Development of *In Vitro* Biocompatibility Models of the Ocular Surface

by

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AUTHOR'S DECLARATION

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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

Statement of contributions

Parts of sections 2.1, 2.2 and 2.5 of this thesis were co-authored with Cameron Postnikoff, Robert Pintwala and Dr Maud Gorbet as a review chapter “Chapter 36: Ocular responses to biomaterials" in the “Handbook of biomaterials biocompatibility”

Abstract

Over 120 million individuals wear contact lenses (CLs) worldwide and compared to non CL wearers, they exhibit a higher risks of eye infection and ocular inflammatory events. As CLs are evolving towards becoming diagnostic and therapeutic devices, there is a growing need for *in vitro* models that can efficiently assess ocular surface toxicity and biocompatibility of the technologies embedded in CL. Hence, understanding the effects of CLs on the health and integrity of ocular surface is imperative. While there exists some *in vitro* ocular cell models for the characterization of cellular mechanisms and biocompatibility, many of these are too complex and costly for rapid testing or don't always allow for a biomaterial to be present. The aim of this thesis was to explore and develop cell culture models that could best mimic some of the interactions between the ocular surface and a biomaterial.

In the first phase of the development of the *in vitro* model, to investigate the role of the ocular surface geometry on corneal cells and understand how curvature affects cell response, HPV-immortalized corneal epithelial cells (HCEC) were grown on flat and curved surfaces. Next, the effect of artificial tear flow (dynamic conditions) in an *in vitro* model was assessed. The OcuCell testing platform, an *in vitro* system which mimics tear flow between an "eyelid" and "eyeball" pieces, was used to determine the role of dynamic conditions when assessing combination of CL and CL cleaning solutions. The Ocucell eye pieces were made of 10% Sylgard 184; the two pieces fit together and permit a CL to be set on the corneal surface of the "eyeball" piece. HCEC were grown to confluence on the outer curved eyeball surface and on the inner concave surface of the eyelid piece and two CL (Etafilcon A and

Balafilcon A) were tested in combination with two CL cleaning solutions, ReNu Fresh (a PHMB-based multi-purpose solution) and ClearCare (a hydrogen peroxide based solution). Experiments were performed under static (no flow) and dynamic conditions. Finally, the effect of crosstalk between two different corneal cell populations in an *in vitro* testing for CL was investigated. A coculture system using HCEC and conjunctival (ICONJ) corneal cells was implemented, where ICONJ cells were grown on a PET transwell insert and HCEC on the well of a tissue culture treated polystyrene plate (TCPS). When cells reached confluency, they were incubated together with the CL combination. Results were compared to CL incubation with single cell population. All experiments were performed for 6 hours and cells were harvested and expression of integrin α_3 and β_1 was characterized by flow cytometry.

Curvature was shown to have a significant effect on corneal epithelial cells where cells grown on the convex and concave pieces exhibited a significant upregulation in α_3 and β_1 integrin expression compared to that of the flat surface. Using the OcuCell test platform, downregulation in integrin expression was observed when HCEC were exposed to various CL-solution combinations. The combination BA-ReNu consistently resulted in significant reduction in integrin α_3 and β_1 when compared to the control lens (lens incubated in PBS), suggesting that the release of MPS components on BA, such as PHMB and borate buffer, affected HCEC. In the mono culture model, no difference was observed with any CL-solution combinations. In the double culture model (where a contact was placed in between the TCPS and insert with same cell population), some downregulation in integrin expression was observed with HCEC but not ICONJ. However, upon co-culture of HCEC with ICONJ,

downregulation of integrin expression was observed in all combinations and more significantly so with BA-ReNu.

In this thesis, the effects of curved versus flat surfaces, dynamic versus static conditions, and mono versus co-culture models, were investigated to understand their potential role when assessing CL biocompatibility. Our results highlight how each experimental *in vitro* model provides different but complementary information about the biocompatibility of the CL and multipurpose cleaning solution combination. The co-culture model also provides *in vitro* evidence of the crosstalk between cells and how this may impact ocular cell response to a biomaterial. The *in vitro* models and methodologies explored in this thesis represent news means to test biocompatibility of CLs, and potentially allow for future testing for ophthalmic materials and contact lens technologies in *in vitro* cell models that are simple and cost effective to allow for fast prototyping and development.

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To Tarique, Amo Majed, Khalto Dhiyaa, Khalto Julliette and Khalo Khattar, thank you for always checking up on me and always letting me know I am loved and cared for.

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Dedication

To my best friend and sister, Lana.

This one is for you.

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List of Abbreviations

µg	Micrograms
µl	Microlitre
AA	Alphafilcon A
AFU	Arbitrary Fluorescent Units
Aldox	Myristamidopropyl Diametylamie
AMO	Abbott Medical Optics
ANOVA	Analysis of Variance
BA	Balafilcon A
BAK	Benzalkonium Chloride
BBS	Borate-Buffered Solution
BCEpiC	Bovine Corneal Epithelial Cells
BEC	Basal Epithelial Cells
CA	Comfilcon A
CC	Clear Care Cleaning & Disinfection Solution
CL	Contact Lenses
EA	Etafilcon A
EDTA	Ethylenediaminetetraacetic
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GA	Galyfilcon A
ICONJ	Human Conjunctival Cells
H_2O_2	Hydrogen Peroxide
HCEC	Human Corneal Epithelial Cells
HCLE	Human Corneal-Limbal Epithelial Cells
HPMC	Hydroxypropylmethylcellulose
KGS	Keratinocyte Growth Supplements
KM	Keratinocyte Medium
LA	Lotrafilcon A
LB	Lotrafilcon B
MDCK	Madin-Darby Canine Kidney Cells
MPS	Multipurpose Solution
MTT	3-(4,5-Dimethylthiazol-2yl)-2,5-Diphenyltetrazolium Bromide
mg	Milligram
ml	Millilitre
OCED	Organization for Economic Co-Operation and Development
OFX	Opti-Free Express
OFR	Opti-Free Replenish

PBS	Phosphate-Buffered Saline
Pen/Strep	Penicillin/Streptomycin
PHMB	Polyhexamethylene Biguanide
PI	Propidium Iodide
PDMS	Polydimethylsiloxane
PQ	Polyquad, Polyquaternium-1
PS	Phosphatidylserine
RGP	Rigid Gas-Permeable
SD	Standard Deviation
SEC	Superficial Epithelial Cells
SEM	Scanning Electron Microscope
SICS	Solution Induced Corneal Staining
SiHy	Silicone Hydrogel
SV-40	Simian Vacuoloting Viris 40
TCPS	Tissue Culture Plate
TEER	Transepithelial Electrical Resistance
TNF	Tumor Necrosis Factor
VA	Vasurflicon A
α	Alpha
β	Beta
° C	Degree Celsius
zonulae occludentes (ZO)	Zonulae Occludentes (ZO)

Chapter 1

Introduction

The field of contact lens research is one of the fastest growing medical markets worldwide. In 2015, the market capitalization was at $1.4B with an expected growth of 6.4% every year[1]. While contact lenses represent the most widely used medical device (estimated 125M wearers worldwide), surprisingly little research has investigated mechanisms related to corneal and conjunctival cell interaction following exposure to contact lens materials[2].

The development of silicone hydrogel (SiHy) contact lens has overcome oxygen transmissibility challenges that other contact lens materials have not offered. However, with the change of material and lens wearers seeking fast and convenient way to clean their contact lenses, new challenges have arisen. Cleaning solutions are used with daily wear lenses and soaked overnight to remove of any deposits of proteins from the tear film as well as any foreign materials from wear. Two main options currently exist, (1) the multipurpose cleaning solution (MPS) which contain specific biocides to kill foreign pathogens during a 4 hour-minimum lens treatment and (2) the hydrogen peroxide (H_2O_2) system which uses H_2O_2 that is neutralized and requires a minimum 6 hour-treatment. There is now evidence that contact lenses have the potential to adsorb components of the multipurpose cleaning solutions (MPS), which can then be released onto the corneal surface post insertion[3,4]. The interaction between the contact lens and the lens care/lens cleaning system has the potential to result in biocompatibility problems and a phenomenon termed solution-induced corneal staining has been observed in vivo with specific combinations of contact lens and lens care system. It is difficult to evaluate the effects of contact lens materials have on the human cornea *in vivo* due to the limitations of the clinical techniques available, which are mainly imaging and thus cannot provide quantitative physiological

information on the health of ocular cells. To develop better ophthalmic solutions and biomaterials, it is important to be able to characterize the interactions between the cleaning solution, contact lens and cells of the ocular surface, namely corneal and conjunctival epithelial cells. *In vitro* models represent an avenue to investigate these interactions in a cost-effective and ethical manner compared to animal models.

Existing *in vitro* models for CL testing have often been designed as cytotoxicity models for lens cleaning solution and provide little information on the biocompatibility of an ophthalmic material such as a CL. This is because the most prevalent *in vitro* testing method in research today uses the infinite sink (no cells) or a static single cell model with often no lens. These approaches do not allow researchers to model for blinking, tear flow exchange, crosstalk between ocular cell population and characterize cell response in the presence of a contact lens[5]. Novel biocompatibility models are being developed to allow us to gain a better understanding of cell-material interactions. Furthermore, recent research also demonstrated that human corneal epithelial cells (HCEC) respond to mechanical stress[6,7]. To better understand mechanisms of biocompatibility on the ocular surface, it is relevant to be able to study the interactions of CL materials with *in vitro* model of the ocular surface under dynamic conditions[8,9]. In an effort to design *in vitro* models that better mimic the interactions that take place at the ocular surface during lens wear, the presence of different ocular surface cells in the model also needs to be considered.

To gain a better understanding of the role of *in vitro* models to assess lens-solution combinations and inform the design of *in vitro* biocompatibility model, the general objectives of this research thesis project were to assess contact lens-solution combination using a static and dynamic *in*

vitro model, and explore *in vitro* the role of crosstalk between corneal and conjunctival epithelial cells. This MASc thesis had three overarching hypotheses:

(1) a change in curvature will lead to cell response which can be assessed with integrin expression,

(2) a dynamic *in vitro* model will result in significant differences in HCEC response to lens-solution combinations compared to a static incubation model,

(3) a co-culture model with conjunctival and corneal epithelial cells will lead to significant differences in cell response to lens-solution combinations compared to a mono-culture model.

The thesis begins by introducing the structure of the eye, ophthalmic materials such as contact lenses and cleaning solution, and various methods of testing ocular biocompatibility. In chapter 3, the effects of curvature and material on cells are investigated. Chapter 4 presents the study with the OucCell system using dynamic and static conditions for testing contact lens-cleaning solution combinations. In chapter 5, three different *in vitro* models (monoculture, double culture and co-culture models) are used to investigate the effects of crosstalk between two cell populations when testing different combinations of contact lens and cleaning solution. Conclusion and recommendations for future work are presented in chapter 6.

Chapter 2

Literature review

2.1 Cornea anatomy [1]

The cornea is a transparent avascular, non-keratinized epithelial structure, forming one-sixth of the area of the outer wall of the eye. It represents the optical interface between the eye and external environment and functions as an optical element and protective barrier. Together with the lens, the primary function of the cornea is to refract light to focus an image on the retina; therefore, they must maintain their transparency, optical physiology, and structure. As depicted in Figure 1, the normal human cornea is 500 μm thick and consists of 5 layers: corneal epithelium, Bowman's layer, stroma, Descemet's membrane, and the corneal endothelial monolayer[1]. The corneal epithelium is a stratified structure, 50 μm thick, consisting of a single layer of squamous superficial epithelial cells (SEC) several layers of intermediate wing cells, and a single layer of columnar basal epithelial cells (BEC)[1] (figure 2.1). Superficial corneal cells provide a substrate for the precorneal tear film, which acts as the primary refracting surface of the eye[1].

[1] Sections of this chapter have been published in "Chapter 36: Ocular responses to biomaterials" in the "Handbook of biomaterials biocompatibility", a review chapter to which I contributed to as co-author with C. Postnikoff, R. Pintwala and M. Gorbet [72]

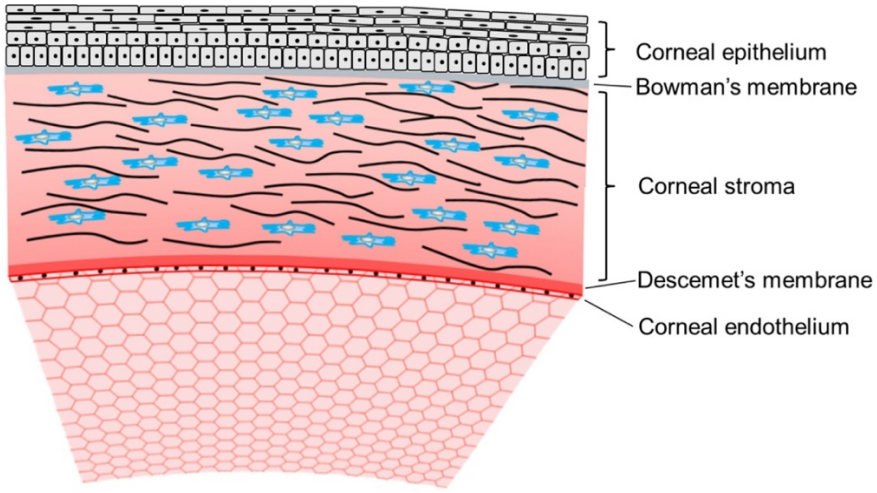


Figure 2.1: Cross-sectional schema of the cornea. Figure reprinted with permission from Chen[2] *et al*.

The cornea is well protected from pathogens and the external environment by tight junctions and the constant cell self-renewal, lacrimation and blinking, antimicrobial enzymes in tears, and nearby antigens, cytokines, inflammatory mediators or leukocytes that enter the cornea via limbic and/or ciliary body vessels[1]. The population of epithelial cells is maintained by the balance between cell divisions at the limbus and basal layers and cell loss or sloughing at the surface (with an epithelial cell turnover rate of approximately 7 days)[1]. The presence of tight junctions in the corneal epithelial layer plays a vital role in the barrier function of the cornea, protecting intraocular structures against diffusion of substances from the tears, transport of ionic or polar molecules, microbial infections, and other environmental stresses[3–5] (figure 2). The tight junctions are formed of occludin and claudins integral transmembrane proteins as well as zonulae occludentes (ZO) membrane-associated proteins (ZO-1, ZO-2, and ZO-3). As opposed to the corneal epithelium, the corneal endothelium has a limited capacity for regeneration. This monolayer of cells plays a key function in regulating water to maintain hydration of the cornea and allows diffusion of nutrients. Cells of the ocular surface and endothelium express integrins

(cell membrane receptors), which play a role in cell adhesion, migration, and maintenance of tissue integrity and which expression can be affected by contact lens biomaterials[3,6,7]. Corneal and conjunctival epithelial cells, stromal cells, and resident antigen-presenting cells also express toll-like receptors (specifically TLR2, 3, 4, 5, and 7), which are involved in inflammatory and immune cell activation and recruitment[8].

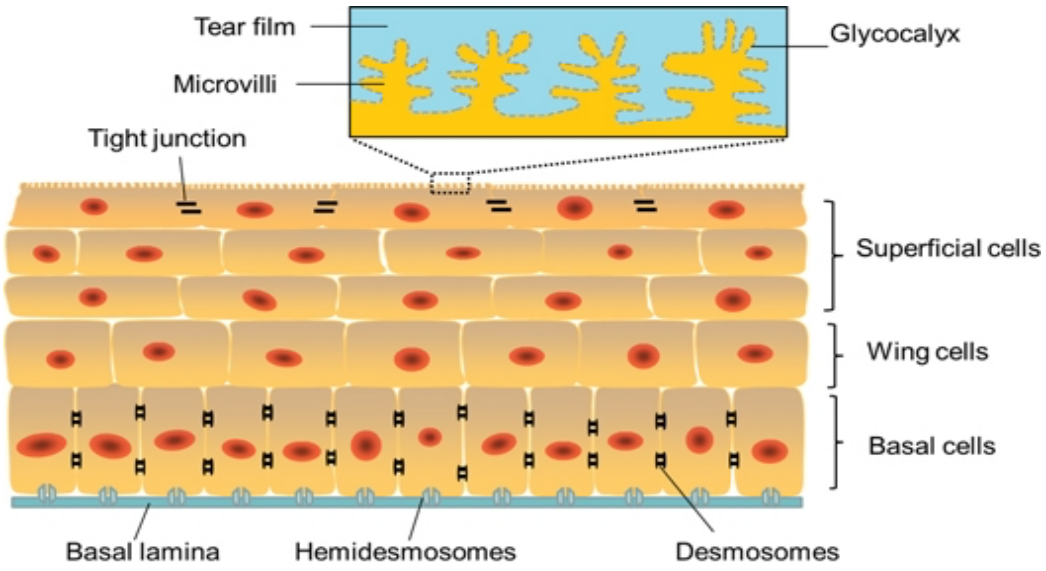


Figure 2.2: Cross-sectional schema of the corneal epithelium Figure reprinted with permission from Chen[2] *et al*.

Corneal epithelial cells contain a family of cell surface receptors known as integrins. There are 24 known alpha subunits and 9 known beta subunits. These subunits can non-covalently join together to form heterodimers. Integrins serve to maintain tissue integrity by aiding in cell adhesion and the formation of adhesion structures. The integrins are able to carry out these functions by ligating with extracellular matrix proteins such as laminin and fibronectin. These proteins contain an RGD sequence that serves as an attachment site for integrins as well as

extracellular matrix molecules. The following paragraph introduces some of the key integrins in the cornea.

$\alpha_3\beta_1$ aids in cell migration by regulating matrix metalloproteinase expression which is responsible for cell movement and spreading[9,10]. It is also important for the stability of hemidesmosomes and maintaining cell-cell junctions[7,11]. **$\alpha_6\beta_4$** is a factor for cell survival[12]. It regulates the progression of the cell cycle[11]. This integrin is phosphorylated during cell migration to prevent the early assembly of hemidesmosomes[11]. It is a strong component of hemidesmosomes, and its expression is essential for the stable adhesion of the epithelium to the basement membrane[7,11,13]. Decreased levels of this integrin result in the disruption of cell adhesion[11,14]. The absence of the β_4 subunit in this integrin results in defective epidermal adhesion and assembly of the basement membrane[11,14]. **$\alpha_5\beta_1$** aids in the process of healing wounds[9,15,16]. Healthy cells express this integrin, increasing fibronectin sensitivity and acting as a chemotactic migratory stimulus[10,15,17]. **$\alpha_9\beta_1$** also aids in the wound healing process[9,11,16]. It is located in the corneal epithelium. The α_9 subunit regulates the protein association of the cytoskeleton, assisting with cell migration[11]. It is an important factor in sustaining cell migration after initiation[11]. **$\alpha_v\beta_6$** is another important factor in wound healing and cell migration[11,16,18]. During wound healing, this integrin is expressed to help maintain the integrity of the tissue[11]. E-Cadherin expression is regulated by this integrin[11]. Decreased levels of $\alpha_v\beta_6$ lead to a loss of junctions[11].

2.2 Conjunctiva anatomy

The conjunctiva is the mucous membrane that is thin, transparent, and vascularized and covers the inner surfaces of the eyelids and extends to the cornea. The conjunctival epithelium

has a stratified structure and similarly to the corneal epithelium, its cells express various integrins and receptors and tight junctions (in the superficial layers). It is involved in different ocular surface diseases, playing an active role in the pathophysiology of common conditions such as dry eye[19]. The conjunctiva covers the anterior sclera by reflecting forwards on the eye at the fornix. It consists of two components, namely: a bulbar component and a palpebral component[20]. The bulbar conjunctiva is a thin, semitransparent, colorless tissue[21]. The sclera up to the corneoscleral junction is covered by the bulbar conjunctiva[21]. The palpebral conjunctiva, on the other hand, is a thick, opaque, red tissue. It is divided into three zones which are: marginal, tarsal, and orbital zones[22]. The keratinization is minimal in the marginal zone which lay between the skin and the conjunctiva. The tarsal conjunctiva is a fibrous relatively smooth layer[21]. The functional shape of the eyelid is provided by the tarsal conjunctiva. In addition, the conjunctiva consists of accessory lacrimal glands, lymphoid tissue, mast cells, and goblet cells. The accessory lacrimal glands and the Meibomian glands exist in both the upper and the lower tarsal conjunctiva, however, they are concentrated in the upper one. The tarsus and the fornix have an abundance of goblet cells (Figure 3)[23]. Conjunctival epithelial cells synthesize inflammatory mediators which will affect both ocular health and vision and play a role in the complex inflammatory system/response of the eye.

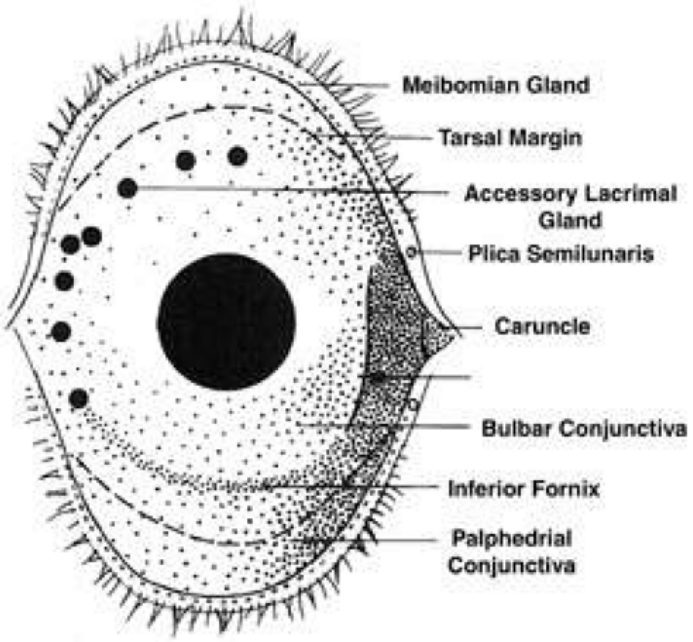


Figure 2.3: Schematic representation of the anatomy of the conjunctiva. Figure reprinted from Lemp[24].

The tear film is responsible for the hydration, lubrication, and nutrition of the ocular surface while providing a barrier to pathogens and particulates. The tear film is viewed as layered consisting of the outermost non-polar lipid layer, the polar lipid layer, the aqueous-mucin layer, and a glycocalyx layer covering the cornea (Figure 4). Components of the tear film are secreted by different epithelial and glandular tissues, such as the meibomian glands (the lipid layer), lacrimal glands (the aqueous layer), and the goblet cells within the conjunctiva (the mucin layer)[25]. Mucins are highly glycosylated proteins that are both secreted and membrane-associated.

