In Vitro Release of Inflammatory Cytokines from Contact Lens Materials

by

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Author's Declaration

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

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

Abstract

Purpose:

The upregulation of inflammatory mediators in the tear film during contact lens wear indicates that lens wear may induce an inflammatory response on the ocular surface. Previous research has investigated the relationship between lens wear and ocular inflammation through analyses of cytokine levels in tear samples. However, there has been little discussion regarding the interaction between contact lens materials and cytokines, and its role in ocular inflammation. This thesis aimed to study the release of cytokines from contact lens materials. Particularly, to determine whether the release of cytokines differed between silicone hydrogel and conventional hydrogel contact lens materials.

Materials and Methods:

The contact lens materials investigated in this thesis include hydrogel (etafilcon A; omafilcon A) and silicone hydrogel (delefilcon A; somofilcon A) materials. Lens materials were incubated in a combined cytokine solution that contained ten recombinant human cytokines. The cytokines of interest for this study were: IL-1β, IL-6, IL-8, and TNF-α.

- **Chapter 3:** A protocol to aid in quantifying cytokines released from contact lens materials was developed. To determine the parameters of the protocol, the effects of changing temperature, incubation period, and volume of diluent 2 on the amount of cytokines released from lens materials were analyzed. Cytokines present in samples were quantified using the Meso Scale Discovery (MSD) cytokine assay.
- **Chapter 4:** The protocol developed in chapter three was used to determine whether the release of cytokines differed between contact lens materials that were pre-exposed to

various ocular conditions. The ocular conditions tested were: 1) contact lens materials incubated in a high cytokine concentration solution; 2) contact lens materials incubated in a cytokine solution for a 12-hour period; 3) contact lens materials incubated in a cytokine solution at 34℃.

- **Chapter 5:** Contact lens materials were first incubated in a single protein solution (lysozyme, lactoferrin, or albumin) for 16-hours. Post incubation, lens materials were soaked in a cytokine solution for a 6-hour period. The protocol developed in chapter three was then used to determine whether the release of cytokines differed between protein-coated contact lens materials.

Results:

In chapter three, the parameters for the protocol were set and the finalized protocol involved incubating cytokine-adsorbed contact lenses in 550μL of diluent 2 for 1 hour at room temperature. In chapter four, the difference in the release of cytokines between delefilcon A and etafilcon A lenses was tested, and it was observed that when the lenses were incubated in a high cytokine concentration solution, etafilcon A released more IL-1β than delefilcon A. In chapter five, it was found cytokine release did not differ between protein-coated delefilcon A and etafilcon A lens materials.

Conclusion:

This thesis contributes to existing knowledge of ocular inflammation related to contact lens wear by providing an insight into lens materials' ability to release IL-1β, IL-6, IL-8, and TNF-α. Future research studies should investigate the effect of released cytokines on the ocular surface.

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Dedication

To my loving family,

Appa, Amma, Thangachi and Thambi

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List of Abbreviations

ACN	Acetonitrile
AK	*Acanthamoeba* Keratitis
CIE	Corneal Infiltrative Events
CLARE	Contact Lens-Associated Red Eye
CLD	Contact Lens Discomfort
CLPU	Contact Lens-Induced Peripheral Ulcer
DAMPs	Damage-Associated Molecular Patterns
DED	Dry Eye Disease
ELISA	Enzyme-Linked Immunoassay
EGF	Epidermal Growth Factor
FDA	Food and Drug Administration
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HEMA	Hydroxyethyl Methacrylate
Ig	Immunoglobulin
IL	Interleukin
IK	Infiltrative Keratitis
kDa	Kilodalton
LTB_4	Leukotriene B_4
MMP-9	Matrix Metalloproteinase 9
MA	Methacrylic Acid
MK	Microbial Keratitis
MSD	Meso Scale Discovery
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate-buffered solution
pH	Potential of Hydrogen
pI	Isoelectric Point
PMNs	Polymorphonuclear leucocytes
PMMA	Polymethyl Methacrylate
RGP	Rigid Gas-Permeable
R.P.M	Revolutions per Minute
sIgA	Secretary IgA
SD	Standard Deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel
TACE	TNF-α Converting Enzyme
TFA	Trifluoroacetic Acid
TFLL	Tear Film Lipid Layer

TLRs	Toll-Like Receptors
TNF	Tumour Necrosis Factor
T_H	Helper T Cells
USAN	United States Adopted Name

Chapter 1: Literature Review

1.1 Cornea

The outer tunic of the eye, shaped like an asymmetric globe, is composed of the cornea and sclera.[1,2] Approximately 85% of the outer tunic is comprised of the opaque sclera, with the remaining 15% made up of the transparent cornea.[1] Both structures meet at a region called the limbus to form the outer coat of the eye.[1] These components act as a structural barrier to enclose other ocular contents as well as provide protection.[1,3] The ability of the cornea and sclera to withstand both intraocular pressure as well as any force exerted onto the eye during ocular movements is made possible through its structure.[1] Both components are made of connective tissue containing collagen fibrils, however, it is the difference in the arrangement of the collagen fibrils that allows for one to appear transparent and the other to be opaque.[1]

The collagen fibrils in the cornea are small and laterally arranged with a high level of organization. Essentially, the role of the cornea is to refract and transmit light.[2] Refraction of light is dependent on the curvature of the anterior and posterior corneal surface, the refractive index as the medium changes from tear film to cornea and then from cornea to aqueous humor, and the thickness of the cornea.[2] It is important for the cornea to maintain a smooth precise curvature shape as it, along with the tear film that lays on top of it, forms the main refractive component of the eye.[1] On the contrary, the collagen in the sclera contains wider fibrils and lacks organization in its arrangement; rather, it follows a more interwoven pattern and thus is opaque in appearance. This appearance allows for prevention of internal light scatter.[1] While the sclera does not necessarily have an optical function, mechanically it is responsible for providing attachment sites for extraocular muscles, withstand any movement induced by internal and external forces and

maintain the shape of the eye.[1,2] Physiologically, it helps maintain ocular pressure by allowing aqueous humour to flow out through outflow channels.[1]

The cornea is made up of 5 layers: corneal epithelium, Bowman's layer, stroma, Descemet's membrane, and the corneal endothelium.[2] The epithelium is on the anterior portion of the cornea and it is in direct contact with the tear film, while the endothelium is on the posterior side and it faces the anterior chamber.[2] The stratified corneal epithelium is a multi-cell layer consisting of nonkeratinized squamous cells on the surface, with wing cells and basal cells beneath it.[2,3] The surface layer of the cell membrane is 2-3 cell layers thick. They consist of cells that are flat and polygonal in shape.[4] Surrounding each cell are apical microvilli and microplicae which are coated in a layer of glycocalyx.[4,5] The projections present on these cells increase the surface area to which the mucinous layer can adhere and thus lay closely along the cell membrane.[4] While substances from the tear film are able to travel through the cells, any movement between the cells is prohibited.[2,4] The apical cell layer is held closely together via tight junctions and desmosomes along the lateral walls of the cells to prevent the movement of tears within intercellular spaces.[2,4] The mid-layer of the epithelium is made of wing cells and is about 2-3 layers thick. Wing cells have lateral processes and are also held together by tight junctions. Finally, the basal cell layer is a single cell layer of columnar epithelium cells. The basal cells are capable of undergoing mitosis.[4,6] The only other cells in the epithelium that can do so are the stem cells and the transient amplifying cells.[4] As they undergo mitosis, they give rise to the wing cells present in the middle layer.[2,4] Basal cells secrete a basement membrane below it, and it is attached via a hemidesmosome system.[2,4] Anchoring fibrils travel through the hemidesmosomes to help hold the basal epithelium to Bowman's layer and the stroma.[2,7] It is important for there to be a tight attachment between the

tissue layer and the corneal epithelium as any breakage in the system could lead to corneal erosion syndromes or epithelial defects.[4]

The layer beneath the epithelium, Bowman's layer, is a nonregenerative acellular layer comprised of collagen fibrils and proteoglycans.[2-4] While it is not a true membrane, its main role is to help the cornea hold its shape.[2-4] The next layer is the corneal stroma, which makes up about 80-85% of the cornea. The precise arrangement of the bundles of collagen fibrils into parallel layers contributes to its transparency.[4,8] Aside from the fibrils, the stroma also contains keratocytes and ground substances such as proteoglycans which helps maintain the tensile strength of the cornea.[2]

The next layer is Descemet membrane, which is also known as the basement membrane of the endothelium layer.[2] The endothelium is a single-cell layer made of flattened cells.[2] In order for the stroma to remain transparent, it needs to be in a state of dehydration.[4] To help with its maintenance, the endothelial cells have a pump-leak system through which fluid flows from the stroma, past the endothelial cells and into the aqueous humour.[4] This process is possible via an osmotic gradient created between the hypo-osmotic stroma and the hypertonic aqueous humour.[4] The organization and composition of the cornea is crucial in order for it to refract and transmit light effectively.[2]

1.2 Tear Film

The ocular surface that comes into contact with the environment is exposed to air pollution, various pathogens and the consequences that come with the change in air temperature[9] and

humidity[9].[10] To protect the eye against these risk factors, the outer eye is covered by a liquid layer known as the tear film.[10] The tear film is quite important to the visual system. Its responsibilities include moisturizing the conjunctiva and the cornea;[11] and nourishing the cornea with its required nutrients[12].[13] Aside from protecting and hydrating the cornea, the tear film is also the first refractive surface light hits and thus it is important for it to be smooth in texture.[14]

The tear film is broadly made up of three layers: the outer lipid layer, aqueous layer, and inner mucous layer.[14] The outermost layer lying along the eye-air interphase, commonly known as the Tear Film Lipid Layer (TFLL), is biphasic as it has both a thick non-polar and a thin polar phase.[10,15] The polar phase lies on the inner side of the TFLL and is created by phospholipid molecules. Meanwhile the nonpolar phase sits on the outer side and is predominantly composed of nonpolar lipids such as wax esters, sterol esters, hydrocarbons, and triglycerides.[15] Primary functions of the TFLL include reducing surface tension of the tear film;[10] preventing evaporation of the aqueous layer;[10,15] ensuring tears remain on the ocular surface without spilling over; [10,15,16] and preventing sebaceous lipids from interacting with the tear film.[15,17] While evaporation of the tear film is influenced by the nonpolar phase, it is the composition and stability of the polar phase that allows for it to hold its function.[15,18] The polar phase of the TFLL contain amphipathic phospholipid molecules which align themselves along the aqueous–lipid interface, allowing for the interaction between nonpolar lipids and aqueous lipids.[15] This interaction helps to reduce surface tension between the tear film and air, eventually reducing evaporation.[19]

The layer below the lipid layer is the aqueous layer, which forms the majority of the tear film's thickness.[20,21] With 98.2% of it being water, the remaining 1.8% of it are solids.[13,22]

Components of the aqueous phase are produced from the lacrimal gland[20,23] and the accessory glands of Krause and Wolfring with contributions also from epithelial cells lining the ocular surface.[20] This phase also acts as a transfer medium for nutrients, such as inorganic salts, glucose, and oxygen to travel to the cornea.[13,20,22] The proteins, peptide growth factors, vitamins, inflammatory response markers, and hormones all have crucial roles in this aqueous phase.[20] The composition of the tear film is quite important, as the ions help maintain the epithelial cells lining the ocular surface.[15,24] The aqueous phase holds several electrolytes, which help create a buffer solution to maintain the tear film pH level.[15,25] These electrolytes include sodium, potassium, magnesium, calcium, and bicarbonate.[15] They also contribute to the osmolality of the tear film.[15,26] Consequences of hyperosmolarity include damage to the ocular surface, as well the development of dry eye syndrome.[15,20,27] Proteins also have a prominent role in the tear film as they assist with ocular defense mechanisms[28] and tear film stability.[20,29] Some of the more abundant proteins found in the tear film include lysozyme, lactoferrin and lipocalin. In the case of an inflammatory response, the tear film will also contain various inflammatory markers which respond to situations such as irritation occurring on the ocular surface or epithelial cell dysfunction.[20,30]

The innermost tear film layer lying along the cornea and tear film interface is the mucous layer.[13] The components of this layer are mainly secreted by the conjunctival goblet cells with contributions also made from the corneal and conjunctival epithelium.[13] When stimulated, conjunctival goblet cells release mucin and glycoproteins onto the cornea.[13] Components of the mucous layer include mucin, immunoglobulins, urea, salts and enzymes.[13,31] The mucins make the relatively hydrophobic cells of the conjunctiva and cornea hydrophilic, which then allows for the aqueous layer to spread across the corneal epithelium.[13] Ocular mucins in particular are responsible

for preventing the adherence of bacteria to the corneal epithelium.[13] Prevention can occur through one of two mechanisms. In one scenario, mucin have the ability to directly bind to the bacteria and thus prevent it from adhering to the epithelium.[13] On the other hand, mucin can also competitively bind to microbial receptors and prevent adherence that way.[13,32] All the components of the mucus come together to provide lubrication to the cornea, defend it against shear forces, and aid in stabilizing the tear film.[13]

While the tear film does have 3 layers, a deeper analysis into the composition of the mucin layer found that there exists a blend between the aqueous and mucin layer.[14] This resulted in recognizing it as a single layer, thus referring to it as the muco-aqueous layer.[14] Consequently, the tear film is recognized to be bi-phasic in nature, with its components being the lipid layer and the muco-aqueous layer.[14]

The composition of the tear film is not always consistent. Due to its high responsiveness to ocular conditions, researchers have analyzed its biochemical properties under these conditions, and that has helped understand what causes disease.[33] The lacrimal gland plays an important role in protecting the health of the ocular surface, as it secretes antimicrobial molecules such as lactoferrin, lysozyme, lipocalin toll-like receptors, and immunoglobulins (Ig) such as IgA and IgG into the tear film.[33,34] Secretary IgA (sIgA) has a crucial role in the adaptive immune response which is responsible for specific immunity.[33] sIgA is a primary immunoglobulin, also commonly referred to as an antibody, which helps to protect against any microorganism that may contact the eye.[13,33,35] In the case that there are bacteria present on the ocular surface, the sIgA binds to the bacteria and stimulates polymorphonuclear leucocytes (PMNs) to conduct phagocytosis to remove

the bacteria from the surface.[35] sIgA acts on bacteria by also creating a coat which results in bacterial agglutination and the bacterial molecules are then neutralized and lysed.[13,36,37] As a result, bacteria can no longer bind onto the corneal epithelial cells.[13,36,37] During an infection it is common to see an increase in specific antibodies, and their presence can be indicative of certain ocular diseases.[33] For example, increased levels in IgA, IgG and IgM can be associated with ocular infections such as acute adenoviral conjunctivitis and acute bacterial conjunctivitis.[33]

Another important factor in the immune system, present in the tear film are the Toll-Like receptors (TLRs).[33] TLRs are located on the corneal epithelial cells and are responsible for recognizing specific pathogen-associated molecular patterns (PAMPs).[33] PAMPs are molecules whose structures have been conserved through evolution and are frequently present in similar pathogens.[33,38] The presence of these molecules initiates adaptive and innate immune responses.[33,38] Commonly found PAMPs in the tear film are flagellin, peptidoglycan, lipoteichoic acid, and lipopolysaccharide.[33,39] Once the TLRs are activated by the PAMPs, they initiate an immune response by signaling for the upregulation of cytokines and chemokines which are both protein molecules.[33] These molecules then signal for inflammatory cells to migrate to the site of infection to fight it.[33] Ocular disorders in which TLRs have been found to have a role, include herpes simplex keratitis, Sjogren's syndrome dry eye, and non-Sjogren's syndrome dry eye.[33]

1.3 Proteins in the tear film:

1.3.1 Cytokines

Cytokines are small molecular proteins, which are responsible for relaying the communication and interaction between cells.[40,41] Although they are secreted by most nucleated

cells [41], the primary sources are helper T cells (T_H) and macrophages.[40] While "cytokine" is the general term used to classify these proteins, terms such as lymphokine, monokine and interleukin (IL) are also used, as these names provide a direct indication of which cell secreted them.[40,41] For example, an interleukin is secreted by leukocytes.[40] The production of cytokines often happens through a cascade.[40] Once the initially released cytokine signals its target cell, that cell then activates its cytokine gene expression, leading to the production of various cytokines.[40] These cytokines will then act similarly to signal other target cells and lead to the production of additional cytokines, and so on.[40] When referring specifically to inflammation, cytokines for the most part can be divided into two categories: proinflammatory (promoting inflammatory responses) and anti-inflammatory (preventing the proinflammatory cytokine activity).[40,41] The balance between both inflammatory responses heavily influences disease management in an individual.[33,41]

Proinflammatory responses are primarily regulated through the presence of IL-1 (α and β) and tumour necrosis factor (TNF) (α and β), as they collectively activate cytokine expression by acting on the endothelium.[41,42] When macrophages or monocytes are triggered by stimuli such as cell injury they secrete IL-1α and β, which then act on IL-1 receptors (IL-1R) to induce inflammatory responses.[40,43,44] While the IL-1α gene is constantly transcribed and produced in its 31 kDa pro-form, the production of IL-1β requires cells to be exposed to pathogen- or damage-associated molecular patterns (PAMPs or DAMPs).[44,45] This exposure then leads to the secretion of IL-1β (35kDa) in its inactive pro-form.[44] The activation of IL-1β involves inflammasomes. Inflammasomes are protein complexes whose components include: a sensor, adaptor and procaspase-1.[46] In the presence of PAMPs and DAMPs, inflammasomes are constructed which then go on to activate caspase-1enzyme (CASP1/ICE).[43,46] Caspase-1 then cleaves the pro-IL-1β

into its active 17 kDa form.[44,46] Once in its active form, IL-1 then goes onto regulate the secretion of several other cytokines such as IL-6, IL-8, and TNF-α.[47,48]

While IL-1β and TNF do act synergistically, IL-1β is also involved in the regulation of its secretion.[33,48,49] Pro-TNF (26 kDa)[50] is a transmembrane protein that is cleaved into soluble TNF (17 kDa) via the TNF-α-converting Enzyme (TACE).[50] Soluble TNF has the ability to travel through blood plasma, thus acting on sites far from the location where it was produced.[50] TNF-α acts on the receptors TNFR1[40] and TNFR2[40] to regulate immune responses and increase pain sensitivity.[40,42]

Another prime cytokine that is often investigated in the tear film is IL-6, as it has a role in ocular inflammation.[51] While several cells are capable of producing IL-6, such as monocytes, vascular smooth muscle cells and osteoblasts, there are only a limited number of cells that express the receptor for IL-6.[51] These include macrophages, neutrophils, and some T-cells.[51] Aside from IL-6 (21-28 kDa) being involved in the differentiation of both B-cells and T-cells, it can also stimulate lymphocyte chemotaxis.[51] Similarly, another common cytokine that plays a crucial role in ocular inflammation is IL-8.[52] IL-8 is recognized as a chemokine, with a molecular weight of 8-10 kDa.[52] Due to its ability to promote proinflammatory responses, its production is maintained at a minimum and thus in healthy tissues, its levels are usually low or undetectable.[52,53] Similar to many cytokines, IL-8 is also produced by monocytes and macrophages. IL-8 is a commonly used chemotactic factor in the immune system. Its presence induces chemotaxis and thus directs the movement of neutrophils, basophils and T lymphocytes to sites of inflammation.[52] Aside from recruiting immune cells, it also has a role in cell adhesion, angiogenesis, and protecting neurons.[52]

1.3.1.1 Cytokine Concentrations in Human Tears

Several studies investigating inflammatory cytokine levels in human tears under various conditions have been carried out. In a study which set out to determine IL-1α, IL-1β, IL-6 and IL-8 levels in normal human healthy tears, Nakamura et al. collected tear samples from 270 healthy participants.[54] Both basal tears (mean of 9μL) and reflex tears (mean of 115 μL) were collected from each eye.[54] Cytokine levels were quantified using an enzyme-linked immunoassay (ELISA) and it was reported that IL-1α levels (mean ± SEM) were 10.9 ± 1.2 pg/mL and 9.3 ± 2.7 pg/mL in basal and reflex tears, respectively. Levels of IL-6, IL-8 and IL-1β in basal tears were reported to be 226.2 ± 29.6 pg/mL, 731.4 ± 116.2 pg/mL, and 12.9 ± 2.3 pg/mL, respectively.[54] Meanwhile in reflex tears, IL-6 and IL-8 levels were reported as 11.6 ± 1.6 pg/mL and 276.1 ± 47.5 pg/mL, respectively.[54] IL-1β levels in reflex tears were not detectable.[54] The purpose of these results and this study was to provide a baseline for normal human tear cytokine concentrations so that they could be used as a comparison when analyzing tears from participants with ocular diseases.[54]

With a similar purpose, a study by Carreño et al. was conducted to determine the concentration of 30 cytokines and chemokines in tears from healthy subjects.[55] Nine participants with no previous history of ocular disease were recruited and tear samples were collected from each eye.[55] Samples were then analyzed through a multiplex bead analysis using the Luminex IS-100.[55] While 30 cytokines and chemokines were analyzed, only 25 of them were detected in the samples.[55] Some of the cytokine concentrations reported include a mean concentration of 47.5 ± 3.3 pg/mL, 101.4 ± 2.8 pg/mL, 130.4 ±12.3 pg/mL and 322.7 ± 33.5 pg/mL for TNF-α, IL-1β, IL-6 and IL-8 respectively.[55]

One study by Thakur et al., investigated the difference in cytokines and lipid inflammatory mediators between tears in closed eyes and open eyes.[56] In the absence of blinking, which is usually when a person is sleeping, the rate of tear flow is reduced. Previous research found that PMNs such as neutrophils were being recruited into tears upon eye closure.[56,57] The method of recruitment however was not clear.[56] Thakur et al. predicted that since IL-8 was a known neutrophil chemokine, it was being produced when the eyes were closed. Apart from IL-8, leukotriene B_4 (LTB_4) is also chemoattractant for neutrophils.[56] It was hypothesized that while the eyes were closed during sleep, there was an upregulation of cytokines and lipid inflammatory mediators.[56] As a result, PMNs were recruited and the production of IgAs were increased.[56] To test this hypothesis, participants with no reported ocular infections were recruited and their open-eye tears and closed-eye tears were collected.[56] Tears for the closed-eye study were collected after 3, 5, and 8 hours of sleep.[56] Cytokines and lipid inflammatory mediators were quantified using ELISA kits. It was reported that there was a significant increase in IL-8 and LTB_4 levels in closed-eye tears after 8 hours of sleep in comparison to open-eye tears ($P < 0.0001$). Levels of IL-8 were found to be significantly higher in tears collected at 8 hours of sleep versus 3 hours ($p < 0.008$).[56] IL-6 levels were reported to be $147.0 \pm$ pg/mL in closed-eye tears and below the limit of detection in open-eye tears.[56] Levels of IL-1β were not detected in both closed- and open-eye tears.[56] The results from their study showed that the upregulation of cytokines and arachidonic acid metabolites stimulated the migration of PMNs into the tear film.[56] It is predicted that granulocyte macrophage colony stimulating factors (GM-CSF) activate these PMNs which then allows for them to express IgA receptors.[56] Through the expression of these receptors, PMNs then have the opportunity to engulf debris or microorganisms present on the ocular surface.[56]

Cytokine concentrations can also vary depending on ocular conditions. A study by Massingale et al. was set out to determine the correlation between cytokine levels and tears of patients with dry eye disease (DED).[47] Non-stimulated tears (20 μL) were collected from one eye from seven healthy participants and seven participants with DED.[47] The tears were then analyzed using Invitrogen's Multiplex Bead Immunoassay, which uses flow cytometry, for a total of 8 cytokines and 1 chemokine.[47] Results showed that there was a significant increase in the proinflammatory cytokines IL-6, IL-1β and TNF-α and the chemokine IL-8 in tears from DED participants (1625.7 ± 430.9 pg/mL, 664.3 ± 148.8 pg/mL, 435.7 ± 145.6 pg/mL and 48508.6 ± 9397.3 pg/mL, respectively) in comparison to tears from healthy participants (632.3 ± 167.9 pg/mL, 436.3±116.7 pg/mL, 25.06 ± 63.2 pg/mL and 16791.4 ± 2841.2 pg/mL, respectively).[47] From this study they concluded that inflammation has a role in DED and the extent of it is dependent on the degree of the disease.[47] In addition, they reported that increased tear inflammatory markers concentration while the eye is in DED state, is due to its upregulation rather than tear film evaporation.[47]

Another ocular condition investigated by researchers interested in cytokine levels was keratoconus.[58] Lema et al. conducted a study to quantify the levels of cytokines, cell adhesion molecules, and matrix metalloproteinase 9 (MMP-9) in patients with keratoconus.[58] The cytokines of interest were IL-4, IL-6, IL-10, and TNF-α.[58] Both, patients with keratoconus and normal healthy subjects, were recruited. Tear samples were collected from the inferior meniscus and analyzed using an enzyme-linked immunoadsorbent assay kit.[58] The results from the study showed that while there was no significant difference between the levels of IL-4 and IL-10 in both keratoconus tears and normal tears, significantly increased levels of IL-6 and TNF-α were found

in patients with keratoconus (values reported as median [quartiles]) (6.7 [4.8-10.8] pg/mL and 3.8 [2.9 – 14.4] pg/mL, respectively) in comparison to normal tear samples (2.2 [1.0 -4.1] pg/mL and 1.8 [1.5-2.3] pg/mL, respectively).[58] The results also showed that MMP-9 levels were significantly higher in keratoconus tears (66.5 [49.2-139.3) ng/mL) in comparison to the normal tears (6.1 [3.9 -8.3] ng/mL).[58] While the roles that these cytokines play in keratoconus were unknown, it was concluded that with an increase in IL-6, TNF-α and MMP-9, inflammatory events take place on the ocular surface in patients with keratoconus.[58]

Together, all the studies mentioned above show that inflammatory events on the ocular surface can be induced by various ocular diseases and conditions. In addition, there is no definitive basal level of tears present as the results in these studies varied due to the methods used to collect tears; participant demographics; and the equipment and assays used to quantify the cytokines from the samples.

