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**Development of *in vitro* Models for Delivery of the Anti-Viral Drug Acyclovir for Ocular HSV-1
Infection Treatment**

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**Development of *in vitro* Models for Delivery of the Anti-Viral Drug
Acyclovir for Ocular HSV-1 Infection Treatment**

Bettina Bareiss

This thesis is submitted as a partial fulfillment of the M.Sc. program in Cellular and
Molecular Medicine

10th March 2010

Cellular and Molecular Medicine

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Abstract

Herpes simplex virus 1 (HSV-1) infection is the main cause of vision loss in the developed world due to the fact that after initial infection, the virus establishes latency and can be reactivated; resulting in a very low success rate of treatment by transplantation (22%). We are developing corneal substitutes suitable for HSV-1 transplantation by incorporating anti-viral drugs such as acyclovir (ACV), into corneal substitutes that could potentially suppress the viral reactivation. Specifically, we examined the feasibility of preventing viral reactivation during surgery, by sustained delivery of ACV introduced during corneal transplantation surgery, through encapsulation of the drug within silica (SiO₂) nanoparticles (NP) incorporated into biosynthetic alternatives to donor corneas. We show that incorporation of NPs did not affect optical clarity of the collagen-based corneal substitutes nor their biocompatibility. NP-encapsulation effectively sustained ACV release from the biosynthetic implants over 10 days, compared to free ACV incorporated directly into the hydrogel constructs. The NP-enabled sustained release resulted in effective prevention of virally-induced cell death, not observed with the free drug. This early model demonstrates the feasibility of using biomimetic corneal substitutes that incorporate a drug release system (*e.g.* silica nanoparticles encapsulating ACV) as future alternatives to human donor tissue grafts, for transplantation of HSV-infected corneas.

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List of Abbreviations

ACV-Acyclovir

ANOVA-analysis of variance

CHEO- Children's Hospital of Eastern Ontario

DMEM-Dulbecco's Modified Eagle's Medium

FBS-fetal bovine serum

GAPDH-glyceraldehyde-3-phosphate dehydrogenase

HCEC- Human corneal epithelial cells

HCl-Hydrochloric acid

HPLC-high performance liquid chromatography

HSV-Herpes simplex virus

KSFM- Keratinocyte Serum-Free Medium

MOI-multiplicity of infection

MPC-2-methacryloyloxyethyl phosphorylcholine

PBS-phosphate buffered saline

PFA-paraformaldehyde

Pfu-plaque forming units

RNA-ribonucleic acid

RT-PCR-reverse transcript polymerase chain reaction

SiO₂ NP-Silica nanoparticles

TEM-Transmission Electron Microscope

UV-ultraviolet

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CHAPTER 1

INTRODUCTION

1.1 Cornea

1.1.1 Cornea structure and function

The cornea is the central, anterior and transparent covering of the eye that protects the inner parts and focuses light onto the retina for vision (Griffith, et al. 2002). The human cornea has a diameter of about 11 mm and a central thickness of approximately 0.5 mm, which increases towards the periphery to around 0.6-0.8 mm (Cassin, et al. 1990). The cornea is composed of 5-7 layers of epithelial cells, Bowman's layer, stroma of extracellular matrix filled with cells, Descemet's membrane, and a monolayer of endothelial cells (Cassin, et al. 1990). The stratified squamous corneal epithelium has a greater regeneration potential and constantly sheds the outermost layer at the epithelium-air interphase. Its regeneration is generated from the basal located epithelium cell layer (Kruse, et al. 1994). This constant regeneration process is important as a defense against invading pathogens that infect the outer epithelium layer, and is a repair mechanism for the frequent mechanical force that is placed on the epithelium through blinking. The underlying Bowman's layer is a membrane-like layer of collagen, protecting the corneal stroma (Bron, et al. 1988). This stroma consists of a hydrated extracellular matrix with collagen Type I making up 71% of the dry weight of the corneal ECM (Newsome, et al. 1981) with keratocytes as the predominant cell type, which are needed for maintenance and repair of this organ (Wilson, et al. 1970). The Descemet's membrane serves as a basement membrane for the endothelial cell layer. The corneal endothelium maintains hydration and the proper thickness of the cornea (Harris, et al. 1967). Furthermore, the endothelium regulates the transport of nutrients

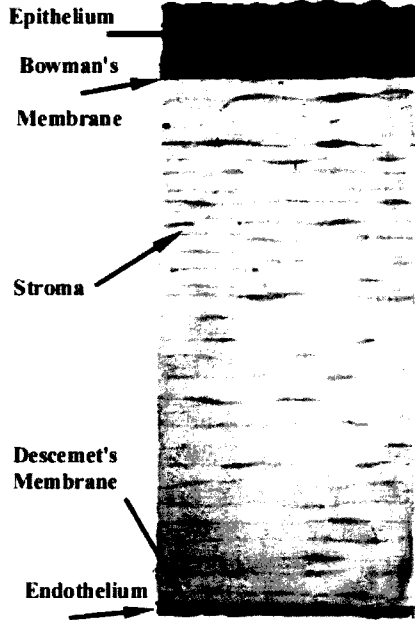
and solutes between the aqueous humour and the corneal stroma. However, the endothelium of the cornea is different when compared to other endothelia found in the human body. Ordinary endothelia comprise inner layers of vessels for transport of blood or lymph liquid, however the corneal endothelium is in touch with aqueous humour and therefore its function, appearance and origin is different. The corneal endothelium in humans does not readily regenerate *in situ*. In case of endothelial apoptosis, the remaining endothelial cells tend to spread out to cover the holes (Joyce, et al. 2003). This can impact the hydration of the cornea and other functions. The cornea is innervated by unmyelinated sensory nerve endings that originate from the trigeminal ganglion (Belmonte, et al. 2007). These nerves contain several subtypes and terminals that makes the cornea sensitive to touch, temperature and chemicals and can trigger a blink reflex (Belmonte, et al. 2007). To maintain the transparency, the cornea has no blood vessels, but rather, obtains its nutrition through diffusion from the aqueous humour within the eye, and the tear film on the outside (Turss, et al. 1972). Additionally, neurotrophins are supplied by innervating nerve fibers that maintain the overall health of the cornea and oxygen supply is provided through the air (Hill, et al. 1963).

The cornea is responsible for the majority of the refraction; including bending and focusing of light that enters the eye (Kikkawa, et al. 1968). In the following path, the lens fine-tunes the focus of the light. After traveling through the vitreous body of the eye, light reaches the receptor system of the retina and is converted to a neuronal signal, carried along the optic nerve, until it reaches the visual complex of the brain (Jay, et al. 2004) (Figure 1).

The refractive index of the cornea is determined and maintained by its shape and transparency. The corneal transparency is due to the small fiber diameter and the organized fibrous collagenous structure of the corneal stroma is essential for mechanical strength for holding the cornea in its shape (Michelacci, et al. 2003). Damage to the cornea through chemicals, injury and scarring, autoimmune disease, or infections can result in loss of

transparency, and subsequently vision loss, or blindness. The gold standard for treatment in these cases is corneal transplantation (Kaufmann, et al. 2009).

1A



1B

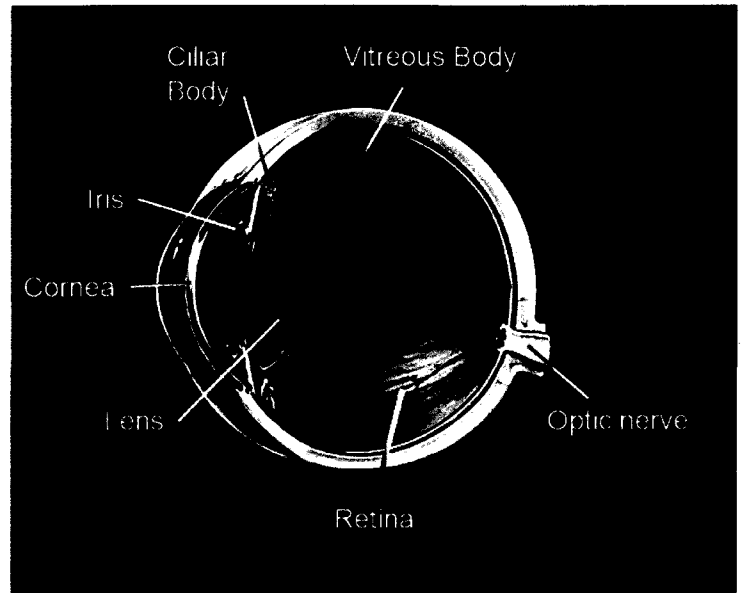


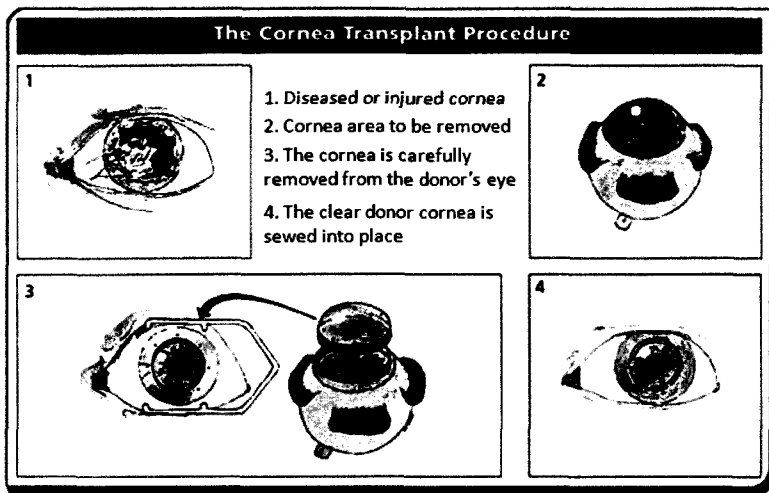
Figure 1: Diagrams of the cornea and eye. 1A shows a cartoon of a cross section of the cornea with the layers anterior to posterior: Epithelium, Bowman's Membrane, Stroma, Descemet's Membrane and Endothelium. 1B demonstrates a cartoon of the eye with Cornea, Iris, Lens, Ciliar Body, Vitreous Body, Retina, and Optic nerve.