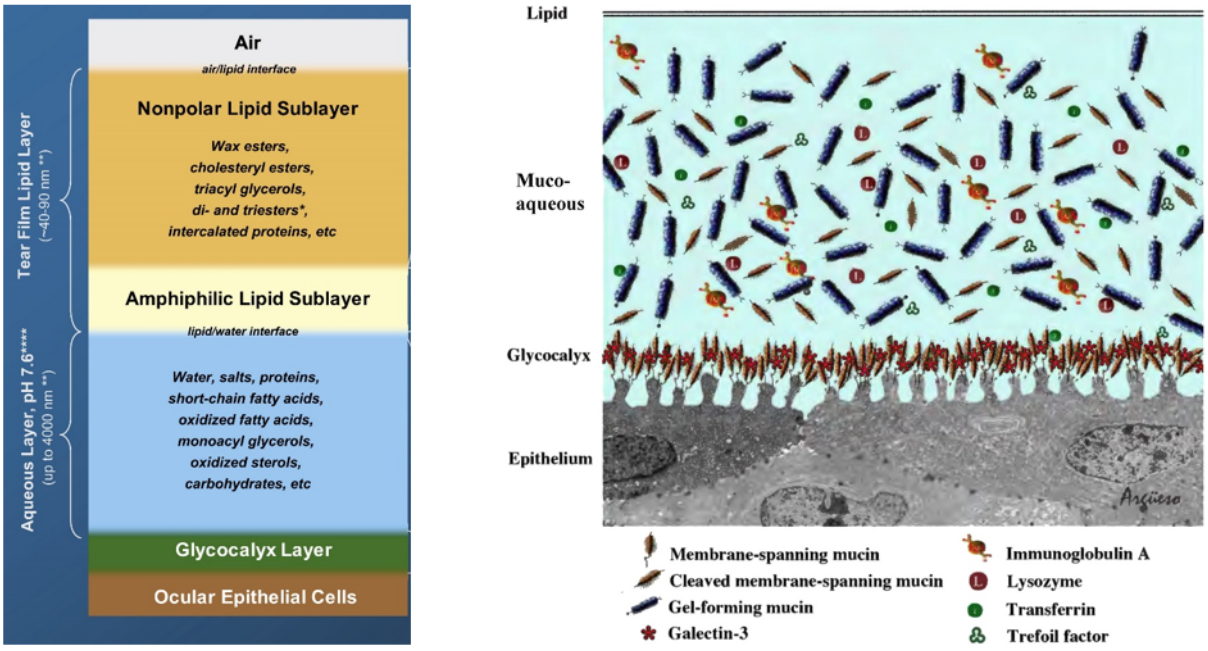


Figure 2.4: The tear film structure. The left scheme presents the overall structure while the right scheme presents a more detailed view of the mucins and galectin of the glycocalyx, soluble mucins, and proteins in the mucoaqueous layer. Figures reprinted from Butovich[26] and Willcox *et al*[27] with permission from Elsevier.

Over 1500 tear proteins have been identified in tears from healthy subjects, with lysozyme, lactoferrin, tear lipocalin, and secretory immunoglobulin A (sIgA) being the main proteins found in the tear fluid[28]. Tears contain cytokines (such as IL-1β, IL-6, IL-8, and IL-12), chemokines (such as CCL2, CXCL1, and CXCL8) and growth factors, which are produced by resident cells of the ocular surface and infiltrating immune cells and play an essential role in corneal homeostasis and inflammatory processes[28–30]. It is important to note that the composition of the open-eye and closed-eye tears are significantly different from each other. Closed-eye tears contain higher levels

of albumin, fibronectin, complement proteins, sIgA, and plasmin compared to open-eye tears, with sIgA representing up to 80% of the total protein content[31,32]. Furthermore, a large number of neutrophils have been observed in closed-eye tears[33,34]. These differences have the potential to impact the ocular response to biomaterials.

With its lack of blood vessels and inherent protection mechanisms, the cornea is considered an immune privilege tissue, whereby to prevent vision loss, minimal inflammation and immune response may occur[35,36]. However, the presence of a biomaterial, such as a contact lens, may affect this protected environment and trigger an inflammatory response that can damage the ocular surface[34,37,38]. This thesis project is interested in developing *in vitro* models of biocompatibility that consider the interplay between corneal and conjunctival cells when assessing a contact lens.

2.3 Contact lens materials

Contact lenses (CL) represent the most widely used medical device (with an estimated 125 million wearers worldwide) and the most common biomaterial to interact with the ocular surface[39]. Soft contact lenses are hydrogel polymers typically made either from poly(2-hydroxyethylmethacrylate) or silicone hydrogels[40]. In order to increase oxygen transmissibility of these materials to reduce the risk of corneal hypoxia, all of these polymers are surface treated with monomers and wetting agents added to their surface[40]. Rigid gas-permeable (RGP) contact lenses used in orthokeratology or with keratoconus patients were originally manufactured with polymethyl methacrylate, which has a low oxygen permeability[41]. To increase oxygen permeability and improve biocompatibility, RGP lenses now use most commonly silicone or fluorosilicone acrylate materials[41].

About 90% of contact lens wearers use hydrogel or silicone hydrogel lenses. A four-part classification system used water content and ionicity to classify contact lenses. This was used to facilitate testing of different lens materials with different lens care systems. It is important to understand how hydrogel materials react with varying care systems as well as with proteins in the tear film. Silicone hydrogel (SiHy) material lenses were originally classified either as group 1, low water and non-ionic, or group 3, low water content and ionic. A fifth category was created to include the unique properties of SiHy lenses and the way they interact with CL solutions and tear film. The current classification system differentiates SiHy lenses based on water content, ionicity, surface treatment, and the presence of hydrophilic monomers or semi-interpenetrating network. Table 1 lists the most common CLs used today and their classifications.

Table 0.1: Most Commonly Used Soft Contact Lenses in Today's Market

Lens Type	*Group 1, Nonionic/Low H_2O*	*Group 2, Nonionic/High H_2O*	*Group 3, Ionic/Low H_2O*	*Group 4, Ionic/High H_2O*
Hydrogel	Polymacon Tetrafilcon A	Omafilcon A Nelfilcon A Alphafilcon A	Phemfilcon A	Ocufilcon D Etafilcon A Methafilcon A

Lens Type	*Group 5A, Ionic/No H_2O Specification*	*Group 5B, Nonionic/ Low H_2O*	*Group 5Cm, Nonionic/Low H_2O/ST (Surface Treatment)*	*Group 5C Nonionic/Low H_2O/Non-ST/Hydrophylic Monomer*	*Group 5 Cr, Nonionic/Low H_2O/Non-ST/Semi-Interpenetrating Network*
Silicone hydrogel	*Delefilcon A	Somofilcon A	Balafilcon A Lotrafilcon B Lotrafilcon A	Samfilcon A Comfilcon A Enfilcon A	Senofilcon A Narafilcon A

*Could also fall into 5C

Table adapted with permission from Kuc[42] *et al*.

2.4 Cleaning solutions

Daily disposable lenses are worn and discarded after each use. Daily wear lenses can be cleaned after every use and be worn for a week, two weeks, or a month before being discarded. Disinfecting these lenses overnight allows for the removal of any deposits of proteins from the tear film as well as any foreign materials. Extended wear lenses allow for continuous, overnight wear by using a material that allows for more oxygen to reach the ocular surface. Improper care of daily wear contacts may result in infection or irritation to the eye, which, in serious cases, can be sight-threatening[43].

Multipurpose cleaning solutions (MPS) and hydrogen-based cleaning solutions are used to clean, disinfect, and store contact lenses. MPS are also being designed to improve lens wear and some have been reported to increase wettability (when containing poloxamine) and protein removal[44]. The procedure for disinfecting contact lenses is to remove the lenses from the eye with clean hands, drop MPS and each side of the lens, and rub for 2-20 seconds, based on the manufacturers' instructions. This is then followed by rinsing the lens with more MPS, then leaving it to disinfect in MPS for 4 to 6 hours. "No rub" solutions instructed to remove the lens from the eye, rinsing on each side, and then leaving it to disinfect in the solution. While no evidence showed an increase in infection with no rub solutions, studies have shown that rubbing reduces the amount of bacteria that adheres to hydrogel lenses[45]. There is now evidence that CLs have the potential to adsorb and subsequently release components of MPS on the ocular surface. The chemical composition of lenses have been shown to result in different levels of adsorption and release of various components of MPS [46,47]. Components of MPS and hydrogen-based cleaning solutions include biocides, surfactants, chelating buffering agents (see Table 2). They are able to remove proteins and lipids and provide broad disinfection against pathogenic

microorganisms while in the lens case to prevent transfer onto the ocular surface upon lens re-insertion.

Biocides are a major component of contact lens disinfecting solutions. They are added to lens care systems to kill bacteria and fungi. The most common biocides in CL solutions are hydrogen peroxide (H_2O_2), polyhexamethylene biguanide (PHMB), polyquaternium-1 (Polyquad), myristamidopropyl diametylamie (Aldox), and alexidine. PHMB is a large molecule with a strong positive charge. It is effective against both gram-negative and gram-positive bacteria. Due to its ionicity and attraction forces, PHMB has a longer "uptake and release time" which may cause eye discomfort to the lens user. It has been seen in studies that corneal staining increases with PHMB[46,47].

Polyquad is another biocide which, like PHMB, is also cationic, leading to a longer uptake and release time. Polyquad is often combined with other biocidal agents, such as alexidine dihydrochloride, in disinfecting systems. Alexidine dihydrochloride and Polyquad are combined as a dual-acting biocide in RevitaLens OcuTec Abbott Medical Optics (AMO) solution. Most hydrogen peroxide systems contain a platinum-coated disk which lowers the concentration of hydrogen peroxide from 30000ppm to below 100ppm within 6 hours to reduce the likelihood of ocular discomfort.

Surfactants, also known as "surface-active agents", have hydrophobic tails and hydrophilic heads. This amphiphilic structure allows them to be soluble in both polar and non-polar. Due to the surfactant's ability to act as both a surface cleaner and a wetting agent, they can remove surface deposits and also enhance contact lens wettability by lowering the amount of surface tension applied to it.

Chelating agents improve the disinfection and cleaning abilities of contact lens solutions by working with preservatives in the solutions. In current MPS, some common chelating/sequestering agents are ethylenediaminetetraacetic acid (ETDA), citrate, and hyrdroxyalkylphosphonate (hydranate).

To maintain the acidity of a solution, a buffering agent is added. This weak acid or base prevents rapid pH changes when another acid or base is added to the solution. Phosphate, borate, and citrate are commonly used buffering agents in contact lens solutions. While phosphate buffers are more physiologically compatible, this makes them easily contaminable and may promote growth to foreign pathogens. On the other hand, borate buffers have a microbiological advantage, however, their antimicrobial properties negatively affect cell viability[42].

Table 0.2: Most commonly used contact lens solutions in today's market designed for soft contact lenses including peroxide and multipurpose systems

Product	*Preservative/Biocide*	*Chelating Agent*	*Surfactant*	*Buffer*	*pH*
Clear care plus (clear care original has no added hydraglyde)	H2O2	None	Pluronic 174R, Hydraglyde (polyoxyethylene polyoxybutylene)	Phosphate (stabilizer) Phosphonic Acid, Sodium chloride	6.7
Oxysept	H_2O_2	None	HPMC (hydroxypropylmethylcellulose)	Phosphate	
Optifree express	Polyquaternium-1 (Polyquad)/Aldox (myristamidopropyl dimethylamine)	Ethylenediamine-Tetraaceticacid, (EDTA)/Sodium citrate	Tetronic 1304	Boric acid, Sorbitol AMP-95	7.8
Optifree repleniSH	Polyquaternium-1/Aldox 0.0005	Sodium citrate	Tearglyde (Tetronic 1304 + Nonanoyl ethylene-diaminetriacetic acid)	Boric Acid	7.8
Optifree pure Moist	Polyquaternium-1/Aldox 0.0006	EDTA/Sodium citrate	Tetronic 1304/Hydraglyde	Boric Acid, Sodium chloride	
Complete revitalens ocutec	alexidine dihydrochloride 0.00016%/polyquaternium-1 0.0003%	EDTA/Sodium Citrate	Tetronic 904	Boric acid, Sodium Borate, Purified Water	
Complete	PHMB	EDTA	Poloxamer 237	Sodium, Phosphate	7.2
Aquify	PHMB	EDTA	Pluronic F127	Sodium Phosphate	7.2
Biotrue	PHMB/PQ-1 PHMB	EDTA	Hyaluronan; poloxamine	Boric acid, Sodium borate Sodium chloride	7.5
Renu fresh	PHMB	EDTA/Hydranate	Poloxamine	Boric acid, Sodium borate, Sodium chloride	7.3

Table adapted with permission from Kuc[42] *et al*.

2.5 Biocompatibility of contact lens and lens care solutions

Biocompatibility is defined as a material's ability to perform without an appropriate host response in a specific situation. Biocompatibility is often discussed alongside cytotoxicity, a material's ability to induce host cell death. Although a biomaterial may not be considered cytotoxic, it is not necessarily biocompatible as it may poorly integrate into the host environment or lose its function. Thus, a new definition of biocompatibility was formed: "a material's ability to perform as desired without negatively affecting the host while generating the most beneficial response relevant to that therapy"[48]. The cleaning solution and the contact lens have become a system that need to be carefully assessed for biocompatibility. As mentioned above, MPS contain biocides which, while killing pathogenic microorganisms in the lens case, may also affect cells from the ocular surface upon release from the contact lens. Thus, lens care solution interactions with contact lens should be taken into account when performing biocompatibility experiments on MPS and other lens care solutions. A seminal paper in 2010 by Powell [49] *et al*. demonstrated that the uptake and release of the biocide PHMB was higher in non-silicone hydrogel and/or ionic lenses (Etafilcon A (EA),Alphafilcon A, Balafilcon Al) than in nonionic SiHy lenses (Lotrafilcon B, Lotrafilcon (LA), Comfilcon A). Aldox, a biocide used in several MPS, was also shown to be uptaken and released at a higher amount than PHMB with all SiHy lenses. Shortly thereafter, Tanti *et al* showed that SiHy contact lenses soaked in Aldox-containing solution (Optifree Express) resulted in low corneal epithelial cell viability and downregulation of integrin expression, similarly to lenses soaked in PHMB-containing solution (ReNu) [50]. It was hypothesized that the biocide release exposed cells to a high concentration of preservative and disinfecting agent, causing increased cell death or metabolic dysfunction[50]. A

following study by Gorbet et al reported that some of the cytotoxic effects of biocides may also be compounded by the buffering agents (phosphate versus borate buffer), further highlighting the complexity of testing cleaning lens solution/contact lens combinations [51].

To demonstrate that the biomaterial or the lens care solution is not cytotoxic, *in vitro* and in *vivo* tests need to be performed. One previous requirement was the ocular irritation test using the Draize scoring system (with rabbit) and histopathology often followed to determine the presence of an inflammatory response, scarring or toxicity.

The Draize rabbit test, developed in 1944, is the only eye toxicity test officially accepted in the Organization for Economic Co-operation and Development (OECD). The in vivo rabbit test can only be performed as a last step after *in vitro* tests have produced negative results. However, there are several structural, physiological, and biochemical differences between the human and the rabbit eye. Rabbits have relatively low tear production, blink frequency, and ocular surface sensitivity. The anatomy of the rabbit eye is also different from the human eye. The reproducibility of the Draize test has also been found to be poor within and among laboratories[52–54]. As such, research into developing models to assess biocompatibility of contact lens and lens care solutions has shifted to focus on the development of better *in vitro* and *ex vivo* models of the cornea. As such, ocular organotypic models avoid sacrificing animals only for eye irritation tests by isolating fresh eyeballs and corneas from various animals, such as bovine, porcine, chicken, and rabbit, obtained from the slaughterhouse or after euthanasia. For example, the bovine corneal opacity permeability tests and the hen's eggs chorioallantonic membrane tests have been used as alternatives for the Draize test[54]. These tests have been successfully validated by the European Center for the Validation of Alternative Methods along with the Interagency Coordinating Committee on the Validation of Alternative Methods. However, these tests still fall

short with the inability to use human tissue as they do not have predictive capacity for effects on the human ocular surface[52].

The Draize test has been used mostly by contact lens and lens care solution manufacturers to meet FDA requirements. Little research has investigated the effects of CL cleaning solutions and CL combinations using animals. A study undertaken by Tchedre[55] et al evaluated the expression of common eye mucins (MUC1 and MUC16) and the effects of boric acid in MPS solution on mucin expression in Wistar rats after drops of MPS were instilled in their eyes. Upon exposure to boric acid for up to 24hrs/10% MPS, MUC1 and MUC16 were downregulated and impacted the integrity of the corneal epithelial surface. With contact lens material and lens care solutions, a lot of effort has been dedicated to develop better cell culture models rather than organotypic models. While it is recognized that in vivo models provide a physiological environment where various responses can be assessed, the challenges around developing a contact lens that fits the eye of the animal and the lack of blinking or closed eye conditions have also represented significant limitations.

2.6 *In vitro* models for lens care solutions and contact lens testing

Different corneal models using cell culture methods have been developed and vary from monolayer cultures to stratified cell cultures, to epithelium-stroma co-cultures, to more complex tissue-engineered three-dimensional corneal equivalents. Due to their ease of use and lower costs (less than complex stratified or co-culture models), monolayer cell in vitro model have been used extensively to characterize cytotoxicity (live/dead assays, cell counts, etc), and investigate changes in cellular metabolism, cytoskeleton and cellular function as well as characterize cell phenotype. Note that some of these cell characterization methods are also applicable to stratified or 3D models but are more complex to implement. *In vitro* models have been developed using

immortalized and primary corneal epithelial cells from rabbits and in the 1990s human-derived cells began being used. While an ideal reconstruction of a human cornea would contain all 3 parts of the cornea, technical limitations have only allowed for the reconstruction of corneal-like epithelium (HCE). Several models of RCE include EpiOcular™, SkinEthic™, and LabCyte CORNEA-MODEL and MCTT HCE™. EpiOcular™ and SkinEthic are commercially available stratified epithelium which have structural, morphological, and functional similarities compared to the human cornea, they are different in that they use keratinocyte and skin epithelial cells, respectively which are a non-corneal cells. Limitations of these commercially available models are the costs per assay as well as the fact the stratified cultures are grown in small inserts (6 mm in diameter), thus making it difficult to test with contact lens/lens care solution combinations and the lack of dynamic conditions (absence of medium replenishment). A majority of the research investigating lens cleaning solutions and lens cleaning solutions in combination with contact lenses has thus been performed on monolayer and stratified *in vitro* models. Table 2 summarizes the studies published in the past 20 years.

Table 0.3: Monolayer and stratified models of the ocular surface using contact lens cleaning solutions and contact lenses

In vitro model	Materials tested	Incubation time (CL/culture)	Tests performed	Findings	Ref # & year
T-hCEC immortalized monolayer	Complete MPS Easy Rub, Opti-Free Express, Opti-Free Replenish, ReNu MultiPlus and Hank's Balanced Salt Solution as control	50% dilution 30 mins – 24 hours	MTT Cell Viability Assay, TUNEL DNA Fragmentation Assay, Fluorescein Permeability Assay, Tight Junction Protein Staining	Significantly lower cell viability, higher rates of apoptosis, as well as significant disruption of ZO-1 and occludin tight junction integrity in cells Opti-Free Express, Opti-Free Replenish, and ReNu MultiPlus compared to controls after 6-hour exposure	2008[56]
Human corneal epithelial cells (HCEpiC), bovine corneal epithelial cells (BCEpiC)	Renu MultiPlus (A), Opti-Free Express (B), AQuify(C), OPTI-FREE RepleniSH (D)	1 or 2 hours at 40%, 60%, 80%, or 100%	ATP quantitation, Resazurin reduction assay, lactate dehydrogenase release	Cellular ATP reduction and resazurin reduction in all concentrations of MPS B and D and only 100 % for A and C	2009[57]
Monolayer and stratified human corneal-limbal epithelial cells (HCLE)	Opti-Free, Opti-Free Express, Opti-Free Replenish, ReNu MultiPlus, Complete Multi-Purpose. Unisol 4 as control	10 min, 20 min, 60 min all in 100% solution	Live/dead assay, flow cytometry (calcein AM and EthD-1), transepithelial resistance assay	**Monolayer Culture** Disruption of cell junctions with all MPS at 60 min treatment). Cells recovered after 24 hours in new medium except for Opti-Free.	2009[58]

In vitro model	Materials tested	Incubation time (CL/culture)	Tests performed	Findings	Ref # & year
				No cell death with Opti-Free, ReNu MultiPlus, Complete Multi-Purpose	
Monolayer and stratified human corneal-limbal epithelial cells (HCLE)	Opti-Free, Opti-Free Express, Opti-Free Replenish, ReNu MultiPlus, Complete Multi-Purpose. Unisol 4 as control	10 min, 20 min, 60 min all in 100% solution	Live/dead assay, flow cytometry (calcein AM and EthD-1), transepithelial resistance assay	**Stratified Culture** Stratified HCLE cells did not have changed morphology or attachment after any exposure time to MPS compared to control	2009[58]
Monolayer of human corneal epithelial SV40 transformed cell line	Biotrue, AQuify, Complete Easy Rub, Opti-Free Express, Opti-Free Replenish	10 or 30 mins at 50%, 75%, and 100%	ZO-1 immunostaining, transepithelial electrical resistance (TEER), electrical cell-substrate impedance sensing, scanning electron microscopy	Disruption in ZO-1 staining for AQuify, Complete Easy Rub, and Opti-Free Express Decreased TEER after exposure to Complete Easy Rub and Opti-Free Express Increased TEER after exposure to AQuify	2010[59]
Monolayer of the Wong Kilbourne derivative of Chang human conjunctival cells (WKD), ATCC CCL-20.2	Low water soft lens (Balafilcon A, BA), high water soft lens (Vasurflicon A, VA), rigid lens (Ocellus) Opti-Free Express, ReNu, Solocare Aqua, Menicare Plus	Lenses for 96 hours of 20 cycles of 100mL of solution at 100% Cells for 24 hours	Cytotoxicity, cell morphology, fluorescence microscopy, flow cytometry (apoptosis) NR Test (cell viability), Chromatin Condensation Evaluation (Apoptosis), DNA Fragmentation Evaluation (Apoptosis)	Both BA and VA lenses with Opti-Free Express alter morphology to round shape, refringent, and nonadherent, strong decrease in cell viability Both BA and VA lenses with ReNu decrease cell viability	2010[60]

In vitro model	Materials tested	Incubation time (CL/culture)	Tests performed	Findings	Ref # & year
				BA lens with Solocare Aqua decrease in cell viability	
Monolayer of human corneal epithelial SV40 transformed cell line	PureVision and Acuvue Advance lenses Biotrue (Borate-buffered solution, BBS) and Aquify MPS	Lenses incubated in solution for 96 hours Cells incubated in 100% solution for 24 hours	Modified MEM elution assay, ZO-1 immunostaining, transepithelial electrical resistance, *In vivo* biocompatibility assessment	Clinically relevant concentrations of boric acid are not cytotoxic in vitro and did not alter ZO-1 distribution of TEER in both lenses and boric acid combinations	2010[61]
HPV immortalized human corneal epithelial cells (HCEC)	Opti-Free Express, Opi-Free RepleniSH, Complete Moisture Plus, ReNu fresh, and SoloCare Aqua Balafilcon A, Lotrafilcon A, Lotrafilcon B, Comfilcon A, and Galyfilcon A lenses	18-24 hr CL incubation, 24 hr culture incubation (0.1-10% concentration)	Cell viability, flow cytometry (α_3 and β_1 integrin expression, caspase activation)	Opti-Free Express and ReNu (all CL) decreased in viability and downregulated α_3 and β_1 at 5% dilution Caspase activation with Opti-Free Express in all lenses except BA lens Complete and Solo showed a decrease of integrin expression at 10% dilution	2011[51]
human corneal–limbal epithelial (HCLE) cells – monolayer/ stratified	A containing no H_2O_2 at pH 7.0, B containing 0.01% H_2O_2 at pH 7.0, (C) containing no	10, 20 or 60 min (0.1% H_2O_2)	Morphology, live/dead assay (florescence microscopy/ flow cytometry, Fluorescein Leakage Test, Transepithelial Electrical Resistance Assay	Swelling was seen in all formulation on monolayer	2011[62]

