ACA as the dependent variable, and lens material and treatment as factors. ACAs from the second objective of this study were reported as mean ± standard deviation where the data were analyzed using a repeated measures analysis of variance (ANOVA), with ACA at each time point as the dependent variable, and lens material and treatment as factors. For both tests, the Tukey's HSD (Honestly Significant Difference) post hoc test was used where $P<0.05$ was considered significant.

3.4 Results

Figure 3-3 illustrates the ACAs for all five lens materials under the three experimental conditions (blister packaging solution, PBS, and PRG4-coated). For all lens

materials, with the exception of balafilcon A, the ACA of the lens measured directly from its blister package was significantly lower compared to the PBS-soaked control lens ($P<0.05$). For balafilcon A, the ACA of PRG4-coated lenses was significantly lower compared to both the blister ($P<0.05$) and PBS control lenses ($P<0.05$). Senofilcon A lenses incubated in PRG4 also revealed a significantly lower ACA compared to the blister ($P<0.05$) and PBS-soaked lenses ($P<0.05$). Analysis of lotrafilcon B lenses revealed that PRG4-coated lenses had a significantly higher ACA when compared to the lenses out of blister pack ($P<0.05$) and the PBS-soaked control lenses ($P<0.05$). The same phenomenon was seen with comfilcon A and etafilcon A lenses where PRG4-coated lenses had a statistically greater ACA than the blister ($P<0.05$) and PBS condition ($P<0.05$).

Figure 3-4 describes the substantivity of PRG4 onto the lens surface. Lenses incubated in a PRG4 and PBS control solution were rinsed in Unisol for eight cycles. The plot for balafilcon A illustrated that the ACAs for PBS incubated lenses remained relatively consistent throughout all cycles with no significant differences ($P>0.05$). However, PRG4-coated lenses demonstrated a gradual increase in ACA towards control values after subsequent rinsing. PBS-soaked senofilcon A lenses also displayed a relatively consistent ACA after each rinse cycle with no significant differences ($P>0.05$). However, the ACA for PRG4-coated lenses increased significantly and was not statistically different compared to PBS control values after one rinse cycle ($P>0.05$). For PBS-soaked lotrafilcon B lenses, although it was not statistically significant ($P>0.05$), the ACA appeared to steadily increase up to the fifth rinse where the contact angle remained relatively consistent for the remaining rinses. The ACA was also seen to be constant when these lenses were coated with PRG4 with

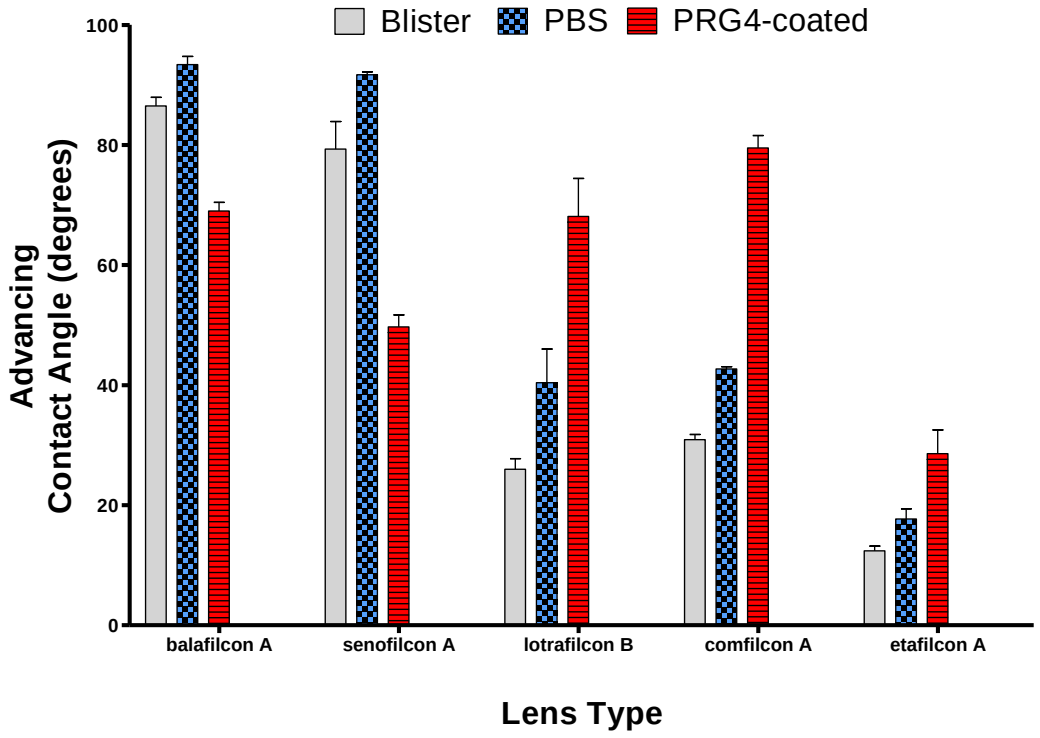

Figure 3-3. The advancing contact angle (ACA) for each lens material on removal from the blister packaging solution, rinsed in phosphate-buffered saline (PBS), and incubated in a PRG4 solution for balafilcon A; senofilcon A; lotrafilcon B; comfilcon A; etafilcon A.

no significant difference between rinses ($P>0.05$). Minor fluctuations in ACAs between rinses for PRG4-coated comfilcon A lenses were observed, though there were no significant differences between these values ($P>0.05$). PBS-soaked comfilcon A lenses, however, exhibited a significant increase in ACA during the cycles of rinses ($P<0.05$). On the other hand, the CH material, etafilcon A, exhibited a relatively constant ACA for all rinses in both PBS and PRG4 treated lenses, though minor fluctuations were observed but not statistically significant ($P>0.05$).

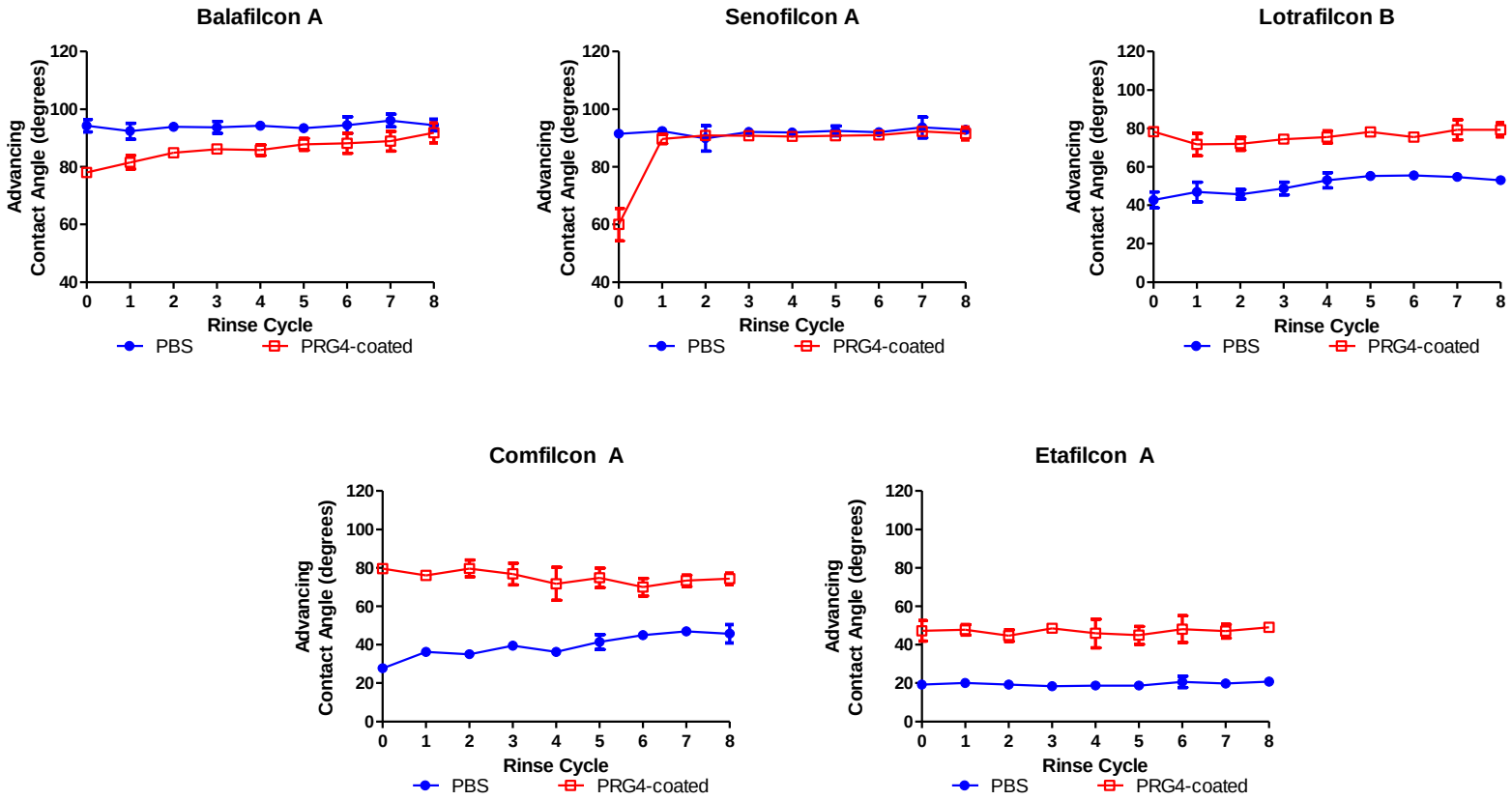

Figure 3-4. The advancing contact angle (ACA) for each lens material rinsed in phosphate-buffered saline (PBS) and incubated in a PRG4 solution after each progressive cycle of rinsing in Unisol for balafilcon A; senofilcon A; lotrafilcon B; comfilcon A; etafilcon A.

3.5 Discussion

SH lenses have been commercially available for over 15 years and hold a substantial share in the contact lens industry. Even though these lenses brought forth the ability for continuous wear and significantly reduced hypoxic complications, symptoms of dryness and discomfort were still being reported by lens wearers, which may potentially be due to the hydrophobic siloxane component of these lenses.[76] The majority of SH lenses now undergo various modifications to enhance wettability and, consequently, lens modification has been an area of growing interest as researchers are exploiting novel techniques to improve material wettability.

The results of this study revealed that the novel incorporation of bovine PRG4 onto the surfaces of commercial SH lenses do affect the materials wettability significantly when compared to the PBS control ($P<0.05$). However, certain lens materials appeared to benefit from the PRG4 inclusion, whereas other materials showed an antagonistic effect. Specifically, PRG4-coated balafilcon A and senofilcon A lens materials showed a significant reduction in the ACA compared to the two controls: blister and PBS. In comparison, lotrafilcon B, comfilcon A, and etafilcon A lens materials all experienced a significant increase in the ACA, which translates to reduced wettability. This observation can be explained by examining the structure of PRG4, which mimics that of a surfactant in that it contains hydrophobic and hydrophilic domains (Figure 1-7).[172] When PRG4 is bound onto a hydrophobic surface, the hydrophobic domains of the glycoprotein will bind onto the surface and expose the hydrophilic domains (Figure 3-5). This behaviour is likely occurring on PRG4-coated balafilcon A and senofilcon A lenses as these lenses are known to have

relatively hydrophobic surfaces based on previous *in vitro* wettability measurements.[53,55,174,175] Therefore, the increase in wettability is likely due to the hydrophilic domains exposed onto the surface allowing the liquid phase to spread with greater ease. Conversely, on relatively hydrophilic surfaces, such as lotrafilcon B, comfilcon A, and etafilcon A, the protein will adopt a more energetically favorable conformation such that the hydrophobic domains are expressed, thus accounting for the increase in ACA (Figure 3-6). Although an increase in ACA translates to poorer wettability, it should be kept in mind that PRG4 can also act as a lubricant to reduce the surface friction of these lenses and possibly improve lens wear comfort, as well.

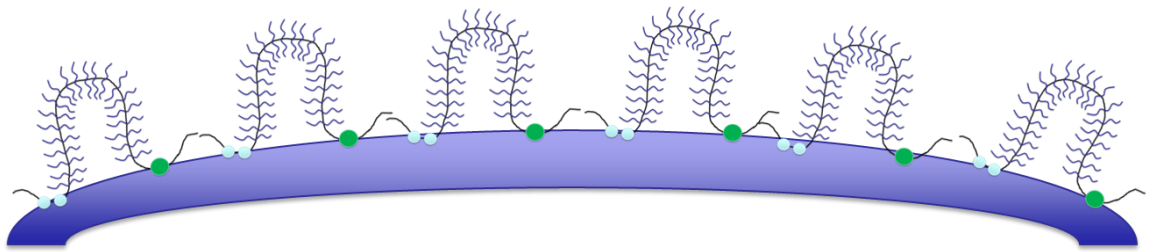

Figure 3-5. Illustration of PRG4 bound onto a hydrophobic surface.

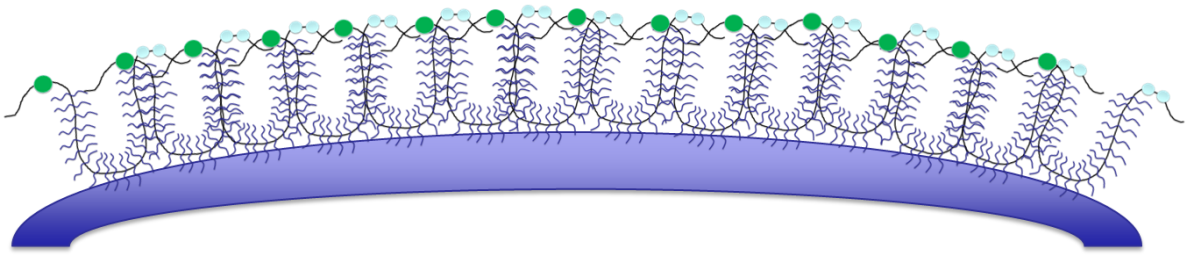

Figure 3-6. Illustration of PRG4 bound onto a hydrophilic surface.

For all lens materials tested, with the exception of balafilcon A, the ACAs for lenses rinsed in PBS were significantly greater than when measured directly out of its blister package. These results mirrored previous studies that investigated the effect the blister solution has on the lens material.[33,55] With dryness and discomfort being the main reasons for contact lens dropout,[176] manufacturers incorporate surfactants and low surface tension molecules into the blister packaging solution with the aim of minimizing these rates by enhancing the material wettability to improve initial in-eye comfort.[173,177-179] Therefore, when these lenses were rinsed in PBS, the blister components were removed and, consequently, the ACA increased significantly and represented the true material wettability. Although balafilcon A lenses did not demonstrate a significantly lower ACA in the blister condition, it did, however, measure a lower ACA on average compared to PBS rinsed lenses. Despite the blister constituents not showing a statistically significant effect, the small decrease in ACA may potentially have clinical significance, such that initial wear comfort is improved. Future studies should investigate the blister constituents on balafilcon A lenses.

