Characterization and Application of a Human Corneal \textit{in vitro} Model for Controlled Release of Ophthalmic Drugs

by

Shahabedin Eslami

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Applied Science
in
Systems Design Engineering

Waterloo, Ontario, Canada, 2015

©Shahabedin Eslami 2015
Author's Declaration

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

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

The cornea acts as a transparent shield protecting the eye from germs, dust and pathogens as well as regulating the ocular environment by controlling the diffusion of external substances like oxygen or drugs via the tear film into the intraocular tissues. The design and development of an intraocular-drug delivery device highly depend upon the understanding of the interactions between the biological system of the eye and the drug-ophthalmic material complex. In vivo models have been used in clinical studies as the necessary and most valid testing platforms for regulatory authorities, but animal experiments have always been widely criticized for ethical and economical reasons. Furthermore, when studying interactions in the eye environment, using animal models has other disadvantages such as difference in blinking patterns, tear composition, and organ size when compared to the human eye. Therefore, in vitro cell culture models using human cells have been considered as a powerful pre-screening tool for drug testing to study interactions with the ocular tissue. In vitro models also provide an experimental environment, in which each parameter can be controlled. With a lower complexity level and less variability, in vitro models allow gaining a better understanding of the parameters involved in the drug delivery system.

Using eye drops for ocular drug delivery to the anterior section of the eye is a significant challenge due the rapid washout in tears and the corneal barrier to drug diffusion. Therefore mimicking this environment in vitro can help in designing more robust drug delivery system for the front of the eye.

This research focuses on the application and characterization of an in vitro human corneal model which mimics in part the in vivo environment to assess corneal interaction with the drug/biomaterial complexes. In this project, an in vitro model using human corneal epithelial cells is used to characterize the drug release profile and transport from commercially available contact lenses. Three model drugs for treatment of glaucoma, eye inflammation and infection respectively were tested in combination with two commercial contact lenses with different surface chemistry and their release profiles were measured. The effects of biological transporters on the controlled release systems were investigated. The role of organic anion transporter protein OATP 2A1 (prostaglandin transporter) in transcellular transport of
Latanoprost was characterized and the presence of its gene in the HPV16 E6/E7 immortalized corneal epithelial cell line was verified by real-time polymerase chain reaction. To assess the role of OATP 2A1, transporters were inhibited by Diclofenac sodium and the release kinetics of Latanoprost from Pure Vision® (balafilcon A) and Acuvue Oasys® (senofilcon A) contact lenses in three in vitro conditions with live corneal cells monolayer, fixed epithelial cells and transporter inhibited epithelial cells were studied.

The presence of corneal epithelial cells in vitro had a significant effect on release kinetics of Latanoprost resulting in a zero-order release profile. Complete inhibition of the OATP 2A1 transporter reduced the release rate of Latanoprost by 30% and 52% from balafilcon A and senofilcon A respectively. Comparison between live, fixed and transport inhibited models indicated that Latanoprost transport occurred mainly through the active transcellular pathway but was not mediated only by OATP 2A1. Other transporters might be involved in the transport and further research is required to identify these.

The effect of initial loading concentration of Latanoprost on release rate and amount of drug eluted from contact lenses was also investigated. Reduction in initial loading concentration of the drug increased the released percentage of the drug from 6.9% to 14.1% for balafilcon A and from 11.4% to 57.3% for senofilcon A. Both silicone hydrogel contact lenses, regardless of initial loading concentrations, released Latanoprost at above therapeutic daily dose up to 96 hours with zero-order constant rate.

The release kinetics of two hydrophilic ophthalmic drugs, Ciprofloxacin HCl and Timolol Maleate also from the soft contact lenses were evaluated in the corneal in vitro model over 48 hours. The effect of corneal epithelial barrier on the release kinetics of these hydrophilic drugs was evaluated. The release kinetics of hydrophilic Ciprofloxacin HCl and Timolol maleate followed a first-order rate. The presence of corneal epithelial cells in vitro had no significant effect on the release rate of hydrophilic drugs. However, presence of cells (live or fixed) acted as a diffusion barrier by decreasing the amount of Ciprofloxacin released from silicone hydrogel contact lenses compared to no-cell in vitro model. No significant difference in released amount of Timolol was observed between live, fixed and no cell in vitro models.

Our results further confirmed the effect of chemical interaction between drug molecules and contact lens polymer on a controlled release system and the importance of selecting the
appropriate in vitro model. Testing conditions and presence of biological barriers provided by cells can have a significant impact on release profile and provided a more realistic test platform for studying the release kinetics for the ocular environment. All in all, this thesis confirms the importance of proper selection of in vitro test models for the assessment of ocular drug delivery system and suggests that commercially available silicone hydrogel ocular materials may be used effectively for the release of hydrophobic therapeutics.
Acknowledgements

First of all I would like to express my heartfelt gratitude to my supervisor, Dr. Maud Gorbet for her leadership, support, invaluable guidance, constructive criticism, and encouragement throughout my two years of study.

I would like to express my sincere gratitude to friend and colleague Saman Mohammadi, who supported me by all means during the course of studying and research and for allowing me to collaborate with him, and to continue the project that he started in the lab.

I would also like to extend my thanks to all my coworkers and students in the MIBS lab particularly Cameron Postnikoff, Robert Pintwala and Sara Molladavoodi who have provided me with support, assistance, and enthusiasm during my research studies.

I would like to acknowledge Dr. Marc Aucoin and Steve George who have supported me in doing this project and helped me to run PCR experiments and using their lab facilities.

I also acknowledge and appreciate Dr. Marc Aucoin and Dr. Jonathan Kofman for taking the time to review my thesis.

I wish to thank the University of Waterloo and NSERC for providing me the opportunity to conduct meaningful research during my graduate studies and for providing financial support to pursue this research project.

Last but not least, I am very grateful for endless support from my lovely wife, Hengameh, who is always standing beside me at any circumstances. I could not complete this study without her understanding and caring. I wish to express my immense gratitude for her patience and moral support.
To My Soulmate Hengameh

Without her my life would be miserable
# Table of Contents

Author's Declaration ........................................................................................................... ii  
Abstract ................................................................................................................................. iii  
Acknowledgements ............................................................................................................. vi  
Table of Contents ................................................................................................................ viii  
List of Figures ....................................................................................................................... xi  
List of Tables ......................................................................................................................... xiii  
List of Abbreviations ........................................................................................................... xiv  
Chapter 1 Introduction ......................................................................................................... 1  
Chapter 2 Background ......................................................................................................... 3  
  2.1 Human Eye ......................................................................................................................... 3  
    2.1.1 Cornea ........................................................................................................................... 4  
  2.2 Ocular Drug Delivery ........................................................................................................ 8  
    2.2.1 Ocular barriers ............................................................................................................. 9  
    2.2.2 Corneal Transporters ................................................................................................. 13  
  2.3 Latanoprost and Glaucoma ............................................................................................. 15  
  2.4 Silicone Hydrogel Contact Lenses as drug delivery systems ....................................... 18  
  2.5 Ocular In vitro Models ..................................................................................................... 21  
    2.5.1 Primary Cell Culture ................................................................................................. 21  
    2.5.2 Immortalized Cell Culture ......................................................................................... 22  
    2.5.3 Corneal Epithelial In vitro Models ........................................................................... 23  
Chapter 3 Characterization of the Human Corneal In vitro Model ....................................... 26  
  3.1 Introduction ....................................................................................................................... 26  
  3.2 Materials and Methods .................................................................................................. 27  
    3.2.1 Preparation of Drug Solution ................................................................................. 27  
    3.2.2 Preparation of Contact Lenses ................................................................................. 27  
    3.2.3 Cell Culture ............................................................................................................. 28  
    3.2.4 Flat Monolayer HCEC Model ............................................................................... 28  
    3.2.5 Fixing cells .............................................................................................................. 28  
    3.2.6 Transport Inhibition ............................................................................................... 29
3.2.7 MTT viability Assay .................................................................................................................. 29
3.2.8 In Vitro Drug Release Experiment .............................................................................................. 30
3.2.9 RNA Isolation and Real Time PCR ............................................................................................ 31
3.2.10 Gel Electrophoresis .................................................................................................................. 31
3.2.11 Data Analysis ............................................................................................................................ 32
3.3 Results ........................................................................................................................................... 33
3.3.1 The effect of active transport on release of Latanoprost .............................................................. 33
3.3.2 Real Time PCR ............................................................................................................................. 36
3.4 Discussion ........................................................................................................................................ 38
Chapter 4 Application of the Human Corneal In vitro Model to assess Latanoprost Controlled Released System .................................................................................................................. 39
4.1 Introduction .................................................................................................................................... 39
4.2 Materials and Methods .................................................................................................................... 40
4.2.1 Preparation of Drug Solution ...................................................................................................... 40
4.2.2 Preparation of Contact Lenses ................................................................................................... 40
4.2.3 Latanoprost Uptake and Release ............................................................................................... 40
4.2.4 Data Analysis .............................................................................................................................. 40
4.3 Results ........................................................................................................................................... 41
4.3.1 Latanoprost Uptake and Release ............................................................................................... 41
4.3.2 Latanoprost Release Kinetics ................................................................................................... 43
4.4 Discussion ........................................................................................................................................ 46
Chapter 5 Release System for Hydrophilic drug .................................................................................... 48
5.1 Introduction .................................................................................................................................... 48
5.2 Materials and Methods .................................................................................................................... 50
5.2.1 Preparation of Drug Solution ...................................................................................................... 50
5.2.2 Calibration curve .......................................................................................................................... 50
5.2.3 Preparation of Contact Lenses ................................................................................................... 52
5.2.4 Hydrophilic Drugs Uptake and Release ..................................................................................... 52
5.2.5 Data Analysis .............................................................................................................................. 52
5.3 Results ........................................................................................................................................... 53
List of Figures

Figure 2-1 Schematic presentation of human eye................................................................. 3
Figure 2-2 A cross-section of the human cornea. ............................................................... 5
Figure 2-3. A proposed model for the structure of the precorneal tear film ...................... 7
Figure 2-4. Drug concentration in the tear film after topical administrations..................... 9
Figure 2-5. Schematic illustration of the biological barriers in the human eye. ...................... 11
Figure 2-6 Cellualr organization of the cornea showing its various transport barriers. ........ 13
Figure 2-7. Schematic representation of the currently known transporters/receptors in the corneal epithelium ................................................................................................. 14
Figure 2-8. Chemical structure of Latanoprost and its respective free acid form............... 17
Figure 2-9 Corneal Models constructed with cell culture techniques................................. 24
Figure 2-10 Step by step construction corneal equivalent.................................................. 24
Figure 3-1. Human corneal epithelial cells were grown on PET membrane insert and were stained with MTT on the 6th day of growth (left). Formazan (purple) crystals inside cells (right) indicate viable cells................................................................. 30
Figure 3-2. Latanoprost release from balafilcon A contact lenses through live, transporter-inhibited and fixed HCEC monolayer models................................................................. 34
Figure 3-3. Latanoprost release from senofilcon A contact lenses through live, transporter inhibited and fixed HCEC monolayer models................................................................. 35
Figure 3-4. Transport inhibition of organic anion transporter protein OATP 2A1 by 100 µM Diclofenac throughout 96 hours of Latanoprost release from balafilcon A and senofilcon A silicone hydrogel contact lenses. ................................................................. 36
Figure 3-5 Real-Time PCR Amplification graphs............................................................... 37
Figure 3-6. Gel Electrophoresis of amplified cDNA samples............................................. 37
Figure 4-1. Latanoprost release from balafilcon A contact lenses through live HCEC monolayer models........................................................................................................... 44
Figure 4-2. Latanoprost release from senofilcon A contact lenses through live HCEC monolayer models........................................................................................................... 45
Figure 5-1. Ciprofloxacin HCl standard curve................................................................. 51
Figure 5-2. Timolol Maleate standard curve................................................................. 51
Figure 5-3 Ciprofloxacin HCl release from balafilcon A contact lenses through live and fixed
HCEC monolayer and No-cell models. ........................................................................... 56
Figure 5-4. Ciprofloxacin HCl release from senofilcon A contact lenses through live and fixed
HCEC monolayer and No-cell models. ........................................................................... 57
Figure 5-5. Timolol Maleate release from balafilcon A contact lenses through live and fixed
HCEC monolayer and No-cell models. ........................................................................... 61
Figure 5-6. Timolol Maleate release from senofilcon A contact lenses through live and fixed
HCEC monolayer and No-cell models. ........................................................................... 62
Figure 7-1. Latanoprost release experiment, through cellulose membrane inserts versus
Polyethylene terephthalate (PET) membrane. ................................................................. 68
Figure 7-2 MTT viability test on live, transporter inhibited and fixed HCEC monolayer models.
.......................................................................................................................................... 69
Figure 7-3 MTT viability test performed on live and fixed HCEC monolayer models after
Ciprofloxacin HCl release experiments. ........................................................................... 70
Figure 7-4 MTT viability test performed on live and fixed HCEC monolayer models after
Timolol maleate release experiments ............................................................................ 70
**List of Tables**