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1.1.2 Corneal transplantation

Severe damage to the cornea can occur due to infection or chemical and mechanical injury, and may result in the need for a corneal transplantation. This is a surgical procedure whereby the damaged cornea is replaced by a donor graft (Forrester, et al. 2004). Trephines are used to remove the central part of the host cornea, which is then excised with scissors and a scalpel, leaving a circular hole in the centre of the cornea. The donor cornea is then cut by a similar method, resulting in a graft called button. Running or interrupted stitches are commonly used to hold the donor cornea in place (Forrester, et al. 2004) (Figure 2). Depending on the severity of the damage, only parts of the cornea may be removed by a deep lamellar keratoplasty (DLK). With respect to a diseased and clouded cornea, the whole thickness of the cornea has to be replaced in order to restore vision. For post-operative treatment, antibiotic ophthalmic solutions are commonly applied to prevent infections. In the following days or weeks, anti-inflammatory ophthalmic solutions and corticosteroids are applied to prevent graft failure.



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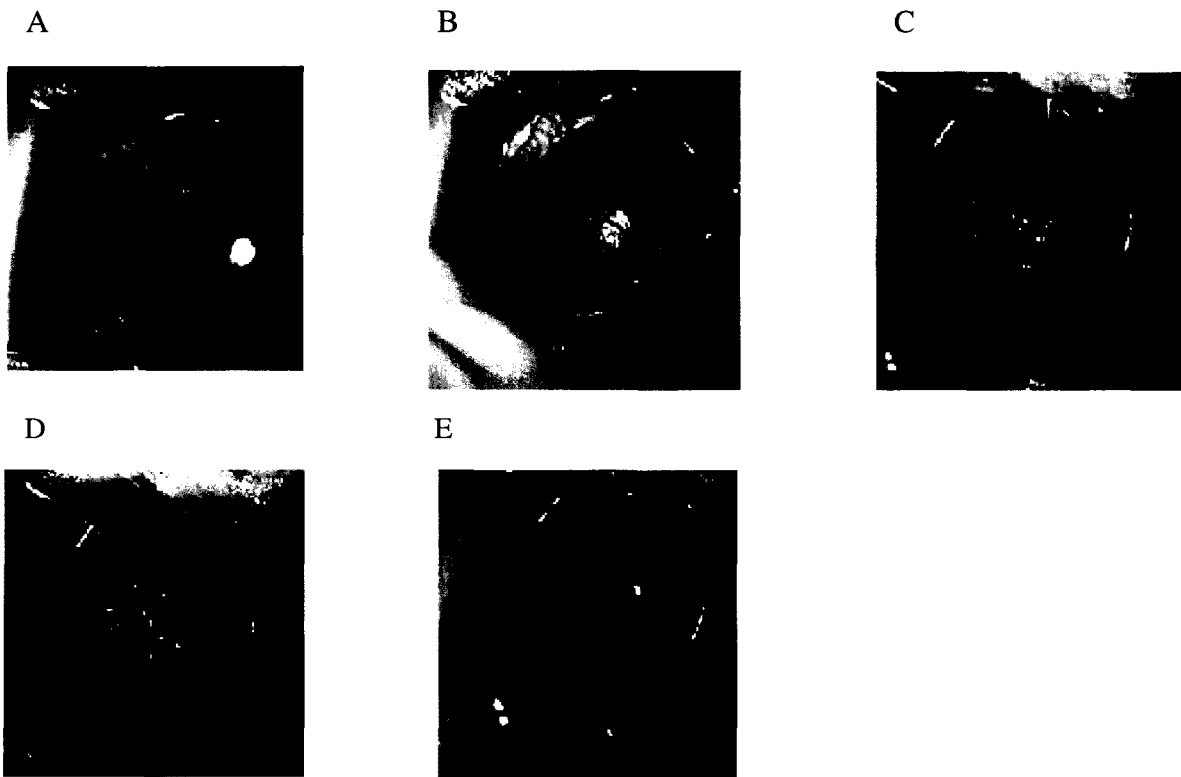
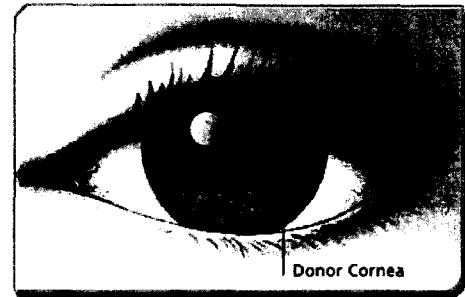


Figure 2: Corneal Transplantation.

1-5: Cartoons of corneal transplantation on human patient, following the Copyright for educational purpose from University of Miami webpage www.bpei.med.miami.edu/.../cornea_transplant.jpg. See text description in figure.

A-E: Corneal Transplantation on a pig. A trephine is used to cut around the "damaged" area of the host/patient cornea (A). This is followed by blunt dissection with a scalpel to excise the damaged tissue (B). A donor cornea is placed where the damaged portion was removed (C) and is held in place by suturing with very fine sutures to hold the graft in place (D). A bandage contact lens is then put in place over the implant for protection during initial healing (E).

1.1.3 Artificial corneas

Corneal transplantation is becoming more and more problematic, since there is a worldwide shortage of donor corneas due to an aging population (Slomovic, et al. 2006). Additionally, exclusion factors for donors are increasing due to the increased number of people having refractive laser surgery, using contact lenses, or being infected with HIV or hepatitis. To overcome these issues, artificial corneas consisting of synthetic materials have been developed (Hicks, et al. 2002, Hicks, et al.2003, Hicks, et al. 2004; Crawford, et al. 2005). The most promising graft candidate was composed of Poly (2-hydroxyethyl methacrylate) (HEMA) and is known as the AlphaCor™ (Hicks, et al. 2003). Unfortunately there was only limited clinical success, and this formulation of implant was not suitable for HSV (Herpes simplex virus) infected patients due to a high frequency of graft failure (Hicks, et al. 2003). Bio-interactive corneal substitutes are based on recombinant human collagen and have been shown to promote corneal regeneration in patients, including re-epithelization and re-innervation (Fagerholm, et al. 2009). However, these implants were developed for transplantation into patients with anterior corneal issues, like epithelial and stromal damage. These patients are considered as moderate risk patients and obtain a partial thickness graft. Therefore, the current constructs are not suitable for full-thickness grafts of high risk patients (*e.g.* patients with HSV).

1.2 HSV

1.2.1 HSV Description

HSV is an enveloped double-stranded DNA (150 kbp) virus and belongs to the herpes virus family Herpesviridae. Furthermore, HSV has a neurophilic character and usually infects mucous tissues found in the mouth, the genital region, and the eye, leading to watery blisters (Ryan, et al.

2004). There are two members, HSV-1 and HSV-2; whereas HSV-1 is associated with facial infection, HSV-2 is mostly found in the genital area (Coen, et al. 1996). Transmission of HSV happens through close contact when the infected person is shedding the virus from skin, saliva, or genital secretions. HSV-1 is often acquired during childhood through oral infection, while HSV-2 can be transmitted sexually or during childbirth from mother to child (Corey, et al. 2009). In severe cases, HSV-2 can lead to encephalitis, since the infant immune system is not developed enough to prevent brain damage. The focus in this study lies in corneal infection with HSV-1. HSV-1 is a very prominent virus, since over 90% of the human population over 60 years of age is serologically positive for HSV-1 antibodies (Toma, et al. 2008). Despite the frequent shedding of HSV-1 DNA in human tears, the incidence of HSV-1 ocular disease is relative low due to an intact immune system of most hosts (Toma, et al. 2008). Possible outcomes of HSV-1 ocular disease could be dependent on genetic makeup of the host, genetic makeup of the virus, and immune status of the host. The clinical signs of a corneal primary infection can be conjunctivitis and keratitis consisting of punctuated ulceration or even nerve damage, and characteristic superficial branching of a corneal ulcer (Ritterband, et al. 1998). Both primary and latent infections with HSV-1 are described in more detail below.

1.2.2 HSV-1 structure

The double stranded HSV-1 DNA genome is surrounded by a capsid consisting of an icosahedral protein cage. The next layer is a lipid bilayer, called an envelope. The space between the capsid and envelope is filled with the tegument (Figure 3) (Modrow, et al. 2003). The tegument consists of a cluster of proteins that help the viral DNA replicate and evade the immune response. For example, the viral protein ICP-47 can block the transporter associated with antigen processing (TAP transporter) (Burgos, et al 2006), which is important for maintaining the stability of the major histocompatibility complex (MHC) class I, before virus particles are transported to the cell

surface to activate Cytotoxic T cells with CD8 surface proteins. Another important tegument protein is the virus enzyme virion host shutoff protein (VHS) for the viral replication. It stops the synthesis of host proteins and degrades hosts mRNA (Matis, et al. 2001; Toma, et al. 2008), thereby favouring viral gene expression and replication.

The HSV genome encodes for proteins involved in building the viral envelope, tegument, and the capsid. They are grouped into Immediate early genes (encodes proteins that regulate the expression of early or late viral genes), Early genes (for synthesis of enzymes involved in DNA replication and production of envelope glycoproteins), and Late genes (encodes virus particle proteins) (Rajcani, et al. 2004).