In vitro model	Materials tested	Incubation time (CL/culture)	Tests performed	Findings	Ref # & year
	H_2O_2 at pH 7.9, and (D) containing 0.01% H_2O_2 at pH 7.9 and ClearCare			0.1% H_2O_2 damaged cells without recovery to stratified HCLE cells. ClearCare (<0.01% H_2O_2) had no effect	
Monolayer of immortalized human corneal epithelial cells	Lotrafilcon A (LA) or balafilcon A (BA) lenses in Unisol, Opti-Free express, ReNu Multiplus, and Complete Moisture Plus	CL incubated for 18 to 24 hrs of 2mL of 100% solutions cells incubated for 24 hrs	MTT assay (viability), flow cytometry (α_3, β_1, and β_4 integrin expression and caspase activation)	Decreased viability for ReNu with both BA and LA lenses Decreased α_3 integrin expression in ReNu- BA lens and OFX BA and LA soaked lenses No difference in Complete for both lenses Increase in caspase for LA lenses	2011[50]
Human corneal epithelial cells ATCC CRL-11135 (HCEC)-monolayer	Opti-Free Replenish (A), ReNu Fresh Multiplus (B), Complete Multipurpose (C)	15 min on cells, recovery rate assessed after 2 hr 10-40% concentration	Cell integrity after exposure and recovery, metabolic rate, Annexin V-FITC/7-AAD staining and flow cytometry	A showed strong cytopathic effect and disruption of monolayer, B had slight, C had none.	2012[63]
IOBA-NHC conjunctival cells monolayer	Complete, Opti-Free	12 hours at 0.1, 0.5, 1, 2.5 %	Flow cytometry (Apoptosis and necrosis) SELDI-TOF-MS and MALDI TOF-MS (protein profiles)	Opti-free solution leads to more negative side effects than Complete MPS (lower protein expression, increased apoptosis and necrosis)	2012[64]
HCEpiC monolayer, stratified layer	Biotrue (A) Retivalens OcuTec (B), Opti-Free (OF)	50% MPS- 2 and 4 hr incubation,	Viability and apoptosis, ZO-1 staining, Electric cell-substrate impedance sensing,	**Monolayer Culture** MPS B-F were reduced in viability	2012[65]

In vitro model	Materials tested	Incubation time (CL/culture)	Tests performed	Findings	Ref # & year
	Evermoist (C), OF Express (D), OF RepleniSH (E), Synergi (F), All in One Light (G)		Transepithelial electrical resistance,	B/C showed tight junction break down	
HCEpiC monolayer, stratified layer	Biotrue (A) Retivalens OcuTec (B), Opti-Free (OF) Evermoist (C), OF Express (D), OF RepleniSH (E), Synergi (F), All in One Light (G)	50% MPS- 2 and 4 hr incubation,	Viability and apoptosis, ZO-1 staining, Electric cell-substrate impedance sensing, Transepithelial electrical resistance,	**Stratified Culture** MPS B-E showed reduction in viability B-E and G showed tight junction break down	2012[65]
Wong-Kilbourne-derived human conjunctival epithelial cells and corneal epithelial cells from Riken Cell Bank – separate monolayer	ReNu(A), Menicare (B), Regard (C), Ephemere (D), OptiFree Express (E) and Boston Simplus (F)	15, 30 and 60 min incubation	Membrane integrity, Alamar Blue, and MTT	E had greatest reduction in viability, others had no change B,C,D and E induced largest decrease in neutral red signal of membrane integrity Cytotoxic ranking: A and F<C<B<D<E	2012[66]
Immortalized human corneal limbal epithelial cells (HCLE)-monolayer	Opti-Free Express, Opti-Free RepleniSH	MPS dilutions 1-20% for 2,6,18 hr on cell culture	Cell number, metabolic activity, confocal imaging, apoptosis	Opti-Free Express showed greater reduction in metabolic activity, change in morphology, higher levels of IL-6 and IL-8	2012[67]

In vitro model	Materials tested	Incubation time (CL/culture)	Tests performed	Findings	Ref # & year
Primary cultured HCE cells, monolayer	ReNu MultiPlus (A), Opti Free EverMoist (B), Solo-care Aqua(C), Complete (D), Unica Sensitive (E), Options Multi(F), Biotrue (G), COMPLETE RevitaLens (H)	Cells incubated for 12 hours at 30% or 50% solution	Microscopic observation, viability, mitochondria enzyme activity, cytokine (IL)-I ~ (IL-IB), IL-6, IL-8, IL-IO, (TNF-a), and IL-12, PCR, cDNA synthesis, and PCR	A,B,E,F,H stimulated the most pro-inflammatory cytokines and cytotoxic effects C,D,G had less of an effect H_2O_2 based cleaning solutions was found to be less harmful to cells	2013[68]
SV040, Chinese hamster fibroblast cells monolayer	Complete Clear Comfort (1), RevitaLens OcuTec (2), Opti-Free Replenish (3) and Biotrue (4) Acuvue Advance, Air Optix, Biofinity, Pure Vision, Acuvue 2	CL incubation100 mL of MPS for 72 hours with gentile shaking at 5%, 25%, 50%, 75%, or 100% Cells incubation 5mins – 25mins	AlamarBlue (MPS-treated cell viability, cytotoxicity), colony formation assay (cell viability), Zonula Occludens-1 immunostaining (tight junctions), transepithelial electrical resistance	MPS3 (MAPD+PQ-1) is more cytotoxic and less compatible with silicone hydrogel than standard hydrogel	2016[69]

In vitro model	Materials tested	Incubation time (CL/culture)	Tests performed	Findings	Ref # & year
Primary HCEC – monolayer	Opti-Free PureMoist, ReNu fresh, Biotrue and Complete	24 hr solution incubation 18 hours on cells, 1%, 5%,10% and 20% concentration	Cytokine release of Il-1β, Il-6, Il-8 and TNF- α, metabolic activity	Opti-Free PureMoist/ ReNu fresh – reduction in metabolic activity (greatest in Opti Free Pure Moist), increase in IL-1β and decrease in IL-6,Il-8 and TNF- α Biotrue- reduction in metabolic activity increase in Il-1β, Il-6, Il-8 and TNF- α Complete- no reduction in metabolic activity/ cytokine release	2017[70]
HaCaT epidermal keratinocytes cell line – 3D model (stratified layer)	Has yet to test with MPS/CL				2019[71]

As presented in Table 2.3, several research groups have investigated the effects of contact lens cleaning solution alone as well as the combination of cleaning solutions with contact lenses *in vitro*. However, most of the work conducted has used monolayer of corneal epithelial cells and has not always provided much assessment outside of cytotoxicity. It is also evident that a discrepancy exists between data using various models, such as for example between multilayer and monolayers as seen with Cavet[65] et al. Furthermore, a majority of the studies have been performed with MPS alone. One problem with such *in vitro* studies is in selecting the concentration of the solution and period of time that cells should be exposed to. This is especially important as it has been recognized that lens composition plays a significant role in uptake and release of MPS components. Very few studies (no more than 2 as shown in the table 3) have also investigated corneal inflammatory markers (such as integrin expression and cytokine release) following exposure to combinations of cleaning solution and contact lenses and even fewer have actually studied the response of conjunctival cells[64]. These studies[50,51,56,58,60,62,65,66,68,69] further highlight how our knowledge of cell response has been mostly limited to viability and metabolism. The lack of characterization and understanding of ocular cell response to the combination of contact lens and cleaning solution *in vitro* may prevent further development and progress in contact lens technology. One may note from the table that there is also a missing component on all the studies with MPS alone or in combination with contact lens: the absence of tear replenishment or of a dynamic system that mimics the physiological environment of the ocular surface that is continuously replenished with new tears. While some *in vitro* models of the ocular surface have added a dynamic aspect to their models (tear replenishment[72]; friction[73], shear stress[9]), these models have not yet been used assess to the biocompatibility of lens/solution combinations.

While *in vitro* cell models are recognized to be the first steps towards understanding mechanisms involved in cell response to drug and biomaterials, current *in vitro* models of the ocular surface have been limited to corneal epithelial cells that poorly mimic the dynamic environment of the anterior eye. This prevents gaining a better understanding of the immunology of these interactions and the effective development and testing of medical technologies and pharmaceutics. There is thus a need to develop an *in vitro* model that can better mimic the ocular environment, taking into account the various cells present at the ocular surface as well as the dynamic environment provided by tear exchange.

Chapter 3

Effect of curvature on integrin expression of corneal epithelial cells

3.1 Introduction

Cells are known to adapt to the environment that they are exposed to and interact with. It has been demonstrated that cellular behavior is affected by the chemistry, geometry and mechanical properties of their surroundings [1]. Physical properties such as material, topography, and curvature, as well as surface chemistry affect cellular behavior in terms of adhesion, growth and cell differentiation [2]. Mechanical properties such as substrate stiffness and roughness also have similar affects [3]. Mechanical stress, such as shear stress, is also able to influence mobility, shape, phenotype and cell orientation [4]. The biochemical signals of a cell from its interactions with the surface ultimately dictates the decisions of a cell [1]. Understanding the response of cells is important when it comes to the design of implants, scaffolds and *in vitro* models. Once the response of cells in different environments has been investigated, the development and optimization of tissue engineering and *in vitro* models are better enabled.

Corneal tissue is the most transplanted tissue worldwide [5]. The cornea is an avascular and curved multilayered ocular tissue. It serves as the functional barrier to protect the inner ocular tissues from chemical and pathogen incursion and is the major element for maintaining a proper light refraction for vision. The conjunctiva is the mucous membrane that that is thin, transparent, and vascularized and covers the inner surfaces of the eyelids and extends to the cornea. Being the outermost layer of the eye, corneal and conjunctival cells are exposed to various stimuli.

In epithelial cells, integrin a_3 and integrin b_1, are involved in cell migration, adhesion, cell spreading and cell-substrate interaction [6]. Integrin b_1 is also essential for maturation and maintenance of corneal structural integrity [7]. HLA-Dr is a transmembrane heterodimer detected in conjunctival epithelial cells. It is upregulated in response to signaling in the instance of inflammatory stimuli and mediators.

Both human corneal epithelial cells (HCEC) and conjunctival cells (ICONJ) are exposed in situ to various curvatures and recent research suggest that they can sense and react to the curvature at the cellular and multicellular levels[8,9]. Aside from Gouveia *et al* who showed cornea cells are able to self-organized in response to surface curvature, limited research exists on the effects of curvature on ocular surface cells [10]. Postnikoff *et al* also developed a stratified, curved, epithelial model which attempted to assess cytotoxicity [11]. However, how curvature may affect integrin expression, which are important cell adhesion and migration proteins, was not characterized in either studies.

Understanding how topographical cues can control cell behavior is a fundamental question of particular interest for the design of *in vitro* models that can better mimic the physiological environment. Therefore, the purpose of this study was to investigate how the geometry of a curved surface and material affects cornea and conjunctival cells using flow cytometry as it allows for quantification of protein expression by fluorescent antibody tagging. The membrane proteins, integrin β_1 and integrin α_3 are important in cell attachment, wound healing, and cell migration [12] and their levels of expression are also a good indication of substrate biocompatibility [13]. Cell viability and integrin expression were used to assess the effects of curvature on ocular surface cell response.

3.2 Materials/Methods

3.2.1 Reagents and Antibodies

Keratinocyte medium (KM) Keratinocyte growth supplements (KGS) and penicillin/streptomycin (Pen/Strep) solution were purchased from ScienCell (Carlsbad, California). All other cell culture reagents, including Dulbecco's Minimum Essential Medium (DMEM), 1:1 DMEM in Ham's F12 nutrient medium, fetal bovine serum (FBS) and TripLE™ Express were purchased from Life Technologies (Burlington, Ontario, Canada). Phosphate buffered saline (PBS) was purchased from Lonza (Allendale, New Jersey). Monoclonal antibodies to β_1 integrin (CD29), α3 integrin (CD49c), ICAM1 (CD54), and HLA-DR were purchased from Becton Dickinson (Mountain View, CA, USA). Sylgard 184 was purchased from Ellsworth (Stoney Creek, ON, Canada). Collagen, Type I from rat tail was purchased from ScienCell.

3.2.2 *In vitro* cell culture: Immortalized human corneal epithelial cells (HCEC) and human conjunctival epithelial cells (ICONJ)

Immortalized human corneal epithelial cells were cultured in KM supplemented with KGS and Pen/Strep. Immortalized human conjunctival epithelial cells (Innoprot, Derio, Spain) were cultured in KM supplemented with KGS, Pen/Strep and 5% FBS. Fresh medium was added every other day and cells were grown to 90% confluency in a tissue cultured treated flask at 37°C, 5% CO_2, and 95% humidity. Adherent cells were removed using TripLE™ Express, a dissociation solution. Cells were routinely observed for any morphological changes. Only cells below passage eleven were used.

3.2.3 Synthesis of Polydimethylsiloxane (PDMS) curved and concave pieces

Polydimethylsiloxane (PDMS), using Sylgard 184, was synthesized using a 10% ratio (base – curing agent) and poured into custom-made moulds. The casting molds for the curved surfaces were designed by Dr. Saman Mohammadi and Joyce Zhang. They were designed to be used on the OcuCell platform and thus the specifications of the design were to mimic the dimension of the cornea (curvature and diameter) (Figure 3.1). The molds were also designed to reduce air bubble formation during injection of the PDMS. The convex surface (also referred to as the cornea piece/mold) had a diameter of 17 mm and a radius of curvature of 9 mm while the concave surface (also referred to as the eyelid piece/mold) had a diameter of 18 mm and a radius of curvature of 9.2 mm.

PDMS (1mL) was also added to 12 well plates for the "flat PDMS" samples. All PDMS samples were then degassed for 10 minutes and incubated at 67°C overnight to cure. Cornea (convex) and eyelid (concave) pieces and the flat PDMS wells were cleaned in 70% ethanol for 30 minutes and then washed with PBS 3 times with a 1-hour incubation each time. PDMS pieces were then UV-sterilized for 20 minutes. PDMS pieces were then plasma treated and were collagen coated with Type I collagen from rat tail (10% solution) for 1 hour and then washed with PBS 3 times and stored under sterile conditions until required for experiments.

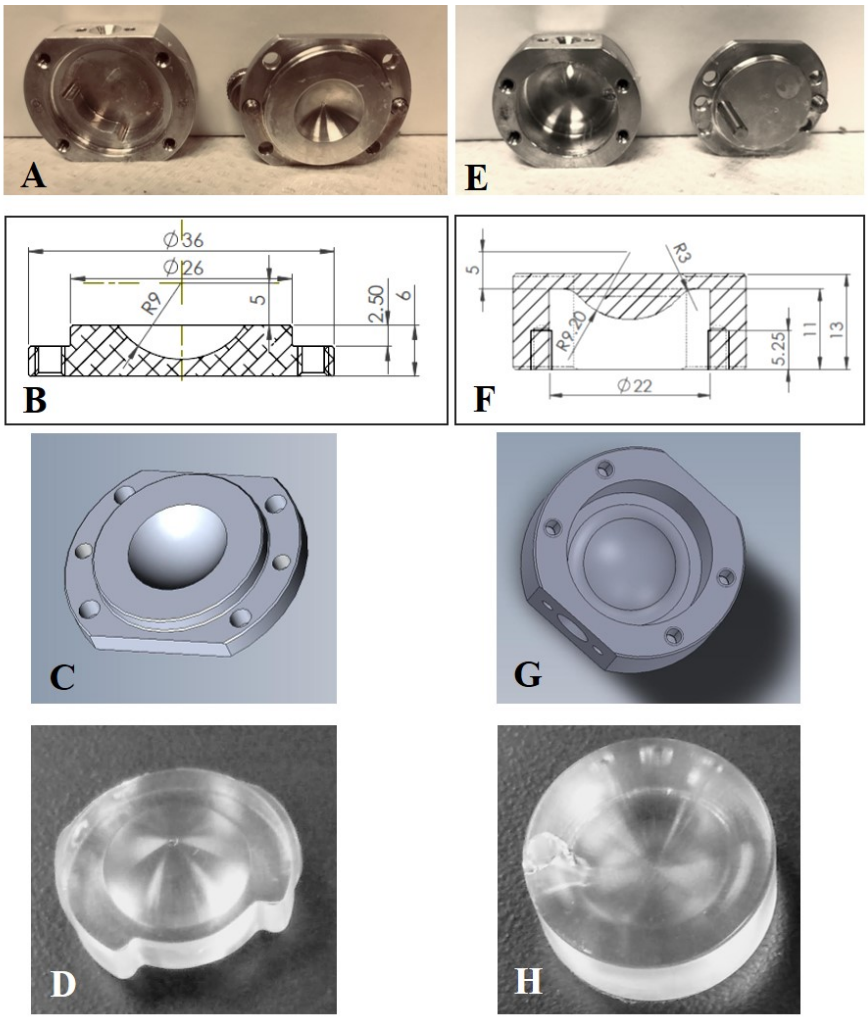


Figure 3.1: Curved casting molds: Cornea mold (A-D) and Eyelid mold (E-H). Casting molds (A and E), CAD drawings with curvature dimension (B and F) and 3D rendering of curved pieces (C and G). PDMS was added to closed molds and allowed to cure overnight at 67°C and PDMS curved surfaces were obtained: cornea piece (convex surface) (D) and eyelid piece (concave surface) (H). Mold design and CAD drawings from Saman Mohammadi and Joyce Zhang.

3.2.4 Seeding the PDMS Eyelid and Cornea pieces and flat PDMS wells

To optimize cell growth on the curved/convex (cornea piece) or concaved (eyelid piece) surfaces, eyelids were seeded with $2.5x10^5$ cells resuspended in 210µL of KM and cornea pieces were placed on top of the eyelid piece and the system was flipped on one side or the other

depending on which surface cells needed to grow on (Figure 3.2). Pieces were submerged in KM and incubated at 37°C for 5 hours to allow the cells to attach to the bottom cornea and eyelid pieces. Tops were removed after 5 hours and cells were then allowed to grow on the bottom surface of the system for 4 days until confluent. Medium was changed every other day. A similar protocol was followed for the flat PDMS in a 12-well plate; the flat PDMS surfaces were seeded with $2.5x10^5$ cells and incubated with 2mL of KM, medium was changed every other day for 4 days.

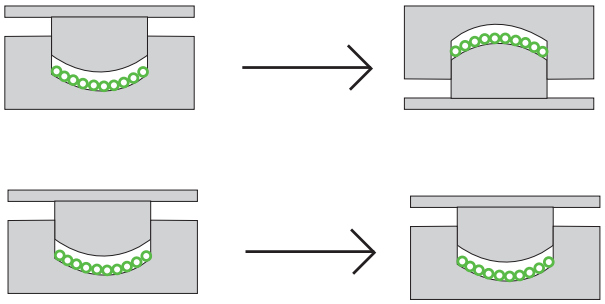


Figure 3.2: Schematic of the cell culture on the PDMS Eyelid and Cornea pieces. Cells were seeded on PDMS pieces. A simple flip in the system allowed to select which side the cells would initially adhere to and then proliferate on.

3.2.5 Seeding the Flat and Curved Tissue Culture Treated Well Plate

Wells of a 12-well tissue culture-treated polystyrene plate (TCPS) were curved using an aluminum mold. The TCPS was gently heated, and the mold was pressed in to create the shape to mimic the curvature of the cornea. TCPS were then plasma treated and were collagen coated with Type I collagen from rat tail (10% solution) for 1 hour and then washed with PBS 3 times and stored under sterile conditions until required for experiments. The flat and curved 12 well

TCPS were seeded at $6x10^4$ cells and incubated with 1mL of KM, medium was changed every other day for 4 days until confluent.

3.2.6 Viability Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) is used to assess cell viability though a reduction reaction. After 4 days of incubation, proliferation and viability was assessed with the MTT metabolic assay, where thiazoyl blue tetrazolium bromide is metabolized by live cells into a solid purple precipitate, formazan crystals. MTT was added to eyelid and cornea pieces and incubated for 2 hours at 37°C and 5% CO_2 to visualize metabolically active cells on the surface (Figure 3.3). The MTT solution was then removed and isopropanol was added to each well and agitated for 2 hours. Aliquots of the dissolved MTT crystal solution was transferred to 96-well plate and read in a UV-Vis spectrophotometer at 595 nm with a reference wavelength of 650 nm. All results are expressed as relative viability compared to control cells; cells incubated in KM in flat TPCS. Cellular metabolic activity determined from the mixed solution is reported in the results section.

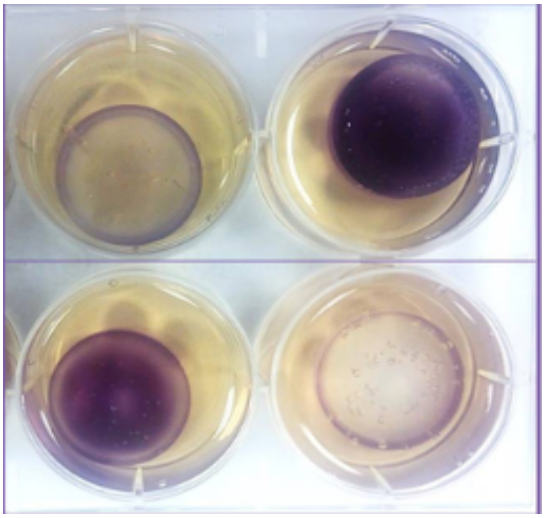


Figure 3.3: Cell growth on curved surface. PDMS corneas and eyelids were made; cells were grown on pieces. MTT was added to visualize coverage.

3.2.7 Flow cytometry

Following the experiments, cells were detached from the PMDS pieces and TCPS in a 20-min incubation with TripLE™ Express. After gentle washing following TripLE™ Express treatment, cells were incubated with fluorescently labelled antibodies against integrin β_1 (CD29), integrin-α_3 (CD49c) and HLA-DR for 30 minutes at room temperature in the dark. Samples were then diluted and fixed using paraformaldehyde (1% final concentration) and analyzed by flow cytometry (BD FACSCalibur, BD Biosciences, San Jose, CA, USA) within 5 days. Samples were acquired on a FACSVantage flow cytometer using CELLQuest Software. At least 5000 events were collected per sample. Analysis was performed with CELLQuest post data acquisition and the geometric mean of fluorescent intensities were recorded and used for statistical analysis

3.2.8 Statistical Analysis

All results are reported as mean ± standard deviation. Samples were compared to cells grown on flat TCPS using t-tests. Significant changes were reported. A p-value less than 0.05 was required for statistical significance.