Table 2-1. Basic classification of the glaucomas .................................................................................. 16  
Table 2-2. FDA classification of soft contact lenses ............................................................................... 19  
Table 2-3. Characteristics of silicone hydrogel contact lenses ............................................................... 20  
Table 3-1 Properties of the Contact Lens Hydrogel Materials ............................................................... 27  
Table 3-2. Average Latanoprost release rate from balafilcon A and senofilcon A silicone hydrogel in 96 hours .................................................................................................................................................... 34  
Table 4-1 Latanoprost uptake into balafilcon A and senofilcon A contact lenses and total amount of drug released after 96 hours through the live HCEC monolayer \textit{in vitro} model ...................................................... 42  
Table 4-2. Latanoprost average release rate from silicone hydrogel contact lenses in 96 hours .......... 45  
Table 5-1. Ciprofloxacin HCl uptake into balafilcon A and senofilcon A contact lenses and total amount of drug released after 48 hours through the live HCEC monolayer \textit{in vitro} model ....... 54  
Table 5-2 Timolol Maleate uptake into balafilcon A and senofilcon A contact lenses and total amount of drug released after 48 hours through the live HCEC monolayer \textit{in vitro} model .......... 59
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AC</td>
<td>anterior chamber</td>
</tr>
<tr>
<td>ACG</td>
<td>angle-closure glaucoma</td>
</tr>
<tr>
<td>ACTB</td>
<td>β-actin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BBS</td>
<td>borate buffered saline</td>
</tr>
<tr>
<td>BAB</td>
<td>blood-aqueous barrier</td>
</tr>
<tr>
<td>BCRP</td>
<td>breast cancer related protein</td>
</tr>
<tr>
<td>BRB</td>
<td>blood-retina barrier</td>
</tr>
<tr>
<td>CO2</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>DMA</td>
<td>N,N-dimethylacrylamide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGDMA</td>
<td>ethyleneglycol dimethacrylate</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>food and drug administration</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HCEC</td>
<td>human corneal epithelial cell</td>
</tr>
<tr>
<td>HEMA</td>
<td>2-hydroxyethylmethacrylate</td>
</tr>
<tr>
<td>HPV</td>
<td>human papilloma virus</td>
</tr>
<tr>
<td>IOP</td>
<td>intraocular pressure</td>
</tr>
<tr>
<td>KGS</td>
<td>keratinocyte growth supplement</td>
</tr>
<tr>
<td>KSFM</td>
<td>keratinocyte serum free medium</td>
</tr>
<tr>
<td>MA</td>
<td>methacrylic acid</td>
</tr>
<tr>
<td>MCT</td>
<td>monocarboxylate transporter</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>mPDMS</td>
<td>monofunctional polydimethylsiloxane</td>
</tr>
<tr>
<td>MRP</td>
<td>multidrug resistant protein</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>NCVE</td>
<td>N-carboxyvinyl ester</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>nonsteroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>NTG</td>
<td>normal tension glaucoma</td>
</tr>
<tr>
<td>NVP</td>
<td>N-vinyl pyrrolidone</td>
</tr>
<tr>
<td>OAG</td>
<td>open-angle glaucoma</td>
</tr>
<tr>
<td>OATP</td>
<td>organic anion transporter protein</td>
</tr>
<tr>
<td>OPT</td>
<td>oligopeptide transporter</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBVC</td>
<td>poly[dimethylsiloxy] di[silylbutanol]bis[vinyl carbamate]</td>
</tr>
<tr>
<td>PC</td>
<td>posterior chamber</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>penicillin/streptomycin</td>
</tr>
<tr>
<td>PET</td>
<td>polyethylene terephthalate</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>pHHEMA</td>
<td>poly-2-hydroxyethyl methacrylate</td>
</tr>
<tr>
<td>PMMA</td>
<td>poly(methylmetacrylate)</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SV</td>
<td>simian virus</td>
</tr>
<tr>
<td>TAE</td>
<td>tris acetate ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>TEGDMA</td>
<td>tetraethyleneglycol dimethacrylate</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>ultraviolet visible</td>
</tr>
</tbody>
</table>

xv
Chapter 1
Introduction

Topical drug delivery to the eye remains a significant challenge for scientists and engineers due to the unique anatomy of the eye and its physiology, that lower the bioavailability of topical ophthalmics administration to less than 5% (Sjöquist et al., 1998). The main route for drug delivery to the anterior eye is the cornea (Gaudana et al. 2009, 2010). The cornea is a transparent functional barrier protecting the eye from infections, the entrance of germs, dust or pathogens as well as regulating the internal environment of the eye by controlling the diffusion of external substances or drugs into the intraocular tissues. Development of new treatments for corneal injuries and the design of effective intraocular drug delivery systems highly depend upon the understanding of how the visual organ works and the biological roles of each tissue in the eye (Castro-Muñozledo, 2008). As part of the development and design of such systems, studying cytotoxicity and biocompatibility of biomaterials/drugs used for the treatment of eye diseases/injuries or controlled released systems is paramount, and relies on comprehension of the biological interactions between biomaterials, the drugs and the biological hosts (Williams, 2014, 2008). In other words, each biomaterial-drug-host complex has its own characteristics and must be studied as a system (Williams, 2014).

To develop drug delivery systems and characterize biocompatibility requires experimental models, such as in vitro, in vivo or ex vivo models, where toxicity, efficacy and other parameters can be tested. Animal models, still used as the most valid test beds for regulatory authorities in clinical studies, have always been criticized due to ethical issues since a high number of animals has to be sacrificed to collect sufficient tissue samples. While in vivo experiments cannot be removed completely to obtain approval for clinical studies, there is a strong call to reduce the number of required in vivo experiments to a minimum (Hornof et al., 2005). Furthermore using animal models to study ocular materials and drug delivery systems has some other disadvantages such as inter-sample variations, different blinking patterns and tear composition than human and different organ size/surface compared to the human eye (Urtti and Salminen, 1993). The development of in vitro
models using human cell lines has thus been considered as a powerful pre-screening tool for studying the tissue barrier function and tissue regulations as well as for testing pharmaceuticals. Furthermore, in vitro models provide controlled experimental conditions, in which each parameter can be regulated to obtain more reproducible results in comparison to the more complex ex vivo studies with dissected animal tissues or in vivo tests (Hornof et al. 2005; Reichl et al. 2011). Therefore in order to use in vitro cell culture instead of animal models and extract meaningful information from in vitro cell interactions with drugs and biomaterials comparable to in vivo and clinical results, it is crucial to design an in vitro model which addresses the key natural characteristics of the extracellular matrix (ECM) as well as the biophysical, biochemical and biomechanical properties of the native cell niche in the tissue (Bacakova et al., 2011; Castro-Muñozledo, 2008).

This research thesis aimed to characterize a corneal in vitro model and assess the role of live corneal epithelial cells in application of this model for testing controlled delivery of ophthalmics. This thesis starts with an introduction to the anatomy of the eye and the cornea, and ocular drug delivery concepts in Chapter 2. In this chapter, ocular barriers and corneal transporters are briefly reviewed; glaucoma, Latanoprost and silicone hydrogel contact lenses are introduced and ocular in vitro models discussed. In Chapter 3, a corneal epithelial in vitro model is described and characterized for ocular transporters. The results on the role of transporters in Latanoprost transcorneal permeation are reported. Chapter 4 presents the release kinetic studies of Latanoprost from silicone hydrogel contact lenses in different corneal in vitro models. Chapter 5 reports the examination of corneal in vitro model for assessment of hydrophilic drugs release from silicone hydrogel contact lenses. Finally, Chapter 6 and Chapter 7 present the conclusions and recommendations for future work, respectively.
Chapter 2
Background

2.1 Human Eye

Eyes are complicated organs through which we sense and comprehend the outside world. As a vital organ, the eye is protected by the surrounding bones in the skull called the bony orbit. The eyeball (globe) is also wrapped in layers of fat and supporting tissues for protection and stability (Lens et al. 2008). Figure 2-1 schematically illustrates the overall topographic anatomy of the human eye. As shown in the figure, the globe is not completely round and is made of two merged unequal spheres joined at the junction of the cornea and the conjunctiva. The sagittal diameter of an adult globe is about 24 mm and its volume is about 6.5 ml. The weight of the globe is about 28 grams. The most outer skeleton of the eye is formed by the fibrous envelope of the cornea and the sclera. The cornea is shaped like an ellipsoid and covers about 20% of the eye total surface. The remainder 80% of the globe surface is covered by the sclera (Rodrigues, 1996).

![Schematic presentation of human eye](http://creativecommons.org/licenses/by/3.0/)

Figure 2-1 Schematic presentation of human eye (adapted with permission from CC: By Holly Fischer, © Regents of the University of Michigan [http://creativecommons.org/licenses/by/3.0/](http://creativecommons.org/licenses/by/3.0/))
The globe can be divided into two asymmetric portions namely the anterior segment and the posterior segment. The posterior segment is larger (two third of the eye globe) than the anterior part and includes the structures posterior to the lens such as the choroid, macula, retina, posterior sclera and optic nerve. (Figure 2-1). The posterior segment is filled by a gel like fluid called the vitreous humor. The anterior segment (also called the front of the eye) consists of the pupil, the crystalline lens, cornea, iris, conjunctiva and ciliary body. The anterior segment is divided by the iris into the anterior and the posterior chambers which are filled by the aqueous humor (Rodrigues, 1996, Lens et al. 2008). The light is first refracted through the cornea and then via the pupil passes through the lens and the vitreous humor until it reaches the visual receptor cells of the retina called rods and cones, which convert the light to nerve impulses. These impulses are transmitted to the brain via optic nerves for further visual processing (Lens et al., 2008). The eye is comprised of several components and layers. However, only the structures relevant to this thesis will be reviewed in the next sections.

2.1.1 Cornea

The cornea is the strongest refractive component of the entire visual system and the main chemical and mechanical barrier of the anterior eye which protects the anterior segment and the intraocular tissues from external substances and contaminations (Lens et al. 2008). The cornea is transparent, avascular and consists of three cellular layers and two acellular supporting membranes with a thickness of ~ 500μm (Huhtala et al., 2008). The outmost apical part consists of 5-6 layers of stratified non-keratinized squamous corneal epithelium cells supported by the Bowman’s membrane basally and covered by the tear film laterally (Lens et al., 2008). The top superficial epithelial cells are flattened and are connected to each other with desmosomes and tight junctions and form a strong diffusional barrier against exogenous materials. Beneath the superficial cells are 2-3 layers of wing cells followed by a monolayer of columnar basal cells attached to the Bowman’s membrane. Proliferation occurs at the basal cells and cells differentiate and stratify as they propagate and move upward to wing cells and then to flattened superficial cells. Corneal epithelial cells eventually shed from the top layer at the surface and will be replaced by the cell layers beneath (Lens et al., 2008). The main role of the Bowman’s membrane is to act as a supporting ECM for
epithelial cells; its surface topography is porous with micro to nano sized pores, bumps and fibers which are stochastically dispersed (Garland et al., 2014). Under the Bowman’s membrane is the stroma which represents ~90 percent of the corneal thickness. It contains arranged collagen fibers, proteoglycans and fibroblastic keratocytes (stromal cells) and lies on the Descemet’s membrane, which separates the stroma from a monolayer of polygonal endothelial cells. Endothelial cells separate the cornea from the aqueous humor and are responsible for keeping the stroma slightly dehydrated and clear. However, the transparency of the cornea also relies on other factors such as fast proliferation of basal epithelial cells, homeostasis and metabolic activity of keratocytes and the endothelium as well. Figure 2-2 presents a cross-section of a human cornea (Barar et al., 2008; Hornof et al., 2005; Huhtala et al., 2008; Reichl et al., 2011). The different layers of the corneal epithelium are clearly distinguishable in Figure 2-2.B (Reichl et al., 2011)

Figure 2-2 A cross-section of the human cornea (A). The cornea consists of the epithelium, the stroma and endothelium cell layers. The corneal epithelium has five to six cell layers (B). Magnified cross-section of corneal epithelial cells illustrating the flattened superficial cell layers, wing cells and columnar monolayer attached to Bowman’s membrane. Proliferation occurs at the basal layer and the cell differentiation happens to wing cells as they grow. At the apical surface, cells become flattened and form a tight barrier (adapted with permission from Reichl et al., 2011)
The corneal surface is covered by a thin film of tear, renewing with a turnover rate of 0.5-2.2 µl/min in humans (Mishima et al., 1966). The tear film is responsible for lubrication of the cornea and since it is located at the air-eye interface, it also provides oxygen and nutrients to it. It partially refracts light and also contributes to light refraction by maintaining the corneal surface clear and distortion free (Rodrigues, 1996). The tear film is also a diffusional barrier against drugs and exogenous materials via lacrimal drainage (Hornof et al. 2005; Lens et al. 2008). The tear film structure is illustrated in the Figure 2-3. The tear film is believed to consist of four immiscible layers (over the last decades, there has been several debates about the layering of the tear film (Butovich et al. 2008; Green-Church et al. 2011; Prydal and Campbell, 1992; Sullivan, 1994). The inner mucin layer is 0.2-0.5 µm in thickness and helps to attach to the hydrophobic ocular surface. The intermediate aqueous layer is 6-7 µm in thickness and contains inorganic salts, glucose, urea, proteins, glycoproteins and surface active polymers. This central layer is responsible for lubrication of the ocular surface while blinking. The outer lipid bilayer with a thickness of ~0.1 µm is composed of an outer nonpolar layer in contact with the air interface guarding against water evaporation and an inner polar layer, responsible of stabilizing the outer layer. It mainly contains low polar lipids and waxy esters (Green-Church et al., 2011).
Figure 2-3. A proposed model for the structure of the precorneal tear film (taken with permission from Butovich et al., 2008)
2.2 Ocular Drug Delivery

In the design and development of ophthalmics, it is critical to consider parameters such as bioavailability (a measurement of the rate and amount of a drug that reaches the target site in an unchanged chemical form via blood circulation system (Griffin and Grady, 2002)), drug delivery system, tolerability and stability of the final drug formulations; the commercial drug system must be an ideal compromise of these parameters (Ali and Lehmussaari, 2006). To deliver the drug to the target site, a drug carrier strategy is needed which leads to design of a drug delivery system. A drug delivery system can also be designed as a controlled release system to minimize the pulsatile variation of drug concentration at the target tissue and keep it at the effective therapeutic levels and below toxic levels in the period of treatment (Figure 2-4) (Novack, 2009). This system must be also stable in the physiological environment and be tolerated by the site of action (target tissue) (Griffin and Grady, 2002).

Due to the unique anatomy and physiology of the eye and the presence of various barriers, ocular drug delivery in a sustained and controlled manner has been a major challenge for scientists and pharmacologists in the design of ophthalmic drug delivery systems (a Urtti, 2006). In general, drug delivery to the eye is classified into anterior and posterior segments. The majority of the anterior eye diseases are usually treated by conventional drug delivery dosage forms such as topically instilled ophthalmic eye drops (solutions), ointments, gels and suspensions (Gaudana et al., 2009). Medications applied to the outer surface of the eye for treatment of infections are also classified into anterior segment drug delivery systems. Injectable drugs, oral doses and ocular inserts are other types of conventional dosage which are used when the active ingredient is topically impermeable or the target site is not accessible by topical administration (Lang, 1995). However, these methods are not efficient and each has drawbacks such as blurring vision, low bioavailability, low drug penetration to the anterior chamber and poor patient compliance. For topically administered ophthalmics, most of the applied dose is rapidly washed out by tear fluid turnover, blinking and through the lacrimal drainage; less than 5% of the drug is actually absorbed to the ocular surface due to lacrimal drainage and tear fluid turnover (Sjöquist et al. 1998). Therefore a frequent dose application by the patient is required, which may increase pulsatile variations in the drug concentration. Increasing the dose or the frequency of administration may
also cause ocular toxicity (Figure 2-4). Low stability and presence of preservatives and penetration enhancers in the formulation of these ophthalmics are also other disadvantages pertaining to the conventional dosages. In the design of ocular drug delivery systems, these problems can be solved with various approaches by extending residence time of the drug, providing controlled release, designing prodrugs, using carriers and transporters/receptors and more local delivery by implants (Ciolino et al., 2009; White et al., 2011a).