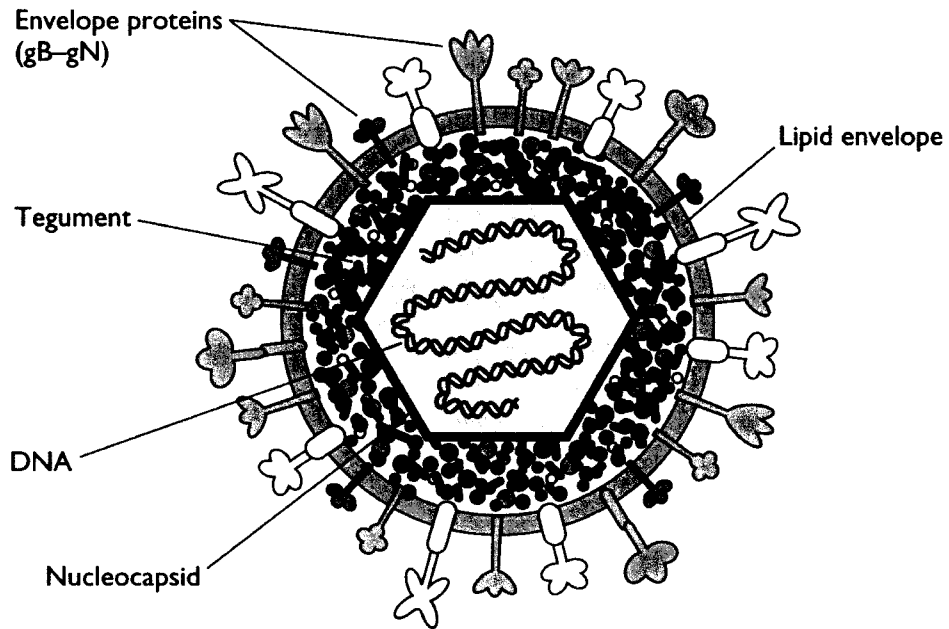


Figure 3: HSV-1 structure. The cartoon illustrates the structural components of HSV-1 with DNA, Nucleocapsid, Tegumentum, Lipid envelope, and Envelope proteins.

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1.2.3 HSV-1 primary infection

The HSV-1 pathogenesis begins with the initial exposure, called the primary infection (Figure 4). First, the virus attaches to cell surface receptors, *e.g.* viral glycoprotein C (gC) binds to the cell receptor heparin sulfate (Spear, et al. 1992; Toma, et al. 2008) which is a polysaccharide that regulates developmental processes, angiogenesis, blood coagulation and tumour metastasis. Furthermore, viral glycoprotein D (gD) binds strongly to the cell receptor herpesvirus entry mediator receptor (HVEM) (Campadelli-Fiume, et al. 2007; Toma, et al. 2008), which belongs to the family of Tumor necrosis factor receptors (TNFR). After this first attachment of the virus to the cell membrane, gD changes its conformation and binds with viral glycoproteins gH and gL, leading to the so called hemifusion state (Subramanian, et al. 2007; Toma, et al. 2008). In this state, the viral envelope merges with the host cell membrane and a hole is created to allow the virus capsid and tegmentum proteins to enter the host cell. Once inside the host cell, the viral capsid is transported along the cytoskeleton to the nucleus, where it attaches to the nuclear entry pore and subsequently inserts the viral DNA in the nucleus through the capsid portal, which is formed by twelve copies of the viral UL6 protein (Yang, et al. 2007; Toma, et al. 2008). Now, the transcription of the immediate-early, early and late genes starts. After 18 hours of primary infection, the late structure proteins are used to form viral capsids and viral envelope proteins are placed on the cell membrane just before the virus is assembled and buds out of the cell (Granzow, et al. 2001).

Primary infection

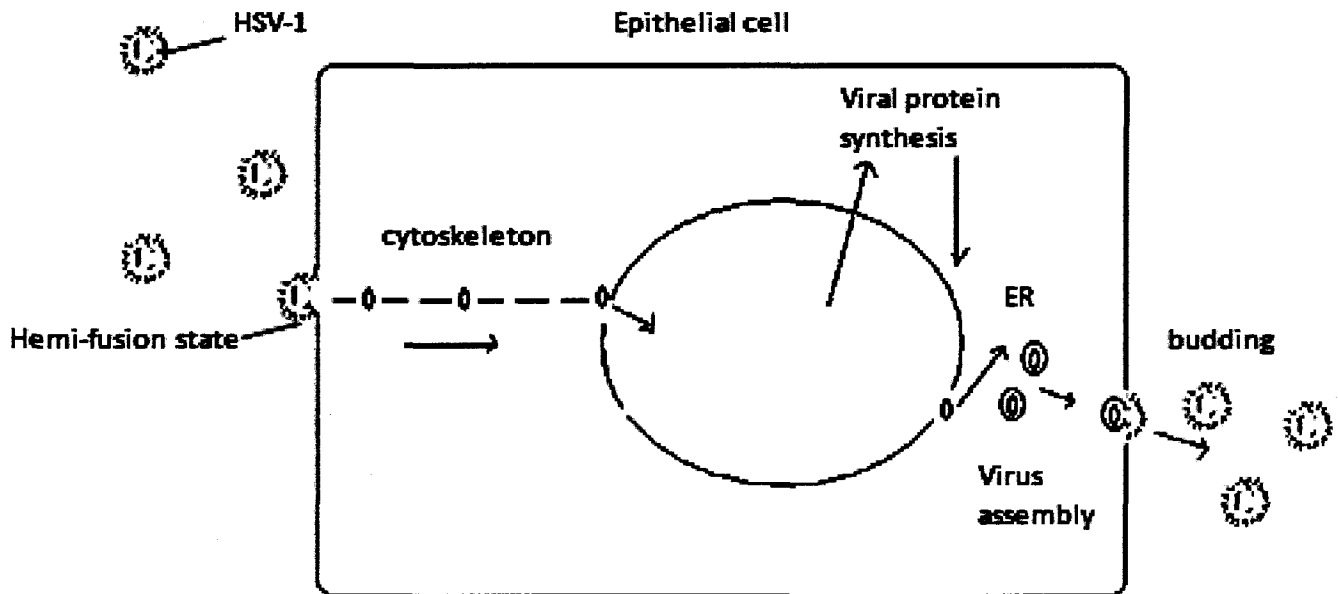


Figure 4: HSV-1 primary infection. It begins with viral attachment to cell surface receptors, fusion with host membrane, followed by viral capsid transport to the host nuclear pores. Then viral gene transcription and protein synthesis happens through the host cell machinery. Produced viral particles are transported to the endoplasmic reticulum. The primary infection ends with exocytosis of the virus particles from the host cell.

1.2.4 Latent HSV-1 infection

Following recovery of the primary infection, the virus can enter nerve endings in the infected tissue through nectin-1 receptors (Stiles, et al. 2010). Then HSV travels through retrograde axonal transport to the trigeminal ganglion, which gives rise to sensory neurons in the cornea (Figure 6) (Theil, et al. 2001). At this stage, the viral DNA is present in the nucleus of the neurons as an HSV genome histone-associated episome, resulting in the latent state (Hengel, et al. 2008). However, there is an intense restriction to viral gene expression since there is no viral replication during the latency phase, therefore no clinical signs are visible and there are no viral antigens expressed by the latently infected host cells. Thus, the host immune system cannot recognize the latently infected cells and eliminate them. However, there is one transcript associated with latency that is not translated into proteins, the Latency Associated Transcript (LAT) (Stevens, et al. 1987), which supposedly prevents apoptosis in the host cell. Even though numerous publications speculate about the mechanisms of latency, the concept is not completely understood (Toma, et al. 2008; Kutluay, et al. 2009; Paladino, et al. 2009).

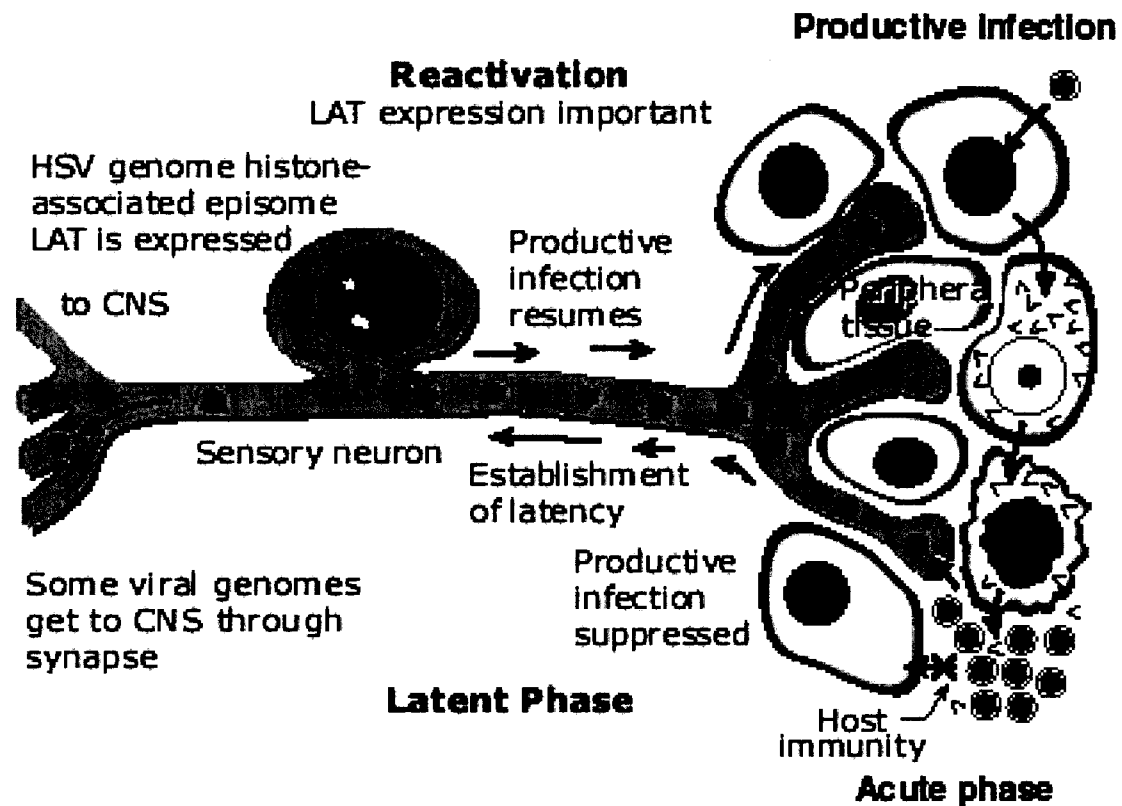


Figure 5: Latent HSV-1 infection. After the productive (primary) infection of HSV-1 and the acute phase with the host immune system in the epithelium, HSV-1 can enter into nerve endings in the peripheral tissue and travel in the axonal transport system of the sensory nerve to the ganglion. Here the virus is in a latent state as a HSV genome histone-associated episome, expressing only LAT. The virus can be reactivated through depletion of LAT, re-infecting the peripheral tissue that is innervated by the neuron. In rare cases, the virus can go the other direction to the CNS, leading to encephalitis.