The second part of this study investigated the substantivity of bovine PRG4 onto the surfaces of four SH and one CH lenses. According to Chang et al.,[172] PRG4 adsorbs strongly onto both hydrophobic and hydrophilic surfaces, which would suggest that PRG4 would remain tightly bound onto the surfaces of all hydrogels, though the results show otherwise. PRG4 coated onto balafilcon A and senofilcon A lens materials improved the wettability compared to out of blister pack and PBS; however, the glycoprotein did not adhere strongly onto the surface after subsequent rinses in Unisol. This lack of adhesion was much more pronounced with senofilcon A, since a single rinse caused the ACA to increase back to

control values. PRG4-coated balafilcon A lenses showed a gradual shedding of the glycoprotein with each rinse, and ACA values approached control values. This discrepancy is likely due to the differences in ionicity between the two materials. Balafilcon A is the only SH material that is ionic because of the incorporation of *N*-vinyl aminobutyric acid (NVA), which has made this lens type prone to protein sorption.[180] Therefore, it is reasonable to assume that the ionicity of balafilcon A retains PRG4 better than that of the non-ionic senofilcon A. Nonetheless, PRG4 demonstrated poor adhesion onto these lenses with relatively hydrophobic surfaces. Conversely, the remaining three lens materials (lotrafilcon B, comfilcon A, etafilcon A) that have relatively hydrophilic surfaces demonstrated greater substantivity after repeated rinses, which was evident based on the ACA remaining relatively constant. Though Chang et al.[172] stated that PRG4 adsorbs strongly onto a hydrophobic surface, they used a simplistic self-assembled monolayer terminating in methyl groups. It is possible that the differences in results seen could be due to the complex chemistry of hydrogel lenses as they contain various components that may influence the adsorption of PRG4. Additionally, PRG4's hydrophilic mucin domain covers a larger surface area compared to its hydrophobic domains, which can potentially allow the protein to adhere onto the lens surface with greater strength. As illustrated in Figures 3-5 and 3-6, the level of attachment appears to be greater and more continuous on hydrophilic surfaces than on hydrophobic surfaces.

One interesting observation seen for comfilcon A lenses is that the ACAs for PBS control lenses increased steadily during the 8 rinse cycles. Comfilcon A is a third generation SH material that is relatively new and few publications have investigated its lens chemistry.

One study by Lorentz et al.[181] showed that repeated exposure to air will cause a significant increase in cholesterol deposition on comfilcon A lenses. Although their study used a novel model blink cell apparatus to mimic air exposure, the sessile drop technique essentially imposes intermittent periods of air exposure during measurements. The gradual rise in ACA has been associated with an increase in surface hydrophobicity, which is likely the cause for increased cholesterol deposition. It has been hypothesized that the hydroxyethyl methacrylate (HEMA) of a lens material may undergo a chain rotation such that the hydrophobic polymers are exposed out on the surface when the lens is not in an aqueous environment.[61] This phenomenon can explain the fluctuations in ACA observed in between rinses for the HEMA-based lenses. Interestingly, when comfilcon A lenses are coated with PRG4, the ACAs remain relatively consistent. This observation may suggest that PRG4 on the surface may potentially prevent these chain rotations from occurring, thus keeping the hydrophobic domains within the lens, and it may possibly decrease lipid deposition, as well.

3.6 Conclusion

In conclusion, our data show that bovine PRG4 can act as a surface wetting agent on lenses with a relatively hydrophobic surface (balafilcon A and senofilcon A). Despite having a positive effect on these lenses, the glycoprotein does not adhere strongly onto the surface. Future work should consider chemically binding PRG4 onto these lens surfaces via 1-[(3-dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride (EDC) reaction, which was successfully demonstrated by Dutta et al.[182] using the peptide melimine. In addition, several studies have explored the enhanced wettability effect of hyaluronic acid (HA) as an internal

wetting agent in contact lenses,[118-120,183] and Morrison et al.[156] have shown that PRG4 and HA have a synergistic effect on boundary lubrication at the cornea-lens interface. It would be interesting to test a lens containing both PRG4 and HA to see the impact it has on wettability, lubricity, and wear comfort. The use of naturally occurring molecules as wettability enhancers has become a growing area of interest. It is likely that PRG4 will continue to be studied for various applications in the future, such as a possible treatment for dry eye disease.

Chapter 4 – Localization of full-length recombinant human proteoglycan 4 in commercial contact lenses using confocal microscopy

4.1 Overview

Purpose: The aim of this study was to determine the location of full-length recombinant human PRG4 (rhPRG4) tagged with fluorescein isothiocyanate (FITC) in various commercial contact lenses, using a confocal laser scanning microscopy (CLSM) technique.

Methods: Four commercially available silicone hydrogel contact lenses (balafilcon A, senofilcon A, comfilcon A, lotrafilcon B) and one conventional hydrogel lens material (etafilcon A) were examined. Purified rhPRG4, expressed in a proprietary Chinese hamster ovary cell line, was provided by Lμbris, LLC and was manually tagged with FITC via amine reaction to obtain a labeling ratio of approximately 5-6 dye/protein. Unconjugated FITC was removed using a Sephadex-G25 resin in phosphate buffered saline (PBS). Lenses were incubated under two conditions: (1) FITC-rhPRG4 solution at 300 μg/ml and (2) PBS for 1 hour at 37°C in darkness with gentle shaking. After incubation, the central 4 mm of each lens was removed and fixed onto a microscope slide and viewed with the Zeiss 510 CLSM using the argon laser at 488 nm. Scans were taken at 1 μm intervals to a maximum depth of approximately 100 μm. Images were processed using the Zen 2009 software.

Results: All lens materials demonstrated sorption of rhPRG4. Senofilcon A materials revealed FITC-rhPRG4 penetrating into the bulk of the lens material uniformly, yet more or less favoring the two surfaces of the material. The same was observed with balafilcon A lenses, with a greater degree of rhPRG4 being seen at the surface compared to senofilcon A. Conversely, rhPRG4 was seen exclusively on the surface of lotrafilcon B and having no presence within the bulk of the lens. For comfilcon A and etafilcon A lenses, rhPRG4 was evenly distributed throughout the bulk of the lens, as well as on surface.

Conclusions: The location of rhPRG4 conjugated with FITC in a contact lens can be successfully visualized using CLSM. The lens chemistry, such as polymer composition, surface treatment and pore size, can influence the sorption of rhPRG4. This sorption profile may potentially impact rhPRG4's lubricating and wetting properties on lenses.

4.2 Introduction

Contact lenses are biomedical devices that are used to correct refractive errors to improve visual acuity. With the increasing trend in the prevalence of myopia,[1] contact lenses have become one of the most commercially successful biomedical products, with over 125 million lens wearers worldwide.[184] Part of their success can be attributed to their continuing evolution in terms of lens chemistry in order to better meet the needs of the wearer.[10,185] Some of these metrics include lower modulus,[186] greater oxygen permeability,[21,22] and enhanced material wettability,[51,165,166] to name a few.

Soft contact lenses are hydrogels, which are polymeric materials that are capable of absorbing large amounts of water, while maintaining their physical shape and properties.[8] The water absorptive characteristics of hydrogels are largely beneficial as contact lens materials as it prevents desiccation of the ocular surface during wear. However, contact lenses are in direct contact with the tear film and tear components, such as proteins and lipids, which are prone to being sorbed into the lens.[67,69,78,187] A significant amount of contact lens research has been dedicated to understanding the sorption of tear film components. Techniques such as radiolabeling,[68,70,188] chromatography[67,189,190] and various other assays[69,191,192] have proven useful in quantifying sorbed tear components. A major limitation to these techniques, however, is that they do not qualitatively elucidate the sorption characteristics within the lens. Imaging techniques, such as atomic force microscopy (AFM),[103,105,193] have provided greater information regarding the deposition of tear components, though these images are limited to the surface of the lens. Luensmann et al.[194,195] have described a novel method to qualitatively localize tear proteins deposited on the

lens surface and within the bulk using confocal laser scanning microscopy (CLSM). These findings demonstrate the importance of studying contact lenses as a whole rather than examining the surface, because the lens bulk constitutes a significant proportion of the lens material. New lenses with unique material chemistry are continually entering the market, thus there is a need to study the interaction between these novel materials and molecules in their surrounding environment.

The tendency for hydrogels to absorb tear film molecules has been the bane of contact lens development, but researchers and lens manufacturers are starting to use this property to their advantage. For example, several studies have examined the potential for contact lenses to be used as a drug delivery vehicle, due to their ability to uptake various drugs.[196-198] Additionally, Johnson & Johnson incorporates a wetting agent known as polyvinylpyrrolidone (PVP) into the bulk of its lenses, which leaches onto the lens surface to improve material wettability.[109] Another molecule that has been investigated for its wetting potential on contact lenses is the glycoprotein proteoglycan 4 (PRG4). Briefly, PRG4 is described as a natural lubricant that acts on articular cartilages to minimize frictional forces.[150,171] Schmidt et al.[145] have shown evidence of PRG4 on the ocular surface and proposed that it may have a significant role in ocular health. Previous studies have exhibited that PRG4 can improve the surface wettability of model[157] and commercially available contact lenses (Chapter 3). However, these findings are only exclusive to the lens surface and provide no insight as to how PRG4 interacts with the lens. Several modern day lenses undergo various modifications, such as surface treatments and inclusion of charged

polymers, and these modifications may potentially impact the wetting and lubricious potential of PRG4.

Recent advances in molecular biotechnology have allowed for the expression of full-length recombinant human PRG4 (rhPRG4) and this form is currently undergoing clinical evaluation to treat for dry eye disease.[199] Samsom et al. have successfully characterized rhPRG4 and concluded that it displays similar levels of post-translational modifications compared to native bovine PRG4.[154] The purpose of this study was to fluorescently tag rhPRG4 with fluorescein isothiocyanate to determine its location within various commercial contact lenses, to understand its interaction with the lens material, using CLSM.

4.3 Materials and methods

4.3.1 Contact lenses

The properties of the tested lens materials were described previously in Chapter 3.3.1 (Table 3-1).

4.3.2 Recombinant human PRG4 and fluorescent labeling

RhPRG4, which was expressed in a proprietary Chinese hamster ovary cell line, was purified and obtained from Lμbris, LLC (Framingham, MA) with a molecular weight of approximately 220 kDa.[154] A rhPRG4 stock solution was prepared at 2 mg/ml in 0.1M sodium carbonate-bicarbonate buffer (pH = 9.1). RhPRG4 was fluorescently labeled in-house with fluorescein isothiocyanate (FITC, Life Technologies, Carlsbad, CA) via an amine reaction. Figure 4-1 illustrates the molecular structure of FITC. This fluorescent dye was

selected because it has high absorptivity, excellent fluorescence quantum yield, and is soluble above pH 6. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, MO, USA) was used to solubilize FITC and an aliquot was added to the rhPRG4 stock to obtain a labeling ratio of 5:1 FITC:rhPRG4, as determined by a spectrophotometer. Vials were covered with aluminum foil to protect from light and allowed to react for 1 hour at room temperature. Unconjugated FITC was separated using a Sephadex-G25 resin (GE Health Sciences, Baie d'Urfe, QC, CA), as seen in Figure 4-2, eluted with PBS at pH 7.4, and concentrated using a molecular weight cutoff (MWCO) 100 kDa centrifugal filter (Sigma-Aldrich, MO, USA).

4.3.3 Preparation of contact lenses

An aliquot of the concentrated FITC-rhPRG4 suspension was added to PBS to obtain a final concentration of 300 µg/ml rhPRG4 as the incubating solution. Lenses were incubated for 1 hour at 37°C with gentle shaking and covered in aluminum foil. Afterwards, the central 4 mm of the lens was removed using a mechanical punch press and mounted onto a glass microscope slide (Fisher Scientific, Pittsburgh, PA). To prevent the sample from drying, 40 µl of PBS was placed onto the lens sample. A glass coverslip (VWR, Bridgeport, NJ) was carefully applied to stabilize the sample while being analyzed, and it was sealed with clear nail polish along the edges to prevent evaporation.

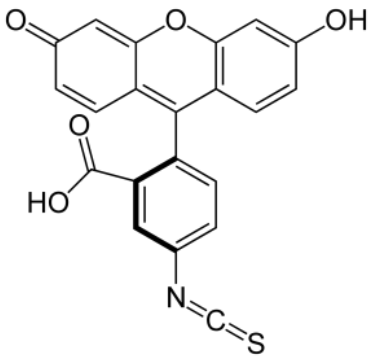

Figure 4-1. Molecular structure of fluorescein isothiocyanate (FITC; MW = 389.39 g/mol). The absorbance and emission spectra for FITC is 495 nm and 521 nm, respectively.

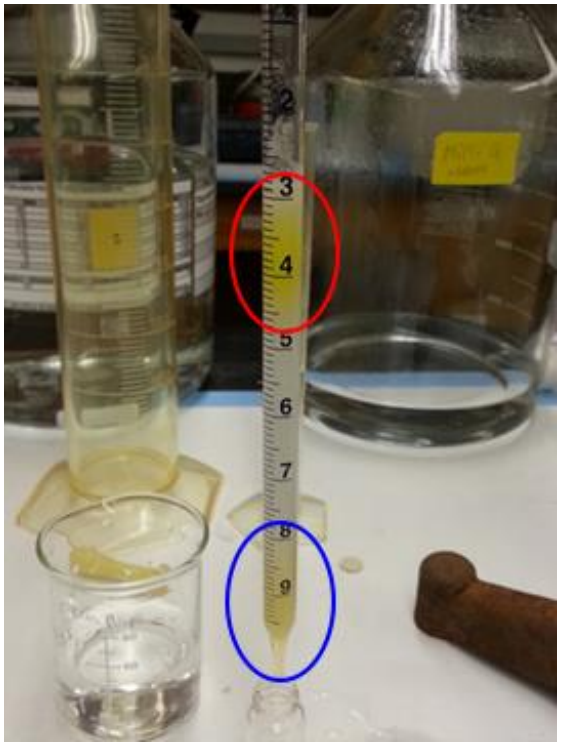

Figure 4-2. Separation of free FITC (red circle) with bound FITC to rhPRG4 (blue circle) using a Sephadex–G25 resin. Separation is based on differences in MW, such that smaller MW molecules will be trapped within the resin while larger MW molecules will be eluted.