Figure 2-4. Drug concentration in the tear film after topical administrations. Graph A represent the conventional frequent application with a high dose which may reach to the toxicity threshold. Graph B shows a low dose application which is not efficient. Graph C represent the ideal dosing at a constant rate which keeps the drug concentration at therapeutic level. (taken with permission from White and Byrne 2010)

2.2.1 Ocular barriers

There are several possible routes of ocular drug delivery into the ocular tissues and various cellular membranes and barriers control the transport of fluids and solutes into the eye as well as effectively hamper the penetration of exogenous molecules, pathogens and unfortunately ocular therapeutics. Hence, in the design of an ocular drug delivery system, the functionality and
characteristics of these biological barriers must be fully understood and addressed (Barar et al., 2008; Hornof et al., 2005; Urtti, 2006). Figure 2-5 illustrates the biological barriers of the eye. Topically administered drugs are adsorbed through the anterior segment including the cornea, sclera and conjunctiva while oral dosage and systematically administered drugs penetrate into intraocular tissues via the blood-retina barrier (BRB) and blood-aqueous barrier (BAB). Intravitreal injection is another route (invasive method) to reach to the vitreous humor. BRB surrounds the posterior segment of the eye and includes two layers of inner retinal capillary endothelial cells and outer retinal pigment epithelium. The presence of tight junctions in the retinal epithelium and endothelium makes the BRB a strict barrier against traverse of nutrients, proteins and small hydrophilic compounds (del Amo and Urtti, 2008; Gaudana et al., 2009; Hughes et al., 2005). The BAB is located at the anterior part of the eye (Figure 2-5) and consists of two cellular layers of the endothelium of the iris/ciliary blood vessels and the non-pigmented ciliary epithelium. This barrier controls the traverse of solutes, large molecules and proteins into the aqueous and vitreous humors and is responsible for keeping the intraocular fluids clear. The small and lipophilic drugs in the vitreous humor are eliminated through the BAB while larger hydrophilic drugs are washed away by aqueous humor turnover (Barar et al., 2009, 2008).
Most of the anterior segment diseases are usually treated by topical administration. Topical penetration routes for ocular drug delivery are classified into corneal and non-corneal (conjunctiva and sclera) routes. The corneal route is the major pathway for topical ocular drug delivery while the non-corneal route is considered to be inefficient for most of ophthalmics since drugs penetrating through the conjunctiva and sclera are eliminated into the systematic circulation via local capillary beds. Although the cornea is the main route of drug penetration, the presence of the corneal epithelium makes it the rate limiting barrier against drugs, and the permeability of compounds highly depends on the size and physiochemical characteristics of the drug molecules.
Large and hydrophilic drugs are mostly absorbed through the conjunctiva and sclera while small hydrophobic drugs penetrate through the cornea into the aqueous humor (Barar et al., 2009, 2008; Hornof et al., 2005; Urtti, 2006). Figure 2-6 illustrates the cellular organization of the cornea with its various transport barriers. As mentioned earlier, the corneal epithelium consists of superficial cell layers, wing cells and basal cells which are connected together with desmosomes and tight junctions making a strong barrier with just 10-20 nm cavities between cell layers. Tight junctions are part of the epithelial cell membranes at the apical side where two cells fuse together and make a tight barrier so that exogenous materials cannot pass between two interacting cells; the superficial cell monolayer then regulates the passage of molecules via transcellular active transport (see below). Desmosomes are molecular complexes of cell adhesion and linking proteins which are responsible for attachment of cell surface adhesion proteins to intracellular keratin cytoskeletal filaments (Hall, 1990; Sunkara and Kompella, 2003).

There are two parallel penetration pathways through the cornea:

- paracellular transport, which is driven by passive diffusion between the cells across the continuous corneal epithelium

- transcellular transport, which is mediated actively by cellular receptors and carriers through the lipid bilayer of the cell membrane.

Paracellular transport is limited by the size and charge of permeating molecule whereas transcellular movement depends on the interaction between transporters/receptors and the drug molecules (Dey et al., 2003). The mechanism of topical drug absorption to the cornea is a combination of both transports although, based on physiochemical characteristics of the drug molecules, one of them is the dominant transport pathway (Huang et al., 1983; Schoenwald and Huang, 1983). Epithelial cells selectively control the transport of hydrophilic compounds that can easily pass through the stroma and endothelial cells. On the other hand, hydrophobic drugs have a higher transcellular permeability through the corneal epithelial cells but have a lower permeability through the stroma (Mannermaa et al., 2006).
Figure 2-6 Cellular organization of the cornea showing its various transport barriers. Topmost layers of epithelial cells with tight junctions make a strong barrier monolayer. The basal endothelial monolayer is more permeable. (taken with permission from Barar et al., 2008)

2.2.2 Corneal Transporters

Membrane transporters/receptors play a critical role in transport processes of drugs, absorption, distribution and elimination of nutrients, ions and solutes in and out of the ocular cells. As mentioned in the previous section, corneal epithelial cells impose a tight barrier against xenobiotics and drugs, protecting the anterior part of the eye while the blood–retina barrier controls the traverse of systematically administered drugs to the posterior segment. By revealing the functional role of transporters/receptors, it has become evident that they are critically important in pharmacokinetics of ophthalmic drugs (Nies et al. 2011). Studying the transport processes existing in ocular barriers and selecting the proper drug delivery strategies utilizing these transporters and receptors can significantly improve the drug bioavailability to the target ocular tissue (Barnstable, 2008).
In ocular tissues, the two principal families of transporters are the efflux transporters (export pumps) and the influx transporters (uptake pumps). Influx transporters mediate the uptake of substances from the extracellular space into cells, and efflux transporters facilitate the secreting of substances out of cells. Figure 2-7 presents the currently known transporters of the corneal epithelium. The influx transporters in the cornea include amino acid transporters (LAT1, ATB0+, and ASCT1), oligopeptide transporters (PepT1, PepT2, PHT1 and PHT2), monocarboxylate transporters (MCT), nucleoside transporters (CNT3) and organic anion transporting polypeptides (OATP2). Most of these influx transporters belong to the solute carrier (SLC) superfamily. The efflux transporters consist of P-glycoprotein (P-gp), multidrug resistant protein (MRP) and breast cancer related protein (BCRP). All of these efflux transporters belong to the ATP binding cassette (ABC) superfamily (Barnstable, 2008; Dey et al., 2003; Mannermaa et al., 2006).

![Figure 2-7](image-url)

**Figure 2-7.** Schematic representation of the currently known transporters/receptors in the corneal epithelium (taken by permission from Barar et al., 2008)
Organic anion transporting polypeptides (OATPs) are a group of membrane carriers with a wide spectrum of amphiphilic substrates. Genes that encode for the OATPs are classified as the SLCO family. The OATP family consists of 11 members including the prostaglandin transporter OATP2A1 which is the shortest protein in its family with only 643 amino acids and is widely expressed in ocular tissues and particularly in human cornea (Kraft et al., 2010; Science and Haven, 1997). Prostaglandins (PGs) play an important therapeutic role in treatment of human ocular hypertension (glaucoma) and lowering the intraocular pressure (IOP) (Kashiwagi et al., 2002; Salvador, 1972). At physiological pH (7.0-7.4), PGs are charged anions and can poorly cross the lipid bilayer of cell membranes. The transport of prostaglandin is mediated by OATP2A1 transporters in the ocular tissues (Science and Haven, 1997; Kraft et al. 2010).

2.3 Latanoprost and Glaucoma

Glaucoma is a disease affecting more than 60 million people worldwide and is known as the second leading cause of irreversible blindness, happening often in elderly people. The number of people suffering from glaucoma is estimated to reach 80 million worldwide by 2020 (Quigley and Broman, 2006). In the United States alone, the number of people with glaucoma is approximately 2.5 million leading to annual US healthcare costs of about $2.5 billion (Mills et al., 2006).

Glaucoma is a group of ocular disorders mainly caused by ocular hypertension leading to progressive optic nerve damage, loss of vision and visual field defects. Glaucoma is most often due to increased IOP (Casson et al., 2012; Green, 1998). IOP is created by a dynamic balance between inflow and outflow of aqueous humor in anterior and posterior chambers. The aqueous humor is a watery fluid which is produced in ciliary body at a rate of ~2.5 µl/min (inflow), occupies the anterior chamber (AC) and posterior chamber (PC) and drains through trabecular meshwork to the canal of Schlemm (outflow). Normal IOP is in a range of 10-21 mmHg but when the balance between inflow and outflow is disrupted, it will lead to an elevated IOP which causes ocular hypertension and eventually optic nerve damage (glaucoma) (Lens et al., 2008).

Glaucoma can be categorized roughly into two main types, Open-Angle Glaucoma (OAG) and Angle-Closure Glaucoma (ACG). However, there are five basic subtype of glaucoma which are
clinically classified and are presented in the Table 2-1 (Casson et al., 2012). Primary open angle glaucoma is the most common form of glaucoma accounting for more than 90% of US cases of glaucoma patients (Mills et al., 2006). OAG has no symptoms other than elevated IOP until the patient loses vision. ACG has acute symptoms such as headache, redness, blurred vision and pain in the eye or even nausea and vomiting (Adatia and Damji, 2005). Although invasive surgical techniques are sometimes used to treat the acute ACG, IOP reduction with medication is the most common treatment strategy for all types of glaucoma, including normal tension glaucoma (NTG) (Casson et al., 2012).

<table>
<thead>
<tr>
<th>Subtypes of Glaucoma</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Open-Angle Glaucoma (POAG)</strong></td>
<td>IOP is elevated but no pathological cause is identified.</td>
</tr>
<tr>
<td><strong>Secondary Open-Angle Glaucoma (SOAG)</strong></td>
<td>IOP is high and the cause of elevated IOP is clinically identified.</td>
</tr>
<tr>
<td><strong>Primary Angle-Closure Glaucoma (PACG)</strong></td>
<td>A subtype of closed angle glaucoma with the elevated IOP but no identifiable cause.</td>
</tr>
<tr>
<td><strong>Secondary Angle-Closure Glaucoma (SACG)</strong></td>
<td>Elevated IOP with Identifiable pathological cause</td>
</tr>
<tr>
<td><strong>Normal Tension Glaucoma (NTG)</strong></td>
<td>A subtype of POAG where IOP is normal and no pathological cause is identified.</td>
</tr>
</tbody>
</table>

Various categories of ocular hypertensive drugs containing active ingredients such as β-blockers, carbonic anhydride inhibitors, prostaglandin analogues and α-adrenergic agonist or combinations of them are used in the form of eye drops for lowering the IOP in early diagnosis of glaucoma (Hariharan et al., 2009). Timolol Maleate, a β-blocker, was the first anti-glaucoma medication introduced in 1978. Later in 80s and early 90s, several other selective and non-selective β-blockers were introduced into the market. Apraclonidine and Brimonidine were α-adrenergic agonists which were introduced in 1988 and 1996 respectively. The effect of prostaglandin analogues on
lowering the IOP was reported first in 1985 and the first most potent prostaglandin prodrug to receive FDA approval in 1996 was Latanoprost in 1996 (Shah et al., 2011). Prostaglandin F2α analogues have shown an effective reduction of the IOP (Hariharan et al., 2009; Russo et al., 2008; Sjöquist and Stjernschantz, 2002a; Sjöquist et al., 1998). Latanoprost is designed as a lipophilic prodrug in which the carboxylic acid moiety in the α-chain has been esterified. Latanoprost is hydrolyzed to the free-acid form to increase the bioavailability of the active drug into the eye (Fig 2-8) by esterases in the cornea. The Latanoprost free-acid is the pharmacologically active form of the drug and increases the outflow of the aqueous humor at the trabecular meshwork (Toris et al., 2008).

![Latanoprost and Latanoprost Acid](image)

22-8. Chemical structure of Latanoprost and its respective free acid form (taken with permission from Sjöquist and Stjernschantz, 2002a)

The marketed anti-glaucoma drug (Xalantan) contains 50 µg/ml of Latanoprost. The effective adult recommended daily dose is one drop per affected eye which contains 1.5µg of Latanoprost (Electronic Medicines Compendium, eMC www.medicines.org.uk). Clinical studies have shown that the maximum concentration of Latanoprost active form (Latanoprost free acid) detected in aqueous humor after 1-2 hour of administration is about 150-300 ng which is only 2% of the applied dose (Sjöquist and Stjernschantz, 2002). However it has been shown that this amount reduces the IOP effectively in open angle glaucoma patients for 24 hours (Diestelhorst et al., 1997; Dubiner et al., 2004).
The poor bioavailability of Latanoprost and the lack of compliance by patients can lead to further vision impairment. Therefore designing a drug delivery system for Latanoprost which can increase the drug ocular surface retention time and delivers the drug on an extended period of time, can increase drug bioavailability while decreasing the frequency of administration and reliance on patient compliance and can improve the prognosis of glaucoma patients.

2.4 Silicone Hydrogel Contact Lenses as drug delivery systems

To overcome the low bioavailability of the topical drug instillation, regardless of corneal barrier and drug properties affecting absorption, one strategy is to increase the residence time of the drug on the ocular surface. This objective has been achieved by various approaches such as increasing the drug solution viscosity, using drug loaded gels, mucoadhesive formulations, ocular inserts, nanoparticles, emulsions and therapeutic contact lenses. (Chaiyasan et al., 2013; Gaudana et al., 2010; Lang, 1995). Among these, using contact lenses as a polymeric reservoir for ophthalmic drugs has been viewed as the most convenient approach due to the high degree of comfort, biocompatibility, and the potential for significantly increasing ocular drug retention time and bioavailability.

Contact lenses were developed more than half a century ago for vision correction and since have also been considered as an alternative for ocular drug delivery. First generation contact lenses were made of poly(methylmetacrylate) (PMMA) which had many disadvantages including low oxygen permeability and low water content (Guillon and Guillon, 1989; Robertson et al., 2007). The next generations of contact lenses were developed based on poly-2-hydroxyethyl methacrylate (pHEMA) (conventional contact lenses) with some also incorporating siloxane groups (silicone hydrogel contact lenses). Silicone hydrogel contact lenses have excellent oxygen permeability, a key characteristic for extended wear of contact lens. However, their hydrophobic surface chemistry can be the cause of discomfort and tear film destabilization. The hydrophilicity (wettability) of silicone hydrogels are significantly improved by surface treatment and incorporation of wetting agents (Stapleton et al., 2006). US food and Drug Administration (FDA) has categorized soft hydrogel contact lenses in four groups based on their water and ionic contents (Table 2-2). Silicone hydrogel contact lenses mostly belong to group I and III, which have a water content of less than
50%. Table 2-3 summarizes the characteristics of common commercially available silicone hydrogel contact lenses (Stapleton et al. 2006).

Contact lenses have been considered for ocular drug delivery for more than 50 years (Sedláček, 1965). Several methods have been used to load and impregnate the contact lens with drugs in order to get the optimum controlled release rate. These methods include drug soaking, molecular imprinting and carrier/surfactant mediated release using nanoparticles and liposomes. for complete reviews please refer to (Bengani et al., 2013; Guzman-Aranguez et al., 2013; Hsu et al., 2014; White et al., 2011). Drug-soaked contact lenses are the most common and easiest technique to obtain a release system for ophthalmics. Commercially available extended wear contact lenses, particularly silicone hydrogels, can be promising platforms to be used for extended release of drug to the anterior eye for treatment of diseases like glaucoma (Bengani et al., 2013; Kim et al., 2008).