1.3.2 Major Tear Film Proteins

Amongst the many proteins in the tear film, lysozyme, lactoferrin and albumin are most commonly investigated proteins in contact lens and dry eye studies.

Lysozyme (14.3 kDa, pI pH 11.4 [59]) is another commonly found protein in the tear film as it constitutes approximately 20-30% of the total protein concentration found in the tears.[60-62] This protein molecule has enzymatic antimicrobial properties and has the ability to kill gram positive bacteria through lytic activity.[60,61,63,64] Bacterial cell walls are composed of peptidoglycan, which consists of 1,4-beta linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine.[60,61,64,65]

Lysozyme breaks the 1,4-beta linkage, which compromises the state of the cell wall.[60] This leads to an increase in internal osmotic pressure which eventually results in the lysis of the bacteria.[60]

Lactoferrin (pI pH 8.7 [59]) is a glycoprotein[63] with a molecular weight of 82 kDa.[66] By weight, it makes up approximately 25% of the total protein found in tears, with tear concentration levels reported to be 2.2 mg/mL.[66,67] While the main source of secretion is the acinar cells found in the lacrimal gland[61], the epithelia on the ocular surface can produce lactoferrin as well.[66,68] In the tear film, lactoferrin can have both anti-microbial and anti-inflammatory properties.[66] Bacteria require iron for growth, and lactoferrin inhibits bacterial growth by binding to the free iron molecules in the tears.[63,66,69] In addition, lactoferrin is also a positively charged molecule at the physiological pH.[66] This property thus allows for it to bind onto negatively charged molecules present on the surface of both gram-negative and gram-positive bacteria. As a result, the cell wall of the bacteria is often compromised.[61,66]

Albumin (66 kDa [70,71]) is a negatively charged serum protein that has a pI pH of 5.2.[59] It is produced by the hepatocytes in the liver.[70,71] As a small molecular protein, it has the ability to cross the blood-tear barrier. Its presence in the ocular environment is thus used to indicate the permeability of the vasculature.[71] Its duties include acting as a transport protein to deliver physiological molecules such as hormones, fatty acids and drugs.[70-72] It also has a role in stabilizing the blood pH levels as well as its osmotic pressure.[70] Reported albumin levels in the tear film ranges from 0.0103 mg/ml to 390 mg/ml.[70,73] Its concentration can vary depending on ocular conditions. For instance, higher levels of albumin can be seen in patients with symptoms of dry eye or in those who wear contact lens.[70]

1.4 Contact lenses

1.4.1 Contact Lens Background and History

Contact lenses are biomaterials primarily used as a medical device to correct refractive errors[74,75], which are worn by over 125 million people globally.[75] The demand for the use of contact lenses goes beyond the purpose of solely using them for correcting vision. With the advancement in research, contact lenses have been used to provide therapeutic uses such as acting as a vehicle to deliver drugs to treat ocular conditions such as glaucoma [75,76] and acting as a bandage to treat corneal diseases such as corneal epithelial disorders.[77] Another common eye condition that is estimated to affect over 1400 million people around the world is myopia.[75,78] The growing interest in myopia control have led researchers to develop contact lenses which can slow progression of myopia.[75,79]

The concept of using contact lenses was first introduced by August Muller in 1889. His idea was that in order for the lenses to be held in place against the surface of the eye, the shape of the lenses should follow that of the outer surface of the eye.[74] The first set of contact lenses that were fitted to a patient was made of a protective glass shell and were referred to as glass scleral lenses.[74,80] These lenses did have their limitations as they were large and allowed only limited tear flow beneath the lens.[74,80] Improvements to the lens model was done by introducing plastics as a material to develop lenses. After trying cellulose nitrate, cellulose acetate and polyvinyl acetate[81], in 1938, polymethyl methacrylate (PMMA) was used to make scleral lenses.[74,80,81] Lenses that were made of PMMA were often referred to as hard lenses.[75] While they were light in weight, these lenses were limited in their hydrophilic properties and did not offer oxygen permeability[75], which led to corneal hypoxia.[74,75,82]

In an attempt to create a lens material that offered greater biocompatibility with the eye, hydrogel soft lenses were invented in the early 1970's.[83] The initial hydrogel material was

fabricated from hydroxyethyl methacrylate (HEMA) which were hydrophilic and were the first contact lens materials to absorb water.[75] While lenses made from HEMA were successful, they did have their drawbacks as they did not offer enough oxygen permeability.[75] The need to improve oxygen permeability led to the eventual development of silicone-based hydrogel lenses. Oxygen is highly soluble in silicone, so the incorporation of silicone into hydrogel lenses eliminated a lot of hypoxia-related ocular conditions.[83] These lenses offer the highest oxygen permeability when compared to all other materials.[75] Conversely, silicone is inherently hydrophobic in nature.[83] The hydrophobicity caused by silicone led to poor lens surface wettability.[75,83] To improve the wettability and add hydrophilicity to the lens, hydrophilic co-monomers were incorporated into the silicone material.[75,83]

1.4.2 Contact Lens Classification

In 1994, The United States Food and Drug Administration (FDA) issued a document that that categorized conventional contact lenses according to two physical properties: water content and ionic charge.[84] The grouping system that was developed is shown in Table 1:

Table 1.4-a: Classification of conventional hydrogel contact lens issued by the FDA[84]

Group	Water Content	Ionic Charge
I	Low – water content	Non-ionic lenses
II	High-water content	Non-ionic lenses
III	Low-water content	Ionic lenses
IV	High-water content	Ionic lenses

Due to the incorporation of monomers and silicone macromers into silicone hydrogel contact lens, a separate grouping system was assigned for silicone hydrogel lenses.[84] The

classification of silicone hydrogel lenses is also dependent on water content (low-water versus high-water content) and ionic charge (non-ionic versus ionic).[84] However, the low-water, non-ionic group is further subdivided into three categories. The subdivision allows for predictions to be made with regards to how the lens material interacts with human lipids and whether the lenses have any surface treatments present.[84] As seen in Table 1.4-b, lenses that are low-water, and non-ionic can be placed into group V-A, V-B1 and B2. While lenses that are surface treated are placed into group V-A, non-surface treated lenses are placed into group V-B, which is then subdivided into V-B1 and V-B2.[84] Non-surface treated V-B lenses that contain hydrophilic monomers are placed into V-B1, and non-surface treated V-B lenses that contain a semi-interpenetrating network are placed into V-B2.[84]

Table 1.4-b: Classification of silicone hydrogel contact lens[84]

Group	Water Content	Ionic Charge
V - A	Low-water content	Non-ionic
V - B1	Low-water content	Non-ionic
V - B2	Low-water content	Non-ionic
V - C	High-water content	Non-ionic
V - D	Both low-water and high-water content	Ionic

The grouping system allows for researchers and product developers to predict and understand how contact lenses will interact with tear film components and preservatives, in terms of uptake and release.[84]

1.4.3 Contact Lens Discomfort

Although wearing contact lenses has its benefits, there are several disadvantages which can lead to the discontinuation of lens wear. One such disadvantage is discomfort. Contact lens discomfort (CLD) is a condition defined by unfavourable ocular sensations due to the lack of compatibility between the eye and contact lenses.[85] CLD may result in visual disruptions, leading to the discontinued use of lenses or decreased wearing times.[85] Symptoms of CLD that have been reported include: dryness, blurry vision, scratchiness, irritation, light sensitivity, and eye soreness.[86,87] While environmental and lens property factors have been reported to be causes of CLD, researchers have also proposed that CLD may be a response to sensitivity relayed by the neural terminals present on the ocular surface.[88] For example, changes to tear composition such as an increase in proteins can be interpreted as a chemical stimuli by a neuroreceptor.[88] In addition, mechanoreceptors and thermoreceptors can become stimulated due to an increased interaction between the ocular surface and lenses and an increase in ocular temperature due to inflammatory responses, respectively.[88] In fact, a study was conducted investigating the relationship between the level of ocular inflammation present during contact lens wear and the level of discomfort felt by lens wearers. Amongst the various cytokines analyzed, it was reported that CLD was associated with an increase in IL-17A levels in contact lens wearers.[89] The discussion around CLD and the mechanism that induces it has led researchers to study whether CLD is a result of subacute inflammation.

1.4.4 Contact lens and Corneal Infiltrative Events

Contact lens-related corneal infiltrative events (CIE) have been of interest to clinicians and researchers for several years as its occurrence in contact lens wearers has been increasing in recent times.[90,91] When inflammatory responses are initiated on the ocular surface, inflammatory

mediators are released which then signal for the infiltration of white blood cells and other PMNs to the site of injury.[92] The term CIE thus refers to the accumulation and aggregation of inflammatory cells on the cornea due to an inflammatory response.[92,93] The term infiltrate refers to any cell, fluid, or substance that has travelled into the gaps present in tissues or cells.[94] These infiltrates can be characterized as small, round, or shapeless, hazy-coloured clumps of inflammatory cells present on the corneal surface.[93] While the categorization of CIEs has been challenged, the proposed grouping of CIE is: sterile vs infectious keratitis.[90,92]

Microbial keratitis (MK) is a form of ocular inflammation which is caused by microbes such as bacteria and fungi present on the ocular surface.[95] Once these microbes make their way into the corneal stroma they can cause inflammation which in extreme cases can lead to structural damage.[95,96] Some risk factors that can lead to MK include: contact lens wear (particularly both overnight and extended wear), poor lens disinfecting practices, ocular surgery, etc.[95] As a result of MK, patients often experience redness, tearing and blurry vision [95], severe hyperemia [93], and tissue necrosis.[93]

Sterile keratitis (or infiltrative keratitis; IK) is a term used to define non-progressive keratitis as its cause is not directly related to a microbial infection.[94,97] If microbes are present in sterile infiltrates, its active growth is usually halted.[93] It is important for clinicians to be able to identify the difference between the two types of infiltrates, as infectious infiltrates require immediate medical attention. They are often distinguished from each other through location, size, shape and colour.[93] Infectious infiltrates are characterized to have diameters larger than 2.0 mm, and are normally located close to the center of the cornea with more widely spread out borders.[93]

Sterile infiltrates, however, are less than 1.0 mm in diameter and are typically located on the outer ends of the cornea, and have more precise edges.[93]

Corneal infiltrates associated with contact lens wear have been the subject of many reports in the literature. Common forms include contact lens-induced peripheral ulcer (CLPU) and contact lens-associated red eye (CLARE).[93] Research has shown that lens modality can be a factor in developing both microbial or sterile keratitis.[93] For instance, the risk of developing microbial keratitis and sterile keratitis in extended-wear soft lens was reported to be 36.8 and 4.6 times higher than in RGP lens wearers respectively.[93,98] Similarly, there was a higher risk of developing microbial and sterile keratitis in daily–wear soft contact lens wearers (4.2 and 2.3 times, respectively) when compared to RGP lenses.[93,98]

Structural and physiological changes to the cornea caused by contact lens wear can eventually lead to the development of CIEs.[93] Contact lens-associated CIEs can be induced in a variety of ways. For example, when the corneal epithelial cells are damaged due to hypoxia and corneal trauma caused by lens wear, they often release proinflammatory cytokines, thus inducing an inflammatory response.[93,99] In other cases, the lenses can act as a hub for bacteria or debris which when transferred to the cornea or held against the corneal surface, can lead to the development of CIEs.[93] In some cases, the contact lens material itself or the deposits present on the lens materials can act as proinflammatory agents and stimulate a proinflammatory response, thus leading to CIEs.[93] Evidently, when the corneal epithelium is subject to any form of mechanical, hypoxic, or toxic condition, they signal for the stromal keratocytes to produce proinflammatory cytokines as an immune response.[93] These cytokines and chemokines, such as

IL-8, in return signal for the infiltration of inflammatory cells to the corneal surface, whose aggregation ultimately lead to CIEs.[93]

1.4.4.1 Contact Lens Induced Inflammation

The relationship between contact lens wear, ocular inflammation and contact lens discomfort has been investigated in several studies. Ocular inflammation is commonly analyzed by quantifying the inflammatory mediators present during contact lens wear. In 2016, Efron proposed the idea that asymptomatic contact lens wear is intrinsically inflammatory.[100] In his article he explained that symptoms from uncomplicated lens wear meet the criteria for both clinical and subclinical definitions of inflammation. The criteria for clinical inflammation included: redness, heat, swelling, pain, and loss of function.[100] The criteria for sub-clinical inflammation included cellular and biochemical reactions.[100] While hydrogel lens wear presented with all signs for both forms of inflammation, silicone hydrogel presented most forms.[100] One notable sign of inflammation that was present in both hydrogel and silicone hydrogel lens wear was the presence of biochemical reactions, specifically the up-regulation of inflammatory mediators.[100]

There have been several studies conducted which investigated the relationship between contact lens wear and the presence of inflammatory mediators. The purpose of these studies was to further understand ocular inflammation as a response to contact lens wear.

In a study conducted by Poyraz et al., the researchers focused on quantifying the concentrations of IL-6 and IL-8 in neophyte contact lens wearers.[101] Recruited participants were either fitted to silicone hydrogel lens or conventional hydrogel lens and were asked to wear the

lens for a 6 month period for 8 hours a day.[101] Tear samples were collected from the participants at the 0, 1, 3- and 6-month time mark. Cytokine concentrations of IL-6 and IL-8 from the tear samples were then analyzed using an ELISA.[101] Tear levels of IL-6 and IL-8 from both lens wear groups were found to have significantly increased at 6 months when compared to the levels reported at the 0 and 1 months marks ($p < 0.001$).[101] The researchers concluded that both silicone hydrogel and hydrogel contact lens wear caused an ocular inflammatory response, specifically an increase in IL-6 and IL-8 in neophyte contact lens wearers over a 6-month period.[101] In another study, Dogru et al., recruited participants and fitted them with senofilcon A silicone hydrogel lenses.[102] Participants were asked to wear the lenses for a 2-week period for 12 hours a day. Tear samples were collected pre-lens wear and post-lens wear.[102] They found that tear IL-6 levels were significantly increased after the 12-week period (post-wear mean: 2505 ± 951 pg/mL; pre-wear mean: 1516 ± 497 pg/mL).[102] The results from this study further support the idea that contact lens wear can induce an increase in cytokine concentration in the eye.

González-Pérez et al. conducted a study focused on determining whether continuous wear of silicone hydrogel lenses and reverse geometry rigid contact lenses influenced tear film inflammatory mediator concentrations.[103] Reverse geometry contact lenses have been used in corneal refractive therapy to induce short-term flattening of the corneal curvature to reduce or slow the progression of myopic refractive error.[103,104] While these treatments do have their advantages, researchers were interested in noting whether the cornea was compromised by inflammation and infections during the corneal flattening treatments.[103] They were interested in analyzing the tear marker concentrations to determine whether there were inflammatory events that occurred after extended wear of silicone hydrogel lens and overnight corneal refractive therapy.[103] The tear

markers of interest were: IL-6, IL-8, epidermal growth factor (EGF), and MMP-9. Results from their study showed that after 12 months of continuous lens wear, there was an increase in tear levels of IL-6, IL-8, MMP-9, and EGF in the overnight corneal refractive therapy group in comparison to the silicone hydrogel lens wear group as well as the no lens wear control group.[103] From these results it was understood that the upregulation of these inflammatory markers was a response to the changes that were occurring on the corneal surface.[103] While the inflammatory response could be a mechanism used to assist with corneal wound healing as well as corneal protection, it is unknown if these inflammatory responses have any long term negative impacts.[103]

Acanthamoeba Keratitis (AK) is an ocular infection caused by the amoebae *Acanthamoeba* that can possibly lead to vision loss.[105] It has been reported that contact lens wear is the leading cause of AK,[105] particularly when associated with the exposure of the lenses to tap water. Common symptoms experienced by affected people include pain, tearing, photophobia, and infiltrates in the stroma.[105] In a study conducted by Carnt et al., they wanted to compare tear cytokine levels between contact lens wearers that had AK and lens wearers without the disease.[106] They also wanted to compare cytokine levels in patients with severe forms of AK disease with those that had a milder form of the disease.[106] Of the various cytokines analyzed, IL-8 was found to be detected more in tear samples collected from patients with AK in comparison to tear samples from non-AK patients ($p = 0.003$).[106] In addition, higher concentrations were found in tear samples from patients who had a more severe form of AK compared to those that had a milder form. Aside from IL-8, IL-22 was also detected more frequently in severe forms of AK than milder forms.[106] Results from all these studies further support the idea that contact lens-induced diseases can impact the ocular environment and thus eventually lead to an inflammatory response.

1.4.4.2 Silicone hydrogel contact lens and CIEs

The development of silicone hydrogel contact lens has its advantages. The presence of silicone in the contact lens material allowed for an increased permeability of oxygen through the lenses to the cornea. It was predicted that with an increase in oxygen permeability, there would be a decrease in the risk of developing hypoxic conditions.[83] However, although risks with hypoxia-related conditions have been reduced, the possibility of developing ocular inflammation and infection remains, as the lack of oxygen is not the only factor contributing to the development of microbial keratitis.[107,108] In fact, reports have shown that the risk of developing CIEs from silicone hydrogel contact lens wear is two times higher than in the lower oxygen permeable contact lens.[109-111] In a meta-analysis conducted by Szczotka-Flynn and Diaz, they reported that there was a two-fold higher risk in developing CIEs when wearing extended wear silicone hydrogel lenses for 30 days in comparison to wearing lower oxygen permeable lenses for 7 days.[107] Researchers were, however, unable to determine whether the increase in CIEs was primarily due to the difference in contact lens material as the length in wear time also had an impact on these results.[107] Furthermore, reports have shown that planktonic bacterial cells adhere more to certain silicone hydrogel lenses in comparison to hydrogel lenses, which can also be a cause for the increase in CIE development.[109,112] In fact, in a study conducted to determine the risk factors associated with CIEs in young soft contact lens wearers, they found that the age of patient, lens care products, years of lens wearing experience, soft contact lens material, and extended wear were all contributing factors.[113] Now, while all these factors have been reported to influence contact lens associated CIEs, the root cause of a higher rate of CIE development in silicone hydrogel lens wear is still unknown.

1.4.5 Protein Deposition Profiles

A lot of research has gone into determining how the materials used in contact lens impacts the development of CIEs. Contact lenses have been reported to be deposited with various proteins, lipids and calcium.[94] When these proteins or lipids denature, the ocular immune system may recognize them as foreign bodies. The immune response will then lead to the upregulation of inflammatory markers eventually causing PMNs to infiltrate into the ocular tissue.[94]

Protein deposition and lipid deposition onto both hydrogel and silicone hydrogel lenses have been discussed in the literature. Properties of the contact lens, such as its hydrophobic or hydrophilic character and the monomer's ionic charge, influence protein deposition profiles on various lens materials. The protein's properties such as its size and isoelectric point also influence protein deposition profiles.[59] For example, the total amount of protein deposited onto worn Group I, II, III lenses has been found to be less than 100 μg, whereas, 400 μg to 2000 μg of protein have been found to be deposited onto most Group IV lenses.[59] Lysozyme deposition also varies between lens types, as twice the percentage of active lysozyme has been found to be on ionic materials in comparison to non-ionic materials.[59]

While the deposition of various proteins such as lysozyme, lactoferrin, and albumin have all been discussed in the literature, there is minimal information with regards to cytokine deposition on contact lens. In 2019, Chao et al., conducted an experiment to determine the levels of cytokines absorbed, or firmly bound by lens materials.[114] The lenses that were investigated in this study were: comfilcon A, balafilcon A, omafilcon A, and etafilcon A. These lenses were soaked into both individual and combined protein solutions of IL-8, MMP-9 or IL-1Ra at

concentrations of either 500 pg/mL or 100 pg/mL.[114] The inflammatory markers of interest were then extracted from the lenses using a 1:1 2% trifluoroacetic acid: acetonitrile. The results from this study showed that in a solution of MMP-9 (500 pg/mL), omafilcon A (466 ± 9 pg/mL) absorbed more MMP-9 in comparison to balafilcon A (437 ± 11 pg/mL) and etafilcon A (428 ± 13 pg/mL).[114] There was more MMP-9 firmly bound to omafilcon A, comofilcon A and balafilcon A in comparison to etafilcon A lenses.[114] With respect to IL-8, they found no differences in absorptions between the lenses, however at 500 pg/mL, more IL-8 was found to be firmly bound to omafilcon A than etafilcon A.[114] It was also reported that there was no difference between the materials with regards to both absorbed and firmly bound concentrations of IL-1Ra.[114] When lenses were placed into a solution that combined all the analytes of interest, it was found that etafilcon A lenses absorbed more IL-8 in comparison to the other lens materials.[114] This study concluded that competitive binding between the various mediators played a role in absorption and extraction by the lens materials.[114]

With minimal information regarding cytokines deposition onto contact lens materials, there is a growing interest in determining whether cytokines bound to contact lens materials play a role in inducing CIEs. The research undertaken in this thesis focuses cytokines released from contact lens materials.

