I, Poonam Chopra, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences/Biopharmaceutics.

It is entitled:
Ocular Iontophoresis of Nanocarriers for Sustained Drug Delivery to the Eye

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Ocular Iontophoresis of Nanocarriers for
Sustained Drug Delivery to the Eye

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ABSTRACT

Transscleral iontophoresis has been shown as a viable modality to deliver therapeutic drugs for the treatment of eye diseases. Although several studies have been conducted to examine the utility of iontophoresis for both charged and neutral molecules, the understanding of the mechanisms controlling the iontophoretic delivery of macromolecules across human sclera is required to optimize this approach. In addition, transscleral iontophoresis is limited by fast clearance of the drug from the eye to the systemic circulation, leading to the requirement of repeated iontophoresis administrations. The present dissertation was an effort to understand the significance of flux enhancing mechanisms to optimize transscleral iontophoretic delivery of macromolecules. Another objective of the present dissertation was to develop a sustained release drug delivery system consisted of mixed micellar carriers of phospholipids and surfactants to increase the residence time of drugs in the eye in transscleral iontophoretic delivery.

Chapter 3 of this dissertation describes the effectiveness of iontophoresis in the delivery of charged macromolecules along with the mechanisms controlling the iontophoretic transport of these macromolecules across human sclera. The results in the transscleral transport study suggested that electroosmosis was the major flux-enhancing mechanism in the iontophoretic transport of macromolecules of low charge to mass ratios such as micellar carriers. However, highly negatively charged macromolecules were more effectively delivered by the direct field effect, electrophoresis.

The feasibility of transscleral delivery of sustained release drug delivery systems composed of micelles by passive and iontophoretic methods was evaluated using
cadaveric human sclera in vitro and the results are described in Chapter 4. The mixed micellar carrier system enhanced the solubility of corticosteroids and effectively transported these corticosteroids across human sclera during cathodal iontophoresis. Drugs were then released in a slow and sustained manner from the carrier system in the sclera after iontophoresic delivery.

In Chapter 5, the influence of drug lipophilicity on the solubilization potential of a mixed micellar carrier system was determined. In addition, the relationship between drug lipophilicities and drug release profiles of the mixed micellar carrier system was also investigated. The results showed that drug solubility enhancement of the micellar carrier system increased with increasing drug lipophilicities. The more lipophilic drugs displayed slower drug release from the sclera compared with the less lipophilic drugs after iontophoresic drug delivery with the mixed micelles.

The feasibility of mixed micelles as a sustained release drug delivery system was evaluated in vivo using mouse as an animal model in Chapter 6. Pharmacokinetics study of a corticosteroid in the ocular tissues after transscleral iontophoresic delivery of the mixed micelles was performed. To evaluate the safety of transscleral iontophoresis of the mixed micelles, histological examination of the eye tissues was performed after the mixed micelle and iontophoresis treatments. Histological analysis showed no significant changes in the structure of the ocular tissues treated with the mixed micelles and iontophoresis.
This thesis is dedicated to my father who believed in me, my wonderful husband and my beautiful and precious daughter, who have been a source of encouragement and inspiration to me throughout my journey.
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CHAPTER 1

Introduction

1.1. Background

1.1.1. Anatomy of the eye

The human eye is a slightly asymmetrical globe with an inch in diameter. The front part of the eye comprises iris, cornea, pupil, and ciliary body, and most of the anterior segment of the eye is filled with a clear liquid called aqueous humor. The aqueous humor is produced by the ciliary body. The vitreous humor is present in the posterior part of the globe and is a clear, avascular and gelatinous fluid [1]. The exterior shell of the eye consists of sclera, choroid and inner retina (Fig. 1.1.1A). The human sclera is a rigid, white, dense, avascular, metabolically inert connective tissue, which performs the important function of the visual integrity of the eye. The sclera covers about 80% of the eyeball and extends posteriorly from the corneal perimeter to the optic foramen and is perforated at the entrance of the optic nerve. The human sclera is thickest 1-1.35 mm at the posterior pole, decreasing gradually to 0.4-0.6 mm at the equator and thinnest 0.3 mm under the rectus muscles [2]. The human sclera mainly consists of collagen fibers packed in lamellar bundles. These collagen fibers exhibit a wide range of diameters from 25-230 nm [3] and are immersed into an amorphous base containing glucoseaminoglycans, proteins and protein-polysaccharide complexes.

The inside lining of the eye is covered by special light-sensing cells that are collectively called the retina. The retina converts light into electrical impulses. Behind
the eye, the optic nerve carries these impulses to the brain. The macula is a small sensitive area within the retina that gives central vision. It is located at the center of the retina and contains the fovea, a small depression or pit at the center of the macula that gives the clearest vision (Fig. 1.1.1 B).
Figure 1.1.1. (A) Anatomy of the eye (B) Cross section of the eye (rear view)

http://www.99main.com/~charlief/Blindness.htm
**Barrier properties of cornea**

In general, the cornea is considered as the main pathway for the permeation of drugs into the eye [4]. The cornea is composed of epithelium, stroma and endothelium. Each layer of the cornea is different in structure for drug permeation. The corneal epithelium has 5-6 cell layers in thickness and is a lipophilic cellular tissue. The tight junctions among the corneal epithelium are formed to restrict paracellular drug permeation from the tear film [5-6]. For transcellular permeation, lipophilicity is a prerequisite and permeation of hydrophilic drugs is limited to the paracellular space [7]. Physiochemical properties of drugs such as octanol-water partition coefficient, molecular size and shape and degree of ionization affect the route and rate of drug permeation in the cornea [8-13]. In general, the permeability of the cornea increases with an increase in the lipophilicity and octanol-water partition coefficient of the permeant. It has been suggested that the optimum octanol-water partition coefficient for corneal drug absorption is around the logarithm of octanol-water partition coefficient = 1 to 2 and further increase in the octanol-water partition coefficient will decrease drug absorption [14]. The molecular weight cut off for the corneal drug permeation was determined to be in the range of 400-600 Da [9]. Corneal epithelium is negatively charged at physiological pH, so the cornea is more permeable to positively charged molecules as compared to the negatively charged molecules [11]. The corneal epithelium is metabolically active with the presence of an enzymatic barrier composed of peptidases, proteases and esterases [15-16]. The stroma present in the cornea is a hydrophilic tissue composed of collagen fibrils, mucopolysacchrides, and 70-80 % water [6]. The stroma acts as barrier to the permeation of highly lipophilic permeants. The corneal endothelium
is considered lipophilic and is composed of hexagonal cells with tight junctions [6, 17]. The tight junctions present in endothelium are wider than the tight junctions in the epithelium, and the permeability of the endothelium has been reported to display strong dependence on both octanol-water partition coefficient and molecular size of the permeants [8].

**Barrier properties of sclera**

The barrier properties of conjunctiva and sclera are of recent interest, due to its larger surface area for drug delivery. Drug delivery applications through these tissues include diffusion of the drug across the conjunctiva and sclera to the anterior segment for the treatment of anterior eye diseases and directly across the sclera to the posterior segment for the treatment of posterior eye diseases [18-19]. Sclera is a porous membrane and studies have shown that molecules up to 150,000 Da could penetrate the sclera. Scleral permeability has been shown to have a strong dependence on the molecular sizes of the permeants; the permeability of human sclera showed an inverse relationship between the scleral permeability and molecular weight of the permeants [20]. The literature study also shows that permeability of sclera has no apparent dependence on the lipophilicity of the permeants [8]. It has been suggested that the size of a permeant has more pronounced effect on its permeability in sclera as compared to the cornea [21] and the sclera was found to be approximately 11 times more permeable to topical compounds as compared to the cornea [22].
1.1.2. Posterior segment ocular diseases

Posterior eye diseases account for the majority of blindness in the United States [23]. Currently, the treatment of posterior eye diseases is limited by the difficulty in delivering the drug effectively to the target tissues in the posterior eye. Patients suffering from posterior eye diseases are in need of therapies which are both effective and noninvasive. These diseases include uveitis, diabetic macular edema (DME), diabetic retinopathy (DR), proliferative vitreoretinopathy (PVR), age-related macular degeneration (AMD), retinitis pigmentosa (RP), choroidal neovascularization (CNV) and glaucoma.

Uveitis is the inflammation of uvea composed of: iris, ciliary body and choroid. Since the retina receives most of its blood supply via the uvea, the inflammation of the uvea may lead to blurred vision, redness of the eye, eye pain and sensitivity to light. Depending on the site of inflammation, uveitis may be classified as anterior uveitis, intermediate uveitis and posterior uveitis. Anterior uveitis, which affects the front part of the eye i.e., iris, is the most common form of uveitis accounting for 50-75% of all cases. Intermediate uveitis refers to the inflammation of tissue in the area just behind the iris and lens of the eye. Posterior uveitis refers to the inflammation of the choroid and accounts for about 25-50% of the cases [24]. The reported number of patients suffering from posterior uveitis is more than 100,000 people in the United States (38 per 100,000 people yearly) [25].

Diabetic retinopathy (DR) is a condition more prevalent in diabetic patients and has been found to be in 12% of patients with type 2 diabetes. In DR, small changes in
the blood vessels at the back of the eye take place which may lead to the loss of vision. When the blood vessels in the retina are damaged, fluid may leak from the blood vessels. When the fluid leaks from these vessels and accumulates in the macula, it leads to the condition called diabetic macular edema (DME). The accumulation of the fluid in the macula of the retina causes its thickening and in turn affects vision.

Age-related macular degeneration (AMD) is a disease associated with aging and is the leading cause of blindness in the aged population. More than 10 million Americans suffer from AMD in the United States alone and approximately 1.7 million among these Americans are over the age of 65. AMD occurs in two forms: wet AMD and dry AMD. In wet AMD, abnormal blood vessels behind the retina start to grow under the macula and often leak blood and fluid, which will eventually damage the macula. Dry AMD occurs when the light-sensitive cells in the macula slowly break down, gradually blurring central vision in the affected eye.

Proliferative vitreoretinopathy (PVR) is a condition in which a scar tissue is formed within the eye due to the complication in patients recovering from surgical repair of retinal detachment. PVR occurs in 8-10% of the patients undergoing primary retinal detachment surgery. Retinitis pigmentosa (RP) is a condition in which the light-sensitive retina of the eye slowly degenerates and eventually results in blindness. RP is an inherited disease caused by a number of genetic defects. The symptoms of RP include poor night vision, loss of side vision and in the advance stages loss of central vision. Choroidal neovascularization (CNV) is a condition in which new blood vessels are
formed in the choroid layer of the eye along with the presence of excessive amounts of vascular endothelial growth factors (VEGF).

Glaucoma is the second leading cause of adult blindness in the United States. High intraocular pressure can lead to glaucoma. Particularly, the elevated intraocular pressure can damage the cells around the optic nerve forming cells. Under this condition, there is loss of peripheral vision followed by the loss in central vision and potentially blindness.

1.1.3. Ocular drug delivery

*Drug administration for the treatment of posterior eye diseases*

Four approaches may be used to deliver drugs to the posterior segment-- topical application, oral delivery, systemic therapy and injections of various types: subconjunctival, intravitreal, sub-tenon, retrobulbar and periocular [26-27]. Since anterior uveitis affects the front of the eye, it can be treated with topical applications of corticosteroids, e.g. hydrocortisone, prednisone and dexamethasone eye drops application 1-2 hr for several days. The approach of topical applications of drugs for the treatment of intermediate and posterior eye diseases is not very effective because it does not lead to therapeutic drug levels in the vitreous, retina or choroid [28]. Intermediate and posterior uveitis usually requires once a month intravitreal injections and / or high systemic dose therapy. Intravitreal injections are invasive and are associated with side effects such as retinal detachment, hemorrhage, infection in the eye and increase in the intraocular pressure. Therefore they are not always well tolerated by patients [29]. In addition, these
injections require the participation of experienced ophthalmologists, resulting in higher healthcare cost. Although systemic administration can deliver drugs to the posterior eye, the large systemic doses often lead to significant side effects such as cataracts, glaucoma, myalgia, weight gain and hypertension etc. [30-31].

Laser surgery is a common therapy used for the treatment of AMD, in which a high energy beam of light is used to destroy the leaky blood vessels at the back of the eye. Laser therapy is associated with the risk of damaging the surrounding healthy tissue and the development of new blood vessels after the treatment, making repeated treatments necessary. For the treatment of diabetic retinopathy, laser therapy was a common treatment. In the advanced disease state, vitrectomy is recommended where the blood filled vitreous is replaced with a clear solution.

Platelet-derived growth factor receptor α (PDGFRα) is found to be associated with PVR. Currently, there is no effective prevention of PVR. Recent study has shown that the combined therapy of encompassing a subset of vitreal growth factors and cytokines is a potential method to prevent PVR [32]. The common method to control CNV is the intravitreal injections of anti-VEGF drugs such as Avastin and Lucentis, to reduce the fluid below the retinal pigment epithelium. These drugs have been shown to improve visual prognosis with CNV, but the recurrence of the disease remains high. There is no effective treatment of retinitis pigmentosa although some studies have suggested that intake of vitamin A, omega-3 fatty acid, and DHA may help in the reduction of progression of the disease. In addition, genetic counseling and testing may help determine whether the children are at risk for this disease for early prevention.
Eye drops containing drugs such as prostaglandins analogues, beta-blockers, alpha-adrenergic and carbonic anhydrase inhibitors are used for the treatment of glaucoma. These eye drops are normally applied to the eye 2-5 times a day and often lead to poor patient compliance.

Ocular drugs available in the market for the treatment of posterior eye diseases

Commercial drugs are available to treat eye diseases such as glaucoma, allergy, inflammation, infection and dry eye syndrome. For the treatment of elevated intraocular pressure, prostaglandin eye drops such as Xalatan®, Lumigan®, Travatan Z® and Rescula®, beta-blockers eye drops such as Timoptic XE®, Istaol® and Betoptic S®, alpha-adrenergic eye drops such as Iopidine®, Alphagan® and Azopt® are available in the market. For inflammatory conditions corticosteroids such as hydrocortisone, prednisolone and dexamethasone are applied topically and commercially available, e.g., Spersadex Comp®. Antibiotics such as azithromycin, fluoroquinolones and neomycin are used for the treatment of ocular infections and an example is Azasite®. TobraDex® is available commercially for the treatment of infectious diseases [33-34]. For the treatment of posterior uveitis, Retisert® (fluocinolone acetonide implant) [35] and Ozurdex® (dexamethasone intravitreal implant) have been used [36]. For the treatment of wet AMD, visudyne® (verteporfin intravenous injection) has been approve by US Food and Drug Administration (FDA) [37]. In addition, recent advances in drug discovery have provided other novel therapeutic drugs such as anti-VEGF Macugen®, a humanized anti-VEGF antibody fragment Leucentis® and a full length humanized
monoclonal antibody against VEGF Avastin® for the treatment of neovascular eye
diseases [38-40]. Macugen® is approved by FDA to treat all types of wet macular
degeneration and is injected into the eye every 6 weeks. Leucentis® is derived from
Avastin and can improve vision in patients suffering from macular degeneration.
Avastin® is a drug approved by FDA in the treatment of colon cancer and has been used
as an off-label drug for the treatment of macular degeneration. Currently, there is no
effective drug delivery method for the treatment of posterior eye diseases even though
new drugs have been discovered and become available for their treatment.

1.1.4. Transscleral iontophoresis

Transscleral iontophoresis is a viable modality to deliver therapeutic drugs for the
treatment of eye disease [41]. Transscleral iontophoresis is a noninvasive technique in
which a low electric current is used to drive a drug into and through body tissue to
enhance the flux of the drug. The flux enhancing mechanisms of iontophoretic drug
delivery include electrophoresis (direct field effect), electroosmosis (convective solvent
flow), and electro-permeabilization (field induced tissue alterations) [42-44]. An
iontophoresis device has two electrodes: one is the donor electrode and the other is the
receiver electrode. The donor electrode holds the drug solution. Depending on the
polarity of the drug, the donor electrode can either have a positive or negative polarity. If
the drug is positively charged, then the donor electrode will be the anode. If the drug is
negatively charged, the donor electrode will be the cathode.
In iontophoretic drug delivery to the eye, safety is the main concern, and there have been a number of transscleral iontophoresis studies focusing on the safety and efficacy of passing an electric current across the eye tissue [45-46]. A study performed by Behar and Cohen showed that iontophoresis is a safe method of drug delivery when current density lower than 50 mA/cm\(^2\) was used for 10 minutes. In this study, methylprednisolone was delivered using transscleral iontophoresis in rabbits. The results showed higher concentrations of the drug in both anterior and posterior segments of the eye than those achieved after i.v. administration. Transscleral iontophoresis has also been used in the delivery of small therapeutic agents such as dexamethasone phosphate and carboplatin into the eye of rabbits as an animal model [47-48]. Besides electrically assisted delivery of small therapeutic drugs into the eye, ocular iontophoresis has been explored for gene therapy, DNA delivery and the delivery of both neutral and charged macromolecules with molecular weights between 4-150 kDa [49-50]. In the transscleral iontophoretic delivery of macromolecules, both electroosmosis and electrophoresis could play a significant role. Although there have been a number of studies dedicated to the transscleral iontophoretic delivery of macromolecules, the optimization of this delivery method requires further understanding of these two mechanisms (electroosmosis and electrophoresis) and their relative contribution to iontophoresis in the eye.
1.1.5. Sustain release ocular drug delivery

*Intraocular implants*

To eliminate the need for repeated drug administration to the eye and the associated adverse effects, controlled drug delivery devices such as ocular implants have been developed. Based on their degradation property, these implants can be classified into two categories: a) biodegradable and b) non-biodegradable [51]. Some examples are: Vitrasert, Posurdex, Retisert and Surmodics which are used for the treatment of CMV, diabetic macular edema, and uveitis, respectively [52-56]. The advantage of these implants is that they provide controlled release of drugs over a long period of time. However, they require intraocular surgery that is associated with potential side effects such as cataract, retinal detachment, and endopthalmitis. Additionally, these implants are not well tolerated by patients due to the application procedure, psychological factors, and possible interference with vision [57-59].