3.3 Results and Discussion

3.3.1 PDMS curing agent concentration

In order to create a convex and concave cornea-shaped mold, a bottom-up approach was used to identify the best substrate, PDMS samples were synthesized in 6-well TCPS plate. The effect of different concentrations of curing agent (from 5 to 25%) on cell viability was assessed. As shown in Table 3.1, a small increase, albeit not statistically significant (p=0.07), in proliferation and metabolism seemed to occur for the stiffer PDMS (higher concentration of curing agent, 25%) compared to the more compliant substrates (lower concentration of curing agent, 5%). Stiffness of a surface has been known to allow cells to move and adhere across the surface and may have contributed to the increase in viability [14]. The high percentage of viable cells on the PDMS indicates that it was not toxic to cells and provided a suitable environment for proliferation. Microscopic observation (illustrated in figure 3.4) of cells revealed higher confluency of cell grown on the stiffer substrates, although it appeared cells may grow on top of each other (as illustrated in figure 3.4B, with darker cells being seen). Based on the viability results and microscopic evaluation (figure 3.4), the 10% curing agent concentration for Sylgard 184 synthesis was thus selected for the rest of the study.

Table 0.1: Effect of curing agent on corneal epithelial cell viability. Cells were grown on PDMS substrate for 4 days and cell viability was assessed using the MTT assay. Viability is reported as percentage relative to control well (TCPS), mean ± Standard Deviation

Percentage of Curing Agent	**Viability**
Tissue culture plate 0%	100%
PDMS 5%	85% **± 3**
PDMS 10%	95% **± 3**
PDMS 15%	87% **± 4**
PDMS 20%	88% **± 3**
PDMS 25%	91% **± 4**

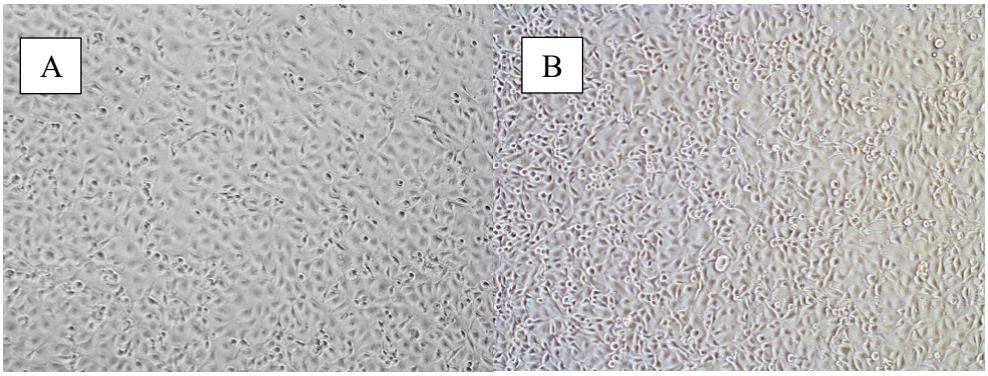


Figure 3.4: Pictures of cells after growth on PDMS substrates. A: 10% curing agent, collagen coated. B: 25% curing agent, collagen coated.

3.3.2 Integrin expression on cells grown on different PDMS curvature

In order to gain an understanding of the effect of curvature (concave and convex) on HCEC, the expression of integrin α_3 (CD49c) and β_1 (CD29) were studied using flow cytometry.

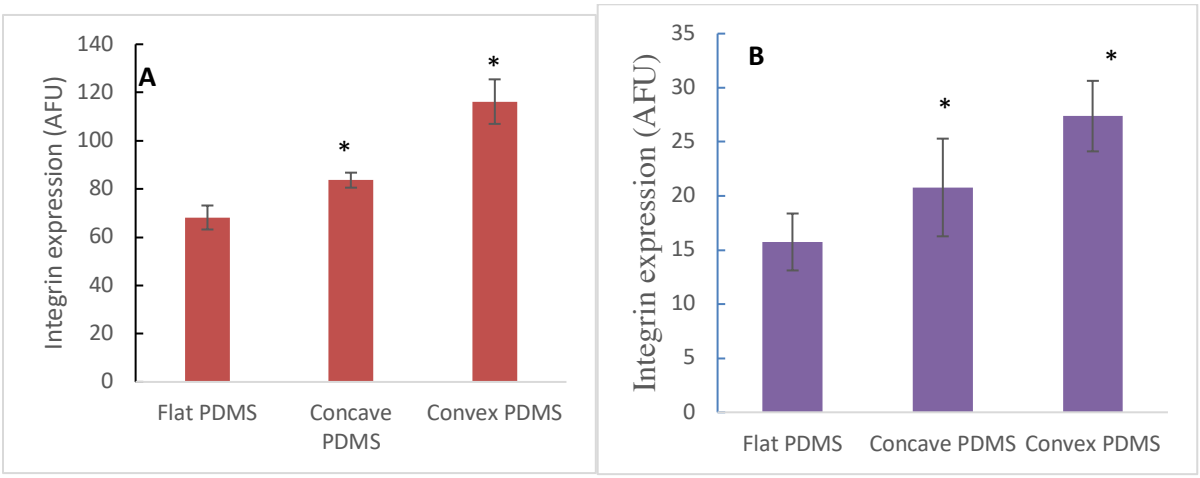


Figure 3.5: Integrin expression of integrin β_1 (A) and integrin a_3 (B) on HCEC cells grown to confluence on various PDMS geometry: flat, concave and convex PDMS pieces. Fluorescence intensities of integrin expression in Arbitrary Fluorescent Unit (AFU) were recorded by flow cytometry. N=4, mean ± SD, * statistically significant from flat PDMS, $p < 0.05$

As shown in Figure 3.5, HCEC grown on the convex and concave pieces showed a significant upregulation in a_3 and β_1 integrin expression compared to that of the flat PDMS. However, interestingly, HCEC grown on the convex PDMS exhibited an upregulation in integrin β_1 but a downregulation of integrin a_3 when compared to cells on the concave PDMS. Integrin- a_3 and β_1 are two important membrane receptors involved in epithelial cell adhesion and spreading [15]. Changes in integrin β_1 may be attributed to the mechanoreceptors [16] and cytoskeletal reorganization [17] in cells. The changes in expression seen between convex and

concave geometry could be attributed to the stress cells might be exposed to when trying to grow upwards on the curve. The upregulation in a_3 on cells growing on the concave piece may have be due to a decrease of cell spreading. The cells may have been forced by their surroundings to grow towards the bottom of the curve and unable to spread outwards which may have caused the upregulation.

No changes in ICAM-1 expression were observed (data not reported). This also confirms previous studies that were conducted in the lab, which indicate that PDMS does not appear to activate corneal epithelial cells [13].

It is also important to note the effect (or lack thereof in this case) of the material between TCPS and PDMS on the corneal epithelial cells. Difference in AFU values were observed between TCPS flat and PDMS flat. Integrin values were 24 ± 2 and 16 ± 3 for $\alpha 3$, and 29 ± 4 and 67 ± 8 for β_1 for TCPS flat and PDMS flat respectively. All experiments were also performed the same days with the same cell passages. The changes in integrin expression between PDMS and TCPS uggests there may be a change in biomaterial compatibility.

3.3.3 Integrin expression on cells grown on different TCPS curvature

The effect of curvature was also studied on a TCPS plate to remove the potential confounding effect of molds being used which may also have a micro-patterning surface effect. The response of conjunctival and corneal epithelial cells was investigated by measuring integrin expression as well as HLA-DR and presented in Table 2. Note that actin staining was also

performed. It was however difficult to acquire clear images due to the curvature of the surface. Images collected can be found in appendix.

Table 3.2: Integrin expression of HCEC and ICONJ cells on flat and convex TCPS surfaces. Fluorescence intensities of integrin expression in Arbitrary Fluorescent Unit (AFU) were recorded by flow cytometry. N=5, mean± SD * statistically significant from flat TCPS, $p < 0.05$

	Integrin β_1	**Integrin a_3**	**HLA-DR**
ICONJ Flat	39.0 ± 2.9	29.5 ± 3.1	12.8 ± 1.8
ICONJ Convex	47.0 ± 4.4*	24.8 ± 2.8	17.8 ± 1.6*
HCEC Flat	28.8 ± 4.4	23.7 ± 2.1	
HCEC Convex	59.4 ± 1.1*	44.5 ± 3.9*	

Similar trends as seen with convex PDMS and upregulation of integrin expression were observed on the convex TCPS. For HCECs on convex TCPS, both integrin a_3 and β_1 expression were significantly upregulated ($p=0.02$). For ICONJ cells, expression of β_1 ($p=0.01$) and HLA-DR ($p=0.02$) also demonstrated a significant difference in upregulation between flat and convex plates. The MHC class II cell surface receptor HLA-DR was also upregulated in ICONJ cells, suggesting that change in curvature may lead to cell activation and induce an inflammatory response in conjunctival cells. Increase in HLA-DR is known to be associated with inflammatory stimulus [17] but had not yet been reported to change with curvature. This warrants further investigation as this would have important implications in the ocular environment where changes in curvatures may occur. While integrin β_1 expression was significantly upregulated on ICONJ cells on the convex surface, no significant changes in α_3 expression were observed in ICONJ cells, which was different from HCEC cells where upregulation of both α_3 and β_1 occurred on HCEC grown on the convex surfaces. These results may suggest that in conjunctival epithelial cells, β_1 may be more on its own rather than in a dimer with α_3 [18] and/or

that HCEC express more β_1. Naylor *et al*. has reported the importance of β_1 in morphology and focal adhesion in epithelial cells [19], which would further support our upregulation results for corneal epithelial cells as morphological changes occur due to the curvature of TCPS (or PDMS). The HCEC adjusted and grew to confluency to fit the curvature of the plate. Topography is generally referring to surface geometry. This includes curvature as well as micro- and nano-scale roughness and topography of the surface. There was an apparent change in AFU signals for the different materials, it could be that the heat-pressed TCPS had the imprint of the micro-topography found on the mold and caused the same reaction in the TCPS as the PDMS. Causing the rise in integrin expression between flat and curved models.

Morphological changes and integrin expression changes related to curvature is essential to explore as keratoconus is an ocular disease that relates to the progressive thinning of the Bowman layer and change in curvature of the cornea to a conical shape [8]. While recent clinical studies have reported increased apoptosis corneal epithelial cells from patients suffering from keratoconus, very few *in vitro* studies have investigated how change in curvature in the eye affect ocular cells [20]. A recent review by Callens *et al* highlight the important role that curvature plays in tissue repair and pathology [21]. Taken together with our results, this would support the importance of further investigation with ocular cells both from a disease perspective and from *in vitro* model design.

3.4 Conclusion

Our study provide evidence that curvature has an effect on corneal and conjunctival cells with cells showing significant changes in integrin expression between flat, concave and convex

surfaces. Our results suggest that geometry should be taken into account when designing new *in vitro* models, and that *in vitro* models may be used to better understand how change in ocular surface geometry (such as during keratoconus or following keratoprosthesis) may affect cell behavior. In future studies, cell apoptosis and necrosis as well as cytokine synthesis should be characterized to evaluate the influence of curvature on cell cycle and the inflammatory response of ocular cells.

Chapter 4

Testing Contact Lens and Cleaning Solution Combinations in Dynamic *In Vitro* Model

4.1 Introduction

The cornea is an avascular multilayered ocular tissue. It serves as the functional barrier to protect the inner ocular tissues from chemical and pathogen incursion and is the major element for maintaining a proper light refraction for vision. Over 125 million individuals wear contact lenses (CL) worldwide [1] and they exhibit a higher rate of eye infection and inflammatory complications compared to non CL wearers[2]. Hence, understanding the effects of CLs on the health and integrity of the cornea is imperative. In the last decade, silicone hydrogel (SiHy) contact lenses were introduced to the market along with several multipurpose solutions (MPS) for ease and effective cleaning and disinfection. To disinfect lenses, biocides such as polyhexamethylene biguanide (PHMB), Aldox or polyquad have been used in MPS while hydrogen peroxide is used in hydrogen peroxide-based lens care systems. Clinical and epidemiological studies have shown that certain combinations of SiHy lenses and MPS are more biocompatible than others[3–6]. There is also evidence that corneal epithelial cell exposure to MPS can cause cell death *in vitro*, either through apoptosis or necrosis [7]. However, with the current methods and technologies available, it remains difficult to evaluate the effects of CL/MPS combinations on the human eye.

Corneal staining with sodium fluorescein (NaFl) is used clinically to assess biocompatibility of lens-solution combinations. However, there is currently a poor

understanding of why hyper-fluorescence and hyper-reflectivity are observed with certain combinations in the eye[8]. In vivo methods to assess biochemical changes or biocompatibility in the cornea include the Draize test and in vivo models with rats and rabbits wearing contact lens. Animal models provide the in vivo ocular environment and more tests are permissible on animals in vivo than in human clinical trials [9]. However, animal studies have also been criticized due to poor repeatability, poor sensitivity, and lack of objectivity [9]. Animal studies have prompted the development of *in vitro* models to test for biocompatibility. Existing *in vitro* models have often been designed as cytotoxicity models for lens cleaning solution and provide little information on the biocompatibility of an ophthalmic material such as a contact lens. Most of the work conducted has used monolayer of cornea cells and has provided varying results across labs. [10,11]

The OcuCell model was developed to address these limitations. Using the initial design from the OcuFlow [12], a simple *in vitro* dynamic model for contact lens testing, the system was modified to allow for cell culture (note that protocol development and initial testing of the model were performed prior to my MASc and are provided in Appendix 1). The OcuCell uses a silicone-based 3D shaped cornea model that is made in a 3D-aluminum mold. The curvature of the model allows the contact lens to be worn in the *in vitro* model similarly to what occurs *in vivo*. The curved monolayer on the cornea model is then mounted on the OcuCell model where medium flows slowly over the system.

We hypothesized that the OcuCell dynamic testing platform may provide a more accurate testing platform to investigate biocompatibility of cleaning solutions and contact lenses. The objective of this study was thus to assess the response of human corneal epithelial

cells when exposed to combinations of contact lens and cleaning solutions under dynamic conditions using the OcuCell testing platform. A SiHy lens (Balaficon A) and a conventional lens (Etafilcon A) were tested with a MPS containing polyhexamethylene biguanide (PHMB (ReNu Fresh) and a hydrogen peroxide cleaning system (Clear Care Triple Action Cleaning). Following a 6-hr exposure to lens-solution combinations, the level of integrin expression on HCEC was characterized using flow cytometry. The membrane proteins, integrin β_1 and integrin α_3 were chosen as they play an important role in cell attachment, wound healing, and cell migration [13] and their levels of expression have also been previously used as an indicator for *in vitro* biocompatibility [7,14–19].

4.2 **Methods**

4.2.1 Reagents and Antibodies

Keratinocyte medium, keratinocyte growth supplements (KGS) and penicillin/streptomycin (Pen/Strep) solution were purchased from ScienCell (Carlsbad, California). All other cell culture reagents, including Dulbecco's Minimum Essential Medium (DMEM), 1:1 DMEM in Ham's F12 nutrient medium, fetal bovine serum (FBS) and TripLE™Express were purchased from Life Technologies (Burlington, Ontario, Canada). Phosphate buffered saline (PBS) was purchased from Lonza (Allendale, New Jersey). Monoclonal antibodies to β_1 integrin (CD29), α_3 integrin (CD49c) and ICAM1 (CD54) were purchased from Becton Dickinson (Mississauga, ON, Canada). Sylgard 184 kit was obtained from Ellsworth Adhesives Canada (Stoney Creek, ON, Canada).

4.2.2 *In vitro* cell culture: Immortalized human corneal epithelial cells (HCEC)

HPV-immortalized human corneal epithelial cells (HCEC) were cultured in keratinocyte medium supplemented with KGS and Pen/Strep (KM). Fresh medium was added every other day and cells were grown to 90% confluency in a tissue cultured treated flask at 37°C, 5% CO_2, and 95% humidity. Adherent cells were removed using TripLE™ Express, a dissociation solution. Cells were routinely observed for any morphological changes. Only cells below passage eleven were used.

4.2.3 Synthesis of OcuCell Samples

Polydimethylsiloxane (PDMS) was synthesized using the Sylgard 184 kit with a 10% ratio (base – curing agent) and then poured into custom-made moulds. The molds were designed to reduce air bubble formation during injection of the PDMS. Samples were then degassed for 10 minutes and cured at 67°C overnight. The next day, cornea and eyelid pieces were soaked in 70% ethanol for 30 minutes and solution was replaced with PBS and changed 3 times for 1 hour each time. PDMS pieces were UV-sterilized for 20 minutes and stored under sterile and dry conditions until required for experiments.

4.2.4 Seeding the Eyelid and Cornea pieces

The OcuCell cornea and eyelid pieces were incubated together at 37°C for 30 minutes with a small volume of PBS (210μL) in between the pieces. PBS was aspirated from OcuCell

cornea and eyelid pieces. To optimize cell growth on the curved/convex (cornea piece) or concave (eyelid piece) surfaces, OcuCell eyelids were seeded with $2.5x10^5$ cells resuspended in 210µL of KM. OcuCell corneas were placed on top of the eyelid piece and the system was flipped on one side or the other depending on which surface cells needed to grow on (chapter 3). Pieces were submerged with KM and incubated at 37°C for 5 hours to allow the cells to attach to the OcuCell corneas and eyelids. Tops were removed after 5 hours and cells were allowed to grow for 4 days until confluent, medium was changed every other day. The cell-laden OcuCell pieces were then used on day 5 for experiments.

4.2.5 Contact Lens and Lens Cleaning Solutions

Silicone hydrogel Balafilcon A (BA; Bausch & Lomb, Rochester, NY, USA) and Etafilcon A (EA; Johnson and Johnson, New Brunswick, New Jersey, USA) were tested. All lenses were obtained in their original manufacturer packaging. Both BA and EA lenses had a base curvature of 8.6 mm, 14.0 mm diameter, and power of 3.00 dioptres. All lenses were used before their expiry date. Whole lenses were used and were not cut before placement in the in vitro model. ReNu Fresh (ReNu, Bausch & Lomb, Rochester, NY, USA) and Clear Care Cleaning & Disinfection Solution (CC, Alcon Canada, Mississauga, ON, Canada) were used under sterile conditions and before their expiry date. The detailed composition of the lens cleaning solutions as reported by the manufacturers can be found in Chapter 5.

The day before the experiment, BA and EA lenses were added to wells containing in 1mL of ReNu or PBS or to the lens basket of Clear Care lens case containing CC solution filled to the line (as per use instructions). Lens treatment was performed for 12 hours aseptically. EA lenses soaked in Phosphate buffered saline was used as a negative control [20]. Previous work has also shown that there was no difference between PBS-soaked EA and BA lenses [7,20] and to accommodate for the number of lenses that could be tested simultaneously with the OcuCell platform, only one lens control was used.

4.2.6 OcuCell Experimental Setup (dynamic testing conditions)

Syringes (10 mL) were filled with KM, connected to the pump with tubing Dow Corning Silastic Laboratory Tubing (0.062" ID x 0.125" OD and 0.032 wall thickness, VWR) and to the syringe pump (Cole-Parmer 75900-50 programmable 6-channel syringe pump) (Figure 4.1) in the incubator (5% CO_2, 90% humidity). The setting up of the OcuCell pieces in the OcuCell testing platform (previously cleaned with 70% ethanol) was performed under sterile conditions. Using sterile tweezers, the cell-laden OcuCell eyelid and cornea pieces were removed from the wells containing KM and carefully inserted in their holders on the Ocuflow module. Contact lenses were added and the cell-laden OcuCell cornea and eyelid pieces on the OcuCell module were then slowly brought closer together and a small drop of KM was added to the system. The tubes were then connected to the OcuCell module and the testing platform was transferred into a plexiglass box and then to the incubator. The pump was placed into the incubator. Then the syringes connected to the tubes were added to the pump and the pump was

started to deliver "tear" flow to the OcuCell module. At a flow rate of 10µL/min. Prior experiments determined this to be the most appropriate flow rate for cell viability (see appendix 1).

Figure 4.1: Cole-Parmer 75900-50 programmable 6-channel syringe pump

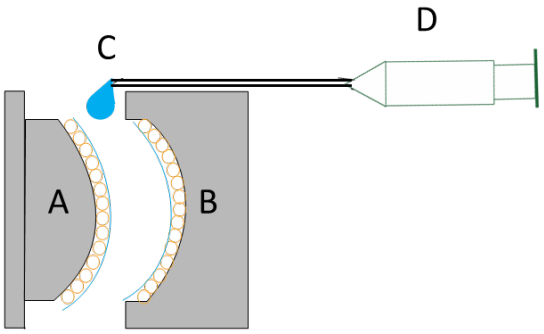


Figure 4.2: Schematic of OcuCell. The OcuCell system includes 2 silicone pieces: the cornea (A) and the eyelid (B) piece with cells grown on each. Artificial tear (KM) (C) flow is introduced through a syringe (D) and rate can be adjusted by setting the pump.

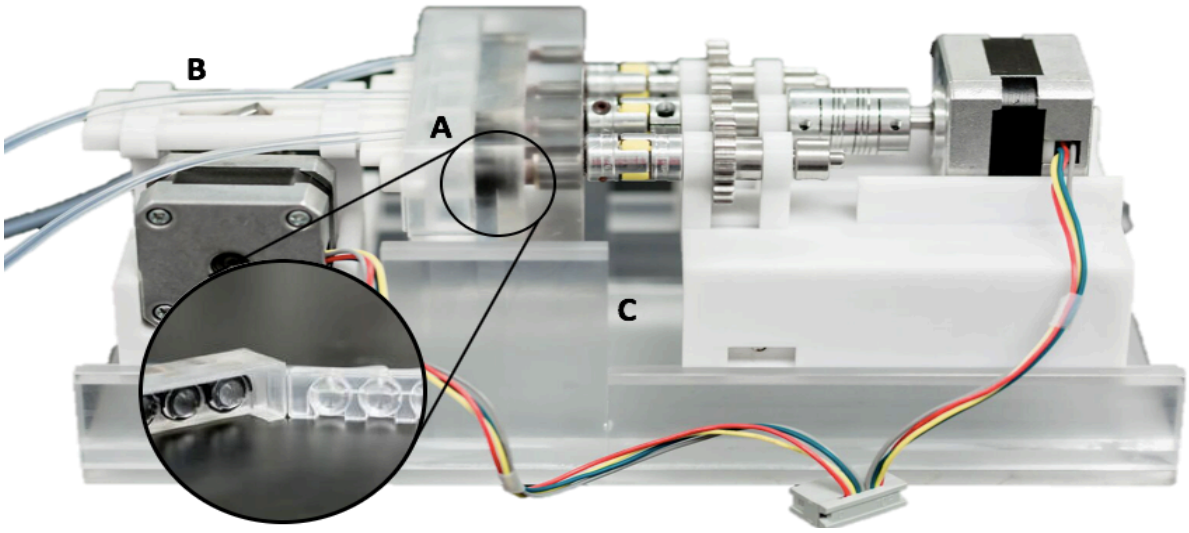


Figure 4.3: Features of the OcuCell *in vitro* eye model: (A) corneal eyepiece and lid housing the contact lens, (B) inlet for tear flow and (C) Collecting plate

4.2.7 OcuCell Experimental Setup (static testing conditions)

Similarly, to the dynamic testing conditions, OcuCell eyelid pieces were set in a 6-well plate, contact lenses were placed in each and 200mL of media was added. A cornea piece was then added on top of the eyelid piece and the pieces were flipped to the cornea side on the bottom. 2mL of media was added and plates were placed in the incubator.