Table 2-2. FDA classification of soft contact lenses (FDA Soft Contact Lens Grouping System 2014, www.fda.gov)

<table>
<thead>
<tr>
<th>FDA-group</th>
<th>Water Content (%)</th>
<th>Ionic content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>below 50%</td>
<td>below 0.2%</td>
</tr>
<tr>
<td>Group II</td>
<td>above 50%</td>
<td>below 0.2%</td>
</tr>
<tr>
<td>Group III</td>
<td>below 50%</td>
<td>above 0.2%</td>
</tr>
<tr>
<td>Group IV</td>
<td>above 50%</td>
<td>above 0.2%</td>
</tr>
<tr>
<td>Group V* (A)</td>
<td>below 50%</td>
<td>below 0.2%</td>
</tr>
<tr>
<td>Group V* (B)</td>
<td>below 50%</td>
<td>above 0.2%</td>
</tr>
<tr>
<td>Group V* (C)</td>
<td>above 50%</td>
<td>below 0.2%</td>
</tr>
</tbody>
</table>

*Group I-IV are conventional hydrogels and group V are silicone hydrogels
Table 2-3. Characteristics of silicone hydrogel contact lenses (adapted with permission from Stapleton et al., 2006)

<table>
<thead>
<tr>
<th>Commercial name (US adopted name)</th>
<th>Acuvue Oasys senofilcon A</th>
<th>Pure Vision balafilcon A</th>
<th>Focus Night&amp;Day Lotrafilcon A</th>
<th>O2Optix Lotrafilcon B</th>
<th>Acuvue Advance Galyfilcon A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>Johnson &amp;Johnson</td>
<td>Bausch &amp; Lomb</td>
<td>CIBA Vision</td>
<td>CIBA Vision</td>
<td>Johnson &amp;Johnson</td>
</tr>
<tr>
<td>Water content (%)</td>
<td>38</td>
<td>36</td>
<td>24</td>
<td>33</td>
<td>47</td>
</tr>
<tr>
<td>Principal Monomer</td>
<td>mPDMS</td>
<td>DMA, TRIS, NVP, TPVC siloxane macromer</td>
<td>DMA, TRIS, NVP, TPVC siloxane macromer</td>
<td>mPDMS, DMA, HEMA siloxane macromer</td>
<td></td>
</tr>
<tr>
<td>Surface modification</td>
<td>Plasma oxidation producing glassy islands</td>
<td>None</td>
<td>25 nm plasma coating with high refractive index</td>
<td>25 nm plasma coating with high refractive index</td>
<td>None Internal wetting agent (PVP)</td>
</tr>
<tr>
<td>FDA group</td>
<td>(I) Low water Non-ionic</td>
<td>(III) Low water Ionic</td>
<td>(I) Low water Non-ionic</td>
<td>(I) Low water Non-ionic</td>
<td>(I) Low water Non-ionic</td>
</tr>
</tbody>
</table>

NVP N-vinyl pyrrolidone; TPVC tris-(trimethylsiloxy)silyl) propylvinyl carbamate; NCVE N-carboxyvinyl ester; PBVC poly[dimethylsiloxyl] di[silylbutanol] bis[vinyl carbamate]; DMA N,N-dimethylacrylamide; HEMA 2-hydroxyethylmethacrylate; MA methacrylic acid; PVP polyvinylpyrrolidone; mPDMS monofunctional polydimethylsiloxane; TEGDMA tetraethyleneglycol dimethacrylate; EGDMA ethyleneglycol dimethacrylate.
2.5 Ocular In vitro Models

The Draize eye irritation test using live rabbits and dogs was developed in the 1940’s and has been the only valid toxicity test accepted by regulatory authorities since 1964. The Draize eye test has been widely criticized due to ethical and cost issues. Ex vivo test models using dissected animal eyes and corneas, are being used as alternatives but suffer from similar criticism as they still lead to animal sacrifices (Castro-Muñozledo, 2008; Draize et al., 1944; Huhtala et al., 2008; York and Steiling, 1997). Cell immortalization and cell culture techniques have enabled the development of in vitro ocular models. Extensive research has led to promising test platforms for assessing the drug permeability, ocular bioavailability and toxicity as well as studying the biological interaction of drug releasing materials (Castro-Muñozledo, 2008). The cornea is the topmost outer surface of anterior eye with the maximum penetration resistance and rate-limiting barrier for permeation to the anterior chamber, therefore corneal models consists mostly of stratified corneal epithelial cells (Castro-Muñozledo, 2008; Postnikoff et al., 2014; Robertson et al., 2011). Other commercial and experimental multilayer corneal constructs containing stroma and endothelium, have also been successfully developed (Castro-Muñozledo, 2008; Huhtala et al., 2008; Reichl 2004; Reichl et al., 2011).

2.5.1 Primary Cell Culture

Corneal cultures using isolated primary cells from humans and animals have been extensively described in the literature (Castro-Muñozledo 2008; Reichl et al. 2011). Techniques for the culture of primary cells have been developed since 1940s and several protocols have been established using rabbit cells because of their rapid proliferation rate and their potential to compare to in vivo test results since most of the in vivo tests are performed in rabbits. However, the main challenges in cultivating primary corneal epithelial cells in vitro are developing appropriate growth conditions for long-term survival and stable expression of differentiated phenotypes and establishing culture conditions close to their natural environment to enable the cells to proliferate and differentiate into a stratified multilayer (Hornof et al., 2005; Castro-Muñozledo, 2008; Reichl et al., 2011). Using primary human corneal primary cells in vitro for assessment of drug
permeability and toxicity is not routine due to the high variability in phenotype, the instability in gene expression of different donor samples, as well as their short life span. Many attempts have been made to resolve the shortcomings of using primary cells by modifying culture condition and culture media to extend the life span, increase the number of sub-cultures and the stability of differentiated phenotypes (Castro-Muñozledo, 2008; Hornof et al., 2005). However, immortalized human cell lines appear to be more suitable for routine and screening tests (Hornof et al., 2005).

2.5.2 Immortalized Cell Culture

Following the development of genetic engineering techniques, a variety of immortalized corneal cell lines has been established from human, rabbit, hamster and rat corneas. Immortalization of mammalian cells provides an alternative to address the high demand for use of animal models in biomedical and pharmaceutical research. Following isolation of cells from the tissues, these techniques establish cell lines which have the ability to grow and differentiate under defined culture conditions for high number of passages without any major instability in phenotype gene expression (Hornof et al. 2005; Castro-Muñozledo 2008; Huhtala et al. 2008). Most of immortalization of corneal epithelial cells is induced with oncogenes such as Simian Virus SV40, SV40-LT antigen and human papilloma virus HPV16 and HPV18. However, immortalization with viral oncogenes has some drawbacks such as the resistance to final differentiation and phenotype instability. Researchers have thus explored another immortalization approach by increasing the telomerase activity of cells. The mechanism of the shortening of the telomere during each cell division works as a mitotic clock and is responsible for controlling the cell life-span. By inactivation of p16, p53 and hTERT gene expression (which are responsible for telomere shortening), corneal epithelial cells are immortalized. Instead of genetic manipulation to immortalize epithelial cells, extended serial subcultures can also lead to isolation of spontaneous immortalized cell lines. However, these procedures are difficult and depend highly on the cell type, animal species and the tissue from which cells are isolated. Using immortalized corneal cell lines in in vitro models has major advantages like extended life span and fast growth with improved conditions that provide a high-throughput tool for screening and assessment of drugs (Castro-Muñozledo 2008).
2.5.3 Corneal Epithelial In vitro Models

On the ocular surface, the cornea is the main route of topical drug absorption with the corneal epithelium being the outer most surface. Therefore, current research in the field of drug delivery of ophthalmics is highly focused on corneal interactions with drugs and biomaterial at the tissue, cellular and molecular levels (Järvinen et al., 1995; Reichl et al., 2011; Sandeman et al., 2003).

Numerous corneal models have been developed by using different cell culturing techniques. These vary from culturing a simple monolayer of epithelium on a permeable membrane (Reichl et al., 2011) to differentiated stratified corneal epithelium models (Postnikoff et al., 2014). Models gain complexity from being a co-culture of epithelium-stroma or epithelium-3T3 fibroblast to the most complex engineered 3D corneal equivalents containing epithelium, stroma and endothelium layers (Castro-Muñozledo, 2008; Germain et al., 2000; Reichl, 2004; Reichl et al., 2011). Figure 2-9 illustrates the variety of different corneal models based on their specificity versus their complexity. These models, constructed with different cell culturing methods have various applications from simple in vitro toxicity test and drug permeability to construction of whole corneal equivalent for tissue implantation. Corneal epithelial monolayer models are mostly used for toxicity and eye irritation tests (Huhtala et al., 2008; Reichl et al., 2011; Toropainen et al., 2001). By developing more complex corneal models consisting of endothelial, stromal and epithelial layers constructed (Figure 2-10) on cell culture inserts, researchers hope to find better correlations between in vivo and in vitro results (Hornof et al. 2005; Huhtala et al. 2008). Various extracellular factors are involved in development of a three dimensional corneal model. As the corneal epithelium is exposed to the air interface in vivo, an air-liquid interface in the in vitro model is critical for differentiation of the corneal epithelium. This interface leads to the development of apical flat cells with desmosomes and tight junctions which carry out the barrier function.
The *in vitro* corneal models used for the assessment of ocular barrier function are made of stratified corneal epithelium multilayers or stratified epithelium multilayers co-cultured with stroma or 3T3 fibroblasts (Ban et al., 2003). Expression of differentiated keratocyte-specific biomarkers is a key indicator of a successful development of a stratified 3D corneal epithelium model. Therefore
modulation of cell proliferation and differentiation using modification of supplemented culture media, co-culture techniques and grafting culture inserts with ECM proteins are established methods by researchers for the development of corneal models mimicking the corneal barrier function. Another element which should not be neglected is the expression of transporters at the gene and molecular levels when these models are used for drug permeation and screening studies (Castro-Muñozledo, 2008; Dey et al., 2003; Forbes and Ehrhardt, 2005; Hornof et al., 2005; Mandery et al., 2010). Postnikoff et al. for the first time developed a curved stratified corneal epithelium in vitro model to assess the ocular biocompatibility of contact lens-multipurpose solution. This 3D corneal epithelium model was reconstructed successfully with the real human eye curvature using immortalized human corneal epithelial cells that proliferated and stratified on a collagen coated cellulosic membrane. This 3D model would fit well the curvature and the area of contact lenses, and is a suitable platform for ocular biocompatibility assessment of contact lenses, multipurpose solutions and any other topical ophthalmics (Postnikoff et al. 2014).

Only a few whole cornea models have been developed so far that can mimic the in vivo environment. Minami et al. have isolated bovine endothelial, stromal and epithelial cells and cultured them in a 3D collagen matrix. In this model, the cells express the cornea specific keratin biomarkers and the epithelium consists of 5–6 layers of stratified differentiated cells. This was the first reconstructed corneal equivalent from primary cells (Hornof et al. 2005). Griffith et al., successfully constructed a functional human cornea from immortalized cell lines consisting of three cellular compartments of epithelium, stroma and endothelium as well as two acellular Bowman’s and Descemet’s membranes. The equivalent cornea mimicked major physiological and morphological functions as well as the transparency of the real human cornea. This in vitro human corneal construct showed similar level of expression of genes and key biochemical markers of human cornea (Li et al., 2005, Griffith, 1999).
Chapter 3
Characterization of the Human Corneal *In vitro* Model

3.1 Introduction

The main objective of this study was to characterize an *in vitro* corneal epithelial cell model previously used in Dr. Gorbet’s lab and further investigate the transport of Latanoprost (a glaucoma drug) released from a contact lens. As previous results by Mohammadi et al., (2014) showed that live cells in the *in vitro* models had a significant effect on the extended release of Latanoprost from a contact lens (Mohammadi et al., 2014), it is hypothesized that active transcellular transport mediated by certain drug transporters in the corneal epithelium is involved in transport of Latanoprost. It is also hypothesized that, for Latanoprost, the transcellular active transport is more important than the passive diffusion in paracellular transport. To test these hypotheses, the presence of organic anion transporting polypeptide 2A1 (OATP2A1), which is responsible for the transport of prostaglandins in the human cornea, was investigated at the gene expression level. To further study the potential role of the OATP2A1 transporters in transcorneal diffusion of Latanoprost, the effect of Diclofenac, a cyclooxygenase inhibitor, on the function of OATP2A1 transporters was studied.
3.2 Materials and Methods

3.2.1 Preparation of Drug Solution

Latanoprost was bought from Cayman Chemical (Ann Arbor, MI, USA) as 5mg aliquot in a solution of methyl acetate. To obtain a solution of 50µg/ml, the 5mg of Latanoprost aliquot was dissolved in 100ml of phosphate buffered saline (PBS) (Lonza, Walkersville, MD) The concentration of the stock drug solution was then measured by enzyme immuno assay (EIA) (see below) and determined to be 161.2±36.5µg/ml. The solution was further diluted and concentration confirmed at 19.3±3.4µg/ml. All Latanoprost solutions were stored at –20 °C.

3.2.2 Preparation of Contact Lenses

Two commercially available silicone hydrogel contact lenses, balafilcon A and senofilcon A were used in this study. The chemical properties of these lenses are presented in the Table 3-1. Lenses were first incubated for 24 hours in PBS to remove any remnants of their packaging solution. The lenses were then incubated in 1.5ml of the drug solution (total amount of Latanoprost available to contact lens was 29 µg) for 24 hours.

| Table 3-1 Properties of the Contact Lens Hydrogel Materials (Mohammadi et al., 2014) |
|---------------------------------|---------------------------------|---------------------------------|
| **Commercial name** (US adopted name) | Acuvue Oasys senofilcon A | Pure Vision balafilcon A |
| Manufacturer | Johnson & Johnson | Bausch & Lomb |
| Water content | 38 | 36 |
| Principal Monomer | mPDMS | NVP + TPVC |
| | + DMA + HEMA + siloxane macromer + | + NVA + PBVC |
| | TEGDMA + PVP | |
| Surface modification | Plasma oxidation producing glassy islands | None Internal wetting agent – (PVP) |
| FDA group | (I) Low water Non-ionic | (III) Low water Ionic |
3.2.3 Cell Culture

The HPV16-E6/E7 immortalized human corneal epithelial cells (HCEC) generously gifted by Dr. Griffith, University of Ottawa Eye Institute, Ottawa, ON, Canada (Griffith et al., 1999) were cultured in a 25cm² treated tissue culture flask containing 10ml of keratinocyte serum free medium (KSFM) supplemented with keratinocyte growth supplement (KGS), and penicillin/streptomycin (Pen/Strep) (ScienCell, Carlsbad, California, USA) at 37°C and 5% carbon dioxide (CO₂). Every other day, the medium was renewed and cells were monitored for any morphological changes. After cells reached 90% confluency, adherent cells were removed following a 15-minute incubation in 3ml TryplExpress (Life Technologies Burlington, Ontario, Canada) dissociation solution at 37°C and 5% CO₂. Dissociated cells were centrifuged at 1200 rpm for 7 minutes and were washed with 10ml medium supplemented with 10% fetal bovine serum (FBS) (Life Technologies Burlington, Ontario, Canada), then were ready to use.

3.2.4 Flat Monolayer HCEC Model.

To develop a transcorneal flat monolayer in vitro model, polyethylene terephthalate (PET) membrane inserts (Millicell PET membrane with a 1.0m pore size, also referred to as culture inserts, Millipore, MA, USA) were set in a 12 well plate. The PET inserts were incubated with 90 µl of 0.05 mg/ml Collagen type I from rat tail (ScienCell Carlsbad, California, USA) for 30 minutes. Following collagen coating, the inserts were washed with 500 µl of PBS (Lonza, Walkersville, MD) and were then seeded with 10⁵ cells per well. The corneal epithelium models were fed with KSFM on each of the basal and apical sides of the cell layers for 6 days in order for the monolayer to form tight junctions. The medium was exchanged every other day.