*Colloidal dosage forms*

Due to the chronic nature of many ocular diseases and the high rate of drug clearance, frequent drug administration is required in the treatment of these ocular diseases. Colloidal dosage forms such as liposomes, nanoparticles, microemulsions and nanoemulsions have been widely studied in pharmaceutical sciences. These dosage forms have also been employed in the field of ocular drug delivery [26]. The advantages of using these colloidal carrier systems in ocular drug delivery include sustained and controlled release of the drugs, reduced frequency of drug administration and their ability
to overcome blood-ocular barriers [60]. Nanoparticles are defined as particles with a diameter of less than 1 \( \mu \text{m} \), and they have been widely studied in the field of ocular drug delivery [26, 61-62]. For example, biodegradable PLGA nanoparticles comprising dexamethasone acetate were administrated by the intravitreal route and were shown to provide significantly higher ocular drug levels for the treatment of cytomegalovirus retinitis (CMV) [63]. In another study, chitosan-sodium alginate nanoparticles were prepared for the topical eye delivery if gatifloxacin. This system showed burst release of the drug followed by sustained release for 24 hr [64].

**History of mixed micellar nanocarriers**

The use of mixed micellar carrier systems to improve therapeutic efficacy of various drugs has been established for different routes of drug administration, e.g., ocular, parenteral, oral, and dermal routes [65-71]. The mixed micellar carrier systems composed of phospholipids and surfactants have been found to exhibit a phase transition from mixed micelles to liposomes on aqueous dilution [72-73]. This phase transition property could be advantageous for sustained drug delivery. In the present dissertation, mixed micellar carrier systems were used as nanoparticles for the delivery of corticosteroids such as dexamethasone and triamcinolone acetonide. These corticosteroids were selected because of their use in the treatments of ocular conditions such as macular edema and uveitis. Dexamethasone has been traditionally used in these treatments because of its anti-inflammatory, angiostatic and anti-permeability properties. Dexamethasone can also decrease intracellular and extracellular edema, and suppress
lymphokine activity. Similarly, triamcinolone acetonide is used for different ocular therapies such as macular edema and neovascularization. Currently, the administration of triamcinolone acetonide is by intravitreal injection, and there is no sustained-release product available for this drug on the market.

Animal model

In the present dissertation work, mouse (C57BL/6) was used as the animal model. Mouse is considered as a well accepted model for ocular research [74-75].
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Significance of the Research, Hypothesis and Objectives

Ocular drug delivery has met with significant challenges due to the unique ocular anatomy and physiology. Because of the presence of ocular barriers, less than 5% of the drug can reach the intraocular tissues after topical drug administration. The treatment of posterior segment eye diseases by the application of intravitreal injections, periocular injections and systemic therapy is hampered by the invasive nature of the treatments and systemic toxicity. Therefore, effective treatment of the posterior segment eye diseases without causing undesirable side effects remains a challenge to pharmaceutical scientists.

In the present dissertation, the objectives were to a) understand the mechanisms controlling iontophoretic transport of macromolecules in human sclera, b) develop a sustained release drug delivery system of drug loaded mixed micelles, and c) study transscleral iontophoretic transport of mixed micelles across human sclera and drug release form the sclera after iontophoresis \textit{in vitro}. Such a sustained drug delivery system involving iontophoresis is noninvasive, can reduce healthcare cost, and eliminate complications related to invasive procedures like intravitreal injections.
The following hypotheses were tested:

Transscleral iontophoretic transport of neutral macromolecules is enhanced by electroosmosis, and iontophoretic transport of charged macromolecules across the sclera is dominated by electrophoresis. A mixed micelles carrier system, composed of phospholipids and bile salt, that can encapsulate corticosteroid drugs by enhancing their solubility, can be transported into human sclera by iontophoresis. Once these carrier systems are transported into the sclera using iontophoresis, there is a slow and sustained release of the drug from these carrier systems.
The specific aims of the research work are as follows:

**Aim 1- Characterization of human sclera by studying the effects of electroosmosis and electrophoresis in the iontophoretic transport of macromolecules**

1.1 Transport study of neutral small and macromolecules was conducted *in vitro.*
1.2 Transport study of charged small and macromolecules was conducted *in vitro.*
1.3 The electrophoretic mobility of charged macromolecules used in the transport study was measured.

**Aim 2- Transscleral iontophoretic transport of mixed micelles *in vitro***

2.1 The physiochemical properties of mixed micellar carrier systems were determined.
2.2 The feasibility of transscleral delivery of mixed micellar carrier across cadaveric human sclera was examined.
2.3 Drug release profiles after the transport experiments were determined *in vitro.*

**Aim 3- Influence of drug lipophilicity on drug release from the sclera after iontophoretic delivery of mixed micellar carrier system to human sclera**

3.1 The influence of drug lipophilicity on the solubilization potential of mixed micellar carrier system was determined.
3.2 The relationship between the drug lipophilicity and drug release profiles of the mixed micellar carrier system was determined *in vitro.*
Aim 4- Feasibility of mixed micelles as a sustained release drug delivery system *in vivo*.

4.1 The amounts of corticosteroid in ocular tissues after transscleral iontophoretic delivery of the mixed micelles were determined.

4.2 The safety of transscleral iontophoresis of the mixed micelles was evaluated by histological examination of the eye tissues after the treatments.

In Aim 1 the mechanisms of iontophoresis involved in the transport of neutral and charged macromolecules through human sclera were studied using side-by-side diffusion cells *in vitro*. In Aim 2 & 3, a nanocarrier mixed micelle drug delivery system in conjunction with iontophoretic delivery was developed and characterized. This carrier system was examined in transscleral iontophoresis study with human sclera *in vitro*. Drugs with different lipophilicities incorporated into the micelles were delivered using the carrier system and iontophoresis, and the relationship between drug lipophilicities and drug release profiles of the mixed micellar carrier system was studied. To test the efficacy of carrier system *in vivo*, experiments were also performed using mouse as an animal model under Aim 4. The results in the present project showed that this method of drug delivery was safe and efficient. This innovative approach could therefore provide a noninvasive sustained release system to deliver corticosteroids for the treatment of eye diseases. Such a development could provide a possible solution to the unmet need of posterior eye drug delivery.
CHAPTER 3

Characterization of Human Sclera by Studying the Effects of
Electroosmosis and Electrophoresis in the Iontophoretic
Transport of Permeants

3.1. Introduction

The treatment of diseases such as age-related macular degeneration, diabetic retinopathy, and uveitis has been a challenge to ophthalmologists [1]. The rapid progress of drug discovery has led to the development of biologicals for the treatment of these eye diseases [2-3]. However, most of these new bioactive agents have high molecular weights and are usually administered via intravitreal injections. As mentioned in the Chapter 1, multiple injections are common in these treatments and increase the potential of adverse effects [4]; hence a safe and effective method is required to deliver these bioactives to the posterior segment of the eye.

The flux enhancing mechanisms (electrophoresis, electroosmosis and electropermeabilization) of iontophoretic delivery have been studied and there are a number of ocular iontophoresis studies focusing on the delivery of therapeutic drugs into the eye and on its safety [5-13]. It has been demonstrated that macromolecules with multiple charges and molecular weight (MW) between 4 to 150 kDa could be transported into the sclera by electrical field [14]. Because both electroosmosis and electrophoresis
could play a significant role in iontophoretic flux enhancement of these macromolecules, the optimization of transscleral iontophoretic delivery of macromolecules requires the understanding of these two mechanisms and their relative contributions in transscleral iontophoresis.

Studies of iontophoresis for ocular delivery of both charged and neutral molecules using rabbit as an animal model have been reported [6, 15-17]. The mechanisms of transscleral iontophoretic transport of small molecules have also been investigated using rabbit sclera [18]. However, human sclera data for studying the transport behavior of charged macromolecules are limited in the literature. A systematic examination of transscleral iontophoretic transport behavior of macromolecules with human sclera is required to optimize transscleral iontophoretic drug delivery.

Bevacizumab (Avastin®), a humanized recombinant monoclonal antibody, is the first anti-angiogenic protein approved by FDA. As an anti-human vascular endothelial growth factor (VEGF) agent, intravitreal injection of bevacizumab has been shown to be beneficial in off-label treatment of age-related macular degeneration, choroidal neovascularization, and diabetic retinopathy [19-20]. Due to the potential adverse effects of intravitreal injections, transscleral iontophoresis could be an alternative method and enhance the delivery of bevacizumab via the transscleral route.

The purpose of the present study was to study the transport of charged macromolecules of 60 to 150 kDa in transscleral iontophoresis using human cadaveric sclera in vitro. First, the effectiveness of iontophoresis in the delivery of these charged macromolecules across human sclera was studied in side-by-side diffusion cells in vitro. Then, the mechanisms controlling the iontophoretic transport of the macromolecules
across human sclera *in vitro* were examined. In addition, effective electrophoretic mobilities of the macromolecules used in the present study were determined using capillary electrophoresis method.
3.2. Materials and Methods

3.2.1. Materials

Phosphate buffered saline (PBS, pH 7.4, consisting of 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride) was prepared by dissolving PBS tablets (Sigma-Aldrich, St. Louis, MO) in distilled, deionized water. $^{14}\text{C}$-tetraethylammonium (TEA, MW 130 Da) bromide (1–5 mCi/mmol) and $^{14}\text{C}$-salicylic acid (SA, 40–60 mCi/mmol, MW 138 Da) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). All radiolabelled chemicals had purity of at least 97%. Fluorescein isothiocyanate (FITC) labelled dextrans (FITC-D) of average MW 4 and 20 kDa (degree of substitution 0.004–0.005 mol FITC/mol of glucose) and bovine serum albumin (BSA, purity > 98%, MW 68 kDa) were purchased from Sigma-Aldrich (St. Louis, MO). Poly (styrene sulfonic acid) sodium salt (PSS, MW 67 kDa, $M_w/M_n < 1.2$) was purchased from Polysciences, Inc. (Warrington, PA). Bevacizumab (BEV Avastin®, 100 mg in 4 mL, MW 149 kDa) was from Genentech, Inc. (Oceanside, CA). Agarose (low EEO, molecular biological grade) was purchased from Fisher Scientific (Fair Lawn, NJ). 0.15 M TEA and SA solutions were prepared by reacting tetraethylammonium hydroxide (20% w/w, Acros Organics, Morris Plains, NJ) with hydrochloric acid and dissolving sodium salicylate (Acros Organics, Morris Plains, NJ) in distilled, deionized water, respectively, and the solutions were adjusted to pH 7.4 with hydrochloric acid or sodium hydroxide.
3.2.2. Preparation of the sclera

Cadaver eyes were obtained from Dayton Eye Bank (Dayton, OH) and Moran Eye Center at the University of Utah (Salt Lake City, UT). The tissues were stored in moisture chamber at 2–4°C not more than six months. Before the experiments the tissue was thawed at room temperature and adhering tissues on the sclera including the retina and choroid were removed with a pair of forceps. The sclera was then rinsed with PBS, cut into small pieces, and equilibrated in PBS at room temperature for 30 min. The use of human tissues was approved by the Institutional Review Board at the University of Cincinnati, Cincinnati, OH.

3.2.3. Passive and iontophoresis transport experiments

Passive transport, anodal iontophoresis, and cathodal iontophoresis were conducted for all the permeants used in the present study. Experiments were first conducted with low MW permeants TEA and SA. These experiments allowed the comparison of the present results using human sclera with those in previous studies using rabbit sclera [18]. Experiments of macromolecules were then studied using BSA, PSS, and BEV. BSA has a low charge to MW ratio similar to those of therapeutic peptides/proteins and PSS has a high charge to MW ratio similar to those of oligonucleotides and small interfering RNAs (siRNA). BEV is a model drug that has clinical significance.

In the experiments, the sclera was sandwiched between the two half-cells of a side-by-side diffusion cell (with an effective diffusion area of approximately 0.2 cm$^2$).
with the choroid side facing the receptor chamber. The diffusion cell was placed in a circulating water bath at 36 ± 1°C. The volume of the donor and receptor solutions was 1.5 mL. PBS was used as the receptor solution for all transport experiments except for BEV, in which 0.05% (w/v) in PBS was used. In the transport studies of TEA and SA, trace amounts of radiolabelled TEA or SA (2–10 μCi/mL) were added into the donor solution of PBS. In the transport studies of BSA and PSS, approximately 1.7 mg/mL of BSA in PBS, and 15 mg/mL PSS in PBS were the donor solutions, respectively. In the transport study of BEV, the donor solution was prepared by diluting Avastin® (25 mg/mL) with PBS containing 0.05% (w/v) BSA to a final concentration of 2.5 mg/mL. In all iontophoresis experiments, a 2-mA current was applied across the sclera with a constant current iontophoretic device (Phoresor II Auto, Model PM 850, Iomed, Inc., Salt Lake City, UT) using Ag/AgCl (cathode) and Ag (anode) as driving electrodes. The electrodes were immersed in the donor and receptor solutions except in the BEV study. In the BEV study, the donor electrode was placed in an electrode chamber containing PBS, which was connected to the donor chamber containing the donor solution through a salt bridge (5% w/v agarose in PBS). The salt bridge was stored in the donor solution before use. This salt bridge setup would prevent the interactions between BEV and the electrode during iontophoresis and the precipitation of BEV in the donor chamber. In all iontophoretic transport experiments, the electrodes were replaced before the AgCl on the Ag/AgCl electrode was depleted to avoid any changes in the pH of the donor and receptor solutions during iontophoresis. The compositions of the donor and receptor solutions could be affected by ion transport and the formation of electrochemical products at the electrodes. For example, significant depletion of the chloride ion in the
donor chamber during anodal iontophoresis could lead to AgCl precipitation in the donor solution. In the anodal iontophoresis experiments, no AgCl precipitation was observed in the donor chamber, indicating the presence of enough chloride ions in the system for the electrochemical reaction at the anode electrode. Ten microliters of the donor solution and 1 mL of the receptor solution were taken at predetermined time intervals for assay. Fresh receptor solution of 1 mL was then added into the receptor to maintain a constant volume in the receptor. The durations of the passive and iontophoretic transport experiments ranged from 2 to 96 hr. In the macromolecule transport experiments, long experiments were performed to study the mechanisms of iontophoresis under steady-state conditions and therefore experiment duration was dependent on the permeants in the protocols.

In an additional study, the same tissues were used in the passive and iontophoresis experiments with small molecules (TEA and SA) without dissembling the diffusion cells. A second passive transport experiment after iontophoresis was conducted with the same tissue so the results of these passive experiments before and after iontophoresis could be compared to examine possible irreversible electro-permeabilization effects of iontophoresis upon cadaver human sclera. Another study was also performed with small molecules (TEA and SA) using 0.15 M TEA and 0.15 M SA in deionized water (instead of PBS) as the donor in the TEA and SA experiments, respectively. These studies provided the same experimental conditions as in a previous rabbit sclera study [18] for direct comparison of the results in the present human and previous rabbit studies. Passive transport experiments of macromolecules FITC-D of molecular weights 4 and 20 kDa with human sclera were also performed. The duration of the experiments was 24–48 hr, and the donor was 2 mg/mL FITC-D in PBS. The results in these experiments were used,
along with the TEA, SA, BSA, PSS, and BEV data, to determine the effective pore size of the transport pathways in the sclera.

3.2.3.1. Assay methods

Radiolabelled TEA and SA samples were analyzed by a liquid scintillation counter (Beckman coulter LS 6500, Fullerton, CA). The samples were mixed with 10 mL of liquid scintillation cocktail (Ultima Gold™, PerkinElmer Life and Analytical Sciences, Shelton, CT) and assayed by the liquid scintillation counter. Samples of DEX 4 kDa were analyzed using a spectrophotometer (UV-mini 1240, Shimadzu, Kyoto, Japan) at 493 nm. FITC-D 20 kDa, BSA, and PSS samples were analyzed using high performance liquid chromatography (HPLC). The HPLC system (Prominence, Shimadzu, Columbia, MD) consisted of CBM-20A system controller, LC-20AT solvent delivery module, SIL-20A autosampler, and SPD-20A UV-Vis detector. A size exclusion column (TSKgel™ G4000 PWXL, 7.8 mm × 300 mm; Tosoh Biosciences, Montgomeryville, PA) was used at room temperature. The mobile phase was distilled, deionized water and was delivered at a flow rate of 0.5 mL/min and detection was at 490 nm for FITC-D 20 kDa and 280 nm for BSA and PSS. The concentration of BEV was quantified by enzyme-linked immunosorbent assay (ELISA) as described previously [21] with modifications. Briefly, samples were diluted with 0.05% (w/v) BSA in PBS and were analyzed in duplicates. 100 μL of each sample was incubated in 96-well plates coated with human immunoglobulin G (IgG)-specific goat IgG (BD Biocoat; BD Biosciences, San Jose, CA) for 1 h at room temperature. Subsequently, the wells were incubated for 1 h with biotinylated recombinant human VEGF165 (Fluorokine
biotinylated human VEGF; R&D Systems, Minneapolis, MN) diluted in 0.05% (w/v) BSA in PBS. This was followed by the addition of 100 μL peroxidase-conjugated streptavidin (BD Biosciences, San Jose, CA) diluted 1:2000 in 0.05% (w/v) BSA in PBS and incubated for 1 h. Peroxidase activity was determined after the incubation with 100 μL peroxidase substrate solutions (BD Biosciences, San Jose, CA) for 10 min. Absorbance was quantified in a SpectraMax 250 (GMI, Inc., Ramsey, MN) microplate reader at 450 nm with the subtraction of reference absorbance at 650 nm. The detection limit of the assay was 30 ng/mL.