4.2.8 Flow cytometry

Following experiments, cells were detached for 20 minutes from the eyelid and cornea pieces with TripLE™ Express, transferred to a centrifuge tube, washed once and resuspended in DMEM/FBS. Cells were then incubated with fluorescently labelled antibodies against integrin β_1 (CD29) and integrin-α_3 (CD49c) and ICAM-1 for 30 minutes at room temperature in the dark. Samples were then diluted with Hepes tyrode buffer and fixed with paraformaldehyde (1% final concentration). Samples were analyzed by flow cytometry (BD FACSCalibur, BD Biosciences, San Jose, CA, USA) within 5 days. At least 5000 events were collected per sample. Analysis was performed with CELLQuest post data acquisition and the geometric mean of fluorescent intensities were recorded and used for statistical analysis

4.2.9 Statistical Analysis

All results are reported as means ± standard deviation. an ANOVA was performed, followed by multiple pair-wise comparisons using the Fisher LSD test using Statistica V13 (StatSoft, Tulsa, OK). A p value of <0.05 was required for statistical significance. The number of experiments was equal to or greater than four with different cell passages.

4.3 **Results**

As shown in Figure 4.4, with cells on the eyelid piece under dynamic conditions (flow), exposure to BA-ReNu led to significant reduction ($p<0.02$) in integrin β_1 expression when

compared to both EA-PBS (control lens) and EA-ReNu. A significant downregulation in α_3 ($p=0.03$) was also observed with BA-ReNu when compared to the control lens. Under static conditions (no flow), a non-statistically significant reduction in β_1 was observed with BA-ReNu and BA-CC when compared to the control (EA-PBS). No changes in α_3 expression were noted under static conditions on cells from the eyelid piece. Regardless of conditions and lens-solution combinations, no changes in ICAM-1 expression were observed (data not reported). When comparing α_3 and β_1 expression between flow and no flow conditions for the same lens-solution combinations, no statistical differences were observed.

Under dynamic conditions for the cornea piece (Figure 4.5), ReNu-cleaned lenses led to receptor downregulation. A significant downregulation of integrin β_1 expression was observed when cells were exposed to BA-ReNu when compared to control ($p=0.004$); the difference almost reached statistical significance with BA-CC ($p=0.058$). Exposure to BA-ReNu also resulted in significant a_3 downregulation ($p<0.04$) compared to control, BA-CC and EA-CC, while almost significant with EA-ReNu ($p=0.06$). EA-ReNu also significantly reduced β_1 expression on HCEC when compared to both control and EA-CC ($p<0.01$). Under static conditions, similar observations were noted with ReNu. Expression of α_3 and β_1 were significantly reduced in the presence BA-ReNu ($p<0.04$) compared to EA-Renu, EA-CC and control. The conditions (dynamic versus static) had a significant effect and when comparing α_3 and β_1 expression between flow and no flow conditions for the same lens-solution combinations, statistical differences were observed with EA-ReNu (for α_3 and β_1), EA-CC (for α_3) and BA-CC (for β_1).

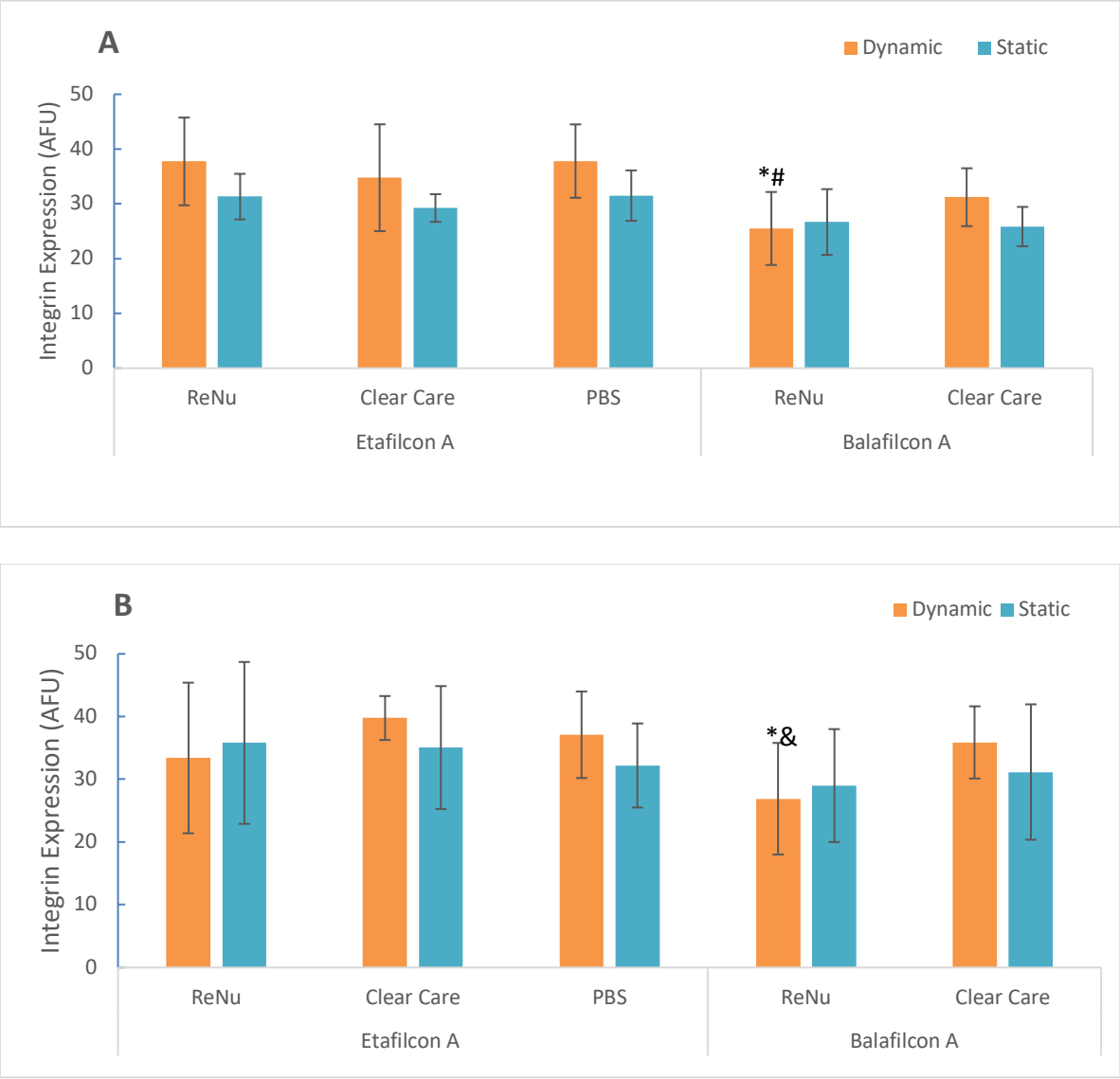


Figure 4.4: Integrin expression (A: Integrin β_1, and B: Integrin α_3) on HCEC on the eyelid piece following exposure to combinations of lens cleaning solution/contact lenses. Contact lenses were incubated with lens cleaning solutions for 12 hrs and placed within OcuCell pieces. HCEC were exposed to lens cleaning solution/contact lens for 6 hours under dynamic conditions in the OcuCell testing platform (flow rate of 10 mL/min) and static conditions (no flow). β_1 and α_3 expressions were measured by flow cytometry and are reported as Arbitrary Fluorescent Units. N= 5, mean ± SD. * statistically significant from control, $p < 0.03$. # statistically significant from EA-ReNu, & statistically significant from EA-CC

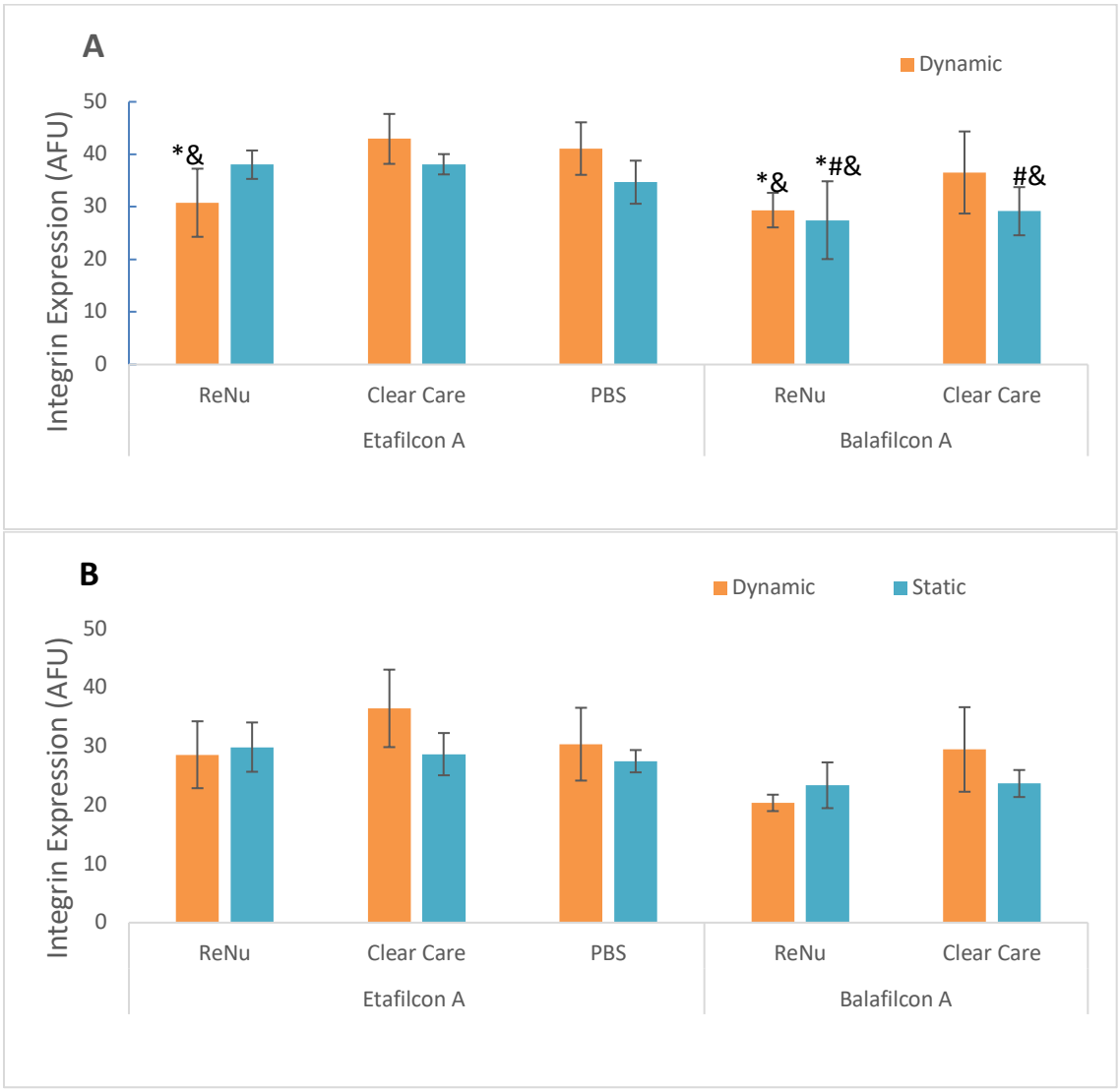


Figure 4.5: Integrin expression (A: Integrin β_1 and B: Integrin a_3) on HCEC on the cornea piece following exposure to combinations of lens cleaning solution/contact lenses. Contact lenses were incubated with lens cleaning solutions for 12 hrs and placed within OcuCell pieces. HCEC were exposed to lens cleaning solution/contact lens for 6 hours under dynamic conditions in the OcuCell testing platform (flow rate of 10 mL/min) and static conditions (no flow). β_1 and α_3 expressions were measured by flow cytometry and are reported as Arbitrary Fluorescent Units. N= 5, mean ± SD. * statistically significant from control, $p < 0.03$. # statistically significant from EA-ReNu, & statistically significant from EA-CC.

To further compare the models and have a more objective view of the importance of dynamic conditions, changes in integrin expression were ranked. Table 4.1 provides a summary of the rankings of the lens-solution combinations based on changes in α_3 and β_1 expression under dynamic and static conditions for both the eyelid and cornea pieces. The most compatible lens and solution combination are associated with a rank of 1 and the least as 4. If combinations rank similar to another combination, their positions would be added and divided by two. The results highlight the consistent trends compared to EA-PBS with reduced integrin expression observed following exposure to ReNu-soaked BA lenses. Most reduced BA-ReNu > EA-RENU> BA-CC > EA-CC least reduced in the dynamic model. In static system the order follows BA-ReNu = BA-CC > EA-CC > EA-ReNu. It further identifies difference that occurs between the two conditions.

Table 4.1: Ranking of integrin downregulation for CL and cleaning solution combinations compared to EA-PBS.

		EA-CC	**BA-CC**	**EA-ReNu**	**BA-ReNu**
Dynamic	Eyelid	3	4.5	4.5	8
	Cornea	2.5	4	6	7.5
	Total	**5.5**	**8.5**	**10.5**	**15.5**
Static	Eyelid	4.5	6.5	2.5	6.5
	Cornea	4	6	4	6
	Total	**8.5**	**12.5**	**6.5**	**12.5**

4.4 Discussion

The OcuCell testing platform was developed to provide a simple dynamic *in vitro* model to investigate biocompatibility of new lens technologies and lens cleaning solution-contact lens combination. The *in vitro* model was previously evaluated using PBS and Benzalkonium chloride (BAK)-soaked lenses and as expected, significant changes in integrin expression and viability were observed with BAK-soaked lenses, suggesting that the OcuCell may be a useful tool to assess cytotoxicity and biocompatibility in a dynamic environment. It was hypothesized that the dynamic conditions would impact ocular cell response *in vitro* as the constant flow would affect the release of compounds that have been adsorbed in the lens during the cleaning cycle as well as expose the cells to a dynamic flow of the compounds being released.

There has been significant controversy around the combination of BA-ReNu due to the solution-induced corneal staining noted clinically [21,22]. The biocide PHMB is part of the chlorhexidine pharmaceutical family that has the ability to fight against a wide range of gram positive and negative bacteria and is the main preservative component in ReNu [23]. The ocular response observed in vivo has been hypothesized to be associated with PHMB that has been adsorbed on the lens and its interactions with corneal epithelial cells [24]. It is now well recognized that the contact lens absorbs MPS components when soaked overnight, which are then released onto the ocular surface post-insertion [25,26]. PHMB has been investigated in a variety of conditions *in vitro* and has been shown to reduce viability, cause higher rates of apoptosis, disrupt tight junction integrity, down regulate integrin expression and increase of proinflammatory cytokines [7,18,20,27–31]. Our results show that under dynamic conditions, ReNu

induced statistically significant changes in integrin β_1 expression in BA and EA lenses compared to PBS-EA lenses.

The decrease in integrin expression of the BA-ReNu combination that is seen in static and dynamic conditions but less so with EA-ReNu and CC combinations can likely be attributed to the variation in the chemical formulas of solutions that affect the uptake and release of biocides and other solutions components from the lens. Studies have shown that PHMB-containing solutions combined with BA contact lenses result in the greatest amounts of biocide absorption. PHMB is a large molecule with a positive charge and has been shown to have a higher absorption along with a slower release rate in ionic lenses than in non-ionic lenses. EA is ionic and high in water content while BA is also high in water content but non-ionic. A study conducted by Powell et al. showed PHMB release of 0.17mg with EA and 0.43mg with BA lenses over 2 hours [32]. This study can thus shed some light on our results as more PHMB being released from BA lenses would likely result in HCEC changes such as reduced integrin expression when comparing different lens types. Furthermore, while PHMB release from EA lenses may be lower, our results indicate that *in vitro* this also resulted in downregulation of integrin. Another contributing factor to the downregulation of integrin induced by ReNu-soaked lenses may be due to the fact that ReNu is a borate-buffered MPS and borate buffer has been shown to impact corneal epithelial cells both *in vitro* [19] and in vivo [33].

The continuous flow of medium being pumped through the OcuCell system may also contribute to some of the difference seen within lens-combinations between the static and dynamic conditions. The constant renewal of medium creates a change in equilibrium in terms

of MPS in the contact lens compared to its surrounding. The environmental osmolarity is constantly renewed by the medium allowing for a continuous/dynamic exchange with the MPS solution in the contact lens and therefore the release of MPS components from the lenses, which can have then affected HCEC in our *in vitro* model. This may be the reason for the toxicity of ReNu being more apparent in the dynamic versus the static system. The dynamic system seems to magnify the effect of ReNu-BA combination compared to EA-PBS than the static system. As BA lenses releases more ReNu, the dynamic system also provides an extra push compared to the static system. The dynamic system also shows the difference between CC and ReNu and shows a significant difference in integrin α_3 and for β_1 (expect for ReNu-EA cornea).

Under static conditions, it was noted that BA-CC also induced a downregulation in integrin expression. This was unexpected as Clear Care is a phosphate buffered solution with H_2O_2 as the active ingredient and previous studies have shown that Clear Care does not reduce viability, nor induce cytokine release or morphological changes [11,34]. H_2O_2 is believed to be quickly metabolized on the ocular surface due to its small molecular weight and is less likely for form a cytotoxic complex [4]. We hypothesized for our results in the static system are likely due to H_2O_2 adsorbed on the contact lens which was not neutralized in the AOSept disc system and is now directly contacting the corneal epithelial cells or being released in a small volume in the static system. As there is no replenishment of this small volume, the active H_2O_2 may damage cells, leading to downregulation of integrin expression [4]. Another hypothesis may be that Pluronic- F127, found in ClearCare, may affect cells. Pluronic F-127 may preferentially absorb on BA and upon release may lead to cell membrane disruption.

4.5 Conclusion

Overall, our results present a novel *in vitro* model, where corneal cells can be exposed to a contact lens-cleaning solution combination in the presence of artificial tear flow using the OcuCell system. This study provides a proof-of-concept of the OcuCell to assess lens-solution biocompatibility, testing two lenses and two lens cleaning solutions. Downregulation in integrin expression was consistently observed with lenses cleaned in the MPS ReNu, which may be due to the release of the biocide PHMB. Future testing with other combinations of contact lenses and lens cleaning solution will also bring further insights into *in vitro* biocompatibility testing. The OcuCell model provides a simple dynamic *in vitro* model of the ocular surface, mimicking some of the dynamic interactions that occur at the interface of corneal epithelial cells and contact lens. This *in vitro* model offers new opportunities for toxicological evaluation of therapeutic, diagnostic and conventional contact lenses and may be able to support the development and testing of new diagnostic lens materials. In the future, the OcuCell system can be further developed to allow extending testing time (such as 12 to 24hrs) and the biocompatibility response with conjunctival cells could also be investigated.

Chapter 5

Effect of crosstalk between cell population: testing contact lens and cleaning solution combination in a monoculture, double culture and co-culture model.

5.1 Introduction

The cornea is a stratified ocular tissue serving as the functional and physical barrier to protect inner ocular tissues from chemical and pathogen incursion and is the major element for maintaining proper light refraction for vision. The eye harbors many bacteria and pathogens that may become infectious to the eye upon loss of epithelium integrity[1] and it is thus important to maintain a healthy ocular environment when a biomaterial, such as a contact lens, is present. Over 125 million individuals wear contact lenses worldwide[2]. While contact lenses are arguably the most successful biomaterial available to the public, lens wearers have been reported to be at increased risk of sight threatening infections and inflammatory events such as microbial keratitis, Acanthamoeba infection, and fungal keratitis[3]. With daily disposable lenses, the contact lenses are discarded each day; however, daily wear contact lenses are worn on a weekly, biweekly, or monthly basis and need to be cleaned daily. By disinfecting the daily wear lenses overnight, proteins, foreign materials or deposits that build up are being removed. Improper care for daily wear contacts may result in infection or irritation to the eye [4].

Multi-purpose solution (MPS) and hydrogen peroxide based (H_2O_2) lens care systems

are used to clean, disinfect, and store contact lenses. Components of MPS include buffers, preservatives, and antimicrobial agents. They are convenient as they require limited contact time with the lenses to provide broad disinfection against pathogenic microorganisms and additive components such as poloxamine have been shown to potentially improve lens wear and protein removal [5–9]. When using a H_2O_2 based lens care system, it is necessary to neutralize the solution to ensure no harm is caused to the eye, which requires a minimum of 6 hrs in the lens disinfection cycle.

There is now evidence that contact lenses have the potential to adsorb components of multipurpose cleaning solutions (MPS) and packaging solutions, which has also been shown a deleterious effects on the ocular surface [10]. The release of adsorbed MPS compounds onto the corneal surface post lens insertion has been hypothesized to be linked to corneal staining and conjunctival hyperaemia following several clinical and epidemiological studies comparing different combinations of SiHy and MPS [11–17]. The concept of lens-MPS biocompatibility has been difficult to define and evaluate clinically as in vivo measurements of biocompatibility remain mostly qualitative (through slit lamp observation) and the mechanisms of corneal staining are not yet well understood [12,13,17,18]. In particular, Solution Induced Corneal Staining (SICS) has been associated more predominantly with one lens-solution combination (Balafilcon A and ReNu/ReNu Fresh) [19]. ReNu Fresh is a MPS which uses dymed (polyaminopropyl biguanide) 0.0001% also known as PHMB as a biocide. The "gold standard" of care has often been recommended to be a solution using hydrogen peroxide (3%) (ClearCare) [20].

In vitro models represent a means to gain a better understanding of lens-cleaning solution interactions. Furthermore, contact lens and MPS effects are mainly studies using corneal epithelial cells, to investigate their effects on the ocular barrier, rather than conjunctival cells. However, studies have shown that corneal cells and conjunctival cells react differently to lens care systems [21–23]. There is thus a need to develop a better understanding of the effects of contact lenses and MPS on the health and integrity of ocular surface cells (namely corneal and conjunctival epithelial cells) and for an *in vitro* model that enable testing in the presence of both cell types.

The objective of this study was to evaluate of the effects of MPS and contact lens combinations on human corneal epithelial cells (HCEC) and immortalized conjunctival fibroblast (ICONJ) cells using three different *in vitro* models: (1) a monolayer of HCEC or ICONJ on a tissue culture treated polystyrene (TCPS) well, (2) a monolayer of HCEC or ICONJ on TCPS well with an insert of the same cell type (double culture) and (3) a coculture model with HCEC on TCPS well and ICONJ on insert. This study used the *in vitro* contact "onlay" model with two different contact lenses and cleaning solutions with a 6-hr incubation time. As integrin β_1 and integrin α_3 are membrane proteins important in cell attachment, wound healing, and cell migration [24] (see chapter 1) and have been previously reported to change upon *in vitro* incubation with contact lenses [21,25–28], their levels of expression following exposure to MPS-soaked lenses were assessed through flow cytometry. Based on previous data and available literature, the hypothesis of this study was that lenses soaked in a PHMB-containing lens cleaning solution will result in altered cell response, all the more so in the co-culture model.