4.3.4 Confocal laser scanning microscopy and image analysis

A CLSM Zeiss 510 equipped with an inverted motorized microscope Axiovert 200M (Zeiss Inc. Toronto, Canada) was used to analyze the lens samples. An argon laser set at a wavelength of 488 nm (50% output and 5% laser transmission) and BP 475-525 nm emission filters were used to scan the central location of the sample to detect the FITC. The 40x water immersion C-Apochromat objective was used to scan the lenses and out of focus rays were filtered by setting the pinhole size to 1 Airy unit. Samples were analyzed using the *z*-stack function where each panel represented 1 µm intervals starting from the anterior towards the posterior surface of the lens. The ZEN software was used to process the panel of images for each lens type. A total of 3 replicates for each lens incubated in FITC:rhPRG4 were examined and a representative image was selected. Control lenses incubated in PBS containing no FITC were included for each respective lens to determine level of lens autofluorescence.

4.4 Results

The findings from this study are depicted in the following series of figures for each lens material. Each figure consists of a representative control scan of lenses incubated in PBS and a test scan with lenses incubated in FITC:rhPRG4 at 300 µg/ml. Each panel, going from left to right, represents 1 µm scan intervals into the sample. The intensity of green light emitted qualitatively indicates the relative amount of FITC:rhPRG4 at that location in the lens.

For all lens materials examined, the control scans consisting of the lens material and rhPRG4 (untagged) did not demonstrate significant fluorescence with minimal background noise. However, lenses incubated with labeled rhPRG4 displayed varying patterns of fluorescence. RhPRG4 absorbed relatively uniform through the bulk of the balafilcon A lens material, though a greater proportion was adsorbed to the anterior and posterior surfaces of the lens (Figure 4-3). Senofilcon A lenses, on the other hand, did not exhibit the same magnitude in fluorescence intensity on the surfaces. These lenses portrayed a uniform distribution of rhPRG4 throughout the bulk and a modest increase in adsorption on the surfaces (Figure 4-4). CLSM scans of treated lotrafilcon B lenses exhibited an interesting phenomenon, where fluorescence signals were exclusive on the lens surfaces and no presence within the lens (Figure 4-5). The sorption profile of rhPRG4 for comfilcon A and etafilcon A are shown in Figures 4-6 and 4-7, respectively. Both lens materials displayed similar trends in sorption, such that there was an even distribution of rhPRG4 both within the lens as well as on the surfaces of the material.

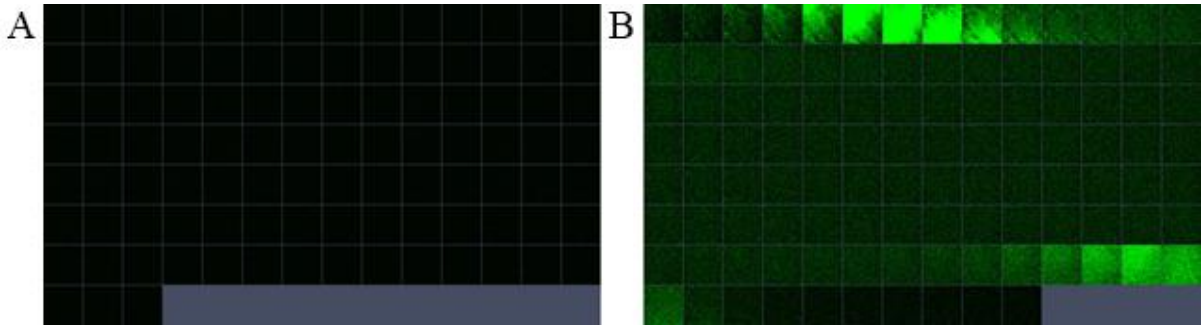

Figure 4-3. Confocal microscopy scans of balafilcon A lenses illustrating the sorption profile of rhPRG4. A) control lens with no FITC; B) test lens with FITC conjugated to rhPRG4.

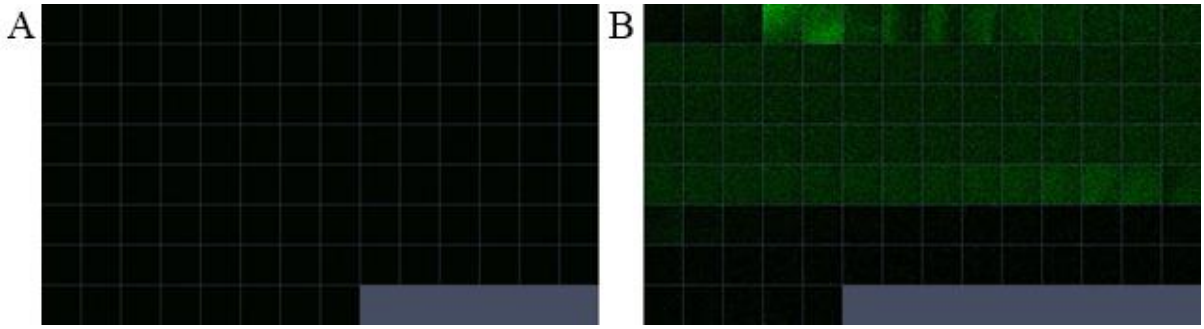

Figure 4-4. Confocal microscopy scans of senofilcon A lenses illustrating the sorption profile of rhPRG4. A) control lens with no FITC; B) test lens with FITC conjugated to rhPRG4.

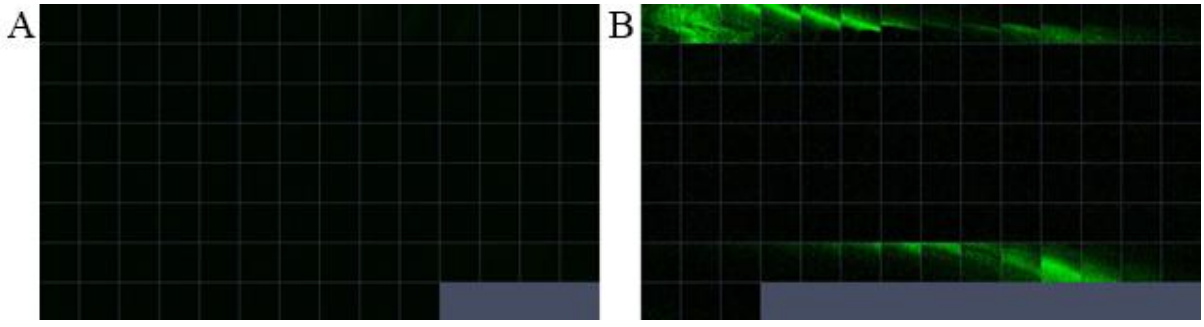

Figure 4-5. Confocal microscopy scans of lotrafilcon B lenses illustrating the sorption profile of rhPRG4. A) control lens with no FITC; B) test lens with FITC conjugated to rhPRG4.

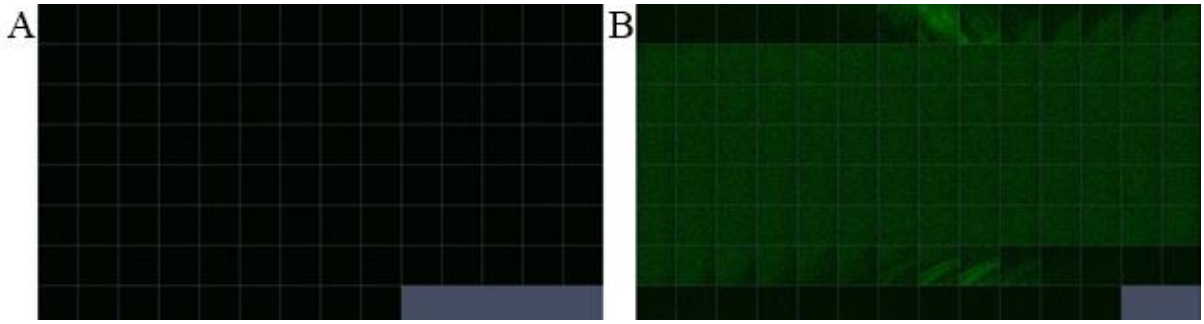

Figure 4-6. Confocal microscopy scans of comfilcon A lenses illustrating the sorption profile of rhPRG4. A) control lens with no FITC; B) test lens with FITC conjugated to rhPRG4.

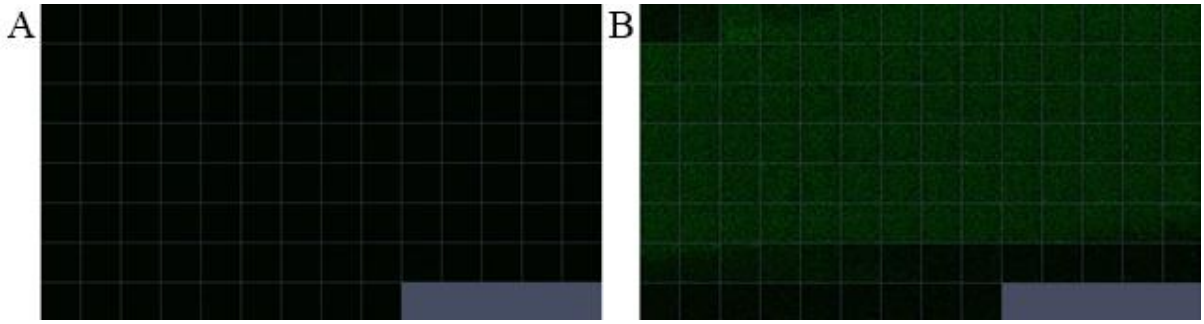

Figure 4-7. Confocal microscopy scans of etafilcon A lenses illustrating the sorption profile of rhPRG4. A) control lens with no FITC; B) test lens with FITC conjugated to rhPRG4.

4.5 Discussion

Confocal microscopy is a highly versatile instrument that has many advantages over traditional light microscope. CLSM provides high resolution images by utilizing the pinhole phenomenon that essentially rejects out-of-focus light rays. Additionally, the focal plane is adjustable, which allows the user to scan within a sample, as opposed to light microscopes that are limited to surface images. Furthermore, CLSM is capable of *in vivo* imaging, which has been used extensively for ocular research.[200-202]

The results from this study have demonstrated that contact lenses can be imaged successfully using CLSM and that rhPRG4 has the capacity to be fluorescently labeled with FITC. There are several studies that have used CLSM to visually track the sorption of tear proteins within various commercial contact lenses.[194,195,203] Their general conclusion was that protein and lens material interaction is a complex relationship that is influenced by several factors, such as lens composition, ionicity, hydrophobicity and presence of a surface treatment. Another important factor that needs to be considered is the pore size within the hydrogel material that can serve as a potential reservoir for molecules to bind in.[204] Several studies have reported different pore sizes within the hydrogels examined, which ranged from 30 Å to over 400 Å.[73,205,206] RhPRG4, with a molecular weight of approximately 220 kDa,[154] is much larger in size compared to typical proteins of the tear film. Nonetheless, sorption of rhPRG4 was seen in the majority of the tested lenses, suggesting that the true hydrogel pore size is likely in the upper limits.

Balafilcon A and lotrafilcon B lenses displayed significantly different sorption profiles of rhPRG4, which can be attributed to the specific surface treatment that each

respective material undergoes. Surface wettability is enhanced by chemically transforming the overlying siloxane polymers into hydrophilic silicate groups through plasma oxidation.[102] The presence of these hydrophilic groups on the lens surface may in part explain why a great proportion of rhPRG4 is found on the surface. Additionally, balafilcon A lenses have an ionic charge due to the presence of monomer *N*-vinyl aminobutyric acid within its structure. Lens ionicity has been shown to be a primary factor for increased protein sorption,[73,207] and rhPRG4 may also experience a net attraction towards these ionic materials.

Lotrafilcon B lenses, on the other hand, undergo a plasma polymerization process that forms a high-index refractive coating on the lens surface.[106] Unlike the surface treatment for balafilcon A lenses, where discontinuous silicate islands are manifested onto the lens surface, plasma polymerization produces a continuous coating that can physically act as a barrier to impede the penetration of biological molecules. Figure 4-5 clearly illustrates that rhPRG4 was exclusively restricted to the lens surfaces with virtually no presence within the bulk of the lens. These findings were also consistent with albumin[194] and lysozyme[195] tested on lotrafilcon B, suggesting that the plasma coating is a superior surface treatment that minimizes protein sorption of varying sizes.

The sorption of rhPRG4 on the remaining three lens materials (senofilcon A, comfilcon A, etafilcon A) appeared relatively similar between lenses in that there was more or less a uniform distribution throughout the bulk and surfaces of the lens. A common characteristic amongst these lens material is that they are not surface treated. Therefore, without an impeding physical barrier, rhPRG4 was shown to absorb freely and uniformly throughout the bulk. The incorporation of polyvinylpyrrolidone (PVP) in senofilcon A lenses

also did not appear to significantly affect the penetration of rhPRG4. However, unlike PVP, which can act as a wetting agent both from within and on the lens surface, it is unknown whether rhPRG4 has any significant wetting properties within the bulk of the lens. The findings from this study show that rhPRG4 penetrated into the matrix of all lenses examined, with the exception of lotrafilcon B, which may effectively reduce its wetting and lubricious potential as a large concentration is being sequestered within the lens. On the other hand, if rhPRG4 is loosely bound to the lens matrix, it has the potential to leach out towards the lens surface, which would be analogous to a contact lens as a drug delivery vehicle. Future work should consider investigating different techniques to fix rhPRG4 onto the lens surface, in addition to determining the level of mobility that rhPRG4 has within the bulk.

Despite the novel use of confocal microscopy to image sorbed molecules within contact lenses, there are a few considerations that need to be addressed while performing this technique. In order to prevent the sample from drying, 40 µl PBS was added to the lens. It is possible that this procedure, although necessary, may potentially affect the sorption of rhPRG4 by displacing its location within the lens. In addition, scans of lotrafilcon B lenses demonstrated streaks of fluorescence on the lens surface. These lenses are known to have one of the highest modulus compared to other lenses, and this artifact may likely be due to the sample's inability to completely flatten when mounted onto the microscope slide. Furthermore, the potential of the fluorescent probes dissociating from the protein and binding non-specifically to the substrate should also be considered. Contact lenses have been shown to take up free fluorescent dye, which can have major implications when interpreting the data.[208] To circumvent this issue, rhPRG4 was manually tagged with FITC in this study and

free FITC was physically separated using a Sephadex-G25 resin (Figure 4-2). Fresh incubating solutions of FITC:rhPRG4 were created each time in order to minimize the chance for dye dissociation. Even when FITC is securely bound to rhPRG4, the influence that the dye has on the molecule must also be considered. Guan et al.[209] investigated the effect of fluorescent labels on protein sorption in hydrogels and found that the attachment of a probe increases the uptake of the protein compared to unlabeled proteins. This observation was more pronounced on hydrophobic materials with a 10-fold difference. Although Guan et al. did not examine FITC, it is possible that FITC can potentially increase the sorption of rhPRG4, which should be taken into consideration while interpreting the results from this study.