Chapter 2: Thesis Rationale

2.1 Objectives

The effect of contact lens wear on inflammatory biomarkers in the tear film has been fairly well documented. With lens wear, there is a risk for corneal infection and inflammation.[97,115,116] Research shows that lens wear can influence tear concentration levels of inflammatory markers.[101,117-120] The amount of inflammatory markers present during pre- and post-lens wear is often quantified by collecting tear samples from lens wearers and analyzing them with antibody-based assays. While such a process provides insight into the relationship between ocular inflammation and contact lens wear, it does not account for the interaction between the markers of interest and lens materials. There has been little discussion regarding the adsorption, deposition, and release of cytokines onto contact lens materials. Studying the interaction between cytokines and lens materials may help understand the mechanisms causing contact lens discomfort, ocular inflammation, and CIEs. In particular, the question of why there is a 2X greater risk of developing CIEs with silicone hydrogel lens wear can be answered.[107]

There were three main objectives in this thesis. The first objective was to develop a protocol that would aid in quantifying the amount of adhered cytokines released from contact lens materials. The second was to determine whether the release of cytokines differed between contact lens materials that were pre-exposed to various ocular conditions. The third objective was to determine whether the release of cytokines differed between protein-coated (lysozyme, lactoferrin, or albumin) contact lens materials.

2.2 Contact Lens of Interest

It was important to investigate both conventional and silicone hydrogel lenses because, while protein deposition occurs on both, their deposition profiles vary greatly. Charge, water content, and surface properties of the lens material influence the extent of deposition. Absorption typically increases with increased water content and favourable electrostatic attractions.[121] To understand how the release of cytokines differs between materials, four different daily disposable lens types were used. The hydrogel contact lenses of interest in this thesis were: etafilcon A (1-Day Acuvue® Moist®) (ionic, high water content) and omafilcon A (Proclear® 1 day) (non-ionic, high water content). The silicone hydrogel lens of interest were: somofilcon A (Clariti ™1 day) (non-ionic, high water content) and delefilcon A (Dailies Total1®) (core material – non-ionic, low water content; surface coating: non-ionic, high water content). Table 2.2-a illustrates some of the main properties of these lens materials.

Table 2.2-a: Properties of Contact Lens Materials Investigated in This Thesis

	United States Adopted Name (USAN)			
	Etafilcon A	**Omafilcon A**	**Somofilcon A**	**Delefilcon A**
Proprietary name	1-Day Acuvue® Moist®	Proclear® 1 day	Clariti ™1 day	Dailies Total1®
FDA category	IV	II	V	V
Ionicity	Ionic	Non-ionic	Non-ionic	Non-ionic
Modality	Daily Disposable	Daily Disposable	Daily Disposable	Daily Disposable
Water Content (%)	58 [122,123]	60[122]	56[123]	Gradient: 33 (core), ≥ 80 (surface)[122,123]
Dk/t @ -3.00D	25.5	28.0	86.0	156.0
Principle Monomer	HEMA + MA[81]	HEMA + PC[81]	Alkyl methacrylates, siloxane monomers, NVP[123]	The core: DMA, TRIS-Am, siloxane macromer The surface: copolymers of polyamidomine and poly(acrylamide-acrylic acid)[123]

HEMA = 2-hydroxyethyl methacrylate; MA = methacrylic acid; DMA = *N,M*-dimethylacrylamide; NVP = *N*-vinyl pyrrolidone; PC = 2-methacryloylethyl phosphorylcholine; TRIS-Am = *N*-[trismethylsiloxy)-silylpropyl]acrylamide.

2.3 Cytokines of Interest

Inflammatory responses, both pro-inflammatory and anti-inflammatory responses, are promoted through cytokine activity.[40,41] Human tears have been analyzed for the presence and basal levels of cytokines and chemokines. Of the 30 cytokines analyzed, 25 were detected.[55] Due to their prominent roles in the inflammatory cascade, this thesis chose to focus on four cytokines: IL-1β, IL-6, IL-8, and TNF-α. When it comes to initiating an inflammatory response, there is a cytokine hierarchy. In a study that looked at the hierarchy of IL-1β, IL-6, IL-17A, and TNF, it was found that IL-1β required the least amount of expression to cause an inflammatory response in the eye.[124] An experimental study showed that small increments of IL-1β levels in the eye can contribute to the development of ocular inflammation over time.[124] IL-1β, a proinflammatory cytokine, is secreted by monocytes, macrophages, dendritic cells, and neutrophils.[125] While IL-1β has a crucial role in initiating inflammatory responses, it can also impose toxic effects inside the human body. Hence, its production is highly regulated.[126] Apart from being involved in infection control and cellular activity, IL-1β can also regulate the secretion of other cytokines such as IL-6, IL-8, and TNF-α.[49,127] While TNF can also initiate ocular inflammation, in comparison to IL-1β, increased amounts of expression is required.[124] TNF-α is involved in the development of inflammatory, neovascular, and neurodegenerative ocular conditions.[128] IL-6 is a cytokine that can be both anti-inflammatory and pro-inflammatory. Although it can protect the eye against infection, it can also cause damage to ocular tissues via neovascularization or increasing the severity of inflammation.[51] Higher levels of IL-6 have been detected in those with a history of CIEs in comparison to tears from healthy participants.[119] IL-8 is a highly regulated pro-inflammatory chemokine.[52] It is expressed at the early stages of inflammation and can sustain its active state for

time periods as long as weeks.[52] As a chemokine, it signals for the migration of neutrophils, basophils and T lymphocytes to sites of inflammation.[52]

Neophyte silicone and conventional hydrogel lens wearers have been found to have increased IL-6 and IL-8 levels after a six month wear period.[101] Due to their presence in ocular inflammation and tears from contact lens wearers, it was thus of interest to study the release of IL-1β, IL-6, IL-8, and TNF-α from contact lens materials. Properties pertaining to the cytokines studied as well as basal levels found in human tears are illustrated in Table 2.3-a.

Table 2.3-a. Properties and Basal Levels in Tears of Cytokines Studied in this Thesis

Cytokine	Molecular Weight	pI point	Basal levels in tears (mean ± SD) (pg/mL)
IL-1β	17 kDa [44]*	~ 5.8 *	58.32 ± 3.61[129], 9.6 ± 7.9[130], 436.3 ± 116.7[47], 12.9 ± 2.3[54], 13.0 ± 4.0[131], 7.42±5.62[132]
IL-6	21-28 kDa[51], 20.3 kDa*	~ 6*	2634.49 ± 251.99[129], 89.5 ± 83.5[130], 632.3 ± 167.9[47], 226.2 ± 29.6[54], 171.8±32.1[131], 13.43±8.74[132]
IL-8	8-10 kDa[52], 8 kDa*	~ 8.5*	729.11 ± 46.26[129], 16791.4 ± 2841.2[47], 731.4 ± 47.5[54], 56.5±33.8[131]
TNF-α	17 kDa[50] 17.5 kDa*	~ 6.9*	252.6 ± 275.3[130] , 250.6 ± 63.2[47], 7.46 ± 8.74[132]

***** Values pertain to the cytokines used in this thesis. They were specified by MSD Scientific Support.

2.4 Hypothesis

In this thesis, *in vitro* studies were set out to investigate the release of adhered cytokines from daily disposable contact lens materials. It is hypothesized that silicone hydrogel lens releases more cytokines than conventional hydrogel lens materials. It is predicted that these cytokines then re-enter the ocular environment and stimulate an inflammatory response, leading to the development of CIEs.

Chapter 3: Protocol Development

3.1 Introduction

While the deposition of proteins, such as lysozyme, onto contact lenses has been investigated in the literature, minimal research with regards to the deposition of cytokines onto contact lenses has been conducted. In 2019, Chao and colleagues investigated whether inflammatory markers were absorbed or bound to contact lens materials. In the study, a 1:1 ratio of 2% trifluoroacetic acid and acetonitrile solution was used to extract cytokines from contact lenses to determine the concentration of absorbed and firmly bound cytokines.[114] While this study focused on studying proteins absorbed to contact lens materials, an earlier study conducted in 2007 by Mann and Tighe focused on analyzing the proteins present in the "tear envelope".[133] This concept refers to a small layer of the tear film which encloses the contact lens the moment it is removed from the lens wearer's eye. The purpose of analyzing the tear envelope was to investigate the relationship between tear film composition and the contact lens surface.[133] In this study, participant-worn lenses were collected, placed into 0.5 mL microcentrifuge tubes and immersed in 5μL of a buffer solution if the lenses were found to be dry.[133] The tube was vortexed and subsequently, a small hole was created at its base. The 0.5 mL lens-containing tube was then placed into a 1.5 mL microtube. Together, they were centrifuged at 3000 r.p.m. for a total of 15 minutes. The eluate collected in the 1.5mL tube was immediately analyzed for proteins on the 2100 Bioanalyzer using the Protein 200 Plus LabChip kits.[133] A similar protocol was used in another study to also analyze proteins in the tear envelope.[134] However, in this study, the contact lens was submerged into 40μL of sodium dodecyl sulphate (SDS) and 2-mercaptoethanol.[134] The lens-containing tube and the carrier tube it was placed in underwent ultracentrifugation at 5000 r.p,m for 10 minutes. The eluate collected in the larger tube was then processed using SDS-PAGE.[134]

These methods were used to analyze the tear film enclosing worn contact lenses. For the studies conducted in this thesis, the main purpose was to quantify the amount of adhered cytokines released by contact lens materials through an *in vitro* model. Thus, the protocol mentioned in the study conducted by Mann and Tighe[133] was adapted to explore the effects of changing various variables, to then finalize a protocol that would provide ideal results for future studies. All the studies conducted in this thesis were done *in vitro.*

The results from the studies conducted below, provided parameters that assisted in developing a protocol that was suitable for quantifying the amount of adhered cytokines released by contact lens materials.

To develop the protocol, the effects of changing three variables were explored: temperature, incubation period, and volume of diluent. Prior to conducting the experiments, it was decided that all studies would be conducted using Diluent 2. Diluent 2, as described by the MSD Scientific Support Group, is a proprietary phosphate-buffered solution (PBS) with a pH of ~ 7.4. It is provided as the assay diluent in the MSD Proinflammatory Panel II (human) Kit which is an immunoassay kit used to quantify cytokines, specifically: IL-1β, IL-6, IL-8 and TNF-α. The Diluent 2 provided, is specifically used to reconstitute the lyophilized calibrator blend, which contains IL-1β, IL-6, IL-8 and TNF-α, to prepare the cytokine solution. Thus, by using the same solution, it helps to minimize any effects that may be due to the interaction between the solvent and the cytokines of interest.

Experiment 1: Temperature

In this study, contact lenses were soaked in a cytokine solution. Following the soaking period, they were immediately placed into a Diluent 2 solution. It was of interest to determine whether incubating the contact lens in Diluent 2 at different temperatures (room temperature and body temperature of 37℃) impacted the amount of cytokines released by the lens materials. In a study conducted by Kessel et al., it was found that human corneal temperatures can range between 36.5℃ to 37 ℃ when the ambient temperature is increased.[135] As contact lenses sit on the ocular surface, whether or not ocular temperature influences the adhered cytokines from coming off the lens surface and back into the tear film was worthy of determination. Thus, for this experiment, the temperatures chosen were the maximum ocular temperature, 37℃, and room temperature.

Experiment 2: Incubation Period

Once the temperature was decided, the next variable explored was the incubation period. The incubation periods of choice were 0 hours or 1 hour. In the case of zero-hours (no incubation), contact lenses were removed from the cytokine solution post soak period and then immediately placed into the Diluent 2 solution to then be vortexed. However, in the case of a one-hour incubation period, once the lenses were removed from the cytokine solution, they were left to incubate in a Diluent 2 solution for 1 hour before being vortexed. The objective was to determine whether increasing the incubation period increased the amount of adhered cytokines released from the contact lenses.

Experiment 3: The volume of Diluent 2 required

The final variable that was analyzed was the volume of Diluent 2 required to aid in the removal of adhered cytokines. There were two volumes tested in this experiment: 200 µL and 550 µL. With a volume of 200 µL, the contact lens material was submerged inside the diluent. With the volume of 550 µL, diluent 2 filled the 0.6mL tube entirely. This experiment helped determine whether increasing the diluent volume would increase the amount of cytokines removed from the contact lenses.

3.2 Temperature Study

3.2.1 Materials and Methods

Contact Lenses of Interest and Cytokine Solution

A total of four different contact lens materials (n = 3 for each lens material in each condition), were investigated in this study. This consisted of two conventional hydrogels (etafilcon A; omafilcon A) and two silicone hydrogels (delefilcon A; somofilcon A). A multi-analyte lyophilized calibrator (Meso Scale Diagnostics, Rockville, MD) containing the cytokines, IL-1β, IL-6, IL-8, and TNF-α, was reconstituted in 7mL of Meso Scale Discovery's Diluent 2 to prepare a cytokine solution. The theoretical concentrations of IL-1β, IL-6, IL-8, and TNF-α in the prepared cytokine solution were reported as 88.57 pg/mL, 112.71 pg/mL, 74.14 pg/mL and 45 pg/mL, respectively.

Sample Preparation

Contact lenses were removed from their packaging solution and immediately rinsed in a PBS solution (Lonza Biosciences, Walkersville, MD). Excess PBS solution was removed on lens

paper (VWR, Radnor, PA). The lenses were individually placed into 5 mL conical-bottom polypropylene tubes (VWR, Radnor, PA) and soaked in 200 µL of the prepared cytokine solution. A control solution was prepared for each lens type in each condition. For this study, controls (n = 1 for each lens material in each condition), were used where the lenses underwent the same procedure. However, the lenses were soaked in 200µL of Diluent 2 rather than a cytokine solution. The purpose of this was to quantify background data produced by the lens. The lenses were left to incubate at room temperature for 6 hours with shaking. Post the 6-hour incubation period, lenses were removed from the polypropylene tubes and immersed into 200 µL of Diluent 2 in a 0.6 mL microcentrifuge tube (Axygen®, Inc, Union City, CA). The lenses were then placed on the Orbital Shaker (VWR, Radnor, PA) to incubate at either room temperature or on the INNOVA 4200 incubator shaker (New Brunswick Scientific Company, Inc., Edison, NJ) at 37°C for 1 hour with shaking. Subsequently, the tubes were vortexed for 5 seconds and pin-sized holes were made at its base. The 0.6 mL lens-containing tubes were then placed into a 2.0 mL microcentrifuge tube (Axygen®, Inc, Union City, CA) which acted as carrier tubes. Together, they were centrifuged using the MiniSpin® (Eppendorf, Mississauga, ON) at 604 relative centrifugal force (RCF) for 15 minutes. Post centrifugation, eluate was collected in each 2.0 mL tube and stored at -80°C. Cytokine quantification was performed at a later date using a multiplex electrochemiluminescence (ECL) assay Human Proinflammatory Panel II (4-Plex) assay (Meso Scale Diagnostics, Rockville, MD) and the MESO QuickPlex SQ 120 Imager (Meso Scale Diagnostics, Rockville, MD). A visual representation of the methodology is shown in Figure 3-1.

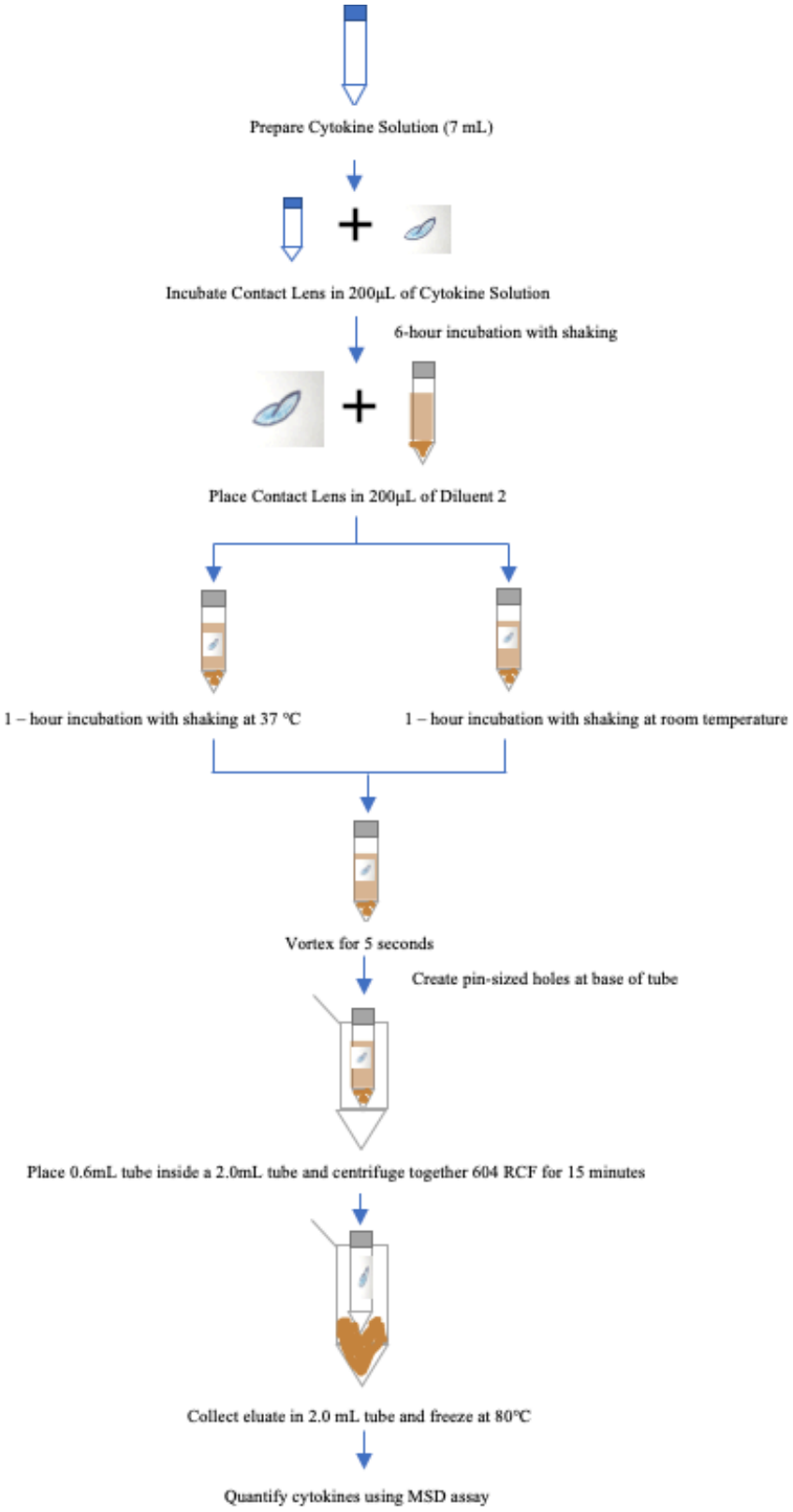

Figure 3-1: Schematic diagram of the methodology used for the temperature study. Created by Nijani.

Statistical Analysis

Data were analyzed using GraphPad Prism V8 software (GraphPad Software Inc., San Diego, CA). A two-way ANOVA with Sidak's multiple comparisons test was performed to determine whether there was a significant difference, within a single lens type, between the cytokines released by hydrogels at the two temperatures of interest. A two-way ANOVA with Tukey's multiple comparisons test was performed to determine whether there was a significant difference between hydrogel materials concerning the amount of cytokines released. Results for both tests were considered significant if $p < 0.05$.

3.2.2 Results

The amount of adhered cytokines released by each hydrogel is shown in Figure 3-2, Figure 3-3, Figure 3-4, and Figure 3-5 and summarized in Table 3.2-a, Table 3.2-b, Table 3.2-c, and Table 3.2-d (reported as mean ± standard deviation). There was no significant difference within a single lens type, between temperatures, with respect to the amount of cytokines released for all four cytokines (adjusted p-value > 0.05). In addition, there was no significant difference between the hydrogel materials concerning the amount of cytokines released (adjusted p-value > 0.05). To calculate the amount of cytokines released by the lens materials in pg/lens the following calculation was made:

Equation 3.2-1: Amount of adhered cytokines released by contact lens materials (pg/mL)

$$\frac{pg}{lens} = \left(\left(concentration\ of\ cytokine\ in\ eluent\ \left(\frac{pg}{mL}\right)\right) - \left(concentration\ of\ cytokine\ in\ lens\ control\ \left(\frac{pg}{mL}\right)\right)\right) * (volume\ of\ Diluent\ 2\ (mL))$$

This equation was used for all studies conducted in this thesis to calculate the amount of cytokines released by contact lens materials in pg/lens.

Table 3.2-a: IL-1β released by contact lens materials incubated at room temperature or 37°C

Lens type	Room temperature, pg/lens	37 °C, pg/lens	Adjusted p-value
Etafilcon A	1.288 ± 1.248	0.787 ± 0.343	0.8168
Omafilcon A	1.102 ± 0.305	1.053 ± 0.535	> 0.9999
Somofilcon A	1.327 ± 0.441	1.129 ± 0.335	0.9926
Delefilcon A	1.693 ± 0.888	1.763 ± 0.183	0.9999

Table 3.2-b: IL-6 released by contact lens materials incubated at room temperature or 37°C

Lens Type	Room temperature, pg/ lens	37 °C, pg/lens	Adjusted p-value
Etafilcon A	1.837 ± 1.697	1.258 ± 0.565	0.8659
Omafilcon A	1.524 ± 0.402	1.522 ± 0.654	> 0.9999
Somofilcon A	2.055 ± 0.663	1.690 ± 0.532	0.9714
Delefilcon A	2.122 ± 0.845	2.208 ± 0.208	0.9999

Table 3.2-c: IL-8 released by contact lens materials incubated at room temperature or 37°C

Lens Type	Room temperature, pg/ lens	37 °C, pg/lens	Adjusted p-value
Etafilcon A	1.070 ± 0.996	0.718 ± 0.324	0.8605
Omafilcon A	1.028 ± 0.237	0.928 ± 0.479	0.9986
Somofilcon A	1.233 ± 0.324	1.020 ± 0.286	0.9740
Delefilcon A	1.355 ± 0.525	1.428 ± 0.210	0.9996

Table 3.2-d: TNF-α released by contact lens materials incubated at room temperature or 37°C

Lens Type	Room temperature, pg/ lens	37 °C, pg/lens	Adjusted p-value
Etafilcon A	0.562 ± 0.534	0.365 ± 0.173	0.8507
Omafilcon A	0.470 ± 0.097	0.501 ± 0.205	0.9999
Somofilcon A	0.649 ± 0.212	0.483 ± 0.134	0.9125
Delefilcon A	0.821 ± 0.369	0.790 ± 0.021	0.9998

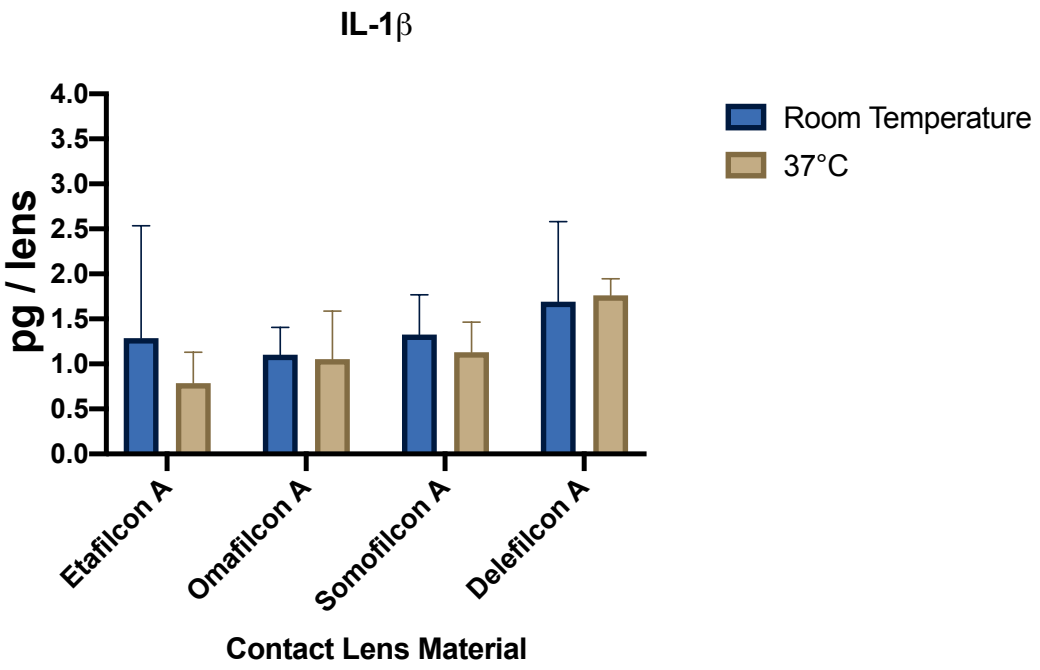

Figure 3-2: Amounts of IL-1β released by contact lens materials incubated at room temperature or 37 ℃.