1.2.5 HSV-1 Reactivation

Various factors at various times can trigger reactivation of HSV from latency (Toma, et al. 2008) which can lead to re-infection of the peripheral tissue innervated by the neurons. Triggers can be stress, immunosuppression, sunlight, postoperative care following surgical corneal trauma and postoperative corticosteroid therapy (Carton, et al. 1952; Hill, et al. 1978; Pazin, et al. 1978; Cleator, et al. 2000). In case of corneal graft failure through HSV reactivation, in patients with herpetic keratitis, the reactivation of the latent HSV may be due to surgical corneal trauma and post-operative corticosteroid therapy. Furthermore, a penetrating corneal transplantation includes the interruption of corneal nerves, which was shown to be correlated with HSV reactivation (Beyer, et al. 1990). Moreover, HSV infection was even shown to be the cause of graft failure in patients undergoing corneal transplantation for other reasons than herpetic keratitis (De Kesel, et al. 2001).

1.2.6 Artificial corneas and HSV

There are an estimated 500,000 cases per year in the US of corneal damage through HSV-1 infection (Khan, et al. 2004). The diseased cornea can be treated with donor corneal transplantation. However, the latent HSV in nerve cells can be reactivated as mentioned above. A combination of reactivated HSV and enzymes that are secreted as part of the immunological response can lead to rejection in both donor transplantations and corneal prostheses. Even though cornea transplants are the most successful organ transplantations performed, the success rate of transplantations in HSV patients is reduced to 22 % after 5 years (Larkin, et al. 1998). In comparison, patients without a HSV history have a graft success rate of 73 % (Coster, et al. 2009)

1.3 Anti-viral drug acyclovir (ACV)

One of the main anti-HSV drugs is acyclovir (ACV). It is a guanosine analogue and one of the most used anti-viral drugs. ACV has a low cytotoxicity due to its selectivity (Coen, et al. 1996). As a pro-drug, ACV has to be activated by the viral thymidine kinase (TK) and is therefore not active in uninfected cells. In further steps, the cellular kinases phosphorylate the activated pro-ACV to be an active guanosine analogue. Now ACV is able to block the viral DNA Polymerase in order to stop viral replication (O'Brien, et al. 1989). Topical application of ACV in the eye in the form of 5% ointment or ophthalmic drops is effective for superficial (dendritic or geographic) HSV keratitis, but ineffective against stromal or deep ocular HSV infection (Fields, et al. 2001; Shimomura, et al. 2008). This is due to the low penetration into the eye, as the eye drops are easily washed away from the cornea during blinking. This occurs before the drug can enter the inner corneal layers. Additionally, ACV can be taken orally as tablets (200 mg, 400 mg, 800 mg, or 1 g); however the bioavailability of the drug taken systemically in the cornea is only 10-20 % (Tyring, et al. 2002). High doses of ACV have also been associated with side effects such as gastrointestinal upset when it is given orally, suppression of the immune system after prolonged use and reported resistance occurs in HIV patients (Coen, et al. 1996). Therefore, a local and direct drug delivery would be favored to prevent expensive long-term medication and to prevent the evolution of ACV-resistant HSV strains through mutation in the viral TK kinase.

1.4 Research Summary

1.4.1 Hypothesis

My hypothesis is that a drug delivery system present within a corneal graft can prevent HSV-reactivation during surgery. Corneal substitutes would be best placed to incorporate such a drug delivery system as nanoparticles encapsulating the drug can be incorporated into these implants while they are being fabricated.

1.4.1 Objective

My research objectives, therefore, were to establish a nanoparticle drug delivery system for ACV, which can be incorporated into corneal substitutes to prevent viral reactivation caused by the surgery itself. These ACV containing corneal substitutes were then tested *in vitro* for their anti-viral activity to determine the feasibility of such constructs for future use in high risk transplant patients with latent HSV, aiding in the prevention of HSV-reactivation triggered by the surgery.

ACV was utilized, since this is the “gold standard” anti-viral drug against HSV infection. The novelty of this delivery system is that the release is sustained and is direct, from the corneal substitute. Since the delivery system is local, a smaller amount of drug is needed.

CHAPTER 2

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Controlled Release of Acyclovir Through Bioengineered Corneal Implants with Silica Nanoparticle Carriers

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Abstract: Herpes simplex virus (HSV) infection is the most common cause of corneal blindness in the Western world. Despite effective anti-viral drugs such as acyclovir (ACV), disease recurrence due to the virus establishing latency within the corneal nerves and possibly cells makes treatment very challenging. Furthermore, although effective, current systemic and topical preparations of anti-viral drugs do not appear to deliver sufficient quantities to the cornea to prevent reactivation. Current treatment for HSV vision loss is transplantation with donor corneas, but the surgery itself can reactivate viruses. We examined the feasibility of preventing viral reactivation during surgery, by sustained delivery of ACV introduced during corneal transplantation surgery, through encapsulation of the drug within silica (SiO₂) nanoparticles (NP) incorporated into biosynthetic alternatives to donor corneas. We show that incorporation of NPs did not affect optical clarity of the collagen-based corneal substitutes nor their biocompatibility. NP-encapsulation effectively sustained ACV release from the biosynthetic implants over 10 days, compared to free ACV incorporated directly into the hydrogel constructs. The NP-enabled sustained release resulted in effective prevention of virally-induced cell death, not observed with the free drug. This early model demonstrates the feasibility of using biomimetic corneal substitutes that incorporate a drug release system (e.g. silica nanoparticles encapsulating ACV) as future alternatives to human donor tissue grafts, for transplantation of HSV-infected corneas.

Key words: acyclovir, bioengineered corneal substitutes, herpes simplex virus, nanoparticles, drug delivery

INTRODUCTION

The Herpes simplex virus (HSV) is a neurotrophic virus that commonly infects the skin and mucous membranes of the mouth, genitalia, and eye. HSV-1 is the strain most commonly associated with corneal infections, and is the major cause of infectious vision loss in the developed world. Approximately 500,000 cases of corneal HSV infections are reported yearly in the US alone. There are 50,000 new and recurrent cases per year (Liesegang, et al. 1989; Liesegang, et al. 2001; Miserocchi, et al. 2007; Toma, et al. 2008), with visual disability reported as high as 40 % (Khan, et al. 2004). The pathogenesis for HSV infection is complex. Initial exposure generally results in a primary infection that can give rise to a variety of clinical symptoms. Symptoms can include surface (epithelial) ulceration and nerve damage, damage to deep stromal structures, corneal perforation, and both necrotizing and non-necrotizing stromal disease. Following recovery from primary infection, the virus establishes latency in the trigeminal ganglion that supplies sensory neurons to the ocular surface (Balfour, et al. 1999) and likely, within the cornea (Liesegang, et al. 2001). The total viral load of HSV-1 genomes in the trigeminal ganglia during latency was reported to be between 1,000 to 10,000 copies (Pevenstein, et al. 1999). Shimomura (2007) reported that corneas with a history of HSV keratitis harbour 1.6×10^4 copies of HSV DNA. Triggers for reactivation are varied and non-specific, and can include stress, immunosuppression, sunlight, and ingestion of certain foods (Liesegang, et al. 1989).

Corneal transplantation is the treatment of choice for HSV-induced corneal blindness that results from opacity due to heavy corneal damage and scarring (Khan,

2004). However, prognosis is very poor with a low success rate at five years (Misericchi, et al. 2007) due to viral reactivation and infection of grafts compared to non-HSV grafts (Khan, et al. 2004). We have previously developed corneal stromal matrix substitutes, based on carbodiimide crosslinked recombinant human collagen, that are biointeractive. These substitutes promoted regeneration of corneal epithelial and stromal layers, as well as corneal nerves in pig models (Liu, et al. 2008; Merrett, et al. 2008). Early results from an on-going Phase I clinical study shows similar results in humans (Fagerholm, et al. 2009). Furthermore, an acellular scaffold implant decreased postoperative inflammation, eliminating the need for sustained steroidal use when compared to allograft transplants (Fagerholm, et al. 2009). More recently, we enhanced the mechanical strength and biostability of such collagen hydrogels by incorporating a second crosslinked polymeric network based upon a synthetic phospholipid, 2-methacryloyloxyethyl phosphorylcholine (MPC). These interpenetrating networks of collagen showed an increase in mechanical strength over crosslinked collagen alone, and an increased stability against enzymatic degradation while retaining their ability to promote regeneration of both corneal cells and nerves (Liu, et al. 2009). The increased stability in a hostile environment of high enzymatic activity commonly found in HSV-infected corneas is important for implant retention and viability.

Acyclovir (ACV) is an anti-viral drug that is used both topically and orally to treat ocular HSV. It is a nucleotide analogue that is specifically activated by HSV-induced thymidine kinase, and inhibits viral DNA polymerase. Non-infected cells are not affected by ACV, as they have no means of activating the drug (Balfour, et al. 1999; Snoeck, et al. 1999). While oral ACV is an effective drug against HSV-1 infections (Bourlais, et al.