The sorption characteristics of rhPRG4 into commercial contact lenses are similar to other reported proteins in the tear film, suggesting that lens material composition plays a significant role in how it sorbs various molecules. Lens characteristics, such as the presence of a surface treatment and ionically charged polymers, can have a major impact on the uptake of rhPRG4 into the material. Future studies involving CLSM should consider pairing the data with quantitative techniques to provide a better understanding of rhPRG4 within the lens material. It would also be noteworthy to track the sorption of rhPRG4 over an extended period of time to provide more information regarding its uptake and release kinetics.

Chapter 5 – Investigating the effects of proteoglycan 4-coated silicone hydrogels on *Staphylococcus aureus* adhesion and viability

5.1 Overview

Purpose: The purpose of this study was to evaluate the effect of proteoglycan 4 (PRG4) on the total adhesion and viability of *Staphylococcus aureus* on various silicone hydrogel lenses.

Methods: Four lens materials (delefilcon A, narafilcon A, lotrafilcon B, comfilcon A) were treated with bovine PRG4 and recombinant human PRG4 (rhPRG4) at 300 µg/ml for 1 hour at 37°C (untreated lenses served as controls). Subsequently, these lenses were exposed to a bacterial suspension containing *S. aureus* radiolabeled with 0.2% v/v ^{3}H-uridine. Total bacterial adhesion was determined using a Beta counter by measuring the counts per minute emitted by the ^{3}H isotope, while the viability was determined by counting the number of colony forming units (CFU) on Tryptic Soy Agar (TSA) nutrient plates.

Results: All lens materials displayed bacterial adhesion with varying levels of viable bacteria. Bovine PRG4 and rhPRG4-treated lenses did not differ significantly from one another ($P>0.05$), though either treatment did not significantly reduce total bacterial adhesion ($P>0.05$). A significant finding was, however, observed with bovine PRG4-coated narafilcon A lenses, where an increase in viable bacteria was observed ($P<0.05$). Additionally, graphical

analysis revealed a moderate trend where lenses treated with bovine and rhPRG4 had more viable bacteria on average compared to uncoated lenses, though this was statistically insignificant ($P>0.05$).

Conclusions: PRG4, both bovine and recombinant, did not display a significant effect in modulating the adhesion of *S. aureus* on silicone hydrogel lenses. However, it is possible that a significant effect may exist in the early stages of contamination, though further work is required to investigate this claim.

5.2 Introduction

Novel biomedical devices are constantly under scrutiny when being evaluated for bacterial contamination. Surgical instruments, for example, are developed with unique designs with the aim of reducing bacterial adhesion.[210] Despite the numerous efforts in preventing contamination, surgical site infections still occur, resulting in approximately 3 deaths for every 100 surgeries.[211] Other than patient mortality, the patient's quality of life is diminished due to prolonged hospitalization associated with these healthcare-associated infections.

Contact lenses are also prone to bacterial contamination. Although the risks are usually not life threatening when compared to post-surgical infections, contaminated lenses can ultimately lead to blindness if left untreated.[212] The most common type of contact lens complication is microbial keratitis (MK), which is an infection of the cornea, and approximately 90% of MK cases are bacterial related.[213] The risk of keratitis increases significantly if the cornea has been compromised, such as from overnight and extended contact lens wear,[214-217] poor hygiene,[218] and non-compliance/lack of knowledge of care regimens.[219] Additionally, contact lenses can act as a substrate for bacteria to bind, thus further increasing the risk for keratitis to occur.[95] The two most common opportunistic pathogens associated with MK are *staphylococci* and *pseudomonas*, which are normally found on skin and in soil, respectively.[85-87] Similar to surgical instruments, new contact lens materials and care solutions are being developed to prevent the initial adhesion of bacteria onto the lens surface, which would essentially prevent the cascade for MK from occurring.

Although the development of soft conventional hydrogel lenses in the 1970's improved wearer comfort compared to rigid gas permeable lenses, an increase in the prevalence of MK was observed.[217,220,221] As newer soft lens modalities were developed to reflect different wear times, it was observed that conventional hydrogel lenses designed for an extended wear basis had the greatest risk for MK relative to the other lens modalities.[217] However, lens wear characteristic are not the only factor that influences bacterial adhesion, lens material also has a significant impact. In the early 2000s, a new line of lenses, known as silicone hydrogels, were developed to address issues of hypoxia associated with conventional hydrogel lenses. The incorporation of siloxane groups into the lens material significantly enhanced oxygen transmissibility to the cornea.[14,18,19] However, the inclusion of silicone increased the lens surface hydrophobicity, which has been shown to significantly increase adsorption of lipids[69-71] and bacterial adhesion,[91,92,98] compared to conventional hydrogel lenses.

Since its introduction in the market, silicone hydrogels have undergone significant changes to their surface characteristic to render them more hydrophilic/less hydrophobic. There currently exist several generations of silicone hydrogels which represent different techniques used to enhance surface hydrophilicity, such as surface treatment,[102] internal wetting agents,[108] and unique blends of polymers.[114,222] Recently, there has been a growing interest in the use of biomolecules to improve lens wettability and wearer comfort. A notable example is the inclusion of hyaluronic acid in lens care solutions and other ophthalmic solutions.[223-225] Other biomolecules, such as proteoglycan 4 (PRG4), are also currently being investigated for their impact on the ocular surface.[145] Briefly, PRG4 is a glycoprotein and a

major lubricant of the body, particularly at the joint where it reduces the friction between the articular cartilages.[150,171] Since its isolation from bovine synovial fluid by Swann et al.,[226] PRG4 has been discovered in various parts of the body, including on the ocular surface,[145] thus giving it relevance as a potential constituent in ophthalmic solutions. In terms of contact lens applications, isolated bovine PRG4 has been shown to reduce friction[159,160] and enhance surface wettability for both model[157] and certain commercial lenses (Chapter 3). Additionally, PRG4 has been shown to exhibit anti-adhesive properties,[227] which is an attractive property given the tendency for tear film proteins and lipids to deposit onto lens surfaces. This anti-adhesive phenomenon has also been applied to bacteria, where PRG4-coated polystyrene surfaces show a reduction in bacterial adhesion.[149] Advancements in molecular biology have allowed for the expression of full-length recombinant human PRG4 (rhPRG4), which is currently available for clinical evaluation. RhPRG4 has been fully characterized by Samsom et al.,[154] and they have shown that it has the same level of *O*-linked glycosylations and lubricious property as that of the bovine PRG4. To date, there is little published regarding the other characteristics that rhPRG4 may possess. Therefore, the aim of this study was to evaluate the effect that bovine PRG4 and rhPRG4 has on bacterial adhesion and viability on silicone hydrogel contact lenses.

5.3 Materials and methods

5.3.1 Contact lenses

The contact lenses used in this study are detailed in Table 5-1. Briefly, two daily disposable [delefilcon A (Alcon, Fort Worth, TX), narafilcon A (Johnson & Johnson, Jacksonville, FL)] and two reusable [comfilcon A (CooperVision, Pleasanton, CA), lotrafilcon B (Alcon, Fort Worth, TX)] silicone hydrogel lenses were evaluated (n=6). All lenses were unworn and rinsed in sterile phosphate buffered saline (PBS) overnight to remove blister package constituents. To our knowledge, there have been no studies reporting the bacterial adhesion on delefilcon A lenses. However, the other three lens materials listed have been shown to report high amounts of bacterial adhesion, which would make these lenses advantageous to study with a PRG4-coating applied.

5.3.2 Bovine and recombinant human proteoglycan 4

Mature bovine stifle joints containing articular cartilage disks were harvested for the extraction of bovine PRG4. The preparation of bovine PRG4 has been previously described.[150,151]

Full-length recombinant human PRG4 (rhPRG4) was expressed in a Chinese hamster ovary (CHO) cell line and was provided by Lμbris, LLC (Framingham, MA). A 0.2 μm polysulfone membrane (Sigma-Aldrich, Oakville, ON) was used to filter both bovine PRG4 and rhPRG4 stock solutions to ensure the removal of any possible bacteria. Bovine PRG4 and rhPRG4 incubating solutions were prepared to 300 μg/ml in PBS and stored at –4°C until usage.

Table 5-1. Properties of the silicone hydrogel lenses evaluated.

Wear Schedule	**Daily Wear**		**Continuous Wear**	
USAN	Delefilcon A	Narafilcon A	Lotrafilcon B	Comfilcon A
Proprietary Name	Dailies Total 1	1-Day Acuvue TruEye	Air Optix Aqua	Biofinity
Manufacturer	Alcon	Johnson & Johnson	Alcon	CooperVision
Water Content	>80% surface; 33% bulk	46%	33%	48%
Surface Treatment	Not disclosed	None	Plasma polymerization	None
Ionicity	Yes	No	No	No
Principal Monomers	Not disclosed	HEMA + DMA + mPDMS + TEGDMA + PVP	DMA + TRIS + siloxane macromer	FM0411M + HOB + IBM + M3U + NVP + TAIC + VMA

DMA (N,N-dimethylacrylamide); **FM0411M** (2-ethyl [2-[(2-methylprop-2-enoyl)oxy]ethyl]carbamate); **HEMA** (poly-2-hydroxyethyl methacrylate); **HOB** ((2RS)-2-hydroxybutyl 2-methylprop-2-enoate); **IBM** (Isobornyl methacrylate); **M3U** (α-[[3-(2-[[2-(methacryloyloxy)ethyl] carbamoyloxy]ethoxy)propyl]dimethylsilyl]-ω-[3-(2-[[2-(methacryloyloxy)ethyl] carbamoyloxy]ethoxy)propyl]poly([oxy[(methyl) [3-[ω-methylpoly(oxyethylene)oxy]propyl]silylene]/[oxy[(methyl)(3,3,3-trifluoropropyl)]silylene]/oxy (dimethylsilylene)])); **mPDMS** (monofunctional polydimethylsiloxane); **NVP** (N-vinyl pyrrolidone); **PVP** (poly(vinylpyrrolidone)); **TAIC** (1,3,5-triprop-2-enyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione); **TEGDMA** (tetraethyleneglycol dimethacrylate); **TRIS** (trimethylsiloxy silane); **USAN** (United States Adopted Name); **VMA** (N-Vinyl-N-methylacetamide).

5.3.3 Bacterial growth and radiolabeling

The bacterial strain that was selected for this study was the gram positive organism *Staphylococcus aureus* (ATCC 6538 – human isolate). Stock cultures of *S. aureus* were stored in tryptic soy broth (TSB; Becton, Dickinson and Company, Franklin Lakes, NJ) containing 10% glycerol at –80°C. Strains were grown on tryptic soy agar plates (TSA; Becton, Dickinson and Company, Franklin Lakes, NJ) for 18h at 37°C. Cells were harvested by centrifugation (3000 RPM, 10 minutes) and re-suspended in sterile PBS. The concentration of the bacterial suspension was adjusted using a spectrophotometer to achieve an optical density (OD) of 1.0 at 660 nm, which is approximately 1.0×10^{10} colony-forming units (CFU)/ml. Strains were grown for 24h at 37°C in 10 ml of TSB containing 0.2% v/v ^{3}H-uridine (Perkin Elmer, Waltham, MA). After incubation, cells were collected by centrifugation and washed three times in sterile PBS to remove any loosely bound isotope on the bacterial membrane. The final concentration of the bacterial suspension was diluted in PBS and adjusted to 1.0×10^{8} CFU/ml by obtaining an OD of 0.1 at 660 nm.

5.3.4 Pre-treatment and challenging lenses

Prior to exposing the lenses to the bacterial suspension, lenses were incubated under three conditions: (1) PBS-control; (2) 300 µg/ml bovine PRG4; (3) 300 µg/ml rhPRG4 for 1h at 37°C with gentle shaking. After incubation, lenses were dabbed onto lens paper (VWR, Mississauga, ON) to remove excess solution.

Control and treated lenses were placed aseptically into a 24-cell culture plate in their respective wells that contained 1 ml of the radiolabeled bacterial suspension. The culture

plate was sealed with Parafilm and incubated at 37°C with gentle shaking for 18 hours. Afterwards, lenses were rinsed three times in 1 ml sterile PBS on a plate shaker for 30 seconds to remove any loosely bound bacteria on the lens surface. Each lens was then transferred to a 5 ml plastic vial (VWR, Mississauga, ON) which contained 1 ml PBS and 3 glass beads (Fisher Scientific, Waltham, MA). Vials were vortexed for 2 minutes to allow the glass beads to mechanically remove all the bacteria from the lenses. The resulting homogenate was then used to determine both the viable and total counts of *S. aureus* for the lens material.

5.3.5 Determination of total counts

Total bacterial counts, which constitute both live and dead cells, were determined in the following procedure: 1) the contact lens and its homogenate and glass beads were transferred to its respective scintillation vial, 2) scintillation fluid was added to each vial and the counts per minute (CPM) were determined using a Beta counter (Beckman Coulter LS6500 Multipurpose Scintillation Counter, Fullerton, CA). In order to convert the CPM generated to cells/lens, a standard curve was plotted with $R^2 > 95\%$.

5.3.6 Determination of viable counts

Viable bacterial counts, which constitute only live cells, were determined in the following procedure: 1) serial dilutions of the homogenate for each lens material were made in a microcentrifuge tube by transferring 100 µl into 900 µl (1:10) of D/E Neutralizing Broth (Fisher Scientific, Waltham, MA), 2) each serial dilution was plated on nutrient agar plates as triplicates consisting of 50 µl aliquots, 3) plates were incubated for 18h at 37°C and the

number of colonies was determined afterwards. Dilutions that contained between 10 and 100 colonies were counted, averaged, and logarithmically converted to cells/lens.

5.3.7 Statistical analysis

All statistical analysis was performed using Statistica 12 (StatSoft Inc, Tulsa, OK). A factorial analysis of variance (ANOVA) was selected with lens material, lens condition, total adhesion, and viable counts as the factors. Post hoc comparisons were conducted with the Tukey Honestly Significant Difference (HSD) test. $P<0.05$ was considered significant for all tests.