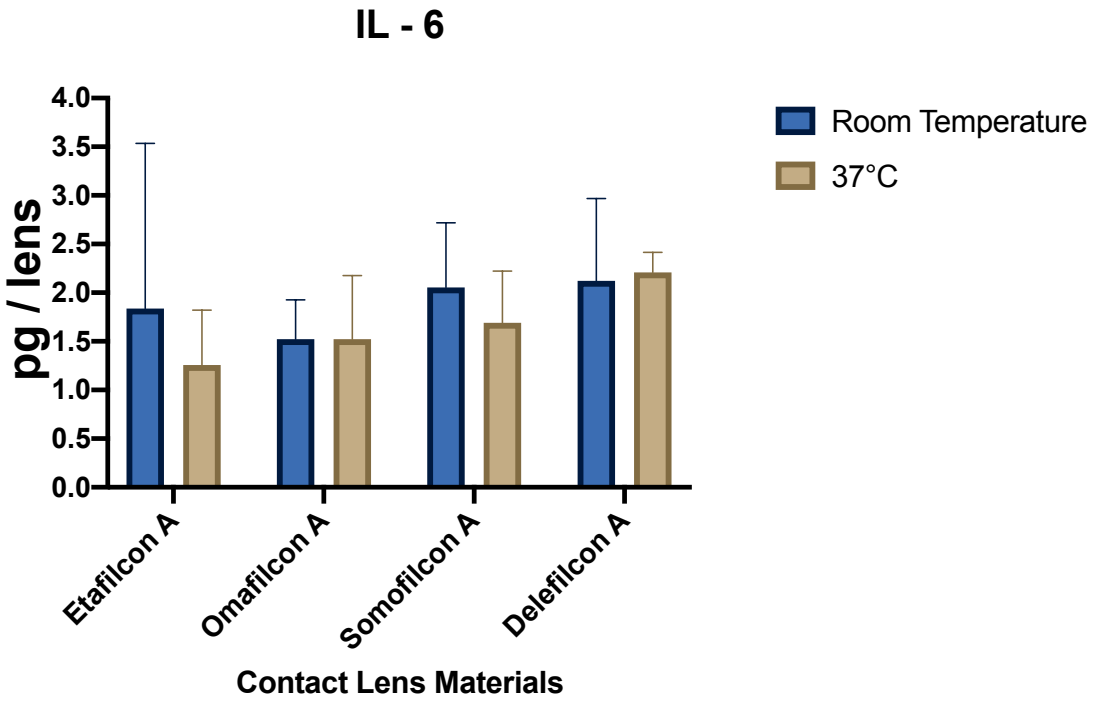

Figure 3-3: Amounts of IL-6 released by contact lens materials incubated at room temperature or 37 ℃.

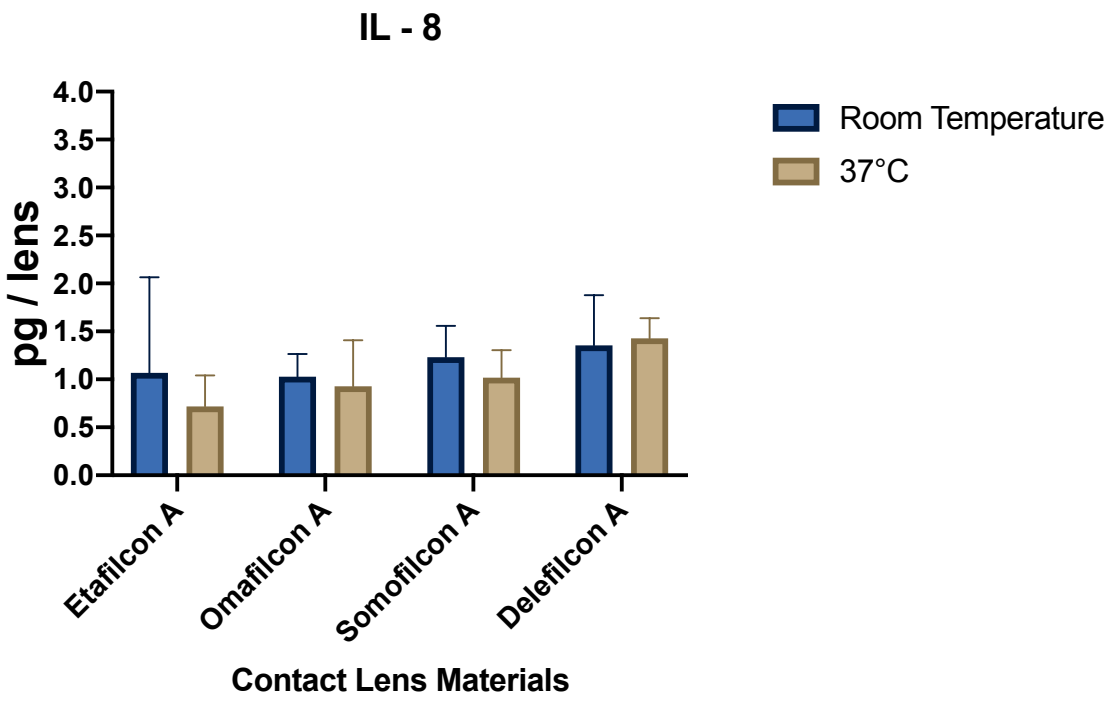

Figure 3-4:Amounts of IL-8 released by contact lens materials incubated at room temperature or 37°C.

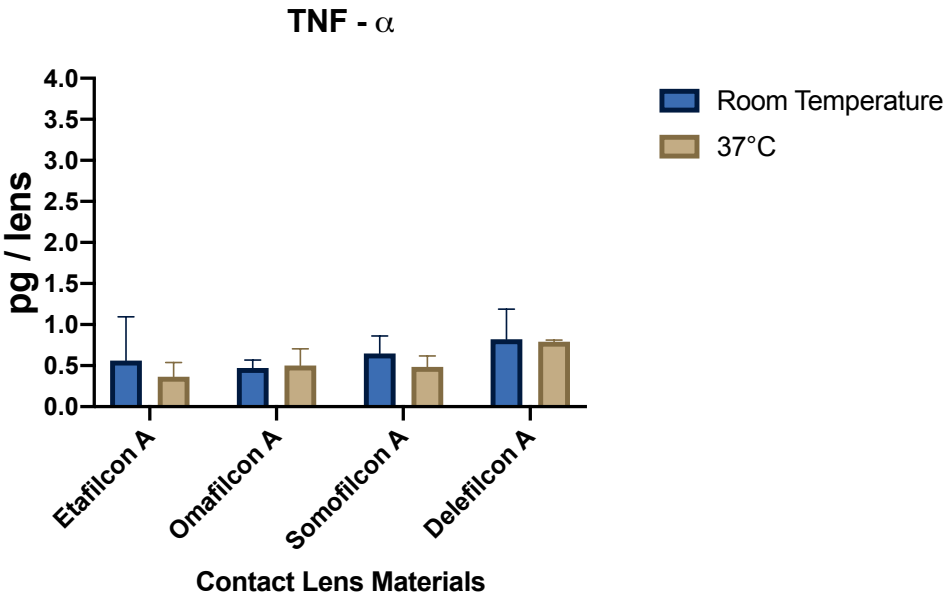

Figure 3-5: Amounts of TNF-α released by contact lens materials incubated at room temperature or 37°C.

3.2.3 Discussion

The purpose of the experiments conducted in this chapter was to develop a protocol that would aid in quantifying the amount of adhered cytokines released by contact lens materials. Protocol development involved analyzing the effects of three variables individually. Based on the results of each study, a final protocol that was best appropriate to study the cytokines of interest was chosen.

In this study, two temperature settings, room temperature and 37°C, were explored to determine which condition would provide a protocol that would result in a release of more adhered cytokines from the lens materials. According to the results shown in section 3.2.2, there was no statistically significant difference between the results obtained for either temperature (adjusted p-value > 0.05). Also, there was no significant difference found between materials at each temperature (adjusted p-value > 0.05).

As the results show, increasing the temperature to the maximum ocular temperature did not result in an increase or decrease in the release of cytokines from the lens materials. Multiple factors influence the adherence of protein molecules to lens materials. In this study, there were four contact lens materials (etafilcon A, somofilcon A, delefilcon A and omafilcon A) and four cytokines (IL-1β, IL-6, IL-8, and TNF-α) of interest. It was interesting to find that the conventional hydrogels, etafilcon A and omafilcon A, behaved similarly to the silicone hydrogels delefilcon A and somofilcon A.

It has been reported in the literature that surface charges of both the protein molecules and lens materials can influence their interaction.[136] Etafilcon A is a negatively charged lens material.

Its ionic character is due to the copolymerization of the negatively charged methacrylic acid (MA) monomer with HEMA.[136] Due to its negative charge, theoretically, it should have a strong electrostatic attraction with positively charged protein molecules. From the cytokines of interest, IL-1β, IL-6, and TNF-α were expected to be negatively charged in the calibrator solution, as their pI points (~5.8, ~6, ~6.9 respectively, as per MSD Scientific Support) are all below the pH of the diluent 2 (7.4, as per MSD Scientific Support) solution and IL-8 was expected to be positively charged as its pI point (~8.5, as per MSD Scientific Support) is above the pH of the diluent 2 solution. It was expected that the etafilcon A lens should have released more IL-1β, IL-6, and TNF-α and less IL-8. Omafilcon A, somofilcon A, and delefilcon A are non-ionic hydrogels and thus, there would be minimal electrostatic attraction present between the surface of the material and the cytokines. Due to these differences, it was expected that etafilcon A would release less IL-8 in comparison to the other lens materials, however, no difference was found between the materials at either temperature (adjusted p-value > 0.05).

Increasing the temperature can also weaken electrostatic attractions. With the use of an incubation temperature of 37°C, it was thought that there would be an increase in the release of cytokines from the lens materials due to a weaker interaction between the surface of the cytokines and the surface of the lens. However, there was no difference in the results with respect to the release of cytokines at either temperature (adjusted p-value > 0.05).

While there was no significant difference between the temperatures for each lens material for all four cytokines, it was necessary to decide on temperature for subsequent studies. Although the results in these experiments did not show any difference, there is a possibility of protein

denaturation at higher temperatures. For future studies, it was important to have minimal interference in the results. Thus, to reduce the possibility of being unable to quantify cytokines due to its structure being denatured, it was decided that an incubation temperature of room temperature would be used for subsequent studies.

3.3 Incubation Period Study

3.3.1 Materials and Methods

Contact Lens of Interest, Cytokine Solution, and Sample Preparation

The contact lens of interest as well as the methods to prepare the cytokine solution are the same as those reported in section 3.2.1. The theoretical concentrations of IL-1β, IL-6, IL-8, and TNF-α in the prepared cytokine solution is reported to be 88.57 pg/mL, 112.71 pg/mL, 74.14 pg/mL and 45 pg/mL respectively. The methodology used to prepare samples for this experiment was similar to that of section 3.2.1. The change made in this experiment was that for the second incubation period, the 0.6 mL lens-containing microtubes were left to incubate at room temperature in 200 µL in one of two conditions, 0 hours (no incubation period) or 1 hour. All other steps remained constant. A visual representation of the experiment is shown in Figure 3-6.

Statistical Analysis

Data were analyzed using GraphPad Prism V8 software (GraphPad Software Inc., San Diego, CA). A two-way ANOVA with Sidak's multiple comparisons test was performed to determine whether there was a significant difference, within a single lens type, between the cytokines released by hydrogels at the two incubation periods of interest. A two-way ANOVA with Tukey's multiple comparisons test was performed to determine whether there was a

significant difference between hydrogel materials with respect to the amount of cytokines released. Amount of cytokines released was calculated using Equation 3.2-1. Results for both tests were considered significant if $p < 0.05$.

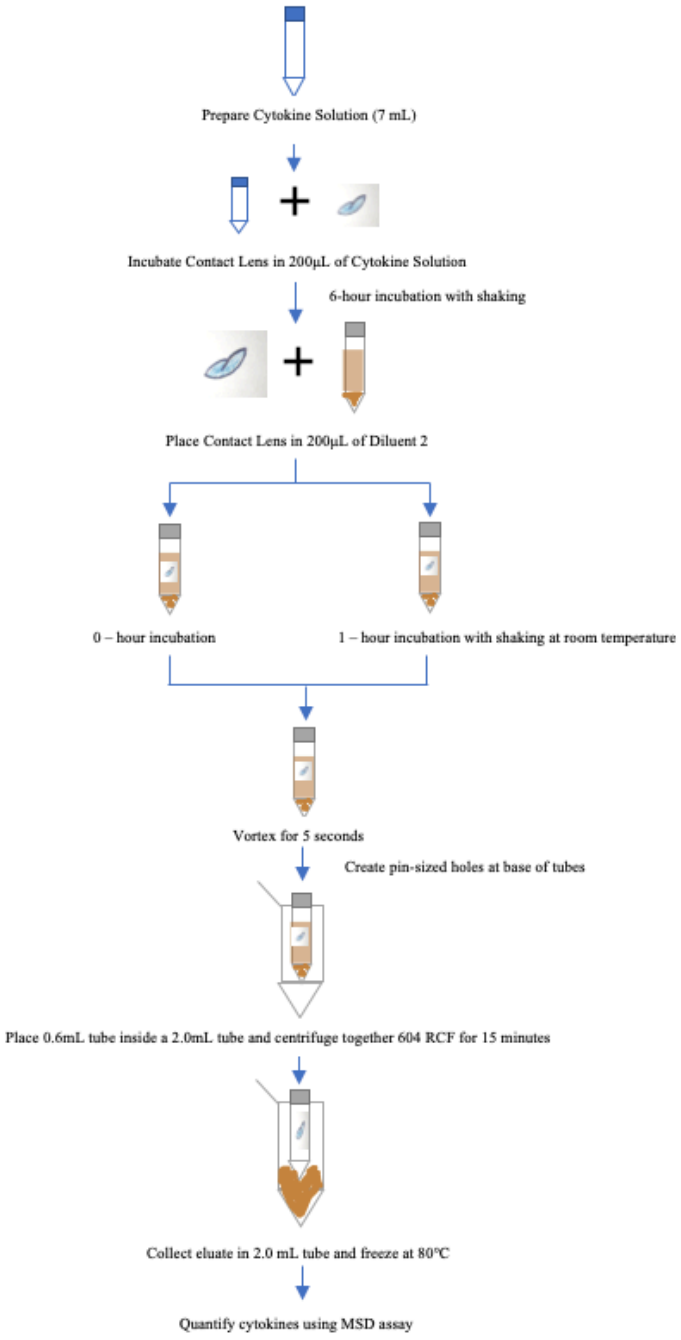

Figure 3-6: Schematic diagram of the methodology used for the incubation period study. Created by Nijani.

3.3.2 Results

The amount of adhered cytokines released by each hydrogel is shown in and summarized in Table 3.3-a, Table 3.3-b, Table 3.3-c, and Table 3.3-d (reported as mean ± standard deviation) and illustrated in Figure 3-7, Figure 3-8, Figure 3-9, and Figure 3-10. There was no significant difference between incubation periods with respect to the amount of cytokines released by each hydrogel for all four cytokines (adjusted p-value > 0.05). In addition, there was no significant difference between the hydrogel materials with respect to the amount of cytokines released (adjusted p-value > 0.05).

Table 3.3-a: Il-1β released by contact lens materials incubated for 0 hours or 1 hour

Lens Type	0 hour, pg/lens	1 hour pg/lens	Adjusted p-value
Etafilcon A	0.578 ± 0.185	1.135 ± 0.718	0.5831
Omafilcon A	0.873 ± 0.797	0.560 ± 0.623	0.9152
Somofilcon A	0.548 ± 0.307	0.599 ± 0.271	> 0.999
Delefilcon A	1.131 ± 0.461	1.039 ± 0.307	0.9991

Table 3.3-b: Il-6 released by contact lens materials incubated for 0 hours or 1 hour

Lens type	0 hour, pg/lens	1 hour pg/lens	Adjusted p-value
Etafilcon A	0.852 ± 0.217	1.389 ± 0.902	0.8195
Omafilcon A	1.091 ± 1.189	0.727 ± 0.758	0.9476
Somofilcon A	0.812 ± 0.443	0.973 ± 0.451	0.9975
Delefilcon A	1.467 ± 0.569	1.340 ± 0.349	0.9990

Table 3.3-c: IL-8 released by contact lens materials incubated for 0 hours or 1 hour

Lens type	0 hour, pg/lens	1 hour pg/lens	Adjusted p-value
Etafilcon A	0.585 ± 0.150	1.057 ± 0.598	0.7819
Omafilcon A	0.756 ± 0.689	0.442 ± 0.453	0.9383
Somofilcon A	0.550 ± 0.165	1.074 ± 1.110	0.7129
Delefilcon A	0.954 ± 0.379	0.845 ± 0.199	0.9988

Table 3.3-d: TNF-α released by contact lens materials incubated for 0 hours or 1 hour

Lens Type	0 hour, pg/lens	1 hour pg/lens	Adjusted p-value
Etafilcon A	0.275 ± 0.078	0.485 ± 0.361	0.7311
Omafilcon A	0.374 ± 0.335	0.252 ± 0.274	0.9495
Somofilcon A	0.230 ± 0.105	0.297 ± 0.138	0.9942
Delefilcon A	0.460 ± 0.213	0.480 ± 0.160	> 0.9999

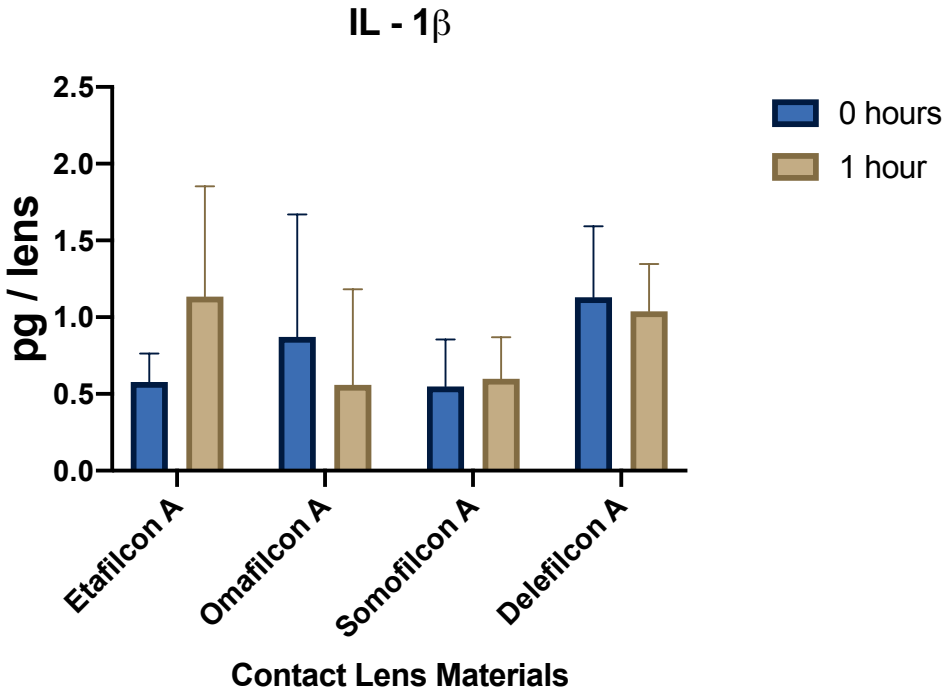

Figure 3-7: Amounts of IL-1β released by contact lens materials incubated for 0 hours or 1 hour.

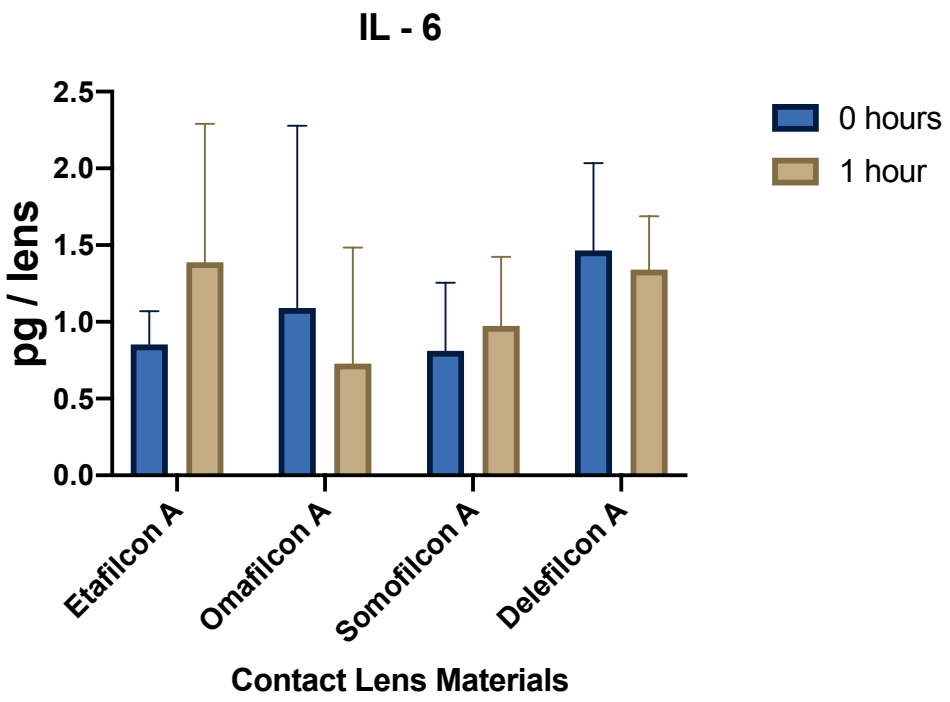

Figure 3-8: Amounts of IL-6 released by contact lens materials incubated for 0 hours or 1 hour.