1998; O'Brien, et al. 1989), it is expensive, and long term use can lead to gastrointestinal upset, as well as suppression of the immune system (Bourlais, et al. 1998; Nagarsenker, et al. 1999). To bypass these problems, ACV can be administered through eye drops. This delivery method demonstrates low efficacy, due to poor drug solubility and lack of penetration to the target sites (Bourlais, et al. 1998; Gulsen, et al. 2004). Topical delivery of ACV through colloidal systems such as encapsulated nanoparticles, microparticles, and liposomes, has improved the delivery of this drug to some degree (Kreuter, et al. 1990; Chetoni, et al. 2004; Marcato, et al. 2008). Efficacy is still limited as the product is quickly discharged from the eye (*i.e.* by blinking or tears) (Bourlais, et al. 1998). Placing the drug carrier within a stable hydrogel, such as a contact lens therefore allows sustained delivery (Gulsen, et al. 2004). It therefore follows that a stable hydrogel, such as our collagen-MPC interpenetrating network that has been developed as a corneal substitute for transplantation, can also serve as a drug carrier. In the case of high risk transplants of HSV-infected eyes, drugs such as ACV could be included within the implant to treat or circumvent HSV-reactivation triggered by the surgery.

In this study, we tested the hypothesis that a nanoparticulate drug delivery system, delivering ACV for example, can be incorporated into our corneal substitutes and can inhibit HSV infection. Such ACV containing corneal substitutes will be suitable for high risk transplant patients with latent HSV, aiding in the prevention of HSV-reactivation triggered by the surgery.

Silica (SiO₂) nanoparticles (NP) (diameter < 100 nm) were selected as a prototype delivery vehicle, as they are simple to prepare and a range of methods for preparing porous SiO₂ NP are available (Kosuge, et al. 1995; Shinoda, et al. 1995; Aznar, et al.

1996; Bourlais, et al. 1998; Temuujin, et al. 2001; Ikeue, et al. 2002; Temuujin, et al. 2003; Liu, et al. 2008). The microemulsion and reversed micelle (with HCl) method (Yamauchi, et al. 1989; Osseo-Asare, et al. 1990; Osseo-Asare, et al. 1999) was selected, as the water-oil microemulsion effectively encapsulates hydrophobic drugs (such as ACV), by dissolving the drug in the oil phase (Gulsen, et al. 2004). We examined the effects of encapsulation of ACV within SiO₂ NP on drug activity. We compared the release kinetics of ACV from NP within collagen-MPC hydrogels, with release of free ACV within similar hydrogels. With respect to their implications in the prevention of HSV-1 re-infection, we demonstrate that the sustained release of NP encapsulated ACV within hydrogels was able to prevent re-infection of corneal cells in culture.

MATERIALS AND METHODOLOGY

Materials

Type I porcine atelocollagen was purchased from Nippon Meat Packers Inc. (Tokyo, Japan). Poly(ethylene glycol) diacrylate (PEGDA, Mn=575), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), ACV, Cyclohexane, tetraethyl orthosilicate (TEOS), 2,2-dimethyl methoxy-2-phenyl acetophenone (DMPA), ammonium persulfate (APS), N,N,N',N'-tetramethyl ethylene diamine (TEMED) and Triton were purchased from Sigma-Aldrich Canada Ltd (Ontario, Canada). 2-animoethyl methacrylate (AEMA) was purchased from PolySciences Inc (PA, USA). N-hydroxyl-succinimide (NHS) was

purchased from Fluka (Buchs, Switzerland). 2-methacryloyloxyethyl phosphorylcholine (MPC) was purchased from Biocompatibles UK Ltd (Surrey, UK). All chemicals, unless otherwise stated, were obtained from Sigma-Aldrich Canada Ltd (Ontario, Canada).

Preparation of Silica Nanoparticles and Acyclovir Encapsulation

For preparation of SiO₂ NP, 6 ml cyclohexane was combined with 2 ml Triton, followed by the addition of a solution of 1.5 ml concentrated hydrochloric acid containing 80 mg ACV. Under magnetic stirring, 0.5 ml TEOS was added drop wise, followed by slow neutralization of the mixture using concentrated ammonia hydroxide. The mixture was stirred for two days at 50 °C. The resulting NPs were washed twice with 50 % ethanol, and then vacuum dried. For controls, SiO₂ NP were prepared as above, but without the addition of ACV. The concentration of free ACV in solution was measured at 252 nm with a Beckman Du-640B spectrophotometer. Particle size and morphology of resultant particles were determined on a Phillips CM120 Transmission Electron Microscope (TEM).

Biocompatibility of Silica Nanoparticles

Immortalized human corneal epithelial cells (HCEC) that possess key morphological and physiological characteristics of primary cells (Araki-Sasaki, et al. 1995) were grown to

80 % confluence in 48-well plates supplemented with Keratinocyte Serum-Free Medium (KSFM; Invitrogen, Burlington, Canada) in a humidified tissue culture incubator. ACV, SiO₂, or SiO₂ encapsulated ACV at concentrations of 0, 10, 25, 50, 100, 250 and 500 µg/ml were introduced into the culture medium and incubated for 24 hours. Cytotoxicity was tested using live/dead staining (Molecular Probes kit) and a MTT assay (Ngamwongsatit, et al. 2008).

To determine whether there was any uptake of NP by HCEC, cells were incubated for 24 hours with 100 µg/ml NPs. They were released from the dishes by light trypsinization, pelleted by centrifugation, rinsed and then fixed in 2% glutaraldehyde in 0.1 M phosphate buffered saline (PBS). They were then processed for transmission electron microscopy (TEM) according to standard procedures at the electron microscopy facility at the Children's Hospital of Eastern Ontario (CHEO) in Ottawa. Samples were examined and micro-graphed using a JEOL 1010 transmission electron microscope.

Collagen-MPC Hydrogels

Collagen-MPC hydrogels were prepared as previously described (Liu, et al. 2009). Briefly, 400 mg of 20 % (w/w) porcine type I acidic atelocollagen solution was buffered with 150 µl of 0.625 M MES buffer in a syringe mixing system. The solution was thoroughly mixed with 100 µl of 20 % (w/v) MPC solution in MES buffer, before adding 6 µl PEGDA solution. 25 µl of 4 % (w/v) APS solution in MES buffer containing TEMED (APS:TEMED=1:0.78,w/w) was added into the collagen-MPC-PEGDA solution

and thoroughly mixed at 0 °C. Calculated volumes of EDC and NHS solution (both at 20 % w/v, molar equivalent ratio EDC:NHS:collagen-NH₂=1.5:1.5:1) were then added to crosslink the collagen and again thoroughly mixed at 0 °C. The final mixture was immediately dispensed into glass plate moulds. The hydrogels were cured at 100 % humidity under nitrogen at room temperature for 16 h, and then at 37 °C for 5 h prior to demoulding in a 0.1 M phosphate buffered saline (PBS) solution.

Effect of Incorporation of Silica Nanoparticles on Hydrogel Optical Properties

Light transmission through hydrogels was measured on a custom-built instrument previously described (Priest, et al. 1998). The measurements were made at 21 °C; Readings were obtained both for white light (quartz-halogen lamp source) and for narrow spectral regions (centered at 450, 500, 550, 600, and 650 nm).

Preparation of Hydrogels Containing Nanoparticle-Encapsulated or Free Acyclovir

To incorporate ACV into collagen-MPC gels, 300 µl ACV solution (0.2 mg ACV in 0.3 ml water) was added to the hydrogel solution prior to addition of APS to cure. To incorporate NP encapsulated ACV, 300 µl ACV SiO₂ NP, (5.31 mg ACV SiO₂ NP, in 0.3 ml water) was added to the mixture prior to APS curing.

Acyclovir Release and Bioactivity of Released Drug

Hydrogels containing either ACV or SiO₂ NP encapsulated ACV were placed in 15 ml PBS for 12 days and assayed for amounts of drug released. On Days 1, 2, 3, 6, 8, 10 and 12, the PBS was collected and replaced by fresh PBS. All PBS time points were analysed by high performance liquid chromatography (HPLC). HPLC was performed using an Agilent 1100 Series apparatus (G1379A Degasser, G1312A Binary Pump, G1387A Autosampler, G1315B Diode Array Detector). Separations were achieved on a reversed-phase C-18 column (Zorbax SB C-18 4.6 mm x 25 cm) at room temperature. The mobile phase, flowing at a rate of 0.5 mL/min., consisted of acetonitrile and water with 1% acetic acid added to improve peak shape. It was ramped from 0% to 50% acetonitrile over 25 min., then to 100% acetonitrile over 20 min., then back to 100% water with acetic acid over 10 min. with a final 5 minutes at this composition. The injection volume was 10.0 µl. Absorbance signals were monitored at 254 nm using 400 nm as a baseline reference. Under these conditions, acyclovir was detected at 11.9 min. and was sufficiently separated from the background peaks. Quantification was based on peak area using a calibration curve constructed by analyzing standard samples of ACV with the identical HPLC method.

To confirm the bioactivity of the released ACV, the ability of the released drug to block the cytopathogenic effects of HSV-1 viruses was compared to that of free drug. Briefly, 2.5 X 10⁴ HCECs were seeded onto 12-well tissue culture plates as previously described and incubated until confluence. 10⁴ pfu/well of HSV-1 was added, along with free ACV at concentrations of 0.5, 1.0, 2.0 and 4.0 µg/ml; or SiO₂ NP encapsulated ACV

at concentrations of 0.5, 1.14, 2.28 and 3.5 µg/ml. After 24 hours, the cultures were fixed with 80% acetone and stained with crystal violet. Infected cells that were dead (or showed gross cytopathic changes) vs healthy, live cells were counted in four randomly selected areas in the field of view for each sample and proportions of infected cells were noted (n=6 samples for each condition). Difference in proportions of infected vs. healthy cells at each dose range was analyzed by ANOVA, followed by a Tukey test, with statistical significance set at $P < 0.05$.