3.2.4. Capillary electrophoresis

Capillary electrophoresis was performed using a Beckman P/ACE MDQ analytical capillary electrophoresis system (Beckman Coulter, Brea, CA) equipped with a diode array detector. The columns were uncoated bare fused silica capillary columns, 30 cm total length and 20 cm length to detector window, and 50 μm I.D. (BIOTAQ Inc., Washington DC) and 75 μm I.D. (Agilent Technologies, Santa Clara CA). The analytes of interest, BSA, PSS and bevacizumab, were evaluated in both columns and the results were combined in the analyses. In the experiments, the capillaries were pretreated with 0.1 M NaOH rinse for 5 min, followed by rinsing with deionized water for 2 min, and followed by running buffer for 1 or 2 min, all at a pressure of 20 psi. Capillary zone electrophoresis (CZE) and dynamic Sieving capillary electrophoresis (DSCE) has been used by using uncoated fused silica 3× extended light path capillary with an i.d of 50 μm with an effective length of 40 cm or 56 cm at voltage of either 10 KV or 20 KV. The
background electrolyte solutions were filtered by Millipore membrane filter before used. Samples were injected into the column hydrodynamically at a pressure of 0.5 psi for 5 s. The applied electrical potential was 10 kV and 6 kV for the 50 and 75 µm I.D. capillary columns, respectively. The inlet of the bare fused silica column was always the anode during capillary electrophoresis except in the preliminary studies. The capillary and sample temperature was maintained at 25 °C. Detection was accomplished using a diode array detector monitoring at 212 and 254 nm. Benzyl alcohol was used as a neutral marker to monitor the electroosmotic flow in the column. Capillary electrophoresis of benzyl alcohol was carried out before and after each experiment and between every other sample run. In a number of experiments, the neutral marker was mixed with the analyte sample so both the maker and analyte were analyzed concurrently to compare the migration times with and without the marker.
3.2.5. Theory and equation

The cumulative amount of a permeant transported across the sclera ($Q$) was plotted against time ($t$). The steady-state flux of the permeant ($J$) was calculated from the slope of the linear portion of the plot ($\Delta Q/\Delta t$) under sink conditions, correcting for the effective diffusion area ($A_D$). The steady-state permeability coefficient ($P$) was calculated by normalizing the flux by the donor concentration ($C_D$). In the iontophoresis experiments, $P$ was the effective permeability coefficient under the particular iontophoresis condition for comparison to the passive control. Enhancement factor ($E$) is calculated by the ratio of permeability coefficient of iontophoretic transport to that of passive transport.

$$J = \frac{1}{A_D} \frac{\Delta Q}{\Delta t} \quad (3.1)$$

$$P = \frac{1}{C_D A_D} \frac{\Delta Q}{\Delta t} \quad (3.2)$$

The steady-state iontophoretic flux ($J_{\Delta \psi}$) of the permeant through a homogeneous porous membrane can be described by the modified Nernst-Planck model [18], a model appropriate for constant current iontophoresis when the electrical resistance of the membrane and the voltage applied across the membrane are relatively constant during iontophoresis.

$$J_{\Delta \psi} = e \left\{ -HD \left[ \frac{dC}{dx} + \frac{CzF}{R_{gas} T} \frac{d \psi}{dx} \right] \pm WvC \right\} \quad (3.3)$$

where $\psi$ is the electric potential in the membrane, $F$ is the Faraday constant, $R_{gas}$ is the gas constant, $T$ is the temperature, $v$ is the average velocity of the convective solvent.
flow, \( \varepsilon \) is the combined porosity and tortuosity factor of the membrane, and \( C, x, z, \) and \( D \)
are the concentration, the position in the membrane, the charge number, and the diffusion coefficient of the permeant, respectively. \( H \) is the hindrance factor for simultaneous Brownian diffusion and migration driven by the electric field and \( W \) is the hindrance factor for permeant transport via convective solvent flow during iontophoresis.

Assuming cylindrical pore geometry in the membrane and using asymptotic centerline approximation, the hindrance factor \( H \) can be expressed as [22]:

\[
H = \frac{6\pi(1-\lambda_i)^2}{K_f}
\]

(3.4)

where \( \lambda_i \) is the ratio of permeant radius \( (r_i) \) to the pore radius \( (R_p) \) and \( K_f \) is:

\[
K_f = \frac{9}{4} \pi^2 \sqrt{2(1-\lambda_i)^{-5/2}} \left[ 1 + \sum_{n=1}^{2} a_n (1-\lambda_i)^n \right] + \sum_{n=3}^{4} a_{n+3} \lambda_i^n
\]

(3.5)

and \( a_1 = -1.217, a_2 = 1.534, a_3 = -22.51, a_4 = -5.612, a_5 = -0.3363, a_6 = -1.216, a_7 = 1.647 \). When \( \lambda_i \) is <0.4, Eq. 4 is equivalent to the commonly used Renkin equation. The effective pore radius of the membrane can be calculated from the ratio of the permeability coefficients of the permeants (of different molecular sizes) obtained from the passive transport experiments using Eqs. 4–6.

\[
\frac{P_i}{P_j} = \frac{H_i D_i}{H_j D_j}
\]

(3.6)

The intrinsic electrophoretic mobilities (\( \mu_i \)) of the analytes were calculated using the migration times observed for each analyte and those of the electroosmotic flow marker.
\[ \mu_i = \frac{IL}{V} \left( \frac{1}{t_i} - \frac{1}{t_m} \right) \]  

(3.7)

where \( l \) and \( L \) are the effective length from the inlet to the window and the total length of the capillary column, \( V \) is the applied voltage, and \( t_i \) and \( t_m \) are the migration times of the analyte and electroosmotic flow marker, respectively.

3.2.6. Statistical analysis

All experiments were conducted with a minimum of four replicates using sclera from different human donors. The means ± standard deviations (SD) of the data are presented, and the statistical differences were determined by Student’s t-test. Differences were considered to be significant at a level of \( p < 0.05 \). Power of the test was also performed in paired comparisons to avoid type II error in testing the null hypothesis.
3.3. Results and Discussion

3.3.1. Passive transport of permeants

The passive permeability coefficients of human sclera for the permeants TEA, SA, FITC-D of MW 4 and 20 kDa, BSA, PSS, and BEV were calculated using Eq. 3.2 and presented in Figure 3.1. TEA and SA have comparable passive permeability coefficients due to their similar MW. Likewise, the passive permeability coefficients of BSA and PSS are approximately the same as they have similar MW. The passive permeability coefficient decreases (from $3 \times 10^{-5}$ to $8 \times 10^{-7}$ cm/s) when the MW of the permeant increases (from around 130 Da to 150 kDa), consistent with previous trends of a general inverse relationship between the permeability coefficient and MW of permeants [23-26].
Figure 3.1. Comparison of the relationships between passive permeability coefficients and permeant MW for human sclera in the present study and those for human, rabbit, and porcine sclera in the literature. Symbols: closed diamonds, experimental passive permeability with human sclera in the present study (mean and standard deviation, \( n \geq 4 \)); open squares, previous human passive permeability data [24]; open triangles, rabbit passive permeability data [23]; open circles, porcine passive permeability data [25].
Figure 3.1 also provides the comparison between passive permeability coefficients of the permeants in the present study and those from previous studies. The passive permeability coefficient values of macromolecules in the present study are close to the values in a previous human sclera study [24], generally lower than those in the rabbit sclera [23], and higher than those in the porcine sclera studies [25].

Figure 3.2 is a plot of the passive permeability coefficient ratio of TEA to the permeants versus permeant MW in the present study. The lines in the figure represent the theoretical calculations of Eqs. 3.4–3.6. Using the experimental passive permeability coefficient ratios of TEA to BSA, TEA to PSS, TEA to BEV, and TEA to the FITC-Ds in Figure 3.2, the average effective pore radius of human sclera was estimated to be around 10–40 nm.
Figure 3.2. Permeability coefficient ratios of permeant vs. permeant MW. Symbols: experimental permeability coefficient ratios of TEA to SA, TEA to BSA, TEA to PSS, TEA to BEV, and TEA to FITC-Ds with MW 4 and 20 kDa (FITC-D 4k and FITC-D 20k). The lines represent the theoretical calculations of the permeant permeability ratio vs. permeant MW at different effective membrane pore radius using Eqs. 4–6.
3.3.2. Iontophoretic transport of small charged permeants

The passive and iontophoretic permeability coefficients of TEA and SA are presented in Figure 3.3. The figure shows significantly higher iontophoretic permeability coefficients of TEA during anodal iontophoresis and of SA during cathodal iontophoresis compared to those of their respective passive transport ($p < 0.05$, Student’s $t$-test), suggesting significant flux enhancement due to iontophoresis. The higher iontophoretic permeability coefficient of TEA during anodal iontophoresis when the donor electrode had the same polarity as the permeant relative to those of passive and cathodal transport suggests that electrophoresis was the main flux enhancing mechanism for transscleral iontophoresis of small charged molecules. The SA results with higher cathodal iontophoretic permeability coefficient relative to those of passive and anodal transport are consistent with this finding. Iontophoresis enhanced the fluxes of TEA and SA by approximately 5- and 3-fold during anodal and cathodal iontophoresis, respectively (Table 3.1). The differences in the iontophoretic transport enhancement of TEA and SA can be attributed to the effect of electroosmosis, consistent with previous finding of transscleral electroosmosis and the negatively charged sclera at physiological pH [18].
**Figure 3.3.** Permeability coefficients of TEA and SA obtained in the passive (open bars), anodal iontophoretic (black bars), and cathodal iontophoretic (gray bars) transport experiments with human sclera. Data represent the mean and standard deviation, \( n \geq 4 \). The Student’s t-test analyses show significant differences between the iontophoresis and passive data (\( p < 0.05 \)) and between the cathodal iontophoresis and anodal iontophoresis data (\( p < 0.05 \)).
**Table 3.1.** Enhancement factors of charged small and macromolecules

<table>
<thead>
<tr>
<th>Experiment</th>
<th>TEA</th>
<th>SA</th>
<th>BSA</th>
<th>PSS</th>
<th>BEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anodal iontophoresis</td>
<td>4.9</td>
<td>0.9</td>
<td>58</td>
<td>7</td>
<td>32</td>
</tr>
<tr>
<td>Cathodal iontophoresis</td>
<td>0.4</td>
<td>3.2</td>
<td>19</td>
<td>12</td>
<td>8</td>
</tr>
</tbody>
</table>

\(^a\) Data represent the mean of \(n \geq 4\) samples.
The effective permeability coefficients of passive and iontophoretic transport of the small permeants for human sclera in the present study are consistent with those obtained previously with rabbit sclera [18], suggesting that the rabbit sclera can be an appropriate model for transscleral drug transport in vitro. In addition, no significant difference ($p > 0.05$) in the passive permeability coefficients of TEA and SA before and after iontophoresis ($3.1 \pm 0.5$ and $3.3 \pm 0.4 \times 10^{-5}$ cm/s, mean $\pm$ SD, $n = 6$, for TEA and SA, respectively, after iontophoresis) was observed. This suggests minimal irreversible impact of the electric field on the barrier properties of the tissue. If electro-permeabilization of the sclera had occurred, the data show that the effect was reversible and the tissue regained its original properties when the application of electric current was terminated.

### 3.3.3. Iontophoretic transport of charged macromolecules

Figure 3.4 presents the cumulative amounts of BSA, PSS, and BEV transported across the sclera in passive transport, anodal iontophoresis, and cathodal iontophoresis. The permeability coefficients are summarized in Figure 3.5. For BSA, higher permeability coefficient was observed during anodal iontophoresis than that during cathodal iontophoresis ($p < 0.05$, Student’s t-test). The observation that the transport of the macromolecule was enhanced even when the polarity of the electrode in the donor was opposite to the charge of the permeant, i.e., anodal iontophoresis of BSA, suggests the dominant effect of electroosmosis for the macromolecule. The significance of electroosmosis on transscleral iontophoretic transport of BSA is similar to the findings in previous skin studies, in which BSA transport was enhanced across human skin by anodal
iontophoresis due to the dominant electroosmosis effect upon macromolecules of low charge density [27]. For BEV, higher permeability coefficient ($p < 0.05$, Student’s t-test) was observed during anodal iontophoresis compared to that during passive transport and cathodal iontophoresis. The significant anodal iontophoretic transport enhancement is likely due to the dominant effect of electroosmosis in the system similar to the result in the BSA transport study. Different from the BSA and BEV results, significantly higher permeability coefficient ($p < 0.05$, Student’s t-test) was observed in cathodal iontophoresis of PSS as compared to those in passive transport and anodal iontophoresis. The higher flux enhancement of PSS during cathodal iontophoresis was likely a result of the dominant effect of electrophoresis (versus dominant electroosmosis for BSA and BEV).
Figure 3.4. Representative plots of cumulative amount transported across human sclera vs. time for (a) BSA, (b) PSS, and (c) BEV in passive transport (squares), anodal iontophoresis (diamonds), and cathodal iontophoresis (triangles). Data represent the mean and standard deviation, $n \geq 4$ for each condition.
Figure 3.5. Permeability coefficients of BSA, PSS, and BEV in the passive (open bars), anodal iontophoretic (black bars), and cathodal iontophoretic (gray bars) transport experiments with human sclera. Data represent the mean and standard deviation, \( n \geq 4 \). The Student’s t-test analyses show significant differences between the iontophoresis and passive data \( (p < 0.05) \) and between the cathodal iontophoresis and anodal iontophoresis data \( (p < 0.05) \).
The iontophoretic transport enhancement of macromolecules depends on the net effect of transport enhancements due to electrophoresis and electroosmosis. At pH 7.4 in the present study, the human sclera was expected to be net negatively charged [18] and electroosmosis favored the transport from the anode to cathode in anodal iontophoresis. The electrophoresis transport enhancement of negatively charged macromolecules is anticipated from the cathode to anode in cathodal iontophoresis, which is a function of the charge to mass ratio of the macromolecule. Given the similar MW of BSA and PSS, the difference in the iontophoretic transport results of BSA and PSS is due to the charge to mass ratio of the macromolecules. BSA has a lower charge to mass ratio than PSS, which is a polyelectrolyte with high negative charges. Other factors that can affect iontophoretic transport of macromolecules but have not been discussed here include molecular shape and electrophoretic and relaxation effects [28]. It should be noted that the fluxes of BSA, PSS, and BEV were enhanced by both anodal and cathodal iontophoresis. The effects of electrophoresis in cathodal iontophoresis of BSA and BEV and the effects of electroosmosis in anodal iontophoresis of PSS could be significant as indicated by the flux enhancement observed in anodal iontophoresis of PSS and cathodal iontophoresis of BSA and BEV. Other mechanisms such as changes in the sclera properties caused by the macromolecules and/or electric field during iontophoresis might be involved.
3.3.4. Electrophoretic mobility measurements

Table 3.2 summarizes the intrinsic electrophoretic mobilities of salicylate, BSA, PSS and BEV calculated using the migration time data and Eq. (3.7). The electrophoretic mobility of salicylate (an anion control) determined using the method in the present study is consistent with the value in the literature (-3.6 x 10^{-4} \text{ cm}^2/\text{s}/\text{V at infinite dilution}) [29]. The electrophoretic mobility of BSA (a macromolecule control) was also similar to the literature value (-2.3 x 10^{-4} \text{ cm}^2/\text{s}/\text{V at ionic strength of 0.01 M}) [30]. The absolute intrinsic electrophoretic mobilities of bevacizumab is significantly lower than those of the small molecules salicylate and macromolecules BSA and PSS.

The results in the present study suggest that although PSS (similar to polynucleotides) has high negative charges, the electrophoretic mobility is significantly lower than those estimated from the polymer structure. This has reduced the effectiveness of iontophoretic delivery of the polyelectrolyte during cathodal iontophoresis. BEV has low effective charges under the physiological condition (~ pH 7.4 and 0.16 M ionic strength). The low electrophoretic mobility of BEV suggests that this macromolecule would not be significantly enhanced by the mechanism of electrophoresis (direct field effect) during iontophoresis. As a result, electroosmosis is likely to be the dominant flux enhancing mechanism in the iontophoretic delivery of these macromolecules. Iontophoretic drug delivery of the anti-VEGF antibody and Fab could be most effective from the anode to cathode (i.e., anodal iontophoresis). These results are consistent with trends observed in transscleral iontophoretic study of these macromolecules described in the Section 3.3.3.
Table 3.2. Intrinsic electrophoretic mobilities of the analytes used in the present study.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Background electrolyte</th>
<th>Intrinsic electrophoretic mobility $(\times 10^{-4} \text{ cm}^2/\text{s/V})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylate</td>
<td>150 mM PBS, pH 7.4</td>
<td>-1.0 ± 0.2</td>
</tr>
<tr>
<td>Salicylate</td>
<td>100 mM PBS, pH 7.4</td>
<td>-3.0 ± 0.3</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>150 mM PBS, pH 7.4</td>
<td>-1.2 ± 0.1</td>
</tr>
<tr>
<td>Polystyrene Sulfonate</td>
<td>150 mM PBS, pH 7.4</td>
<td>-6.0 ± 0.2</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>100 mM PBS, pH 7.4</td>
<td>-0.7 ± 0.2</td>
</tr>
</tbody>
</table>

*Mean ± SD, n ≥ 3.
3.3.5. Transscleral drug delivery

Several studies have shown that transscleral iontophoresis is safe and efficient for ocular drug delivery and could take advantage of the large surface area of the sclera [12, 16]. Previous studies have shown that the sclera allows the penetration of macromolecules such as FITC-D of 70–150 kDa [23, 25] and transscleral delivery depends on the size and shape of the permeant [24, 31-32]. Consistent with the previous findings, the results in the present study show a similar relationship between passive permeability coefficient and molecular size for the charged permeants. The present iontophoresis data suggest that iontophoretic flux enhancement of small charged permeants was governed by electrophoresis along with small contribution of electroosmosis. For BSA and BEV, macromolecules with low charge to mass ratios, electroosmosis is the major contributing mechanism in the iontophoretic transport of the macromolecules. Anodal iontophoresis could be a promising strategy to deliver BEV through human sclera for the treatment of retinal eye diseases. This is consistent with the findings of neutral macromolecules in a previous study [25].