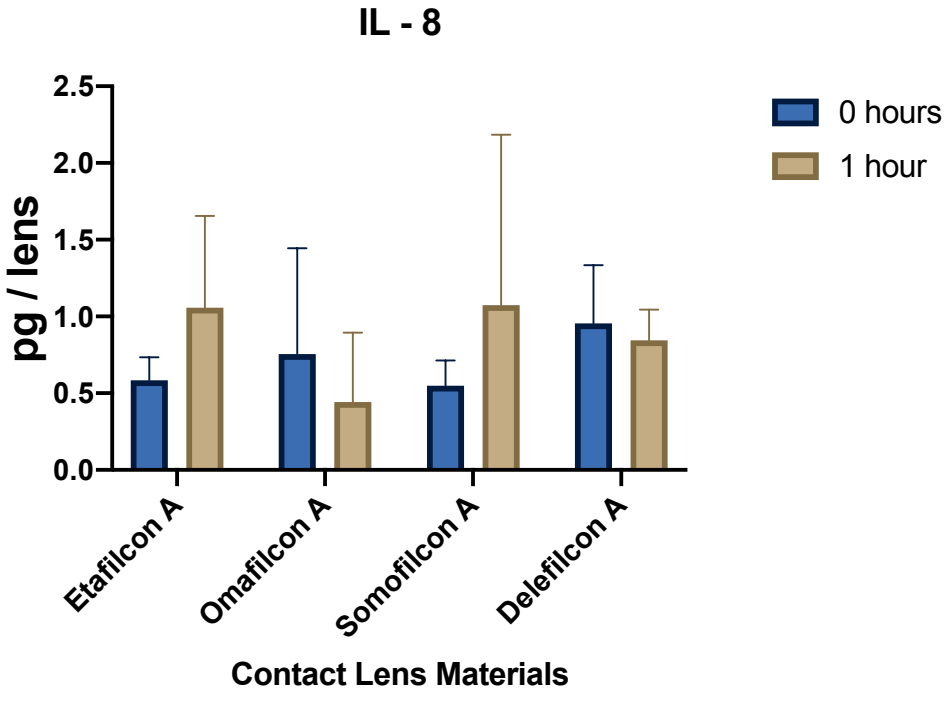

Figure 3-9: Amounts of IL-8 released by contact lens materials incubated for 0 hours or 1 hour.

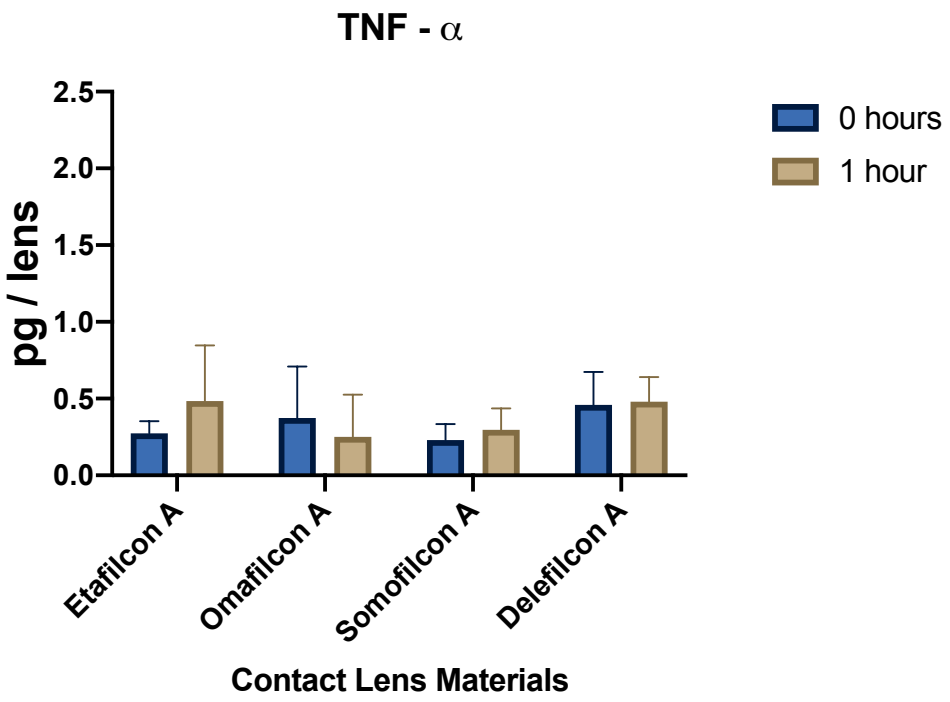

Figure 3-10: Amounts of TNF-α released by contact lens materials incubated for 0 hours or 1 hour.

3.3.3 Discussion

The purpose of this investigation was to determine whether increasing the incubation period would result in an increase or decrease of adhered cytokines released from the contact lens materials. The two incubation periods tested were 0 hours and 1 hour. As results show in section 3.3.2, there was no statistically significant difference between the results obtained for either incubation periods. There was also no significant difference between lens materials.

As mentioned previously, various factors can influence the binding of cytokines to lens materials. It has been reported in the literature that competitive adsorption of proteins onto the surface may exist due to the Vroman effect.[114,137] This phenomenon refers to the displacement of proteins adsorbed to a surface by various other proteins which may have a higher affinity for the surface in comparison to the initially absorbed proteins.[114,137] The displacement of proteins takes

place over time. Due to factors such as molecular weight, certain proteins are less mobile than others and thus will not bind to the surface of the material as rapidly as the more mobile proteins. However, over time, proteins with a higher affinity for the surface will begin to displace proteins with lower affinity.[114,137] The loosely bound proteins will then be released back into the solution.[114]

In this study, it was interesting to see whether increasing the incubation period would possibly allow for the Vroman effect to take place and thus have an effect on cytokines released from the materials. For example, Il-8 is a positively charged cytokine in Diluent 2. Thus, it would have a greater affinity for the negatively charged etafilcon A material in comparison to the other cytokines. With time, the IL-8 may displace the negatively charged cytokines, resulting in fewer amounts of IL-8 being released from the lens material. However, such a difference was not seen. A longer incubation period or a higher concentration of cytokines may be required for such an effect to take place. Nonetheless, in this study, there was no significant difference seen between the incubation periods.

To conduct the subsequent experiments, it was necessary to decide on an incubation period. An incubation period of one hour was chosen. A decision was made to use the one-hour incubation period to allow for any loosely adhered cytokines to be released from the contact lens materials.

3.4 Volume Study

3.4.1 Materials and Methods

Contact lens of interest, Cytokine Solution, Sample Preparation and Statistical Analysis

The contact lens of interest as well as the methods to prepare the cytokine solution are the same as those reported in section 3.2.1. The theoretical concentrations of IL-1β, IL-6, IL-8, and

TNF-α in the prepared cytokine solution were reported as 67.29 pg/mL, 91.57 pg/mL, 71.43 pg/mL and 45.29 pg/mL respectively. The methodology used to prepare samples for this experiment was similar to that of section 3.2.1. The change that was made in this experiment is that for the second incubation period, the 0.6 mL lens-containing microtubes were left to incubate at room temperature for 1 hour in one of two volumes of diluent 2, 200µL or 550 µL. In addition, for tubes with 550µL, the centrifugation step was repeated twice. This was due to the larger diluent 2 volume, as one centrifugation cycle was not sufficient to collect the full 550µL in the 2.0mL tube. A visual representation of the experiment is shown in Figure 3-11. Data were analyzed using GraphPad Prism V8 software (GraphPad Software Inc., San Diego, CA). A two-way ANOVA with Sidak's multiple comparisons test was performed to determine whether there was a significant difference, within a single lens type, between the cytokines released by hydrogels at the two Diluent 2 volumes of interest. A two-way ANOVA with Tukey's multiple comparisons test was performed to determine whether there was a significant difference between hydrogel materials concerning the amount of cytokines released. Amount of cytokines released was calculated using Equation 3.2-1. Results for both tests were considered significant if $p < 0.05$.

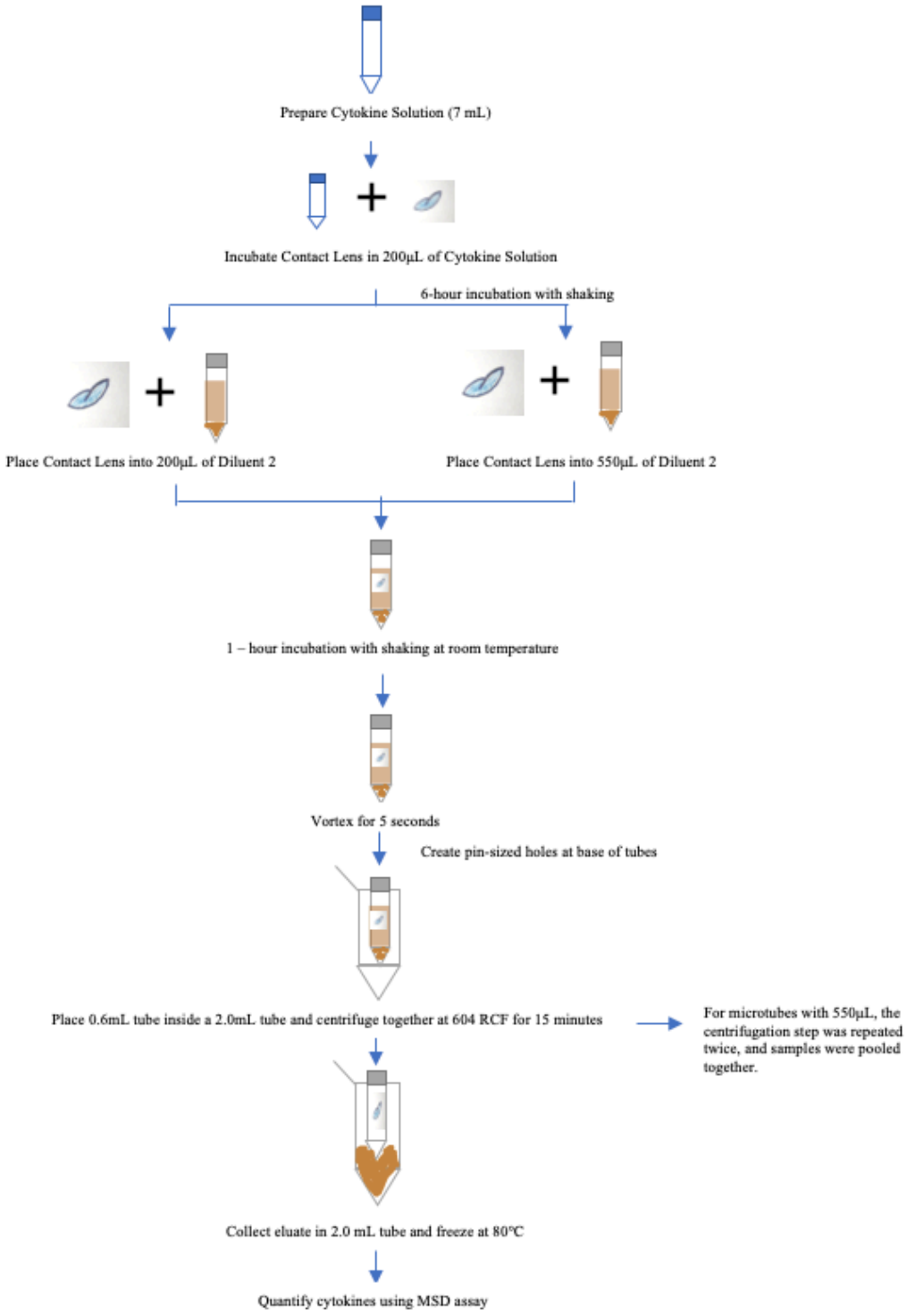

Figure 3-11: A schematic diagram illustrating the methodology used for the Volume Study. Created by Nijani.

3.4.2 Results

The amount of adhered cytokines released by each hydrogel is shown in Figure 3-12, Figure 3-13, Figure 3-14, and Figure 3-15 and is summarized in Table 3.4-a, Table 3.4-b, Table 3.4-c and Table 3.4-d (reported as mean ± standard deviation). There was no significant difference between volumes with respect to the amount of cytokines released by each hydrogel for all four cytokines (adjusted p-value > 0.05). When comparing the release of cytokines between contact lens materials, there was a significant increase of IL-6 released by etafilcon A in comparison to somofilcon A ($p = 0.0473$) and omafilcon A ($p = 0.0438$) when a diluent volume of 550 µL was used.

Table 3.4-a: IL-1β released by contact lens materials incubated in 200µL or 500 µL of Diluent 2.

Lens Type	200 µL, pg/lens	550 µL, pg/lens	Adjusted p-value
Etafilcon A	0.910 ± 0.404	0.966 ± 0.626	0.9998
Omafilcon A	0.608 ± 0.212	0.406 ± 0.185	0.9738
Somofilcon A	0.361 ± 0.061	0.191 ± 0.108	0.9863
Delefilcon A	0.671 ± 0.319	1.042 ± 0.972	0.8092

Table 3.4-b: IL-6 released by contact lens materials incubated in 200µL or 500 µL of Diluent 2.

Lens Type	200 µL, pg/lens	550 µL, pg/lens	Adjusted p-value
Etafilcon A	1.160 ± 0.508	1.297 ± 0.722	0.9849
Omafilcon A	0.196 ± 0.271	0.429 ± 0.110	0.3999
Somofilcon A	0.622 ± 0.099	0.441 ± 0.172	0.9587
Delefilcon A	0.837 ± 0.361	0.787 ± 0.138	0.9997

Table 3.4-c: IL-8 released by contact lens materials incubated in 200µL or 500 µL of Diluent 2.

Lens Type	200 µL, pg/lens	550 µL, pg/lens	Adjusted p-value
Etafilcon A	0.892 ± 0.427	0.894 ± 0.499	>0.9999
Omafilcon A	0.683 ± 0.203	0.309 ± 0.085	0.4181
Somofilcon A	0.447 ± 0.085	0.343 ± 0.132	0.9865
Delefilcon A	0.715 ± 0.365	0.469 ± 0.087	0.7688

Table 3.4-d: TNF-α released by contact lens materials incubated in 200µL or 500 µL of Diluent 2.

Lens Type	200 µL, pg/lens	550 µL, pg/lens	Adjusted p-value
Etafilcon A	0.534 ± 0.238	0.532 ± 0.349	> 0.9999
Omafilcon A	0.402 ± 0.127	0.172 ± 0.054	0.4247
Somofilcon A	0.285 ± 0.048	0.241 ± 0.080	0.9968
Delefilcon A	0.412 ± 0.200	0.284 ± 0.034	0.8563

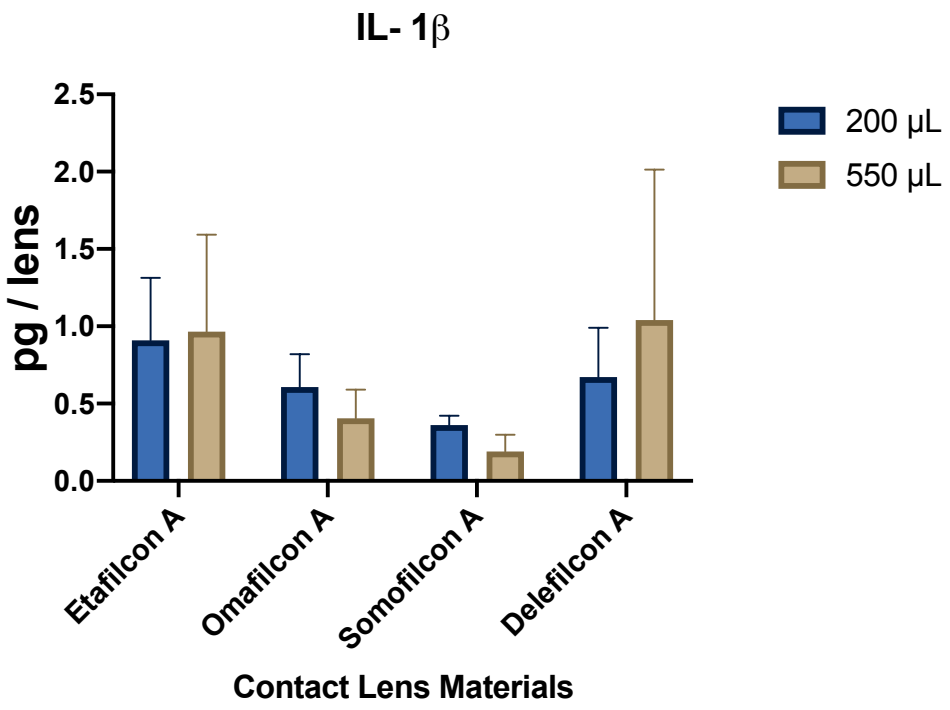

Figure 3-12: Amounts of IL-1β released by contact lens materials incubated in 200µL or 500 µL of Diluent 2.

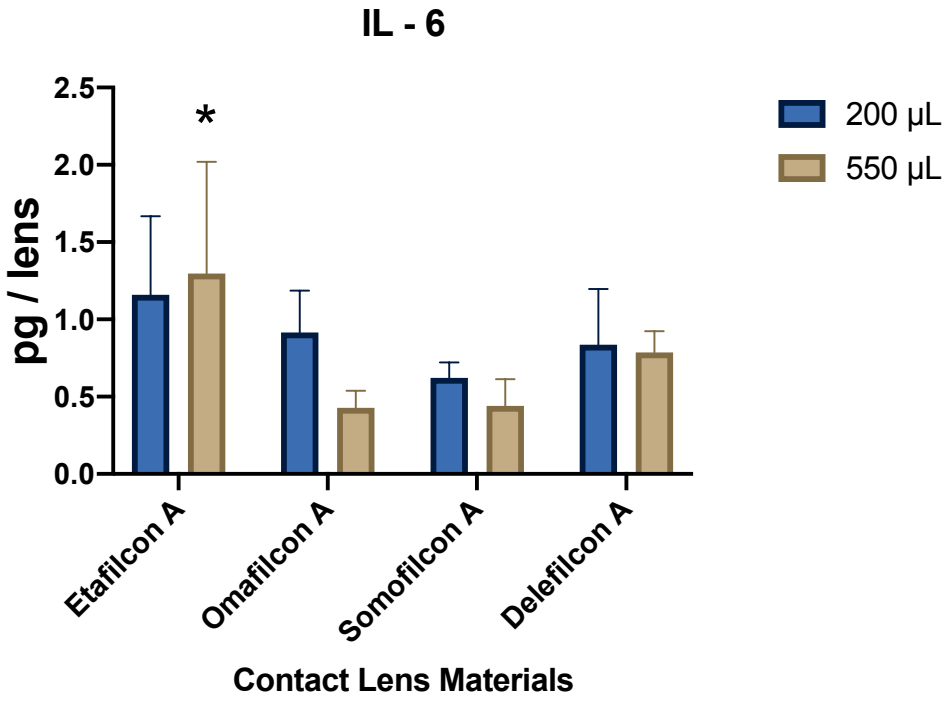

*Figure 3-13: Amounts of IL-6 released by contact lens materials incubated in 200µL or 500 µL of Diluent 2. * A significant increase was seen with etafilcon A in comparison to somofilcon A and omofilcon A when the lens was incubated in 500µL of Diluent 2.*

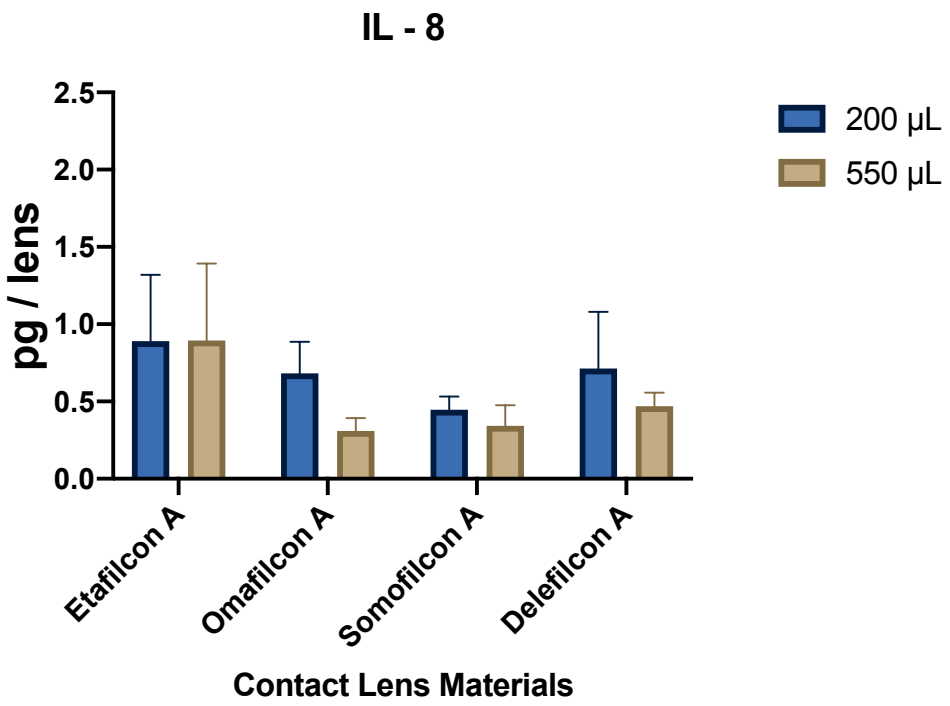

Figure 3-14: Amounts of IL-8 released by contact lens materials incubated in 200µL or 500 µL of Diluent 2.

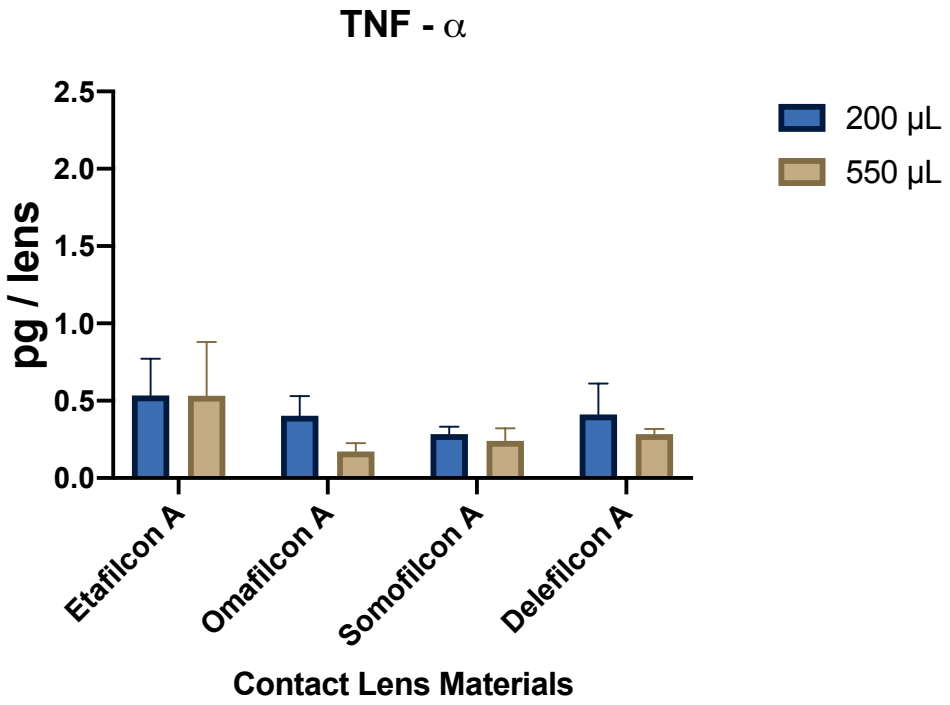

Figure 3-15: Amounts of TNF-α released by contact lens materials incubated in 200µL or 500 µL of Diluent 2.