Efficacy of Released ACV in Preventing Re-infection over 12 days

Collagen-MPC gels containing either free ACV or SiO₂ NP encapsulated ACV were placed on top of HECE cell cultures of 80% confluence, allowing for release of the drug. After time points Day 1, Day 2, Day 3, Day 6, Day 8, Day 10 and Day 12, hydrogels were placed on top of corneal epithelial cells and were infected with HSV-1 viruses at a ratio of 0.1 MOI (10^4 pfu HSV) to simulate viral re-infection (or re-activation). The cells, viruses, and hydrogel samples were re-incubated for a further 48 hours, after which all samples were fixed in 80 % acetone and stained with crystal violet (n=6 samples for each condition). Uninfected cells and virus-infected but untreated cells served as controls. Cultures were imaged on a Nikon inverted microscope, and proportions of live to dead cells (including cells with gross cytopathic changes) were counted from an average of four randomly selected areas of each sample. Differences in ACV release efficacy, as

indicated by the proportion of live cells, were analyzed statistically, using a one-way ANOVA. Statistical significance was set at $P < 0.05$.

Efficacy of Released ACV in Reducing Viral Copies in Treated Cells: Real-time Reverse Transcription PCR (qRT-PCR)

Total RNA was extracted from four different HCEC samples, using an RNeasy mini kit (Qiagen) after 3 days of infection. The samples comprised HCEC infected with 10^4 pfu HSV-1 that were untreated, treated with hydrogel containing ACV only, or treated with hydrogel containing SiO₂ NP encapsulated ACV; and a negative control of uninfected cells. Reverse transcription of total RNA was performed using a SMART™ PCR cDNA Synthesis Kit (Clontech) to generate cDNA, according to the manufacturer's instructions. qRT-PCR was performed in special optical tubes in 96-well microtitre plates (Perkin-Elmer/Applied Biosystems) using an iCycler (Bio-Rad). Fluorescent signals were generated using a Quantitect SYBR Green PCR kit (Qiagen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a stably expressed housekeeping gene was used to normalize HSV gene expression. GAPDH sense and antisense primer sequences used were: 5-ATGTGTCCGTCGTGGATCTGA-3 and 5-TTGAAGTCGCAGGAGACAACCT-3, respectively. The HSV gene was analyzed using HSV-1 sense and antisense primer sequences 5-CCGTCAG CACCTTCATCGA-3 and 3-CTGATGTGCCTCCAGGTCGC-5 obtained from the diagnostic laboratory at CHEO. The HSV and GAPDH genes were amplified using the

above mentioned primers and a cDNA template from the four different groups. Target samples were added in individual reactions to a total volume of 25 μ l and no cDNA was added to the negative control. For each amplification using realtime PCR, the protocol included 10 min at 95 °C and 40 cycles of 15 s at 95 °C, 1 min at 55 °C and 1 min at 72 °C, followed by a melting curve. All qRT-PCR experiments were run in triplicate. The iCycler software (Bio-Rad) detected the threshold cycle (CT) for each amplicon. Normalization was performed using the 2^{-2DDC T} method (Livak, et al. 2001).

RESULTS

Silica Nanoparticles

No NP were formed at a pH of < 2 or > 7. Additionally, the proportion of oil to water had to be beyond 1:7 to allow for NP formation. For encapsulation of ACV, the optimum encapsulation parameters were a pH between 5-6 and a water:oil ratio of 1:10. TEM imaging of the resulting SiO₂ NP showed that they were electron dense spheroids, with mean diameters of 25 \pm 5 nm (Fig. 1).

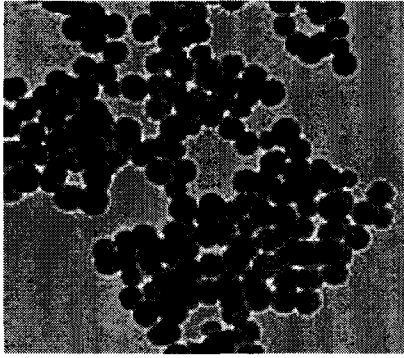


Figure (1). TEM image of SiO₂ nanoparticles, showing their spherical shape. Average diameter of particles is 25 ± 5 nm.

Effects of SiO₂ on Cell Viability

Concentrations of ACV, SiO₂ NP, and SiO₂ encapsulated ACV dispersed in culture medium did not have any cytopathic effects on cultured HCECs, up to 250 $\mu\text{g/ml}$. At 500 $\mu\text{g/ml}$, however, the NP aggregated and there was lifting of cells observed (live/dead staining and MTT; data not shown). TEM observations showed that the presence of NP, up to concentrations of 100 $\mu\text{g/ml}$, mostly remained extracellular (Fig. 2A). The few NP that were internalized were found in membrane-bound structures (Fig. 2B), *e.g.* lysosomes. However, the cell cytoplasm remained electron lucent with intact organelles, confirming that they were still healthy.

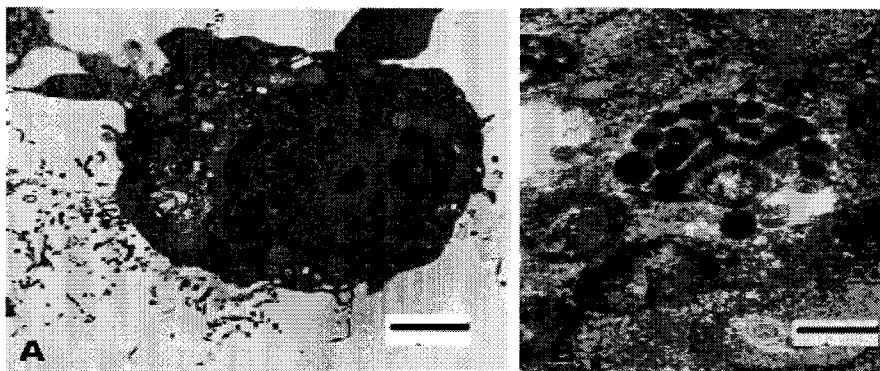


Figure (2). *Effects of SiO₂ nanoparticles on cell viability. HCECs were incubated with 100 μg/ml SiO₂ and processed for transmission electron microscopy (TEM) to visualize uptake of NP, seen as electron dense spheres. Scale bars, A, 2μm; B, 200 nm.*

Effect of SiO₂ Nanoparticles on Hydrogel Transparency

The incorporation of SiO₂ NP did not adversely affect the transmission of light through hydrogels (Table 1). Transmission values at all wavelengths tested was over 90 %, meeting the reported value of about 87 % reported for healthy human corneas (Beems, et al. 1990). As our NP average about 25 nm in size, below the wavelength of visible light (<280 nm; sunlight has an averaged wavelength of 560 nm), they do not cause perceptible light scattering and therefore do not affect transparency (Fresta, et al. 2001).

Sample	Collagen-MPC					
Wavelength (nm)	White	450	500	550	600	650
Transmission	91.6	99.7	99.6	99.4	98.8	97.4
Sample	SiO ₂ NP Dispered in Collagen-MPC					
Wavelength (nm)	White	450	500	550	600	650
Transmission	95.7	92.7	99.6	99.4	98.8	97.4

Table 1. Optical Properties of Biomimetic Corneas. Collagen-MPC alone and Loaded with SiO₂ NP were Measured for Transmission at Different Wavelengths of Visible Light (450, 500, 550, 600 and 650 nm) Sample Collagen-MPC.

Release of ACV from SiO₂ Nanoparticles within Hydrogels, and Bioactivity of Released ACV

Free ACV incorporated within collagen-MPC hydrogels showed rapid and complete drug release after 2 days (Fig. 3). By comparison, SiO₂ encapsulated ACV within collagen-MPC hydrogels showed a gradual release over 12 days, shown by HPLC analysis.

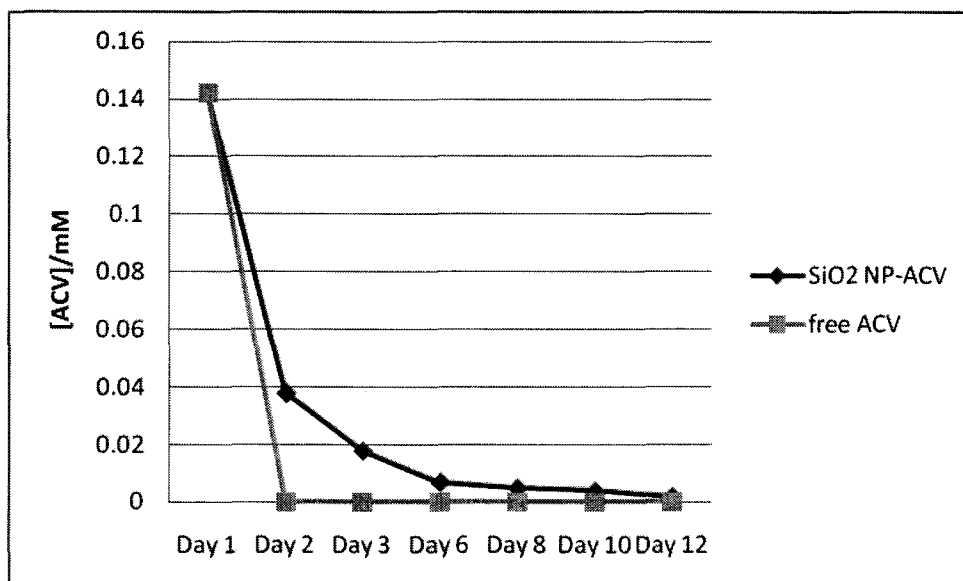


Figure (3). Release profile of ACV encapsulated within SiO₂ nanoparticles that have been incorporated into collagen-phosphorylcholine hydrogels compared with that of free ACV within the similar hydrogels, as determined by HPLC over the course of 12 days.