5.4 Results

The results obtained from this study are reported in Table 5-2 as mean ± standard deviation cells/lens (Log10). Figures 5-1 and 5-2 were plotted to graphically visualize the data for trends.

5.4.1 Bacterial adhesion and viability on uncoated materials

All four lens materials displayed contamination when incubated in the bacterial suspension for 18h. The total bacterial adhesion was significantly greater than viable counts for delefilcon A ($P<0.01$), narafilcon A ($P<0. 01$), and comfilcon A ($P<0.01$). Lotrafilcon B lenses, on the other hand, did not show a statistical difference between total adhesion and viable counts ($P=0.50$). Additionally, statistical analysis revealed that the total bacterial adhesion did not differ significantly between materials ($P<0.05$). However, graphical analysis

indicated that lotrafilcon B lenses had slightly more bacterial adhesion on average compared to delefilcon A, narafilcon A, and comfilcon A. For viable counts, there was no significant difference between delefilcon A, narafilcon A, and comfilcon A lenses ($P>0.05$), however, lotrafilcon B lenses did demonstrate significantly more viable bacteria compared to the other three materials ($P<0.01$).

Table 5-2. The total adhesion and viable counts of *S. aureus* on different silicone hydrogel lenses treated with bovine and rhPRG4.

			Bovine PRG4 Cells/Lens (Log10)			rhPRG4 Cells/Lens (Log10)		
Lens Treatment	Test Type	Material	N	Mean	SD	N	Mean	SD
Coated	Total Adhesion	Delefilcon A	6	6.0401	0.3329	6	5.8359	0.2093
		Narafilcon A	6	6.1749	0.3401	6	5.8181	0.0793
		Lotrafilcon B	6	6.4662	0.2268	6	6.5847	0.1274
		Comfilcon A	6	6.3122	0.1582	6	5.6749	0.3973
Coated	Viable Counts	Delefilcon A	6	4.2911	0.1613	6	4.7774	0.4459
		Narafilcon A	6	4.8196	0.3231	6	4.9212	0.2498
		Lotrafilcon B	6	5.8390	0.1454	6	6.3021	0.0240
		Comfilcon A	6	4.7662	0.1994	6	4.7953	0.2774
Un-Coated	Total Adhesion	Delefilcon A	6	6.1204	0.3983	6	5.7124	0.1676
		Narafilcon A	6	6.1131	0.4612	6	5.8567	0.2602
		Lotrafilcon B	6	6.3729	0.2053	6	6.3830	0.2760
		Comfilcon A	6	5.8356	0.5035	6	5.7456	0.4768
Un-Coated	Viable Counts	Delefilcon A	6	4.0300	0.3450	6	4.3446	0.2101
		Narafilcon A	6	4.1695	0.0803	6	4.5994	0.2157
		Lotrafilcon B	6	5.8195	0.0978	6	5.7641	0.2370
		Comfilcon A	6	4.6653	0.2097	6	4.6000	0.6321

5.4.2 Effect of bovine PRG4 on bacterial adhesion and viability

Lenses treated with bovine PRG4 were assessed for their effect on bacterial adhesion and viability. In terms of total bacterial adhesion, bovine PRG4-coated lenses did not differ significantly compared to the uncoated controls ($P>0.05$). There was no statistical significance in total adhesion between PRG4-coated delefilcon A, narafilcon A, lotrafilcon B, and comfilcon A lenses ($P>0.05$).

Bovine PRG4-treated delefilcon A, lotrafilcon B, and comfilcon A lenses showed no statistical difference in viable bacteria compared to uncoated lenses ($P>0.05$). However, narafilcon A lenses reported a statistically greater amount of viable bacteria after incubating in bovine PRG4 compared to the control ($P=0.04$) (Figure 5-1).

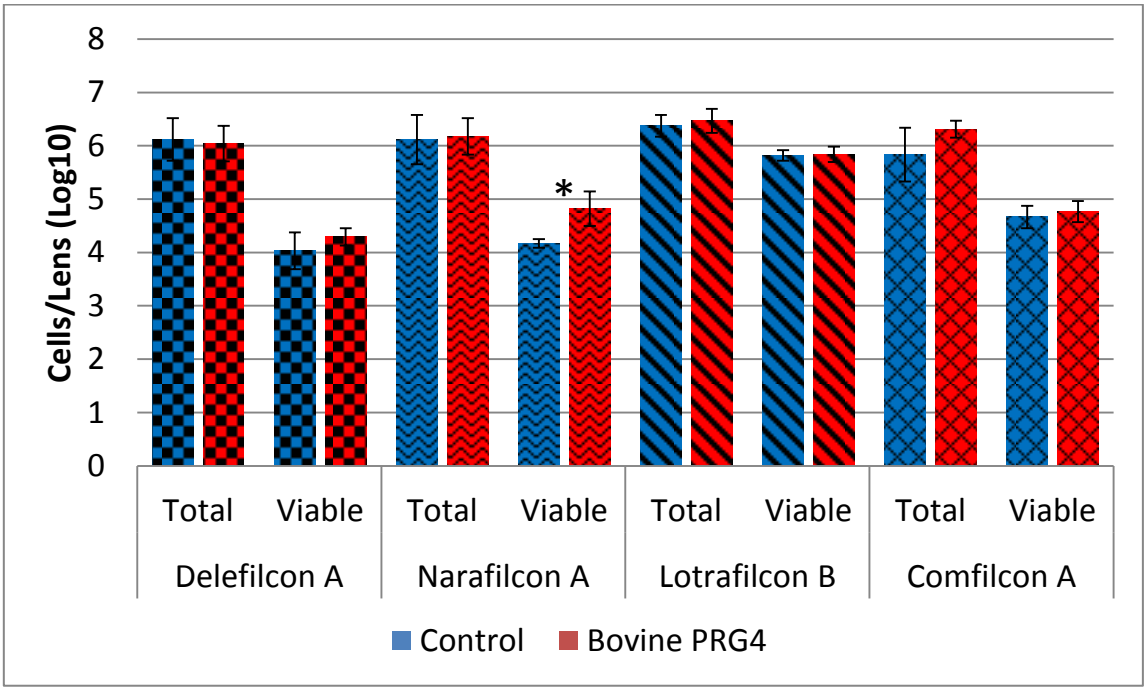

Figure 5-1. Graphical representation of *S. aureus* total adhesion and viable counts on uncoated and bovine PRG4-coated silicone hydrogel lenses. *Significance ($P<0.05$) between control and bovine PRG4-treated narafilcon A lenses for viable bacteria.

5.4.3 Effect of recombinant human PRG4 on bacterial adhesion and viability

Lenses treated with rhPRG4 were assessed for their effect on bacterial adhesion and viability. Total bacterial adhesion on rhPRG4-coated lenses did not differ significantly from their uncoated controls (P>0.05). When total adhesion was compared between the four different lenses, there was no statistical significance in total adhesion between rhPRG4-coated delefilcon A, narafilcon A, lotrafilcon B, and comfilcon A lenses (P>0.05).

RhPRG4-treated delefilcon A, narafilcon A, lotrafilcon B, and comfilcon A lenses showed no statistical difference in viable bacteria compared to their uncoated lens controls (P>0.05). Additionally, viable bacterial counts did not differ significantly between rhPRG4-treated delefilcon A, narafilcon A, and comfilcon A lenses (P>0.05) (Figure 5-2).

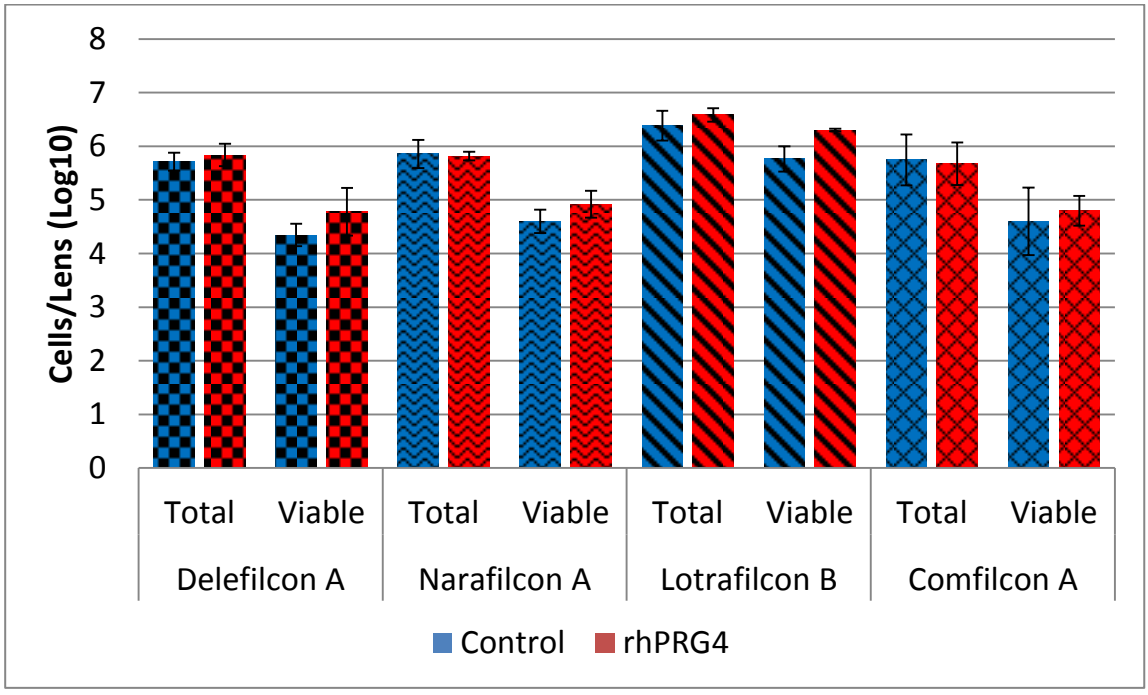

Figure 5-2. Graphical representation of *S. aureus* total adhesion and viable counts on uncoated and rhPRG4-coated silicone hydrogel lenses.

5.4.4 Comparison between recombinant human- and bovine PRG4-coated lenses

Statistical analysis revealed that lenses coated with rhPRG4 and bovine PRG4 did not differ significantly from one another when determining both total bacterial adhesion ($P>0.05$) and viable counts ($P>0.05$).

5.5 Discussion

The present study was the first to report the effects of PRG4 lens coatings on various commercial silicone hydrogel lenses against *S. aureus*. The advent of silicone hydrogels brought forth lenses with superior oxygen permeability, but it inadvertently increased the risk of bacterial contamination.[84,95,98] It has been shown that bacterial adhesion is greater on silicone-based lenses than on conventional hydrogels, which is most likely due to the incorporation of the hydrophobic siloxane monomers.[91,92,98] If left unnoticed and untreated, lens bacterial contamination can become a significant risk factor for MK, which in turn can opacify the cornea and ultimately lead to loss of vision and/or blindness.[228] Lens manufacturers have developed various cleaning solutions with several potent biocides in an attempt to reduce rates of MK, however, dead bacterial cells on the lens are still antigenic and can elicit an immunological response that leads to "sterile" keratitis or an inflammatory response.[229] A more effective strategy would be to prevent the initial adhesion of bacteria onto a contact lens, thus preventing the cascade for contact lens-related MK from occurring.

It was hypothesized that contact lenses treated with a PRG4-coating would discourage the adhesion of bacteria. Studies have shown that PRG4 can enhance the lubricity

and wettability of materials through its extensive *O*-linked glycosylations and hydrated sugars.[154,156,157,159] Recent data have also revealed that PRG4 contains anti-adhesive properties due to the steric repulsive forces contained within the glycosylated chains.[147,148] Aninwene et al.[149] were the first to report the anti-adhesive effect of PRG4 against *S. aureus* on polystyrene surfaces, which had inspired for the need to evaluate its effect on contact lenses. Furthermore, rhPRG4 has been recently expressed and characterized,[154] and it was also evaluated to determine if it exhibits the same properties as bovine PRG4.

The findings from this study have shown that both PRG4 and rhPRG4 did not differ significantly from one another; moreover, neither coating had a significant effect against total *S. aureus* adhesion. Despite Aninwene et al.[149] reporting a significant reduction in bacterial binding to PRG4-coated surfaces, there are several factors that may account for this discrepancy. Perhaps the most significant factor is the different substrates used. A polystyrene surface was used for their study, whereas contact lenses, which are essentially hydrogel polymers, were used in the current study. These two substrates have completely different properties from one another, which would ultimately dictate how PRG4 and bacteria interact with the material.[230] For instance, the hydrogel lens is capable of absorption into the matrix due to large cavities designated for water molecules to occupy,[8] whereas most polystyrene materials are not significantly absorptive. This characteristic has major implications on the effective concentration of PRG4 in the lens and the effect it may have on bacteria, as the concentration of PRG4 would be greater on the surface of polystyrene than on a hydrogel.

Another important factor that needs to be considered is the type of analysis used. In this study, a novel radiolabeling technique was used to quantify the total bacteria on each lens, whereas Aninwene et al. measured the OD of the lens homogenate. Radiolabeling provides greater sensitivity[231] and, therefore, a better measure of total bacteria bound to the contact lens material (both living and dead bacterial cells) than the OD technique. A possible issue with OD reading is that bacterial by-products can potentially influence the OD measurement value; thus resulting in misleading interpretations.

Furthermore, the experimental design used in this study allows significantly more time for bacterial contamination of the lens than for PRG4 uptake onto the lens. In order to remain consistent with previous PRG4 studies lenses were incubated in PRG4 for 1 hour, whereas in bacterial studies lenses are challenged for at least 18 hours.[96,232,233] It is possible that PRG4 may have an anti-adhesive effect against *S. aureus* in the early stages (i.e. the first hour or two). However, this effect may have eventually become subdued as the lenses are exposed to the bacterial suspension for a much longer duration, which would allow for a greater proportion of bacteria to adhere onto the lens surface over time. Further work investigating PRG4 and bacterial adhesion kinetics is required to support this claim.