3.2.5 Fixing cells

To investigate the role of metabolically active cells versus a physical barrier of cell monolayer to drug permeation, a set of experiments was designed to compare Latanoprost release from a contact lens through a fixed and a live monolayer corneal model. In the fixed monolayer, cells are dead and thus neither metabolic activity nor active transport occurs. The only transport would be a passive paracellular diffusion. Cells were fixed for 24 hours with a 2% paraformaldehyde solution in PBS.
3.2.6 Transport Inhibition

The nonsteroidal anti-inflammatory drugs (NSAIDs) are inhibitors of both isoforms of cyclooxygenase, COX-1 and COX-2, which are the rate limiting enzymes for the synthesis of prostaglandins (Cryer and Feldman, 1998). Diclofenac, a NSAID, can inhibit completely the OATP 2A1 transporter responsible for the transport of prostaglandins in cells (Mandery et al., 2010). Diclofenac sodium salt (Sigma-Aldrich Catalogue No. 93484) was dissolved in KSFM at the concentration of 100 µM (Mandery et al., 2010). To inhibit the potential prostaglandin transporters in the corneal epithelial cells, at day 5, some of the corneal epithelial monolayers were incubated in 1.5 ml of the Diclofenac solution for 24 hours before the release experiments.

3.2.7 MTT viability Assay

The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay measures mitochondrial activity and is based on the conversion of MTT into formazan crystals by living cells. For most cells, the total mitochondrial activity is related to the number of viable cells, thus this assay is broadly used to assess viability and cytotoxic effects of drugs on cell lines (Cree, 2011; Mosmann, 1983). To perform the MTT assay, the tetrazolium salt was dissolved in pre-warmed KSFM at 0.5 mg/ml and then sterilized through a 0.2µm pore sized filter. 0.5ml and 1ml of MTT solution was applied to the basal and apical surfaces of the monolayer respectively. After three hours of incubation at 37°C and 5% CO₂, the inserts were covered with the dark purple formazan crystals (Figure 3-1). The formazan crystals were dissolved in 1.5ml of isopropanol on the apical and basal side of the cell culture inserts. The inserts with isopropanol were left on a shaker for 2 hours in order to ensure complete dissolution of the formazan. The apical and basal formazan solutions were mixed and an aliquot of formazan solution was read on a UV-Vis plate reader at wavelengths of 595nm and 620nm.
Figure 3-1. Human corneal epithelial cells were grown on PET membrane insert and were stained with MTT on the 6th day of growth (left). Formazan (purple) crystals inside cells (right) indicate viable cells.

3.2.8 *In Vitro* Drug Release Experiment

Three *in vitro* models were used to assess the effect of active transport on the release kinetics of Latanoprost from commercially available contact lenses. The *in vitro* models used diffusion through a monolayer of human corneal epithelial cells (HCECs), through a transport inhibited HCECs monolayer and a fixed monolayer of HCECs. Aliquots of 50µl were taken from the bottom of the *in vitro* models and replaced by fresh culture medium. Samples were taken at 1, 4, 8, 12, 24, 48, 72 and 96 hours. Collected samples were analyzed by an enzyme immuno-assay (EIA) for Latanoprost (Cayman Chemical, Ann Arbor, MI, USA). Following the EIA kit instructions, each collected sample was analyzed in duplicate and at two different dilutions. Each set of experiments was repeated 3 times independently on different days. Drug concentrations were calculated based on the previously published study from Gorbet’s research laboratory (Mohammadi et al., 2014). Briefly, samples were taken from the bottom of the wells and replaced by fresh medium at each time point. In order to consider the dilution rate and to calculate the absolute amount of drug released at each point, mass balance principles were applied. To estimate the actual concentration at each time point, the equation below was used:

\[ C_{a,i} = C_i + k \sum_{j=1}^{i-1} C_j \]
$C_{a,i}$ is the actual concentration at the time point $t_i$, $C_i$ is the measured concentration at the time point $t_i$, $k$ is the fraction of the total medium volume at the bottom of the wells which was aliquoted. $\sum_{j=1}^{i-1} C_j$ is the summation of concentrations measured before time point $t_i$. The effect of dilution on the diffusion rate of the drug was assumed to be insignificant due to the small difference in the total volume of medium after sampling (Mohammadi et al., 2014).

### 3.2.9 RNA Isolation and Real Time PCR

Total RNA from corneal epithelial cells grown to confluence in a 25 cm$^2$ flask was extracted with an RNA isolation kit (Illustra RNAspin Mini; GE Healthcare, Little Chalfont, Buckinghamshire, UK) based on the company’s protocol. RNA samples were stored at -80°C until analysis.

The reverse transcription of extracted RNA (2 µl each) was performed using Superscript III kit (Invitrogen, Carlsbad, CA) based on the company’s protocol. The cDNA was amplified in a sequence detection system (ABI 7900HT; Applied Biosystems) at 45 cycles of 50°C for 2 minutes, followed by denaturation at 95°C for 15 seconds, and then annealing at 60°C for 1 minute. Predesigned primers and TaqMan probes for SLCO2A1 (OATP2A1) and the housekeeping genes $\beta$-actin (ACTB) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used (Hs00194554_m1, Hs00200670_m1, Hs00357333_g1, Hs00266705_g1; Applied Biosystems) (Kraft et al., 2010; Mandery et al., 2010).

### 3.2.10 Gel Electrophoresis

Agarose gels (1.5%) were obtained by dissolving 0.75 g agarose in 50 ml of Tris Acetate EDTA (TAE) buffer heated to its boiling point. After complete dissolution of agarose, the solution was cooled down for 5 minutes and 0.2 µl GelRed dye (Biotium, Inc. Hayward, CA USA) was added. The solution was cast into the mold plate and the gel film was formed within 30 minutes. Amplified PCR cDNA samples were mixed with DNA dye (bromophenol blue) at a ratio of 1:5. The agarose gel was placed into a Bio-Rad mini sub-cell and filled with Tris Acetate EDTA buffer to cover the entire gel surface. 18 µl of cDNA samples with dye were loaded in each well and
electrophoresis was run at 85V and 300 mA until the samples had migrated to within ¾ of the positive electrode end of the gel. The gel was removed and photographed under UV lamp.

3.2.11 Data Analysis

All results were expressed as the mean ± standard deviation of three independent experiments. Statistical analysis was performed by two-way analysis of variance (ANOVA) using Minitab Express™ Software. A p value of less than 0.05 was required for statistical significance.
3.3 Results

3.3.1 The effect of active transport on release of Latanoprost

The release profiles of Latanoprost from balafilcon A through live, transporter-inhibited and fixed monolayer in vitro models are presented in Figure 3-2. The maximum amount of Latanoprost released from balafilcon A after 96 hours was 1.8±0.2µg in the live HCEC monolayer model. The least amount of Latanoprost (0.3±0.0µg) was released in the monolayer model where cells had been fixed. As can be seen on Figure 3-2, the release profiles of Latanoprost in all three models were linear with approximately constant rates (R²>0.99). Table 3-2 reports the Latanoprost release rate from balafilcon A. The average release rates for balafilcon A in live, inhibited and fixed models were 19.1±1.8, 13.3±3.1 and 2.9±0.3 ng/hour respectively. Inhibiting transporters in the HCEC monolayer led to approximately 36±1% reduction in the total amount of Latanoprost released from balafilcon A after 24 hours (1.2±0.3 µg), a 30% reduction in the rate of release (Table 3-2). The release rate and the amount of released drugs were significantly affected by inhibition of transporters in immortalized HCECs compared to live HCEC monolayer model (p <0.0001). Figure 3-4 presents the rate of inhibition calculated based on the amount of released drug through live, transport inhibited and fixed cell models over 96 hours of experiment. As seen in the figure, after 24 hours, the percentage of inhibition remained constant up to 48 hours and started to unexpectedly be less effective after that. Fixing the cells also led to 83±1.5% decrease in the amount of released Latanoprost (~85% decrease in the release rate from balafilcon A).
Figure 3.2. Latanoprost release from balafilcon A contact lenses through live, transporter-inhibited and fixed HCEC monolayer models. Cells were fixed in 2% paraformaldehyde. Lenses were soaked for 24 hours in drug solution (29 µg Latanoprost) and then overlaid on the monolayer for 96 hours, n = 3, Mean ±SD.
* Significantly different compared to transport inhibited model (p< 0.0352)

Table 3.2. Average Latanoprost release rate from balafilcon A and senofilcon A silicone hydrogel in 96 hours

<table>
<thead>
<tr>
<th>Contact lens</th>
<th>Average Release Rate (ng/hour)$§$</th>
<th>In vitro Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Live</td>
<td>Transport Inhibited</td>
</tr>
<tr>
<td>balafilcon A</td>
<td>19.1±1.8*</td>
<td>13.3±3.1</td>
</tr>
<tr>
<td>senofilcon A§</td>
<td>34.6±6.7*</td>
<td>16.6±2.7</td>
</tr>
</tbody>
</table>

* Significantly different compared to transport inhibited model (p<0.0001)
§ Significantly different compared to balafilcon A (p=0.0095)

Figure 3-3 presents the release profiles of Latanoprost from senofilcon A through the live, transport inhibited and the fixed corneal *in vitro* models. The total amount of Latanoprost released from senofilcon A in the live monolayer model was 3.2±0.6 µg after 96 hours; this significant increase in release is almost twice the amount released from balafilcon A in the same *in vitro* model (p=0.0322). The total amounts of drug released from senofilcon A after 96 hours were 1.6±0.3 µg
and 0.2±0.1 μg in the transport inhibited and the fixed cell models respectively. Similar to balafilcon A, the release profiles of Latanoprost in all three models were linear throughout the 96 hours of the experiments with a constant release rate (R²>0.99) (Table 3-2). The average rate of release for senofilcon A through live, inhibited and fixed cell models were 34.6±6.7, 16.6±2.7 and 2.4±0.6 ng/hour respectively. Inhibiting transporters in the HCEC monolayer by 100μM of Diclofenac sodium after 12 hours decreased the total amount of Latanoprost released from senofilcon A by approximately 58±3% and remained constant up to the end of the experiments (see Figure 3-4). This translated to a 52% reduction in the average rate of release as shown in Table 3-2. Fixing the cells caused a 91±3.4% reduction in total amount of released drug from senofilcon A (a reduction of 93% in release rate).

![Figure 3-3. Latanoprost release from senofilcon A contact lenses through live, transporter inhibited and fixed HCEC monolayer models. Cells were fixed in 2% paraformaldehyde. Lenses were soaked for 24 hours in drug solution (29 µg Latanoprost) and then overlaid on the monolayer for 96 hours. Release experiments were conducted in three separate dates n = 3, Mean ± SD. * Significantly different compared to transport inhibited model at all time points after 12 hours (p<0.0005)](image)

Overall, inhibition of transporters in immortalized HCECs significantly affected the release rate and the amount of released drugs compared to live HCEC monolayer model (p<0.0001). It is also
important to highlight that in all 3 models, Latanoprost release from balafilcon A was significantly lower than senofilcon A ($p=0.0322$). Analysis of variances (ANOVA) indicated that contact lens materials and in vitro models had significant effects on the release kinetics of Latanoprost ($p<0.0001$); however the interaction effect of contact lens type and in vitro model was not significant ($p =0.062$).

![Graph](image)

**Figure 3-4.** Transport inhibition of organic anion transporter protein OATP 2A1 by 100 µM Diclofenac throughout 96 hours of Latanoprost release from balafilcon A and senofilcon A silicone hydrogel contact lenses. Inhibition percentage is calculated based on the difference between the average released amount of drug from lenses through live and inhibited monolayer in vitro models, $n = 3$, Mean ±SD

### 3.3.2 Real Time PCR

The real-time polymerase chain reaction amplification graphs for the organic anion transporter protein genes SLCO 2A1 responsible for the expression of the OATP 2A1 protein (prostaglandin transporter) are presented in Figure 3-5. Comparison of the SLCO 2A1 to the housekeeping genes indicated that SLCO 2A1 cDNA was amplified, confirming the presence of this gene in the immortalized human corneal epithelial cell line. The gel electrophoresis results from the amplified cDNA samples (Figure 3-6) further highlight the presence of the OATP 2A1 gene in the human corneal epithelial cell line.
Figure 3-5 Real-Time PCR Amplification graphs. SLCO2A1 (light green), Beta Actin (Blue), GAPDH (Orange). 2µl of cDNA sample were run at 2 dilutions (1X and 10X). 2µl of RNA samples were used as negative control.

Figure 3-6. Gel Electrophoresis of amplified cDNA samples. Sample 2 is 10X dilution of sample 1. 2µl of RNA samples were used as negative control.
3.4 Discussion

In our three cell models, the release profiles of Latanoprost from both contact lenses were linear, indicative of a controlled release with constant rate. These results are in agreement with results presented previously by Mohammadi et al. (2014). In this study, the amount of drug released in 48 hours from silicone hydrogels loaded with ~185µg Latanoprost increased from 0.3-0.5 µg in a no-cell model to 2.5-4.5 µg in a model using a monolayer or a multilayer of corneal epithelial cells.

Lower amounts of Latanoprost were released from contact lenses when cells were fixed or inhibited. These results thus confirm that the presence of live cells in the in vitro model significantly affects the rate of release and amount of eluted Latanoprost from balafilcon A and senofilcon A. Considering the hydrophobic nature of Latanoprost and its high affinity for the lens polymer, it can be postulated that an active transport mechanism mediated by cell transporters is involved in the transport of Latanoprost in the corneal in vitro model. OATP2A1 is a well-known transporter responsible for transport of prostaglandins (Kraft et al., 2010; Mandery et al., 2010) and its presence in the HPV16 E6/E7 immortalized human corneal epithelial cells was confirmed by RT-PCR. Furthermore, the significant decrease in the rate and amount of released Latanoprost following inhibition of this transporter with 100µM of Diclofenac sodium confirmed the role of active transcellular transport in the experimental model. Based on previous work by (Mandery et al., 2010), Diclofenac sodium at 100µM is expected to completely inhibit OATP2A1 transporters in HCEC. However, only a 40 to 50% reduction in transport was achieved suggesting that other transporters may be responsible for active transport of Latanoprost through the cornea. The fact that over 90% of Latanoprost transport was inhibited by paraformaldehyde fixation indicates that cellular active transport governs transcorneal permeation of the hydrophobic Latanoprost.

The zero order release rates observed in this study also suggest that loading of Latanoprost prodrug in balafilcon A and senofilcon A can potentially provide a simple platform for a sustained controlled release system which may be able to deliver Latanoprost to the eye at a constant rate within the therapeutic range and during an extended period of time. Further investigation is needed to determine effective dosing for extended drug delivery purposes, which will be discussed in the next chapter.
Chapter 4
Application of the Human Corneal *In vitro* Model to assess Latanoprost Controlled Released System

4.1 Introduction

This part of the project was defined with the perspective of designing a controlled release device using commercially available extended-wear contact lenses loaded with lower amount of Latanoprost (low dose therapeutic extended-wear contact lens). Mohammadi et al. 2014 previously showed a linear release of Latanoprost from three silicone hydrogel contact lenses (balafilcon A, senofilcon A and galyfilcon A) in the presence of live cells. While 95% of the Latanoprost in solution (131 µg/ml) was uptaken in lenses, less than 4% of the uptaken drug was released. This is due to the high affinity of the hydrophobic drug to the contact lens. Despite overall low release from the lenses, this amount was still much higher than the required therapeutic level (150-300ng) of Latanoprost. In this study, it was hypothesized that by loading lower concentration of Latanoprost on contact lenses, the therapeutic amount of the drug could still be released and in turn increase the efficacy and economy of the controlled release system. To test this hypothesis, the uptake and release kinetics of silicone hydrogel contact lenses loaded with lower amount of Latanoprost over an extended period of time was assessed.
4.2 Materials and Methods

4.2.1 Preparation of Drug Solution

The drug solutions with concentrations of 19.3, 4.8 and 2.9 µg/ml were obtained by dilution of Latanoprost stock solution (as explained previously in section 3.2.1).