HSV-1 viruses showed a statistically significant ($P < 0.05$; by ANOVA/Tukey tests) dose dependence response to ACV. At 1.0 to 1.5 and 2.0 to 2.5, SiO₂ encapsulated ACV showed significantly higher anti-viral activity, but it should be noted that the released ACV doses were slightly higher than the free ACV (Fig. 4). At the highest dose, 3.5 $\mu\text{g/ml}$ of released ACV was compared to 4 $\mu\text{g/ml}$ of free ACV (Fig. 4). Nevertheless, these results demonstrate fairly comparable efficacy between encapsulated and free drug, showing that the nanoparticle-encapsulated drug retained its full bioactivity.

Free ACV incorporated within collagen-MPC hydrogels showed rapid and complete drug release after 24 hours. By comparison, SiO₂ encapsulated ACV within collagen-MPC hydrogels showed a gradual release over 12 days, shown by an anti-viral assay (Fig. 5). Free ACV within the hydrogels was rapidly depleted, and thus had no effect on viral

activity. In the free ACV case, the number of live cells remaining was not significantly different from those of untreated, infected cells after 24 hours. SiO₂ encapsulated ACV within the hydrogels however, was able to interfere with virus activity, and enhance cell survival until 10 days. Fig. 5 B, C, and D demonstrate furthermore the cell morphology of healthy and HSV-infected cells.

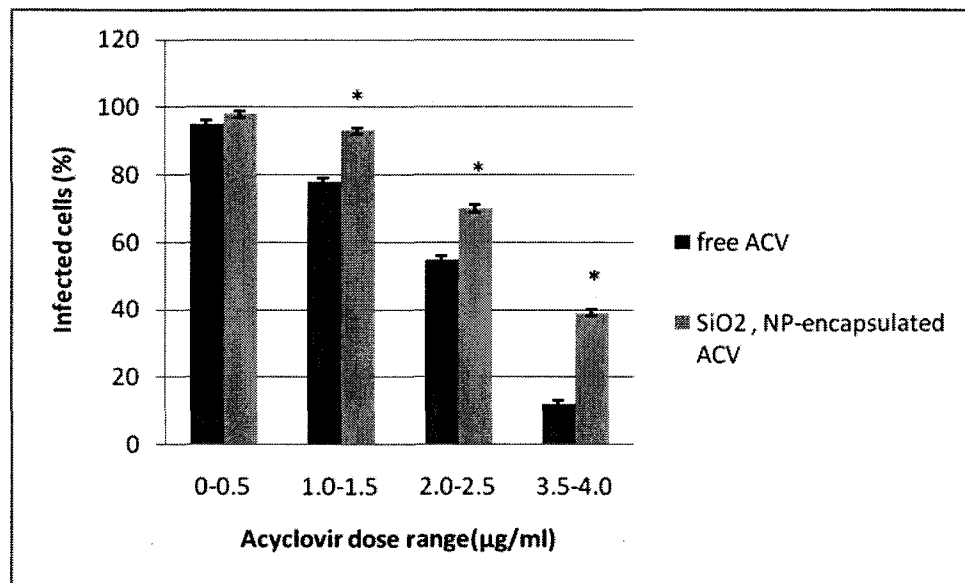


Figure (4). Anti-HSV activity of SiO₂ nanoparticle released ACV compared to comparable free ACV concentrations. There was residual unreleased ACV from the nanoparticle-encapsulated drug, so narrow dose ranges were compared instead of exact doses. * Indicates significant difference (*P* value < 0.001) from free ACV within one dosage range.

Effects of Released ACV in Reducing Viral Copies in Treated Cells: Real-time PCR

Real-time PCR runs showed the relative expression change of HSV compared to the control of virus-infected cells after three days of washing the hydrogels (Table 2). Cells infected with HSV and treated with ACV loaded directly into hydrogels showed a 4002-

fold lower expression in HSV gene expression compared to infected cells, showing viral suppression by the drug. However, when infected cells were treated with ACV released from nanoparticles within the hydrogels, relative gene expression was 7131-fold lower than the infected cells control. These data clearly demonstrates that prolonged ACV release from SiO₂ NP and hydrogel leads to a greater anti-HSV activity.

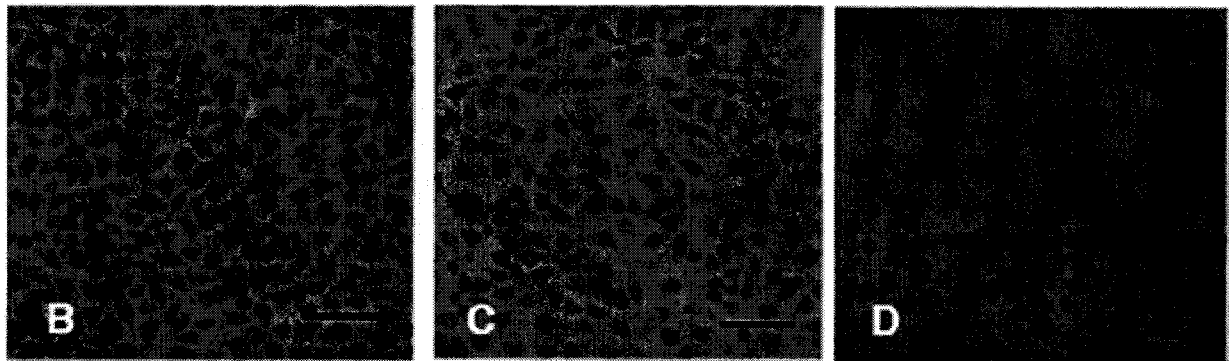
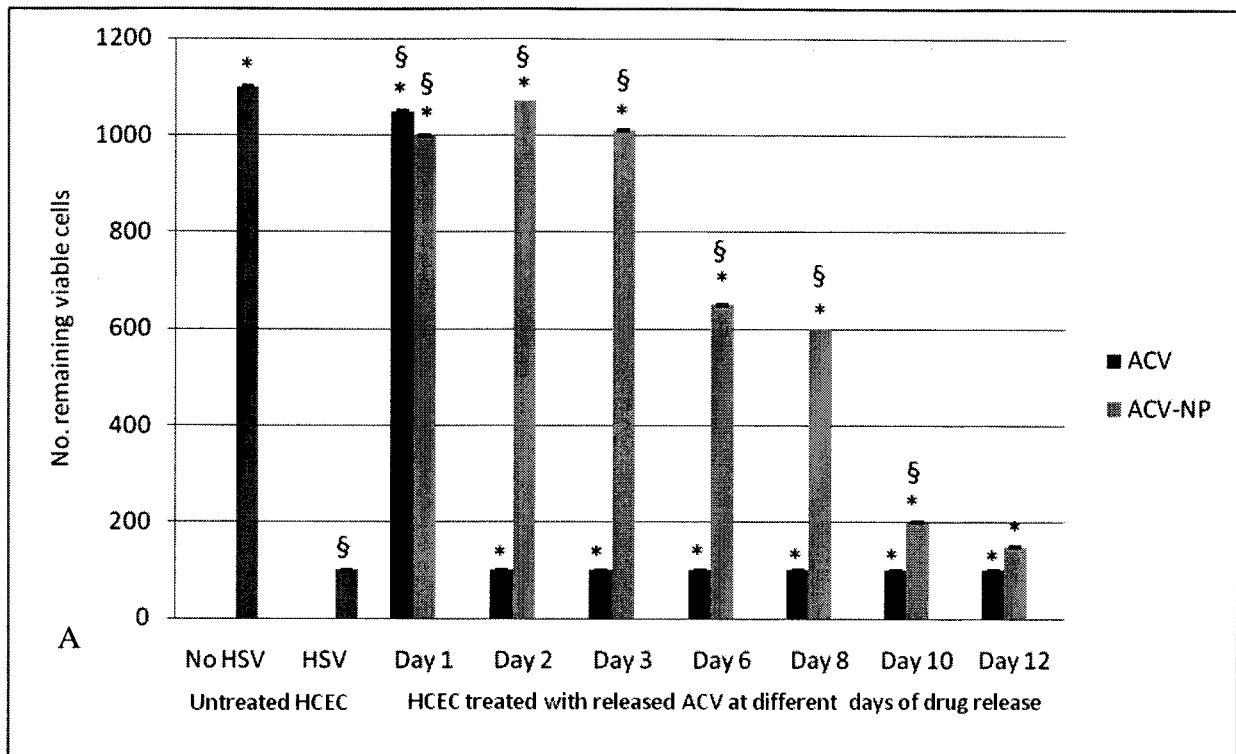


Figure (5). A) Comparison of efficacy in inhibiting HSV-1 viruses of free vs. SiO₂ nanoparticle-encapsulated ACV released from collagen-MPC hydrogels over 12 days. Hydrogels were placed in PBS to allow release of ACV. At different time points after the start of release (days 1, 2, 3, 6, 8, 10 and 12) they were placed on top of corneal epithelial cells that were infected with 10⁴ pfu of HSV-1. Counts of surviving cells indicating efficacy of remaining ACV in the hydrogels at each time point were analysed by ANOVA followed by a Tukey-test. *,§ indicates significant difference (P value < 0.001) between each sample and the negative control of uninfected HCEC, or positive control of HSV-1 infected, untreated HCEC, respectively. (B) Healthy, uninfected HCECs showing a flattened, cobble-stoned morphology when confluent. The same morphological appearance is observed for HSV-1 exposed cells treated with ACV in doses that block viral cytopathic activity (C). HSV-1 infected cells are rounded, with pyknotic nuclei that are typical of dying cells (D). Scale bars, 30 μm.

Relative fold change of virus transcripts			
	HSV untreated	HSV + ACV in hydrogel	HSV + ACV-SiO ₂ in hydrogel
HSV-1	1	-4002	-7131
GAPDH	1	1	1

Table 2. Relative fold change of virus transcripts. HCEC infected with Virus was used to Normalize GAPDH Housekeeping Gene in all Samples. HSV-1 Primers were used to Determine Viral Copy Fold Changes in Samples Compared to Control.