To our knowledge, this was the first study that evaluated bacterial viability in PRG4-coated contact lenses. The data showed that both bovine PRG4 and rhPRG4 did not have a statistically significant effect on bacterial viability on the tested lenses, with the exception of narafilcon A lenses, which showed a statistically significant increase in viable bacteria with the bovine PRG4 coating. Interestingly, all PRG4-coated lenses (bovine and recombinant) demonstrated more viable bacteria, on average, compared to the uncoated controls, as seen in

Figures 5-1 and 5-2, but these differences were not significant. It is possible that the PRG4, both recombinant and bovine, are being utilized by the bacteria as a potential food source. Silicone hydrogel lenses are not known to adsorb large amounts of protein,[67] but the proteins that deposit are highly denatured.[77,78] It is possible that in proteins such as PRG4, denaturation changes the conformational state of the protein so that it is less stable and easier for bacteria to feed off of because amino acid units are more susceptible. Previous studies have examined the effect of worn lenses on bacterial viability and shown that the wearing of lenses does not have a significant influence,[234-236] however, these results may have been confounded by the variety of proteins present within the tear film that have bactericidal properties, such as lysozyme and lactoferrin.[237,238] That being said, the synergistic effects of lysozyme and lactoferrin may have been opposed by other tear proteins, such as albumin, which has been claimed to promote bacterial survival.[82,232] Based on the results of this study, it is possible that PRG4 may behave similarly to albumin in that it promotes bacterial survival, given that no bactericidal proteins are present. Regardless, further work is required to investigate this observation.

Delefilcon A lenses (Dailies Total 1) are a relatively new line of daily disposable silicone hydrogel lenses with water gradient technology.[239,240] To our knowledge, this was the first study reporting the bacterial adhesion and viability on these lenses when unworn and uncoated. It was shown that the level of bacterial adhesion and viability on delefilcon A was statistically similar to narafilcon A and comfilcon A materials. Lotrafilcon B lenses did demonstrate, on average, more bacterial adhesion and viable bacteria compared to the other 3 lens materials, which is consistent with other studies.[96,97,232] It is believed that bacterial

adhesion is also affected by water content, where low water content lenses promote adhesion and bacterial viability.[92,95] These findings are consistent with the lotrafilcon B material tested in this study as it had the lowest water content overall.

Despite PRG4 not having a significant effect in reducing bacterial adhesion, it should also be noted that it does not have a significant adverse effect on total adhesion. A study conducted by Subbaraman et al.[232] has shown that a protein coating onto a lens surface can significantly increase bacterial binding, which may potentially increase the risk of MK from developing. PRG4 is essentially a protein and one would expect an increase in bacterial adhesion onto these lenses, though this was not the case. Therefore, the fact that there was not a significant effect between *S. aureus* and PRG4 is a positive result in itself because it suggests that the protein is relatively inert to this bacterial strain, which is encouraging considering PRG4 may one day be used on patients. Future studies should examine other bacterial strains, such as *Pseudomonas aeruginosa*, and determine the effect PRG4 has, if any, on these microbes.

Although there appears to be a trend suggesting an increase in bacterial viability, these lenses are directly exposed to a concentrated bacterial suspension and allowed to freely incubate for 18 hours, without interference from eyelid sweeps and bacteriostatic tear proteins. These settings are exaggerated and not truly representative of the level of contamination on worn lenses. Nonetheless, by investigating extreme conditions of lens bacterial contamination, we are able to determine a baseline value that can be used to conduct future experiments accordingly. In conclusion, PRG4 still has the potential to be a significant

factor in preventing bacterial adhesion, but further investigation is required, particularly work examining the kinetics of early bacterial contamination in an *in vivo* like environment.

Chapter 6 – General Discussion and Conclusion

This thesis has provided some insight into the interaction of a novel glycoprotein with a variety of commercial contact lens materials. Proteoglycan 4 (PRG4) has been previously studied under numerous applications;[136,146-149,152,153,155] though its unique interaction with contact lenses was first reported in great detail in this thesis. Several independent metrics were evaluated, which will be summarized in this chapter.

The objective of Chapter 3 was to investigate the potential for PRG4 to act as a wetting agent "enhancer" on contact lens surfaces. The lens materials examined in this study comprised a wide array of lenses consisting of unique surface chemistries and properties. Specifically, lenses with relatively hydrophobic surfaces showed improved wettability with the inclusion of PRG4. Conversely, incorporation of PRG4 onto relatively hydrophilic lenses exhibited a reduction in surface wettability. This interesting phenomenon is likely due to changes in the conformational state of PRG4 depending on the surface that it is bound to. Essentially, PRG4 is a surfactant where it possesses both hydrophobic and hydrophilic domains, which are the *N*- and *C*-terminals and mucinous core, respectively.[172] The end terminal regions of PRG4 bound onto hydrophobic surfaces which allowed for the hydrophilic mucin domain to be expressed onto the surface, increasing wettability. The opposite occurred with hydrophilic surfaces, where by the mucin domain bound onto hydrophilic surfaces, thus exposing the hydrophobic terminal ends onto the surface and decreasing wettability.

The second part of this study was to assess the stability of PRG4 onto these lens surfaces. Previous studies have reported that PRG4 binds tightly onto all surfaces,[172] however, an unexpected phenomenon was observed where PRG4 did not demonstrate the same effect on all of the lenses examined in this study. The results of this study demonstrated that PRG4 adsorbed relatively tightly onto hydrophilic surfaces, but exhibited poor adhesion onto hydrophobic surfaces. The central mucin domain undergoes extensive glycosylation and comprises a significant proportion of PRG4.[128] The differences in surface area between the end terminals and mucin domain could potentially be the reason for the differences in adhesion observed, as a larger surface area can provide a stronger anchor onto the lens surface.

The sorption profile of PRG4 as it interacted with various lenses was further explored in Chapter 4. Full-length recombinant human PRG4 (rhPRG4) was evaluated in this study, as it was recently expressed successfully in ovary cells of Chinese hamsters. Three important conclusions were: 1) rhPRG4 is capable of being fluorescently tagged; 2) rhPRG4's location within contact lenses can be viewed using confocal laser scanning microscopy; 3) the composition of the lens material largely influences the sorption profile of rhPRG4. Due to the extensive levels of glycosylation that proteoglycans undergo during post-translational modifications, their relative size compared to other proteins is much larger. RhPRG4 is approximately 220 kDa while other proteins, such as lysozyme, are approximately 14 kDa in size.[154] Despite its large size, rhPRG4 was able to penetrate into all the lenses examined, with the exception of lotrafilcon B due to its specific surface treatment that impedes penetration of

molecules. However, these findings raise the question of whether rhPRG4 within the matrix of lenses has any beneficial effect, and this question warrants further evaluation.

In the fifth chapter of this thesis, the anti-bacterial binding property of PRG4 was evaluated. Specifically, the effect of PRG4 on the adhesion and viability of *Staphylococcus aureus* was investigated on a variety of silicone hydrogels of different wear modalities. Both bovine PRG4 and rhPRG4 were examined in this study, and the findings suggest that neither demonstrated a significant effect on total bacterial adhesion or bacterial viability. Despite no statistical significance, lenses coated with PRG4 were found to support slightly more viable bacteria on average compared to the control lenses. It was speculated that PRG4 sorbed onto silicone hydrogel lenses may denature to a certain extent, where it can then act as a potential food source for the bacteria to thrive on.[82] Despite these supposedly unfavourable findings, it should be kept in mind that the experimental *in vitro* conditions were exaggerated to mimic extreme lens contamination that would normally not be representative to *in vivo* clinical conditions. In addition, the fact that PRG4 does not significantly increase bacterial adhesion is a noteworthy observation in itself. Generally, a protein coating on contact lenses has been shown to increase bacterial adhesion,[232] though this was not the case for PRG4. One can interpret the lack of a significant effect as a positive result, as these findings suggest that PRG4 had no impact with *Staphylococcus aureus.* PRG4 did not demonstrate any detrimental bacterial effects, which is encouraging if PRG4 is to be used with commercial lenses in the future.

The overall findings from this thesis provide strong evidence for the potential use of PRG4 with commercial contact lenses. Contact lens wearers may experience symptoms of

contact lens discomfort due to lens wear. However, it is possible that certain lens modalities may benefit substantially with the incorporation of PRG4. If a lens was capable of slowly releasing PRG4 onto the ocular surface to reduce symptoms of contact lens discomfort, then the patient's wear comfort may increase as a result. Of all the lenses examined, balafilcon A shows the highest potential in releasing PRG4 over time as it was shown that the protein slowly shed off the lens surface after each rinse. The use of PRG4 with balafilcon A as a possible drug delivery device should be considered, given that the material undergo certain modifications to enable a controlled and sustained release of PRG4.

Another modality that would benefit from the inclusion of PRG4 would be daily wear lenses. Some lenses (balafilcon A & senofilcon A) examined did not adsorb PRG4 tightly onto its lens surface. However, the initial wettability for these materials was significantly enhanced, which may potentially translate to a sensation of comfort when first worn. Lens manufacturers are known to include various wetting agents and surfactants within the blister solution that contact lenses are packaged in.[177-179] Although these agents are often washed off relatively quickly during wear, lens wearers are aware of the initial comfort that they provide, and it is possible that PRG4 can also serve as a possible wetting agent in the blister solution.

Lastly, PRG4 was seen to adsorb relatively tightly onto lotrafilcon B, comfilcon A, and etafilcon A lenses. Although wettability was reduced for these lenses, the inclusion of PRG4 for these materials should still be considered, as wettability is only one factor out of many that may contribute to comfort. Another factor that has been shown to correlate with comfort is lubricity.[241,242] Since PRG4 is primarily a boundary lubricant, it is possible that PRG4 can reduce the friction on these lens surfaces.[156] Since these materials are meant to be

worn on a continuous basis, the fact that PRG4 adheres tightly onto the lens surface is a positive characteristic. Additionally, the findings from the bacterial studies indicate that PRG4 had no impact on *Staphylococcus aureus*. However, there is a slight possibility that over an extended period of time (i.e. 2-4 weeks), an increase in viable bacteria may be observed. Therefore, the use of continuous wear lenses should be cautioned until further studies are conducted.

To conclude, the use of PRG4 for contact lenses, as well as with ophthalmic products, shows promise. However, these findings are still preliminary and further research is required to have PRG4's full potential come to fruition. Nonetheless, this thesis has formed the groundwork for PRG4 and contact lens research, and the next section will propose a series of future experiments that can build upon this thesis and further the development of safe, comfortable contact lenses.

Chapter 7 – Future Studies

The incorporation of PRG4 with commercial contact lenses is an innovative and novel area of research that has the potential to address multiple issues regarding lens wear. The findings from this thesis have constructed a basic foundation for PRG4 and contact lens research, which can be built upon with future studies. Therefore, in the current chapter, several future experiments have been proposed in an attempt to strengthen our understanding of the interaction between PRG4, the contact lens material, and the surrounding ocular environment.

Determining the optimal PRG4 concentration for contact lenses and assessing its toxicity on corneal and conjunctival epithelial cells

All lenses that were evaluated in this thesis were incubated in some form of PRG4 at a concentration of 300 µg/ml. This value was chosen because it is the approximate concentration of PRG4 in healthy human synovial fluid.[243] Additionally, previous studies have used this concentration to study the lubricious effect of PRG4 on contact lenses.[145,156,159,160] By keeping the concentration of PRG4 consistent, we can interpret the data reliably between studies. Although the findings from this thesis have shown that 300 µg/ml is sufficient to alter the lens surface wettability, it would be valuable to examine lower concentrations and observe if the same effect is seen. From an economical perspective, these studies would be beneficial because if less PRG4 is required to elicit a significant effect, it would effectively lower the cost of PRG4 per lens.

If PRG4 were to be included in the blister package or in various ophthalmic products, it must first be cleared by the FDA and deemed safe for human usage. Unfortunately, the majority of research has been *in vitro* and, to our knowledge, there have been no published *in vivo* studies. It would be interesting to evaluate the effect PRG4 has on the ocular surface, specifically targeting the corneal and conjunctival epithelial cells. Despite recent reports of PRG4 on the ocular surface,[145] it is likely present at much lower quantities compared to the concentration used for *in vitro* studies. It is possible that a concentration above a certain threshold may yield cytotoxic results on the ocular surface, or perhaps elicit an immunological response. Therefore, future studies should consider conducting PRG4 dose-and-response experiments with ocular tissues to determine its optimal and safe concentration for human use.

<u>Developing a novel technique to radiolabel PRG4 to quantify its sorption within contact lenses</u>

Ever since its discovery in bovine synovial fluid, our understanding of PRG4 has grown exceptionally. However, it is still considered a novel molecule and cannot be acquired commercially. One of the difficulties that arise when experimenting with PRG4 is the lack of a standard protocol for quantification. This realization came to fruition during Chapter 4 of this thesis where rhPRG4 was fluorescently labeled with FITC. Although the location of rhPRG4 within various lenses was determined, the fluorescent intensity data cannot accurately measure the amount sorbed. Therefore, there is a need to develop and optimize a radiolabelling procedure for PRG4 since lenses sorb varying amounts due to a variety of

factors, such as the presence of a surface treatment, hydrophobicity, polymer composition, and pore sizes. ^{125}I has been used to radiolabel lysozyme[68,244,245] for previous studies; therefore, it is possible that this isotope can also be used to radiolabel PRG4.

Investigating the effect of PRG4-coated lenses on an artificial tear solution

Although PRG4 is primarily a lubricant within the body, it has been shown to have additional properties, such as the ability to prevent the adhesion of various molecules.[147,148] The anti-adhesive property of PRG4 was briefly alluded to in Chapter 5 of this thesis, where its effect on bacterial adhesion on contact lenses was evaluated. It would be interesting to expand upon that study by examining the effect PRG4-coated lenses have on an artificial tear film, specifically on the adhesion of proteins and lipids. It can be argued that deposition onto a contact lens can be beneficial or harmful to the lens wearer, though it largely depends on the molecule adsorbed. Nonetheless, by minimizing the deposition of all molecules, it would essentially lower the chances of any complication from occurring.

Appendix A – Covalent binding of proteoglycan 4 onto a commercial silicone hydrogel contact lens

Overview

Purpose: The purpose of this study was to covalently tether bovine proteoglycan 4 (PRG4) onto the surface of a commercially available silicone hydrogel lens.

Methods: Senofilcon A lenses (Johnson & Johnson) were examined in this study (n=3). All lenses were unworn and rinsed in phosphate buffered saline (PBS) to eliminate blister pack constituents from the lens surface. Lenses were first treated with 4-azidobenzoic acid (ABA) dissolved in methanol for 1 hour at room temperature, air dried, and irradiated under ultraviolet light for 20 minutes. Loosely bound ABA on the lens surface was removed by rinsing the lens in methanol. Lenses were then incubated with 1-3-dimethylaminopropyl-3-ethylcarbodiimide hydrochloride (EDC) dissolved in MilliQ for 1 hour at room temperature. Lenses were rinsed with MilliQ to remove loosely bound EDC from the lens surface. Once the lens surface had been functionalized with ABA and EDC, lenses were incubated in a bovine PRG4 suspension at 300 µg/ml for 24 hours at 37°C. Lens surface wettability and PRG4 substantivity were determined using an Optical Contact Angle analyzer instrument where the sessile drop technique was performed to measure advancing contact angle. Negative controls (lenses incubated in PBS; n=3) and positive controls (lenses incubated in bovine PRG4; n=3) were also included.