5.2 Materials/Methods

5.2.1 Reagents and Antibodies

Keratinocyte medium (KM) Keratinocyte growth supplements (KGS) and penicillin/streptomycin (Pen/Strep) solution were purchased from ScienCell (Carlsbad, California). All other cell culture reagents, including Dulbecco's Minimum Essential Medium (DMEM), 1:1 DMEM in Ham's F12 nutrient medium, fetal bovine serum (FBS) and TrypLE Express were purchased from Life Technologies (Burlington, Ontario, Canada). Phosphate buffered saline (PBS) was purchased from Lonza (Allendale, New Jersey). Monoclonal antibodies to β_1 integrin (CD29), α3 integrin (CD49c) and ICAM1 (CD54 were purchased from Becton Dickinson (Mountain View, CA, USA). 24 mm diameter polyethylene terephthalate (PET) hanging inserts, 1 μm pore size, were purchased from Millipore (USA).

5.2.2 Contact Lens and Lens Cleaning Solutions

Daily-wear silicone hydrogel balafilcon A (BA; Bausch & Lomb, Rochester, NY, USA) and etafilcon A (EA; Johnson and Johnson, Vistakon, FL, USA) were tested. All lenses were obtained in their original manufacturer packaging. Both BA and EA lenses had a base curvature of 8.6 mm, 14.0 mm diameter, and power of 3.00 dioptres. All lenses were used before their expiry date. Whole lenses were used and were not cut before placement on the cultures.

Phosphate buffered saline was used as a negative control "lens solution" and had been previously evaluated as being biocompatible *in vitro* [29]. Contact lenses were incubated overnight (12 hours) in ReNu Fresh (Bausch & Lomb, Rochester, NY, USA), 1mL in wells of TCPS, or Clear Care (Alcon, Forth Worth, Texas, USA), in the AO disc lens case filled to the line. The composition of the two lens cleaning solutions is presented in Table 5.1.

Table 0.1: Composition of lens cleaning solutions

Product	Preservative/Biocide	Chelating Agent	Surfactant	Buffer	pH
Clear Care Plus	H_2O_2 (3%)	None	Pluronic 174R, Hydraglyde (polyoxyethylene, polyoxybutylene)	Phosphate (stabilizer) Phosphonic Acid, Sodium chloride,	6.7
ReNu Fresh	PHMB aka DYMED 0.0001%	EDTA/Hydranate	Poloxamine	Boric acid, Sodium borate, Sodium chloride	7.3

5.2.3 *In vitro* cell culture: Immortalized human corneal epithelial cells (HCEC) and human conjunctival epithelial cells (ICONJ)

Immortalized human corneal epithelial cells (HCEC) were cultured in KM supplemented with KGS and Pen/Strep. Immortalized human conjunctival epithelial cells (ICONJ, Innoprot, Derio, Spain) were cultured in KM supplemented with KGS, Pen/Strep and 5% FBS. Fresh medium was added every other day and cells were grown to 90% confluency in a tissue cultured treated flask at 37°C, 5% CO_2, and 95% humidity. Adherent cells were then detached using the dissociation solution TripLE™Express (Sigma-Aldrich,

Oakville, Ontario, Canada) and seeded as described below. Cells were routinely observed for any morphological changes. Only cells below passage eleven were used.

5.2.4 Seeding of insert and wells and incubation with contact lens

For individual cell culture plate (ie, one cell type only in plate, either HCEC or ICONJ), HCEC and ICONJ cells were seeded in a 12-well tissue culture treated polystyrene (TCPS) at $6x10^4$ cells per well with 1mL of their respective cell medium. Medium was changed every other day for 4 days. On the 5^{th} day, when cell had reached confluence, media was changed, and contact lenses soaked in MPS or PBS were added face-down (concave surface facing upwards); lenses were fully immersed in media (1mL) (Figure 5.1). MPS-soaked lenses were incubated with cells for 6 hours.

For double culture (HCEC+HCEC or ICONJ+ICONJ) or co-culture (HCEC-plate +ICONJ - insert), HCEC and ICONJ cells were seeded onto a 12-well TCPS and cells were also seeded on inverted PET inserts at $6x10^4$ cells (Figure 5.2). Cells on inverted inserts were allowed to adhere for 5 hours before being flipped into the respected well culture (Figures 5.2). As with the monoculture TCPS model, medium (1mL) was changed every other day between plate and insert for 4 days until the cells reached confluence. On the 5^{th} day, media was changed, the insert was quickly lifted and MPS or PBS soaked lenses was added face-down (concave surface facing upwards), the cell-laden insert was carefully lowered, and the system was incubated for 6 hours (Figure 5.1B and 5.1C).

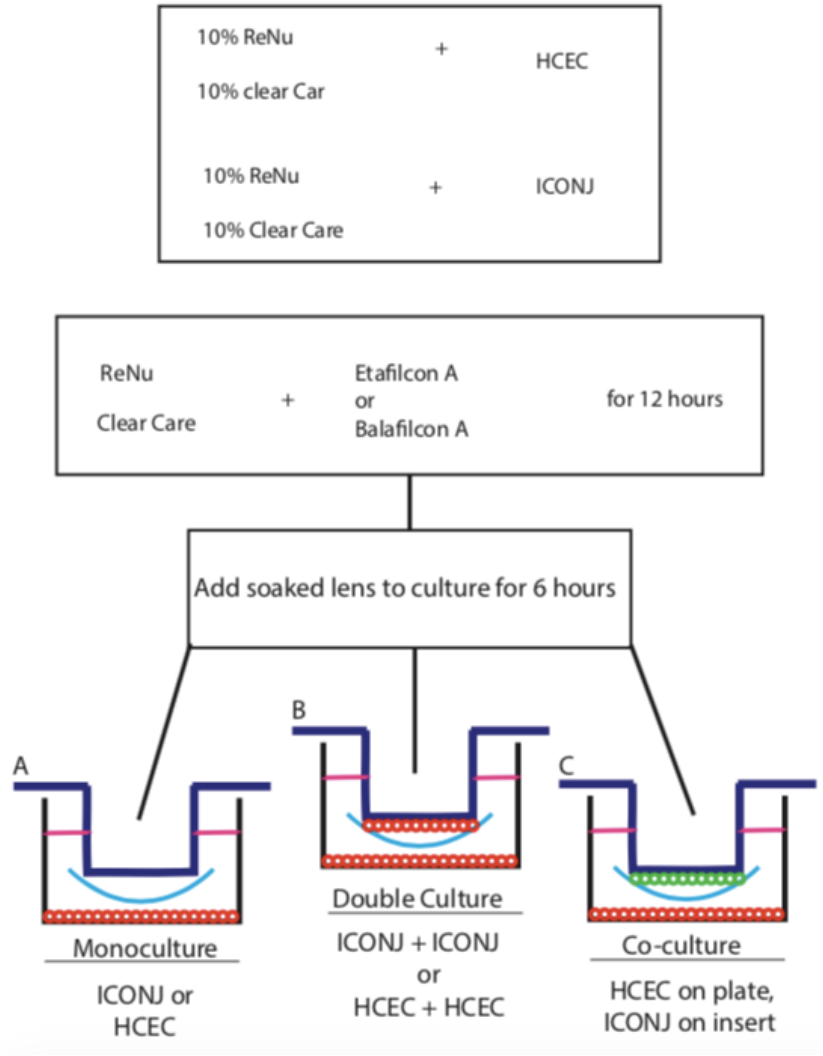


Figure 5.1: Flow chart of steps. The 3 different models being used, monoculture of ICONJ or HCEC cells (A), double culture of both ICONJ or HCEC cells on PET insert and TCPS well (B) or co-culture model with HCEC cells on TCPS well and ICONJ cells on PET insert (C).

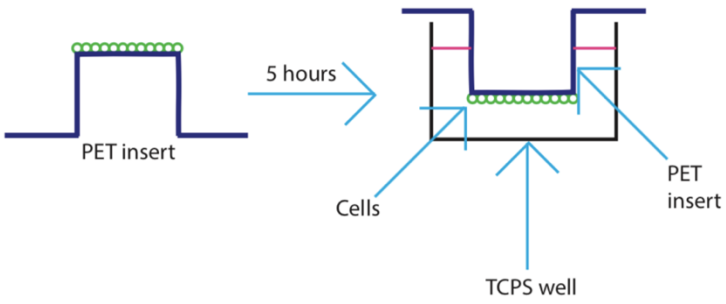


Figure 5.2: Inverted seeding and flip of PET insert into TCPS well

5.2.5 Flow cytometry

Following incubation, cells were detached for 20 minutes from the TCPS wells and PET inserts (TripLE™ Express (Sigma-Aldrich, Oakville, Ontario, Canada). After gentle washing following TripLE™ Express treatment, cells were incubated with fluorescently labelled antibodies against integrin β_1 (CD29), integrin-α_3 (CD49c), ICAM-1 (CD54) and HLA-Dr (BD Biosciences, USA) for 30 minutes at room temperature in the dark. Samples were then diluted and fixed using paraformaldehyde (1% final concentration) and analyzed by flow cytometry (BD FACSCalibur, BD Biosciences, San Jose, CA, USA) within 5 days. Samples were acquired on a FACSVantage flow cytometer using CELLQuest Software. At least 5000 events were collected per sample. Analysis was performed with CELLQuest post data acquisition and the geometric mean of fluorescent intensities were recorded and used for statistical analysis

5.2.6 Statistical Analysis

All results are reported as means ± standard deviation. an ANOVA was performed, followed by multiple pair-wise comparisons using the Fisher LSD test using Statistica V13 (StatSoft, Tulsa, OK). A p value of <0.05 was required for statistical significance. The number of experiments was equal to or greater than three with different cell passages.

5.3 Results and Discussion

As shown in Table 5.2, cells exposed to PBS-soaked Etafilcon A lens showed no significant difference in integrin expression when compared to no-lens. Similar results have been observed with other contact lenses soaked in PBS [25] and suggest that the results presenting herein indicate that the changes expression of cells is induced by the products released from the cleaning solution-soaked lenses and not the presence of a lens itself. It is also important to note the marginal difference between TCPS and PET material. There is a small but not statistically significant increase in integrin expression seen in all integrin expression. This could be due to difference in cell interactions with the material (tissue-culture treated polystyrene versus polyethylene terephthalate), or to cells being exposed to growing against gravity (growing on the bottom of the insert) or cells being exposed the air-liquid interface as no medium was added to the top of the insert. A noteworthy change is present in α_3 integrin expression in the co-culture itself where a significant downregulation was observed on HCEC when cultured in the presence of ICONJ cells. Conversely a significant downregulation in β_1 integrin expression was observed on ICONJ cells co-cultured with HCEC. This suggests that even in the absence of inflammatory stimulus or

foreign material, mediators are being released and cross talk within the co-culture is happening and leading to significant changes in cell membrane receptor expression.

Table 5.2: Integrin expression on cells grown on TCPS (cell culture plate) and PET (insert) in the presence or absence of a PBS-soaked lens Etafilcon A lens (PBS-EA). Results are reported as Arbitrary Fluorescent Units (AFU), mean ± standard deviation, N=4

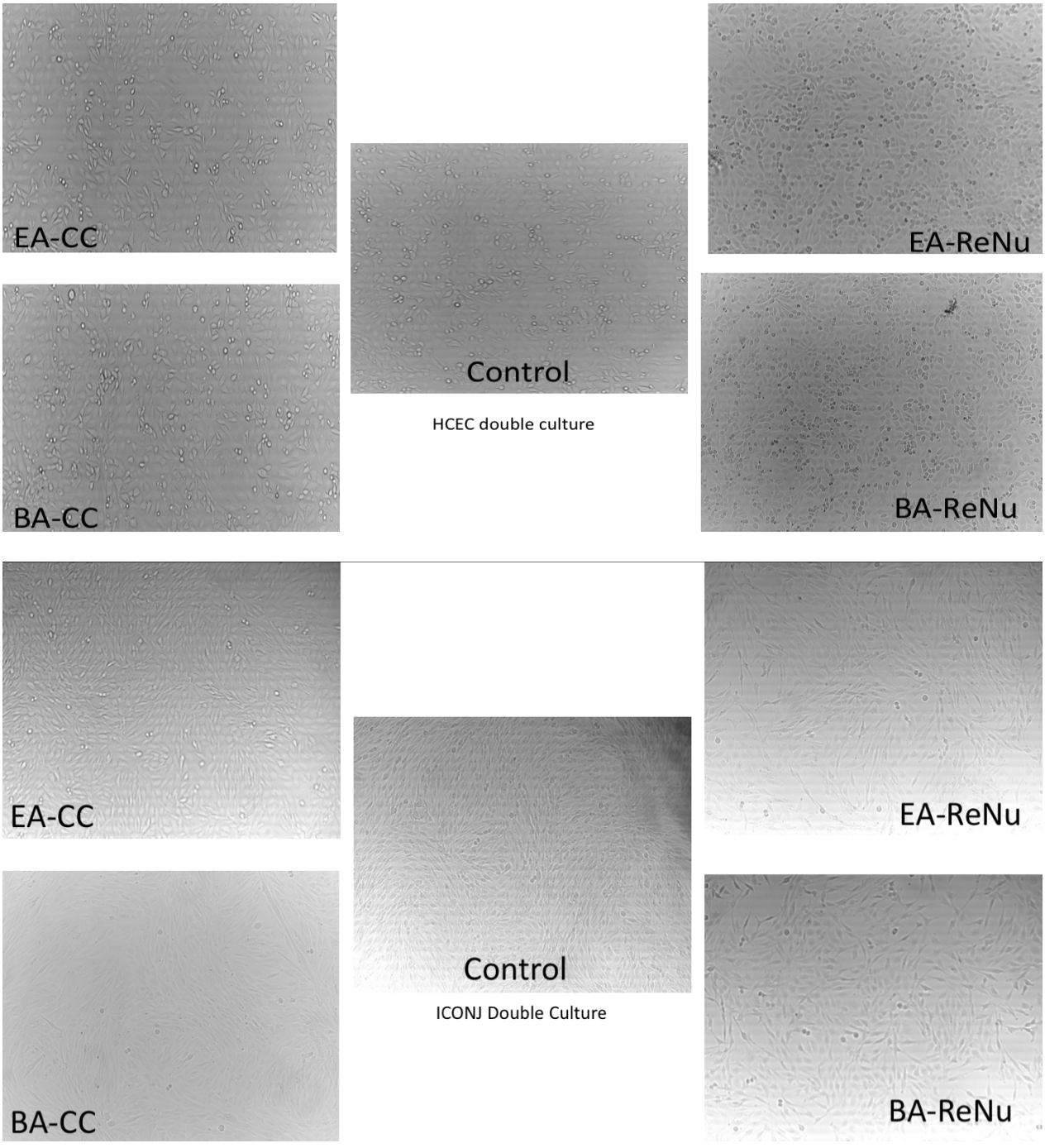
		β_1 integrin		α_3 integrin	
Condition	**Substrate**	**No lens (AFU)**	**PBS-EA (AFU)**	**No lens (AFU)**	**PBS-EA (AFU)**
HCEC	TCPS	44 ± 3	46 ± 2	37 ± 2	42 ± 4
+HCEC	PET	55 ± 5	54 ± 4	47 ± 5	45 ± 2
ICONJ	TCPS	62 ± 7	57 ± 5	14 ± 2	16 ± 4
+ICONJ	PET	71 ± 3	69 ± 7	27 ± 3	32 ± 3
HCEC	TCPS	38 ± 5	35 ± 2	21 ± 3*	23 ± 4*
+ICONJ	PET	46 ± 7*	44 ± 5*	25 ± 4	28 ± 4

* significantly different from same cells grown in a double culture model, $p<0.03$

Upon cell interactions with contact lenses, some changes in cell morphology were observed during incubation with BA- ReNu for both HCEC and ICONJ cells, as illustrated in Figure 5.3. Cells appeared more round than the control and with ICONJ cells, empty space was visible, indicating the potential loss of cells. This was also noted with EA-ReNu and ICONJ cells. Microscopically, it appears that ReNu is more toxic to cells in both EA and BA lenses. It should also be noted that the in combination with BA lenses, both CC and ReNu seem to harm cells more than with EA lenses. It is important to note the difference of integrin expression between TCPS and PET material. Overall, there is an increase in both integrin expression in both HCEC and ICONJ cells between TCPS and PET. This is likely attributed

to the change in material surface chemistry, topography and materials rigidity between PET versus TCPS. The increase may also be attributed to gravity acting on cells growing upside down on the PET insert.

Figure 5.3: Microscopic pictures taken of double culture models. Solution soaked contact lenses were incubated with cells for 6 hours. Control: no lens/no solution



The response of HCEC to lens solution combinations in the three *in vitro* models is reported in Figure 5.4. In the HCEC monoculture, regardless of contact lens material or lens cleaning solution, no change in integrin β_1 and α_3 expression were observed. This is in contrast to what was previously observed in Tanti [27] et al, where after an 8hr incubation with BA-ReNu, a 25% reduction in integrin expression was observed. Such a difference in results can likely be explained by the shorter contact time (6hrs versus 8hrs) as well as the difference in the surface area of exposure (12 well plate versus 24 well plate) and volume of medium added and thus dilution of potential cytotoxic compounds. On the other hand, in the double culture model (where a lens was inserted between cells grown on TCPS and a transwell insert), significant changes in α_3 integrin expression on HCEC on TCPS ($p<0.0001$) were observed for all combinations. Significant downregulation in β_1 integrin ($p<0.003$) was also induced by the presence of EA-ReNu and BA-CC. Furthermore, exposing HCEC to lens solution combination in the presence of ICONJ cells (in the co-culture model) resulted in a significant downregulation of both integrins β_1 and α_3 when compared to control (EA-PBS). More than a 30% reduction in expression was observed on HCEC exposed to BA-ReNu. When comparing mono and double culture to the co-culture model, it is evident that the presence of ICONJ in the *in vitro* model had a significant impact on HCEC response to the lens-solution combination. In all cases, a significant downregulation in integrin expression on HCEC was present when comparing mono to co-culture system. This suggests that the conjunctival cells present in the model may be releasing inflammatory mediators, which result in the downregulation of integrin expression. The change in integrin expression in the co-culture model during incubation with contact lenses provides further evidence of crosstalk

between ICONJ and HCECs (which had been observed in Table 5.2). Paduch et al[30] investigated corneal and conjunctival cell response in co-culture and also found a change in inflammatory cytokine and nitric oxide secretion between mono and co-culture models. Upon co-culture in a similar setup than our model (HCEC on TCPS and ICONJ on PET insert), their results suggests that a situation of inflammation may lead to increase release of IL-1β, IL-6 and IL-10[30]. How the release of potential inflammatory mediators leads to downregulation in integrin expression will require further investigation as there is currently limited research reporting quantitative evaluation of integrin expression on HCEC and ICONJ. Noteworthy is the fact that the downregulation was mostly observed in HCEC (see Figure 5.5 for integrin expression on ICONJ cells) which also suggests that different mechanisms regulated integrin expression in HCEC and ICONJ cells.

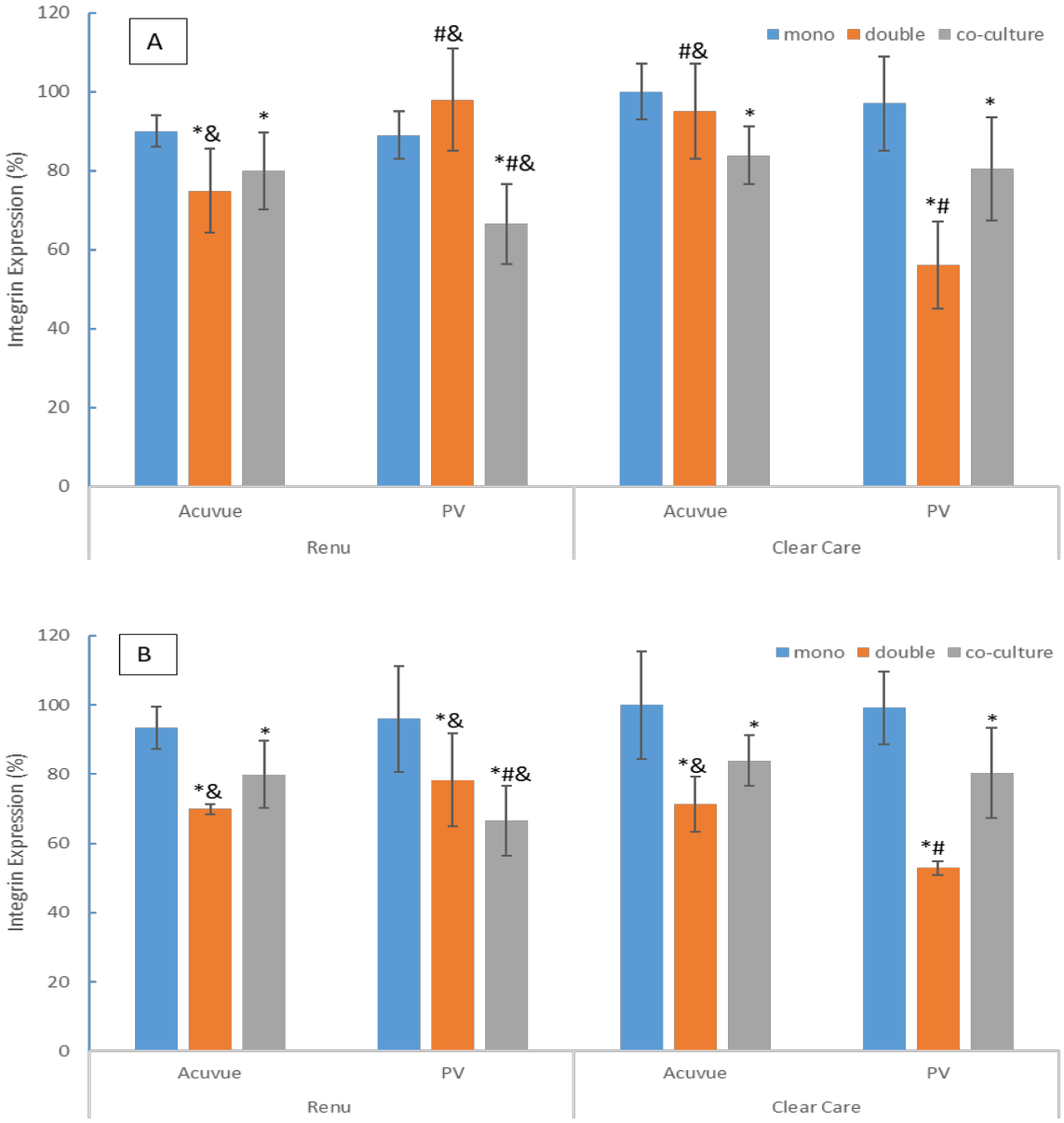


Figure 0.4: Integrin expression (A: Integrin β_1, and B: Integrin α_3) on HCEC on TCPS following exposure to combinations of lens cleaning solution/contact lenses in various cell culture models. Contact lenses were incubated with lens cleaning solutions for 12 hrs. HCEC were exposed to lens cleaning solution/contact lens for 6 hours in mono, double culture or co-culture (with ICONJ) model. β_1 and α_3 expressions were measured by flow cytometry and are reported as a percentage relative to

geometric mean expression of cells exposed to control lens (EA-PBS). N= 3 to 5 mean ± SD. * statistically significant from control, p <0.03., # statistically significant from EA-ReNu, & statistically significant from BA-CC.