3.4.3 Discussion

The purpose of this experiment was to determine whether increasing the volume of Diluent 2 would result in an increase or decrease of adhered cytokines released from the contact lens materials. It was important to determine a suitable volume of diluent for this protocol because certain contact lens materials may require more or fewer volumes of extraction buffer depending on the amount of proteins they absorb. For example, when extracting lysozyme from contact lens materials, the volume of extraction buffer (TFA/ACN) differs depending on the lens material. While silicone hydrogel materials require 1.5 mL, group IV lens materials, such as etafilcon A, requires 4 mL of the buffer solution.[138] The volumes of Diluent 2 that were investigated in this study were 200µL and 500µL.

As the results show in section 3.4.2, there was no statistically significant difference between the incubation periods for all four lens materials. This means that either of the volumes investigated of Diluent 2 could be used as a diluent to collect cytokines released from lens materials. However, there was a significant difference between the amount of IL-6 released by etafilcon A compared to somofilcon A and omafilcon A when a diluent volume of 550 µL was used. A possible explanation for this is the difference in ionic properties between the lenses. While etafilcon A is a negatively charged material, omafilcon A and somofilcon A are non-ionic. In diluent 2, Il-6 is a negatively charged protein and thus it will have a repulsive electrostatic force with the surface of etafilcon A material in comparison to omafilcon A and somofilcon A. For this reason, more IL-6 may have been released by etafilcon A.

While there was no significant difference between the amount of cytokines released for either volume, it was necessary to decide on a volume of diluent 2 that would be best suitable for

the protocol. In this protocol, the subsequent step post-incubation period is to vortex the microcentrifuge tube. To reduce the possibility of losing cytokines to the sides of the tube during the vortex step, it was decided that a volume of 550µL would be best suitable for this protocol. A volume of 550µL fills the entirety of a 0.6mL microcentrifuge tube and thus reduces the possibility of having cytokines adhering to the sides of the microcentrifuge tube during the vortex step.

3.5 Conclusion

This concludes the experiments conducted to develop a protocol to quantify the amount of adhered cytokines released by contact lens materials. The final protocol involves incubating the cytokine adsorbed contact lenses in 550µL for 1 hour at room temperature. A visual representation of the final protocol is shown in Figure 3-16.

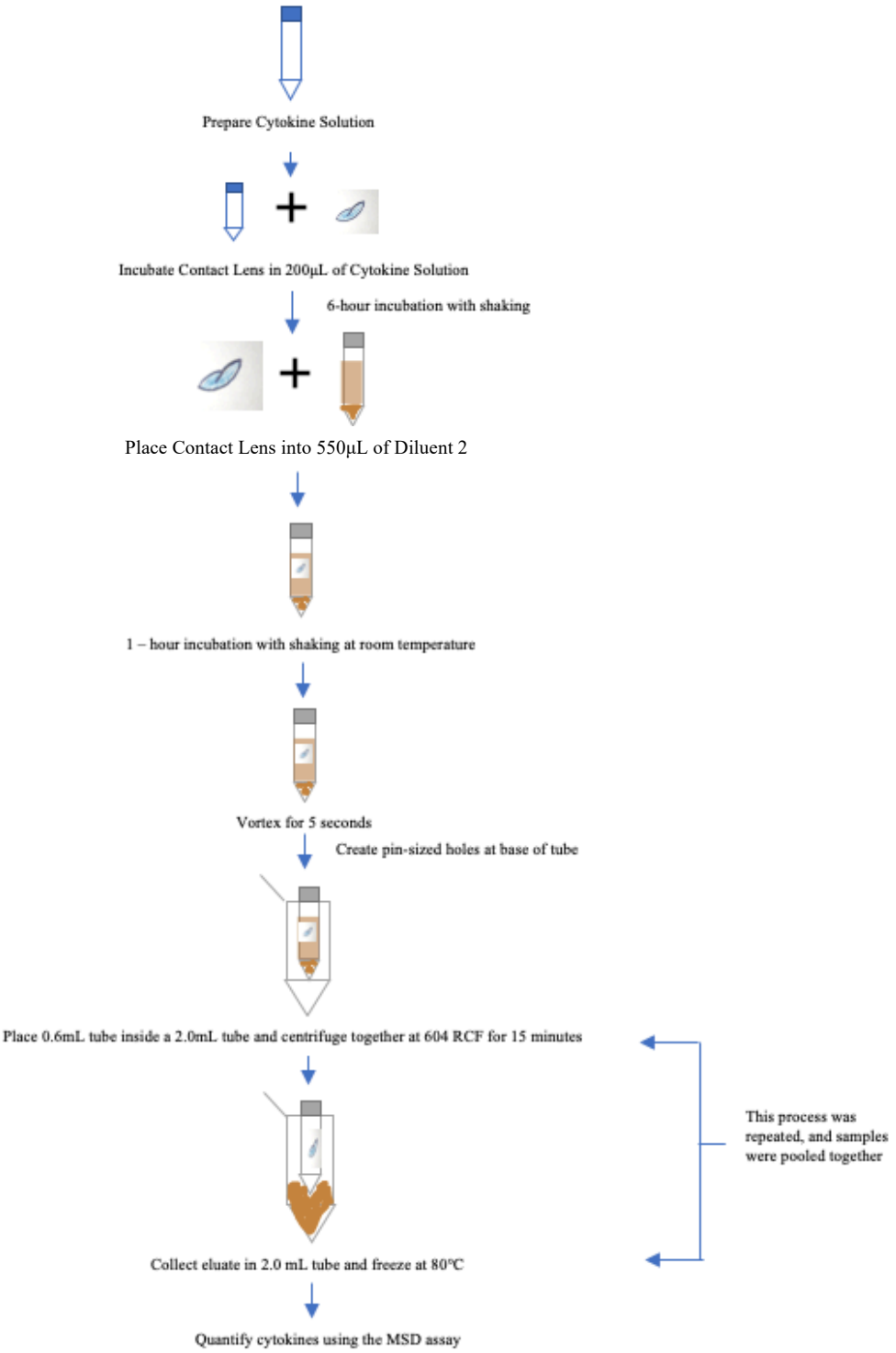

Figure 3-16: A schematic diagram illustrating the finalized protocol. Created by Nijani.

Chapter 4: Investigating the Difference Between Treated Conventional and Silicone Hydrogel Lens and Their Release of Adhered Cytokines

4.1 Introduction

Incorporating silicone hydrogel polymers into contact lens materials solved a major concern in the contact lens industry. As discussed previously, pHEMA hydrogels did not provide enough oxygen permeability required for lens wear longer than 24 hours.[75] Due to oxygen's solubility in silicone, incorporating silicone into hydrogel material allowed for an increase in oxygen transmissibility, which resulted in the decrease of hypoxia-related ocular conditions.[83] However, while lens-induced hypoxic conditions did decrease[83], there was a 2X higher risk in developing CIEs with the use of silicone hydrogel lenses.[107] While factors such as bacterial bioburden on the eyelid margins and tear film instability have been associated with the occurrence of CIEs in silicone hydrogel lens wearers [109], the root cause is still unknown.

In this thesis, the amount of adhered cytokines released from contact lens materials were quantified. The purpose of this was to be able to use this information in future studies to determine whether the cytokines adhered to contact lens materials were triggering an inflammatory response on the corneal surface. The experiments in this chapter were focused on investigating whether the release of cytokines differed between different contact lens materials. The results may help understand whether ocular inflammation and CIEs are influenced by the inherent properties of contact lens materials. The results between contact lens materials were compared, as protein deposition profiles have been reported to differ depending on the materials.[59] For instance, amongst the four FDA lens materials groups used to describe hydrogel lens materials, Group I materials reportedly deposit the least amount of protein, while Group IV deposit the most.[59] Furthermore, when lens materials of high water content are compared for protein deposition, the

lens material with charged polymers will show greater protein deposition.[121] This is due to the addition of electrostatic attraction present between the protein molecule and the surface (i.e. Group IV (high-water content, ionic lens material) will deposit higher amounts of protein in comparison to Group II (high-water content, non-ionic lens material)).[121]

For the most part, the accumulation of protein, particularly lysozyme, found on silicone hydrogel material is less compared to that found on conventional hydrogels.[139] When lysozyme deposition was compared between worn hydrogel (etaficon lens; 14-day daily wear lens) and silicone hydrogel (lotrafilcon and balafilcon; 30 day continuous wear) it was found that etafilcon deposited a significantly higher amount of lysozyme (985±241μg/lens) compared to lotrafilcon (3±1 μg/lens) and balafilcon (10±3 μg/lens).[139] Due to differences in protein deposition between lens materials, this chapter focused on utilizing the protocol developed from the previous chapter to analyze two different contact lens materials: etafilcon A, an ionic hydrogel material, and delefilcon A, a non-ionic silicone hydrogel material. Experiments were conducted to mimic certain conditions of contact lens wear.

The first condition of interest was to expose contact lenses to a high concentration of cytokines. Multiple clinical studies have analyzed the cytokines present in the tear film during contact lens wear under various conditions.[101-103,106] In the case of ocular inflammation, such as *Acanthamoeba* keratitis there is an upregulation of cytokines on the ocular surface during contact lens wear. [106] Thus, to mimic an ocular environment under inflammatory conditions, contact lenses were soaked in a high cytokine solution. The purpose of this experiment was to determine whether

the release of adhered cytokines from the lens materials exposed to a high cytokine concentration differed between materials.

The second condition that was investigated was the contact lens wear time. How long individuals wear contact lenses throughout the day can vary. In the previous chapter, contact lenses were soaked in the cytokine solution for 6 hours, thus mimicking a wear time of 6 hours. For this experiment, a 12-hour wear time was selected instead. The purpose of this experiment was to rather see whether the amount of adhered cytokines released, differed amongst lens materials if the wear time was increased.

The third condition that was investigated was ocular temperature. In previous experiments, the contact lens was incubated in cytokine solutions at ambient temperature. To further understand how contact lens materials would respond to cytokines in an ocular setting, contact lenses were incubated in a cytokine solution at 34℃ to stimulate average ocular temperature. In the previous chapter, for protocol development, an ocular temperature of 37℃ and room temperature were tested. The purpose of testing a higher ocular temperature was to create parameters which would test extreme conditions. By doing so, it would help understand whether an increased ocular temperature resulted in an increase in release of cytokines from contact lens materials. However, for this study, the purpose was to determine whether the amount of adhered cytokines released differed amongst lens materials if they were exposed to cytokines at an average ocular temperature of 34℃.

4.2 Materials: Contact Lens of Interest

A total of 2 different contact lens materials were investigated in this chapter. This consisted of one conventional hydrogel lens and one silicone hydrogel lens. The conventional hydrogel lens of interest was etafilcon A and the silicone hydrogel lens of interest was delefilcon A.

4.3 Statistical Analysis

Data in this chapter were analyzed using GraphPad Prism 8. Unpaired t-tests were performed to compare the amount of cytokines released by etafilcon A and delefilcon A. Results were considered significant if p-value < 0.05. Amount of cytokines released was calculated using Equation 3.2-1. Results are reported as mean ± SD.

4.4 Incubating Contact Lens Materials in High Cytokine Concentration Solution

4.4.1 Materials and Methods

Cytokine Solution and Sample Preparation

A multi-analyte lyophilized calibrator (Meso Scale Diagnostics, Rockville, MD) containing the cytokines, IL-1β, IL-6, IL-8, and TNF-α, was used. This calibrator differs from that used in section 3.2.1 as this calibrator contained a total of 14 cytokines at high concentrations. For this study, a cytokine solution was prepared for each of the two lens types. Each calibrator was reconstituted in 1 mL of Diluent 2 to prepare a cytokine solution. The theoretical concentrations of IL-1β, IL-6, IL-8, and TNF-α in the prepared cytokine solutions were reported as 5462.5 pg/mL, 2712.5 pg/mL, 2600 pg/mL, 5312.5 pg/mL, respectively. The methodology used to prepare samples for this experiment followed that illustrated in Figure 3-16. A sample size of $n = 4$ was used for this study. For the control, lenses were left to incubate in a 200µL of Diluent 2 rather than

in a cytokine solution. Prior to testing, high cytokine solution samples underwent a 1:10 dilution with Diluent 2. This dilution ensured samples would be within the detection range set by the MSD imager.

4.4.2 Results

In this study, etafilcon A lens material released more IL-1β (127.7 ± 29.38 pg/lens) than delefilcon A (69.86 ± 35.74 pg/lens; p = 0.0465). There were no significant differences between etafilcon A and delefilcon A with respect to the amount of IL-6 (p-value = 0.0542), IL-8 (p-value = 0.0924), and TNF-α (p-value = 0.1477) released. The amount of cytokines released by the two lens type are shown in Table 4.4-a and illustrated in Figure 4-1, Figure 4-2, Figure 4-3, and Figure 4-4. Samples were diluted prior to testing. Thus, to determine the amount of cytokines released by contact lens materials, concentration values provided by the MSD imager for diluted samples were first multiplied by 10. These values were then inserted into Equation 3.2-1.

Table 4.4-a: Cytokines released by Contact Lens Material incubated in a high cytokine concentration solution.

Lens Type	IL-1β, pg/lens	IL-6, pg/lens	IL-8, pg/lens	TNF - α, pg/lens
Etafilcon A	127.7 ± 29.38	51.66 ± 12.95	57.17 ± 15.51	85.68 ± 28.85
Delefilcon A	69.86 ± 35.74	29.23 ± 13.62	33.13 ± 18.37	51.85 ± 28.74

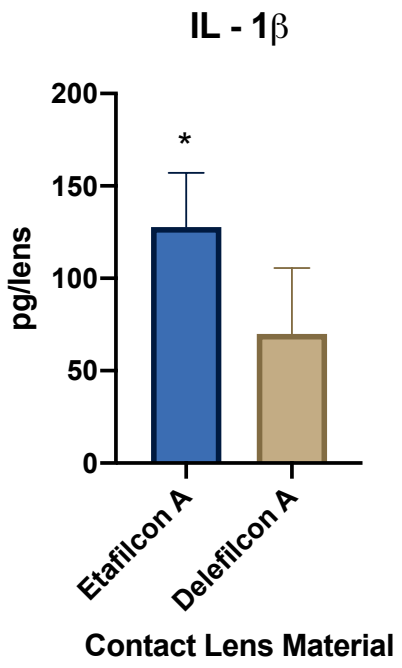

*Figure 4-1: Amount of IL-1β released by contact lens materials that were incubated in a high cytokine concentration solution. * Significance between etafilcon A and delefilcon A lens material (p = 0.0465).*

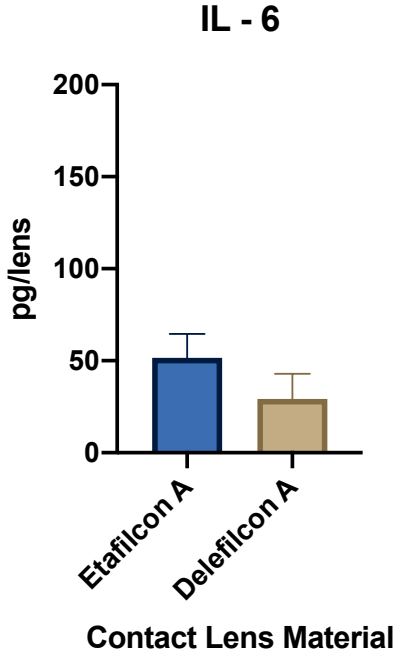

Figure 4-2: Amount of IL-6 released by contact lens materials that were incubated in a high cytokine concentration solution.

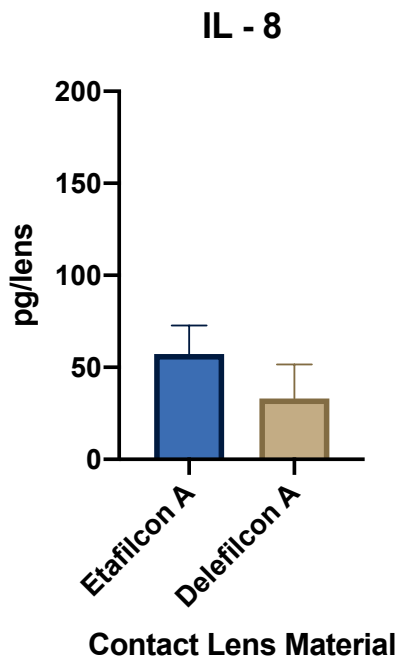

Figure 4-3: Amount of IL-8 released by contact lens materials that were incubated in a high cytokine concentration solution.

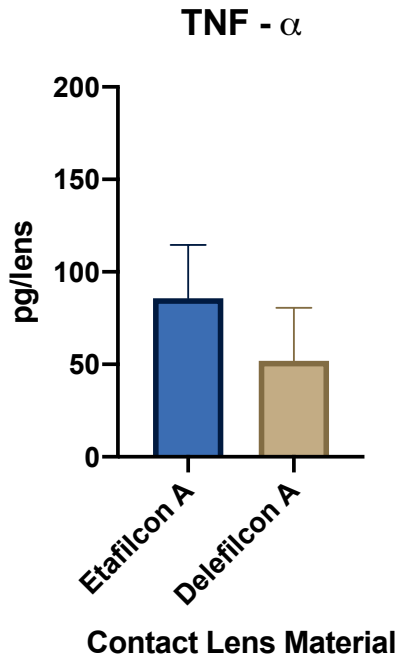

Figure 4-4: Amount of TNF-α released by contact lens materials that were incubated in a high cytokine concentration solution.

4.5 Incubating Contact Lens Materials in a Cytokine Solution for a 12-hour period

4.5.1 Materials and Methods

Cytokine Solution and Sample Preparation

A multi-analyte lyophilized calibrator containing the cytokines, IL-1β, IL-6, IL-8, and TNF-α, was used. This calibrator was similar to the ones used in section 3.2.1. The calibrator was reconstituted in 2 mL of Diluent 2 to prepare a cytokine solution of concentration 310 pg/mL, 394.5 pg/mL, 259.5 pg/mL, and 157.5 pg/mL, for each cytokine, respectively. The methodology used to prepare samples for this experiment followed that shown in Figure 3-16 with one minor change. The lenses (n = 3 per lens type) were incubated in a cytokine solution for a 12-hour period. For the control, lenses were left to incubate in a 200μL of Diluent 2 rather than a cytokine solution.

4.5.2 Results

There were no significant differences between etafilcon A and delefilcon A with respect to the amount of IL-1β ($p = 0.5208$), IL-6 ($p = 0.6556$) IL-8 ($p = 0.4773$), and TNF-α ($p = 0.5002$) released. Results were considered significant if $p > 0.05$. The amount of cytokines released by the two lens type are shown in Table 4.5-a and illustrated in Figure 4-5, Figure 4-6, Figure 4-7, and Figure 4-8. Results are reported as mean ± standard deviation (pg/lens). Results were considered significant if $p < 0.05$.

Table 4.5-a: Cytokines released by Contact Lens Material incubated in a cytokine solution for a 12-hour period

Lens Type	IL-1β	IL-6	IL-8	TNF - α
Etafilcon A	4.222 ± 0.9722	5.712 ± 1.431	3.350 ± 0.7627	1.437 ± 0.2818
Delefilcon A	5.501 ± 2.998	6.802 ± 3.656	4.579 ± 2.607	1.881 ± 0.9992

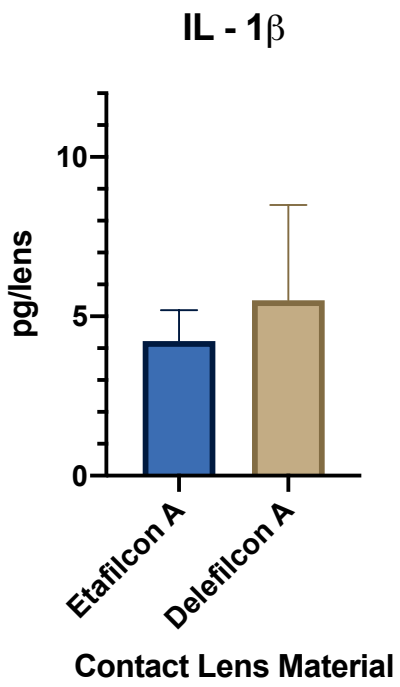

Figure 4-5: Amount of IL-1β released by contact lens materials that were incubated in a cytokine solution for a 12-hour period.

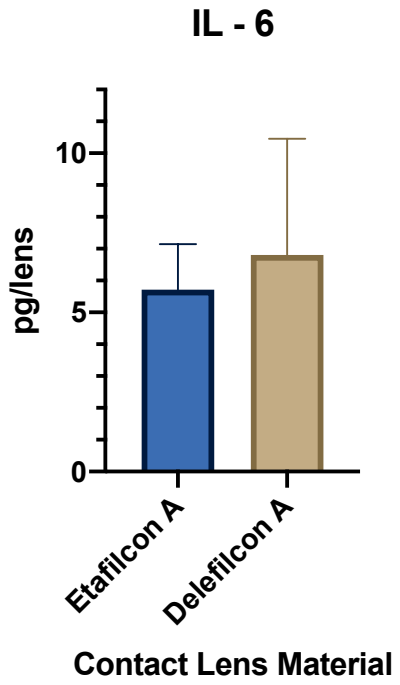

Figure 4-6: Amount of IL-6 released by contact lens materials that were incubated in a cytokine solution for a 12-hour period.

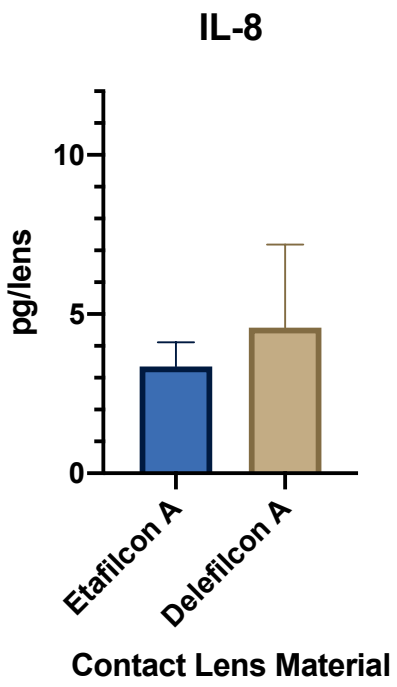

Figure 4-7: Amount of IL-8 released by contact lens materials that were incubated in a cytokine solution for a 12-hour period.

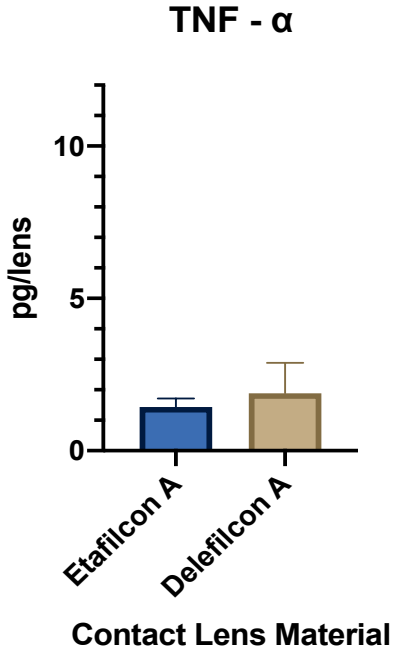

Figure 4-8: Amount of TNF-α released by contact lens materials that were incubated in a cytokine solution for a 12-hour period.