DISCUSSION

Our results show that SiO₂ NP produced by the water-oil microemulsion method can effectively encapsulate hydrophobic ACV, and release the drug in a sustained manner over 12 days. We also showed that SiO₂ NP are biocompatible and non-cytotoxic to HCECs in culture, up to concentrations of 250 µg/ml. At 500 µg/ml, some cytotoxic effects were observed that were presumably related to the observed clumping of the NP at higher concentrations. TEM observations showed that at a non-cytotoxic level of 100 µg/ml, most NP were localized external to the cell plasma membranes. Only a small number of particles were seen within membrane bound structures that resembled lysosomes. However, the cells in which these NP were localized still had electron lucent cytoplasm and intact organelles, indicative of healthy cells.

When incorporated into collagen-MPC hydrogels, the NP did not impair optical clarity. On the contrary, implants with NP showed high light transmission (92.7-99.6 %) that was comparable to control hydrogels without NP (91.6-99.7 %). This was within the acceptable range of about 87 % reported for human corneas (Livak, et al. 2001). The high optical clarity was most likely due to the small size of the 20-30 nm diameter particles, which are weakly interacting with light. However, we also found that the optical clarity is very dependent upon the dispersement of the NP.

The release of free ACV incorporated into collagen-MPC hydrogels occurred over 2 days. Release of SiO₂ NP-encapsulated ACV within collagen-MPC hydrogels, however, occurred over 12 days. In addition, the released ACV from hydrogel-NP constructs retained its bioactivity. These results show that this drug encapsulation method

and the further incorporation into collagen-MPC hydrogels did not adversely affect ACV bioactivity.

Previous studies have shown very clearly that the use of micro- and nano-particulate carriers (*e.g.* PECA nanospheres (Beems, et al. 1990), Eudragit microparticles (Shinoda, 1995), or liposomes (Chetoni, et al. 2004)) have in topical applications been successful in extending drug release time and therefore availability. However, *in vivo* applications are still problematic as both drugs and their carriers are rapidly cleared from the ocular surface by tearing and blinking. Hence, incorporation of carriers into a retentive ophthalmic ointment (Chetoni, et al. 2004) or into contact lenses (Aznar, et al. 1996) have been proposed as means of extending drug availability to the ocular surface. However, to remedy HSV-induced vision loss, instead of treating the cornea with drugs and performing allograft surgery as two separate modalities, both with limited success rates, our results indicate that it would be possible to incorporate the drug/carrier directly into biosynthetic substitutes of human donor corneas (Fagerholm, et al. 2009; Liu, et al. 2009), to allow for the drug to be released after implantation to prevent viral re-activation and re-infection. Incorporating the drug into a graft would circumvent drug availability issues to all the corneal layers. Moreover, the presence of the drug at the time of surgery would serve a prophylactic function.

In the present study, nanoparticle-encapsulated ACV released from collagen-MPC hydrogels that we previously designed as corneal substitutes (Liu, et al. 2009) showed sufficient antiviral activity, even after 10 days of continuous release, to significantly increase the viability of cultured human corneal epithelial cells that were infected with HSV-1 viruses. Correspondingly, real-time PCR showed that the sustained release of

ACV was able to block HSV-1 infection of corneal epithelial cells in culture, as evidenced by the extremely low viral copies within treated corneal cells (Table 2).

Although further optimization (*e.g.* change in drug carrier, size of the carrier, drug incorporation method, most appropriate anti-viral drug/prodrug) is required to maximize the sustained release of drug and treatment efficacy, we have nevertheless demonstrated the feasibility of incorporation of a drug-release system into corneal substitutes designed as alternatives to donor human tissues for corneal transplantation of HSV-infected eyes. With future development, this could become one method to counter potential HSV reactivation, triggered by the stress induced by the transplantation procedure and post-operative steroid treatment.

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CHAPTER 3

GENERAL DISCUSSION

3.1 Significant scientific contributions

Recurring HSV-1 reactivation in the cornea can be devastating for patients, as this can lead to vision loss and blindness. Corneal grafts are the only treatment option in these severe cases. The reactivation of HSV-1 can lead to graft failure after the corneal transplantation and the patient may remain blind. Many groups have been devising delivery systems for anti-viral drugs in ocular drops or ophthalmological gels, however without significant success (Richards, et al. 1983; Kreuter, et al. 1990; Bourlais, et al. 1998; Chetoni, et al. 2004; Gulsen, et al. 2004; Marcato, et al. 2008).

In Chapter 2, I described a novel drug delivery system which uses silica nanoparticles to encapsulate the anti-HSV drug ACV. This encapsulated drug was then placed in artificial corneas to establish a drug delivery system. This is the first time that a combination of corneal surgery and anti-viral drug delivery against HSV-1 has been proposed. Others have tried only the incorporation of carriers into ophthalmic ointments, or contact lenses (Richards, et al. 1983; Kreuter, et al. 1990; Bourlais, et al. 1998; Chetoni, et al. 2004; Gulsen, et al. 2004; Marcato, et al. 2008). The issue of the released drug being depleted through blinking and the tear film is alleviated by the direct release from the implant. Drug release from contact lens material is still under investigation and

seems to be suitable for superficial infection in the epithelium. However, these implants need to be further tested *in vivo*. A critical important consideration in successful HSV-1 treatment is the prevention of viral activation by the surgery itself immediate effect after surgery. Incorporation of a drug carrier that could extend drug release and may be able to prevent graft failure due to the reactivated virus is possible in bioengineered corneas, as opposed to donor human tissue. The availability of ACV throughout all layers of the cornea is critical to prevent viral reactivation in any one layer of the cornea that could lead to graft failure. Long-term drug medication might still be required for prevention; but this is not the focus of the current study.

As these experiments were limited to *in vitro* work, it is important to discuss the strategies that should be implemented for future progression to *in vivo* work (see below).

3.2 Issues for future consideration

3.2.1 Design of an *in vivo* HSV-1 animal model

In order to help HSV-1 patients, it will be important to develop an *in vivo* model. Several points must be considered. First, choosing the species to create a model will be important. Both mice and rabbits are established animal models with similarities in HSV-1 establishment of latency and reactivation patterns (Laycock, et al. 1991; Marquart, et al. 2001; Bloom, et al. 2004; Kaufman, et al. 2008). Furthermore, their cornea is similar to

the human cornea and transplantation models are developed (Forrester, et al. 2004). However, from preliminary data I gathered thus far (data not shown), rabbit corneal transplantations might need the assistance of a 3rd eyelid suture to take the pressure off of the full-thickness artificial corneal graft. 3rd eyelids that slide up over the surface of the eye are found in domestic animals like cats, dogs, or rabbits. A suture can be placed through this lid and tighten on the other edge of the eye. However, this might interfere with the HSV infection model, and humans would not be subjected to this 3rd eye lid suture. For an application in rabbits, the artificial corneas tested thus far would need further improvement with respect to stiffness and elasticity. In another preliminary animal model, BALB/c mice were infected with HSV-1 on the ocular surface. However, the virus strain revealed itself as being extremely aggressive, since all mice died of encephalitis even when subjected to the smallest viral concentration. This could be due to BALB/c mice having an immune response that is biased towards Th2 immune response with more bias towards antigen producing plasma cells, which makes them susceptible for viral infection (Culley, et al. 2006). The HSV-1 strain I used was a clinical isolate and more aggressive than laboratory strains. Toma *et al.* 2008 reviewed that different strains of HSV-1 can differ in terms of their pattern of disease, severity of infection, and the numbers of latent virus copies per neuron. Therefore, other mouse strains that are more Th1 biased including Interferon- γ production (making them more resistant for HSV-1 infection, but still susceptible enough for a latent infection) should be investigated.

Another important issue to consider is the method of viral reactivation. The trigger for a HSV reactivation is important, as the stress of surgery may not be enough to reactivate the virus. Ophthalmic steroids are known to cause viral reactivation, however

they are also known to cause graft failure. Since the goal is to sustain the implant and only reactivate the virus, other methods should be considered: heat shock (Sawtell, et al. 1992; Gebhardt, et al. 2004), Sodium butyrate (Neumann, et al. 2007), or UV light (Harbour, et al. 1983; Shimeld, et al. 1990). Furthermore, the timeline for testing the reactivation will be important to optimize for HSV-1 patients, since the delivery time of anti-virals will be limited. In this case it is also important to consider possible clinical situations with the severity of HSV in patients, since not all patients have a devastating outcome. Therefore, diverse methods of reactivating virus with diverse time lines and severity of reactivation must be tested.

The artificial corneal implants will also need further optimization in terms of resistance against enzymatic degradation. Although we have tested enzymatic degradation against collagenases *in vitro* (data not shown), HSV infection is associated with a copious amount of enzymatic activity from the infected tissue and immune response. Therefore the implants need to be extremely resistant to this type of hostile environment.

3.2.2 Contact lens for further treatment

Modified artificial corneas will be designed for HSV-1 patients, whose corneal tissue is already damaged and clouded beyond repair. These constructs will be designed to prevent graft failure immediately after the corneal surgery. If there is a case of reactivation at a later time point, additional treatments will need to be considered. One

possibility is the use of a contact lens which is modified in a similar method as the artificial corneas (*i.e.* loaded with antiviral drugs). These contact lenses can be applied as a prophylactic treatment or at the first signs of reactivation of the virus, to prevent the devastating destruction of the cornea.

3.3 Conclusion

Corneal HSV-1 infection is included in the high risk group for corneal transplantations. The reactivation of the virus is often triggered by surgery or post-operative treatment which can lead to graft failure. I have demonstrated suitable anti-viral modification for corneal substitutes to potentially prevent viral infection *in vitro*. These investigations have contributed to the development of a possible new therapeutic method for treating patients requiring corneal transplantation due to HSV-1 infection. While further development and testing in animals is needed, I have nevertheless demonstrated the feasibility of this potential new method for treatment of HSV-1 corneal infections that require grafting.

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