Results: Negative control lenses soaked in PBS had an average contact angle of approximately 90°, which remained relatively consistent throughout 8 cycles of rinses in Unisol saline. Positive control lenses that were incubated in bovine PRG4 demonstrated a significant reduction in contact angle at approximately 60° initially ($P<0.05$). However, contact angle values returned to control values after 1 rinse in Unisol. Lenses that underwent covalent attachment initially demonstrated a reduction in contact angle at approximately 80°. However, after 1 rinse in Unisol the contact angle values were not significantly different compared to control values. This finding remained consistent for the remainder of Unisol rinses.

Conclusion: The findings from this study have shown that the proposed methodology to covalently bind PRG4 onto the surface of senofilcon A lenses was not effective. It is likely that silicone hydrogel lenses do not have sufficient hydroxyl groups on the lens surface to catalyze the covalent reaction. Additionally, the overall procedure imposed significant stress as lenses were repeatedly swelled and air dried. The incorporation of PRG4 during lens synthesis should be considered as an alternative.

Introduction

Silicone hydrogel contact lenses were developed to alleviate symptoms of corneal hypoxia during lens wear.[21,22] Although these lenses demonstrated superior oxygen permeability, the incorporation of siloxane polymers into the hydrogel network inadvertently increased the overall hydrophobicity of the material.[100] Several lens manufacturers have developed techniques to render the lens surface more hydrophilic and wettable to support a stable overlying tear film.[14,100] Surface treatments were observed in the first generation silicone lenses (balafilcon A and lotrafilcon A), where plasma technology was used to chemically transform the hydrophobic siloxane into a variant that was hydrophilic.[101,102,106] However, plasma treatment of lenses is a relatively expensive procedure, which in turn is reflected in the cost of the lens. Alternative methods of improving lens wettability have been implemented, which can be seen in subsequent generations of silicone hydrogel lenses. Nonetheless, research is ongoing, with new and innovative techniques being proposed regularly to improve the lens material wettability and, ultimately, comfort.

PRG4 has the potential to improve lens wettability,[157] as well as lubricity.[159,160] The findings in this thesis (Chapter 3) have shown that bovine proteoglycan 4 (PRG4) can improve the surface wettability of select silicone hydrogel lenses – balafilcon A and senofilcon A. However, PRG4 did not adhere strongly onto the lens surface as it was quickly removed during rinses in Unisol. Simply soaking the lenses in PRG4 did not adequately fix the glycoprotein onto the surface.

Previous studies have examined the use of chemical reagents to covalently tether peptides onto surfaces containing hydroxyl groups.[182,246] Since soft contact lenses are

primarily composed of the polymer 2-hydroxyethyl methacrylate (HEMA), which contains a free hydroxyl group, as shown in Figure A-1, covalent attachment onto a contact lens surface is possible. Therefore, the objective of this pilot study was to evaluate a method of covalently binding PRG4 onto the surface of a silicone hydrogel lens and assess the PRG4 stability by measuring the advancing contact angle over time, using a sessile drop technique.

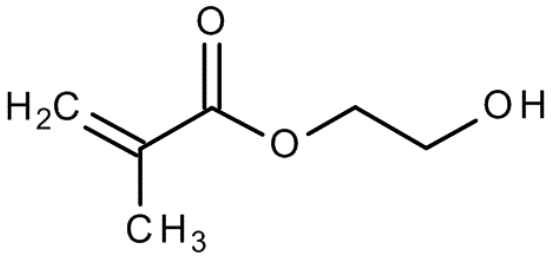

Figure A-1. Chemical structure of 2-hydroxyethyl methacrylate (HEMA).

Materials and Methods

Contact lens

The senofilcon A lens material (Johnson & Johnson) was selected for this experiment because it 1) represents a silicone hydrogel lens that has a relatively poor surface wettability when assessed by contact angle analysis; 2) does not strongly adsorb PRG4 onto its surface; and 3) is not surface treated. Three experimental lens conditions were conducted: PBS-soaked negative control, PRG4-soaked positive control, and covalent attachment of PRG4 onto the lens surface. All lenses (n=3) were unworn and rinsed overnight in phosphate buffered saline (PBS) to remove blister pack residues from the lens surface.

Covalent binding reagents

The aromatic azide, 4-azidobenzoic acid (ABA; TCI Organic Chemicals), was selected to initiate the covalent binding mechanism by acting as a cross-linking agent on the lens surface. ABA was dissolved in methanol (10 mM) and allowed to react with the hydroxyl groups on the lens surface for 1 hour at room temperature. Lenses were then removed from their vials and air dried before undergoing irradiation under ultraviolet (UV) light for 20 minutes. Lens surfaces were rinsed 3 times with methanol to remove loosely bound ABA and treated with 1-3-dimethylaminopropyl-3-ethylcarbodiimide hydrochloride (EDC; Alfa Aesar) dissolved in MilliQ at 27 mg/ml for 1 hour at room temperature. MilliQ was used to rinse the lens surface to remove loosely bound EDC on the surface.

Bovine proteoglycan 4

Proteoglycan 4 (PRG4) was extracted and purified from bovine knee caps, as previously described.[150] The PRG4 suspension was prepared at 300 µg/ml in PBS (pH 7.2) and stored at 4°C until usage. Lenses with surfaces functionalized with ABA and EDC were incubated in the PRG4 suspension for 24 hours at 37°C with gentle shaking. Lenses that did not undergo the covalent attachment procedure were simply soaked in PRG4 at 300 µg/ml for 1 hour at 37°C with gentle shaking.

Sessile drop technique

Refer to Chapter 3.3.4 of this thesis.

PRG4-substantivity

Refer to Chapter 3.3.5 of this thesis.

Data analysis

Refer to Chapter 3.3.6 of this thesis.

Results

The results from this study are illustrated in Figure A-2. Control lenses that were soaked in PBS maintained a consistent contact angle of approximately 90° throughout the 8 cycles of rinses. Lenses that were soaked non-specifically in bovine PRG4 recorded an initial contact angle of approximately 60°, which was significantly less than the control value ($P<0.05$). However, when these lenses were rinsed, the contact angle increased and was not statistically significantly different from the control values for the remainder of rinses. Similarly, lenses that underwent covalent attachment of PRG4 onto the surface initially showed a significant reduction in contact angle (initial contact angle ~80°) compared to control values ($P<0.05$). However, these values increased to control values after one rinse, and the contact angle measurements were not significantly different from the control for the remainder of rinses.

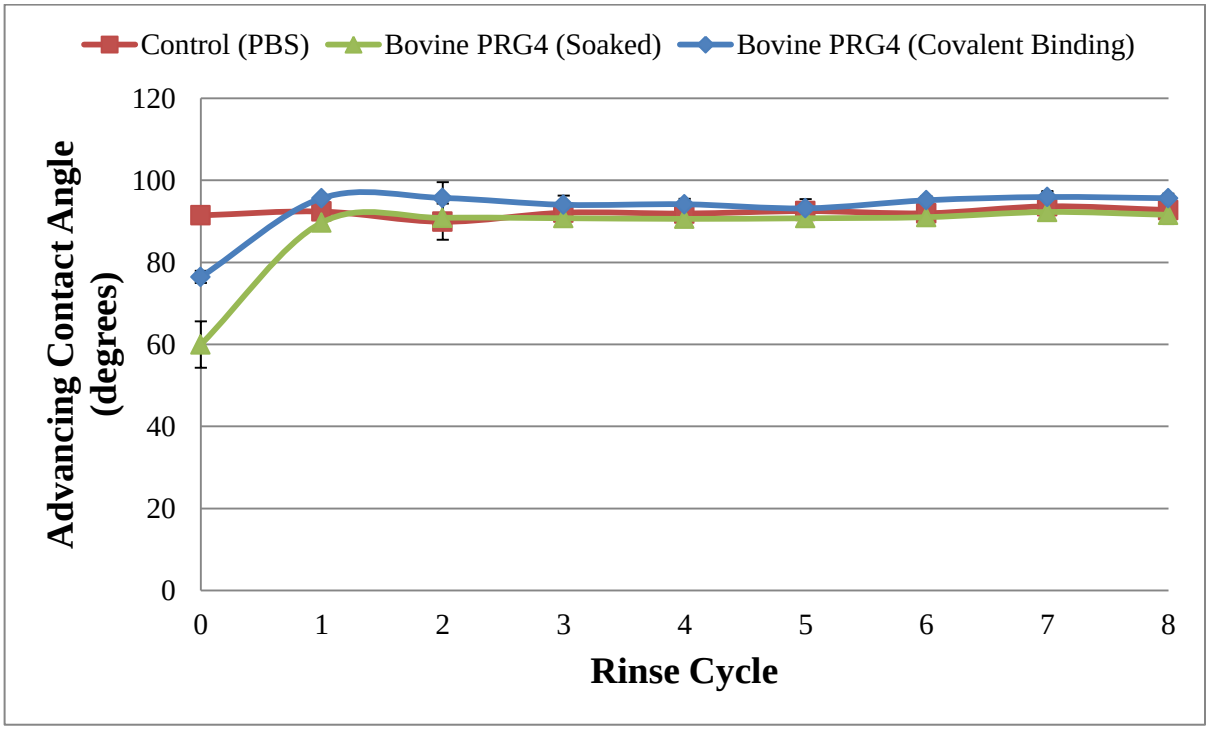

Figure A-2. The advancing contact angles over 8 cycles of rinses in Unisol for senofilcon A lenses under three experimental conditions: A) control – PBS; B) bovine PRG4 – soaked; C) bovine PRG4 – covalent binding.

Discussion

The objective of this pilot study was to assess the viability of a novel method to covalently attach PRG4 onto the surface of a silicone hydrogel lens – senofilcon A. The findings from this study suggests that the proposed methodology utilizing the reagents ABA and EDC was ineffective at chemically binding and fixing PRG4 onto the lens surface. Although Chen et al.[246] reported successful attachment of their peptide, they performed the covalent reaction onto a glass surface, whereas a silicone hydrogel lens was used in this study. Glass surfaces contain an abundance of hydroxyl groups that are free to react with

ABA. Commercial contact lenses, on the other hand, may not have the same degree of hydroxyl groups exposed on the surface. Furthermore, it is likely that the hydroxyl groups of HEMA were orientated towards the bulk of the lens while the lenses were submerged in solution, because this arrangement is energetically favourable.[61] Without a sufficient amount of hydroxyl groups present on the surface, the reaction becomes limited. In addition, it is also difficult to isolate the covalent reaction exclusively on the lens surface because hydrogel lenses have significant absorptive capabilities. It is possible that the chemical reagents are taken up by the lens matrix, which would decrease its effective concentration on the surface. Lastly, the protein suspension for covalent attachment may need to be concentrated and in excess to ensure a high rate of attachment. Dutta et al.[182] evaluated different concentrations of peptide and determined that 3 mg/ml was the most optimal; however, approximately 150 µg/ml was measured on the surface. In this study, the PRG4 suspension was at 300 µg/ml, which may be insufficient considering that only a fraction of that amount may be present on the surface.

Although the proposed methodology is theoretically sound, it does not take into account the soft and delicate nature of a contact lens. Overall, the process of functionalizing the surface with ABA and EDC imposed significant stress on the lens, which resulted in a low yield of lenses that remained intact. The solvents used in dissolving the binding reagents swelled the lens significantly, and the lenses then underwent periods of air drying either under UV light exposure and the sessile drop technique. It was interesting to observe that lenses that underwent covalent attachment reported a higher contact angle (~80°) compared to lenses that were simply soaked in PRG4 (~60°). This discrepancy in contact angle could

be due to hysteresis, since the physical stressed endured by lenses undergoing covalent attachment may change its surface properties.

In conclusion, the results from this pilot study have shown that the proposed method of covalently attaching PRG4 onto the surface of senofilcon A lens was not effective and requires significant revisions and optimizing. It appears that post-surface modifications on commercial lenses are difficult to achieve; therefore, the chemical incorporation of PRG4 during lens production should be considered as an alternative.

Appendix B – Comparing the wettability effects of bovine PRG4 and full-length recombinant human PRG4 on contact lenses

Overview

Purpose: The purpose of this study was to evaluate and compare the effect of bovine proteoglycan 4 (PRG4) and recombinant human PRG4 (rhPRG4) on the wettability of various commercial contact lenses.

Methods: Four commercially available silicone hydrogel lenses [balafilcon A (Bausch + Lomb), senofilcon A (Johnson & Johnson), comfilcon A (CooperVision), lotrafilcon B (Alcon)] were examined in this study. All lenses (n=3) were rinsed in phosphate-buffered saline (PBS) overnight to remove blister pack residues. Lenses were incubated under five conditions: (1) PBS; (2) bovine PRG4; (3) rhPRG4 + 0.1% Tween 20; (4) rhPRG4; (5) PBS + 0.1% Tween 20. Solutions were prepared at 300 µg/ml and incubated at 37°C for 1 hour with gentle shaking. Lens surface wettability was determined using an Optical Contact Angle analyzer that measured the advancing contact angle via the sessile drop technique.

Results: Lenses soaked in bovine PRG4 significantly affected the wettability for all lens materials ($P<0.05$). Significant reductions were observed when lenses, with the exception of senofilcon A, were incubated in rhPRG4 + 0.1% Tween 20 ($P<0.05$); however, these values

were not statistically different to lenses incubated in PBS + 0.1% Tween 20 ($P>0.05$). Balafilcon A and senofilcon A lenses treated with rhPRG4 did not demonstrate a significant change in contact angle compared to the PBS control ($P>0.05$). Conversely, rhPRG4-coated comfilcon A and lotrafilcon B lenses did exhibit a significant increase in contact angle compared to the PBS-soaked controls ($P<0.05$) and were not statistically different to the bovine PRG4 treated lenses ($P>0.05$).

Conclusion: Bovine PRG4 and rhPRG4 displayed significantly different effects on the wettability of balafilcon A and senofilcon A lenses. The inclusion of Tween 20 in rhPRG4 significantly influenced lens wettability measurements. Future production of rhPRG4 containing Tween 20 should be reconsidered. Additionally, differences in the wetting properties between bovine PRG4 and rhPRG4 should be further investigated.