The model permeants employed in the present study have similar MW as those of therapeutic proteins/ peptides and oligonucleotides that could be used in the treatment of posterior eye diseases. It is therefore believed that proteins of up to 150 kDa can be effectively delivered by anodal iontophoresis but highly negatively charged macromolecules such as oligonucleotides and siRNA are more effectively delivered by cathodal iontophoresis. As electroosmosis can be affected by formulation factors such as solution ionic strength and pH and the presence of surfactants, a key to effective
iontophoretic delivery of charged macromolecules is to understand the interplay of these factors on transscleral electroosmosis in future studies.
3.4. Conclusions

The iontophoretic transport of macromolecules has been studied by using human sclera in vitro. Transport of the macromolecules was significantly affected by electroosmosis as such the transport of negatively charged BSA was enhanced from the anode to cathode. The effect of electroosmosis in iontophoretic transport of polyelectrolyte such as PSS was relatively less important than that of electrophoresis. No irreversible effect of the electric field upon the barrier properties of human sclera was observed under the experimental conditions studied. The iontophoresis results with human sclera are comparable to those of rabbit sclera. Transscleral iontophoresis of biologicals could provide new therapeutic approaches for treating posterior eye diseases. Understanding the different mechanisms involved in transscleral iontophoresis of these macromolecules would help researchers optimize this drug delivery method.
3.5. References


CHAPTER 4

Sustained Release Micellar Carrier Systems for Iontophoretic Transport of Dexamethasone across Human Sclera

4.1. Introduction

Posterior eye diseases account for the majority of blindness in the United States [1]. For example, it was reported that the number of patients suffering from posterior uveitis was more than 100,000 people in the United States (38 per 100,000 people yearly) [2]. The use of dexamethasone and other corticosteroids as anti-inflammatory agents to inhibit the inflammation in the ocular tissue has been well documented [3-5]. Topical administration of corticosteroids is commonly used to treat anterior eye diseases [6-7], but is not effective in the treatment of posterior eye diseases due to the low levels of drugs delivered to the posterior eye from the administration site [8-9]. A limitation of transscleral iontophoresis for the treatment of chronic eye disease is the fast clearance of the drug from the eye to the systemic circulation such as through episcleral vessels and conjunctival lymphatic system, leading to the requirement of repeated iontophoresis administration. Also, short duration transscleral iontophoresis is not expected to directly deliver drugs to the posterior segment of eye due to the long transport path length from the application site to the back of the eye [10] and the ocular dynamic barrier [11]. Therefore, a sustained release carrier system for transscleral iontophoresis that can maintain therapeutic concentrations of the drug in the eye is desirable.
Nanoparticulate drug delivery systems such as micellar carriers have been studied to increase the solubility of poorly water soluble drugs [12-14]. The application of these carrier systems to improve therapeutic efficacy of various drugs has been established for different routes of drug administration, e.g., ocular, parenteral, oral, and dermal routes [15-21]. Micellar carrier systems have the properties such as being charged and nanosize for transscleral iontophoresis. In addition, mixed micelles composed of egg lecithin and taurocholate have been found to exhibit a phase transition from mixed micelles to liposomes upon aqueous dilution [22-23]. This phase transition property could be advantageous for sustained drug delivery.

The objective of the study performed in the present chapter was to investigate the properties of sustained release drug delivery systems of micelles for transscleral iontophoresis. Particularly, dexamethasone (DEX) was chosen as the model drug and DEX-loaded simple and mixed micellar carrier systems prepared using taurocholate and egg lecithin were the model micellar systems investigated. The physical properties of these micellar carrier systems, such as drug encapsulation, size, charge, conductivity, viscosity, and osmolarity, were characterized. The feasibility of transscleral delivery of these carrier systems by passive and iontophoretic transport methods was evaluated using cadaveric human sclera in vitro. Drug release profiles were determined after the transport experiments in vitro to examine the potential of these micelles as novel sustained release systems for poorly water soluble drugs.
4.2. Materials and Methods

4.2.1. Materials

$^3$H-dexamethasone was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA) with purity of at least 97%. Sodium taurocholate (TA) was purchased from Sigma-Aldrich (St. Louis, MO). Lecithin (LE, from eggs, purity >90%) was purchased from Indofine Chemical (Hillsborough, NJ). Dexamethasone was purchased from Letco Medical (Decatur, AL). Filter membrane (0.22 μm) was purchased from Fisher Scientific (Pittsburgh, PA). Dialysis membrane of molecular weight cutoff 1000 Da (MWCO 1000) was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Phosphate-buffered saline (PBS, pH 7.4, consisting of 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride) was prepared by dissolving PBS tablets (Sigma-Aldrich, St. Louis, MO) in distilled, deionized water. High performance liquid chromatography (HPLC) grade acetonitrile was purchased from Pharmaco-AAPER (Shelbyville, KY).

4.2.2. Preparation of micellar carrier systems

Mixed micellar solution at a ratio of LE to TA of 1:4 (mole ratio) was prepared by first dissolving an appropriate amount of TA in PBS to obtain a clear solution and then adding an appropriate amount of LE to make the mixed micelle solution of desired total lipid concentration. The concentration of the mixed micelle solution was 95 mg/mL in all experiments unless otherwise stated. DEX was formulated in the mixed micelles at two different concentrations: mixed micelles containing saturated (SMM) and
unsaturated (UMM) DEX. In the preparation of SMM, excess amounts of DEX were added to the mixed micelle solution followed by equilibration at 36±1 °C for 24 to 48 h. After equilibration, the mixture was filtered through 0.22 μm filter membrane to obtain clear SMM solution. In the preparation of UMM, an appropriate amount of DEX was added to the mixed micelle solution to a final DEX concentration of 0.1 mg/mL. Since both simple and mixed micelles coexisted in the mixed micellar carrier system, saturated simple micellar carrier system (SSM) was also prepared and tested in the present study. SSM was prepared by dissolving an appropriate amount of TA in PBS followed by saturating the system with excess DEX. The solution was equilibrated in a circulating waterbath at 36±1 °C for 24 to 48 hr. After equilibration, the undissolved drug was separated from the solution by filtration using 0.22 μm filter membranes. The concentration of TA in the SSM solution was 28 mg/mL in all experiments unless otherwise stated. This concentration was selected because it was the same TA concentration as of the simple micelles co-existing in the 95 mg/mL mixed micelle system according to calculations based on a previous study [24]. Control solution of saturated DEX was prepared by adding an excess amount of DEX in PBS followed by equilibration in a circulating waterbath at 36±1 °C for 24 to 48 h. After equilibration, the undissolved drug was separated from the solution by filtration using 0.22 μm filter membranes. The concentration of DEX in the solution (control) was found to be 0.1 mg/mL.
4.2.3. Preparation of the sclera

Cadaver eyes were obtained from National Disease Research Interchange (NDRI, Philadelphia, PA). The tissues were stored in moisture chambers at 4 °C. Before the experiments, the tissue was cleaned at room temperature and adhering tissues on the sclera including the retina and choroid were removed with a pair of forceps. The sclera was then rinsed with PBS, cut into approximate size, and equilibrated in PBS at room temperature for 30 min before its use. The use of human tissues was approved by the Institutional Review Board at the University of Cincinnati, Cincinnati, OH.

4.2.4. Characterization of micellar carrier systems

4.2.4.1. Drug encapsulation: solubility study

Different concentrations of SMM and SSM were prepared using the method described in Section 4.2 to study the solubility of DEX in the micellar carrier systems. The total lipid concentrations (TA and LE) of SMM were 25, 40, 55 and 95 mg/mL in the SMM experiments, and the concentrations of TA were 29, 39, 47, 70 and 95 mg/mL in the SSM experiments. After equilibrating SMM and SSM solutions with excess amounts of DEX in a circulating waterbath at 36±1 °C for 24 to 48 h, the undissolved drug was separated from the solution by filtration using 0.22 μm filter membranes. Aliquots of the filtered micelle solutions were then subjected to appropriate dilution with the mobile phase used in the HPLC assay. The diluted samples were assayed for the drug using an HPLC system (Prominence, Shimadzu, Columbia, MD) consisted of CBM-20A system.
controller, LC-20AT solvent delivery module, SIL-20A autosampler, and SPD-20A UV–Vis detector. The separation was performed with Microsorb C18 column (150 mm×4.6 mm; Varian, Inc., Palo Alto, CA) at room temperature. The mobile phase consisted of acetonitrile and water (65:35, v/v) and was delivered at a flow rate of 1.0 mL/min. The detection wavelength for DEX was 284 nm. Each sample was analyzed at least twice, and the averages were used to calculate the concentrations of solubilized drug in the different systems.

4.2.4.2. Drug encapsulation: dialysis membrane study

Drug encapsulation in the micellar systems was also measured in a passive transport experiment with the MWCO 1000 dialysis membrane in a circulating waterbath at 36±1 °C. The dialysis membrane was mounted between the two half-cells of a side-by-side diffusion cell with an effective diffusion area of 0.64 cm². The donor solutions were prepared by adding trace amounts of radiolabelled DEX (0.2 μCi/mL) into SMM, UMM, and the control solutions. The receptor solution was PBS. The volume of the donor and receptor solutions was 1.5 mL. The duration of the transport experiments was 90 min. Samples of 10 μL and 1 mL were withdrawn from the donor and receptor compartments at predetermined time intervals, respectively. The volume of the receptor was maintained constant by the addition of 1 mL fresh PBS after each sampling. The samples were then mixed with 10 mL of liquid scintillation cocktail (Ultima Gold™, PerkinElmer Life and Analytical Sciences, Shelton, CT) and assayed by a liquid scintillation counter (Beckman Coulter LS 6500, Fullerton, CA). The apparent flux (J)
was calculated from the slope \((\Delta Q/\Delta t)\) of the linear region of the plot of the cumulative amount \((\Delta Q)\) of the permeant transported across the membrane into the receptor chamber versus time \((t)\) divided by the effective diffusion area \((A_D)\).

\[
J = \frac{1}{A_D} \frac{\Delta Q}{\Delta t} \tag{4.1}
\]

The apparent permeability coefficient \((P)\) was calculated by normalizing the apparent flux by the donor concentration of the permeant \((C_D)\).

\[
P = \frac{1}{C_D A_D} \frac{\Delta Q}{\Delta t} \tag{4.2}
\]

Encapsulation efficiency \((EE)\) is defined as the fraction of the drug encapsulated into the mixed micelles with respect to the total amount of the drug in the micelle solution. As only the free drug can permeate across the dialysis membrane, EE was determined by:

\[
EE = 1 - \frac{P_{mm}}{P_{aq}} \tag{4.3}
\]

where \(P_{mm}\) is the apparent permeability coefficient of the drug determined in the transport experiment of the micellar carrier system and \(P_{aq}\) is the apparent permeability coefficient of the drug determined in the control transport experiment.

4.2.4.3 Measurements of conductivity, viscosity, osmolarity, size, and zeta potential
The conductivity, viscosity, osmolarity, effective size and zeta potential of the micellar carrier systems were measured at room temperature using a conductivity meter (510 series, Oakton Instruments, Vernon Hills, IL), Ostwald viscometer (Barnstead International/Thermo scientific, Dubuque, Iowa), osmometer (Model 3300, Advanced Instruments Inc, Norwood, MA), and Malvern Zetasizer ® (Nano ZS, Malvern Instruments Ltd, Worcestershire, UK), respectively.

4.2.5. Transscleral transport study

Cadaveric human sclera was sandwiched between the two half cells of a side-by-side diffusion cell with the choroid side facing the receptor. The diffusion cells had effective diffusion area of 0.2 cm$^2$ and the temperature was maintained by a circulating waterbath at 36±1 °C. The volume of the donor and receptor solutions was 1.5 mL. Prior to the transport experiments, trace amounts of radiolabelled DEX (0.5–1 μCi/mL) were added into SMM, UMM, SSM, and the control solutions in the donor chamber. PBS was the receptor solution in all the transport experiments. Passive transport, anodal iontophoresis (anode in the donor), and cathodal iontophoresis (cathode in the donor) experiments were performed. In the iontophoresis experiments, 2 mA current was applied across the sclera with a constant current iontophoretic device (Phoresor II Auto, Model PM 850, Iomed, Inc., Salt Lake City, UT) using Ag/AgCl (cathode) and Ag (anode) as the driving electrodes. The duration of the transport experiment was 20 min. Samples of 10 μL donor solution and 1 mL receptor solution were taken at predetermined time intervals for assay. Fresh PBS of 1 mL was then added into the receptor to maintain
a constant volume in the receptor. The samples were mixed with 10 mL of the liquid scintillation cocktail and assayed by the liquid scintillation counter. In these transscleral transport experiments, the instantaneous fluxes were calculated from the changes in the cumulative amount of the permeant transported across the sclera into the receptor chamber and the effective diffusion area for all time points using Eq. 4.1. The instantaneous fluxes at the last two time points in the transport experiments were averaged, and the apparent permeability coefficient (flux normalized by the donor concentration) was calculated by dividing the average flux by the donor concentration using Eq. 4.2. It was important to characterize the transscleral transport behavior of the micellar carrier systems under a controlled in vitro setting in these experiments although this setting might not predict transscleral drug delivery in vivo due to the absence of blood vasculature and lymphatic clearance. In a separate study, transport experiments of DEX in SMM were conducted on the same sclera tissue using a three-stage protocol: first passive transport, cathodal iontophoresis, and second passive transport under the same experimental conditions of the transport experiments described above. The results of the first and second passive transport experiments were compared to examine possible irreversible electropermeabilization effects of iontophoresis upon human sclera.

4.2.6. Drug release study

Drug release study was carried out following the transscleral transport study with the same diffusion cells and sclera. Particularly, at the end of the transport experiments, both the donor and receptor solutions were removed and 1.5 mL of fresh PBS was added into the receptor chamber to start the release study. A stopper was used on the donor
chamber to seal the donor chamber and maintain the pressure in the chamber. The donor and receptor chambers were not rinsed before the start of the release study to avoid potential washout of the drug from the sclera. The drug release study was performed for 6 days. At predetermined time intervals, 1 mL of receptor solution was taken from the receptor for assay followed by replenishing the receptor with 1 mL fresh PBS. The samples were mixed with 10 mL of the liquid scintillation cocktail and assayed by the liquid scintillation counter. While the results in the in vitro drug release study might be different from those in vivo due to the absence of clearance in vitro, the study was performed to assess the sustained release properties of the micellar carrier systems before future in vivo studies.

4.2.7. Drug extraction study

Drug extraction study of the sclera was performed following the drug release study to determine the amount of DEX remained in the sclera after the drug release study. At the end of the drug release experiment, the sclera was removed from the side-by-side diffusion cell assembly and was immersed in 2 mL ethanol solution in a vial for 24 hr. After 24 hr of extraction, 1 mL of sample was withdrawn from the vial, mixed with 10 mL of liquid scintillation cocktail, and assayed for DEX by the liquid scintillation counter. The total amounts of DEX loaded into the sclera with passive and iontophoretic delivery were calculated as the sum of the amounts of DEX released from the sclera in the drug release study and the amounts of DEX remained in the sclera.
4.2.8. Statistical analysis

All experiments were conducted with a minimum of three replicates using sclera tissues from different donors. The means ± standard deviations (SD) of the data are presented. Data were compared using Student's t-test. Differences were considered to be significant at a level of $p \leq 0.05$. 
4.3. Results and Discussion

4.3.1. Characterization of micellar carrier systems

Figure 4.1 presents the amounts of DEX solubilized in different concentrations of SMM and SSM. With an increase in the concentration of total lipids in the SMM system from 25 mg/mL to 95 mg/mL, the solubility of DEX increased linearly from 0.17 mg/mL to 0.45 mg/mL. According to these data, the concentration of DEX in SMM in the transport experiments was 0.45 mg/mL, which was within the clinical dose range in practice [25]. For SSM, the solubility of DEX in SSM increased from 0.24 mg/mL to 0.61 mg/mL at TA concentration from 29 mg/mL to 95 mg/mL. In SMM, both simple and mixed micelles existed and contributed to the solubilization of DEX. As stated in Section 4.2, the concentration of simple micelles of TA in the SMM was ~28 mg/mL. From the slope in Figure 4.1, the amount of DEX solubilized by simple micelles of TA was calculated to be 0.23 mg per 28 mg TA, corresponding to 8.2 µg per mg TA simple micelle. The amount of DEX solubilized by the mixed micelles estimated by subtracting the amount of DEX solubilized by TA simple micelles from the total drug solubilized in SMM was 0.22 mg per 95 mg total lipids. This suggests that ~50% of the drug was solubilized by the simple micelles in SMM. From the y-intercept in Figure 4.1, the aqueous solubility of DEX in the absence of micelles was approximately 0.07 mg/mL, not significantly different from the solubility of DEX determined in the control (0.1 mg/mL).