4.2.2 Preparation of Contact Lenses

Two commercially available silicone hydrogel contact lenses, balafilcon A and senofilcon A were used in this study. The chemical properties of these lenses were presented in the Table 3-1. Lenses were first incubated for 24 hours in PBS to remove any remnants of their packaging solution. The lenses were then incubated in 1.5ml of the drug solution (total amounts of Latanoprost available to contact lenses were 29.0, 7.2 and 4.3 µg) for 24 hours.

4.2.3 Latanoprost Uptake and Release

After incubation with the drug solutions, the lenses were set on the in vitro corneal model. The volume of liquid in the insert was 0.5ml and 1.0ml in the bottom well. The release was studied for up to 96 hours. Aliquots of 50µl were taken from the bottom of the in vitro models and replaced by fresh culture medium. Samples were taken at 4, 12, 24, 48, 72 and 96 hours. Collected samples were analyzed by an EIA kit for Latanoprost (Cayman Chemical, Ann Arbor, MI, USA). Following the EIA kit instructions, each collected sample was analyzed in duplicate and at two different dilutions. To determine the uptake amount by the contact lenses, samples were also collected from the soaking drug solution as well as the remaining drug solutions after soaking the lenses. Each set of experiment were repeated for three times independently. Drug concentrations were calculated based on the equation explained previously in Chapter 3.

4.2.4 Data Analysis

Results presented here are the mean±standard deviation of three independent experiments. Statistical analysis of the data was performed by two-way analysis of variance (ANOVA) using Minitab Express™ Software. A p level of less than 0.05 was required for statistical significance.
4.3 Results

4.3.1 Latanoprost Uptake and Release

The Latanoprost uptake and release data for both balafilcon A and senofilcon A contact lenses are presented in Table 4.1. The theoretical expected concentration of the drug solution and actual initial concentrations of drug loading solution measured by EIA are also presented in this table. As shown in the table, more than 95% of Latanoprost available to the lenses was uptaken in 24 hours regardless of initial concentration. The maximum drug loaded was 28.9 μg (99.5±0.1% uptake) for balafilcon A with initial loading amount of 29.0±5.1 μg Latanoprost. The minimum drug loaded was 4.2 μg with initial loading amount of 4.3±0.3 μg Latanoprost. As expected, there was a significant effect of initial loading concentrations on the uptake amount of Latanoprost to contact lenses (p=0.0001) but there was no effect of contact lens materials (p=0.6316).

The maximum amount of drug released after 96 hours was 4.1±0.3 μg which represented 57.2±3.5% of the uptaken drug by senofilcon A with the initial loading amount of 7.2±0.6 μg. The minimum amount released after 96 hours was 0.5±0.1 μg from balafilcon A loaded with 4.3±0.3 μg. After 96 hours, senofilcon A released significantly more Latanoprost than balafilcon A regardless of initial amount of loading (p=0.0007). The amounts of drug released from both silicone hydrogel contact lenses with initial loading conditions of 29.0 and 7.2 μg were significantly higher than the amount released from these lenses loaded with 4.3 μg (p=0.007); regardless of contact lens type. There was no significant difference in the release amount between initial loading conditions of 29.0 and 7.2 μg (p=0.7920).
Table 4-1 Latanoprost uptake into balafilcon A and senofilcon A contact lenses and total amount of drug released after 96 hours through the live HCEC monolayer *in vitro* model. Lenses were soaked for 24 hours in different concentrations of Latanoprost solutions and then drug uptake into the lens were measured. Uptake experiments were conducted in three separate dates (n = 3, Mean ± SD) except for drug solutions containing 7.2 µg Latanoprost (n=2, Mean ± SD)

<table>
<thead>
<tr>
<th>Theoretical concentration(^1) (µg Latanoprost/ml)</th>
<th>10</th>
<th>6</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual loading concentration(^2) (µg Latanoprost/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loading dose (µg)</td>
<td>19.3 ± 3.4</td>
<td>4.8 ± 0.4</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>29.0 ± 5.1</td>
<td>7.2 ± 0.6</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uptake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>balafilcon A</td>
<td>99.5±0.1</td>
<td>6.1±0.6</td>
<td>1.8±0.2</td>
</tr>
<tr>
<td>senofilcon A</td>
<td>98.6±0.6</td>
<td>11.3±2.2$</td>
<td>3.2±0.6$</td>
</tr>
<tr>
<td>Release(^3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>balafilcon A</td>
<td>95.7±0.9</td>
<td>14.2±6.5</td>
<td>97.8±0.9</td>
</tr>
<tr>
<td>senofilcon A</td>
<td>96.0±1.0</td>
<td>57.3±3.5$</td>
<td>97.4±0.2</td>
</tr>
<tr>
<td>Release(^4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>balafilcon A</td>
<td>6.9±0.1</td>
<td>4.1±0.3$</td>
<td>4.2±0.0</td>
</tr>
<tr>
<td>senofilcon A</td>
<td>97.4±0.2</td>
<td>32.2±11.9$</td>
<td>1.4±0.5$*</td>
</tr>
</tbody>
</table>

1 Expected (nominal) concentration based on the manufacturer’s label
2 Concentration of the solution measured by EIA
3 The release percentage was calculated based on the ratio of the released amount over the uploaded amount of drug per contact lens.
4 Significantly different compared to balafilcon A (p < 0.0001)
5 Significantly different compared to 7.2 and 29.0 µg loading amount (p < 0.003)
4.3.2 Latanoprost Release Kinetics

Figure 4-1 and 4-2 illustrate the 96-hour release profiles of Latanoprost from balafilcon A and senofilcon A contact lenses loaded with different amount of drugs (Table 4-1). The release data for initial loading amount of 7.2 µg at the time point of 72 hours are not included due to sampling errors for both balafilcon A and senofilcon A contact lenses. The trend of release is presented by dash lines connecting data of between 48 and 96 hours’ time points for this condition.

As shown in Figure 4-1, the lenses which were loaded with 7.2 and 29.0 µg of Latanoprost showed linear release kinetics ($R^2>0.98$). No significant difference in the amount of drug released was observed ($p=0.5480$) at all time-points. On the other hand, the release rate for the contact lens loaded with the lowest amount of Latanoprost (4.3µg) decreased after 24 hours and reached an equilibrium showing a first-order decay rate ($R^2 = 0.9492$). At 24 hours, all the amount of Latanoprost released for all three initial loading concentrations were above clinical therapeutic dose. The average release rates of balafilcon A with different initial loading concentrations are presented in Table 4-2. The maximum average rate of release for balafilcon A was 36.5 ng/hour with the initial loading of ~7.2 µg Latanoprost whereas the minimum average release rate for balafilcon A was 7.3ng/hour with the initial loading amount of ~4.3 µg.
Figure 4-1. Latanoprost release from balafilcon A contact lenses through live HCEC monolayer models. Lenses were soaked for 24 hours in drug solutions containing 4.3, 7.2 and 29.0 µg Latanoprost and then overlaid on the monolayer for 96 hours. Release experiments were conducted on three separate dates n = 3, Mean + SD except for drug solutions containing 7.2 µg Latanoprost n=2, Mean ± SD * Significantly different after 24 hr compared to 7.2 and 29.0 µg loading amount (p<0.0214)

The release profile of Latanoprost from senofilcon A contact lenses loaded with different amount of drugs in a time course of 96 hours are presented in Figure 4-2. The Latanoprost release profiles for senofilcon A have a similar trend compared to the release behavior of balafilcon A, regardless of the rate and the amount of drug released. The release kinetics of senofilcon A with initial loading amount of 29.0 and 7.3 µg were zero-order (R²>0.98) with average rates of 44.2 and 36.4 ng/hour respectively (Table 4-2). However, the kinetics of Latanoprost release from senofilcon A with 4.3 µg loading amount was a first-order decay (R²=0.68) depleting the drug after 24 hours of release with the average rate of 14.6 ng/ml.

Statistical analysis indicated that there was a significant effect of lens type on release kinetics (p<0.0001) and initial loading concentration significantly affected the rate of release from silicone hydrogel contact lenses. (p=0.0221)
Figure 4-2. Latanoprost release from senofilcon A contact lenses through live HCEC monolayer models. Lenses were soaked for 24 hours in drug solutions containing 4.3, 7.2 and 29.0 µg Latanoprost and then overlaid on the monolayer for 48 hours. Release experiments were conducted on three separate dates n = 3, Mean ± SD except for drug solutions containing 7.2 µg Latanoprost n=2, Mean ± SD

* Significantly different after 24 hours compared to 7.2 and 29.0 µg loading amount (p<0.0406)

Table 4-2. Latanoprost average release rate from silicone hydrogel contact lenses in 96 hours

<table>
<thead>
<tr>
<th>Contact Lens</th>
<th>Initial loading amount (µg)</th>
<th>average release rate (ng/hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>balafilcon A</td>
<td>4.3</td>
<td>7.3±1.4*</td>
</tr>
<tr>
<td>senofilcon A</td>
<td></td>
<td>14.6±5.3§*</td>
</tr>
<tr>
<td>balafilcon A</td>
<td>7.2</td>
<td>36.5±19.6</td>
</tr>
<tr>
<td>senofilcon A</td>
<td></td>
<td>44.2±2.6§</td>
</tr>
<tr>
<td>balafilcon A</td>
<td>29.0</td>
<td>19.1±1.8</td>
</tr>
<tr>
<td>senofilcon A</td>
<td></td>
<td>34.6±6.7§</td>
</tr>
</tbody>
</table>

§ Significantly different compared to balafilcon A (p<0.0417)
* Significantly different compared to 7.2 and 29.0 µg loading amount (p<0.0221)
4.4 Discussion

This study was undertaken with the ultimate goal of designing a controlled release device using marketed contact lenses for extended release of Latanoprost for treatment of glaucoma. The short retention time on ocular surface and poor patient compliance due to the frequent dose requirement of topical eye drops are two of the main challenges in topical ophthalmic administration (Urtti, 2006). Using silicone hydrogel contact lenses has always been considered as an alternative solution to overcome these challenges (Kim et al., 2010, 2008; Mohammadi et al., 2014; Peng and Chauhan, 2011; Peng et al., 2012).

In this study, the impact of loading lower amount of drug on the rate and the amount of drug released in an extended delivery time (4 days) was assessed. The results showed that the therapeutic dose has been reached in 24 hours with all three initial loading amounts. However, the release rates for the low loading amount (4.2 µg) were not constant throughout the entire release period for both balafilcon A and senofilcon A reaching a plateau after 24 hours of release. Comparison between the uptake and the release amounts for this particular condition indicates that lenses have not been depleted, with only up to 32% being released. This saturation might be due to the equilibrium between the cellular active transport of Latanoprost and chemical interaction forces between hydrophobic Latanoprost molecules and the contact lens polymer matrix. This could be a disadvantage for an extended drug delivery device. On the other hand, the release rates for both contact lenses loaded with 29 and 7.2 µg of Latanoprost were approximately constant throughout the entire experiment giving a zero order release. The maximum released amount of Latanoprost from both contact lenses in these conditions were in the range of ~3.2–4.1 µg which are comparable to the released amount achieved by Mohammadi et al., 2014 although these amounts of Latanoprost are eluted in 96 hours rather than 24 hours.

Surprisingly, the release results showed a higher amount of drug eluted from senofilcon A loaded with 7.2 µg compared to senofilcon A loaded with 29.0 µg Latanoprost. While this indicates that soaking the senofilcon A lens in 7.2 µg of Latanoprost provides a drug delivery system just as effective as soaking the lens into 29 µg, the statistical difference observed may highlight some of the experimental variations that are observed with measuring concentration.
of Latanoprost. The high dilutions that are necessary to measure Latanoprost using the EIA are associated with high standard variations and may be at the origin of the currently observed statistical difference. Similar large standard deviations were noted by Mohammadi et al., (2014). Further experiments will be required to confirm the difference observed in these experiments.

The fact that, regardless of initial loading concentrations, senofilcon A released significantly higher amount of Latanoprost compared to balafilcon A is in agreement with previous results by Mohammadi et al. (2014). Our results with lower uploaded amounts indicates a release of more than 55% of uptaken drug into contact lenses, a significant improvement over the 4% obtained previously in our lab, suggesting that a simple, economical and cost effective drug delivery system can be obtained with silicone hydrogels and Latanoprost.

In this study, we were able to obtain sustained release of Latanoprost in vitro for up to 4 days. There is currently limited data available on the effect of extended release of glaucoma drug on maintaining IOP at a healthy level as only the effects of drops have been studied in human. Previous work by Peng et al. (2012) demonstrated that using 4-day extended release of Timolol from drug/vitamin E loaded contact lenses on beagle dogs in vivo resulted in an IOP as low as the one associated with daily use of eye drops. The IOP was also significantly below the IOP baseline value for glaucoma eyes in the beagle dogs (Peng et al., 2012). In our experiments, the average rate of release for balafilcon A ranged from 19.1 to 36.5 ng/hour for initial loading of 29.0 and 7.2 µg respectively while rates for senofilcon A were 34.6-44.2 ng/hour. Over a 4-day period of contact lens wear, these rates of Latanoprost release can potentially satisfy the therapeutic daily dose of 150-300 ng/day and effectively lower IOP. Further investigations are necessary to confirm these release kinetics and their effectiveness in ex vivo and in vivo conditions or in a more complex in vitro model mimicking the dynamic environment of ocular surface.
Chapter 5
Release System for Hydrophilic drug

5.1 Introduction

Timolol is a non-selective β-blocker which has been used widely for treatment of glaucoma and IOP hypertension for over 30 years (Neufeld et al., 1983). Timolol is a replacement for prostaglandin analogues or is prescribed in combination with other prostaglandin analogues like Latanoprost (Volotinen et al., 2011). Timolol reduces the IOP by controlling the formation of aqueous humor and is used conventionally in the form of eye drops (Neufeld et al., 1983). The Timolol molecule by itself is categorized as a lipophilic drug but it is commercially available in the hydrophilic form of 0.5% Timolol maleate gel forming solution in order to increase its duration of action on the ocular surface (Shedden et al., 2001).

Ciprofloxacin HCl is a new generation of antibacterial agents which is very active against a broad range of Gram-positive and Gram-negative bacteria and has been widely prescribed in the form of eye drops as an antibiotic agent for ocular infections. Ciprofloxacin HCl is also a hydrophilic molecule (Campoli-Richards et al., 1988).