Introduction

Recombinant technologies have allowed for the expression of valuable proteins in large quantities, which would otherwise be naturally produced in miniscule amounts. One notable example is the production of recombinant human insulin to treat individuals with insulin-dependent diabetes.[247] Briefly in recombinant techniques, the DNA of the protein of interest is isolated from producing cells and cloned through PCR amplification. Cloned DNA strands are then transplanted into a suitable host organism that is capable of producing large and stable amounts of mRNA, which can then be effectively translated into the desired protein.[248] The use of recombinant proteins has made significant contributions to both the biological and biomedical sciences.

Proteoglycan 4 (PRG4) is a glycoprotein that functions as a lubricant to reduce friction in joints. Ever since it was discovered in bovine synovial fluid, extensive research has been conducted to better understand and characterize the glycoprotein. Acquisition of PRG4 for studies required harvesting and extraction from bovine knee caps. However, this process has low throughput, as it yields low amounts of PRG4 and purification reagents are costly. Initial attempts at expressing recombinant PRG4 proved difficult due to truncation of its central mucin domain, which undergoes extensive glycosylation during its post-translational modifications.[152] Recent advances in protein expression, however, have successfully expressed full-length recombinant human PRG4 (rhPRG4) by transfecting the human PRG4 gene into Chinese hamster ovary cells. Samsom et al.[154] have characterized rhPRG4 and concluded that the recombinant variant displays appropriate levels of glycosylation compared to native bovine PRG4, which is responsible for the chemical

properties intrinsic to PRG4. They also confirmed that rhPRG4 and bovine PRG4 have similar lubricious effects in various friction tests, though its wetting effects have not been compared and evaluated. Therefore, the aim of this study was to measure and compare the wettability of various commercial lenses treated with both bovine PRG4 and rhPRG4.

Materials and Methods

Contact lenses

Four silicone hydrogel materials [balafilcon A (Bausch + Lomb), senofilcon A (Johnson & Johnson), comfilcon A (CooperVision), lotrafilcon B (Alcon)] were investigated in this study. Prior to treatment, lenses were rinsed in phosphate-buffered saline (PBS) to remove components from the blister pack from the lens surface.

Bovine proteoglycan 4 and recombinant human proteoglycan 4

Refer to sections 3.3.3 and 5.3.2.

Incubating solutions and conditions

Lens materials were incubated in five different conditions: (1) PBS; (2) bovine PRG4; (3) rhPRG4 + 0.1% Tween 20; (4) rhPRG4; (5) PBS + 0.1% Tween 20. Concentrations were maintained at 300 µg/ml and were incubated at 37°C for 1 hour with gentle shaking.

Sessile drop technique

Refer to section 3.3.4.

Data analysis

Refer to section 3.3.6.

Results

The results from this study are illustrated in the following figures. Bovine PRG4, rhPRG4, and rhPRG4 + 0.1% Tween 20 were evaluated and compared for their wettability effects on various commercial lenses. All lenses soaked in bovine PRG4 displayed a significantly different contact angle compared to the PBS soaked controls ($P<0.05$). When lenses were incubated in rhPRG4, the contact angles for comfilcon A and lotrafilcon B lenses were significantly greater ($P<0.05$) compared to their PBS controls. No differences were observed with balafilcon A and senofilcon A lenses with their respective PBS controls ($P>0.05$).

Balafilcon A, comfilcon A, and lotrafilcon A lenses that were incubated in rhPRG4 + 0.1% Tween 20 demonstrated a significantly lower contact angle compared to the PBS controls ($P<0.05$). However, there was no statistical difference between these values and the values obtained from lenses soaked in PBS + 0.1% Tween 20 ($P>0.05$). Senofilcon A lenses incubated in rhPRG4 + 0.1% Tween 20 and PBS + 0.1% Tween 20 were also not statistically significantly different compared to the PBS soaked controls ($P>0.05$).

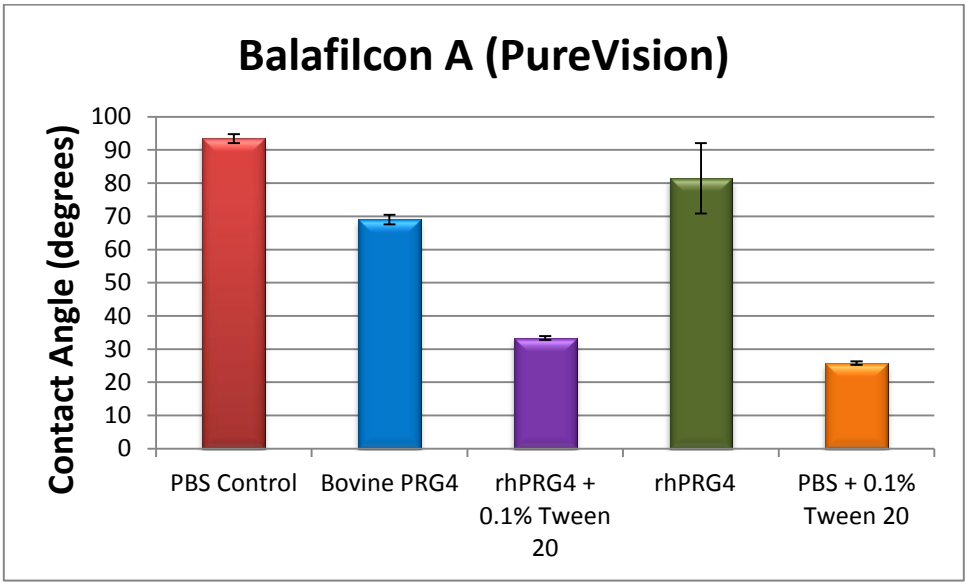

Figure B-1. Advancing contact angle measurements for balafilcon A lenses in 1) PBS; 2) bovine PRG4; 3) rhPRG4 + 0.1% Tween 20; 4) rhPRG4; 5) PBS + 0.1% Tween 20.

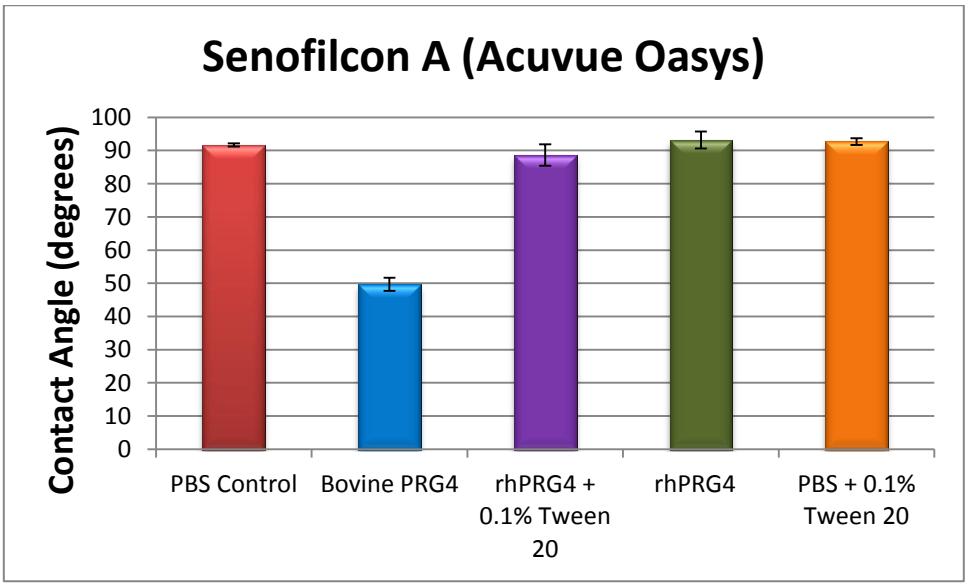

Figure B-2. Advancing contact angle measurements for senofilcon A lenses in 1) PBS; 2) bovine PRG4; 3) rhPRG4 + 0.1% Tween 20; 4) rhPRG4; 5) PBS + 0.1% Tween 20.

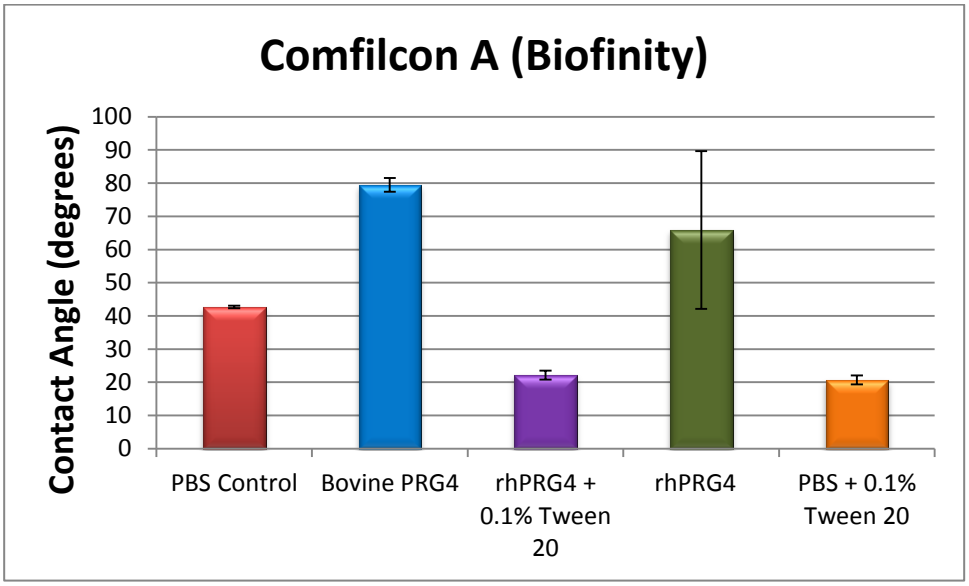

Figure B-3. Advancing contact angle measurements for comfilcon A lenses in 1) PBS; 2) bovine PRG4; 3) rhPRG4 + 0.1% Tween 20; 4) rhPRG4; 5) PBS + 0.1% Tween 20.

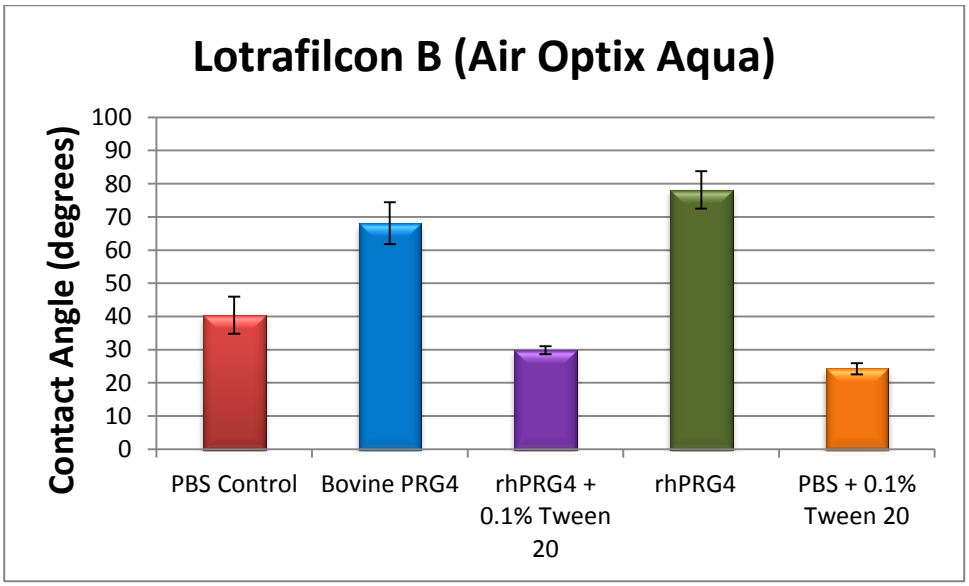

Figure B-4. Advancing contact angle measurements for lotrafilcon B lenses in 1) PBS; 2) bovine PRG4; 3) rhPRG4 + 0.1% Tween 20; 4) rhPRG4; 5) PBS + 0.1% Tween 20.

Discussion

The findings from this study have shown that there are differences between native bovine PRG4 and its recombinant form in how it affects lens surface wettability. Three experimental conditions were examined: bovine PRG4, rhPRG4, and rhPRG4 + 0.1% Tween 20. Original production of rhPRG4 yielded small amounts that were not viable from a commercial and research standpoint; though, the inclusion of Tween 20 during production can significantly increase yields to over 1 g/L and improve overall stability of the protein.[249] However, Tween 20 is essentially a surfactant that could potentially have a significant influence on wettability measurements. Data involving rhPRG4 + 0.1% Tween 20 have shown that it significantly enhances the material wettability for balafilcon A, comfilcon A, and lotrafilcon B lenses. However, when the negative controls consisting of PBS + 0.1% Tween 20 were examined, the same phenomenon (enhanced wettability) was observed. When examining the wettability effect of rhPRG4 without Tween 20, it did not reproduce the same wetting enhancements compared to rhPRG4 + 0.1% Tween 20. The discrepancy in these results suggests that Tween 20 largely influences the wettability measurements, despite its presence at a low concentration. Future production of rhPRG4 with Tween 20 should be carefully reconsidered, as the wetting effects observed can be misinterpreted as an intrinsic property of rhPRG4.

Interestingly, rhPRG4 (without Tween 20) and bovine PRG4 did not exhibit similar wetting effects on two of the lenses examined, despite Samsom et al.[154] reporting that the two share appropriate levels of glycosylation and frictional results. Specifically, rhPRG4 had no significant effect in lowering the contact angle for balafilcon A and senofilcon A lenses.

These two materials, however, demonstrated significantly reduced contact angles when incubated in bovine PRG4. Since these materials have relatively hydrophobic surfaces, it was believed that PRG4 undergoes structural rearrangement such that the hydrophobic *N*- and *C*-terminal domains adsorb onto the lens while its hydrophilic regions are exposed on the surface. Much of the focus and difficulties in producing rhPRG4 have been due to expressing rhPRG4's central hydrophilic mucin domain in ensuring that it is properly glycosylated because this is where its chemical properties are derived from. However, proper expression and characterization of the *N*- and *C*-terminals should not be neglected as these regions potentially serve as anchors for the glycoprotein to render hydrophobic surfaces more hydrophilic. Further characterization of rhPRG4, particularly at the hydrophobic terminal domains, is required in order to identify any additional differences it may have with bovine PRG4.

In conclusion, the ability to express rhPRG4 signifies the monumental advancements achieved in recombinant technology. The potential for rhPRG4 to be included in various ophthalmic products and future studies is promising considering the high yields obtained during production. The fact that comfilcon A and lotrafilcon B lenses yielded similar wettability results when treated with both bovine PRG4 and rhPRG4 is encouraging, though there is no absolute explanation as to why the same effect was not observed for the other two lens materials. Therefore, rhPRG4 still requires further analysis and characterization in order to consistently elicit the same properties as bovine PRG4.

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