The amount of DEX encapsulated in the UMM system cannot be determined in the DEX solubility study, so transport experiments with dialysis membrane were
performed. The passive permeability coefficients of DEX in SMM, UMM, and control solutions across the dialysis membrane were calculated using Eq. 4.2. The passive permeability coefficient of DEX in the control (2.3 × 10^{-5} cm/s) was approximately 3 times higher than that of SMM (5.6 × 10^{-6} cm/s) and UMM (5.8 × 10^{-6} cm/s). The passive permeability coefficient in the control experiment was higher than those of SMM and UMM because DEX loaded in the mixed micelles could not permeate through the MWCO 1000 dialysis membrane [24]. As only free DEX can diffuse across the membrane into the receptor, this allows the determination of the encapsulation efficiencies of the carrier systems using Eq. 4.3. The encapsulation efficiencies of DEX in SMM and UMM calculated were approximately 75%. These results are consistent with the approximately four times increase in the solubility of DEX in 95 mg/mL SMM from its aqueous solubility (0.1 mg/mL).
Figure 4.1. Solubility of DEX as a function of total lipid concentration in the saturated mixed micelles (SMM, closed diamonds) and saturated simple micelle (SSM, closed triangles) systems.
Table 4.1. Properties of the micellar carrier systems and the control in the present study\(^a\)

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Conductivity (mS)</th>
<th>Viscosity (mPa.s)</th>
<th>Osmolarity (mOsm)</th>
<th>Hydrodynamic diameter (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSM</td>
<td>15.1 ± 0.2</td>
<td>1.18 ± 0.03</td>
<td>370 ± 11</td>
<td>-(^b)</td>
<td>-67.0 ± 1.1</td>
</tr>
<tr>
<td>SMM</td>
<td>13.8 ± 0.6</td>
<td>1.64 ± 0.02</td>
<td>394 ± 10</td>
<td>4.4 ± 0.2</td>
<td>-61.1 ± 2.0</td>
</tr>
<tr>
<td>UMM</td>
<td>14.1 ± 0.3</td>
<td>1.64 ± 0.01</td>
<td>420 ± 9</td>
<td>4.7 ± 0.2</td>
<td>-52.0 ± 2.0</td>
</tr>
<tr>
<td>Control</td>
<td>14.8 ± 0.4</td>
<td>1.06 ± 0.01</td>
<td>303 ± 13</td>
<td>-(^b)</td>
<td>-(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SD, n ≥ 3. \(^b\) Not applicable.
Table 4.1 summarizes the physical properties of the micellar carrier solutions and the saturated DEX control solution tested in the present study. The physical properties were determined to understand the iontophoretic transport and drug release mechanisms of the micellar carrier systems. The results show that the conductivities of SSM, SMM, and UMM solutions are essentially the same. These results are also not significantly different from that of the control despite the relatively high concentration of micelles (28 mg/mL for SSM and 95 mg/mL for SMM and UMM) used in the present study. This can be attributed to the background electrolyte being the main conducting ions in the SSM, SSM, and UMM solutions and the increase in the viscosity of the solutions in the presence of the micelles. The viscosities of SMM and UMM were approximately 1.4 times higher than that of SSM, suggesting that SMM has a larger impact on solution viscosity than SSM. The viscosity of SSM solution was approximately 1.2 times that of the control. The osmolarities of SSM, SSM, and UMM solutions were between 370-420 mOsm, indicating that these micelle solutions were slightly hypertonic.

The effective sizes (i.e., hydrodynamic diameter) of mixed micelles SMM and UMM were 4.4 and 4.7 nm, respectively (Table 4.1), whereas that of the simple micelle SSM was below the detectable range of the instrument. This is consistent with the general view that simple micelles are smaller than the mixed micelles. In addition, the sizes of the micelles in SMM and UMM were studied upon dilution with PBS. The results show that dilution led to an increase in the size of SMM and UMM (to ~20 nm at 20-fold dilution) followed by a slight decrease in size upon further dilution due to the formation of monodisperse vesicles. This trend is consistent with a previous report that the size of TA/lecithin mixed micelles first increases and then decreases with dilution.
The zeta potentials of the micellar carrier systems were also determined, and the results show that the micellar carriers were net negatively charged in PBS under the experimental conditions in the present study. Overall, SMM and SSM differed in their properties including micellar carrier sizes and solution viscosity. These differences could influence transscleral transport, drug release, and scleral loading of DEX of these micellar carrier systems as discussed in the following sections.

4.3.2. Transport study with SMM and SSM

Figures 4.2A and 4.2B show the cumulative amounts of DEX transported across human sclera in the passive and iontophoretic transport experiments with SMM and SSM solutions. Before the discussion of the SMM data, it should be realized that both simple and mixed micelles exist in the SMM solution due to the relatively high concentration of TA compared to LE (4:1 mole ratio) in the micellar system (also see Section 4.3.1); these simple micelles likely contribute to the behavior of DEX delivery in the passive and iontophoretic transscleral transport experiments. For both SSM and SMM, higher cumulative amounts of DEX transported across the sclera were observed during cathodal iontophoresis as compared to their passive counterparts. These results are consistent with the negative charges and zeta potential of SMM and SSM (Table 4.1) and demonstrate the direct electric field effect of iontophoresis (i.e., electrorepulsion or Nernst-Planck effect) [27] on these carrier systems. For SMM, the higher cumulative amounts of DEX delivered in the anodal iontophoresis experiment than those in passive delivery were likely attributed to the contribution of electroosmosis. The electroosmosis effect was
expected to be larger for the mixed micelles in SMM than for SSM due to the difference in their sizes (Table 4.1) [28]. Accordingly, the cumulative amounts of DEX in anodal and cathodal iontophoresis of DEX in SMM were not significantly different. In summary, the observed iontophoresis enhancement of both simple and mixed micelle systems suggests that these carrier systems can be efficiently delivered across human sclera using iontophoresis as compared to passive delivery.

Figure 4.2C shows the cumulative amounts of DEX transported across human sclera in the passive and iontophoretic transport experiments with the control solution. In these control experiments, significantly higher cumulative amount of DEX transported across the sclera was observed during anodal iontophoresis as compared to those of passive transport and cathodal iontophoresis. The enhancement of DEX transport observed in the anodal iontophoresis control experiment is consistent with the dominant effect of electroosmosis upon DEX transport across the negatively charged human sclera under the condition in the present study.
Figure 4.2. Cumulative amounts of DEX transported across human sclera versus time in the passive (closed diamonds), cathodal iontophoresis (closed squares), and anodal iontophoresis (closed triangles) transport experiments of (A) SMM, (B) SSM, and (C) control. Data represent the mean and standard deviation, n ≥ 3.
Figure 4.3. Apparent permeability coefficients of DEX of SMM, SSM, and the control in the cathodal iontophoresis (open bars), passive (black bars), and anodal iontophoresis (gray bars) transport experiments with human sclera. Data represent the mean and standard deviation, n ≥ 3.
To compare transscleral transport of SMM and SSM, the apparent permeability coefficients of human sclera for DEX in SMM, SSM, and the control were calculated by normalizing the flux with the donor concentration (Figure 4.3). The passive permeability coefficient of DEX in SMM was lower than those of SSM and the control. This is consistent with the larger effective size and hydrodynamic diameter of SMM as compared to that of SSM (see Section 4.3.1). DEX in the control experiments had the highest passive permeability coefficient due to its molecular size and diffusion coefficient in the sclera compared to those of the carrier systems. Similarly, the higher cathodal iontophoretic permeability coefficient of DEX in SSM than that of SMM could be partly attributed to the smaller effective sizes of SSM than SMM.

It should be pointed out that the conductivity results of SMM, SSM, and control solutions (Table 4.1) suggest similar voltage drop across the sclera in the present constant current iontophoretic transport experiments. Therefore, DEX iontophoretic transport in the SMM, SSM, and control solutions was likely under similar iontophoretic driving forces (i.e., constant current iontophoresis of constant electrical potential). This allows the direct comparison of the permeation data to understand the interplay of electrophoresis and electroosmosis of the micellar systems upon flux enhancement in the present study. In addition, the osmolarity results in Table 4.1 suggest that convective solvent driven transport due to the water concentration gradient across the sclera (the difference in osmolarities of the receptor solution and the SMM, SSM, and UMM donor solutions) in the transport experiments was not likely to be significant. The effective sizes and charges of the micellar carriers and their encapsulation capacity are the primary factors influencing iontophoretic transscleral transport in the present study.
In the study using the three-stage transport protocol of passive transport followed by cathodal iontophoresis and then second passive transport, no significant difference was observed in the passive permeability coefficients of SMM before and after iontophoresis \((4.5 \pm 0.8 \times 10^{-6} \text{ and } 6.2 \pm 1.1 \times 10^{-6} \text{ cm/s, mean } \pm \text{ SD, } n = 4, \text{ respectively})\). The essentially same permeability coefficients in the first and second passive stages suggest no irreversible electropermeability effect on the barrier properties of human sclera for micellar transport under the iontophoretic conditions in the present study.

### 4.3.3. Drug release study with SMM and SSM

The effects of iontophoretic delivery on drug release from the sclera were first investigated. As shown in Figures 4.4A and 4.4B, drug release from the sclera in the SMM and SSM experiments was enhanced after cathodal iontophoresis as compared to those after anodal iontophoresis and passive transport. These results are consistent with the findings in the transport study (Section 4.3.2) that cathodal iontophoresis can effectively enhance transscleral delivery of these micellar carrier systems. Conversely, in the control experiment without the carrier systems (Figure 4.4C), drug release was enhanced after anodal iontophoresis as compared to those after passive and cathodal iontophoretic transport, consistent with electroosmotic enhancement of DEX during anodal iontophoresis. Both these trends can be attributed to the ability of iontophoresis in enhancing drug loading into the tissue compared to that of passive delivery, and hence providing better sustained drug delivery from the sclera. The effects of iontophoresis upon drug loading will be discussed in the next section (Section 4.3.4).
To examine the effects of micellar carrier systems on the rate of DEX released from the sclera, the results in Figures 4.4A and 4.4B were compared with those in Figure 4.4C. Figure 4.4C shows that more than 60% of DEX was released within two hr after passive delivery whereas Figures 4.4A and 4.4B show only 14% and 16% of DEX were released in the same time period after passive delivery of SMM and SSM, respectively. Similarly, drug release after iontophoretic transport of the micellar carrier systems was generally slower as compared to that of the control after iontophoresis. For example, approximately 33% of DEX was released in one day after cathodal iontophoresis of SMM while the release of DEX was 63% in one day after anodal iontophoresis of the control. These results suggest that SMM and SSM carrier systems exhibit sustained release properties by providing slower DEX release than the control. SMM and SSM also have different sustained drug delivery profiles. A comparison of drug release from the sclera after cathodal iontophoresis of SMM with that of SSM shows that approximately 55% of DEX was released in two days with SMM, less than the approximately 80-90% of DEX released with SSM in the same time period. These results suggest that SMM provides better sustain release properties than SSM.
Figure 4.4. Cumulative amounts of DEX released from human sclera versus time in the release studies performed after the passive (closed diamonds), cathodal iontophoresis (closed squares), and anodal iontophoresis (closed triangles) transport experiments of (A) SMM, (B) SSM, and (C) control. Data represent the mean and standard deviation, $n \geq 3$. 
4.3.4. Drug extraction study with SMM and SSM

Figure 4.5 presents the total amounts of DEX loaded in the sclera at the end of the transport experiments. The results show that the amounts of DEX loaded in the sclera after cathodal iontophoretic transport of SMM and SSM were significantly larger than those after passive transport of SMM and SSM. The two- to four-fold higher drug loading into the sclera after iontophoretic transport than passive delivery supports the hypothesis that iontophoresis enhances the loading of charged micellar carrier systems into the sclera and hence provides enhanced sustained transscleral drug delivery after iontophoresis. This hypothesis is also consistent with the sclera transport data observed in Section 4.3.2 in which higher apparent permeability coefficients were observed during cathodal iontophoresis (SMM and SSM) as compared to their permeability coefficients in passive delivery. On further comparison of enhanced drug loading due to iontophoresis, the enhancement of drug loading was larger for SMM than for SSM after cathodal iontophoresis. This might be related to the higher DEX concentration in the donor in the transport experiment of SMM than that of SSM (0.45 mg/mL versus 0.23 mg/mL, respectively).

To investigate the effects of the micellar carriers on scleral drug loading, the amounts of DEX loaded in the sclera after SMM and SSM passive delivery were compared with that of the control. The amount of DEX loaded into the sclera after passive drug delivery in the control experiment was significantly smaller than those in SMM and SSM. This suggests that the improved aqueous solubility of DEX in the micellar carrier systems enhanced drug loading into the sclera in passive transscleral delivery. Similar findings of the micellar carrier systems being more effective in loading
the drug into the sclera in iontophoretic delivery as compared to iontophoretic delivery of the control were also observed. Together, these results demonstrate the advantages of iontophoretic delivery and the micellar systems for sustained ocular drug delivery. It should be pointed out that the amounts of drug loaded into the sclera from iontophoresis correspond to tissue concentration as high as or higher than the drug concentration in the donor chamber of the transport study within the uncertainties of the dimensions of the sclera mounted on the diffusion cells. This is probably due to iontophoretic enhanced drug loading into the membrane [29] saturating the sclera under these conditions. In addition, drug delivery from the small amount of residual donor solution on the surface of the sclera after the removal of the donor solution in the release study could also contribute to this observation.
Figure 4.5. Amounts of DEX loaded in human sclera after cathodal iontophoresis (open bars), passive (black bars), and anodal iontophoresis (gray bars) transport experiments with SMM, SSM, and control. Data represent the mean and standard deviation, $n \geq 3$. 
4.3.5. Transport and release studies with UMM

UMM that contained the same amount of DEX as the control (0.1 mg/mL) was used to study the effects of micellar DEX concentration upon transscleral drug transport and drug release from the sclera. As shown in Table 4.1 and Section 4.3.1, the physical properties and encapsulation efficiency of UMM and those of SMM are not significantly different, suggesting that the encapsulation of DEX into the micelles did not significantly alter the physical properties of the micelles. The encapsulation of DEX into the micelles also did not significantly impact transscleral transport of DEX in the micellar carrier systems. For example, the passive ($5.7 \times 10^{-6}$ cm/s) and cathodal iontophoretic ($1.3 \times 10^{-5}$ cm/s) permeability coefficients of sclera for DEX in UMM are essentially the same as those in SMM, respectively.

A comparison of the UMM results with those of the control (saturated DEX solution without the micelles) shows that the passive and iontophoretic permeability coefficients of DEX in UMM are significantly lower than those of the control. This is consistent with UMM being larger in hydrodynamic size than free DEX. Despite the lower permeability coefficients of DEX in UMM relative to the control, UMM shows higher sclera loading than those of the control after passive and iontophoretic delivery.

Figure 4.6 compares the results of drug release from the sclera in UMM with that of the control (results from Figure 4.4). From the release profiles in the figure, approximately 25% of DEX was released from the sclera within 2 hr after passive delivery of UMM, which is significantly smaller than the more than 60% DEX released within the same period after passive delivery in the control experiment. This suggests
interactions between the micellar carrier system and the sclera that contributed to the observed slower drug release. Figure 4.6 also demonstrates the effect of cathodal iontophoresis upon the drug release profiles of UMM. Iontophoresis of UMM enhanced the total amount of DEX released from the sclera over that after passive delivery.
Figure 4.6. Cumulative amounts of DEX released from human sclera in the release studies performed after the passive (open diamonds) and cathodal iontophoretic (closed diamonds) transport experiments of UMM. The results of the control after passive delivery (open triangles) are presented again for comparison. Data represent the mean and standard deviation, n ≥ 3.
4.3.6. Mechanisms of micellar carrier sustained delivery

Micellar carrier-sustained DEX delivery could be related to two main factors: improved sclera loading and delayed drug release from the sclera. The increased DEX loading in the sclera due to higher aqueous solubility of DEX in the micellar carrier systems and enhanced delivery of DEX into the sclera can result in an increase in the total amount of DEX released from the sclera compared with the control. Particularly, the effect of drug loading upon sustained drug delivery is demonstrated in the iontophoresis experiments. After cathodal and anodal iontophoresis that enhanced drug loading, the amounts of DEX released were generally larger and slower than those of passive delivery.

For delayed drug release, the mechanisms can be through carrier and drug interactions with the sclera and slower micellar carrier diffusion than that of free DEX in the sclera. For instance, interactions between these micellar carrier systems and the sclera such as micelle binding to the tissue can result in delayed drug release from the sclera. In addition, the larger effective sizes of the micellar carriers in SMM and SSM and the resultant slower diffusion of these carriers in the sclera relative to those of free DEX would lead to delayed drug release. As evidenced in the results from the UMM studies (Figure 4.6), such delayed drug release contributed to the sustained release properties observed in the present study. However, this effect is probably not as significant as sclera loading effect (Figures. 4.4 and 4.6). Enhanced sclera loading due to the increase of DEX solubility in SMM and SSM is believed to be the main mechanism for the differences observed between the release profiles of SMM and SSM versus that of the control. Another factor that is worth mentioning is the higher viscosity
of SMM and UMM than SSM and the control (Table 4.1) that can also contribute to the different sustained release profiles of DEX in SMM compared to SSM and the control.