Like any other topical ophthalmics, the main challenges for Ciprofloxacin HCl and Timolol maleate ocular delivery are their bioavailability and short residence time on ocular surface. Moreover due their hydrophilic nature, they would mainly permeate through the paracellular route because they cannot distribute into the cellular lipid bilayer membrane (Sakanaka et al., 2006). Therefore extensive attempts have been made to implement a therapeutic contact lens system for extended release of these hydrophilic drugs using different techniques and strategies. However the biggest obstacle in this effort, the burst release of the drug, seems to remain unconquerable. The majority of these studies examined the release kinetics of drug loaded contact lenses in a fixed-volume in vitro model containing PBS or water, which is far from the ocular environment conditions (Holden et al., 2012; Hui et al., 2014, 2012; Kim et al., 2008; Nguyen et al., 2012; Peng et al., 2012).
The main objectives of this part of the project were to evaluate the release kinetics of hydrophilic drug eluting from silicone hydrogel contact lenses \textit{in vitro} in the presence of corneal epithelial cells and compare them to their release kinetics in a fixed-volume \textit{in vitro} model with no cells. The validity of the corneal \textit{in vitro} model as the test platform for screening of the drug eluting biomaterials, against the hydrophilic drugs was also assessed. Previous work by Mohammadi et al., (2014) had only investigated the hydrophilic derivative of Latanoprost, Latanoprost free-acid. Alternative low cost and fast analytical technique (spectrophotometry) allowing the real-time measurements of the drug concentration were other factors leading us to switch to hydrophilic drugs other than Latanoprost free-acid, which requires an expensive and laborious EIA analytical method.

To address these objectives, we assessed and statistically compared the uptake and the release kinetics of balafilcon A and senofilcon A silicone hydrogel contact lenses uploaded with Ciprofloxacin HCl and Timolol maleate in three \textit{in vitro} models: no cell, live cell and fixed cell.
5.2 Materials and Methods

5.2.1 Preparation of Drug Solution

The drug solutions were prepared by dissolving of Ciprofloxacin HCl (Sigma-Aldrich) and Timolol Maleate (Sigma-Aldrich) in cell culture water (Lonza, Walkersville, MD). The expected concentration of Ciprofloxacin HCl stock drug solution was 3000 µg/ml and the expected concentration of Timolol Maleate was 1000 µg/ml based on the drug amount claimed on the labels by the manufacturers. The actual concentrations were measured by spectrophotometry and are presented in Table 5.1 and Table 5.2. Stock solutions were stored at -20 °C.

5.2.2 Calibration curve

In order to calculate the drug concentrations based on the light absorbance using a UV-Visible spectrophotometry technique, a standard curve by serial dilutions of drugs at concentrations of 1, 2, 3, 5, 7, 10, 12, 15 and 20 µg/ml was developed. Absorbance of Ciprofloxacin HCl and Timolol Maleate were measured at the wavelengths of 274 and 295 nm respectively. The calibration curves for Ciprofloxacin HCl and Timolol Maleate are presented in Figure 5-1 and 2.
Figure 5-1. Ciprofloxacin HCl standard curve. Drug absorbance was read at $\lambda=274$ nm.

Figure 5-2. Timolol Maleate standard curve. Drug absorbance was read at $\lambda=295$ nm.
5.2.3 Preparation of Contact Lenses

Two commercially available silicone hydrogel contact lenses, balafilcon A and senofilcon A were used in this study. The chemical properties of these lenses were presented in Table 3-1. Lenses were first incubated for 24 hours in PBS to remove any remnants of their packaging solution. The lenses were then incubated in 1.5ml of the drug solution for 24 hours.

5.2.4 Hydrophilic Drugs Uptake and Release

After a 24 hour incubation in PBS, contact lenses were soaked in 1.5ml of drug solution for 24 hours. The lenses were put on three release models (No cell, Fixed and Live monolayer). The volume of liquid in the cell insert was 0.5ml and 1.0ml in the bottom well. The release was studied for up to 48 hours. Aliquots of 100 µl were taken from the bottom of the in vitro models and replaced by fresh culture medium. Samples were taken at 1, 4, 8, 12, 24 and 48 hours. Collected samples were analyzed via a UV-Vis spectrophometry (SPECTRA max PLUS 384 Made in USA) at wavelengths of 274 nm and 295 nm for Ciprofloxacin HCl and Timolol Maleate respectively. To determine the uptake amount by the contact lenses, samples were also collected from the soaking drug solution as well as the remaining drug solutions after soaking the lenses. Each set of experiment were repeated 3 times independently. Drug concentrations were calculated based on the equation explained previously in Chapter 3.

5.2.5 Data Analysis

Results presented here are the mean±standard deviation of three independent experiments. Statistical analysis of data was performed by two-way analysis of variance (ANOVA) using Minitab Express™ Software. A p value of less than 0.05 was required for statistical significance.
5.3 Results

5.3.1 Ciprofloxacin HCl Uptake and Release

The uptake and release amount of Ciprofloxacin HCl from balafilcon A and senofilcon A with initial loading concentration are presented in Table 5-1. The total Ciprofloxacin HCl uptake by balafilcon A was 4672.8±60.4µg after 24 hours which was 14.1±6.1% of the total available drug to the contact lens. The total amount of the drug uptaken by senofilcon A was 4726.2±105.6 µg or 43.6±1.4% of the available drug in the 1.5 ml doping solution. The Ciprofloxacin uptake by senofilcon A was significantly higher than that of balafilcon A ($p<0.0001$). The maximum amounts of Ciprofloxacin HCl released from balafilcon A after 48 hours were 145.2±20.1, 143.9±16.1 and 186.2±31.0 µg in the live monolayer, fixed monolayer and no-cell models respectively, whereas the maximum released drug from senofilcon A were 45.1±2.2, 42.2±0.3 and 58.3±2.6 µg for live, fixed and no-cell models respectively. While senofilcon A uptake was higher than balafilcon A, the amounts of Ciprofloxacin HCl released from senofilcon A in all three models were significantly lower than from balafilcon A ($p<0.0001$). While there was no significant difference in Ciprofloxacin HCl released between the live and fixed model ($p=0.7867$), the released amount of Ciprofloxacin HCl was significantly higher in the no-cell model compared to live and fixed models regardless of contact lens material ($p=0.0170$).
Table 5-1. Ciprofloxacin HCl uptake into balafilcon A and senofilcon A contact lenses and total amount of drug released after 48 hours through the live HCEC monolayer \textit{in vitro} model. Lenses were soaked for 24 hours in drug solutions (3000 µg/ml) and then drug uptake into the lens were measured. Uptake and release experiments were conducted in three separate dates \( n = 3 \), Mean + SD

<table>
<thead>
<tr>
<th>Theoretical concentration(^1) (µg Ciprofloxacin/ml)</th>
<th>3000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual loading concentration(^2) (µg Ciprofloxacin/ml)</td>
<td>2942.0±70.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>In vitro Model</th>
<th>Live Monolayer</th>
<th>Fixed Monolayer</th>
<th>No-cell*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uptake(^$)</td>
<td>Release(^$)</td>
<td>Release(^$)</td>
</tr>
<tr>
<td></td>
<td>(%)</td>
<td>(µg)</td>
<td>(%)</td>
</tr>
<tr>
<td>balafilcon A</td>
<td>14.1±6.1</td>
<td>4672.8±60.4</td>
<td>3.1±0.4</td>
</tr>
<tr>
<td>senofilcon A(^$)</td>
<td>43.6±1.4</td>
<td>4726.2±105.6</td>
<td>0.9±0.1</td>
</tr>
</tbody>
</table>

\(^1\) Expected (nominal) concentration based on the manufacturer’s label.

\(^2\) Concentration of the solution measured by Spectrophotometry

* Significantly different compared to fixed and live models \( p<0.0170 \)

\(^\$\) Significantly different compared to balafilcon A \( p<0.0001 \)
The release profile of Ciprofloxacin HCl from balafilcon A through no-cell, live and fixed monolayer *in vitro* models are presented in Figure 5-3. As can be seen on Figure 5-3, the release profiles of Ciprofloxacin in all three models were not linear, indicating that the rate of release from contact lens is not constant regardless of the *in vitro* model. The amount of drug release in the no-cell model was significantly higher and a faster equilibrium can be observed in this model. In the no-cell model, about ~70% of the maximum released amount of Ciprofloxacin HCl was eluted in the media in the first 8 hours of the experiments while only ~45% and ~53% of the maximum released amounts were depleted from balafilcon A lenses in this period of time in the live and fixed models respectively.

The release profile of Ciprofloxacin HCl from senofilcon A through no-cell, live and fixed monolayer *in vitro* models are presented in Figure 5-4. Similarly to balafilcon A, the release profiles of Ciprofloxacin HCl from senofilcon A did not present a zero-order release regardless of the *in vitro* models used, although the amount of drug release in the no-cell model was significantly higher compared to the live and fixed models. In the no-cell model about ~66% of the maximum released amount of the drug was eluted in the first 12 hours of the experiments while only ~47% and ~57% of the maximum released amounts were released from senofilcon A in the live and fixed models respectively after 12 hours.
Figure 5-3 Ciprofloxacin HCl release from balafilcon A contact lenses through live and fixed HCEC monolayer and No-cell models. Cells were fixed in 2% paraformaldehyde. Lenses were soaked for 24 hours in drug solution (3000 µg/ml) and then overlaid on the monolayer for 48 hours. Release experiments were conducted in three separate dates n = 3. Mean ± SD.

* Significantly different than fixed and live models (p<0.0094)
Figure 5-4. Ciprofloxacin HCl release from senofilcon A contact lenses through live and fixed HCEC monolayer and No-cell models. Cells were fixed in 2% paraformaldehyde. Lenses were soaked for 24 hours in drug solution (3000 µg/ml) and then overlaid on the monolayer for 48 hours. Release experiments were conducted in three separate dates \( n = 3 \), Mean + SD.

* Significantly different than fixed and live models \( (p<0.0015) \)

### 5.3.2 Timolol Maleate Uptake and Release

The uptake and release amount of Timolol maleate from balafilcon A and senofilcon A lenses with their initial loading concentrations are presented in Table 5-2. The total Timolol available to contact lenses was 16677±18.8 µg and the total uptake by balafilcon A after 24 hours was 1591.1±72.2µg or 10.9±3.5% of the total available drug. The total amount of the drug uptaken by senofilcon A was 1602.4±170.7 µg or 10.6±5.5% of available drug to the lens. There was no significant difference in Timolol uptakes between senofilcon A and balafilcon A \( (p=0.6108) \). The total amounts of Timolol released from balafilcon A after 48 hours were 56.9±17.7, 37.1±6.7 and 35.2±15.5µg in the live, fixed and no-cell models respectively, whereas the maximum released amount of the drug from senofilcon A were 45.3±19.2, 28.5±9.4 and 29.3±15.6µg for live, fixed and no-cell models respectively. There was a
significant difference between contact lens material in the amount of released Timolol regardless of the in vitro model used ($p<0.0001$). No significant difference in the released amount of the drug after 48 hours was observed between the no cell, live and fixed models, regardless of contact lens material ($p=0.2391$).
Table 5-2 Timolol Maleate uptake into balafilcon A and senofilcon A contact lenses and total amount of drug released after 48 hours through the live HCEC monolayer *in vitro* model. Lenses were soaked for 24 hours in drug solutions (1000 µg/ml) and then drug uptake into the lens were measured. Uptake experiments were conducted in three separate dates n = 3, Mean + SD.

<table>
<thead>
<tr>
<th>Theoretical concentration¹ (µg Timolol/ml)</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual loading concentration² (µg Timolol/ml)</td>
<td>1118.2±12.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>In Vitro Model</th>
<th>Live Monolayer</th>
<th>Fixed Monolayer</th>
<th>No-cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uptake (µg)</td>
<td>Release (%)</td>
<td>Release (µg)</td>
</tr>
<tr>
<td>balafilcon A</td>
<td>10.9±3.5</td>
<td>1591.1±72.2</td>
<td>3.6±1.1</td>
</tr>
<tr>
<td>senofilcon A§</td>
<td>10.6±5.5</td>
<td>1602.4±170.7</td>
<td>2.8±1.2</td>
</tr>
</tbody>
</table>

¹ Expected (nominal) concentration based on the manufacturer’s label.

² Concentration of the solution measured by Spectrophotometry

§ Release significantly different compared to balafilcon A (p<0.0001)
The release profile of Timolol maleate from balafilcon A through no-cell, live and fixed monolayer *in vitro* models are presented in Figure 5-5. As can be observed, the release rates of Timolol in all *in vitro* models are not constant. The rate of release diminishes after 12 hours approaching the equilibrium regardless of lens type and *in vitro* models used; this indicates a first-order release rate. However, unlike Ciprofloxacin HCl release results, there was no significant difference in the amount of Timolol released in the no-cell model compared to the fixed and live models, but a faster rate to reach the saturation can be observed in this model compared to fixed and live models. In the no-cell model about \( \sim 83\% \) of the maximum released amount of Timolol was eluted in the beginning of the experiments (4 hours) while only \( \sim 47\% \) and \( \sim 46\% \) of the maximum released amounts were depleted from balafilcon A lenses in this period of time in the live and fixed models respectively. The release profile of Timolol from senofilcon A through no-cell, live and fixed monolayer *in vitro* models are presented in Figure 5-6. Similar to balafilcon A, the release profiles of Timolol from senofilcon A do not present a constant rate of release regardless of the *in vitro* models tested. However, the amount of drug released in the no-cell model was significantly higher for this lens with an initial burst of \( \sim 62\% \) of the total released drug while, after 4 hours, \( \sim 36\% \) and \( \sim 35\% \) of the maximum released amounts were released from balafilcon A in the live and fixed models respectively.
Figure 5-5. Timolol Maleate release from balafilcon A contact lenses through live and fixed HCEC monolayer and No-cell models. Cells were fixed in 2% paraformaldehyde. Lenses were soaked for 24 hours in drug solution (1000 µg/ml) and then overlaid on the monolayer for 48 hours. Release experiments were conducted in three separate dates n = 3, Mean ± SD.
* Significantly different than fixed and live models (p<0.0164)
5.4 Discussion

In this part of the project, we assessed in vitro the release kinetics of drug eluting silicone hydrogel contact lenses loaded with hydrophilic Ciprofloxacin HCl and Timolol Maleate in the presence of corneal epithelial cells and compared them to their release kinetics in a fixed-volume in vitro model with no-cells. The main problem reported extensively by studies on drug-soaked commercially marketed contact lenses is the release time, which is limited to minute to hours (Campoli-Richards et al. 1988; Karlgard et al. 2003; Hui et al. 2008; Nguyen et al. 2012). Several strategies have been explored to improve the release kinetics of commercially available contact lenses such as loading Vitamin E as a diffusional barrier to
slow down the release and extend the drug delivery (Hsu et al., 2013; Hui et al., 2014; Peng et al., 2012).

Hui et al. have investigated the uptake and release of Ciprofloxacin HCl by commercially available conventional and silicone hydrogel contact lenses. Three different contact lenses were soaked in 6000 µg of the drug (2 ml of 0.3% Ciprofloxacin solution in PBS). The contact lenses released an average amount of 133 µg/lens but the drug release reached a plateau within 10 minutes. Despite attempts to modify Ciprofloxacin release kinetics using molecular imprinting techniques with a model silicone hydrogel contact lens (Hui et al., 2014), fixed-volume in vitro results in borate buffered saline (BBS) showed a maximum release of 361±5µg of Ciprofloxacin over a 6 day period where ~87% of the drug was released in the first day by an initial burst. While our results tend to agree with Hui et al as for the fact that a plateau is reached early on, our slightly longer time of release before saturation may be due the difference in material chemistry (balafilcon A and senofilcon A were not tested in that study) as well as the difference in release medium (PBS versus KSFM). Mohammadi et al have previously reported a small difference in release between PBS and KSFM.