As shown in Figure 5.5, similarly to HCEC results, exposing ICONJ cells in monoculture to contact lenses cleaned in ReNu or Clear Care did not lead to any significant changes in integrin expression (only β_1 integrin expression are reported as α_3 expression was not recorded in mono culture in these experiments). In the double culture model of ICONJ (ICONJ on TCPS and insert with lens in between), a small non-statistically significant downregulation was observed in some cases with ICONJ cells on the insert. Note that similar results were observed with ICONJ cells on TCPS but are not reported as the ICONJ cells response on the insert is more relevant for comparison with the co-culture system where ICONJ cells are present on the insert and not on TCPS. As observed with HCEC and discussed earlier, crosstalk between conjunctival and corneal epithelial cells is present in the co-culture system and interactions with contact lens material soaked in lens cleaning solution appears to lead to significant cell disruption in the co-culture model, especially in the case of BA-ReNu. Results indicate a 30% downregulation of integrin expression in co-culture versus non-significant downregulation in other models: mono culture (no downregulation) or double culture (a 10% decrease). As previously discussed in Chapter 4, the downregulation in integrin expression is likely induced by the release of PHMB and borate buffer present in the ReNu MPS solution and that is adsorbed by the BA lenses during the cleaning cycle and released upon contact/incubation in the *in vitro* model[31]. Based on our knowledge, this is the first time that such results are reported in a co-culture *in vitro* model.

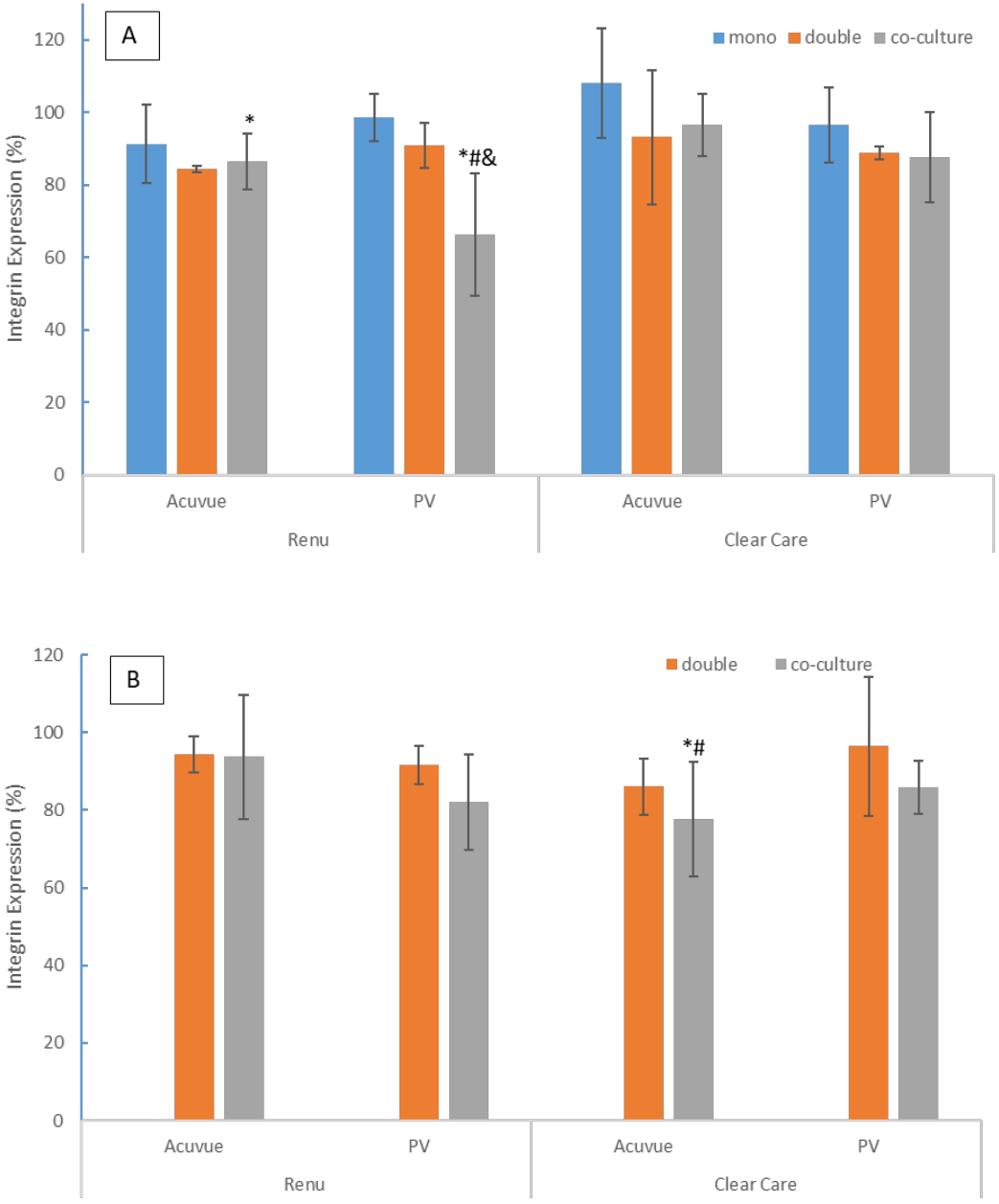


Figure 5.5: Integrin expression (A: Integrin β_1, and B: Integrin α_3) on ICONJ cells on PET insert (double and co-culture) and TCPS (mono culture) following exposure to combinations of lens cleaning solution/contact lenses in various cell culture models. Contact lenses were incubated with lens cleaning solutions for 12 hrs. ICONJ cells were exposed to lens cleaning

solution/contact lens for 6 hours in mono, double culture or co-culture (with HCEC) model. β_1 and α_3 expressions were measured by flow cytometry and are reported as a percentage relative to geometric mean expression of cells exposed to control lens (EA-PBS). N= 3 to 5 mean ± SD. * statistically significant from control, $p < 0.03$., # statistically significant from EA-ReNu, & statistically significant from BA-CC

As a control, experiments were also performed with exposing cells to diluted solutions. ReNu and ClearCare (following an overnight cycle in the lens case system as previously described for lenses) were added to the monoculture (10% final concentration). No significant changes in integrin expression were observed with ClearCare, further confirming that during the cleaning cycle, the H_2O_2 in solution was fully neutralized by the AOSept disc. This further indicates that the downregulation in integrin expression is due to the interaction with the lens and compounds released by the lenses during the incubation in the various models. HCEC exposure to ReNu resulted in a 20% downregulation in integrin expression (Table 5.3). Previous studies, including our own[25], have reported similar results. Tanti et al reported a greater downregulation in downregulation, however incubation was performed for 24 hours while in our study, cells were only exposed to ReNu (10%) for 6 hours[27]. It is interesting to note that neither ReNu nor Clear Care affect ICONJ cells. This further highlight the difference in inflammatory mechanisms in ocular cells and the needs for further testing in co-culture models to gain a better understanding of the potential inflammatory response of ocular cells as a system.

Table 5.3: Cell response to exposure to lens cleaning solution (10% final dilution). ReNu and Clear Care (after cycle in the AOSEPt system) solutions were added to cells for 6 hours compared to no solution, and integrin expression was assessed by flow cytometry. N=2± SD

	Condition	**Integrin α_3**	**Integrin β_1**
HCEC	ReNu 10%	78% ± 5	80% ± 3
HCEC	Clear Care 10%	98% ± 2	96% ± 6
ICONJ	ReNu 10%	98% ± 5	97% ± 6
ICONJ	Clear Care 10%	105% ± 4	93% ± 4

5.4 Conclusion

Currently, there is no single *in vitro* test that has been accepted as an alternative to the Draize test and the prospect of eliminating whole animal testing remains a challenge as *in vitro* models that can better mimic interaction with the ocular surface are being developed. This study is the first step to building a more complete *in vitro* model with including two cell population present on the ocular surface. Our results provide evidence that a co-culture model using conjunctival and corneal epithelial cells may be better able to assess biocompatibility of lens-cleaning solution combination compared to a mono-culture cell model. Significant downregulation in integrin expression was observed with the BA-ReNu combination, suggesting that the uptake and release of PHMB may play a role in the observed cell response. Future work should focus on the crosstalk between corneal and conjunctival cell response to identify the inflammatory mediators inducing changes in conjunctival and corneal epithelial cells. It would also be important to characterize cell death and apoptosis. Further studies should also be performed with other lens-solution combinations to confirm the effect observed in the co-culture system with the two lens-solution combination studied. This novel co-culture

incubation model may allow to better understand the inflammatory response induced by contact lens and lens cleaning solutions.

Chapter 6

Conclusion and recommendation for future work

6.1 Conclusion

In chapter 3, the hypothesis was that a change in curvature would result in increased stress on cells and inlead to cell response which can be assessed by measuring integrin expression. In this chapter, the importance of material and curvature as it relates to corneal and conjunctival epithelial cells was demonstrated. An upregulation in integrin expression was observed with cells grown on convex versus concave or flat surfaces, with no difference between TCPS and PDMS material. These results have important implications in the design of in vitro model (ie the geometry of the model matters) as well as for research in disease such as keratoconus where the curvature of the cornea changes over time.

For chapter 4, it was hypothesized that a dynamic in vitro model would result in significant difference in HCEC response to lens-solution combinations compared to a static incubation model. This led testing the OcuCell platform under dynamic and static conditions. The dynamic model led to a greater difference when testing combination of contact lens-cleaning solution combination compared to the static model.

Lastly, in chapter 5, it was hypothesized that a co-culture model with conjunctival and cornea cells would result in significant difference in HCEC and ICONJ responses to lens-solution combinations compared to single cell population model. The experiments presented in Chapter 5 with monoculture, double culture and co-culture models demonstrated the importance of cell crosstalk in response to a biomaterial. Integrin expression showed a

greater downregulation in integrin expression when exposed to the same combination of contact lens-cleaning solution compared to mono and double culture. These results highlight that to gain a better understanding of lens-solution interactions at the ocular surface, co-culture models should be further explored.

In all our in vitro models, the combination of BA-ReNu consistently led to reduced integrin expression compared to our control lens. This is in agreement with many other in vitro studies and suggest that the release of PHMB and other components of ReNu significantly affect corneal and conjunctival epithelial cells in vitro. Our studies also identified that under certain experimental conditions, lens combination with ClearCare were not as biocompatible as expected *in vitro*, suggesting that H_2O_2 adsorbed on contact lens material may not have been neutralized during the overnight treatment with the AOSEPT disk.

6.2 Future Work

One of the limitations of this study was from only characterizing integrin expression. While flow cytometry allows for rapid analysis of multiple characteristics for each cell by quantification of fluorescence intensities. Cytokine synthesis and cell apoptosis and necrosis should be measured to further understand how curvature may affect the cell cycle and inflammatory response of the ocular surface. Microtopography also needs to be assessed on the curve samples. Further research is also needed with the co-culture model to better characterize the cell response and identify the inflammatory mediators that are being released and lead to a downregulation of integrin expression. It would also be worthwhile to

determine if the co-culture leads to change in cell apoptosis as well as changes in tight junctions. In terms of building a more accurate static model of the ocular surface, it may also be important to consider the role of neutrophils in the eye and how they interact with conjunctival and corneal cells as well as ophthalmic materials. The current *in vitro* model can be used with neutrophils as they could be easily added to the co-culture model.

The OcuCell platform represent a simple and cost-effective system for dynamic conditions. From a design perspective, to help during experimental setup, it would be important to consider the following modifications:

- Changing the hole tear fluid comes out from to be a diagonal instead of horizontal. This would allow easier insertion and potentially less cells to be washed off by flow.
- Decreasing the size of cornea and eyelid slightly to fit into a 12 well plate, this would allow for easier growth for cells as well as less media used.
- Find a material better than PDMS that allows for greater cell growth and adherence

To better mimic the ocular surface, the OcuCell platform could also be used to grow conjunctival cells on the eyelid piece of the OcuCell and would thus provide a co-culture model to test ophthalmic materials in a dynamic system. Flow time should also be extended for 12-24 hours and test various ophthalmic materials and combinations of CL and cleaning solutions.

Further investigation should also be performed for contact lens and cleaning solution combination. First, it would be important to test other lens-solution combinations, such as other SiHy and conventional lenses as well as other MPS (with or without PHMB as the biocide) to investigate how our models are able to characterize cell interaction with CL combination *in vitro*. Future work could look at Clear Care to understand the mechanisms for integrin downregulation when in combination with BA lenses, especially since cells exposed only to ClearCare solution exhibited no decrease in integrin expression. It would also be useful to characterize the rate release for components of cleaning solutions such as PHMB and pluronic from the contact lens to better able to correlate the cell response to the release in the *in vitro* model. This could provide answers to the difference of BA-CC to EA-CC when compared to EA-PBS, since H_2O_2 cleaning solution is considered the gold standard and non-toxic to the eye.

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Chapter 2

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Figure 2.1 and 2.2

TOPICAL REVIEW • OPEN ACCESS

Biomaterials for corneal bioengineering

Zhi Chen[1], Jingjing You[2,3], Xiao Liu[1], Simon Cooper[2], Christopher Hodge[2,4], Gerard Sutton[8 8 8,2,3,4,5], Jeremy M Crook[8 8 8,1,6,7] and Gordon G Wallace[8 8 8,1]

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Abstract

Corneal transplantation is an important surgical treatment for many common corneal diseases. However, a worldwide shortage of tissue from suitable corneal donors has meant that many people are not able to receive sight-restoring operations. In addition, rejection is a major cause of corneal transplant failure. Bioengineering corneal tissue has recently gained widespread attention. In order to facilitate corneal regeneration, a range of materials is currently being investigated. The ideal substrate requires sufficient tectonic durability, biocompatibility with cultured cellular elements, transparency, and perhaps biodegradability and clinical compliance. This review considers the anatomy and function of the native cornea as a precursor to evaluating a variety of biomaterials for corneal regeneration including key characteristics for optimal material form and function. The integration of appropriate cells with the most appropriate biomaterials is also discussed. Taken together, the information provided offers insight into the requirements for fabricating synthetic and semisynthetic corneas for *in vitro* modeling of tissue development and disease, pharmaceutical screening, and *in vivo* application for regenerative medicine.

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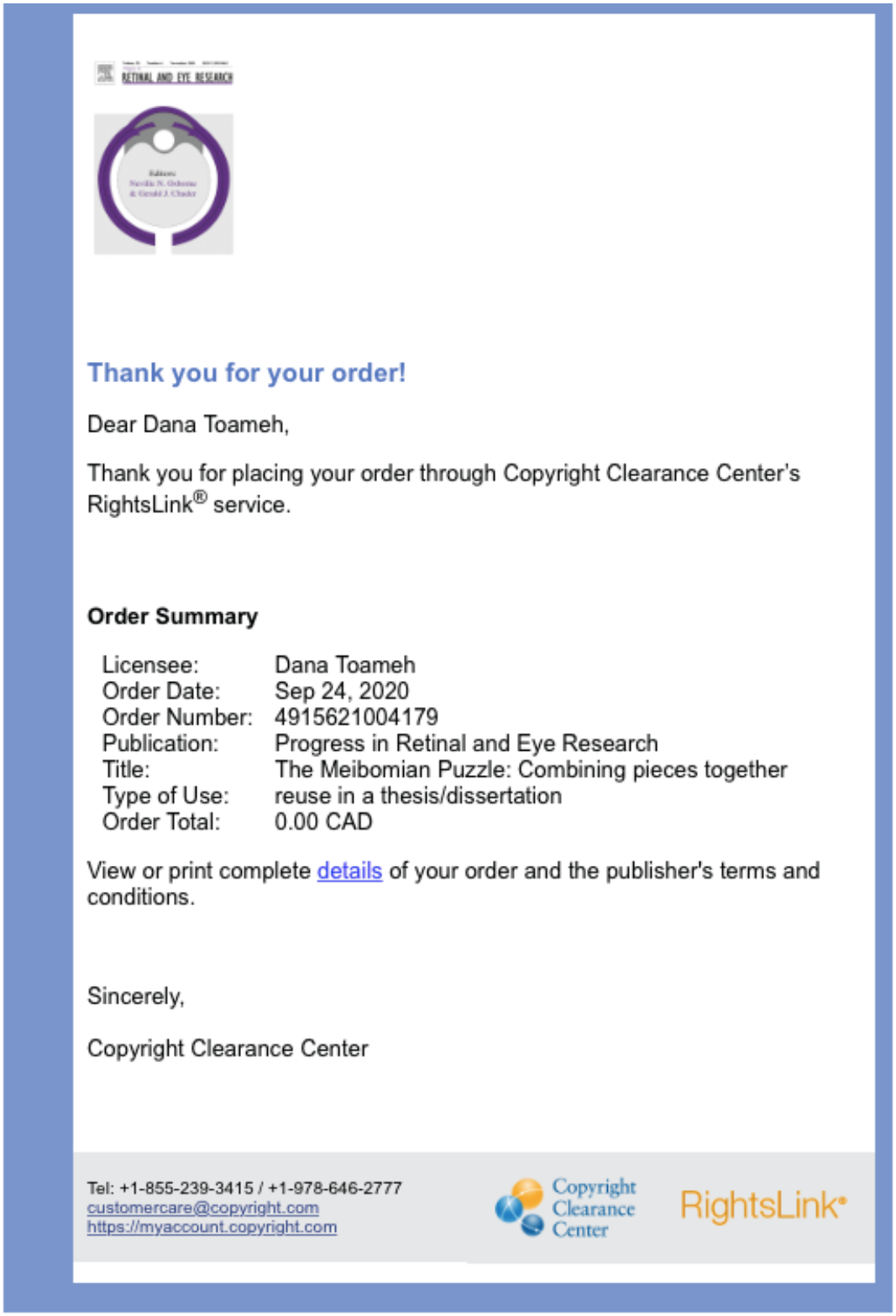

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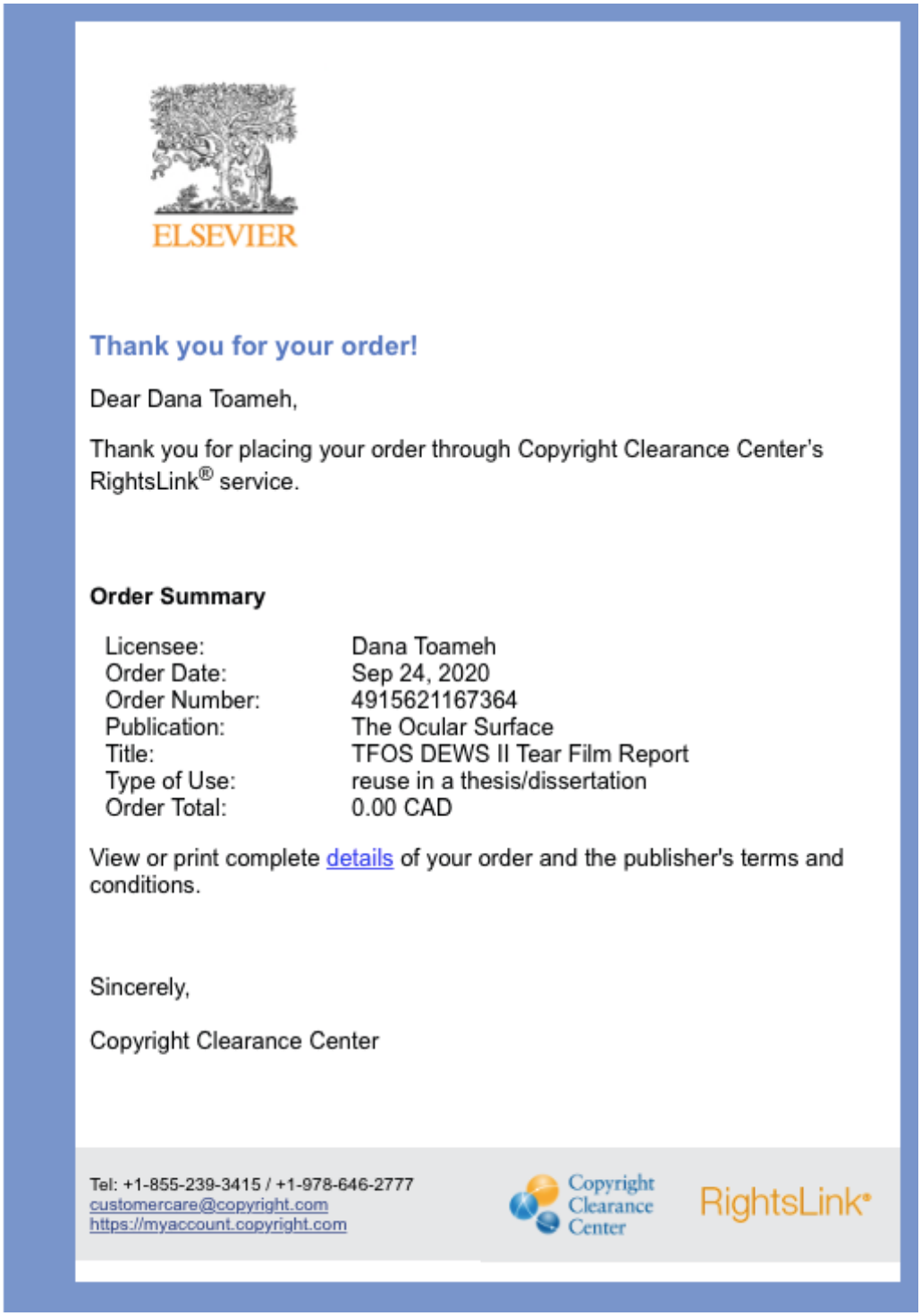

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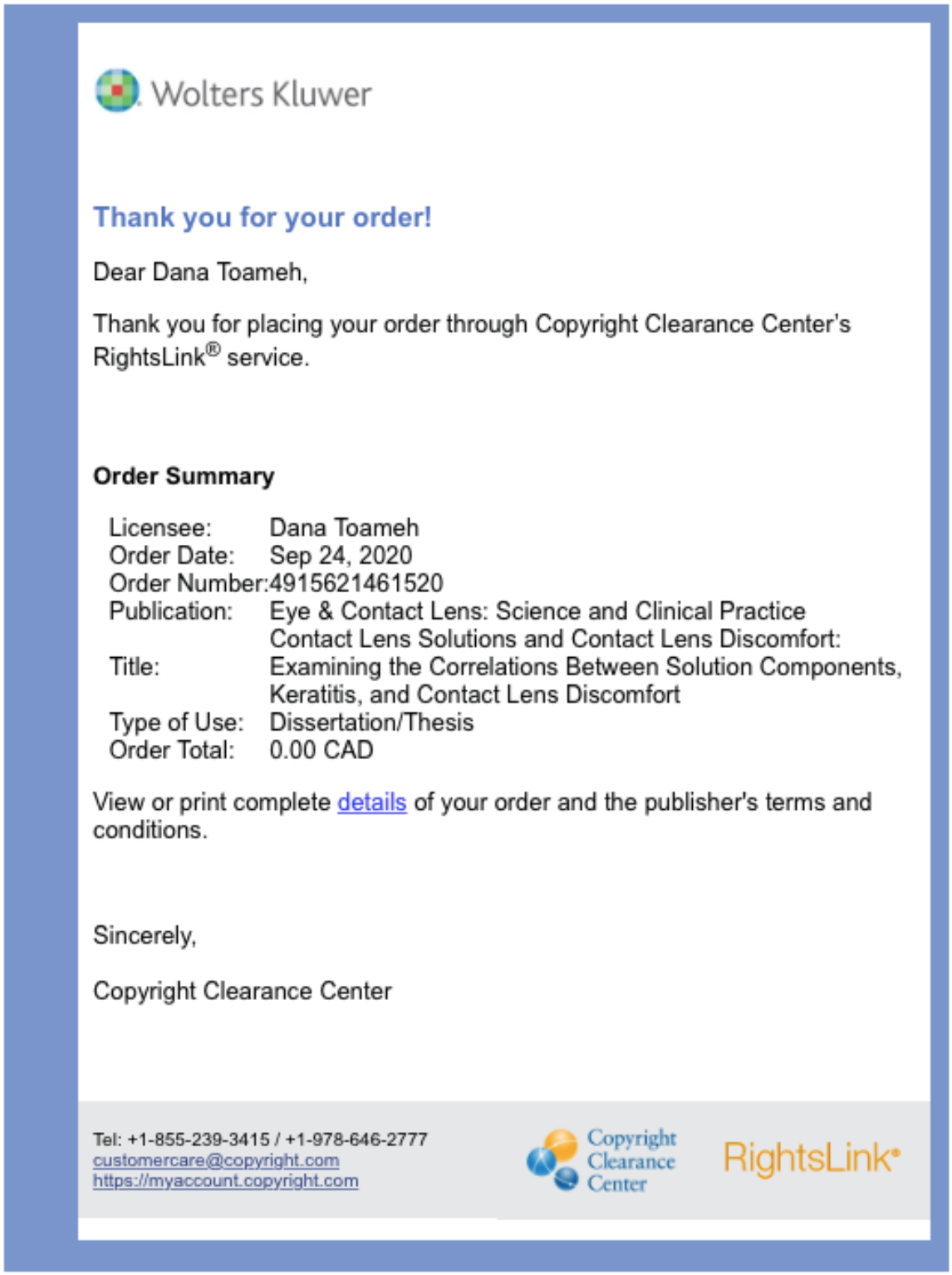

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Appendices

APPENDIX 1

PROOF OF CONCEPT OF OCUCELL DYNAMIC SYSTEM

Cells were grown on OcuCell pieces for 4 days. The OcuCell was run for 2 and 6 hours at 2 and 10µl/min. Etafilcon A contact lenses were soaked in PBS and then 0.001% BAK (diluted in PBS) for 24 hours to remove any remnants of their packaging solution. BAK was used as a positive control as it is known to be cytotoxic. On day 4 of cell growth, the PBS or BAK soaked lens were put on the cornea and eye lid piece for 2 or 6 hours. The control was no lens and only medium was changed for these OcuCell pieces. Experiments were performed under dynamic conditions (using the OcuCell platform platform) and statically.