4.3.7. Potential application of transscleral iontophoresis of the micellar carriers

Sustained release drug delivery systems can be a therapeutic modality for the treatment of chronic ocular disorders such as chronic inflammatory eye diseases [30]. Micellar carrier systems are generally considered as safe [31], can be tailored in a variety of ways to enhance the aqueous solubility of lipophilic drugs, and provide a sustained drug delivery effect. Physical enhancement methods such as iontophoresis have been employed in ocular drug delivery. For example, transscleral iontophoresis was shown to have the potential to enhance the transport of small and macromolecules across the sclera [32]. The results in the present study suggest that the studied micellar carrier systems can be delivered across the sclera by transscleral iontophoresis. Particularly, transscleral iontophoresis of the micellar carrier systems was shown to enhance drug loading into the sclera and prolong drug delivery from the sclera. The combination of micellar nanocarriers and transscleral iontophoresis could therefore maintain higher drug concentrations at the site of application, provide sustained release from this site to the site of drug action in the eye, and overcome the need of frequent drug dosing and injections in the eye for the desired therapeutic effect. This drug delivery platform can be a promising noninvasive strategy in the treatment of posterior eye diseases. It could be more cost-effective than frequent intravitreal injections, a procedure that generally requires the involvement of a skilled ophthalmologist or retina specialist.
It should be pointed out that the present study was conducted under the *in vitro* setting. Cautions must be exercised in the interpretation of the results in the present study as it may not predict transscleral drug delivery in practice *in vivo* due to the absence of blood vasculature and lymphatic clearance in the present experiments *in vitro*. Further pharmacokinetic studies of iontophoresis and micellar carriers are required to demonstrate the feasibility of these systems *in vivo*.

In addition, the safety and toxicity of the mixed micelles used and those of the combination use of micelles and iontophoresis have not been evaluated in the present *in vitro* study. Although various studies have demonstrated the safety of mixed micellar carriers in cell lines [33-34] in the concentration range and LE to TA molar ratio similar to those in the present study, it would be difficult to predict their effects in the eye. In a preliminary study to assess the safety of the mixed micelles in the subconjunctival space using mouse eyes, no irritation and toxicity were observed for one week after dosing (unpublished data). Despite these results, future studies are required to fully evaluate the safety of the proposed micellar carrier systems for transscleral iontophoretic delivery.
4.4. References


CHAPTER 5

Influence of Drug Lipophilicity on Drug Release from Sclera after Iontophoretic Delivery of Mixed Micellar Carrier System to Human Sclera

5.1. Introduction

In the previous chapter, a mixed micellar carrier system composed of egg lecithin (LE) and taurocholate (TA) of 1:4 mole ratio was developed to enhance the aqueous solubility of dexamethasone (DEX). This study showed that the combination of micellar nanocarriers and transscleral iontophoresis could enhance drug delivery into the sclera and provide sustained release of the drug [1]. The mixed micellar system prepared with LE/TA 1:4 (mole ratio) had both simple and mixed micelles coexisted in the carrier system and both these carriers are responsible for the enhancement of DEX solubilization and in the sustained release of DEX after iontophoretic delivery into and across the sclera. Because it has been reported in the literature that phospholipids (egg phophatidylcholine) play an important role in the solubilization of poorly water soluble drug in mixed micelles and an increase in LE composition (i.e., mole ratio) in the micelles enhances the solubility of the drug in the micelles [2], mixed micellar carrier systems prepared with higher LE/TA mole ratios are believed to provide better sustained drug delivery with transscleral iontophoresis. However, the interplay of drug
lipophilicity, drug solubilization, and sustained drug release of these mixed micelles in transscleral delivery is not well understood.

The objectives of the present study were to (a) determine the influence of drug lipophilicity on the solubilization potential of a mixed micellar carrier system (LE/TA 1:1, mole ratio) and (b) study the relationship between drug lipophilicity and drug release profiles of the mixed micellar carrier system. Dexamethasone (DEX), triamcinolone acetonide (TRIAM), and β-estradiol (E2β) were the model drugs selected in this study, which represented a range of lipophilicity indicated by the logarithm of octanol/water partition coefficient (log Ko/w) from 1.8 to 3.5. Table 5.1 summarizes the physicochemical properties of these model permeants.
**Table 5.1.** Physicochemical properties of the drugs used in the present study.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Molecular Formula</th>
<th>Molecular Weight (g/mol)</th>
<th>log K&lt;sub&gt;o/w&lt;/sub&gt;&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>Aqueous Solubility (mg/mL)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEX</td>
<td>C&lt;sub&gt;22&lt;/sub&gt;H&lt;sub&gt;29&lt;/sub&gt;FO&lt;sub&gt;5&lt;/sub&gt;</td>
<td>392.47</td>
<td>1.8</td>
<td>0.1</td>
</tr>
<tr>
<td>TRIAM</td>
<td>C&lt;sub&gt;24&lt;/sub&gt;H&lt;sub&gt;31&lt;/sub&gt;FO&lt;sub&gt;6&lt;/sub&gt;</td>
<td>434.5</td>
<td>2.53</td>
<td>0.021</td>
</tr>
<tr>
<td>E2β</td>
<td>C&lt;sub&gt;18&lt;/sub&gt;H&lt;sub&gt;24&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>272.38</td>
<td>3.5</td>
<td>0.003</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values obtained from the Estimation Programs Interface (EPI) database.

<sup>b</sup> logarithm of octanol/water partition coefficient.
5.2. Materials and Methods

5.2.1. Materials

$^{3}$H-DEX ($6,7^{3}$H(N)−, 23.1 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA) with purity of at least 97%. $^{3}$H-TRIAM (0.8 Ci/mM) and $^{3}$H- E2β (2,4-$^{3}$H, 24.5 Ci/mmol) were purchased from Moravek Biochemicals and Radiochemicals (Brea, CA). TA was purchased from Sigma–Aldrich (St. Louis, MO). LE (from eggs, purity > 90%) was purchased from Indofine Chemical (Hillsborough, NJ). DEX, TRIAM, and E2β were purchased from Letco Medical (Decatur, AL).

Phosphate-buffered saline (PBS: 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4) was prepared using distilled, deionized water and PBS tablets as described by the manufacturer (Sigma-Aldrich, St. Louis, MO). PBS with 2% (w/v) bovine serum albumin (BSA) was prepared by dissolving BSA (purity > 98%, MW 68 kDa, Sigma-Aldrich, St. Louis, MO) in PBS. Filter membranes (MSE syringe filters, 0.22 µm) were purchased from Fisher Scientific (Pittsburgh, PA). Ethanol (denatured, anhydrous ethyl alcohol) was purchased from Fischer Scientific (Rochester, NY). High performance liquid chromatography (HPLC) grade acetonitrile was purchased from Pharmaco-AAPER (Shelbyville, KY). All materials were used as received.

5.2.2. Preparation of mixed micellar and control solutions

The mixed micellar solutions at LE to TA ratio of 1:1 (mole ratio) were prepared by first dissolving appropriate amounts of TA in PBS to obtain clear solutions and then adding appropriate amounts of LE to the solutions to achieve final total lipid
concentration of 95 mg/mL. In the preparation of drug-loaded mixed micellar solutions, excess amounts of drugs (DEX, TRIAM, and E2β) were added to the mixed micellar solutions followed by equilibration of the solutions at 36 ± 1°C for 48 to 96 h. After equilibration, the mixtures were filtered through the 0.22 μm filter membranes to obtain clear solutions.

Control solutions of saturated DEX, TRIAM, and E2β were prepared by adding excess amounts of drugs in PBS followed by equilibration in a circulating waterbath at 36 ± 1°C for 48 to 96 h. After equilibration, the undissolved drugs were separated from the solutions by filtration using the 0.22 μm filter membranes.

5.2.3. Characterization of micellar carrier systems

To determine the solubilities of drugs in the mixed micellar solutions and controls, aliquots of the filtered micelle and control solutions were subjected to appropriate dilution with the HPLC mobile phase. The diluted samples were then assayed for the drugs using a HPLC system. The HPLC system (Prominence, Shimadzu, Columbia, MD) consisted of CBM-20A system controller, LC-20AT solvent delivery module, SIL-20A autosampler, and SPD-20A UV-Vis detector. The separation was performed with Microsorb C18 column (150 mm × 4.6 mm; Varian, Inc., Palo Alto, CA) at room temperature. For the assays of DEX and TRIAM, a mobile phase consisting of a mixture of distilled deionized water and acetonitrile (35:65, v/v) was delivered at a flow rate of 1.0 mL/min and the drugs were detected at 284 nm wavelength. For the assay of E2β, a mobile phase consisting of a mixture of distilled deionized water and acetonitrile (50:50,
v/v) was delivered at a flow rate of 1.0 mL/min and the detection wavelength was 204 nm.

To determine the effective sizes and zeta potentials of the mixed micellar carrier systems, Malvern Zetasizer® (Nano ZS, Malvern Instruments Ltd, Worcestershire, UK) was used. The measurements were carried out with the mixed micellar solutions of DEX, TRIAM, and E2β in triplicate at 25°C.

5.2.4. Preparation of the sclera

Human cadaver eyes were obtained from National Disease Research Interchange (NDRI, Philadelphia, PA). The tissues were stored in moisture chambers at 4°C. Before the experiments, the sclera was soaked in PBS at room temperature and the adhering tissues on the sclera including the retina and choroid were removed with a pair of forceps. The sclera was then rinsed with PBS, cut to appropriate sizes, and equilibrated in PBS at room temperature for 30

5.2.5. Transscleral transport study

Cadaveric human sclera was sandwiched between the two half-cells of a side-by-side diffusion cell with the choroid side facing the receptor. The diffusion cells have an effective diffusion area of 0.2 cm² and the temperature was maintained by a circulating waterbath at 36 ± 1°C. The volume of the donor and receptor solutions was 1.5 mL. Prior to the transport experiments, the donor solutions were prepared by adding trace
amounts of radiolabelled DEX, TRIAM, and E2β (0.5-1 μCi/mL) into the DEX, TRIAM, and E2β mixed micelle or control solutions, respectively. PBS was the receptor solution in all the transport experiments. Passive transport, anodal iontophoresis, and cathodal iontophoresis experiments were performed. In the iontophoresis experiments, 2 mA direct current was applied across the sclera with a constant current iontophoretic device (Phoresor II Auto, Model PM 850, Iomed, Inc., Salt Lake City, UT) using Ag/AgCl (cathode) and Ag (anode) as the driving electrodes. The duration of the transport experiment was 20 min. At predetermined time intervals, samples of 10 μL donor solution and 1 mL receptor solution were taken for assay. Fresh PBS of 1 mL was then added into the receptor to maintain a constant volume in the receptor. The samples were mixed with 10 mL of liquid scintillation cocktail (Ultima Gold™, PerkinElmer Life and Analytical Sciences, Shelton, CT) and assayed by a liquid scintillation counter (Beckman Coulter LS 6500, Fullerton, CA).

To analyze the transscleral transport data, the apparent flux (J) was calculated from the change in the cumulative amount (ΔQ) of the permeant transported across the sclera into the receptor chamber over time (t) divided by the effective diffusion area (A_D) using Eq. (1).

\[
J = \frac{1}{A_D} \frac{\Delta Q}{\Delta t} \tag{5.1}
\]

The apparent permeability coefficient (P) was defined as the flux normalized by the donor concentration and was calculated by dividing the apparent flux by the donor concentration of the permeant (C_D):

\[
P = \frac{1}{C_D A_D} \frac{\Delta Q}{\Delta t} \tag{5.2}
\]
5.2.6. Drug release study

Drug release study was carried out following the transscleral transport study. Particularly, at the end of the transport experiments of DEX and TRIAM, both the donor and receptor solutions were removed and fresh 1.5 mL of PBS was added into the receptor chamber to start the release study. A stopper was put on the donor chamber to maintain the pressure in the donor chamber. For E2β, the same drug release study method as that of DEX and TRIAM was used except that 1.5 mL of 2% (w/v) BSA in PBS was added into the receptor chamber instead of PBS. BSA was used in the receptor solution to prevent the binding of E2β to the glass diffusion cells. The duration of the drug release study was 6 days. At predetermined time intervals, 1 mL of solution was taken from the receptor for assay followed by replenishing the receptor with 1 mL fresh PBS (or 2% BSA in PBS). The samples were mixed with 10 mL of the liquid scintillation cocktail and assayed by the liquid scintillation counter.

Extraction of drugs from the sclera was performed following the drug release study to determine the amounts of DEX, TRIAM, and E2β remained in the sclera at the end of the drug release study. After the last sample was taken in the drug release experiment, the sclera was removed from the side-by-side diffusion cell assembly and was immersed in 2 mL ethanol in a vial for 24 hr. After 24 hr of extraction, 1 mL of sample was withdrawn from the vial, mixed with 10 mL of liquid scintillation cocktail, and assayed for DEX, TRIAM, and E2β using the liquid scintillation counter. The total amounts of DEX, TRIAM, and E2β loaded into the sclera by passive and iontophoretic delivery were calculated as the sum of the amounts of DEX, TRIAM, and E2β released
from the sclera in the drug release study and the amounts of DEX, TRIAM, and E2β remained in the sclera.

5.2.7. Statistical analysis

All experiments were conducted with a minimum of three replicates using sclera from different eye donors. The means ± standard deviations (SD) of the data are presented. Statistical differences were determined by Student’s $t$-test. Differences were considered to be significant at a level of $p < 0.05$. 
5.3. Results

5.3.1. Characterization of mixed micellar carrier systems

Table 5.2 summarizes the solubilities of the drugs in the mixed micellar carrier systems. The aqueous solubilities of DEX, TRIAM, and E2β without the mixed micelles (control solutions) were 0.1, 0.02, and 0.003 mg/mL, respectively. These aqueous solubility values are essentially the same as those reported in the literature (EPI Suite) (Table 5.1). The amounts of DEX, TRIAM, and E2β solubilized in the mixed micellar carrier systems were 0.74, 0.22, and 0.13 mg/mL, respectively. Compared to the aqueous solubilities measured in the controls, the mixed micelles significantly increased the solubilities of the drugs. Figure 5.1 presents the ratios of drug solubilities in the micellar carrier solutions to those in the controls. The enhancement in drug solubility of the mixed micellar carriers was from approximately 7 to 33-fold when the log Ko/w of the drugs increased from 1.8 to 3.5.

Table 5.2 also presents the hydrodynamic diameters and zeta potentials of the mixed micelles under the experimental conditions in the present study. The mean hydrodynamic diameters of the mixed micelles ranged from 4.6 to 5.3 nm. The mixed micelles of DEX were smaller than those of TRIAM and E2β. The zeta potential results of the mixed micelles indicate that the micellar carriers were net negatively charged in PBS, and the absolute values of their zeta potentials were in the order of DEX > TRIAM > E2β.
Table 5.2. Properties of drug-loaded mixed micellar carrier systems investigated in the present study.  

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Solubility (mg/mL)</th>
<th>Hydrodynamic Diameter (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEX</td>
<td>0.74 ± 0.02</td>
<td>4.6 ± 0.2</td>
<td>-68.0 ± 1.4</td>
</tr>
<tr>
<td>TRIAM</td>
<td>0.22 ± 0.01</td>
<td>5.3 ± 0.2</td>
<td>-56.0 ± 1.0</td>
</tr>
<tr>
<td>E2β</td>
<td>0.13 ± 0.01</td>
<td>5.1 ± 0.3</td>
<td>-44.4 ± 1.3</td>
</tr>
</tbody>
</table>

a Mean ± SD, n ≥ 3

b Solubility of drugs was measured in the mixed micellar carrier systems at a total lipid concentration of 95 mg/mL.
Figure 5.1. Drug solubilization enhancement (ratios of solubilities of DEX, TRIAM, and E2β in the mixed micellar carrier systems to controls vs. drug log Ko/w.)
5.3.2. Transport study

Table 5.3 summarizes the apparent permeability coefficients of human sclera for DEX, TRIAM, and E2β in the passive and anodal and cathodal iontophoretic transport experiments with the mixed micellar and control solutions. In the control experiments, significantly higher iontophoretic permeability coefficients were observed during anodal iontophoresis as compared to those of passive transport; anodal iontophoresis enhanced the apparent permeability coefficients of DEX, TRIAM, and E2β all by approximately 3-fold versus passive transport. This is consistent with the effect of electroosmosis upon DEX, TRIAM, and E2β transport across the negatively charged sclera without the mixed micelles.

In general, iontophoresis enhanced the apparent permeability coefficients of DEX, TRIAM, and E2β in the mixed micellar carrier solutions as compared to those in passive transport. The higher cathodal iontophoresis permeability coefficients of the drugs (except TRIAM) in the mixed micellar carrier systems are consistent with the negative zeta potentials of the mixed micelles and the direct electric field effect (i.e., electrorepulsion) as a flux enhancing mechanism of the micellar carrier systems. Cathodal iontophoresis enhanced the apparent permeability coefficients of DEX and E2β by approximately 2-fold and 5-fold, respectively versus their passive transport. The higher anodal iontophoretic permeability coefficients of the drugs in the mixed micellar carrier systems during anodal iontophoresis compared to those of passive transport were likely attributed to electroosmotic transport across the negatively charged transport pathway in the sclera. Anodal iontophoresis enhanced the apparent permeability
coefficients of DEX, TRIAM, and E2β by approximately 2-, 3.5- and 5-fold, respectively, versus passive transport.
Table 5.3. Apparent permeability coefficients ($\times 10^{-5}$, cm/s) of DEX, TRIAM, and E2β across human sclera in passive transport, anodal iontophoresis, and cathodal iontophoresis experiments$^a$.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>DEX</th>
<th>TRIAM</th>
<th>E2β</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Passive</td>
<td>Cathodal</td>
<td>Anodal</td>
</tr>
<tr>
<td>Mixed micelles</td>
<td>0.6 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Control</td>
<td>1.2 ± 0.6</td>
<td>1.2 ± 0.7</td>
<td>3.0 ± 1.5</td>
</tr>
</tbody>
</table>

$^a$ Mean ± SD, n $\geq$ 3
The iontophoretic permeability coefficients of DEX, TRIAM, and E2β reveal no relationship between drug lipophilicity and transscleral iontophoretic transport of the mixed micellar carrier systems. The transport behavior of these mixed micelles does not seem to be influenced by the lipophilicity of the drugs loaded into the carriers. Iontophoretic transport therefore was likely controlled by the properties of the mixed micelles such as their sizes and charges. Comparing the passive and iontophoresis results of DEX in the present study of 1:1 LE/TA (mole ratio) mixed micelles to those in a previous study using 1:4 LE/TA (mole ratio) mixed micelles [1] suggests that the transport properties of the 1:1 and 1:4 LE/TA mixed micellar carrier systems are similar.