4.6 Incubating Contact Lens Materials in a Cytokine Solution at 34°C

4.6.1 Materials and Methods

Cytokine Solution, and Sample Preparation

A multi-analyte lyophilized calibrator containing the cytokines, IL-1β, IL-6, IL-8, and TNF-α, was used. This calibrator was similar to the ones used in section 3.2.1. The calibrator was reconstituted in 2 mL of Diluent 2 to prepare a cytokine solution of concentration 310 pg/mL, 394.5 pg/mL, 259.5 pg/mL, and 157.5 pg/mL, for each cytokine, respectively. The methodology used to prepare samples for this experiment followed that shown in Figure 3-16 with one minor change. The lenses (n = 3 per lens type) were incubated in a cytokine solution at 34°C with shaking. For the control, lenses were left to incubate in a 200µL of Diluent 2 rather than a cytokine solution.

4.6.2 Results

There were no significant differences between etafilcon A and delefilcon A with respect to the amount of IL-1β (adjusted p-value = 0.7205), IL-6 (adjusted p-value = 0.5868), IL-8 (adjusted p-value = 0.7403), and TNF-α (adjusted p-value = 0.6624) released. The amount of cytokines released by the two lens type are shown in Table 4.6-a and illustrated in Figure 4-9, Figure 4-10. Figure 4-11, and Figure 4-12. Results are reported as mean ± standard deviation (pg/lens). Results were considered significant if $p < 0.05$.

Table 4.6-a: Cytokine released by contact lens materials incubated in a cytokine solution at 34°C.

Lens Type	IL-1β, pg/lens	IL-6, pg/lens	IL-8, pg/lens	TNF-α, pg/lens
Etafilcon A	3.298 ± 1.543	4.346 ± 1.897	2.608 ± 1.139	1.140 ± 0.5605
Delefilcon A	2.905 ± 0.8693	3.619 ± 0.9778	2.331 ± 0.7283	0.9656 ± 0.3144

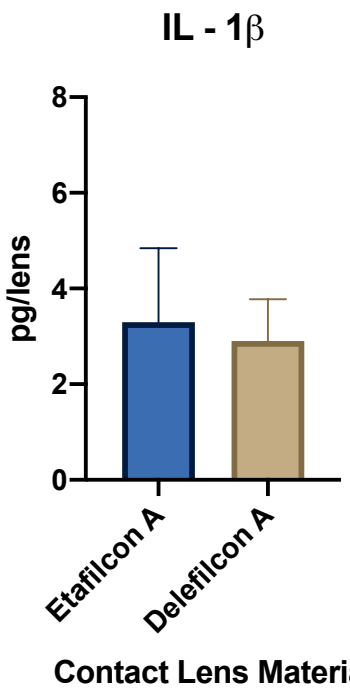

Figure 4-9: Amount of IL-1β released by contact lens materials incubated in a cytokine solution at 34°C.

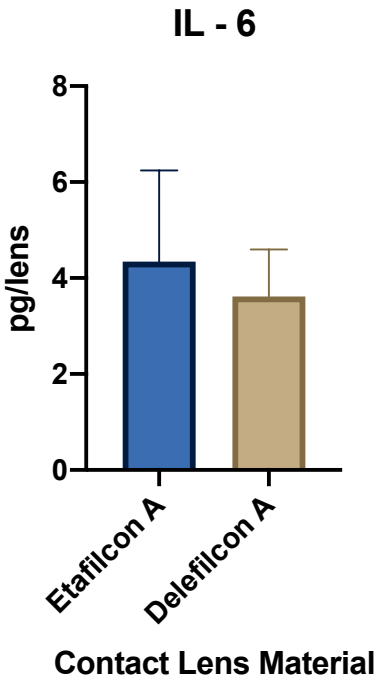

Figure 4-10: Amount of IL-6 released by contact lens materials incubated in a cytokine solution at 34°C.

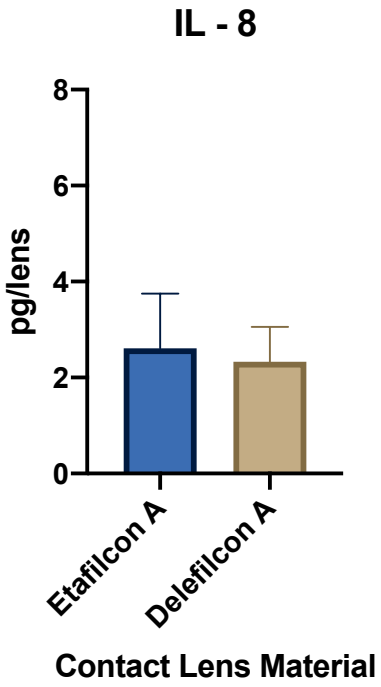

Figure 4-11: Amount of IL-8 released by contact lens materials incubated in a cytokine solution at 34°C.

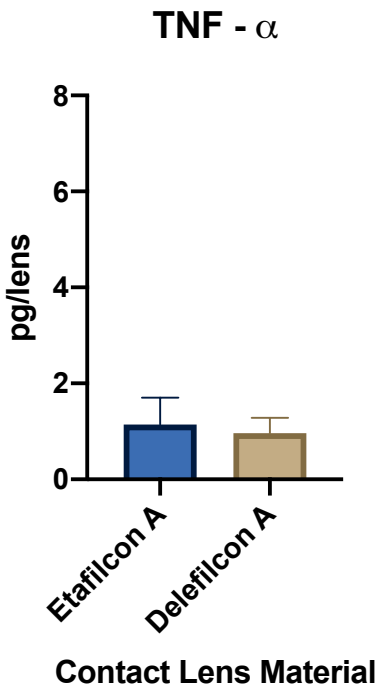

Figure 4-12: Amount of TNF-α released by contact lens materials incubated in a cytokine solution at 34°C.

4.7 Discussion

In this chapter, the experiments conducted focused on determining whether there was a significant difference between lens materials with respect to the amount of adhered cytokines released by lens materials exposed to different conditions. The lens materials of interest were conventional hydrogel, etafilcon A, and silicone hydrogel, delefilcon A.

The first experiment conducted focused on incubating contact lens materials in a high cytokine concentration solution, as cytokine levels are substantially upregulated during inflammation compared to basal levels. Thus, testing under these conditions stimulated a contact lens wear during an inflammatory response. As mentioned previously, multiple factors influence protein adsorption onto lens materials. With a higher concentration of cytokines present in the solution, there is a possibility for competitive binding between the cytokines. It may be that at higher concentrations, cytokines with a higher affinity more easily displace the cytokines which have a lower affinity for the lens material.

The results in section 4.4.2 show that there was a significant increase in the amount of IL-1β released by the etafilcon A lens in comparison to delefilcon A ($p = 0.0465$). While there was no significant increase for the other three cytokines, graphically, a similar pattern is observed. Although it is evident that etafilcon A released more cytokines than delefilcon A, the differences between the results may be due to the amount of cytokines that were initially adhered to the contact lens materials. It has been reported in the literature that pore size, among various other factors, may affect protein uptake by the contact lens materials.[60] With lens materials, larger pore sizes are associated with higher water content.[140] While etafilcon A is classified as a high-water content material, delefilcon A's water content is described to be a gradient. While the surface is greater

than 80%, the core of the lens is 33% water.[122,123] It could be that Etafilcon A was able to uptake more cytokines due to its larger pore size in comparison to the Delefilcon A lens. As a result of more proteins adsorbed, the Etafilcon A lens may have been able to release more cytokines which is shown in Figure 4-1.

The second experiment conducted focused on examining whether there were significant differences between lens materials for the amount of adhered cytokines released by lens materials exposed to cytokines for 12 hours. The purpose of this experiment was to mimic longer lens wear using the *in vitro* model. However, as seen in section 4.5.2 there was no significant difference between the amount of cytokines released by etafilcon A and delefilcon A ($p > 0.05$). A factor that may have affected the release of cytokines is protein denaturation. Protein denaturation can be a consequence of the protein adhering to a surface or material. For instance, lysozyme deposited onto lens materials have been found to be denatured.[139] With the prolonged incubation period, the cytokines may have denatured over time and thus were not able to be released from the lens material. Thus, with a longer soaking time for cytokine uptake (12-hours), there was no difference in the amount of adhered cytokines released by the lens materials.

The third experiment conducted focused on examining whether significant differences between lens materials for the amount of adhered cytokines released by lens materials exposed to cytokines at 34°C (ocular temperature). The purpose of this experiment was to mimic lens wear by incubating it in a cytokine solution at ocular temperature. The change in temperature affects the equilibrium water content of a contact lens material.[141] In fact, the increase in water content can lead to an increase in the amount of protein adsorbed.[121] By incubating the contact lens material in

a cytokine solution at a higher temperature, there was a possibility for the lens to adhere more or fewer cytokines and as a result release more or fewer cytokines. However, as seen in section 4.6.2, there was no significant difference between the lenses with respect to the amount of cytokines they released.

In this chapter, one study showed that there is a significant difference between contact lens materials with respect to the amount of IL-1β released when incubated in a high cytokine concentration solution. The remainder of the studies showed that there was no significant difference between the materials with respect to the amounts of cytokines released. However, the results do demonstrate that contact lens materials exposed to various conditions can adsorb cytokines and release them as well.

Chapter 5: Investigating the Differences Between Protein Coated Conventional and Silicone Hydrogel Lens and Their Release of Adhered Cytokines

5.1 Introduction

The purpose of chapter 4 was to determine whether adhered cytokine concentrations differed between contact lens materials under various ocular conditions using the protocol prepared in chapter 3. In these experiments, lenses were free to interact with cytokines with minimal interference. Interaction between cytokines and lenses were minimized to the inflammatory markers present in the calibrator blend. When a contact lens is inserted into the eye, the environment it is introduced to is much different. It has been well established that once a contact lens is inserted into the eye, it adsorbs various tear proteins and lipid molecules.[139,142-144] Protein molecules which are adsorbed onto contact lens materials include lysozyme[145,146], lactoferrin[146,147], lipocalin-1[146], immunoglobulin G[148], and etc.

Due to lysozyme, lactoferrin, and albumin being three of the most abundant proteins found in the tear film, their deposition onto contact lens materials has been thoroughly investigated. The adsorption and movement of protein into the matrix of the contact lens material is largely due to the surface charge and water content of the lens material.[121] While several studies have investigated individual protein deposition profiles onto contact lens materials, one study observed the competitive adsorption between proteins onto lens materials. When the competitive adsorption between lysozyme and lactoferrin on silicone hydrogel materials was examined, it was found that the results varied depending on the material.[147] For instance, while lysozyme and lactoferrin deposition on lotrafilcon B was affected by competitive adsorption, deposition on senofilcon A and balafilcon A was not.[147] In another study, researchers analyzed the deposition of albumin on

the lysozyme-coated contact lens and found that etafilcon A material deposited significantly lower amounts of albumin onto lysozyme coated lenses (58 ± 12 ng/lens) compared to uncoated lenses (84 ± 5 ng/lens; $p = 0.02$).[149]

The purpose of the experiments conducted in this chapter was to study the release of adhered cytokines from lysozyme, lactoferrin, or albumin coated lenses. Whether or not results differed between contact lens materials was also determined. The lens materials of interest in this study were etafilcon A, an ionic hydrogel material, and delefilcon A, a non-ionic silicone hydrogel material.

5.2 Materials and Methods

Contact lens of interest and Cytokine Solution

A total of 2 different contact lens materials ($n = 3$ for each lens material in each condition) were investigated in this study. This consisted of one conventional hydrogel lens (etafilcon A) and one silicone hydrogel lens (delefilcon A). A multi-analyte lyophilized calibrator containing the cytokines, IL-1β, IL-6, IL-8, and TNF-α was used. The calibrator blend used in this study was the high cytokine concentration blend. This blend was also used in section 4.4.1. For this study, a cytokine solution was prepared for each of the two lens types. Each cytokine solution was prepared by pooling two calibrator blends (reconstituted with 1.5 mL of Diluent 2 each) together. Thus, each cytokine solution prepared for each lens type had a final volume of 3 mL. The theoretical concentrations of IL-1β, IL-6, IL-8, and TNF-α in each solution were 3641.67 pg/mL, 1808.33 pg/mL, 1733.33 pg/mL, and 3541.67 pg/mL, respectively. For the control solution, lenses were soaked in 200μL of Diluent 2 rather than in a cytokine solution.

Lysozyme, Lactoferrin and Albumin preparation

The proteins of interest in this study were lysozyme, lactoferrin, and albumin. The concentrations of the prepared protein solutions were as followed: 1.9 mg/mL of lysozyme (Lysozyme from chicken egg; Sigma Aldrich, Oakville, ON), 1.9 mg/mL of lactoferrin (Lacromin-Recombinant Human Holo Lactoferrin; InVitria, Junction City, KS) and 0.5 mg/mL of albumin (Bovine Serum Albumin; Sigma Aldrich, Oakville, ON). All proteins were dissolved in a PBS solution.

Sample Preparation

Contact lenses were removed from their packaging solution and were immediately rinsed in PBS solution. The excess PBS solution was removed from the lens by holding the lenses against lens paper. The lenses were then individually placed into polypropylene tubes containing 1mL of either a lactoferrin, albumin, or PBS solution. For the lysozyme solution, a volume of 6mL and 1.5mL was used for etafilcon A and delefilcon A lenses, respectively. Individual lenses were incubated in the protein solutions at room temperature for 16 hours with shaking. There were two control solutions prepared in this study. A lens from each lens type was incubated in 1mL of PBS to later be incubated in a cytokine solution (to compare the release of cytokines from a lens with no protein coat) or a diluent 2 solution (to calculate background data). At the end of the 16-hour incubation period, lenses were rinsed twice in a PBS solution. The lenses then underwent the finalized procedure mentioned in Figure 3-16 using the prepared cytokine solution**.** Prior to testing, high cytokine solution samples underwent a 1:10 dilution with Diluent 2.

5.3 Results and Statistical Analysis

The amount of adhered cytokines released by various protein-coated contact-lens are described in Table 5.3-a, Table 5.3-b, Table 5.3-c, and

Table 5.3-d. Results are also illustrated in Figure 5-1, Figure 5-2, Figure 5-3 and Figure 5-4. Samples were diluted prior to testing. Thus, to determine the amount of cytokines released by contact lens materials, concentration values provided by the MSD imager for diluted samples were first multiplied by 10. These values were then inserted into Equation 3.2-1. A two-way ANOVA statistical test was conducted with lens type and protein as factors. Tukey's multiple comparisons test was undertaken to compare results within a single lens type and a Sidak's multiple comparisons test was undertaken to compare results between lens types. There was no statistically significant difference between the lens materials with respect to the amount of cytokines released ($p > 0.05$). There was also no significant difference within a lens material with respect to the different proteins it was coated with ($p > 0.05$). Results were considered significant if $p < 0.05$.

Table 5.3-a: IL-1β released by protein-coated contact lens materials (pg/lens)

Lens Type	Lysozyme	Lactoferrin	Albumin	PBS
Etafilcon A	87.035 ± 23.100	38.293 ± 17.517	63.382 ± 19.774	77.139 ± 30.342
Delefilcon A	83.692 ± 72.139	84.622 ± 30.410	73.500 ± 35.651	66.183 ± 6.465
Adjusted p-value	>0.9999	0.4026	0.9943	0.9923

Table 5.3-b: IL-6 released by protein-coated contact lens materials (pg/lens)

Lens Type	Lysozyme	Lactoferrin	Albumin	PBS
Etafilcon A	39.900 ± 9.580	19.251 ± 8.475	30.958 ± 9.267	37.622 ± 12.378
Delefilcon A	42.688 ± 35.121	41.021 ± 13.447	39.286 ± 16.875	35.765 ± 2.692
Adjusted p-value	0.9993	0.4005	0.9547	0.9999

Table 5.3-c: IL-8 released by protein-coated contact lens materials (pg/lens)

Lens Type	Lysozyme	Lactoferrin	Albumin	PBS
Etafilcon A	36.709 ± 10.699	17.013 ± 7.899	28.643 ± 9.290	33.550 ± 11.919
Delefilcon A	39.780 ± 35.502	37.428 ± 14.740	33.805 ± 16.008	30.731 ± 1.723
Adjusted p-value	0.9990	0.4682	0.9924	0.9993

Table 5.3-d: TNF-α released by protein-coated contact lens materials (pg/lens)

Lens Type	Lysozyme	Lactoferrin	Albumin	PBS
Etafilcon A	77.148 ± 20.058	36.805 ± 16.938	58.242 ± 19.253	69.278 ± 27.266
Delefilcon A	79.676 ± 70.776	77.672 ± 30.320	72.467 ± 32.278	61.505± 5.805
Adjusted p-value	>0.9999	0.4803	0.9761	0.9976

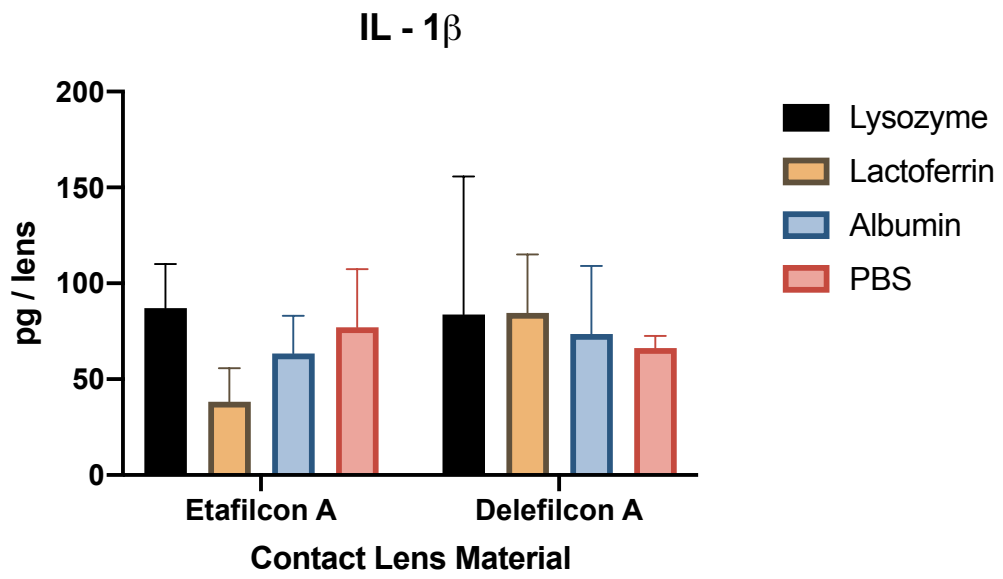

Figure 5-1: Amounts of IL-1β released by lysozyme, lactoferrin, albumin or PBS coated contact lens materials

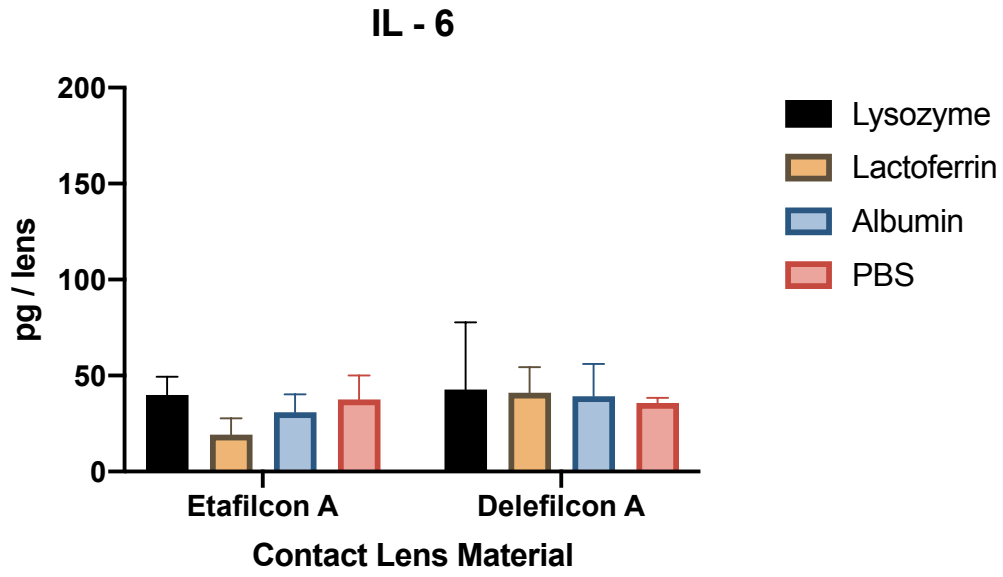

Figure 5-2: Amounts of IL-6 released by lysozyme, lactoferrin, albumin or PBS coated contact lens materials

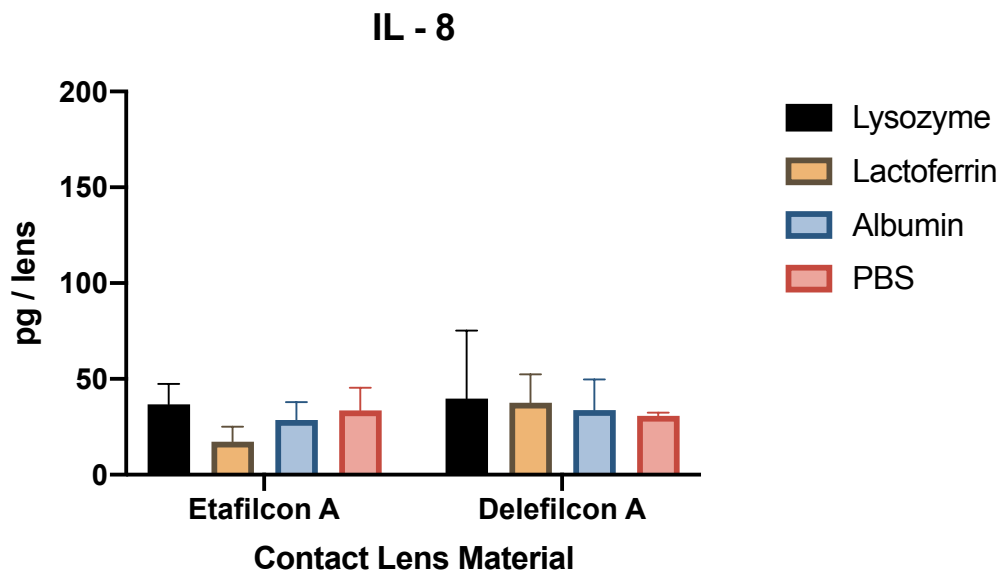

Figure 5-3: Amounts of IL-8 released by lysozyme, lactoferrin, albumin or PBS coated contact lens materials

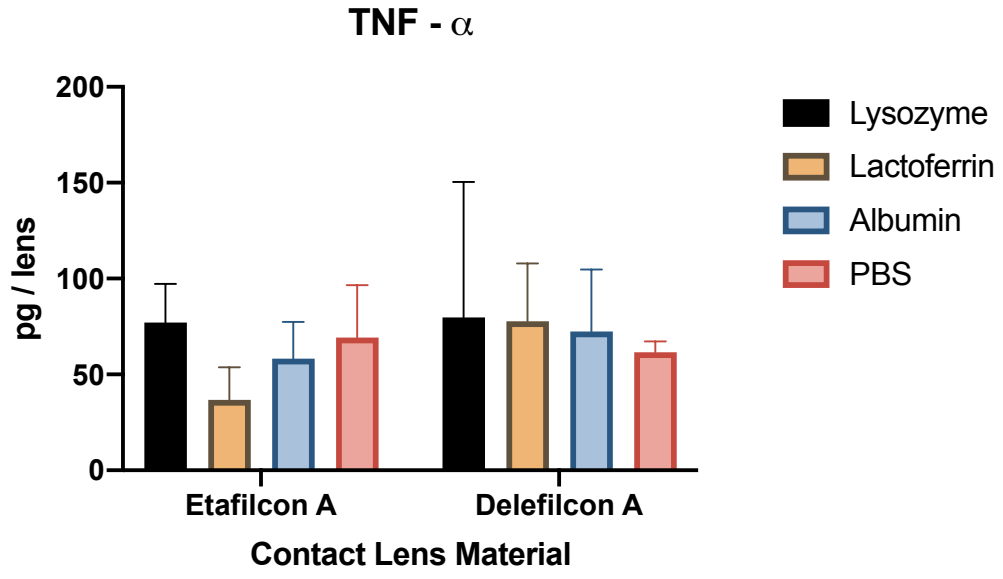

Figure 5-4: Amounts of TNF-α released by lysozyme, lactoferrin, albumin or PBS coated contact lens materials

5.4 Discussion

In this chapter, the conducted studies showed the amount of cytokines released by contact lens materials that were coated with lysozyme, lactoferrin, albumin, or the control, PBS. The contact lens materials of interest were an FDA group IV lens material, etafilcon A, and a group V lens material delefilcon A. Multiple factors such as the surface charge and water content of the lens material, the temperature, and pH of the solution, and protein characteristics can all influence protein adsorption.[136] This in return will affect the release of proteins from the lens material.