Viability Assay: MTT is used to assess cell viability though a reduction reaction. After 4 days of incubation, Proliferation and viability was assessed with the MTT metabolic assay, where thiazoyl blue tetrazolium bromide is metabolized by live cells into a solid purple precipitate, formazan crystals. MTT was added to eyelid and cornea pieces and incubated for 2 hours at 37°C and 5% CO_2 to visualize metabolically active cells. Confluency of cell growth was observed. The MTT solution was then removed and isopropanol was added and agitated for 2 hours. The solutions in was read in a UV-Vis spectrophotometer at an optical density of 595 nm with a reference at 650 nm. All results are expressed as relative viability compared to control cells; cells incubated in KM and in the absence of flow from OcuCell and a contact lens.

Flow cytometry (refer to chapter 4 for methodology). Note AFU can not be directly compared with chapter 4 as different flow cytometry settings were used during this preliminary work which took place in 2018-early 2019.

Preliminary testing of OcuCell Cornea and Eyelid with lens soaked in PBS on static model

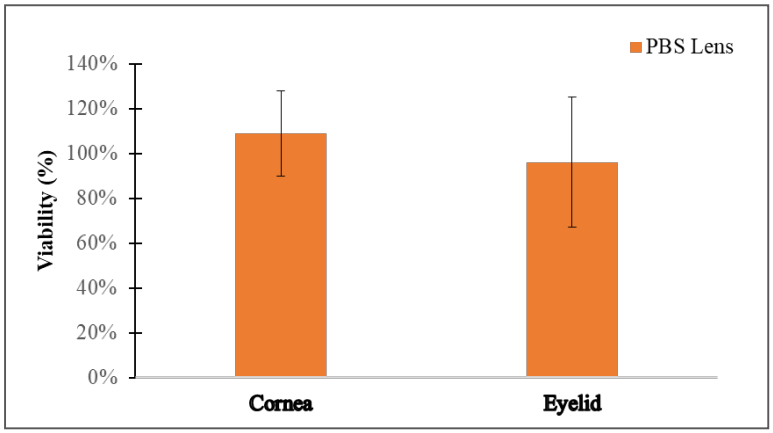


Figure 1: HCEC viability on OcuCell pieces (Static Conditions (no flow). Contact lenses soaked in PBS were added to the OcuCell pieces for 2 hours and Viability was assessed by MTT and is expressed relative to no lens. N=3 average ± SD.

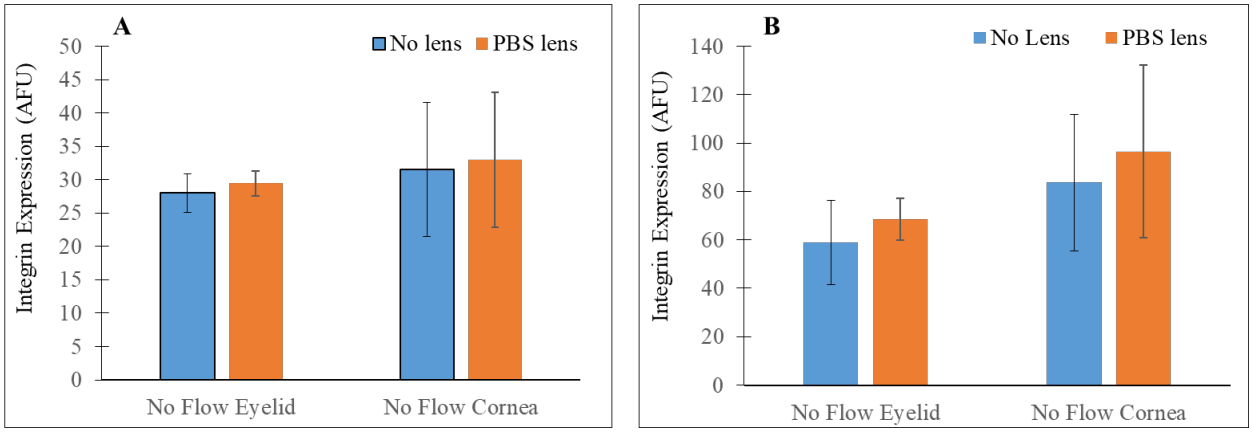


Figure 2: Integrin expression on HCEC grown on OcuCell pieces (Static Conditions, no flow). Contact lenses soaked in PBS were added to the OcuCell pieces for 2 hours and integrin expression (A: α_3 and B:β_1) was measured using flow cytometry N=4 ± SD.

In this proof of concept study, the OcuCell pieces were not placed on the OcuCell dynamic platform, they were tested under static conditions. Viability was also tested at 6 hours and the same trend is seen (data not shown). Viability of the PBS soaked lenses confirms that PBS is biocompatible with cells and we are able to continue testing using this model. While there is a slight elevation of integrin expression in the no lens control and PBS lens, these were not significant.

Preliminary testing of OcuCell Cornea and Eyelid under dynamic conditions (no lens)

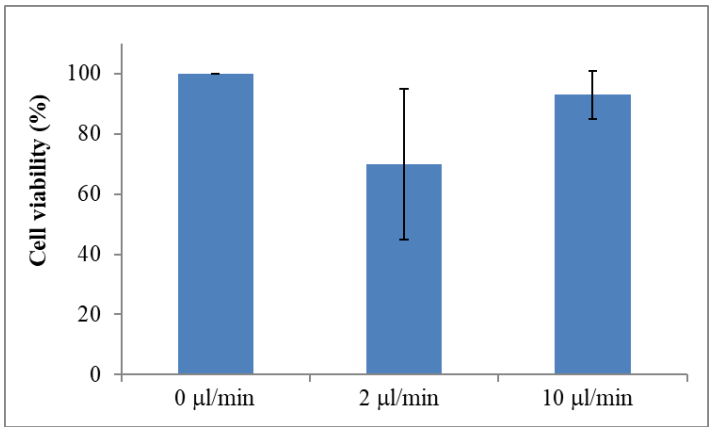


Figure 3: HCEC viability on OcuCell pieces under static and flow conditions. Viability was assessed by MTT and is expressed relative to no flow. N=3 average ± SD.

Due to large variations with lower flow, 10 µl/min was chosen for dynamic conditions.

Testing with positive and negative controls: BAK and PBS lens

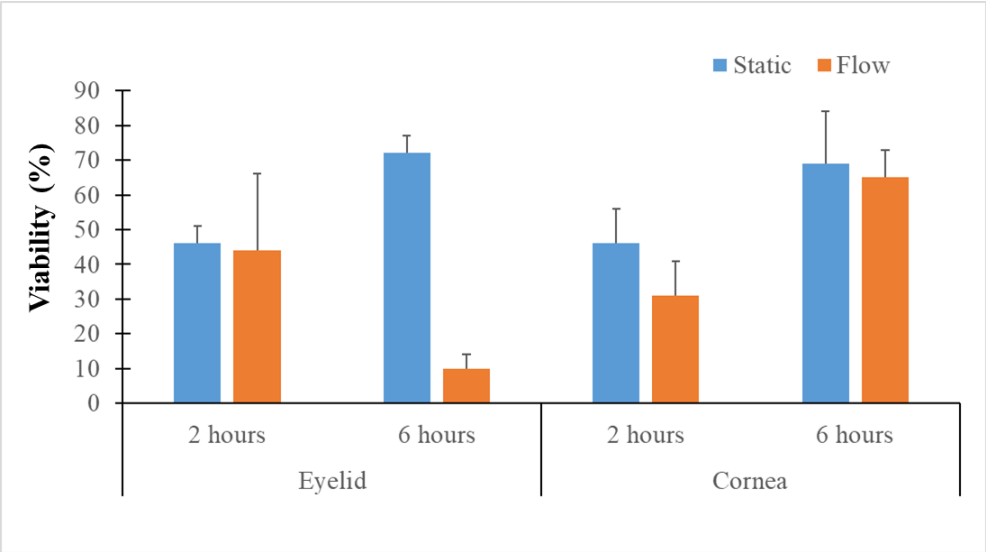


Figure 4: HCEC viability on OcuCell pieces under static and flow conditions (10 µl/min) in presence of BAK-soaked lenses. Viability was assessed by MTT and is expressed relative to PBS-soaked lens. N=3 average ± SD.

Table 1: Integrin expression on HCEC on OcuCell pieces exposed to PBS and BAK-soaked lenses under static and flow conditions. Cells on the OcuCell cornea pieces were subjected to a flow rate of 10 µL/min (flow conditions) or incubated statically (no flow conditions) for 2hrs in the presence of a PBS and BAK soaked lens. Integrin β_1 and integrin α_3 were measured using flow cytometry.

	Integrin α_3	Integrin β_1
Flow Eyelid BAK	33 ± 4.	50 ± 8
Flow Back PBS	29 ± 8	66 ± 10
Flow Cornea BAK	31 ± 5	62 ± 12
Flow Cornea PBS	29 ± 5	71 ± 12
No Flow Eyelid BAK	27 ± 5	44 ± 9
No Flow Eyelid PBS	30 ± 2	68 ± 9
No Flow Cornea BAK	32 ± 7	58 ± 12
No Flow Cornea PBS	33 ± 10	96 ± 36

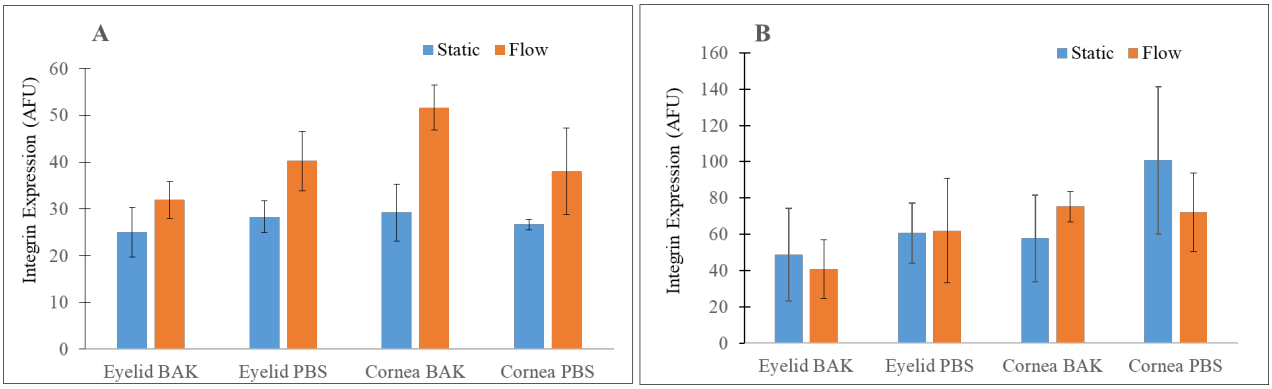


Figure 5: Integrin expression on HCEC on OcuCell pieces exposed to PBS and BAK-soaked lenses under static and flow conditions. Cells on the OcuCell cornea pieces were subjected to a flow rate of 10 µL/min (flow conditions) or incubated statically (no flow conditions) for 2hrs in the presence of a PBS and BAK soaked lens. Integrin α_3 (A) and integrin β_1 (B) were measured using flow cytometry.

APPENDIX 2

PILOT EXPERIMENTS FOR ACTIN STAINING ON FLAT AND CURVED SURFACES

Figure 1: A: ICONJ **B:** HCEC cells grown on flat TCPS. Fixed cells were stained with FITC-Phalloidin to view actin filaments and microscopic pictures were taken.

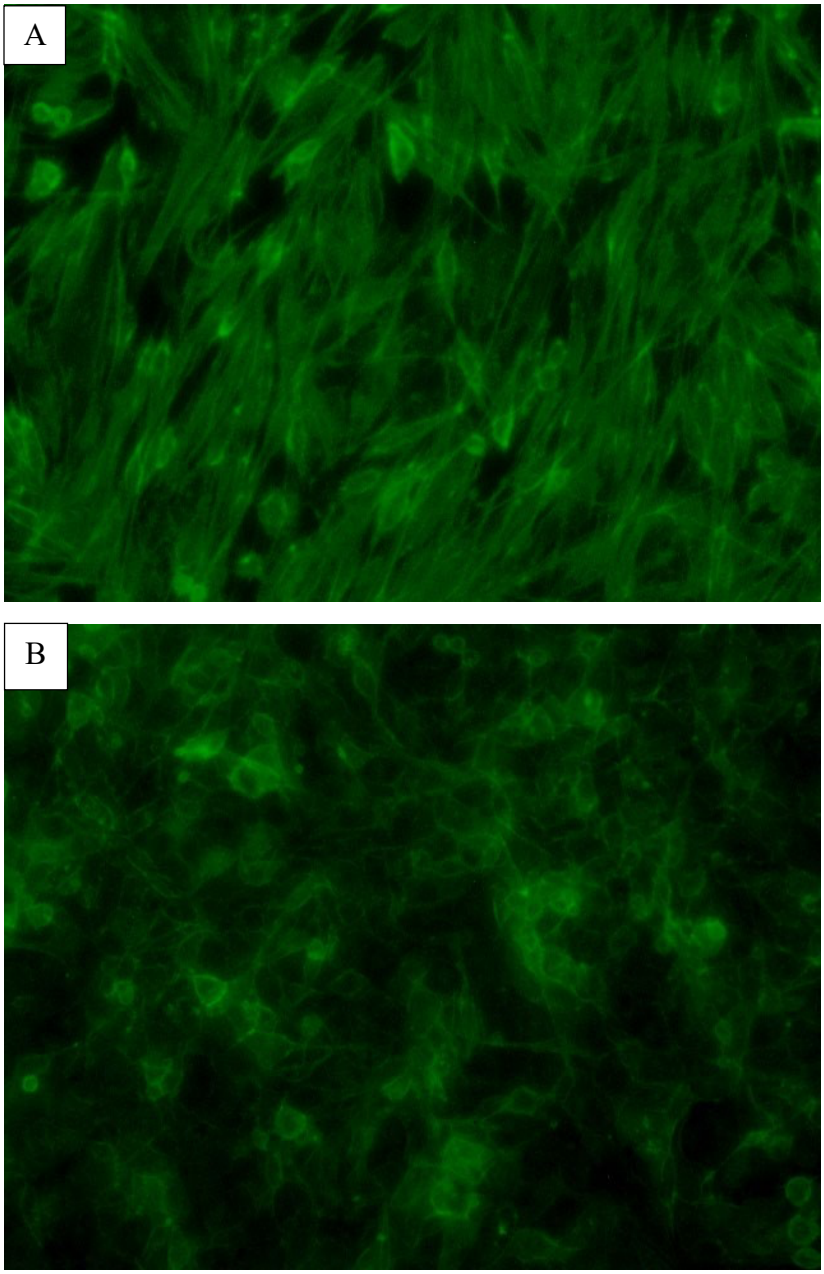
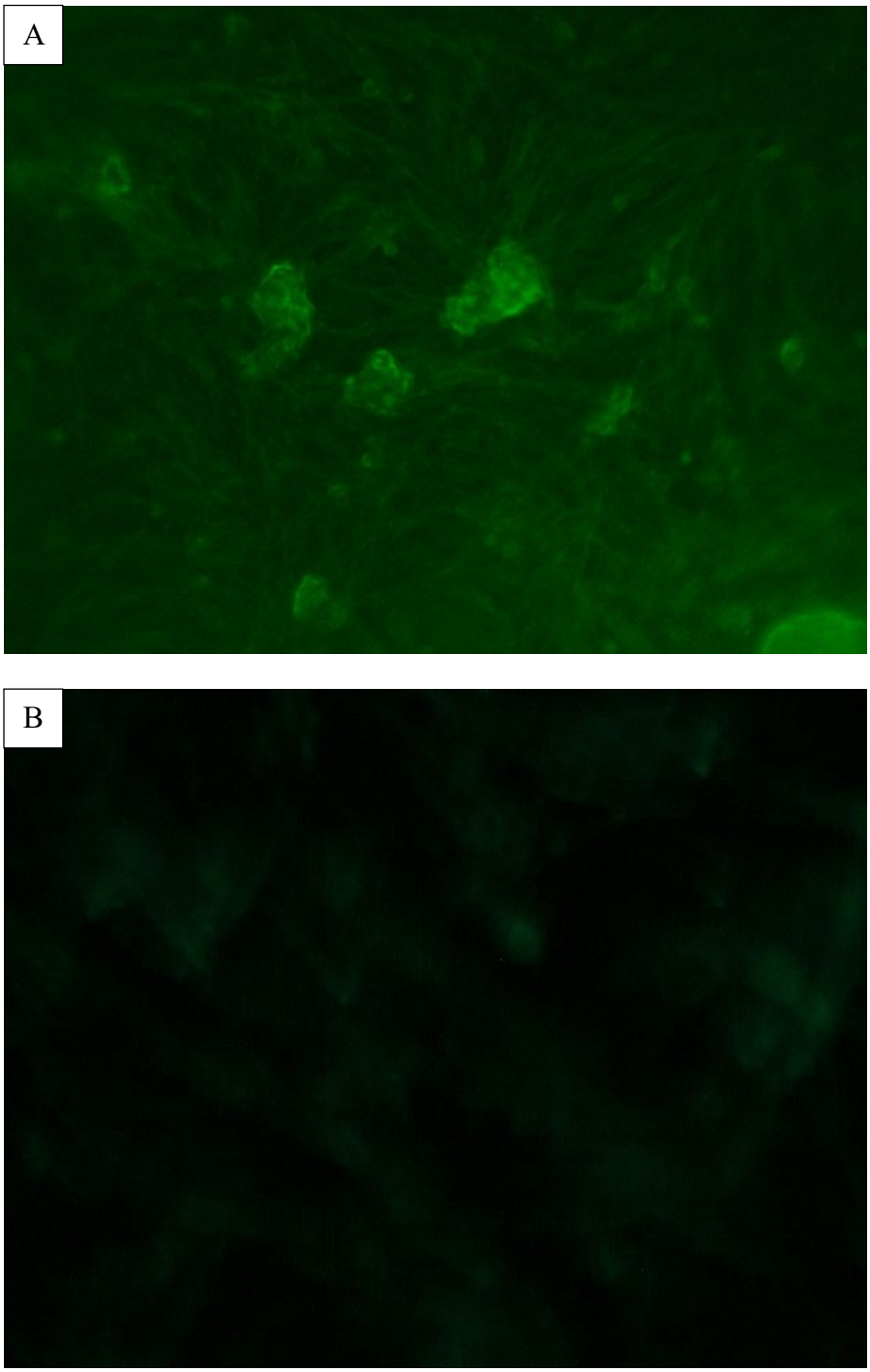


Figure 2: A: ICONJ **B:** HCEC cells grown on curved TCPS. Fixed cells were stained with FITC-Phalloidin to view actin filaments and microscopic pictures were taken. Microscope was unable to focus properly on the curved surface.



APPENDIX 3

PIOLT STUDY USING INFLMATORY MARKERS AND IL-8 IN COCULTURE

Table 1: Cells were grown on TCPS and insert as mentioned in Chapter 5. Lipopolysaccharide (LPS), interferon-α (IFNα) and Interleukin-8 (IL-8) stimulation was added to cells for 2 hours and an Elisa for Il-8 was performed.

Condition	Values
HCEC Control	0.80
ICONJ Control	0.92
LPS + HCEC + ICONJ (5ug)	1.73
LPS HCEC	1.14
LPS ICONJ	2.10
INFα HCEC	0.81
INFα ICONJ	0.84
INFα + HCEC + ICONJ (5ug)	1.61
INFα + HCEC + ICONJ (10ug)	1.66
IL-8 + HCEC + ICONJ (10ug)	1.41
HCEC + ICONJ (co-culture) control	1.20

Preliminary analysis shows LPS as an inflammatory activator for IL-8 and is stronger for ICONJ cells than HCEC. INFα shows no change in expression for monocultures compared to their respective controls. However, an increase is seen in the co-culture model. This study indicated crosstalk between ICONJ and HCEC cells as more of a difference is seen in co-culture than monoculture models. More testing is needed to understand the reason for this change.