5.3.3. Drug release study

Drug release from the sclera was investigated after the passive and iontophoretic transport study of DEX, TRIAM, and E2β. Figures 5.2A, B and C show the cumulative amounts of DEX, TRIAM, and E2β released from the sclera after passive and iontophoretic delivery, respectively. As shown in the figures, drug release from the sclera in the micellar carrier experiments was enhanced after both cathodal and anodal iontophoretic delivery, and anodal iontophoresis provided better drug release enhancement than cathodal iontophoresis for TRIAM and E2β. In the control experiments without the mixed micelles, drug release was enhanced after anodal iontophoresis as compared to those after passive transport and cathodal iontophoresis, consistent with the permeability enhancement observed in the transport study.
The results in the drug extraction study of the sclera at the end of the drug release study showed that less than 12% of the total drugs loaded into the sclera remained in the tissue after the release study (except E2β, which was less than 25%). For example, the amounts of drugs extracted from the sclera at the end of the drug release study were 18.2 ± 5.2, 3.1 ± 0.4, and 3.0 ± 2.5 µg/cm² for DEX, TRIAM, and E2β, respectively, after anodal iontophoretic delivery with the mixed micelles (versus 183, 194, and 41 µg/cm² total amounts loaded into the sclera for DEX, TRIAM, and E2β, respectively). This indicates that most of the drugs loaded into the sclera were released from the sclera over the 6-day period in the drug release study. The sclera extraction study together with the drug release study show that substantial amounts of drugs were loaded into the sclera after iontophoretic delivery compared to those after passive delivery, and drug delivery with the mixed micellar carrier systems (both iontophoretic and passive delivery) provided higher drug loading into the sclera than those of the controls without the mixed micelles.

To examine the effect of drug lipophilicity on drug release from the sclera after passive, anodal and cathodal iontophoretic drug delivery with the mixed micelles, the cumulative amounts of drugs released at the 48-hr time point as percent of total amounts of drugs loaded into the sclera were plotted against the log Ko/w values of the drugs in Figure 5.3A and compared. As shown in the figure, approximately 70-78%, 50-73%, and 40-53% of DEX, TRIAM, and E2β were released from sclera 48 hr (2880 min) after transscleral passive and iontophoretic delivery with the mixed micelles, respectively. These results suggest that the rates of drug release decreased with increasing lipophilicity of the drugs after they were delivered into the sclera. This can be attributed to the higher
partition coefficients of the more lipophilic drugs to the mixed micelles. Without the mixed micelles, greater than 80% DEX, TRIAM, and E2β were released from the sclera in the same 48 hr (2880 min) period, again demonstrating the role of the mixed micelles in the sustained release of DEX, TRIAM, and E2β from the sclera.

To examine the effect of drug lipophilicity on drug loading into the sclera with the mixed micellar carrier systems after passive, anodal and cathodal iontophoretic delivery, the ratios of scleral drug loading with the mixed micellar systems to the controls were plotted against the log Ko/w values of the drugs in Figure 5.3B and compared. These ratios do not show any particular trend of tissue loading behavior of the drugs based on their lipophilicities. For example, after passive transport, the ratio of scleral loading of DEX, TRIAM, and E2β was not significantly different from each other (p > 0.05). This is consistent with the results observed in the transport study, in which the drug delivery behavior does not seem to be influenced by drug lipophilicity. However, once the drugs were delivered into the tissue, the amounts of the drugs released from the sclera were dependent on drug lipophilicity as shown in Figure 5.3A (slower drug release with increasing drug lipophilicity).
Figure 5.2. Cumulative amount of DEX (A), TRIAM (B), and E2β (C) released from human sclera versus time in the release study performed after passive (closed diamonds), cathodal iontophoresis (closed squares) and anodal iontophoresis (closed triangles) of mixed micelles and passive (open diamonds), cathodal iontophoresis (open squares) and anodal iontophoresis (open triangles) of the controls. Data represent the mean and standard deviation, n ≥ 3.
Figure 5.3. (A) Percent of DEX, TRIAM, and E2β released from sclera at time point (2880 min) 48-hr versus drug log Ko/w in the drug release study and (B) loading enhancement of the drugs into the sclera by the mixed micelles compared with the controls versus drug log Ko/w after passive transport (diamonds), cathodal iontophoresis (squares), and anodal iontophoresis (triangles). Data represent the mean and standard deviation, n ≥ 3.
5.4. Discussion

5.4.1. Effects of drug lipophilicity and transscleral delivery

Corticosteroids such as DEX and TRIAM have traditionally been used as potent anti-inflammatory agents in the treatment of ocular diseases [3-6]. Problems associated with the delivery of these drugs include their poor aqueous solubility and poor permeability across ocular tissues. E2β is more lipophilic than DEX and TRIAM and was used as a model drug to investigate the effect of drug lipophilicity upon the drug delivery behavior of the mixed micelles in the present study. Using DEX, TRIAM, and E2β, the relationship between drug lipophilicity and the solubilization potential of the mixed micellar systems was studied. The impacts of drug lipophilicity on drug loading into the sclera and the drug release profiles of these mixed micellar carrier systems from the tissue were evaluated to understand the interplay among drug lipophilicity, drug solubilization in the mixed micelles, and the release profiles of sustained drug delivery from the sclera.

Mixed micellar carriers have a hydrophobic core for drug loading and lipophilic drugs usually have stronger interactions with this hydrophobic core than less lipophilic drugs. This attributes to the increase in the solubilization potential of the mixed micelles to the more lipophilic drugs observed in the present study. Different from drug solubilization, transscleral drug transport with the mixed micellar carrier systems was observed not to be impacted by the lipophilicity of the drugs in the micellar systems but the physical properties of the mixed micelles such as their charges. Similarly, the scleral drug loading results of these mixed micellar carrier systems reveal no relationship
between drug loading into the sclera and drug lipophilicity. After the drugs were loaded into the sclera, a relationship between scleral drug release and drug lipophilicity was observed. The slower drug release from the sclera with increasing drug lipophilicity may be due to the stronger drug-to-micelle and/or drug-to-sclera interactions of the more lipophilic drug. Among the transscleral delivery protocols investigated, drug loading was enhanced after iontophoretic drug delivery with the mixed micelles as compared to their passive counterparts. The higher drug loading into the sclera can be attributed to enhanced drug delivery due to iontophoresis. This trend was consistent with our previous study in which improved sclera loading was observed after transscleral iontophoretic delivery of a drug [1], suggesting transscleral iontophoretic delivery as an efficient method of drug loading into the tissue.

It should be noted that high molecular weight drugs may not follow the same trend observed between drug lipophilicity and scleral drug release observed in the present micellar carrier study. In a separate study with cyclosporine A (CysA), a highly lipophilic large molecule, and the mixed micellar carrier system, the solubility enhancement of CysA was ~ 35 times higher than its aqueous solubility but no CysA was detected in the receptor chamber (PBS with 2% (w/v) BSA) in the transport and drug release experiments (data not shown). This is believed to be due to the high lipophilicity and/or high molecular weight of CysA. In another study with salicylate, a negatively charged small molecule, and the mixed micellar carrier system, although salicylate can be delivered effectively into and across the sclera with and without the mixed micelles, sustained drug release from the sclera was not observed (data not shown). This is believed to be due to the polar nature of salicylate, and hence low encapsulation
efficiency into the mixed micelles. Together, these results demonstrate the limitations of the micellar carrier system as an effective system for the sustained delivery of drugs of large molecular sizes such as CysA and high polarities such as salicylate. The method of combined iontophoresis and mixed micellar carriers in the present study is useful mainly for transscleral delivery of drugs with small molecular sizes (250-500 Da) and moderate lipophilicity.

5.4.2. Effects of mixed micellar carrier

Mixed micelles have been shown to increase the bioavailability of poorly water soluble drugs [2, 7-8]. The mixed micellar carrier system used in the present study was composed of 1:1 LE/TA (mole ratio), which was different from those of 1:4 LE/TA (mole ratio) investigated previously. Although the hydrodynamic diameter and zeta potential of the present 1:1 LE/TA mixed micelles with DEX are not significantly different from those of the previous 1:4 LE/TA DEX mixed micelles (~ 4.4 nm versus 4.3 nm and -68.0 mV versus -61.1 mV, respectively), the 1:1 LE/TA mixed micellar system has higher solubilizing capacity than those in the previous study [1]; the mixed micellar carrier system prepared using 1:1 LE/TA in the present study enhanced the solubility of DEX more than seven times as compared to its aqueous solubility whereas the 1:4 LE/TA mixed micellar system in the previous study enhanced the solubility of DEX by only four times [1]. The approximately 2-fold increase in DEX solubility in the 1:1 LE/TA mixed micellar system indicates that an increase in the concentration of LE in the mixed micelles provides better solubilization of DEX in the micelles. This difference can be
attributed to the increase in the volume of the hydrophobic lipid core of the micelles
and/or decrease in the concentration of simple micelles in the 1:1 LE/TA mixed micellar
system compared to the 1:4 LE/TA system. In spite of the higher DEX loading into the
micelles, the apparent permeability coefficients of DEX were not significantly different
in the 1:1 LE/TA and 1:4 LE/TA micellar carrier systems (p > 0.05), probably due to the
similar sizes and zeta potentials of the mixed micelles in these carrier systems. On
comparing the data in the present study with those in the previous study [1], the more
than 2-fold difference in the amounts of DEX loaded into the sclera after anodal
iontophoretic transport of 1:4 LE/TA and 1:1 LE/TA mixed micellar carrier systems
suggests that the 1:1 LE/TA carrier system can provide better sustained release delivery
than the 1:4 LE/TA carrier system in transscleral anodal iontophoresis. The improvement
in drug loading and sustained release of the drugs from the sclera after anodal
iontophoresis due to the higher drug solubility in the 1:1 LE/TA mixed micellar system in
transscleral iontophoretic delivery can be an advantage over the 1:4 LE/TA mixed
micellar system developed and investigated in the previous study [1].
5.5. Conclusion

Mixed micellar systems are well recognized for their abilities to enhance the solubility and delivery of poorly water soluble drugs. The drugs investigated in the present study were selected based on their lipophilicity. Mixed micellar carrier systems of 1:1 LE/TA were developed to enhance the solubility of these lipophilic drugs for transscleral iontophoretic delivery, and the effects of drug lipophilicity upon transscleral transport, drug release, and scleral drug loading of the mixed micellar carrier systems were studied. The following are the main findings in the present study. The present results suggest that the solubilizing potential of the mixed micellar carrier systems for the drugs increased with increasing lipophilicity of the drugs. The 1:1 LE/TA mixed micellar carrier systems have higher solubilizing potential than the previously investigated 1:4 LE/TA mixed micelles. The drugs in the 1:1 LE/TA carrier systems were efficiently transported into human sclera and their permeation into the sclera was not affected by drug lipophilicity. The scleral drug loading data indicate that iontophoresis enhanced drug loading into the sclera. The release profiles of the drugs were related to the lipophilicity of the drugs in the mixed micellar carrier systems. The release study showed prolonged scleral drug release with increasing lipophilicity of the drugs. These mixed micellar carrier systems can be a useful drug delivery strategy in transscleral delivery of lipophilic drugs to provide sustained drug release from the sclera. The loading of drugs into the sclera can be amplified by using iontophoresis. Thus, the combination of iontophoresis and mixed micellar carriers has the potential to be an alternative to the existing drug delivery methods for the treatment of chronic ocular disorders.
5.6. References


CHAPTER 6

Transscleral Iontophoretic Delivery of Triamcinolone Acetonide-Loaded Mixed Micellar Carrier System \emph{in vivo}

6.1. Introduction

Triamcinolone acetonide (TRIAM) is widely used in the treatment of posterior ocular diseases such as uveitis, inflammatory macular edema, diabetic retinopathy, retinal venous occlusion, and choroidal neovascularization [1-5]. Although TRIAM is suggested to be effective in the treatment of these ocular diseases, ocular delivery of TRIAM is limited by its poor water solubility. In clinical practice, TRIAM suspensions, such as Trivaris (80 mg/mL, Allergan, Inc. USA) and Triesence (40 mg/mL, Alcon, Inc. USA), are FDA approved for intravitreal injections [4]. Due to the invasive nature and multiple applications of intravitreal injections, complications such as cataract, retinal detachment, vitreous hemorrhage, and endophthalmitis, have been reported [6]. Although various implantable devices with sustained drug release properties have been studied [7], there is no sustained release product for TRIAM available in the market.

The studies described in the previous chapters (4 and 5) contribute to the understanding of a novel approach combining transscleral iontophoresis and mixed micellar carriers, to be an efficient delivery system for poorly water soluble corticosteroids \emph{in vitro}. It was shown that transscleral iontophoresis of the micellar
carrier systems enhanced drug loading into the sclera and provided sustained drug release from the sclera [8]. It was also deduced from this previous study that the solubilization capacity of the mixed micellar carrier system increased with increasing lipophilicity of the drugs. Particularly, the solubilization potential of mixed micellar nanocarriers for TRIAM was higher than that of (less lipophilic) dexamethasone. In addition, transscleral iontophoresis of the mixed micelles provided prolonged scleral release of the drugs, and the rate and extent of drug release were related to drug lipophilicity [9]. This novel approach of combining electric current and nanoparticles in ocular delivery was hypothesized to provide sustained transscleral drug delivery in vivo.

The study in the present chapter is a continuing effort of our investigation of transscleral iontophoretic delivery of mixed micellar carrier systems for sustained ocular drug delivery. The objectives of the present preclinical study were to (a) examine the feasibility of TRIAM mixed micelles prepared using sodium taurocholate (TA) and egg lecithin (LE) as a sustained release drug delivery system in vivo using mouse as an animal model, and (b) evaluate the safety of transscleral iontophoresis of the mixed micelles by histological examination of the eye tissues after the treatments.
6.2. Material and Methods

6.2.1. Materials

\(^3\)H-Triamcinolone acetonide (0.8 Ci/mM) was purchased from Moravek Biochemicals and Radiochemicals (Brea, CA). Sodium taurocholate (TA) was purchased from Sigma–Aldrich (St. Louis, MO). Lecithin (LE, from eggs, purity > 90%) was purchased from Indofine Chemical (Hillsborough, NJ). Triamcinolone acetonide (TRIAM) was purchased from Letco Medical (Decatur, AL). Filter membrane (MSE syringe filters, 0.22 µm) was purchased from Fisher Scientific (Pittsburgh, PA). Phosphate-buffered saline (PBS, pH 7.4, consisting of 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride) was prepared by dissolving PBS tablets (Sigma-Aldrich, St. Louis, MO) in distilled, deionized water. Ethanol (denatured, anhydrous ethyl alcohol) was purchased from Fischer Scientific (Rochester, NY). Paraformaldehyde (PFA) solution of 4% (w/v) was prepared by diluting 16% (w/v) PFA solution (EM grade, Electron Microscopy Sciences, Hatfield, PA) in PBS.

6.2.2. Animals

Mice (6-12 weeks, C57BL/6) supplied from Harlan Sprague Dawley (Indianapolis, IN) were used in this study. All experiments were conducted in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and with approval
of the Institutional Animal Care and Use Committee at the University of Cincinnati (Cincinnati, OH).

6.2.3. Preparation of mixed micellar carrier system

The mixed micellar carrier and control solutions were prepared by the procedures described previously [8]. Briefly, the mixed micellar carrier solution was prepared by dissolving an appropriate amount of TA and LE in PBS at a ratio of 1:1 (mole ratio) to achieve the total lipid concentration of 95 mg/mL. An excess amount of TRIAM was then added to the mixed micelle solution followed by equilibration in a circulating waterbath at 36 ± 1°C. After equilibration, the mixture was filtered through the 0.22 µm filter membrane to obtain clear saturated mixed micelle solution. The concentration of TRIAM in the mixed micelle solution was determined previously to be 0.22 mg/mL [9]. Control solution (without mixed micelles) of saturated TRIAM was prepared by adding an excess amount of TRIAM in PBS followed by equilibration in a circulating waterbath at 36 ± 1°C. After equilibration, the undissolved drug was separated from the solution by filtration using the 0.22 µm filter membrane. The concentration of TRIAM in the control solution without the mixed micelles was found previously to be 0.02 mg/mL [9]. Trace amounts of ³H-TRIAM were then added into the mixed micelle and control solutions before the passive and iontophoretic transport study.
6.2.4. Passive and iontophoretic transscleral delivery \textit{in vivo}

The mice were anesthetized by interperitoneal injection of 10 mg/kg xylazine (Phoeinix Pharmaceuticals, inc., St. Joseph, MO) and 80 mg/kg ketamine (Phoeinix Pharmaceuticals, inc., St. Joseph, MO) before the experiments. The animals were divided into two groups: Groups A and B. In Group A, animals were treated with TRAIM in the mixed micellar carrier system, and in Group B, control solution of TRAIM without the mixed micelles was used for the treatment. Both eyes of the animals were used in the present study, but they were treated with different protocols (e.g., iontophoresis delivery and passive delivery) or used for different time points (30 min, 2 h, 4 h and 6 h after treatments).