In this study, it was of interest to determine whether delefilcon A would release more or fewer cytokines in comparison to etafilcon A lens material when pre-coated with various proteins. It was also of interest to determine whether the type of protein coat influenced the release of cytokines within a single lens type. However, as the results in section 5.3 demonstrate, there was no significant difference between the two lens materials with respect to the amount of cytokines released. While both lenses have different characteristics, in terms of ionicity, where etafilcon A is a negatively charged ionic material and delefilcon A is a non-ionic material, it was interesting to find that both materials released cytokines in a similar manner. Protein adsorption is dependent on the affinity of a protein for the lens surface. As results show in section 5.3, there was also no significant difference between the amount of cytokines released by the lens materials, regardless of whether the lenses were coated with a protein (lysozyme, lactoferrin, albumin) or not (PBS). Both etafilcon A and delefilcon A may have a higher affinity for the coated proteins and as a result, displacement of these proteins by cytokines did not occur.

These results illustrate that cytokines are released by both lens materials irrespective of the other proteins adsorbed to the lens. This indicates how cytokines may be released in an ocular

environment. In the ocular environment, while the lens may be deposited with various other proteins, these depositions may have minimal effects on the release of adhered cytokines.

Chapter 6: Conclusions and Future Work

6.1 Summary

This thesis adds to the current discussion surrounding contact lens wear and its impact on ocular inflammation. Biomarkers found in tears can be used to analyze ocular conditions such as dry eye disease[150,151], keratoconus[152], ocular discomfort, and corneal infiltrative events caused by contact lens wear.[89,119] Experimental studies have explored the connections between cytokine levels and tear samples from contact lens wearers, but minimal research has been conducted to determine whether cytokines adhered to contact lens materials also impact the tear film of contact lens wearers. While risk factors such as wear modality, gender, ocular, and general health have been found to be associated with contact lens-related CIEs[153], the role of cytokines adsorbed by lens materials is unclear. The experiments conducted in this thesis sought to determine whether the release of adhered cytokines on lenses differed between lens materials. Information on the amount of cytokines released by the lens may provide an insight into whether lens wear has the potential to induce an inflammatory response.

This thesis focused on three objectives: 1) develop a protocol that would aid in quantifying the amount of adhered cytokines released by contact lens materials; 2) determine whether the release of cytokines differed between contact lens materials that were pre-exposed to various ocular conditions; and 3) determine whether the release of cytokines differed between protein-coated (lysozyme, lactoferrin or albumin) contact lens materials.

In chapter three, the parameters for a protocol to quantify the released cytokines were set after examining the effects of changing temperature, incubation period, and volume of diluent 2

followed by determining which change would be best suitable. The finalized protocol involved incubating cytokine-adsorbed contact lenses in 550μL of diluent 2 for 1 hour at room temperature.

The outline of the protocol developed in chapter three was adapted from a study conducted by Mann and Tighe in 2007.[133] In their study, they focused on analyzing proteins present in the tear envelope. The tear envelope was defined to be a small layer of tear film which enclosed the contact lens material the moment it was removed from the lens wearer's eyes.[133] The purpose of this was to further understand the relationship between tear film composition and the contact lens surface. For the studies conducted in this thesis, while the protocol from the study conducted by Mann and Tighe was adapted, the definition of the "tear envelope" was slightly changed. In the studies conducted in this thesis, the focus was to quantify the adhered cytokines released by the contact lens materials. Due to protocol limitations, it was difficult to determine whether cytokines were adhered to the surface or the matrix of the lens materials. Thus, rather than focusing primarily on the surface of the lens material, the focus was shifted to analyzing and quantifying the amount of cytokines released by the entire lens material. Thus, this included quantifying cytokines present in the small layer of cytokine solution surrounding the contact lens material, as well as that present in the matrix of the lens.

In order to investigate the amount of cytokines present in the "tear envelope" defined by Mann and Tighe, alterations would have to be made to the protocol developed in chapter three. In a study conducted by VanDerMied *et al.*, it was concluded that Schirmer strips may be used to quantify cytokines and MMPs from tear samples.[154] Perhaps, this technique may also be used to assess the quality of the "tear envelope" present around the contact lens materials. Once contact

lens materials are removed from the cytokine solution, a Schirmer strip may be used to dab the surface of the contact lens materials to remove the "tear envelope". Then, using an appropriate extraction buffer, cytokines can be extracted from the Schirmer strip and later be quantified using an immunoassay. This procedure may provide an overall estimation regarding the quantity and type of cytokines present in the tear envelope.

Protein deposition profiles on lens materials can often be predicted using the characteristics (e.g. surface charge) of both the protein molecules and the lens materials. In this thesis, four cytokines (IL-1β, IL-6, IL-8 and TNF-α) and their interaction with two lens types, etafilcon A and delefilcon A, were assessed.

The charge of a protein molecule may depend on the pH of the solution in which it is present. In the studies conducted in this thesis, the solution used to collect the released cytokines from contact lens materials was Diluent 2. Diluent 2, as described by the MSD scientific support group, is a proprietary PBS solution with a pH of ~ 7.4. Using this pH value, as well as the pI points of the various cytokines, the charge of the cytokines can be predicted. The pI points of IL-1β, IL-6, and TNF-α (~ 5.8, ~ 6, ~ 6.9 respectively, as per MSD Scientific Support) are all below the pH of the diluent 2 solution. Thus, these cytokines are expected to be negatively charged in the calibrator solution. Meanwhile, IL-8 has a pI point of ~ 8.5 (as per MSD Scientific Support). Due to this value being greater than the pH of the diluent 2 solution, IL-8 is expected to possess a positive charge. Using this information, from a charge perspective, the cytokine's affinity for the different lens materials may be predicted. Now, while etafilcon A is a negatively charged lens material, delefilcon A is a non-ionic hydrogel and thus its interaction with cytokines may not be

due to electrostatic attractions. However, with etafilcon A lenses, due to its negatively charged character, it can be predicted that this particular lens material may have a stronger attraction for IL-8 and weaker attraction for the negatively charged IL-1β, IL-6, and TNF-α. The interaction between the cytokines and lens material due to charge could have impacted the results attained in this thesis in multiple ways. For example, with a weaker electrostatic attraction, etafilcon A lens materials may have been able to release a greater amount of IL-1β, IL-6, and TNF-α compared to IL-8. Or, perhaps due to a greater electrostatic attraction for IL-8, etafilcon A lens materials may have been able to adhere to IL-8 more than IL-1β, IL-6, and TNF-α. This in return may have resulted in a higher release of IL-8 due to there being a higher concentration of this particular cytokine being present in the matrix of the lens material. From an adherence perspective, it can be predicted that etafilcon A lens material may have adsorbed more IL-8 in comparison to IL-1β, IL-6, and TNF-α.

Another factor that may influence the interaction between cytokines and lens materials is the pore size of the contact lens material and the size of the cytokines. It has been noted in literature that the pore size of lens material may be associated with its water content.[140] With greater water content, the lens material may have larger pore sizes.[140,155] Due to its high-water content, etafilcon A lens materials can be expected to have larger pore sizes in comparison to delefilcon A lens whose water content is described to behave in a gradient pattern (core: 33%; surface >80%).[122,123] With its larger pores, etafilcon A may allow for cytokines to travel further into its matrix in comparison to delefilcon A. As a result, the total amount of cytokines released by both lens types may vary. For instance, due to etafilcon A's ability to adsorb more cytokines, it may in return release more cytokines than deleficon A.

Pore sizes may also influence the type of cytokine that may be allowed to travel through the matrix of the lens. For example, when compared to the other cytokines investigated in this thesis, IL-8 is a relatively small cytokine. As seen in Table 2.3-a, it has a molecular weight of 8 kDa. With a relatively small molecular weight, IL-8 may be expected to travel further and more easily through the core of the lens material in comparison to a larger cytokine such as IL-6 (20.3 kDa). Using cytokine sizes and lens pore sizes as factors, it can be predicted that a higher concentration of IL-8 may be adsorbed and thus released by etafilcon A lens in comparison to IL-6. Due to their similarities in molecular weights, IL-1β and TNF-α (17 and 17.5 kDa, respectively) can be expected to behave similarly with respect to how far it may penetrate into the etafilcon A lens material.

Although multiple factors influence the adsorption and release of proteins to contact lens materials, the most commonly discussed factor is the surface charge of both the protein molecule and the lens material of interest. While electrostatic attractions can be used to predict protein deposition patterns, there are cases where it is not as direct. For instance, pHEMA lenses that are incorporated with MA (a negatively charged group) are found to deposit a higher level of lysozyme (a positively charged protein) due to the electrostatic attraction between them.[136] However, protein deposition can still occur despite the surface of the protein and material holding similar charges. The adsorption of a negatively charged protein molecule onto a negatively charged material can be encouraged if there are portions of positively charged areas present on the material.[136] Proteins also have the ability to re-arrange their structure when they come into contact with synthetic surfaces.[141] To remain in a soluble state in an aqueous solution, proteins fold such that their non-polar amino acid groups are tucked in, on the inner side of the protein structure, and the polar

amino acid groups are found on the outer surface.[141] However, this arrangement can change depending on the properties of the synthetic surface to which it is exposed. When a protein molecule comes into contact with a hydrophobic surface, the molecule may re-fold into an arrangement that allows for its non-polar amino acid groups to interact with the hydrophobic surface, and for the hydrophilic amino acid groups to interact with and face the direction of the solvent.[141] If a hydrophilic surface is presented, re-arrangement occurs in a manner where the non-polar amino acid groups are tucked inside the protein structure, and the outer surface is composed of polar amino acids which are then free to interact with the hydrophilic surface through hydrogen bonds and salt-bridge associations.[141] This re-folding process may explain the results seen in chapter four. Aside from the significant increase in the release of IL-1β by etafilcon A compared to delefilcon A from section 2.2.2, there was no significant difference between the amount of cytokines released between the etafilcon A and delefilcon A lens materials. With a re-arrangement in structure, the adsorption of cytokines onto both materials may have been favoured such that irrespective of charge, both lens materials released cytokines in similar manners.

The experiments conducted in chapter 5 focused on the interaction between cytokines and protein-coated contact lens materials. The three proteins of interest were: lysozyme, lactoferrin, and albumin. The literature shows that an initial protein coating has the ability to influence the subsequent protein adhesion.[144] For instance, a lysozyme-coating has been reported to reduce the subsequent adsorption of albumin onto etafilcon A lens material.[149] In a protein solution, proteins possessing a higher affinity for the material will bind onto its surface.[144] The increase in protein deposition can reduce the hydrophilicity or hydrophobicity of a material depending on its initial state.[144] This change then allows for it to attract protein molecules it was not attracted to

previously.[144] As the deposition of the subsequent protein molecule increases, the material begins to lose its affinity for the first protein molecule, decreasing its deposition in return.[144] Keeping this mechanism in mind, it was of interest to determine whether the presence of a protein layer influenced the release of subsequently adhered cytokines. The results from this chapter showed that the effects of a lysozyme coat on etafilcon A and delefilcon A lens materials were similar to those of a lactoferrin, albumin, and a PBS coat. It was demonstrated that the presence or absence of a protein coat did not influence the release of cytokines from lens materials. As well, neither did the composition of the lens material.

HEMA, the major monomer found in etafilcon A[81] is hydrophilic in nature.[156] Thus, etafilcon A material is projected to possess hydrophilic properties. Delefilcon A is an FDA group V lens (silicone hydrogel). However, due to its water gradient technology, the composition of the core of the material varies from that present on the surface. While the core of delefilcon A encompasses a low water content silicone hydrogel material, its surface is composed of hydrophilic polymers which include polyamidoamide and poly(acrylamide-acrylic) acid.[156] When surface levels of silicon were measured in various silicone hydrogel lens materials, the lowest percentage of silicon was found in delefilcon A and lotrafilcon A.[156] Since silicon is hydrophobic in nature, the lack of surface-expressed silicon found in delefilcon A[156] may cause it to have less hydrophobic properties on its surface. With a hydrophilic surface and a lack of silicon,[156] it is possible that delefilcon A interacted with cytokines similarly to etafilcon A. This resulted in both materials releasing similar levels of cytokines.

The effects of contact lens materials on tissue-grown corneal epithelial cells have been investigated, and it was found that the presence of lens materials did not greatly influence or affect cytokine production.[157] It was concluded that the direct interaction between the contact lens and the corneal epithelial cells is unlikely to induce the production of inflammatory markers.[157] With the cause of adverse events due to contact lens wear still in question, the cytokines released from contact lens materials may be inducing an inflammatory response. The results from this thesis provide evidence that cytokines can be released from contact lens materials. It is unknown, however, if this release occurs over some time, or at once. In the case that there is a burst of cytokines from lens materials, the ocular immune system may recognize it as an inflammatory response. For instance, IL-1β is known to trigger inflammatory responses.[41] In the case that IL-1β is released from lens materials into the tear film at once, inflammatory cells may be recruited to the site of infection and the upregulation of subsequent cytokines in the inflammatory cascade may be initiated.[158] If cytokines are released over time, with the constant recruitment of inflammatory cells to the surface of the cornea, the cells may aggregate, and that may lead to the eventual production of CIEs.

As shown in Table 2.3-a**,** there is a large range in basal levels of cytokines found in tears. Disruption to homeostatic levels of cytokines may be enough to induce an inflammatory response, as there is no clear level of inflammatory markers required to cause inflammation. For an individual whose basal level of IL-1β is 436.3 pg/mL[47], a release of 3.298 pg (from Table 4.6-a) may not disrupt homeostatic levels and lead to an inflammatory response. However, individuals whose average basal level of IL-1β is 7.42 pg/mL[132], the addition of 3.298 pg may be recognized

by the host defense system as an inflammatory response, consequently leading to an upregulation of subsequent cytokines.

While the results do not provide much evidence of lens material-dependent cytokine release or sufficient reason for why there exists a two-fold increased risk in developing CIEs with silicone hydrogel lens wear[107], this thesis provided an insight into lens materials' ability to release cytokines.

6.2 Limitations

The experiments conducted in this thesis did have various limitations. While the experiments focused on the amount of cytokines released, the amounts adsorbed before their release were not measured. This information would have aided in determining the efficiency of the protocol developed in chapter 3. In addition, it may have also indicated whether lens materials had the ability to release all adsorbed cytokines. Details with regards to the amount of cytokines adsorbed may also help determine the kinetics of cytokine release from lens materials. While both lens materials in chapters four and five seem to have released similar amounts of cytokines, it is unknown what percentage of cytokines were released from each material. As an FDA group IV lens material, etafilcon A has the potential to deposit a large amount of proteins.[121] It is possible that while delefilcon A released a large portion of adhered cytokines, etafilcon A may have released a smaller percentage, thus leaving a larger amount of cytokines still deposited onto the lens.

Due to the limitations present in the studies conducted, it was also difficult to determine the direct relationship between charge and pore size of lens material and the release of cytokines. To further understand this relationship, the following studies may be performed. Firstly, it would be of interest to use a calibrator blend that contained one particular type of cytokine as opposed to a blend containing multiple cytokines (as used in this thesis). Next, to investigate the role a cytokine's charge had on its release from lens materials, it would be of interest to study the release of IL-8 (a positively charged cytokine in diluent 2) from an ionic lens material such as etafilcon A and a non-ionic lens material such as omafilcon A. Theoretically, due to its negative surface charge, etafilcon A should release a lower concentration of IL-8 in comparison to omafilcon A. If there is a great difference in results, the impact of the electrostatic attraction between the lens material and cytokine will be known. Then, to analyze the role a cytokine's molecular weight has on its release from lens materials, it would be of interest to study IL-8 (8 kDa) and IL-6 (20.3 kDa) and its interaction with a non-ionic lens material that has a relatively large pore size such as omafilcon A. The purpose of using a non-ionic lens material is to not allow for difference in charges to influence the final result. Results from this study may provide details with regards how the difference in molecular weight influences a proteins ability to be released by lens materials.

The primary focus of chapter 3 was to develop a protocol that would assist with quantifying the number of adhered cytokines released by contact lens materials. However, the process of developing a protocol was met with its limitations.

Firstly, there were three variables of interest in chapter three. For each variable, two conditions were tested using four lens types. For these studies, a sample size of three lenses per

lens material per testing condition was used. Due to the novelty of the study, there were minimal studies available in literature expressing appropriate sample sizes for such experiments. Thus, taking cost and supplies into consideration, a sample size of three was chosen. However, a small sample size may have impacted the statistical analysis. For instance a small sample size may cause the study to have low power.[159] One possible consequence of a study having low power is that the study may produce false negative results.[159] With low power, statistical differences between testing conditions such as temperature (room temperature vs 34 ℃) may not be detected. Hence, increasing the sample size to four may increase the power of the study. As a result, there may be less variability in the results and the statistical impact due to sample size will decrease.

Next, there was a limitation placed on an intermediate step in this study. In chapter three, lens materials were soaked in a cytokine solution. Post the incubation period, tweezers were used to remove the lens materials and the lenses were then immediately placed into a 0.6μL tube filled with diluent 2. This condition introduced the possibility of the lenses transporting excess solution in addition to adhered cytokines. To minimize solution transfer, contact lens materials were held against the side of the polypropylene tube to drain excess solution. However, this process does not eliminate the possibility of transferring solution completely. In this scenario, it is possible that the cytokines quantified may have also come from the solution surrounding the lens material in addition to those that were originally adhered to the lens. For future studies, in order to reduce the transfer of excess solution, it would be appropriate to rinse the lenses in a PBS solution the moment the lenses are removed from the cytokine solution. Once the excess solution is removed from the lens, the lenses may then be placed into the 0.6μL microcentrifuge tube. With this additional

rinsing step, the source of the cytokines in the final eluate can be narrowed down to the lenses rather than the lenses and excess solution.

In this study, a lyophilized calibrator blend prepared by Meso Scale Discovery was used. The blend used in chapter three contained a total of ten cytokines, four of which were quantified in this thesis. In this blend, the initial concentration of the cytokines varied. For example, as seen in section 3.2.1 the theoretical concentration of IL-1β, IL-6, IL-8, and TNF-α in the prepared cytokine solution were reported as 88.57 pg/mL, 112.71 pg/mL, 74.14 pg/mL and 45 pg/mL, respectively. Any result that implied an increased release of one cytokine over another may primarily be due to the unequal distribution of the cytokines in this blend rather than the lens materials having a higher affinity for one cytokine over another.

The application of the protocol is also limited by the variables investigated during protocol development. In chapter 3, three variables were investigated and for each variable, two conditions were tested. To develop a protocol, it would be optimal to test multiple conditions prior to deciding on a most suitable protocol. For example, to determine the incubation period, 0-hours and a 1-hour incubation times were tested. It is unknown whether incubating the lens for a longer period of time, such as 24 hours, would result in a greater release of cytokines. While testing multiple time periods would assist in determining optimal conditions, it was not feasible given my timelines for completion.

6.3 Future Work

In this thesis, there was a focus on examining the release of protein inflammatory markers, specifically cytokines, from contact lens materials. Due to their key role in ocular inflammation the cytokines of interest were: IL-1β, IL-6, IL-8 and TNF-α. However, several other cytokines and chemokines also have crucial roles in the ocular inflammatory process. Increased levels of IL-17A have been found in contact lens wearers who experienced contact lens discomfort[89], so determining the levels of IL-17A released by lens materials would be beneficial as well. Similarly, increased levels of MMP-9 were found in those who wore reverse geometry contact lens, but not in those who wore silicone hydrogel lens or no lens.[103] Hence, it would also be of interest to study MMP-9 levels as well.

Several studies in literature have reported that the risk of developing CIEs from silicone hydrogel contact lens wear is two times higher than in lower oxygen permeable contact lenses.[109-111] The purpose of the studies conducted in this thesis was to investigate whether the release of cytokines differed between conventional and silicone hydrogel lens material. If so, the increased risk of developing CIEs with silicone hydrogel lens may be due to it releasing more cytokines and thus inducing CIEs. While that was the focus of this thesis, there was a limitation placed on the conclusions that could be drawn. In the studies conducted, there was a focus placed on daily disposable lenses. However, the higher risk of developing CIEs with silicone hydrogels was seen with extended wear lenses (worn for a 30 day period in comparison to the lower oxygen permeable lenses worn for 7 days).[107] Under these conditions, the exposure time of cytokines and tear film to lenses may have played a role in inducing CIEs onto the corneal surface. To mimic such conditions, and further understand how extended wear silicone hydrogel lenses may influence CIE

development, minor changes can be made to the protocol developed in chapter three. Firstly, rather than incubating contact lens in a cytokine solution for a 6-hour period, perhaps the incubation period can be extended to 7-14 days. In addition, every 24 hours, a 50µL sample of the cytokine solution should be collected. Following the incubation period, the lenses should undergo the cytokine removal process. The results from this study may help understand whether cytokine deposits accumulate onto the lens overtime, and whether there exists a maximum amount of cytokines that can be adhered to and thus released by lenses. The purpose of collecting a 50µL sample of the cytokine solution every 24 hours is to observe whether cytokines are being released back into the solution by the lenses over the incubation period. If the concentration of the cytokines decreases overtime, it may be due to the lens adsorbing the cytokines. However, if the concentration of cytokines plateaus over a period of time, it may be due to the lens's inability to adsorb any additional cytokines due to saturation. Alternatively, if the cytokine concentration fluctuates, it may be due to the process of adsorption and release reaching equilibrium. The results from these studies may help further understand how extended lens wear influences the release of cytokines by lens materials.

This thesis focused on *in vitro* studies to provide an insight into the amount of cytokines released by contact lens materials. With the protocol developed in chapter 3, future studies could aim to determine the amount of cytokines released by patient-worn lenses. Perhaps, this may aid in quantifying the amounts of cytokines released back into the tear film. A comparison between the amount of cytokines present in post-lens wear tears and the amount released from patient-worn lenses may answer whether the upregulation of inflammatory markers during contact lens wear is caused by an inflammatory response due to the released cytokines.

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