Our no-cell, live and fixed model results are comparable with previously published in vitro release results for Timolol (Saettone et al. 1996; Kim et al. 2008; Peng et al. 2012) whereby it was observed that the release rate of hyrophilic Timolol from silicone hydrogels follows a first order kinetics. The difference in results between rate and amount of released drug are due to the difference in release media, contact lens material and in vitro experimental models.

Comparison of ciprofloxacin HCl and Timolol maleate release profile with Latanoprost indicates that presence of live cells in the model do not change the order of release rate to a constant zero-order decay. Since hydrophilic drugs permeate via paracellular routes by passive diffusion rather than active cell transport, presence of cells in the release model act as a diffusion barrier and prolongs the release kinetics of hydrophilic drugs (Sakanaka et al., 2006). The similarities in results between the live and fixed cell models for Timolol and Ciprofloxacin from both contact lens materials further supports this hypothesis.

Our in vitro results agree with works of others (Hui et al., 2014, 2012, 2008; Nguyen et al., 2012) that the release kinetics of hydrophilic drugs from silicone hydrogels follow the first-
order decay rate. However, our *in vitro* release experiments in the presence of corneal epithelial cells, which act as an active barrier also highlight that the release of hydrophilic drugs from commercial contact lenses is prolonged when tested with realistic diffusion and permeation conditions. While the difference may be small and has minimal impact on the poor outcome of soaking contact lenses in hydrophilic drugs, our data further highlight the difference that the presence of cells has when assessing drug release *in vitro*. 
Chapter 6
Conclusions

The cornea is the main barrier against the permeation of ocular drugs to the anterior chamber. Hydrophobic and hydrophilic drugs permeate through different transcorneal routes. In feasibility assessment of drug delivery contact lenses, the role of these barriers and the biological interaction of corneal cells with the drug/lens complex must be taken into account. In this thesis, the presence of the prostaglandin transporter OATP 2A1 in immortalized human corneal epithelial cell line has been verified. The role of drug transporters in transcorneal permeation becomes important when hydrophobic drugs interact with the cornea. The active transcellular transport plays an important role in transcorneal permeation of hydrophobic molecules. Fixing cells almost entirely abolish transcorneal permeation of the hydrophobic Latanoprost; ~90% decrease in the permeation of the drug was observed compared to the live cell model. Inhibition of the prostaglandin transporter OATP 2A1 significantly decreased the rate and the amount of Latanoprost released from both contact lens materials tested. Only 50% of Latanoprost transport was inhibited suggesting that the active transcellular transport of Latanoprost may not be mediated only by OATP 2A1. Other transporters might be involved in transport of Latanoprost as well. From our in vitro experiments, it appears that a combination of mechanisms are involved which are driven mainly by transcellular molecular mediated active transport and partially by passive diffusion-solution through lipid bilayers of cell membranes. These mechanisms govern the total permeation of hydrophobic drugs across the corneal epithelium.

Proper dosing and a sustainable zero-order release rate are the ultimate goals in designing therapeutic contact lenses. Silicone hydrogels showed a zero-order release profile for Latanoprost when assessed in vitro in the presence of corneal epithelial cells. Initial loading concentration of the drug had a significant effect on the rate of release.
The uptake of hydrophilic drugs by silicone hydrogels were significantly lower than the uptake of hydrophobic Latanoprost, and the released amount of hydrophilic drugs were significantly higher than Latanoprost. Hydrophilic drugs have lower affinity to silicone hydrogels than hydrophobic molecules. Our results further confirm that chemical interaction between drug molecules and contact lens polymer must be taken into account in the design of the controlled release system. As well, the testing conditions and presence of biological barriers provided by cells can produce a more realistic test platform for studying the release kinetics for the ocular environment. All in all, this thesis confirms the importance of proper selection of *in vitro* test models for the assessment of ocular drug delivery system.
Chapter 7
Recommendations for Future Works

This research contributes a small building block to the massive construction of ocular drug delivery science but leaves also us with many unanswered questions. The following are few recommendations for future research.

To further characterize the corneal in vitro model and assess the corneal permeation, it is recommended to test more combination of hydrophobic ophthalmics and silicone hydrogel lenses, this may give more solid ground to stand on and make more inferences on mechanism of corneal drug transport. It is also recommended to characterize the human corneal epithelial cell line for drug metabolism enzymes and esterase activity of the enzymes on prodrugs.

To obtain more valid in vitro release data, it is recommended to measure the Latanoprost and other drugs release rates in an in vitro model with a dynamic condition mimicking the ocular environment. For instance in order to have an in vitro model which has a better prediction of in vivo results, it is recommended to study the release rate of the drugs from different contact lens materials on a stratified curved corneal 3D construct and test them under the effect of tear replenishment with the newly developed microfluidics tear replenishment system (TRS) at the MIBS lab.

Performing ex vivo experiments with excised animal cornea may help to assess the corneal penetration rate as well as provide comparison points to evaluate the validity of in vitro results. Ex vivo experiment can also be performed in a dynamic environment.

Mathematical modeling of contact lens release mechanism in the corneal in vitro model may provide a predictive tool for the design of therapeutic contact lens as well as for prediction of transcorneal drug diffusion.
Appendix A Complementary Experiments

In order to understand the effect of membrane material used in cell culture inserts on the release profile of Latanoprost, a set of experiments was designed in absence of contact lenses. 0.5 ml of 161µg/ml Latanoprost solution in PBS was added on top of a monolayer grown on PET and cellulose inserts and 1ml of KSFM to the bottom. 100µl of samples were taken every 30 minutes up to 4 hours and then after 12 and 24 hours. Samples were analyzed using EIA kit.

**Figure 7-1.** Latanoprost release experiment, through cellulose membrane inserts versus Polyethylene terephthalate (PET) membrane, monolayer of (HCECs) with no contact lens, and drug solution on top with initial concentration of 161 µg/ml. All membranes were collagen-coated prior to cell culture and release experiments. n=2, Mean ±SD
Figure 7-2 MTT viability test on live, transporter inhibited and fixed HCEC monolayer models. Cells were incubated with 100µM Diclofenac sodium for 24 hours to inhibit the transporters. Cells were fixed in 2% paraformaldehyde solution in PBS for 24 hours. n=3, Mean ±SD
Figure 7-3 MTT viability test performed on live and fixed HCEC monolayer models after Ciprofloxacin HCl release experiments. Ciprofloxacin HCl concentration was 3 mg/ml in the No-Lens model. Cells were fixed in 2% paraformaldehyde solution in PBS for 24 hours. n=3, Mean ±SD

Figure 7-4 MTT viability test performed on live and fixed HCEC monolayer models after Timolol maleate release experiments. Timolol Maleate concentration was 1 mg/ml in the No-Lens model. Cells were fixed in 2% paraformaldehyde solution in PBS for 24 hours. n=3, Mean ±SD
Appendix B
Copyright Permissions

Thesis/Dissertation Reuse Request
Taylor & Francis is pleased to offer reuses of its content for a thesis or dissertation free of charge contingent on resubmission of permission request if work is published.
Thesis/Dissertation Reuse Request

Taylor & Francis is pleased to offer reuses of its content for a thesis or dissertation free of charge contingent on resubmission of permission request if work is published.
Thesis/Dissertation Reuse Request

Taylor & Francis is pleased to offer reuses of its content for a thesis or dissertation free of charge contingent on resubmission of permission request if work is published.
Title: Ocular novel drug delivery: impacts of membranes and barriers
Author: Jaleh Barar, Ali Reza Javadzadeh, Yadollah Omidi
Publication: Expert Opinion on Drug Delivery
Publisher: Taylor & Francis
Date: May 1, 2008
Copyright © 2008 Taylor & Francis

Thesis/Dissertation Reuse Request
Taylor & Francis is pleased to offer reuses of its content for a thesis or dissertation free of charge contingent on resubmission of permission request if work is published.
# ELSEVIER LICENSE TERMS AND CONDITIONS

This is an Agreement between Shahabedin Eslami ("You") and Elsevier ("Elsevier"). It consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

| Supplier | Elsevier Limited  
The Boulevard, Langford Lane  
Kidlington, Oxford, OX5 1GB, UK |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Registered Company Number</td>
<td>1982084</td>
</tr>
<tr>
<td>Customer name</td>
<td>Shahabedin Eslami</td>
</tr>
</tbody>
</table>
| Customer address | 200 University Avenue West  
Waterloo, ON N2L 3G1 |
| License number | 3735061324784 |
| License date | Oct 23, 2015 |
| Licensed content publisher | Elsevier |
| Licensed content publication | Survey of Ophthalmology |
| Licensed content title | Ocular and Systemic Pharmacokinetics Of Latanoprost in Humans |
| Licensed content author | Birgitta Sjöquist, Johan Stjernschantz |
| Licensed content date | August 2002 |
| Licensed content volume number | 47 |
| Licensed content issue number | n/a |
| Number of pages | 7 |
| Start Page | S6 |
| End Page | S12 |
| Type of Use | reuse in a thesis/dissertation |
| Portion | figures/tables/illustrations |
| Number of figures/tables/illustrations | 1 |
| Format | electronic |
| Are you the author of this Elsevier article? | No |
| Will you be translating? | No |
| Original figure numbers | figure 5 |
| Title of your thesis/dissertation | Characterization and Application of a Human Corneal in vitro Model for Controlled Release of Ophthalmic Drugs |
| Expected completion date | Oct 2015 |
| Estimated size (number of pages) | 80 |
INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows: "Reprinted from Publication title, Vol. edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier material may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC or publisher's behalf).
13. Objecting to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.
14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion.

76
discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. Translation: This permission is granted for non-exclusive world English rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. Posting licensed content on any Website: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the website must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com. All content posted to the website must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable. The website shall be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. For journal authors: the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.). Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by uploading a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- after the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license – this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published
published journal article (PJA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review coordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others’ research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and coursework programs), and inclusion of the article for grant funding purposes.

Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark link, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier’s posting policy for further information.

18. For book authors: the following clauses are applicable in addition to the above. Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. Posting to a repository: Authors are permitted to post a summary of their chapter only in their institution’s repository.

19. Thesis/Dissertation: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for ProQuest/UMI to supply single copies, on demand, of the complete thesis. Should you wish to publish your thesis commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author’s choice of Creative Commons user license. See our open access license policy for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: The CC-BY license allows users to copy, create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

CC BY NC SA: The CC BY-NC-SA license allows users to copy, create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and
provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial use of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee. Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. Other Conditions:

v1.8

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.
# ELSEVIER LICENSE TERMS AND CONDITIONS

Oct 23, 2015

This is an Agreement between Shahabedin Esfami ("You") and Elsevier ("Elsevier"). It consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Elsevier Limited</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Boulevard, Langford Lane</td>
<td>Kidlington, Oxford, OX5 1GB, UK</td>
</tr>
<tr>
<td>Registered Company Number</td>
<td>1982084</td>
</tr>
<tr>
<td>Customer name</td>
<td>Shahabedin Esfami</td>
</tr>
<tr>
<td>Customer address</td>
<td>200 University Avenue West Waterloo, ON N2L 3G1</td>
</tr>
<tr>
<td>License number</td>
<td>3735070380222</td>
</tr>
<tr>
<td>License date</td>
<td>Oct 23, 2015</td>
</tr>
<tr>
<td>Licensed content publisher</td>
<td>Elsevier</td>
</tr>
<tr>
<td>Licensed content publication</td>
<td>The Ocular Surface</td>
</tr>
<tr>
<td>Licensed content title</td>
<td>Silicone Hydrogel Contact Lenses and the Ocular Surface</td>
</tr>
<tr>
<td>Licensed content author</td>
<td>Fiona Stapleton, Serina Stretton, Eric Papas, Cheryl Skotnitsky, Deborah F. Sweeney</td>
</tr>
<tr>
<td>Licensed content date</td>
<td>January 2006</td>
</tr>
<tr>
<td>Licensed content volume number</td>
<td>4</td>
</tr>
<tr>
<td>Licensed content issue number</td>
<td>1</td>
</tr>
<tr>
<td>Number of pages</td>
<td>20</td>
</tr>
<tr>
<td>Start Page</td>
<td>24</td>
</tr>
<tr>
<td>End Page</td>
<td>43</td>
</tr>
<tr>
<td>Type of Use</td>
<td>reuse in a thesis/dissertation</td>
</tr>
<tr>
<td>Portion</td>
<td>figures/tables/illustrations</td>
</tr>
<tr>
<td>Number of figures/tables/illustrations</td>
<td>1</td>
</tr>
<tr>
<td>Format</td>
<td>both print and electronic</td>
</tr>
<tr>
<td>Are you the author of this Elsevier article?</td>
<td>No</td>
</tr>
<tr>
<td>Will you be translating?</td>
<td>No</td>
</tr>
<tr>
<td>Original figure numbers</td>
<td></td>
</tr>
<tr>
<td>Title of your thesis/dissertation</td>
<td>Characterization and Application of a Human Corneal in vitro Model for Controlled Release of Ophthalmic Drugs</td>
</tr>
<tr>
<td>Expected completion date</td>
<td>Oct 2015</td>
</tr>
<tr>
<td>Estimated size (number of pages)</td>
<td>80</td>
</tr>
</tbody>
</table>
INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
   "Reprinted from Publication title, Vol. edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier OR APPLICABLE SOCIETY COPYRIGHT OWNER." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion.
discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. Translation: This permission is granted for non-exclusive work English rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. Posting licensed content on any Website: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com. All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. For journal authors: the following clauses are applicable in addition to the above:

Preprints:
A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.). Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscripts:
- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- after the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:
- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published
journal article.

**Published journal article (PJA):** A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review coordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

**Subscription Articles:** If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others’ research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

**Gold Open Access Articles:** May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier’s posting policy for further information.

18. For book authors the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. Posting to a repository: Authors are permitted to post a summary of their chapter only in their institution’s repository.

19. Thesis/Dissertation: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

**Elsevier Open Access Terms and Conditions**

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author’s choice of Creative Commons user license. See our open access license policy for more information.

**Terms & Conditions applicable to all Open Access articles published with Elsevier:**

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author’s honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

**Additional Terms & Conditions applicable to each Creative Commons user license:**

**CC BY:** The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at [http://creativecommons.org/licenses/by/4.0](http://creativecommons.org/licenses/by/4.0).

**CC BY-NC-SA:** The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at [http://creativecommons.org/licenses/by-nc-sa/4.0](http://creativecommons.org/licenses/by-nc-sa/4.0).

**CC BY-NC-ND:** The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and
provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee. Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. Other Conditions:

v1.8

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

---

Permission to use figures

Anne Huhtala <annemhuhtala@gmail.com>
To: s2eslami@uwaterloo.ca

Dear Shahabedin Eslami,

I give you the permission to use figures 2 and 3 from my e-book chapter "Corneal Models for the Toxicity Testing of Drugs and Drug Releasing Devices" that was published in Topics in Multifunctional Biomaterials & Devices, Vol. 1, Ed. N. Ashammakh, 2008, Chapter 2, pp. 1-23.

I kindly ask you to contact Editor, Dr. Nureddin Ashammakh to get the publisher's permission to use the figures.

Sincerely,
Dr. Anne Huhtala
Tampere, Finland
Bibliography


Hsu, K.-H., Gause, S., Chauhan, A., 2014. Review of ophthalmic drug delivery by contact


Williams, D.F., 2014. There is no such thing as a biocompatible material. Biomaterials 35, 5.