A custom-designed applicator was used in the passive and iontophoretic delivery experiments. As shown in Figure 6.1, this applicator was constructed using a 10 µL pipette tip, which had an orifice of approximately 0.8 mm$^2$ at the end of the tip. A small piece of cotton gauze was placed in the pipette tip. For iontophoretic drug delivery, an Ag/AgCl electrode was also inserted into the cotton gauze placed in the pipette tip. Immediately before the experiments, the mixed micelle or the control solution of 100 µL was loaded in the applicator as the donor.

Different protocols for iontophoresis were first tested in the preliminary experiments to determine the parameters necessary for the \textit{in vivo} experiments (e.g., the polarity of the electric current and the duration of the iontophoresis treatment). In these preliminary experiments, both anodal (anode electrode in the donor) and cathodal iontophoresis (cathode electrode in the donor) at electric current of 1 or 2 mA were
tested. The duration of the iontophoresis treatments varied from 5 to 20 min. The preliminary results (data not shown) showed that larger amounts of drug were delivered after cathodal iontophoresis of the mixed micelles and after anodal iontophoresis of the control solution. Therefore, in the present study, cathodal iontophoresis was used for the delivery of the mixed micelles, and anodal iontophoresis was used for the delivery of the control solution. Iontophoresis treatment of 1 mA for 10 min was selected in the present study to produce sufficient drug delivery for pharmacokinetic study and to minimize potential damages to the ocular tissues due to the high current density of the electric current applied.

In the passive and iontophoresis experiments, the applicator loaded with the test solution (the mixed micelles or the control solution) was placed on the intact mouse conjunctiva on the sclera adjacent to the limbus. Finger pressure was used to seal the applicator against the eye to prevent leakage of drug solution from the applicator. The duration of the passive drug delivery protocol was 10 min for both the mixed micellar carrier system and the control. For iontophoretic drug delivery, the Ag/AgCl electrode in the applicator was attached to a constant current iontophoretic device (Phoresor II Auto, Model PM 850, Iomed, Inc., Salt Lake City, UT). An alligator clip connected to the iontophoretic device was used as the return electrode and was clamped on a piece of Kimwipes® saturated with PBS on the mouse ear opposite to the treated eye to complete the electric circuit. Cathodal iontophoresis of 1 mA for 10 min and anodal iontophoresis of 1 mA for 10 min were used as the iontophoresis protocols for the mixed micelle and control solutions, respectively.
Figure 6.1. Schematic presentation of the applicator used in the passive and iontophoretic delivery experiments.
At the end of the passive and iontophoretic delivery experiments (i.e., the predetermined time points after the passive and iontophoresis treatments), the animals were euthanized by carbon dioxide followed by cervical dislocation (secondary physical method). After euthanization, the eyes of the animals were removed and blotted slightly using Kimwipes® to remove the solutions on the eye surface. The eyes were then rinsed three times in PBS and dissected with surgical scissors and forceps. The cornea, the lens, and the sclera with the conjunctiva and retina attached were collected, respectively. Aqueous humor/vitreous was collected using Kimwipes®. All the samples were placed in scintillation vials followed by the addition of 100 µL absolute ethanol for drug extraction. After 30 min extraction, the samples were mixed with 10 mL of liquid scintillation cocktail (Ultima Gold™, Perkin-Elmer Life and Analytical Sciences, Shelton, CT) and assayed by a liquid scintillation counter (Beckman Coulter LS 6500, Fullerton, CA). The amount of TRIAM (µg) in each sample was calculated using the specific activity of the drug.

6.2.5. Histology study

In the histology study, 12 mice were divided into 3 groups (4 mice in each group). The first group was treated with TRIAM mixed micelles using passive delivery, second group was treated with TRIAM mixed micelles using cathodal iontophoresis, and third group was treated with control without mixed micelles using anodal iontophoresis. In all animals, one eye was treated with the mixed micelles and/or iontophoresis and the other eye was not treated as a reference (no treatment reference). The passive and
iontophoretic delivery protocols used in the histology study were the same as those used in the transscleral delivery experiments (iontophoresis treatment of 1 mA for 10 min and passive delivery for 10 min).

All the mice were sacrificed immediately after the treatment and the eyes were enucleated. The eyes were rinsed three times with PBS and were immediately fixed in 4% PFA solution. After the fixation of eyes, dehydration procedure was accomplished by passing the tissue through a series of solutions of increasing alcohol concentrations of 30%, 50%, 70%, 80%, 90%, 95%, and 100%. Tissue clearance procedure was performed by passing the tissue through xylene/citrasol. After the dehydration and clearance procedures, the tissue was placed into an embedding mold, and melted paraffin was poured into the mold to form a block. The blocks were allowed to cool for sectioning. The tissues embedded in the paraffin blocks were sectioned with a standard steel microtome (Microm HM325, GMI Inc, Minnesota). The specimens obtained after section cutting were stained with hematoxylin-eosin for histological assessment using a light microscope (Nikon Eclipse E800, Nikon Instruments Inc. Melville, NY).

6.2.6. Statistical analysis

The amounts of TRIAM in the ocular tissue samples were expressed as the mean ± standard deviation (SD). The amounts of TRIAM determined in the iontophoresis and control experiments were compared using the non-parametric Mann-Whitney test. Differences between the groups were considered as significant at $p < 0.05$. 
6.3. Results and Discussion

6.3.1. Ocular delivery of TRIAM

The amounts of TRIAM in the cornea, aqueous/vitreous, and sclera/retina samples at 30 min, 2 hr, 4 hr, and 6 hr after the passive and iontophoretic delivery of the control and mixed micelle solutions are presented in Figure 6.2. These amounts of TRIAM are within the safe range of dose observed in clinical practice [10]. Figure 6.2A shows the amounts of TRIAM delivered to the cornea after passive and iontophoretic delivery. As shown in the figure, larger amounts of TRIAM were observed in the cornea after anodal iontophoretic delivery of the control as compared to those of passive delivery of the control at 30 min, 2 hr and 4 hr after the treatments. With the mixed micellar carrier system, larger amounts of TRIAM were observed in the cornea as compared to those of the control in both passive and iontophoretic delivery. The amounts of TRIAM decreased over time in the tissue. Figure 6.2B presents the amounts of TRIAM in the aqueous/vitreous humor in the study. The figure shows that the amounts of TRIAM in the aqueous/vitreous humor after passive delivery of the control were smaller than those of iontophoresis under the same condition. When time progressed after passive and iontophoretic delivery of control solution, the amounts of TRIAM decreased. With the passive delivery of mixed micelles, the amounts of TRIAM in the aqueous/vitreous humor were significantly higher (e.g., at 4 hr and 6 hr, \( p \leq 0.05 \)) as compared to the amounts of TRIAM after iontophoretic delivery of the control. Cathodal iontophoresis of mixed micellar carrier system further increased the amounts of TRIAM delivered into the aqueous/vitreous humor. Figure 6.2C shows similar trends of TRIAM in the sclera/retina layer as those observed in the cornea and aqueous/vitreous humor.
6.3.2 Effects of iontophoresis and mixed micelles

In general, based on the method of delivery and tested solutions (Figures 6.2A, B, and C), the amounts of TRIAM were in the order of iontophoresis of mixed micelles > passive mixed micelles > iontophoresis of control > passive control. The amounts of TRIAM in the aqueous/vitreous humor and sclera/retina at 4 hr after iontophoretic transport of the control were approximately 2.5-fold higher as compared to those after passive delivery of the control, demonstrating the enhancement of iontophoresis in transscleral drug delivery to the ocular tissue. However, at 6 hr after the treatments with the control, the amounts of TRIAM in these tissues were not significantly different ($p > 0.05$) between the iontophoretic and the passive delivery groups. These results show that although iontophoresis enhanced the delivery of drug in control solution to these ocular tissues at the earlier time points, TRIAM was cleared quickly in the these ocular tissues irrespective of the method of drug delivery (passive or iontophoretic delivery) at the later time point (e.g., 6 hr after the treatment).

Passive delivery of the mixed micellar carrier system provided approximately 6-9 fold increase in the amounts of TRIAM in the aqueous/vitreous humor and sclera/retina versus the iontophoretic delivery of the control at 6 hr after treatment. With iontophoresis, the amounts of TRIAM in the aqueous/vitreous humor and sclera/retina at 6 h after iontophoretic delivery of the mixed micelle solution were approximately 13-17 fold of those after iontophoretic delivery of the control. In other words, the amounts of TRIAM were 2-fold higher at 6 hr after the iontophoretic delivery of the mixed micellar system versus those after passive delivery. The larger amounts of TRIAM in these ocular tissues after iontophoretic delivery of the mixed micelles is consistent with the findings
observed in our previous studies [8-9], suggesting transcleral iontophoresis as an efficient method of drug delivery. The larger amounts and longer sustained release of TRIAM in these ocular tissues after the treatment with the mixed micelles as compared to the control are related to the sustained release properties of the mixed micellar carrier system. However, the amount of TRIAM at 24 hr after iontophoretic delivery of the mixed micelles was below the detection limit (data not shown). In our previous in vitro study, significant amounts of drugs were released from the sclera 48 hr after iontophoretic delivery with mixed micelles. The shorter retention of TRIAM observed in the present in vivo study than the previous in vitro study could be related to drug clearance in the ocular tissues in vivo such as clearance via episcleral and conjunctival venous blood flow, conjunctival lymphatic drainage, and choroidal blood flow [11-12].
Figure 6.2. Amounts of TRIAM in the (A) cornea, (B) aqueous/vitreous humor and (C) sclera/retina at 30 min, 2 hr, 4 hr, and 6 hr after passive control (closed diamonds), iontophoresis control (closed square), passive mixed micellar carrier system (open diamonds) and iontophoresis mixed micellar carrier system (open square) delivery. Data represent the mean and standard deviation, n ≥ 3.
6.3.3. Histology study

The effects of the mixed micellar formulation and transscleral iontophoresis on the ocular tissue were evaluated by histological study of the eye tissues. The histological samples of the treated groups were compared with the histology of the blank (Figure 6.3A, no treatment) eyes. The results in Figure 6.3 show no sign of histological damages or no visible lesions in either the retina or sclera after the treatment with the mixed micelle solution alone (Figure 6.3B) and in combination with transscleral iontophoresis (Figure 6.3C). No abnormalities due to the interaction between the surfactants used in the preparation of the mixed micelles and the eyes as well as thermal damages due to iontophoresis on the eyes were observed. Based on the histological observations, it is believed that this drug delivery system is a safe method to administer TRIAM to achieve a prolonged therapeutic level of TRIAM in the eye tissues. However, long term toxicity and irritation studies should be conducted before this method can be tested in humans.
Figure 6.3. Histological micrographs (A) blank eye, (B) passive mixed micelle treatment (C) cathodal iontophoretic mixed micelle treatment (n ≥ 4). The passive and iontophoretic treatment using mixed micelles were performed at sclera layer.
6.3.4. Practical application of transscleral iontophoresis of mixed micellar carrier system

The use of TRIAM for the treatment of retinal diseases is gaining popularity among the ophthalmic community [5]. It has been suggested that TRIAM can increase the tight junction integrity and inhibit vascular endothelial growth factors in ocular tissues [7]. Although TRIAM is an effective drug to suppress ocular inflammation, the methods of drug delivery for TRIAM in clinical practice are associated with side effects [6] [13]. In our previous study, mixed micelles were shown to be a sustained release drug delivery system that can be efficiently delivered into cadaveric human sclera using iontophoresis [8]. The present study is a first step to examine transscleral iontophoretic delivery of TRIAM using the mixed micellar carrier system in vivo. The present results suggest that sustained delivery of a corticosteroid can be achieved in the intermediate and posterior segments of mouse eye after transscleral iontophoresis of the mixed micellar carrier system. However, in the present in vivo study, drug delivery was not sustained in the ocular tissues as those observed in the previous in vitro study due to clearance such as blood vasculature and lymphatic clearance in transscleral delivery [14-15]. This suggests that drug clearance in ocular tissues is an important factor to consider in achieving long term sustained delivery of drugs in the posterior segment of the eye. Regardless, the combination of iontophoresis with mixed micellar carriers is an effective drug delivery method compared to either iontophoresis of the drug solution or passive delivery of the mixed micellar drug system alone. This finding warrants further evaluation of the drug delivery system in feasibility studies, e.g., in a diseased animal model, for the treatment of posterior eye diseases.
6.4. References


CHAPTER 7

Summary and Future Directions

7.1. Summary

Ocular iontophoresis is a non-invasive technique, which has been investigated for drug delivery into different parts of the eye for the treatment of ocular diseases. Recent ocular iontophoresis studies have investigated the delivery of small and large therapeutic agents. To improve ocular iontophoresis, better understanding of the mechanisms of transscleral iontophoresis is needed.

The studies described in the present dissertation contributed to the field of ocular drug delivery by providing new information on the mechanisms of iontophoretic delivery of macromolecules across sclera. Anodal and cathodal constant current iontophoresis experiments were conducted with excised human sclera in side-by-side diffusion cells in vitro using neutral and charged macromolecules. Both anodal and cathodal iontophoresis experiments showed significant enhancement of transscleral delivery of the macromolecules compared to their passive transport. The experimental data with excised human sclera in vitro suggested that iontophoretic flux enhancement of small charged permeants was governed by electrophoresis. For the iontophoretic transport of negatively charged macromolecules with low charge to mass ratio, electroosmosis was shown to be a major mechanism in the iontophoretic transport of the macromolecules. However, for iontophoretic transport of polyelectrolyte, the contribution of electroosmosis was not as
important as that of electrophoresis. The understanding of the different mechanisms involved in transscleral iontophoresis could allow us to optimize the delivery of macromolecules such as proteins, peptides, oligonucleotides, siRNA.

Another challenge facing transscleral iontophoresis is the fast clearance of drug from the eye to the systemic circulation. It would be a major advance in the field to be able to provide sustained release of the drug following ocular iontophoresis to reduce the frequency of iontophoresis treatment. A major objective of this dissertation was to characterize a mixed micelle nanocarrier system for sustained release ocular drug delivery with transscleral iontophoresis. These nanoparticles were characterized with respect to their solubility potential, size, zeta potentials, viscosity and osmolarity. It was hypothesized that the solubility enhancement of poorly water soluble corticosteroids using mixed micellar carriers would lead to further enhancement in drug delivery using transscleral iontophoresis. Experiments involving constant current transscleral iontophoresis of 2 mA were performed with excised human sclera in side-by-side diffusion cells in vitro using mixed micellar carrier systems as donor. The results showed that these micellar carrier systems efficiently enhanced corticosteroid transport across sclera using iontophoresis. These mixed micellar carrier systems were also found to sustain the delivery of corticosteroids after passive and iontophoretic delivery across the sclera.

The present dissertation also studied ocular iontophoresis of corticosteroids dexamethasone, triamcinolone acetonide and model drug estradiol in vitro to determine the influence of drug lipophilicity on the solubilization potential of mixed micellar carrier system, transport properties and drug release profiles after transscleral iontophoretic
delivery. Our study showed that passive and iontophoretic transport of the carrier system was not affected by drug lipophilicity but the release profiles of the drugs were related to the lipophilicity of the drugs in the mixed micellar carrier systems. More lipophilic drugs showed slower release from the sclera which might be due to the stronger interaction of the lipophilic drug with the micelles and/or with the sclera.

Upon successful demonstration of the effects of the mixed micellar system in sustained release ocular drug delivery with iontophoresis in vitro, a mixed micellar system of triamcinolone acetonide was tested in vivo in an animal model. Passive and iontophoretic delivery of the mixed micellar carrier system was investigated in mice. In addition, the safety of transscleral iontophoresis of the mixed micelles was also evaluated by histological examination of the eye tissues after the treatments. In the in vivo drug delivery experiments, the combination of iontophoresis with mixed micellar carriers was found to be a more effective drug delivery method compared to either iontophoresis of the drug solution without mixed micelles or passive delivery of the mixed micellar drug system alone; this is consistent with the results observed in the in vitro study. However, drug delivery in the in vivo study was not sustained in the ocular tissues as those observed in the in vitro study, probably due to ocular clearance such as blood vasculature and lymphatic clearance in transscleral delivery. The histology study showed no signs of damage or no visible lesions present on the tissue after the iontophoresis or mixed micelle treatments. The demonstration of the role of clearance on the sustained release properties of the mixed micellar nanocarrier system and the safety of sustained release drug delivery system were significant findings in this in vivo study.
7.2. Future work

The two major challenges facing ocular drug delivery are to deliver the drugs into posterior tissues of the eye using noninvasive methods and provide sustained release of the drugs following their delivery so that the frequency of the treatments can be reduced. Our present research suggests transscleral iontophoresis as an efficient method for the delivery of sustained release mixed micellar nanocarriers carrying poorly water soluble corticosteroids into the sclera. According to the *in vivo* data presented in this dissertation, the fast clearance of nanosize mixed micellar carrier systems was attributed to the barrier for prolonged drug delivery in the eye. Accordingly, an aim of the future studies will be to investigate the effect of the size of biodegradable polymeric micelles, e.g., poly (ethylene glycol)-poly(aspartate hydrazide) block copolymers and poly (D,L-lactide), upon sustained release drug delivery in transscleral iontophoresis. It is believe that these polymeric micelles can enhance the solubility of poorly water soluble corticosteroids, and transscleral iontophoresis may provide efficient delivery of these carriers into the sclera. Secondly, the effect of vasoconstrictor in transscleral delivery could also be studied to reduce ocular clearance and thereby enhance drug retention in the sclera for sustained drug delivery to the posterior ocular tissues.